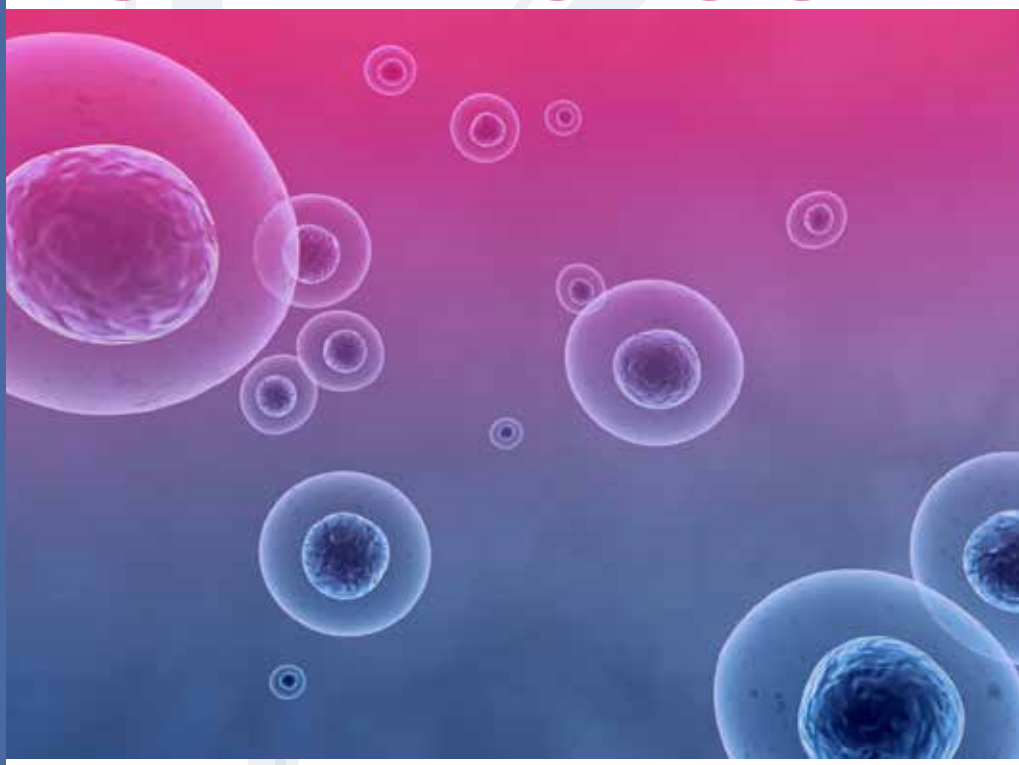
SCRIPTA VARIA 137

Edited by EDWARD M. DE ROBERTIS
MARCELO SÁNCHEZ SORONDO

CELL BIOLOGY AND GENETICS



Workshop | 23-24 October 2017
Casina Pio IV | Vatican City

Libreria Editrice Vaticana Vatican City 2018

Cell Biology and Genetics

Pontificiae Academiae Scientiarvm Scripta Varia 137

The Proceedings of the Workshop on

Cell Biology and Genetics

23-24 October 2017

Edited by
Edward M. De Robertis
Marcelo Sánchez Sorondo

EX AEDIBVS ACADEMICIS
IN CIVITATE VATICANA • MMXVIII

The Pontifical Academy of Sciences
Casina Pio IV, 00120 Vatican City
Tel: +39 0669883195 • Fax: +39 0669885218
Email: pas@pas.va • Website: www.pas.va

ISBN 978-88-7761-112-3

PONTIFICIA ACADEMIA SCIENTIARVM
LIBRERIA EDITRICE VATICANA

VATICAN CITY

Here I would recall the balanced position of Saint John Paul II, who stressed the benefits of scientific and technological progress as evidence of "the nobility of the human vocation to participate responsibly in God's creative action", while also noting that "we cannot interfere in one area of the ecosystem without paying due attention to the consequences of such interference in other areas". He made it clear that the Church values the benefits which result "from the study and applications of molecular biology, supplemented by other disciplines such as genetics, and its technological application in agriculture and industry". But he also pointed out that this should not lead to "indiscriminate genetic manipulation" which ignores the negative effects of such interventions. Human creativity cannot be suppressed. If an artist cannot be stopped from using his or her creativity, neither should those who possess particular gifts for the advancement of science and technology be prevented from using their God-given talents for the service of others. We need constantly to rethink the goals, effects, overall context and ethical limits of this human activity, which is a form of power involving considerable risks.

Pope Francis, *Laudato Si'*, §131.

Saint John Paul II at the foundational meeting of the Academia de Ciencias de América Latina (ACAL) at the Vatican in 1982. Professor Carlos Chagas filho as President of the Pontifical Academy of Sciences called a meeting to promote science in Latin America. In the photo Dr. Chagas (left) is introducing Sergio Mascarehnas (right) to Pope John Paul II. As a result of the meeting at the Casina Pio IV, headquarters of the Pontifical Academy of Sciences, the attendees constituted ACAL. Founding members were Carlos Chagas, Jorge E. Allende, Héctor Croxatto, Leopoldo de Meis, Sonia Dietrich, Patricio J. Garrahan, Armando Gómez Poyou, G. B. Marini-Bettolo, Sergio Mascarenhas, A. Paes Carvalho, Carlos Monge, M. Peixoto, Marcel Roche, Fernando Rosas, P. Rudomín, Andrés Stopanni, Jorge Villegas y Raimundo Villegas. Thirty-five years later the PAS welcomed many members of ACAL for the Cell Biology and Genetics Workshop recorded in these pages.


E. DE ROBERTIS

Contents

Biomedicine Session

Closing Session

Objectives of the Workshop

Bishop Sánchez Sorondo,
President Bifano,
Academicians,
Ladies and gentlemen,

The objective of this Workshop on Cell Biology and Genetics is to bring together researchers from the Pontifical Academy of Sciences and of the Academia de Ciencias de América Latina to report on the latest discoveries in Cell Biology and Biomedicine. The co-organizers, Vanderlei Bagnato, Rafael Vicuña and I are very grateful to PAS and Pope Francis for opening their doors to us.

ACAL was founded 35 years ago here at the Casina Pio IV during a special meeting called by then PAS President Prof. Carlos Chagas filho. This was a visionary decision at a time when the power of science and technology in improving the daily lives of the people was not self-evident as it is today, when we are living through the synergistic convergence of biotechnology, molecular medicine and information technology.

During the past 35 years science has made enormous progress. In Latin America ACAL had a very beneficial influence, facilitating interactions between scientists of many nations. One practical example is the case of Uruguay, which lacked an Academy of Sciences, and the government legislated one at the express advice of the authorities of ACAL. Under the administration of President Claudio Bifano, in the last few years ACAL has elected 38 new academicians representing a young generation of scientific leaders. Some of them are here today, and are the future of ACAL.

Latin America has enormous human capital, for it has an educated and highly civilized population. There is a long history of contributions to research, especially in biology. This has included Nobel Prizes awarded to Bernardo Houssay and Luis Leloir for work done in Buenos Aires. New institutes have been created that have powerful scientific capabilities, although many challenges remain. Latin American biology has benefitted from programs such as the Pew Fellows and HHMI Scholars and bilateral student exchanges that did not exist previously.

We have here the leaders in Cell Biology of the vast Latin American sub-continent. We look forward to learning about the latest advances in cell biology and biomedicine.

From whom much is given much is expected. Your research papers will be published as a book in *Acta Vaticana Scripta Varia*. We are grateful that so many of you have already provided your chapters and remind the others to send us their manuscripts within the next month. At PAS the old expression of "presenting a paper" is meant literally, and is based on its 400 years of experience. Your published scientific studies will be – to the Church and the world – a window on the current state of research in the field.

We have a limit of 25 minutes per presentation, followed by 10 minutes discussion. The Chairpersons will enforce it strictly.

There will be ample time for further discussions during our meals in the wonderful atmosphere of this magical Casina Pio IV. We hope these convivial interactions will result in new ideas for the closing session, which is dedicated to a general discussion on how we can improve Latin American science looking forward. The debate should not center on present difficulties, but rather focus on positive recommendations to improve science for the next few decades.

Topics to be discussed could include intra-continental horizontal scientific exchanges between Latin countries, the formation of human capital, the role of scientific societies and of national academies, the possible role of the Organization of American States, Pan-American congresses, short-term Ph.D. student exchanges with more developed countries, and the impact of information technology and artificial intelligence on molecular medicine and society.

In addition to the speakers and their spouses, who are very welcome to attend the discussions, the United States Department of State is represented by Dr. Franklin Carrero-Martínez, Deputy Science and Technology Adviser to the Secretary of State. We also want to recognize Dr. Mario Zurita from Mexico, who was President of the Latin American Society of Developmental Biology.

The Final Statement of this Workshop will be prepared by *rapporteur* Rafael Vicuña from Chile. I encourage you all to give Rafael a paragraph or two summarizing the suggestions you consider most useful for Latin American scientific development. It is very important that we produce an optimistic and forward-looking set of final recommendations from this meeting.

Once again, we would like to thank Monsignor Sánchez Sorondo, his indefatigable secretary Simonetta Ulisse, and PAS and ACAL for bringing the two academies together. The PAS has a uniquely universal perspective and hopefully their present efforts will once again give rise to many decades of building bridges through Latin American science.

Edward M. De Robertis

Programme

Monday, 23 October 2017

OPENING SESSION

9:00 *Word of Thanks*
Claudio Bifano | ACAL President
Objectives of the Workshop
Edward De Robertis | Uruguay/USA
9:15 *Presentation of the PAS-ACAL Encounter*
H.E. Msgr. Marcelo Sánchez Sorondo | Chancellor PAS

BIOPHYSICS AND MEMBRANE BIOLOGY SESSION
Chair: Edward De Robertis

9:20 *Biophysics and Biochemistry in Singulo: When Less is More*
Carlos Bustamante | Peru/USA
9:45 Discussion
9:55 *Transient Receptor Potential Channels as Sensors of Heat and Pain*
Ramón Latorre | Chile
10:20 Discussion
10:30 Coffee Break
11:00 *Is the Inflammatory Response the Cause of Tissue Dysfunction in Chronic Diseases?*
Juan Carlos Sáez | Chile
11:25 Discussion
11:45 *Free Radicals, Oxidants and Antioxidant Systems in Physiology and Disease*
Rafael Radi | Uruguay
12:10 Discussion
12:20 *The Role of Glucosylation and Demannosylation on the Quality Control of Glycoprotein Folding in the Endoplasmic Reticulum*
Armando J. Parodi | Argentina
12:45 Discussion
12:55 Lunch at the Casina Pio IV

GENETICS SESSION
Chair: Rafael Vicuña

15:00 *Design of Novel Regulatory Gene Circuits in Plant Cells Using Recombinase Serine Integrases*
Elibio Rech | Brazil
15:25 Discussion
15:35 *Molecular Mechanisms of Cell Adaptation to Hypoxia*
Pablo Wappner | Argentina
16:00 Discussion
16:10 *How Chloroplast-derived Signals Influence Leaf Developmental Fate; the Voice of the Slave*
Patricia León | Mexico
16:35 Discussion
16:45 *Nitrogen Fixation and Tropical Crops*
María Luisa Izaguirre | Venezuela
17:10 Discussion
17:20 Coffee Break
17:40 *Molecular Genetics of Plant Cell Resistance to Stress*
Luis R. Herrera Estrella | Mexico
18:05 Discussion

CELL SIGNALING AND DEVELOPMENTAL BIOLOGY SESSION
Chair: Salvador Moncada

18:15 *On the Origin of Life on Earth*
Rafael Vicuña | Chile
18:40 Discussion
18:50 *Regulation of Protein Degradation by Wnt Signaling*
Edward De Robertis | Uruguay/USA
19:15 Discussion
19:25 Dinner at the Casina Pio IV

Tuesday, 24 October 2017

NEUROBIOLOGY SESSION
Chair: Rafael Radi

9:00 *Sound Perception: From the Ear to the Brain and Back*
Ana Belén Elgoyhen | Argentina
9:25 Discussion
9:35 *New Insights About the Biology of Zika Virus Using Human Induced Pluripotent Stem Cell*
Stevens Rehen | Brazil
10:00 Discussion

BIOMEDICINE SESSION
Chair: Vanderlei Bagnato

10:10 *Endothelial Cells in Physiology and Medicine*
Salvador Moncada | Honduras/UK
10:35 Discussion
10:45 Coffee Break
11:15 *Physiopathology of the Cerebrovascular System: Advances from Genomics*
Conrado J. Estol | Argentina
11:40 Discussion
11:50 *The Methylome as an Example of the Contribution of Basic Research to Solving Medical Problems in the Third World*
Rafael J. Apitz-Castro | Venezuela
12:15 Discussion
12:25 *Applications of Physics to Cell Biology*
Vanderlei S. Bagnato | Brazil
13:00 Lunch at the Casina Pio IV
15:00 *RNA Processing, Ageing and Neurodegeneration*
Francisco E. Baralle | Argentina/Italy
15:25 Discussion
15:35 *Translating the 'Sugar Code' into Immune and Vascular Programs: from Basic Discovery to Drug Design*
Gabriel A. Rabinovich | Argentina
16:00 Discussion
16:10 Coffee Break

CLOSING SESSION

Chairs: Claudio Bifano (Venezuela) and Luis Davidovich (Brazil)

16:50 General Discussion: Improving Scientific Cooperation in Latin America
Academia de Ciencias de América Latina 35
Claudio Bifano | Venezuela
Intracontinental Scientific Collaborations
Luiz Davidovich | Brazil
Science and Technology Initiatives of the Department of State of the USA
Franklin A. Carrero-Martinez | Puerto Rico/USA
Generating the Final Statement of the PAS-ACAL Encounter
Rafael Vicuña | Chile

19:40 Dinner at the Casina Pio IV

List of Participants

Dr. Rafael J. Apitz-Castro
Canciller, Academia de Ciencias de América Latina (ACAL), Venezuela

Prof. Vanderlei S. Bagnato
University of Sao Paulo
Department IFSC - Physics
Brazil | PAS Academician

Dr. Francisco E. Baralle
PI RNA Biology Group
Former Director-General
International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy

Dr. Claudio Bifano
President, Academia de Ciencias de América Latina (ACAL), Venezuela

Dr. Carlos Bustamante
Professor Molecular and Cell Biology, Chemistry, and Physics,
UC Berkeley, USA

Dr. Franklin A. Carrero-Martínez
Deputy Science and Technology Adviser to the Secretary,
acting Office of the Science and Technology Adviser to the Secretary (E/STAS) US Department of State, USA

Prof. Luiz Davidovich
Universidade Federal do Rio de Janeiro, Instituto de Física,
Departamento de Física Matemática,
Brazil

Prof. Edward De Robertis
University of California, Los Angeles
Howard Hughes Medical Institute,
USA | PAS Academician

Dr. Ana Belén Elgoyhen
Laboratorio de Fisiología y Genética de Audición INGEBI-CONICET,
Buenos Aires, Argentina

Dr. Conrado J. Estol
Director, Stroke Unit, Guemes Clinic,
University of Buenos Aires School of Medicine, Argentina

Dr. Luis R. Herrera Estrella
Dirección Langebio
Cinvestav IPN
Irapuato, Guanajuato, Mexico

Dr. María Luisa Izaguirre
Full Professor Emeritus Vice Chancellor of the Latin American Academy of Science, Venezuela

Prof. Ramón Latorre
Profesor y Director
Centro Interdisciplinario de Neurociencia,
Universidad de Valparaíso, Chile

Dr. Patricia León
Universidad Nacional Autónoma de México,
Department of Plant Molecular Biology, Mexico

Prof. Sir Salvador Moncada
Cancer Domain Director Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health; Manchester Cancer Research Centre Building, University of Manchester, Manchester, UK

Dr. Armando J. Parodi
Laboratorio de Glicobiología
Fundación Instituto Leloir
Buenos Aires, Argentina

Dr. Gabriel A. Rabinovich
National University of Córdoba (Argentina)
School of Exact and Natural Sciences
University of Buenos Aires
Immunopathology Lab (IBYME)
Buenos Aires, Argentina

Dr. Rafael Radi
Professor and Chair of Biochemistry, Director, Center for Free Radical and Biomedical Research, Departamento de Bioquímica
Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

Prof. Elibio Rech
EMBRAPA Genetic Resources and Biotechnology
Laboratory of Synthetic Biology
Brasília, Brazil

Dr. Stevens Rehen
Head of Research - D'Or Institute for Research and Education
Professor of Biomedical Sciences - Federal University of Rio de Janeiro, Brazil

Dr. Juan Carlos Sáez
Departamento de Fisiología,
Pontificia Universidad Católica de Chile, Santiago, Chile

H.E. Msgr. Marcelo Sánchez Sorondo
Chancellor, The Pontifical Academy of Sciences, Vatican City

Prof. Rafael Vicuña
Pontificia Universidad Católica de Chile, Facultad de Ciencias Biológicas, Departamento de Génetica Molecular y Microbiología, Santiago, Chile | PAS Academician

Dr. Pablo Wappner
Director, Laboratorio de Genética y Fisiología Molecular,
Instituto Leloir,
Buenos Aires, Argentina

Observers

José Luis M. Martinez LC
Mario Zurita

Biophysics and Membrane Biology Session

Biochemistry and Biophysics *in Singulo*: When Less is More

Carlos Bustamante[1] **and Sara Tafoya**[2]

Hay dos panes. Usted se come dos Yo ninguno,
Consumo promedio: un pan por persona.
Nicanor Parra

Function Begets Structure

Were it not for its abundance all around us, life is the most remarkable and in many respects, the strangest phenomenon in nature. First, there is its elaborate structure and organization, unlike anything else around us. This makeup is not only complex but responds to a unique organizational principle, one that has no parallel in natural history. Indeed, biological systems are exceptional in that, every part of a living organism is there to fulfill a purpose and to perform a function. Its shape, dimensions, structures, and organizational details, all respond to a larger and complex *inner program* that must be ultimately capable of perpetuating the organism itself.

A rock has an inner structure too; moreover, it has shape, dimensions, physical and chemical properties. However, these properties are not there to fulfill any task. To be sure, there are physical and chemical laws that are responsible for the organization an inner structure of that rock. At the appropriate length scale, it is seen that atoms are not placed in a disordered or random fashion in its interior, and the physical and chemical properties of the rock can all be related and rationalized by its internal structure. But that rock has no program, no plan associated with it. It did not have to be of that size, to have that composition, to have that shape. There is no requirement or necessity to any of its properties. Non-living matter lacks function, purpose. In fact, function and purpose are the properties that make biological systems unique. A living system is *matter with purpose.*

Function occupies, therefore, a unique place in biology, for it is the central organizational principle of a living system. Out of the fundamental tendency of matter to self-organize, the mechanism of evolution sculpts the structure to fulfill the function. As a result, there is a *necessity* to every

[1] Department of Molecular and Cell Biology, Department of Physics, Department of Chemistry and Biophysics Graduate Group University of California, Berkeley, CA.
[2] Biophysics Graduate Group University of California, Berkeley, CA.

part of a biological organism that has no equivalent in non-living matter. This reasoning leads us to formulate a natural corollary of the fundamental organizational principle of living matter: *Function begets structure*. It is in this sense that I interpret the concept of necessity in Jacques Monod's celebrated monograph, *Chance and Necessity* (Monod J., 1970). Chance, because matter organizes through the random process of self-assembly; necessity, because those structures were either kept or discarded throughout evolution by the requirement to fulfill a function.

Structure and function are, thus, the two faces of the biological coin. Accordingly, biologists have made enormous efforts to uncover the structures of proteins, nucleic acids, and their complexes, with the ultimate hope of understanding their function. Indeed, few advances in biology have been more impressive than those of the methods of structural determination – X-ray crystallography and NMR – during the last four decades. Because of these efforts, the Protein Data Bank contains today more than 100,000 proteins whose structures are represented at atomic or near-atomic resolution. In many cases, the structure itself provides sufficient hints as to its function, but more often than not such insight is not enough. Function directs not only the structure of the basic parts, but their interaction in larger and more complex molecular organizations and, moreover, the structures can at best provide only a static picture of the parts.

In contrast, methods to follow the dynamics of these structures have developed comparatively more slowly. The reason can be traced to the nature of dynamics itself. The moving parts that make the functional structure do so *in time;* at the molecular length and nano to millisecond time scales at which they occur, all processes are stochastic in nature because they involve the thermal, spontaneous crossing of energy barriers. As a result, even if one starts with a synchronized population of molecules, the random nature of their time trajectories ensures that the population, when allowed to evolve in time, will soon lose coherence. Consequently, the signal extracted from the evolving population becomes the property over the individual molecular trajectories. And averaging can be very misleading.

In Multiplo or in Singulo, How to Study Function?

As an example, take the case of a chemical transformations such as A + B → C. The study of such systems has been traditionally dominated by the use of ensemble or "bulk" methods. In this approach, many (on the order of Avogadro's number) molecules of type A are mixed with a similar number of molecules of type B to produce an Avogadro order of magnitude

number of molecules C. In a typical experiment of this type, it is possible to follow the curse of the reaction if a property associated with one of the chemical species in the reaction can be measured in a concentration depended manner. In this way, the progress of the reaction can be monitor in real time in terms of the gradual disappearance of components A or B or the appearance of component C. We will refer to this approach as an "*in multiplo*" method, to distinguish it from the "*in singulo*" approaches that are the subject of this article.

Ensemble or "bulk" methods such as these have been extremely successful in chemistry and biochemistry. And, indeed, there are a number of advantages to *in multiplo* studies: First, these methods are robust because the measured signals are proportional to the number of molecules present in the reaction vessel at that moment and, at most times, their number is large. Second, the signals are ensemble averages of the individual molecular properties; thus, they appear to vary smoothly in time as the reaction proceeds, displaying little or no contribution of fluctuations. In fact, in macroscopic experiments of this type signal fluctuations relative to the magnitude of those signals are negligible since they decrease as $1/(N)^{1/2}$, where N is the number of molecules in the ensemble.

As an example, imagine an experiment in which we follow in real-time the unfolding of an ensemble of protein molecules by monitoring the changes in the intrinsic fluorescence of the solution upon the addition of 9 M urea. The protein denaturation process that ensues in these conditions may, for instance, expose to the solvent tryptophan residues that were initially buried in the hydrophobic interior of the folded molecules to the solvent, leading to a redshift in the emitted fluorescence. By monitoring the increase of emission at ~340 nm we could follow in real time the denaturation of the protein. The observed signal is an ensemble average of the contributions of all molecules in the solution at any given time and, as such, they are seen to vary smoothly in time with little or no fluctuations. The picture that emerges from a bulk measurement of this type is that of 'idealized molecules' all of which follow in synchrony a deterministic, well-defined dynamics in which denaturation proceeds monotonically, exposing more and more of their interior to the solvent as a function of time.

However, such picture could not be further from the truth. Upon addition of urea, each molecule of the sample will follow its own and independent dynamics that would be uncorrelated to those of the other molecules. Some of them would begin to denature immediately upon getting in contact with the chaotropic agent, while others would remain folded for a

varying time before beginning to denature. In this process, some molecules may even move backward temporarily, regaining some structure before eventually becoming completely denatured and so on. The signal measured in bulk is thus the sum of all these simultaneous contributions. Its variation in time represents the dynamics of the "mean" of the population and, as such, it does not reflect the dynamics of *any* of the individual molecules in the sample.

Moreover, even if the protein solution is not subjected to such strong perturbation as the addition of a strong denaturing agent so as to initiate a time dependent process, proteins in this example may co-exist in two different folded states with different structure and correspondingly different tryptophan emission signals. Once again, the signal measured in bulk will be an average of the contributions of each individual molecule in solution. This average again does not represent appropriately those of any of the populations that coexist in solution (Figure 1, left panel). Alternatively, the molecule may exist in a number of closely related sub-structures with correspondingly slightly different fluorescence signals. In this case, the bulk average signal is closer to the signals of the individual populations, yet it does not convey the existence of multiple closely related structures (Figure 1, central panel). Finally, the molecule may exist in a well-defined structure characterized by a narrow emission distribution. Only in this case the average signal provides information that describes most of the structures present in solution (Figure 1, right panel). But note that even in this case, were the conditions to change and result in some kind of molecular process (denaturation, binding, dimerization, etc.), the average signal will again fail to faithfully describe the true dynamics of the process. Therefore, despite their power and robustness bulk or *in multiplo* measurements represent a great compromise in the information they provide about dynamic processes in molecular ensembles.

The alternative is to follow and study the molecules one at a time. *In singulo* methods provide a way to follow not the value of the ensemble average of a molecular property as a function of time, but the dynamics of one of the molecules in the ensemble. The resulting signals in these experiments are widely varying and dominated by fluctuations, and do not display the smoothly changing characteristics of the population average. But they do provide direct access to the dynamics of the process.

The two most widely used single-molecule methods to date are based on the relatively recent ability to detect single molecule fluorescence and to manipulate individual molecules through force spectroscopy. More re-

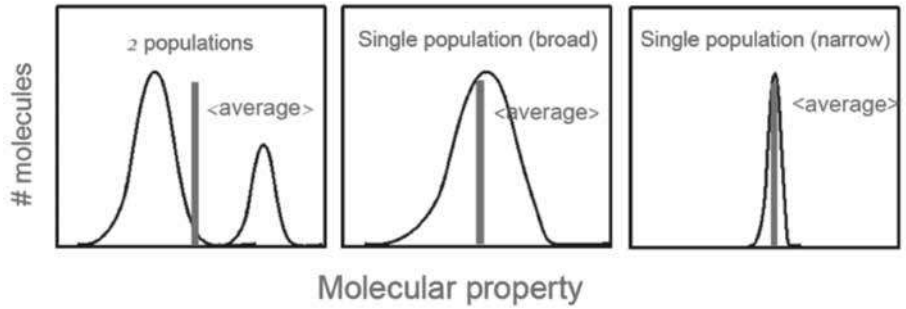


Figure 1. A protein molecule in solution may exist in two different conformations at equilibrium with each other: A ‹=========›A'. When some molecular property of an ensemble of such molecules is being measured, the signal may correspond to multiple populations (left panel), to a single broad population (middle panel) or a to a single, narrow population (right panel).

cently, these two approaches have been combined in the same experiment, providing additional information over what can be learned from each measurement separately. In this article, we will concentrate on describing some of the developments in single molecule force spectroscopy.

As pointed out in the example above single-molecule methods are uniquely informative for kinetic processes, because such processes are stochastic. Each step in a reaction has a different rate; each intermediate species has a different lifetime. For each single kinetic step the lifetimes of each species are distributed exponentially and can take any given value in an unpredictable way. Moreover, as illustrated in that example, although many molecules can be started at the same time in the reaction (by adding urea, for example), after a few steps some will be on the third step, while others will be far ahead. The states of the molecules rapidly become unsynchronized relative to each other. Sequencing a nucleic acid by determining the identity of the end nucleotide as a function of time can only be done one molecule at a time. Watching each step in the synthesis of a protein requires following a single ribosome de-coding a single messenger RNA molecule in real time.

Furthermore, molecules may visit, very rarely, extreme states that are therefore only scantily represented in the ensemble; as a result, the averaged bulk signal provides little or no information about such rare states. In contrast, during *in singulo* experiments it is possible that at some point the molecule visits such extreme state and the signal detected during that period will reflect the properties associated with that state.

Because of their nature, and in contrast to those derived from ensemble methods, signals obtained in single molecule measurements are small – their tiny magnitudes requiring highly specialized instrumentation – rapidly varying, and highly fluctuating. The magnitudes of these fluctuations are typically of the order of the signals themselves. These fluctuations arise because, kept at a temperature T, the molecule is an open thermodynamic system that can exchange energy and matter with a large thermal bath. Each degree of freedom of the molecule can exchange energy with this bath. This constant exchange of energy, in turn, is responsible for inducing transitions across energy barriers that appear as fluctuations of the signal in single molecule experiments.

Why Single Molecule Methods?

We have seen that the use of single molecule methods arose as a methodological necessity to overcome the perils of population averaging that plague ensemble or *in multiplo* approaches. However, there is a more fundamental reason for their use and for wishing to have a description of biomolecular interactions at the single molecule level. The cell, seen as a reactor vessel, is a place where single molecule behavior matters. In fact, a back of the envelope calculation shows that in an *E. coli* cell a single molecule of any kind (a repressor protein for example) is at the concentration of ~ 1.6 nM, because of the tiny volume of that cell. Since the dissociation constants of most DNA binding proteins to their cognate site are in the sub-nano Molar or even pico Molar regime, it follows that the cell does not need to have present too many of these molecules around to ensure occupancy of the cognate site more than 99% of the time. In consequence, many fundamental cellular processes (replication, transcription, chromosomal segregation, etc) are carried out by very few molecules and, therefore, are subjected to the random fluctuations observed when studying individual molecules and possess none of the deterministic and smooth temporal characteristics of *in multiplo* measurements. Therefore, there is great interest to understand the role of these fluctuations in molecular processes and how the cell extracts robustness out of noise.

In the next sections, we will make a brief exposition of the critical results in the development of single molecule force spectroscopy. The intention is not to make a review of the main results, but simply to illustrate the type of experiments that have become possible by these pioneering experiments.

Mechanical Experiments

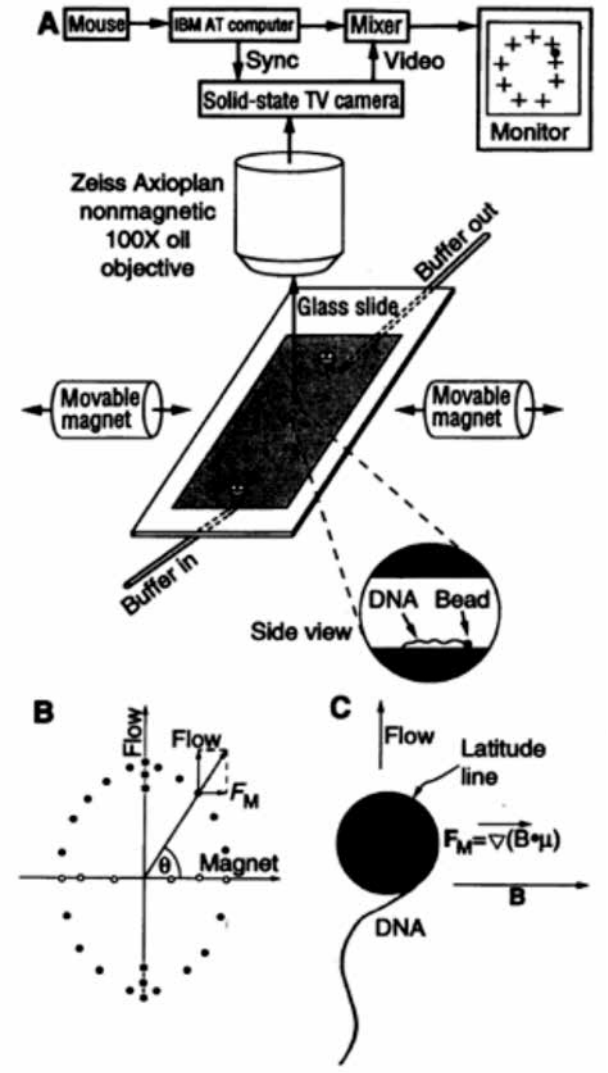


Figure 2. (A) Experimental set up to measure the elastic behavior of single DNA molecules using magnetic and flow or hydrodynamics forces. The magnetic force is increased or decreased by moving the magnets closer or farther away from the molecule. (B) Ellipse generated by applying the maximum magnetic force and increasing the hydrodynamic force applied along a perpendicular direction. (C) Accounting for possible rotational orientation of the bead due to the alignment of the dipole moment with the gradient of the magnetic field. Adapted from Smith *et al*., 1992.

The first experiments in single molecule force spectroscopy involved the direct manipulation of DNA using gravitational force (Bustamante *et al.*, 1991) and magnetic force via magnetic tweezers (Smith *et al.*, 1992). In the latter experiments, a molecule of DNA was attached by one of its ends to the surface of a microscope slide and to a magnetic bead by the other. The authors applied varying magnetic forces to the bead along the + and – x-axis, and varying hydrodynamic forces along the + and – y-axis (Figure 2A). As a result, the bead described an ellipse with the major axis centered along the Y-axis, as the molecule is increasingly extended by the combined magnitude of the hydrodynamic and magnetic forces (Figure 2B). In these experiments, the magnetic force was determined by detaching the bead from the molecule and by measuring the speed of the free bead moving under the same magnetic forces applied to the molecule. Using the known radius of the bead, the authors applied Stokes' law to determine the magnetic force for each point on the ellipse. Since the angle of elevation q for each position on the ellipse (Figure 2B) depends on the relative magnitude of the magnetic and hydrodynamics forces at that position, knowing the

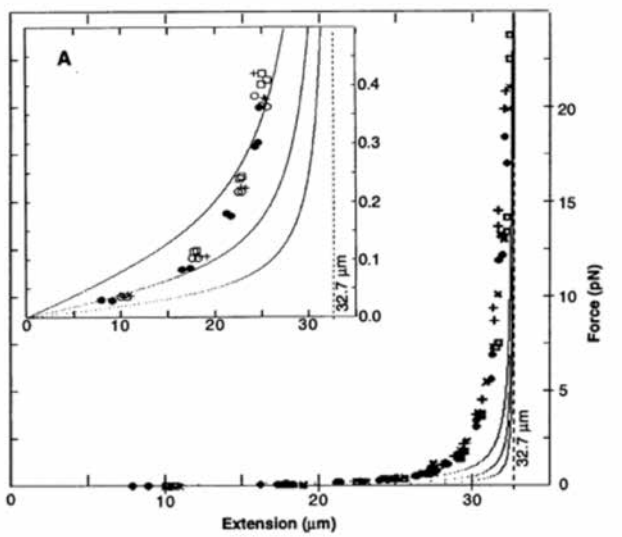


Figure 3. Force extension curves of 4 DNA lambda dimers. (Contour length 32.7 μm. Notice the highly non-linear behavior of the molecule. The solid lines are fittings to the FJC model for different values of the Kuhn segment length. None of the curves describe correctly the elastic behavior of the molecule. Inset: Enlargement of the experiment – theory discrepancy region. Adapted from Smith *et al.*, 1992.

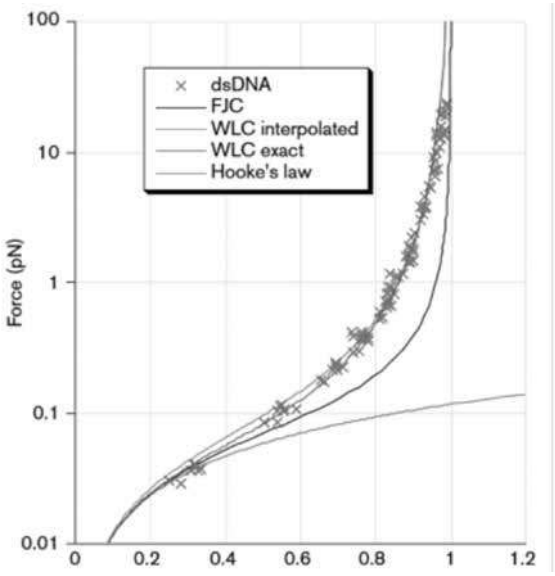


Figure 4. Force versus extension data (red crosses) for lamda phage dsDNA (48,502 bp) pulled by magnetic beads in 10 mM Na+ buffer [4]. The data are fit to a WLC model solved numerically (WLC exact) or using Equation 1 (WLC interpolated), both assuming P = 53 nm. The FJC curve assumes b = 2P = 106 nm. The Hooke's law force curve is from Equation 50. Notice the excellent agreement between experiment and theory with the WLC model. Adapted from Bustamante *et al.*, 2000.

magnetic component it is possible to calculate the resultant total force acting on the molecule. These experiments were directed towards characterizing the elasticity of a polymer molecule. Figure 3 depicts the force vs extension curves obtain from 4 DNA dimers of bacteriophage l (32.7 mm long). These experiments revealed the highly non-linear elastic response of DNA and provided the best experimental test to date of the competing models of polymer statistics, the Freely Jointed Chain (FJC) and the Worm-like Chain (WLC). It was, thus, possible to obtain a quantitative description of the elastic response of the DNA molecule under the application of external force using the WLC (Bustamante *et al.* 1994, Marko and Siggia, 1995). The expression, derived in two different regimes of force and extrapolated to force values in between is:

$$\frac{FP}{k_B T} = \frac{1}{4(1-\frac{x}{L})^2} + \frac{x}{L} - \frac{1}{4} \quad (1)$$

where F is the external force acting on the molecule, L its contour length, assumed to be non-extensible, x the force-dependent end-to-end distance of the molecule, P its persistence length of the molecule and kB Boltzmann constant. This analysis showed that the WLC is the best model to describe the elasticity of the DNA molecule (See Figure 4).

Just a few years later, Strick *et al.* (Strick *et al.* 1996) showed that it was possible to study the torsional properties of a double strand DNA molecule similarly tethered between a surface and a magnetic bead, with the molecule torsionally constrained at both ends. The authors demonstrated that the molecule when over- or undertwisted forms plectonemes and becomes shorter as long as the tension applied to the molecule is below certain threshold. That threshold, the authors showed, was different for overtwisting than for undertwisting (Figure 5).

Having shown that it was possible to mechanically manipulate a single molecule of DNA, scientists soon sought to apply a similar strategy to study the mechanical unfolding and refolding of single protein molecules. Simultaneously, three groups accomplished this task on the giant muscle protein titin. Using an atomic force microscope-based manipulator, Rief

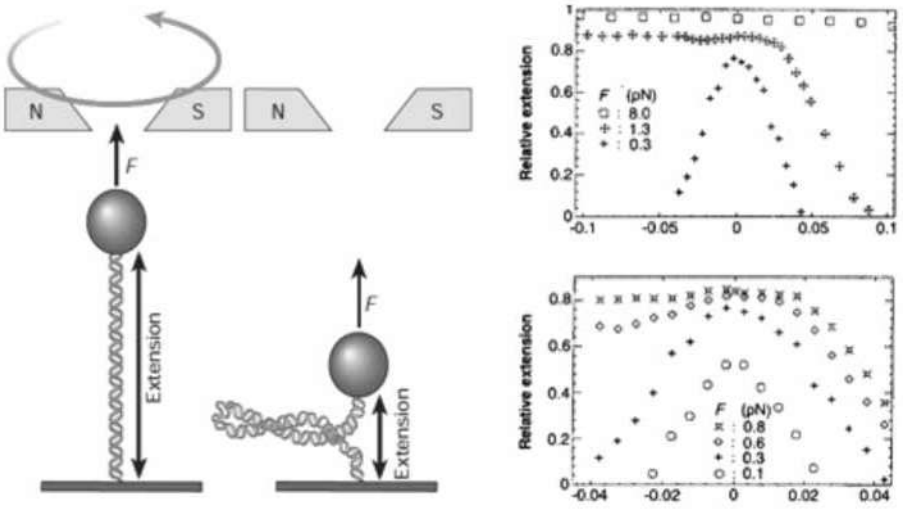


Figure 5. (Left) Use of magnets to over- or under-twist DNA to form plectonemes. (Right) Relative extension vs. degree of supercoiling σ at various forces: F= 8 pN, 1.3 pN, and 0.3 pN. (C) F=0.8 pN, 0.6 pN, 0.3 pN, and 0.1 pN. Notice the symmetric behavior under σ → - σ at smaller forces and the transition to an extended state at greater forces (starting at 0.45 pN for negative supercoil and at 3 pN for positive supercoil). Adapted from Strick *et al.*, 1996.

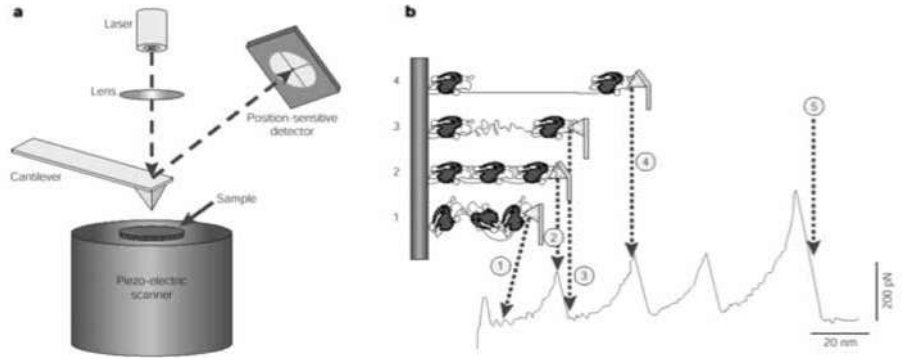


Figure 6. Use of the atomic force microscope (AFM) as a nano-manipulator. a. The principal AFM components. A laser beam is focused onto the back of a cantilever that ends with a nanometre-scale tip. The reflection and corresponding position of the tip is detected by a position-sensitive photodiode. A piezo-electric scanner moves the sample in all directions, enabling the tip to scan topography or to extend molecules attached to the surface. b. Diagrams and force-extensions curves showing the mechanical unfolding of repeating immunoglobulin-like domains. As the distance between the surface and tip increases (from state 1 to state 2), the molecule extends and generates a restoring force that bends the cantilever. When a domain unfolds (state 3), the free length of the protein increases, relaxing the force on the cantilever. Further extension again results in a restoring force (state 4). The last peak represents the final extension of the unfolded molecule before detachment from the SFM tip (state 5). Adapted from Fisher *et al*., 1999.

et al. (Rief *et al*., 1997) showed that it was possible to unfold mechanically the individual Ig127 domains of titin in a sequential manner, giving rise to a characteristic "saw tooth" pattern in a force vs. extension plot (Figure 6b bottom). In parallel, two other groups (Kellermayer *et al*. 1997 and Tskhovrebova *et al*. 1997) utilized optical tweezers to unfold isolated molecules of titin. These studies showed that it was possible to use force as a mean to investigate the folding ßà unfolding equilibrium of individual protein molecules.

The initial force spectroscopic experiments described above, established the bases for the development of a number of single molecule assays of nucleic acid and protein processing enzymes, respectively.

Enzymatic Assays

Many nucleic acid processing enzymes behave as molecular motors, converting part of the energy of the downhill reaction they catalyze into the generation of force and/or torque. The forces or torques generated in

the course of the chemical reaction are responsible for the directionality of their motion. In this way, the operation of molecules not normally recognized as molecular motors *per se*, such as DNA polymerases, RNA polymerases, ribosomes as well as molecules whose functions are mechanical in the cell, such as helicases and translocases, have been studied by means of these methods (for a recent review see Bustamante *et al.* 2011). It should be noticed that in these reactions, force or torque are themselves *products* of the reaction. As such, it follows from Le Chatelier's principle, that application of force or torque to these reactions will lead necessarily to a change in the rate of and, possibly, to alternative pathways for those reactions.

Two examples of these types of studies are illustrated in Figure 7. On the left panel, an assay developed to follow transcription by single molecules or RNA polymerase is depicted (Yin *et al.* 1995, Wang *et al.* 1998). The enzyme is attached non-specifically to the glass slide and the downstream end of the DNA template is tethered to a microscopic bead held in an optical trap. RNA polymerase is known to be an extremely processive enzyme and, therefore, this geometry makes it possible to follow the progress of the enzyme by the shortening of the DNA tether or, equivalently, by the movement of the bead away from its initial position in the trap. The experiment can be done in two modes. In *constant force mode*, a feedback circuit is used to move the trap closer to the enzyme as transcription progresses, so as to maintain the tension on the template constant. It is against

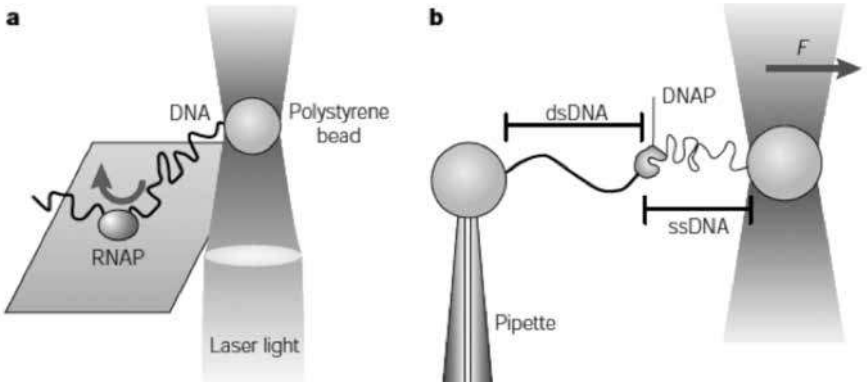


Figure 7. Experimental set ups to investigate the function of nucleic acid based enzymes using optical tweezers. (A) Assay to follow transcription by RNA polymerase from E. Coli. (B) A single molecule assay to follow the progress of single molecules of T7 DNA polymerase molecules. Adapted from Wuite *et al.*, 2000.

this force that the polymerase molecule must do work to transcribe. In *passive mode*, the trap position is kept fixed and, as transcription proceeds and as the tether shortens, the enzyme must work against an increasing force. Using this method Wang *et al*. showed that RNA polymerase can generate up to 20 pN of force and that the processive translocation by the enzyme is interspersed by periods of inactivity or pauses.

The right panel in Figure 7 depicts an assay designed to follow the activity of a non-processive enzyme such as T7 DNA polymerase (Wuite *et al.*, 2000). In this experiment, a single stranded DNA molecule is tethered between two beads, one a top a pipette and the other held in an optical trap. A short RNA primer is then added to allow molecules of T7 DNA polymerase to elongate its 3' end, converting the single stranded DNA into a double stranded tether. This geometry in which the DNA template is held by both ends instead of the enzyme is used in this case because of the distributive nature of DNA polymerases, which copy only a few tens or at most hundreds of bases of DNA before they detach. Because the elastic behaviors (extension at a given force) of single stranded and double stranded DNA are very different, holding the tension constant at a pre-established value using a feedback circuit implies that the end-to-end distance of the tether must either get larger or shorter, depending on whether at that force ssDNA is shorter or larger than dsDNA respectively. Because the enzymes detach from the DNA after short polymerization runs and before new ones bind again, the data depicts periods of activity interspersed with

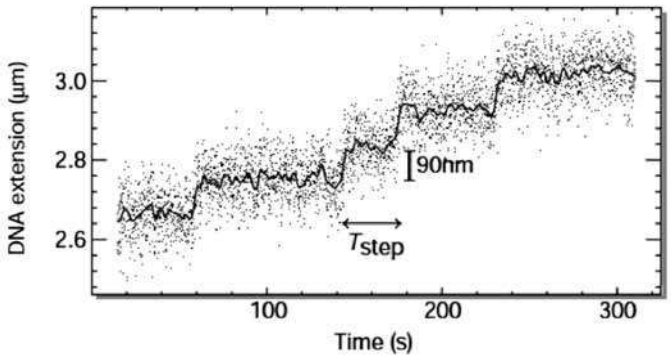


Figure 8. Change in DNA extension over time observed upon adding Topoisomerase II to a single molecule of DNA previously supercoiled with magnetic tweezers. Adapted from Strick *et al.*, 2000.

periods of inactivity. From these, it is possible to calculate the average processivity of the motors. Interestingly, in this experiment it was found that when the tension in the tether is increased above 40 pN, the polymerase molecule starts moving 'backwards' converting the dsDNA into ssDNA. It was shown that at this tension, the deformation of the DNA template strand signals the enzyme to send the 3' end of the growing chain to the exonucleolysis active site where it is shorten as an editing response.

It can be seen that in both of these assays, the elastic behavior of the DNA (Equation 1) is essential to convert the end-to-end distance of the tether (x) into a contour length (L) in nm or base pairs, as long as the persistence length of the DNA molecule and the force F applied to it are known.

Likewise, the studies of DNA torsion and the formation of plectonemic DNA led to the development of assays to investigate the activity of topoisomerases (Strick *et al.*, 2000). Indeed, when topoisomerase II is added to a molecule containing plectonemes in the magnetic tweezers assay (Figure 5, left), 90-nm changes in extension are seen, corresponding to the removal of two turns, as predicted by the model (Figure 8).

A Case Study in Detail

We will finish this brief review of the developments of single molecule force spectroscopy with a somewhat more detailed description of experiments designed to understand the operation of a motor whose function is to pump the DNA of a virus into its pre-assembled capsid. The motor that sits at the base of the capsid in bacteriophage phi29 is a homopentamer that belongs to the Rec A superfamily of ring ATPases. These molecules are known to perform many important transport functions in prokaryotic and eukaryotic cells. The experimental set up is depicted in Figure 9A. A single capsid already in the process of packaging the DNA is attached to a bead held in an optical trap. The distal end of the DNA is then attached to another bead held in a second trap. Upon addition of ATP, it is possible to follow the packaging process by measuring the end-to-end distance of the tether. In early studies (Moffitt *et al.*, 2009), it was found that the motor packages the DNA using a two-phase cycle: a "dwell" phase during which the DNA remains stationary, followed by a "burst" phase during which 10 base-pairs (bp) of DNA are rapidly translocated into the head (Figure 9A). This observation immediately suggested a model in which each burst is composed of five 2 bp translocation sub-steps, one sub-step per subunit. This mechanism would be consistent with the number of base pairs (two) packaged per hydrolyzed ATP derived from bulk experiments. Surprising-

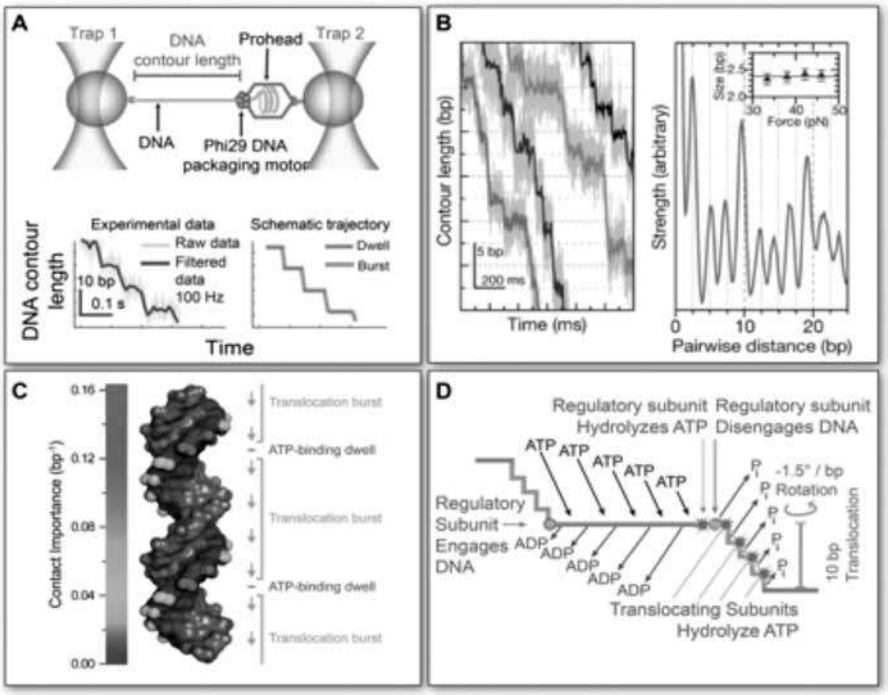


Figure 9. (A) (Top) Single-molecule experimental geometry & (Bottom) experimental data and schematic representation. (B) (Left) Representative packaging traces collected with external loads of 40 pN at saturating [ATP]. Data in light grey are plotted at 1.25 kHz whereas data in colour are boxcar-filtered and decimated to 100 Hz. (Right) Average pairwise distribution of packaging traces. Adapted from Moffitt *et al*., 2009. (C) Relative importance of the motor-DNA contacts. Units reflect the distance at which the transversal probability of the packaging motor in a given DNA construct drops to 50%. Adapted from Aathavan *et al*., 2009. (D) Model of the mechano-chemical cycle indicating the location of all chemical transitions in the regulatory and translocating subunits, as well as DNA rotation. Adapted from Chistol *et al*., 2012.

ly, however, single molecule packaging trajectories obtained subsequently with high-resolution optical tweezers, revealed that the "burst" was comprised actually of four 2.5 bp sub-steps (Figure 9B). This observation indicated that one of the subunit does not play a mechanical role, a result completely inaccessible through ensemble measurements. In this same study, the authors showed that decreasing the concentration of ATP lengthened the dwells, indicating that nucleotide binding takes place during this phase of the cycle. It had been previously shown (Chemla *et al*., 2005) that the only chemical transition capable of supporting the work done by the mo-

tor near its stall force (~ 57 pN) is the release of inorganic phosphate after ATP hydrolysis.

In a later study (Chistol *et al.*, 2012), the authors used non-hydrolyzable nucleotide analogs (ATPgS and AMP-PNP) to stop the motor at specific chemical transitions of the catalytic cycle. They were able to show that ATP binding and ADP release occur in an interlaced manner during the dwell phase, while ATP hydrolysis and Pi release occur in the burst phase (Figure 9D). Moreover, ATP hydrolysis and Pi release are also interlaced, with the release of inorganic phosphate coupled to DNA movement (the power stroke). Furthermore, this study provided insight into the role of the non-translocating subunit. Experiments in which one subunit was temporarily inactivated by binding to the non-hydrolyzable analog, ATPgS, showed that the non-translocating subunit must hydrolyze ATP also to fulfill a critical regulatory role in the dwell-burst cycle (Figure 9D). Thus, this study resolved the apparent contradiction between single molecule and bulk studies on the number of base pairs packaged per hydrolyzed ATP: four are used to performed a mechanical task and one to perform a regulatory one.

At this point, however, the origin of symmetry breaking among motor subunits remained unknown. In a previous single-molecule study, the packaging motor was challenged to translocate a variety of modified DNA substrates (Aathavan *et al.*, 2009). These experiments helped to determine the nature of the motor-DNA interactions throughout the mechano-chemical cycle (Figure 9C). During packaging, the motor makes specific electrostatic contacts every 10 bp with a pair or phosphates in the DNA backbone during the dwell. In contrast, the motor makes mostly nonspecific, steric contacts to propel the DNA during the four power strokes of the burst phase. In view of these results, Chistol *et al.* proposed (Chistol *et al.* 2012) a model in which the subunit that makes these specific contacts is the non-translocating subunit and that the binding to the DNA phosphate is the event that confers it its regulatory role (Figure 9D). Two questions remain unanswered: Is the identity of the special, non-translocating subunit retained from cycle to cycle? And if so, how does the motor deal with the difference between the 10-bp motor burst size and the 10.4-bp DNA helical pitch?

In a later study (Liu *et al.*, 2014), the authors developed a "rotor bead" assay capable of measuring the three-dimensional trajectory of DNA during packaging. These experiments provided the first experimental demonstration that the motor rotates the DNA as it packages it. To answer whether rotation is a passive response to the supercoiled organization of the

DNA inside the capsid, the authors used "trepanated" proheads (wherein the DNA does not accumulate due to the perforation of the proheads). Significantly, in experiments performed with "trepanated" proheads, the DNA was still observed to rotate by about -1.5°/bp, indicating that the motor actively rotates the DNA during translocation. Interestingly, this figure accounts for the mismatch between the 10 bp burst and the 10.4 bp DNA helical pitch, as it is the exact amount of rotation (~ 15 degrees in 10 bp) necessary for the same subunit to contact the DNA phosphates ever 10 bps and to retain its regulatory role over consecutive cycles (Figure 9D). Furthermore, it was long known that packaging slows down as the head fills with DNA and the internal pressure opposing the motor increases. However, the mechanism by which head filling regulates motor velocity was unclear. Therefore, Liu *et al.* also examined ϕ29 packaging dynamics at different stages of head filling and found that multiple aspects of the motor's mechano-chemical cycle are altered in the process. First, the motor displays long-lived pauses. Second, the motor burst size is reduced from 10 bp to 9 bp, yet still making four steps per burst. Remarkably, the decrease in the burst size observed with head filling is accompanied by a corresponding increase in the magnitude of DNA rotation in the precise amount needed for the regulatory subunit to engage the DNA phosphate and retain its identity at the end of each burst throughout packaging. In other words, DNA rotation changes to preserve the coordination between the regulatory and translocating subunits.

The results described above evidence the importance of the motor's inter-subunit coordination to complete its biological task, and invite an obvious question: how does such coordination emerge from stochastic, elementary molecular interactions? A series of single-molecule experiments are currently being performed to identify the structural elements and interactions that enable this coordination. In particular, a highly-conserved trans-acting arginine residue that mediates communication between two adjacent subunits is being extensively studied. The hope is to understand the role of this trans-acting residue in establishing *global* inter-subunit coordination from *local* interactions.

Epilog

The advent of single molecule methods has brought about a profound change in biochemical and biophysical research. Most of our knowledge of Chemistry and Biochemistry has been derived from bulk or *in multiplo* approaches. However, in the short years that have elapsed since the per-

formance of the first single molecule experiments, scientists have learned that by avoiding the perils of ensemble or *in multiplo* averaging, it is possible to have more direct access to the molecular mechanisms underlying biochemical processes, thus fulfilling the epistemological need in Biology to understand function in order to rationalize structure. Moreover, the idea that forces and torques develop in the course of chemical reactions – postulated almost 130 years ago by Svante Arrhenius – while generally accepted, had remained only a reasonable conjecture. Today, single molecule force spectroscopy methods have made forces and torques part of the vocabulary of biochemists and biophysicists, as these quantities have become experimental observables and the mechanical nature of many biochemical processes is increasingly being recognized. In the course of these advances, our view of the cell has itself began to evolve: it can no longer be seen as a small 'bag' with a concentrated solution of macromolecules undergoing second order reactions. The picture that emerges, looks instead more like a complex of interconnected factories, each responsible for an essential cellular function and made up of highly coordinated molecular machines. This framework represents, in turn, an important conceptual step in the hope of scientists to turn Biology into a truly quantitative science.

Bibliography

Monod J. *El Azar y la Necesidad*, Barral Editores, 1970 Barcelona.

Bustamante C, Marko JF, Siggia ED, Smith SB. Entropic Elasticity of Lambda-phage DNA. *Science*. 265:1599-1600 (1994).

Marko JF, Siggia ED. Statistical Mechanics of Supercoiled DNA. *Phys. Rev. E*. 52:2912-2938 (1995).

Bustamante C, Cheng W, and Mejia Y. Revisiting the Central Dogma one Molecule at a Time. *Cell*. 144:480-497 (2011).

Strick TR, Allemand JF, Bensimon D, Bensimon A, Croquette V. The Elasticity of a Single Supercoiled DNA Molecule. *Science*. 271:1835-1837 (1996).

Smith SB, Finzi L, Bustamante C. Direct Mechanical Measurements of the Elasticity of Single DNA Molecules by Using Magnetic Beads. *Science*. 258:1122-1126 (1992).

Bustamante C, Finzi L, Sebring PE, Smith SB. Manipulation of Single DNA Molecules and Measurement of Their Elastic Properties under an Optical Microscope. Optical Methods for Ultrasensitive Detection and Analysis: Techniques and Applications, *SPIE Proceedings Series*. 1435:179-187 (1991).

Bustamante C, Smith SB, Liphardt J, Smith D. Single-molecule Studies of DNA Mechanics. *Curr. Opin. Struct. Biol*. 10(3):279-285 (2000).

Smith SB, Cui Y, Bustamante C. Overstretching B-DNA: The Elastic Response of Individual Double-stranded and Single-stranded DNA Molecules. *Science*. 271:795-799 (1996).

Strick TR, Croquette V, Bensimon D. Single-molecule Analysis of DNA Uncoiling by a Type II Topoisomerase. *Nature*. 404:901-904 (2000).

Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub, HE. Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM. *Science*. 276:1109-1112 (1997).

Fisher TE, Oberhauser AF, Carrion-Vazquez M, Marszalek PE, Fernandez JM. The Study of Protein Mechanics with the Atomic Force Microscope. *Trends in Biochemical Sciences.* 24:379-384 (1999).

Kellermayer MSZ, Smith SB, Granzier HL, Bustamante C. Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers. *Science*. 276:1112-1116 (1997).

Tskhovrebova L, Trinick J, Sleep JA, Simmons RM. Elasticity and Unfolding of Single Molecules of the Giant Muscle Protein Titin. *Nature*. 387:308-312 (1997).

Wuite, G. J., Smith, S. B., Young, M., Keller, D. & Bustamante, C. Single-molecule Studies of the Effect of Template Tension on T7 DNA Polymerase Activity. *Nature*. 404:103-106 (2000).

Yin H, Wang MD, Svoboda K, Landick R, Block SM, Gelles J. Transcription Against an Applied Force. *Science*. 270:1653-1657 (1995).

Wang MD, Schnitzer MJ, Yin H, Landick R, Gelles J, Block SM. Force and Velocity Measured for Single Molecules of RNA Polymerase. *Science*. 282:902-907 (1998).

Chemla YR, Aathavan K, Michaelis J, Grimes S, Jardine PJ, Anderson DL, Bustamante C. Mechanism of Force Generation of a Viral DNA Packaging Motor. *Cell*. 122(5):683-692 (2005).

Moffitt JR, Chemla YR, Aathavan K, Grimes S, Jardine PJ, Anderson DL, Bustamante C. Intersubunit Coordination in a Homomeric Ring ATPase. *Nature*. 457:446-450(2009).

Aathavan K, Politzer A, Kaplan A, Moffitt JR, Chemla YR, Grimes S, Jardine P, Anderson D, Bustamante C. Substrate Interactions and Promiscuity in a Viral DNA Packaging Motor. *Nature*. 461:669-673 (2009).

Chistol G, Liu S, Hetherington CL, Moffitt JR, Grimes S, Jardine PJ, Bustamante C. High Degree of Coordination and Division of Labor Among Subunits in a Homomeric Ring ATPase. *Cell*. 151:1017-1028 (2012).

Liu S, Chistol, G, Hetherington CL, Tafoya S, Aathavan K, Schnitzbauer J, Grimes S, Jardine P, Bustamante C. A Viral Packaging Motor Varies Its DNA Rotation and Step Size to Preserve Subunit Coordination as the Capsid Fills. *Cell*. 157(3):702-713(2014).

Sensing Pain and Temperature

Ramon Latorre, Karen Castillo, and Ignacio Díaz-Franulic[1]

Introduction

Sensing pain and temperature is essential for life. However, the molecular nature of nociceptors or temperature receptor was a mystery until the end of the last century. Sherrington (Sherrington, 1906) postulated the existence of sensory receptors specialized in sensing noxious stimuli, and he called them nociceptors. In the skin, nociceptors are located in some Aδ small myelinated axons that respond to intense mechanical but not to thermal or chemical stimuli. Others Aδ fibers respond to noxious mechanical stimuli and noxious heat (~43°C). Unmyelinated C fibers give rise to nociceptors able to sense noxious mechanical, thermal and chemical stimuli but with a lower thermal threshold (~43°C). The conduction velocity of Aδ and C is very different, 20 m/s and 1 m/s, respectively. When the noxious stimulus is applied to a mix Aδ and C group of nociceptors, the subject may report two distinct pains separated in time: a "fast" or "first" pain and a "slow" or "second" pain (Patton, 1989). Isolation of fibers innervating the glabrous skin of the monkey's hand indicates the presence of fibers responding to warming pulses from a base temperature of 34°C (innocuous warm) with a conduction velocity of 1.2 m/s suggesting that warm fibers are C-type fiber (Patton, 1989). The same region of the monkey skin gives rise to about the same number of fibers responding to a cold stimulus. Temperature sensing and pain converge in the same type of neurons in the dorsal root (DRG) and the trigeminus (TG) ganglia innervating the body and the head, respectively.

What are the cellular and molecular basis of thermosensation? What is the relationship between pain and temperature sensing? Not so many years ago researchers thought that the possibilities of finding an answer to this question were rather slim given the intensive properties of temperature. Temperature is a physical property that does not depend on the size of the system or the amount of material in the system. This situation, however, changed dramatically with the cloning and biophysical characteriza-

[1] Centro Interdisciplinario de Neurociencia de Valparaíso, Facultad de Ciencias, Universidad de Valparaíso, Valparaíso, Chile.

tion of a heat-activated channel belonging to Transient Receptor Potential Vanilloid family, TRPV1 (Caterina *et al.*, 1997), followed by the discovery of the cold receptor channel Transient Receptor Potential Melastatin 8, TRPM8 (McKemy *et al.*, 2002; Peier *et al.*, 2002a). TRPV1 was expression cloned taking advantage that it is a capsaicin receptor activated by noxious heat (>42°C). TRPM8, on the other hand, is a sensor of innocuous cold (10-28°C) and activated by menthol. These findings triggered the search for other detectors of thermal stimulus in the nervous system with the outcome that in the vanilloid family TRPV2 (Caterina *et al.*, 1999), and TRPV4 (Guler *et al.*, 2002) were also found to be activated by heat but with different temperature thresholds. TRPV3 is also a temperature-dependent channel but expresses mainly in keratinocytes (Peier *et al.*, 2002b; Smith *et al.*, 2002; Xu *et al.*, 2002). When expressed heterologously, TRPV2 shows activity only when raising the temperature above 52°C and TRPV3 and TRPV4 respond to warm temperatures in the 26-34°C range. Temperature-sensitive TRP channels (thermoTRPs) are also present in the melastatin (TRPM2-5) in the ankyrin (TRPA1) and the classical or canonical (TRPC5) TRP channel subfamilies. TRPM2 is a Ca^{2+}-permeable cationic channel activated by adenosine diphosphoribose (Perraud *et al.*, 2001; Sano *et al.*, 2001) able to detect temperatures in the non-painful warmth range (Togashi *et al.*, 2006). TRPM3 is a channel strongly activated by pregnenolone sulfate (Wagner *et al.*, 2008) and by noxious temperatures (35-43°C) and its activation provokes pain (Vriens *et al.*, 2011). TRPM4 has two splice variants (a and b) with widely different properties. TRPM4a is a Ca^{2+} permeable cationic channel (Xu *et al.*, 2001), and TRPM4b is a Ca^{2+}-activated but Ca^{2+}-impermeable monovalent cation channel (Nilius *et al.*, 2005). Temperatures in the range of 15-35°C activate TRPM4b and TRPM5(Talavera *et al.*, 2005). Talavera *et al.* (Talavera et al., 2005) suggested that TRPM5 is also involved, albeit indirectly, in taste perception since *Trpm5* knockout mice show a markedly decreased gustatory nerve response to sweet compounds. In the TRP classical or canonical family only TRPC5 was found to be temperature sensitive (Zimmermann *et al.*, 2011). TRPC5 responds to cold temperatures in the 37-25°C range and its activation by cold is potentiated by PLC-coupled and Gq-linked receptors (Zimmermann *et al.*, 2011). The only member of the TRP Ankyrin subfamily, TRPA1 is a heat receptor in amphibians, reptiles, and birds (Viswanath *et al.*, 2003; Gracheva *et al.*, 2010). However, in mammals, the temperature sensitivity of this channel is still under debate. Thus, TRPA1 is an extreme cold receptor in rodents (it responds to painful cold, <15°C)

(Story *et al.*, 2003; Chen *et al.*, 2013), some reports describe the human TRPA1 as temperature insensitive (Caterina *et al.*, 1999; Chen *et al.*, 2013) and in reconstituted systems hTRPA1 is activated by cold and heat (Moparthi *et al.*, 2016).

From this long list of thermoTRPs in many cases, the genetic evidence indicates that not all are involved in thermosensation. Although there is no doubt that TRPV1 is involved in inflammatory thermal hyperalgesia, it shares its role in detecting noxious temperatures with TRPM3 (Vriens *et al.*, 2011). TRPV2 appear not to play a part in heat sensation given that *Trpv2* knockout mice show normal heat responses (Park *et al.*, 2011). Heat responses are also normal in the double *Trpv3*, and *Trpv4* knockout mice (Huang *et al.*, 2011) and the recent results obtained in McNaughton laboratory strongly suggest that the culprit in detecting warm temperatures is TRPM2 (Tan and McNaughton, 2016). The involvement of TRPC5 is temperature sensing is also controversial since deletion of TRPC5 in mice does not produce behavioral changes related to cold temperatures (Palkar *et al.*, 2015). TRPM8 is involved in sensing innocuous cool temperatures (Dhaka *et al.*, 2007), but the role of TRPA1 as the noxious cold-sensor is debatable (Bautista *et al.*, 2006; Kwan *et al.*, 2006; Knowlton *et al.*, 2010). Here, we discuss the biophysics and the characteristics as a polymodal receptor of the first cloned thermoTRP, TRPV1.

TRPV1

Structure

TRPV1 is a cationic channel with a high Ca^{2+} permeability that was expression cloned (Caterina *et al.*, 1997). The isolated cDNA from encoded a capsaicin receptor from sensory neurons from the DRG. TRPV1 is tetramer in which each subunit contains, like voltage-dependent K+ channels, six transmembrane segments (S1-S6), with the N- and C-terminus located towards the cell cytoplasm, and the N-terminus is rich in ankyrin repeat domains (Fig. 1A & B). The group of Julius was able to obtain the structure of TRPV in the closed configuration (APO structure; no agonists added) at 3.4 Å resolution using cryo-microscopy (Liao *et al.*, 2013) (Fig. 1A & D). TRPV1 channels show a central pore formed by segments S5 and S6 and this ion pathway is structurally related to the pore of K^+ channels. A loop connects S5 with the pore helix, which is continued by a "pore loop" that gives rise to the selectivity filter ending in S6 (Fig. 1C & D). In the periphery of the pore and making contact with the adjacent subunit S1-S4 forms a structure equivalent to the voltage sensor in K^+ (Fig. 1A &

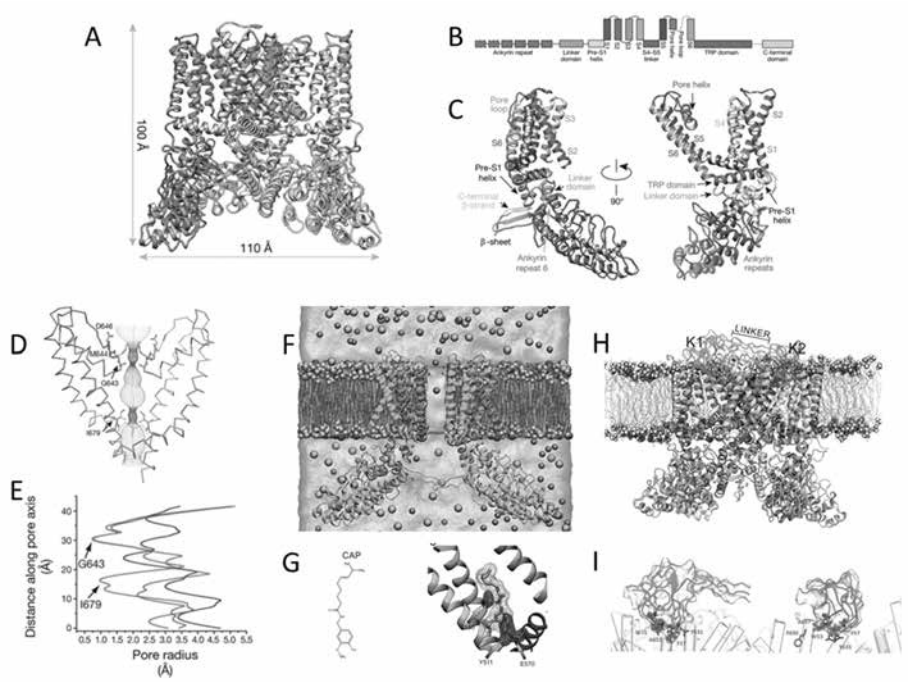


Figure 1. (A) Ribbon representation of the TRPV1 channel obtained by cryo-EM. (B) Scheme of the structural domains forming each monomer of the TRPV1 channel. C) Different views of a TRPV1 channel subunit, in color code of B (D) The permeation pathway of the TRPV1 channel in their closed (APO) conformation obtained by the HOLE suite of the VMD software where front and back subunits were removed for better visualization. Key residues for ion conduction (D646, M644) and those forming the channel activation gates (G643 and I679) are depicted in licorice representation and pointed by arrows. (E) The pore radius along the channel axis was obtained by the HOLE algorithm for the APO/closed (red), capsaicin-bound (green), and RTX-/DkTx-bound (blue) structures of the TRPV1 channel. (F) Side view of the TRPV1 channel (blue) embedded in a POPC membrane in the presence of capsaicin (red) and 100mM of KCl G) Chemical structure of capsaicin (left). Closer view of capsaicin at the vanilloid binding pocket. Key residues for capsaicin binding Y511 and E570 are pointed by arrows. (H) Side view of the TRPV1 channel (Brown) embedded in a POPC membrane in the presence of the Double Knot Toxin (DkTx) (Green). The DkTx peptide is formed by two functional domains, termed Knot 1 and Knot 2, connected by a short linker. (I) A closer view of the TRPV1/DkTx interaction surface. Amino acids at the TRPV1 channel and DkTx peptide are highlighted in red and green respectively.

C). However, this domain appears not to be involved in voltage sensing but instead constitutes the site of binding of agonists and antagonists (for a review see (Diaz-Franulic *et al.*, 2016a)). The intracellular N-terminus is composed by six ankyrin repeats, a linker domain and a pre S1 helix and the C-terminus although not completely resolved in the structure shows

a α-helix, the TRP domain, almost parallel to the plane of the membrane near the S4-S5 linker and a coiled-coil structure (Fig. 1C). The TRPV1 closed structure indicates the presence of two constrictions at the level of the S6 residue I679 (inner gate), and just before the internal entrance of the selectivity filter, G643 (outer gate) (Liao *et al.*, 2013) (Fig. 1D). The presence of capsaicin allowed to obtain the partially TRPV1 open channel structure (Cao *et al.*, 2013). In the partially open structure, only the internal gate is dilated (Fig. 1E), and we (Diaz-Franulic *et al.*, 2016b) have speculated that it may represent the formation of a TRPV1 channel that underwent a transition towards a desensitized state (14). The fully TRPV1 open structure was obtained in the presence of resiniferatoxin and double knot toxin (RTX/DkTx; see below) (Cao *et al.*, 2013).

Gating

TRPV1 is polymodal receptor able to integrate multiple different stimuli (Trevisani *et al.*, 2002). Capsaicin (Caterina *et al.*, 1997), toxins such as resiniferatoxin (Szallasi and Blumberg, 1989; Caterina *et al.*, 1997) and double-knot toxin (Bohlen *et al.*, 2010), protons (Tominaga *et al.*, 1998), voltage (Voets *et al.*, 2004), temperature (Caterina *et al.*, 1997), ions (Ahern *et al.*, 2005; Jara-Oseguera *et al.*, 2016), cannabinoids (Zygmunt *et al.*, 1999; Smart *et al.*, 2000), and lipids (Reviewed in (Diaz-Franulic *et al.*, 2016a)) strongly modulated the activity of TRPV1 (Table).

Agonists and toxins

Agonists and toxins activate TRPV1 channels by interacting with well-defined binding sites and open the channel in an allosteric fashion (Matta and Ahern, 2007). The TRPV1 channel in birds is capsaicin insensitive (Wood *et al.*, 1988) and Jordt and Julius (Jordt and Julius, 2002) found that transferring S2 through S4 from rTRPV1 to chick TRPV1 confer capsaicin sensitivity to the latter. Capsaicin binds to a hydrophobic pocket with heads down orientation, that the vanilloid ring interacts with E570 at the S4-S5 linker, and that Y511 in S3 and T550 in S4 are also important in determining capsaicin binding (Fig. 1F & G) (Darre and Domene, 2015; Poblete *et al.*, 2015; Yang *et al.*, 2015). Notably, a single capsaicin-bound subunit can induce TRPV1 channel maximal open probability albeit with an increase in the capsaicin dissociation constant (Hazan *et al.*, 2015). Resiniferatoxin (RTX), a compound a plant origin (*Euphobia poissonii*) is about 1000-fold hotter than pure capsaicin (Szallasi and Blumberg, 1989; Szolcsanyi *et al.*, 1991). RTX binds 20-times tighter than capsaicin to

TRPV1 (EC50 = 39 nM and 712 nM, respectively; (Caterina *et al.*, 1997). RTX binds to the capsaicin-binding pocket and also interacts with Y511 and T550 (Cao *et al.*, 2013). Two inhibitor cysteine knots (ICK) domains, peptides found in a wide variety of poisonous animals (Craik *et al.*, 2001), joined by a loop give origin to DxTx, and by interacting with the external aspect of the TRPV1 pore, DxTx keeps the TRPV1 channel open (Fig. 1H & I) (Bohlen *et al.*, 2010; Bohlen and Julius, 2012).

Protons

In TRPV1, protons potentiate the effect of temperature and capsaicin (Tominaga *et al.*, 1998; Jordt *et al.*, 2000; Welch *et al.*, 2000; Ryu *et al.*, 2003; Ryu *et al.*, 2007). Jordt *et al.* (Jordt *et al.*, 2000) found that a negatively charged residue located in the linker between S5 and the pore region, E600, is one important TRPV1 molecular determinant of the channel pH sensitivity. Charge reversal (E600K) leftward shifted the normalized current vs pH curve suggesting that E600 is a sort safety valve that keeps the TRPV1 channel closed at physiological pH and temperature. The mutant E600K is active at 37°C promoting cell death of TRPV1 transfected cells. Exchange of different external segments between the pH-insensitive TRPV2 and TRPV1 led to the conclusion that the S3-S4 linker and the pore helix were involved in the direct activation of TRPV1 by low pH (Ryu *et al.*, 2007). Moreover, the point mutants V538A in the S3-S4 linker and T633L in the pore helix abolished TRPV1 pH sensitivity. Since neither V538 nor T633 are titratable residues, their involvement in pH sensitivity is most likely in the coupling between proton sensor activation and gate activation. These mutations did not alter the channel activation mediated by capsaicin or the heat response confirming the allosteric nature of the TRPV1 gating (see below).

Temperature and voltage

One of the most intriguing aspects of the behavior of thermoTRPs is their ability to sense temperature with exquisite sensitivity. The TRPV1 thermodynamic properties can be obtained to a first approximation using a two-state model in which the channel transit between a closed (*C*) and an open (*O*) state defined by an equilibrium constant K_{eq}

$$C \overset{K_{eq}}{\leftrightarrow} O$$

where the free energy difference (ΔG) between the two conformations is given by

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

$$\Delta G = -RT ln K_{eq} \qquad (2)$$

where ΔH and ΔS are the enthalpy and the entropy change, respectively and T is the temperature. Since in a two-state model, the open probability (Po) of the channel is

$$P_o = \frac{}{1+K_{eq}} = \frac{}{1+e^{-\frac{\Delta G}{RT}}} \qquad (3)$$

by introducing eqs (1) and (2) into eq. (3) and measuring Po experimentally as a function of T, we can obtain ΔH and ΔS. In the case of TRPV1, the van't Hoff plot of Keq yields a ΔH = 150 kcal/mol and a ΔS = 470 cal/mol x K (Liu *et al.*, 2003). These values are quite large suggesting a process involving significant conformational changes and the positive sign of ΔS implies that the open state has a greater entropy (is more disordered) than the closed state. Note that the entropic contribution, $T\Delta S$, is also large and positive (137 kcal/mol). Therefore, ΔG is in a range that ensures reversibility of the closed-open reaction and decreases as temperature is increased. In the case of cold receptors such as TRPM8 and TRPV1 enthalpies and entropies changes are also large but negative, and ΔG decreases as temperature is decreased (Brauchi *et al.*, 2004; Karashima *et al.*, 2009). On the other hand, large enthalpy change implies considerable thermal coefficients, Q_{10}. For TRPV1 and TRPM8 the $Q_{10}s$ of the macroscopic currents have been found to be ~40 and 25, respectively. Thus, thermosTRP channels are about ten times more sensitive than other membrane proteins including other ion channels and pumps which Q_{10}. is about 3.

The above analysis considers that ΔH and ΔS are temperature independent, but Clapham and Miller (Clapham and Miller, 2011) have argued that a large and positive heat capacity change, ΔC_p, should accompany the transition from closed to open if temperature promotes large conformational changes of the channel-forming protein. If this is the case, ThermoTRP channels should be activated by cold and by heat and the temperature dependence of lnK_{eq} should be described by a parabola that is concave upwards. The present data show that ThermoTRPs do not have the characteristics expected from the ΔC_p hypothesis and several arguments have been given against it (e.g., (Qin, 2014)). However, measure-

ments of ΔC_p during channel activation are needed to have a definitive test of this proposition.

Depolarizing voltages activate TRPV1 and how voltage and temperature interact to activate the channel has been the matter of some debate. The question whether these two stimuli are strictly coupled or their cross-talk is allosteric can be answered by performing the appropriate biophysical experiments. In the case of TRPV1 and TRPM8 voltage is a partial activator of TRPM8 and TRPV1 implying that the opening reaction in voltage independent. Allosteric models of the type previously proposed to explain the activation by Ca^{2+} and voltage of the Ca^{2+}- and voltage-activated K+channel (Horrigan and Aldrich, 2002) can account for temperature, voltage, and agonist modulation of these two thermoTRPs (Brauchi *et al.*, 2004; Matta and Ahern, 2007; Raddatz *et al.*, 2014). In these models voltage and temperature sensors are considered to be in separate modules, and they can activate the channel independently. The communication be-

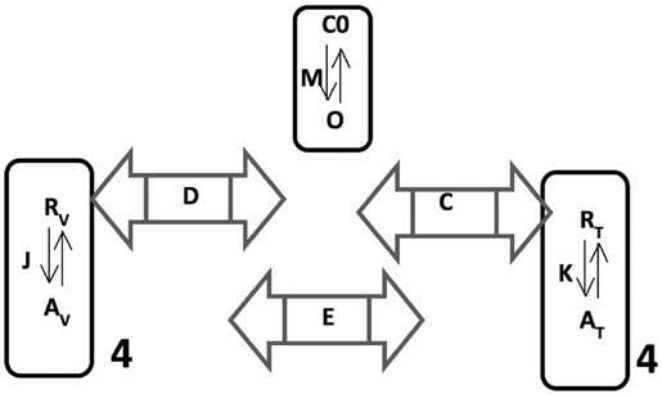


Figure 2. Two-tiered allosteric model. This model considers the existence of four voltage and four temperature sensors that can be activated independently, and each voltage sensor contributes with equal amounts of energy to displace the closed to open equilibrium. The pore has two conformations, closed (*C*) and open (*O*). The equilibrium constant for the closed to open reaction is *M*, defined for resting voltage and temperature sensors. M is voltage- and temperature-independent. The voltage sensor equilibrium between resting (R_V) and active (A_V) conformations is governed by the constant, defined for closed pore and resting temperature sensor. The temperature sensor resting (R_T) to active (A_T) reaction is controlled by equilibrium constant *K*, defined for closed pore and resting voltage sensor. When all the voltage sensors are active, the pore opening reaction is favored by the allosteric factor D and the equilibrium constant becomes LD^4. In the same way, when temperature sensor is active, the pore opening reaction is favored by the allosteric factor C. When both sensors are all active, the pore opening equilibrium constant is LC^4D^4. Factor E accounts for the interaction between voltage and temperature sensors.

tween sensors is allosteric, and the channel can open even in the absence of stimuli. Activation of sensors increases the open probability because the coupling existing between the sensor activation and the pore opening decreases the free energy to undergo the transition from closed to open.

Given the tetrameric nature of the channel, an allosteric model that best combines simplicity and tractability is one based on the tetrameric nature of the channel considers four voltage sensors and four temperature sensors. In this two-tiered allosteric model, the pore opening reaction is coupled to the temperature, and voltage sensors by allosteric factors C and D, respectively (Fig. 2). For example, the best fit to the TRPM8 *Po* vs voltage data obtained in 20-10°C range using a two-tiered model is obtained with $C = 52$ and $D = 2.3$ (Raddatz *et al.*, 2014). Thus, when all the TRPM8 temperature and voltage sensors are activated the equilibrium constant M that defines the C-O equilibrium becomes MC^4D^4 increasing M by $2x10^8$-fold. Most importantly, at a high negative voltage, when all voltage sensors are at rest TRPM8 *Po* becomes voltage independent but in this voltage range increases about 100-fold when the temperature is decreased from 28 to 15°C indicating that temperature alone can activate the channel (Raddatz *et al.*, 2014). Whether if the observations made using TRPM8 can be extrapolated to TRPV1 remain to be determined experimentally but what is clear is that TRPV1 is a polymodal receptor with allosteric gating (Matta and Ahern, 2007).

Molecular determinants of temperature sensing

Neither the voltage nor the temperature sensor has been identified unequivocally. In TRPV1 several domains have claimed to be involved in temperature sensing. Brauchi *et al.* (Brauchi *et al.*, 2006) swapped the C-terminus domains of TRPM8 and TRPV1, and the resultant chimeras have a phenotype corresponding to the C-terminal exchanged: the TRPV1 channel containing the C-terminus of TRPM8 became a cold receptor. Chimeric constructs produced by exchanging different segments of TRPV1 and TRPV2 protein gave as a result that thermal sensitivity in these two channels was determined by a region connecting the Ankyrin repeats to S1, denominated the membrane proximal domain (MPD) (Yao *et al.*, 2011). The linker between S5 and the pore helix (pore turret) has also been involved in the high thermal sensitivity of TRPV1 since its total or partial deletion eliminated or decreased channel temperature dependence (Yang *et al.*, 2010; Cui *et al.*, 2012). However, these results are controversial since TRPV1 channels with a deleted turret are still activated by temper-

ature (Yao *et al.*, 2010). Additionally, using random and targeted mutagenesis, Grandl *et al.* (Grandl *et al.*, 2010) identified three residues implicated in heat activation, N652 and Y653 in selectivity filter-S6 linker and N628 in the pore helix.

Nature has provided us with TRPV1 orthologs channels with markedly different temperature sensitivity. The thirteen-lined ground squirrels and Bactrian camels express TRPV1 orthologs that although normal in their response to capsaicin or protons are not temperature-dependent in the 25-45°C range (Laursen *et al.*, 2016). These difference in temperature sensitivity with the hTRPV1 or rTRPV1 has profound physiological consequences since allows squirrels and camels to adapt to hot environments. Notably, a single amino acid substitution in the first Ankyrin domain N126S can confer to the squirrel TRPV1 a rTRPV1 phenotype.

The problem in identifying a *bona fide* temperature sensor resides in the fact that mutations that alter TRPV1 heat sensitivity are spread throughout the channel-forming protein (Winter *et al.*, 2013) making difficult to decide which residues form part of the sensor and which correspond to the coupling system that communicates the sensor with the pore gates. However, the evidence indicates that the response to temperature in proteins is nonhomogeneous and the most disordered segments are more sensitive to thermal changes and able to absorb most efficiently the thermal energy from the solvent (Mandel *et al.*, 1996; Lewandowski *et al.*, 2015). Does heat diffuse from the heat sink (temperature sensor) following pre-determined pathways? If we can identify these corridors, do they lead to the channel gates? To answer these question, we have used an *in silico* method denominated anisotropic thermal diffusion (ATD) that consists in injecting vibrational energy into each residue of the protein kept at a very low temperature and measuring the response of the rest of the amino acids (Burendahl and Nilsson, 2012; Liu *et al.*, 2013; Mino-Galaz, 2015). The thermal map constructed in this manner shows how the thermal energy diffuses along the protein structure. We have applied the ATD method to a single TRPV1 subunit considering that the heat sink resides in the neighbourhood of the MPD, a domain considered to be a possible thermal sensor (see above) (Yao *et al.*, 2011). If this domain is "heated", thermal energy is transfer to the C-terminal, and from there heat flows to S5, to the pore helix, finally reaching S6 where the two channels gates are located (Diaz-Franulic *et al.*, 2016b). Thus, the ATD method allowed to us to identify a putative allosteric pathway through which thermal energy diffuses preferentially and suggests that the communication between the sensor and the gates do not

necessarily imply large conformational changes in the protein structure. How we reconcile then this result with the large change in enthalpy determined from the van't Hoff plot? Below we propose an alternative explanation for the large ΔH involved in the closed-open transition. In the case of TRPM8, the channel deactivation time course proceeds as bi-exponential decay and the time constant of both processes are temperature-dependent. A closed-closed-open kinetic scheme predicts a double exponential with the proviso that the forward rate constant in the close-open transition is different from zero (Goldman, 1991). Assuming that the closed-closed and the closed-open transition are related to the slow and fast relaxation, respectively, we demonstrated that the overall Q_{10} of the deactivation reaction is the product of the Q_{10} of the slow (α) and fast backward rate (β) constants. I.e., $Q_{10overall\ reaction} = Q_{10\alpha}\ Q_{10\beta}$. Therefore, two reactions with a relatively low Q_{10} (low ΔH, small conformational changes) may produce a very *large* Q_{10} (large ΔH) of the overall equilibrium.

Ions

Ahern *et al.* (Ahern *et al.*, 2005) showed that Mg^{2+} sensitize TRPV1 at physiological concentrations (1-3 mM). Notably, TRPV1 channels previously activated by PKC, show a temperature threshold decrease in a Mg^{2+}-dependent manner. It decreases from 47°C in the absence of the divalent cation to about 35°C when the final external concentration of Mg^{2+} is 10 mM. Concentrations of Mg^{2+} > 10 mM directly activate TRPV1 channel apparently by binding to the same negatively charged residues involved in the activation and sensitization of the channel by protons, namely E600 and E648 located in the pore-forming loop (Ahern *et al.*, 2005). Cao *et al.* (Cao et al., 2014) have argued that the lowering of the temperature threshold induced by Mg^{2+} is the result of a direct and selective interaction of the divalent cation with the heat-sensing machinery. They based their contentions on the fact that prolonged application of Mg^{2+} or Ba^{2+} desensitizes heat activation but not capsaicin activation. However, that Mg^{2+} can activate the channel directly was recently confirmed by Jara-Oseguera *et al.* (Jara-Oseguera *et al.*, 2016). Sodium, on the other hand, appears to be a negative modulator of TRPV1 channel activity since the removal of extracellular Na+ elicited an increase in the internal Ca^{2+} concentration and an outward current with a reversal potential corresponding to that of a cationic channel (Ohta *et al.*, 2008). Na^{+} binds to a site in the pore-forming loop of TRPV1 involving E600 and E648 (Ohta *et al.*, 2008; Jara-Oseguera *et al.*, 2016). Remarkably, the absence of external Na^{+} not only opens the

TRPV1 channel at physiological temperatures but also the channel becomes only marginal temperature dependent (Jara-Oseguera *et al.*, 2016). Thus, the Na^+ modulator site stabilizes the TRPV1 channel in a closed state(s) and is strongly coupled to the temperature-dependent transitions. The effects of external Na^+ on the temperature-dependent transitions can be understood assuming that Na^+ binding to the extracellular site modifies temperature-sensor activation and pore opening allosterically. Since protons and Na+ appear to interact with the same site, it is reasonable to conclude that Na+ acts by suppressing or decreasing channel activity under physiological conditions.

