Scientific Report

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2013

DE DUVE INSTITUTE

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de Duve Institute

Ludwig Institute for Cancer Research

The de Duve Institute

Emile Van Schaftingen

Originally named *International Institute of Cellular and Molecular Pathology* (abbreviated ICP), the de Duve Institute was founded in 1974 by Professor Christian de Duve to develop basic biomedical research with potential medical applications. The guiding principles on which the institute was founded were certainly important for de Duve's own research career, sketched by our colleague and former member of the directorate Fred Opperdoes (see pages 9-12). *Excellence and freedom* of the researchers to choose their own line of investigation are perfectly illustrated by de Duve's dramatic reorientation of his research from biochemical problems linked to insulin's action towards an exploration of the cell leading him to the lysosomes and the peroxisomes. This freedom has a corollary, which is the responsibility to help make these discoveries translate into *medical progress*. Having worked as a team leader at the border between biochemistry and cell biology, de Duve insisted also on the importance of *collaborative work* and valued *interdisciplinary* research.

The main commitment of the members of the de Duve Institute is research. Discovery is the endpoint of their efforts and the only element taken into account for their evaluation. The Institute functions in symbiosis with the Faculty of Medicine of the Université de Louvain and many of its senior members hold a Faculty position and have teaching appointments. The influx of doctoral students and postdoctoral fellows from the University is also a key element in the success. The University hospital (Cliniques Universitaires St-Luc) is located within walking distance of the Institute, which also facilitates collaborations with clinicians.

Benoît Van den Eynde

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions

has been pursued ever since. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is extremely close and the sharing of resources is considerable. The Director of the Brussels Branch of the Ludwig Institute is also a member of the de Duve Institute Directorate.

Miikka Vikkula

The de Duve Institute is managed by a directorate, presently composed of E. Van Schaftingen, B. Van den Eynde, M. Vikkula and F. Lemaigre. The directorate is appointed by the Board of directors, which comprises eminent members of the Belgian business and finance world, as well as the Rector of the University of Louvain, and three other members of the University. About 170 researchers work in the de Duve Institute and in the Ludwig Institute, assisted by a technical and administrative staff of about 80 members. The de Duve Institute has the ambition of pursuing research projects of high quality under conditions that allow original, long-term projects to be pursued. Research is funded by public bodies, national and international, as well as by private donations. Most funds are awarded on a competitive basis. The Institute has an endowment, the strenghtening of which is a goal of the Development and Expansion Council of the de Duve Institute. This endowment is a source of key financing for priority issues, such as the creation of new laboratories for promising young researchers. We expect that the quality of our researchers, supported by sound organisational approaches, will enable the de Duve Institute to stand at the forefront of European Research. We are extremely grateful to all those who support the institute. We wish to address our peculiar thanks to the former president of our Board of Directors, Mr Norbert Martin, for his tireless help during more than 20 years and to his successor, Mr Luc Bertrand.