Coda

A large variety of noxious stimuli including heat, acid and chemical compounds activate the TRPV1 channel, which contained in nociceptors mediated the depolarization of the specialized primary afferent neurons. The action potentials promoted by the neuron depolarization propagate to the central nervous system informing about the damage evoking a pain sensation. In particular, TRPV1 is involved in the hyperalgesia that occurs during inflammation (Caterina and Julius, 2001; Julius and Basbaum, 2001). Contained in different structures, the sensors for the different stimuli communicate each other allosterically contributing to decrease the threshold for channel activation.

Table. ThermoTRP Channel Pharmacology

Thermo TRP channel	Agonist	Antagonist	Toxin
TRPV1	**Capsaicin Resiniferatoxin Anandamide Piperine Olvanil Oxytocin Eugenol Lysophosphatidic acid (LPA) Camphor Gingerol Cannabidiol Zingerone Isovelleral Polygodial**	**Capsazepine Agatoxin Resolvin D2 4-tert-butylphenyl)-4-(3-chloropyridin-2- y l)piperazine-1-carboxamide (BCTC) Omega-9 fatty acid ABT-102 Yohimbine Thapsigargin**	**Vanillotoxins (VaTX 1-3) Resiniferatoxin (RTX) Double Knot Toxin (DkTx) BmP01 RhTx (red head centiped toxin) AG489 toxin AG505 toxin**

TRPM2	**Adenosine diphosphate ribose (ADPR)** **Adenosine diphosphate ribose-2'-phosphate (ADPRP)** **2'-Deoxyadenosine 5'-diphosphoribose (2'-deoxy-ADPR)** **Reactive oxygen species (ROS)**	**Flufenamic acid** **Curcumin** **2-aminoethoxydiphenyl borate (2-APB)**	
TRPM3		**Pirimidona** **Mefenamic acid** **Flavanones** **Naringenin** **Hesperetin** **Eriodictyol** **Isosakuranetin** **Liquiritigenin**	
TRPM8	**Menthol** **Eucalyptol** **Menthone** **Icilin** **Geraniol** **Cannabigerol** **Linalool** **Hydroxy-citronellal** **Testosterone** **Eugenol** **Frescolat ML** **Menthyl lactate** **L-carvone** **Isopulegol** **Cryosim-3**	**BCTC** **thio-BCTC** **Capsazepine** **Cannabidiol** **AMTB** **Arylglycines** **Carboxamide compounds** **2-(Benzyloxy) benzamides** **PBMC** **Cinnamaldehyde**	

TRPV1: (Caterina and Julius, 1999; Szallasi *et al.*, 1999; Caterina *et al.*, 2000; Tafesse *et al.*, 2004; Kitaguchi and Swartz, 2005; Siemens *et al.*, 2006; Vriens *et al.*, 2009; Bohlen *et al.*, 2010; Park *et al.*, 2011; Meotti *et al.*, 2014; Yang *et al.*, 2015; Morales-Lazaro *et al.*, 2016; Nersesyan *et al.*, 2017).

TRPM2: (Hill *et al.*, 2004; Togashi *et al.*, 2008; Kheradpezhouh *et al.*, 2016; Fliegert *et al.*, 2017).

TRPM3: (Holzer and Izzo, 2014; Uchida *et al.*, 2016; Thiel *et al.*, 2017)

TRPM8: (McKemy *et al.*, 2002; Peier *et al.*, 2002a; Behrendt *et al.*, 2004; Madrid *et al.*, 2006; Bodding *et al.*, 2007; Cahusac and Noyce, 2007; Malkia *et al.*, 2007; De Petrocellis *et al.*, 2008; DeFalco *et al.*, 2011; Knowlton *et al.*, 2011; Zhu *et al.*, 2013; Borrelli *et al.*, 2014; Asuthkar *et al.*, 2015; Yang *et al.*, 2017)

References

Ahern, G.P., I.M. Brooks, R.L. Miyares, and X.B. Wang. 2005. Extracellular cations sensitize and gate capsaicin receptor TRPV1 modulating pain signaling. *J Neurosci*. 25:5109-5116.

Asuthkar, S., P.A. Elustondo, L. Demirkhanyan, X. Sun, P. Baskaran, K.K. Velpula, B. Thyagarajan, E.V. Pavlov, and E. Zakharian. 2015. The TRPM8 protein is a testosterone receptor: I. Biochemical evidence for direct TRPM8-testosterone interactions. *J Biol Chem*. 290:2659-2669.

Bautista, D.M., S.E. Jordt, T. Nikai, P.R. Tsuruda, A.J. Read, J. Poblete, E.N. Yamoah, A.I. Basbaum, and D. Julius. 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell*. 124:1269-1282.

Behrendt, H.J., T. Germann, C. Gillen, H. Hatt, and R. Jostock. 2004. Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. *Br J Pharmacol*. 141:737-745.

Bodding, M., U. Wissenbach, and V. Flockerzi. 2007. Characterisation of TRPM8 as a pharmacophore receptor. *Cell Calcium*. 42:618-628.

Bohlen, C.J., and D. Julius. 2012. Receptor-targeting mechanisms of pain-causing toxins: How ow? *Toxicon*. 60:254-264.

Bohlen, C.J., A. Priel, S. Zhou, D. King, J. Siemens, and D. Julius. 2010. A bivalent tarantula toxin activates the capsaicin receptor, TRPV1, by targeting the outer pore domain. *Cell*. 141:834-845.

Borrelli, F., E. Pagano, B. Romano, S. Panzera, F. Maiello, D. Coppola, L. De Petrocellis, L. Buono, P. Orlando, and A.A. Izzo. 2014. Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a Cannabis-derived non-psychotropic cannabinoid. *Carcinogenesis*. 35:2787-2797.

Brauchi, S., P. Orio, and R. Latorre. 2004. Clues to understanding cold sensation: thermodynamics and electrophysiological analysis of the cold receptor TRPM8. *Proc Natl Acad Sci U S A*. 101:15494-15499.

Brauchi, S., G. Orta, M. Salazar, E. Rosenmann, and R. Latorre. 2006. A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *J Neurosci*. 26:4835-4840.

Burendahl, S., and L. Nilsson. 2012. Computational studies of LXR molecular interactions reveal an allosteric communication pathway. *Proteins*. 80:294-306.

Cahusac, P.M., and R. Noyce. 2007. A pharmacological study of slowly adapting mechanoreceptors responsive to cold thermal stimulation. *Neuroscience*. 148:489-500.

Cao, E., M. Liao, Y. Cheng, and D. Julius. 2013. TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature*. 504:113-118.

Cao, X., L. Ma, F. Yang, K. Wang, and J. Zheng. 2014. Divalent cations potentiate TRPV1 channel by lowering the heat activation threshold. *J Gen Physiol*. 143:75-90.

Caterina, M.J., and D. Julius. 1999. Sense and specificity: a molecular identity for nociceptors. *Curr Opin Neurobiol*. 9:525-530.

Caterina, M.J., and D. Julius. 2001. The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci*. 24:487-517.

Caterina, M.J., A. Leffler, A.B. Malmberg, W.J. Martin, J. Trafton, K.R. Petersen-Zeitz, M. Koltzenburg, A.I. Basbaum, and D. Julius. 2000. Impaired

nociception and pain sensation in mice lacking the capsaicin receptor. *Science*. 288:306-313.

Caterina, M.J., T.A. Rosen, M. Tominaga, A.J. Brake, and D. Julius. 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*. 398:436-441.

Caterina, M.J., M.A. Schumacher, M. Tominaga, T.A. Rosen, J.D. Levine, and D. Julius. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*. 389:816-824.

Clapham, D.E., and C. Miller. 2011. A thermodynamic framework for understanding temperature sensing by transient receptor potential (TRP) channels. *Proc Natl Acad Sci U S A*. 108:19492-19497.

Craik, D.J., N.L. Daly, and C. Waine. 2001. The cystine knot motif in toxins and implications for drug design. *Toxicon*. 39:43-60.

Cui, Y., F. Yang, X. Cao, V. Yarov-Yarovoy, K. Wang, and J. Zheng. 2012. Selective disruption of high sensitivity heat activation but not capsaicin activation of TRPV1 channels by pore turret mutations. *J Gen Physiol*. 139:273-283.

Chen, J., D. Kang, J. Xu, M. Lake, J.O. Hogan, C. Sun, K. Walter, B. Yao, and D. Kim. 2013. Species differences and molecular determinant of TRPA1 cold sensitivity. *Nat Commun*. 4:2501.

Darre, L., and C. Domene. 2015. Binding of Capsaicin to the TRPV1 Ion Channel. *Mol Pharm*. 12:4454-4465.

De Petrocellis, L., V. Vellani, A. Schiano-Moriello, P. Marini, P.C. Magherini, P. Orlando, and V. Di Marzo. 2008. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *J Pharmacol Exp Ther*. 325:1007-1015.

DeFalco, J., M.A. Duncton, and D. Emerling. 2011. TRPM8 biology and medicinal chemistry. *Curr Top Med Chem*. 11:2237-2252.

Dhaka, A., A.N. Murray, J. Mathur, T.J. Earley, M.J. Petrus, and A. Patapoutian. 2007. TRPM8 is required for cold sensation in mice. *Neuron*. 54:371-378.

Diaz-Franulic, I., J. Caceres-Molina, R.V. Sepulveda, F. Gonzalez-Nilo, and R. Latorre. 2016a. Structure-Driven Pharmacology of Transient Receptor Potential Channel Vanilloid 1. *Mol Pharmacol*. 90:300-308.

Diaz-Franulic, I., H. Poblete, G. Mino-Galaz, C. Gonzalez, and R. Latorre. 2016b. Allosterism and Structure in Thermally Activated Transient Receptor Potential Channels. *Annu Rev Biophys*. 45:371-398.

Fliegert, R., A. Bauche, A.M. Wolf Perez, J.M. Watt, M.D. Rozewitz, R. Winzer, M. Janus, F. Gu, A. Rosche, A. Harneit, M. Flato, C. Moreau, T. Kirchberger, V. Wolters, B.V.L. Potter, and A.H. Guse. 2017. 2'-Deoxyadenosine 5'-diphosphoribose is an endogenous TRPM2 superagonist. *Nat Chem Biol*. 13:1036-1044.

Goldman, L. 1991. On the extraction of kinetic rate constants from experimental data. *Biophys J*. 60:519-523.

Gracheva, E.O., N.T. Ingolia, Y.M. Kelly, J.F. Cordero-Morales, G. Hollopeter, A.T. Chesler, E.E. Sanchez, J.C. Perez, J.S. Weissman, and D. Julius. 2010. Molecular basis of infrared detection by snakes. *Nature*. 464:1006-1011.

Grandl, J., S.E. Kim, V. Uzzell, B. Bursulaya, M. Petrus, M. Bandell, and A. Patapoutian. 2010. Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. *Nat Neurosci*. 13:708-714.

Guler, A.D., H. Lee, T. Iida, I. Shimizu, M. Tominaga, and M. Caterina. 2002. Heat-evoked activation of the ion channel, TRPV4. *J Neurosci*. 22:6408-6414.

Hazan, A., R. Kumar, H. Matzner, and A.

Priel. 2015. The pain receptor TRPV1 displays agonist-dependent activation stoichiometry. *Sci Rep*. 5:12278.

Hill, K., C.D. Benham, S. McNulty, and A.D. Randall. 2004. Flufenamic acid is a pH-dependent antagonist of TRPM2 channels. *Neuropharmacology*. 47:450-460.

Holzer, P., and A.A. Izzo. 2014. The pharmacology of TRP channels. *Br J Pharmacol*. 171:2469-2473.

Horrigan, F.T., and R.W. Aldrich. 2002. Coupling between voltage sensor activation, Ca2+ binding and channel opening in large conductance (BK) potassium channels. *J Gen Physiol*. 120:267-305.

Huang, S.M., X. Li, Y. Yu, J. Wang, and M.J. Caterina. 2011. TRPV3 and TRPV4 ion channels are not major contributors to mouse heat sensation. *Mol Pain*. 7:37.

Jara-Oseguera, A., C. Bae, and K.J. Swartz. 2016. An external sodium ion binding site controls allosteric gating in TRPV1 channels. *Elife*. 5.

Jordt, S.E., and D. Julius. 2002. Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell*. 108:421-430.

Jordt, S.E., M. Tominaga, and D. Julius. 2000. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci U S A*. 97:8134-8139.

Julius, D., and A.I. Basbaum. 2001. Molecular mechanisms of nociception. *Nature*. 413:203-210.

Karashima, Y., K. Talavera, W. Everaerts, A. Janssens, K.Y. Kwan, R. Vennekens, B. Nilius, and T. Voets. 2009. TRPA1 acts as a cold sensor in vitro and in vivo. *Proc Natl Acad Sci U S A*. 106:1273-1278.

Kheradpezhouh, E., G.J. Barritt, and G.Y. Rychkov. 2016. Curcumin inhibits activation of TRPM2 channels in rat hepatocytes. *Redox Biol*. 7:1-7.

Kitaguchi, T., and K.J. Swartz. 2005. An inhibitor of TRPV1 channels isolated from funnel Web spider venom. *Biochemistry*. 44:15544-15549.

Knowlton, W.M., A. Bifolck-Fisher, D.M. Bautista, and D.D. McKemy. 2010. TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo. *Pain*. 150:340-350.

Knowlton, W.M., R.L. Daniels, R. Palkar, D.D. McCoy, and D.D. McKemy. 2011. Pharmacological blockade of TRPM8 ion channels alters cold and cold pain responses in mice. *PLoS One*. 6:e25894.

Kwan, K.Y., A.J. Allchorne, M.A. Vollrath, A.P. Christensen, D.S. Zhang, C.J. Woolf, and D.P. Corey. 2006. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*. 50:277-289.

Laursen, W.J., E.R. Schneider, D.K. Merriman, S.N. Bagriantsev, and E.O. Gracheva. 2016. Low-cost functional plasticity of TRPV1 supports heat tolerance in squirrels and camels. *Proc Natl Acad Sci U S A*. 113:11342-11347.

Lewandowski, J.R., M.E. Halse, M. Blackledge, and L. Emsley. 2015. Protein dynamics. Direct observation of hierarchical protein dynamics. *Science*. 348:578-581.

Liao, M., E. Cao, D. Julius, and Y. Cheng. 2013. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature*. 504:107-112.

Liu, B., K. Hui, and F. Qin. 2003. Thermodynamics of heat activation of single capsaicin ion channels VR1. *Biophys J*. 85:2988-3006.

Liu, J., G.J. Tawa, and A. Wallqvist. 2013. Identifying cytochrome p450 functional networks and their allosteric regulatory elements. *PLoS One*. 8:e81980.

Madrid, R., T. Donovan-Rodriguez, V. Meseguer, M.C. Acosta, C. Belmonte, and F. Viana. 2006. Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. *J Neurosci*. 26:12512-12525.

Malkia, A., R. Madrid, V. Meseguer, E. de la Pena, M. Valero, C. Belmonte, and F. Viana. 2007. Bidirectional shifts of TRPM8 channel gating by temperature and chemical agents modulate the cold sensitivity of mammalian thermoreceptors. *J Physiol*. 581:155-174.

Mandel, A.M., M. Akke, and A.G. Palmer, 3rd. 1996. Dynamics of ribonuclease H: temperature dependence of motions on multiple time scales. *Biochemistry*. 35:16009-16023.

Matta, J.A., and G.P. Ahern. 2007. Voltage is a partial activator of rat thermosensitive TRP channels. *J Physiol*. 585:469-482.

McKemy, D.D., W.M. Neuhausser, and D. Julius. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*. 416:52-58.

Meotti, F.C., E. Lemos de Andrade, and J.B. Calixto. 2014. TRP modulation by natural compounds. *Handb Exp Pharmacol*. 223:1177-1238.

Mino-Galaz, G.A. 2015. Allosteric communication pathways and thermal rectification in PDZ-2 protein: a computational study. *J Phys Chem B*. 119:6179-6189.

Moparthi, L., T.I. Kichko, M. Eberhardt, E.D. Hogestatt, P. Kjellbom, U. Johanson, P.W. Reeh, A. Leffler, M.R. Filipovic, and P.M. Zygmunt. 2016. Human TRPA1 is a heat sensor displaying intrinsic U-shaped thermosensitivity. *Sci Rep*. 6:28763.

Morales-Lazaro, S.L., I. Llorente, F. Sierra-Ramirez, A.E. Lopez-Romero, M. Ortiz-Renteria, B. Serrano-Flores, S.A. Simon, L.D. Islas, and T. Rosenbaum. 2016. Inhibition of TRPV1 channels by a naturally occurring omega-9 fatty acid reduces pain and itch. *Nat Commun*. 7:13092.

Nersesyan, Y., L. Demirkhanyan, D. Cabezas-Bratesco, V. Oakes, R. Kusuda, T. Dawson, X. Sun, C. Cao, A.M. Cohen, B. Chelluboina, K.K. Veeravalli, K. Zimmermann, C. Domene, S. Brauchi, and E. Zakharian. 2017. Oxytocin Modulates Nociception as an Agonist of Pain-Sensing TRPV1. *Cell Rep*. 21:1681-1691.

Nilius, B., J. Prenen, J. Tang, C. Wang, G. Owsianik, A. Janssens, T. Voets, and M.X. Zhu. 2005. Regulation of the Ca2+ sensitivity of the nonselective cation channel TRPM4. *J Biol Chem*. 280:6423-6433.

Ohta, T., T. Imagawa, and S. Ito. 2008. Novel gating and sensitizing mechanism of capsaicin receptor (TRPV1): tonic inhibitory regulation of extracellular sodium through the external protonation sites on TRPV1. *J Biol Chem*. 283:9377-9387.

Palkar, R., E.K. Lippoldt, and D.D. McKemy. 2015. The molecular and cellular basis of thermosensation in mammals. *Curr Opin Neurobiol*. 34:14-19.

Park, U., N. Vastani, Y. Guan, S.N. Raja, M. Koltzenburg, and M.J. Caterina. 2011. TRP vanilloid 2 knock-out mice are susceptible to perinatal lethality but display normal thermal and mechanical nociception. *J Neurosci*. 31:11425-11436.

Patton, H.D., Fuchs, A:F., Hille, B. Scher, A.M., Steiner, R. 1989. Textbook of Physiology. Excitable cells and Neurophysiology. WB Saunders Co, Philadelphia.

Peier, A.M., A. Moqrich, A.C. Hergarden, A.J. Reeve, D.A. Andersson, G.M. Story, T.J. Earley, I. Dragoni, P. McIntyre, S. Bevan, and A. Patapoutian. 2002a. A TRP channel that senses cold stimuli and menthol. *Cell*. 108:705-715.

Peier, A.M., A.J. Reeve, D.A. Andersson, A. Moqrich, T.J. Earley, A.C. Hergarden, G.M. Story, S. Colley, J.B. Hogenesch, P. McIntyre, S. Bevan, and A. Patapoutian. 2002b. A heat-sensitive TRP chan-

nel expressed in keratinocytes. *Science*. 296:2046-2049.

Perraud, A.L., A. Fleig, C.A. Dunn, L.A. Bagley, P. Launay, C. Schmitz, A.J. Stokes, Q. Zhu, M.J. Bessman, R. Penner, J.P. Kinet, and A.M. Scharenberg. 2001. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature*. 411:595-599.

Poblete, H., I. Oyarzun, P. Olivero, J. Comer, M. Zuniga, R.V. Sepulveda, D. Baez-Nieto, C. Gonzalez Leon, F. Gonzalez-Nilo, and R. Latorre. 2015. Molecular determinants of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) binding to transient receptor potential V1 (TRPV1) channels. *J Biol Chem*. 290:2086-2098.

Qin, F. 2014. Temperature sensing by thermal TRP channels: thermodu¿ynamics basis and molecular insights. *Curr. Top. Membranes*. 74:19-50.

Raddatz, N., J.P. Castillo, C. Gonzalez, O. Alvarez, and R. Latorre. 2014. Temperature and voltage coupling to channel opening in transient receptor potential melastatin 8 (TRPM8). *J Biol Chem*. 289:35438-35454.

Ryu, S., B. Liu, and F. Qin. 2003. Low pH potentiates both capsaicin binding and channel gating of VR1 receptors. *J Gen Physiol*. 122:45-61.

Ryu, S., B. Liu, J. Yao, Q. Fu, and F. Qin. 2007. Uncoupling proton activation of vanilloid receptor TRPV1. *J Neurosci*. 27:12797-12807.

Sano, Y., K. Inamura, A. Miyake, S. Mochizuki, H. Yokoi, H. Matsushime, and K. Furuichi. 2001. Immunocyte Ca2+ influx system mediated by LTRPC2. *Science*. 293:1327-1330.

Sherrington, C.S. 1906. The integrative action of the nervous system. New Have, Yale University Press.

Siemens, J., S. Zhou, R. Piskorowski, T. Nikai, E.A. Lumpkin, A.I. Basbaum, D. King, and D. Julius. 2006. Spider toxins activate the capsaicin receptor to produce inflammatory pain. *Nature*. 444:208-212.

Smart, D., M.J. Gunthorpe, J.C. Jerman, S. Nasir, J. Gray, A.I. Muir, J.K. Chambers, A.D. Randall, and J.B. Davis. 2000. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol*. 129:227-230.

Smith, G.D., M.J. Gunthorpe, R.E. Kelsell, P.D. Hayes, P. Reilly, P. Facer, J.E. Wright, J.C. Jerman, J.P. Walhin, L. Ooi, J. Egerton, K.J. Charles, D. Smart, A.D. Randall, P. Anand, and J.B. Davis. 2002. TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature*. 418:186-190.

Story, G.M., A.M. Peier, A.J. Reeve, S.R. Eid, J. Mosbacher, T.R. Hricik, T.J. Earley, A.C. Hergarden, D.A. Andersson, S.W. Hwang, P. McIntyre, T. Jegla, S. Bevan, and A. Patapoutian. 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*. 112:819-829.

Szallasi, A., and P.M. Blumberg. 1989. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience*. 30:515-520.

Szallasi, A., T. Szabo, T. Biro, S. Modarres, P.M. Blumberg, J.E. Krause, D.N. Cortright, and G. Appendino. 1999. Resiniferatoxin-type phorboid vanilloids display capsaicin-like selectivity at native vanilloid receptors on rat DRG neurons and at the cloned vanilloid receptor VR1. *Br J Pharmacol*. 128:428-434.

Szolcsanyi, J., A. Szallasi, Z. Szallasi, F. Joo, and P.M. Blumberg. 1991. Resiniferatoxin. An ultrapotent neurotoxin of capsaicin-sensitive primary afferent neurons. *Ann N Y Acad Sci*. 632:473-475.

Tafesse, L., Q. Sun, L. Schmid, K.J. Valenzano, Y. Rotshteyn, X. Su, and D.J. Kyle.

2004. Synthesis and evaluation of pyridazinylpiperazines as vanilloid receptor 1 antagonists. *Bioorg Med Chem Lett*. 14:5513-5519.

Talavera, K., K. Yasumatsu, T. Voets, G. Droogmans, N. Shigemura, Y. Ninomiya, R.F. Margolskee, and B. Nilius. 2005. Heat activation of TRPM5 underlies thermal sensitivity of sweet taste. *Nature*. 438:1022-1025.

Tan, C.H., and P.A. McNaughton. 2016. The TRPM2 ion channel is required for sensitivity to warmth. *Nature*. 536:460-463.

Thiel, G., S. Rubil, A. Lesch, L.A. Guethlein, and O.G. Rossler. 2017. Transient receptor potential TRPM3 channels: Pharmacology, signaling, and biological functions. *Pharmacol Res*. 124:92-99.

Togashi, K., Y. Hara, T. Tominaga, T. Higashi, Y. Konishi, Y. Mori, and M. Tominaga. 2006. TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. *EMBO J*. 25:1804-1815.

Togashi, K., H. Inada, and M. Tominaga. 2008. Inhibition of the transient receptor potential cation channel TRPM2 by 2-aminoethoxydiphenyl borate (2-APB). *Br J Pharmacol*. 153:1324-1330.

Tominaga, M., M.J. Caterina, A.B. Malmberg, T.A. Rosen, H. Gilbert, K. Skinner, B.E. Raumann, A.I. Basbaum, and D. Julius. 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*. 21:531-543.

Trevisani, M., D. Smart, M.J. Gunthorpe, M. Tognetto, M. Barbieri, B. Campi, S. Amadesi, J. Gray, J.C. Jerman, S.J. Brough, D. Owen, G.D. Smith, A.D. Randall, S. Harrison, A. Bianchi, J.B. Davis, and P. Geppetti. 2002. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nat Neurosci*. 5:546-551.

Uchida, K., L. Demirkhanyan, S. Asuthkar, A. Cohen, M. Tominaga, and E. Zakharian. 2016. Stimulation-dependent gating of TRPM3 channel in planar lipid bilayers. *FASEB J*. 30:1306-1316.

Viswanath, V., G.M. Story, A.M. Peier, M.J. Petrus, V.M. Lee, S.W. Hwang, A. Patapoutian, and T. Jegla. 2003. Opposite thermosensor in fruitfly and mouse. *Nature*. 423:822-823.

Voets, T., G. Droogmans, U. Wissenbach, A. Janssens, V. Flockerzi, and B. Nilius. 2004. The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature*. 430:748-754.

Vriens, J., G. Appendino, and B. Nilius. 2009. Pharmacology of vanilloid transient receptor potential cation channels. *Mol Pharmacol*. 75:1262-1279.

Vriens, J., G. Owsianik, T. Hofmann, S.E. Philipp, J. Stab, X. Chen, M. Benoit, F. Xue, A. Janssens, S. Kerselaers, J. Oberwinkler, R. Vennekens, T. Gudermann, B. Nilius, and T. Voets. 2011. TRPM3 is a nociceptor channel involved in the detection of noxious heat. *Neuron*. 70:482-494.

Wagner, T.F., S. Loch, S. Lambert, I. Straub, S. Mannebach, I. Mathar, M. Dufer, A. Lis, V. Flockerzi, S.E. Philipp, and J. Oberwinkler. 2008. Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic beta cells. *Nat Cell Biol*. 10:1421-1430.

Welch, J.M., S.A. Simon, and P.H. Reinhart. 2000. The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from the activation by either acid or heat. *Proc Natl Acad Sci U S A*. 97:13889-13894.

Winter, Z., A. Buhala, F. Otvos, K. Josvay, C. Vizler, G. Dombi, G. Szakonyi, and Z. Olah. 2013. Functionally important amino acid residues in the transient receptor potential vanilloid 1 (TRPV1) ion channel--an overview of the current mutational data. *Mol Pain*. 9:30.

Wood, J.N., J. Winter, I.F. James, H.P.

Rang, J. Yeats, and S. Bevan. 1988. Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci*. 8:3208-3220.

Xu, H., I.S. Ramsey, S.A. Kotecha, M.M. Moran, J.A. Chong, D. Lawson, P. Ge, J. Lilly, I. Silos-Santiago, Y. Xie, P.S. DiStefano, R. Curtis, and D.E. Clapham. 2002. TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature*. 418:181-186.

Xu, X.Z., F. Moebius, D.L. Gill, and C. Montell. 2001. Regulation of melastatin, a TRP-related protein, through interaction with a cytoplasmic isoform. *Proc Natl Acad Sci U S A*. 98:10692-10697.

Yang, F., Y. Cui, K. Wang, and J. Zheng. 2010. Thermosensitive TRP channel pore turret is part of the temperature activation pathway. *Proc Natl Acad Sci U S A*. 107:7083-7088.

Yang, F., X. Xiao, W. Cheng, W. Yang, P. Yu, Z. Song, V. Yarov-Yarovoy, and J. Zheng. 2015. Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nat Chem Biol*. 11:518-524.

Yang, J.M., F. Li, Q. Liu, M. Ruedi, E.T. Wei, M. Lentsman, H.S. Lee, W. Choi, S.J. Kim, and K.C. Yoon. 2017. A novel TRPM8 agonist relieves dry eye discomfort. *BMC Ophthalmol*. 17:101.

Yao, J., B. Liu, and F. Qin. 2010. Pore turret of thermal TRP channels is not essential for temperature sensing. *Proc Natl Acad Sci U S A*. 107:E125; author reply E126-127.

Yao, J., B. Liu, and F. Qin. 2011. Modular thermal sensors in temperature-gated transient receptor potential (TRP) channels. *Proc Natl Acad Sci U S A*. 108:11109-11114.

Zhu, B., M. Xia, X. Xu, D.W. Ludovici, M. Tennakoon, M.A. Youngman, J.M. Matthews, S.L. Dax, R.W. Colburn, N. Qin, T.L. Hutchinson, M.L. Lubin, M.R. Brandt, D.J. Stone, C.M. Flores, and M.J. Macielag. 2013. Arylglycine derivatives as potent transient receptor potential melastatin 8 (TRPM8) antagonists. *Bioorg Med Chem Lett*. 23:2234-2237.

Zimmermann, K., J.K. Lennerz, A. Hein, A.S. Link, J.S. Kaczmarek, M. Delling, S. Uysal, J.D. Pfeifer, A. Riccio, and D.E. Clapham. 2011. Transient receptor potential cation channel, subfamily C, member 5 (TRPC5) is a cold-transducer in the peripheral nervous system. *Proc Natl Acad Sci U S A*. 108:18114-18119.

Zygmunt, P.M., J. Petersson, D.A. Andersson, H. Chuang, M. Sorgard, V. Di Marzo, D. Julius, and E.D. Hogestatt. 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*. 400:452-457.

Does Inflammatory Response Cause Tissue Dysfunction in Chronic Diseases?

Juan C. Sáez[1]

Introduction

It is well accepted that protein subunits called connexins (Cxs) form gap junctions, which are membrane specializations made up of aggregates from a variable number of intercellular communication channels called *gap junction channels*. Each channel is formed by two hemichannels (Figure 1), which correspond to Cx oligohexamers that could be homomeric or heteromeric. While gap junctions formed by the same type of hemichannels are homotypic, those formed by different types of hemichannels are heterotypic. However, Cxs show different affinities when interacting with other Cxs, hence reducing the number of different possible hemichannel and gap junction channels that they can form.

Gap junctional communication can be established between cells of the same (homocellular) or different (heterocellular) type. While gap junction channels serve for direct communication between the cytoplasm of contacting cells, hemichannels serve as membrane pathways between intra and extracellular compartments. Vertebrates also express a more recently discovered protein family composed of three members termed pannexins (Panx1-3). Since evidence for Panx-formed gap junctions is very limited and most available data demonstrate that Panxs form hemichannel-like structures, the currently accepted name for these structures is Panx channels (Sosinsky *et al.*, 2011) (Figure 1). Although our knowledge regarding the permeability properties of these channels is still limited, it is well established that most cells express at least one Panx type, which is similar to what is known for Cxs.

Gap junction channels, Cx hemichannels and Panx1 channels are permeable to ions and small molecules and thus, are involved in coordinating electrical and metabolic responses among members of a cell community. Therefore, groups of cells communicated via gap junctions and hemichan-

[1] Departamento de Fisiología, Facultad de Biología, Pontificia Universidad Católica de Chile, Alameda 340, Santiago Chile. Instituto Interdisciplinario de Neurociencias de la Universidad de Valparaíso, Valparaíso, Chile.

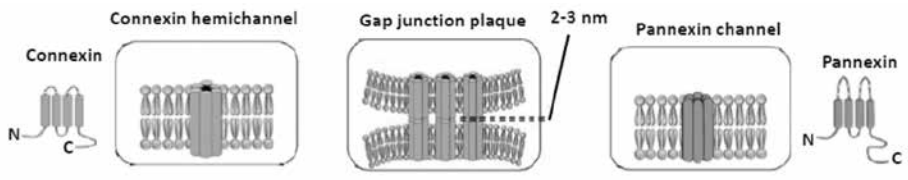


Figure 1. Scheme of a gap junction plaque containing three gap junction channels traversing two lipid bilayers, each corresponding to the cell membrane of adjacent cells (Center panel). A virtual gap of 2-3 nm is depicted between the hemichannels constituting each gap junction channel. A connexin hemichannel (Left panel) and a pannexin channel (Right panel) on the cell surface, each composed of six protein subunits termed connexins (Far left) and pannexins (Far right), respectively. Connexins and pannxins have similar membrane topology with the N and C terminal domains located in the intracellular space and each protein presents four transmembrane domains.

nels represent functional units for many tissue responses, rather than single cells. For instance, hepatic vasopressin receptors are primarily expressed by cells located closer to the blood exit (Terminal hepatic venules), and not in cells found closer to the blood entrance (Terminal portal venules) of each hepatic semi-acinus. And in the whole liver loaded with a fluorophore sensitive to changes in intracellular free Ca2+ concentrations ([Ca2+]i), perfusion with vasopressin-containing solution generates Ca2+ waves that propagate from terminal hepatic venules to terminal portal venules, which is a retrograde direction compared to that of blood flow (Nathanson *et al.*, 1995). These Ca2+ waves are the result of a regenerative process involving the intercellular transfer of a Ca2+-releasing molecule likely to be IP3 (Sáez *et al.*, 1989) that in the liver is generated upon vasopressin perfusion. Thus, IP3 is likely to be generated in cells with higher vasopressin density located close to terminal hepatic venules and would diffuse to neighboring cells generating a Ca2+ wave that propagate to terminal portal venules coordinating many Ca2+-dependent metabolic responses. The possibility that cells that express more vasopressin receptors release a signaling molecule to the extracellular milieu such as ATP, known to cause intracellular free Ca2+ increases, is unlikely because such putative molecule would be washed out from the acini by the blood flow before reaching cells located in the reverse direction of the blood flow. Nevertheless, Ca2+ waves generated by ligands that bind to membrane receptors also exit, and can co-exist with gap junction mediated Ca2+ waves, but the contribution of each would vary under different physiological conditions. Other well-known function of gap junctions is that found in astroglial cell syncytia, which un-

der high neuronal activity serve as spatial buffer through which neurotoxic neurotransmitters (e.g., glutamic acid) and depolarizing ions such as K+ are diluted. This contributes to maintaining the extracellular homeostasis for adequate neuronal performance (Theis and Giaume, 2012).

The aforementioned membrane channels are also expressed by cells that do not form solid tissues such as polymorphonuclear cells, macrophages and lymphocytes, as well as metastatic cells (Sáez *et al.*, 2003). These cells can establish homocellular or heterocellular contact at very specific places and moments in their trafficking domains. For instance, dendritic cells and T cells establish physical contact during antigen presentation as well as during extravasation from lymphatic vessel as well as blood flow to specific tissues. In immune cells, the expression of Cxs as well as the formation of functional gap junction channels and hemichannels have been so far shown to be transiently upregulated by different cytokine mixtures and extracellular ATP (Sáez *et al.*, 2014).

In addition to forming gap junction channels and hemichannels, Cxs have also been detected in the inner membrane of mitochondria, forming ion permeable channels that affect the functional activity of this subcellular organelle (Boengler and Schulz, 2017). Moreover, two channel-independent functions of Cxs have been documented. One of them pertains to cell-cell adhesion proteins accomplished through strong head to head interactions of Cxs as part of gap junction channels. This interaction is so strong that it can only be interrupted in the degradation pathways of gap junction channels, which are internalized as annular rings into one of two interacting cells. Another channel-independent function of Cxs is that they act as membrane transducers since they can be detected in the nucleus interacting with intracellular proteins that regulate gene expression such as the transcriptional factor β-catenin (Chang *et al.*, 2014).

Cell-cell communication under chronic pathological conditions

A common factor of chronic diseases is a small but persistent increase in [Ca2+]i. For example, this type of [Ca2+]i increase is found in myofibers of *mdx* mice, a model of Duchenne muscular dystrophy or in cells of a dysfernilopathy (Cea *et al.*, 2016a; 2016b). Although this change is not very dramatic, it could be sufficient for maintaining higher activity of several intracellular signaling pathways that progressively generate pro-inflammatory bioactive molecules (e.g., oxygen free radicals, cytokines and eicosanoid products). The outcomes of these bioactive products include the activation of the inflammasome, mitochondrial dysfunction, modifications in gene

expression and cell death. However, the origins of the increase in [Ca2+] i have remained elusive, and could either be from Ca2+ influx and/or release from intracellular stores.

Numerous studies have tried to identify selective membrane ion channels as putative pathways for increases in Ca2+ inflow, but their blockade has not shown to render significant protection. Recently, it has been demonstrated that several non-selective channels are expressed *de novo* or are upregulated under chronic inflammatory conditions. These channels include Cx hemichannels, Panx1 channels, P2X7 receptors and TRPV2 channels (Cea *et al.*, 2013; 2016a; 2016b; Cisterna *et al.*, 2016; Balboa *et al.*, 2017). Interestingly, several of these channels are permeable to Ca2+. For instance Cx26, Cx32 and Cx43 hemichannels are permeable to Ca2+ (Sánchez *et al.*, 2008; Schalper *et al.*, 2010; Fiori *et al.*, 2012). At least for Cx26 and Cx43 hemichannels, it seems that no additional protein is involved in allowing Ca2+ diffusion across the cell membrane, because hemichannels reconstituted in liposomes have been shown to permeate Ca2+ (Schalper *et al.*, 2010; Fiori *et al.*, 2012). Similarly, the P2X7 receptor and TRPV2 channels are permeable to Ca2+, and could also contribute to increasing [Ca2+]i (Iwata *et al.*, 2009; Young *et al.*, 2012). In addition, increases in [Ca2+]i can induce Ca2+ release from intracellular stores, which further increases intensity and/or duration of the rise in intracellular free Ca2+ concentrations. This response could reduce the extracellular Ca2+ concentration, which increases the open probability of Ca2+ permeable channels, including TRP channels, P2X receptors and several Cx hemichannels. Additionally, greater [Ca2+]i increases the open probability of Panx1 channels (Sáez and Leybaert, 2017), which together with Cx hemichannels allow ATP release to the extracellular milieu, favoring the activation of P2Y and P2X7 receptors. While P2Y receptors desensitize with prolonged or high ATP concentrations the P2X7 receptor does not desensitize contributing to a feedforward mechanism (Figure 2). Accordingly, the inactivation of P2X7 receptors, TRPV2 channels or Cx hemichannels provides protection to skeletal myofibers of mdx mice, as discussed by Cea and collaborators (Cea *et al.*, 2016a) (Figure 2).

Consequently, the resulting increase in [Ca2+]i activates several inflammatory signaling pathways that generate oxygen free radicals known to activate Cx hemichannels (Retamal *et al.*, 2006; Ramachandra *et al.*, 2007; Figueroa *et al.*, 2013) as well as the inflammasome, which yields pro-inflammatory cytokines (e.g., IL-1β) (Cea *et al.*, 2013; Cea *et al.*, 2016a). Then, pro-inflammatory cytokines released by parenchymal cells, as well as

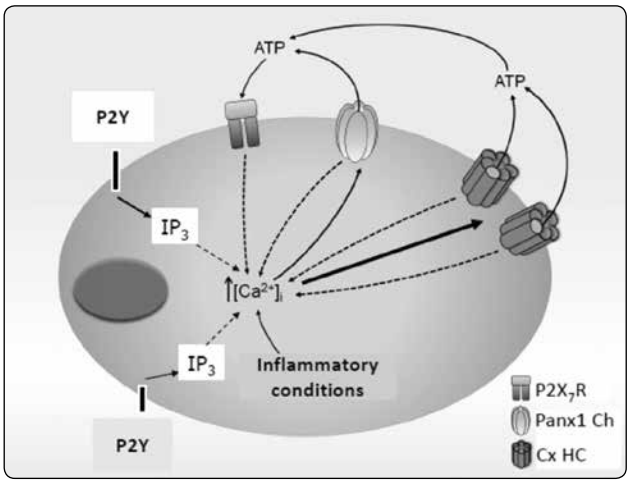


Figure 2. Scheme of a reverberant mechanism of Ca2+ influx and ATP release that participates in inflammatory responses in different cell types. When a cell is inflamed molecular triad composed of Panx1 channel (Panx1 Ch), connexin hemichannels (Cx HCs) and P2 receptors (P2X7R and P2Y) is expressed. While P2Y receptors activated by extracellular ATP causes the release of Ca2+ from intracellular stores via IP3, all other channels are permeable to Ca2+ and are activated by elevated intracellular Ca2+, causing more ATP release via Panx1 Ch and Cx HCs. Also, an increase in intracellular Ca2+ can induce Ca2+ release from intracellular stores.

infiltrated inflammatory cells, generate another positive feedback because the resulting pro-inflammatory cytokines also increase the open probability of Cx hemichannels via p38 kinase and iNOS (Retamal *et al.*, 2007). Subsequently, the nitric oxide generated via iNOS could cause S-nitrosylation of Cxs increasing the open probability of Cx hemichannels (Retamal *et al.*, 2006; Figueroa *et al.*, 2013), which seems to be a gating mechanism because the amount of hemichannels present in the cell surface is not significantly affected (Retamal *et al.*, 2006). However, increases in Cx hemichannel activity induced by oxygen deprivation, metabolic inhibition or pro-inflammatory cytokines seem to be explained by greater amounts of Cxs in the cell membrane (Retamal *et al.*, 2006; Orellana *et al.*, 2010; Orellana *et al.*, 2011).

In parallel to increases in Cx hemichannel activity promoted by pro-inflammatory agents, reductions in gap junctional communication between parenchymal cells have consistently observed (Retamal *et al.*, 2007; Orellana *et al.*, 2010; Hernández-Salinas *et al.*, 2013; Figueroa *et al.*, 2012;Ye *et al.*, 2014), and neurons become more susceptible to insults including oxidant

agents (Ye *et al.*, 2014). The relevance of recoupling to protect cells from insults has been limited by the lack of strategies that increase gap junctional communication, without having any effect on hemichannels. However, the importance of Cx channels in some diseases has been demonstrated by using constitutive Cx knockout or cells specific Cx knockout mice. But, this strategy does not differentiate between hemichannels and gap junction channels, unless the cell type does not form one channel type, as in denervated skeletal muscles that express only hemichannels (Cea *et al.*, 2013). Nevertheless, inhibition of Cx hemichannels without effects on gap junction channels has been accomplished selective inhibitor including mimetic peptides and organic molecules (Willebrords *et al.*, 2017). Given that increases in Cx hemichannels in chronic diseases might require long-term treatments, the use of peptides might have certain limitations due to degradation and possible immune responses. Alternatively the use of organic molecules that selectively block hemichannels, and do not affect gap junction channels might be a choice of preference. For instance, boldine has been shown to block Cx hemichannels and Panx1 channels without inhibitory effects on gap junction channels (Hernández-Salinas *et al.*, 2013; Yi *et al.*, 2017). Recently, we demonstrated that chronic treatment with boldine prevents to a great extent morphological and functional kidney alterations induced by diabetes (Hernández-Salinas *et al.*, 2013), as well as the neuronal suffering in an animal model for Alzheimer's disease (Yi *et al.*, 2017) two chronic diseases that develop inflammation. Unfortunately, boldine is not a selective molecule, since it also affects several other membrane proteins (O'Brien *et al.*, 2006) and in the same line of analysis the pharmacologic and toxic doses for boldine are too close to each other, which is an important limitation for human use. Nevertheless, bioinformatic studies using the crystal structure of Cx26 channels (Maeda *et al.*, 2009) and boldine as well as other non-selective gap junction channel/hemichannel blockers, such as carbenoxolone, were used as pharmacophore and has led to the discovery of a potent and selective Cx hemichannel blocker with EC50 around 10 nM (further described in Chilean and International patent applications). Consistent with the high affinity and selectivity (it does not block gap junction channels of Panx1 channels), the pharmacologic and toxic doses are apart by at least three orders of magnitude. In addition, animal models of acquired or inherited chronic diseases with significant inflammatory responses (mdx mice) recover their organ function (muscle strength) when the compound called D4 is administered for 3-4 weeks. Complete recovery occurs if the disease affects a tissue with regenerative

properties (e.g. skeletal muscles). In mice treated acutely or chronically (kindling) with epileptogenic agents (e.g., PTZ or ketamine), we found high Cx hemichannel and Panx1 channel activity in glial cells and treatment with D4 also drastically prevent the manifestation of seizures and neuroinflammation. Therefore, the aforementioned finding indicate that tissue dysfunction (skeletal muscle or brain) in chronic diseases results from the inflammatory response in which Cx hemichannels play a critical role. Manuscripts containing these results are under preparation and will soon be reported elsewhere.

References

Balboa, E., Saavedra, F., Cea, L.A., Vargas, A.A., Ramírez,V., Escamilla, R., Sáez, J.C., and Regueira, T. (2017) Sepsis-induced channelopathy in skeletal muscle is associated with expression of non-selective channels. *Shock*. May 30. doi: 10.1097/SHK.0000000000000916. [Epub ahead of print]

Boengler, K., & Schulz, R. (2017). Connexin 43 and mitochondria in cardiovascular health and disease. *Adv Exp Med Biol. 982*, 227-246.

Cea, L.C., Cisterna, B.A., Puebla, C., Frank, M., Figueroa, X.F., Cardozo, C., Willecke, K., Latorre, R., & Sáez, J.C. (2013). *De novo* expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. *Proc. Natl. Acad. Sci. USA, 110*, 16229-16234.

Cea, L.A., Puebla, C., Cisterna, B.A., Escamilla, R., Vargas, A.A., Frank, M., Martínez-Montero, P., Prior, C., Molano, J., Esteban-Rodríguez, I., Pascual, I., Gallano, P., Lorenzo, G., Pian, H., Barrio, L.C., Willecke, K., & Sáez, J.C. (2016a). Fast skeletal myofibers of mdx mouse, model of Duchenne muscular dystrophy, express connexin hemichannels that lead to apoptosis. *Cell Mol Life Sci., 73*, 2583-2599.

Cea, L.A., Bevilacqua, J.A., Arriagada, C., Cárdenas, A.M., Bigot, A., Mouly, V., Sáez, J.C., & Caviedes, P. (2016b). The absence of dysferlin induces the expression of functional connexin-based hemichannels in human myotubes. *BMC Cell Biol., 17*, Suppl 1:15. doi: 10.1186/s12860-016-0096-6.

Cisterna, B.A., Vargas, A.A., Puebla, C., & Sáez, J.C. (2016). Connexin hemichannels explain the ionic imbalance and lead to atrophy in denervated skeletal muscles. *Biochim Biophys Acta-Molecular Basis of Diseases*, *1862*, 2168-2176.

Chang, S.F., Chen, L.J., Lee, P.L., Lee, D.Y., Chien, S., & Chiu, J.J. (2014). Different modes of endothelial-smooth muscle cell interaction elicit differential β-cateninphosphorylations and endothelial functions. *Proc Natl Acad Sci USA*, *111*, 1855-1860.

Figueroa V., Sáez P.J., Salas J.D., Jara O., Martínez A.D., Sáez J.C., & Retamal M.A. (2012). Linoleic acid induces opening of connexin 26 hemichannels through a PI3K/Akt/Ca2+-dependent pathway. *Biochem Biophys Acta – Biomembranes, 1828*, 1169-1179.

Figueroa, X.F., Lillo, M.A., Gaete, P.S., Riquelme, M., & Sáez, J.C. (2013). Diffusion of nitric oxide across cell membranes of the vascular wall requires specific connexin-based channels. *Neuropharmacology, 75*, 471-478.

Fiori, M.C., Figueroa, V., Zoghbi, M.E., Sáez, J.C., Reuss, L., & Altenberg, G.A.

(2012). Calcium transport through purified connexin 26 hemichannels. *J. Biol. Chem., 287*, 40826-40834.

Hernández-Salinas, R., Vielma, A.Z., Arismendi, M.N., Boric, M.P., Sáez, J.C., & Velarde, V. (2013). Boldine prevents renal alterations in diabetic rats. *J Diabetes Res.* 593672. doi: 10.1155/2013/593672.

Ishikawa, M., Iwamoto, T., Nakamura, T., Doyle, A., Fukumoto, S., & Yamada, Y. (2011). Pannexin 3 functions as an ER Ca2+ channel, hemichannel, and gap junction to promote osteoblast differentiation. *J Cell Biol., 193*, 1257-1274.

Iwata, Y., Katanosaka, Y., Arai, Y., Shigekawa, M., & Wakabayashi, S. (2009). Dominant-negative inhibition of Ca2+ influx via TRPV2 ameliorates muscular dystrophy in animal models. *Hum Mol Genet., 18*, 824-834.

Le, H.T., Sin, W.C., Lozinsky, S., Bechberger, J., Vega, J.L., Guo, X.Q., Sáez, J.C., & Naus, C.C. (2014). Gap junction intercellular communication mediated by connexin 43 in astrocytes is essential for their resistance to oxidative stress. *J Biol Chem., 289*, 1345-1354.

Maeda, S., Nakagawa, S., Suga, M., Yamashita, E., Oshima, A., Fujiyoshi, Y., & Tsukihara, T. (2009). Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature*, 458, 597-602.

Nathanson, M., Burgstahler, A.D., Mennone, A., Fallon, M.B., González, C., & Sáez, J.C. (1995) Organization of intercellular Ca2+ waves in rat liver. *Amer. J. Physiol., 269*, G167-G171.

Orellana, J.A., Hernández, D.E., Ezan, P., Velarde, V., Bennett, M.V.L. Giaume, C., & Sáez, J.C. (2010). Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels. *Glia, 58*, 329-343.

O'Brien, P., Carrasco-Pozo, C., & Speisky, H. (2006). Boldine and its antioxidant or health-promoting properties. *Chem Biol Interact., 159*, 1-17.

Orellana, J.A., Froger, N., Ezan, P., Jiang, J.X., Bennett, M.V., Naus, C.C., Giaume, C., & Sáez, J.C. (2011a). ATP and glutamate hemichannels mediate neuronal death through released via astroglial connexin 43 activation of pannexin 1 hemichannels. *J Neurochem., 118*, 826-840.

Orellana, J.A., Shoji, K.F., Abudara, V., Ezan, P., Amigou, E., Sáez, P.J., Jiang, J.X., Naus, C.C., Sáez, J.C., & Giaume, C. (2011b). Amyloid β-induced death in neurons involves glial and neuronal hemichannels. *J Neurosci.*, *31*, 4962-4977.

Ramachandran, S., Xie, L.H., John, S.A., Subramaniam, S., & Lal, R. (2007). A novel role for connexin hemichannel in oxidative stress and smoking-induced cell injury. *PLoS One, 2*(8), e712.

Retamal, M.A., Froger, N., Palacios-Prado, N., Ezan, P., Sáez, P.J., Sáez, J.C. & Giaume, C. (2007). Cx43 hemichannels and gap junction channels in astrocytes are oppositely regulated by pro-inflammatory cytokines released from activated microglia. *J. Neurosci., 27*, 13781-13792.

Retamal, M.A., Cortés, C.J., Reuss, L. Bennett, M.V.L., & Sáez, J.C. (2006). S-nitrosylation and permeation through connexin 43 hemichannels in astrocytes: induction by oxidant stress and reversal by reducing agents. *Proc. Natl. Acad. Sci. USA, 103*, 4475-4480.

Sáez, J.C., Connor, J.A., Spray, D.C., & Bennett, M.V.L. (1989). Hepatocyte gap junctions are permeable to the second messengers inositol 1,4,5-trisphosphate and calcium ions. *Proc. Natl. Acad. Sci. U.S.A., 86*, 2708-2712.

Sáez, J.C., & Leybaert, L. (2014). *Hunting for connexin hemichannels. FEBS Letters, 588*, 1205-1211.

Sáez, P.J., Shoji, K.F., Aguirre, A., & Sáez,

J.C. (2014). Regulation of hemichannels and gap junction channels by cytokines in antigen-presenting cells. *Mediators Inflamm.,* 742734. doi: 10.1155/2014/742734.

Sánchez, H.A., Orellana, J.A., Verselis, V.K., & Sáez J.C. (2009). Metabolic inhibition increases the activity of connexin-32 hemichannels which are permeable to Ca2+. *Am. J. Physiol., 297*, C665-C678.

Sosinsky, G.E., Boassa, D., Dermietzel, R., Duffy, H.S., Laird, D.W., MacVicar, B., Naus, C.C., Penuela, S., Scemes, E., Spray, D.C., Thompson, R.J., Zhao, H.B., & Dahl, G. (2011). Pannexin channels are not gap junction hemichannels. *Channels* (Austin), *5*, 193-197.

Schalper, K.A., Lee, S-C; Altenberg, G.A., Nathanson, M.H., & Sáez J.C. (2010). Connexin 43 is a pH-gated plasma membrane calcium channel. *Am. J. Physiol., 299*, C1504-C1515.

Theis, M., & Giaume, C. (2012). Connexin-based intercellular communication and astrocyte heterogeneity. *Brain Res., 1487*, 88-98.

Willebrords, J., Maes, M., Crespo Yanguas, S., & Vinken, M. (2017). Inhibitors of connexin and pannexin channels as potential therapeutics. *Pharmacol Ther., 180*, 144-160. doi: 10.1016/j.pharmthera.2017.07.001.

Yi, C., Ezan, P., Fernández, P., Schmitt, J., Sáez, J.C., Giaume, C., & Koulakoff A. (2017). Inhibition of glial hemichannels by boldine treatment reduces neuronal suffering in a murine model of Alzheimer's disease. *Glia, 65*, 1607-1625.

Young, C.N., Brutkowski, W., Lien, C.F., Arkle, S., Lochmüller, H., Zabłocki, K., & Górecki, D.C. (2012). P2X7 purinoceptor alterations in dystrophic mdx mouse muscles: relationship to pathology and potential target for treatment. *J Cell Mol Med.*, *16*, 1026-1037.

Free Radicals, Oxidants and Antioxidant Systems in Physiology and Disease

Rafael Radi[1]

Introduction

Free Radicals and Oxidants in Human Biology

Free radicals and oxidants are continuously produced in cells and tissues as a consequence of normal metabolism. These reactive species include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitric oxide ($\ddot{Y}NO$) and peroxynitrite ($ONOO^-$), all of which are short-lived species (*i.e.* biological t1/2 range from μs in the case of $O_2^{\bullet -}$ to s in the case of $^{\bullet}NO$). While at low levels free radicals and oxidants participate in redox signaling activities that promote cell proliferation and adaptation to the environment, high levels result in accumulation of oxidative modifications of biomolecules, which are associated to the disruption of redox homeostasis, oxidative damage and cell death. Free radicals and oxidants participate in the process of normal aging and also in pathology such as in the development of acute and chronic disease conditions such as inflammation, atherosclerosis, hypertension and neurodegeneration. Free radicals and oxidant exposure to human tissues also arises from external sources such as UV radiation and smog and also secondary to xenobiotic metabolism. In essence, we are exposed to either endogenous or exogenous free radicals and oxidants throughout life!

Footprints of oxidative damage in human cells and tissues can be assessed by a variety of methods including immunochemical and analytical techniques, where stable products derived from oxidant reactions with target molecules can be identified and quantitated. For example, protein tyrosine nitration (Fig. 1A) (Radi, 2013a), an oxidative posttranslational modification in proteins secondary to the reactions of $^{\bullet}NO$-derived oxidants such as peroxynitrite, has been assessed in tissues with antibodies that recognize 3-nitrotyrosine in a large variety of disease conditions (*e.g.*

[1] Departamento de Bioquímica and Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay. e-mail: rradi@fmed.edu.uy

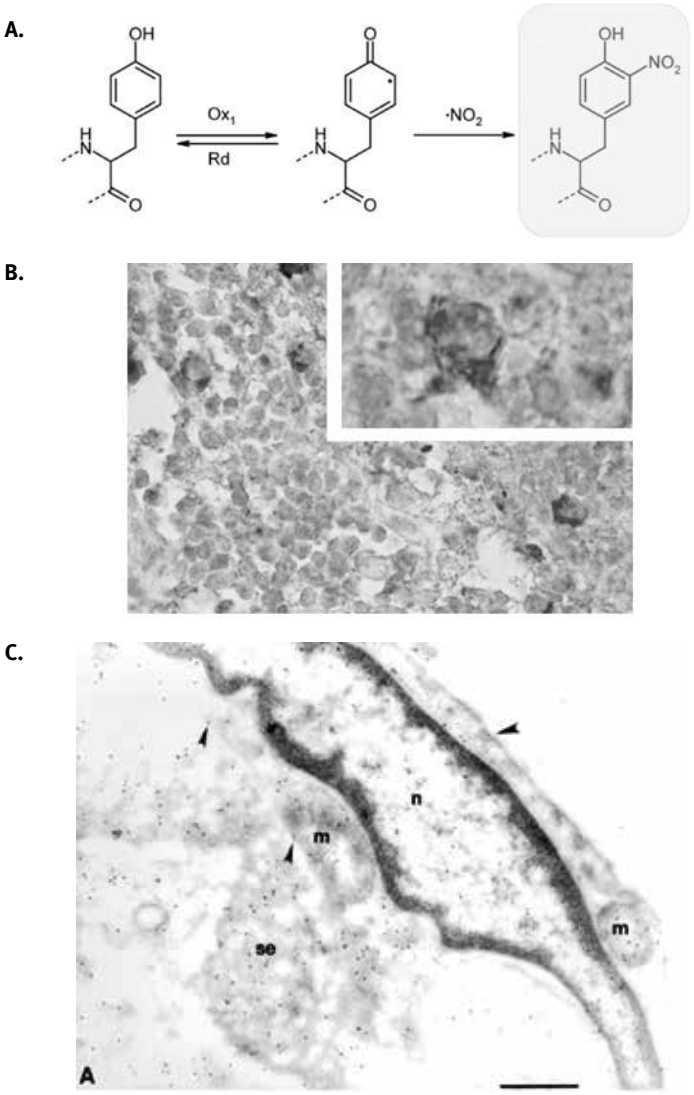


Figure 1. Protein 3-nitrotyrosine: biochemical mechanisms of formation and immunodetection in tissues. A. Formation of 3-nitrotyrosine is a two-step process that involves the one-electron oxidation of tyrosine to tyrosyl radical followed by the combination reaction with nitrogen dioxide (Reproduced with modifications from Radi, 2013a). B. Immunodetection of 3-nitrotyrosine in human mediastinal lymph nodes undergoing an inflammatory process. Heavy immunostain is observed in macrophages (see inset) (Reproduced from Brito *et al.*, 1999). C. Immunoelectromicroscopy revealing protein-3-nitrotyrosine in the aorta of an old rat. At the the vascular endothelium the highest label density is over mitochondria (m). Bar, 0.5 µm. Original magnifications: ×22,000. (Reproduced from van der Loo *et al.*, 2000).

inflammation, Fig. 1B) (Brito *et al.*, 1999) and the process of aging (*e.g.* in the vasculature, Fig. 1C) (van der Loo *et al.*, 2000).

Mitochondria: a key source and target of free radicals and oxidants

Under normal conditions > 99% of molecular oxygen that is consumed by most mammalian cells is utilized in the process of respiration at the terminal oxidase of the mitochondrial electron transport chain, cytochrome c oxidase, where it is reduced by four electrons to water. The process of cell respiration is coupled to the mitochondrial generation of ATP and, as such critical, for bioenergetics. Nevertheless, even under normal conditions, a small percentage of electrons flowing through the electron transport chain component "leak out" directly to molecular oxygen, causing its monovalent reduction to $O_2^{\bullet -}$. Thus, mitochondria constitute a constant source of $O_2^{\bullet -}$, much of which is readily converted to H_2O_2, *via* the enzymatic action of a family of metalloenzymes, the superoxide dismutases (SOD) (Fridovich, 1997), which are present either in the matrix (MnSOD) or the intermembrane space (CuZnSOD). SODs readily catabolize $O_2^{\bullet -}$ in a near-diffusion controlled reaction (Eq. 1):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad \text{Eq. 1}$$

Some of the $O_2^{\bullet -}$ may escape the action of SOD and selectively react and inactivate one of the rate-limiting enzymes of the Krebs cycle aconitase, or with .NO to yield peroxynitrite (*vide infra*).

Once formed, H_2O_2 can diffuse out of the mitochondria, react with mitochondrial targets or be decomposed by mitochondrial peroxidatic systems including peroxiredoxins and glutathione peroxidase. Thus, overall mitochondria have an array of antioxidant enzyme systems (SODs, peroxiredoxins, glutathione peroxidase) that maintain both $O_2^{\bullet -}$ and H_2O_2 under low steady state concentrations (possibly in the nM range for $O_2^{\bullet -}$- and μM range for H H_2O_2) (Winterbourn, 2008). Under conditions of mitochondrial aging or mitochondrial dysfunction observed in disease states secondary to a variety of challenges and mediators, mitochondrial $O2^{\bullet -}$/ H_2O_2 fluxes can enhance several fold and lead to disruption of mitochondrial redox homeostasis, oxidative damage, apoptotic signaling and even bioenergetics failure (Szabo *et al.*, 2007) (Fig. 2). In this sense, restoration of the mitochondrial redox-bioenergy homeostasis becomes a central ther-

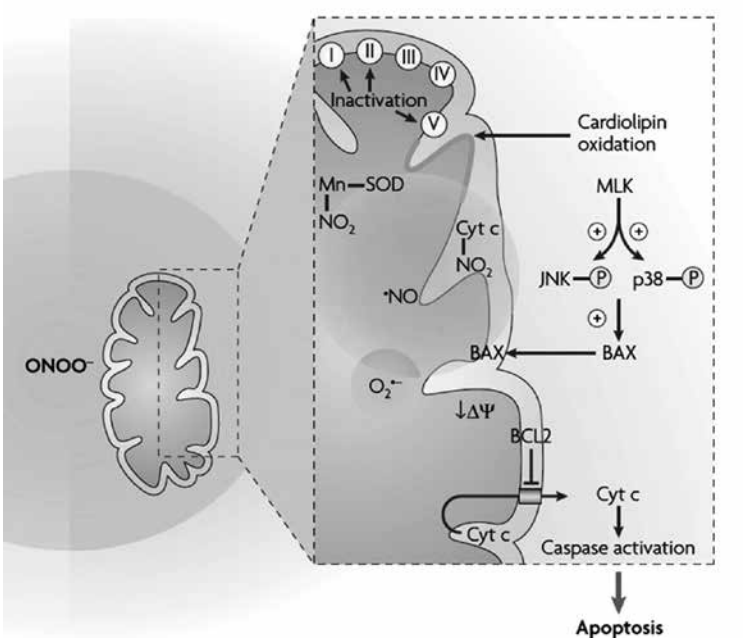


Figure 2. Formation and reactions of peroxynitrite in mitochondria. Nitric oxide diffusion to mitochondria results in the formation of peroxynitrite due to the fast reaction with mitochondrial $O_2^{\bullet-}$. Peroxynitrite may disrupt mitochondrial redox and energy homeostasis *via* inactivation of aconitase and respiratory chain complexes. Additionally, peroxynitrite promotes the nitration and inactivation of MnSOD rendering the organelle even more susceptible to excess $O_2^{\bullet-}$ generation. Cytochrome c may be also nitrated and elicit a gain-of-peroxidase function that contributes to cardiolipin oxidation. Overall, the conditions of oxidative stress created in the organelle facilitate the opening of the permeability transition pore with the release of pro-apoptotic factors to the cytosol. Mitochondrial peroxiredoxins attenuate peroxynitrite toxicity *via* its two-electron reduction to nitrate. Mitochondrial-targeted antioxidants may exert also protective actions against the toxicity generated by excess mitochondrial oxidant formation. (Reproduced from Szabo *et al.*, 2007).

apeutic goal in disease conditions in which mitochondrial oxidant formation and mitochondrial dysfunction play central roles in pathogenesis (Murphy and Smith, 2007).

NADPH Oxidases: from pathogen killing to redox signaling

Other central sources of cellular oxidants are constituted by the NADPH oxidases, redox enzymes present in the plasma membrane of many cell types, notably phagocytic cells. NADPH oxidases were initially described in neutrophils and macrophages where upon appropriate stimuli

(*e.g.* phagocytosis of invading pathogens) cytosolic components migrate to the plasma membrane leading to enzyme activation and generation of large fluxes of $O_2^{\bullet -}$ towards the phagosome or extracellular space for periods that range from 30-120 min in a process described as the "oxidative burst". The primary role of this NADPH oxidase-derived $O_2^{\bullet -}$ is mainly directed to kill invading bacteria and parasites, by oxidative mechanisms that also involve the secondary formation of stronger oxidants such as HOCl or peroxynitrite in the case of the neutrophils or macrophages, respectively (Winterbourn, 2008; Prolo *et al.*, 2014). Thus, phagocyte NADPH oxidase (NOX-2) mainly plays a role in the cytotoxic cellular immune response; this contention can be corroborated with the observations of the larger tendency to infections in animal NOX-2 KO models (Nathan and Shiloh, 2000) or in patients suffering chronic granulomatous disease that have a genetic defect in the NOX-2 gene (Babior and Curnutte, 1987). More recent work has shown that the NOX enzymes (other isoforms) are largely distributed among many cell types, serving cell signaling and cytoregulatory purposes (*e.g.* NOX-1 in vascular endothelial cells).

Nitric Oxide and Peroxynitrite: from signal transduction to cytotoxicity

Nitric oxide is mainly formed enzymatically by the action of nitric oxide synthases (NOS) in a reaction that utilizes as substrates L-arginine, molecular oxygen and NADPH (Knowles and Moncada, 1994). The enzyme utilizes tetrahydrobiopterin as cofactor and its activity, especially in the constitutive forms, is regulated by calcium. There are three main isoforms, endothelial, neuronal and inducible NOS, that play central roles in vasodilation, neurotransmission and immune responses, respectively.

Nitric oxide is a small, neutral and hydrophobic molecule that can readily permeate across lipid bilayers. Its signaling actions mainly depend on its reversible reaction with the heme group of guanylate cyclase leading to enzyme activation and promoting the generation of cGMP. Another important reversible reaction, is its interaction with cytochrome c oxidase (in competition with molecular oxygen) that leads to modulation of respiratory rates. The region of action of $^{\bullet}NO$ is typically within 100-1000 μm, being mainly a paracrine or autocrine mediator. Once it reaches a blood vessel, it is readily consumed by red blood cells *via* the oxy-hemoglobin-mediated oxidation of $^{\bullet}NO$ to nitrate.

A key aspect connecting $^{\bullet}NO$ with redox biology relates to its fast reaction with $O_2^{\bullet -}$. Indeed, $^{\bullet}NO$ reacts with $O_2^{\bullet -}$ at diffusion-controlled rates

to yield the potent oxidizing and nitrating species, peroxynitrite (Eq. 2) (Beckman *et al.*, 1990; Radi *et al.*, 1991)

$$^{\bullet}NO + O_2^{\bullet -} \rightarrow ONOO^- \qquad \text{Eq. 2}$$

The anionic form of peroxynitrite is in equilibrium with peroxynitrous acid (p*Ka ca.* 6.8), what makes that both species coexist under biologically-relevant conditions. Equation 2 clearly shows that $O_2^{\bullet -}$ controls the biological $t_{1/2}$ of $^{\bullet}NO$ and decreases its bioactivity. For example, in hypertension the bioavailability of endothelial cell-derived $^{\bullet}NO$ is decreased by enhanced formation rates of $O_2^{\bullet -}$ in the vasculature. A similar $^{\bullet}NO$ /$O_2^{\bullet -}$ interplay, although in a much more moderate manner, is observed during normal vascular aging (var der Loo *et al.*, 2000). In addition, the formation of peroxynitrite helps to rationalize much of the toxic actions of $^{\bullet}NO$ under inflammatory conditions and how $^{\bullet}NO$, not being *per se* a strong oxidant,

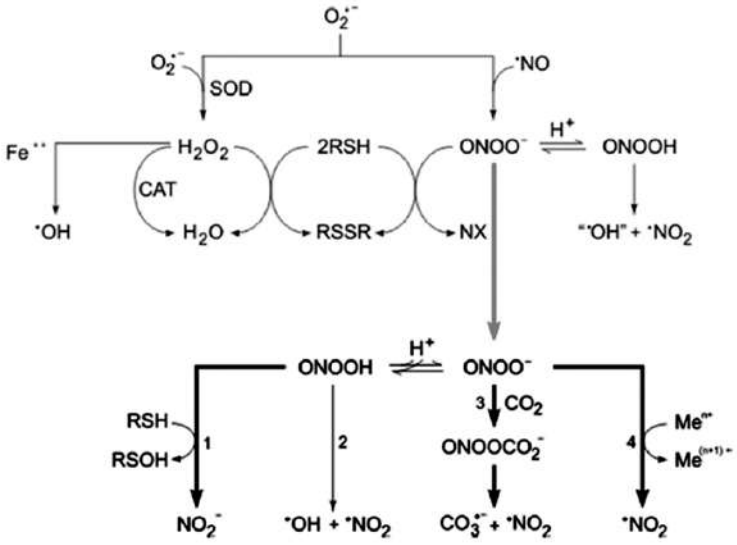


Figure 3. Peroxynitrite reaction pathways. Nitric oxide can kinetically outcome the SODs for the reaction with $O_2^{\bullet -}$ and yield peroxynitrite. The red arrow connects an early scheme proposing the basic biochemistry of peroxynitrite (Radi *et al.*, 1991) with our current knowledge of the pathways of peroxynitrite reactions involving two-electron oxidation of thiols (I), proton-catalyzed homolysis (II), reaction with CO_2 (III) and one-electron oxidation of transition metal centers. The relative contribution of each of the four pathways is kinetically controlled, with homolysis being a marginal route. (Reproduced from Radi, 2013b).

can participate in oxidation and nitration reactions in the pathogenesis of disease and the process of aging. Peroxynitrite can promote direct one- and two-electron oxidations in biomolecules such as transition metal-containing centers and thiols, respectively. It can also evolve to secondary free radicals such as carbonate radicals ($CO_3^{\bullet -}$) and nitrogen dioxide ($^{\bullet}NO_2$) following its fast reaction with carbon dioxide (Fig. 3) (Radi, 2013b); marginal amounts of hydroxyl radical ($^{\bullet}OH$), a potent oxidizing intermediate, can be also formed by homolytic cleavage of ONOOH. Peroxynitrite is typically more reactive than other oxidants such as H_2O_2. In fact, a seminal report showed that the second order rate constant at pH 7.4 and 37°C of peroxynitrite with typical thiols is at least three orders of magnitude higher than that with H_2O_2 (*e.g. ca.* 5,000 M-1s-1 vs. 5 M-1s-1) (Radi, 1991). Recent work has shown that some proteins contain "fast reacting thiols" that can

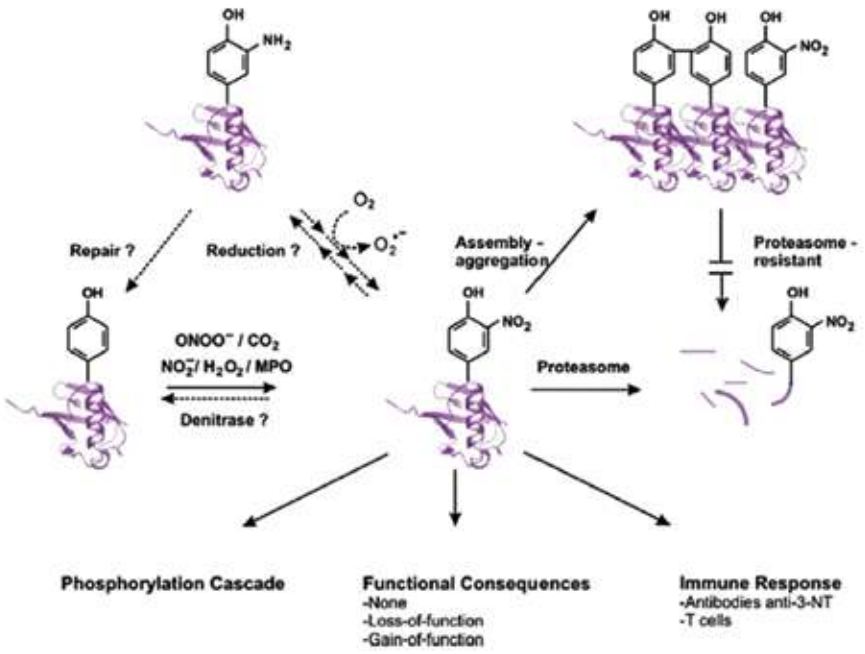


Figure 4. Formation and consequences of protein tyrosine nitration. Peroxynitrite-dependent and independent mechanisms of protein tyrosine nitration are indicated as well as the functional impact that nitration may have. Nitration of tyrosine residues many times is associated to tyrosine dimerization (and cross linking), as both nitration and dimerization required the intermediacy of tyrosyl radical. The mechanisms of turn over and removal of nitrated tyrosines from proteins are still not fully defined. (Reproduced from Souza *et al.*, 2008).

react with H_2O_2, peroxynitrite and even lipid hydroperoxides at significant faster rates than with typical thiols, notable examples being the enzymes of the peroxiredoxin family (Trujillo *et al.*, 2016). It is likely that peroxiredoxins not only contribute to catalytically detoxify peroxide, but also can represent "redox relays" in redox signaling processes (Stocker *et al.*, 2017).

In addition to the direct thiol oxidation reactions, the free radicals arising from peroxynitrite participate in another paradigmatic process, namely the formation of 3-nitrotyrosine. Indeed, the one-electron oxidation of tyrosine leads to tyrosyl radical that readily reacts with $^{\bullet}NO_2$ to yield a nitrated product. Protein 3-nitrotyrosine is a hallmark of the reactions of $^{\bullet}NO$-derived oxidants in vivo; moreover, nitration of key tyrosine residues can affect protein structure and function, alter tyrosine kinase-dependent signal transduction cascades, elicit immunogenic responses and affect protein turnover rates (Fig. 4) (Souza *et al.*, 2008; Radi, 2013a). Oxidized and nitrated proteins can be recognized and removed by proteolytic systems, including the proteasome, and favor protein turnover. Still, it is clear that during the aging process oxidized proteins (and other products such as oxidized lipids and DNA) accumulate and lead to an alteration of proteostasis (Lopez-Otín *et al.*, 2013).

Enzyme antioxidant networks, low molecular weight antioxidants and redox-based therapeutics

Antioxidant enzyme systems in mammalian cells are represented by the SODs, peroxiredoxins and glutathione peroxidases present both in the cytosol and mitochondria (Winterbourn, 2008). For some of these enzymes, extracellular forms (*e.g.* ECSOD) also play important roles in the modulation of tissue redox homeostasis. Another important H_2O_2 catabolizing enzyme is catalase, mainly present in peroxisomes. Altogether the array of antioxidant enzyme system can cope, at least partially, with enhanced formation rates of $O2_2^{\bullet -}$, H_2O_2 and even peroxynitrite (this last is also readily decomposed by peroxiredoxins). In the case of the peroxiredoxins and glutathione peroxidases, these enzymes need to be coupled to reducing systems such as thioredoxin or GSH to restore the enzymes back to the reduced (native) state. The reduction process ultimately depends on the correct supply of NADPH. Some low molecular weight compounds such as GSH, ascorbate, uric acid, α-tocopherol and ubiquinol also act as antioxidants intercepting reactive radicals intra- or extra-cellularly and at the plasma or mitochondrial membranes. It is important to indicate that while the mammalian antioxidant enzyme network plays usually homeostatic and tissue

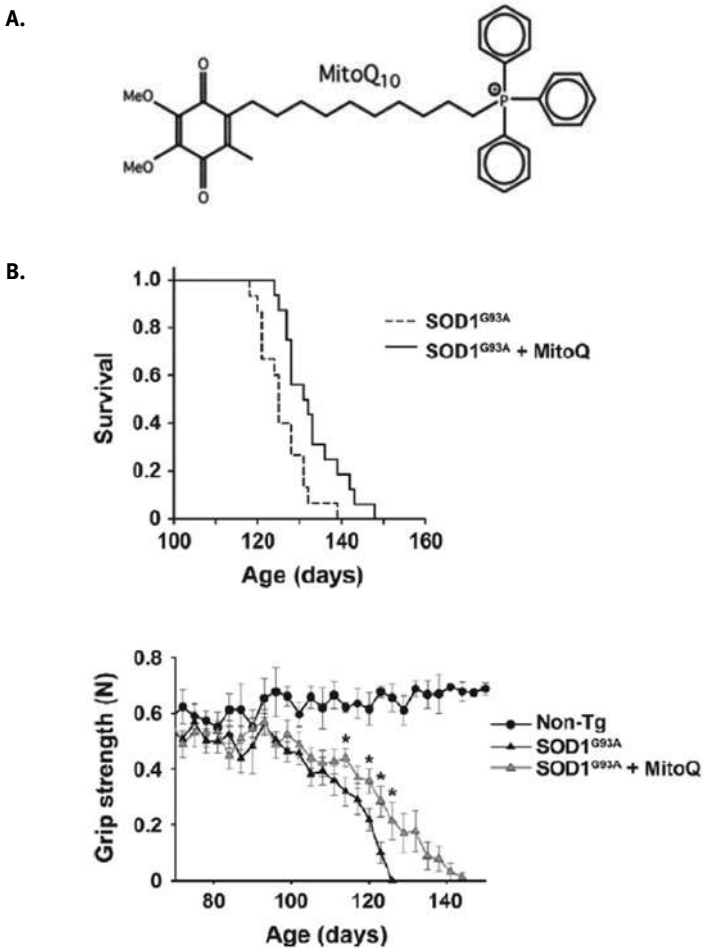


Figure 5. Mitochondrial-targeted redox active compound mitoQ in the treatment of an animal model of ALS. A. MitoQ structure. B. Oral administration of mitoQ after initiation of symptoms resulted in increased survival and improved grip strength test in the hSOD1 G93A mice. (Reproduced from Miquel *et al.*, 2014).

protective roles, in the case of invading pathogens (*e.g.* bacteria, parasites), their antioxidant enzyme systems (usually different to those of the host tissue) are utilized to evade the cytotoxic response from inflammatory cells and have been recently identified as virulence factors (Piacenza *et al.*, 2009). Thus, pathogens have evolved antioxidant enzyme systems to neutralize the oxidative response of the host (in good part mediated by NADPH oxidase

and NOS-dependent processes) (Nathan and Shiloh, 2000) and may be suitable as therapeutic targets for the combat of infections.

Genetic or pharmacological up-regulation of antioxidant enzyme systems has proven in many cases to confer protection to cells, tissues and organs in different disease models. Importantly, while for many years it was thought in the field that consumption or administration of redox active natural (*e.g.* polyphenols) or synthetic compounds (*e.g.* Mn-porphyrins) provided additional direct antioxidant protection to cells or tissues, it has now become very clear, that many of the therapeutic cytoprotective effects of tested redox active compounds rely on "indirect" effects *via* redox signaling (*e.g. via* the redox sensitive transcription factors Nrf-2 or Foxo) and secondary upregulation of enzyme systems (Fiuza *et al.*, 2015) An interesting aspect of the concept of redox-based therapeutics relates to the effect of redox active compounds that help to restore mitochondrial function and redox homeostasis in disease states. For instance, we have successfully used a mitochondria-targeted ubiquinol (mitoQ) in an animal model of motor neuron degeneration (resembling the development of ALS in humans, the hSOD1 G93A transgenic mice model) (Miquel *et al.*, 2014). Administration of mitoQ in the drinking water of animals *after* the initiation of the symptoms, resulted in better muscle mitochondria function, a slowing of disease progression, and extension of life span, compared to the transgenic animals with placebo (Fig. 5). This and several other recent studies reaffirm the idea of mitochondria-targeted redox-based therapeutics as a sound strategy to treat disease conditions where excess oxidant formation and mitochondrial dysfunction play a contributory role. Also, control and maintenance of cell and tissue redox homeostasis within physiological boundaries through life-style patterns that include regular exercise and balanced diet as well as by emerging preventive and therapeutic interventions becomes essential to promote healthy aging. By optimizing mitochondrial redox functions and antioxidant enzyme networks in human cells, tissues and organs, the unavoidable and continuous exposure to endogenous and exogenous oxidants is better managed, minimizing the inherent "risks" of aerobic life.

Acknowlegments

This work was supported by grants from Universidad de la República (CSIC and Espacio Interdisciplinario) and Agencia Nacional de Investigación e Innovación, Uruguay. Additional funding was obtained from PEDECIBA, the Howard Hughes Medical Institute, the National Institutes of Health and the Alexander Von Humboldt Foundation.

I am grateful to all my present and past co-investigators in Montevideo, Uruguay as well as international collaborators that have contributed to the work presented in this manuscript.

References

Babior, B.M. and Curnutte J.T. (1987), Chronic granulomatous disease – pieces of a cellular and molecular puzzle, *Blood Rev*. 1(4):215-8.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman BA. (1990), Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*. 87(4):1620-4

Brito, C., Naviliat. M., Tiscornia, A., Vuillier, A., Gualco, G., Dighiero, G., Radi, R. and Cayota, A. (1999), Peroxynitrite inhibits t-lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death, *J Immunol* 162(6):3356-66.

Fiuza, B., Subelzú, N., Calcerrada, P., Straliotto, M.R., Piacenza, L., Cassina, A., Rocha, J.B., Radi, R., de Bem, A.F., Peluffo, G. (2015), Impact of SIN-1-derived peroxynitrite flux on endothelial cell redox homeostasis and bioenergetics: protective role of diphenyl diselenide via induction of peroxiredoxins, *Free Radic Res.*49(2):122-32.

Fridovich, I. (1997), Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem*., 272(30):18515-7.

Knowles, R.G., Moncada, S. (1994), Nitric oxide synthases in mammals. *Biochem J.* 298 (Pt 2):249-58.

Miquel, E., Cassina, A., Martínez-Palma, L., Souza, J.M., Bolatto, C., Rodríguez-Bottero, S., Logan, A., Smith, R.A., Murphy, M.P., Barbeito, L., Radi, R., Cassina, P. (2014), Neuroprotective effects of the mitochondria-targeted antioxidant MitoQ in a model of inherited amyotrophic lateral sclerosis, *Free Radic Biol Med*. 70:204-13.

Murphy, M.P., Smith, R.A. (2007), Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol*. 47:629-56.

Nathan C, Shiloh MU. (2000), Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*. 97(16):8841-8.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G. (2013), The hallmarks of aging, *Cell*. 2013 Jun 6;153(6):1194-217.

Piacenza, L., Zago, M.P., Peluffo, G., Alvarez, M.N., Basombrio, M.A. and Radi, R. (2009), Enzymes of the antioxidant network as novel determiners of *Trypanosoma cruzi* virulence, *Int. J. Parasitol*. 39: 1455-1464.

Prolo, C., Alvarez, M.N., Radi, R. (2014), Peroxynitrite, a potent macrophage-derived oxidizing cytotoxin to combat invading pathogens, *Biofactors.* 40(2): 215-25.

Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991), Peroxynitrite oxidation of sulfhydryls: the cytotoxic potential of superoxide and nitric oxide, *J. Biol. Chem*. 266: 4244-4250.

Radi, R. (2013a), Protein Tyrosine Nitration: Biochemical Mechanisms and Structural Basis of Functional Effects, *Acc. Chem. Res*. 46 (2):550-9.

Radi, R. (2013b), Peroxynitrite, a stealthy biological oxidant, *J. Biol. Chem*. 13;288(37):26464-72.

Stöcker, S., Van Laer, K., Mijuskovic, A., Dick, T.P. (2017), The Conundrum

of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins asRedox Relay Hubs. *Antioxid Redox Signal*. doi: 10.1089/ars.2017.7162.

Szabó, C., Ischiropoulos, H. and Radi, R. (2007), Peroxynitrite: biochemistry, pathophysiology and development of therapeutics, *Nature Reviews Drug Discovery.* 6: 662-680.

Trujillo, M., Alvarez, B., Radi, R. (2016), One- and two-electron oxidation of thiols: mechanisms, kinetics and biological fates. *Free Radic Res*. 50(2):150-71.

van der Loo, B., Labugger, R., Skepper, J.N., Bachschmid, M., Kilo, J., Powell, J.M., Palacios-Callender, M., Erusalimsky, J.D., Quaschning, T., Malinski, T., Gygi, D., Ullrich, V., Lüscher, T.F. (2000), Enhanced peroxynitrite formation is associated with vascular aging, *J Exp Med*. 192(12):1731-44.

Winterbourn, C.C. (2008), Reconciling the chemistry and biology of reactive oxygen species, *Nat Chem Biol*. 4(5):278-86.

How Glycans in Glycoproteins Provide the Cell with Information on the Folding Status of the Protein Moieties

Armando J. Parodi[1]

Introduction

About a third (9000) of eukaryotic proteins follows the secretory pathway and approximately 80% of them are *N*-glycosylated in the endoplasmic reticulum (ER) lumen. This is a highly crowded environment (about 200 mg protein/ml) containing several conventional chaperones belonging to the Hsp70 (BiP) or Hsp90 (GRP94) families and several protein disulfide isomerases that promote the correct disulfide bond formation in a highly oxidizing milieu. *N*-glycosylation consists in the transfer in most species of the glycan Glc3Man9GlcNAc2 (G3M9) from a polyprenol lipid (dolichol) to the amidic nitrogen in the side chain of an Asn unit (see Fig 1A for the processing and lectin recognition of *N*-glycans in the early secretory pathway and Fig 1B for the structure of the glycan transferred to proteins). The amino acid must be in the sequence Asn-Xxx-Ser/Thr to be glycosylatred, where Xxx can be any amino acid except for Pro. Secretory proteins acquire their tertiary structures and in most cases also their quaternary ones in the ER lumen. Glycoproteins that fail to properly fold or, in the case of protein complexes, to acquire their quaternary structures are retrotranslocated to the cytosol to be degraded by the proteasomes. Two questions do emerge, therefore: first, how do cells distinguish between folding intermediates, irreparably misfolded glycoproteins or incompletely assembled complexes nevertheless formed by correctly folded subunits from properly folded glycoproteins or assembled complexes so as to prevent the Golgi exit of the former and allow secretion of the latter? And second, how do cells distinguish between folding intermediates from irreparably misfolded glycoproteins so as to allow the former additional possibilities for proper folding in the ER lumen but to send the latter to proteasomal degradation? It must be noted that folding intermediates are structurally undistinguishable from irreparably misfolded species. As will be

[1] Fundación Instituto Leloir, Buenos Aires.

further developed below, glycans in glycoproteins are the main participants in the answer to both questions (Parodi, 2000; Caramelo and Parodi, 2008; Caramelo and Parodi, 2015; D´Alessio *et al.*, 2010).

1. Glycan Processing. The ER Glucosidases

The constancy in the composition and structure of the glycan initially transferred in most species (Fig. 1B) sharply contrasts with the wide structural variation of *N*-glycans present in fully mature glycoproteins at their final destinations. The reason for this is that glycans are processed by removal and addition of several monosaccharides along the secretory pathway, mainly at the Golgi apparatus. On the one hand, the great variety of different glycans is well suited for the many recognition phenomena occurring at the cell surface in which glycan structures play central and discriminating roles. For instance cell differentiation, migration and proliferation are mediated or modulated by *N*-glycans. On the other hand, the glycan structures that are common to all glycoproteins and species at early secretion stages provide tools required for a process that is common to all glycoproteins, namely the acquisition of their native tertiary and in some cases also their quaternary structures in the ER (Larkin and Imperiali, 2011).

Processing of the protein-linked glycan in the ER involves two glucosidases, a glucosyltransferase and several mannosidases. Glucosidase I (GI), an α(1,2) exoglucosidase, is a type II membrane protein belonging to the glycosylhydrolase 63 family that is associated to the Sec61 translocon, a feature that ensures removal of the outermost glucose (residue n, Fig. 1B) immediately after glycan transfer to protein (Dejgaard *et al.,* 2010). Glucosidase II (GII) is a heterodimeric soluble protein belonging to the glycosylhydrolase family 31, as it displays the consensus sequence (G/F)(L/I/V/M) WXDMNE. It is responsible for removal of the two innermost glucose units (residues m and l, Fig. 1B). Subunit GIIα bears the catalytic activity and no retention/retrieval KDEL-like sequence. The latter is present at the C-terminus of the GIIβ subunit. GIIβ has a mannose receptor homologue (MRH) C-terminal domain, capable of oligomannosyl glycan recognition. Similar domains are present in other proteins of the secretory pathway as in the UDP-GlcNAc:lysosomal enzyme GlcNAc 1-P transferase (the first enzyme responsible for the creation of the Man 6-P epitope in lysosomal hydrolases), in receptors responsible for driving lysosomal hydrolases from the trans Golgi network to lysosomes and in a lectin (Osp9/OS9) involved in driving irreparably misfolded glycoproteins from the ER to proteasomal degradation in the cytosol (see below)(D'Alessio and Dahms, 2015; Olson

et al., 2015; Trombetta *et al.*, 1996). However, all mentioned MRH domains show different specificities towards oligomannosyl ligands displaying different structures. The GIIβ MRH domain greatly enhances, both *in vivo* and *in vitro*, the catalytic activity present in the GIIα subunit and its affinity towards *N*-glycans decreases as the number of mannose units diminishes (Hu *et al.*, 2009; Stigliano *et al.*, 2009). As expected, therefore, a decrease in the mannose content of *N*-glycans greatly reduces GIIα activity (Stigliano *et al.,* 2011). The main residue influencing it appeared to be residue k (Fig 1B). Concerning the relative speed of removal of both glucoses, that of the first cleavage (removal of residue m, Fig. 1B) is faster than that of the second one (removal of residue l, Fig. 1 B) (Totani *et al.*, 2006). This fact would provide better chances of interaction of glycoproteins with ER lectins calnexin (CNX) and calreticulin (CRT) (see below).

2. The Lectins (CNX and CRT)

Both CNX (65 kDa) and CRT (46 kDa) are ER permanent resident proteins that display an N-terminal signal peptide and a β-sandwich N-terminal domain that is similar to that of leguminous lectins and bears the lectin activities. In both proteins the lectin domains are followed by Pro-rich domains, called P-domains, and by acidic C-terminal domains. A transmembrane sequence appears in CNX before the cytosolic C-terminal domain. CRT is a soluble protein having a KDEL-like retention/retrieval sequence at its C-terminus, whereas CNX shows an RKPRRE ER-localization sequence at its C-terminus. The P-domain is characteristic of these two proteins. In the case of CNX it protrudes about 140 Å from the lectin domain and consists of four copies of motif 1 (IxDP(D/E)(A/D)xKP(D/E)DWD(D/E) followed by four copies of motif 2 (GxWxx-PxIxNPxY) in an 11112222 array. The motifs are present in four modules with a head to tail arrangement. The CRT P-domain is shorter, with only three copies of similar motifs. The P-domains are extremely flexible and are capable of embracing bound glycoproteins, thus preventing their aggregation. Both CNX and CRT strictly require the presence of a single glucose unit in the glycans for binding, but mannoses also contribute to the binding energy (Kb=2.2 104 M-1; 56 104 M-1 and 102 104 M-1 for Glcα1,3Man, Glcα1,3Manα1,2Man and Glcα1,3Manα1,2Manα1,2Man, respectively). 2-deoxyGlcα1,2Man is not recognized by CRT, thus showing that the equatorially oriented 2-hydroxyl group in glucose is required for binding (Kapoor *et al.,* 2003). Although CNX and CRT glycan binding features are identical, *in vivo* they bind an only partially overlapping

set of glycoproteins, the former preferring glycans located close to the ER inner membrane and the latter mainly binding glycans located distally to it (Hebert *et al.,* 1997). As further described below, BiP and CNX/CRT cooperate to assist the entire folding pathway of glycoproteins. Early folding intermediates preferentially interact with BiP and more advanced intermediates are recognized by the lectins (Labriola *et al.,* 2011; Molinari and Helenius, 2000; Wang *et al.,* 2005). It should be mentioned that some microorganisms only have one of the lectins. For instance trypanosomatid protozoa only have CRT, while the fission yeast *Schizosaccharomyces pombe* genome only codes for CNX.