Frédéric Lemaigre

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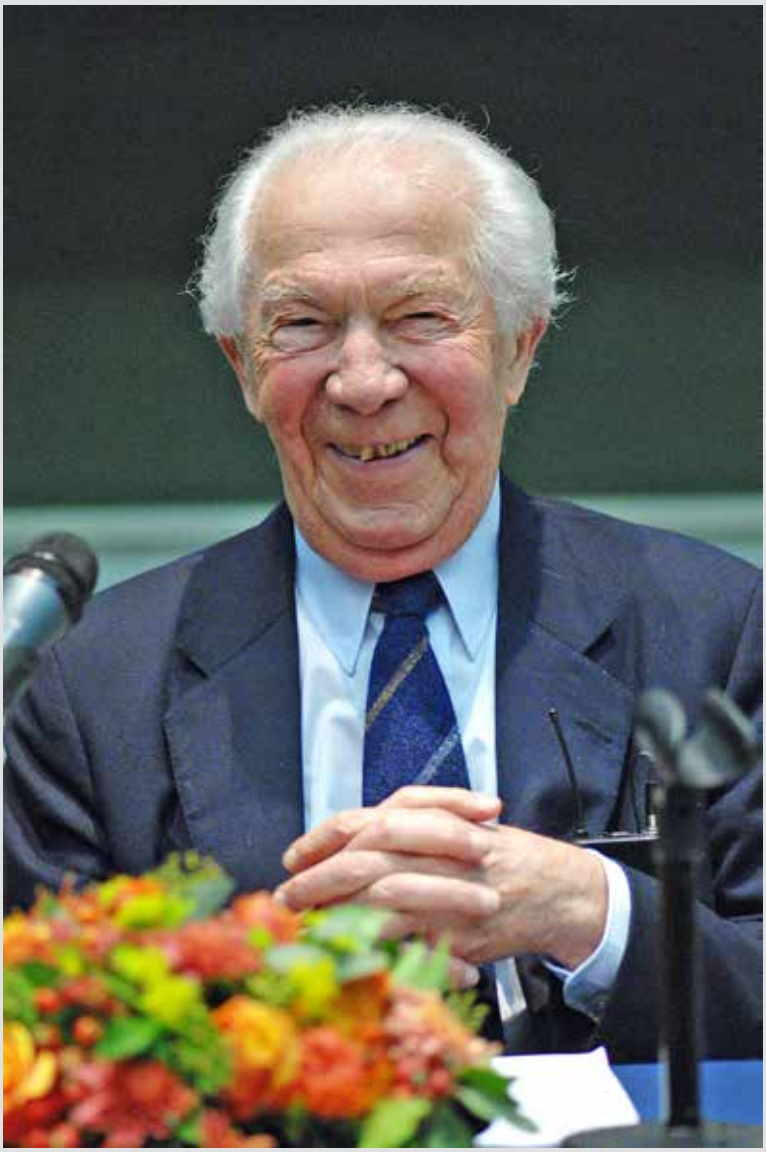

Photo courtesy UCL

Christian de Duve

October 2, 1917 - May 4, 2013

A Feeling for the Cell: Christian de Duve (1917–2013)

Fred Opperdoes
Emeritus Professor, Université catholique de Louvain
Former Member of the Directorate, de Duve Institute
Reprinted from: PLoS Biol 11(10): e1001671. doi: 10.1371/journal.pbio.1001671

Christian de Duve was an internationally renowned cell biologist whose serendipitous observation while investigating the workings of insulin led to groundbreaking insights into the organization of the cell. The observation, which he once described as "essentially irrelevant to the object of our research," ultimately led him to discover two organelles, the lysosome and the peroxisome, for which he shared the Nobel Prize in Physiology or Medicine in 1974 with Albert Claude and George E. Palade.

Born in 1917 in the United Kingdom to Belgian parents who had fled the devastation of the Western Front during the First World War, Christian de Duve spent his early life in the village of Thames Ditton near London. After the war, in 1920, he and his family returned to Belgium and the young Christian went to school in the Flemish city of Antwerp. He embarked upon his career as a researcher when he enrolled as a medical student at the francophone branch of the Catholic University of Louvain in Belgium (1934–1941). He could speak four languages, a skill that would later help save his life.

de Duve decided to specialize in endocrinology and joined the laboratory of the Belgian physiologist J. P. Bouckaert, where he started his research under the difficult circumstances of the Second World War when facilities and financial support for basic research were very limited. Drafted by the Belgian army, he served as a medical officer in France where he was taken prisoner of war by the Germans. Thanks to his excellent knowledge of German and Flemish, de Duve was able to outwit the enemy and escape back to Belgium, where he immediately returned to his research.

As a young researcher, he initially concentrated on the storage and retrieval of glucose, the body's principal energy source, which is regulated by the pancreatic hormone insulin. In doing so, he discovered that a commercial preparation of insulin happened to be contaminated with another pancreatic hormone, the insulin antagonist glucagon, an insight that led to a better understanding of the mode of action of these two hormones.

After the war, Christian de Duve developed an interest in metabolism to gain a better understanding of the exact mode of action of insulin and glucagon. But his knowledge of biochemistry was still limited, so he decided to widen his horizons. He certainly must have had a gift for selecting the very best laboratories of those days. First, he spent almost a year in Hugo Theorell's laboratory at the Nobel Medical Institute in Stockholm; subsequently, he crossed the ocean and went to Gerty and Carl Cori's laboratory at Washington University in St. Louis where he also had the opportunity to collaborate with Earl Sutherland. Later, all four would become Nobel Prize winners. The Nobel prize for Physiology or Medicine was received

by Carl and Gerti Cori in 1947 for their research on glycogen metabolism, Hugo Theorell in 1951 for his discoveries on oxidation enzymes and Earl Sutherland in 1971 for the discovery of cyclic AMP.

In 1947, de Duve returned to Belgium to take up the position of professor at the medical school in Louvain and to continue his research on insulin and glucagon. Beginning in 1950, however, he devoted himself more and more to subcellular biochemistry, a new field that he would pioneer together with Albert Claude and George Palade, both in New York. This change of direction was inspired by a chance observation that would change our understanding of the structural and functional organization of the cell: de Duve noticed that the activity of the liver enzyme glucose-6-phosphatase was mostly associated with a sedimentable cell fraction called microsomes which mainly contained vesicles of the endoplasmic reticulum. (Subsequent analyses established glucose-6-phosphatase as the 'marker' enzyme for the endoplasmic reticulum.) Oddly enough, acid phosphatase, which he assayed as a control enzyme during these studies, was latent — it could be activated by various treatments, all of which affected membrane integrity.

Together with his collaborators in Louvain, de Duve concentrated on refining the techniques of differential and isopycnic centrifugation for separating cell constituents, which had been developed by Albert Claude at the Rockefeller Institute (now Rockefeller University) in New York, to isolate the various cellular compartments and to establish their contributions to cell metabolism. Using this approach while working at the Catholic University of Louvain in 1955, de Duve and collaborators reached the conclusion that acid phosphatase and other hydrolases with an acid pH optimum were enclosed in a new organelle, which they proposed to call 'lysosome'. Later, together with Alex B. Novikoff of the Albert Einstein College of Medicine in New York, de Duve first saw lysosomes when he made electron micrographs of highly purified cell fractions. These organelles turned out to be loaded with hydrolytic enzymes for the digestion of a multitude of macromolecules taken up by the cell. Thus lysosomes, as we now know, function as the "stomach" of the cell's digestive system. A few years later he discovered a second organelle, which was characterized by the marker enzyme catalase and was loaded mostly with enzymes involved in a dangerous form of oxidation, releasing toxic peroxides, explaining why such reactions should be enclosed within a separate membrane-bound compartment. For this organelle de Duve coined the name peroxisome.

The discovery of these new subcellular organelles paved the way for others to identify lysosomal and peroxisomal diseases. Both are inherited metabolic disorders — mutations or deletions of specific genes that code for either lysosomal or peroxisomal proteins, which results in a dysfunctional cell organelle.

From 1962 onwards, because of his collaborative work with Claude and Palade in New York, de Duve divided his time between the Catholic University of Louvain and the Rockefeller Institute, where he was appointed Andrew W. Mellon professor (1962–1987). In 1968, Belgian politicians decided to split the bilingual Catholic University of Louvain along language lines. The francophone part was moved 30 km south to French-speaking Wallonia, (l' Université catholique de Louvain in Louvain-la-Neuve), and de Duve moved to Brussels with the new medical school, which was created at a separate campus from the new francophone university. Immediately after receiving the Nobel Prize, de Duve, together with several of his Belgian colleagues, created a new biomedical institute on the Brussels

campus: the International Institute of Cellular and Molecular Pathology (ICP), an institute of translational medicine that was re-named the de Duve Institute on the occasion of his 80th birthday in 1997. de Duve modeled the ICP after the Rockefeller University by giving an absolute priority to scientific excellence and a total freedom of research to its investigators. An experienced administrator, he both ran the institute and acted as fundraiser, a skill he had acquired while in the United States.

In addition to the Nobel Prize, Christian de Duve was the recipient of the Canada Gairdner International Award (1967), the Dr. H. P. Heineken Prize (1973) from the Royal Netherlands Academy of Arts and Sciences, and the E. B. Wilson Award of the American Society for Cell Biology (1989). In 1975, he was elected a foreign associate of the National Academy of Sciences of the United States of America. Eighteen universities around the world awarded him honorary degrees. He was also a member of the Royal Academies of Medicine and the Royal Academy of Sciences, Arts, and Literature of Belgium; the Pontifical Academy of Sciences of the Vatican; the American Academy of Arts and Sciences; the Academy of Sciences of Paris; the Deutsche Akademie der Naturforscher Leopoldina; and the Royal Society of London. In 1989, he was raised to the rank of Viscount by King Baudouin of Belgium.