CRT is one of the main ER calcium buffers as it accounts for about half the cation content of the organelle. The acidic C-terminal domain of the protein can bind about 25 calcium ions with low affinity (Kd about 1 mM). The CRT lectin and calcium buffering activities are mutually independent (Conte *et al*., 2007). CRT KO is embryonically lethal in mice (E14,5) and this effect is due to a deficient development of the heart (Mesaeli *et al.,* 1999). This effect can be prevented by expressing a constitutively active calcineurin in that organ. This result shows that CNX can compensate for the absence of CRT in the Glycoprotein Folding Quality Control (GFQC, see below), and stress the role of CRT in the regulation of cellular calcium homeostasis. Contrary to what happens with CRT, CNX KO mice are viable, although the animals have a dysmyelination phenotype in the peripheral and central nervous systems. Their immune system is not affected, they are fertile and have a normal lifespan (Kraus *et al*., 2010).

3. Two Enzymes Interacting with CNX/CRT

Two enzymes have been reported to interact with CNX/CRT. The first one (ERp57) is a member of protein disulfide isomerase family (Jessop *et al*., 2009). The domain organization of this enzyme is similar to that of PDI (protein disulfide isomerase proper), that is, four thioredoxin domains (a, b, b´, a´), in which a and a´ display the redox motif CGHC. Whereas in the PDI proper the b´ domain displays a hydrophobic patch responsible for binding protein substrates, the ERp57 b´ domain has a cluster of positively charged amino acids that interact with the negatively charged tip of CNX/CRT P-domains. This interaction is responsible for presentation of most substrates to ERp57. CNX-ERp57 interaction is modulated by a disulfide bond located at the P-domain (this bond is absent from CRT). Deletion of the disulfide bridge leads to a five-fold increase in the binding constant. The affinities between CNX/CRT and ERp57 are quite moderate (6 and

7 μM, respectively). Its fast off-rate (Koff > 1000 s-1) probably implies that ERp57 can rapidly act in several glycoprotein-lectin complexes. Additionally, it may be speculated that the high flexibility of the P-domains allows ERp57 to scan for far-located disulfide bonds. As will be further developed below, glycoprotein-CNX/CRT association results in increased folding efficiency, decreased aggregation and facilitation of proper disulfide bonding, this last effect being a consequence of CNX/CRT-ERp57 association.

The tips of the P-domains also mediate the interaction between the lectins and cyclophilin B (CypB), an ER member of the peptidyl prolyl *cys-trans* isomerase family, thus facilitating proper folding of CNX/CRT-bound glycoproteins (Kozlov *et al.,* 2010). A cluster of positively charged amino acids in CypB mediates its interaction with the lectins, with a Kd of about 10 μM. It may be speculated that CypB-CNX/CRT interaction improves the intrinsic low catalytic efficiency of the isomerase. Both CypB and ERp57 are very abundant species and how the balance between ERp57-CNX/CRT and CypB-CNX/CRT complexes is regulated is unknown.

4. The Protein Conformation Sensor (UGGT)

UGGT stands for UDP-Glc:glycoprotein glucosyltransferase. This is an ER resident, soluble, quite large enzyme (1555 residues in humans) that displays a hydrophobic N-terminal signal peptide and a C-terminal KDEL-like retention/retrieval sequence (except for the *Trypanosoma cruzi* enzyme that lacks that sequence)(Conte *et al.*, 2003; Fernández *et al.*, 1996; Trombetta and Parodi, 1992). The catalytic activity resides in the C-terminal domain (about 20% of the molecule) that is highly homologous to enzymes of the glycosyltransferase family 24. The rest (80% of the molecule) is believed to be responsible for sensing the conformation of the glucose acceptor glycoproteins. The enzyme requires calcium ions at millimolar concentrations for activity and has an optimal neutral pH for activity. It is highly specific for UDP-Glc as sugar donor (UDP-Gal, TDP-Glc and ADP-Glc proved to be ineffective) (Fernández *et al.*, 1984). Contrary to GII (UGGT´s opposing activity), removal of mannose units from arms B and C (Fig. 1B) does not affect UGGT activity (Stigliano *et al.*, 2011). The enzymatic activity was first detected in *T. cruzi*: as Man9GlcNAc2 (M9) and not G3M9 is transferred to protein in this protist, detection of the transient presence of protein-linked Glc1Man9GlcNAc2 (G1M9) upon labeling cells with [14C]glucose implied the occurrence of a direct glycoprotein glucosylation reaction (Parodi and Cazzulo, 1982). Further studies showed that a similar reaction occurred in mammalian, plant and fungal

cells: on complete deglucosylation of the transferred glycan (G3M9) by GI and GII, a glucose unit may be transferred directly from UDP-Glc, thus recreating a G1M9 glycan structurally identical to that produced by partial deglucosylation of the transferred glycan (Parodi *et al.,* 1983; Trombetta *et al.,* 1989). On devising an assay for purifying the enzyme, it was observed that incubation of rat liver microsomes with UDP-[14]Glc plus a glycoprotein displaying M9, Man8GlcNAc2 (M8) and Man7GlcNAc2 (M7) as putative acceptors (thyroglobulin), only resulted in a successful single glucose transfer reaction when the glycoprotein had been previously denatured with 8 M urea and not allowed to properly fold when withdrawing the drug (Trombetta *et al.*, 1989). In fact, it was shown that UGGT not only recognized the protein conformation of the acceptor glycoprotein but also the innermost GlcNAc unit of the glycan (Sousa and Parodi, 1995). This residue is normally occluded by neighboring amino acids in native conformations. The use of synthetic glycopeptides of increasing length displaying well characterized three dimensional structures (up to 64 amino acids with the glycopeptide M9-Asn chemically attached to C7) as substrates showed that UGGT maximum kinetic efficiency coincided with a maximum capacity for 8-anilino-1-naphtalene sulfonate (ANS) binding (Caramelo *et al.*, 2003). This compound specifically binds hydrophobic patches. Optimal glycopeptides displayed the so called molten globule conformations, already having most secondary and some tertiary native structures but exposing hydrophobic patches as amino acid side chains are not yet buried in the molecule interior. Furthermore, partially assembled glycoprotein complexes containing a suboptimal number of correctly folded subunits were found to act as UGGT acceptors (Keith *et al.,* 2005). In this case the hydrophobic surfaces exposed by the lack of the full subunit complement were reported to be the structural features recognized by UGGT. Not surprisingly, M9 linked to a hydrophobic peptide served as an acceptor, provided it had a suitable length (Taylor *et al.*, 2003). Moreover, the same glycan linked to a hydrophobic, non-proteinaceous aglycone also served as an acceptor (Totani *et al.,* 2009). In addition, UGGT proved to be an extremely sensitive sensor of glycoprotein conformations as it differentially glucosylated glycoproteins only slightly differing in their tertiary structures (Caramelo *et al.,* 2004). The above mentioned UGGT acceptor substrate conformational requirements that were found in cell-free assays indicated that probably glycoproteins in their last folding stages, that is, in stages exposing hydrophobic patches, are UGGT optimal substrates in the live cell. In fact, *in vivo* experiments confirmed this idea as a glycoprotein displaying

several disulfide bonds was monoglucosylated by UGGT only when all or most of such bonds had been already formed (Labriola *et al.,* 1999).

UGGT KO in mice is embryonically lethal at E13 but it is not essential for survival in plants (Molinari *et al.,* 2005). In the case of *S. pombe,* UGGT is required for viability under extreme ER stress conditions (Fanchiotti *et al.*, 1998).

There are two homologues coding for UGGT-like proteins in Euteleostomi, which is a successful clade that includes more than 90% of the living species of vertebrates, and at least in some species of nematodes belonging to the genus Caenorhabditis. Recent studies showed both mammalian proteins (called UGGT1 and UGGT2) were enzymatically active (Takeda *et al.,* 2014).

The tertiary structure of UGGT from a thermophilic yeast (*Chaetomium thermophilum*) has been solved recently by X-ray crystallography (Roversi *et al.,* 2017). The enzyme has four thioredoxin-like domains arranged in a long arc that terminates in two β-sandwiches tightly clasping the C-terminal glucosyltransferase domain. Cryo-EM reconstruction revealed an extreme interdomain flexibility. It was speculated that the intrinsic UGGT flexibility of the thioredoxin domains endow the enzyme with the promiscuity required to reglucosylate many substrates of different forms and shapes and/or with the ability to reglucosylate *N*-glycans located at variable distances from the misfolded site.

5. An Overall Picture of the Quality Control of Glycoprotein Folding (QCGF)

Information provided above and additional non-mentioned results suggest the following mechanism for the QCGF (see Fig. 1A): as a protein enters the ER lumen through the Sec61 translocon it may be *N*-glycosylated by the octameric oligosaccharyltransferase complex. The protein moiety interacts then with the classical chaperone BiP that assist the first glycoprotein folding stages. The sequential action of GI and GII then generates monoglucosylated species that are recognized by the unconventional chaperone-lectins CNX/CRT. It may be speculated that at this stage glycoproteins are already displaying a molten globule-like conformation and that they are exposing hydrophobic patches. The lectin-glycoprotein interaction prevents the Golgi exit of folding intermediates, of irreparably misfolded glycoproteins and of incompletely assembled glycoprotein complexes. In addition it enhances folding efficiency. The glycoprotein-lectin P-domain embrace may be in part responsible for this last effect as it prevents aggregation. In addition CNX/CRT lectin-associated enzymes

ERp57 and CypB may help in forming the correct disulfide bonds and in *cis-trans* proline isomerization. Upon GII removal of the innermost glucose (residue l, Fig. 1B), the glycoprotein is liberated from the lectin anchors. If properly folded it is allowed to continue the secretory pathway but if not it may be reglucosylated by UGGT, thus recreating the CNX/CRT-glycoprotein interaction. Cycles of reglucosylation-deglucosylation catalyzed by the opposing activities of UGGT and GII would continue until the glycoprotein adopts its native final tertiary structure (or in the case of protein complexes also the correct subunit composition). Finally, if cells realize that the glycoprotein is irreparably misfolded or unable to form a complex with the expected subunit complement, it is sent to proteasomal degradation by the ERAD process (see below).

6. ERAD or the Way to Gently Send Misfolded Glycoproteins to the Gallows

As mentioned above, *N*-glycans in the ER may be demannosylated in addition to deglucosylated. The former process is much slower than the latter. In fact, demannosylation constitutes a "mannose time-clock" that identifies glycoproteins staying for rather long time periods in the ER lumen, as misfolded proteins and incompletely assembled glycoprotein complexes do.

There are two ER α-mannosidases in the yeast lumen. ER α-mannosidase I removes residue i (Fig. 1B), thus generating M8B (the mannose residue is removed from arm B). A second α-mannosidase, called Htm1p then removes residue k (Fig. 1B) and M7BC is thus generated. This last mannosidase was found to be forming a complex with PDI proper. The oxidoreductase enhanced the mannosidase activity of Htm1p and participated in the recognition of ERAD substrates (Gauss *et al.*, 2011). The α(1,6)-linked mannose unit exposed upon removal of both mannoses (residue j Fig. 1B) is then recognized by a lectin (called Osp9 in yeast and OS9 in mammals) that bears a MRH domain (Hosokawa *et al.*, 2009; Mikami *et al.,* 2011). Contrary to what happens with GIIβ MRH domain, that in Osp9/OS9 preferentially binds M7BC instead of M9. The lectin drives then the glycoprotein bearing demannosylated M7BC *N*-glycans to proteasomal degradation. There is an ER α-mannosidase I in the ER of mammalian cells as well as other similar proteins called EDEMs 1, 2 and 3 that carry out mannose trimming. The latter have extremely low α-mannosidase activities, even though they have all the amino acids required for removing α-linked mannose units. ER α-mannosidase I and EDEM 1 are single-pass

type II membrane proteins, whereas EDEMSs 2 and 3 are soluble proteins. It is unclear for the moment which of the four mannosidases removes each individual mannose unit, but as in the case of yeasts, the final product to be degraded exposes residue j (Fig. 1B) (Avezov *et al.*, 2008; Hosokawa *et al.*, 2010; Ninagawa *et al.*, 2014; Tamura *et al.*, 2011). On the other hand, a prolonged permanence in the ER leads to removal of residue g (Fig. 1B). Absence of that residue prevents UGGT-mediated addition of glucose units, thus resulting in the exit of glycoproteins from CNX/CRT cycles and in facilitating ERAD (Frenkel *et al.*, 2003).

The slow sequential removal of residues i and k in mammalian cells provides two checkpoints to ensure that only irreparably misfolded glycoproteins are derived for degradation. In the case of the budding yeast (*S. cerevisiae*), practically all *N*-glycans are rapidly demannosylated to M8B even those present in correctly folded glycoproteins. The slow removal of the second residue (residue k, Fig. 1B) by Htm1p provides in this case only one checkpoint. The fission yeast *S. pombe* has the same complement of ER α-mannosidases as the budding one but in this case both demannosylation reactions are slow processes (Movsichoff *et al.*, 2005).

The balance between GII and UGGT activities is pivotal in determining glycoprotein binding to CRT/CNX. As mentioned above, *in vivo* observations indicate that UGGT activity is not affected by removal of mannose residues, whereas that of GII sharply decreases. This would ensure the ER retention of glycoproteins showing folding difficulties as the half-lives of monoglucosylated increase upon progressive mannose removal (Stigliano *et al.*, 2011). Nevertheless, this may also negatively affect the passage of terminally misfolded proteins to the ERAD machinery. Here lies one major puzzle, since the QCGF and ERAD machineries should discriminate folding intermediates within a productive pathway from terminally misfolded proteins. A mistake in any other way may have dangerous consequences. Interestingly, even though UGGT delays the secretion of immature glycoproteins, the enzyme does not affect the processing of misfolded species by ERAD (Tannous *et al.*, 2015). This implies that somehow the ERAD machinery can extract very efficiently the substrates from CNX/CRT cycles.

Retrotranslocation of misfolded glycoproteins from the ER lumen to the cytosol to be eventually degraded by proteasomes ultimately depends of protein complexes some of whose components are integral ER membrane proteins (Ruggiano *et al.,* 2014). Depending on the location of the misfolded domain, proteins are delivered to proteasomal degradation

through different complexes. Thus, ER membrane proteins with folding defects in the cytosolic portion of the molecule (ERAD-C) are extracted from the ER membrane by the so called Doa10 complex, whereas those in which the folding defect is present in the lumenal (ERAD-L) or membrane (ERAD-M) portions of the molecules utilize the Hrd1 complex. Both Doa10p and Hrd1p are integral membrane proteins showing E3 ubiquitin-ligase activity in their cytosolic portions. In addition, other proteins both lumenal and cytosolic, form part of the complexes. Some of them are chaperones; others display E2 ubiquitin-conjugating activity or participate in misfolded protein recognition. For instance, glycoproteins exposing α(1,6)-linked mannosyl units bound to Osp9/OS9 are driven to degradation only if present in unstructured polypeptides, a feature recognized by Hrd3p, a protein found in the Hrd1 complex. Finally, there are protein components common to both complexes, as for instance p97 in mammals, which is responsible for the membrane extraction of misfolded proteins in an ATP-dependent manner. It should be mentioned that the pore by which ERAD-L substrates are actually transported to the cytosol has not been unequivocally identified yet but it has been established that to be able to be translocated to the cytosol, misfolded lumenal proteins must be previously unfolded in the ER lumen, a process involving conventional chaperones and protein disulfide isomerases.

N-glycans are removed from the protein moieties prior to proteasomal degradation. It appears that a cytosolic peptide:*N*-glycanase (PNGase) plays an important role in both removing the glycan and constructing an efficient pre-degradation complex. The PNGase recognizes only misfolded or denatured glycoproteins and the enzyme is bound to the proteasome by subunits S4 and HR23B as a complex with cytosolic protein Cdc48, a component of both ERAD complexes. Degradation of the released glycan occurs in two stages. First, partial cleavage occurs between the chitobiose core via a cytosolic endo-β-N-acetylglucosaminidase or possibly a neutral-pH cytoplasmic chitobiase. A cytosolic α-mannosidase cleaves up to four mannose residues to generate Man5GlcNAc (residues b, c, d, e, f and g, Figure 1B). This glycan is then taken into the lysosome for final degradation to monosaccharides (Joshi *et al.,* 2005; Li *et al.,* 2005; Moore, 1999; Suzuki *et al.,* 2002).

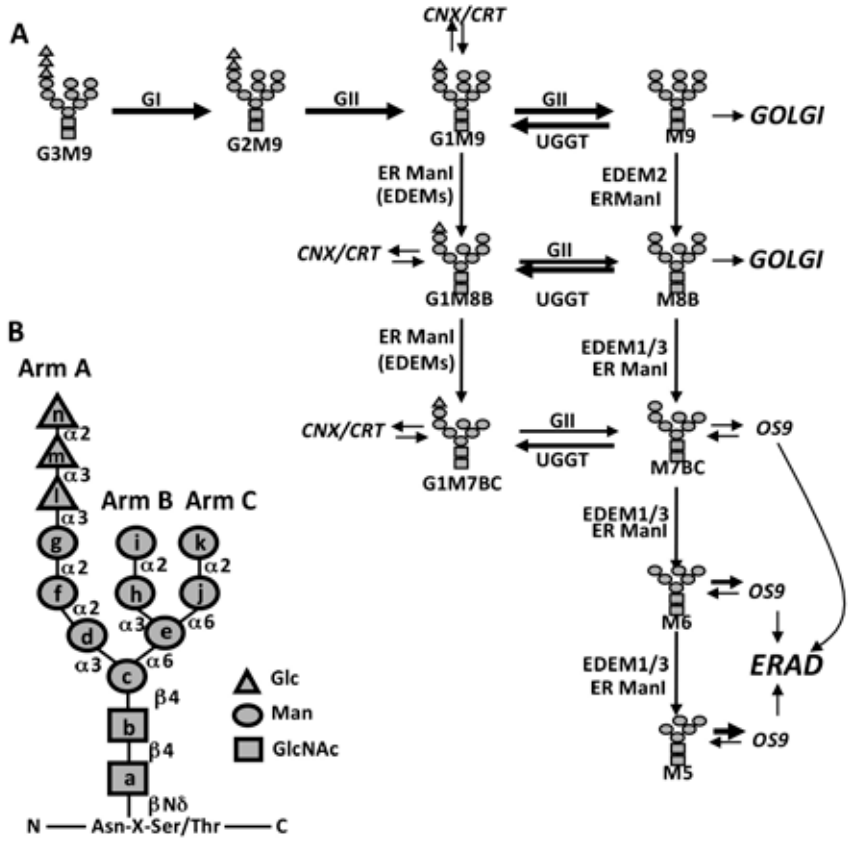


Figure 1. A. Processing of *N*-glycans in the endoplasmic reticulim; B. Structure of the glycan transferred to proteins in most eukaryotic cells (Glc3Man9 GlcNAc2).

Bibliography

Avezov, E., Frenkel, Z., Ehrlich, M., Herscovics, A. and Lederkremer, G. Z. (2008), Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for *N*-glycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation, *Mol. Biol. Cell 19*, 216-25.

Caramelo, J.J. and Parodi, A.J. (2008), Getting in and out from calnexin/calreticulin cycles, *J. Biol. Chem. 283*, 10221-25.

Caramelo, J.J. and Parodi, A.J. (2015), A sweet code for glycoprotein folding, *FEBS Lett.* 589, 3379-87.

Caramelo, J.J., Castro, O. A., Alonso, L.G., de Prat-Gay, G. and Parodi, A.J. (2003), UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates, *Proc. Natl. Acad. Sci. U.S.A. 100*, 86-91.

Caramelo, J.J., Castro, O.A., de Prat-Gay, G. and Parodi, A.J. (2004), The endoplasmic reticulum glucosyltransferase recognizes nearly native glycoprotein folding intermediates, *J. Biol. Chem. 279*, 46280-5.

Conte, I.L., Keith, N., Gutierrez-Gonzalez, C., Parodi, A.J. and Caramelo, J.J. (2007), The interplay between calcium and the *in vitro* lectin and chaperone activities of calreticulin, *Biochemistry 46*, 4671-80.

Conte, I.L., Labriola, C., Cazzulo, J.J. Docampo, R. and Parodi, A.J. (2003), The interplay between folding-facilitation mechanisms in *Trypanosoma cruzi* endoplasmic reticulum, *Mol. Biol. Cell 14*, 3529-40.

D'Alessio, C. and Dahms, N.M. (2015), Glucosidase II and MRH-domain containing proteins in the secretory pathway, *Curr. Protein & Pept. Sci. 16*, 31-48.

D'Alessio, C., Caramelo, J.J. and Parodi, A.J. (2010), UDP-glc:glycoprotein glucosyltransferase-glucosidase II, the ying-yang of the ER quality control, *Semin. Cell Dev. Biol. 21*, 491-99.

Dejgaard, K., Theberge, J.F., Heath-Engel, H., Chevet, E., Tremblay, M.L. and Thomas, D.Y. (2010), Organization of the Sec61 translocon, studied by high resolution native electrophoresis, *J. Proteom. Res. 9*, 1763-71.

Fanchiotti, S., Fernandez, F., D'Alessio, C. and Parodi, A. J. (1998), The UDP-Glc:Glycoprotein glucosyltransferase is essential for *Schizosaccharomyces pombe* viability under conditions of extreme endoplasmic reticulum stres, *J. Cell Biol. 143*, 625-35.

Fernández, F., Jannatipor, M., Hellman, U., Rokeach, L.A. and Parodi, A.J. (1996), A new stress protein: synthesis of *Schizosaccharomyces pombe* UDP-Glc:glycoprotein glucosyltransferase mRNA is induced under stres conditions but the enzyme is not essential for viability, *EMBO J. 15*, 705-13.

Fernández, F.S., Trombetta, S.E., Hellman, U. and Parodi, A.J. (1994), Purification to homoheneity of UDP-Glc:glycoprotein glucosyltransferase from *Schizosaccharomyces pombe* and apparent absence of the enzyme from *Saccharomyces cerevisiae*, *J. Biol. Chem. 269*, 30701-6.

Frenkel, Z., Gregory, W., Kornfeld, S. and Lederkremer, GZ. (2003) Endoplasmic reticulum-associated degradation of mammalian glycoproteins involves sugar chain trimming to Man6-5GlcNAc2, *J . Biol. Chem. 278*, 34119-24.

Gauss, R., Kanehara, K., Carvalho, P., Ng, D.T. and Aebi, M. (2011), A complex of Pdi1p and the mannosidase Htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum, *Mol. Cell 42*, 782-93.

Hebert, D.N., Zhang, J.X., Chen, W., Foellmer, B. and Helenius, A. (1997), The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin, *J. Cell Biol.. 139*, 613-23.

Hosokawa, N., Kamiya, Y., Kamiya, D., Kato, K. and Nagata, K. (2009) Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed *N*-glycans, *J. Biol. Chem. 284*, 17061-8.

Hosokawa, N., Tremblay, L.O., Sleno, B., Kamiya, Y., Wada, I., Nagata, K., Kato, K. and Herscovics, A. (2010), EDEM1 accelerates the trimming of alpha1,2-linked mannose on the C branch of *N*-glycans, *Glycobiology. 20*, 567-75.

Hu, D., Kamiya, Y., Totani, K., Kamiya, D., Kawasaki, N., Yamaguchi, D., Matsuo, I., Matsumoto, N., Ito, Y., Kato, K. and Yamamoto, K. (2009), Sugar-binding activity of the MRH domain in the ER alpha-glucosidase II beta subunit is important for efficient glucose trimming, *Glycobiology 19*, 1127-35.

Jessop, C.E., Tavender, T.J., Watkins, R. H., Chambers, J.E. and Bulleid, N.J. (2009), Substrate specificity of the oxidoreductase ERp57 is determined primarily by its interaction with calnexin and calreticulin, *J. Biol. Chem. 284*, 2194-202.

Joshi, S., Katiyar, S. and Lennarz, W.J. (2005) Misfolding of glycoproteins is a prerequisite for peptide:*N*-glycanase mediated deglycosylation, *FEBS Lett. 579*, 823-26.

Kapoor, M., Srinivas, H., Kandiah, E., Gemma, E., Ellgaard, L., Oscarson, S., Helenius, A. and Surolia, A. (2003), Interactions of substrate with calreticulin, an endoplasmic reticulum chaperone, *J. Biol. Chem. 278*, 6194-200.

Keith, N., Parodi, A.J. and Caramelo, J.J. (2005), Glycoprotein tertiary and quaternary structures are monitored by the same quality control mechanism, *J. Biol. Chem. 280*, 18138-41.

Kozlov, G., Bastos-Aristizabal, S., Maattanen, P., Rosenauer, A., Zheng, F., Killikelly, A., Trempe, J.F., Thomas, D.Y. and Gehring, K. (2010), Structural basis of cyclophilin B binding by the calnexin/calreticulin P-domain, *J. Biol. Chem. 285*, 35551-7.

Kraus, A., Groenendyk, J., Bedard, K., Baldwin, T.A., Krause, K.H., Dubois-Dauphin, M., Dyck, J., Rosenbaum, E.E., Korngut, L., Colley, N.J., Gosgnach, S., Zochodne, D., Todd, K., Agellon, L.B. and Michalak, M. (2010), Calnexin deficiency leads to dysmyelination, *J. Biol. Chem. 285*, 18928-38.

Labriola, C., Cazzulo, J.J. and Parodi, A.J. (1999), *Trypanosoma cruzi* calreticulin is a lectin that binds monoglucosylated oligosaccharides but not prorein moieties of glycoproteins, *Mol. Biol. Cell 10*, 1381-94.

Labriola, C.A., Giraldo, A.M., Parodi, A.J. and Caramelo, J. (2011,) Functional cooperation between BiP and calreticulin in the folding maturation of a glycoprotein in *Trypanosoma cruzi*, *Mol. Biol. Parasit. 175*, 112-7.

Larkin, A., and Imperiali, B. (2011), The expanding horizons of asparagine-linked glycosylation, *Biochemistry. 50*, 4411-26.

Li, G., Zhou, C.X., Zhao, G., Schindelin, H. and Lennarz, W.J. (2005), Multiple forms of interaction of the deglycosylation enzyme, mouse peptide:*N*-glycanase with proteasome, *Proc. Natl. Acad. Sci. U.S.A. 102,* 15809-14.

Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K.H., Opas, M., MacLennan, D.H. and Michalak, M. (1999,) Calreticulin is essential for cardiac development, *J. Cell Biol. 144*, 857-68.

Mikami, K., Yamaguchi, D., Tateno, H., Hu, D., Qin, S.Y., Kawasaki, N., Yamada, M., Matsumoto, N., Hirabayashi, J., Ito, Y. and Yamamoto, K. (2010,) The sugar-binding ability of human OS-9 and its involvement in ER-associated degradation, *Glycobiology.* 20, 310-21.

Moore S.E.H. (1999) Oligosaccharide transport: pumping waste from the ER into lysosomes. *Trends Cell Biol. 9,* 441-46.

Molinari, M. and Helenius, A. (2000), Chaperone selection during glycoprotein translocation into the endoplasmic reticulum, *Science 288*, 331-3.

Molinari, M., Galli, C., Vanoni, O., Arnold, S.M. and Kaufman, R.J. (2005), Persistent glycoprotein misfolding activates the glucosidase II/UGT1-driven calnexin cycle to delay aggregation and loss of folding competence, *Mol. Cell 20*, 503-12.

Movsichoff, F., Castro, O.A. and Parodi, A.J. (2005), Characterization of *Schizosaccharomyces pombe* ER α-mannosidase. A re-evaluation of the role of the enzyme on ER-associated degradation, *Mol. Biol. Cell 16*, 4714-24.

Ninagawa, S., Okada, T., Sumitomo, Y., Kamiya, Y., Kato, K., Horimoto, S.,

Ishikawa, T., Takeda, S., Sakuma, T., Yamamoto, T. and Mori, K. (2014), EDEM 2 initiates mammalian glycoprotein ERAD by catalyzing the first mannose-trimming step, *J. Cell Biol. 206*, 347-56.

Olson, L J., Orsi, R., Peterson, F.C., Parodi, A.J., Kim, J.J., D'Alessio, C. and Dahms, N M. (2015), Crystal structure and functional analyses of the lectin domain of glucosidase II: insights into oligomannose ecognition, *Biochemistry 54*, 4097-111.

Parodi, A.J. (2000), Protein glucosylation and its role in protein folding, *Annu. Rev. Biochem. 69,* 69-93.

Parodi, A.J. and Cazzulo, J.J. (1982), Protein glycosylation in *Trypanosoma cruzi*. II. Partial characterization of protein-bound oligosaccharides labeled "*in vivo", J. Biol.* Chem. *257*, 7641-5.

Parodi, A.J., Mendelzon, D.H. and Lederkremer, G.Z. (1983), Transient glucosylation of protein-bound Man9GlcNAc2, Man8GlcNAc2 and Man7GlcNAc2 in calf thyroid cells. A possible recognition signal in the processing of glycoproteins*, J. Biol. Chem. 258*, 8260-5.

Roversi, P., Marti, L., Caputo, A.T., Alonzi, S.S., Hill, J.C., Dent, K.C., Kumar, A., Levasseur, M.D., Lia, A., Waksman, T., Basu, S., Soto Albrect, Y., Qian, K., McIvor, J.P., Lipp, C.B., Siliqi, D., Vasiljevik, S., Mohammed, S., Lukacik, P., Walsh, M.A., Santino, A. and Zitzmann, N. (2017), Interdomain conformational flexibility underpins the activity of UGGT, the eukaryotic glycoprotein secretion checkpoint, *Proc. Natl. Acad. Sci. U.S.A. 114,* 8544-49.

Ruggiano, A., Foresti, O. and Carvalho, P. (2014), ER-associated degradation, protein quality control and beyond*, J. Cell Biol. 204*, 869-79.

Sousa, M. and Parodi, A.J. (1995), The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase, *EMBO J. 14*, 4196-203.

Sousa, M.C., Ferrero-Garcia, M.A. and Parodi, A.J. (1992), Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase, *Biochemistry 31*, 97-105.

Stigliano, I.D., Alculumbre, S.G., Labriola, C.A., Parodi, A J. and D'Alessio, C. (2011), Glucosidase II and *N*-glycan mannose content regulate the half-lives of monoglucosylated species *in vivo*, *Mol. Biol. Cell. 22*, 1810-23.

Stigliano, I.D., Caramelo, J.J., Labriola, C A., Parodi, A.J. and D'Alessio, C. (2009), Glucosidase II beta subunit modulates *N*-glycan trimming in fission yeasts and mammals, *Mol. Biol. Cell 20*, 3974-84.

Suzuki, T., Yano, K. Sugimoto, S., Kitajima, K., Lennarz, W.J., Inoue, S. Inoue, Y. and Emory, Y. (2002), Endo-beta-N-acetylglucosaminidase, an enzyme involved in processing of free oligisaccharides in the cytosol, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9691-96.

Takeda, Y., Seko, A., Hachisu, M., Daikoku, S., Izumi, M., Koizumi, A., Fujikawa, K., Kajihara, Y. and Ito, Y. (2014, Both isoforms of human UDP-glucose:glycoprotein glucosyltransferase are enzymatically active, *Glycobiology*. 24, 344-50.

Tamura, T., Cormier, J.H. and Hebert, D.N. (2011), Characterization of early EDEM1 protein maturation events and their functional implications, *J. Biol. Chem. 286*, 24906-15.

Tannous, A., Patel, N., Tamura, T. & Hebert, D.N. (2015), Reglucosylation by UDP-glucose:glycoprotein glucosyltransferase 1 delays glycoprotein secretion but not degradation, *Mol. Biol. Cell. 26*, 390-405.

Taylor, S.C., Thibault, P., Tessier, D.C., Bergeron, J J. and Thomas, D.Y. (2003,) Glycopeptide specificity of the secretory protein folding sensor UDP-glucose

glycoprotein:glucosyltransferase, *EMBO Rep. 4*, 405-11.

Totani, K., Ihara, Y., Matsuo, I. and Ito, Y. (2006), Substrate specificity analysis of endoplasmic reticulum glucosidase II using synthetic high mannose-type glycans, *J. Biol. Chem. 281*, 31502-8.

Totani, K., Ihara, Y., Tsujimoto, T., Matsuo, I. and Ito, Y. (200,) The recognition motif of the glycoprotein-folding sensor enzyme UDP-Glc:glycoprotein glucosyltransferase, *Biochemistry. 48*, 2933-40.

Trombetta, E.S., Simons, J.F. and Helenius, A. (1996), Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit, *J. Biol. Chem. 271*, 27509-16.

Trombetta, S.E. and Parodi, A.J. (1992) Purification to apparent homogeneuty and partial characterization of rat liver UDP-Glc:glycoprotein glucosyltransferase, *J. Biol. Chem. 267*, 9236-40.

Trombetta, S.E., Bosch, M. and Parodi, A.J. (1989) Glucosylation of glycoproteins by mammalian, plant, fungal and trypanosomatid protozoa microsomal membranes, *Biochemistry 28*, 8108-16.

Wang, N., Daniels, R. and Hebert, D N. (2005), The cotranslational maturation of the type I membrane glycoprotein tyrosinase: the heat shock protein 70 system hands off to the lectin-based chaperone system, *Mol. Biol. Cell 16*, 3740-52.

Genetics Session

Design of Novel Regulatory Gene Circuits In Plant and Mammalian Cells Utilizing Recombinase Serine Integrases and Sustainable Intensification of S&T In Latin America

Elibio Rech

Introduction

Early humans domesticated plant and animal species based on ancient empirical concepts (Darwin,1868; 1876). In 1886, Mendel established a new paradigm of hereditary laws (Mendel, 1866; 1870; 1950) based on genotypic and phenotypic traits of cross-compatible species, establishing a complex breeding technology that is currently utilized for the development of most food and livestock-derived products. Recently, studies on deciphering the double-helical structure (Watson & Crick, 1953a,b) and how to restrict DNA (Arber, 2012) have established the foundation of recombinant DNA technology. A new era is paving the way for genetic manipulation of important traits among all the kingdom's organisms, allowing for the development of innovative and widely utilized products for the agricultural, industrial and pharmaceutical production sectors (Mc Elroy, 2003, 2004; ISAAA, 2016).

Currently, it is possible to state that the world has reached the mature stage of recombinant DNA technology, which, in turn, may allow us to envisage the capacity to generate a significant number of novel processes and products for the benefit of human beings and sustainable utilization and conservation of biodiversity and the environment as a whole. One possibility was recently termed "synthetic domestication of useful traits" (Rech and Arber, 2013). Although technical issues are still a challenge, the wide potential of progress is accelerating. Nevertheless, ethics and a comprehensive regulatory system ought to be discussed to guarantee deeper and higher standards of deregulation before any product reaches the market.

For critical evaluation and discussion, I would like to present remarks and insights considering three main points: 1) examples of the utilization of recombinant DNA technology and genetic engineering, including sustainable utilization of biodiversity based on a "humanitarian concept"; 2)

how and why to intensify science and technology (S&T) in Latin America; and 3) equalization of components for progressive development.

The principal conclusion is that the introduction of S&T into the production sectors is one of the key components of progress, including conservation and utilization of biodiversity and reduction of inequity in low-income segments. However, this progress is beneficial only if it is intrinsically related and equalized with components such as human health, high-quality education, food security, environmental focus and added market value.

1. Recombinant DNA technology and genetic engineering

Currently, available tools and technologies for synthetic domestication of useful traits have opened the door for another breakthrough in scientific progress (Cermak *et al.*, 2011; Doyon *et al.*, 2011; Gibson *et al.*, 2008; Rech & Arber, 2013; Cong *et al.*, 2013; Boch *et al.*, 2009; Carlson & Lancto, 2016). Making use of genome editing (Boch *et al.*, 2009; Mali *et al.*, 2013; Li *et al.*, 2013; Jiang *et al.*, 2013; Cong *et al.*, 2013; Doudna & Charpentier, 2014; Carlson & Lancto, 2016), a template founded on molecular synthesis, assembly and synthetic biology (Yu *et al.*, 2006; Gibson *et al.*, 2008; 2010; Annaluru *et al.*, 2014; Hutchison *et al.*, 2016), has allowed for the sustainable prospection and manipulation of innovative traits found in biodiversity. As an example, I will comment on three studies, which may serve as practical examples.

1.1. Production of synthetic spider fibre

Spider silk fibre has been noted for its unique physical and mechanical properties and has been recognized as a protein-based nanomaterial (Lewis, 2006; Silva & Rech, 2013). To this end, the functional genome of different Brazilian spiders' silk glands have been studied, considering evolution and evaluating the potential development of novel biopolymers through synthetic biology (Bittencourt *et al.*, 2010; Prosdocimi *et al.*, 2011). Drawing on genome and transcriptome data, it has become possible to design *de novo* proteins and produce synthetic spider-like fibres in bacteria. This has led to the development of alternative strategies for the production of synthetic silk proteins using recombinant DNA technology (Teulé *et al.*, 2009; Murad & Rech, 2011). A complete procedure was developed for the artificial spinning of fibres made from recombinant proteins based on chimeric or native synthetic spider silk-like sequences produced through genetic engineering in *E. coli*. The strategy is to build large synthetic spider

silk-like tandem repeat sequences from small double-stranded monomer DNAs flanked by compatible but non-regenerable restriction sites.

1.2. Engineering soybean seeds as a scalable platform to produce a microbicide against HIV

There is an urgent need to provide effective anti-HIV microbicides to resource-poor areas worldwide. Some of the most promising microbicide candidates are biotherapeutics targeting viral entry. To provide biotherapeutics to poorer areas, it is vital to reduce their cost. Cyanovirin-N (CV-N), an 11 009 Da protein isolated from cultures of the cyanobacterium *Nostoc ellipsosporum*, is a potent lectin capable of irreversibly inactivating diverse strains of HIV (types 1 and 2) and simian immunodeficiency virus (Boyd *et al.*, 1997). We report the production of biologically active recombinant cyanovirin-N (rCV-N), an antiviral protein, in genetically engineered soybean seeds. Pure, biologically active rCV-N was isolated with a yield of 350 μg/g of dry seed weight. rCV-N purified from soya is active in anti-HIV assays, with an EC50 of 0.82-2.7 nM (compared to 0.45-1.8 nM for *E. coli*-produced CV-N). Standard industrial processing of soya bean seeds to harvest soya bean oil does not diminish the antiviral activity of recovered rCV-N, allowing the use of industrial soya bean processing to generate both soya bean oil and a recombinant protein for anti-HIV microbicide development (O'Keefe *et al.*, 2015). Expression of rCV-N was achieved using specific regulatory sequences within soybean seed tissues (Rech *et al.*, 2008). One of the difficulties in fully evaluating CV-N as a microbicide has been the high cost of production. CV-N produced in soybean seeds addresses this critical requirement, and soya beans should be further evaluated as a production system to produce other suitable candidate microbicides for further preclinical evaluation and, possibly, clinical testing in humans. Development of a suitable expression source for the manufacture of an anti-HIV topical microbicide requires a low-cost methodology to have the broadest utility in the areas of the world most affected by HIV (Essex, 1996; Gartner *et al.*, 1986; O'Keefe *et al.*, 2009). Suitable microbicide candidates must meet an array of criteria. Although potential microbicides meeting some of these functional criteria are available, few microbicides have been able to be produced at sufficiently low cost.

1.3. Regulatory gene circuits utilizing serine integrases

Although single genetic switches and circuits are in the early stage of development, it is possible to envisage a not-too-distant future in which

multiple switches become the norm, allowing increasingly precise control of gene regulation and expression in plants and mammalian cells for the development of innovative processes and products for the benefit of human beings and the environment. Studies on gene regulation have demonstrated effective control of the RNA polymerase flux by utilizing different serine integrases, which are capable of catalysing unidirectional inversion of DNA to turn on/off regulatory genes in prokaryotic cells (Schwille, 2011; Nandagopal & Elowitz, 2011; Bonnet *et al.*, 2013; Yang *et al.* 2014; Nielsen *et al.*, 2016; Weinberg *et al.*, 2017). A simplified mathematical model was proposed to explain and define core features that are demanded and sufficient for the regulation of both 'forward' and 'reverse' integrase reactions (Pokhilko *et al.*, 2016). In this model, the substrates of the 'forbidden' reactions (between attL and attR in the absence of RDF and between attP and attB in the presence of RDF) are trapped as inactive protein-DNA complexes, ensuring that these 'forbidden' reactions are extremely slow. The model is in good agreement with the observed *in vitro* kinetics of recombination by fC31 integrase, and it defines the core features of the system that are necessary and sufficient for directionality. However, knowledge about the functionality of integrases in eukaryotic cells is still limited. Here, we show the remarkable functional capability of bacteriophages' serine integrases acting in plant and mammalian cells. A co-transformation plasmid system was utilized for *in vitro* evaluation of different integrases in *Arabidopsis thaliana* protoplasts, bovine fibroblasts and human cells. The first plasmid contained the codon-optimized integrase 2 gene and integrase 5 gene sequences under inducible promoters. The second plasmid was a reporter plasmid that contains the *gfp* gene under the 35SCaMV promoter placed in reverse complement orientation and flanked by the attB and attP sites of both integrases. Once the integrases were expressed, the promoter sequence was flipped to its correct orientation, promoting GFP (green fluorescent protein) expression. The results obtained demonstrated that the promoter was correctly flipped, which, in turn, led to RNA polymerase flux through the DNA molecule and GFP expression, as detected by fluorescence microscopy and flow cytometry. The promoter inversion was detected by PCR and sequencing analyses. We anticipate our results to be an initial point for development of more complex models of gene regulation in plants using synthetically engineered integrases. Currently, we have been utilizing recombinases, such as serine integrases, to determine the endogenous nonessential genes within genomes and to design and provide systems to control endogenous and exogenous gene regulation through the

development of synthetic genetic circuits, which, through external chemical, physical and/or biological inducers, are capable of switching specific traits 'on' and 'off' in model eukaryotic organisms.

1.4. Genetically Modified Crops (GMC)

Contributions of recombinant DNA technology directed to develop genetically modified crops for food security, sustainability and climate change include increasing crop productivity, conserving biodiversity from land ploughing and cultivation and reducing CO2 emissions. However, higher costs for deregulation of transgenic crops still remain a constraint to adoption, which is particularly important for many developing countries, which are thus denied the opportunity of using genetically modified crops aiming at food, feed, and fibre security. A report and analysis by the Council for Agricultural Science and Technology (CAST, 2016) on the impact of asynchronous approvals for GMOs on agricultural sustainability, trade and innovation also indicate that there are large volumes of trade worth billions of dollars at risk. The challenges to overcome require intensification of cooperative partnership between public and private sectors (ISAAA, 2016).

Examples of GMC and traits being evaluated under field conditions in the pipeline for the benefit of farmers and consumers are as follows (ISAAA, 2016):

1. Staple crops such as beta-carotene-enriched golden rice are being tested in the Philippines and Bangladesh (as a sustainable contribution to reduce vitamin A malnutrition).
2. Bunchy top virus-resistant bananas are being grown in Uganda.
3. Drought-tolerant, altered oil content and grain composition are being field-tested in Australia and Brazil.
4. Drought-tolerant sugarcane is being grown in India and Indonesia.
5. Omega-3-enriched camelina is being grown in the EU.

2. Why and how to intensify science and technology (S&T) cooperation in Latin America?

Latin America has a significant percentage of its economic-based income generated in the agricultural production sector and is also a mega-biodiverse region. Therefore, it is opportune to mention that conservation and sustainable utilization of biodiversity constitute the physical basis for agriculture, which in turn relies on water availability, genetic resources, soil conservation, climate stability and nutrient recycling, among other

physical and chemical factors (Soares-Filho *et al.* 2006; Gullison *et al.* 2007; Rosenzweig and DeFriesa 2010).

Brazil's agricultural structure may serve as a case study for different agricultural income segments and its correlation with the urgent necessity and opportunity to establish a new configuration and equalization for the sustainable intensification of food production, conservation of biodiversity and reduction of inequity.

An analysis by Fundação Getúlio Vargas (Lopes *et al.,* 2010, 2011; Alves *et al.*, 2017) of data collected by the agriculture census in Brazil (Instituto Brasileiro de Geografia e Estatística – IBGE, 2006) indicated a total of 5,175,636 farms divided into three classes. Class C was defined as having annual incomes between US$5,650.00 and US$24,950.00. Classes A/B and D/E were defined as having annual incomes above and below the mentioned income limits, respectively. As evidenced by the analytical data of the production structure, it is noted that introduction of technologies into the production sector is determined to be one of the key steps in reducing inequity in a sustainable manner, with a significantly higher impact mainly in the low-income classes (D/E income < US$5,650.00/year) (Rech and Lopes, 2013).

Producers are surrounded by a social and economic environment that discriminates against the low-income class segment. In short, low-income farmers commercialize production (output) at lower prices and purchase inputs at high prices. Therefore, utilization of technology implies an inadequate ratio of inputs at higher cost to generate an output that is not profitable. As a consequence, the technology is barely adopted. In contrast, without technology, there is no way to increase the revenue per hectare and open the door to alleviate poverty in agriculture (Alves, 2017; personal communication).

The importance of political initiatives and efforts to elevate and sustain the low-income class segment in Brazilian agriculture over recent decades is recognized. Indeed, the current programmes have contributed to these efforts. However, the advances achieved so far are limited and have not overcome current restrictions in a sustainable manner. This is probably because the current programmes lack the capacity to reach the vulnerable class segments in rural areas in an appropriate format.

In spite of that, technology alone will not save the farmers and reduce inequity unless it is equalized with other imperative components as a foundation such as human health, high-quality education, food security, the environment and added market value in order to establish the equalization

of intrinsic and inter-dependent related components to intensify progress in the agriculture sector in Brazil. This, in turn, can also be expanded and/or adapted to the Latin American region as a whole.

We believe that this example might be utilized as a conceptual basis for a wider discussion in Latin America and establishment of equalization and functional and progressive operative models to effectively contribute to reducing inequity and unsustainability.

3. Equalization of components for progressive development

Over time, developed countries were able to build an equalized foundation to support and generate a consequent exponential ratio of development based on S&T. In contrast, most developing countries have been unable to establish an equalized operational foundation, manifesting an inability for sustainable progressive development, including reduction of inequity.

Below, the equalized components required:

Education.S&T.human health.environment.adding value = progress⋆

⋆general supporting mechanisms: Acceleration of operative synergies between north-south-east-west; funded long-term bottom-up science and technology consortiums; active networking tools (education; training; trained teachers and schools; knowledge dissemination; high-risk support programmes; innovative and emerging research themes; clear objectives, definitions and deliverables; intensification of multi- and interdisciplinary science and technology research; wide engagement of the next generation of young researchers; intensification of scientific and technological research for peaceful aims and the public interest; establishment of research consortiums that operate jointly, from the lab to the market; promotion of knowledge exchange, new joint research and new communication strategies).

Conclusion

The introduction of technologies into the production sectors constitutes one of the key components in the promotion of systemic and sustainable progress advances in Latin America. Intensification of S&T collaboration should be an important approach. A viable option to also reduce inequity is if the envisaged policies are applied in consonance with the equalization of other components, such as human health, high-quality education, food security, the environment and added market value. A foundation for con-

servation of biodiversity and preservation of knowledge in local communities, in line with regulatory and ethical concepts directing that potentially derived products be carefully and responsibly evaluated before being made commercially available, is needed.

Bibliography

Alves E., Souza G., Marra R. (2017) Uma viagem pelas regiões e estados guiada pelo Censo Agropecuário 2006. *Revista de Política Agrícola*, ano 26, n. 1:113-150.

Annaluru N., Muller H., Mitchell L.A., Ramalingam S., Stracquadanio G., Richardson S.M., Dymond J.S., Kuang Z., Scheifele L.Z., Cooper E.M., Cai Y. Zeller K., Agmon N., Han J.S., Hadjithomas M., Tullman J., Caravelli K., Cirelli K., Guo Z., London V., Yeluru A., Murugan S., Kandavelou K., Agier N., Fischer G., Yang K., Martin J.A., Bilgel M., Bohutskyi P., Boulier K.M., Capaldo B.J., Chang J., Charoen K., Choi W.J., Deng P., DiCarlo J.E., Doong J., Dunn J., Feinberg J.I, Fernandez C., Floria C.E., Gladowski D., Hadidi P., Ishizuka I., Jabbari J., Lau C.Y.L., Lee P.A., Li S., Lin D., Linder M.E., Ling J., Liu J, Liu J., London M., Ma H., Mao J.M., McDade J.E., McMillan A., Moore A., Yu A.T., Koszul R., Bader J.S., Boeke J.D., Chandrasegaran S. (2014) Total Synthesis of a Functional Designer Eukaryotic Chromosome. *Science*, 344: 55-58.

Arber W. (2012) Genetic variation and Molecular Darwinism. In: Meyers, R.A. (Ed.). *Systems Biology: Advances in Molecular Biology and Medicine*. Verlag: Wiley-VCH, p. 145-168.

Bittencourt D., Dittmar K., Lewis R.V., Rech E.L. (2010) MaSp2-like gene found in the Amazon mygalomorph spider *Avicularia juruensis*. Comparative Biochemistry and Physiology. B, *Biochemistry & Molecular Biology*, 155:419-426.

Bonnet J., Yin P., Ortiz M.E., Subsoontorn P., Endy D. (2013) Amplifying genetic logic gates. *Science*, 340:599-603.

Boyd M.R., Gustafson K.R., McMahon J.B., Shoemaker R.H., O'Keefe B.R., Mori T., Gulakowski T.R.J., Wu L., Rivera M.I., Laurencot C.M., Currens M.J., Cardellina J.H. II, Buckheit R.W. Jr. Nara P.L., Pannell L.K., Sowder R.C., Henderson L.E. (1997) Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120; potential applications to microbicide development. *Antimicrob. Agents Chemotherapy*, 41:1521-1530.

Boch J., Scholze H., Schornack S., Landgraf A., Hahn S., Kay S., Lahaye T., Nickstadt A., Bonas U. (2009) Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. *Science*, 326:1509-1512.

Council for Agricultural Science and Technology – CAST. (2016). (http://www.cast-science.org/publications/). QTA2016-2, 12 pp.

Carlson D.F., Lancto C.A. (2016) Production of hornless dairy cattle from genome-edited cell lines. *Nature Biotechnology*, 34:479-481.

Cermak T., Doyle E.L., Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove A.J., Voytas DF. (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Research*, 39: p.e82-e82.

Cong L., Ann Ran F., Cox D., Lin S., Barreto R., Habib N., Hsu P.D., Wu X., Jiang W., Marraffini A., Zhang F. (2013) Multiplex genome engineering using

CRISPR/Cas systems. *Science*, 339:819-823.

Darwin C. (1868) *The Variation of Animals and Plants Under Domestication*. London, UK: John Murray.

Darwin C. (1876) *The Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. London, UK: John Murray.

Doudna J.A., Charpentier E. (2014) The new frontier of genome engineering with CRISPR-Cas9, *Science*, 346, Issue 6213, DOI: 10.1126/science.1258096

Doyon Y, Vo T.D., Mendel M.C., Greenberg S.H., Wang J., Xia D.F., Miller J. C., Urnov F.D. Urnov, Gregory P.D., Holmes M.C. (2011) Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nature Methods*, 8:74-79.

Essex M. (1996) Retroviral vaccines: challenges for the developing world. *AIDS Research Human Retroviruses*, 12:361-363.

Gartner S.P., Markovits D.M., Kaplan M.H., Gallor R.C., Popovic M. (1986) The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science*, 233:215-219.

Gibson D.G., Benders G.A., Andrews-Pfannkoch C., Denisova E.A., Baden-Tillson H., Zaveri J., Stockwell T.B., Brownley A., Thomas D.W., Algire M.A., Merryman C., Young L., Noskov V.N., Glass J., Craig Venter J., Hutchison III C.A., Smith H.O. (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science*, 319:1215-1220.

Gibson D.G., Glass J., Lartigue C., Noskov V.N., Chuang Ray-Yuan, Algire M.A., Benders G.A., Montague M.G., Ma L., Moodie M.M., Merryman C., Vashee S., Krishnakumar R., Assad-Garcia N., Andrews-Pfannkoch C., Denisova E.A., Young L., Qi Zhi-Qing, Segall-Shapiro T.H., Calvey C.H., Parmar P.P., Hutchison III C.A., Smith H.O., Craig Venter J. (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. *Science*, 329: 52-56.

Gullison R.E., Frumhoff P.C., Canadell J.G., Fieldm C.B, Nepstad D.C., Hayhoe K. (2007) Tropical forests and climate policy. *Science*, 316:985-986.

Hutchison III C.A., Chuang Ray-Yuan Noskov V.N., Assad-Garcia N., Deerinck T.J., Ellisman M.H., Gill J., Kannan K., Karas B.J., Ma L., Pelletier J.F., Qi Zhi-Qing, Richeter R.A., Strychalski E.A., Sun L., Suzuki Y., Tsvetanova B., Wise K.S., Smith H.O., Glass J., Merryman C., Gibson D.G., Craig Venter J. (2016) Design and synthesis of a minimal bacterial genome. *Science*, 351: p. aad6253-aad6253.

ISAAA (2016). Global Status of Commercialized Biotech/GM Crops: 2016. *ISAAA Brief* No. 52. ISAAA: Ithaca, NY. ISBN: 978-1-892456-66-4.

Jiang W., Zhou H., Bi H., Fromm M., Yang B., Weeks D.P. (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research*, 41: p.e188-e188.

Lewis R.V. (2006) Spider silk: Ancient ideas for new biomaterials. *Chemical Reviews*, 106:3762-3774.

Lopes I.V., Rocha D.P., Lopes M.R. (2010) *Quem produz o que no campo: quando e onde II*. Fundação Getúlio Vargas e Confederação Nacional da Agricultura e Pecuária, Fundacão Getúlio Vargas, Instituto Brasileiro de Economia e Confederação da Agricultura e Pecuária do Brasil, 192 p.

Lopes, I.V., D.P. Rocha, Lopes, M.R. (2011) *Os perfis das classes de renda rural no Brasil*. Fundação Getúlio Vargas e Confederação Nacional da Agricultura e Pecuária, Fundação Getúlio Vargas e Instituto Brasileiro de Economia.

Li, J.-F. *et al.* (2013) Multiplex and homologous recombination-mediated genome

editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology*, 31:688-691.

Mali P., Yang, Kevin L., Esvelt M., Aach J., Guell M., DiCarlo J.E., Norville J.E., Church G.M. (2013) RNA-Guided human genome engineering via Cas9. *Science*, 339:823-826.

MC Elroy D. (2003) Sustaining agbiotechnology through lean times. *Nature Biotechnology*, 21:996-1002.

MC Elroy D. (2004) Valuing the product development cycle in agricultural biotechnology – what's in a name. *Nature Biotechnology*, 22:817-822.

Mendel G. (1866) Versuche über Pflanzen-Hybriden. *Journal of Hereditary*, 42:3-47.

Mendel G. (1870) *On Hieraclum-hybrids obtained by artificial fertilisation* (English translation). In: Stern C, Sherwood ER (eds) *The origin of genetics: a Mendel source book*, 1966. W.H. Freeman, San Francisco, pp 49-55.

Mendel G. (1950) Gregor Mendel's Letters to Carl Nägeli. *Genetics* 35(5, pt 2):1-29 (Originally published as: *Abhandlungen der Mathematisch-Physischen Klasse der Königlich Sächsischen Gesellschaft der Wissenschaften* 29: 189-265, 1905. Reprinted in *Carl Correns, Gesammelte Abhandlungen zur Vererbungswissenschaft aus periodischen Schriften*, 1899-1924. (Fritz V. Wettstein ed.) Berlin, Julius Springer, 1924:1237-1281.

Murad A.M., Rech E.L. (2011) Molecular dynamics simulations of the minor ampullate spidroin modular amino acid sequence from *Parawixia bistriatra*: insights into silk tertiary structure and fibre formation. *Journal of Molecular Modeling*, 17:1183-1189.

Nandagopal N., Elowitzm B. (2011) Synthetic Biology: Integrated Gene Circuits. *Science*, 333:1244-1248.

Nepstad D., MCGrath D.G., Alencar A., Barros A.C., Carvalho G., Santilli M., Diaz, M.D.C. (2002) Frontier Governance in Amazonia. *Science*, 295:629-631.

Nielsen A.A.K., Der B.S., Shin J., Vaidyanathan P., Paralanov V., Strychalski E.A., Ross D., Densmore D., Voigt C.A. (2016) Genetic circuit design information. *Science* 352: issue 6281, DOI: 10.1126/science.aac7341.

O'Keefe B., Vojdani F., Buffa V., Shattock R.J., Montefiori D.C., Bakke J., Mirsalise J., d'Andreae A.-L., Hume S.D., Bratcherf B., Saucedo C.J., McMahon J.B., Pogue G.P., Palmer K.E. (2009) Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. *Proceedings of the National Academy of Sciences USA*, 106:6099-6104.

O'Keefe B.R., Murad A.M., Vianna G.R., Ramessar K., Saucedo C.J., Wilson J., Cunha N.B., Guerra A.C., Lacorte C., Madeira L., MCMahon J.B., Rech E.L. (2015) Engineering soya bean seeds as a scalable platform to produce cyanovirin-N, a non-ARV microbicide against HIV. *Plant Biotechnology Journal*, 13:884-892.

Pokhilko A., Zhao J., Stark W.M., Colloms S.D., Ebenhoh O. (2017) A simplified mathematical model of directional DNA site-specific recombination by serine integrases. *Journal of Royal Society Interface*, 14: 20160618. doi.org/10.1098/rsif.2016.0618.

Prosdocimi F., Bitteconcourt D., Da Silva F.R., Kirst M., Motta P.C., Rech E.L. (2011) Spinning gland transcriptomics from two main clades of spiders (order: Araneae) – insights on their molecular, anatomical and behavioral evolution. *PLoS One*, 6: Issue 6; e21634.

Rech E.L., Lopes M.R. (2012) Insights into Brazilian agricultural structure and sustainable intensification of food production. *Food and Energy Security*, 1:77-80.

Rech E.L., Vianna G.R., Aragao F.J. (2008) High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nature Protocols* 3:410-418.

Rosenzweig C., DeFriesa R. (2010) Toward a whole-landscape approach for sustainable land use in the tropics. *Proceedings of National Academy of Sciences USA* 107:19627-19632.

Schwille, P. (2011) Bottom-Up Synthetic Biology: Engineering in a Tinkerer's World. *Science*, 333:1252-1254.

Silva L.P., Rech E.L. (2013) Unravelling the biodiversity of nanoscale signatures of spider silk fibres. *Nature Communications*, 4, 3014, DOI:10.1038/ncomms4014.

Soares-Filho B.S., Nepstad D.C., Curran L.M., Cerqueira G.C., Garcia R.A., Ramos C.A.O., Voll E.L., MCDonald A., Lefebvre P., Schlesinger P. (2006) Modelling conservation in the Amazon basin. *Nature*, 440: 520-523.

Teule F., Copper A.R., Furin W.A., Bittencourt D., Rech E.L., Brooks A. LEWIS R.V.A. (2009) A protocol for the production of recombinant spider silk-like proteins for artificial fiber spinning. *Nature Protocols*, 4:341-355.

Watson J.D., Crick F.H.C. (1953a) Molecular structure of nucleic acids. A structure for deoxyribose nucleic acid. *Nature*, 171: 737-738.

Watson J.D., Crick F.H.C. (1953b) The structure of DNA. *Cold Spring Harbor Symposium Quantitative Biology*, 18:123-131.

Weinberg B.H., Hang Pham N.T., Caraballo L.D., Lozanoski T., Engel A., Bhatia S., Wong W.W. (2017) Large-scale design of robust genetic circuits with multiple inputs and outputs for mammalian cells. *Nature Biotechnology*, doi:10.1038/nbt.3805

Yang L., Nielsen A.A.K., Fernandez-Rodriguez J., McClune C.J., Laub M.T., Lu T.K., Voigt C.A. (2014) Permanent genetic memory with >1-bute capacity. *Nature Methods*, 11:1261-1266.

Yu W., Lamb J.C., Han F., Birchler J.A. (2006) Telomere-mediated chromosomal truncation in maize. *Proceedings of the National Academy of Sciences USA*, 103:17331-17336.

Molecular Mechanisms of Cell Adaptation to Hypoxia

Pablo Wappner[1]

Introduction

Hypoxia (low oxygen tension) modulates many human pathological and physiological conditions that include angiogenesis, erythropoiesis, vasodilation, and at a cellular level, metabolism, cell proliferation and apoptosis among others [1, 2]. These adaptive responses rely on modifications of the transcriptional profile, which are mediated mostly by a single transcription factor named Hypoxia Inducible Factor (HIF) [3]. HIF is a α/β heterodimer of basic Helix Loop Helix-PAS proteins (bHLH-PAS), in which the β- subunit is constitutive, and the α-subunit is regulated by oxygen at several different levels that include protein degradation, transcription, translation, subcellular localization and post-translational modifications [4, 5].

Oxygen-dependent degradation of the α-subunit is clearly the principal mechanism governing HIF regulation: When O_2 is available (normoxia) HIFα is rapidly destroyed at the 26S proteasome, while in hypoxia HIFα is protected from degradation, accumulates at the cytoplasm, enters the nucleus, heterodimerizes with the β-subunit, and induces hypoxia-dependent gene expression [6]. Oxygen-dependent degradation of HIFα depends on the hydroxylation of two specific prolyl residues of this subunit. This HIFα post-translational modification is required for binding of the Von Hippel Lindau (VHL) tumor suppressor factor, which is in turn the substrate recognition subunit of a multimeric E3 ubiquitin ligase complex that promotes HIFα proteasomal degradation [7].

Importantly, prolyl hydroxylation of the α-subunit is catalyzed by a specific prolyl-4-hydroxylase that utilizes molecular oxygen and 2-oxoglutarate as substrates for the hydroxylation reaction, so in hypoxia hydroxylation is prevented, the α-subunit is not degraded at the 26S proteasome, and HIFα accumulates to promote hypoxia-specific transcription [8].

This hypoxia core machinery, which is composed of the transcription factor HIF, the prolyl-4-hydroxylase and VHL, is evolutionary conserved in

[1] Instituto Leloir, Buenos Aires (1405), Argentina

animals from nematodes to humans [9]. In my laboratory we utilize the fruit fly *Drosophila melanogaster* as a genetic model to investigate novel modes of cell adaptation to hypoxia and new mechanisms of HIF regulation.

1. The transcriptional response to hypoxia in Drosophila melanogaster

Over the last 15 years we and other laboratories have defined an oxygen-responsive system in *Drosophila* that is homologous to that of mammalian HIF [10]. The fruit fly homologs of HIFα and HIFβ are respectively the bHLH-PAS proteins Sima and Tango [11]. While Tango protein accumulates constitutively, Sima is oxygen-regulated through protein degradation at the 26S proteasome, degradation being dependent on a specific prolyl-4-hydroxylase named Fatiga and a multimeric E3 ubiquitin ligase that contains a VHL homolog (dVHL) [11]. In hypoxia, Sima degradation is prevented, so the protein accumulates in cell nuclei promoting hypoxia-dependent transcription. Oxygen-dependent regulation of gene expression mediates adaptation to hypoxic conditions through a wide variety of physiological mechanisms that include body growth reduction, oxygen- dependent ramification of the larval respiratory (tracheal) system and a metabolic switch from aerobic mitochondrial respiration to anaerobic glycolysis [12].

2. Utilizing *Drosophila* to seek for new mechanisms of adaptation to hypoxia. Genome-wide screen for novel HIF regulators

Having defined the HIF system in *Drosophila*, we sought to identify novel mechanisms of HIF regulation by taking advantage of the genetic tools available in this species. To this end, we designed an RNAi-based genome-wide screen to seek for novel HIF regulators [13]. We worked in collaboration with the *Drosophila RNAi Screening Centre* at Harvard medical School, where a collection of double stranded RNAs for silencing each of the genes of the *Drosophila* genome in cultured cells is available. We first verified that in a *Drosophila* cell line (Schneider, S2 cells) Sima is degraded in normoxia and accumulates in hypoxia. Next, we generated a luciferase-based transcriptional reporter that is induced in hypoxia in a HIF-dependent manner. The reporter is based on an enhancer of the murine Lactate Dehydrogenase-A (LDH-A) gene, which was cloned upstream of the firefly luciferase open reading frame. The plasmid encoding this reporter was stably transfected in S2 cells along with a constitutive reporter in which an Actin A5 promoter drives the expression of *Renilla* luciferase. This stably transfected S2 cell line behaved as expected: Firefly

luciferase activity was strongly induced at 1% O_2 for 6 or 24 hours, while activity of *Renilla* luciferase remained constant. Treatment of these cells with the iron chelator desferroxyamine (DFO) mimicked the hypoxic response, since the HIF-prolyl hydroxylase requires Fe^{2+} for catalysis. Importantly, treatment of these cells with double stranded RNAs against *sima* or *tango* (positive controls) largely blocked firefly luciferase hypoxic (or DFO-dependent) induction [13].

Thus, we were in a position to conduct the RNAi-based genome-wide screen for novel HIF regulators. S2 cells carrying the HIF-responsive reporter were treated with the whole collection of dsRNAs that target each of the genes of the *Drosophila* genome (nearly 22,000 genes). The screen was carried out is triplicate, comparing cells exposed to DFO with untreated normoxic controls. After three rounds of selection, 31 hits of the screen could be confirmed. Whereas 11 of these 31 hits correspond to previously characterized elements of the HIF system including Sima and Tango, 20 genes had not been associated before to hypoxia biology.

3. The novel regulators of HIF

The 20 new HIF regulators are genes involved in various cellular functions, including transcription, protein translation [14], chromatin remodeling [15], RNA processing, and remarkably, the Argonaute1 (Ago1), the fly homolog of mammalian Ago2, essential for miRNA function. While these findings opened several lines of research in the lab that continue nowadays, the current presentation will focus on the results derived from the discovery that Ago1 and the miRNA machinery regulate HIF-dependent responses to hypoxia.

First, we checked that not only Ago1, but also other elements of the canonical miRNA machinery like Drosha, Dicer and GW182, are also necessary for hypoxia-dependent transcription. All these elements are essential for HIF-dependent transcription. Later, we verified that hypoxia-dependent induction of endogenous HIF target genes – and not only expression of the HIF reporter – was affected after silencing the miRNA machinery in S2 cultured cells. Finally, we confirmed that elements of the miRNA machinery are required not only in S2 cells, but also *in vivo*, in fly embryos, for hypoxia-dependent gene expression [13]. Taken together, our results suggested that one or more *Drosophila* miRNAs are necessary for maximal hypoxia-dependent gene expression, so we sought to search for these specific miRNAs.

3.1. Screen for miRNAs required for HIF-dependent gene expression

To search for miRNAs required for maximal HIF activity, we utilized a transgenic fly line bearing a LacZ hypoxia-inducible reporter. In this reporter, the same hypoxia-responsive promoter that controlled luciferase expression in the cell line utilized for the genome-wide screen was cloned upstream of bacterial LacZ, so that in embryos or larvae exposed to hypoxia (typically 3% to 5% O_2) β-galactosidase is strongly expressed. Interestingly, induction of this reporter is remarkably high at the tracheal (respiratory) system [10]. To screen for hypoxia-relevant miRNAs in the above transgenic line, we undertook an over-expression strategy in which each of the miRNAs of the *Drosophila* genome was overexpressed in the embryonic tracheal system [16]. The rationale was that, if one or more miRNAs are required for maximal HIF activity, overexpression of these miRNAs might perhaps provoke exacerbated HIF activity in non-hypoxic conditions or in conditions of very mild hypoxia. We collaborated with the lab of Norbert Perrimon at Harvard Medical School, who had generated the whole collection of transgenic fly lines that overexpress each of the miRNAs encoded in the *Drosophila* genome, which they sent to our lab in Buenos Aires.

Hence, each of the *Drosophila* miRNAs was overexpressed at 11% O_2 (very mild hypoxic conditions) in embryos carrying the LacZ hypoxia-inducible reporter. While at 11% O_2, wild type control embryos do not express the reporter, overexpression of miR-190, miR-274, miR-280 or miR-985 rendered strong expression of the hypoxia-inducible reporter. These results suggest that the above four miRNAs can enhance HIF-dependent transcription. The rest of my presentation will focus on miR-190 as a novel HIF regulator.

3.2. miR-190 enhances HIF-dependent responses to hypoxia and is required for maximal HIF-dependent gene expression.

miR-190 is encoded in intron 13 of the gene *rhea*. *Drosophila* Rhea is homologous to a mammalian protein called Talin, which is essential for integrin function and its interaction with the cytoskeleton. Having established that overexpression of miR-190 can trigger HIF-dependent transcription, we next assessed as whether loss of miR-190 compromises HIF activity in hypoxia. This was indeed the case, since in larvae homozygous for miR-190 mutant alleles, hypoxic induction of the LacZ reporter was impaired. Quantitative RT-PCR analysis revealed that hypoxia-dependent induction of HIF endogenous target genes, such as *hsf* and hphB, was also

impaired in miR-190 homozygous mutants, suggesting that this miRNA is necessary for maximal HIF-dependent responses to hypoxia.

As a next step, we investigated as whether miR-190 mediates not only HIF-dependent molecular effects, but also biological responses to hypoxia, as for example ramification of the tracheal (respiratory) system in 3rd instar larvae, a physiological adaptation analogous to mammalian angiogenesis. This was indeed the case, as miR-190 overexpression enhanced tracheal terminal ramification. Taken together these results indicate that miR-190 is necessary for HIF-dependent responses to hypoxia.

3.3. Searching for miR-190 molecular targets

Having established that miR-190 is necessary for maximal HIF activity, we next sought to define its molecular targets. miRNAs usually function as mRNA translational inhibitors or mediate mRNA degradation. Given that miR-190 enhances HIF-dependent molecular and biological effects, we reasoned that its molecular target is most likely a direct or indirect inhibitor of HIF. Several miRNA target prediction algorithms indicated that the Fatiga prolyl-4-hydroxylase mRNA might include two miR-190 binding sites on its 3' untranslated region (3'UTR). Since anti-Fatiga antibodies are not currently available, we were not in a position to directly assess Fatiga protein levels following miR-190 overexpression or abrogation. We therefore utilized a Fatiga activity transgenic reporter in which the Sima (HIFα) oxygen-dependent degradation domain ODDD is fused to GFP on its N-terminus. When the activity of Fatiga is high, GFP-ODDD is rapidly degraded, and when its activity is low, GFP-ODDD accumulates [16]. This reporter was ubiquitously expressed in transgenic fly lines under control of an ubiquitin promoter, and in the same transgenic line, miR-190 was overexpressed only at the posterior compartment of the wing imaginal disc, under control of an *engrailed-Gal4* driver. Thus, we compared the signal derived from the GFP-ODDD reporter at the posterior versus anterior compartment of the wing imaginal disc. The posterior wing disc compartment, which overexpressed miR-190, displayed much higher GFP-ODDD signal than the anterior control compartment, suggesting that miR-190 might restrict the expression of Fatiga.

The above results suggested that miR-190 might bind the 3'UTR of *fatiga* mRNA and mediate its translational repression. To investigate if *fatiga* is indeed a direct miR-190 molecular target, we fused the *fatiga* 3'UTR to the firefly luciferase open reading frame on its 3' end, and expressed this construct in cell culture under control of a Cu^{2+}-inducible promoter.

When miR-190 was co-expressed in these cells, luciferase activity was strongly reduced, suggesting that this miRNA binds *fatiga* 3'UTR, and inhibits luciferase translation. A mutagenized version of the luciferase construct, in which miR-190 binding sites at the *fatiga* 3'UTR were altered, was insensitive to miR-190 overexpression, confirming that this miRNA directly regulates the translation of *fatiga* mRNA.

3.4. miR-190 is induced in hypoxia

Having determined that miR-190 is a novel player of the HIF machinery, it was interesting to define if it is itself oxygen-regulated, so we measured miR-190 levels in 3rd instar larval extracts prepared from normoxic or hypoxic individuals. We found clear induction of miR-190 in hypoxia and interestingly, this induction was not altered in larvae expressing an RNAi against Sima. Thus, miR-190 is induced by hypoxia in a HIF-independent manner. Taking into consideration that miR-190 is encoded in an intron of the rhea gene, we also investigated possible oxygen regulation of *rhea* gene expression. Quantitative RT-PCR analysis revealed that rhea mRNA levels are strongly induced in larvae exposed to hypoxia, and that this induction does not depend on Sima either. Likewise, miR-190 precursor levels (pre-miR-190) are also induced by hypoxia in a Sima/HIF-independent manner. Taken together, these results suggest that miR-190 is regulated by oxygen through the transcription of the *rhea* gene, in whose intron the miRNA is encoded. We therefore propose that miR-190 enhances HIF-dependent responses to hypoxia by inhibiting the prolyl-4-hydroxylase Fatiga. In normoxia, miR-190 dependent inhibition of Fatiga is lifted, thereby contributing to Fatiga-dependent inhibition of the hypoxic response.

4. miR-190, a conserved miRNA with potential role in human cancer

To our knowledge, this is the first report of a miRNA that directly downregulates an oxygen sensing prolyl-4-hydroxylase. miR-190 is broadly conserved in evolution, not only within the *Drosophilid* lineage, but also in distant taxa, including mammals. In most mammalian species, two miR-190 family members occur, miR-190a and miR-190b. The miR-190a locus lies in an intron of *talin2* (*TLN2*), which encodes a high molecular weight cytoskeletal protein. Remarkably, *Drosophila melanogaster* miR-190 is encoded in an intron of the gene *rhea*, the homolog of *talin2 (TLN2)*. Intron 53 of human *TLN2-001* (which is 12,893 nucleotides long) and intron 14 of *rhea-RB* (which is 356 nucleotides long) only share sequence

similarity within the miR-190 locus, reflecting the physiological relevance of this miRNA and perhaps some biological link with Rhea/Talin2. Interestingly, human PHD3 (also known as EGLN3), which is one of the three mammalian homologs of *Drosophila* Fatiga, has a predicted binding site for miR-190a, according to the miRNA target prediction databases TargetScan (www.targetscan.org) and miRDB (mirdb.org), even though with a relatively low score in both cases. Thus, it is possible that miR-190-dependent regulation of HIF-prolyl hydroxylases is conserved in evolution.

We found that Drosophila miR-190 is induced in hypoxia. Interestingly, mammalian miR-190 is upregulated in different types of cancer, including hepatocellular carcinoma, primary myelofibrosis, pancreatic, breast, rectal and papillary thyroid cancer. Hypoxic microenvironment is a common feature of many solid tumors, and most primary human cancers and their metastases exhibit increased levels of HIFα. Given that miR-190 is upregulated in diverse cancer types, our findings open the possibility that miR-190 contributes to HIFα stabilization in cancer cells, thereby enhancing tumor progression. Importantly, strengthening the notion of a possible involvement of miR-190 in mammalian responses to low oxygen, miR-190 is induced by hypoxia in a rat model of hypoxic pulmonary artery hypertension (PAH).

5. Conclusion

The results reported here about the participation of miR-190 in HIF regulation increase our understanding of the network controlling HIF-dependent responses to hypoxia, and open the possibility of analyzing the regulation exerted by additional miRNAs which may be part of this complex network.

References

1. Kaelin, W.G., Jr. and P.J. Ratcliffe, Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell,* 2008. 30(4): p. 393-402.
2. Kim, J.W., *et al.*, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*, 2006. 3(3): p. 177-85.
3. Semenza, G.L., HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev*, 2010. 20(1): p. 51-6.
4. Centanin, L., T.A. Gorr, and P. Wappner, Tracheal remodelling in response to hypoxia. *J Insect Physiol*, 2010. 56(5): p. 447-54.
5. Jaakkola, P., *et al.*, Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, 2001. 292(5516): p. 468-72.

6. Schofield, C.J. and P.J. Ratcliffe, Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol*, 2004. 5(5): p. 343-54.
7. Kaelin, W.G., Jr., Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer*, 2002. 2(9): p. 673-82.
8. Epstein, A.C., *et al.*, C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*, 2001. 107(1): p. 43-54.
9. Gorr, T.A., M. Gassmann, and P. Wappner, Sensing and responding to hypoxia via HIF in model invertebrates. *J Insect Physiol*, 2006. 52(4): p. 349-64.
10. Lavista-Llanos, S., *et al.*, Control of the hypoxic response in Drosophila melanogaster by the basic helix-loop-helix PAS protein similar. *Mol Cell Biol*, 2002. 22(19): p. 6842-53.
11. Centanin, L., P.J. Ratcliffe, and P. Wappner, Reversion of lethality and growth defects in Fatiga oxygen-sensor mutant flies by loss of hypoxia-inducible factor-alpha/Sima. *EMBO Rep*, 2005. 6(11): p. 1070-5.
12. Centanin, L., *et al.*, Cell autonomy of HIF effects in Drosophila: tracheal cells sense hypoxia and induce terminal branch sprouting. *Dev Cell*, 2008. 14(4): p. 547-58.
13. Dekanty, A., *et al.*, Drosophila genome-wide RNAi screen identifies multiple regulators of HIF-dependent transcription in hypoxia. *PLoS Genet*, 2010. 6(6): p. e1000994.
14. Bertolin, A.P., *et al.*, Musashi mediates translational repression of the Drosophila hypoxia inducible factor. *Nucleic Acids Res*, 2016. 44(16): p. 7555-67.
15. Perez-Perri, J.I., *et al.*, The TIP60 Complex Is a Conserved Coactivator of HIF1A. *Cell Rep*, 2016. 16(1): p. 37-47.
16. De Lella Ezcurra, A.L., *et al.*, miR-190 Enhances HIF-Dependent Responses to Hypoxia in Drosophila by Inhibiting the Prolyl-4-hydroxylase Fatiga. *PLoS Genet*, 2016. 12(5): p. e1006073.

How Chloroplasts-Derived Signals Influence Leaf Developmental Fate; the Voice of the Slave

Patricia León

Introduction

One of the characteristics of plants is the presence of specialized organelles known as plastids that carry out particular functions, depending on the tissue and the environmental conditions. Similar to mitochondria, plastids are the result of an endosymbiosis that occurred around 1.2 billion years ago between a non-photosynthetic mitochondria-containing eukaryote and a free-living cyanobacteria (Gould *et al.*, 2008). From the different plastid types the most prominent is the chloroplast, which is present in the green tissues of plants and whose principal function is the acquisition of carbon molecules through the photosynthetic process (Pribil *et al.*, 2014). In contrast, non-photosynthetic plastids include amyloplasts and elaioplasts present in root tissues, cotyledons and seeds with a storage function. Others plastids have the capacity to accumulate high levels of carotenoid pigments and are called chromoplasts. Chromoplasts are found in diverse tissues such as flowers and fruits and are responsible for the orange and yellow colors present in petals and fruits from different plants, and their function is related to attraction for pollinators and to seed dispersal (López-Juez and Pyke, 2005). All the different plastid types originate from an undifferentiated progenitor, the proplastid, present in meristematic cells of plants through a particular differentiation process. In addition to the specific roles that the different plastids have, these organelles are also indispensable for plant development because they are the site of synthesis of many important metabolites that have biological and biotechnological importance, including several plant hormones, vitamins (E and A), pigments, and hundreds of secondary compounds derived from the isoprenoid pathway. Diverse secondary metabolites play essential roles in basic plant metabolism and are also important for interactions with the environment, including defense and adaptation. Thus, a deep understanding of how chloroplasts differentiate and their relation to overall plant development is a field of interest in our laboratory. In this manuscript I will present some of our work regarding the participation of signals produced by the chloroplast that play

a function in modulating leaf development and nuclear gene expression. I will describe our findings that support that these signals play an important role transmitting information of the developing plastid to the nucleus, the nature of these signals and finally, the possible function of this signal in plant development.

1. Chloroplast and nucleus communication

Although important progress has been accomplished in the past decade in understanding the process of how chloroplast differentiates, the molecular aspects remain elusive. Based on recent proteomic analysis it has been estimated that chloroplast requires around 3000 proteins for its differentiation and function (van Wijk and Baginsky, 2011). In agreement with their origin, plastids contain their own genome, but during evolution the size of this molecule was largely reduced, encoding presently only an average of 120 genes (Timmis *et al.*, 2004). Thus, the majority of the proteins required for chloroplast functionality and differentiation are encoded in the genome, translated in the cytoplasm and post-translationally imported into the organelle to perform their function. In this form, the nucleus tightly regulates chloroplast function and development through what is known as anterograde regulation. Several nuclear regulators, including the red (phytochromes) and blue (cryptochromes) photoreceptors, have been shown to modulate these early events of organelle differentiation.

However, key subunits of photosynthetic complexes and some central regulatory proteins such as the RNA polymerase are still encoded in the chloroplast genome. This introduces the necessity to coordinate the expression of both genomes for the synthesis of diverse protein complexes essential for chloroplast development and functionality. Diverse experimental data have demonstrated that chloroplasts emit multiple signals depending on their developmental and metabolic status, which have the capacity to modulate the expression of nuclear genes and in this form regulate the stoichiometric assembly of nuclear-encoded and plastid-encoded proteins. This signaling process from the plastids to the nucleus is commonly referred to as retrograde communication (Chan *et al.*, 2016). This communication is central not only for the functionality of the differentiated organelle but also during the different stages of its development. In this scenario, signals emerge from the organelle under differentiation that have the capacity to modulate the expression of diverse nuclear genes. Thus, development is fine-tuned by balancing anterograde and retrograde signals. Since plastids and, in particular, chloroplasts serve as a central hub of

diverse metabolic pathways, they integrate a diversity of signals and in this sense they have the capacity to influence cell and organismal homeostasis. Moreover, plastids have been demonstrated to produce compounds that serve as long distant signals for communication between different plants (Dudareva *et al.*, 2013).

1.1 Retrograde signaling

Understanding how chloroplasts communicate their developmental and functional status to the nucleus has been a long-standing interest for many groups. The use of mutants with compromised chloroplast function and differentiation has been a successful approach to identify components of this process. In the past years important progress has been achieved with the characterization of different mutants. These studies have demonstrated a complex scenario that involves not one but multiple signals and pathways that initiate in the chloroplasts and result in the regulation of different sets of nuclear genes. Although the existence of multiple signals and retrograde pathways imposes a challenge for their molecular understanding, it provides enormous flexibility for adaptation to rapid changes in the environment and developmental needs, as they can modify the expression at the transcriptional and posttranscriptional level of different genes, depending on the specific requirements. As a matter of simplification, two different types of retrograde signals are currently recognized; those that are produced during the development of the organelle (biogenic) and those produced in response to environmental changing conditions in the fully differentiated organelle (operational), including light quality or abiotic stresses (Pogson *et al.*, 2008). While biogenic retrograde signals are central for the correct coordination of the assembly of chloroplast components, operational retrograde signals are essential in the adjustment of organelle functionality and the responses to environmental changes.

1.2 The Arabidopsis clb5 mutant impairs biogenic retrograde signaling

We have been interested in dissecting the molecular bases of biogenic retrograde communication and with this objective we have isolated diverse mutants that affect chloroplast differentiation at different stages, which we have named *clb,* for chloroplast biogenesis. One of these mutants (*clb5*) was of particular interest because it affects chloroplast biogenesis at early stages as well as the expression of diverse nuclear- and chloroplast-encoded genes (Gutiérrez-Nava *et al.*, 2004). In addition, a characteristic present in *clb5* that differs from most other *clb* mutants is that this plant displays

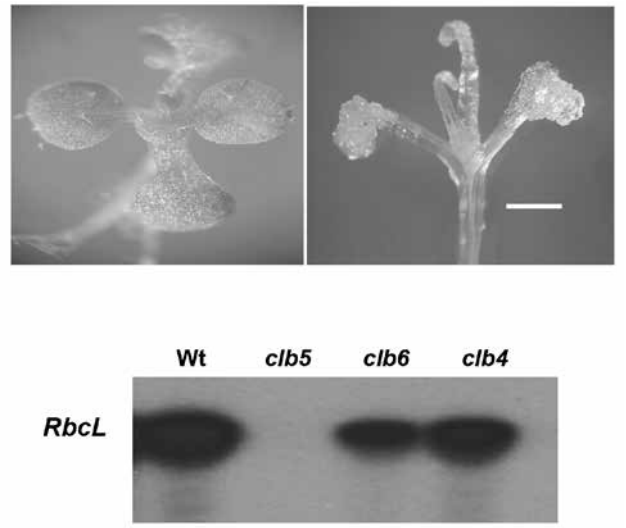


Figure1. *clb5* mutant phenotypes. a) Leaf phenotype of the wild-type (Wt) and *clb5* mutant (*clb5*). b) Expression of *RbcL* gene in three clb albino mutants.

altered leaf morphology (Figure 1). This leaf displays radial symmetry and with the use of leaf developmental markers we have shown display defects in the abaxial/adaxial polarity as well as problems in cell expansion (Aveñdano-Vazquez *et al.*, 2014). Molecular characterization of this mutant demonstrated that the gene affected in *clb5* corresponds to the *ZDS* that encodes for the zeta carotenoid desaturase biosynthetic enzyme, involved in the synthesis of carotenoids (Aveñdano-Vazquez *et al.*, 2014).

1.3 Linear carotenoids affect leaf morphology and nuclear gene expression

Carotenoids are terpenoids derived from the isoprenoid pathway synthesized in the chloroplasts (McQuinn *et al.*, 2015). These molecules are essential for plants with diverse functions. Carotenoids are accessory pigments of the photosynthetic apparatus with light absorption and photoprotection functions. These molecules are also important in human health, because they are precursors of vitamin A (retinol). Finally, carotenoids serve as precursors of an important number of molecules termed apocarotenoids that are diverse in function and structure and include hormones (ABA and strigolactones), flavors, scents and also as retrograde signaling molecules (Cazzonelli, 2011; Ramel *et al.*, 2012; McQuinn *et al.*, 2015). Carotenoid

biosynthesis takes place in plastids and derives from the 5-carbon precursors isopentenyl diphosphate and its isomer dimethylallyl diphosphate synthesized from the 2-C methyl-D-erythriol 4-phosphate (MEP) pathway. Condensation of these two blocks gives rise to the precursor molecule for all carotenoids, geranylgeranyl diphosphate (GGPS), a molecule of 20-carbons. From this point carotenoids are synthesized by the condensation of two GGPS to produce the linear carotenoid *all-trans* lycopene. Next *all-trans* lycopene undergoes cyclization producing a and b carotenoids (Figure 2).

The gene affected in the *clb5* mutant corresponds to the second desaturase enzyme required in the synthesis of the linear *all-trans* lycopene

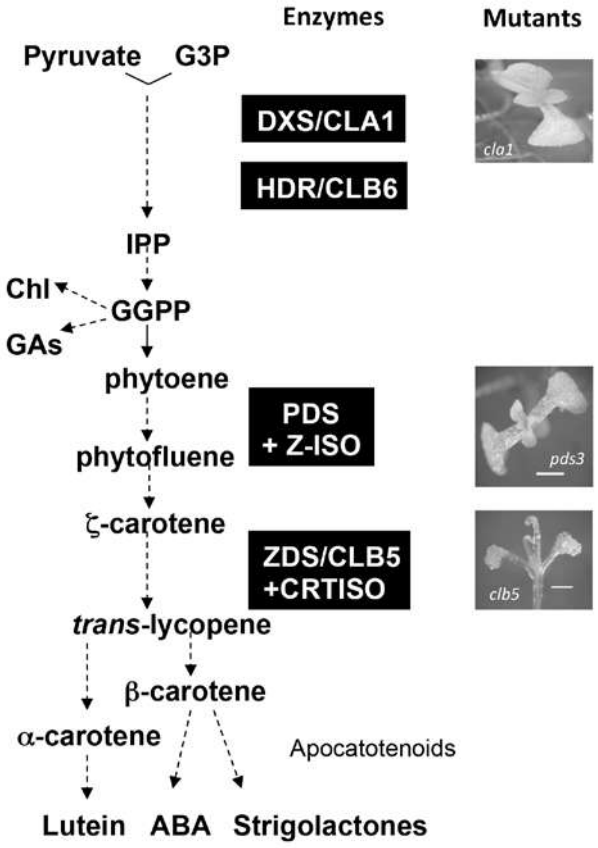


Figure 2. Morphology of the leaf phenotype in mutants affected in carotenoid biosynthesis. Simplified carotenoid biosynthetic pathway. Selected enzymes that participate in the pathway and their corresponding mutants. Only clb5 display defects in the leaf morphology.

(Figure 2). Our analysis demonstrated that although other mutants in the carotenoid pathway have albino phenotypes and alterations in the chloroplast development as *clb5* does, none of them affect leaf morphology and the expression of nuclear and chloroplast genes to the extent observed in the *clb5* mutant (Aveñdano-Vazquez *et al.*, 2014). Moreover, inhibition of the activity of the previous enzyme of the carotenogenic pathway (PDS), using inhibitors such as fluridone or through mutation (*clb5pds3*) restores the leaf morphology of the *clb5*. These results demonstrate that the upstream accumulation of the linear carotenoids, phytofluene and z-carotene, alters chloroplast, leaf development and the expression of chloroplast- and nuclear-encoded genes. To further understand the role of this signal that was named ACS1 we directed our attention to carotenoids and their derivative molecules.

2. Nature of the signal

2.1 ACS1is an Apocarotenoid generated by CCD4 enzyme

Carotenoids are precursors of molecules known as apocarotenoids, produced by their enzymatic or non-enzymatic oxidative cleavage. Many apocarotenoids have important functions as hormones, signaling molecules and retrograde signals (Hou *et al.*, 2016). The enzymes involved in apocarotenoid synthesis are the carotenoid cleavage digoxygenases (CCDs) that are an evolutionary conserved family of proteins composed in *Arabidopsis thaliana* of 9 members (Auldridge *et al.*, 2006). Since apocarotenoids have been shown to act as retrograde signals, we evaluate the morphology of the leaves in double mutants between *clb5* and some CCDs. This analysis demonstrated that the lack of one of these enzymes (CCD4) results in a partial restoration of leaf morphology in the double *clb5ccd4* mutant. This result supports the idea that ACS1 is an apocarotenoid produced through the action of CCD4. CCD4 is a chloroplast-localized enzyme that has been shown to cleave carotenoids, particularly b-carotenoids, at the 9,10 double bond position. However, the specificity of this enzyme is not yet fully clear. The results obtained in our analysis support that CCD4 has the capacity to cleave linear *cis*-carotenoids and generate the ACS1 signal. To further analyze this possibility, we analyze the phenotypic impact that alterations in the CCD4 levels have, using the *ccd4* mutant and over-expressing plants (OECCD4). We observe that the *ccd4* mutant has a phenotype similar to wild-type plants, except that the shape of the leaves in this mutant is altered compared to wild-type plants. Also, over expression of CCD4 results in alterations in the leaves morphology. Altogether this data strongly supports

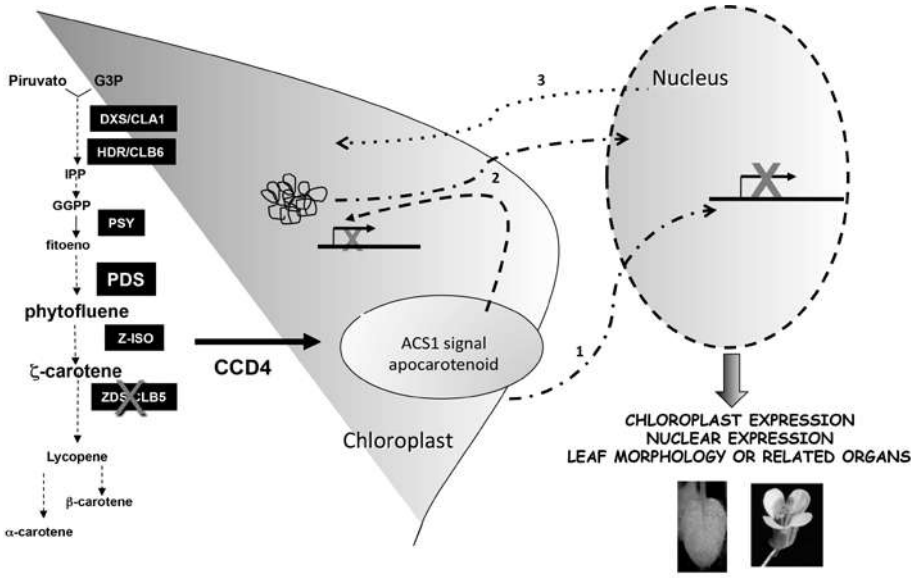


Figure 3. Working model in which the ACS1 signal is generated from the accumulation of phytofluene and/or ζ-carotene intermediates by the action of the CCD4 enzyme. This signal can directly affect nuclear gene expression (1) or through and indirect pathway that involves plastid gene expression (2). The generation of this signal might result from specific plastid developmental alteration in response to external stimuli or a specific nuclear factor (3).

a role of CCD4 in modulating leaf morphology and suggests that ACS1 might function as a retrograde signal that could be produced under specific conditions for this regulation (Figure 3).

3. Biological function of the ACS1 signal in plants

3.1 ACS1 signal could be synthesized in particular tissues and developmental stages

Although the nature of the ACS1 signal as an apocarotenoid has been established, the specific signal molecule has not yet been identified. However, a central question is to understand the biological function that this signal has during normal plant development and where it is generated. To address these aspects diverse approaches have been taken. The first is to define the expression pattern of the known genes and proteins that participate in the synthesis of ACS1. Transgenic plants expressing GUS marker under the regulation of ZDS and CCD4 regulatory regions have been

obtained. The characterization of such lines permitted to demonstrate that the expression of both genes displays particular expression patterns during plant development. These results support the possibility that ACS1 can be produced in specific moments and/or regions of the differentiating leaf. It also showed that the expression of ZDS and CCD4 expand beyond photosynthetic tissues as it is detected in some non-photosynthetic tissues such as flowers. These findings also demonstrate that both genes are expressed in other plastid types in addition to chloroplasts, an aspect that requires analysis in the future.

3.2 Impact of the ACS1 signal

A second strategy to understand the possible role of ACS1 included the characterization of the genes regulated by this signal. With this objective a wide-genome transcriptome analysis of the *clb5* mutant using RNAseq was carried out and compared to that of the *pds3* and the double *clb5ccd4* mutants. PDS3 encodes for the enzyme involved in the previous biosynthetic step to ZDS and, as all mutants in the pathway, has an albino phenotype but in contrast to *clb5* displays normal leaf morphology. In the case of the *clb5ccd4* double mutant we have previously demonstrated that the absence of the *CCD4* gene results in a partial suppression of the leaf phenotype of the *clb5* mutant, supporting the idea that the genes that are important for leaf morphology in *clb5* would be restored in this double mutant. Comparative analysis of these transcriptomes demonstrated that mutations in the ZDS gene result in mis-expression of hundreds of genes. More importantly, the expression level of most of those genes was restored in the double *clb5ccd4* mutant. Bioinformatic analysis of these genes will permit to pinpoint some pathways affected by this mutation and suggest possible roles of the ACS1 signal.

3.3 Open questions of the role of the ACS1 signal

This work provided biological evidences that support that linear carotenoids are key intermediates in the generation of apocarotenoid molecules with signaling functions capable of altering leaf morphology in response to the state of plastid development or its differentiation status (Figure 3). The accumulation of this putative signal results in a leaf with radial symmetry affected in polarity (Aveñdano-Vazquez *et al.*, 2014). Interestingly, other groups have also reported defects in leaf morphology in plants with defects in chloroplast development. For example, the *scabra3* mutant displays leaves with important serrations in contrast to wild-type leaves. The gene affect-

ed in this mutant encodes the nuclear-encoded plastid RNA polymerase RpoTp, required for the transcription of the plastid genes (Hricová *et al.*, 2006). It is interesting that some of the nuclear factors mis-expressed in *scabra3* are also affected in the *clb5* mutant, supporting possible connections between these responses (Hricová *et al.*, 2006). Other studies have shown that inhibition of chloroplast translation using drugs such as spectinomycin or lincomycin result in diverse defects in leaf morphology, including radialized leaves (Tameshige *et al.*, 2013; Tiller and Bock, 2014). Our comparative analysis demonstrated similarities with the leaf morphology of the *clb5* mutant and those obtained by the inhibition of the chloroplast translation, suggesting a possible relation between these responses. Future characterization of these leaves using particular markers will provide a better understanding of the possible relation between these responses.

4. Concluding remarks

The acquisition of plastids by photosynthetic lineages has dictated the evolution of these organisms. Today plastids play essential roles as metabolic hubs of the plant cell. However, during evolution these organelles have co-evolved with their host establishing complex regulations and specific differentiation patterns of these organelles. Plastid development is regulated by environmental cues as well by the differentiation status of the tissues where they reside. In addition, these organelles have developed mechanisms that impact not only the physiological status of the tissue but also influence the morphogenetic program of the plant. This complex dialog takes advantage of the enormous diversity of molecules produced by these organelles such as carotenoids and their derivative-molecules apocarotenoids. Our work and others have provided the bases for the understanding of one of those signaling mechanism that plays a novel role in leaf development. These findings have opened a new field regarding the impact of plastids in plant development.

Bibliography

Auldridge, M.E., Block, A., Vogel, J.T., Dabney-Smith, C., Mila, I., Bouzayen, M., Magallanes-Lundback, M., DellaPenna, D., McCarty, D.R., and Klee, H.J. (2006). Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *Plant J* 45, 982-993.

Aveñdano-Vazquez, A.O., Cordoba, E., Llamas, E., San Roman, C., Nisar, N., De la Torre, S., Ramos-Vega, M., Gutierrez-Nava, M.D., Cazzonelli, C.I., Pogson,

B.J., and Leon, P. (2014). An Uncharacterized Apocarotenoid-Derived Signal Generated in zeta-Carotene Desaturase Mutants Regulates Leaf Development and the Expression of Chloroplast and Nuclear Genes in Arabidopsis. *Plant Cell* 26, 2524-2537.

Cazzonelli, C. (2011). Carotenoids in nature: insights from plants and beyond. *Functional Plant Biology* 38, 833-847.

Chan, K.X., Phua, S.Y., Crisp, P., McQuinn, R., and Pogson, B.J. (2016). Learning the Languages of the Chloroplast: Retrograde Signaling and Beyond. *Annu Rev Plant Biol* 67, 25-53.

Dudareva, N., Klempien, A., Muhlemann, J.K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *The New phytologist* 198, 16-32.

Gould, S.B., Waller, R.F., and McFadden, G.I. (2008). Plastid evolution. *Annu Rev Plant Biol* 59, 491-517.

Gutiérrez-Nava, M.L., Gillmor, C.S., Jiménez, L.F., Guevara-García, A., and León, P. (2004). CHLOROPLAST BIOGENESIS genes act cell and noncell autonomously in early chloroplast development. *Plant Physiol* 135, 471-482.

Hou, X., Rivers, J., Leon, P., McQuinn, R.P., and Pogson, B.J. (2016). Synthesis and Function of Apocarotenoid Signals in Plants. *Trends Plant Sci*.

Hricová, A., Quesada, V., and Micol, J.L. (2006). The SCABRA3 nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in Arabidopsis. *Plant Physiol* 141, 942-956.

López-Juez, E., and Pyke, K.A. (2005). Plastids unleashed: their development and their integration in plant development. *Int J Dev Biol* 49, 557-577.

McQuinn, R.P., Giovannoni, J.J., and Pogson, B.J. (2015). More than meets the eye: from carotenoid biosynthesis, to new insights into apocarotenoid signaling. *Curr Opin Plant Biol* 27, 172-179.

Pogson, B.J., Woo, N.S., Forster, B., and Small, I.D. (2008). Plastid signalling to the nucleus and beyond. *Trends Plant Sci* 13, 602-609.

Pribil, M., Labs, M., and Leister, D. (2014). Structure and dynamics of thylakoids in land plants. *J Exp Bot* 65, 1955-1972.

Ramel, F., Birtic, S., Cuine, S., Triantaphylides, C., Ravanat, J.L., and Havaux, M. (2012). Chemical quenching of singlet oxygen by carotenoids in plants. *Plant Physiol* 158, 1267-1278.

Tameshige, T., Fujita, H., Watanabe, K., Toyokura, K., Kondo, M., Tatematsu, K., Matsumoto, N., Tsugeki, R., Kawaguchi, M., Nishimura, M., and Okada, K. (2013). Pattern Dynamics in Adaxial-Abaxial Specific Gene Expression Are Modulated by a Plastid Retrograde Signal during Arabidopsis thaliana Leaf Development. *PLoS genetics* 9, e1003655.

Tiller, N., and Bock, R. (2014). The Translational Apparatus of Plastids and Its Role in Plant Development. *Molecular plant*.

Timmis, J.N., Ayliffe, M.A., Huang, C.Y., and Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nature reviews. *Genetics* 5, 123-135.

van Wijk, K.J., and Baginsky, S. (2011). Plastid proteomics in higher plants: current state and future goals. *Plant Physiol* 155, 1578-1588.

Effects of Plant Associated Bacteria on Crop Yields: The Case of Tropical N2-Fixing Legumes

María Luisa Izaguirre-Mayoral,[1] George Lazarovits[2] and Bikash Baral[3]

Introduction

The world population continues to grow and is expected to reach 9.7 billion by 2050. Currently, 975 million people are chronically undernourished and about one billion are affected by malnutrition. Approximately 24 million children face daily hunger due to food security issues (UN, 2013; García *et al.*, 2017). Therefore, food production will have to be increased by 70-100% to feed the world and to achieve 'food sovereignty'. This term was first coined by La Via Campesina in 1996, and means "the right of people to healthy and culturally appropriate food produced through ecologically sound and sustainable methods, and their right to define their own food and agriculture systems" (Nyéléni Declaration, 2015). To fulfil this goal, the 1st step should be the modification of the prevailing unsustainable farming practices. In the case of high input farmers, the heavy use of pesticides, fertilizers, unnecessary irrigation, intensive ploughing and large-scale monocropping systems are significant contributors to land degradation and climate change that is triggering the degradation of soil and water systems, erosion and salinization, accompanied by the loss of soil fertility leading to greater use of chemical fertilizers. In the case of small scale low input farmers, lack of accessibility to chemical fertilizers and seeds selected, based on agroecologically adapted cultivars, as well as lack of irrigation to compensate seasonal droughts, are considered as the pivotal factors underlying low crop yields. At the same

[1] Academia de Ciencias de América Latina, Palacio de las Academias, Avenida Universidad, Apartado Postal 1121-A, Distrito Federal, Venezuela. E-mail: mlizaguirre@gmail.com.

[2] A&L Biologicals, Agroecological Research Services Centre, London, ON, Canada, N5V 3P51, Department of Biology, University of Western Ontario, London, ON, Canada.

[3] Department of Biochemistry, University of Turku, FIN-20014 Turku, Finland.

time, such low-input agricultural systems foster reduced negative impacts on the environment.

Nitrogen (N)-fertilizer provides plants with the N required for photosynthetic CO2 fixation, growth and seed yields. The category of N as the most essential macronutrient for plants is supported by the highly significant correlations between crop yields and N fertilization reported in Australia (r2=0.65), China (r2=0.96), Latin America (r2=0.94) and USA (r2=0.67) (Hatfield and Prueger, 2004). The fact that plants absorb soil available N exclusively through their roots only in the form of urea or nitrates spawned a massive N-based fertilizer industry worldwide in 1982. Predictions for 2018 point toward: (1) an annual growth rate demand of N fertilizers of 3.3% in Latin America plus the Caribbean, 0.5% in North America and 4.6% in Sub-Saharan Africa, with the greatest use of N-fertilizers taking place in Asia (57.7%) and in Latin America plus the Caribbean (17.6%); and (2) an increase from 3.7% in 2014 to 9% in 2018 in the potential N balance as % of global total demand (FAO, 2015a; b). Moreover, predictions for chemical fertilizer consumption in 2018 indicate a greater annual growth rate demand for phosphorous (P) above N fertilizers in West Asia (3 fold), Central Europe (2.2 fold), North Africa (1.6 fold) and Eastern Europe and Central Asia combined (1.4 fold), with the greatest P consumption occurring in Asia (57.6%) and in Latin America plus the Caribbean (26.1%) (FAO, 2015b). FAO also pointed out that to support agriculture in 2018, manufactures worldwide will have to produce an estimate of 4430 tons of urea (the main source of anthropogenic N for the manufacturing of N-based fertilizers) and 1591 tons of phosphoric acid (as P2O5) more than produced in 2017. However, the practice of applying industrially produced fertilizers to augment agricultural yields has proven to incur severe economic, agronomic and environmental penalties as crops do not take up more than 50% of the added chemical N and P fertilizer (Aulakh *et al.*, 2017). The rest ends up becoming pollutants to soils and surface and subsurface water in the form of nitrites and residual phosphates. Air pollution with nitrous oxide, a potent ozone depleting-greenhouse gas, is also strongly correlated with the increase in the global input of N-fertilizers to agricultural soils (Kim *et al.*, 2017). The manufacture of NPK-15:8:15 fertilizer in Iran consumes 1660 MJ ton-1 of nonrenewable fossil fuels (Farahani *et al.*, 2017).

Combing all these factors, there is an urgent need for agricultural systems that are productive as well as sustainable, which reduce the need for external inputs and do not compromise the environment and public health. This main objective of a highly productive agriculture with mini-

mum damages to the biosphere was included within the framework of the Sustainable Development Goals in the United Nations 2030 Agenda for Sustainable Development, written 25-27 September 2015. Goal 2 instructs UN members to "End hunger, achieve food security and improve nutrition and promote sustainable agriculture". Goal 13 encourages Nations to "take urgent action to combat climate change and its impacts". This relatively new approach to increase crop yields via a sustainable intensification of agriculture is gaining supporters worldwide. However, as highlighted by Bernard and Lux (2017), sustainable agriculture associated with high crop yields could also become a "Trojan horse paving the path for many kinds of technologies, including genetically modified organisms". In any case, sustainable agriculture involves the successful management of agricultural resources to satisfy changing human needs, while maintaining or enhancing the environmental quality and conserving natural resources. Within this idea, developing efficient biofertilizers for crops has become the focus of scientists around the world as a strategy for sustainable management of soil N and P, with lower collateral damage to the environment.

A widely-accepted definition of biofertilizer is "a substance which contains living microorganisms which, when applied to seeds, plant surfaces or soil, colonize the rhizosphere or the interior of the plant, promoting growth by increasing the supply or availability of nutrients to the host plant". Thus, environmental contamination by chemical fertilizer derivatives can be reduced by adoption of biofertilizers as a management practice that increase the nutrients' accessibility for plant use, enhances plants' ability to uptake nutrients, and more closely matches the nutrient applications with agronomic needs. The effectiveness of biofertilizers is based on the adaptive capacity of plants to establish associations with host immunity evader or suppressor rhizospheric, endospheric, phyllospheric and endophytic Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria species displaying N2 fixing and/or P-solubilizing abilities. Microbes are the predominant form of life on the planet and have evolved to exhibit a wide range functional and metabolic diversity that vastly exceeds that of all other known organisms. Therefore, the aim of this paper will be the discussion of the pros and cons of the biotechnologies associated to biofertilization as a key factor for the implementation of a sustainable agriculture under N and/or P deficient soil limiting conditions. Due to the complexity of the microbiome associated with plants, the analyses will concentrate on rhizobia-nodulated N2-fixing tropical legume crops.

Biological N2 fixation

Within the phylum of Gram-negative proteobacteria, the subphylum α-proteobacteria includes the members of the N2-fixing genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Ensifer, Mesorhizobium* and *Rhizobium* capable of using atmospheric N2 through a process known as biological nitrogen fixation (BNF). This involves the conversion of atmospheric N2 to NH3, catalyzed by the highly conserved enzyme nitrogenase (Masepohl, 2017). This agriculturally important N2 fixing bacterial group has an ability to establish symbiosis with legumes belonging to a single phylogenetic genera, the Fabaceae, comprising three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) (Kaschuk

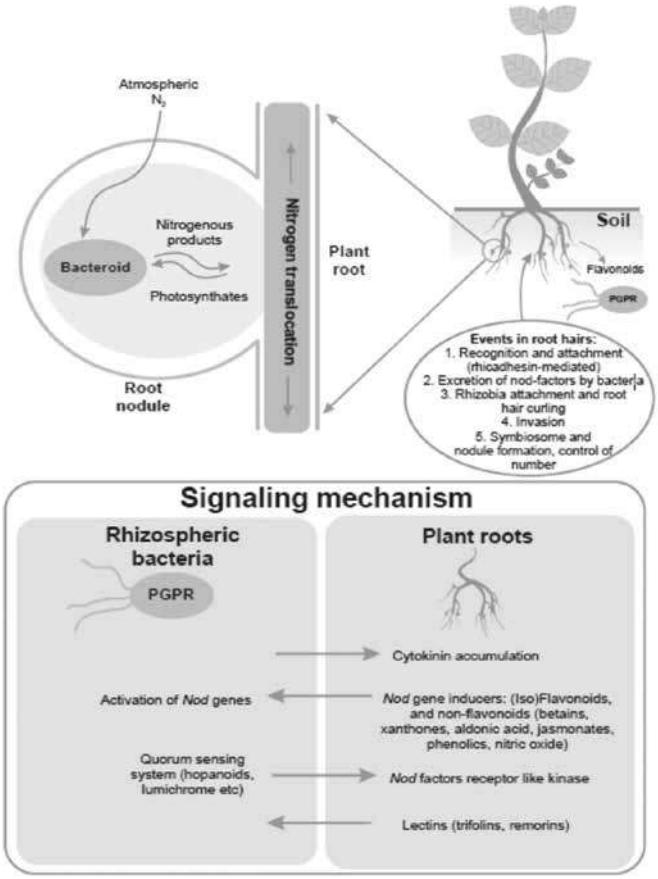


Figure 1. Signaling mechanism between the rhizobia and the plant.

and Hungria, 2017). As reviewed by Baral *et al.*, (2016), the evolution of this complex symbiotic trait between legumes and N2-fixing rhizobia involves the legume's recruitment of the mechanism that perceives mycorrhizal lipo-chito-oligosaccharides and mutations in the regulation of nitrate-regulated gene expression. In brief (Fig. 1), the biogenesis of the symbiosis starts with the recognition by the soil rhizobia strains of (iso) flavonoids that exist in the seed coat or are excreted by roots, followed by the attachment of the free-living rhizobia to plant root hairs (Lopez *et al.*, 2017) and the initiation of the nodule meristem formation. Detailed description of the signaling exchanges between the rhizobia and the plant, activation of genes encoding the symbiosis and onset of the BNF were published by Baral *et al.*, (2016). Within the root nodules the colonizing rhizobia undergo a dedifferentiation process to bacteroids linked to the activation of the N2 fixing genes and triggering the onset of the nitrogenase activity. Rhizobia-nodulated tropical crop legume species belonging to the Fabaceae generally form a determinate type of root nodules where the fixed N2 is metabolically converted into ureides [allantoin ($C_4H_6N_4O_3$) and allantoic acid ($C_4H_8N_4O_4$)] to be exported to aerial organs in exchange for photosynthates as carbon (C) source for the bacteria (Baral and Izaguirre-Mayoral, 2017). Once in the mature leaves, ureides are catabolized to NH_3 to satisfy the N demand of the plant for growth a reproduction. The biochemistry and genetics underlying BNF were reviewed by (Saha *et al.*, 2017). Interestingly, data collected by the Sample Analysis at Mars (SAM) instrument on the Curiosity Rover and analyzed by NASA showed $\delta^{15}N$ values corresponding to N2 fixation processes during the early geological history of the planet Mars (Stern *et al.*, 2017).

Plants growth promoting bacteria (PGPB)

Legumes also host a consortium of agriculturally important β- and γ-proteobacteria as well as the taxonomically diverse group of plant growth promoting bacteria (PGPB), all positively strengthening the performance of abiotically-challenged plants (Forni *et al.*, 2017; Turan *et al.*, 2017). Most recognized PAB belong to the genera *Acinetobacter, Alcaligenes*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Bradyrhizobium, Burkholderia*, *Curtobacterium, Enterobacte*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia*. Amid PGPB, the rhizoplane, rhizospheric and endophytic species that have the greatest agricultural impacts include: *Azotobacterchroococcum, Azospirillum spp., Azospirillum brasilense, Herbaspirillum seropedicae*, *Bacillus amylolique-*

faciens, B. pumilus, B. simplex, B. thuringiensis, Enterobacter aerogenes, Moraxella pluranimalium, Pseudomonas putida strain FBKV-2, *P. stutzeri* and *Pantoea alhagi*. In general, all rhizobiales and PGPB promote crop yields via N2 fixation, synthesis of indole acetic acid (IAA) and the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Vergani *et al.*, 2017) that decreases the amount of ACC available for the synthesis of ethylene in stressed plants (Checcucci *et al.*, 2017; Defez *et al.*, 2017; Mehnaz and Lazarovits, 2017). Plant growth and yield improvement by PGPB also involve the solubilization of soil macro and micronutrients – otherwise unavailable to plants – as well as the pathogen antagonistic features such as production of siderophores, antibiotics, bacteriocins, proteinaceous toxins and lytic enzymes (Tariq *et al.*, 2017). As shown by Püschel *et al.*, (2017), a strongly positive correlation exists between total plant P content and the percentage of N derived from the atmosphere (%Ndfa). This clearly documents the importance of plant P nutrition for BNF efficiency.

Crop biofertilization

Grain legumes are major agriculture crops which provide an important source of proteins for human and animals and are widely used as green manures, as cash crops and forages under a wide range of agro-climatic conditions. Grain legumes are also important sources of carbohydrates, fibers and micronutrients especially iron and zinc as well as of potentially disease-preventing and health-promoting compounds (Zargar *et al.*, 2017). Legumes are additionally known as the most efficient system for BNF, displaying high %Ndfa. Amid grain legumes, soybean is the most important legume crop in the world due to the high nutritional quality of its leaves and grains and for the use of soybean oil as an alternative to fossil oil. Annual average soybean yield is close to 3 t·ha-1 in USA and Brazil, a value three fold higher than the grain yields recorded in most African countries (Munthali *et al.*, 2017). Common bean, cowpea, mung bean and yardlong bean are preferred by small scale farmers due to their edible high iron-protein and antioxidant nutritional quality of leaves and seeds as well as the plants' tolerance to drought and salinity (Izaguirre-Mayoral *et al.*, 2017). Grain yields of common bean ranks from 0.3-2.2 t·ha-1 in sub-Saharan African countries, 2.1-3.1 t·ha-1 in Brazil, and 0.6-1.4 t·ha-1 in Chiapas and Veracruz (Mexico) (Diaz *et al.*, 2017; Gereziher *et al.*, 2017; Nascente *et al.*, 2017; Pereira *et al.*, 2017; Valle *et al.*, 2017). Cowpea grain yields oscillate between 0.3-0.8 t·ha-1 in Brazil (de Oliveira *et al.*, 2017), 1.5-1.8t·ha-1 in Ghana (Daramy *et al.*, 2017), 0.6-1.2 t·ha-1 in Nigeria (Okunade *et al.*,

2017), 0.8-1.3 t·ha-1 in Uganda (Ddamulira *et al.*, 2017) and 0.3-0.9 t·ha-1 in Zimbabwe (Manzeke *et al.*, 2017). In all cases, variations in grain yield were attributed to the prevailing local agricultural practices and the genotype x environment interactions.

Brazil, Argentina and Uruguay are the largest soybean producing countries in South America, with Brazil being the largest common bean producer. In these countries, as well as in the USA, the pre-inoculation of legume seeds with rhizobia inoculants is considered as the most important among the agricultural practices. Introduction of this biotechnology in Sub-Sahara Africa has been sponsored by the Melissa and Bill Gates Foundation for the last 10 years in a coordinated project denoted as N2Africa. Peat or liquid rhizobia-based commercial biofertilizers are widely used by high input commercial farmers, with soybean, cowpea and bean crops obtaining between 24-89% of their N requirements from BNF (Bello *et al.*, 2017) depending on the levels of mineral soil N, prevailing environmental conditions during the cropping seasons, agronomic management, effectiveness of the nodulating rhizobial strains and plant genetic traits (Santachiara *et al.*, 2017). In Africa, Stadler *et al.*, (2017) reported an overall rhizobia inoculation increased grain legume yield of 115 kg·ha-1, with 75% of rhizobia inoculated fields having predicted responses between 102 and 172 kg·ha-1. Although this may seem to be a moderate gain, the low cost of inoculants (~6-10 USA $ per hectare) means that this biotechnology is predicted to provide economic benefits to small-scale farmers. Examples are outlined in Table 1.

In South America, high-quality commercial rhizobia inoculants are provided by several companies. In Argentina, Rizobacter, Fragaria and Monsanto Bioag are the major biofertilizer and adjuvant manufactured. At present, through its dealers, Rizobacter products are available in countries like Paraguay, Bolivia, Brazil, Botswana, Namibia, South Africa and Zambia. In Bolivia, Fertimax seems to be the major biofertilizer factory. Local production of rhizobial inoculants in Brazil is carried out by the companies Farroupilha (also merged to Lallemande), Microquimica, Stoller, Grap, Biosoja, Bioagro and Total Biotecnología, whereas in Uruguay the companies Lage (recently merged to Lallemande) and Calister supply the local rhizobia inoculants. In Colombia, 22 companies produce and/or commercialize different consortia of rhizobium and PGPB containing biofertilizers.

During the last decade accumulating evidence indicates that a positive synergism exists between PGPB consortia and legume crop yields, when compared with single bacteria inoculations (Table 2). Moreover, inocula-

Crop	Country	Control	Rh^+	References
		Grain yield ($t \cdot ha^{-1}$)		
Africanyam	Nigeria	2.5	2.8(s)	(Binang *et al.*, 2017)
Bambara groundnut	Nigeria	1.3	1.3	(Binang *et al.*, 2017)
Bebi bean	Nigeria	1.0	1.0	(Binang *et al.*, 2017)
Chickpea	Pakistan	1.8	2.1(s)	(Nawaz *et al.*, 2017)
Common bean	Ethiopia	1.3	2.1(s)	(Argaw, 2017)
Common bean	Ethiopia	1.4	2.5(s)	(Tarekegn *et al.*, 2017)
Cowpea	Brasil	1.2	1.4(s)	(Batista *et al.*, 2017)
Cowpea	Burkina Faso	0.3	0.7(s)	(Haro *et al.*, 2017)
Cowpea	Ethiopia	2.3	3.1(s)	(Tarekegn *et al.*, 2017)
Cowpea	Mozambique	1.0	1.4(s)	(Kyei-Boahen *et al.*, 2017)
Faba bean	Australia	1.8	3.5(s)	(Denton *et al.*, 2017)
Faba bean	Egypt	2.6	4.7(s)	(Youseif *et al.*, 2017)
Faba bean	Ethiopia	3.5	5.0(s)	(Argaw and Mnalku, 2017)
Groundnut	Chad, Cameroon	1.5	2.7(s)	(Gomoung *et al.*, 2017)
Mucuna bean	Nigeria	1.5	2.8(s)	(Binang *et al.*, 2017)
Peanut	Brazil	1.4	1.8(s)	(Santos *et al.*, 2017)
Peanut	Ethiopia	2.0	3.8(s)	(Argaw, 2017)
Soybean	Ethiopia	1.6	2.7(s)	(Muleta *et al.*, 2017)
Soybean	Ethiopia	1.5	3.3(s)	(Getachew *et al.*, 2017)

Means in a row followed by (s) are significantly different to each other

Table 1. Examples of grain yields in control (uninoculated) and rhizobia-inoculated (Rh+) legume crops assessed in different nations.

tion with different mixtures of *Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Bradyrhizobium. Burkholderia, Clostridium, Enterobacter, Gluconacetobacter, Herbaspirillum*, *Klebsiella, Pseudomonas* and *Rhizobium* strains, some displaying high N2 fixation rates, was shown to promote legume as well as non-legume crop fitness to N and P deficient soils (Saha *et al.*, 2017). The consortium of *Achromobacter xylosoxidans, Bacillus cereus, B. subtilis* and *B. thur-*

Crops	Control	Rh^+	Rh^+ + PGPB	References
	Grain yield ($t \cdot ha^{-1}$)			
Black gram	0.7c	0.9b	0.9a	(Kudi *et al.*, 2017)
Commonbean	1.5b	1.5b	2.3a	(Souza *et al.*, 2017)
Peanut	0.7c	0.7b	0.9a	(Kudi *et al.*, 2017)
Soybean	2.1c	2.4b	2.5a	(Jaybhay *et al.*, 2017)

Means in a row followed by different letters are statistically different to each other

Table 2. Examples of grain yields in legume crops uninoculated (control), rhizobia inoculated (Rh+) or inoculated with a mixture of Rh+ and phosphorus-solubilizing plant growth promoted bacteria (PGPB).

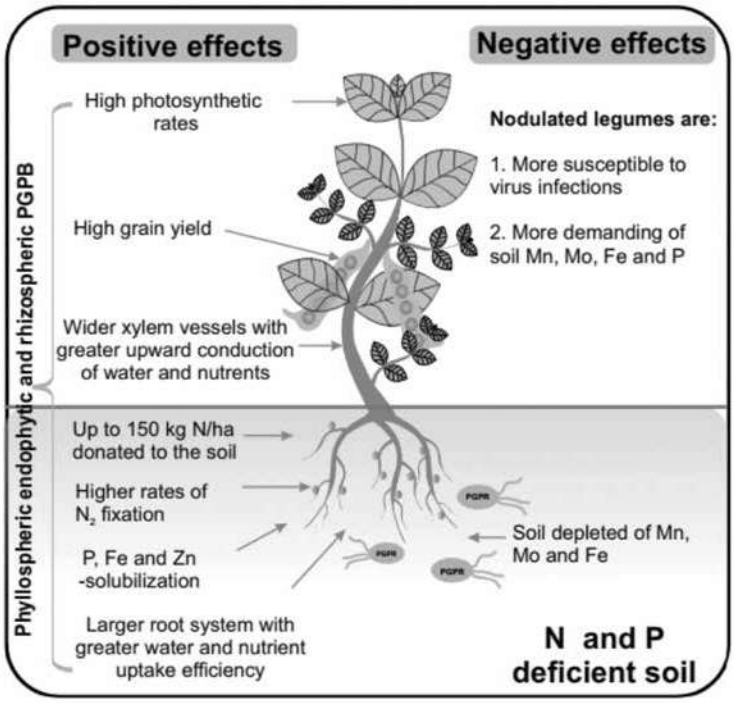


Figure 2. Effects of inoculation with effective growth promoting bacteria (PGPB) at the soil and plant levels.

ingiensis was also shown to protect crop legumes against the infection by the pathogen *Fusarium solani* (Egamberdieva *et al.*, 2017). Therefore, there is an increasing tendency to include PGPB in the rhizobia inoculants. The positive and negative effects elicited by PGPB on plants are outlined in Fig. 2.

Major constraints to the use of biofertilizers

The use of biofertilizers to enhance crop yields is far from being a foolproof biotechnology, facing at least two important constraints.

Virus infections

For large and small-scale soybean farmers, high yields are critical to maintain acceptable profit margins. However, the incidence of insect pests and virus diseases hamper legume grain yields resulting in significantly lower farm income for rural and urban economies (Anderson *et al.*, 2017; Beatrice *et al.*, 2017; Guy, 2014; Hema *et al.*, 2014; Odedara and Kumar, 2017; Ramesh *et al.*, 2017; Zanardo and Carvalho, 2017). Rhizobia-inoculated N2-fixing nodulated legumes have been found to have a much greater susceptibility to virus infections due to the blockage of the plant defence mechanisms induced by the colonizing rhizobia (Izaguirre-Mayoral and Garrido, 2010; Lopez *et al.*, 2017). The rhizobial cells attempting to colonize plants are first recognized as pathogens by the plant immune surveillance system (Izaguirre-Mayoral and Garrido, 2010), leading to a complex array of global as well as specific defence responses, which are often associated with plant cell apoptosis and the subsequent arrest of the invader (Lopez *et al.*, 2017). Thus, for the rhizobia to enter the root via the root hairs, the hypersensitive response is switched off by the excreted bacteria Nod factors. However, this suppression of immunity renders the plant open to infections by viruses.

In N2-fixing grain legumes, viruses hijack the plant cellular machinery for viral replication, triggering the plant defence mechanisms to repress intracellular virus movements via the cell plasmodesmata (Izaguirre-Mayoral and Garrido, 2010). In the case of systemic viral infections, the virus hindering effects on the early events of nodulation result in reduced plant growth and nodulation (Lopez *et al.*, 2017). Infection by the virus particles and their replication inside the nodules causes major ultrastructural alterations in the cells containing the symbiosomes and this in turn delays the onset of nitrogenase activity and reduces the activity of several key nodule enzymes (Izaguirre-mayoral *et al.*, 1994; Izaguirre-Mayoral *et al.*, 1992). In the case of seed-borne legume viruses, reported mainly among the RNA

viruses belonging to the genera *Como-* and *Sobemovirus,* there is almost a total absence of nodulation on the uppermost root zone, and some limited secondary nodulation on the rest of the root. Adding external flavonoids does not bring about any further positive effects due to virus-induced deformations of root hairs impeding the docking of the free living soil rhizobia (Lopez *et al.*, 2017).

The immunity blocked situation in biofertilized legume crops is further complicated by the field prevalence of more than 145 viruses in 27 groups infecting 281 species in 64 genera of the Leguminosae, all displaying distinctive modes of transmission and different strategies to achieve successful spread and reproduction in plants. Worldwide, virus infection caused 1.4% losses out of the 245 million tons attainable soybean production in 2002-2003, while in the USA, virus infections caused approximately $35 million loss to soybean producers (Reddy, 2015). Economic analyses of the impact of *Aphis glycines* (Hemiptera: Aphididae), the vector for a large number of legume infecting viruses worldwide, on soybean production predicted that $3.6 to $4.9 billion annual losses, depending upon the cost of insecticide applications, the size of the aphid outbreak and the price elasticity of soybean supply (Marchi-Werle *et al.*, 2017). To make the situation even worse, the commercially available legume genotypes or cultivars being released in most developing and under developed countries are highly susceptible to many viruses transmitted by seeds and/or insects (Meziadi *et al.*, 2017; Rubiales *et al.*, 2015). The seed-borne *Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), *Cowpea mild chlorotic virus* (CMMV), *Cowpea mild mottle virus* (CMMV), *Southern bean mosaic virus* (SBMV), *Soybean mosaic virus* (SMV) and *Tobacco streak virus* seem to be the most important viral pathogens of legume crops, with a worldwide distribution and high virulence (Izaguirre-Mayoral *et al.*, 2017; Lopez *et al.*, 2017; Nordenstedt *et al.*, 2017; Patil *et al.*, 2017; Smith *et al.*, 2017). The SMV is transmitted by up to 30% of the seed from SMV-infected plants and by aphids in a non-persistent manner decreasing the content and quality of the seed oil and causing up to 100% grain loss as well as severe seed mottling (Nandakishor *et al.*, 2017). Seven USA G1-G7 strains and 21 Chinese SMV strains of SMV are reported so far (Yang *et al.*, 2014). Yield losses up to 40% are reported in BPMV-infected soybean crops (Byamukama *et al.*, 2011; Ugwuoke and Onyeke, 2010) and BPMV-infected plants are more prone to undergo infection by *Phomopsis longicolla* (Soto-Arias and Munkvold, 2011). Up to 80% loss is also reported in the case of infection by *Yellow mosaic virus* (YMV), another major disease of

soybean (Kumar *et al.*, 2014). Infection by CMMV and *Cucumber mosaic virus* (CMV) caused an average reduction of 65% and 60%, respectively, in six Nigerian soybean lines (Arogundade and Balogun, 2010). Damage to soybean from a CMM-*like virus* has been of increasing concern in Argentina, Brazil, Mexico and the USA. Moreover, worldwide there is an increasing number of reports on new viruses infecting legumes, more potentially destructive new or previously reported viruses, new recombinant virus strains exhibiting different pathogenicities, RNA viruses undergoing high mutation rates during replication affecting the heterogeneity of the virus population, as well as the prevalence of mixed viral infections in agricultural fields (Irizarry *et al.*, 2016; Jiang *et al.*, 2017; Varsani *et al.*, 2017). Also, the quasi species cloud size is known to be affected by the virus shifting among different host legume species, making the possibility of obtaining virus-free seeds very low (Navas-Castillo *et al.*, 2011).

In summary, avoiding epidemics of legume infecting virus diseases constitutes a challenge due to the complexity of the three-cornered pathosystems (virus, vector and host) involved and their interactions with the environment. Effective control of virus vectors such as aphids, thrips, scales, whiteflies and leaf beetles can be achieved by either seed coating or spraying insecticides like thiamethoxam and oxydemeton methyl on the crops (Swathi and Gaur, 2017). However, applications of insecticides and herbicides, although a proven means to increase crop yields, are known to hinder BNF (Ing. Agr. Carlos Labandera, Uruguay, personal communication). A new chemical encapsulated/controlled release formulation of p-Imidacloprid (1-(6 chloro-3-pyridinyl methyl)-N- nitroimidazolidin-2-ylideneamine), prepared using amphiphilic polymers derivatives from polyethylene glycol and aliphatic diacid, was shown not to hinder BNF and grain yield (Adak *et al.*, 2012). In all cases, pest control represents an extra cost to farmers and is linked to environmental pollution by the applied chemicals. Another option to control the incidence of virus infections is to block the systemic spread of the viruses and the appearance of symptoms by planting transgenic legume plants expressing the transgenic products that induce resistance to different viruses (Kreuze and Valkonen, 2017; Tavazza *et al.*, 2017). The adoption of the OMG technology however, has been disputed for almost two decades and the planting of transgenic plants is not allowed by law in many countries. The possibility, although more complicated, of using biological control by bacteria such as *Pseudomonas aeruginosa* on soybean crops to counteract infection by the *Soybean stunt virus* (Khalimi and Suprapta, 2011) should not be ruled out. At present, *Bacillus thuringiensis*

and Baculovirus are the most widely, biologically safest and successfully used bioinsecticide in the integrated pest management programs in the world (Deshayes *et al.*, 2017).

Biofertilizers production and storage conditions

The commercial production of biofertilizers requires the previous isolation and characterization of microbial strains displaying plant growth promoting qualities in association to local legume cultivars under the prevailing soil properties and environmental conditions. The selected PGPB are replicated in large fermenters under strict sterile conditions to be finally packaged as liquid or organic-carrier formulated biofertilizers. The most commonly used formulations are solid or peat-based inoculants that can be purchased for seed or direct soil applications. For pre-inoculation of seeds the peat based biofertilizers are mixed with an adhesive product to ensure the adherence of the PGPB cells to the seed coats. However, for peat formulations it is highly recommended that the peat be gamma-irradiated to eliminate peat-carried microorganisms that can outcompete rhizobia for available nutrients in the carrier media. The final product must be registered and certified for use with crops destined for animal and/or human consumption.

Peat-irradiated biofertilizers have excellent shelf life of several months when storage is at room temperature of <25°C. Such carriers also allow for elevated levels of viability of the PGPB on the seed coat as well as in the upper soil horizons after sowing. Another benefit is the efficient bacterial holding capacity of the peat when rainfall occurs within 72 hours following sowing. However, during storage the solid aerobic materials impose on the PGPB a substantial cost for colony growth due to the energy required to manage intracellular oxygen via multiple oxygen protection strategies such as the production of extracellular polymers as a barrier to O2 diffusion, an increase in the cell size, as well as higher carbohydrate consumption for respiratory protection (Inomura *et al.*, 2017). Liquid biofertilizers are available in sterile broth cultures mixed with sterile water and these are usually sprayed into the seed furrow at planting. In the case of pre-inoculation of seeds, the diluted bacterial broth is mixed with a water-soluble adhesive product at the time of sowing. Liquid biofertilizers must be kept refrigerated during shipment and storage, thus their availability through normal distribution channels is limited. Under no circumstances should biofertilizers be exposed to direct sunlight since ultraviolet rays and heat will kill the bacteria.

The effectiveness of the biofertilizer will be determined by the ability of the PGPB to compete and survive in the soil, as well as the efficiency of the rhizobia to colonize roots. This is determined by the expression of genes that regulate cell-cell communication via quorum sensing (Masepohl, 2017). However, the screening of isolated rhizobia strains against local crop varieties/landraces for improved BNF is an expensive and time-consuming process in terms of reagents and equipment, technical expertise, labor, accuracy and the need for non-fixing reference plants. A new technique to screen rhizobia inoculants for BNF activity based on measuring Gln output using the *GlnLux* biosensor, which consists of *Escherichia coli* cells auxotrophic for Gln and expressing a constitutive *lux* operon, was developed by (Thilakarathna *et al.*, 2017). On the other hand, biofertilizers are generally not compatible with insecticide/fungicide seed treatments, unless the rhizobial strains in the inoculants were pre-selected to withstand the seed coating chemicals. This complicates and delays the selection and evaluation of rhizobial strains to be included in the production of biofertilizers. Additionally, there is overwhelming evidence that applying rhizobia inoculants to soils that were previously biofertilized provides no yield benefit. Also, there is a consensus that under ample N and P supply, the benefits of rhizobial and PGPB inoculations, in terms of improved plant nutrient acquisition and growth, are reduced or eliminated completely. Therefore, bacterial inoculants can contribute to increase agronomic efficiency by reducing production costs and environmental pollution, once the use of chemical fertilizers is reduced or eliminated.

The approach to use biofertilizers as the source on macro and microelements for crop production requires very close collaboration between the private sector and scientific community to ensure the quality of the commercial inoculants. Applications of biofertilizers to seeds or soil may have collateral harmful aspects related to the presence in the product of potential pathogenic microorganisms as PGPB or as contaminants. According to South America's leading trading block (MERCOSUR), regulations to export or import biofertilizers within the region require that the product be certified by a governmental laboratory as harboring at least 108 PGPB cells and less than 103 cell contaminants per gram or milliliter of product, and that the contaminants must be ones that are not hazardous to human or animal health. Nevertheless, there are still more than 90% of microbial diversity and microbial activities influencing bio-sphere functioning to be discovered that may exert positive effects on agriculture, food and environmental protection via new improved biotechnological developments. As

indicated by Trivedi *et al.* (2017), there are about 54,000 complete genome sequences of microbes available in public domains, with a vast library of over 250 million genes to be prospected for biotechnological applications.

Final remarks

Agriculture has been central to the success of *Homo sapiens*. However, expansion of conventional agricultural practices to meet future food demands is not going to be economically or environmentally feasible. Thus, to improve farm productivity and food quality in a sustainable manner, the adoption of the biofertilizers technology must be encouraged by governments and supported by a high level of scientific research. Through recent years, the use of multiple compatible PGPB consortia was shown to play a pivotal role on the improve growth, vigor, nutrient use efficiency and biotic/abiotic stress tolerances of crops in a positive environmental, social and economic manner. Therefore, the integration of biofertilizers carrying highly efficient N2-fixing PGPB and the selection of matching microbe-optimized crops for different soil types is the ultimate goal to enhance eco-friendly crop yields for the human food security, worldwide.

Despite recent scientific advances on the understanding of the microbiome associated with legume crops and the promising results from extensive field trials, the utilization of biofertilizers is still far from reaching small-scale farmers in non-developed nations worldwide. There is also an urgent need for plant breeders to work together with soil microbiologists on the creation of new legume cultivars that are better adapted to prevailing environmental conditions and that can establish efficient associations with PGPB formulations in biofertilizers. The success of biofertilizers as crop yield promoters depends on low-input farmers having access to certified virus-free seeds of the improved cultivars and to their abilities to ensure that soils have sufficient micronutrients in terms of manganese, zinc, and molybdenum required for the establishment of successful plant-microbe interactions. Concomitantly, it is of great importance that small-scale farmers are properly trained on the handling and application of biofertilizers to seeds or soils. Local retailers also need to be informed on the conditions under which biofertilizers must be handled and stored. The lack of field experts on these aspects may cause the failure of the deployment of biofertilizers and create a negative image for farmers as to the further use this biotechnology.

Acknowledgements

M.L.I-M. is grateful to Monsignor M. Sánchez-Sorondo and Dr. E. de Robertis for the excellent organization of the Workshop on *Cell Biology and Genetics*, sponsored by the Pontifical Academy of Science, at the Vatican, October 23-24, 2017.

References

Adak, T., Kumar, J., Dey, D., Shakil, N.A., Walia, S., 2012. Residue and bio-efficacy evaluation of controlled release formulations of imidacloprid against pests in soybean (*Glycine max*). *J. Environ. Sci. Heal.* – Part B 47, 226-231. doi:10.1080/03601234.2012.634368.

Anderson, N.R., Irizarry, M.D., Bloomingdale, C.A., Smith, D.L., Bradley, C.A., Delaney, D.P., Kleczewski, N.M., Sikora, E.J., Mueller, D.S., Wise, K.A., 2017. Effect of soybean vein necrosis on yield and seed quality of soybean. *Can. J. Plant Pathol.* 39, 334-341. doi:10.1080/07060661.2017.1354333.

Argaw, A., 2017. Organic and inorganic fertilizer application enhances the effect of *Bradyrhizobium* on nodulation and yield of peanut (*Arachis hypogea* L.) in nutrient depleted and sandy soils of Ethiopia. *Int. J. Recycl. Org. Waste Agric.* 6, 219-231. doi:10.1007/s40093-017-0169-3.

Argaw, A., Mnalku, A., 2017. Effectiveness of native *Rhizobium* on nodulation and yield of faba bean (*Vicia faba* L.) in Eastern Ethiopia. *Arch. Agron. Soil Sci.* 63, 1390-1403. doi:10.1080/03650340.2017.1287353.

Arogundade, O., Balogun, O.S., 2010. Influence of *Cowpea mottle virus* and *Cucumber mosaic virus* on the growth and yield of six lines of Soybean (*Glycine max* L.). *J. Agric. Sci.* 2, 72-78.

Aulakh, M.S., Garg, A.K., Manchanda, J.S., Dercon, G., Nguyen, M.L., 2017. Biological nitrogen fixation by soybean and fate of applied 15N-fertilizer in succeeding wheat under conventional tillage and conservation agriculture practices. *Nutr. Cycl. Agroecosystems* 107, 79-89. doi:10.1007/s10705-016-9816-8.

Baral, B., Izaguirre-Mayoral, M.L., 2017. Purine-derived ureides under drought and salinity, In: Sparks, D.L. (Ed.), *Advances in Agronomy*. Academic Press, pp. 167-204. doi:10.1016/bs.agron.2017.07.001.

Baral, B., Teixeira da Silva, J.A., Izaguirre-Mayoral, M.L., 2016. Early signaling, synthesis, transport and metabolism of ureides. *J. Plant Physiol.* 193, 97-109. doi:10.1016/j.jplph.2016.01.013.

Batista, E.R., Guimaraes, S.L., Bonfim-Silva, E.M., de Souza, A.C.P., 2017. Combined inoculation of rhizobia on the cowpea development in the soil of Cerrado. *Rev. Ciência Agronômica* 48, 745-755.

Beatrice, M., Susan, N.-M., Paul, N., Fred, T., Magdalena, W., Papias, B., Elisiana, K., Michael, K., Deusdedith, M., 2017. Viruses infecting common bean (*Phaseolus vulgaris* L.) in Tanzania: A review on molecular characterization, detection and disease management options. *African J. Agric. Res.* 12, 1486-1500. doi:10.5897/AJAR2017.12236

Bello, S.K., Yusuf, A.A., Cargele, M., 2017. Performance of cowpea as influenced by native strain of rhizobia, lime and phosphorus in Samaru, Nigeria. *Symbiosis*. doi:10.1007/s13199-017-0507-2. doi:10.1007/s13199-017-0507-2.

Bernard, B., Lux, A., 2017. How to feed the world sustainably: an overview of the discourse on agroecology and sus-

tainable intensification. *Reg. Environ. Chang.* 17, 1279-1290. doi:10.1007/s10113-016-1027-y.

Binang, W., Ojikpong, T., Takim, F., 2017. Nodulation, biomass production and yield of some indigenous legumes as influenced by *Rhizobium* inoculation in the Rainforest Agro-ecological zone of Nigeria. *J. Appl. Life Sci. Int.* 11, 1-9. doi:10.9734/JALSI/2017/32624.

Byamukama, E., Robertson, A.E., Nutter, F. W., J., 2011. Quantifying the within-field temporal and spatial dynamics of Bean pod mottle virus in Soybean. *Plant Dis.* 95, 126-136. doi:10.1094/PDIS-07-09-0469

Checcucci, A., Azzarello, E., Bazzicalupo, M., Carlo, A. De, Emiliani, G., Mancuso, S., Spini, G., Viti, C., Mengoni, A., 2017. Role and regulation of ACC deaminase gene in *Sinorhizobium meliloti*: Is it a symbiotic, rhizospheric or endophytic gene? *Front. Genet.* 8, 6. doi:10.3389/fgene.2017.00006.

Daramy, M.A., Sarkodie-Addo, J., Dumbuya, G., 2017. Effect of nitrogen and phosphorous fertilizer application on growth and yield performance of cowpea in Ghana. *J. Exp. Biol. Agric. Sci.* 5, 038-044.

Ddamulira, G., Santos, C.A., Alanyo, M., Ramathani, I., Maphosa, M., 2017. Maturity, protein content and yield stability of cowpea in Uganda. *South African J. Plant Soil* 34, 255-261. doi:10.1080/02571862.2016.1274919.

de Oliveira, L.B., Barros, R.L.N., de Magalhães, W.B., Medici, L.O., Pimentel, C., 2017. Cowpea growth and yield in sole crop and intercropped with millet . *Rev. Caatinga* 30, 53-58.

Defez, R., Andreozzi, A., Bianco, C., 2017. The overproduction of indole-3-acetic acid (IAA) in endophytes upregulates nitrogen fixation in both bacterial cultures and inoculated rice plants. *Microb. Ecol.* 74, 441-452. doi:10.1007/s00248-017-0948-4.

Denton, M.D., Phillips, L.A., Peoples, M.B., Pearce, D.J., Swan, A.D., Mele, P.M., Brockwell, J., 2017. Legume inoculant application methods: effects on nodulation patterns, nitrogen fixation, crop growth and yield in narrow-leaf lupin and faba bean. *Plant Soil* 1-15. doi:10.1007/s11104-017-3317-7.

Deshayes, C., Siegwart, M., Pauron, D., Froger, J.-A., Lapied, B., Apaire-Marchais, V., 2017. Microbial pest control agents: Are they a specific and safe tool for insect pest management? *Curr. Med. Chem.* 24, 2959-2973. doi:10.2174/0929867324666170314144311.

Diaz, L.M., Ricaurte, J., Cajiao, C., Galeano, C.H., Rao, I., Beebe, S., Raatz, B., 2017. Phenotypic evaluation and QTL analysis of yield and symbiotic nitrogen fixation in a common bean population grown with two levels of phosphorus supply. *Mol. Breed.* 37. doi:10.1007/s11032-017-0673-1.

Egamberdieva, D., Wirth, S.J., Shurigin, V. V., Hashem, A., Abd Allah, E.F., 2017. Endophytic bacteria improve plant growth, symbiotic performance of Chickpea (*Cicer arietinum* L.) and induce suppression of Root rot caused by *Fusarium solani* under salt stress. *Front. Microbiol.* 8, 1887. doi:10.3389/fmicb.2017.01887.

FAO., 2015a. Sustainable crop production intensification (SCPI) in FAO. *Sustain. Intensif. FAO.* URL http://www.fao.org/agriculture/crops/thematic-sitemap/theme/spi/scpi-home/framework/sustainable-intensification-in-fao/en/ (accessed 9.15.17).

FAO., 2015b. Agroecology for food security and nutrition. *Proc. FAO Int. Symp.* URL http://www.fao.org/publications/card/en/c/d1f541b5-39b8-4992-b764-7bdfffb5c63f/ (accessed 9.15.17).

Farahani, S.S., Rajabipour, A., Keyhani, A., Sharifi, M., 2017. Energy use and economic analysis of NPK-15:8:15 fertilizer granulation process in Iran. *J. Saudi Soc.*

Agric. Sci. 16, 265–269. doi:10.1016/j.jssas.2015.09.001.

Forni, C., Duca, D., Glick, B.R., 2017. Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. *Plant Soil* 410, 335-356. doi:10.1007/s11104-016-3007-x.

García, T.H., Zapata, M.R., Pardo, C.G., 2017. La malnutrición un problema de salud global y el derecho a una alimentación adecuada. *Rev. Investig. y Educ. en Ciencias la Salud* 2, 3-11.

Gereziher, T., Seid, E., Bisrat, G., 2017. Performance evaluation of common bean (*Phaseolus vulgaris* L.) varieties in Raya Valley, Northern Ethiopia. *African J. Plant Sci.* 11, 1-5. doi:10.5897/AJPS2016.1464.

Getachew, Z., Abera, G., Beyene, S., 2017. *Rhizobium* inoculation and sulphur fertilizer improved yield, nutrients uptake and protein quality of soybean (*Glycine max* L.) varieties on Nitisols of Assosa area, Western Ethiopia. *African J. Plant Sci.* 11, 123-132. doi:10.5897/AJPS2017.1519.

Gomoung, D., Mbailao, M., Toukam, S.T., Ngakou, A., 2017. Influence of cross-inoculation on groundnut and Bambara groundnut-*Rhizobium* symbiosis: Contribution to plant growth and yield in the field at Sarh (Chad) and Ngaoundere (Cameroon). *Am. J. Plant Sci.* 8, 1953-1966. doi:10.4236/ajps.2017.88131.

Guy, P.L., 2014. Viruses of New Zealand pasture grasses and legumes: A review. *Crop Pasture Sci.* 65, 841-853. doi:10.1071/CP14017.

Haro, H., Sanon, K.B., Le Roux, C., Duponnois, R., Traoré, A.S., 2017. Improvement of cowpea productivity by rhizobial and mycorrhizal inoculation in Burkina Faso. *Symbiosis* 1-14. doi:10.1007/s13199-017-0478-3.

Hatfield, J.L., Prueger, J.H., 2004. Nitrogen over-use, under-use, and efficiency. *Crop Sci.* 1-15.

Hema, M., Sreenivasulu, P., Patil, B.L., Kumar, P.L., Reddy, D.V.R., 2014. Tropical food legumes: Virus diseases of economic importance and their control, In: *Advances in Virus Research*. pp. 431–505. doi:10.1016/B978-0-12-801246-8.00009-3.

Inomura, K., Bragg, J., Follows, M.J., 2017. A quantitative analysis of the direct and indirect costs of nitrogen fixation: a model based on *Azotobacter vinelandii*. *ISME J.* 11, 166–175. doi:10.1038/ismej.2016.97.

Irizarry, M.D., Pathology, P., Groves, C.L., Pathology, P., 2016. Re-emergence of *Tobacco streak virus* infecting Soybean in the United States and Canada. *Plant Heal. Prog.* 17, 92-94.

Izaguirre-Mayoral, M.L., Brito, M., Baral, B., Garrido, M.J., 2017. Silicon and nitrate differentially modulate the symbiotic performances of healthy and virus-infected Bradyrhizobium-nodulated cowpea (*Vigna unguiculata*), Yardlong Bean (*V. unguiculata*subsp. sesquipedalis) and Mung Bean (*V. radiata*). *Plants* 6. doi:10.3390/plants6030040.

Izaguirre-mayoral, M.L., Carballo, O., Mallorca, M.S. De, Marys, E., 1994. Symbiotic nitrogen fixation and physiological performance of bean (*Phaseolus vulgaris* L.) plants as affected by *Rhizobium* inoculum position and *Bean rugose mosaic virus* infection. *J. Exp. Bot.* 45, 373-383. doi:10.1093/jxb/45.3.373.

Izaguirre-Mayoral, M.L., Garrido, M.J., 2010. Propyl gallate, a free radical scavenger, counteracts the benefits of exogenously applied salicylic acid and aggravates the deleterious effects of the *Southern bean mosaic virus* in *Rhizobium*-nodulated *Phaseolus vulgaris* plants. *Arch. Phytopathol. Plant Prot.* 43, 1643-1657.

Izaguirre-Mayoral, M.L., Uzcategui, R.C.D.E., Mallorca, M.S.D.E., 1992. Physiological and biochemical aspects of symbiotic nitrogen fixation in Cowpea (*Vigna*

unguiculata (L.) Walp .var .Tuy) plants infected by *Cowpea mosaic virus*. *J. Exp. Bot*. 43, 455-462.

Jaybhay, S.A., Taware, S.P., Varghese, P., 2017. Microbial inoculation of *Rhizobium* and phosphate-solubilizing bacteria along with inorganic fertilizers for sustainable yield of soybean [*Glycine max* (L.) Merrill]. *J. Plant Nutr.* 40, 2209-2216. doi:10.1080/01904167.2017.1346678.

Jiang, H., Li, K., Dou, D., Gai, J., 2017. Characterization of a *Soybean mosaic virus* variant causing different diseases in *Glycine max* and *Nicotiana benthamiana*. *Arch. Virol.* 162, 549-553. doi:10.1007/s00705-016-3123-1.

Kaschuk, G., Hungria, M., 2017. Diversity and importance of diazotrophic bacteria to agricultural sustainability in the tropics, In: *Diversity and Benefits of Microorganisms from the Tropics*. Springer International Publishing, Cham, pp. 269-292. doi:10.1007/978-3-319-55804-2_12.

Khalimi, K., Suprapta, D.N., 2011. Induction of plant resistance against *Soybean Stunt Virus* using some formulations of *Pseudomonas aeruginosa*. *Int. Soc. Southeast Asian Agric. Sci.* 17, 98-105.

Kim, H., Park, D., Yoon, S., 2017. pH control enables simultaneous enhancement of nitrogen retention and N2O reduction in *Shewanella loihica* strain PV-4. *Front. Microbiol.* 8, 2–10. doi:10.3389/fmicb.2017.01820.

Kreuze, J.F., Valkonen, J.P.T., 2017. Utilization of engineered resistance to viruses in crops of the developing world , with emphasis on sub-Saharan Africa. *Curr. Opin. Virol.* 26, 90-97. doi:10.1016/j.coviro.2017.07.022.

Kudi, V.K., Singh, J.K., Choudhary, M., Koodi, H.L., 2017. Effect of *Rhizobium*, PSB and fertility levels on nutrient contents and their removal and yield of blackgram (*Vigna mungo* L.) under custard apple (*Annona squamosa* L.) based agri-horti system in Vindhyan region of Uttar Pradesh. *Int. J. Chem. Stud.* 5, 378-381.

Kumar, B., Talukdar, A., Verma, K., Girmilla, V., Bala, I., Lal, S.K., Singh, K., Sapra, R.L., 2014. Screening of soybean [*Glycine max* (L.) Merr.] genotypes for *Yellow mosaic virus* (YMV) disease resistance and their molecular characterization using RGA and SSRs markers. *Aust. J. Crop Sci.* 8, 27-34.

Kyei-Boahen, S., Savala, C.E.N., Chikoye, D., Abaidoo, R., 2017. Growth and yield responses of cowpea to inoculation and phosphorus fertilization in different environments. *Front. Plant Sci.* 8, 1-13. doi:10.3389/fpls.2017.00646.

Lopez, M., Muñoz, N., Lascano, H.R., Izaguirre-Mayoral, M.L., 2017. The seed-borne *Southern bean mosaic virus* hinders the early events of nodulation and growth in *Rhizobium*-inoculated *Phaseolus vulgaris L. Funct. Plant Biol.* 44, 208-218.

Manzeke, M.G., Mtambanengwe, F., Nezomba, H., Watts, M.J., Broadley, M.R., Mapfumo, P., 2017. Zinc fertilization increases productivity and grain nutritional quality of cowpea (*Vigna unguiculata* [L.] Walp.) under integrated soil fertility management. *F. Crop. Res.* 213, 231-244. doi:10.1016/j.fcr.2017.08.010.

Marchi-Werle, L., Pereira, R.R., Reese, J.C., Heng-Moss, T.M., Hunt, T.E., 2017. Yield response of tolerant and susceptible soybean to the soybean *Aphid*. *Agron. J.* 109, 1-7. doi:10.2134/agronj2016.11.0631.

Masepohl, B., 2017. Regulation of nitrogen fixation in photosynthetic purple nonsulfur bacteria, in: *Modern Topics in the Phototrophic Prokaryotes*. Springer International Publishing, Cham, pp. 1-25. doi:10.1007/978-3-319-51365-2_1.

Mehnaz, S., Lazarovits, G., 2017. Rhizotrophs: Plant growth promotion to bioremediation. *Microorg. Sustain.* 2, 1-14. doi:10.1007/978-981-10-4862-3.

Meziadi, C., Blanchet, S., Geffroy, V., Pflieger, S., 2017. Genetic resistance against viruses in *Phaseolus vulgaris* L.: State of the art and future prospects. *Plant Sci.* 265, 39-50. doi:10.1016/j.plantsci.2017.08.009.

Muleta, D., Ryder, M.H., Denton, M.D., 2017. The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia. *Soil Sci. Plant Nutr.* 1-11. doi:10.1080/00380768.2017.1370961.

Munthali, M., Nalivata, P., Makumba, W., Mbewe, E., Manase, H., Oduor, G., Macharia, M., Kayuki, K., 2017. Optimizing nutrient use efficiency and returns from Soybean production under smallholders in three agro-ecologies of Malawi. *Agric. Sci.* 8, 801–815. doi:10.4236/as.2017.88059.

Nandakishor, H.V, Kumar, P., Mane, S.S., 2017. Transmission studies of *Soybean mosaic virus*. *Int. J. Curr. Microbiol. Appl. Sci.* 6, 867-869.

Nascente, A.S., Carvalho, M. da C.S., Melo, L.C., Rosa, P.H., Nascente, A.S., Carvalho, M. da C.S., Melo, L.C., Rosa, P.H., 2017. Nitrogen management effects on soil mineral nitrogen, plant nutrition and yield of super early cycle common bean genotypes. *Acta Sci. Agron.* 39, 369. doi:10.4025/actasciagron.v39i3.32781.

Navas-Castillo, J., Fiallo-Olivé, E., Sánchez-Campos, S., 2011. Emerging virus diseases transmitted by whiteflies. *Annu. Rev. Phytopathol.* 49, 219-248. doi:10.1146/annurev-phyto-072910-095235.

Nawaz, F., Khan, N., Shah, J.A., Khan, A., Liaqat, A., Ullah, S., Ur, A., Khalil, R., Jan, T., Ali, M., Ali, M., 2017. Yield and yield components of chickpea as affected by various levels of FYM and *Rhizobium* inoculation. *Pure Appl. Biol.* 6, 346-351.

Nordenstedt, N., Marcenaro, D., Chilagane, D., Mwaipopo, B., Rajamaki, M.L., Nchimbi-Msolla, S., Njau, P.J.R., Mbanzibwa, D.R., Valkonen, J.P.T., 2017. Pathogenic seedborne viruses are rare but *Phaseolus vulgaris* endornaviruses are common in bean varieties grown in Nicaragua and Tanzania. *PLoS One* 12, 1-18. doi:10.1371/journal.pone.0178242.

Nyéléni Declaration, 2015. Declaration of the international forum for agroecology. *Int. Declar. Agroecol.* http://www.foodsovereignty.org/wp-content/uploads/2015/02/Download-declaration-Agroecology-Nyeleni-2015.pdf.

Odedara, O.O., Kumar, P.L., 2017. Incidence and diversity of viruses in cowpeas and weeds in the unmanaged farming systems of savanna zones in Nigeria. *Arch. Phytopathol. Plant Prot.* 50, 1-12.

Okunade, D.A., Adekalu, K.O., Raphael, O.D., 2017. Cowpea response to compaction and mulching on a sloping loamy sand soil in Southern Nigeria. *Agric. Eng. Int. CIGR J.* 19, 26-32.

Patil, A.S., Thankappan, R., Mehta, R., Yadav, R., Kumar, A., Mishra, G.P., Jain, R.K., 2017. Evaluation of transgenic peanut plants encoding coat protein and nucleocapsid protein genes for resistance to tobacco streak virus and peanut bud necrosis virus. *J. Environ. Biol.* 38, 187.

Pereira, H.S., Alvares, R.C., Silva, F.D.C., Faria, L.C. de, Melo, L.C., 2017. Genetic, environmental and genotype x environment interaction effects on the common bean grain yield and commercial quality. *Semin. Ciências Agrárias* 38, 1241. doi:10.5433/1679-0359.2017v38n3p1241.

Püschel, D., Janoušková, M., Voříšková, A., Gryndlerová, H., Vosátka, M., Jansa, J., 2017. Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition. *Front. Plant Sci.* 8, 1-12. doi:10.3389/fpls.2017.00390.

Ramesh, S.V., Chouhan, B.S., Gupta,

G.K., Husain, S.M., Chand, S., 2017. Genomic sequence characterization of Begomovirus infecting soybean and molecular evolutionary genomics of Legume *Yellow mosaic viruses* (LYMVs). *Plant Omics* 10, 88-96. doi:10.21475/poj.10.02.17.pne391.

Reddy, P.P., 2015. Impacts on crop protection, in: *Climate Resilient Agriculture for Ensuring Food Security*. Springer India, New Delhi, pp. 107-113. doi:10.1007/978-81-322-2199-9.

Rubiales, D., Fondevilla, S., Chen, W., Gentzbittel, L., Higgins, T.J.V., Castillejo, M.A., Singh, K.B., Rispail, N., 2015. Achievements and challenges in legume breeding for pest and disease resistance. *CRC. Crit. Rev. Plant Sci*. 34, 195-236. doi:10.1080/07352689.2014.898445.

Saha, B., Saha, S., Das, A., Bhattacharyya, P.K., Basak, N., Sinha, A.K., Poddar, P., 2017. Biological nitrogen fixation for sustainable agriculture, In: *Agriculturally Important Microbes for Sustainable Agriculture*. Springer Singapore, Singapore, pp. 81-128. doi:10.1007/978-981-10-5343-6_4.

Santachiara, G., Borrás, L., Salvagiotti, F., Gerde, J.A., Rotundo, J.L., 2017. Relative importance of biological nitrogen fixation and mineral uptake in high yielding soybean cultivars. *Plant Soil* 1-13. doi:10.1007/s11104-017-3279-9.

Santos, C.E. de R. e S., Silva, V.S.G. da, de Freitas, A.D.S., da Silva, A.F., Bezerra, R. de V., de Lyra, M. do C.C.P., Ferreira, J. da S., 2017. Prospecting of efficient rhizobia for peanut inoculation in a Planosol under different vegetation covers. *African J. Microbiol. Res.* 11, 123-131. doi:10.5897/AJMR2016.8355.

Smith, C.M., Gedling, C.R., Wiebe, K.F., Cassone, B.J., 2017. A sweet story: *Bean pod mottle virus* transmission dynamics by Mexican bean beetles (*Epilachna varivestis*). *Genome Biol. Evol.* 9, 714–725. doi:10.1093/gbe/evx033.

Soto-Arias, J.P., Munkvold, G.P., 2011. Effects of virus infection on susceptibility of soybean plants to *Phomopsis longicolla*. *Plant Dis.* 95, 530-536. doi:10.1094/PDIS-10-10-0767.

Souza, P.F.N., Silva, F.D.A., Carvalho, F.E.L., Silveira, J.A.G., Vasconcelos, I.M., Oliveira, J.T.A., 2017. Photosynthetic and biochemical mechanisms of an EMS-mutagenized cowpea associated with its resistance to *Cowpea severe mosaic virus*. *Plant Cell Rep.* 36, 219-234.

Stadler, M., Schilt-van Ettekoven, C., Giller, K.E., van Heerwaarden, J., 2017. N2-Africa, Putting nitrogen fixation to work for smallholder farmers in Africa. *N2Africa Pod*. 43, 1-8.

Stern, J.C., Sutter, B., Jackson, W.A., Navarro-Gonzalez, R., McKay, C.P., Ming, D.W., Archer, D.P., Glavin, D.P., Fairen, A.G., Mahaffy, P.R., 2017. Nitrogen on Mars: Insights from curiosity. *Lunar Planet. Sci*. XLVIII, 13-14.

Swathi, M., Gaur, N., 2017. Effect of border crops and insecticides on management of Whitefly, *Bemisia tabaci* (Gennadius) transmitted *Yellow mosaic virus* in Soybean. *Int. J. Curr. Microbiol. Appl. Sci.* 6, 613-617.

Tarekegn, Y., Bekele, B., Tewodros, A., 2017. Influence of Bradyrhizobia inoculation on growth, nodulation and yield performance of cowpea varieties. *African J. Agric. Res.* 12, 1906-1913. doi:10.5897/AJAR2017.12273.

Tariq, M., Noman, M., Ahmed, T., Manzoor, N., Zafar, M., 2017. Antagonistic features displayed by Plant Growth Promoting Rhizobacteria (PGPR): A review. *J. Plant Sci. Phytopathol.* 1, 38-43.

Tavazza, M., Lucioli, A., Ilardi, V., 2017. Gene silencing provides efficient protection against plant viruses, in: Dalmay, T. (Ed.), *Plant Gene Silencing: Mechanisms and Applications.* pp. 193-208.

Thilakarathna, M.S., Moroz, N., Raizada, M.N., 2017. A biosensor-based leaf

punch assay for glutamine correlates to symbiotic nitrogen fixation measurements in legumes to permit rapid screening of Rhizobia inoculants under controlled conditions. *Front. Plant Sci.* 8, 1714. doi:10.3389/fpls.2017.01714.

Trivedi, P., Schenk, P.M., Wallenstein, M.D., Singh B.K., 2017. Tiny microbes, big yields: enhancing food crop production with biological solutions. *Microbial Biotechnol* 10, 999-1003.

Turan, M., Yildirim, E., Kitir, N., Unek, C., Nikerel, E., Ozdemir, B.S., Güneş, A., N.E.P, M., 2017. Beneficial role of plant growth-promoting bacteria in vegetable production under abiotic stress, in: *Microbial Strategies for Vegetable Production*. Springer International Publishing, Cham, pp. 151-166. doi:10.1007/978-3-319-54401-4_7.

Ugwuoke, K.I., Onyeke, C.C., 2010. *Bean pod mottle virus* (BPMV) (Genus Comovirus): A limiting factor in the production of Soybean (*Glycine max* (L.) Merril) in humid tropical zone of Nigeria. *Bio-Research* 8, 571-578. doi:10.4314/br.v8i1.62533.

UN, 2013. World population projected to reach 9.6 billion by 2050 with most growth in developing regions, especially Africa – says UN. UN Dep. Econ. Soc. Aff. URL http://www.un.org/en/development/desa/news/population/un-report-world-population-projected-to-reach-9-6-billion-by-2050.html (accessed 9.10.17).

Valle, T., Hugo, O., Salinas, L., Lezama, Z., Sánchez, V., Rodríguez, R., Raúl, J., 2017. Producción de genotipos de frijol negro en condiciones de humedad residual y sequía terminal. *Terra Latinoam.* 35, 29-39.

Varsani, A., Roumagnac, P., Fuchs, M., Navas-Castillo, J., Moriones, E., Idris, A., Briddon, R.W., Rivera-Bustamante, R., Murilo Zerbini, F., Martin, D.P., 2017. Capulavirus and Grablovirus: two new genera in the family Geminiviridae. *Arch. Virol.* 162, 1819-1831. doi:10.1007/s00705-017-3268-6.

Vergani, L., Mapelli, F., Marasco, R., Crotti, E., Fusi, M., Di Guardo, A., Armiraglio, S., Daffonchio, D., Borin, S., 2017. Bacteria associated to plants naturally selected in a historical PCB polluted soil show potential to sustain natural attenuation. *Front. Microbiol.* 8, 1385. doi:10.3389/fmicb.2017.01385.

Yang, Q.-hua, Li, K., Zhi, H.-jian, Gai, J.-yi, 2014. Genetic diversity of Chinese *Soybean mosaic virus* strains and their relationships with other plant Potyviruses based on *P3* Gene sequences. *J. Integr. Agric.* 13, 2184-2195. doi:10.1016/S2095-3119(13)60653-X.

Youseif, S., Abd El-Megeed, F., Saleh, S., 2017. Improvement of Faba bean yield using *Rhizobium* /*Agrobacterium* inoculant in low-fertility sandy soil. *Agronomy* 7, 2. doi:10.3390/agronomy7010002.

Zanardo, L.G., Carvalho, C.M., 2017. *Cowpea mild mottle virus* (*Carlavirus*, Betaflexiviridae): A review. *Trop. Plant Pathol.* DOI:10.1007/s40858-017-0168-y. doi:10.1007/s40858-017-0168-y.

Zargar, S.M., Mahajan, R., Nazir, M., Nagar, P., Kim, S.T., Rai, V., Masi, A., Ahmad, S.M., Shah, R.A., Ganai, N.A., Agrawal, G.K., Rakwal, R., 2017. Common bean proteomics: Present status and future strategies. *J. Proteomics* http://dx.doi.org/ 10.1016/j.jprot.2017.03.019. doi:10.1016/j.jprot.2017.03.019.

Molecular Networks That Regulate the Acquisition of Desiccation Tolerance in Plant Seeds

Luis Herrera-Estrella[1]

Introduction

Desiccation tolerance (DT) is defined as the ability to survive the loss of most (>95%) cellular water without the occurrence of lethal damage. DT organisms orchestrate a complex number of responses to protect cellular structures and prevent damage to proteins and nucleic acids. Early land plants evolved mechanisms to survive harsh drying environments to successfully exploit different ecosystems on land. Therefore, it has been postulated that the initial evolution of vegetative DT, in both vegetative and reproductive stages, was a crucial step required for the colonization of land by primitive plants of a freshwater origin (Mishler & Churchill, 1985).

DT is a remarkable process that allows plant seeds in the dry state to remain viable for centuries or even thousands of years (Sallon *et al.*, 2008). It has been postulated that seed DT evolved by rewiring the regulatory and signaling networks that controlled vegetative DT, which itself emerged as a crucial adaptive trait of early land plants. Phylogenetic analyses suggest that vegetative DT was initially present in less complex plants such as bryophytes, but was then lost in the evolution of vascular plants, when this complex trait was reconfigured to be active during seed rather than vegetative development (Oliver *et al.*, 2000). Interestingly, however, at least eight independent cases of evolution (or re-evolution) of vegetative DT occurred in angiosperms and one in gymnosperms (Oliver *et al.*, 2000).

The independent regain of vegetative DT in different angiosperm clades suggests that, in spite of being quite complex processes, both vegetative and seed DT might be controlled by one or a few regulatory networks composed of a discrete number of transcription factors (TFs). Understanding the networks that regulate seed desiccation tolerance in model plant systems will provide the tools to understand an evolutionary process that

[1] Laboratorio Nacional de Genómica para la Biodiversidad del Centro de Investigaciones y de Estudios Avanzados del IPN.

played a crucial role in the diversification of flowering plants. In this paper, I will present the work done in our laboratory to identify regulatory networks involved in the acquisition of DT in Arabidopsis seeds.

Desiccation tolerance in plant seeds

In angiosperms, DT is acquired at the seed maturation stage, which involves a complex regulatory network that activates a large subset of genes involved in a number of mechanisms that influence seed survival in the dry state (Righetti, K. *et al.*, 2015). The set of genes required for seed DT includes genes encoding protective proteins such as late embryogenesis abundant (LEA) and heat shock proteins (HSPs), enzymes involved in scavenging reactive oxygen species and the biosynthesis of protective compounds such as oligosaccharides, and antioxidants such as tocopherols and flavonoids.

In Arabidopsis, embryo development and seed maturation, including the acquisition of DT, is orchestrated by a set of four master regulators: LEAFY COTYLEDON 1 (LEC1), a CCAAT-box binding factor, and three B3 domain-containing proteins16 ABSCISIC ACID INSENSITIVE 3 (ABI3), FUSCA 3 (FUS3) and LEC2. In addition to controlling embryo formation and seed maturation, these master regulators also repress the expression of genes required for the transition from embryonic to vegetative development (Nambara *et al.*, 1992; Luerßen *et al.*, 1998; Stone *et al.*, 2001). Although the role of these master regulators during seed maturation is globally similar, some of their functions are very specific. For example, in contrast to mutations in *LEC1*, *ABI3* or *FUS3* that drastically affect DT21,22, *lec2* mutants do not present this effect22,23. Interestingly, ectopic expression of *LEC1*, *FUS3* or *ABI3* in single or double mutant backgrounds of the other two regulators activated some processes of seed maturation, such as lipid and seed storage protein accumulation, but not DT, suggesting that all three regulators are required to activate DT. Genetic evidence suggests that downstream of *LEC1*, *FUS3*, *ABI3* and *LEC2*, other TFs play important roles in the network that regulates specific aspects of embryo development and seed maturation and, in particular, seed DT24. Although several processes involved in seed maturation and their regulatory mechanisms have been studied in Arabidopsis (Fait, A. *et al.*, 2005) and Medicago (Verdier, J. *et al.,* 2013), the regulatory interactions activating DT remain largely unknown.

Transcriptional networks regulating desiccation tolerance

To identify the regulatory networks involved in the acquisition of DT downstream of *LEC1*, *FUS3* and *ABI3*, we designed a comparative tran-

scriptomic analysis between the seed desiccation intolerant (DI) lines *lec1-1*, *fus3-3* and *abi3-5*, and the DT line *lec2-1*. Since *lec1* and *lec2* have similar phenotypes, including morphological alterations during embryo development and reduced accumulation of storage compounds, while they differ in DT, a comparative analysis should permit the identification of those genes directly involved in DT and are activated in *lec2* but not in *lec1*. To obtain a global view of the transcriptional differences between DI and DT lines during seed maturation, we constructed libraries for RNA-sequencing at three developmental stages, namely 15 DAF (days after flowering), a developmental stage previous to drastic water loss, 17 DAF, when rapid water loss starts, and 21 DAF, when the seed is completely dry.

We identified differences between tolerant and intolerant Arabidopsis mutants at each of the tested developmental stages. A large subset of genes is upregulated in *lec2-1*, *lec1-1*, *abi3-5* and *fus3-3*, but not *abi3-1*, and likely represents genes that are activated as part of the direct transition from embryo to vegetative growth in these mutants, as opposed to activation during the entrance to dormancy and DT. A second subset of DEGs, which are drastically repressed in all DI mutants, could represent genes that are directly or indirectly relevant for the acquisition of DT in Arabidopsis seed. These downregulated genes were enriched in the following GO categories:

- In molecular function: oxidoreductase activity and nutrient reservoir;
- In biological process: stress responses, lipid and carbohydrate biosynthesis and seed development;
- In responses to stimuli: ABA and stress responses, such as water, oxidative and temperature stress.

The finding that overrepresented processes of downregulated genes specific for DI lines are related to stress responses and cell protection categories, and that the number of genes for these categories increase at 17 and 21, when the seeds were in rapid water loss, confirmed that DI mutants fail to activate mechanisms required to acquire DT in the seed.

Our transcriptomics data provided information on the genes possibly involved in DT acquisition. However, they do not provide evidence as to the regulatory networks that connect them. In order to identify these regulatory pathways and predict novel genes involved in DT, we constructed two co-expression networks using two curated datasets obtained from 169 seed-specific CEL files from 24 ATH1 microarray experiments, a general co-expression regulatory network of all genes represented in the ATH1 microarray (FullSeedNet) and a TFs-only co-expression network (TFsSeedNet)27. When mapped to the TFsSeedNet, TF genes that fail to be

activated in DI mutants at 15, 17 and 21 DAF formed two main co-expression subnetworks, which we termed snetTFsDT1 and snetTFsDT2. We then searched the FullSeedNet for the non-TF genes that are co-ex-

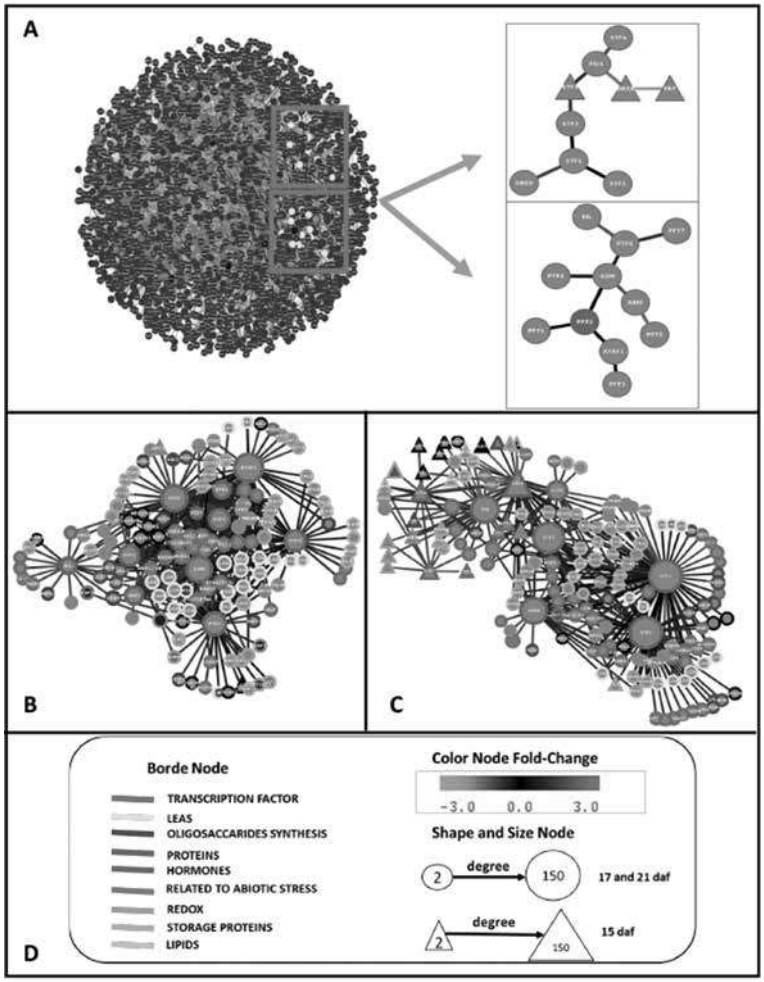


Figure 1. Transcriptional networks regulating seed desiccation tolerance. A) General transcription network including all genes that are expressed in seeds; red and green dots indicate genes that are overexpressed or repressed in desiccation tolerant mutants, respectively, and yellow dots represent genes TF genes that fail to be activated in these mutants. B) Subnetwork1 regulating the accumulation of reserve compounds a subset of stress-related genes. C) Subnetwork2 regulating the expression of large set of genes involved in stress tolerance, D) Panel of indicators that explain colors and figures included in panels B and C.

pressed with the TFs in snetFullDT1 and snetFullDT2, and that should represent their putative targets for transcriptional activation. snetFullDT1 was composed of a total of 280 genes, for which the most significantly enriched categories included nutrient reservoir activity and lipid storage. The second subnetwork (snetFullDT2) was composed of 317 genes, which represent 17% of all downregulated genes from time-specific tolerance differences and was enriched in genes in genes belonging to redox activity, LEA protein and response to abiotic stimulus categories. Interestingly, snetFullDT2 was specifically activated at 17 DAF and became more complex at 21 DAF, which corresponds to the developmental stages at which rapid water, or total water loss occurs (González-Morales, 2016).

Functional characterization of regulatory networks

TFs identified as major nodes in our DT subnetworks should regulate the expression of target genes directly involved in DT, such as those involved in oligosaccharide biosynthesis or encoding LEA proteins. We found that knockout mutants in these genes had a reduction in germination of 20 to 30% with respect to their WT controls. These results suggest that target genes of the major nodes of snetFullDT2 do indeed play important roles in DT.

If some of the TFs identified as major nodes in snetFullDT1 and snetFullDT2 act downstream of ABI3, FUS3 and LEC1 and play important roles in activating effector genes involved in DT, overexpression of these

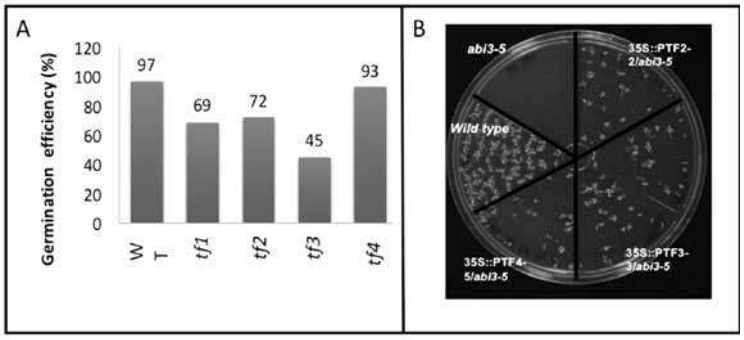


Figure 2. Functional characterization of major nodes of one of the subnetworks regulating seed desiccation tolerance. A) Effect of knockout mutants in 4 TF identified as major nodes in subnetwork2 on seed germination after 4 weeks in the desiccated state. B) Effect of constitutive expression of three TF identified as major nodes in subnetwork2 on the tolerance to desiccation of the desiccation sensitive *abi3-5* mutant.

TFs in a DI mutants, such as *abi3-5*, should partially revert the desiccation intolerance phenotypes of these mutants. To test this, we expressed *AGL67*, *DREB2D* and *ERF23* from snetTFDT1 and *PLATZ1*, *PLATZ2* and *DREB2G* from snetTFDT2 under the control of a constitutive promoter in the *abi3-5* background. Dry seeds were collected and stored for 1, 2 and 4 weeks and then tested for germination efficiency. As previously reported, *abi3-5* seeds rapidly lose viability after desiccation, and after 2 weeks of storage germination was reduced to less than 10%. In contrast, seeds from *abi3-5/35S::PLATZ1, abi3-5/35S::AGL67* and *abi3-5/35S::DREB2G* lines showed a germination rate of 25, 30 and 12%, respectively after 4 weeks of storage demonstrating that overexpression of these TFs is able to partially rescue DT in the desiccation intolerant seeds of the *abi3-5* mutant (González-Morales, 2016).

Conclusions

Our comparative transcriptomic analysis of desiccation-tolerant and -intolerant mutants identified biological processes important for DT acquisition in seeds and coincides with findings from previous reports (3, 37). However, there is no information available on TFs that regulate the genetic networks involved in the acquisition of seed DT. In this work, we identified TF genes that are major nodes in the subnetworks related to DT. The importance of these TFs in the acquisition of seed DT was confirmed by the observation that seed viability was reduced in knockout mutants of some of these TFs, such as PLATZ1, PLATZ2, AGL67, and ATAF1. Moreover, network prediction of the potential targets of the TFs identified as major regulatory nodes was confirmed by the observation that the expression of several of the putative targets of PLATZ1 was indeed upregulated in PLATZ1-overexpressing lines. These data support the notion that TFsSeed-subNetDT1 and TFsSeedsubNetDT2 downstream of LEC1, FUS3, and ABI3 are essential for the acquisition of DT in seeds. It remains to be determined whether the regulatory subnetworks that regulate, at least in part, DT in Arabidopsis seeds are or are not conserved in other angiosperms. In this regard, it is quite suggestive that several of the nodes in TFsSeed-subNetDT1 and TFsSeed-subNetDT2 have seed-specific expression in different plant species. For example, PLATZ1 orthologues are specifically expressed during seed maturation in rice, soybean, and maize. Interestingly, maize PLATZ1 is orthologous; in addition to being expressed during seed maturation, it is also strongly induced by drought stress. Therefore, we propose that recruitment of the DT regulators that act downstream of

LEC1 and ABI3 for expression in vegetative tissues might be responsible for the independent re-evolution of vegetative DT during plant evolution. This opens up the possibility that a core DT regulatory network could indeed have been conserved throughout plant evolution. Further research will be needed to explore whether DT is orchestrated by regulatory networks in which at least a common core of TFs has been conserved during plant evolution and to determine how it has been rewired several times to be activated in seeds and in vegetative tissues.

Finally, it is worth mentioning the potential biotechnological applications through the activation of one or more of the major nodes of snetTFsDT1 and snetTFsDT2 in vegetative tissues, by gene transfer or genome editing, in order to confer inducible drought tolerance to crop plants.

Bibliography

1. Mishler, B.D. & Churchill, S.P. Transition to a land flora: Phylogenetic relationships of the green algae and bryophytes. *Cladistics* 1, 305-328 (1985).
2. Sallon, S. *et al*. Germination, genetics, and growth of an ancient date seed. *Science* 320, 1464 (2008).
3. Oliver, M.J., Tuba, Z. & Mishler, B. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology* 151, 85-100 (2000).
4. Righetti, K. *et al.* Inference of Longevity-Related Genes from a Robust Coexpression Network of Seed Maturation Identifies Regulators Linking Seed Storability to Biotic Defense-Related Pathways. *Plant Cell* 27, 2692-708 (2015).
5. Nambara, E., Naito, S. & McCourt, P. A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new abi3 allele. *The Plant Journal* 2, 435-441 (1992).
6. Luerßen, H., Kirik, V., Herrmann, P. & Miséra, S. FUSCA3 encodes a protein with a conserved VP1/ABI3-like B3 domain which is of functional importance for the regulation of seed maturation in Arabidopsis thaliana. *The Plant Journal* 15, 755-764 (1998).
7. Stone, S.L. *et al.* LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences* 98, 11806-11811 (2001).
8. Fait, A. *et al*. Arabidopsis Seed Development and Germination Is Associated with Temporally Distinct Metabolic Switches. *Plant Physiology* 142, 839-854 (2006).
9. Verdier, J. *et al*. A regulatory network-based approach dissects late maturation processes related to the acquisition of desiccation tolerance and longevity of Medicago truncatula seeds. *Plant Physiol* 163, 757-74 (2013).
10. González-Morales SI, *et al*. Regulatory network analysis reveals novel regulators of seed desiccation tolerance in Arabidopsis thaliana. Proc Natl Acad Sci U S A. 113(35):E5232-41 (2016).