In the last period of his life, this tireless researcher again shifted his scientific interest, this time from cell biology to the chemical mechanisms that might have led to the origin of life on Earth. A talented writer, he wrote many influential books on this subject as well as on biology and evolution. In total, he wrote more than a dozen books in English and in French, including *A Guided Tour of the Living Cell; Blueprint for a Cell: the Nature and Origin of Life; Vital Dust: Life as a Cosmic Imperative; Life Evolving: Molecules, Mind, and Meaning; and Singularities: Landmarks on the Pathways of Life*.

Dr. Christian de Duve remained active until the very end of his life, as the photograph taken in his last year demonstrates. He finished his last book *Sept vies en une: Mémoires d'un Prix Nobel* only a few months before he passed away. When he felt that both his health and strength were rapidly subsiding, he decided to end his life at the age of 95. He chose to die by an act of euthanasia, while surrounded by his children.

Christian de Duve was a brilliant scientist, mentor and writer. He will be greatly missed by the scientific community.

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Belgian Cancer Plan, Ministry of Health

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Centre du Cancer - Cliniques St Luc

Cystinosis Research Foundation

European Research Council

Fédération Wallonie-Bruxelles

Fondation contre le Cancer

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Acknowledgements

In 2012, the de Duve Institute has attracted major gifts from several foundations, companies and individuals who have been very generous. These sponsors are providing the resources that enable our scientists to better understand and treat diseases that afflict people around the world. Gifts are the lifeblood of new research initiatives and private resources are crucial in underwriting the costs of new laboratories. On an annual basis, fund-raising from private sources has increased during the past decade over levels achieved previously and now supports about 10 % of the Institute's budget.

The appeal for sponsoring postdoctoral fellowships was also widely followed. In 2012 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Vitalina Gryshkova,

the «Michel de Visscher» fellowship to Anubha Saxena,

the «Maurange» fellowship to Paulina Aleksandrowicz and Harakleia Episkopou,

and other fellowships have been awarded by the Institute to Stefania Cane, Susanna Infantes, Audrey de Rocca Serra.

We express our gratitude to all who contributed to the financing of post-doctoral fellows and state-of-the art research laboratories at the de Duve Institute, ensuring that this institute will remain at the top of the field in biomedical research.

Luc Bertrand
President of the Development and Expansion Council

Doctoral theses (PhD) in 2012 - 2013

10 • 1 • 2012
Dan SU, MEXP
Modulation by LDV infection of macrophage receptors involved in endotoxin shock and blood autoimmune disease
Promoter: J.-P. Coutelier

28 • 2 • 2012
Mélanie UEBELHOER, GEHU
Pathogenic Effects of Mutations causing Venous Malformation
Promoter: M. Vikkula

16 • 3 • 2012
Laurent BULTOT, PHOS
AMPK phosphorylates and inactivates liver glycogen synthase but does not phosphorylate myosin regulatory light chains
Promoter: M. Rider

23 • 4 • 2012
Frédéric SORGELOOS
Evasion of antiviral innate immunity by Theiler's virus L* protein
Promoter: T. Michiels

15 • 5 • 2012
Muriel LEMAIRE
Roles of chitinases and Th17/Th1 cytokines in mouse experimental asthma
Promoter: J.-C. Renauld

11 • 6 • 2012
Sarah CARPENTIER, CELL
Phosphoinositide 3 kinase type III and dynamin are critical for apical endocytic recycling in kidney proximal tubular cells
Promoter: P. Courtoy

22 • 6 • 2012
Melisa GUALDRON-LOPEZ, TROP
Study of the molecular mechanism involved in recycling of matrix protein receptor, PEX5, during glycosome biogenesis in *Trypanosoma brucei*.
Promoter: P. Michels

26 • 6 • 2012
Nicolas PARMENTIER, LICR
Production d'un peptide antigénique par l'insulin-degrading enzyme
Promoter: B. Van den Eynde

28 • 6 • 2012
Emeline de VIRON
Predicting resistance to chemotherapy in chronic lymphocytic leukemia - towards a role of PLK2 and miR-27 in oncogenesis
Promoter: F. Bontemps

2 • 7 • 2012
Ana BRENNAND, TROP
Characterization of proteins involved in turnover of glycosomes in *Trypanosoma brucei*
Promoter: P. Michels

17 • 7 • 2012
Laurent DETALLE , MEXP
Experimental model of autoimmune thrombocytopenia in the mouse: analysis of autoreactive antibodies and of T lymphocyte-dependent pathogenic mechanisms
Promoter: J.-P. Coutelier

31 • 08 • 2012
Laurence DESMYTER, GEHU
Multigenic etiology of cleft lip and palate
Promoter: M. Vikkula

10 • 09 • 2012
Nicole REVENCU, GEHU
Rasa1-Related Phenotypes And Possible pathophysiological Mechanism
Promoter: M. Vikkula

25 • 10 • 2012
Katleen DENONCIN, BCHM-GRM
Dissecting the pathways that promote protein folding in the Escherichia coli periplasm
Promoter: J.-F. Collet

8 • 11 • 2012
Anne-Christine HICK, CELL
Paracrine communications control reorganization of epithelial masses into polarized monolayers
Promoter: C. Pierreux

9 • 11 • 2012
Roxana-Irina ALBU, SIGN
N-Glycosylation and Tandem Cytokine Receptor Modules Are Required for Efficient Traffic and Function of The Thrombopoietin Receptor
Promoter: S. Constantinescu

Scientific prizes and Awards in 2012 - 2013

To Emile Van Schaftingen - 2012
Chaire Francqui au Titre Belge aux Facultés Universitaires de Namur

To Jean-Christophe Renauld - 2012
Prix Scientifique Wivine et Jacques Allard-Janssen 2012

To Laurent Knoops - 2013
Prix Lambertine-Lacroix

To Laurence Boon and Miikka Vikkula - 2013
Prix InBev-Baillet Latour 2013 pour la Recherche Clinique

The J.-F. Heremans Lecture

Following the untimely death, on 29th October, 1975, of our colleague Joseph-Félix Heremans, a J.-F. Heremans Memorial Lecture was created, to be given every other year by a prominent international scientist.

1976 Sir Gustav NOSSAL • The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

1978 Henry KUNKEL • Rockefeller University, New York, USA

1980 Michael SELA • Weizmann Institute of Science, Israel

1982 Jean DAUSSET • Nobel Laureate • Institut de Recherches sur les Maladies du Sang, Hôpital Saint-Louis, Paris, France

1984 Avrion MITCHISON • University College of London, UK

1986 Jan KLEIN • Max-Planck-Institut für Biologie, Tübingen, Germany

1988 Zanvil COHN • Rockefeller University, New York, USA

1990 André CAPRON • Institut Pasteur de Lille, France

1992 Jean-Charles CEROTTINI • Lausanne Branch Ludwig Institute for Cancer Research, Switzerland

1994 Fritz MELCHERS • Basel Institute for Immunology, Switzerland

1996 Lorenzo MORETTA • University of Genova, National Institute for Cancer Research (Immunopathology laboratories), Italian Society of Immunology and Immunopathology, Italy

1998 Charles WEISSMANN • Universität Zürich, Institut für Molecularbiologie, Switzerland

2000 Antonio LANZAVECCHIA • Institute for Research in Biomedicine, Bellinzona, Switzerland

2002 Salvador MONCADA • The Wolfson Institute for Biomedical Research, University College London, UK

2004 Alain FISCHER • Groupe Hospitalier Necker-Enfants Malades, Paris, France

2006 Harvey LODISH • Whitehead Institute for Biomedical Research and Massachussetts Institute of Technology, Cambridge, MA, USA

2008 Jean-Laurent CASANOVA • Hôpital Necker-Enfants Malades, Paris, France

2010 Jules HOFFMANN • Nobel Laureate • Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France

2012 Marc TESSIER-LAVIGNE • President, The Rockefeller University, New York, USA

Plenary lectures 2012

September

Almut SCHULZE
Gene Expression Analysis LaboratoryCancer Research UK, London Research Institute, UK
Balancing macromolecule biosynthesis, bioenergetics and antioxidant production to support cell growth and cancer cell survival

Sebahat OCAK
Pôle pneumologie, ORL et Dermatologie, CHU Mont-Godinne, Belgium
Molecular determinants of lung cancer progression: functional implications of the polymeric immunoglobulin receptor and the focal adhesion kinase