Cell Signaling and Developmental Biology Session

Current Thoughts on the Origin of Life on Earth

Rafael Vicuña[1]

The origin of life on earth is perhaps the biggest pending question in contemporary science and constitutes a challenge to researchers from all fields. About thirty-five years ago, after denoting the origin of life as a "happy accident", Francis Crick affirmed that "the origin of life appears at the moment to be almost a miracle, so many are the conditions which would have had to have been satisfied to get it going" (Crick, 1981). In spite of significant progress made in recent decades – mainly in the field of prebiotic chemistry – we are forced to admit that we still do not know much about how matter came to be organized in such a way as to give rise to cellular life. As stated recently by E. Smith and H. Morowitz (2016), "the wide range of opinions expressed in the scientific community makes it clear that we do not yet have a paradigm, even in its broadest outlines, to explain in any detail the origin of life".

And lack of knowledge, as expected, gives rise to all sorts of controversies. How early did life appear on earth? Did life emerge in some kind of primordial soup or in hydrothermal vents in the ocean floor? Or was it perhaps in inland hydrothermal fields? Did life instead come from space? Was the first metabolism autotrophic or heterotrophic? Was there an RNA world first or did metabolism come first? Were lipid vesicles required for either option? Are we ever going to know the origins of chirality and of the genetic code? But above all, how did inert matter organize to give rise to the first organisms?

Prior to attempting an answer to some of these questions, it is worthwhile focusing on the transition between inert matter and life. This is an issue that is very seldom discussed in the literature. Most of the times, the emphasis seems to be placed in the material components of living organisms, giving the impression that all that it is required to get life going are lipid vesicles and some sort of information polymer that can self-replicate inside them.

[1] Pontificia Universidad Católica de Chile.

However, it is fairly obvious that life entails something more than biomolecules. It is the organization pattern of the latter, more than their identity, which is critical for life. On the other hand, common sense suggests that entities that are half inert or half alive are difficult to perceive. Accordingly, we must assume that life is something that appears when a certain threshold between inert mater and a living organism is crossed. Now, what do we mean by living organism?

At the risk of appearing somewhat chauvinistic, to respond to this inquiry I will make use of the approach offered by the Chilean scientists Humberto Maturana and Francisco Varela, namely, autopoiesis, or self-production. As defined by these scientists, "an autopoietic system consists of a network of processes of production, in which the function of each component is to participate in the production or transformation of the other components in the network, including the production of an external limit that specifies the domain of the autopoietic organisation" (Maturana and Varela, 1973). I am certainly aware that this definition is incomplete. For example, it does not specify that the system sustains itself autonomously far from equilibrium; neither does it mention the property of Darwinian evolution that is so distinctive of living beings. However, it is undeniably a good start when attempting to define an organism. In keeping with this reasoning, I would not consider self-replicating polymers floating in a primordial soup to be alive, not to mention the unlikelihood of this scenario. In contrast, I would definitely consider a single cell that is autonomous in obtaining its energy and nutrients from the environment to be a living organism, or more precisely in this case, a microorganism.

Having clarified this crucial issue, the following step would be to try to envision the transition from inert matter to life in the early earth. Most likely, and this is pure conjecture, primeval life must have been much simpler than extant life. It is difficult to conceive that inert matter crossed at once the threshold to life to become a microorganism such as *Mycoplasma genitalium*, the simplest bacterium that can be grown in the lab (Glass *et al.*, 2006). How much simpler can life get? We just don't know, but we can speculate. In my opinion, nobody has done this exercise better than Szostak, Bartel and Luisi, whom about 15 years ago proposed the theoretically simplest cell (Szostak *et al.*, 2001). According to these authors, this hypothetical minimal cell consists in a vesicle that contains a self-replicating ribozyme that also replicates a second ribozyme that is involved in the formation of the lipid membrane, thus closing the autopoietic circuit. The transition from non-life to a minimal organism of this sort would still

be a puzzle (think for example of the provision of activated nucleotides), although perhaps more easy to solve.

Let us now say a few words about the antiquity of life. Isotopic data is one of the main criteria employed to unveil the first appearance of life on earth, since metabolic processes enrich for C12 over C13. There are reports showing enrichment in C12 in 3.8 billion year (b.y.) old rocks which are found in Akilia, a small island off the southwest coast of Greenland (Rosing, 1999; Ohtomo *et al.*, 2014; Bell *et al.*, 2015). These are the oldest rocks on earth and the authors provide arguments claiming that this enrichment is due to biological activity. Lately, however, C12 enrichment has lost some credibility as a reliable biosignature since a growing body of evidence appears to show that light carbon can be generated abiotically as well (Moorbath, 2005; Fedo *et al.*, 2006). Also, there are claims asserting that the carbon in these rocks may have been deposited at some unknown time after their formation. This debate is still open.

On the other hand, there is evidence provided by microfossils. Also in Greenland, in the Southwestern region, there is the so-called Isua Belt, where fossils of stromatolite-like structures have been observed in 3.7 b.y. old rocks (Nutman *et al.*, 2016). The structures are conical in shape, with sedimentary layers in between the cones. The biogenicity of these stromatolites is based on their laminated growth pattern, trace element composition and the presence of dolomite, which requires microbial activity for precipitation. Also, there is a very recent report describing the presence of biosignatures of microorganisms that are about 3.8 b.y. old and possibly 4.280 b.y. old in sedimentary rocks from the Nuvvuagittuq belt in Quebec, Canada, which are interpreted as seafloor-hydrothermal vent-related precipitates (Dodd *et al.*, 2017). The iron-oxide tubes and the haematite filaments observed in these rocks are morphologically similar to tubes and filaments formed by bacterial cells in present-day hydrothermal settings. Carbonate rosettes made of isotopically light carbon, which presumably derives from the oxidation of organic matter by iron III are also observed. With respect to microfossils of individual bacteria whose authenticity has not been contested, the most ancient ones are found in 3.4 b.y. old rocks from Western Australia (Wacey *et al.*, 2011).

In summary, even though there are still some disagreements among investigators regarding the isotopic data, it can be concluded with fair certainty that there was already complex cellular life at least 3.8 b.y. ago. It is therefore reasonable to reason that simpler forms of life appeared very early on earth, perhaps as soon as the environment would allow it. This has been

a matter of dispute for ever, because early life would have in turn required a very early condensation of the atmospheric water vapor followed by a rapid cooling of the oceans and lagoons.

For a long time, it was thought that the oceans were formed by the end of the Hadean eon, about 4 b.y. ago. However, analyses of 4.3 b.y. old zircons found in Jack Hills, Australia, indicate that they formed much earlier (Mojzsis *et al.*, 2001). Zircons are tiny crystals of zirconium silicate that also contain trace amounts of uranium. They can be dated by the decay of uranium to lead, whereas their oxygen isotopic composition reveals whether they were formed in a dry or a wet environment. Based on these criteria, it can be stated that these zircons from Australia crystalized in liquid water. Other groups that conducted more detailed studies on the oxygen isotopic composition of these zircons have questioned the early ocean theory. They have recently proposed that zircons found in Australia could have crystalized in melt sheets caused by the intense meteorite bombardment of the early earth (Kenny *et al.*, 2016). Once again, we will have to wait for further data to know with certainty when the oceans were actually formed. Interestingly, there is a recent report on the presence of C12-enriched graphite encased in an undisturbed 4.1 b.y. old zircon, which constitutes additional support for the very early onset of life on earth (Bell *et al.*, 2015).

A key question in the origin of life field is the scenario in which this major event took place. One option is a lagoon or the ocean, where a primordial soup might have developed. Other likely scenarios are hydrothermal vents in the ocean floor, terrestrial hot springs, water drops suspended in the atmosphere, etc. On the other hand, there is always the possibility that life may have arrived from other planet. Hereafter, a few words will be devoted to the primordial soup scenario and most of the remaining text will focus on hydrothermal vents.

The primordial soup theory was independently proposed by Alexander Oparin in Russia and by John Haldane in the UK, almost one century ago. This theory states that organic compounds formed on earth, together with those brought to our planet by meteorites, self-organized to give rise to the first heterotrophic microorganisms. The late Stanley Miller, whom with his seminal work in experimental prebiotic chemistry which began in 1953 gave support to this theory, was a strong advocate of the primordial soup idea (Lazcano and Miller, 1996).

However, the primordial theory has some drawbacks. The most important one is that life implies disequilibrium and the opportunities for

disequilibrium offered by the soup are not only limited, but also transient. In addition, most prebiotic chemistry supporting the primordial soup theory has no parsimony with extant life. Indeed, no known form of life uses compounds such as cyanide, formamide or cyanimide as substrates for carbon or nitrogen fixation. Nor does any known form of life use UV radiation or lightning as a source of energy.

In recent years, on the other hand, the hypothesis of submarine hydrothermal vents has gained considerable attention as a probable scenario for the origin of life on earth. There are two types of hydrothermal vents in the ocean floor (Martin *et al.*, 2008; Bradley, 2009). One is represented by the so-called black smokers, discovered by the submergible Alvin in 1977 near the Galápagos Islands. At these sites, seawater percolates through fissures or cracks present in the ocean crust in the vicinity of subduction zones. The water reaches the magma and is heated up to 400°C and then reemerges carrying dissolved salts that precipitate when the hot water mixes with the cold ocean water, giving the impression of a chimney discharging underwater "black smoke".

According to Günther Wächtershäuser (2007), life started in these black smokers, with the formation of organic molecules that thereafter established a surface metabolism supported by energy deriving from the formation of pyrite, which is a very exergonic reaction. Reactions conducted in the laboratory under hydrothermal prebiotic conditions that give support to this theory include the formation of aminoacids, lipids, thioesters, peptide bonds, etc. They are catalyzed by metals such as iron, nickel and cobalt that are present in pyrite. According to Wächtershäuser and followers, the first microorganisms were autotrophic and they utilized the reversed Krebs cycle for CO2 fixation. The main criticisms against the black smoker scenario are that prebiotic synthesis of organic compounds relies mainly on CO rather than CO2 fixation. The concentration of the former in the Hadean Ocean is presumed to have been negligible. In addition, no known form of life is powered by energy deriving from pyrite formation.

There are also the Lost City-type hydrothermal vents, discovered more recently in the Atlantic Ocean, although they are present elsewhere. In this case, the water warms up as it percolates the ocean down to depths of about 5 km, without reaching the magma. As the ocean water flows underground, it collects the products of a geologic process termed serpentinization, which include some hydroxides, hydrogen, methane and other hydrocarbons. Thereafter, the alkaline fluid with temperatures in the range of 50 to 90°C mixes with the acidic cold water, leading to the precipitation

of carbonates, silicates, clays and sulfurs of iron and nickel. The chimneys formed are porous and can reach up to 60 m high.

Serpentinization is a geologic process that typically takes place in ultramafic rock (Schulte, 2006; Russell, 2010; Konn *et al.*, 2015). At high pressure and temperatures around 150°C, olivine reacts with water producing serpentinite, magnesium hydroxide and hydrogen, among other products. Hydrogen is also produced in other reactions, whereas methane is produced by reduction of CO2. Serpentinization is a highly exergonic process. In summary, water oxidizes iron II to iron III, the water is reduced to H2 with its oxygen being retained in the rock as iron oxides. The resulting solution is warm, alkaline and reducing.

As the alkaline hydrothermal fluid arises through the porous towers, it establishes a natural disequilibrium with the ocean water, which is cold and acidic. The resulting natural proton gradient resulting in the micro compartments of the mound is very similar to the one that is established in the mitochondria or in a bacterial cell, where protons are pumped to the outside. It is this disequilibrium, namely temperature, pH and redox, what makes so attractive the Lost City-type vents as a conceivable scenario for the origin of life (Martin and Russell, 2007; Sojo *et al.*, 2016). Accordingly, the following process is proposed to have taken place throughout thousands of years (Allen, 2010): in a first step, the hydrothermal fluid flows through the porous rock and the pH gradient is dissipated by the free entrance of protons to the inorganic vesicle and the free exit of hydroxyl ions to the ocean water. Subsequently, the porosity of the vesicle is diminished because some organic compounds that are being synthesized prebiotically are deposited in the vesicle wall. In a third step, the porosity is decreased further and protons are actively pumped out of the vesicle by some unknown mechanism. Finally, a vesicle is formed with an organic external boundary. The vesicle becomes independent of the hydrothermal fluid and the pH gradient is generated only by proton pumping. Needless to say, this is all very hypothetical and there is no experimental proof whatsoever for this hypothesis.

The very first stages of prebiotic organic synthesis in the hydrothermal mound of Lost City-type vents may have proceeded as follows (Russell *et al.*, 2014): there is H2 and CH4 being produced by serpentinization, both flowing into the porous rock. There is also CO2 in the ocean water. This CO2 is reduced to CO by H2, whereas CH4 is oxidized to methanol or methanethiol by an oxidant such as nitrite or nitrate. Thereafter, the latter reacts with CO to give acetate. Metals such as nickel and iron that are

present in the inorganic walls of the micro compartments play a key role in catalyzing these reactions. The life of methanogens in Lost City vents today is basically supported by this type of redox reactions.

The transition element sulphides, hydroxides and oxides that are present in the porous rocks in these vents possess structures that are very similar to prosthetic groups present in extant metalloenzymes (Russell *et al.*, 2014). Among them, the mineral greigite, with a structure very similar to the metal prosthetic group of the enzyme acetyl CoA synthase, and violarite, similar to the metal prosthetic group of CO dehydrogenase. They both contain nickel, in addition to iron and sulfur. As stated previously, these and other minerals present in the rocks are proposed to have played a critical role as early catalysts in prebiotic reactions.

The Lost City-type hydrothermal vents scenario is supported by prebiotic chemistry conducted under conditions proper of these vents. Representative reactions that might be relevant to proto metabolism include the formation of sugars, fixation of CO2 and nitrogen, oligomerization of aminoacids and nucleotides, vesicle formation, etc. (Russell *et al.*, 2014). Most of these reactions are catalyzed by metal centers. Based on reactions of these types, it has been proposed that the first organisms were autotrophs and used the acetyl CoA or Wood-Ljungdahl pathway to fix CO2 (Russell and Martin, 2004; Nitschke and Russell, 2013). This ancient pathway has a strong resemblance with the hypothetical prebiotic chemistry that might have taken place in the hydrothermal mound.

The Wood-Ljungdahl pathway is present in methanogenic (Archea) and acetogenic (Bacteria), although with distinctive features in each domain. Reduction of CO2 to CO catalyzed by carbon monoxide dehydrogenase (CODH) is similar in both, suggesting that it is a primitive pathway. In contrast, the reductive pathway leading to the formation of a methyl group from CO2 exhibits several differences, mainly with respect to the cofactors involved: in Archaea they are of the pterin-type, whereas in acetogens the cofactor is tetrahydrofolate (Nitschke and Russell, 2013). In addition, both domains contain proton motive force generating steps, which constitutes a very unique attribute of this CO2 fixation pathway. Thus, in methanogens, the transfer of a methyl group from one cofactor to another prior to its reduction to methane releases enough energy to pump protons or Na+ ions via the membrane enzyme methyl transferase (Mtr). In acetogens, oxidation of ferredoxin by the NiFe Ech hydrogenase drives the extrusion of Na+ or H+ ions across the membrane. In both cases, ATP is thereafter synthesized via the standard rotor-stator ATP synthase (Sojo *et al.*, 2016).

Let us now zoom into the reduction of CO2 by H2 to give formate, which is the first step of the pathway leading to methane in methanogens or to the methyl group of acetate in acetogens. Under standard conditions, this reaction is not spontaneous. The extant enzyme, formate dehydrogenase, which contains either molybdenum or tungsten plus iron-sulfur clusters, solves this problem by electron bifurcation. The question arises as to how this reaction may have proceeded in the Hadean Ocean, since based on standard reduction potentials, molecular hydrogen is incapable of reducing CO2 to formate. However, with H2 dissolved in serpentinization water at pH 10 and CO2 dissolved in ocean water at pH 6.0, the reduction potential of CO2 becomes higher (more positive) than that of H2, making the reduction of CO2 possible. It is likely that the thin iron sulfur membranes in the mound could sense this different redox potential and transfer the electrons from H2 to CO2 (Herschy *et al.*, 2014).

The most convincing argument in favor of the Wood-Lungdhal pathway as the most primitive route to fix CO2 is based on parsimony: selection imposes some sort of link between the primitive geochemical processes and the ensuing metabolism. Extant methanogens and acetogens gain all their carbon and energy from the reduction of CO2 by H2, which relies on a vectorial ion flux analogous to the one existing in a Lost City-type hydrothermal vents. But there are further reasons to assign the Wood-Ljungdahl pathway a key role in early life. They are the following: a) it is the only CO2 fixation pathway that is present in both Archaea (methanogens) and Bacteria (acetogens); b) it is the only exergonic CO2 fixation pathway. In both domains, the energy is used to generate a chemiosmotic gradient; c) proteins in the pathway have numerous prosthetic groups containing Fe, Ni and sulfur; d) microbial methanogenesis was already functioning 3.5 b.y. ago (Ueno *et al.*, 2006).

Interestingly, Weiss *et al.* (2016) recently published a paper in which they analyzed 6.1 million protein-coding genes from sequenced prokaryotic genomes in order to reconstruct the microbial ecology of LUCA, the last universal common ancestor. The data shows that LUCA was anaerobic, thermophilic, fixed CO2 using the Wood-Ljungdahl pathway and was also able to fix N2. In addition, according to this study, LUCA's biochemistry was replete with iron-sulfur clusters, transition metals and radical reaction mechanisms.

Another quite attractive feature of an origin in Lost City-type hydrothermal vents is that the porous rocks provide a fitting scaffold for the self-organization of the first organic molecules (Martin and Russell,

2003). As mentioned before, the sustained flow of serpentinization fluid across the mineral compartments establishes a permanent redox, pH and temperature gradient, with a persistent source of electrons for a copious prebiotic chemistry in catalytic surfaces of iron sulfide containing other catalytic metals. Thus, an initial step of prebiotic chemistry inside the micro compartments could have been followed in a subsequent step by an RNA world, then a DNA world with the establishment of LUCA, which would not still be free living. The following step might have been the development of cell membrane biochemistry for Archaea and Bacteria, to end up with cells that are able to detach from the mound and become free living microorganisms. There are innumerable questions still pending. But still, it is perhaps the best hypothesis we have today for the origin of life on earth.

Nick Lane, of the most enthusiastic supporters of this theory, recently attempted a response to the old question regarding whether the emergence of life is inevitable or a fluke (Lane, 2012). His contention is as follows: "I'm the first to admit that there are many gaps to fill in, many steps between an electrochemical reactor that produces organic molecules and a living, breathing cell. But consider the bigger picture for a moment. The origin of life needs a very short shopping list: rock, water and CO2. Water and olivine are among the most abundant substances in the universe. Many planetary atmospheres in the solar system are rich in CO2, suggesting that it is common too. Serpentinization is a spontaneous reaction, and should happen on a large scale on any wet, rocky planet. From this perspective, the universe should be teeming with simple cells – life may indeed be inevitable whenever the conditions are right. It's hardly surprising that life on Earth seems to have begun almost as soon as it could".

Precisely regarding the possibility that serpentinization may be a common process in the universe, the recent finding in the Saturn moon Enceladus is highly appealing (Waite *et al.*, 2017). Enceladus has a rocky core completely covered by liquid water, which is frozen on the surface. The Cassini spacecraft discovered large plumes of water vapor erupting from the icy crust and detected hydrogen gas in these plumes, which most likely arises from hydrothermal reactions in the ocean floor. The presence of liquid water in this moon constitutes additional evidence for hydrothermal activity. This is indeed a very exciting finding and we should not be surprised if in the near future evidence for microbial life is found in this particular moon or elsewhere in the universe.

Bibliography

Allen, J.F. (2010). Redox homeostasis in the emergence of life. On the constant internal environment of nascent living cells. *J. Cosmol.* 10, 3362-3373.

Bell, E.A., Boehnke, P., Harrison, T.M. and Mao, W.L. Potentially biogenic carbon preserved in a 4.1 billion-year-old zircon. (2015). *Proc. Natl Acad. Sci. USA* 112, 14518-1452.

Bradley, A.S. (2009). Expanding the limits of life. *Sci. Am.* 301(6), 62-69.

Crick, F. (1982). *Life itself: Its origin and nature*. Simon and Shuster, USA.

Dodd, M.S., Papineau, D., Grenne, T., Slack, J.F., Rittner, M, Pirajno, F., O'Neil, J. and Little, C.T.S. (2017). Evidence for early life in Earth's oldest hydrothermal vent precipitates. *Nature* 543, 60-65.

Fedo, C.M., Whitehouse, M.J., and Kamber, B.S. Geological constraints on detecting the earliest life on Earth: a perspective from the Early Archaean (older than 3.7 Gyr) of southwest Greenland. (2006). *Phil Trans. R. Soc. B* 361, 851-867.

Glass, J.I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M.R., Maruf, M., Hutchison III, C.A., Smith, H.O. and Venter, J.C. (2006). Essential genes of a minimal bacterium. *Proc. Natl. Acad. Sci.* USA 103, 425-430.

Herschy, B., Whicher, A., Camprubi, E., Watson, C., Dartnell, L., Ward, J., Evans, J.R.G. and Lane, N. (2014). An origin-of-life reactor to simulate alkaline hydrothermal vents. *J. Mol. Evol.* 79, 213-227.

Kenny, G.G., Whitehouse, M.J., and Kamber, B.S. (2016). Differentiated impact melt sheets may be a potential source of Hadean detrital zircon. *Geology* 44, 435-438.

Konn, C., Charlou, J.L., Holm, N.G. and Mousis, O. (2015). The production of methane, hydrogen, and organic compounds in ultramafic-hosted hydrothermal vents of the Mid-Atlantic Ridge. *Astrobiology* 15, 381-399.

Lane, N. (2012). Life: Inevitable or fluke? *New Scientist*, June issue, 32-37.

Lazcano, A. and Miller, S.L. (1996). The origin and early evolution of life: Prebiotic chemistry, the pre-RNA world, and time. *Cell*, 85, 793-798.

Martin, W. and Russell, M.J. (2003). On the origin of cells: An hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautorophic prokaryotes, and from prokaryotes to nucleated cells. *Philos Trans R Soc Lond B Biol Sci* 358, 27-85.

Martin, W., Baross, J., Kelley, D. and Russell, M.J. (2008). Hydrothermal vents and the origin of life. *Nature Rev. Microbiol.* 6, 805-814.

Martin, W. and Russell, M.J. (2007). On the origin of biochemistry at an alkaline hydrothermal vent. *Phil. Trans. R. Soc.* B 362, 1887-1925.

Maturana H., Varela F. (1973). *De máquinas y seres vivos: una teoría sobre la organización biológica*. Editorial Universitaria, Santiago, Chile.

Mojzsis, S.J., Harrison, T.M. and Pidgeon, R.T. (2001) Oxygen-isotope evidence from ancient zircons for liquid water at the Earth's surface 4,300 Mya ago. *Nature* 409, 178-181.

Moorbath, S. (2005). Dating earliest life. *Nature* 434, 155.

Nitschke, W. and Russell, M.J. (2013). Beating the acetyl coenzyme A-pathway to the origin of life. *Philos. Trans. R. Soc. Lond.* B 368, 20120258.

Nutman, A.P., Bennett, V.C., Friend, C.R.L., van Kranendonk, M.J. and Chivas, A.R. (2016). Rapid emergence of life shown by discovery of 3,700-million-year-old microbial structures. *Nature* 537, 535-538.

Ohtomo, Y., Kakegawa, T., Ishida, A., Nagase, T. and Rosing, M.T. (2014). Evidence for biogenic graphite in early Archaean Isua metasedimentary rocks. *Nat. Geosci.* 7, 25-28.

Rosing, M.T. (1999). 13C-Depleted carbon microparticles in > 3700-Ma sea-floor sedimentary rocks from west greenland. *Science* 283, 674-676.

Smith, E. and Morowitz, H.J. (2016). *The origin and nature of life on earth*. Cambridge University Press, UK.

Russell, M.J. and Martin, W. (2004). The rocky roots of the acetyl-CoA pathway. *Trends Biochem. Sci.* 29, 358-363.

Russell, M.J., Hall, A.J. and Martin, W. (2010). Serpentinization as a source of energy at the origin of life. *Geobiology* 8, 355-371.

Russell, M.J., Barge, L.M., Bhartia, R., Bocanegra, D. *et al.* (2014). The drive to life on wet and icy worlds. *Astrobiology* 14, 308-343.

Schulte, M., Blake, D., Hoehler, T. and McCollom, T. (2006). Serpentinization and its implications for life on the early Earth and Mars. *Astrobiology* 6, 364-376.

Sojo, V., Herschy, B., Whicher, A., Camprubí, E. and Lane, N. (2016) The origin of life in alkaline hydrothermal vents. *Astrobiology* 16, 181-197.

Szostak, J.W., Bartel, D.P. and Luisi, P.L. (2001). Synthesizing life. *Nature* 409, 387-390.

Ueno, Y., Yamada, K., Yoshida, N., Maruyama, S. and Isozaki, Y. (2006). Evidence from fluid inclusions for microbial methanogenesis in the early Archaean era. *Nature* 430, 516-519.

Wacey, D., Kilburn, M., Saunders, M., Cliff, J. and Brasier, M.D. (2011). Microfossils of sulphur-metabolizing cells in 3.4-billion-year-old rocks of Western Australia. *Nature Geoscience* 4, 698-702.

Wächtershäuser, G. (2007). On the chemistry and evolution of the pioneer organism. *Chemistry & Biodiversity* 4, 584-602.

Weiss, M.C., Sousa, F.L., Mrnjavac, N., Neukirchen, S., Roettger, M., Nelson-Sathi, S. and Martin, W.F. (2016). The physiology and habitat of the last universal common ancestor. *Nature Microbiol.* 1, 16116.

Waite, J.H., Glein, C.R., Perryman, R.S., Teolis, B.D., Magee, B.A., *et al.*, (2017). Cassini finds molecular hydrogen in the Enceladus plume: Evidence for hydrothermal processes. *Science* 356, 155-159.

Introducing Developmental Biology to Brazil

Nicole Le Douarin[1]

I am unable to join my colleagues of PAS today for the Workshop on *Cell Biology and Genetics*. I would still like to share some experiences with Latin America because I participated in a spontaneous endeavour that extended over more than ten years. The aim was to facilitate the introduction in Brazil of a life science domain, Developmental Biology.

The origin of this venture goes back to 1997. At the end of one of my lectures at the Collège de France, in Paris, a freshly recruited Professor of the Faculty of Medicine in the Federal University of Rio de Janeiro, Vivaldo Moura Neto, who was spending a research period in the laboratory of Professor F. Gros, came to me and said: "Developmental Biology is poorly (or even not at all) represented in most Universities in Brazil. I would like to do something to change this regrettable situation. Would you accept to help me in this endeavour? What I would like to do is to arouse the interest of young scientists for this dynamic field of Biological Sciences, about which they have no experience, while they are often embarked in teaching it to students".

I said yes to Vivaldo: how could one reject such a proposition aimed at serving a scientific field that you enjoy?

Today I wish to give you a brief account of the common venture that Vivaldo and I have regularly conducted every year from 1998 to 2016.

The first project was to organize two week sessions for students interested in the subject and selected by Vivaldo. In these courses, in addition to teaching the principles of Developmental Biology in their most modern aspects, a practical course would be organized. The aim was first to make the students familiar with the different animal models used in active laboratories over the world in this particular discipline and to teach them how to perform some of the classical experiments these models have contributed to.

In a second step, the project was to organize symposia on emerging subjects in Developmental Biology to which selected scientists from abroad would be invited.

[1] With the collaboration of Vivaldo Moura-Neto.

The first action, concerning the animal models that developmental biologists use, was not the simplest one. Sea urchins and amphibian embryos like frogs and triton had been the research material from the dawn of embryonic development studies. In addition, the chick embryo has remained a classical model to approach the development of higher vertebrates: available for experimentation in the egg during the entire period of development, it is ideal for studying many aspects of morphogenesis and cell differentiation.

However with the rise of interest in molecular genetics, new organisms were added to this menagerie. The Drosophila melanogaster fly, and the microscopic Caenorhabditis elegans worm were selected for the possibilities they offer to genetic and molecular approaches of development. As far as vertebrates are concerned, a small fish (the so-called Zebrafish) entered the game, and research on a mammalian species became concentrated on the mouse model because researchers invented a way to target mutations on a gene of their choice.

I tried to find, in my country and abroad, scientists who were ready to enter this venture with us. Dr Babinet from the Pasteur Institute came with a technician, with the mice and all the necessary material (in addition to the skills and knowledge) required to "construct" a transgenic mouse.

From the Carnegie Institution for Embryology in Baltimore USA, Dr Marnie Halpern and her technician came carrying a bucket of water in the airplane containing dozens of zebrafishes for their demonstrations and for the students' experiments!

Two CNRS researchers from the University of Toulouse carried several Xenopus frogs for them to lay eggs, in order to reproduce, for the students, the famous Spemann experiment.

From my own lab, several researchers came to illustrate the kind of work feasible on the avian model; namely to show how one can construct chimeric embryos between chick and quail species and what problems of development can be addressed with this approach.

At that time, the Federal University of Rio was equipped with extremely poor microscopes. Professor Vivaldo Moura-Neto managed to obtain the loan of dissecting microscopes from the firms that sell them, for the duration of the course.

In addition to this practical work, part of the day was devoted to talks delivered by renown biologists invited from abroad, e.g.: Professor Antonio Coutinho, from the Gulbenkian Institute in Lisbonne, Professor David Sabatini, from NYU, New York, Dr Françoise Dieterlen, CNRS, Paris, Dr

Anne-Marie Duprat, CNRS, Toulouse, and many others…

What about the financial support for such an endeavour? On my side, the travel expenses of the French participants were covered by the CNRS and INSERM. Professor Coutinho and the US participants found support from their own resources. Professor Vivaldo Moura-Neto was able to get funds to cover the living expenses of all the participants.

These international efforts were acknowledged by the creation of a UNESCO Chair in Developmental Biology in 1998: Catedra de UNESCO de *Biologia da Forma e do Desenvolvimento*, Universidade Federal do Rio de Janeiro.

The follow up of this endeavour had two aspects: the organisation of similar courses to initiate research in Developmental Biology in several universities in Brazil, and the organisation of symposia on emergent topics in Developmental Biology.

Interestingly, the second series of practical courses, that took place in various universities, were taught by students of the first years who benefited from the pioneering sessions initiated in Rio by the international team and who had embarked on development studies.

The UNESCO chair, as a "label", was critical in gathering the conditions for the organisation of these courses and of international conferences. This endeavour coincided, over the years, with the spectacular improvement (which today is unfortunately over) of economic conditions in Brazil.

One of the consequences of these courses was that several Brazilian students became interested in Developmental Biology and went abroad (Europe, namely France, and USA) to prepare a PhD or as Post-docs. Another was that many Brazilian young researchers decided to work in the field of Developmental Biology in several universities all over the country.

We really hope that economic conditions will improve in Brazil, a condition for the continuation of these efforts.

Regulation of Protein Degradation by Wnt Signaling

Edward M. De Robertis

Introduction

Protein degradation plays a key role in cellular homeostasis. The topic of how cellular components are turned over has been one of great interest to members of the Pontifical Academy of Sciences. Academician Christian De Duve discovered that membrane proteins and external nutrients acquired through endocytosis are digested in acidic vesicular organelles called lysosomes (De Duve and Wattiaux, 1966). Cytosolic proteins are mostly degraded in proteasomes, which consisting of cytosolic cysteine proteases. Proteins are targeted to proteasomes after being modified by a small 76 amino acid protein called ubiquitin (Ciechanover, 2005). When a protein is marked by a chain of ubiquitin molecules, called Lysine-48-linked polyubiquitin, it was thought to be invariably degraded in proteasomes. Ubiquitin is also involved in the targeting of membrane proteins for trafficking into lysosomes either by addition of a monoubiquitin or of Lys63-linked polyubiquitin. I had the privilege of discussing with De Duve and Ciechanover here at the Casina Pio IV the relationship between the lysosomal and proteasomal pathways, and both Nobel laureates thought there was none.

In this paper I will present work from our laboratory showing that cytosolic proteins phosphorylated by Glycogen Synthase Kinase 3 (GSK3) can be degraded inside lysosomes, and that the transition from proteasomal to lysosomal degradation is regulated by an extracellular growth factor called Wnt.

Three issues will be addressed:

1) Wnt signaling requires sequestration of GSK3 inside late endosomes, also called multivesicular bodies.
2) Wnt/GSK3, also called canonical Wnt signaling, was long known to signal through the stabilization of a protein called β-Catenin. We have found that Wnt stabilizes many additional proteins, probably in the thousands, causing protein stabilization.
3) The translocation of Wnt receptors complexes carries bound to them not only the cytosolic β-Catenin destruction complex, but also proteins

that have been phosphorylated by GSK3 and marked by Lys48-linked polyubiquitin.

These studies lead to the conclusion that the proteasomal and lysosomal degradation pathways are not independent of each other as previously thought, and that the switch between them is physiologically regulated by the Wnt signaling factor.

1. Wnt signaling causes the sequestration of GSK3 inside multivesicular bodies

1.1. Wnt signaling in development and disease

Our laboratory is interested in the formation of morphogen signaling gradients during vertebrate development. During the cleavage of the *Xenopus laevis* frog egg there is a rotation of the egg cortex such that one side the embryo develops a dorsal crescent of less pigmented cells. These less pigmented cells will invariably become the dorsal (back) side while the darker cells will develop into the ventral (belly) side of the body. The initial asymmetry in the embryo is a strong Wnt signal triggered by the cortical rotation that stabilizes β-Catenin on the dorsal side. As development proceeds, other gradients are formed and lead to the morphogenesis of a perfect embryo time after time.

The canonical Wnt/GSK3 signaling pathway was discovered by Roel Nusse and is now known to be a key regulator of tissue regeneration, stem cells, and cancer (Logan and Nusse, 2004; Nusse and Clevers, 2017). Multiple mutations in this pathway, in genes such as Axin, APC and β-Catenin, cause stabilization of β-Catenin, increasing cell proliferation and leading to cancer.

1.2. Wnt decreases GSK3 activity

The Wnt growth factor binds to the cell surface co-receptors LRP6 (LDL-receptor related protein 6) and Frizzled. Activated receptors are phosphorylated by GSK3 and other kinases and recruit a cytosolic β-Catenin destruction complex consisting of Axin, Adenopolyposis Coli (APC), Dishevelled (Dvl), GSK3 and Casein Kinase 1 (CK1). In the absence of Wnt, the amino terminal region of β-Catenin is phosphorylated first by CK1 and then by three phosphorylations by GSK3. This generates what is called a phosphodegron. When regions of proteins are modified by multiple phosphates in Serines or Threonines in close vicinity, these phosphodegrons are recognized by ubiquitin ligases that catalyze the polyubiquitination of these proteins, targeting them for degradation in proteasomes

(Ciechanover, 2005). The phosphorylation of β-Catenin by GSK3 makes it an unstable protein. However, in the presence of Wnt β-Catenin is no longer phosphorylated and becomes stabilized. The accumulation of newly made β-Catenin causes its translocation into the nucleus where it binds to T-Cell Factor (TCF) on DNA, eliciting the many transcriptional effects of Wnt signaling on gene expression.

The mechanism by which Wnt signaling blocks GSK3 phosphorylations was an enigma. We performed measurements of the enzymatic activity of GSK3 using lysates containing the detergent Triton X-100 and were surprised to find that Wnt addition did not inhibit GSK3 enzyme activity (Taelman *et al.*, 2010). How could this be?

1.3. GSK3 is sequestered into multivesicular endosomes

We then remembered the classical work of Stanley Cohen, who had treated cultured cells with labelled Epidermal Growth Factor (EGF) and found that this growth factor became localized inside multivesicular bodies a few minutes after the endocytosis of its receptor (McKanna *et al.*, 1979). If GSK3 were to follow its receptors into the endolysosomal pathway, its sequestration inside membrane-bounded organelles could explain the reduced activity GSK3 in the cytosol while maintaining total levels of enzyme activity in detergent-treated lysates (Figure 1).

When cultured cells were treated with purified preparations of Wnt3a, after 10 minutes we observed the formation of prominent vesicular structures visible by phase-contrast optical microscopy. Thus, Wnt causes a large increase in endocytosis. When stained with antibodies against endogenous GSK3, it was clear that large amounts of GSK3 are translocated from the cytosol into these vesicles. Cryoimmuno electron microscopy, done in collaboration with David D. Sabatini of New York University, showed that GSK3 was localized inside the intraluminal vesicles (ILVs) of multivesicular bodies. Protease protection assays in cells made permeable with Digitonin showed that GSK3 and Axin were translocated into membrane-bounded organelles by Wnt signaling (Taelman *et al.*, 2010; Vinyoles *et al.*, 2014).

MVBs form during the normal progression of intracellular membrane traffic. Early endosomal vesicles pinch off the plasma membrane. In the case of Wnt, it was known endocytosis is required for signaling (Blitzer and Nusse, 2006) and that components of the receptor complex and the cytosolic destruction complex accumulated in the cell membrane and early endosome (Bilic *et al.*, 2007). However, the decrease of GSK3 activity in the cytoplasm requires its sequestration inside the ILVs of MVBs. Once

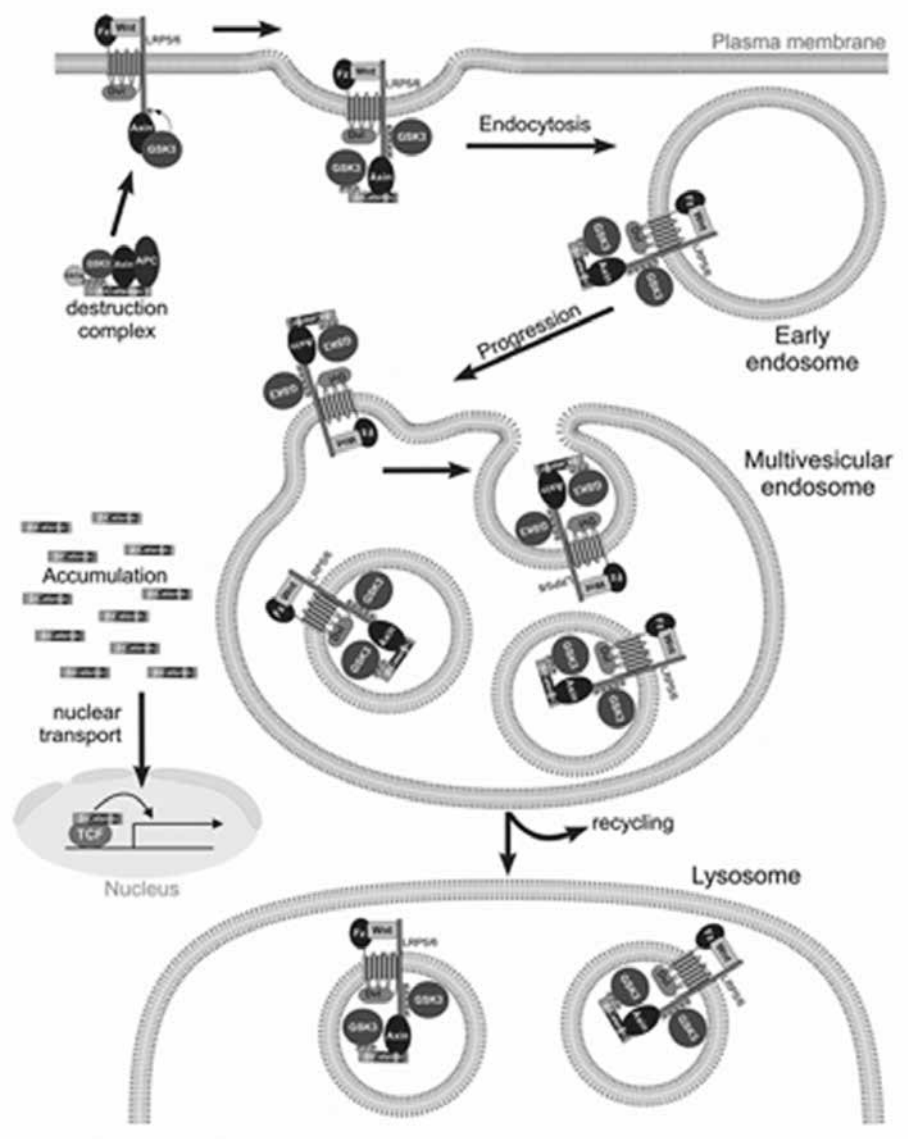


Figure 1. The Wnt growth factor signals through the sequestration of GSK3 and Axin inside multivesicular bodies. The activated Wnt receptors bind to Axin, a scaffold protein that brings bound to it APC, Dvl, phospho-β-Catenin. All these proteins, together with the Wnt coreceptors Frizzled and LRP6, are substrates for phosphorylation by GSK3. Following endocytosis, GSK3 becomes engulfed inside the intraluminal vesicles of multivesicular endosomes. This process is called microautophagy. The sequestration of GSK3 causes many proteins in the cytosol to become stabilized since GSK3 phosphodegrons are no longer formed. From Taelman *et al.*, *Cell* 2010.

inside, the enzyme becomes separated from its cytosolic substrates by two membranes: the ILV membrane and the late endosome/lysosome limiting membrane (Taelman *et al.*, 2010) (Figure 1). The outside-inside formation of ILVs requires great effort by the cell (Piper and Katzmann, 2007). All eukaryotic cells contain an elaborate ESCRT machinery (Endosomal Sorting Complexes Required for Transport), sometimes called Vps (vacuolar protein sorting) proteins that are necessary for membrane invagination into late endosomes.

All plasma membrane proteins must pass through the intraluminal vesicle step before they can enter the lysosome for degradation. Most growth factor receptors, such as the EGF receptor, use endolysosomes to downregulate receptor activity (Katzman *et al.*, 2002). The case of Wnt is different, because the sequestration of GSK3 and Axin constitutes the signal itself. Indeed, inhibiting the activity of the ESCRT proteins HRS/Vps27 or Vps4 blocks canonical Wnt/GSK3 signaling (Taelman *et al.*, 2010).

In conclusion, Wnt signaling requires the sequestration of a cytosolic protein kinase, GSK3, inside endosomes.

1. Wnt regulates the stability of many proteins

1.1. How many proteins are regulated by Wnt/GSK3?

GSK3 is a very abundant protein kinase, and has the peculiarity of being constitutively active (Wu and Pan, 2010). Most other protein kinases require an activation step before they can add phosphates to proteins using ATP as substrate. GSK3 has many other substrates in addition to β-Catenin (Jope and Johnson, 2004; Kim *et al.*, 2009). This raised the question of how many proteins might be stabilized by Wnt/GSK3.

As shown in Figure 2, GSK3 has a preference for pre-phosphorylated substrates. The priming phosphorylation can be introduced by many different kinases such as MAPK, CK1, CDK or PKA. Once phosphorylated, the substrate is recognized by the priming phosphate site in GSK3. The enzyme scans the protein and if a serine or threonine is found in the fourth position upstream of the priming phosphate, another phosphorylation is introduced (Cohen and Frame, 2001). This is a processive mechanism, so that if another Ser/Thr site is found four amino acids upstream, an additional phosphate is introduced until a Ser/Thr is no longer found.

To investigate how many proteins might conceivably be regulated by Wnt/GSK3 we analyzed the human entire proteome. We found that 20% of human proteins contain three or more consecutive GSK3 sites. This is much more than what might be expected by chance alone. The complete

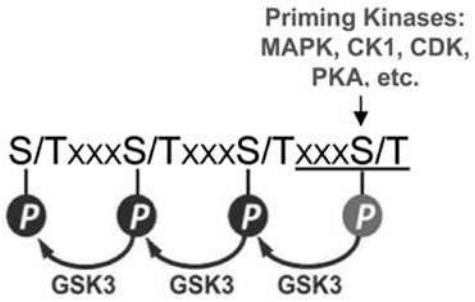


Figure 2. GSK3 is a processive kinase that preferentially adds a phosphate to Ser/Thr amino acids located four residues towards the amino terminus of a pre-phosphorylated site. An amazing 20% of human proteins contain three or more putative GSK3 phosphorylation sites.

list of these proteins is available at: http://www.hhmi.ucla.edu/derobertis/EDR_MS/GSK3%20Proteome/Table_1-full_table.xls

Determining whether a certain protein has possible GSK3 sites is a good predictor of whether it might be stabilized by Wnt signaling. We have investigated some such proteins and found that several are indeed regulated by Wnt addition. Examples include: MITF (Microphthalmia transcription factor, a key oncogene of melanocytes) (Ploper *et al.*, 2015); Tau (a microtubule-associated protein involved in Alzheimer's disease) (Dobrowolski *et al.*, 2012); HDAC4 (histone deacetylase 4) (Taelman *et al.*, 2010); Smad1 (a transcription factor activated by BMP signaling) (Fuentealba *et al.*, 2007); Smad4 (a transcription factor shared by the TGFβ and BMP pathways) (Demagny *et al.*, 2014).

We conclude that Wnt regulates the degradation of a plethora of other proteins in addition to β-Catenin.

1.2. Wnt signaling regulates total cellular protein stability

The effect of Wnt on protein stability is massive. In pulse-chase experiments with radioactive Methionine (30 minute pulse followed by chase in unlabeled medium containing a 5-fold excess of cold Methionine) the half-life of total cellular proteins in human 293 cells was extended by 25% (Taelman *et al*, 2010). This effect is so marked that it increased cell size measured by flow cytometry (Acebron *et al.*, 2014). We confirmed these observations and found that HeLa cells increase 14% in size after treating with Wnt for 48 hours and demonstrated that this increase requires the ESCRT machinery (Kim *et al.*, 2015).

This increase in protein stability has been designated Wnt-STabilization Of Proteins (Wnt/STOP) by Christof Niehrs (Acebron *et al.*, 2014). Wnt signaling is maximal during the G2/M phase of the cell cycle (Davidson *et al.*, 2009), leading to the interesting proposal that Wnt/STOP provides a means for cells to increase their volume by preventing protein degradation just prior to mitosis (Acebron *et al.*, 2014).

These experiments indicate that Wnt, through the sequestration of GSK3 in multivesicular endosomes, is a potent regulator of cellular protein degradation.

2. Wnt signaling translocates Lys48-linked polyubiquitinated proteins normally degraded in proteasomes into the lysosomal pathway

2.1. An unexpected result

Since GSK3 should be inhibited by Wnt, we expected that during Wnt signaling there would be less protein polyubiquitination due to lower levels of GSK3 phosphodegrons. To our surprise, we found that total polyubiquitinylated proteins accumulated after one or two hours of Wnt signaling. This accumulation could be blocked by GSK3 inhibitors. Furthermore, the increase was particularly in Lys48-linked polyubiquitin, which is the form that normally targets proteins to the proteasome. Wnt did not affect proteasomal activity, so we tested whether these proteins might be channeled into the lysosomal pathway by Wnt treatment (Kim *et al.*, 2015).

Chloroquine is an anti-malarial drug that inhibits lysosomal hydrolases by making lysosomes more alkaline. It inhibits lysosomal degradation downstream of the formation of MVBs. Indeed, lysosomes are expanded by Chloroquine and fill up with intraluminal vesicles. Chloroquine treatment greatly increased the accumulation of Lys48 polyubiquitinated proteins. This indicated that proteins normally targeted to proteasomes were being degraded in endolysomes.

2.2. Cross-talk between proteasomal and lysosomal protein degradation

After addition of Wnt protein polyubiquitinated proteins accumulated in vesicles marked by the MVB marker Vps4. Protease protection assays in Digitonin-permeabilized cells showed that the polyubiquitinated proteins were sequestered inside membrane-bounded organelles. Multiple Wnt/STOP substrates normally degraded in proteasomes – such as β-Catenin, Smad1, Smad4 – were carried into MVBs together with GSK3 and polyubiquitin (Kim *et al.*, 2015) (Figure 3).

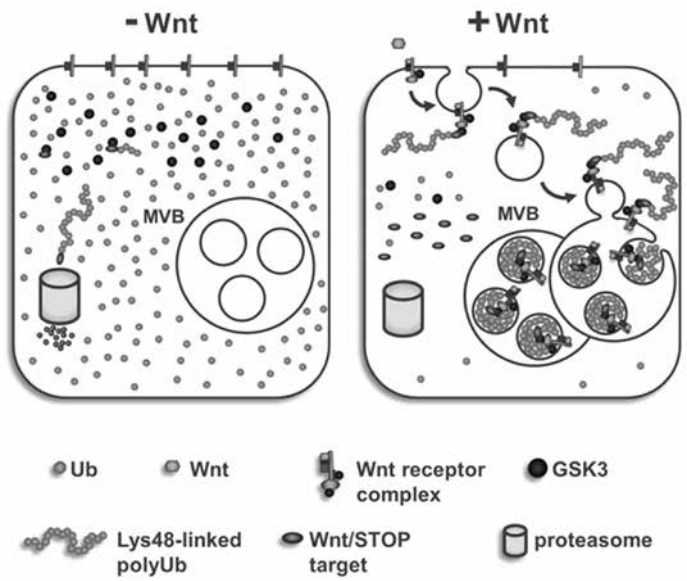


Figure 3. The lysosomal and proteasomal protein degradation systems crosstalk in a Wnt-regulated manner. During Wnt signaling Lys48-linked polyubiquitinated proteins, normally degraded in the proteasome, are translocated into MVB/lysosomes by microautophagy. The sequestration of polyubiquitin chains is so marked that it causes a reduction in free mono-ubiquitin, which is the substrate that marks proteins for degradation. The decrease in mono-ubiquitin results in generalized inhibition of protein degradation during the first two hours of Wnt signaling. From Kim *et al.* 2015.

2.3. A transient decrease in mono-ubiquitin levels strengthens protein stabilization

The sequestration of polyubiquitin inside MVBs is so strong that total levels of cellular mono-ubiquitin were decreased by two thirds during the first two hours following Wnt addition (Kim *et al.*, 2015). Although mono-ubiquitin levels then recovered, two hours represents a long time in cell signaling. Since mono-ubiquitin is the universal substrate for marking proteins for degradation, this reduction in its levels should enhance protein stabilization during the initial hours of Wnt signaling. Protein stabilization by Wnt would result from two converging mechanisms. First, GSK3 sequestration in MVBs will result in fewer phosphodegrons in cytosolic Wnt/STOP targets. Second, the decrease in mono-ubiquitin will inhibit degradation of most ubiquitinated proteins.

Cells regulate ubiquitin homeostasis highly, and a decrease in ubiquitin levels as the one caused by Wnt signaling is very unusual. Further, ubiquitin is recycled by de-ubiquitinases before cytosolic proteins are degraded in proteasomes or membrane proteins engulfed in multivesicular endosomes. Therefore, finding massive accumulation of ubiquitin in endolysosomes was very surprising.

The main discovery from these investigations was that the proteasomal and lysosomal pathways are not independent of each other as previously thought. Remarkably, the physiological choice between proteasomal and lysosomal degradation is controlled by the Wnt extracellular signal.

Conclusions

Organisms use a small number of cell signaling pathways for cell-cell communication. The study of cell signaling is very important in developmental and cancer biology. The Wnt pathway is particularly interesting because it is involved in the initiation of many tumors and also provides the first asymmetry during development of the amphibian embryo. The studies discussed here are at the intersection of the cell biology of membrane trafficking, cell signaling and the regulation of cellular protein degradation. They revealed that the Wnt signaling pathway uses the normal membrane trafficking machinery of endocytosis to generate a signal that results from the sequestration of GSK3 and Axin inside multivesicular endosomes. The removal of GSK3 from cytosol stabilizes a multitude of proteins, leading to a marked increase in protein stability and cell size. GSK3 has many substrates within the Wnt receptor complex and is translocated together with it, as well as with many of its cytosolic substrates, inside the intraluminal vesicles of multivesicular endosomes. This includes GSK3 substrates that have been already polyubiquitinated by Lys48 chains. Wnt causes an unsuspected switch of protein degradation from the proteasome to the lysosome, two pathways that were thought to be independent of each other.

Bibliography

Acebron, S.P., Karaulanov, E., Berger, B.S., Huang, Y.L., and Niehrs, C. (2014). Mitotic Wnt signaling promotes protein stabilization and regulates cell size. *Mol. Cell. 54*, 663-674.

Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M. and Niehrs, C. (2007). Wnt induces LRP6 Signalosomes and promotes Dishevelled-dependent LRP6 phosphorylation. *Science 316*, 1619-1622.

Blitzer, J.T., and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. *BMC Cell Biol. 7*, 28.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol. 6*, 79-87

De Duve, C. and Wattiaux, R. (1966). Functions of lysosomes. *Annu. Rev. Physiol. 28*, 435-492.

Davidson, G., Shen, J., Huang, Y.L., Su, Y., Karaulanov, E., Bartscherer, K., Hassler, C., Stannek, P., Boutros, M., and Niehrs, C. (2009). Cell cycle control of Wnt receptor activation. *Dev. Cell 17*, 788-799.

Demagny, H., Araki, T., and De Robertis, E.M. (2014). The tumor suppressor Smad4/DPC4 is regulated by phosphorylations that integrate FGF, Wnt and TGF-β signaling. *Cell Rep. 9*, 688-700.

Dobrowolski, R., Vick, P., Ploper, D., Gumper, I., Snitkin, H., Sabatini, D.D., and De Robertis, E.M. (2012). Presenilin deficiency or lysosomal inhibition enhances Wnt signaling through relocalization of GSK3 to the late-endosomal compartment. *Cell Rep. 2*, 1316-1328.

Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E.M., and De Robertis, E.M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell 131,* 980-993.

Jope, R.S., and Johnson, G.V. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci. 29*, 95-102.

Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell. Biol. 12*, 893-905 (2002).

Kim, N.G., Xu, C., and Gumbiner, B.M. (2009). Identification of targets of the Wnt pathway destruction complex in addition to β-Catenin. *Proc. Natl. Acad. Sci. USA 106*, 5165-5170.

Kim, H., Vick, P., Hedtke, J., Ploper, D. and De Robertis, E.M. (2015). Wnt signaling translocates Lys48-Linked polyubiquitinated proteins to the lysosomal pathway. *Cell Reports 11*, 1151-1159.

Logan, C.Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol. 20*, 781-810.

Nusse, R. and Clevers, H. (2017). Wnt/β-Catenin signaling, disease, and emerging therapeutic modalities. *Cell 169*, 985-999.

Piper, R.C. and Katzmann, D.J. (2007) Biogenesis and function of multivesicular bodies. *Annu. Rev. Cell Dev. Biol. 23*, 519-547.

Ploper, D., Taelman, V.F., Robert, L., Perez, B.S., Titz, B., Chen, H-W., Graeber, T.G., von Euw, E., Ribas, A., and De Robertis, E.M. (2015). MITF drives endolysosomal biogenesis and potentiates Wnt signaling in melanoma cells. *Proc. Natl. Acad. Sci. USA 112*, E420-429.

Taelman, V.F., Dobrowolski, R., Plouhinec, J.L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., and De Robertis, E.M. (2010). Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell 143*, 1136-1148.

Vinyoles, M., Valle-Perez, B.D., Curto, J., Vinas-Castells, R., Alba-Castellon, L., de Herreros, A.G., and Dunach, M. (2014). Multivesicular GSK3 sequestration upon Wnt signaling is controlled by p120-Catenin/Cadherin interaction with LRP5/6. *Mol. Cell 53*, 444-457.

Wu, D., and Pan, W. (2010). GSK3: a multifaceted kinase in Wnt signaling. *Trends Biochem. Sci. 35*, 161-168.

Neurobiology Session

Receptors That Regulate the Physiology of Audition

Ana Belén Elgoyhen[1]

Introduction

Sensory systems respond to stimuli from the surrounding world and use specialized receptor cells at the periphery to translate those stimuli into electrical signals that neurons can interpret. Further processing of sensory stimuli by the central nervous system generates a representation of the outer world called a percept. In everyday life, one is bombarded with acoustic stimuli of different intensities, frequencies and temporal structures. Some may be very salient and easily detected, some may require refined cerebral processing to extract the necessary information about the environment and some may be deleterious for the normal function of the ear. The neural circuits that compute the acoustic information are located in the brainstem and in higher auditory nuclei up to the auditory cortex. But the sophisticated machinery responsible for the detection of all different sounds and the conversion from mechanical energy intro electrical potentials is located in the inner ear, within a bony structure called the 'cochlea'.

Sound detection begins when sound waves strike the eardrum, which transmits that physical stimulus to the organ of Corti within the cochlea, the sensory epithelium of the mammalian inner ear. The mechanoreceptor cells of the organ of Corti then transform this mechanical input into electrical signals that are sent to the central nervous system by the auditory nerve (Hudspeth, 1997). The cochlea is a complicated labyrinth where fluid movements are produced by acoustic stimulus. The organ of Corti is the epithelium within the cochlea where sensory cells, supporting cells and also synaptic connections to and from the brain, all interact to make hearing happen. The organ of Corti lies over an acellular membrane, called the basilar membrane, with unique mechanical properties. The basilar membrane has the capacity to vibrate in response to fluid movements in the cochlea and propagate this mechanical energy to the organ of Corti. A very stereotyped organization in the cochlea determines that some regions

[1] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Dr Héctor N Torres (CONICET-UBA), Buenos Aires, Argentina.

are more sensitive to low frequency sounds and others to high frequency sounds. This spatial segregation mirrors in all cerebral nuclei responsible for processing acoustic information and is called 'tonotopy'.

1. Hair cells of the cochlea

Hair cells of the inner ear are very few, when compared to the millions of photoreceptors of the retina: approximately 16,000 sensory hair cells in the human cochlea. Mammalian hair cells do not regenerate after damage, thus the importance of protecting the inner ear from insults such us exposure to loud sound (Brigande and Heller, 2009; Lim, 1986), which leads to pathologies such as hearing loss and tinnitus (Eggermont and Roberts, 2004; Elgoyhen and Langguth, 2010). Two types of sensory cells in the organ of Corti are unique to the mammalian sensory system: inner hair cells (IHC) and outer hair cells (OHC). Hair cells are organized in a tonotopic fashion (arranged by frequency sensitivity): those sensitive to high frequency sound are at the basal end nearer to the tympanic middle ear and those sensitive to low frequency are at the apical end of the coiled cochlea (Hudspeth, 1997). Both IHC and OHC are capable of performing mechanotransduction, but whereas IHC are responsible for relaying acoustic information to the brain, OHC have the fundamental capacity to amplify low intensity stimuli to make them detectable. Hair cells present a polarized structure, with hair-like stereocilia in their apical end, and synaptic connections on the base. Between 20 and 300 stereocilia exist in each hair cell, the number varying depending on the location within the cochlea, but always in a three-row array distribution with different heights (Hudspeth, 1989). Each stereocilia is formed by a pack of actin filaments and inserts in the apical end of the hair cell within the so-called cuticular plate. Stereocilia are deflected by sound waves propagating inside the cochlea, not individually but concertedly in a block, ensuring activation of the mechanotransduction apparatus.

2. Mechanotransduction

The centerpiece in this complex process is an extensively investigated cationic channel with large conductance and mechanosensitive characteristics (Fettiplace and Hackney, 2006). It has been shown that movements of the stereocilia produce a large inward current in hair cells, with an ultrafast temporal signature. Unfortunately, and even in this post-genomic era, this channel still lacks molecular identification (Corey and Holt, 2016; Wu and Muller, 2016). Newly presented evidence indicates that members of the

transmembrane channel-like family would be, at least, part of the channel complex (Pan *et al.*, 2013). Several pieces of the mechanotransduction puzzle have been identified in the past years. Channels are located at the tip of the stereocilia and gating occurs when a spring-like element is stretched due to the stereocilia deflection. Detailed electron micrographs show the presence of connecting threads between each stereocilia and its neighbor from a different row (Kachar *et al.*, 2000). They are called 'tip links' and calcium-buffering agents (such as BAPTA) produce a reversible but complete elimination of this structure. Genetic screening of patients with Usher syndrome, a devastating sensory disorder, helped determine the molecular identity of the tip links: cadherin-23 and protocadherin-15 (El-Amraoui and Petit, 2005). They are transmembrane proteins presenting long extracellular domains with several cadherin repeats and are located at the tip links.

When mechanical force is applied, either by experimental means or by acoustically evoked fluid movements in the cochlea, stereocilia do not flex but pivot at their base and once force ceases, stereocilia return to their resting position. This mechanical deflection elicits ionic currents at the hair cell, by gating of the mechanosensitive channel. One of the main difficulties in the identification of these channels is that there are very few in each cell. Evidence from variance analysis indicates that one or few channels are located at the tip of each stereocilium, making about 20 per hair cell (Beurg *et al.*, 2009). Mechanotransduction current increases with deflections towards the tallest stereocilia and decreases in the opposite direction. The conductance of the channel changes along the tonotopical position within the cochlea, suggesting differential requirements at different frequencies.

3. The afferent synapse

All aspects of sound, including intensity, pitch, and location of the source are imparted to the brain through the afferent synapse on IHC. In all these situations synaptic vesicles are exocytosed with high temporal precision and without pause (Matthews and Fuchs, 2010). Several synaptic specializations suggest that this synapse evolved in a unique manner to cope with these tasks. One of its main features is a specialized synaptic organelle, called the 'ribbon', that appears as a dense structure attached to the membrane of IHC in electron-micrographs, always surrounded by vesicles. Other sensory synapses in the retina and vestibular organs present similar characteristics

Classically, in chemical synapses, action potentials propagate into a synaptic terminal generating calcium entry through calcium channels, which

in turn catalyses the fusion of neurotransmitter-filled vesicles. The IHC afferent synapse (also called ribbon synapse) is different. Like in other ribbon synapses, synaptic release occurs in response to graded changes in presynaptic membrane potential, not action potentials (Matthews and Fuchs, 2010). These changes are driven by the mechanotransduction current, and therefore produced by acoustic stimuli. IHC present voltage-activated calcium channels of the L-type, which allow calcium in the cell and trigger the release of synaptic vesicles (Brandt *et al.*, 2003). The characteristics of these channels, low voltage threshold and little inactivation, allow IHC to transmit sensory information in a very precise manner.

Typically, between 10-20 afferent neurons innervate each IHC with individual synaptic contacts, operating independently of each other. These synapses present both overlapping and differential features. The intensity of the sound is encoded in the rate at which vesicles are exocytosed, or equivalently, the firing rate of the postsynaptic neurons forming the auditory nerve. Different neurons contacting a given IHC present different basal activities, different thresholds for sound activation, and they do not saturate at the same intensity (Kiang *et al.*, 1962). Therefore, it is thought that even if synapses belong to the same IHC, they would operate differently by mechanisms that are not totally established yet. The IHC afferent synapse is glutamatergic, and is mediated by AMPA type receptors present in the postsynaptic boutons of auditory nerve neurons (Glowatzki and Fuchs, 2002). No NMDA component has been found in the normal operation of the synapse, although in certain conditions of intense acoustic stimulation NMDA receptors are transiently expressed (Puel *et al.*, 1995). As in other synapses in the auditory pathway, neurons show synaptic adaptations to efficiently respond to high frequency stimuli. For instance, synaptic currents are fast, with decay times of < 0.5 ms, and neurons show low input resistance which ensures fast synaptic potentials, little synaptic integration, and high fidelity responses in a cycle-by-cycle manner.

The specialized synaptic organelle present in hair cells, the ribbon, also called synaptic rod or body, is a matter of intense investigation. It is an electro-dense body in micrographs, surrounded by clear-core synaptic vesicles. It is thought that by concentrating vesicles in the active zones, the ribbon would increase the supply rate, supporting the capacity of this synapse to continuously release neurotransmitter (Matthews and Fuchs, 2010). Molecular components of the release machinery are only partially shared with those of central nervous system synapses. Synaptotagmin, the canonical calcium sensor present in synaptic vesicles, does not intervene

in exocytosis at hair cells. Instead, another candidate called otoferlin, with various calcium binding domains has been identified in a genetic screening from hearing impaired patients (Roux *et al.*, 2006). Otoferlin presents high affinity for calcium and also binds to the SNARE complex in a calcium dependent manner, two fundamental properties required for a calcium sensor candidate.

The afferent neurons that have been described so far contact only IHC, are classified as type I, and represent 95% of all afferents. There is a minor percentage of neurons, called type II, that receive inputs from several OHC with *en passant* synapses (Berglund and Ryugo, 1987). Glutamate also mediates synaptic transmission at these synapses and OHC also present synaptic ribbons, although with different shapes. It is unlikely that type II neurons also encode acoustic information, given that the activity level in these synapses is very low and insufficient to drive neurons to fire in basal conditions (Weisz *et al.*, 2009). It has been suggested that type II neurons may have a role in coding pain in the ear.

4. Outer hair cells and amplification

When sound reaches the cochlea, it produces mechanical vibrations. These are sensed and transduced into an electrical response by motion of the hair bundles of hair cells and activation of the mechanically-gated ion channels. In addition, the hair cells perform work and deliver energy to the system, thus increasing the magnitude of their mechanical input. This amplification of the stimulus constitutes a positive feedback that enhances the sensitivity of hearing, leading to amplification of the incoming sounds, increasing the gain of mechanical inputs and fine-tuning the basilar membrane (Dallos, 2008; Hudspeth, 2008).

In mammals, OHCs provide the feedback underlying cochlear amplification. Two alternative mechanisms for amplification have been described: an old one, also shared by non-mammalian vertebrates, where amplification results from a nonlinearity in the transduction mechanism itself (Chan and Hudspeth, 2005; Jia and He, 2005; Kennedy *et al.*, 2005) and a newer one in which the hair cell receptor potential drives a novel motile process within the lateral membrane of the OHC soma (Brownell *et al.*, 1985; Dallos, 2008). In the latter case, a process known as somatic electromotility (Dallos, 2008), hyperpolarization causes the cell to expand along its longitudinal axis and depolarization causes it to contract. Somatic electromotility of OHCs, as the basis for cochlear amplification, is a mammalian novelty and is mediated by the motor-protein prestin (Zheng *et al.*, 2000).

Mammalian prestin shows strong signatures of positive selection, most likely underlying the acquisition of amino acid substitutions to account for the motor function (Franchini and Elgoyhen, 2006).

5. The efferent synapse

Sensory systems of different modalities bring information about the environment to the brain. Top-down influences from the CNS, originating in the auditory brainstem, go all the way to the cochlea, and control sensitivity. This efferent innervation presents several morphological and functional peculiarities. The principal origin of the efferent neurons is the medial olivary complex (MOC) in the auditory brainstem. Several small nuclei that are responsible for computing auditory information originating from both ears are located in the brainstem. Neurons typically project axons across the midline to innervate nuclei in the contralateral side. The main effect of the MOC efferent innervation is to inhibit cochlear responses by decreasing the gain of the cellular amplifier (Guinan, 1996). MOC neurons innervate directly OHC and produce a net hyperpolarization once activated. MOC stimulation reduces the response at the best frequency measured either at the compound (Galambos, 1956) or single unit response of afferent fibers (Wiederhold and Kiang, 1970), IHC receptor potential (Brown and Nuttal, 1984) or basilar membrane motion (Murugasu and Russell, 1996). The strength of cochlear inhibition is proportional to the rate of MOC activity (Galambos, 1956; Gifford and Guinan, 1987; Wiederhold and Kiang, 1970). These effects most likely result from an inhibition of the motor function of OHCs, which is required for sensitive IHCs responses, thus indicating that MOC activity reduces amplification. Efferent inhibition also affects the cochlear tuning mechanism. The ultimate effect and functional role/s of MOC activity on audition is still a matter of active research. This include, the control of the dynamic range of hearing (Guinan, 1996), improvement of signal detection in background noise (Dolan and Nuttall, 1988; Kawase *et al.*, 1993; Winslow and Sachs, 1988), mediating selective attention (Delano *et al.*, 2007; Oatman, 1976), and protection from acoustic injury (Liberman, 1991; Rajan, 2000; Taranda *et al.*, 2009).

The neurotransmitter involved in the MOC-OHC synapse is acetylcholine (ACh) and the receptor mediating inhibition was a matter of debate over years due to its mixed nicotinic and muscarinic pharmacology (Fuchs, 1996). The cloning of two new receptor subunits of the cholinergic nicotinic family settled the discussion. These new subunits, named a9 and a10, form ionotropic receptors with the same mixed pharmacological

fingerprint of that of hair cells (Elgoyhen *et al.*, 1994, 2001). Receptors formed by a9 and a10 subunits are cationic and present a high calcium permeability. Once ACh is released from efferent neurons, it opens the postsynaptic receptors in hair cells and it is the large calcium influx that subsequently activates a calcium-dependent small potassium conductance, SK2, producing cellular hyperpolarization (Fuchs and Murrow, 1992). It is still to be proven if calcium entering through a9a10 receptors is sufficient to activate the SK2 channels or if alternatively, calcium coming from the extracellular space triggers the release of more calcium from intracellular stores (Lioudyno *et al.*, 2004). Evidence from electron micrographs indicate that this latter possibility is very likely, due to the existence of an endoplasmic organelle in close proximity to the plasma membrane in the basal end of the OHC (Fuchs *et al.*, 2014).

It is important to note that OHC are not the only target of MOC fibers. During postnatal development of the altricial rodent cochlea, IHC transiently receive cholinergic innervation which is also mediated by a9a10 receptors (Katz *et al.*, 2004). During this period, comprising the first two weeks after birth, mice are deaf, but spontaneous electrical activity in the organ of Corti has been shown to occur. IHC are able to fire calcium action potentials, driving neurons in the auditory pathway to fire rhythmically. This activity is determinant for the normal maturation of synapses and circuits of the entire auditory pathway and ceases after the onset of hearing (Kandler, 2004; Kandler *et al.*, 2009). Activity of the MOC innervation is also inhibitory during this developmental critical period and controls the excitability of IHC (Glowatzki and Fuchs, 2000).

6. Pathology of the auditory system

Millions of people around the world have hearing loss or associated conditions, such as tinnitus, otitis media and Ménières disease. Hearing impairment is one of the most common sensory disabilities, and may drastically limit the quality of life, with an incidence of 1:1000 in newborns. It becomes increasingly prevalent with age. Hearing loss affects approximately 17 in every 1000 children under the age 18, approximately 314 in 1000 adults over age 65, and 40-50% of people 75 and older. Hearing loss is a social and economic burden since it can cause considerable difficulties in communication with the outside world in general and lead to sadness, depression, anxiety, social isolation, and insecurity (Cohen *et al.*, 2005). Hearing loss is caused by several environmental and genetic factors and the proportion attributed to inherited causes is thought to be at least 50%

(Cryns and Van Camp, 2004; Vrijens *et al.*, 2008). Among the environmental factors leading to hearing loss is the exposure to overly loud sounds. It is now well established that overexposure to loud sounds causes trauma to the ear, deteriorating our ability to hear (Saunders *et al.*, 1985). This effect can be temporary (like after attending a rock concert) or permanent (as a result of recurrent loud noise over long periods of time). The consequences of acoustic trauma are diverse but include stereocilia disarrangement, synaptic terminal swellings, and even hair cell death (Liberman and Kiang, 1978). This latter one is the most critical, given that mammalian hair cells do not regenerate and therefore, loss of cells determines loss of hearing capacity. One important function of the MOC efferent system is the protection to trauma produced by overly loud sounds (Maison and Liberman, 2000; Taranda *et al.*, 2009). Stimulation of the MOC fibers during sound overexposure produces a reduction of sensitivity loss. Therefore, drugs that activate the MOC system might result beneficial to protect the inner ear (Elgoyhen *et al.*, 2009).

Summary

This manuscript provides a succinct overview of different processes occurring in the auditory periphery that are essential to the sense of hearing. Given the peculiarities of this sensory modality, detection of the simplest sound requires a very complex series of cellular events. The biophysical and physiological details of phenomena such as mechanotransduction, non-exhausting afferent synaptic transmission and efferent control of the hearing sensitivity are only starting to emerge.

References

Berglund, A.M., & Ryugo, D.K. (1987). Hair cell innervation by spiral ganglion neurons in the mouse. *J Comp Neurol, 255*, 560-570.

Beurg, M., Fettiplace, R., Nam, J.H., & Ricci, A.J. (2009). Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. *Nat Neurosci, 12*, 553-558.

Brandt, A., Striessnig, J., & Moser, T. (2003). CaV1.3 channels are essential for development and presynaptic activity of cochlear inner hair cells. *J Neurosci, 23*, 10832-10840.

Brigande, J.V., & Heller, S. (2009). Quo vadis, hair cell regeneration? *Nat Neurosci, 12*, 679-685.

Brown, M.C., & Nuttal, A.L. (1984). Efferent control of cochlear inner hair cell responses in guinea-pig. *J. Physiol. (Lond.), 354*, 625-646.

Brownell, W., Bader, C., Bertrand, D., & de Ribaupierre, Y. (1985). Evoked mechanical responses of isolated cochlear hair cells. *Science, 227*, 194-196.

Cohen, S.M., Labadie, R.F., & Haynes, D.S. (2005). Primary care approach to hearing loss: the hidden disability. *Ear*

Nose Throat J, 84, 26, 29-31, 44.
Corey, D.P., & Holt, J.R. (2016). Are TMCs the Mechanotransduction Channels of Vertebrate Hair Cells? *J Neurosci, 36*, 10921-10926.
Cryns, K., & Van Camp, G. (2004). Deafness genes and their diagnostic applications. *Audiol Neurootol, 9*, 2-22.
Chan, D.K., & Hudspeth, A.J. (2005). Ca2+ current-driven nonlinear amplification by the mammalian cochlea in vitro. *Nat Neurosci, 8*, 149-155.
Dallos, P. (2008). Cochlear amplification, outer hair cells and prestin. *Curr Opin Neurobiol, 18*, 370-376.
Delano, P.H., Elgueda, D., Hamame, C.M., & Robles, L. (2007). Selective attention to visual stimuli reduces cochlear sensitivity in chinchillas. *J Neurosci, 27*, 4146-4153.
Dolan, D.F., & Nuttall, A.L. (1988). Masked cochlear whole-nerve response intensity functions altered by electrical stimulation of the crossed olivocochlear bundle. *J Acoust Soc Am, 83*, 1081-1086.
Eggermont, J.J., & Roberts, L.E. (2004). The neuroscience of tinnitus. *Trends Neurosci, 27*, 676-682.
El-Amraoui, A., & Petit, C. (2005). Usher I syndrome: unravelling the mechanisms that underlie the cohesion of the growing hair bundle in inner ear sensory cells. *J Cell Sci, 118*, 4593-4603.
Elgoyhen, A.B., Katz, E., & Fuchs, P.A. (2009). The nicotinic receptor of cochlear hair cells: a possible pharmacotherapeutic target? *Biochem Pharmacol, 78*, 712-719.
Elgoyhen, A.B., & Langguth, B. (2010). Pharmacological approaches to the treatment of tinnitus. *Drug Discov Today, 15*, 300-305.
Fettiplace, R., & Hackney, C.M. (2006). The sensory and motor roles of auditory hair cells. *Nat Rev Neurosci, 7*, 19-29.
Franchini, L.F., & Elgoyhen, A.B. (2006). Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Mol Phylogenet Evol, 41*, 622-635.
Fuchs, P.A., Lehar, M., & Hiel, H. (2014). Ultrastructure of cisternal synapses on outer hair cells of the mouse cochlea. *J Comp Neurol, 522*, 717-729.
Fuchs, P.A., & Murrow, B.W. (1992). A novel cholinergic receptor mediates inhibition of chick cochlear hair cells. *Proc. R. Soc. Lond. B, 248*, 35-40.
Galambos, R. (1956). Suppression of auditory nerve activity by stimulation of efferent fibers to the cochlea. *J. Neurophysiol., 19*, 424-437.
Gifford, M.L., & Guinan, J.J., Jr. (1987). Effects of electrical stimulation of medial olivocochlear neurons on ipsilateral and contralateral cochlear responses. *Hear Res, 29*, 179-194.
Glowatzki, E., & Fuchs, P. (2000). Cholinergic synaptic inhibition of inner hair cells in the neonatal mammalian cochlea. *Science, 288*, 2366-2368.
Glowatzki, E., & Fuchs, P. (2002). Transmitter release at the hair cell ribbon synapse. *Nature Nerosci, 5*, 147-154.
Guinan, J.J. (1996). Physiology of olivocochlear efferents. In Dallos, Popper, & Fay (Eds.), *The Cochlea* (pp. 435-502). New York: Springer-Verlag.
Hudspeth, A. (1997). How hearing happens. *Neuron, 19*, 947-950.
Hudspeth, A.J. (2008). Making an effort to listen: mechanical amplification in the ear. *Neuron, 59*, 530-545.
Jia, S., & He, D.Z. (2005). Motility-associated hair-bundle motion in mammalian outer hair cells. *Nat Neurosci, 8*, 1028-1034.
Kachar, B., Parakkal, M., Kurc, M., Zhao, Y., & Gillespie, P.G. (2000). High-resolution structure of hair-cell tip links. *Proc Natl Acad Sci USA, 97*, 13336-13341.
Kandler, K. (2004). Activity-dependent organization of inhibitory circuits: les-

sons from the auditory system. *Curr Opin Neurobiol, 14*, 96-104.

Kandler, K., Clause, A., & Noh, J. (2009). Tonotopic reorganization of developing auditory brainstem circuits. *Nat Neurosci, 12*, 711-717.

Katz, E., Elgoyhen, A.B., Gomez-Casati, M.E., Knipper, M., Vetter, D.E., Fuchs, P.A., & Glowatzki, E. (2004). Developmental regulation of nicotinic synapses on cochlear inner hair cells. *J Neurosci, 24*, 7814-7820.

Kawase, T., Delgutte, B., & Liberman, M.C. (1993). Antimasking effects of the olivocochlear reflex. II. Enhancement of auditory-nerve response to masked tones. *J Neurophysiol, 70*, 2533-2549.

Kennedy, H.J., Crawford, A.C., & Fettiplace, R. (2005). Force generation by mammalian hair bundles supports a role in cochlear amplification. *Nature, 433*, 880-883.

Kiang, N.Y., Watanabe, T., Thomas, E.C., & Clark, L.F. (1962). Stimulus coding in the cat's auditory nerve. Preliminary report. *Ann Otol Rhinol Laryngol, 71*, 1009-1026.

Liberman, M.C. (1991). The olivocochlear efferent bundle and susceptibility of the inner ear to acoustic injury. *J Neurophysiol, 65*, 123-132.

Liberman, M.C., & Kiang, N.Y. (1978). Acoustic trauma in cats. Cochlear pathology and auditory-nerve activity. *Acta Otolaryngol Suppl, 358*, 1-63.

Lim, D.J. (1986). Effects of noise and ototoxic drugs at the cellular level in the cochlea: a review. *Am J Otolaryngol, 7*, 73-99.

Lioudyno, M., Hiel, H., Kong, J.H., Katz, E., Waldman, E., Parameshwaran-Iyer, S., Glowatzki, E., & Fuchs, P.A. (2004). A "synaptoplasmic cistern" mediates rapid inhibition of cochlear hair cells. *J Neurosci, 24*, 11160-11164.

Maison, S.F., & Liberman, M.C. (2000). Predicting vulnerability to acoustic injury with a noninvasive assay of olivocochlear reflex strength. *J Neurosci, 20*, 4701-4707.

Matthews, G., & Fuchs, P. (2010). The diverse roles of ribbon synapses in sensory neurotransmission. *Nat Rev Neurosci, 11*, 812-822.

Murugasu, E., & Russell, I.J. (1996). The effect of efferent stimulation on basilar membrane displacement in the basal turn of the guinea pig cochlea. *J Neurosci, 16*, 325-332.

Oatman, L.C. (1976). Effects of visual attention on the intensity of auditory evoked potentials. *Exp. Neurol., 51*, 41-53.

Pan, B., Geleoc, G.S., Asai, Y., Horwitz, G.C., Kurima, K., Ishikawa, K., Kawashima, Y., Griffith, A.J., & Holt, J.R. (2013). TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron, 79*, 504-515.

Puel, J., Safieddine, S., Gervais dÀldin, C., & Pujol, R. (1995). Synaptic regeneration and functional recovery after excitotoxicity injury in the guinea pig cochlea. *Cr Acad Sci Ser III Sci Vie, 318*, 67-75.

Rajan, R. (2000). Centrifugal pathways protect hearing sensitivity at the cochlea in noisy environments that exacerbate the damage induced by loud sound. *Journal of Neuroscience*, 6684-6693.

Roux, I., Safieddine, S., Nouvian, R., Grati, M., Simmler, M.C., Bahloul, A., Perfettini, I., Le Gall, M., Rostaing, P., Hamard, G., Triller, A., Avan, P., Moser, T., & Petit, C. (2006). Otoferlin, defective in a human deafness form, is essential for exocytosis at the auditory ribbon synapse. *Cell, 127*, 277-289.

Saunders, J.C., Dear, S.P., & Schneider, M.E. (1985). The anatomical consequences of acoustic injury: A review and tutorial. *J Acoust Soc Am, 78*, 833-860.

Taranda, J., Maison, S.F., Ballestero, J.A.,

Katz, E., Savino, J., Vetter, D.E., Boulter, J., Liberman, M.C., Fuchs, P.A., & Elgoyhen, A.B. (2009). A point mutation in the hair cell nicotinic cholinergic receptor prolongs cochlear inhibition and enhances noise protection. *PLoS Biol,* 7, e18.

Vrijens, K., Van Laer, L., & Van Camp, G. (2008). Human hereditary hearing impairment: mouse models can help to solve the puzzle. *Hum Genet, 124*, 325-348.

Weisz, C., Glowatzki, E., & Fuchs, P. (2009). The postsynaptic function of type II cochlear afferents. *Nature, 461*, 1126-1129.

Wiederhold, M.L., & Kiang, N.Y.S. (1970). Effects of electrical stimulation of the crossed olivocochlear bundle on cat single auditory nerve fibres. *J. Acoust. Soc. Amer., 48*, 950-965.

Winslow, R.L., & Sachs, M.B. (1988). Single-tone intensity discrimination based on auditory-nerve rate responses in backgrounds of quiet, noise, and with stimulation of the crossed olivocochlear bundle. *Hear Res, 35*, 165-189.

Wu, Z., & Muller, U. (2016). Molecular Identity of the Mechanotransduction Channel in Hair Cells: Not Quiet There Yet. *J Neurosci, 36*, 10927-10934.

Zheng, J., Shen, W., He, D.Z., Long, K.B., Madison, L.D., & Dallos, P. (2000). Prestin is the motor protein of cochlear outer hair cells. *Nature, 405*, 149-155.

Zika Virus: From Neglected Disease to World Threat

Pítia F. Ledur[2] and Stevens K. Rehen[1,2]

Introduction

Zika virus (ZIKV) was very much unknown to the world until 2015, when an outbreak in Brazil was connected to malformations such as microcephaly (Noronha *et al.*, 2016; Calvet *et al.*, 2016). Further investigations revealed that the virus could also cause Guillain-Barre syndrome in adults, an autoimmune disorder that damages the peripheral nervous system, triggering numbness, weakness, and ultimately even paralysis (Muñoz *et al.*, 2016). Formerly, ZIKV was known to cause only mild symptoms such as fever and rash. Intriguingly, ZIKV had been discovered almost 70 years earlier, in 1947 to be precise. The virus was first isolated from a sentinel monkey in Uganda, Africa, during a Yellow Fever study (Dick *et al.*, 1952; Dick 1952), and though its existence was known of, only 14 cases had been described before 2007 (Ledur *et al.*, in preparation). Then, a subtle upsurge happened, with a little over 50 cases being described on the Yap Islands and in Gabon altogether (Duffy *et al.*, 2009; Grard *et al.*, 2014). While this number might seem quite small, the Yap Island studies indicated that 74% of 557 residents assessed had IgM antibodies against the virus (Duffy *et al.*, 2009). Also, a former study in Africa had revealed that 40% of Nigerians had neutralizing antibodies against ZIKV (Fagbami 1979), indicating that even though the number of cases described was small, ZIKV infection seemed more common than thought before. The silent infection did not seem to be alarming, as its consequences were mainly rash, fever, joint pain and conjunctivitis (Duffy *et al.*, 2009; Campos *et al.*, 2015; Hayes 2009). The next noticeable manifestation of the virus happened in 2013-2014 in French Polynesia, raising the number of cases to almost 30,000 (Jouannic *et al.*, 2016). The virus was then connected to autoimmune and neurological symptoms.

When ZIKV reached Brazil, it spread quickly, and the increase in the number of cases was linked to a simultaneous escalation in microcephaly

[1] Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Brazil.
[2] D'Or Institute for Research and Education, Rio de Janeiro, Brazil.

occurrences (Mlakar *et al.*, 2016). It did not take longer for studies to confirm the ability of the virus to cross the placental barrier and to infect progenitor brain cells (Noronha *et al.*, 2016; Garcez *et al.*, 2016; Cugola *et al.*, 2016). Then, ZIKV gained the attention of the world, while the scientific community focused on trying to understand the threat.

We will consider here three main topics about Zika virus:

1) What do we know about the basic biology of ZIKV?
2) What are the consequences of ZIKV infection?
3) Which factors can be playing a role in the more harmful ZIKV episodes?

1. ZIKV Biology

1.1. ZIKV origin and infection mechanisms

Zika virus is a member of the Flaviviridae family. This viral family is known for having single-stranded, positive sense RNA as its genetic material, and for being involved by a lipid envelope. Flaviviruses encode ten proteins, and use the host cell machinery for their replication cycle. Most Flaviviruses are arboviruses, transmitted by mosquitoes and ticks, and many of them are pathogenic to humans. Examples other than Zika virus include Dengue virus, West Nile virus, Japanese encephalitis virus, yellow fever virus, and hepatitis C virus. The mechanism of action of Flaviviruses, once inside the cell, is usually very similar. They release their genetic material (RNA) in the cells cytoplasm, and the RNA associates with ribosomal proteins in the endoplasmic reticulum, for translation into proteins required for viral replication. This process requires the use of energy from the cell, stressing mitochondria as well as the reticulum. The overload eventually causes the production of reactive oxygen and nitrogen species (ROS and RNS, respectively), and the collapse of the organelles. ROS and RNS that are released can have other consequences, such as damaging the DNA from the host cell. DNA damage can then lead to cell death. The outcome for infected cells is usually fatal (Smit *et al.*, 2011; Fernandez-Garcia *et al.*, 2009). Some of the particularities of ZIKV when compared to other Flaviviruses is the capacity to cross the placental barrier (Noronha *et al.*, 2016; Miner *et al.*, 2016). Moreover, ZIKV has a tropism for neural progenitor cells, mainly neural stem cells (NSCs) and glial cells, making it dangerous during neurodevelopment (Retallack *et al.*, 2016; C. Li *et al.*, 2016).

1.2. ZIKV cell entry

The way ZIKV enters the cell has been proposed to involve phosphatidylserine receptors such as the ones from the TIM and TAM families. These receptors have been described as entry points for Dengue virus (Meertens *et al.*, 2012), therefore they were natural candidates when investigating ZIKV entry mechanisms. Further investigation of specific receptors within TIM and TAM family members revealed AXL, a receptor tyrosine kinase, as an important candidate involved in ZIKV cell entry (S. Liu *et al.*, 2016; Meertens *et al.*, 2017; Hamel *et al.*, 2015). AXL receptor, as well as other members from the TAM family, are required for phagocytosis of apoptotic cells (Lemke & Burstyn-Cohen 2010). ZIKV seems to infect human umbilical vein endothelial cells more efficiently than other flaviviruses such as dengue or West Nile virus, and the way ZIKV enters these cells was proposed to involve AXL as well as its ligand Gas6 (Richard *et al.*, 2017). Some groups, however, have disagreed on AXL as the main entry port for ZIKV, as the use of AXL deficient mice (Axl-/-) has not avoided infection. Also, deletion of the AXL gene in neural cell types did not change infectivity nor virus-induced cell death in neural progenitor cells and brain organoid models (Z.-Y. Wang *et al.*, 2017; F. Li *et al.*, 2017; Wells *et al.*, 2016). Controversies in the literature remain, although the understanding of how ZIKV invades cells is fundamental for drug design. There is still work ahead to solve the puzzle of ZIKV cell entry.

1.3. ZIKV strains

After the most recent South American outbreak, genetic and phylogenetic investigations revealed that there are two main circulating strains of ZIKV, the African and the Asian (Faye *et al.*, 2014; L. Wang *et al.*, 2016). Asian ZIKV is the strain involved in recent outbreaks. Tough they are very similar, the differences in sequences between the two strains could be related to cell tropism and virulence, as African ZIKV seems to be more aggressive and less selective (Anfasa *et al.*, 2017; Simonin *et al.*, 2016; Govero *et al.*, 2016). It has been shown that a mutation in the NS1 protein of Asian ZIKV could be behind an augmented infectivity in mosquitoes, facilitating the human cycle of the virus (Y. Liu *et al.*, 2017). Another mutation, in the prM protein of the most recent Asian ZIKV isolates has been named responsible for the increased severity in the microcephaly phenotype (Yuan *et al.*, 2017). It is likely that the virus evolved towards increased neurotropism, especially since microcephaly cases have been linked to the Asian ZIKV strain (Yuan *et al.*, 2017; L. Wang *et al.*, 2016). We should bear

in mind that viruses in general, especially RNA viruses, are very prone to mutations, so we cannot exclude the possibility of new strains arising in a near future.

2. ZIKV consequences

2.1. Microcephaly and Guillain-Barre Syndrome

Microcephaly is a neurodevelopmental disease caused by an impairment in cell proliferation and by increased cell death of cortical progenitors and their derived cells. Therefore, cell biology has been extremely helpful in uncovering ZIKV consequences. Induced pluripotent stem cells (iPSCs) have been used to generate human brain cell types such as neural stem cells (NSCs) and glial cells, as well as three-dimensional models such as neurospheres and brain organoids. Organoids are structures that can mimic the organization and the function of real organs *in vitro* (Clevers 2016), which made them fundamental in understanding ZIKV mode of action. Such models were employed by the first papers that came out elucidating the causality between ZIKV and microcephaly; the virus was shown to increase cell death, reducing viability and affecting organoid growth {Garcez: 2016iq}. Further investigations revealed that ZIKV could target neural precursor cells, affecting cell cycle and neuronal differentiation pathways (Garcez *et al.*, 2017), as well as causing premature differentiation of precursor cells (Gabriel *et al.*, 2017).

Guillain-Barre syndrome is an autoimmune disorder in which the immune system attacks healthy cells from the peripheral nervous system. It is characterized by muscle weakness, which could evolve into acute paralysis (Cao-Lormeau *et al.*, 2016). It often occurs after a bacterial or viral infection, and it had been associated with other flaviviruses in the past (Ravi *et al.*, 1994; Sulekha *et al.*, 2004; Ralapanawa *et al.*, 2017; Oehler *et al.*, 2015). After outbreaks of ZIKV, a marked increase in Guillain-Barre Syndrome cases occurred in Brazil as well as in Colombia, the Dominican Republic, El Salvador, Honduras, Suriname and Venezuela, in addition to French Polynesia (Santos *et al.*, 2016). The way ZIKV causes Guillain-Barre is unknown, though the fact that the disease is triggered by infections suggests that it is caused by an improper immune response.

2.2 Other diseases?

Even though Guillain-Barre Syndrome and microcephaly have been shown to be serious consequences of ZIKV infection, a question remains regarding asymptomatic babies who are born from infected mothers. Since

ZIKV can cross the placental barrier and has proved neurotropism, what consequences can be expected for babies that are born apparently normal? Could they suffer from other diseases that affect the neurological system later in life? And if they are in fact normal, what makes them immune to the virus effects? These questions remain to be answered.

3. ZIKV and environmental factors

After 2015-16, ZIKV cases seem to have drastically dropped. Also, the increase in microcephaly cases in Brazil was an outlier when compared to other countries. Even in Brazil, the northeast was the only region that had a number of microcephaly incidents higher than what the World Health Organization (WHO) considers to be the endemic range of microcephaly (the northeast had 48 cases/10,000 newborn babies versus 0.5-20 cases/10,000 livebirths considered endemic by the WHO) (de Oliveira *et al.*, 2017). The numbers are quite puzzling, so theories emerged that maybe ZIKV alone was not the sole responsible for the surge in malformations; perhaps environmental factors also played a role. One of the first hypothesis was about the use of pyriproxyfen, a larvicide added to drinking water in order to prevent mosquito proliferation. Pyriproxyfen is a growth hormone analog, considered safe by the WHO, and that has been used in Israel, Spain, South Africa and other places. However, the unparalleled use of pyriproxyfen in drinking water in Brazil became a concern as it raised questions that it could be causing or enhancing the incidence in microcephaly. Recent studies with zebrafish, a developmental model, have tested if early exposure to pyriproxyfen could cause developmental changes. No neurodevelopmental defects were observed (Truong *et al.*, 2016; Dzieciolowska *et al.*, 2017). However, no combination of pyriproxyfen exposure and zika virus infection has been tested at this point, as there could be an additive effect. Still, other environmental factors cannot be excluded. Another hypothesis could be the genetic background of infected people, though this also remains to be shown.

Conclusions

ZIKV was discovered in 1947, but it was not until the recent outbreaks that the scientific community joined efforts to study it, trying to understand the connection between the virus and more serious consequences such as malformations and Guillain-Barre Syndrome. The amount of papers published about ZIKV sharply increased in recent years, advancing the understanding of the virus and its mechanisms. Though much has

been learned, controversies and questions remain. Researchers worldwide should keep working to help better understand the virus mode of action and, mostly, to help avoid future outbreaks.

References

Anfasa, F. *et al.*, 2017. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. *Host-Microbe Biology*, 2(4), pp. e00292-17.

Calvet, G. *et al.*, 2016. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. *The Lancet. Infectious diseases*, 16(6), pp. 653-660.

Campos, G.S., Bandeira, A.C. & Sardi, S.I., 2015. Zika Virus Outbreak, Bahia, Brazil. *Emerging Infectious Diseases*, 21(10), pp. 1885-1886.

Cao-Lormeau, V.M. *et al.*, 2016. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *Lancet (London, England)*, 387(10027), pp. 1531-1539.

Clevers, H., 2016. Modeling Development and Disease with Organoids. *Cell*, 165(7), pp. 1586-1597.

Cugola, F.R. *et al.*, 2016. The Brazilian Zika virus strain causes birth defects in experimental models. *Nature*, 534(7606), pp. 267-271.

de Oliveira, W.K. *et al.*, 2017. Infection-related microcephaly after the 2015 and 2016 Zika virus outbreaks in Brazil: a surveillance-based analysis. *Lancet (London, England)*, 390(10097), pp. 861-870.

Dick, G.W.A., 1952. Zika virus. II. Pathogenicity and physical properties. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 46(5), pp. 521-534.

Dick, G.W.A., Kitchen, S.F. & Haddow, A.J., 1952. Zika virus. I. Isolations and serological specificity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 46(5), pp. 509-520.

Duffy, M.R. *et al.*, 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. *The New England journal of medicine*, 360(24), pp. 2536-2543.

Dzieciolowska, S. *et al.*, 2017. The larvicide pyriproxyfen blamed during the Zika virus outbreak does not cause microcephaly in zebrafish embryos. *Scientific reports*, 7, p. 40067.

Fagbami, A.H., 1979. Zika virus infections in Nigeria: virological and seroepidemiological investigations in Oyo State. *The Journal of hygiene*, 83(2), pp. 213-219.

Faye, O. *et al.*, 2014. Molecular Evolution of Zika Virus during Its Emergence in the 20th Century B. Bird, ed. *PLOS Neglected Tropical Diseases*, 8(1), pp. e2636-10.

Fernandez-Garcia, M.-D. *et al.*, 2009. Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell. *Cell Host and Microbe*, 5(4), pp. 318-328.

Gabriel, E. *et al.*, 2017. Recent Zika Virus Isolates Induce Premature Differentiation of Neural Progenitors in Human Brain Organoids. *Cell Stem Cell*, 20(3), pp. 397-406.e5.

Garcez, P.P. *et al.*, 2017. Zika virus disrupts molecular fingerprinting of human neurospheres. *Scientific reports*, 7, p. 40780.

Garcez, P.P. *et al.*, 2016. Zika virus impairs growth in human neurospheres and brain organoids. *Pan American Health Organization / World Health Organization*, 352(6287), pp. 816-818.

Govero, J. *et al.*, 2016. Zika virus infection damages the testes in mice. *Nature*, 540(7633), pp. 438-442.

Grard, G. *et al.*, 2014. Zika Virus in Gabon (Central Africa) – 2007: A New Threat from Aedes albopictus? R. Charrel, ed. *PLOS Neglected Tropical Diseases*, 8(2), pp.e2681–6.

Hamel, R. *et al.*, 2015. Biology of Zika Virus Infection in Human Skin Cells. *Journal of virology*, 89(17), pp. 8880-8896.

Hayes, E.B., 2009. Zika Virus Outside Africa. *Emerging Infectious Diseases*, 15(9), pp. 1347-1350.

Jouannic, J.-M. *et al.*, 2016. Zika virus infection in French Polynesia. *The Lancet*, 387(10023), pp. 1051-1052.

Lemke, G. & Burstyn-Cohen, T., 2010. TAM receptors and the clearance of apoptotic cells. *Annals of the New York Academy of Sciences*, 1209(1), pp. 23-29.

Li, C. *et al.*, 2016. Zika Virus Disrupts Neural Progenitor Development and Leads to Microcephaly in Mice. *Stem Cell*, 19(1), pp. 120-126.

Li, F. *et al.*, 2017. AXL is not essential for Zika virus infection in the mouse brain. *Emerging microbes & infections*, 6(3), p. e16.

Liu, S. *et al.*, 2016. AXL-Mediated Productive Infection of Human Endothelial Cells by Zika Virus Novelty and Significance. *Circulation research*, 119(11), pp. 1183-1189.

Liu, Y. *et al.*, 2017. Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. *Nature Publishing Group*, 545(7655), pp. 482-486.

Meertens, L. *et al.*, 2017. Axl Mediates ZIKA Virus Entry in Human Glial Cells and Modulates Innate Immune Responses. *CellReports*, 18(2), pp. 324-333.

Meertens, L. *et al.*, 2012. The TIM and TAM Families of Phosphatidylserine Receptors Mediate Dengue Virus Entry. *Cell Host and Microbe*, 12(4), pp. 544-557.

Miner, J.J. *et al.*, 2016. Zika Virus Infection during Pregnancy in Mice Causes Placental Damage and Fetal Demise. *Cell*, 165(5), pp. 1081-1091.

Mlakar, J. *et al.*, 2016. Zika Virus Associated with Microcephaly. *The New England journal of medicine*, 374(10), pp. 951-958.

Muñoz, L.S., Barreras, P. & Pardo, C.A., 2016. Zika Virus-Associated Neurological Disease in the Adult: Guillain-Barré Syndrome, Encephalitis, and Myelitis. *Seminars in reproductive medicine*, 34(5), pp. 273-279.

Noronha, L. de *et al.*, 2016. Zika virus damages the human placental barrier and presents marked fetal neurotropism. *Memórias do Instituto Oswaldo Cruz*, 111(5), pp. 287-293.

Oehler, E. *et al.*, 2015. Increase in cases of Guillain-Barré syndrome during a Chikungunya outbreak, French Polynesia, 2014 to 2015. *Eurosurveillance*, 20(48), pp. 30079-2.

Ralapanawa, D.M.P.U.K., Kularatne, S.A.M. & Jayalath, W.A.T.A., 2017. Guillain-Barre syndrome following dengue fever and literature review. *BMC Research Notes*, pp. 1-5.

Ravi, V. *et al.*, 1994. Association of Japanese encephalitis virus infection with Guillain-Barré syndrome in endemic areas of south India. *Acta neurologica Scandinavica*, 90(1), pp. 67-72.

Retallack, H. *et al.*, 2016. Zika virus cell tropism in the developing human brain and inhibition by azithromycin. *Proceedings of the National Academy of Sciences*, 113(50), pp. 14408-14413.

Richard, A.S. *et al.*, 2017. AXL-dependent infection of human fetal endothelial cells distinguishes Zika virus from other pathogenic flaviviruses. *Proceedings of the National Academy of Sciences of the United States of America*, 114(8), pp. 2024-2029.

Santos, Dos, T. *et al.*, 2016. Zika Virus and the Guillain-Barré Syndrome - Case Series from Seven Countries. *The New England journal of medicine*, 375(16), pp. 1598-1601.

Simonin, Y. *et al.*, 2016. Zika Virus Strains Potentially Display Different Infectious

Profiles in Human Neural Cells. *EBioMedicine*, 12, pp. 161-169.

Smit, J. *et al.*, 2011. Flavivirus Cell Entry and Membrane Fusion. *Viruses*, 3(12), pp. 160-171.

Sulekha, C., Kumar, S. & Philip, J., 2004. Guillain-Barre syndrome following dengue fever. *Indian pediatrics*, 41(9), pp. 948-950.

Truong, L. *et al.*, 2016. Assessment of the developmental and neurotoxicity of the mosquito control larvicide, pyriproxyfen, using embryonic zebrafish. *Environmental pollution (Barking, Essex: 1987)*, 218, pp. 1089-1093.

Wang, L. *et al.*, 2016. From Mosquitos to Humans: Genetic Evolution of Zika Virus. *Cell Host and Microbe*, 19(5), pp. 561-565.

Wang, Z.-Y. *et al.*, 2017. Axl is not an indispensable factor for Zika virus infection in mice. *Journal of General Virology*, 98(8), pp. 2061-2068.

Wells, M.F. *et al.*, 2016. Genetic Ablation of AXL Does Not Protect Human Neural Progenitor Cells and Cerebral Organoids from Zika Virus Infection. *Stem Cell*, 19(6), pp. 703-708.

Yuan, L. *et al.*, 2017. A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Pan American Health Organization / World Health Organization*.

Biomedicine Session

Pentose Phosphate Pathway Dependent Inflammation: Key Factor in Glucose Associated Vascular Damage

Carlos Sánchez Ferrer,[1] Concepción Peiró[2] and Salvador Moncada[3]

Introduction

Diabetic vasculopathy is considered a chronic low-grade inflammatory disease (Biondi-Zoccai *et al.*, 2003). Enhanced circulating concentrations of pro-inflammatory cytokines, such as interleukin 1β (IL1β) or tumor necrosis factor α (TNFα), have been found both in patients with type 1 and type 2 diabetes (Pickup *et al.*, 2000; Erbagci *et al.*, 2001).

Hyperglycaemia is recognized as an independent risk factor for cardiovascular disease. It has been proposed that high glucose *per se* induces oxidative stress and inflammation that results in vascular damage, due to an excess entry of glucose into the cell leading to the over-production of superoxide anions in the mitochondria (Giacco and Brownlee, 2010). However, clinical trials have shown that strict control of glycaemia leads only to a modest reduction in macrovascular diabetic complications (Hayward *et al.*, 2015). Thus, the link between hyperglycaemia and atherosclerosis is still not fully understood (Chait and Bornfeldt, 2009), and the role of mitochondria-generated superoxide as the culprit of diabetes induced vascular damage is now questioned (Sharma, 2015).

High glucose per se does not induce inflammation in human vascular cells but potentiates the inflammatory responses evoked by cytokines

We have demonstrated that exposure to high concentrations of glucose does not cause inflammation in human vascular cells unless they are primed with an inflammatory stimulus such as IL1β or TNFα (Lafuente *et al.*, 2008; Azcutia *et al.*, 2010). Thus, increasing glucose up to 22 mmol/l,

[1] Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, 29029 Madrid, Spain.

[2] Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, 29029 Madrid, Spain.

[3] Manchester Cancer Research Centre, University of Manchester, Wilmslow Road, Manchester M20 4QL, UK.

which is twice the plasma concentration considered to be indicative of diabetes (11.1 mmol/l) was unable to modify iNOS gene expression, protein concentration, or activity (Lafuente *et al.*, 2008), as well as the expression of adhesion molecules or the leukocyte adhesion to endothelial monolayers (Azcutia *et al.*, 2010).

Although high glucose alone did not have any effect on the parameters measured, it significantly enhanced the effects of cytokines. Thus, in human vascular smooth muscle and endothelium, respectively, the effects of IL1α on the induction of the inducible isoform of nitric oxide synthase (iNOS) or the expression of the adhesion molecules ICAM-1 and VCAM-1 were significantly enhanced in the presence of 22 mmol/l glucose by a mechanism involving an increased activity of the nuclear factor κB (NFκB) (Lafuente *et al.*, 2008; Azcutia *et al.*, 2010). We therefore hypothesized that a pro-inflammatory pre-conditioning was required to allow the entry of glucose in high concentrations to exert its possible deleterious effects.

IL1β facilitates glucose entry in vascular smooth muscle cells

Interestingly, elevation of extracellular glucose did not result in increased uptake by human vascular smooth muscle cells (Peiró *et al.*, 2016). Indeed, non-inflamed cells exhibit a glycolytic profile in line with previous findings (Suzuki *et al.*, 2001), with no changes observed in glucose consumption and lactate generation when the extracellular glucose was elevated. This can be explained by the saturation of its transport; the glucose transporter GLUT1, predominant in vascular cells, has a high affinity and low capacity (K_m of 1 to 7 mmol/L), operating near its maximal capacity at physiological concentrations of glucose (Mann *et al.*, 2003).

However, treating vascular cells with IL1β increased the transport of glucose in a concentration-dependent manner (Peiró *et al.*, 2016). The enhanced glucose uptake was associated with newly synthesized GLUT1 and increased number of transporters in the cell membrane, similar to what occurs in immune cells following inflammatory activation (MacIver *et al.*, 2006). There was, in addition, an increase in both the consumption of glucose and the generation of lactate, which was proportional to the concentration of glucose in the extracellular medium (Peiró *et al.*, 2016). Therefore, the cytokine was required to achieve extra glucose entry and consumption, which was proportional to the extracellular concentration of glucose (Figure 1).

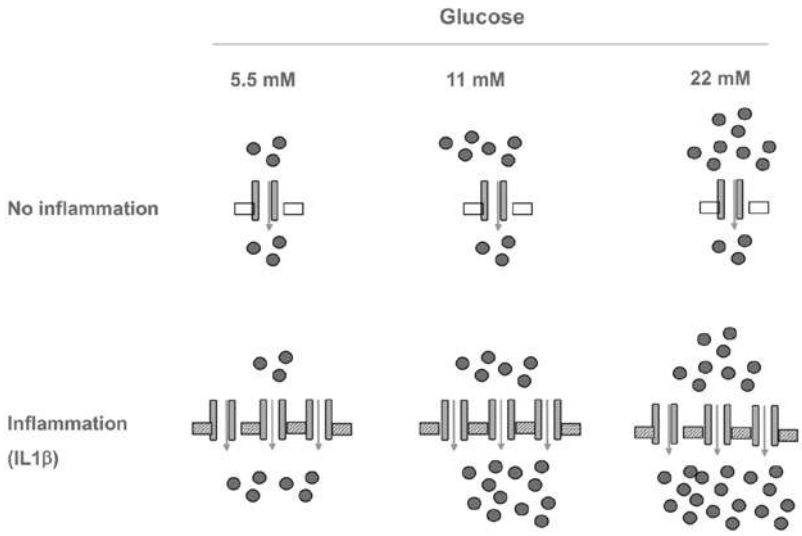


Figure 1. Inflammation of vascular cells results in enhanced GLUT1 expression and glucose uptake.

Increase of glucose entry is not able by itself to induce vascular cell inflammation

The next step was to investigate whether the increase of intracellular glucose was sufficient to induce inflammation. We found that over-expressing functional GLUT1 did not lead to high glucose consumption or inflammation (Peiró *et al.*, 2016). Interestingly, in animals genetically modified to over-express GLUT1 in vascular smooth muscle, inflammatory responses were only observed when vascular damage was superimposed (Adhikari *et al.*, 2011). On the other hand, blockade of mitochondrial respiration by sodium azide enhanced glucose consumption and lactate production to a greater degree than the activation with IL1β (Peiró *et al.*, 2016). This is because the inhibition of mitochondrial respiration is able to increase glucose transport through the translocation of pre-existing GLUT1 to the cell membrane (Behrooz and Ismail-Beigi, 2009). Despite this enhanced glucose consumption, no evidence of glucose-dependent inflammation was observed (Peiró *et al.*, 2016). Therefore, the mere increase of glucose consumption and glycolytic metabolism is not in itself sufficient to trigger inflammation in vascular cells.

IL1β modifies the intracellular metabolic profile of glucose, activating the pentose phosphate pathway (PPP)

We then investigated whether, in addition to promoting glucose entry, the stimulation with IL1b could modify the metabolism of glucose in vascular cells. We observed that the glucose metabolized via the tricarboxylic acid cycle was only slightly increased by the cytokine treatment (Peiró *et al.*, 2016), independently of the extracellular glucose concentration. This argues against the hypothesis suggesting a main role for mitochondria-derived superoxide in the glucose-induced vascular damage (Giacco and Brownlee, 2010). In fact, we found that the PPP is the main route through which high glucose exacerbated inflammation in vascular cells. Specifically, high glucose concentrations led to higher expression of glucose-6-phosphate dehydrogenase (G6PD) and augmented PPP activity, but only in cells activated with IL1β (Peiró *et al.*, 2016). Thus, the cytokine was also required for the diversion of the excess intracellular glucose through this metabolic pathway. Indeed, previous results in vascular cells suggest that the PPP activity, which is low in basal conditions, can be activated during inflammation (Suzuki *et al.*, 2001).

IL1β and high glucose synergize to over-activate NADPH oxidase

The PPP is a main source for NADPH, which can be used as a cofactor of gluthatione reductase for the regeneration of reduced gluthatione (GSH) or as a substrate for NADPH oxidase for the release of free radicals (Figure 2). This pro-oxidant enzyme has been suggested to play a key role in diabetes-associated atherosclerosis (Gray *et al.*, 2013). In our cells, treatment with IL1b activated NADPH oxidase, which was necessary for the induction of inflammation via the production of reactive oxygen species (Peiró *et al.*, 2016). Simultaneously, IL1b increased also GSH content, most probably as a compensatory mechanism against enhanced free radical formation in inflammation (Urata *et al.*, 1996). In the presence of high glucose and PPP over-activation, however, NADPH oxidase activity by IL1b was further increased while the protective GSH was diminished (Peiró *et al.*, 2016). NADPH oxidase requires higher concentrations of NADPH to be active, as the Michaelis constant for this enzyme is 5-fold higher than for glutathione reductase (Matsui *et al.*, 2005). Thus, the over-activation of the PPP would increase the utilization of NADPH by NADPH oxidase and the excess of free radical generation would further contribute to exhausting GSH.

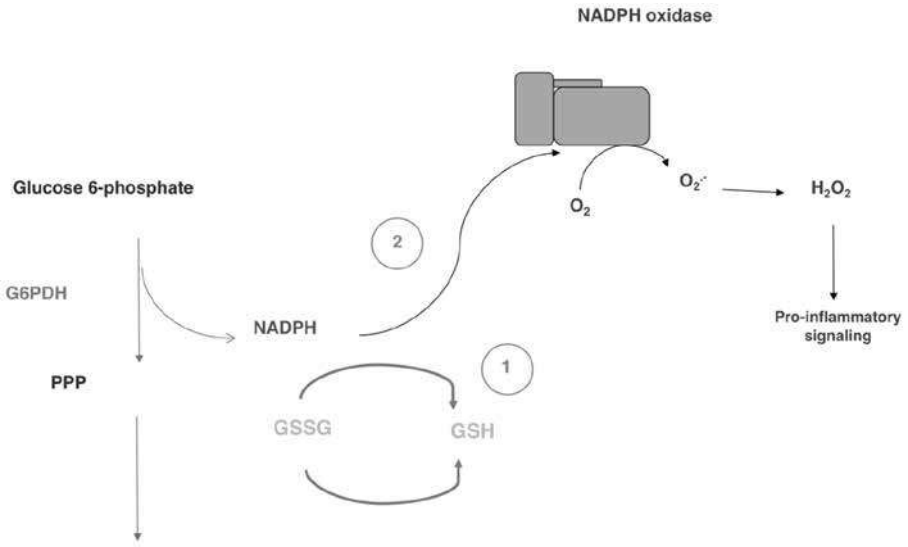


Figure 2. Enhanced PPP activity and NADPH production lead to GSH production (1) and/or to alternative NADPH oxidase activation (2).

Other studies have reported in cardiovascular cells a link between the PPP and NADPH oxidase activation in the context of hyperglycemia and diabetes (Serpillon *et al.*, 2009; Yamashita *et al.*, 2014), our data clearly singled out the role of this pathway as responsible for the exacerbated inflammation and vascular dysfunction induced by high glucose. Thus, silencing G6PD in abrogated not only the over-activation of NADPH oxidase but also the subsequent exacerbated inflammation. This may help to explain a number of other experimental and clinical observations. It has been known for many years that diabetic patients have a decreased tissue concentration of GSH (Seltzer, 1957), while defects in GSH-dependent antioxidant enzymatic activity have been related to diabetes-associated atherosclerosis (Lewis *et al.*, 2007). Moreover, a lower susceptibility to cardiovascular disease has been described in patients with G6PD deficiency (Cocco *et al.*, 1998; Meloni *et al.*, 2008), while mice with genetic deficiency in G6PD are protected against atherosclerosis (Matsui *et al.*, 2006). From a translational approach, it is likely that anti-inflammatory treatment, as adjunct to glucose control, will prove of benefit for the prevention or treatment of

cardiovascular complications in diabetes. This has been suggested by the use of the IL-1 receptor antagonist anakinra in an animal model of diabetes (Vallejo *et al.*, 2014) and, more recently, by the clinical demonstration that administration of the anti-IL1b monoclonal antibody Canakinumab to patients at risk of having a cardiovascular event, lowers the rate of recurrence independent of lipid lowering. (Ridker *et al.*, 2017).

Conclusions

Therefore, the over-activation of the PPP is a crucial mechanism by which high glucose exacerbates vascular cell damage. Activation of vascular cells by pro-inflammatory cytokines allows excess glucose to enter the cell and be redirected via this metabolic route, creating a situation in which free radical formation exceeds the capacity of the cell to regenerate GSH (Figure 3). This pro-oxidant environment increases vascular inflammation and as result, induced the vascular damage associated to hyperglycaemia. Furthermore, from a therapeutic point of view, our results emphasize the need not only to control glycaemia but also to reduce inflammation in order to prevent the harmful effect of high glucose in vascular cells.

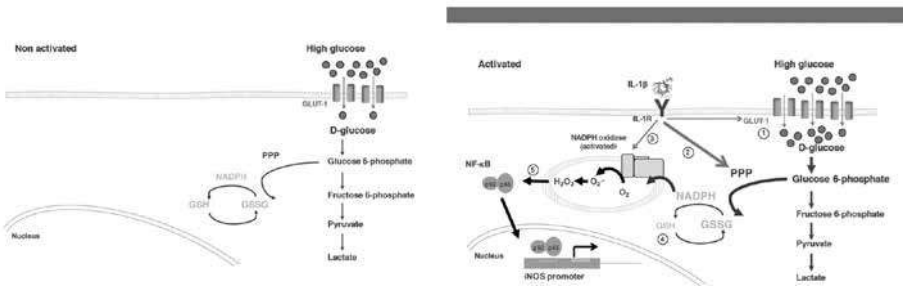


Figure 3. Mechanism by which high glucose potentiates inflammation in vascular cells. In the absence of inflammation (Top), glucose uptake is limited and the PPP activity is directed towards GSH formation. The activation by the cytokine (Bottom) results in the entry of excess glucose via GLUT1 transporters that is derived to the PPP; the enhanced NADPH production fuels NADPH oxidase activity, leading to further oxidation and inflammation.

Bibliography

Adhikari, N., Basi, D.L., Carlson, M., Mariash, A., Hong, Z., Lehman, U., Mullegama, S., Weir, E.K., Hall, J.L. (2011) Increase in GLUT1 in smooth muscle alters vascular contractility and increases inflammation in response to vascular injury. *Arterioscler. Thromb. Vasc. Biol.* 31, 86-94.

Azcutia, V., Abu-Taha, M., Romacho, T., Vázquez-Bella, M., Matesanz, N., Luscinskas, F.W., Rodríguez-Mañas, L., Sanz, M.J., Sánchez-Ferrer, C.F., Peiró, C. (2010) Inflammation determines the pro-adhesive properties of high extracellular D-glucose in human endothelial cells *in vitro* and rat microvessels *in vivo*. *PLoS ONE*. 5:e10091.

Behrooz, A. and Ismail-Beigi, F. (1999) Stimulation of glucose transport by hypoxia: signals and mechanisms. *News Physiol. Sci*. 14, 105-110.

Biondi-Zoccai, G.G., Abbate, A., Liuzzo, G., and Biasucci, L.M. (2003). Atherothrombosis, inflammation, and diabetes. *J. Am. Coll. Cardiol*. 41,1071-1077.

Chait, A., Bornfeldt, K.E. (2009) Diabetes and atherosclerosis: is there a role for hyperglycemia? *J. Lipid Res*. 50, Suppl: S335-S339.

Cocco, P., Todde, P., Fornera, S., Manca, M.B., Manca, P., Sias, A.R. (1998) Mortality in a cohort of men expressing the glucose-6-phosphate dehydrogenase deficiency. *Blood*. 91,706-709.

Erbagci, A.B., Tarakcioglu, M., Coskun, Y., Sivasli, E., Sibel Namiduru, E. (2001) Mediators of inflammation in children with type I diabetes mellitus: cytokines in type I diabetic children. *Clin Biochem*. 34, 645-650.

Giacco, F., Brownlee, M. (2010) Oxidative stress and diabetic complications. *Circ. Res.* 107,1058-1070.

Gray, S.P., Di Marco, E., Okabe, J., Szyndralewiez, C., Heitz, F., Montezano, A.C., de Haan, J.B., Koulis, C., El-Osta, A., Andrews, K.L., Chin-Dusting, J.P., Touyz, R.M., Wingler, K., Cooper, M.E., Schmidt, H.H., Jandeleit-Dahm, K.A. (2013) NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. *Circulation*. 127, 1888-902.

Hayward, R.A., Reaven, P.D., Wiitala, W.L., Bahn, G.D., Reda, D.J., Ge, L., McCarren, M., Duckworth, W.C., Emanuele, N.V. VADT Investigators. (2015) Follow-up of glycemic control and cardiovascular outcomes in type 2 diabetes. *N. Engl. J. Med*. 372, 2197-2206.

Lafuente, N., Matesanz, N., Azcutia, V., Romacho, T., Nevado, J., Rodríguez-Mañas, L., Moncada, S., Peiró, C., Sánchez-Ferrer, C.F. (2008) The deleterious effect of high concentrations of D-glucose requires pro-inflammatory preconditioning. *J. Hypertens*. 26, 478-485.

Lewis, P., Stefanovic, N., Pete, J., Calkin, A.C., Giunti, S., Thallas-Bonke, V., Jandeleit-Dahm, K.A., Allen, T.J., Kola, I., Cooper, M.E., de Haan, J.B. (2007) Lack of the antioxidant enzyme glutathione peroxidase-1 accelerates atherosclerosis in diabetic apolipoprotein E-deficient mice. *Circulation*.115, 2178-2187.

MacIver, N.J., Jacobs, S.R., Wieman, H.L., Wofford, J.A., Coloff, J.L., Rathmell, J.C. (2006) Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. *J. Leukoc. Biol*. 84, 949-957.

Mann, G.E., Yudilevich, D.L., Sobrevia, L. (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol. Rev*. 83, 183-252.

Matsui, R., Xu, S., Maitland, K.A., Hayes, A., Leopold, J.A., Handy, D.E., Loscalzo, J., Cohen, R.A. (2005) Glucose-6 phosphate dehydrogenase deficiency decreases the vascular response to angiotensin II. *Circulation*. 112, 257-263.

Matsui, R., Xu, S., Maitland, K.A., Mastroianni, R., Leopold, J.A., Handy, D.E., Loscalzo, J., Cohen, R.A. (2006) Glucose-6-phosphate dehydrogenase deficiency decreases vascular superoxide and atherosclerotic lesions in apolipoprotein E(-/-) mice. *Arterioscler. Thromb. Vasc. Biol*. 26, 910-916.

Meloni, L., Manca, M.R., Loddo, I.,

Cioglia, G., Cocco, P., Schwartz, A., Muntoni, S., Muntoni, S. (2008) Glucose-6-phosphate dehydrogenase deficiency protects against coronary heart disease. *J. Inherit. Metab. Dis.* 31, 412-417.

Peiró, C., Romacho, T., Azcutia, V., Villalobos, L., Fernández, E., Bolaños, J.P., Moncada, S., Sánchez-Ferrer, C.F. (2016) Inflammation, glucose, and vascular cell damage: the role of the pentose phosphate pathway. *Cardiovasc. Diabetol.* 1, 15, 82.

Pickup, J.C., Chusney, G.D., Thomas, S.M., Burt, D (2000). Plasma interleukin-6, tumour necrosis factor alpha and blood cytokine production in type 2 diabetes. *Life Sci.* 67, 291-300.

Ridker, P.M., Everett, B.M., Thuren, T., MacFadyen, J.G., Chang, WH., Ballantyne, C., Fonseca, F., Nicolau, J., Koenig, W., Anker, S.D., Kastelein, J.J.P., Cornel, J.H., Pais, P., Pella, D., Genest, J., Cifkova, R., Lorenzatti, A., Forster, T., Kobalava, Z., Vida-Simiti, L., Flather, M., Shimokawa, H., Ogawa, H., Dellborg, M., Rossi, P.R.F., Troquay, R.P.T., Libby, P., Glynn, R.J. CANTOS Trial Group. (2017) Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N. Engl. J, Med.* 377, 1119-1131.

Seltzer, H.S. (1957) Blood glutathione in mild diabetes mellitus before treatment and during sulfonylurea-induced hypoglycemia. *Proc. Soc. Exp. Biol. Med.* 95, 74-76.

Serpillon, S., Floyd, B.C., Gupte, R.S., George, S., Kozicky, M., Neito, V., Recchia, F., Stanley, W., Wolin, M.S., Gupte, S.A. (2009) Superoxide production by NAD(P)H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac dysfunction. The role of glucose-6-phosphate dehydrogenase-derived NADPH. *Am. J. Physiol. Heart Circ. Physiol.* 297, H153-H162.

Sharma, K. (2015) Mitochondrial hormesis and diabetic complications. *Diabetes*. 64, 663-672.

Suzuki, L.A., Poot, M., Gerrity, R.G., Bornfeldt, K.E. (2001) Diabetes accelerates smooth muscle accumulation in lesions of atherosclerosis: lack of direct growth-promoting effects of high glucose levels. *Diabetes*. 50, 851-860.

Urata, Y., Yamamoto, H., Goto, S., Tsushima, H., Akazawa, S., Yamashita, S., Nagataki, S., Kondo, T. (1996) Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1beta and tumor necrosis factor-alpha in mouse endothelial cells. *J. Biol. Chem*. 271, 15146-15152.

Vallejo, S., Palacios, E., Romacho, T., Villalobos, L., Peiró, C., Sánchez-Ferrer, C.F. (2014) The interleukin-1 receptor antagonist anakinra improves endothelial dysfunction in streptozotocin-induced diabetic rats. *Cardiovasc. Diabetol.* 13, 158.

Yamashita, A., Zhao, Y., Matsuura, Y., Yamasaki, K., Moriguchi-Goto, S., Sugita, C., Iwakiri, T., Okuyama, N., Koshimoto, C., Kawai, K., Tamaki, N., Zhao, S., Kuge, Y., Asada, Y. (2014) Increased metabolite levels of glycolysis and pentose phosphate pathway in rabbit atherosclerotic arteries and hypoxic macrophage. *PLoS One*. 9, e86426.

Cerebrovascular Disease: The Emerging Role of Genomics

Conrado J. Estol

Burden of vascular disease

Vascular disease accounts for almost 30% of approximately 160,000 deaths occurring worldwide every day. Coronary artery disease (CAD) and cerebrovascular disease (stroke) are the main manifestations and represent the first and second cause of death respectively.[1]

In addition, stroke is the first cause of disability and a leading cause of dementia. During recent years in China and other regions in Asia, vascular disease has overcome infectious disease (HIV, malaria, TB) as the main cause of death. This change has been associated to an occidentalization of the local culture where for the first time there is a high proportion of obese children. It is of concern that recent data shows an increase in the incidence of both CAD and stroke including countries in which a decline had been noted in previous years. An increase in life expectancy, poor control of vascular risk factors and inadequate lifestyle habits are the culprits of these negative changes. Cerebrovascular disease affects people of all ages and although a greater proportion of cases occur after the sixth decade, 25% of events affect younger patients and even adolescents. The perception that stroke is an "old age" disease may have contributed to its being somewhat delayed in the field of genetics. Although lagging behind discoveries in cardiology, major advances have occurred in genomic stroke research over the last few years.

Cerebrovascular disease in emerging regions

Approximately 15 million strokes occur worldwide every year (a proportion are recurrent strokes) and 6 million cause death. Of all strokes, 85% occur in emerging regions where stroke incidence has increased 100% over the last decade.[2, 3, 4]

Prevalence is lower compared to high income countries because the fatality rate is higher and stroke occurs one decade earlier. When silent strokes are counted, these events are more frequent than myocardial infarctions. The risk of a recurrent stroke after a first episode varies between 8 to 12% in the first year. Stroke is the cerebral expression of the same arterial

disease affecting the aorta and the coronary, renal and leg arteries therefore stroke patients are at high risk of general vascular death. In fact, cerebrovascular disease shares similar vascular risk factors, which occur with moderate variations throughout all race/ethnicities worldwide.[5, 6]

Missed effects of prevention

If only what is known about risk factor control was widely applied, the incidence of stroke could decrease up to 80%.[7, 8, 9, 10]

Most stroke risk factors are modifiable. The main risk factors responsible for the disease include smoking, sedentary lifestyle, inadequate nutrition, hypertension, hypercholesterolemia and diabetes. Unfortunately, and due to a variety of reasons, we are far from reducing cerebrovascular disease and in fact, an increase in cases has been recently observed.

Pathophysiology (mechanisms) of cerebrovascular disease

Stroke is a heterogeneous disease compared to CAD. Stroke has two major forms represented by ischemia and hemorrhage. Ischemic stroke is caused by occlusion of small and large vessels secondary to focal atherosclerosis or from cardio, aortic or artery to artery embolism. Hemorrhagic stroke is due to hypertensive hemorrhages and to bleeding from vascular malformations. In the 80s Caplan published an editorial entitled "What is wrong with Mr Jones", alluding to the fact that lumping cerebrovascular disease in one large group would never allow the appropriate treatment of the underlying etiology.[11]

Caplan promoted a "splitter" diagnostic effort to correctly assign a subtype mechanism (hemodynamic versus embolic) and etiology (small and large vessel, cardioembolic, dissection, angeitis, etc). To complicate things further, small vessel disease is different when diffusely affecting the basal ganglia and peri-ventricular hemispheric regions (so called leucoaraeosis) and when causing the typical lacunar infarction at the thalami or internal capsule and those that occur in the brainstem. Small vessel disease develops as lipohyalinosis that progressively occludes vessels smaller than 1 mm in diameter. However, some vessels progress to occlusion manifesting as lacunar infarctions but others develop small dilatations, namely Charcot-Bouchard aneurysms that rupture causing hypertensive intracerebral hemorrhages. What defines the evolution towards ischemic and hemorrhagic manifestations is as yet unknown. These are all different manifestations of small vessel disease as large artery internal carotid stenosis is probably different from middle cerebral artery (also a "large" vessel) stenosis. Cerebral blood vessels

have a somewhat different anatomy when compared to their extracranial counterparts. They lack the external elastic lamina, the muscular layer is thinner (as is the adventitia) and cellular gaps at the internal elastic lamina are larger. All the aforementioned characteristics explain in part why stroke is a complex and heterogeneous disease with multiple different subgroups making it more difficult as a candidate to identify DNA variations responsible for stroke in general.

Clinical tools for the diagnosis and treatment of stroke

When government policies are implemented to treat large numbers of people, then a population-based approach could identify a significant number of individuals at risk based on common vascular scores such as the popular Framingham risk score and others. People that smoke, are overweight, have high blood pressure, diabetes and high cholesterol fulfill score values indicative of a high vascular risk.

However, some people at risk may not be identified with these score system and some considered at high vascular risk may not develop a stroke. This scenario prompted the search for individualized diagnostic tools that could identify more precisely those at high vascular risk. For this purpose, one of the most interesting concepts developed over the last few years is that of accelerated vascular aging. A Doppler technique was developed to measure atherosclerotic plaque load in the carotid artery, which has been recently validated as a reliable indicator of vascular risk.[12]

People in the highest quartile of plaque load had significantly more vascular events compared to other groups. This method offers the opportunity to quantitate carotid plaque area or volume which can be sequentially measured following treatment with statins and thus assess the progression or regression of the atherosclerotic plaque. Depending on age and the amount of plaque measured, a decision for treatment with statins will also be possible. We reported the experience at our Vascular Disease Prevention Clinic where up to 80% of patients had decisions for statin treatment made based on plaque measurement results.[13]

In the Bioimage study plaque load was compared with the coronary calcium risk score and was shown to be equally predictive but at a much lower cost and without the radiation exposure needed to complete the coronary multislice CT.[14]

Vascular rigidity measured with pulse wave velocity is another parameter indicative of arterial status or "vascular age". The greater vessel stiffness is, the more severe the damage noted in the cerebral microcirculation.[15]

Tailored and aggressive medical treatment of the artery can reverse the atherosclerotic process in almost 90% of patients evaluated, and non responders can be identified to include them in specific treatment groups or clinical trials.[16]

Despite major advances in stroke prevention applying all the above knowledge, a greater treatment and prevention effect could be achieved if the individual predisposition for infarction, hemorrhage or drug response was determined. Interestingly, over the last few years many studies on molecular genetics have opened this new window of opportunity under the term Precision Medicine.

Precision medicine

In January of 2015 President Obama first declared the goal to incorporate Precision medicine into routine medical practice. The NIH supported and followed this proposal with the Initiative Cohort Program to study and define the interactions between diseases and the personal genotype of the general population.

Although DNA structure has been known for decades, only very recently the availability of computers with a capacity to process large amounts of data has unraveled the different alterations in genes associated with various diseases. Next generation sequencers allow scanning the entire genome and exome to search for DNA variants that result in abnormal phenotypes.

The completely new field of genetic testing in vascular disease has unfolded over the last decade with a continued exponential growth. The oncology field led this new diagnostic armamentarium with the companion diagnostic biomarkers presently available for precise treatment choices in a large proportion of cancers. This allowed for precise selection of a drug or combination of drugs identified as effective against the specific molecular genetics of a given tumor. The knowledge of physicians in practice lags behind these notorious advances and will probably show a longer latency than that usually observed with novel tools. This occurs because understanding the opportunities offered by genetics require complex and extensive knowledge that most practicing physicians have not acquired during training.

Over the last 5 to 10 years large research efforts have yielded the finding of more than 10 million SNPs with a greater than 5% minor allele frequency (i.e. responsible for rather common diseases).

Genomic studies

Only recently it became possible to analyze greater than 100 thousand SNPs in a genome. The design of studies comparing SNPs of patients and controls without a specific hypothesis in common diseases with common DNA variants is known as genome wide association studies (GWAS). As the name implies, the main shortcoming of GWAS is that they show association rather than causal relations. These SNPs have been used in case-control association studies and led to the identification and study of a series of candidate genes. The advent of whole genome and exome sequencing opens an opportunity to study rare variants that are causal to a disease. Using the technique of Mendelian randomization adds a further step to support association in genomic studies.[17]

As an example, Mendelian randomization was used to prove the causal relation between homocysteine and stroke, which had the confounding of negative trials with vitamin B and folic acid which reduced homocysteine but failed to reduce vascular events. The methylenetetrahydrofolate reductase (MTHFR) genotype was studied in relation to homocysteine levels and stroke risk to confirm that events were related to changes in homocysteine induced by MTHFR activity. Stroke risk was found to be similar for different genotype groups and proportional to the effects of MTHFR on homocysteine levels.[18]

However, Mendelian inheritance of one determinant gene occurs in less than 7% of stroke patients, the majority of which follow a non Mendelian inheritance pattern.

Monogenic diseases

These are diseases with Mendelian inheritance of a single locus gene that has a major effect. A few cardiac diseases responsible for the most frequent causes of sudden death are clear examples of monogenic diseases. Hypertrophic cardiomyopathy represents the leading cause of death in the young, especially for those that are athletes or exercise intensively. More than 900 mutations that affect function of the sarcomere have been identified in 12 genes. Among other mutations causative of sudden death is the long QT syndrome which has variants related to death in boys during exercise (specially swimming), another during emotional or auditory stimuli and a third during sleep. Genetic variants predictive of arrhythmias provided new insight on the role of genetic testing.[19] Van Driest *et al.* studied rare Mendelian disease genes for Brugada syndrome and long QT syndrome in 2000 patients to observe for the occurrence of related clinical phenotypes.

Rare SCN5A and KCNH2 genes arrhythmia variants were found in 200 individuals.[20]

However there was no correlation with clinical arrhythmia phenotypes (symptoms and EKGs) suggesting precaution about informing patients of potentially lethal diseases when these variants are found in the general, unselected population. The latter is an excellent example of the overestimation of genetic penetrance when not all genotype carriers manifest the predicted phenotype. Research usually starts with a particular phenotype to then identify the causing genotype when it should be the other way around to determine what is the phenotypic presentation of a given genotype and how strong is the relationship between genotype and phenotype.

Some diseases follow Mendelian laws and are related to single genes with a large effect capable of causing a stroke syndrome. A paradigmatic example is CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopaty) with an alteration detected in the NOTCH 3 gene in chromosome 19 (with more than 170 mutations detected to date). CADASIL presents on average at age 50 in patients with a history of migraine with aura and multiple recurring strokes leading to dementia. There is no treatment for CADASIL but investigation of unaffected relatives may identify some that could benefit from genetic counseling. One study evaluated 8 stroke genes in patients that exclusively had small vessel pathology. The overall presence of disease causing mutations and CADASIL mutations were more frequent in patients compared to controls but were not significantly associated with stroke risk. The authors concluded that when syndromic and family features are not present, screening for Mendelian stroke genes among the general population with small vessel disease is unlikely to yield diagnostic utility.[21]

On the other hand, Fabry disease, an X linked lysosomal storage disorder, is due to a deficiency in the enzyme alfa galactosidase A secondary to a pathogenic mutation in the GLA gene. Fabry disease can now be treated with enzyme replacement therapy in a bimonthly intravenous treatment with agalsidase alfa and beta. Patients with Fabry disease have kidney and cutaneous alterations, painful peripheral neuropathy and strokes. Therapy has been effective for non-neurological manifestations but has not shown a clear effect on stroke prevention.[22]

A study in animals found one gene that was responsible for 80% of blood vessel collaterals.[23] This information, if confirmed in humans, could help decision making for reperfusion treatments with thrombolytics and thrombectomy devices.

Familial hypercholesterolemia

This severe pathology is underdiagnosed and represents a paradigmatic example of monogenic disease. Various gene mutations have been identified as causative including the LDLR (receptor), APOB, PCSK9, and LDLRAP1 (recessive) mutations. The LDLR heterozygous mutation occurs in 1/500 individuals and increases 100% the risk for early death.[24]

Brown Goldstein disease causes a very high LDL level strongly associated with a larger number of fatal vascular events that usually occur at a young age. Even for a similar given level of elevated LDL, patients carrying the mutation have a much higher risk of vascular events compared to patients without it.

Five statin therapy trials (ASCOT, JUPITER, WOSCOPS, CARDIA and BIOIMAGE) were evaluated selecting genetic risk groups. A polygenic risk score (PRS) using 57 DNA variants was used to compare patients in the top quintile of the PRS versus those without the risk. Analysis of all studies revealed an absolute vascular event risk reduction of 3.6% in the PRS group compared to 1.3% in those without the risk. In WOSCOPS, patients with the same LDL level had a relative risk reduction in vascular end points of 44% when positive for the PRS versus 24% in the group without genetic risk. Each increase in one standard deviation in the PRS was associated with a 10% increase in plaque burden. This probably explains the greater therapeutic effect of statins in those with a higher PRS.

Lipoprotein (a) is a molecule of low density lipoprotein linked to apolipoprotein (a). Various sequencing studies of hundreds of thousands SNPs in greater than ten thousand patients and controls have revealed that the LPA gene and that the copy number polymorphisms of the Kringle IV type II (KIV2) of the LPA gene very strongly correlated with CAD independent of lipoprotein (a) levels. This suggests that genetic testing could be more effective to determine vascular risk than plasma lipoprotein (a) levels which have shown variable correlation with cardiovascular disease in different clinical studies. A reason could be that the number of KIV2 repeats influences the size of the apolipoprotein (a) in the LDL particle resulting in smaller fractions that are more pathogenic than their larger counterparts.[25] Interestingly, in more than one of the lipid studies, it was found that the effect of statins was independent of LDL levels supporting the non-cholesterol related (i.e. pleiotropic) effects of statins to reduce cardiovascular events.[26]

Apolipoprotein E4 has been well known for its association with Alzheimer's disease, although many studies over the last decades have

shown that it is also associated with CAD. Further, it has been proven to decrease survival in patients with MI and this effect was decreased by statin treatment independent of lipid levels, despite the evidence supporting decreased statin effect in APOE4 patients.[27]

Statins are HMG CoA reductase inhibitors that also reduce products in the mevalonate pathway such as ubiquinone and other isoprenoids that cause muscular side effects. These patients (probably <5% which is a much smaller proportion than the patients who complain of muscle pain attributed to statin therapy) undergo ubiquinone (coenzyme Q10) depletion and could have signs of myopathy, rhabdomyolysis and myalgias. This subset of patients may benefit from coQ10 supplements or ezetimibe statin replacement treatment. A few studies have found a genetic susceptibility to statin induced myopathy. Various HMG CoA reductase SNPs have also been correlated with a lower statin efficacy. Genetic variations in the hepatic CYP 450 system explain 10 to 50% variations in statin efficacy. Various different CYP systems regulate the different statins.[28]

An SNP at the SLCO1B1 gene on chromosome 12 showed association with simvastatin myopathy. This gene regulates the hepatic uptake of statins and the effect of the genotype depends on whether statins are lipophilic (simvastatin, atorvastatin) which represent the most likely to cause myalgia and those that are hydrophilic (pravastatin and rosuvastatin) that are least likely to result in side effects. The recently approved PCSK9 inhibitors will present as effective replacement for statins in intolerant patients at high cardiovascular risk.[29], [30] PCKS9 increase plasma LDL by causing degradation of the LDLR (which captures plasma LDL into tissues). Loss of function PCSK9 mutations are associated with reduced cardiovascular mortality. However, statin therapy is associated with an increase in plasma PCSK9 yet, for unknown reasons, their use results in lower LDL levels.

Polygenic diseases

Polygenic diseases occur due to numerous modest effects of DNA variants in multiple genes. The effect of each individual variant is small (as opposed to the large effect of each variant – mutation – in monogenic diseases) and at least in part results from the interaction with environmental and lifestyle variables. The frequency of variants could be common (1 to 5%) or rare (<1%) leading to the common disease-common variant or common disease-rare variant alternatives. This knowledge has been applied to test association between these variants and different types of strokes in gene association studies. More than 4000 disease-associated SNPs have been

identified with these studies. This methodology has become very popular as reflected by 500 studies published in 2000 compared to 8500 in 2010.[31]

Because ischemic and hemorrhagic strokes are common but caused by many different etiologies with multiple genetic associations it is unlikely that present genotyping studies could identify a single DNA variant to explain the entire spectrum of cerebrovascular disease. If large GWAS are undertaken selecting specific stroke subtypes, causal variants are more likely to be found. Genome wide association studies include large numbers of affected individuals with a known phenotype and control groups without the disease. GWAS identify large number of single nucleotide polymorphisms that in general account for a small fraction of stroke. The question evaluated is whether there is an SNP difference between affected and healthy individuals. An array or chips with a selection of tens or hundreds of thousands of SNPs (instead of the millions existing in a genome) are tested. However, each SNP evaluated represents an independent test implying that a significance at $p<0.05$ results in hundreds or even thousands of potential findings by chance when, for example, a million SNPs are tested. For this reason, the Bonferroni correction statistical test is needed when samples of this size are evaluated.

In one study (METASTROKE collaboration) 12,000 affected individuals and 60,000 controls – all of European ancestry – were studied. The genes PITX2 and ZFHX3 were associated with cardioembolic stroke with an increase in risk of 20-40% (likely mediated by atrial fibrillation) and the locus 9p21 – association shown but no genome wide significance – and gene HDAC9 were associated with large vessel disease. A variant in the PKCR gene identified in Japanese and Chinese populations has been associated with small vessel disease. All these findings highlight the need to a priori identify and select the different stroke subtypes when performing genetic studies.[32]

No improvement in the capacity to discriminate high from low stroke risk using C statistics was found when considering classic vascular risk factors in the equation including the DNA variants. Nor was there a change in risk noted in patients with a strong family history of stroke. The latter implies that rare DNA variants (mutations?) with significant phenotype effects are not identified in the genomic risk scores. A few shortcomings of GWAS include that since the risk associated with SNPs is small, thousands of causal SNPs are needed to at least double the risk. Also, most genomic variant studies have been performed in individuals of European ancestry.

One study of the expression of 40 genes could differentiate large artery from cardioembolic stroke with a 95% sensitivity and specificity.[33]

Kathiseran *et al.* performed a polygenic SNPs CAD risk score in 55,000 patients from three different studies and correlated the findings with a lifestyle score (healthy or unhealthy). Patients with a high genetic risk score (i.e. those in the upper quintile) had a 91% higher CAD risk independent of lifestyle. Those with a healthy lifestyle had a lower vascular risk independent of the genetic risk. Among patients with a high genetic risk, those with favorable lifestyles had an almost 50% lower relative risk of coronary events. Benefit from a healthy lifestyle was observed at all levels of genetic risk but the maximal absolute reduction occurred among patients at the highest genetic risk.[34]

If gene expression profiles are identified, then a faster definition of stroke clinical subtypes and TIA patients will be possible, saving valuable time in the emergency room to decide the most effective treatment and increasing the patient's chances to have less severe sequelae. The GRECOS project identified 256 SNPs in 115 candidate genes. The population studied was 1492 patients with a 6% stroke recurrence in the first year. The rs1800801 of the MGP gene related to arterial calcification resulted in a hazard ratio of 1.33. The genetic score of those patients carrying the variant re-classified stroke risk from low at 1.9% to a high recurrence risk at 12.6%.[35]

In another study, one copy of the risk allele at chromosome 9p21 increases CAD 20 to 40% reaching a 66% risk increase for patients in the top quintile.[36] At least one study has shown that the number of 9p21 locus risk alleles also correlated with the occurrence of intracranial aneurysms.

Identifying the variants associated with the various types of cerebral aneurysms (berry, saccular, giant) could help identify most patients before their symptomatic stage.[37] Many other chromosomal loci and genes have been associated with intracranial aneurysms as well with cavernous malformations.

A study of polymorphisms in patients with MI that developed ventricular fibrillation identified the locus 21q21 (CXADR) which increased almost 200% the risk for this arrhythmia.[38]

Pharmacogenomics

Although the term "pharmacogenetics" was coined in the late 50s, this field has only shown an order of magnitude growth recently. Many drugs used to treat cardiovascular disease are difficult to adjust to a therapeutic range (warfarin, acenocumarol), do not exert the expected effect (be-

ta-blockers, aspirin, clopidogrel) or are associated with toxic side effects (statins, antiplatelets). All these manifestations depend on DNA variants that have been identified in multiple GWAS analysis.

More than 300 drugs have been studies and DNA variants found that enhance or diminish their therapeutic or side effects. A relevant example is clopidogrel, an antiplatelet medication used after stent placement, and is regulated by the hepatic cytochrome enzyme system which generates a 10 to 15% active metabolite. Many drugs, such as omeprazole, compete for this pathway and could result in altered active form production. Some patients carry a reduced or loss of hepatic function CYP2C19 DNA variant (of 25 variant alleles described) resulting in the lack of production of the active clopidogrel metabolite and thus substantially increasing (up to 3 times) the risk of stent occlusion that is associated with myocardial infarction or death.[39, 40]

Since up to 25% of patients could be clopidogrel resistant, the high mortality that affects almost 50% of patients with stent thrombosis justifies genetic evaluation to select alternate anti-thrombotic treatments. There is also a gain of function variant of CYP2C19 that doubles the risk of bleeding associated with clopidogrel use. Although questioned by the AHA, in 2010 the FDA added a "boxed warning" for clopidogrel and patients identified as poor metabolizers.

Aspirin resistance is likely mediated by multiple COX-1 and COX-2 SNPs as well as in the platelet receptors P2Y1 and P2Y12 genes. Unfortunately there is no reliable in vitro test to determine aspirin resistance.

Another example among frequently used vascular drugs, is the anticoagulant warfarin. Despite the advent of novel oral anticoagulants that do not require laboratory-based dose adjustments, warfarin (and acenocumarol) continues to be the most widely prescribed anticoagulant. However, a large proportion of patients require many days to reach the therapeutic range and even with appropriate intake adherence they fluctuate between sub and supra-therapeutic anticoagulant range, increasing their risk for thrombotic and hemorrhagic complications. A diet rich in vitamin K (green leaf and other vegetables) accounts for part of the variation in treatment response. At least 25 genes have been identified in relation to warfarin metabolism. However, the VKORC1 and CYP2C9 genes have been shown to explain most of the variability in therapeutic range of many patients in whom no other explanation is found. A CYP2C9 variant explains why some patients require a significantly longer time to achieve a therapeutic range. The VKORC1 gene in chromosome 16p11.2 encodes the Vitamin K epoxide reductase enzyme affected by warfarin.[41]

The widely used beta blockers metoprolol and carvedilol also have significant variations in efficacy induced by the CYP2D6 polymorphism that result in poor or ultra-rapid metabolizers.

Molecular tests that provide genetic information have become the most popular studied defined as "companion diagnostics" although determination of proteins, metabolites and other tracers also fulfill the criteria. The first successful example of companion diagnostics occurred in the oncology field where 40% of drugs are now prescribed in the form of companion diagnostics for targeted therapy. The applications have extended to at least 100 drugs in various medical specialties and are expected to continue growing. The development of companion diagnostics will decrease clinical trial time and expense, will hasten approval procedures by regulators also ensuring less side effects and greater therapeutic efficacy and will decrease payers cost by avoiding unnecessary treatments.[42]

Genomics of the fibrous plaque

Carotid large artery disease is defined as the causal pathology of stroke in approximately 25% of patients. However, artery to artery embolism from non-occlusive carotid artery plaques could be responsible for a sizable proportion of the so-called cryptogenic strokes (i.e. stroke of unknown cause). This carotid plaque burden can nowadays be quantitatively measured and represents a valid phenotype of carotid artery disease. Other carotid artery phenotypes such as ulcers, dissections and intima media thickness have a more complex relation with stroke occurrence and are thus less likely to yield positive results to define the risk associated with carotid artery pathology. Large scale GWAS studies identified 2 sites close to the PIK3CG and EDNRA genes both associated with CAD, arterial plaque and carotid intima media thickness.[43]

The OCT4 gene has a significant influence in plaque vulnerability through various mechanisms. It induces programming somatic cells into pluripotential stem cells, it causes a decrease in the plaque's necrotic core, a reduction of intraplaque hemorrhage and the conversion of smooth muscle cells into a thicker fibrous cap, all of which make the arterial plaque less vulnerable.[44]

Variants were also identified in the ALOX5 and ALOX5AP genes resulting in greater plaque load and instability, with this effect being reversed with a diet high in omega 3 fatty acids.[45]

A new association with large artery stroke was found for the HDAC9 gene in the 7p21.1 chromosome with a potential for treatment with HDCA9 inhibitors.[46]

RNA expression analysis offers a unique opportunity to identify those plaques that are not highly stenotic but vulnerable from those that could cause greater stenosis but will remain clinically stable.

In 1995 a seminal article described the arterial "vulnerable plaque". It made reference to plaque behavior in the coronary arteries. The plaque has a complex matrix with the participation of numerous cell types, oxidative products, neovascularization, metalloproteases, cholesterol, calcium and necrosis in different proportions. The MGP gene correlates with the degree of artery calcification and the risk of stroke recurrence. The Toll-like Receptor 4 (TLR4), an immune response stimulator, has been detected in atherosclerotic plaques mediating local inflammation.[47] It is clear now that the process reflects immunologically-mediated inflammation and a recent study (CANTOS) for the first time showed a decrease in vascular end points following treatment of vascular patients with monoclonal antibodies.[48] This plaque is covered by a fibrous cap under different circumstances can rupture inducing a thrombotic cascade with occlusion of the affected vessel. However, the process mostly occurs in non stenosing plaques that cover the vessel's wall and thus cannot be detected with cardiac stress tests (exercise test) or with usual Doppler methods. The relevance of this mechanism is that close to 90% of all cardiac and cerebral infarcts occur in previously non stenotic plaques that rupture. Defining which plaques are more "vulnerable" could represent a major step in vascular disease prevention. Future genetic studies should be design with this important aim.

Future studies of carotid endarterectomy specimens should search for gene expression signatures directly in the tissue to unravel the mechanisms of vulnerable plaque manifestation.

The near future

There are now at least 70,000 new bioinformatics software tools available in the web. The number of such programs exponentially grew from 30 new ones per week in 2013 to 200 per week in 2016. Five million researchers worldwide cannot handle the amount of information available if not processed in large software platforms already available. This market is expected to have a 20% mean annual growth rate from US$ 8.6 billion in 2015 to 38.6 billion in 2024 (Credence Research). The amount of data generated is so big that data analysis and interpretation is the most likely limiting step in the advancement of genomic understanding.

Medical education covering all phases of genetics should be rapidly undertaken and is already overdue. Physicians should acquire a some-

what more than basic knowledge on the terminology and meaning of the genomics and related omics (transcriptomics, proteinomics, metabolomics) fields. They should be able to select the most appropriate testing for their patient and also to interpret results sorting relevant from non-relevant information. The capacity to interpret genomic study and trial results, to adequately select the study needed in a given patient, to interpret the multiple mutations of DNA variant results and to appropriately advice patients, will become the cornerstone of medical practice in an almost present future.

Genetic testing will allow practice of the most individualized (precise) medicine. This approach demands the definition of clear phenotypic subgroups, the identification of cerebrovascular disease mechanisms and different etiologies and a thorough evaluation of all family members. The completion of large-scale whole genome sequencing studies in the next few years will unravel the presently missing DNA alterations that define the entire spectrum of cerebrovascular disease.[49]

References

1. Mozaffarian D, Benjamin EJ, Go AS, *et al*. Heart disease and stroke statistics. 2016 update. A report from the AHA. *Circulation* 2015; 138:e38-360.
2. Feigin VL. Stroke epidemiology in the developing world. *Lancet* 2005; 365(9478):2160-1.
3. Chokalingam A, Chalmers J, Lisheng L *et al*. Prevention of cardiovascular diseases in developing countries: agenda for action. *Journal of Hypertension* 2000; 18:1705-1708.
4. Strong K, Mathers C, Leeder S, and Beaglehole R. Preventing chronic diseases: how many lives can we save? *Lancet* 2005; 366: 1578-1582.
5. Yusuf S. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study). *Lancet* 2004; 364:937-952.
6. Feigin VL. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurology* 2003; 2(1):43-53.
7. Estol CJ. World Stroke Day, October 2007: Panamerican Congress of Neurology at Santo Domingo. *Int J Stroke* 2008; 3:145-149.
8. Hackam DG, Spence JD. Combining multiple approaches for the secondary prevention of vascular events after stroke: a quantitative modeling study. *Stroke* 2007;38(6):1881-5.
9. Joubert J, Reid C, Joubert L, Barton D, Ruth D, Jackson D, *et al*. Risk factor management and depression post stroke: the value of an integrated model of care. *Journal of Clinical Neuroscience* 2006; 13:84-90.
10. Chiuve SE, McCullough ML, Sacks FM, Rimm EB. Healthy lifestyle factors in the Primary prevention of coronary heart disease among men: benefits among users and nonusers of lipid lowering and antihypertensive medications. *Circulation* 2006, 114(2):160-167.
11. Caplan LR. We need to return to the question: What is wrong with Mr. Jones? *Neurology* 1988; 38:791-3.

12. Spence JD, Eliasziw M, DiCicco M, *et al*. Carotid Plaque Area: A Tool for Targeting and Evaluating Vascular Preventive Therapy. *Stroke* 2002; 33:2916-2922.
13. Estol CJ, Lavalle Cobo A, Gonzalez Ruiz MI, *et al*. Carotid wall plaque area measurement in vascular risk stratification. *Soc Arg Cardiol* 2015; http://www.sac.org.ar/40-congreso-argentino-de-cardiologia/ediciones-anteriores/; 42.
14. Baber U, Mehran R, Sartori S, *et al*. Prevalence, Impact and Predictive Value of Detecting Subclinical Coronary And Carotid Atherosclerosis in Asymptomatic Adults. *JACC* 2015; 65(11): 1065-74.
15. Mitchell GF. Effects of central arterial aging on the structure and function of the peripheral vasculature: implications for end-organ damage. *J Appl Physiol* 2008; 105(5):1652-60.
16. Spence JD, Solo K. Resistant Atherosclerosis: The Need for Monitoring of Plaque Burden. *Stroke* 2017; 48:1624-1629.
17. Nitsch D. Limits to causal inference based on Mendelian randomization: a comparison with randomized controlled trials. *Am J Epidemiol*. 2006; 163(5):397-403.
18. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. *Lancet* 2005; 365:224-32.
19. Rostanski SK, Marshall RS. Precision Medicine for Ischemic Stroke. *JAMA Neurology* 2016; 73(7):773-774 .
20. Van Driest SL, Wells QS, Stallings S, *et al*. Association of arrhythmia-related genetic variants with phenotypes documented in electronic medical records. *JAMA* 2016; 315(1): 47-57.
21. Chong M, Yusuf S. Mendelian Genes and Risk of Intracerebral Hemorrhage and Small-Vessel Ischemic Stroke in Sporadic Cases. *Stroke*, Journal of the AHA 2017; 48(8):2263-2265.
22. Mehta A, Beck M, Elliott P, for the Fabry Outcome Survey Investigators, *et al*. Enzyme replacement therapy with agalsidase alfa in patients with Fabry's disease: an analysis of registry data. *Lancet* 2009; 374:1986-96.
23. Sealock R, Zhang H, Lucitti JL, Moore SM, Faber JE. Congenic fine-mapping identifies a major causal locus for variation in the native collateral circulation and ischemic injury in brain and lower extremity. *Circ Res* 2014; 114(4):660-671.
24. Betteridge DJ, Broome K, Durrington PN, Mann JI, Miller JP, Neil HAW, *et al*. Risk of fatal coronary heart disease in familial hypercholesterolaemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. *BMJ* 1991; 303(6807):893-6.
25. Damani SB, Topol EJ. Emerging Genomic Applications in Coronary Artery Disease. *JACC*: Cardiovascular Interventions 2011; 4(5): 473-82.
26. Ridker PM, Danielson E, Fonseca FA, *et al*., for the JUPITER Study Group. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *NEJM* 2008; 359:2195-207.
27. Donnelly LA, Palmer C, Whitley AL, Lang CC, Doney AS, Morris AD, *et al*. Apolipoprotein E genotypes are associated with lipid-lowering responses to statin treatment in diabetes: a Go-DARTS study. Pharmacogenet Genomics 2008;18(4):279-87.
28. Jones PH. Comparing HMG-CoA reductase inhibitors. *Clin Cardiol* 2003;26(1 Suppl 1):15-20.

29. Sabatine MS, Giugliano RP, Pedersen TR, for the FOURIER Steering Committee and Investigators. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *NEJM* 2017; 376:1713-1722.
30. Bitzur R, Cohen H, Kamari Y, Harats D. Intolerance to Statins: Mechanisms and Management. *Diabetes Care* 2013; 36(Suppl 2): S325-S330.
31. Yu W, Gwinn M, Clyne M, Yesupriya A, Khoury MJ. A navigator for human genome epidemiology. Candidate gene association studies use previously identified gene variants that are tested following a disease hypothesis and are likely to show causative relations. *Nat Genet* 2008; 40(2):124-5.
32. Traylor M, Farrall M, Holliday EG. METASTROKE Collaboration. *Lancet Neurology* 2012; 11:951-61.
33. Jickling GC, Xu H, Stamova B, Ander BP, Zhan X, Tian Y, *et al.* Signatures of cardioembolic and large-vessel ischemic stroke. *Ann Neurol* 2010; 68(5):681-92.
34. Khera AV, Drake I, Kathiresan S. Genetic Risk, Adherence to a Healthy Lifestyle, and Coronary Disease. *NEJM* 2016; 375:2349-2358.
35. Fernández-Cadenas I, Montaner J. GRECOS Project (Genotyping Recurrence Risk of Stroke). *Stroke* 2017; 48:1147-1153.
36. Samani NJ, Erdmann J, Hall AS, *et al.* Genomewide association analysis of coronary artery disease. *NEJM* 2007; 357:443-53.
37. Gretarsdottir S, Thorleifsson G, Manolescu A, Styrkarsdottir U, Helgadottir A, Gschwendtner A, *et al.* Risk variants for atrial fibrillation on chromosome 4q25 associate with ischemic stroke. *Ann Neurol* 2008; 64:402–9.
38. Bezzina CR, Pazoki R, Bardai A, *et al.* Genome-wide association study identifies a susceptibility locus at 21q21 for ventricular fibrillation in acute myocardial infarction. *Nat Genet* 2010; 42:688-91.
39. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, *et al.* Cytochrome p-450 polymorphisms and response to clopidogrel. *NEJM* 2009;360(4):354-62.
40. Wallentin L, James S, Storey RF, Armstrong M, Barratt BJ, Horrow J, *et al.* Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with ticagrelor versus clopidogrel for acute coronary syndromes: a genetic substudy of the PLATO trial. *Lancet* 2010;376:1320-8.
41. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature* 2004;427(6974):541-4.
42. Agarwal A, Ressler D, Snyder G. The current and future state of companion diagnostics. *Pharmacogenomics and Personalized Medicine* 2015; 8: 99-110.
43. Bis JC, Kavousi M, Franceschini N, Isaacs A, Abecasis GR, Schminke U, *et al.* Meta-analysis of genome-wide association studies from the CHARGE consortium identifies common variants associated with carotid intima media thickness and plaque. *Nat Genet* 2011; 43(10):940-7.
44. Cherepanova OA, Gomez D, Shankman LS, *et al.* Activation of the pluripotency factor OCT4 in smooth cells is atheroprotective. *Nature Medicine* 2016; 2:657-665.
45. Helgadottir A, Manolescu A, Thorleifsson G, Gretarsdottir S, Jonsdottir H, Thorsteinsdottir U, *et al.* The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 2004; 36(3):233-9.

46. Bellenguez C, Bevan S, Gschwendtner A, Spencer CC, Burgess AI, Pirinen M, *et al*. Genomewide association study identifies a variant in HDAC9 associated with large vessel ischemic stroke. *Nat Genet* 2012; 44:328-33.
47. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* 2002; 105(10):1158-61.
48. Ridker PM, Everett BM, Thuren T for the CANTOS Trial Group. Anti-inflammatory Therapy with Canakinumab for Atherosclerotic Disease. *NEJM* 2017; 377:1119-1131.
49. Manolio TA, Collins FS, Cox NJ, *et al*. Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747-53.

Reflections on the Contribution of Basic Research to Solving Medical Problems in the Third World. The Methyloma as an Example

Rafael Apitz-Castro

Introduction

Methyloma constitute the central core of the Epigenetics regulatory mechanisms. These are, by definition, metabolic processes capable of modulating the functional behavior of genetic imprints, without altering the DNA sequences. In other words, they perform non-genetic modifications of the genome and, in consequence, were named the Epigenome.

Genome and Epigenome works together, and this crucial relationship is the subject of a superb effort in basic research, especially in the last two decades, in order to obtain precise information on its role in human disease. Soon after the human genome sequence was completed, it was clear that a map of the genome-wide modifications made to DNA and the protein scaffold that supports it was also needed. Thus, in 2005 the idea of developing an "epigenomic" map started making noise. The blueprint for the Human Epigenomic Map came to life in 2010 as the International Human Epigenomic Consortium (IHEC). The Roadmap Epigenomics Project is the equivalent of the Human Genome Project (Roadmap Epigenomic Consortium, 2015).

Although DNA methylation is probably the most detailed studied epigenetic modification of mammalian DNA, these mechanisms are also responsible for epigenetic modification, mainly through acetylation of Histones, the proteins involved in the protection and packaging of the genetic material in the basic chromatin unit or nucleosome (Pelizzola and Ecker, 2011). Moreover, a post-transcriptional level of regulation is conferred by small, noncoding RNAs termed micro-RNAs (miRs).1 Although these regulatory systems use distinct mechanisms, there is a good deal of functional overlap and crosstalk among them (Shahbazian and Grunstein, 2007).

[1] See also Dr. De Robertis' presentation.

Histone modifications

Histone proteins constitute the major organizational and regulatory units of Chromatin. They serve, in eukaryotes, not only for packaging of the genetic material, but also offer the structural basis for regulation of processes such as replication, transcription and repair of DNA.

A Chromatin unit consists of a protein octamer composed of two molecules of each isomorphic histone. These are identified as H2A, H2B, H3 and H4, and 147 base pairs of DNA are wrapped around this supramolecular arrangement to form nucleosome cores (Shahbazian and Grunstein, 2007). Usually, the modification site is on the amino-terminal histone tails. Different modifications, which include acetylation, methylation, and phosphorylation, among others, are common (Li *et al.*, 2007).

Such modifications can induce structural changes in the nucleosome through change in the electrostatic charge of the protein, or modulating the organization of the complex. Different enzymes are in charge of the post-translational modifications of chromatin sub-units. These modifications are, in general, made on the amino-terminal histone tails (Vaquero *et al.*, 2003). Such modifications may alter the nucleosome structure in several ways: a) modulating the organization of the supramolecular structure of chromatin, b) changing the electrostatic charge of the proteins and therefore its interaction with DNA, or c) recruiting other proteins that could act to remodel chromatin. In relation to its transcriptional state, chromatin can be said to have an actively-transcribed euchromatin and an inactive heterochromatin. The former is characterized by high levels of acetylation and trimethylation, while heterochromatin shows low levels of acetylation, while maintaining high levels of methylation (Li *et al.*, 2007).

Histone acetylation occurs mainly on lysine residues, resulting in neutralization of the positive charge and decreasing its DNA affinity. Acetylation is carried out by a group of enzymes called histone acetyltransferases (HATs), represented by two main subtypes, type A (five families) and type B (four families) (Bannister and Kouzarides, 2011).

Acetyl residues removal is effected by histone deacetylases (HDACs), reestablishing the positive lysine charge and restoring the DNA binding affinity. HDACs comprises a family of isoenzymes, classified as I, II or III, according to homologous sequence domains. Interestingly, several inhibitors of HDAC activity have been studied in regard of their potential effects as anticancer drugs; however, the major concern is their lack of specificity (Bieliauskas and Pflum, 2008; Karytinos *et al.*, 2009).

DNA modifications

DNA methylation is the process by which a methyl group is added to the 5 position of cytosine bases of DNA to form 5-methylcytosine. So far, it is the only known epigenetic mechanism that directly modifies the DNA molecule (Pelizzola and Ecker, 2011). Although it is a covalent modification of the DNA molecule, that can be inherited, it is also subject to dynamic changes which are linked to diet and other environmental influences. DNA methylation occurs almost exclusively in the context of CpG dinucleotides, which are distributed in clusters named CpG islands.

These GpC sites are distributed throughout the genome in genic and intergenic regions. Genic regions include the promoter and gene body, while the intergenic ones include distal regulatory complexes as well as repetitive elements (Moore *et al.*, 2013). In general, promoter methylation is repressive and its extent is inversely related to the density of CpG units in the promoter. In this regard, promoters are classified in two groups a) those with low CpG density (LCG) and b) those with high CpG density (HCG), which are the most abundant (Saxsonov *et al.*, 2006). HCG promoters contains clusters of GpC sites (CpG islands) that are mostly devoid of methylation, thus, they are hypomethylated promoters. These promoters are expressed by cells of almost all tissues and are considered housekeeping genes. LCG promoters are, in general, devoid of CpG islands and are associated with tissue-specific gene regulation. Methylation of gene bodies is related to fine-tuning of promoter methylation patterns, while methylation in intergenic regions mainly affects distal regulatory elements (enhancers and repetitive elements) (Hon *et al.*, 2013).

The precise mechanisms for translation of the methylation code into gene expression or repression are not yet well known. However, at least two plausible mechanisms have been proposed: a) transcription of the downstream gene is inhibited by methylation of cytosine in the promoter regions, due to blockade of the binding of transcription factors; b) on the other hand, 5-methylcytosine is recognized by DNA methyl-binding proteins (MBP) involved in chromatin remodeling, necessary for gene inhibition or activation.

In mammals, DNA methylation is a highly organized process, set up early in development, which involves genome-wide demethylation and *de novo* methylation. The first episode of DNA methylation erasure occurs in the blastocyst, following fertilization, while on implantation, the embryo undergoes a wave of de novo methylation. This process establishes a

new methylation pattern, which is copied during division of somatic cells (Martinez *et al.*, 2015; Xin-Jian *et al.*, 2011). After fertilization, most of the paternal genome is rapidly demethylated, through an active enzymatic demethylation process, while the maternal genome undergoes passive, replication dependent demethylation over subsequent cleavage divisions. After implantation, a reprograming, that is, a phase of global *de novo* methylation, re-establishes the DNA patterns that will be maintained, in large part, in somatic tissues. After implantation, there are no additional global changes in DNA methylation, and all other modifications (methylation/demethylation) seem to be sequence-specific directed (Bergman and Cedar, 2013; Guo *et al.*, 2014; Smith *et al.*, 2014).

The enzymatic machinery involved in DNA methylation belongs to a family of DNA methyltransferases, which are responsible for maintaining and/or adding DNA methylation marks (DNMTs). The DNMT family comprises four distinct members, based on sequence homology within their C-terminal catalytic domains: DNMT1, DNMT3A, DNMT3B and DNMT3L. In mammals, DNMT1 is a maintenance methyltransferase which, together with the BMP UHRF1, preferentially adds methyl groups to hemi-methylated double-stranded DNA during replication, whereas DNMT3A and B are responsible for de novo DNA methylation during gamete development and blastocyst implantation, respectively. DNMT3L is devoid of catalytic activity and seems to be a regulator of the activity of DNMT3A, as well as DNMT3B (Bergman and Cedar, 2013; Goll and Bestor, 2005; Chen and Riggs, 2011). DNA demethylation, on the other hand, can be passive or active. Passive DNA demethylation occurs mainly through exclusion of DMNT1 and UHRF1 from the nucleus, resulting in replication-dependent loss of methylation marks (mainly associated to the maternal genome). There is considerable evidence to support the existence of active demethylation in zygotes and primordial germ cells (PGCs) (Guo *et al.*, 2014; Chen and Riggs, 2011). However, no single enzyme is yet known that directly demethylates 5mC to cytosine in the nucleosome. In this regard, several indirect demethylation mechanisms have been proposed (TET proteins, DNA glycosylases, etc) (Chen and Riggs, 2011; Mayer *et al.*, 2000; Auclair and Weber, 2012).

Even from this brief and necessarily very general description of the epigenetic landscape, it is clear that alteration in almost any single step in the molecular mechanisms involved will potentially affects the correct gene expression. This can lead to congenital anomalies, defined as structural or functional anomalies that can be identified at birth or can sometimes only

be detected later in infancy or – as happens with cardiovascular disease, diabetes and many other pathologies – in the adult organism (Martinez *et al.*, 2015; Fernandez *et al.*, 2010).

Role of environmental factors

In this regard, it is of paramount importance the concept that the appropriate functionality of the epigenomic landscape is highly dependent on ambient influences. That is to say that, besides "pure" genetic factors – such as inherited genes that code for an anomaly, or as a result of sudden, not well-understood gene mutations – environmental factors such as socioeconomics and demographic, prenatal maternal health, exposure to pesticides and certain medications and, particularly nutritional deficiencies, play an important role in the methylome functionalism.

Figure 1 depicts an oversimplified scheme that shows the relationships between the underlying epigenetic landscape, represented at the left side, and the environmental factors to which human beings are exposed. As stat-

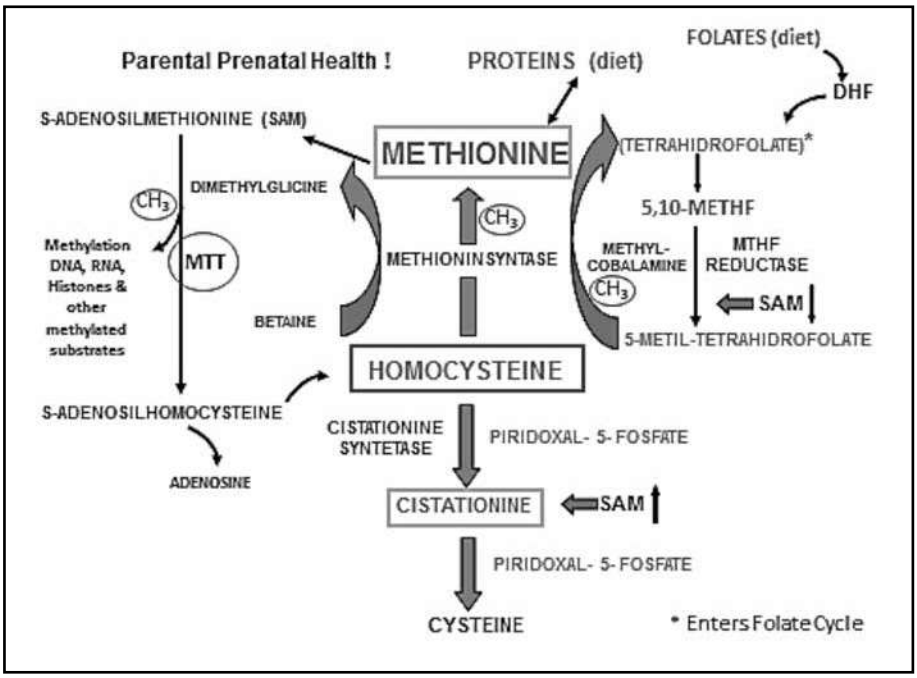


Figure 1. Simplified scheme to show the relationship between ambiental factors and the methylation pathway that leads to epigenetic modifications of the genetic landscape.

ed by Professor Melody Goodman, "*Your zip code is a better predictor of your health than your genetic code*".

Seen under this perspective, we may think of two, non-excluding alternatives, through which health care may benefit from basic research: a) Taking appropriate actions to counteract the effect of negative environmental factors, and b) Applying the wondrous progress in cell and molecular biology from the last three decades, specially in the area of genetics, to develop therapeutic protocols directed to correct nature mistakes.

Clearly, the link between the methylome and the environment is the biochemical pathway centred in the homocysteine-methionine cycle. Folate, from the diet, constitutes the main, if not the only source of methyl groups, and methionine, which is an essential aminoacid, can only be obtained from animal proteins in the diet.

In the period from 1960 to 1965 a series of communications appeared, which gave evidence of a possible relationship between fetal malformation and defective folate metabolism in the mother. Indeed, it was the result of serendipity and basic research (Tiersch, 1952; Goetsch, 1962).

Briefly, in 1952 Thiersch published a controversial paper in which he studied the ability of aminopterin (5-aminopteroyl glutamic acid) to induce abortion in human beings. Ten years latter, in 1962, Goetsch published results from an evaluation of aminopterin as an abortifacient. The paper was followed by a discussion authored by Dr. Russell R. de Alvarez (Goetsch, 1962), in which Alvarez stated that Thiersch's paper was not a publication from the Pacific Northwest Obstetrical and Gynecological Association, and that on the contrary, they did not condone nor recommend the use of antifolates for the production of therapeutic abortion. What is even more interesting is that Alvarez describes the role of folic acid in terms of its function as a methyl group donor for the synthesis of nucleic acids and underlines the requirement of an exogenous source of folic acid. In this regard, the effect of folate deficiency on the development of rat embryos was initially described in 1960 (Nelson, 1960). Although more than 50% of congenital anomalies cannot be associated, so far, with a specific cause, it is estimated that a number of environmental factors would play an important role in not less than 15% of congenital anomalies, considered multifactorial. These factors include: socioeconomic and demographic factors (maternal nutritional status, diabetes, alcoholism, smoking, drugs), exposure to teratogenic chemicals, and maternal infections, among others. Neural tube defects (NTD), cardiovascular system defects, Down syndrome, and cleft lip and / or palate anomalies represent the largest proportion of birth defects. These

anomalies, classified as multifactorial, have been associated with pre-conceptional folate deficiency, combined, especially in third world countries, with general nutritional deficiency. It is noteworthy that even in countries where the general population has adequate food intake, folate deficiency is present (Brent, 2004; Molloy *et al.*, 2008; Padmanabhan *et al.*, 2013; Bailey and Berry, 2005; Ganu *et al.*, 2012; Khoshnood *et al.*, 2015). Moreover, it has been shown that in an inbred C57BL/6 mouse model, exposed to low dietary folate beginning in utero and throughout life, leads to alteration in paternal sperm DNA methylation and is associated with negative reproductive outcomes, including birth defects in the offspring (Lambrot *et al.*, 2013). Based on the compelling experimental evidence, in 1992, the U.S. Public Health Service recommended that all women of childbearing age consume 400 μg of folic acid daily (Centers for Disease Control and Prevention, 1992). This led to the implementation of a public health program that has shown an excellent cost-benefit ratio: the mandatory implementation of fortification of consumer food with folic acid. Several advantages are noted: a) is not discriminatory, b) covers the preconception period, specially important for women in reproductive age and c) is easily implemented and have an excellent cost/benefit ratio. This program has been compulsorily adopted by 81 countries (Crider *et al.*, 2011). In the region, only Venezuela has not implemented the mandatory fortification program (Apitz-Castro, 2015). In this regard, it is relevant to note that in Chile and Costa Rica, where the program has been in effect since 2000, the incidence of NTDs is less than 5/10,000 live births, while in Venezuela it is 20.3/10,000 live births, and combined NTDs/Heart defects reach 50/10,000 live births, an unacceptably high incidence (Apitz-Castro, 2016).

The second alternative, mentioned above, is based on the accelerated progress in genetic research in the last 20 years. This opened a wide range of possible protocols intended to introduce beneficial genetic modifications by directly manipulating the genetic machinery. Different names have been adopted for these approaches: personalized, individualized, stratified, precision, genomic medicine, among others. The 2020 Advisory Group of the European Union defined personalized medicine as "a medical model using characterization of individuals phenotypes and genotypes for tailoring the right therapeutic strategy for the right person at the right time" (European Union: Council Conclusions, 2015). It is clear that this approach is not applicable to any public health program.

So far, personalized medicine has been translated into practice predominantly through the use of genetic diagnostic tests connected to a distinct

molecular characteristic. Moreover, due to the actual costs, it is ethically discriminatory. Methodological advances, however, may change those concerns. An example is the introduction of a very precise gene editing technique known as CRISPR/Cas9. It was adapted from a naturally-occurring genome-editing system in bacteria. A detailed description of the technique is out of the scope of these reflections. Briefly, a Cas endonuclease protein forms a complex with a guide RNA molecule and localizes to a target sequence following simple RNA/genomic DNA base pairing rules. The target DNA sequence must be both complementary to the guide RNA and also contain a short DNA sequence that is required for compatibility with the particular Cas protein being used. A main concern with CrisPR/Cas9 gene editing is the tendency to cut the genome at sites similar in sequence to the targeted sequence (off-target effects). However, recent studies focused on eliminating such effects show notable progress in lowering the frequency of the off-target effects (Lander, 2016; Komor *et al.*, 2017; Slaymaker *et al.*, 2016; Kleinstiver *et al.*, 2016). Clearly, this approach will be of great impact in the advance of personalized medicine, but by definition it focuses on the individual patient. In order to make personalized medicine effective at the population level, these genomic techniques must be standardized and somehow integrated into public health systems that allow a more equitable distribution of the enormous potential benefits derived from basic research (Keeler *et al.*, 2017; Wolpe and Rommelfangen, 2017). In this regard, a promising approach may be the use of genetic manipulation to modify negative ambiental factors such as vectors and/or parasites.[2]

Such an approach has recently been used to fight malaria, a disease which affects more than 250 million people worldwide. In one study, a symbiotic bacterium strain (genus *Serratia*), which stably colonizes anopheles midgut, female ovaries and male accessory glands, was genetically engineered for secretion of anti-plasmodium effector proteins (Wang *et al.*, 2017). Another study focused on genetically-modified mosquitoes to alter expression of their own anti-Plasmodium immune genes. Those genetically-modified mosquitoes showed increased immune activity against Plasmodium and an unexpected changes in mating patterns: a mating preference of genetically-modified males for wild-type females, whereas wild-type males prefer genetically-modified females. It is expected that these mating changes will foster the spread of the genetic modification in the mosquito population (Pike *et al.*, 2017).

[2] See also Dr Rech's presentation.

Conclusions

It seems clear then, from this brief example, that besides the knowledge derived from basic science research, the impact of it on individual well-being and on the wide area of public health is of paramount importance. Although basic researchers and clinicians tend to have different approaches to scientific problems, there are many advantages from successful collaborations between them. While a basic scientist would ask questions such as why and how a phenomenon occurs, a clinician is generally more concerned with the usefulness of the answer for the patient's problem. In developing countries, it is necessary to change the attitude towards interdisciplinary cooperation, starting during the formative years of training. It requires that universities and research institutions diversify training pedagogies to promote interactive activities that bring young clinical and basic researchers together, stimulating collaborative behaviour from the beginning of their careers.

In this regard, it is very important that the scientific community use all its power to change the widespread conception of the useless spending of financial resources in basic research, which, as we know, is a common denominator within most governments, especially in developing countries.

Besides its role in connecting researchers in Latin America, ACAL should also work at the higher political levels to convince politicians of the benefits of investing and encouraging education in science and promote the creation of research facilities on Translational Medicine. Further, ACAL has a growing roster of first-class scientists active in research, working in their countries and/or in more developed countries, who constitute the moving force to accomplish the main purpose of the Academy, defined by the founder members as: "La Academia tiene como objeto promover, estimular y fomentar el cultivo de las ciencias matemáticas, físicas, químicas, de la tierra y de la vida y sus aplicaciones en beneficio del desarrollo y de la integración humana, cultural y social de América Latina y el Caribe" (ACAL, Acta Constitutiva y Estatutos, 1982).

References

Academia de Ciencias de America Latina, https://acal-scientia.org

Apitz-Castro, R. (2015) Los niveles de ácido fólico en la población venezolana y su impacto en el área de salud pública. (2015), *An. Venez. Nutr.* 28(1):21-27.

Apitz-Castro, R. (2016) Estimación de la prevalencia de defectos del desarrollo embrionario en Venezuela: Resultados del primer estudio multicéntrico en el país (junio 2013-junio 2014). *Gaceta Médica de Caracas* 124(2):102-110.

Auclair, G., Weber, M., (2012) Mechanisms of DNA methylation and demethylation in mammals. *Biochimie* 94:2202-2211.

Bailey, L., Berry, R. (2005) Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births and miscarriage. *Am. J. Clin. Nutr.*, 1213S-1217S.

Bannister, A.J., Kouzarides, T. (2011) Regulation of chromatin by histone modifications. *Cell Res.* 21:381-395.

Bergman, Y., Cedar, H. (2013) DNA methylation dynamics in health and disease. *Nature Struct & Molecular Biol.* 20(3):274-281.

Bieliauskas, A.V., Pflum, M.K.H. (2008) Isoform-selective histone deacetylase inhibitors. *Chemical Soc. Rev.* 37(7):1402-1413.

Brent, R. (2004) Enviromental cause of human congenital malformations: The pediatrician's role in dealing with this complex clinical problems caused by a multiplicity of environmental and genetics factors. *Pediatrics* 113:957-968.

Centers for Disease Control and Prevention. (1992) Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Recomm. Rep.* 41:1-7.

Chen, Z., Riggs, A.D. (2011) DNA methylation and demethylation in mammals. *J. Biol. Chem.* 286(21):18347-18353.

Crider, K.S., Bailey, L.B., Berry, R.J. (2011) Folic acid food fortification – Its history, effect, concerns and future directions. *Nutrients*, 3:370-384.

European Union: Council conclusions on personalised medicine for patients. http://eur-lex.europa.eu/legal-content/ (2015), Accessed 9 July 2017.

Fernandez, A.Z., Siebel, A.L., El-Osta, A., (2010), Atherogenic factors and their epigenetic relationship. *Int. J. Vasc. Med.* 2010:1-7.

Ganu, R., Harris, R., Collins, K., Aagaard, K., (2012) Maternal diet: A modulator for epigenomic regulation during development in nonhuman primates and humans. *Int. J. Obesity* 2(Suppl): S14-S18.

Goetsch, C. (1962) An evaluation of aminopterin as an abortifacient. *Am. J. Obst. & Gynec.* 83(11):1474-1477.

Goll, M.G., Bestor, T.H. (2005) Eukaryotic citosine methyltransferases. *Ann. Rev. Biochem.* 74:481-514.