Daniel OLIVE
INSERM UMR 891, Institut Paoli-Calmettes, Marseille, France
Functional expression of BTLA and HVEM on human Vg9Vd2 T cells and non hodgkin lymphomas

Hara EPISKOPOU
National Hellenic Research Foundation, Athens, Greece
Study of the molecular DNA damage response mechanisms induced by alkylating drugs

Wen-Hui LIEN
Laboratory of Mammalian Cell Biology & Development, Rockefeller University, New York, USA
Epigenetic and Transcriptional Regulation of Hair Follicle Stem Cells

October

Guy CORNELIS
Biozentrum, Universität Basel, Switzerland & FUNDP, Namur
Capnocytophaga canimorsus; cave canem !

Christophe CAUX
Centre Léon-Bérard, Lyon, France
Cooperation between plasmacytoid DC and Regulatory T cells in escape to immuno-surveillance in breast carcinomas

Romé VOULHOUX
CNRS Marseille, Université de la Méditerranée, Aix-Marseille II, France
Towards a better understanding of the bacterial type II secretion pathway

November

Tom J. SILHAVY
Department of Molecular Biology, Princeton University, New Jersey, USA
Divide and conquer: Genetic dissection of the -barrel assembly machine

Frédéric BARRAS
Laboratoire de Chimie Bactérienne du CNRS Aix-Marseille, France
Biosynthesis of Fe-S centers, essential and fragile protein cofactors, in Escherichia coli

Dianne COOPER
William Harvey Research Institute, Barts and the London School of Medicine and Dentistry
Galectins in inflammation: sweet or sour?

J.F. Heremans Memorial Lecture
Marc TESSIER-LAVIGNE
President, The Rockefeller University, New York
Wiring the brain: molecular control of axon growth and pruning

Edward KAROLY
Associate Study Director at Metabolon
Metabolomics for advancing biomedical research

December

Alan KORMAN
Bristol-Myers Squibb, Redwood City, CA, USA
Antibody blockade of multiple negative T cell regulators contributes to anti-tumor responses

Ralph Willemsen
APO-T, Amersfoort, The Netherlands
MAGE-A targeted therapy

Plenary Lectures 2013

January

Cédric CORTIJO
Ecole Polytechnique Fédérale de Lausanne, Switzerland
Planar Cell Polarity Controls Pancreatic Beta Cell Differentiation and Glucose Homeostasis

February

Jan WEHKAMP
Department of Internall Medicine and Clinical Pharmacology, Robert Bosch Hospital, Stuttgart, Germany
Relevance and mechanisms of antimicrobial deficiency in the intestinal tract

Amand A. LUCAS
Université de Namur
A fascination for the Double Helix- Optical simulations of the Diffraction of X-rays by DNA

Violaine HAVELANGE
St-Luc Hospital & de Duve Institute, MEXP, Brussels
Implication of microRNAs in acute myeloid leukemia

March

Akira ASAI
Center for Drug Discovery, Graduate School of Pharmaceutical Sciences University of Shizuoka, Japan
Lead discovery and optimization of novel anti-cancer agents

Isabelle MANFROID
ULg, GIGA Research Institute, Liège
From pancreas development to beta cell regeneration in zebrafish

Parmjit JAT
Department of Neurodegenerative Disease, Institute of Neurology, University College London, UK
Dissecting the molecular basis and the signalling pathways underlying cellular senescence

April

Florian KURSCHUS
Universitätsmedizin der Johannes Gutenberg-Universität Mainz, Germany
The role of IL-22 in CNS autoimmunity

Carlo CROCE
Department of Molecular Virology, Immunology and Medical Genetics, Director, Human Cancer Genetics Program, Ohio State University, Columbus, OH, USA
Causes and consequences of microRNA dysregulation in cancer

Francesca SPAGNOLI
Max Delbrück Center for Molecular Medicine, Berlin, Germany
Pancreas development: from fate specification to tissue morphogenesis

May

Maria ASCIERTO
National Institute of Health, Bethesda, MD, USA
Molecular portrait of cancer immune responsiveness: impact on clinical outcome

Isabelle LECLERCQ
UCL, Pôle d'Hépato-Gastroentérologie, Institut de Recherche Expérimentale et Clinique, Brussels
Liver progenitor cells and regeneration of the diseased liver

Jean-Noël OCTAVE
UCL, Institute of Neurosciences, Brussels
Cholesterol, Neuronal activity and Alzheimer disease

Ezra BURSTEIN
UT Southwestern, Dallas, Texas, USA
COMMD1 plays an anti-inflammatory role in vivo and is linked to ulcerative colitis risk

Lawrence PFEFFER
University of Tennessee, Memphis, Tennessee, USA
New therapeutic targets in cancer: Lessons learned from the IFN system

PhD Day, all the graduate students of the de Duve Institute present their work either as a talk or a poster.