Guo, H., Zhu, P., Yan, L. *et al.* (2014) The DNA methylation landscape of human early embryos. *Nature* 511(7511):606-610.

Hon, G.C., Rajagopal, N., Shen, Y., McCleary, D.F., *et al.* (2013) Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. *Nat. Genet.* 45:1198-1206.

Karytinos, A., Forneris, F., Profumo, A., *et al.* (2009) A novel mammalian flavin-dependent histone demethylase. *J. Biol. Chem.* 284(26):17775-17782.

Keeler, A.M., ElMallah, M.K., Flotte, T.R. (2017) Gene therapy 2017: Progress and future directions. *Clin Transl Sci.* 10:242-248.

Khoshnood B., Loane M., de Walle H., Arriola L., *et al.* (2015) Long term trends in prevalence of neural tube defects in Europe: Population based study. *BMJ*, 351:h5949 (doi:10.1136/bmj.h5949).

Kleinstiver, B.P., Pattanayak, V., Prew, M.S., Tsai, S.Q., *et al.* (2016) High-fidelity CRISPR-Cas9 nuclease with no detectablegenome-wide off-target effects. *Nature* 529(7587):490-495.

Komor, A.C., Badran, A.H., Liu, D.R. (2017) CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell* 168(1-2):20-36.

Lambrot, R., Xu, C., Saint-Phar, S., Chountalos, G., *et al.* (2013) Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nature Com-*

mun 4:2889 doi: 10.1038/ncomms3889.
Lander E.S. (2016) The heroes of CRIS-PR. *Cell* 164(1-2):18-28.
Li, B., Carey, M., Workman, J.L. (2007) The role of chromatin during transcription. *Cell*, 128:707-719.
Martinez, S.H., Gay, M.S., Chang, L. (2015) Epigenetic mechanisms in heart development and disease. *Drug Disc. Today*, 20(7):799-811.
Mayer, W., Niveleau, A., Walter, J., *et al.* (2000) Demethylation of the zygotic paternal genome. *Nature* 403:501-502.
Molloy, A.M., Brody, L.C., Mills, J.L., Scott, J.M., *et al.* (2008) The search for genetic polymorphisms in the homocysteine/folate pathway that contribute to the etiology of human neural tube defects. *Birth Defects Res. A Clin. Mol. Teratol.* 82:701-709.
Moore, L.D., Le, T., Fan, G., (2013) DNA methylation and its basic function. *Neuropsychopharmacology* 38:23-38.
Nelson, M.M. (1960) *Congenital malformations*. Ciba Fdn. Symposium, London.
Padmanabhan N., Jia D., Geary-Joo C., Wu X. *et al.* (2013) Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. *Cell* 155:81-93.
Pelizzola, M., Ecker, J.R. (2011) The DNA methylome. *FEBS Lett.*, 585:1994-2000.
Pike, A., Dong, Y., Dizaji, N.B., Gacita, A., *et al.* (2017) Changes in the microbiota cause genetically modified anopheles to spread in a population. *Science*, 357:1396-1399.
Roadmap Epigenomics Consortium (2015) Integrative analysis of 111 reference human epigenomes. *Nature*, 518:317-330.
Saxsonov, S., Berg, P., Brutlag, D.L. (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc. Natl. Acad. Sci. USA*, 103:1412-1417.
Shahbazian, M.D., Grunstein, M. (2007) Functions of site-specific histone acetylation and deacetylation. *Ann. Rev. Biochem.*, 76:75-100.
Slaymaker, I.M., Gao, L., Zetsche, B., Scott, D.A., *et al.* (2016) Rationally engineered Cas9 nucleaseswith improved specificity. *Science* 351(6268):84-88.
Smith, Z.D., Chan, M.M., Humm, K.C., *et al.* (2014) DNA methylation dynamics of the human preimplantation embryo. *Nature* 511(7511):611-615.
Tiersch, J.B. (1952) Therapeutics abortions with a folic acid antagonist, 4-aminopteroylglutamic acid (4-amino P.G.A.) administered by the oral route. *Am. J. Obst. & Gynec.* 63(6):1298-1304.
Vaquero, A., Loyola, A., Reinberg, D. (2003) The constantly changing face of chromatin. *Science Aging Knowledge Environ.* 14:RE4.
Wang, S., Dos-Santos A.L.A., Huang, W., Lin, K.C., *et al.* (2017) Driving mosquito refractoriness to Plasmodium falciparum with engineered symbiotic bacteria. *Science* 357:1399-1402.
Wolpe, P.R. and Rommelfanger, K.S. (2017) Ethical principles for the use of human cellular biotechnologies. *Nature Biotechnology* 35(11): 1050-1058.
Xin-Jian He, Taiping Chen, Jian-Kang Zhu (2011) Regulation and function of DNA methylation in plants and animals. *Cell Research* 21:442-465.

Photodynamic Reactions: Cancer and Microbiological Control

Vanderlei Salvador Bagnato, Cristina Kurachi, Natalia Mayumi Inada, Lilian Tan Moriyama and Sebastião Pratavieira[1]

1. Introduction

While the understanding of cell biology is growing in content and importance, Biophotonics, an area that uses light as a tool in Life Sciences, is a promising area having biomolecules as targets. Biophotonics has been presenting attractive strategies to solve relevant health care problems. In general terms, Biophotonics uses light-tissue interactions to detect or to treat diseases. The therapeutic indications include cancer and infectious diseases, with potential application in cosmetics. Except for the use of Biophotonics in cosmetics, the first two applications are considered important challenges of modern medicine. While the number of people with cancer is growing in absolute terms, mainly due to the increase in life expectancy, infections are becoming more resistant to conventional antibiotics due to the higher incidence of antibiotic-resistant microorganism species. Meanwhile, several innovative technologies to deal with those problems are appearing. Unfortunately, the new way comes with an inconvenient cost/benefit for society. If on the one hand we are doing great in developing and promoting cures, on the other hand, the advanced technologies for health care are becoming less available due to their prohibitive costs, especially considering the scarce availability of resources and the dramatic situation in developing countries. Biophotonics is, among the present possibilities, a terrific opportunity to solve this problem.

Photodynamic reactions

Photodynamic reactions are based on the photoactivation of a specific molecule, called photosensitizer, presenting higher concentration at the target cells, which result in the production of oxidative species. The illumination parameters as wavelength, irradiance, and fluence are chosen depending on the optical properties of the photosensitizer, mainly its absorbance and quantum efficiency to produce oxidative species. The photosensitizer

[1] Instituto de Física de São Carlos, University of São Paulo, Brazil.

at the excited singlet state can result in its conversion to the triplet state, and at this condition may react with the molecular triplet oxygen present at the cell microenvironment, producing the singlet oxygen. The produced oxidative species are highly reactive showing a low action distance and short lifetime. In other words, wherever it is produced, the photodynamic reaction is highly localized and only occurs during illumination. The oxidative result of the photodynamic reactions make it appropriate to be used in the elimination of undesirable cells or microorganisms, and is a photonic technique that can be an alternative option for the treatment of tumors, premalignant and infected lesions. The technique is a local treatment alternative that has been indicated for cancer and infectious diseases, and it is clearly becoming an important alternative for present challenges.

The technique used to treat cancer is known as Photodynamic Therapy (PDT). PDT is similarly used in wet age-related macular degeneration, acne, psoriasis, and atherosclerosis.

When photodynamic reactions are used to kill microbial cells, including bacteria, fungi and viruses, the technique is called Photodynamic inactivation (PDI), antimicrobial photodynamic therapy (aPDT) or photodynamic antimicrobial chemotherapy (PACT). The PDI can be used to kill microorganisms in the human body or in the environment.

Nowadays, many experimental and clinical studies are performed to develop and improve the photosensitizer molecule, the light sources and irradiation strategies, and the protocols to use photodynamic reactions.

The use of photosensitizers as compounds for the diagnosis and treatment of several conditions in dermatology has been frequently driving research. To cite the Hermann von Tappeiner experiments (1), an important contribution for the field of Photodynamic Therapy when he was the Director of the Pharmacological Institute of the University of Munich, Germany, in the 20th century. The findings of his student, Oscar Raab, who discovered the cellular phototoxicity in one of his experiments, led them to introduce the term "Photodynamic" in a publication of 1904 (1). The photodynamic action phenomenon was observed during the lethal effect of light and the dye acridine hydrochloride upon the unicellular organism *Paramecium caudatum*.

In 1903 Niels Ryberg Finsen was awarded the Nobel Prize in Physiology or Medicine for his contribution for this field, treating 800 patients who were suffering from *Lupus vulgaris* disease. Since these historic moments, the experiments and application of Photodynamic Therapy have spread around the world, with several basic and applied studies in countless fields.

The photodynamic action occurs when a molecule (photosensitizer, abbreviated as PS) has the property to transfer an electron to molecular oxygen or the triplet PS transfer energy directly to molecular oxygen. Following the absorption of photons, the ground state PS with two electrons with opposite spins (singlet state) in the low-energy molecular orbital can react directly with a substrate or transfer energy to molecular oxygen, starting the photodynamic reaction. It can also return to the ground state releasing energy as photons, a radiative process known as fluorescence, also detailed in Jablonski's diagram (2). The intersystem crossing is the most important process for PDT and it occurs because it gives to the photosensitizer (PS) its triplet state. Despite the fact that this state has less energy than the singlet state, it has a higher lifetime (microseconds). In this situation, the excited electron in the PS at the triplet state may slowly change its spin orientation, emitting its energy as phosphorescence. The PS in its triplet state may also interact with molecules in the environment. Basically, the photodynamic process is divided in two types. When the electron transfer occurs directly from PS at triplet state to molecular oxygen, sometimes through proton transfer, the reactive oxygen species (free radicals) such as superoxide anion ($O2^{\bullet -}$) is generated as is, consequently, another species such as hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). This is known as Type 1 reaction. The triplet state can transfer its energy directly to oxygen and produce singlet oxygen (Type II reaction). Both types of PDT processes can occur simultaneously, depending on the type of the PS, the substrate, the amount of oxygen and the light parameter (3).

Porphyrins, chlorines, and phthalocyanines are the three main groups of photosensitizers (PSs) studied. Porphyrins are the most frequently used PSs, but their systemic administration shows an important adverse factor in Dermatology. Due to the high accumulation by the skin and slow drug clearance, porphyrins lead to prolonged photosensitization of the organism after application (4). These compounds have characteristics suitable for use in PDT due to high molar extinction coefficients, high absorptivities in the region of the "therapeutic window" (600-800 nm) and with high quantum yields of singlet oxygen production. The 5-aminolevulinic acid (5-ALA) is a precursor for a protoporphyrin IX (PpIX), formed by 5-ALA synthase from glycine and succinylCoA, the rate-limiting step of the heme biosynthesis (5). Once this step is bypassed by the exogenous administration of 5-ALA, porphyrins and heme accumulate mainly in the malignant or abnormal tissue, where the metabolic activity is different.

Nanotechnology and new topical formulations can also be explored in PDT to increase tissue interaction and penetration. Nanoparticles are the new way to improve PS delivery. Several research groups are looking for ways to overcome some of these challenges.

2. Photodynamic action

Photodynamic action on biological tissues with the available photosensitizers is a result of a cellular and/or vascular damage. The cellular damage is present when the photosensitizer is linked to a biomolecule of the cell membranes or organelles, or if the molecule is internalized and free in the cytoplasm, but in this last case, the photodynamic action is much less effective. As mentioned before, the action volume of the singlet oxygen is highly localized, so its efficacy on inducing cell death is dependent on the photosensitizer cell location and concentration. Biological cells can be driven to three types of cell death: necrosis, apoptosis, and autophagy (3,6,7).

Necrosis is mainly observed when there is a high concentration of the photosensitizer at the cell membrane, and the photodynamic action results in the membrane breakdown. Presently this is the main mechanism of cell

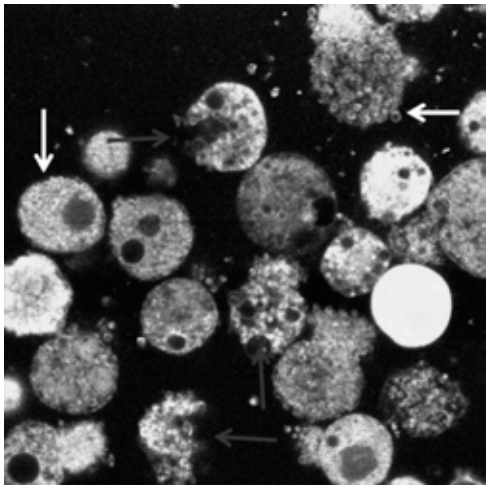


Figure 1. Amoeba cells previously incubated with curcumin solution for 10 minutes and imaged with fluorescence microscopy. The microscope white light has a high intensity at the blue spectrum, in this case it is performing PDT irradiation. This is a frame of a movie taken while imaging the cells. The yellow arrow indicates an intact amoeba cell with a high concentration of curcumin, observed by its green fluorescence. The white arrow indicates a cell presenting several surface bubbles resulting from PDT damage at its wall, and the red arrows present three examples of cells showing wall destruction and intracellular material release to the microenvironment.

death when the injected porphyrin is used, and also for microbial control, destroying the bacterial and fungal membranes and walls. Figure 1 shows a microscopy image of amoeba cells, previously incubated with curcumin, during PDT irradiation. The cell wall disruption can be observed firstly as cell blebbing and later with the intracellular material released.

Apoptosis is another type of cell death that can be produced by the PDT action. This is a type of programmed cell death. While necrosis results from an acute cellular injury, apoptosis is a highly regulated and controlled process. This death response produced by PDT is usually observed when the photosensitizer is presented to the mitochondria or other organelles without extreme damage of the cell membrane. This is the most observed cell death when using the pro-drug ALA or MAL producing the protoporphyrin IX (PpIX), an endogenous photosensitizer.

Autophagy or autophagocytosis is a regular destructive mechanism that can be induced by PDT. Normally, it occurs when the cell degrades and recycles cellular components, but for reasons still not well defined, it can also result in cell death or morbidity. PDT seems also to induce these autophagy mechanisms, resulting in cell death.

Besides a cellular response, PDT may also present a vascular response. This is observed when using photosensitizers that are delivered by intravenous injection. In this case, depending on the drug-light interval, a higher amount of the photosensitizer may still be present at the vascular network. When the tumor is irradiated, these photosensitized vessels show relevant damage on the endothelial cells, resulting in a microvasculature shutdown, and consequently anoxia and lack of blood circulation (8,9). At the same time the vascular response also contributes to tumor destruction: since there is no further tumor nutrition, there is a decreased amount of oxygen, diminishing the photodynamic reaction.

Another PDT action that has been indicated is the improvement of an immune response. Since PDT causes acute inflammation, as a result of tissue necrosis and microvasculature shutdown, there is increased expression of heat-shock proteins, leukocytes invasion and infiltration of the tumor, and there might also be an increased presentation of tumor-derived antigens to T cells (10).

3. Photosensitizers and light sources for PDT

Chromophores are natural light absorbers in human tissue there. The most common are melanin and hemoglobin. They were the first investigated pigments for light interaction in human tissue, but they are not nat-

ural photosensitizers. Photosensitizers are normally exogenous from the tissue and introduced for a specific use. There are many photosensitizing drugs used now days, with the most common one being hematoporphyrin-derivatives. There is however a large collection of drugs, a few them from natural extracts. Photosensitizers can be categorized by direct chemical structure and come from several broad families. Certainly the most important family discovered is based on hematoporphyrin (Hp) and its derivatives. After purification and manipulation, HpD is transformed into commercial products variously called Photofrin, Photosan and Photocan. These products are composed of differing fractions of porphyrin monomers, dimers and oligomers which are required for successful therapy. Depending on the purification steps these commercial products may not be identical, though clinically, they appear equivalent. However, this statement must be made with extreme caution. By adding, subtracting or substituting structures on the porphyrins ring additional photosensitizers can be created. The prodrug d-aminolevulinic acid when administered, even topically, will alter the natural heme synthesis feedback loop to create enough excess protoporphyrin for clinical utility. Mother nature has given us the magnificent series of chemical events called photosynthesis. Clearly, light energy is well used in this process. Chlorophyll-like substances termed chlorines have excellent photosensitizing properties as expected. Speaking of natural products, Curcumin, a yellow-orange dye extracted from the rhizomes of the Curcuma longa, has been used as a photosensitizer in many microbiological control applications, with excellent results. The pharmacological properties of curcumin include: antitumor, anti-inflammatory, antioxidant and antimicrobial. Various animal and cell culture studies have shown that Curcumin is nontoxic in the dark. Its absorption is maximum around 430 nm, and small concentrations are sufficient to observe photo-inactivation effects. As a result, curcumin is an excellent photosensitizer for treatment of localized superficial infections in the mouth or on the skin

To be considered as an ideal photosensitizer agent, the following characteristics are desired:

- It should be chemically pure and easy to synthesize; (11)
- It should have a strong absorption peak between 700 and 800 nm;
- It should have no dark toxicity and fast clearance from normal tissues;
- It should have high selectivity to tumor cells;
- It should be stable and soluble in a body's fluids and in injectable solvents.

The choice of the most suitable light source for the PDT procedure is based on the chosen PS. The light source must emit light at a wavelength that matches the absorption band of the PS molecule, thus being able to take PS molecules from the fundamental energetic state to excited states which will lead to type I and type II reactions.

Most part of the PS absorbs visible light and in this range of wavelength light has a limited penetration in the biological tissue, this way, light delivery is also a fundamental aspect of PDT. Depending on the location of the target lesion, the light source needs to have a specific design in order to make the treatment viable.

Lasers, lamps, LED-based systems and even sunlight have been used for PDT and the choice for each system is more related to the intended used than to the superiority of one over the others (12-14).

Lasers were the first light sources used for PDT and are still the most used light sources, since they can emit monochromatic light with high intensity, and they can be coupled with optical fibers, which allow for the treatment of internal organs and massive tumors by interstitial illumination. Moreover, monochromaticity helps to better calculate light dosimetry for PDT (13,15). Figure 2 shows some existing optical fibers with different geometries designed especially for PDT applications.

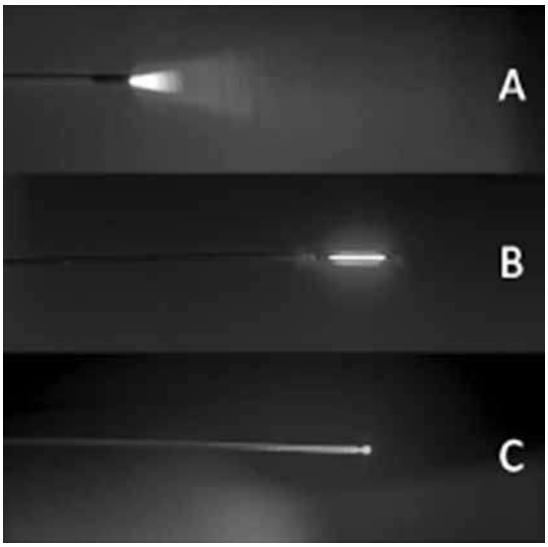


Figure 2. Three examples of optical fibers for PDT applications. A. Flat top; B. Catheter based; C. Spherical.

Figure 3. LED based devices for PDT that allow the treatment of different anatomic areas.

Lamps and LED sources are usually easier to operate and the light can be directly delivered to lesions located on the skin, oral cavity, etc. While lamps usually require the use of filters to narrow the emitted wavelength, LEDs are available in almost all wavelengths emitting in narrow band and are good substitutes for lasers. Another advantage of LEDs is that the diodes can be arranged in arrays, allowing the treatment of large areas. Nowadays it is possible to find LEDs coupled with optical fibers, although the coupling efficacy is not as good as lasers. Figure 3 shows examples of LED-based devices developed for the PDT treatment of different anatomic areas with different sizes.

Daylight PDT have been reported as suitable and effective for the treatment of actinic keratosis and cancerization fields using topical application of MAL, being well tolerated by the patients and nearly painless (14,16,17). Moreover, obviously there are no costs related to light source. The main complication of this PDT modality is the light dosimetry, because sunlight varies greatly with the seasons and the geographical location (18).

4. PDT in tumors: indications and procedures

PDT is a minimally invasive technique for the treatment of malignant and pre-malignant lesions. Since the PS preferentially accumulates in the abnormal cells, a selective treatment can be achieved. PDT has been clinically tested as a potential tool for the treatment of several malignant and pre-malignant lesions, such as: superficial and nodular BCC (19-21), Barrett esophagus (22,23), unresectable cholangiocarcinoma (24), actinic keratosis (16,25-27), actinic cheilitis (28), psoriasis (29), Bowen disease (30-32), head and neck cancer (33), prostate cancer (34-36), bladder cancer (38,38), non-small cell lung cancer (39) and brain tumors (40,41).

As PDT is based on simple concepts, it could be used in any type of well-oxygenated lesion for which it is possible to deliver PS and light.

However, in practice, it is not that simple. Due to the limitation imposed by light penetration, dark lesions, such as melanoma or pigmented basal cell carcinoma, among others, are not adequate for PDT. Also, bulky or internal tumors are also challenges for an adequate and uniform irradiation.

One of the first applications of PDT for cancer treatment was for head and neck tumors. Depending on its size, mainly its thickness, PDT could be indicated as a curative or palliative treatment. In this last case, the objective was to control or decrease the tumor volume in patients where surgical resection, radiotherapy or chemotherapy could not be performed. Two types of photosensitizers were used for head and neck cancers, porphyrin and chlorine, both previously administered with an intravenous injection, 24-48 hours and 4-8 hours, respectively, before PDT irradiation. The most important side effect of systemic PDT is eye and skin photosensitivity that can last up to one month for porphyrin compounds. PDT can be performed without anesthesia, and the delivered energy fluence depends on the tumor type, site and geometry, drug-light interval. Other types of medication, such as analgesics and anti-inflammatory, can be prescribed by the physician based on patient needs; there is no reported evidence of adverse effects of PDT in combination with these drugs.

The main clinical applications of PDT aim at skin lesions by means of the application a topical agent which promotes production of PpIX. Topical PDT has been the most used procedure for the treatment of superficial and nodular basal cell carcinoma (BCC), Bowen's disease and actinic keratosis. The topical PDT procedure is based on the topical application of an ointment containing the precursors (ALA or MAL) of the endogenous photosensitizer, PpIX.

Basic clinical procedure of topical PDT using ALA or MAL for the treatment of BCC lesions

When a patient with a skin lesion is referred to be treated with topical PDT, the lesion is usually scarified, to remove crusts and a certain layer of stratum corneum, allowing better permeation and penetration of both cream and light.

The cream containing the PpIX precursor is then applied on the lesion as a layer about 1 mm thick and an occlusive bandage is placed on the lesion to keep the cream in place and to avoid undesired light exposure during the drug-light interval. After about three hours the bandage is removed, the lesion is cleaned with gauze and is ready to receive illumination. Figure 4 shows the steps of a typical clinical PDT procedure for skin cancer using topical medication.

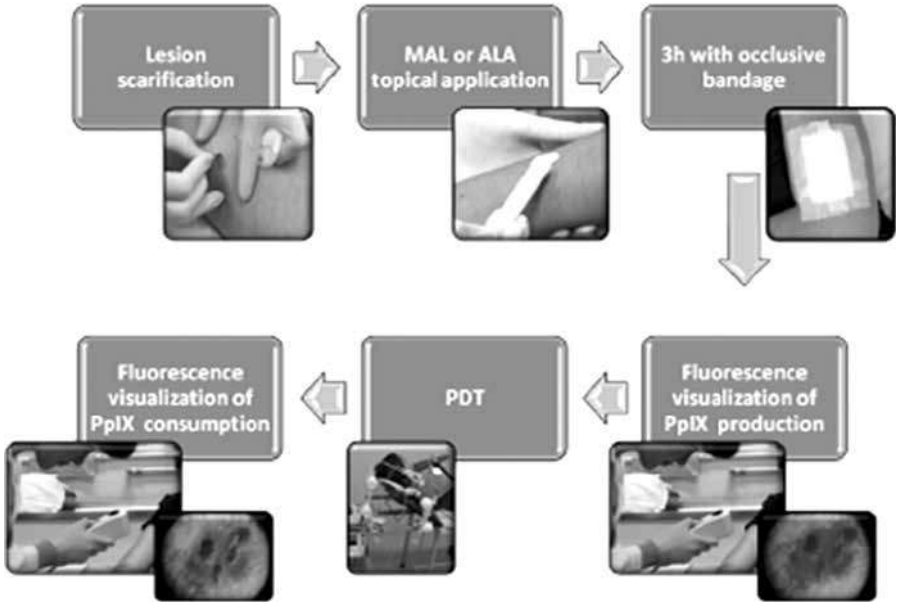


Figure 4. Step sequence of a typical clinical procedure of topical PDT for the treatment of a non-melanoma skin cancer lesion.

It is relevant to mention that fluorescence visualization of PpIX is an important step of the treatment (42). When UV-blue light is incident on PpIX it emits a reddish fluorescence that can be easily seen and distinguished from the normal non-sensitized tissue using adequate optical filters. The red fluorescence emission is indicative that the photosensitizer PpIX has been produced by the cells and the tissue is ready to undergo PDT.

Typical light parameters range from 80 to 200 mW/cm2 with total delivered dose from 80 to 150 J/cm2, hence the irradiation takes from 10 to 30 minutes to be completed.

At the end of the treatment the medical professional can check the fluorescence again and the red emission will be much less intense than the one visualized before the illumination. It is an expected effect which means that the PpIX present in the lesion was consumed (photodegraded) during illumination, leading to the photodynamic reactions responsible to promote cell death and hence tumor death.

Topical PDT is a simple procedure, it can be performed in an ambulatory setting and after the treatment the patient receives instructions related to pain control and post-procedure care and is then released.

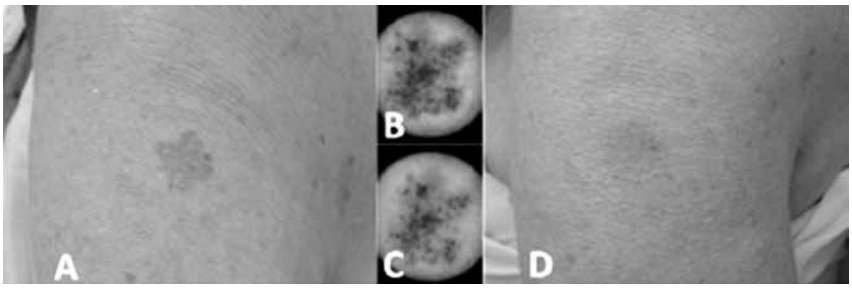


Figure 5. (A) Superficial basal cell carcinoma on the arm. (C) PpIX production after 3 hours of MAL cream incubation. (C) Superficial PpIX photobleaching. (D) Clinical response after 6 months of two MAL-PDT sessions.

PDT has good cosmetic results and an example of the treatment of a BCC lesion is presented in Figure 5.

The procedures described here are the most common, however, nowadays it is possible to find many other types of clinical protocols like ambulatory PDT (light is delivered at very low intensity for a long time and this application can be performed at home) (43).

5. Microbiological control

In addition to being used as therapeutic for oncological diseases, the PDT can be used to treat infections. Antimicrobial Photodynamic Therapy (APDT), Antimicrobial Photodynamic Inactivation (API) is a promising therapeutic modality for infectious disease. It requires the absorption of a photosensitizer (PS) and the application of a light source in the PS absorption band in the presence of oxygen. Photosensitized bacteria absorb light at the adequate wavelength in the presence of oxygen, resulting in reactive oxygen species that cause microbial death. The absorption of photons by PS takes electrons to an excited state in the presence of oxygen and reacts with molecules through the transfer of electrons or hydrogen (type I reaction) or by transferring energy to oxygen (type II reaction) to produce reactive oxygen species (ROS), toxic to microorganisms. Microbial targets in PDT make unlikely the development of antimicrobial resistance.

The antibiotic era represents the history of the therapeutics of antimicrobial drugs with recognized problems relating to the irrational use of these drugs and development of drug resistant pathogenic bacteria over the past fifty years. Studies of bacterial resistance mechanisms to API have

been evaluated. APDI produces ROS toxic to microbial biomolecules which can inhibit its growth. However, the chances of bacteria developing resistance to API are highly unlikely (44). Bacteria cannot develop resistance to PDT due to factors such as local treatment.

Considering the importance of the development of new therapies due to antimicrobial resistance and side effects of existing drugs, we will demonstrate the importance of applications with the use of PDT for microbial decontamination and treatment infections. The projects cited provide current and future possibilities of treatment and prevention of infectious diseases. This research is characterized by interaction with the scientific sector (CEPOF – Biophotonics) and device (MMOptics) and photosensitizer (PDTPharma) companies. Scientific research generates a flow of information between research groups, hospitals, dental offices and companies. *In vitro*, *in vivo* tests and clinical trials were performed for the development of these microbial researches. The outcome of these studies has legitimized new forms of decontamination and infections treatment.

Antimicrobial Photodynamic Inactivation in vitro

In vitro studies suggest new protocols to detailed antimicrobial effects using API as an alternative treatment of infections. Research developed at the Institute of Physics of São Carlos (IFSC / USP) in the field of blood decontamination, treatment of upper respiratory tract infection, oral infections and wound decontamination involve investigating the effects of PS delivery and light penetration into infected tissues. Studies involving nanoparticles, pharmaceutical forms, dose distribution of light and development of new devices have been developed.

Optimization of PDT protocols is performed from studies using pathogenic strains of fungi, yeasts, bacteria and clinical isolates from infections.

The adverse effects that can occur during and after therapy are analyzed, since in vitro tests such as red cell hemolysis in PDT for blood (45,46) and in vivo tests such as histomorphological analysis of tissue cells infected with microorganisms have been standard safety protocol (47,48). The components of the decontamination medium are simulated in vitro considering the interaction with PS and the possibilities of blocking of API by components of the medium such as blood plasma proteins.

Porphyrin and its derivates, curcumin, chlorine and indocyanine green have been used in these studies of optimization of API parameters (49-51). The efficiency of APDT in the death of oral bacteria *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans* in vitro

using toluidine blue, methylene blue and Laser He-Ne was demonstrated since 1993 by Wilson *et al.* (52).

In addition to the effect tests of the synthesis of new photosensitizers, biodegradable and non-biodegradable nanoparticles have been used as vehicles for PS delivery in vitro studies. Considering that for the photodynamic reaction to occur it is necessary to form O2, the PS released from nanoparticles after being irradiated to produce O2. For the PSs bound to nanoparticles the oxygen diffuses through these to form O2. The importance of the uptake of PS has been evaluated by studies comparing the biodistribution of these nanoparticles.

Microbial biofilm

The use of planktonic cells may not simulate all conditions encountered in vivo. Microorganisms can be grouped into complex communities called biofilms. Biofilms consist of a community of microorganisms arranged in layers of cells and surrounded by a polymer matrix. Some PSs may have limited ability to act on biofilms due to the difficulty of penetration of the compound into the deeper cellular layers.

There are projects developed in CEPOF that aim to study the formation and development of microbial biofilms contained in different PI systems. In order to evaluate these effect on the biofilm restructuring of microbial strains, biofilm growth, microbial interactions and their metabolic activities are evaluated (53,54). This destructuring may be related to the thickness, age, density and live cells of the biofilm.

Dovigo *et al.* (55) verified that the combination of Photogem® with 37.5 J/cm2 reduced the viability of fungal cells in biofilms in 1 Log. This small reduction was also observed in other IP studies of biofilm such as using erythrosine with green LED in *C. albicans* and *C. dubliniensis*. Mima *et al.* (56) showed that the application of Photogem® and 37.5J/cm2 on the biofilm of prosthetic surfaces provided the disinfection of prostheses with different *Candida* strains. The microbial reductions are higher in single-species biofilms when compared to multi-species biofilms. However, the sensitivity of microbial biofilm to multiply types of treatments increase when compared with using one therapeutic option. Figures obtained by scanning electron microscopy showed that methylene blue-mediated photosensitization occurred predominantly in the outermost layers of *C. albicans* biofilms. For this reason, the use of a greater biofilm photosensitization time has been recommended in order to obtain more PS penetration.

Considering that bacterial biofilms can be formed in materials used in patients that need assistance in breathing and feeding, with a goal to

improving the quality of life of these people the CEPOF promotes the decontamination of endotracheal tubes with microbial biofilm in hospital patients by PDT using curcumin.The complete absence of cell metabolism was observed in no experimental condition after PDT. However, since PDT can cause a decrease in the number of cells adhered to biofilms, it is possible to suppose that repeated applications of the therapy or treatment associations can potentiate the death effect on biofilms.

Antimicrobial Photodynamic Therapy in vivo

Based on results obtained by in vitro studies it is possible to establish API protocols for animal models of infections. Our research group has been describing animal models to study the use of PDT in infections. Photogem® and 305 J/cm2 in APDT were used for treatment of infections caused by *Candida albicans* in animal models of oral candidosis (47). For the inactivation of *Streptococcus pneumonia* in animals, these are infected by intranasal instillation containing Log8 colony forming units of the microorganism per mouse and the treatment performed with indocyanine green at 100 μM and 780 nm at 120 J/cm2 by extracorporeal illumination. However, studies in animal models have considered the efficiency of light penetration and not only the absorption band of the PS (57).

Clinical trials

In vitro and in vivo studies have indicated the development of new therapeutic modalities for the treatment of infections such as oral candidosis, onychomycosis, pressure ulcer and bacterial pharyngitis.

Streptococcal pharyngitis

The clinical treatment for pharyngotonsillitis proposed by the São Carlos Institute of Physics includes the development of a PDT protocol, a curcumin formulation and an oropharyngeal lighting device which provides high effectiveness in the treatment of acute streptococcal pharyngitis (Figure 6). Curcumin excited by light emits a characteristic green fluorescence (Figure 7). From the initial results of this study it was concluded that PDT is an efficient therapeutic option for the treatment of these infections. PDT can be performed in acute or chronic cases in children, adults, pregnant women and the elderly. In addition to a high rate of complete response to treatment, great patient acceptance was observed due to the procedure being performed in less than 10 minutes and presenting no side effects (58,59).

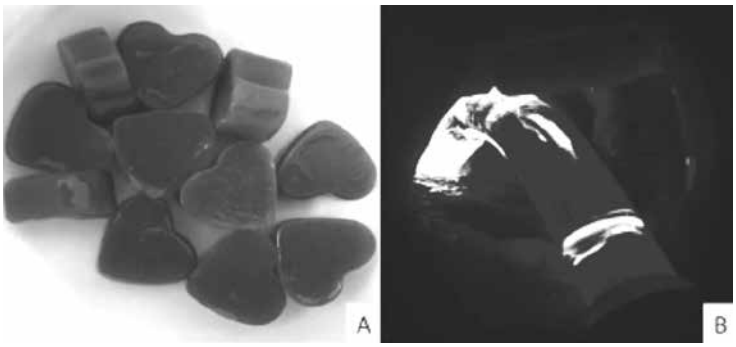


Figure 6. Panel A: Curcumin gum developed and produced by the Brazilian company PDTPharma (Cravinhos, São Paulo, Brazil). Panel B: Anatomic prototype developed by the São Carlos Institute of Physics in collaboration with MMOptics (São Carlos, São Paulo, Brazil) and how the patient illumination is performed for the Streptococcal pharyngitis treatment.

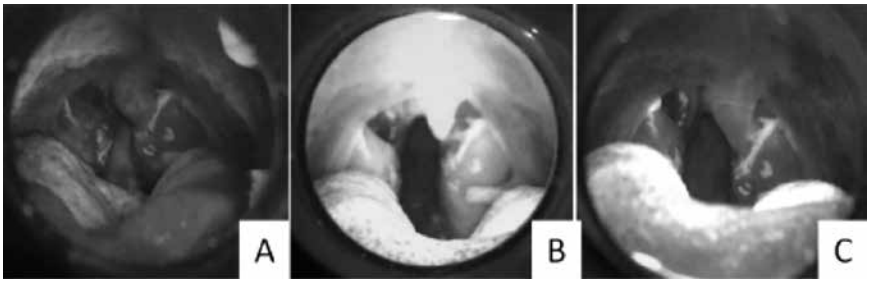


Figure 7. Widefield imaging targeting curcumin fluorescence. Panel A: tissue autofluorescence without curcumin. Panel B: tissue fluorescence just after oral administration of the gum containing curcumin, with high lighting in the throat. Panel C: Fluorescence immediately after PDT showing lower curcumin fluorescence, directly associated with ROS production and bacterial inactivation.

Onychomycosis

Patients have the disease for years, with a history of using different treatments. Inappropriate diagnosis and inappropriate use of antifungals and their intolerance by patients are some of the reasons for relapse of the disease. Silva, AP *et al.* (60) performed a clinical study with 90 patients with diagnostic suspicion of onychomycosis showing excellent results. The tests were performed with Photogem® with 630 nm and Curcumin using a LED device emitting at 470 nm, both with irradiance of 100 mW/cm2. Curcumin in the gel formulation (1.5%) was applied topically to the nail,

and then protected from light for one hour. Six sessions of PDT were applied and obtained an excellent clinical response.

Decubitus ulcer

Our research group developed a clinical protocol combining PDT, laser therapy and cellulose biomembrane that allowed the bacterial inactivation and resolution of the cicatricle process, obtaining promising results in the treatment of these lesions (61).

Dentistry

PDT has been used in dentistry to promote reduction of number of live microorganisms preventing periodontal diseases with decontamination of peri-implantitis, dentures, carious tissue and biofilms of root canal. APDT may be an adjuvant treatment including for *Candida albicans* found in endodontic infections and may be highly effective in cases of endodontic failure due to resistant microorganisms (62).

Aedes aegypti vector control

The vector *Aedes aegypti* has become responsible for the transmission of diseases such as Dengue, Chikungunya and Zika. The partial results of this work performed in CEPOF revealed that curcumin, a nontoxic substance of vegetable origin and widely used as a food additive, is efficient to be used in low concentrations in environments with low and/or high illumination by PI (26). In addition to presenting excellent performance as photoactivatable larvicide the use of curcumin is an economically viable and sustainable strategy (27).

A customized dosimetry for each target (microbial control and tumor treatment) and new delivery systems anatomically designed for basic and clinical studies also need to be improved. To reach these aims new tools from basic and applied science are being implemented to reach even more specialized clinical protocols.

6. Challenges and perspectives

Photodynamic therapy has been presented as a highly attractive technique for local treatment of several tissue conditions as cancer, potentially malignant lesions, field cancerization, infectious diseases, microbial control in distinct tissue conditions and medical devices, and vector control of tropical diseases, among others.

The main advantages of PDT when compared to conventional therapies are: a) selectivity; b) less relevant adverse effects; c) ambulatory proce-

dure; d) non-toxic drug when non-irradiated; e) most PDT light sources are portable; and f) lower costs.

There are still challenges to overcome in order to have a more diffuse and worldwide PDT application. Concerning cancer treatment, the main limitations are bulky and dark tumors and diverse PDT response. A higher volumetric PDT response can be only achieved when using intratumoral irradiation with the use of interstitial fibers, several PDT sessions, combination with other therapies, and development of photosensitizers that are excited by infrared light. A diverse response is the reason why a standard protocol cannot be used for all cases. Due to the inherent behavior of diverse characteristics and status of the organism, the tumor and the cells, the individual optical properties of the target tumor, and individual tumor molecular biology, it is expected that the photosensitizer concentration and light distribution within each tumor and among distinct tumors will be different. For this reason, a customized PDT planning is envisaged, choosing the most ideal photosensitizer for the target tumor. Non-invasive and fast response techniques for photosensitizer detection, and determination of tissue optical properties have been investigated.

For microbial control applications, several research groups have been working on the development of new protocols and instrumentation targeting a great variety of microorganisms and infectious sites. Microorganism cells show a huge diversity of biochemical, metabolism and morphology characteristics, resulting in different interaction and photosensitizer internalization mechanisms. Biofilm has an organized architecture that increases the complexity of planning a PDT protocol for infected tissue or medical device.

Chemists and physicists have been working on the design of new molecules to improve photosensitizer-cell interaction and infrared PDT irradiation, as well as on delivery strategies, mainly based on nanosystems for a more intelligent and selective drug distribution.

Different and customized illumination strategies, using diverse fiber optic tips, irradiance parameters, and combination with other techniques have been under investigation to improve PDT response.

Present and preliminary results show the potential of PDT becoming a first option technique for several diseases, especially considering low-resources sites.

Acknowledgments

Authors greatly acknowledge financial support provided by FAPESP (CEPOF 13/07276-1, INCT 2014/50857-8), CNpq (INCT 2014/50857-

8), MCTI/FINEP/MS/SCTIE/DECIT (01.130430.00/2013) and MCTIC/FNDCT-CNPq/MEC-CAPES/MS-Decit/nº 14/2016.

References

1. Ackroyd R, Kelty C, Brown N, Reed M. The history of photodetection and photodynamic therapy. *Photochem Photobiol* [Internet]. 2001 Nov;74(5):656-69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11723793
2. Dai T, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, St. Denis TG, *et al*. Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. *Frontiers in Microbiology.* 2012.
3. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part one: photosensitizers, photochemistry and cellular localization. *Photodiagnosis Photodyn Ther* [Internet]. 2004 Dec [cited 2014 Jul 13];1(4):279-93. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1572100005000074
4. Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: Update. *British Journal of Dermatology*. 2008. p. 1245-66.
5. Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. *J Photochem Photobiol B Biol.* 1992;14:275-92.
6. Henderson BW, Dougherty TJ. How Does Photodynamic Therapy Work? *Photochemistry and Photobiology*. 1992. p. 145-57.
7. Allison RR, Moghissi K. Photodynamic therapy (PDT): PDT mechanisms. *Clin Endosc.* 2013;46(1):24-9.
8. Zhou X, Chen B, Hoopes PJ, Hasan T, Pogue BW. Tumor Vascular Area Correlates with Photosensitizer Uptake: Analysis of Verteporfin Microvascular Delivery in the Dunning Rat Prostate Tumor. *Photochem Photobiol* [Internet]. 2006;82(5):1348. Available from: http://doi.wiley.com/10.1562/2006-03-25-RA-858
9. Buzzá HH, Zangirolami AC, Davis A, Gómez-García PA, Kurachi C. Fluorescence analysis of a tumor model in the chorioallantoic membrane used for the evaluation of different photosensitizers for photodynamic therapy. *Photodiagnosis Photodyn Ther.* 2017;19:78-83.
10. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumour immunity. *Nature Reviews Cancer*. 2006. p. 535-45.
11. Allison RR, Sibata CH. Oncologic photodynamic therapy photosensitizers: A clinical review. *Photodiagnosis and Photodynamic Therapy.* 2010. p. 61-75.
12. Wilson BC, Patterson MS. The physics of photodynamic therapy. *Phys Med Biol.* 1986 Apr;31(4):327-60.
13. Brancaleon L, Moseley H. Laser and non-laser light sources for photodynamic therapy. *Lasers Med Sci.* 2002;173-86.
14. Wiegell SR, Hædersdal M, Philipsen PA, Eriksen P, Enk CD, Wulf HC. Continuous activation of PpIX by daylight is as effective as and less painful than conventional photodynamic therapy for actinic keratoses; a randomized, controlled, single-blinded study. *Br J Dermatol.* 2008;158(4):740-6.
15. Wilson BC, Patterson MS. The physics, biophysics and technology of

photodynamic therapy. *Phys Med Biol.* 2008 May 7;53(9):R61-109.
16. Wiegell SR, Hædersdal M, Eriksen P, Wulf HC. Photodynamic therapy of actinic keratoses with 8% and 16% methyl aminolaevulinate and home-based daylight exposure: a double-blinded randomized clinical trial. *Br J Dermatol.* 2009;160(6):1308-14.
17. Rubel DM, Spelman L, Murrell DF, See JA, Hewitt D, Foley P, *et al.* Daylight photodynamic therapy with methyl aminolevulinate cream as a convenient, similarly effective, nearly painless alternative to conventional photodynamic therapy in actinic keratosis treatment: A randomized controlled trial. *Br J Dermatol.* 2014;171(5):1164-71.
18. Cantisani C, Paolino G, Bottoni U, Calvieri S. Daylight-Photodynamic Therapy for the Treatment of Actinic Keratosis in Different Seasons. *J Drugs Dermatol.* 2015;14(11):1349-53.
19. Ramirez DP, Kurachi C, Inada NM, Moriyama LT, Salvio AG, Vollet Filho JD, *et al.* Experience and BCC subtypes as determinants of MAL-PDT response: preliminary results of a national Brazilian project. *Photodiagnosis Photodyn Ther.* Elsevier B.V.; 2014 Mar;11(1):22-6.
20. Buzzá HH, Silva AP da, Vollet Filho JD, Ramirez DP, Trujillo JR, Inada NM, *et al.* Photodynamic therapy: Progress toward a scientific and clinical network in Latin America. *Photodiagnosis Photodyn Ther* [Internet]. Available from: http://www.sciencedirect.com/science/article/pii/S157210001530020X
21. Wang H, Xu Y, Shi J, Gao X, Geng L. Photodynamic therapy in the treatment of basal cell carcinoma: a systematic review and meta-analysis. *Photodermatol Photoimmunol Photomed.* 2015;31(1):44-53.
22. Qumseya BJ, David W, Wolfsen HC. Photodynamic therapy for Barrett's esophagus and esophageal carcinoma. *Clin Endosc.* 2013;46(1):30-7.
23. Wolfsen HC, Woodward TA, Raimondo M. Photodynamic therapy for dysplastic Barrett esophagus and early esophageal adenocarcinoma. *Mayo Clin Proc.* 2002;77(11):1176-81.
24. Gao F, Bai Y, Ma SR, Liu F, Li ZS. Systematic review: Photodynamic therapy for unresectable cholangiocarcinoma. *Journal of Hepato-Biliary-Pancreatic Sciences.* 2010. p. 125-31.
25. Ericson MB, Sandberg C, Stenquist B, Gudmundson F, Karlsson M, Ros a-M, *et al.* Photodynamic therapy of actinic keratosis at varying fluence rates: assessment of photobleaching, pain and primary clinical outcome. *Br J Dermatol* [Internet]. 2004 Dec [cited 2014 Oct 1];151(6):1204-12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15606516
26. von Felbert V, Hoffmann G, Hoff-Lesch S, Abuzahra F, Renn CN, Braathen LR, et al. Photodynamic therapy of multiple actinic keratoses: reduced pain through use of visible light plus water-filtered infrared A compared with light from light-emitting diodes. *Br J Dermatol* [Internet]. 2010 Sep [cited 2014 Jun 25];163(3):607-15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20426780
27. Kalka K, Merk H, Mukhtar H. Photodynamic therapy in dermatology. *J Am Acad Dermatol* [Internet]. 2000 Mar;42(3):389-413-6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10688709
28. Yazdani Abyaneh MA, Falto-Aizpurua L, Griffith RD, Nouri K. Photodynamic therapy for actinic cheilitis: A systematic review. *Dermatologic Surg.* 2015;41(2):189-98.

29. Almutawa F, Thalib L, Hekman D, Sun Q, Hamzavi I, Lim HW. Efficacy of localized phototherapy and photodynamic therapy for psoriasis: A systematic review and meta-analysis. *Photodermatology Photoimmunology and Photomedicine.* 2015. p. 5-14.
30. Morton C a, Whitehurst C, McColl JH, Moore J V, MacKie RM. Photodynamic therapy for large or multiple patches of Bowen disease and basal cell carcinoma. *Arch Dermatol* [Internet]. 2001 Mar;137(3):319-24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11255332
31. Choudhary S, Nouri K, Elsaie ML. Photodynamic therapy in dermatology: A review. *Lasers Med Sci*. 2009;24(6):971-80.
32. Babilas P, Karrer S, Sidoroff A, Landthaler M, Szeimies R-M. Photodynamic therapy in dermatology – an update. *Photodermatol Photoimmunol Photomed* [Internet]. 2005 Jun;21(3):142-9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15888131
33. Biel M a. Photodynamic therapy in head and neck cancer. *Curr Oncol Rep* [Internet]. 2002 Jan;4(1):87-96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11734119
34. Arumainayagam N, Moore CM, Ahmed HU, Emberton M. Photodynamic therapy for focal ablation of the prostate. *World J Urol*. 2010;28(5):571-6.
35. Moore CM, Pendse D, Emberton M. Photodynamic therapy for prostate cancer – a review of current status and future promise. *Nat Clin Pract Urol*. 2009;6(1):18-30.
36. Moore CM, Nathan TR, Lees WR, Mosse CA, Freeman A, Emberton M, et al. Photodynamic therapy using meso tetra hydroxy phenyl chlorin (mTHPC) in early prostate cancer. *Lasers Surg Med*. 2006;38(5):356-63.
37. Waidelich R, Beyer W, Kn??chel R, Stepp H, Baumgartner R, Schr??der J, et al. Whole bladder photodynamic therapy with 5-aminolevulinic acid using a white light source. *Urology* [Internet]. 2003 Feb [cited 2014 Jun 25];61(2):332-7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0090429502021647
38. Yavari N, Andersson-Engels S, Segersten U, Malmstrom P-U. An overview on preclinical and clinical experiences with photodynamic therapy for bladder cancer. *Can J Urol*. 2011;18(4):5778-86.
39. Maziak DE, Markman BR, MacKay JA, Evans WK. Photodynamic therapy in nonsmall cell lung cancer: a systematic review. *Ann Thorac Surg*. 2004;77(4):1484-91.
40. Akimoto J. Photodynamic Therapy for Malignant Brain Tumors. *Neurol Med Chir* (Tokyo). 2016;56(4):151-7.
41. Friesen SA, Hjortland GO, Madsen SJ, Hirschberg H, Engebraten O, Nesland JM, *et al*. 5-Aminolevulinic acid-based photodynamic detection and therapy of brain tumors (review). *International Journal of Oncology*. 2002. p. 577-82.
42. Blanco KC, Moriyama LT, Inada NM, Kurachi C, Salvio AG, Leite EJ dos S, *et al*. Fluorescence Guided PDT for Optimization of Skin Cancer Treatment [Internet]. *Frontiers in Physics*. 2015. Available from: http://www.frontiersin.org/Journal/Abstract.aspx?s=106&name=biomedical_physics&ART_DOI=10.3389/fphy.2015.00030
43. Moseley H, Allen JW, Ibbotson S, Lesara, McNeilla, Camacho-Lopez MA, *et al*. Ambulatory photodynamic therapy: a new concept in delivering photodynamic therapy. *Br J Dermatol*. 2006;154(4):747-50.

44. Kashef N, Hamblin MR. Can microbial cells develop resistance to oxidative stress in antimicrobial photodynamic inactivation? *Drug Resist Updat*. 2017;31:31-42.
45. Becker TL, Paquette AD, Keymel KR, Henderson BW, Sunar U. Monitoring blood flow responses during topical ALA-PDT. *Biomed Opt Express* [Internet]. 2010 Jan;2(1):123-30. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3028487&tool=pmcentrez&rendertype=abstract
46. Douplik A, Stratonnikov A, Zhernovaya O, Loshchenov V. Modifications of Optical Properties of Blood during Photodynamic Reactions In vitro and In vivo. *Advanced Optical Flow Cytometry: Methods and Disease Diagnoses*. 2011. p. 627-98.
47. Mima EG de O, Pavarina AC, Dovigo LN, Vergani CE, Costa CA de S, Kurachi C, *et al*. Susceptibility of *Candida albicans* to photodynamic therapy in a murine model of oral candidosis. *Oral Surgery Oral Med Oral Pathol Oral Radiol Endodontology*. Elsevier Inc.; 2010;109(3):392-401.
48. Maia LGM, Alves AVF, Bastos TS, Moromizato LS, Lima-Verde IB, Ribeiro MAG, *et al*. Histological analysis of the periodontal ligament and alveolar bone during dental movement in diabetic rats subjected to low-level laser therapy. *J Photochem Photobiol B Biol*. 2014;135:65-74.
49. Dovigo LN, Pavarina AC, Carmello JC, MacHado AL, Brunetti IL, Bagnato VS. Susceptibility of clinical isolates of Candida to photodynamic effects of curcumin. *Lasers Surg Med*. 2011;43(9):927-34.
50. Geralde MC, Pratavieira S, Bagnato VS. Stability of Indocyanine Green for clinical use. *Proceedings Volume 10417, Medical Laser Applications and Laser-Tissue Interactions VIII*; 104170S (2017); doi: 10.1117/12.2282148.
51. Araújo NC, Fontana CR, Bagnato VS, Gerbi MEM. Photodynamic Effects of Curcumin Against Cariogenic Pathogens. *Photomed Laser Surg* [Internet]. 2012;30(7):393-9. Available from: http://online.liebertpub.com/doi/abs/10.1089/pho.2011.3195
52. Wilson M, Mia N. Sensitisation of Candida albicans to killing by low-power laser light. *J Oral Pathol Med*. 1993;22(8):354-7.
53. Quishida CC, Carmello JC, Mima EG, Bagnato VS, Machado AL, Pavarina AC. Susceptibility of multispecies biofilm to photodynamic therapy using Photodithazine®. *Lasers Med Sci*. 2015;30(2):685-94.
54. Panhóca VH, Geralde MC, Corrêa TQ, Carvalho MT, Souza WO, Bagnato VS. Enhancement of the Photodynamic Therapy Effect on Streptococcus Mutans Biofilm. *J Phys Sci Appl*. 2014;4(2):107-14.
55. Dovigo LN, Pavarina AC, Ribeiro DG, Adriano CS, Bagnato VS. Photodynamic inactivation of four Candida species induced by Photogem®. *Brazilian J Microbiol*. 2010;41(1):42-9.
56. Mima EGDO, Pavarina AC, Ribeiro DG, Dovigo LN, Vergani CE, Bagnato VS. Effectiveness of photodynamic therapy for the inactivation of Candida spp. on dentures: in vitro study. *Photomed Laser Surg*. 2011;29(12):827-33.
57. Geralde MC, Leite IS, Inada NM, Salina ACG, Medeiros AI, Kuebler WM, et al. Pneumonia treatment by photodynamic therapy with extracorporeal illumination – an experimental model. *Physiol Rep*. 2017;5(5):e13190.
58. Blanco KC, Inada NM, Carbinatto FM, Giusti AL, Bagnato VS. Treatment of Recurrent Pharyngotonsillitis by Photodynamic Therapy. *Photo-*

diagnosis Photodyn Ther. Elsevier B.V.; 2017;

59. Blanco, Kate & L Giusti, Andre & , Santos & Inada, Natalia & Bagnato,V. (2017). Curcumin gum: use of photodynamic therapy in children with acute pharyngotonsillitis. *International Journal of Current Medical and Pharmaceutical Research*. 10.24327/23956429.ijcmpr20170003.
60. Silva AP Da, Kurachi C, Bagnato VS, Inada NM. Fast elimination of onychomycosis by hematoporphyrin derivative-photodynamic therapy. *Photodiagnosis Photodyn Ther* [Internet]. Elsevier B.V.; 2013;10(3):328-30. Available from: http://dx.doi.org/10.1016/j.pdpdt.2013.01.001
61. Coelho VHM, Alvares LD, Carbinatto FM, de Aquino Junior AE, Angarita DPR et al. (2017) Photodynamic Therapy, Laser Therapy and Cellulose Membrane for the Healing of Venous Ulcers: Results of a Pilot Study. *J Nurs Care* 6:387. doi:10.4172/2167-1168.1000387
62. Araújo NC, Fontana CR, Bagnato VS, Gerbi MEM. Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. *Lasers Med Sci* [Internet]. 2014;29(2):629-35. Available from: http://link.springer.com/10.1007/s10103-013-1369-3
63. de Souza LM, Pratavieira S, Inada NM, Oliveira KT, Kurachi C, Bagnato VS, Evaluation of photodynamic effects of curcumin against the dengue vector – *Aedes aegypti* (Diptera: Culicidae). *Photodiagnosis and Photodynamic Therapy*, Volume 12, Issue 3, 346-347.
64. de Souza LM, Pratavieira S, Inada NM, Kurachi C, Corbi J, Guimarães FEG, *et al*. Efficacy of photodynamic therapy against larvae of *Aedes aegypti*: confocal microscopy and fluorescence-lifetime imaging. *Proc SPIE 8947, Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues XII* [Internet]. 2014. p. 89472D-89472D-9. Available from: http://dx.doi.org/10.1117/12.2040420

RNA Processing, Ageing and Neurodegeneration

Francisco Ernesto Baralle[1]

Introduction

RNA splicing, a fundamental and unexpected step in RNA metabolism, was observed for the first time 40 years ago. In the genetic translation code, deciphered in bacteria between the 1950s and 1960s, the gene to be read had a contiguous sequence that was transcribed to mRNA and this in turn translated into protein. Nobody imagined for over a decade that genes could be discontinuous. A peculiar RNA processing phenomena was described in the adenovirus 5'UTR in the late 1970s; three pieces of noncoding sequences were joined together and the sequences between them eliminated to form the viral mRNA (Chow *et al.*, 1977).

This was soon followed by the observation that the recently cloned globin mRNA did not hybridized in a single band in a Southern blot but there were clearly other sequences, interrupting the coding sequences (Jeffreys and Flavell, 1977). These genomic nucleotide fragments were even longer than the coding sequences. Finally, after the, at the time, laborious process of cloning higher eukaryotic genes, the sequencing of these clones showed that the presence of noncoding sequences that interrupted the coding sequences was the rule rather than the exception. The name of introns and exons was adopted for the intervening sequences and the coding sequences respectively and the prediction made that the protein coding content of genomes would comprise only a fraction of the total DNA (Gilbert, 1978). The process of cutting the introns and joining the exons was called splicing. This discovery had immediate ramifications regarding biology, namely, how are the exons identified?

The first gene sequences showed, surprisingly, poor sequence homology at the junctions of introns and exons. The only universally conserved nucleotides were the GU at the 5' site of the intron and AG at its 3' site (Breathnach *et al.*, 1978)1978 Obviously, these elements would be insufficient to direct the splicing process which is usually error free. In the following years other features were uncovered that increased the accuracy

[1] ICGEB-Padriciano 99-Trieste.

of exon/intron junction definition resulting in the so-called consensus sequences around the exon/intron junctions (splice sites) and the intronic polypirimidine tract near the 3' splice site (Shapiro and Senapathy, 1987).

In the early 1980s it was discovered that there were variations in exon selection and that several mRNA isoforms could be produced from one pre-mRNA (Kornblihtt *et al.*, 1984), this process was called alternative splicing and was followed by the discovery both *in vitro* and *in vivo* that auxiliary sequences overlapping with the coding sequences in the genomic context of an alternative spliced exon (Mardon *et al.*, 1987; Reed and Maniatis, 1986). Subsequently, specific cis-acting elements within these fragment were identified that according to their location and effect on splicing were called exon splicing enhancers or silencers (ESE and ESS respectively) and intron splicing enhancers and silencers (ISE and ISS respectively) whose effects are transduced through their interaction with RNA binding proteins (RBPs). Akin to the splice site consensus motifs these elements are not particularly conserved. The reader can start to guess that talk of a univocal splicing code will be difficult. In addition to the cis-acting element, other processes that influence splicing outcome exist. They include the effect on splicing of the RNA structure (Buratti and Baralle, 2004), the transcription rate (Kornblihtt, 2007), chromatin remodelling and epigenetic modifications (Luco *et al.*, 2011).

Alternative splicing is a ubiquitous regulatory mechanism that single genes utilize to produce more than one mRNA transcript. Alternative transcripts can differ in untranslated regions affecting mRNA stability, localization, or translation or can be translated into different protein isoforms with diverse functions and/or localizations. Alternative splicing is not an exception: in humans more than 90% of the genes are estimated to undergo alternative splicing. For a review see (Baralle and Giudice, 2017). From the ~20,000 human protein-coding genes, high-resolution mass spectrometry analyses have shown that ~37% of them generates multiple protein isoform (Kim *et al.*, 2014) evidencing alternative splicing contribution to protein diversification. Furthermore, the physiological importance of alternative splicing is highlighted by the enormous number of human diseases caused by mutations in *cis*-acting RNA-sequence elements, *trans*-acting splicing factors or spliceosome components.

Alternative splicing is a main player in cell linage and tissue-identity acquisition and maintenance, cell differentiation, and tissue/organ development. Molecular understanding of developmental transitions has also revealed important bases of pathological mechanisms in diseases where

normal networks are mis-regulated. Multiple mechanisms regulate splicing in nature, in particular during development. While strong splice sites (more similar to consensus sequences) lead to constitutive mRNA splicing, weak splice sites are more different to consensus sequences, thus less efficiently recognized by the splicing machinery and recognition is highly dependent on the cellular context (alternative splicing). There are different classes of alternative splicing events and are classified based on the location of the splice sites: insertion of alternative cassette exons or mutually exclusive exons, selection between alternative 5' or 3' splice sites, intron retention, and alternative usage of polyadenylation sites. Usage of weak splice sites is controlled by *cis*-regulatory sequences in precursor mRNAs (pre-mRNAs) (exonic splicing enhancers and silencers and intronic splicing enhancers and silencers) and *trans*-acting factors mainly RNA-binding proteins (RBPs) that by binding to these elements promote or inhibit site recognition. There are a myriad of splicing factors, positive and negative many of them belong to the RNA binding proteins family, the positive factors in splicing are often the Serine-Arginine (SR) proteins and the negative factors are Heterogeneous Nuclear Ribonucleoproteins (hnRNPs). TDP 43 is a member of this latter family that our laboratory characterized as an hnRNP with a role in splicing events leading to Cystic Fibrosis (Buratti and Baralle, 2001). Further studies showed that TDP 43 is involved in multiple processes in RNA metabolism ranging from splicing, micro RNA processing mRNA transport and stability. In addition it shuttles between nucleus and cytoplasm and regulates its cellular levels by a novel mechanism involving an unusual splicing event in the 3'UTR of the pre mRNA. A decade ago TDP 43 has jumped to fame when was identified as the main component of the protein inclusions seen in the brain of people affected by Amyotrophic Lateral Sclerosis (ALS) and Fronto Temporal Lobar Degeneration (FTLD) (Neumann *et al.*, 2006).

TDP 43 role in ALS

Histological analysis of patient's brain showed TDP 43 inclusions in many neurons some in the nucleus, some in the cytoplasm and even in their axons. TDP 43 is a mostly nuclear protein seen diffusely in the nucleoplasm. However most of the cells containing inclusions have nucleus that were negative for TDP 43 staining. Two hypothesis were proposed for the pathogenesis of ALS one that is due to the toxicity of the inclusions and the other one that the lack of TDP 43 in the nucleus provokes a loss of function in the multiple processes that this protein is involved in such as

RNA splicing, mRNA transport and stability, miRNA processing (Buratti and Baralle, 2012). We have gathered a substantial body of evidence that shows that the latter hypothesis may be the correct one. In fact depletion of TDP 43 in tissue culture cells, obtained by siRNA treatment, produces a series of changes that affect the viability of the cells and splicing variations are clearly visible. Furthermore removing the gene in mice produces disorganization of the embryo and is an early lethal mutation. The fruit fly Drosophila has a homolog of TDP 43 called TBPH, deletion of the gene allows the development of the larva up to the pupa but the adult fly has serious locomotion defects that do not allow its exit from the pupa. If the fly is removed manually can be seen that is unable to move properly and die within 1-2 days. This data pointed to a lack of function model of the disease. This is what should be expected if the aggregates sequester TDP 43 and prevent its functionality. We decided to model the aggregation process in tissue culture cells and in flies.

Modelling ALS like TDP 43 aggregation

Besides the animal models, several attempts were done to mimic the TDP-43 aggregation in cells in culture. Such models are valuable to investigate the impact of aggregation on the cellular metabolism, as well as to evaluate new therapeutic strategies to overcome aggregation.

It was early observed that the TDP-43 C-terminal tail contains a Q/N rich region that is involved in the protein-protein interactions (D'Ambrogio *et al.*, 2009) Moreover, it was showed that expression of C-terminal fragments of TDP-43 is sufficient to generate cytoplasmic aggregates (Igaz *et al.*, 2009). The importance of the Q/N rich region within the C-terminal tail of TDP-43 in the self-aggregation process was also confirmed (Fuentealba *et al.*, 2010). The majority of the mutations that have been found in ALS patients are localized in the C-terminal tail and the aggregation tendency is enhanced by these ALS-linked TDP-43 mutations (Budini *et al.*, 2012b). In addition, the protein is cleaved, generating C-terminal fragments that are associated with cellular toxicity and/or increased TDP-43 mislocalization (Zhang *et al.*, 2009). Based on these findings, and with the aim of looking for methodologies that could model the disease, our laboratory developed a cellular model of aggregation using a 30 amino acid TDP-43 C-terminal peptide to promote TDP-43 aggregation (Budini *et al.*, 2012a; Budini *et al.*, 2012b).

The introduction of tandem repeats of TDP-43 Q/N rich amino acid sequence 331-369 (12xQ/N) linked to EGFP reporter is able to trigger

the formation of predominantly cytoplasmic aggregates, capable of sequestering either exogenous or endogenous full-length TDP-43, recapitulating some of the features of the inclusions present in patients, such as ubiquitination and phosphorylation.

However, there was no detectable splicing function deterioration in the presence of these TDP-43 aggregates induced by EGFP-12xQ/N, suggesting that they were not efficient enough in trapping endogenous TDP-43 to cause a loss of function in the short interval measured in a cell system. In fact, it can be seen in that there is still TDP-43 present in the nuclei of EGFP-12xQ/N expressing cells.

In order to generate a model that could accomplish the nuclear loss of function of TDP-43 that is characteristic of ALS, a new variant of the previous model was generated. This new model is based on the TDP-43 molecule itself linked to the tandem repeats 12xQ/N (TDP-12xQ/N).

The TDP-12xQ/N model was shown to induce TDP-43 aggregation that was accompanied by TDP-43 nuclear depletion and consequent alteration of its splicing function (Budini *et al.*, 2015).

Our cell-based models of ALS are useful tools for the identification of active agents capable of reducing TDP-43 inclusions. In fact as a proof of principle we have tested a series of tricyclic anti depressive drugs that showed a reasonable activity eliminating aggregates by stimulation of the cell clearing systems and restoring TDP 43 functionality. This model is now used in a wide new molecule screening for drugs and is showing optimal results.

We have now also generated an animal model based on this 12xQ/N construct. We have used in the first instance *Drosophila melanogaster* a powerful model to study human neurodegenerative diseases. Several characteristics make *Drosophila* the organism of choice. Among them, the short generation time (approximately 10 days) and short life span (around 60 to 80 days). In particular these features make *Drosophila* amenable to study age-related disorders. In addition, approximately 75% of human genes known to be associated with disease have a *Drosophila* ortholog. In most neurodegenerative disease, specific neuronal regions begin to degenerate late in life. In order to study this, several methods are available to express genes in a spatially and temporally restricted manner. Moreover, synaptic activity can be measured using electrophysiological and imaging techniques from the neuromuscular junction and adult central nervous system, making *Drosophila* particularly amenable to study motor neuron diseases, such as ALS.

The Drosophila 12xQ/N transgene showed that its locomotion was compromised with aging and its lifespan was shorter. The animals with the severe locomotion phenotype present a sharp atrophy and retraction of the Neuro-Muscular Junction. The onset of the locomotion phenotype around day 15 happens in mature flies, a similarity with the rather early onset of ALS in humans. This time coincides with a physiological; evolutionary conserved four-fold reduction in the levels of brain TDP43 (TBPH in flies) relative to the one-day-old fly (Cragnaz *et al.*, 2015).

Currently we are extending this investigation to mice where we have also detected a programmed fourfold reduction of TDP 43 in the brain between 10- and 90-day-old animals. We are also investigating the mechanism of this reduction that is tissue specific. In liver the levels are maintained through the lifetime of the animal, in fact there is a mild constant reduction in brain and a sharp reduction in muscle. The modulation of TDP 43 expression seems to be done through an increase of methylation in the promoter of the gene with age specifically in those tissues where there is a decrease of protein levels. As expected there is an inverse correlation with the time and size of the reduction: With age TDP 43 levels are lower in muscle<brain<liver while the degree of methylation goes the opposite way muscle>brain>liver.

In conclusion we have identified two potential therapeutic approaches for TDP 43 proteinopathies. The most promising one is the new drugs that in our cellular model are non-toxic and are very efficient in aggregate clearance and restoration of TDP 43 functionality. The potential of increasing the production of TDP 43 by interfering with the promoter methylation remains to be explored, in the hope that the higher levels will overcome the loss of functionality.

References

Baralle, F.E., and Giudice, J. (2017). Alternative splicing as a regulator of development and tissue identity. *Nature reviews Molecular cell biology 18*, 437-451.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., and Chambon, P. (1978). Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. *Proceedings of the National Academy of Sciences of the United States of America 75*, 4853-4857.

Budini, M., Buratti, E., Stuani, C., Guarnaccia, C., Romano, V., De Conti, L., and Baralle, F.E. (2012a). Cellular model of TAR DNA-binding protein 43 (TDP-43) aggregation based on its C-terminal Gln/Asn-rich region. *The Journal of biological chemistry 287*, 7512-7525.

Budini, M., Romano, V., Avendano-Vazquez, S.E., Bembich, S., Buratti, E., and Baralle, F.E. (2012b). Role of selected mutations

in the Q/N rich region of TDP-43 in EGFP-12xQ/N-induced aggregate formation. *Brain research 1462*, 139-150.

Budini, M., Romano, V., Quadri, Z., Buratti, E., and Baralle, F.E. (2015). TDP-43 loss of cellular function through aggregation requires additional structural determinants beyond its C-terminal Q/N prion-like domain. *Human molecular genetics 24*, 9-20.

Buratti, E., and Baralle, F.E. (2001). Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9. *The Journal of biological chemistry 276*, 36337-36343.

Buratti, E., and Baralle, F.E. (2004). Influence of RNA secondary structure on the pre-mRNA splicing process. *Molecular and cellular biology 24*, 10505-10514.

Buratti, E., and Baralle, F.E. (2012). TDP-43: gumming up neurons through protein-protein and protein-RNA interactions. *Trends in biochemical sciences 37*, 237-247.

Chow, L.T., Gelinas, R.E., Broker, T.R., and Roberts, R.J. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell 12*, 1-8.

Cragnaz, L., Klima, R., De Conti, L., Romano, G., Feiguin, F., Buratti, E., Baralle, M., and Baralle, F.E. (2015). An age-related reduction of brain TBPH/TDP-43 levels precedes the onset of locomotion defects in a Drosophila ALS model. *Neuroscience 311*, 415-421.

D'Ambrogio, A., Buratti, E., Stuani, C., Guarnaccia, C., Romano, M., Ayala, Y.M., and Baralle, F.E. (2009). Functional mapping of the interaction between TDP-43 and hnRNP A2 in vivo. *Nucleic acids research 37*, 4116-4126.

Fuentealba, R.A., Udan, M., Bell, S., Wegorzewska, I., Shao, J., Diamond, M.I., Weihl, C.C., and Baloh, R.H. (2010). Interaction with polyglutamine aggregates reveals a Q/N-rich domain in TDP-43. The Journal of biological chemistry *285*, 26304-26314.

Gilbert, W. (1978). Why genes in pieces? *Nature 271*, 501.

Igaz, L.M., Kwong, L.K., Chen-Plotkin, A., Winton, M.J., Unger, T.L., Xu, Y., Neumann, M., Trojanowski, J.Q., and Lee, V.M. (2009). Expression of TDP-43 C-terminal Fragments in Vitro Recapitulates Pathological Features of TDP-43 Proteinopathies. *The Journal of biological chemistry 284*, 8516-8524.

Jeffreys, A.J., and Flavell, R.A. (1977). The rabbit beta-globin gene contains a large large insert in the coding sequence. *Cell 12*, 1097-1108.

Kim, M.S., Pinto, S.M., Getnet, D., Nirujogi, R.S., Manda, S.S., Chaerkady, R., Madugundu, A.K., Kelkar, D.S., Isserlin, R., Jain, S., *et al.* (2014). A draft map of the human proteome. *Nature 509*, 575-581.

Kornblihtt, A.R. (2007). Coupling transcription and alternative splicing. *Advances in experimental medicine and biology 623*, 175-189.

Kornblihtt, A.R., Vibe-Pedersen, K., and Baralle, F.E. (1984). Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. *The EMBO journal 3*, 221-226.

Luco, R.F., Allo, M., Schor, I.E., Kornblihtt, A.R., and Misteli, T. (2011). Epigenetics in alternative pre-mRNA splicing. *Cell 144*, 16-26.

Mardon, H.J., Sebastio, G., and Baralle, F.E. (1987). A role for exon sequences in alternative splicing of the human fibronectin gene. *Nucleic acids research 15*, 7725-7733.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., *et al.* (2006). Ubiquitinated TDP-43 in frontotempo-

ral lobar degeneration and amyotrophic lateral sclerosis. *Science 314*, 130-133.

Reed, R., and Maniatis, T. (1986). A role for exon sequences and splice-site proximity in splice-site selection. *Cell 46*, 681-690.

Shapiro, M.B., and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic acids research 15*, 7155-7174.

Zhang, Y.J., Xu, Y.F., Cook, C., Gendron, T.F., Roettges, P., Link, C.D., Lin, W.L., Tong, J., Castanedes-Casey, M., Ash, P., *et al*. (2009). Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proceedings of the National Academy of Sciences of the United States of America 106*, 7607-7612.

Galectin-1: An Emerging Target in Cancer Immunotherapy and Autoimmune Inflammation

Gabriel Adrián Rabinovich[1]

1. Galectins: Professional decoders of glycan-containing information

The physiologic roles of glycans are highly diverse and include critical functions in cell-cell communication and adhesion, membrane topology, cellular signaling and immunomodulation. Programmed remodeling of cell surface glycans through the synchronized action of glycan-modifying enzymes (glycosyltransferases and glycosidases) is a typical hallmark of immune cell activation, differentiation, trafficking and homeostasis. The responsibility of decoding the biological information encrypted by these glycan structures relies on endogenous glycan-binding proteins or lectins, whose expression patterns are regulated during the course of innate and adaptive immune responses (1).

Galectins, a family of soluble glycan-binding proteins, play multiple roles in innate and adaptive immunity (2). To date, fifteen galectins have been identified in mammals, most with wide tissue distribution, including galectin (Gal)-1 and -3, although some members of the family are preferentially expressed in certain tissues (e.g. Gal-12 in adipose tissue, Gal-7 in the skin and Gal-4 in the gastrointestinal tract). From a structural standpoint, galectins share a common fold and at least one conserved carbohydrate-recognition domain (CRD) of about 130 amino acids that mediates carbohydrate recognition. A traditional classification based on structural similarities includes: a) 'proto-type' galectins (Gal-1, -2, -5, -7, -10, -11, -13, -14 and -15) which have one CRD and may exist as monomers or dimers; b) 'tandem repeat-type' galectins (Gal-4, -6, -8, -9 and -12) which contain two different CRDs separated by a linker of up to 70 amino acids and c) the 'chimera-type' Gal-3 which contains a CRD connected to

[1] Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), C1428 Buenos Aires, Argentina; Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428 Buenos Aires, Argentina. Correspondence: gabriel.r@ibyme.conicet.gov.ar (G.A. Rabinovich).

a non-lectin N-terminal region (2). Most galectins are either bivalent or multivalent with regard to their carbohydrate-binding properties, which enable interaction with several binding partners and activation of different signaling pathways; one-CRD galectins can dimerize, two-CRD galectins are at least bivalent, and Gal-3 can form oligomers upon binding to multivalent glycosylated receptors (2).

Although galectins do not contain a classical secretory signal required for externalization, some members are released through an unusual route, independent of the endoplasmic reticulum (ER)/Golgi pathway (2). Once outside the cells, galectins bind to multiple glycosylated ligands and convey glycan-encoded information into immune cell programs including immune cell activation, differentiation, migration and apoptosis (2). Mechanistically, these proteins may function by forming ordered arrays of lectin-glycan structures – often termed 'lattices' – on the cell surface or through direct engagement of specific cell surface glycoconjugates. However, this view is limited to those galectins that are secreted; some members of the family may function primarily within the intracellular compartment. This includes Gal-3 and Gal-10, which play critical intracellular roles either by modulating signaling pathways and pre-mRNA splicing or by controlling the suppressive capacity of regulatory T (Treg) cells (2, 3).

The minimal structure recognized by galectins is the disaccharide N-acetyllactosamine (Galb1,4GlcNAc; LacNAc) which is found in *N*- and *O*-linked glycans and may be presented as multiple units (poly-LacNAc) on cell surface glycoproteins. However, considerable differences exist in glycan-binding preferences among individual members of the galectin family, which might underscore the basis for functional divergences in biological activity. These variations in glycan recognition are mainly associated with the extent of N-glycan branching, the multiplicity of LacNAc residues and/or the modification of terminal saccharides (i.e. sialylation or fucosylation)(4,5). Interestingly, different galectins may selectively co-opt different glycosylated receptors despite their similar glycan structure, suggesting the importance of both protein-glycan and protein-protein recognition systems to galectin-receptor interactions (2). Here I focus on Gal-1 as a representative member of the galectin family and discuss its contribution of to immune tolerance and homeostasis and its therapeutic value in chronic inflammatory microenvironments and cancer.

2. Galectins in immune cell homeostasis

Studies performed over the past decade have illuminated several pathways involved in immune regulation, tolerance and homeostasis. In this

sense, Gal-1 has emerged as a novel regulatory checkpoint molecule that controls activation, trafficking, differentiation, exhaustion and survival of a variety of lymphoid and myeloid cell populations including T cells, B cells, dendritic cells (DCs), macrophages, neutrophils and myeloid-derived suppressor cells (MDSCs) (6).

2A. Effector T cells

Effector T cells are key players in steering the immune responses to execute helper and cytotoxic functions. The differentiation of naïve T cells into fully functional effector CD4 T cells is characterized by the acquisition of new profiles of cytokine production and trafficking patterns with specialized pro-or anti-inflammatory activities. On the other hand, effector CD8 T cells display full cytotoxic activity and interferon (IFN)-g production. Interestingly, Gal-1 controls activation, differentiation, viability and trafficking of both CD4 and CD8 effector T cells (6). Early studies demonstrated the ability of Gal-1 to induce apoptosis of activated, but not resting T cells through binding to LacNAc residues on CD45, CD43 and CD7 glycoproteins through caspase-dependent or independent mechanisms (7-9). Remarkably, we found that Gal-1 acts by selectively deleting Th1 and Th17 cells thus providing a rational explanation for the Th2 bias observed following administration of this lectin (10). In fact, Th1 and Th17 effector cells but not Th2 cells share the repertoire of glycans that are important for Gal-1 binding and apoptosis (particularly high frequency of core 2-O-glycans and low amounts of a2,6-linked sialic acid) (10). Additionally, Gal-1 inhibits T-cell adhesion and trafficking across the extracellular matrix (ECM), suppresses production of pro-inflammatory cytokines including IFN-g and tumor necrosis factor (TNF) and enhances secretion of IL-10, a typical anti-inflammatory cytokine through mechanisms that are independent of its pro-apoptotic activity (11-14). Furthermore, Gal-1 may also antagonize T-cell activation (15) and inhibit differentiation of naïve T cells into Th17 cells (16), highlighting multiple strategies used by this lectin to limit effector T-cell responses.

2B. Regulatory T cells

Regulatory T cells are key players in maintaining the balance between immune activation and tolerance. These cells can silence exuberant or undesired immune responses by restraining inflammation to self antigens, commensal microbiota, allergens, and pathogens, thus preventing autoimmune and autoinflammatory disorders. The so-called, inducible CD4+

regulatory T cells (iTregs) are generated outside the thymic compartment to regulate peripheral immune tolerance, whereas thymus-derived naturally-occurring CD4+ regulatory T cells (nTregs) are generated within the thymus. Depending on whether they stably express the forkhead box P3 (Foxp3) transcription factor, iTregs may be divided into two subsets: the classical TGF-b-induced CD4+Foxp3+ Tregs and the CD4+Foxp3- type 1 regulatory T (Tr1) cells. *In vitro and in vivo* Gal-1 treatment promotes the expansion of both Foxp3+ and Foxp3(-) Tregs and contributed to their immunosuppressive activity (14, 17-20). Targeted disruption of Gal-1, using biochemical or genetic approaches, attenuated the suppressive effects of human and mouse Tregs, suggesting the involvement of this lectin in Treg cell-mediated immunosuppression (17-20). This effect was verified in models of autoimmune retinal disease (14), fetomaternal tolerance (19), cardiac inflammation (21) and cancer (18, 22). Thus, Gal-1 contributes to differentiation, expansion and function of different Treg populations. Given the contribution of other galectin family members to T-cell biology (2), further studies are warranted to investigate their coordinated effects and selective contributions to modulation of effector and regulatory T cell balance.

2C. Natural killer cells

Natural killer (NK) cells, a type of group 1 innate lymphocytes (ILCs), are characterized by their potent cytotoxic activity and secretion of pro-inflammatory (IFN-g and TNF) cytokines. They are best known for killing virally-infected cells, and detecting and controlling early signs of cancer. Although not studied in such detail as T cells, Gal-1 may also influence NK cell biology by modulating trafficking and function of these cells. In fact, targeted deletion of Gal-1 gene expression in tumor cells resulted in augmented NK cell recruitment (23) and enhanced cytotoxic activity of these cells (24).

2D. B cells

In addition to their well-established roles in antigen recognition and processing and their unique function as precursors of immunoglobulin-producing plasma cells, B cells may also become regulatory suppressing immunopathology and silencing the expansion of pathogenic T and B cells. Within the B cell compartment, early studies from our group showed that activated B cells synthesize and secrete elevated levels of Gal-1 to curtail the survival of neighboring activated T cells (25). Moreover, Gal-1 was found to be up-regulated in immunoglobulin (Ig)M+ memory B cells and induced B-cell apoptosis through inhibition of Akt phosphorylation and

up-regulation of the BH3-only protein Bim (26). Interestingly, although Gal-1 suppressed mature B cell signaling and contributed to B cell death, it increased plasma cell differentiation and survival, suggesting that this lectin may be part of the intracellular signaling machinery that governs B cell fate (27). Finally, elegant studies reported that, during B-cell development, Gal-1 secreted by bone marrow stromal cells acts as a pre-B cell receptor ligand to modulate B-cell maturation through mechanisms involving displacement of Gal-1/glycan lattices and shift toward a glycosylation-independent Gal-1-mediated protein-protein interaction (28). These studies suggest that Gal-1 may play different regulatory roles during the lifespan of B cells, targeting their maturation, differentiation, signaling, and survival.

2E. Dendritic cells

In spite of their traditional role in orchestrating adaptive immunity, conventional DC subsets, including myeloid DCs and plasmacytoid DCs, can also attenuate inflammatory reactions by promoting T cell anergy or by favoring the expansion, differentiation and/or recruitment of Tregs. Within the DC compartment, we found that both human and mouse DCs differentiated or matured in a Gal-1-enriched microenvironment acquired a distinctive regulatory or tolerogenic signature characterized by abundant secretion of IL-27 and IL-10 (29). When transferred *in vivo*, Gal-1-conditioned DCs promoted T-cell tolerance in antigen-specific settings, blunted Th1 and Th17 responses and halted autoimmune inflammation through mechanisms involving DC-derived IL-27 and T cell-derived IL-10 (29). Interestingly, we found that *Trypanosoma cruzi*, a parasite responsible of Chagas' disease instructs differentiation of tolerogenic DCs and blunts Th1 and CD8+-mediated anti-parasite immunity (30). Moreover, Gal-1 also represses antibacterial programs and promotes tolerogenic circuits during infection with *Yersinia enterocolitica*, a highly prevalent enteropathogenic bacterium (31). Accordingly, Thiemann et al. demonstrated that this lectin inhibits tissue emigration of immunogenic, but not tolerogenic DCs, through ECM through mechanisms involving differential expression of core-2 *O*-glycans on CD43 on immunogenic versus tolerogenic DCs (32). However, not only exogenous but also endogenous Gal-1 can endow DCs with tolerogenic capacity. In fact, Gal-1-expressing DCs greatly contributed to the resolution of antigen-specific and autoimmune diseases (29). Supporting these findings, shRNA-mediated knockdown of human Gal-1 on DCs or experiments using Gal-1 KO (*Lgals1*-/-) DCs, revealed the importance of this lectin in DC inhibition of T cell responses (29, 33).

2F. Monocytes, macrophages and microglia

Cells of the monocyte/macrophage lineage are characterized by their functional diversity and plasticity. In response to a broad spectrum of stimuli (e.g. TLR agonists, microbial products or cytokines), tissue macrophages may undergo M1 (pro-inflammatory or classically-activated) or M2 (anti-inflammatory or alternatively-activated) polarization, leading to exacerbation or alleviation of immune responses. We found upregulated expression of Gal-1 in activated macrophages (34) which contributes to immunoregulation by tilting the cytokine balance toward an M2 profile, characterized by low nitric oxide production and up-regulation of arginase activity (35). This inhibitory function was confirmed in human monocytes, which upon exposure to Gal-1, exhibited a dose-dependent reduction of IFN-γ-induced MHC II expression and MHC II-dependent antigen presentation (36). Supporting these findings, exposure to Gal-1 inhibited arachidonic acid release and prostaglandin E2 (PGE2) synthesis (37) and favored the conversion of macrophages toward a pro-resolving phenotype, characterized by CD11blow surface expression, up-regulated activity of the 12/15-lipoxygenase (a pro-resolving enzyme), loss of phagocytic capacity and diminished TNF and IL-1 secretion (38), suggesting that exogenous Gal-1 may promotion of a pro-resolving state within the monocyte/macrophage compartment. In this regard, one might speculate that phagocytic clearance of dying cells or cells exposing phosphatidylserine as a result of Gal-1 treatment (39), might also contribute to the generation of anti-inflammatory phagocytes and restoration of immune cell homeostasis. Interestingly, in a model of autoimmune neuroinflammation, we found that astrocytes produce high amounts of Gal-1, which was sufficient to convert inflammatory M1-type microglia toward a neuroprotective anti-inflammatory M2-type phenotype. Mechanistically, astrocyte-derived Gal-1 bound to core 2-*O*-glycans on CD45, retained this glycoprotein on the surface of microglia cells and increased its phosphatase activity, thereby promoting microglia de-activation (40). This effect involved modulation of the p38 MAPK, cyclic adenosine monophosphate (cAMP) response element-binding (CREB) and NF-kB-dependent pathways and suppression of pro-inflammatory cascades mediated by iNOS, TNF and the chemokine (C-C motif) ligand 2 (CCL2) (40). As a result, alternatively-activated M2 microglia prevented inflammation-induced neurodegeneration and abrogated the demyelination process (40).

2G. Neutrophils and myeloid-derived suppressor cells

Polymorphonuclear neutrophils (PMN) play diverse roles in immune cell homeostasis by positively or negatively regulating innate immunity. Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature and mature myeloid cells that expand and accumulate under pathological conditions, such as cancer, acute and chronic infections, trauma and autoimmune diseases. These cells have been identified in most patients and experimental models based on their ability to dampen effector T cell responses. Early studies showed that Gal-1 inhibits extravasation and trafficking of polymorphonuclear neutrophils (PMN) to inflamed tissues (37, 41). Moreover, Gal-1 also contributes to expand a subset of brain-infiltrating MDSCs with powerful immunosuppressive activity in a model of glioblastoma (42). Finally, we recently identified a circuit linking microbiota, tumor-promoting inflammation and immunosuppression via a galectin-dependent mechanism. Briefly, in Toll-like receptor (TLR)5-responsive tumors, systemic IL-6 induced in response to TLR5 signaling drove the mobilization of granulocyte MDSCs, which in turn promoted expansion of Gal-1-secreting γδ-T lymphocytes, disarming antitumor immune responses and accelerating malignant progression in ovary tumors (43).

2H. Eosinophils

Eosinophils play an important role in the body's response to allergic reactions, asthma, and infection with helminth parasites. Recent studies revealed dose- and *N*-glycan-dependent effects of Gal-1 on eosinophils. Whereas low concentrations of this lectin promoted eosinophil adhesion and inhibited eotaxin-1-induced migration, exposure to higher concentrations resulted in ERK1/2-dependent apoptosis and disruption of F-actin cytoskeleton (44). *In vivo*, allergen-challenged Gal-1-deficient mice showed increased recruitment of eosinophils to the airways and developed airway hyperresponsiveness relative to wild-type mice (44). Thus, Gal-1 has emerged as a novel regulatory checkpoint which disarms both innate and adaptive immune cell programs.

3. Tumors hijack Gal-1 to evade immune responses: a novel target for cancer immunotherapy

Tumors employ different strategies to evade immune responses that limit the efficacy of cancer immunotherapeutic strategies. These include co-option of immune checkpoint molecules, secretion of immunosup-

pressive cytokines and recruitment and expansion of different regulatory cell populations including Tregs, tolerogenic DCs, M2 macrophages and MDSCs (45). The broad immunosuppressive activities of Gal-1 together with its up-regulated expression in the tumor microenvironment, prompted the investigation of its roles in tumor immunity. Using the B16 melanoma model in early studies we demonstrated the ability of Gal-1 to evade antitumor T cell responses. Targeted inhibition of Gal-1 gene expression in melanoma cells unleashed, otherwise repressed CD4+ and CD8+ T cell responses, resulting in inhibition of tumor growth in syngeneic mice (46). Supporting these observations, disruption of Gal-1 ligands using peracetylated 4-fluoro-glucosamine (4-F-GlcNAc), a metabolic inhibitor of LacNAc biosynthesis, decreased tumor growth in melanoma by stimulating antitumor immunity (47), emphasizing the critical role of Gal-1-glycan interactions in undermining antitumor responses. Supporting these observations further studies in classical Hodgkin lymphoma (cHL), showed the ability of Gal-1 to blunt antitumor immunity by favoring Th2 and Treg responses (22). Interestingly, expression of Gal-1 was found to be up-regulated by oncogenic viruses, suggesting a cross-talk between tumorigenesis and immune escape. In fact, an Epstein Bar virus-driven AP-1-dependent enhancer regulates Gal-1 expression in cHL (22), whereas the Kaposi's sarcoma-associated herpes virus (KSHV) controls Gal-1 expression in human Kaposi's sarcoma cells (23). These findings were further substantiated in models of lung cancer and neuroblastoma indicating that tumor-derived Gal-1 contributes to tumor-immune escape by shaping lymphoid and myeloid cell compartments (49, 50). Expanding these findings to other tumor types, our studies in the 4T1 triple negative breast cancer model showed that Gal-1 also contributes to systemic immunosuppression and metastasis by enhancing the number and function of Tregs (18). Moreover, genetic ablation of Gal-1 in a model of pancreatic ductal adenocarcinoma (PDAC; EIa-myc mice) dampened tumor progression by stimulating a tumor-associated T-cell response (51). Recently, this immune evasive program was elegantly demonstrated in models of glioblastoma, although in this particular tumor type, modulation of the functionality of NK cells was observed (24). Thus, Gal-1 may de-activate both innate and adaptive arms of antitumor immunity, leading to increased tumor progression. However, although Gal-1 is typically up-regulated in cancer cells, in some tumor types immune and/or stromal cells appear to be the main Gal-1 source. Particularly, in ovarian cancer models gd-T lymphocytes were found to be major Gal-1 source that contribute to systemic immunosuppression (43). Also, in human

chronic lymphocytic leukemia (CLL), Gal-1 was found to be predominantly secreted by accompanying myeloid cells (nurse-like cells, macrophages and DCs) and contributes to establish the appropriate microenvironmental conditions required for leukemic progression (51). These results emphasize the common role of Gal-1 in conferring immune privilege to both solid and hematologic tumors. Moreover, recent studies indicated that, in addition to its immunosuppressive activities, Gal-1 also modulates endothelial cell biology by enhancing vascular endothelial growth factor-2 (VEGFR2) signaling, preserving angiogenesis in anti-VEGF refractory tumors (52). Thus, Gal-1 blockade attenuates tumor growth and/or metastasis by potentiating antitumor responses and suppressing aberrant angiogenesis in a variety of cancers, highlighting its potential therapeutic activity either alone or in combination with other r immunotherapeutic modalities.

4. Gal-1-driven immunosuppressive circuits promote resolution of autoimmune inflammation

The inhibitory activities of Gal-1 in lymphoid and myeloid programs, including modulation of T-cell survival, expansion of Tregs and MDSCs, polarization of macrophages toward an M2 profile and induction of tolerogenic DCs, suggested its potential immunosuppressive activity in autoimmune conditions. In fact, systemic Gal-1 therapy or its genetic delivery to sites of inflammation, suppressed clinical, histopathological and immunological manifestations of autoimmune disease in a large number of experimental models, including collagen-induced arthritis, experimental (CIA) autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis (EAU), experimental diabetes, inflammatory bowel disease (IBD), concanavalin-A-induced hepatitis, experimental autoimmune orchitis (EAO) and graft versus host disease (GvHD). Mechanistically, Gal-1 therapy promoted immunosuppression by tilting the cytokine balance toward a Th2 profile, augmenting Treg cell frequency, modulating T-cell survival and polarizing macrophages/microglia toward an M2 anti-inflammatory profile (10, 12, 14, 29, 53, 54).

5. Conclusions and future directions

Advances in deciphering the information encoded by the 'glycome' – the entire repertoire of complex sugar structures expressed in cells and tissues – has created major interest in its potential biotechnological and pharmaceutical applications, but progress has been hampered by the inherent difficulties in studying the structure-function relationship of these com-

plex molecules. However, recent findings provide clear-cut evidence that protein-glycan interactions critically regulate the physiology of immune cells and trigger a broad spectrum of immunoregulatory effects. Following this assumption, glycan-binding proteins such as galectins have been postulated as potential targets of novel anti-inflammatory, immunomodulatory and anti-cancer therapies. This article underscored pioneer findings and recent advances on the biology of Gal-1, the first discovered member of the galectin family, within lymphoid and myeloid compartments, highlighting important pathways by which Gal-1-glycan complexes control tumor immunity and autoimmune inflammation. Thus, targeting Gal-1-glycan interactions may contribute to suppress tumor growth and metastasis by boosting antitumor immune responses. On the other hand, stimulation of Gal-1 signaling may contribute to suppress autoimmune reactions by promoting resolution of exuberant T-cell responses. However before these therapeutic strategies can be fully embraced there is still a lot to be learned on the contribution of galectin-glycan interactions to immune cell homeostasis, the precise mechanisms and redundancy of galectin-driven circuits, the tissue-specific roles of galectins *in vivo*, their complementation with other regulatory checkpoints (e.g. PD-1/PD-L1, CTLA-4) and the potential undesired effects. Whereas several galectin-tailored agents have been

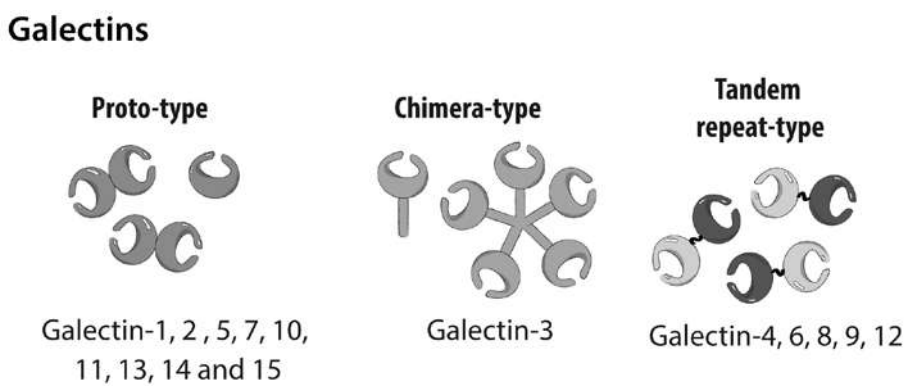


Figure 1. Biochemical structure of galectins. Structure and classification of galectins. Galectins are subdivided into three groups: prototype galectins, which contain one carbohydrate recognition domain (CRD) and can dimerize; tandem-repeat type galectins, which contain two distinct CRDs in a single polypeptide chain and are inherently bivalent; and the unique chimera-type galectin-3, which contains a CRD connected to a non-lectin N-terminal region responsible for oligomerization.

designed for the treatment of cancer, asthma, fibrosis and autoimmunity, including synthetic glycan inhibitors, natural polysaccharides, peptidomimetics and biological agents, progress thus far might represent only the starting point of a therapeutic potential that awaits future discovery.

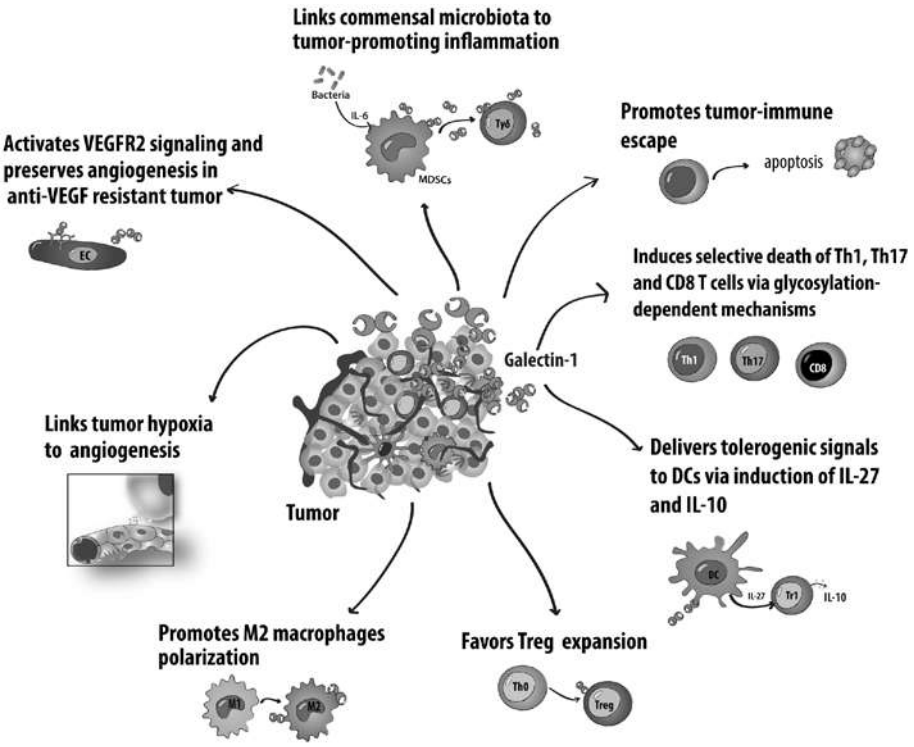


Figure 2. Galectin-1-driven regulatory pathways in the tumor microenvironment. Galectin-1 promotes immune evasive programs in different tumor types through activation of multiple tolerogenic mechanisms. Galectin-1 tilts the balance of the immune response toward a Th2 profile by selectively deleting Th1, Th17 and CD8+ T cells. Moreover, it drives the differentiation of regulatory T cells (Tregs), endows dendritic cells (DCs) with tolerogenic potential, polarizes macrophages toward an anti-inflammatory M2-type profile and inhibits NK cell recruitment. In addition, this lectin is a key player of a regulatory circuit that links commensal microbiota, systemic inflammation and tumor growth through mechanisms involving expansion of myeloid-derived suppressor cells (MDSCs) and gd-T cells. Interestingly, galectin-1-glycan interactions can also couple tumor hypoxia to vascularization and preserve angiogenesis in tumors refractory to anti-vascular endothelial growth factor (VEGF) treatment.

Acknowledgements

I would like to express my sincere thanks to all members of my lab for their hard and collaborative work, to our colleagues and collaborators and to funding agencies that support our work including Fundación Sales (Argentina), Fundación Bunge y Born (Argentina), Agencia Nacional de Promoción Científica y Tecnológica (Ministerio de Ciencia, Tecnología e Innovación Productiva; Argentina), Universidad de Buenos Aires (Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), National Cancer Institute (NCI, NIH; USA) and Kenneth Rainin Foundation (USA).

Bibliography

1. Rabinovich, G.A. and Croci, D.O. (2012) Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. *Immunity* 36, 322-335.
2. Cerliani, J.P. *et al.* (2017). Translating the 'Sugar Code' into Immune and Vascular Signaling Programs. *Trends Biochem Sci*. 42, 255-273.
3. Liu, F.T. and Rabinovich, G.A. (2005). Galectins as modulators of tumour progression. *Nat. Rev. Cancer* 5, 29-41.
4. Hirabayashi, J. *et al.* (2002). Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim. Biophys. Acta* 1572, 232-254.
5. Rabinovich, G.A. and Toscano, M.A. (2009) Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat. Rev. Immunol.* 9, 338-352.
6. Sundblad V *et al.* (2017). Galectin-1: A Jack-of-all-Trades in the Resolution of Acute and Chronic Inflammation. *J Immunol.* 199:3721-3730.
7. Rabinovich, G.A. *et al.* (1997). Specific inhibition of lymphocyte proliferation and induction of apoptosis by CLL-I, a beta-galactoside-binding lectin. *J Biochem*. 122:365-373.
8. Stillman, B.N., *et al.* (2006) Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J. Immunol.* 176, 778-789.
9. Matarrese, P., *et al.* (2005) Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J. Biol. Chem*. 280, 6969-6985.
10. Toscano, M.A., *et al.* (2007) Differential glycosylation of Th1, Th2 and Th-17 effector cells selectively regulates susceptibility to cell death. *Nat. Immunol.* 8, 825-834.
11. Rabinovich, G.A. *et al.* (1999). Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1. *Immunology.* 97, 100-106.
12. Rabinovich, G.A., *et al.* (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J. Exp. Med*. 190, 385-398.
13. van der Leij. J. *et al.* (2007). Strongly enhanced IL-10 production using stable galectin-1 homodimers. *Mol. Immunol.* 44, 506-513.
14. Toscano, M.A., *et al.* (2006) Galectin-1 suppresses autoimmune retinal disease by promoting concomitant

Th2- and T regulatory-mediated anti-inflammatory responses. *J. Immunol.* 176, 6323-6332
15. Chung, C.D. *et al.* (2000). Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction. *J. Immunol.* 165, 3722-3729.
16. de la Fuente, H. *et al.* (2014). The leukocyte activation receptor CD69 controls T cell differentiation through its interaction with galectin-1. *Mol Cell Biol.* 34, 2479-2487.
17. Garin, M.I., *et al.* (2007) Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 109, 2058-2065.
18. Dalotto-Moreno, T., *et al.* (2013) Targeting galectin-1 overcomes breast cancer-associated immunosuppression and prevents metastatic disease. *Cancer Res.* 73, 1107-1117.
19. Blois, S.M., *et al.* (2007) A pivotal role for galectin-1 in fetomaternal tolerance. *Nat. Med.* 13, 1450-1457.
20. Cedeno-Laurent, F., *et al.* (2012) Galectin-1 triggers an immunoregulatory signature in Th cells functionally defined by IL-10 expression. *J. Immunol.* 188, 3127-3137.
21. Seropian, I.M. *et al.* (2013). Galectin-1 controls cardiac inflammation and ventricular remodeling during acute myocardial infarction. *Am J Pathol.* 182, 29-40.
22. Juszczynski, P., *et al.* (2007) The AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical Hodgkin lymphoma. *Proc. Natl. Acad Sci. U S A.* 104, 13134-13139.
23. Croci, D.O., *et al.* (2012) Disrupting galectin-1 interactions with N-glycans suppresses hypoxia-driven angiogenesis and tumorigenesis in Kaposi's sarcoma. *J. Exp. Med.* 209, 1985-2000.
24. Baker, G.J., *et al.* (2016) Natural killer cells require monocytic Gr-1(+)/CD11b(+) myeloid cells to eradicate orthotopically engrafted glioma cells. *Oncoimmunology* 5, e1163461.
25. Zuñiga, E., *et al.* (2001) Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis. *J. Leukoc. Biol.* 70, 73-79.
26. Tabrizi, S.J., *et al.* (2009) T cell leukemia/lymphoma 1 and galectin-1 regulate survival/cell death pathways in human naive and IgM+ memory B cells through altering balances in Bcl-2 family proteins. *J. Immunol.* 182, 1490-1499.
27. Anginot, A., *et al.* (2013) Galectin 1 modulates plasma cell homeostasis and regulates the humoral immune response. *J. Immunol.* 190, 5526-5533.
28. Bonzi, J., *et al.* (2015) Pre-B cell receptor binding to galectin-1 modifies galectin-1/carbohydrate affinity to modulate specific galectin-1/glycan lattice interactions. *Nat. Commun.* 6, 6194.
29. Ilarregui, J.M., *et al.* (2009) Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat. Immunol.* 10, 981-991.
30. Poncini, C.V., *et al.* (2015) *Trypanosoma cruzi* Infection imparts a regulatory program in dendritic cells and T Cells via galectin-1-dependent mechanisms. *J. Immunol.* 195, 3311-3324.
31. Davicino, R.C., *et al.* (2017). Galectin-1-Driven Tolerogenic Programs Aggravate *Yersinia enterocolitica* Infection by Repressing Antibacterial Immunity. *J Immunol.* 2199, 1382-1392.
32. Thiemann, S., *et al.* (2015) Galectin-1 regulates tissue exit of specific dendritic cell populations. *J. Biol. Chem.* 290, 22662-22677.
33. Mobergslien, A. and Sioud, M. (2012) Galectin-1 and -3 gene silencing in

immature and mature dendritic cells enhances T cell activation and interferon-gamma production. *J. Leukoc. Biol.* 91, 461-467.
34. Rabinovich, G., *et al.* (1996) Regulated expression of a 16-kd galectin-like protein in activated rat macrophages. *J. Leukoc. Biol.* 59, 363-370.
35. Correa, S.G., *et al.* (2003) Opposite effects of galectin-1 on alternative metabolic pathways of L-arginine in resident, inflammatory, and activated macrophages. *Glycobiology* 13, 119-128.
36. Barrionuevo, P., *et al.* (2007) A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a non-apoptotic ERK-dependent pathway. *J. Immunol.* 178, 436-445.
37. Rabinovich, G.A., *et al.* (2000) Evidence of a role for galectin-1 in acute inflammation. *Eur. J. Immunol.* 30, 1331-1339.
38. Rostoker, R., *et al.* (2013) Galectin-1 induces 12/15-lipoxygenase expression in murine macrophages and favors their conversion toward a pro-resolving phenotype. *Prostaglandin other Lipid Mediat.* 107, 85-94.
39. Stowell, S.R., *et al.* (2009) Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane. *Mol. Biol. Cell* 20, 1408-1418.
40. Starossom, S.C., *et al.* (2012) Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. *Immunity* 37, 249-263.
41. Cooper, D., *et al.* (2008) Novel insights into the inhibitory effects of Galectin-1 on neutrophil recruitment under flow. *J. Leukoc. Biol.* 83, 1459-1466.
42. Verschuere, T., *et al.* (2014). Glioma-derived galectin-1 regulates innate and adaptive antitumor immunity. *Int J Cancer.* 134, 873-884.
43. Rutkowski, M.R., *et al.* (2015) Microbially driven TLR5-dependent signaling governs distal malignant progression through tumor-promoting inflammation. *Cancer Cell* 27, 27-40.
44. Ge, X.N., *et al.* (2016) Regulation of eosinophilia and allergic airway inflammation by the glycan-binding protein galectin-1. *Proc. Natl. Acad. Sci. U S A.* 113, 4837-4846.
45. Rabinovich, G.A., *et al.* (2007). Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol.* 25, 267-296.
46. Rubinstein, N., *et al.* (2004) Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 5, 241-251.
47. Cedeno-Laurent, F., *et al.* (2012) Metabolic inhibition of galectin-1-binding carbohydrates accentuates antitumor immunity. *J. Invest. Dermatol.* 132, 410-420.
48. Banh, A., *et al.* (2011) Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. *Cancer Res.* 71, 4423-4431.
49. Soldati, R., *et al.* (2012). Neuroblastoma triggers an immunoevasive program involving galectin-1-dependent modulation of T cell and dendritic cell compartments. *Int J Cancer.* 131, 1131-11141.
50. Martínez-Bosch, N., *et al.* (2014). Galectin-1 drives pancreatic carcinogenesis through stroma remodeling and Hedgehog signaling activation. *Cancer Res.* 74, 3512-3524.
51. Croci, D.O., *et al.* (2013) Nurse-like cells control the activity of chronic lymphocytic leukemia B cells via galectin-1. *Leukemia* 27, 1413-1416
52. Croci, D.O., *et al.* (2014) Glycosyla-

tion-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. *Cell* 156, 744-758.
53. Perone, M.J., *et al.* (2006). Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. *J. Immunol.* 177, 5278-5289.
54. Santucci, L., *et al.* (2003). Galectin-1 suppresses experimental colitis in mice. *Gastroenterology*. 124, 1381-1394.

Closing Session

Academia de Ciencias de América Latina. Un reconocimiento, una realidad y un propósito

Claudio Bifano

Al cumplirse 35 años de la creación de la ACAL, y siendo este el primer evento internacional que se organiza después de algunos años de suspensión forzosa de actividades, quisiera hacer un breve reconocimiento a nuestros predecesores y dejar planteado el propósito que puede marcar el rumbo de nuestra Academia.

Altos valores científicos y humanos fueron los que, en 1982, impulsaron a un distinguido grupo de investigadores a concretar la creación de la ACAL como un espacio de asociación humana, científica y social para América Latina. Supieron hacer realidad un instrumento de integración basado en la creatividad científica y la excelencia educativa como valores que habían tenido el privilegio de disfrutar en las mejores universidades de sus países y refinar más tarde en los más prestigiosos centros de estudio del mundo.

Habiendo conocido personalmente algunos de ellos, no dudo en afirmar que tenían una visión de la ciencia como un fin en sí mismo y como un medio para avanzar hacia la formación de una comunidad latinoamericana basada en la libertad y el respeto mutuo, como principios esenciales de convivencia y el progreso. Una iniciativa noble, no cabe la menor duda, alejada de las tentaciones del populismo politiquero de líderes ignorantes e improvisados.

La ACAL fue pues concebida como un espacio de encuentro multinacional, que invitaba a la comunidad científica latinoamericana a canalizar iniciativas de progreso para el bienestar de los pueblos de la región. Un propósito que pretendía dar, en aquel presente y en los años a seguir, una señal de la importancia de la ciencia para el bienestar y el progreso de las sociedades latinoamericanas. Fueron varios reconocidos científicos latinoamericanos, capaces de convertir buenas intenciones en importantes acciones, quienes tejieron ese sueño.

Hoy, quienes tenemos la fortuna de ser herederos de aquel empeño, recordamos con afecto y reconocimiento a los fundadores de la Academia, doctores Carlos Chagas, Jorge E. Allende, Héctor Croxatto, Leopoldo de

Meis, Sonia Dietrich, Patricio J, Garrahan, Armando Gómez Poyou, Giovanni Battista Marini-Bettolo, Sergio Mascarehnas, Antonio Paes Carvalho, Carlos Monge, Mauricio Peixoto, Marcel Roche, Fernando Rosas P. Pablo Rudomín, Andrés Stoppani, Raimundo Villegas y Jorge Villegas.

Algunos de ellos nos han dejado, otros siguen con nosotros y su ejemplo nos anima a seguir adelante, con la esperanza de estar a la altura del reto que nos han dejado. En representación de todos ellos y de los investigadores que se han ido integrando a la ACAL, me referiré brevemente al primer presidente, Dr. Carlos Chagas Filho, y al primer Canciller, Dr. Raimundo Villegas.

Se ha escrito mucho sobre la trayectoria científica y humanística del Profesor Chagas Filho, y sus reseñas biográficas coinciden en resaltar sus extraordinarias cualidades. La promesa que hizo al asumir la Presidencia de la Pontificia Academia de Ciencias en 1972: "Lo que quiero al sumir esta función es muy claro. Trataré de cambiar esta Academia de ser cuerpo de gran prestigio a uno de gran acción", la cumplió con creces a lo largo de los dieciséis años de su ejercicio. Dos de sus grandes logros, de alcance y resonancia mundial, fueron la revisión del caso de Galileo, que recibió unánime apoyo internacional y la campaña contra el uso de armas atómicas. El impacto que tuvo esa campaña contribuyó al logro de un equilibrio de fuerzas entre las principales potencias del mundo para garantizar la paz y obtuvo el respaldo unánime de las Naciones Unidas. También destacan sus continuas reflexiones filosóficas y sociológicas sobre la ciencia y su papel en el mundo moderno, vinculándola siempre a la promoción del ser humano.

Menos se ha escrito sobre el Dr. Raimundo Villegas a quien tuve la fortuna de conocer personalmente. Me produce mucha alegría tener la oportunidad de rememorar hoy su compromiso con el país, su calidad humana y, por supuesto, su incansable dedicación al oficio de hombre de ciencia. Raimundo Villegas fue un líder de la ciencia venezolana y latinoamericana que compartió su vida entre su laboratorio y la preocupación por el desarrollo de la ciencia en su país y Latinoamérica. Hablar de él es hablar de talento, de esfuerzo generoso para la formación de generaciones nuevas de profesionales de científicos y el afán por fortalecer la ciencia en la Región; en suma, es hablar de compromiso con el futuro.

Su periplo como investigador comenzó en el Instituto de Investigaciones Médicas, Fundación Luis Roche.[1] A la caída de la última dictadura

[1] Este Instituto se creó en Caracas en 1952 con la finalidad de "realizar toda clase de investigaciones científicas con fines humanitarios, especialmente en el campo de la medicina, y de manera muy particular sobre las enfermedades endémicas en el país y

formal sufrida por el país pasó a formar parte del Instituto Venezolano de Investigaciones Científicas (IVIC) y culminó su carrera en el Instituto de Estudios Avanzados IDEA, creado por el mismo.

Además de sus muchas e importantes contribuciones científicas, su curriculum muestra un considerable número de aportes que evidencian un gran interés por el Desarrollo de la Ciencia en América Latina. En ellas habla del presente y el futuro de la ciencia en la Región, dejando siempre importantes sugerencias y valiosas propuestas que lo muestran como un hombre de ciencia integral, con preocupaciones que abarcaban espacios mucho mayores a los límites de su laboratorio.

Al hablar de la relación entre el desarrollo de un país y su progreso científico, afirmaba de manera explícita que "la escasez de investigadores con espíritu docente constituye nuestra primera causa de atraso. Faltan quienes transformen las vocaciones latentes en el deseo consciente e irresistible de hacer ciencia". Entendiendo por investigadores con espíritu docente a aquellos "…con vocación y capacidad para orientar y enseñar el arte de la investigación científica". Al referirse al número de investigadores aseveraba que "Si consideramos como investigadores científicos solamente a los hombres y mujeres que saben y comprenden las leyes de la naturaleza, están capacitados en las técnicas de la investigación, tienen entusiasmo, tenacidad y talento creador, entonces el número de investigadores es muy bajo…".

Cuando habla del sistema educativo como obstáculo al progreso de la ciencia, no duda en afirmar que "Nuestras escuelas, liceos y Universidades son esencialmente centros de distribución de información. La enseñanza debería estar orientada a producir personas cultas imaginativas, capaces de progresar intelectualmente y con un conocimiento experto en alguna especialidad. Al educar a los niños y a los jóvenes deberíamos cuidarnos de no destruir su imaginación".

Con personas de esa sensibilidad social y calibre científico se construyó la ACAL, no para satisfacer alguna ambición personal o para cumplir un compromiso de ocasión. Fue un proyecto que contó con un fuerte y claro

en las demás regiones tropicales". Fue dirigida por el Dr. Marcel Roche y en ella se trabajó en anemias nutricionales, diabetes, bocio endémico y bilharziasis. Fue la primera Institución venezolana en asignar un sueldo a personas que trabajaban exclusivamente en investigación. Terminó sus actividades en 1958, al trasladarse la mayoría de los investigadores al Instituto Venezolano de Investigaciones Científicas (IVIC) que había surgido de la reorganización del Instituto Venezolano de Neurología e Investigaciones Cerebrales creado por el Dr. Humberto Fernández Morán en 1954.

liderazgo científico, con recursos económicos que el gobierno venezolano y algunos entes privados aportaron con ocasión de celebrarse el Bicentenario del Nacimiento de Simón Bolívar y se acordó que Venezuela fuera el país sede, no solo porque la iniciativa de crearla proviniera de científicos venezolanos, sino como un reconocimiento al significativo avance de la actividad científica que el país mostraba en ese momento.

La Academia de Ciencia de América Latina comenzó a funcionar con el patrocinio la Fundación Simón Bolívar para la Academia de Ciencias de América Latina FSB-ACAL, creada con la finalidad de coadyuvar con el funcionamiento de la Academia con el apoyo personal de don Alberto Vollmer, que me atrevo a calificar como un mecenas de la ciencia venezolana.

Se organizaban reuniones científicas anuales en distintas ciudades de la región. Entre ellas, Asunción, Bogotá, Buenos Aires, Caracas, Lima, México, Montevideo, Rio de Janeiro, Santiago, Sao Paulo y Viña del Mar, apoyadas conjuntamente por el organismo nacional de ciencia y tecnología del país, las academias nacionales, instituciones de investigación, universidades y, por supuesto, de la Fundación Simón Bolívar para la Academia de Ciencias de América Latina. En la década de los ochenta la ACAL organizó varios cursos de capacitación que abarcaron temas como Fijación biológica del nitrógeno; Energía solar, Transporte de crudos pesados; Modelos matemáticos de producción de cultivos; Nutrición y desarrollo del niño en América Latina y muchos más, en los que participaron jóvenes investigadores de los Estados Unidos, Brasil, Colombia, México, Argentina y Venezuela.

Esos cursos contaban con el financiamiento de los Organismos Nacionales de ciencia y tecnologías, e internacionales como la UNESCO, y su Oficina Regional de Ciencia y Tecnología para América Latina y el Caribe ORCYT-UNESCO, el Consejo Internacional de Uniones Científicas ICSU y la Academia Mundial de la Ciencia TWAS.

Con la finalidad de dar a conocer el potencial científico de la región, se propuso organizar un Directorio de Instituciones Científicas Latinoamericanas y del Caribe, que comunicaba a través de un boletín trimestral titulado Ciencia en América Latina.

Entre los años ochenta y 2013, los presidentes de la ACAL fueron Carlos Chagas Filho, Hugo Aréchiga y Fidel Ramón. El canciller fue Raimundo Villegas y el co-canciller, Guillermo Whittembury. La sede de la Academia fue el Instituto de Estudios Avanzados IDEA y el número de miembros llego a superar los doscientos.

A partir del primer decenio de este siglo algunos hechos incidieron negativamente en el desarrollo de las actividades de la ACAL. El nuevo

gobierno que llegó al poder en Venezuela en 1999 disminuyó el apoyo que recibía del Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT) y en el 2008 la dirección del Instituto de Estudios Avanzados (IDEA), donde funcionaba la Academia, le retiró los espacios que ocupaba desde el año de su creación. Lo peor ocurrió en 2012, cuando el Dr. Villegas sufrió un accidente que limitó seriamente sus capacidades de llevar a cabo sus actividades. En 2013 el presidente, Dr. Fidel Ramón, informó que no seguiría en el cargo por haber terminado su periodo, quedando el co-canciller como único responsable de la ACAL. De hecho, el último informe de actividades de ACAL que hemos encontrado es del año 2006. El Dr. Raimundo Villegas falleció en el 2014. Dada esta situación, y a solicitud del Dr. Whittembury, la Academia de Ciencias Físicas Matemáticas y Naturales de Venezuela ofreció un espacio a la ACAL en el Palacio de las Academias, como nueva sede.

La realidad actual

En 2015 comenzó el proceso de reactivación de la ACAL. De manera resumida podemos decir que en este momento la ACAL está en funcionamiento con normalidad. Se ha reconstruido la infraestructura básica que hace posible su trabajo y permite mantener contacto con todos los académicos. Además, gracias a la Fundación ACAL-FSB, dispone de los recursos necesarios para cubrir los costos relacionados con sus actividades. Pero quizás lo más importante sea el incremento en el número de académicos y académicos correspondientes. Es de destacar que, una vez más, ese incremento refleja la realidad de la ciencia en Latinoamérica, tanto en lo que se refiere a su dispar desarrollo entre los países, el desigual impulso de las diferentes áreas de la ciencia y los efectos negativos de la emigración de talentos que estamos tratando de capitalizar incorporando cada vez más investigadores latinoamericanos residentes en otros países del mundo.

ACAL cuenta con un grupo directivo muy entusiasmado e institucionalmente comprometido y un Consejo Académico que igualmente apoya y garantiza su buen funcionamiento. ¡Queda aún mucho por hacer!

El compromiso que hemos asumido

En líneas generales, el compromiso es contribuir a la consolidación de una comunidad científica conformada por investigadores latinoamericanos, residentes o no en países de la Región, basada en una alta calidad científica y preferiblemente interesada en los problemas y en las realidades de nuestros países. Tomándome la libertad de parafrasear las palabras del Dr.

Chagas Filho, lo que queremos lograr es que esta Academia se convierta en una Institución de gran prestigio por las acciones que emprenda.

A manera de ejemplo, contribuir a la construcción de capacidades científicas; interactuar con representantes de organismos gubernamentales de C y T para generar conciencia sobre la importancia de la ciencia y de los científicos locales para el desarrollo de sus respectivos países; conformar una red de investigadores activos para compartir información sobre posibilidades de cooperación, disponibilidad de equipos y recursos científicos. Establecer vías de comunicación con científicos latinoamericanos residenciados en diferentes países del mundo que faciliten a jóvenes investigadores, la realización de estudios superiores, pasantías de investigación en proyectos de investigación. Fomentar el análisis y la discusión crítica de las políticas nacionales para la ciencia, tecnología e innovación, a través de documentos, planteamiento de ideas, propuestas y recomendaciones para su desarrollo, así como sobre temas de investigación prioritarios para nuestros países.

Definir los propósitos de una Academia no es particularmente difícil. Lo difícil, como siempre, es llevarlos a término. Huelga decir que el futuro de cualquier otra institución depende mucho del interés de sus miembros. Pero en el caso de ACAL, cumplir con algunas de las metas planteadas no debería ser particularmente difícil, porque el número y la calidad de sus miembros permiten ser optimista. Hasta ahora hemos tenido una muy buena respuesta al llamado de postulaciones para nuevos miembros de la ACAL, lo cual es indicativo del interés de los académicos por la Academia. Esto da pie para pensar que podemos tener respuestas similares de los académicos al planearles nuevas proyectos o acciones de carácter general o que, con el respaldo de la ACAL, puedan ser importantes para sus respectivos países.

Pensamos en la promoción de actividades colaborativas con académicos latinoamericanos residenciados fuera de sus países de origen; en la organización de un banco de datos contentivo de la descripción de las líneas de investigación de los académicos, publicaciones recientes; en el lanzamiento de un premio para investigadores jóvenes, al igual que lo hacen instituciones similares a la nuestra; en la posibilidad de establecer las figuras de Miembros protectores de la ACAL a Fundaciones o Empresas interesadas en el avance de la ciencia y Universidades e Institutos de investigación interesados en la enseñanza de la ciencia; en la realización de simposios anuales en diferentes países latinoamericanos, con ocasión de la reunión del Comité Académico que se realizaran bajo los auspicios de Academias Nacionales de Ciencia; en la modernización de la plataforma digital de ACAL,

que permita en tiempo real la realización de cursos cortos o reuniones de trabajo entre grupos de trabajo con intereses comunes para incrementar la interacción entre grupos afines de investigación y abrir espacios a colaboraciones interdisciplinarias.

Estos son las ideas que maneja el Consejo Directivo, con el apoyo del Consejo Académico, con miras al futuro a corto, mediano y largo plazo. Poniendo a disposición de la Institución nuestras modestas capacidades, esperemos lograr cumplirlas por lo menos en parte, en el tiempo que no corresponda dirigirla.

Meeting Global Challenges for Science through Intercontinental Collaboration in Latin America

Luiz Davidovich

Introduction

The workshop on *Cell Biology and Genetics*, held at the Pontifical Academy of Sciences on October 23-24, 2017, celebrated the 35th anniversary of the Academia de Ciencias de America Latina (ACAL), created in 1982 under the sponsorship of the Pontifical Academy of Sciences, then presided by Carlos Chagas Filho, with the aim of promoting and contributing to the advancement of mathematical, physical, chemical, earth, and life sciences, and to their application to the development and integration of Latin America and the Caribbean. These objectives, clearly identified at that time, are still up-to-date, when there is pressing need for a Latin-American scientific agenda.

Big continental challenges require evidence-based governance. Academies of Science have an important role to play, in advising governments and promoting intercontinental collaborations. Relevant topics include food security, water and energy supply, aging, diseases, sanitation, climate change, air pollution, natural disasters, science education, sustainable use of biodiversity, management of huge urban agglomerations, social inclusion with eradication of poverty and reduction of inequality.

Latin America has some clear competitive advantages, as compared to other regions of the world: the planet's largest carbon sink, huge water basins, huge biodiversity. It also suffers from common adversities: Strong social inequality, high vulnerability to natural disasters, deficient educational systems, few innovative industries, and exports dominated by commodities. Also, Latin America has a small number of researchers. Brazil has around 700 researchers per million inhabitants, Argentina around 1100, while the correspondent number for the European Union and the United States is around 3500, attaining 7000 in Korea, 7500 in Denmark, 5200 in Japan.

Intercontinental collaboration would make it easier to tackle these common problems, and to take advantage of the huge territorial wealth. Latin America and the Caribbean are home to 34% of the world's plant species and 27% of the mammals: it is a true world biodiversity superpow-

er! Furthermore, 20% of its territory has been set aside for conservation, much more than the developing world average of 13%.

1. Biodiversity-based biotechnology: an interdisciplinary road to sustainable development

Conservation alone is not however a viable solution: it is important to offer a profitable alternative to deforestation. And the very nature of the conserved regions suggests a possible road to a sustainable future: investment in biodiversity-based biotechnology.

The pioneer work of Schultes (Schultes, 1962) and Gottlieb (Seidl, 1995) on Amazon medicinal plants has contributed to the understanding of that region's huge potential.

An example is the plant *Endopleura Uchi* (Nunomura, 2009), traditionally used for the treatment of inflammations. The word "uchi" means "yellow" in an Indian dialect. This is the color of the fruit of this plant. Bark tea of this plant is used in traditional medicine as an anti-inflammatory and also against tumors and uterine infections. These medicinal properties come from *bergenin*, a substance isolated from the bark (Nunomue, 2009), and also from the yellow fruit (Magalhaes, 2007). See Figure 1 for its structure.

Bergenin, purified at more than 95%, is being sold in Brazil by Merck for 300 dollars per milligram. One could compare this to the price of gold, bought in Brazil for 40 dollars per gram. For the same weight, Bergenin is ten thousand times more expensive than gold!

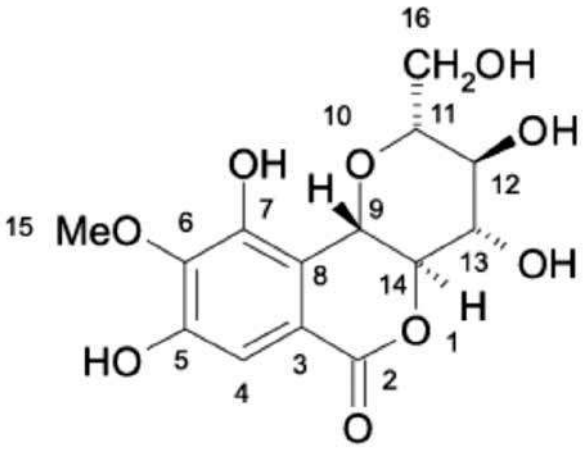


Figure 1. Bergenin structure.

An intercontinental platform to explore the region's biodiversity in a sustainable way, associated to focused training of human resources and promotion of biotechnology startups and pharmacological industry, would correspond to Project Apollo in the United States! Remember that the space race motivated the United States to reformulate its basic education and its industrial profile, culminating on July 20, 1969, when the first human beings landed on the Moon, thus fulfilling the project put forward by John F. Kennedy in 1961. Latin America needs a daring project of this sort, not in space, but on earth: biodiversity is our Moon…

2. Inventing a better future for Latin America

Paving the road to a better future should involve a collaborative strategy in Latin America and the Caribbean. This strategy should include:

- Science and technology capacity building as a shared regional responsibility.
- Digital libraries of science and technology with universal access.
- Virtual networks of excellence linking scientific talents of entire regions and the globe, with a multidisciplinary approach.
- Science education "beyond the school".
- Reformulation of higher education structure and programs towards more interaction between disciplines, new teaching technologies, more optional disciplines, and more time for innovative activities.
- New funding mechanisms for support of science and technology in developing nations; they should include:
- Institutional funds for supporting centers of excellence and multi-user labs of national and regional character.
- Regional program funds, with a competitive grant system.

Some of these items are partially contemplated by organizations like IANAS (InterAmerican Network of Academies of Science), founded in 2004, which includes all countries in the American continent. It has the following as its main objectives:

- To assist in building national scientific capacities by strengthening science and technology relationships among the countries of the Americas.
- To cooperate in building capacities of the Academies of the region, through exchange of information and experience.
- To aid in the creation of new Academies in those countries of the Americas desiring assistance in the establishment of a Science Academy.

- To influence scientific decision-making processes in the Americas, with the goal of promoting prosperity and equity in the hemisphere.

With these objectives, IANAS has conducted six regional programs: Water Management, Food Security, Women for Science, Science Education, Energy, and Capacity Building.

The scientific agenda for Latin America is extensive and challenging. Important developments, requiring strong intercontinental collaboration, should include high-seas research, satellites for prospection of territory and communications, pharmaceuticals based on the continent's biodiversity, biotechnology for food security, broad availability of high-speed internet, research on neglected tropical diseases, new technologies for social innovation.

The participation of ACAL, either by creating its own programs, or mobilizing Latin-American scientists towards contributing to IANAS programs, would be a valuable asset to the region, which faces tremendous challenges to overcome poverty, strong inequality, unstable political systems, and commodities-dominated economies with poor sustainability. Science and technological innovation are necessary ingredients in the path to sustainable development, encompassing social, economic, and nature aspects. A collaborative approach among Latin-American countries would make it easier to pursue this demanding and exciting path.

Bibliography

Schultes, R.E. (1962), The role of ethnobotanist in the search of new medicinal plants, *Lloydia 25*, 257-266.

Seidl, P.R., Gottlieb, O.R., and Kaplan, M.A.C. (1995), Editors, *Chemistry of the Amazon: Biodiversity, Natural Products, and Environmental Issues*, Volume 588 of American Chemical Society Symposium series.

Nunomura, R.C.S., Oliveira, V.G., Da Silva, S.L., and Nunomura, S.M. (2009), Characterization of bergenin in *Endopleura uchi* bark and its anti-inflammatory activity, *J. Braz. Chem. Soc.* 10, 1060-1064.

Magalhães, L.A., Lima, M.P., Marinho, H.A., and Ferreira, A.G. (2007)., *Acta Amaz. 37*, 447-450.

Declaración: Taller de Biología Celular y Genética

Casina Pio IV, 23-24 Octubre 2017

Introducción

Durante los días 23-24 de octubre del 2017 tuvo lugar un taller sobre Biología Celular y Genética en la Casina Pío IV, sede de la Pontificia Academia de Ciencias (PAS) de El Vaticano. El principal objetivo de la reunión fue congregar a miembros de la Academia Pontificia junto a integrantes de la Academia de Ciencias de América Latina (ACAL) para discutir avances recientes en biología celular, genética y biomedicina. El hecho que la ACAL haya sido fundada hace 35 años durante una sesión de la PAS agregó un particular significado al taller, más aún si se tiene en cuenta que la ACAL acaba de reiniciar un período de vigorosa renovación.

Latino América cuenta con un gran capital humano, siendo sectores de su población altamente educados. Existe una larga tradición de contribuciones importantes al conocimiento científico, lo que queda de manifiesto en los Premios Nobel adjudicados a Bernardo Houssay y a Luis Leloir por investigaciones llevadas a cabo en Buenos Aires y a Mario Molina, de México, por explicar los mecanismos químicos que afectan el espesor de la capa de ozono. En años recientes se han creado nuevos Institutos y Centros de Excelencia científica, aunque aún se observan muchas limitaciones.

El taller comprendió un total de 20 presentaciones en las áreas de biofísica y biología de la membrana celular, comunicación intercelular y biología del desarrollo, neurobiología, biomedicina y genética. También hubo ponencias sobre colaboraciones científicas dentro del Continente, sobre iniciativas en ciencia y tecnología del Departamento de Estado de los EEUU y sobre la situación actual de la ACAL. Adicionalmente, se dedicó una sesión completa a discutir sobre mecanismos de cooperación en Latino América y la formación de redes con países del hemisferio norte.

A partir de las presentaciones y discusiones llevadas a cabo en el taller, hemos llegado a una serie de conclusiones que son demostrativas del gran nivel que muestran algunas áreas de las ciencias biológicas en la región. Adicionalmente, puesto que esta reunión proporcionó una ocasión privilegiada para analizar maneras de potenciar el desarrollo de las ciencias biológicas en los países Latino Americanos, nos hemos permitido proponer una

serie de recomendaciones que pueden ser de potencial utilidad para los científicos y también para autoridades responsables de las políticas públicas.

Avances científicos que destacaron durante el taller

Un amplio rango de adelantos quedó de manifiesto durante el taller, los que fueron expuestos por especialistas en distintos campos de la biología celular. Así, a nivel de la membrana celular, se discutió cómo funcionan los receptores transientes de calor y dolor (Ramón Latorre); cómo las señales inflamatorias son transportadas dentro de las células (Juan Carlos Saez); cómo canales iónicos son abiertos por estímulos mecánicos para permitir la audición (Ana Belén Elgoyhen). En biofísica y bioquímica, los tópicos presentados incluyeron: motores moleculares que empaquetan ADN en cabezas virales (Carlos Bustamante); nuevas recombinasas de bacteriófagos que permiten diseñar circuitos genéticos en plantas (Elibio Rech); de qué modo los radicales superóxido causan nitraciones perjudiciales en las proteínas (Rafael Radi); cómo es que los niveles diabéticos de glucosa en células endoteliales conducen a inflamaciones moderadas a través de radicales libres (Salvador Moncada); cómo es que el plegamiento de proteínas glicosiladas es controlado por la adición o remoción de glucosa (Armando Parodi); la bioquímica de las formas más primitivas de vida en la tierra (Rafael Vicuña); regulación de la degradación de las proteínas por microautofagia mediante el factor de crecimiento Wnt (Edward de Robertis); cómo es detectada la hipoxia por la maquinaria molecular involucrada en el cáncer (Pablo Wappner). En genética de plantas, tópicos cubiertos incluyeron: cómo los cloroplastos envían señales al núcleo para regular su propia síntesis (Patricia León); cómo las bacterias del género Rhizobium, que fijan nitrógeno y solubilizan fosfatos, produce un mayor rendimiento de los cultivos (María Luisa Izaguirre) y cómo la expresión forzada de tres factores de transcripción genera plantas resistentes a la desecación (Luis Herrera-Estrella). En biomedicina se presentaron avances en: la replicación del virus Zika en células madre glial radiales humanas (Stevens Rehen); cómo la genómica y la medicina personalizada contribuyen a disminuir la incidencia de derrame cerebral (Conrado Estol); cómo los suplementos dietarios ayudan a prevenir defectos en el tubo neural (Rafael Apitz-Castro); cómo nuevas aplicaciones del láser a través de biofotónica son efectivas para el tratamiento de cáncer a la piel, verrugas, úlceras y otras enfermedades cutáneas a muy bajo costo (Vanderlei Bagnato); cómo agregados proteicos de un factor de corte y empalme del ARN están presentes en un 97% de pacientes con esclerosis lateral amiotrófica (Francisco Baralle) y

cómo inmunoterapias de anticuerpos monoclonales dirigidas a moléculas que evaden la respuesta inmune tales como PD-1 y galectina-1 están revolucionando el tratamiento del cáncer (Gabriel Rabinovich).

Conclusiones y recomendaciones

1. Existe un consenso general en que los gobiernos de países Latino Americanos no reconocen el crítico rol que tiene la ciencia en el desarrollo cultural y socio-económico. En consecuencia, los programas de gobierno por lo general carecen de políticas orientadas a fortalecer la investigación científica y la formación de investigadores. Esta situación impacta de varias formas: hay deficiencias en la enseñanza de la ciencia en todos los niveles educacionales, los recursos asignados a la investigación científica son insuficientes, las iniciativas que apunten a conectar a los científicos con el sector productivo son escasas, etc.

2. Una consecuencia adicional de esta situación es que los tamaños de las comunidades científicas de los países Latino Americanos en relación a sus respectivos números de habitantes son pequeñas, sobre todo si se comparan con aquellos de los países desarrollados. Más aún, hay países de Latino América cuya contribución a la productividad científica mundial es insignificante.

3. Debido a esta falta de apoyo de los gobiernos, muchos científicos jóvenes emigran a países del hemisferio norte donde encuentran las condiciones adecuadas para expresar sus vocaciones. La mayoría de estos científicos se establecen en el extranjero, perdiendo progresivamente contacto con sus colegas nacionales. Se propone que la ACAL tome el liderazgo en evaluar la magnitud de esta diáspora y luego genere redes conducentes al surgimiento de nuevas iniciativas que beneficien a los países Latino Americanos, tales como colaboraciones en investigación, períodos de entrenamiento para estudiantes graduados, participación en comités de tesis, promoción de nuevos programas de investigación en la región, etc.

4. Las políticas de Estado tendientes a proveer recursos e infraestructura destinados a repatriar científicos jóvenes son inadecuadas. Es muy importante establecer programas nacionales de largo plazo dirigidos a la asignación de recursos e infraestructura requeridos para facilitar y promover investigación de alto nivel e internacionalmente competitiva por

parte de científicos jóvenes. La ciencia y la tecnología debieran ser vistas como pilares fundamentales del desarrollo cultural y socio-económico en Latino América.

5. Hay también un amplio acuerdo en que organismos internacionales como la OEA, UNESCO y el Departamento de Estado de los EEUU podrían contribuir a apoyar la ciencia como eje de desarrollo en Latino América. Se propone además que la ACAL, con su vocación continental, debiera tomar la delantera en contactar estos organismos y trabajar con ellos en la generación de estrategias adecuadas para lograr este objetivo.

6. ACAL es una sociedad civil independiente de los gobiernos, formada por y para los científicos. Está idealmente bien posicionada para reactivar la participación de la OEA para promover intercambios horizontales entre países Latino Americanos. En particular, se requieren becas para estadías breves de estudiantes de doctorado. En el pasado la OEA tuvo programas para promover intercambios científicos, los que lamentablemente se descontinuaron. ACAL debiera intentar convencer a los Ministros de Ciencia y Tecnología de los países de la OEA, los que se reúnen periódicamente, de la urgente necesidad de comprometerse con programas STEM (Science, Technology, Engineering and Mathematics). La ciencia es una actividad no política y es esencial para el progreso.

7. El Instituto Médico Howard Hughes (HHMI) tuvo durante 20 años un programa de becas que transformó la ciencia biomédica en Latino América entrenando a muchos científicos que hoy ocupan posiciones de liderazgo en sus países. Se constata con tristeza que dicho programa llegó a su término. Los participantes en el taller, muchos de los cuales recibieron apoyo de HHMI, estimaron que este es el programa más efectivo en términos económicos de todos aquellos impulsados por el HHMI. Se expresó la esperanza de que dicho programa para académicos Latino Americanos sea reactivado en el futuro. Otro programa que está actualmente en curso y está teniendo un gran impacto en biomedicina es el PEW Latin American Fellows Program. Éste asigna recursos por un período de dos años de postdoctorado en los EEUU y provee fondos para la instalación de los laboratorios cuando los científicos retornan a sus países. Los 180 becarios PEW que ya han hecho esto último están cambiando la investigación biomédica en Latino América. En una actitud muy generosa, la PEW Charitable Trusts preparó un

informe al respecto especialmente para ser analizado en este taller. Este informe es agregado como anexo a esta declaración.

8. Independientemente de las medidas que aquí se proponen, los científicos debieran hacer un especial esfuerzo en crear vínculos con distintos sectores de la sociedad. Así, deben interactuar con los medios de difusión con el fin de contribuir a la educación científica del público en general. También se deben relacionar con el sector privado para explorar opciones de innovación en los procesos productivos. Considerada su alta relevancia, una relación fluida con las autoridades del sector salud es particularmente recomendada. Experiencias exitosas en estos ámbitos constituirán los mejores argumentos para convencer a los gobiernos de que es crucial apoyar a la ciencia en estos días que vivimos una convergencia impresionante entre la biotecnología, la medicina molecular y la bioinformática.

9. En medio de estos desafíos, se presentaron algunos casos muy interesantes que han tenido lugar en la región. Por ejemplo, Brasil inauguró un plan dirigido a vincular a los científicos con los políticos. Un resultado muy beneficioso de esta iniciativa es que se evitó un recorte al presupuesto de la ciencia, el que de otro modo seguramente hubiese ocurrido. Una carta dirigida al Presidente de la República firmada por varios Premios Nobel puede haber contribuido a esta determinación. Por otra parte, el tamaño de la comunidad científica en Uruguay ha venido creciendo en forma sostenida y se ha puesto en práctica un sistema nacional de evaluación de los investigadores. También se ha creado recientemente una Academia de Ciencias. Más aún, la comunidad científica del Uruguay ha jugado un papel decisivo en la implementación de un plan nacional dirigido a sustituir combustibles fósiles por fuentes renovables de energía. Los ejemplos paradigmáticos de Brasil y Uruguay son muy estimulantes y sugieren fuertemente que políticas públicas sobre ciencia que son bien diseñadas se traducen en breve plazo en beneficios para la sociedad.

Las investigaciones presentadas en el taller van a ser publicadas en un volumen de *Acta Vaticana Scripta Varia*, lo que permitirá ofrecer a la Iglesia y a todo el mundo una ventana por la que podrán asomarse al nivel de la Biología Celular de Latino América. La maravillosa atmósfera de la Casina Pio IV contribuyó a generar discusiones que esperamos tengan un impac-

to en el desarrollo científico del vasto sub Continente Latino Americano. Los científicos participantes desean expresar su gratitud a Su Santidad el Papa Francisco y a la Academia Pontificia de Ciencias por esta maravillosa experiencia.

Apéndice:

Declaración del Programa Pew Latin American Fellows emitida con motivo del taller de Biología Celular y Genética que tuvo lugar en El Vaticano los días 23-24 de Octubre del 2017,

por Rebecca Rimel y Kara Coleman, de la Pew Charitable Trusts

La Pew Charitable Trusts tiene una larga tradición en cuanto a proveer fondos para el entrenamiento de jóvenes científicos. Fundada en 1985, el Programa Pew Biomedical Scholars ofrece apoyo económico sin restricciones a Profesores Asistentes en los EEUU, dándoles libertad para que ensayen nuevas ideas que si bien pueden involucrar ciertos riesgos, tienen la posibilidad de producir grandes dividendos. Como parte de este programa, todos los beneficiados asisten a un congreso anual, ocasión que contribuye a la promoción de enlaces comunitarios, formación de redes y colaboraciones. Históricamente, estas reuniones anuales se han realizado en el Caribe o en Centro América, lo que llevó a los becarios Pew, en una reunión en México el año 1989, a discutir sobre las inequidades respecto a la disponibilidad de recursos y apoyo disponible a los investigadores Latino Americanos respecto a aquellos de los EEUU.

En dicho congreso realizado en México el año 1989, los investigadores se aproximaron a Rebecca Rimel, presidenta y gerente general de Pew Charitable Trusts y fundadora del Programa Biomedical Scholars, y a Torsten Wiesel, médico, entonces jefe del programa, para expresarles sus preocupaciones. Mientras ambos discutían acerca de la mejor manera de apoyar la ciencia en Latino América, se dieron cuenta de que no solo era importante proveer de recursos para entrenar científicos jóvenes, sino además era fundamental estimularlos a que continuaran sus carreras en sus países de origen. En ese tiempo Latino América estaba experimentando un éxodo de investigadores talentosos que querían perfeccionarse en el extranjero, pero que más tarde no volvían a sus respectivos países. Rimel y Wiesel determinaron que cualquier programa de apoyo a la ciencia en la región debía enfocarse prioritariamente al retorno de científicos a Latino América.

En el año 1991, Pew Charitable Trusts lanzó el Programa Pew Latin

American Fellows. Hoy día, el programa ofrece 10 becas por año, cada una de las cuales provee fondos por dos años para entrenamiento en los EEUU. Además, se otorga un pago adicional a cada becario que retorna a Latino América a instalar su propio laboratorio. Estos recursos son empleados para contribuir a la adquisición de equipamiento de laboratorio. En los 26 años transcurridos desde el inicio del programa, se han asignado 262 becas a científicos de 10 países Latino Americanos. Entre aquellos que han terminado su entrenamiento en los EEUU, más de un 70 por ciento han escogido volver a Latino América donde hoy dirigen sus propios laboratorios.

Una encuesta sobre el programa que se completó el año 2013 mostró el impresionante impacto que el programa ha tenido en comunidades científicas de Latino América. En total, los 151 encuestados que respondieron haber entrenado a 1.469 jóvenes - tecnólogos, estudiantes graduados, postdoctorados y científicos visitantes - llegando a un promedio de unos 10 científicos entrenados por becario. A la vez, luego de sus períodos como becarios Pew, ellos habían publicado el impresionante número de 2.237 artículos científicos, dando un promedio de unos 15 artículos por encuestado, y muchos han sido reconocidos con diversos honores. Entre éstos últimos se cuentas varios que han recibido el Premio Bernardo Houssay, premios de The World Academy of Sciences (TWAS), la beca John Simon Guggenheim Memorial Foundation, subsidios como el prestigioso Howard Hughes Medical Institute International (HHMI) Research Scholar, y la beca L'Oreal-UNESCO para Mujeres en Ciencia. Notablemente, varios han sido elegidos miembros de la Academia Latino Americana de Ciencias (ACAL). Adicionalmente, los ex becarios Pew continúan siendo miembros activos de la comunidad Pew, contribuyendo a la vinculación de las futuras generaciones con el programa Pew, a través de la promoción de postulaciones a becas y revisando postulaciones como parte de los comités regionales.

La comunidad científica Latino Americana continúa creciendo y fortaleciéndose. Por ejemplo, el número de doctorados científicos aumentó diez veces entre los años 2000 y 2010, mientras que el número de artículos publicados por científicos peruanos se triplicó en el mismo decenio. Sin embargo, como ocurre con todas las comunidades científicas, hay todavía espacios de crecimiento, para lo cual se vislumbran varias oportunidades. En primer lugar, es importante que las inversiones en investigación biomédica básica sean vistas como prioritarias, tanto a nivel de los gobiernos como de las universidades. Por otra parte, puede ser muy atractivo financiar proyectos de investigación que sean exploratorios más que aplicados,

considerando las inestabilidades económicas y sociales de la región. En particular, la importancia de la investigación para la comprensión de procesos celulares básicos no puede ser subestimada. Por ejemplo, estudios del sistema celular que degrada las proteínas condujeron al desarrollo de terapias para el cáncer. Un estimulante ejemplo de inversión efectiva en Latino América es la Fundación de Investigación de Sao Paulo, una agencia estatal que asigna más del 37 por ciento de sus fondos a la investigación básica. Esta región produce más de la mitad de las publicaciones científicas de Brasil. Puesto que toma tiempo a la investigación básica producir sus dividendos, asignar recursos para realizar investigación exploratoria es la mejor estrategia para gatillar descubrimientos innovadores que van a revolucionar la salud humana. Inversiones en infraestructura de laboratorios, equipos y salarios también son importantes para impulsar un sostenido progreso de la investigación.

Además del apoyo de los gobiernos y las universidades, es importante que fundaciones y organizaciones filantrópicas de la región inviertan en ciencia. En muchos países Latino Americanos, el porcentaje del producto geográfico bruto dedicado a investigación y desarrollo es menor al 1 por ciento, comparado que el 2-3 por ciento de los países desarrollados. En estas circunstancias, hay una clara necesidad de apoyo por parte de otras entidades de financiamiento para que contribuyan al progreso. En Argentina, la Pew ha establecido asociaciones exitosas con dos fundaciones de Buenos Aires: la Fundación Bunge y Born y la Fundación Williams. En conjunto, estas fundaciones proporcionan fondos adicionales a los de la Pew para la repatriación de los becarios que retornan a Argentina. Estas asociaciones son mutuamente beneficiosas: los becarios Pew reciben fondos adicionales para iniciar sus actividades y la Argentina puede reclutar científicos talentosos para sus instituciones utilizando estas oportunidades de fondos adicionales. Las fundaciones de otros países podrían considerar impulsar un modelo similar con el fin de reclutar talentos y aumentar la provisión de recursos disponibles.

Finalmente, el entrenamiento científico en países de menor infraestructura continúa siendo una oportunidad para la inversión. A lo largo del período de tiempo en que ha funcionado el programa Pew, el número de postulaciones de Argentina, Brasil, Chile y México ha aumentado en forma sostenida, observándose también una mejora en la calidad de los postulantes. Sin embargo, países tales como Colombia, Perú y aquellos de Centro América continúan a la zaga en las postulaciones. Muchos de los que están interesados en recibir instrucción doctoral en el extranjero lo hacen por-

que consideran limitadas sus opciones en sus respectivos países. En la medida que los científicos continúen dejando sus países, ello agravará la ya frágil situación de la ciencia. Estimulamos acciones tales como colaboraciones regionales, compartir equipamiento, recibir a estudiantes y postdoctorados e invitar a científicos a presentar seminarios. Estos pequeños pasos pueden ayudar a fortalecer las comunidades científicas y la infraestructura en toda la región.

Mientras la ciencia Latino Americana mira hacia el futuro, la inversión en investigación y entrenamiento es la mejor manera de apoyar a los investigadores talentosos y comprometidos, como asimismo para estimular los avances científicos y la innovación.

Workshop sobre Biologia Celular e Genética: Declaração Resumida

Casina Pio IV, 23 e 24 de outubro de 2017

Introdução

Um workshop sobre "Biologia Celular e Genética" foi realizado nos dias 23 e 24 de outubro de 2017 em Casina Pio IV, a sede da Academia Pontifícia de Ciências (PAS) do Vaticano. O principal objetivo da reunião foi reunir os membros da Academia Pontifícia com cientistas da Academia de Ciências da América Latina (ACAL) para informar os últimos avanços em biologia celular, genética e biomedicina. O fato da ACAL ter sido fundada há 35 anos durante uma sessão especial da PAS acrescenta um significado especial a este workshop, especialmente porque a ACAL entrou recentemente em um novo período de forte renovação.

A América Latina tem um capital humano imenso, com uma população altamente instruída e civilizada de grande potencial. Há uma longa história de contribuições à pesquisa, especialmente em biologia, que incluem Prêmios Nobel concedidos a Bernardo Houssay e Luis Leloir por trabalho realizado em Buenos Aires. Nos últimos anos, novos institutos foram criados com recursos científicos poderosos, embora haja muitos desafios.

O workshop abrangeu um total de vinte apresentações nos campos da biofísica e biologia da membrana, sinalização celular e biologia do desenvolvimento, neurobiologia, biomedicina e genética. Houveram também relatos sobre colaborações científicas intracontinentais, iniciativas de ciência e tecnologia pelo Departamento do Estado dos EUA e o estado e as atividades atuais da ACAL. Além disso, houve uma sessão especialmente dedicada ao debate sobre a cooperação científica na América Latina e a rede de relacionamentos com os países do Hemisfério Norte.

Das apresentações e debates durante o workshop, obtivemos um conjunto de conclusões científicas importantes que sugerem o refinamento proeminente alcançado em algumas áreas das ciências biológicas na região. Além disso, uma vez que esta reunião proporcionou uma ocasião única para analisar os caminhos adequados para o aprimoramento da ciência para o desenvolvimento dos países da América Latina, nós oferecemos um conjunto de recomendações potencialmente úteis para cientistas e formuladores de políticas públicas.

Novos avanços científicos revelados durante o workshop

Uma ampla gama de avanços em Biologia Celular foi coberta no workshop pelos especialistas no campo. No nível da membrana celular: como funcionam os canais receptores transitórios de calor e dor (Ramón Latorre); como hemicanais de junção gap mediam os sinais inflamatórios (Juan Carlos Saez); e como os canais de íons mecanicamente fechados que mediam a percepção auditiva (Ana Belén Elgoyhen) foram debatidos. Os temas de Biofísica e Bioquímica apresentados abrangeram: motores moleculares que embalam moléculas simples de DNA em cabeças de vírus bacterianos (Carlos Bustamante); recombinações de novos bacteriófagos que permitem a concepção de novos circuitos genéticos nos vegetais (Elibio Rech); como os radicais livres de superóxidos provocam a nitrosilação prejudicial de proteínas (Rafael Radi); como os níveis de glicose diabética nas células endoteliais causam inflamação de baixo grau por meio dos radicais livres (Salvador Moncada); como o desdobramento de proteínas glicosiladas é controlada pela adição e remoção de glicose (Armando Parodi); a bioquímica das primeiras formas de vida (Rafael Vicuña); como a degradação de proteínas pelos lisossomos por microautofagia é regulada pelo fator de crescimento Wnt (Edward De Robertis); e como a hipóxia é percebida por maquinaria molecular envolvida em câncer (Pablo Wappner). Na genética vegetal, os debates abrangeram: como o cloroplasto sinaliza ao núcleo para regular a sua própria síntese (Patricia León); como a bactéria Rizóbio que fixa o nitrogênio e solubiliza o fósforo aumenta a produção de culturas (María Luisa Izaguirre) e como a expressão forçada de três fatores de transcrição geram vegetais resistentes à dessecação (Luis Herrera-Estrella). Na biomedicina, os temas abrangeram: a reprodução do vírus da Zika nas células-tronco da glia radial humana (Stevens Rehen); como a genômica e a medicina personalizada ajudam a reduzir a incidência de acidente vascular cerebral (Conrado Estol); como a complementação dietética ajuda a impedir os defeitos do tubo neural (Rafael Apitz-Castro); como novas aplicações a laser por meio de biofotônico trata com eficácia os cânceres de pele, verrugas, úlceras e outras doenças dermatológicas a um custo muito baixo (Vanderlei Bagnato); como agregados proteicos de um fator de splicing de RNA são formados em 97% dos casos de Esclerose Lateral Amiotrófica (Francisco Baralle) e como a imunoterapia que visa moléculas de evasão imune tal como PD-1 e Galetina-1 com anticorpos monoclonais está revolucionando o tratamento de câncer (Gabriel Rabinovich).

Conclusões e recomendações

1. Há um consenso geral com respeito à falta de reconhecimento por parte dos governos dos países latino-americanos do papel crítico que a ciência desempenha no desenvolvimento socioeconômico cultural. Como resultado, as políticas para o fortalecimento de pesquisas científicas e formação de jovens cientistas geralmente inexistem nos programas dos governos. Esta situação tem um impacto grave em vários aspectos, isto é, há deficiências na educação científica em todos os níveis, fundos alocados para pesquisas científicas são insuficientes, iniciativas que visam ligar a comunidade científica com o setor produtivo são quase inexistentes, etc.

2. Uma outra consequência da condição acima é que o tamanho relativo das comunidades científicas nos países da América Latina, quando medido em relação às suas respectivas populações gerais, é muito pequeno em comparação aos dos países desenvolvidos. Ademais, há diversos países da América Latina cujas contribuições para a produtividade científica mundial são virtualmente insignificantes.

3. Devido a esta falta de apoio muitos jovens cientistas deixam seus países de origem para se estabelecerem em países do hemisfério norte, onde encontram condições adequadas para manifestar suas vocações. Muitas vezes esses cientistas permanecem no exterior, perdendo progressivamente o contato com colegas em seus países de origem. Propõe-se que a ACAL tenha um papel de liderança na avaliação da magnitude desta diáspora científica e, portanto, gere redes de contatos profissionais importantes para novas iniciativas que irão beneficiar os países da América Latina, tais como colaborações de pesquisa, períodos de formação para estudantes de pós-graduação, participação em comitês de tese, promovendo novos programas de pesquisa na região, etc.

4. Há, também, amplo consenso que os organismos internacionais, tais como a Organização dos Estados Americanos (OAS), a UNESCO e o Departamento de Estado dos EUA, poderiam contribuir para a ciência como motor de desenvolvimento na América Latina. Propõe-se, também, que a ACAL, com sua vocação continental, tome a frente no contato com estes organismos internacionais e trabalhe com eles em estratégias adequadas para atingir este objetivo.

5. A ACAL é uma sociedade civil formada por cientistas para cientistas e independente de qualquer governo. Foi primorosamente situada para reativar a participação da OAS na promoção de intercâmbios horizontais entre os países da América Latina. Especialmente, bolsas de estudo de curta duração para estudantes de Ph.D. são muito necessárias. No passado, a OAS ofereceu programas para promover intercâmbios científicos, mas, infelizmente, não tem mais. A ACAL deve tentar convencer os Ministros da Ciência e da Tecnologia da OAS, os quais se reúnem periodicamente, da necessidade urgente de retomar os programas STEM (Ciência, Tecnologia, Engenharia e Matemática). A ciência é apolítica e essencial para o progresso da sociedade no cenário mundial contemporâneo.

6. Durante 20 anos, o Instituto Médico Howard Hughes (HHMI) manteve um programa de Estudos que transformou a ciência biomédica na América Latina ao alavancar muitos pesquisadores que atualmente ocupam posições de liderança em seus próprios países. O término do programa foi observado com tristeza. Os participantes do workshop, muitos dos quais foram ou são subsidiados pelo HHMI, sentiram que este era o programa mais eficaz em termos integrais já financiado pelo HHMI. Esperança de que o programa de Estudos seja reativado no futuro foi o que se viu. Um programa que está em andamento e com um enorme impacto na biomedicina é o Programa para Bolsistas Latino Americanos PEW. Ele oferece uma bolsa de pós-doutorado de dois anos para estudar nos EUA e recursos financeiros de start-up para retornar às instituições do país de origem. Os 180 acadêmicos PEW que já retornaram estão mudando a ciência biomédica latino-americana. As Fundos Beneficentes PEW muito generosamente elaboraram um relatório especificamente para este Workshop. Este relatório encontra-se no Apêndice abaixo e vale à pena ser lido porque ele descreve, com dados impressos, o grande progresso ocorrido na ciência na América Latina nos últimos 25 anos.

7. Independentemente do resultado das iniciativas propostas, os cientistas devem envidar um esforço especial na criação de ligações com os vários setores da sociedade. Eles têm que interagir com a mídia para contribuir com a educação do público em geral. Eles também têm que se relacionar com o setor privado, explorar perspectivas de inovação nos processos produtivos. Devido a sua alta relevância social, uma relação fluente com autoridades do setor da saúde é especialmente relevante. Experiências

bem-sucedidas nesses campos constituirá os melhores argumentos para convencer os governos que apoiar a ciência é indispensável hoje em dia, uma vez que estamos vivendo uma incrível convergência de biotecnologia, medicina molecular e bioinformática.

8. Em meio a esses desafios, alguns casos interessantes também foram apresentados. Por exemplo, o Brasil embarcou em um plano que visa expandir as interações entre cientistas e políticos. Como resultado, o orçamento para as atividades relacionadas à ciência não sofreu a redução que teria sido esperada de outra forma. Uma carta endereçada ao Presidente e assinada por diversos ganhadores do Prêmio Nobel pode ter contribuído para este resultado. Por outro lado, no Uruguai o tamanho da comunidade científica vem aumentado de forma constante e um sistema nacional para avaliação de cientistas foi estabelecido. Além disso, recentemente foi criada uma Academia de Ciência. Ademais, a comunidade científica no Uruguai tem tido um papel decisivo na implementação do plano nacional que objetiva substituir combustíveis fósseis por fontes renováveis de energia. Estes exemplos de paradigmas do Brasil e Uruguai requerem otimismo e constituem a melhor prova de que políticas públicas bem concebidas na ciência são imediatamente traduzidas em benefícios sociais.

9. A pesquisa apresentada no workshop será publicada como um volume da *Acta Vaticana Scripta Varia*, o que proporcionará ao mundo e à Igreja uma janela para os notáveis avanços recentes na Biologia Celular. A maravilhosa atmosfera na Casina Pio IV contribuiu para os debates que esperamos tenham efeitos duradouros no desenvolvimento científico do vasto subcontinente Latino Americano. Os participantes ficaram muito gratos à Academia Pontifícia de Ciências e ao Papa Francisco por esta experiência única.

Apêndice:

Declaração sobre o Programa para Bolsistas Latino Americanos PEW para o Workshop no Vaticano sobre Biologia Celular e Genética

por Rebecca Rimel e Kara Coleman das Fundos Beneficentes PEW

As Fundos Beneficentes PEW tem um longo histórico de financiar a formação de jovens pesquisadores científicos. Fundado em 1985, o Pro-

grama de Estudos Biomédicos PEW fornece financiamento irrestrito para pesquisas para professores assistentes nos Estados Unidos (EUA), dando-lhes total liberdade para testar ideias criativas, potencialmente de risco que têm a possibilidade de retornar grandes dividendos. Como parte deste programa, todos os atuais bolsistas comparecem à reunião anual, um aspecto do programa que serve para promover o desenvolvimento de uma comunidade, rede de contatos e colaboração. Historicamente, estas reuniões anuais são realizadas no Caribe ou América Central e isto levou os Acadêmicos Pew, em uma reunião no México em 1989, a debater suas observações sobre a injustiça dos recursos e subsídio disponíveis aos seus conterrâneos latino-americanos em comparação com os pesquisadores nos EUA.

Na reunião realizada no México em 1989, os acadêmicos abordaram Rebecca Rimel, a presidente e CEO das Fundos Beneficentes PEW, fundadora do Programa de Estudos Biomédicos PEW e Torsten Wiesel, Médico, o então presidente do programa, sobre as suas preocupações. Durante o debate entre a Sra. Rimel e o Dr. Wiesel sobre a melhor abordagem para dar suporte à ciência na América Latina, percebeu-se que era importante financiar não apenas a formação de cientistas talentosos da América Latina, mas que também era crucial incentivá-los a continuar as suas pesquisas nos próprios países de origem. Na época, a ciência na América latina sofria com o êxodo de pesquisadores talentosos que buscavam formação internacional e não voltavam para investir na infraestrutura científica local. Rimel e Wiesel determinaram que qualquer programa para promover a ciência nessas regiões deveria focar na repatriação dos cientistas para a América Latina como prioridade máxima.

Em 1991, as Fundos Beneficentes PEW lançaram o Programa para Bolsistas Latino Americanos PEW. Hoje em dia, o programa fornece 10 bolsas de estudo por ano, sendo que cada uma financia dois anos de formação de pós-doutorado nos EUA. Um pagamento adicional é concedido a cada bolsista que retornar à América Latina para iniciar o seu próprio laboratório. Estes fundos são usados para ajudar a comprar o tão necessário equipamento para iniciar o laboratório. Nos 26 anos de história do programa, 262 bolsas de estudo foram concedidas a cientistas de 10 países da América Latina. Dos bolsistas que terminaram a formação nos EUA, quase 70% optaram por retornar para a América Latina onde agora administram seus próprios laboratórios.

Um levantamento do programa concluído em 2013 revelou o notável impacto que o programa tem tido sobre as comunidades científicas na América Latina. No total, 151 respondentes relataram que formaram

1.469 pessoas, técnicos, estudantes de pós-graduação e de pós-doutorado e cientistas visitantes, numa média total de quase 10 cientistas formados por cada ex-aluno. Os ex-alunos publicaram impressionantes 2.237 artigos para periódicos, resultando em uma média de 15 artigos por respondente, após sua bolsa de estudo Pew e muitos obtiveram inúmeras honrarias. Existem vários ganhadores do Prêmio Bernardo Houssay, dos prêmios da Academia Mundial de Ciências, da bolsa de estudo da Fundação Memorial John Simon Guggenheim, do prêmio Acadêmico Internacional de Pesquisa do Instituto Médico Howard Hughes e da bolsa de estudo da UNESCO-L'Oreal para Mulheres na Ciência. Notadamente, diversos foram apresentados na Academia Latino Americana de Ciências. Além disto, ex-alunos do programa Pew continuam a ser membros ativos na comunidade Pew, onde apadrinham as futuras gerações de participantes no programa Pew ao promoverem a chamada de solicitações para jovens cientistas e participarem na análise das solicitações como parte dos comitês regionais.

A comunidade científica na América Latina continua a crescer e a se fortalecer, por exemplo, o número de doutorados científicos procurados aumentou cerca de 10 vezes na Argentina entre 2000 e 2010 e o número de artigos científicos triplicou entre os cientistas peruanos no mesmo período. No entanto, assim como com todas as comunidades de pesquisa, ainda há espaço para melhoria e existem diversas janelas críticas de oportunidades. Primeiro, é importante que os investimentos em pesquisas biomédicas básicas sejam vistos como prioridade de financiamento, tanto em nível de governo quanto de universidade. Pode ser muito desafiador financiar projetos exploratórios de pesquisa, em vez de aplicados, diante da instabilidade econômica e da inquietação social. No entanto, a importância da pesquisa para a compreensão dos processos celulares básicos não pode ser subestimada. Por exemplo, estudos sobre o sistema celular que degradam proteínas acabaram levando ao desenvolvimento de uma terapia do câncer. Um próspero exemplo na América Latina de um investimento eficaz é a Fundação de Amparo à Pesquisa do Estado de São Paulo, uma agência estadual que direciona mais de 37% do seu financiamento à pesquisa básica. Esta região produz mais da metade dos trabalhos científicos produzidos no Brasil. Embora leve tempo para a pesquisa básica render dividendos, fornecer financiamento aos cientistas para realizar pesquisas exploratórias é o melhor caminho para trazer à luz descobertas inovadoras que irão revolucionar a saúde humana. Os investimentos em unidades laboratoriais, equipamentos e salários também são importantes para sustentar um próspero empreendimento de pesquisas.

Além do apoio da universidade e do governo, é importante para as fundações e filantropias locais investir na ciência em suas regiões. Em muitos países da América Latina, a porcentagem do produto interno bruto gasto em pesquisa e desenvolvimento é inferior a 1%, em comparação com os 2% ou 3% nos países mais desenvolvidos. Sob estas circunstâncias, há uma necessidade clara de apoio proveniente de outras entidades financiadoras para impulsionar o progresso. Na Argentina, Pew estabeleceu parcerias de sucesso com duas fundações em Buenos Aires: Fundación Bunge y Born e Fundación Williams. Juntas, estas fundações fornecem fundos adicionais de repatriação aos bolsistas Pew que retornam para a Argentina. A parceria é mutuamente benéfica: os bolsistas Pew recebem fundos adicionais para start-up e a Argentina é capaz de recrutar cientistas altamente talentosos para suas instituições promovendo mais uma oportunidade de financiamento. Fundações de outros países poderiam considerar um modelo semelhante a fim de recrutar cientistas talentosos para suas regiões e fornecer o tão necessário apoio.

Finalmente, a formação científica em países onde a infraestrutura é menos desenvolvida continua a ser uma oportunidade de investimento. Ao longo dos muitos anos em que o programa Pew vem sendo realizado, o número de solicitações de candidatos da Argentina, Brasil, Chile e México vem aumentando de forma consistente, juntamente com uma melhoria paralela na qualidade do candidato. No entanto, países como a Colômbia, Peru e os da América Central continuam atrás nas solicitações de candidatos. Muitos dos interessados em buscar educação superior vão para o exterior para obter um Ph.D. por causa das opções limitadas em seus países. Como os cientistas continuam a deixar os seus países de origem, isso irá exacerbar o já frágil estado científico. Incentivamos as colaborações regionais, compartilhamento de equipamentos, hospedagem de estudantes e pós-doutorados ou convite a cientistas para apresentarem seminários. Estes pequenos passos podem ajudar a fortalecer as comunidades científicas e as infraestruturas por toda a região.

À medida que a ciência latino-americana olha para o futuro, investimentos em pesquisas e formação são o caminho para apoiar seus pesquisadores talentosos e empenhados e estimular os avanços científicos e a inovação.

Workshop on Cell Biology and Genetics: Summary Statement

Casina Pio IV, 23-24 October 2017

Introduction

A workshop on the subject "Cell Biology and Genetics" was held on 23-24 October 2017 at the Casina Pio IV, the headquarters of the Pontifical Academy of Sciences (PAS) in The Vatican. The main purpose of the meeting was to bring together members of the Pontifical Academy with scientists from the Academia de Ciencias de América Latina (ACAL) to report on recent advances in cell biology, genetics and biomedicine. The fact that ACAL was founded 35 years ago during a special session of the PAS added a particular significance to this workshop, especially because ACAL has recently entered in a new period of vigorous renovation.

Latin America has enormous human capital, with a highly educated and civilized population of great potential. There is a long history of contributions to research. This includes Nobel Prizes awarded to Bernardo Houssay and Luis Leloir for work done in Buenos Aires and to Mario Molina from Mexico for explaining the chemical mechanisms that affect the thickness of the ozone hole. New institutes have been created in recent years that have powerful scientific capabilities, although many challenges remain.

The workshop encompassed a total of twenty presentations in the fields of biophysics and cell membrane biology, cell signaling and developmental biology, neurobiology, biomedicine and genetics. There were also reports about intracontinental scientific collaborations, science and technology initiatives of the US Department of State and the current state and activities of ACAL. In addition, there was a special session devoted to discuss scientific cooperation in Latin America and networking with countries in the Northern Hemisphere.

From the presentations and discussions during the workshop, we have garnered a set of major scientific conclusions that indicate the prominent refinement attained in some areas of the biological sciences in the region. In addition, since this meeting provided a unique occasion to analyze ways to enhance science development of Latin American countries, we provide a set of recommendations that are potentially useful for scientists and public policy makers.

New scientific advances as revealed during the workshop

A wide range of advances in Cell Biology by experts in the field was covered at the workshop. At the cell membrane level: how transient receptor channels for heat and pain work (Ramón Latorre); how gap junction hemichannels mediate inflammatory signals (Juan Carlos Saez); and how mechanically-gated ion channels mediate auditory perception (Ana Belén Elgoyhen) were discussed. In Biophysics and Biochemistry topics presented included: molecular motors that package single DNA molecules into bacterial virus heads (Carlos Bustamante); novel bacteriophage recombinases that allow the design of new genetic circuits in plants (Elibio Rech); how superoxide free radicals cause detrimental nitration of proteins (Rafael Radi); how diabetic glucose levels in endothelial cells cause low grade inflammation via free radicals (Salvador Moncada); how the folding of glycosylated proteins is controlled by the addition and removal of glucose (Armando Parodi); the biochemistry of early life forms (Rafael Vicuña); how protein degradation in lysosomes by microautophagy is regulated by the Wnt growth factor (Edward De Robertis); and how hypoxia is sensed by a molecular machinery involved in cancer (Pablo Wappner). In plant genetics, discussions included: how chloroplasts signal to the nucleus to regulate their own synthesis (Patricia León); how Rhizobium bacteria that fix nitrogen and solubilize phosphorous increase crop production (María Luisa Izaguirre) and how forced expression of three transcription factors generates desiccation-resistant plants (Luis Herrera-Estrella). In biomedicine, topics included: the replication of Zika virus in human radial glia stem cells (Stevens Rehen); how genomics and personalized medicine help decrease the incidence of cerebrovascular stroke (Conrado Estol); how dietary supplementation helps prevent neural tube defects (Rafael Apitz-Castro); how new applications of lasers through biophotonics effectively treats skin cancers, warts, ulcers and other dematologic diseases at very low cost (Vanderlei Bagnato); how protein aggregates of an RNA splicing factor are formed in 97% of cases of Amyotrophic Lateral Sclerosis (Francisco Baralle) and how immunotherapy targeting immune-evasion molecules such as PD-1 and Galectin-1 with monoclonal antibodies is revolutionizing cancer treatment (Gabriel Rabinovich).

Conclusions and recommendations

1. There is general consensus regarding the lack of recognition from governments of Latin American countries of the critical role science plays in both cultural and socio-economic development. As a result, policies for strengthening scientific research and training of young scientists are

generally lacking in government programs. This situation has a severe impact on various grounds, i.e. there are deficiencies in science education at all levels, funds allocated for scientific research are insufficient, initiatives aiming at linking the scientific community with the productive sector are almost nonexistent, etc.

2. An additional consequence of the above condition is that the relative size of the scientific communities in Latin American countries - when measured in relation to their respective general populations - is very small when compared to those of developed countries. Moreover, there are several Latin American countries whose contribution to the world's scientific productivity is virtually negligible.

3. Due to this lack of support many young scientists depart from their home countries and settle in countries of the Northern hemisphere, where they find the proper conditions to express their vocations. Most often these scientists remain abroad, progressively losing contact with colleagues in their home countries. It is proposed that ACAL should play a leading role in assessing the magnitude of this scientific diaspora and thereafter generate networks leading to new initiatives that will benefit Latin American countries, such as research collaborations, training periods for graduate students, participation in thesis committees, promoting novel research programs in the region, etc.

4. State Policies to provide the resources and infrastructure required to repatriate young scientists are inadequate. It will be important to develop long-term national programs to provide the infrastructure and resources needed to facilitate and promote high quality, internationally competitive research programs by young scientists. Science and technology should be viewed as fundamental engines for cultural and socio-economic development in Latin America.

5. There is also ample agreement in that international organisms such as the Organization of American States (OAS), UNESCO and the U.S. Department of State could contribute to science as an engine for development in Latin America. It is also proposed that ACAL, with its continental vocation, should take the lead in contacting these international organisms and work with them on suitable strategies to pursue this objective.

6. ACAL is a civil society formed by scientists for scientists and is independent from any government. It is ideally situated to reactivate the participation of the OAS in promoting exchanges horizontally between Latin American countries. In particular, short-term fellowships for Ph.D. students are greatly needed. In the past OAS had programs to promote scientific exchanges, but sadly no more. ACAL should attempt to convince the Ministers of Science and Technology of OAS, who meet periodically, of the urgent need to re-engage in STEM (Science, Technology, Engineering and Mathematics) programs. Science is non-political and essential for progress.

7. The Howard Hughes Medical Institute had during the past 20 years a Scholars program that transformed biomedical science in Latin America by elevating many researchers who presently occupy leadership positions in their own countries. It was noted with sadness that the program was terminated. The workshop participants, many of whom were or are supported by HHMI, felt that this was the most effective dollar-for-dollar program ever funded by HHMI. Hope was expressed that the Latin Scholars program might be reactivated in future. A program that is ongoing and is having a huge impact in biomedicine is the PEW Latin American Fellows Program. It provides a two-year postdoctoral stipend to study in the U.S. and start-up funds to return to home country institutions. The 180 PEW scholars that have already returned are changing Latin American biomedical science. The PEW Charitable Trusts very generously prepared a report specifically for this Workshop. This report is provided in the Appendix below.

8. Regardless the outcome of the proposed initiatives, scientists should make a special effort in creating links with the various sectors of society. They must interact with the media to contribute to the education of the general public. They must also relate with the private sector, exploring prospects for innovation in the productive processes. Due to its high social relevance, a fluent relationship with authorities of the health sector is particularly relevant. Successful experiences in these fields will constitute the best arguments to convince governments that supporting science is indispensable today as we are living through an amazing convergence of biotechnology, molecular medicine and bioinformatics.

9. In the midst of these challenges, some interesting cases were also presented. For example, the Brazilian Academy of Sciences embarked on a plan aimed at expanding the interactions between scientists and politicians. As a result, the budget for science-related activities did not experience a decrease that would have been expected otherwise. A letter addressed to the President that was signed by several Nobel laureates may have contributed to this effect. On the other hand, in Uruguay the size of the scientific community has been increasing steadily and a national system for evaluation of scientists has been established. In addition, an Academy of Sciences has been recently created. Furthermore, the scientific community in Uruguay has had a decisive role in implementing the national plan aimed at replacing fossil fuels by renewable sources of energy. These paradigmatic examples of Brazil and Uruguay call for optimism and strongly suggest that soundly designed public policies in science promptly translate into social benefits.

The research presented at the workshop will be published as a volume of *Acta Vaticana Scripta Varia*, which will provide the world and the Church with a window into the remarkable recent advances in Cell Biology. The wonderful atmosphere at the Casina Pio IV contributed to discussions that hopefully will have long-lasting effects in the scientific development of the vast Latin American subcontinent. The participants were most grateful to the Pontifical Academy of Sciences and Pope Francis for this unique experience.

Appendix

Statement on the Pew Latin American Fellows Program for the Workshop at the Vatican on Cell Biology and Genetics

by Rebecca Rimel and Kara Coleman of the Pew Charitable Trusts

The Pew Charitable Trusts has a long-standing history of providing funding for the training of young scientific researchers. Founded in 1985, the Pew Biomedical Scholars Program provides unrestricted research funding for assistant professors in the United States (U.S.), giving them free reign to test out creative, potentially risky ideas that have the possibility to return large dividends. As part of this program, all current grantees attend an annual meeting—an aspect of the program that serves to pro-

mote community building, networking, and collaboration. These yearly meetings have historically been held in the Caribbean or Central America, and this led Pew Scholars, at a meeting in Mexico in 1989, to discuss their observations about the inequity of resources and support available to their Latin American contemporaries compared with researchers in the U.S.

At the 1989 Mexico meeting, the scholars approached Rebecca Rimel, the president and CEO of the Pew Charitable Trusts and founder of the Pew Biomedical Scholars Program, and Torsten Wiesel, M.D., then chair of the program, about their concerns. As Ms. Rimel and Dr. Wiesel discussed the best approach to supporting science in Latin American, they realized that it was not only important to provide funding to train talented scientists from Latin America, but that it was also crucial to incentivize them to continue their research in their home countries. At the time, Latin American science was suffering from an exodus of talented researchers seeking international training, who did not later return to invest in the local scientific infrastructure. Rimel and Wiesel determined that any program to further science in these regions should focus on the repatriation of scientists to Latin America as a top priority.

In 1991, The Pew Charitable Trusts launched the Pew Latin American Fellows Program. Today, the program provides 10 fellowships per year, each of which gives funding for two years of postdoctoral training in the U.S. An additional payment is awarded to each grantee who returns to Latin America to start his or her own lab. These funds are used to help purchase much-needed start-up equipment for the laboratory. In the program's 26-year history, 262 fellowships have been awarded to scientists from 10 countries in Latin America. Of those who have finished their training in the U.S., close to 70 percent have chosen to return to Latin America where they now run their own laboratories.

A program survey completed in 2013 revealed the remarkable impact the program has had on scientific communities in Latin America. In total, the 151 respondents reported training 1,469 individuals—technicians, graduate students, postdoctoral fellows, and visiting scientists—amounting to an average of nearly 10 scientists trained per alumnus. Alumni have published an impressive 2,237 journal articles, resulting in an average of 15 articles per respondent, following their Pew fellowship and many have earned numerous honors. There are multiple recipients of the Bernardo Houssay Prize, The World Academy of Sciences prizes, the John Simon Guggenheim Memorial Foundation fellowship, the Howard Hughes Medical Institute International Research Scholar award, and the L'Oreal-UN-

ESCO fellowship for Women in Science. Notably, several have been inducted into the Latin American Academy of Sciences. Additionally, Pew program alumni continue to be active members in the Pew community, ushering in future generations of participants in the Pew program by promoting the call for applications to young scientists and participating in application review as part of regional committees.

The scientific community in Latin America continues to grow and strengthen—for instance, the number of scientific doctorates pursued increased about 10-fold in Argentina between 2000 and 2010, and numbers of scientific articles tripled for Peruvian scientists in the same time period. However, as with all research communities, there is still room for improvement, and several critical windows of opportunity exist. First, it is important that investments in basic biomedical research be viewed as a funding priority at both the government and university level. It can be very challenging to fund exploratory, rather than applied, research projects in the face of economic instability and social unrest. However, the importance of research to understand basic cellular processes cannot be understated. For instance, studies on the cellular system that degrades proteins ultimately led to the development of a cancer therapy. One thriving Latin American example of an effective investment is the São Paulo Research Foundation, a state agency which directs upwards of 37 percent of its funding to basic research. This region produces more than half of the scientific papers that come out of Brazil. While it takes time for basic research to yield dividends, providing scientists with the funding to perform exploratory research is the best way to spark groundbreaking discoveries that will revolutionize human health. Investments in lab facilities, equipment, and salaries are also important to sustaining a thriving research enterprise.

In addition to university and government support, it is important for local foundations and philanthropies to invest in the science in their region. In many Latin American countries, the percentage of gross domestic product spent on research and development is less than 1 percent, compared with 2-3 percent in more developed countries. Under these circumstances, there is a clear need for support from other funding entities to drive progress. In Argentina, Pew has established successful partnerships with two foundations in Buenos Aires: Fundación Bunge y Born and Fundación Williams. Together, these foundations provide additional repatriation funds to Pew fellows returning to Argentina. The partnership is mutually beneficial: the Pew fellows receive additional start-up funds, and Argentina is able to recruit highly talented scientists to their institutions by promoting the

additional funding opportunity. Foundations in other countries could consider a similar model in order to recruit talented scientists to their region, and provide much-needed support.

Finally, scientific training in countries where the infrastructure is less developed continues to be an opportunity for investment. Over the many years that the Pew program has been in place, the number of applications from Argentina, Brazil, Chile, and Mexico has steadily increased—along with a parallel improvement in applicant quality. However, countries such as Colombia, Peru, and those in Central America continue to lag behind in applications. Many who are interested in pursuing higher education go abroad to obtain a Ph.D. because of limited options at home. As scientists continue to leave their home countries, it will exacerbate the already fragile scientific state. We encourage regional collaborations, equipment sharing, hosting students and postdocs, or inviting scientists to present seminars. Such small steps can help to strengthen the scientific communities and infrastructures across the region.

As Latin American science looks toward the future, investment in research and training is a way to support its talented and committed researchers and stimulate scientific advances and innovation.