June

Pierre COURTOY
de Duve Institute, CELL, Brussels
How did Christian de Duve get the Nobel Prize?

Michael EHRMANN
Center for Medical Biotechnology, University Duisburg-Essen, Germany & School of Biosciences, Cardiff University, Wales, UK
Functional potential of antagonistic principles in biological systems

Vincent DETOURS
IRIBHM - ULBLeader of the Computational Biology Group
A-to-I RNA editing in breast cancer

Christine JACOBS-WAGNER
Yale University (CT) and Howard Hughes Medical Institute (MD), USA
A glimpse into the amazing cell biology of bacteria

Matthias ERNST
Ludwig Institute for Cancer Research, New York Branch & Walter and Eliza Hall - Institute of Medical Research, Melbourne, Australia
Exploiting Stat3 signalling for therapeutic purposes in gastrointestinal tumourigenesis

Research Groups

Genetics of human cardiovascular anomalies, cleft lip and palate and cerebral tumors

Miikka Vikkula

The aim of our research is to understand the molecular mechanisms underlying a variety of disorders of the cardiovascular and skeletal systems, as well as certain cancers. We are especially interested in evaluating the contribution of genetic variation to human disease. The bases of many disorders remain unknown, and current treatments are therefore aimed at alleviating symptoms. Identification of the primary causes as well as modulating factors would allow for the development of treatments that are more specific and "curative". As this research is based on human DNA extracted from blood and tissue samples from patients, the group works closely with several clinicians and multidiciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques universitaires St-Luc; Vascular Anomalies Center, Children's Hospital, Boston, USA; Consultation des Angiomes, CHU de Caen, and Centre labiopalatin, Cliniques Universitaires St-Luc).

Venous malformations and glomuvenous malformations ("glomangiomas")

M. Uebelhoer, J. Soblet, P. Brouillard, M. Amyere, HL. Nguyen, A. Mendola, N. Limaye, L.M. Boon and M. Vikkula, in collaboration with B.R. Olsen, Harvard Medical School, Boston, USA; J.B. Mulliken and S. Fishman, Children's Hospital, Boston, USA; A. Dompmartin, CHU, Caen, France..

Venous malformations (VM) are bluish-purple cutaneous and mucosal vascular lesions. They are often congenital, but can appear later in life. They have a tendency to grow slowly with the growth of the child. Glomuvenous malformations (GVM, "glomangiomas") are a special subtype of venous anomaly. They are clinically similar to VMs, but our clinico-genetic study has defined criteria by which they can be distinguished (Boon et al, 2004).

We previously discovered that rare, hereditary venous malformations are caused by an activating mutation in the endothelial cell receptor tyrosine kinase TIE2/TEK (Vikkula et al, 1996). The use of high-throughput screening technologies was instrumental in the identification of several novel mutations amongst affected families. Despite the ubiquitous presence of these germline mutations, the lesions they cause are localized. We therefore hypothesized that lesion-development requires a somatic second hit to locally disrupt the normal allele of TIE2. We obtained proof for this from one lesion, in which the ligand-binding region of the wild-type allele was deleted somatically, causing a local loss of its ability to function [1]. At least 50% of the far more common sporadic VMs are also caused by somatic mutations in TIE2 [1] (Soblet et al, 2013), as are lesions of Blue Rubber Bleb Nevus syndrome, a rare congenital disorder whose etiology was unknown (Soblet et al, In Preparation). To understand how TIE2 mutations cause VMs, we carried out functional analyses of the effects of VM-causative mutations on endothelial cells, in vitro and in vivo (Fig 1). We find that the aberrant activation of AKT by VM-causative mutations inhibits the transcription factor FOXO1, resulting in a lack of the major smooth muscle cell recruitment factor PDGFB [2]. This would account for the sparse, irregular mural layer characteristic of VM. The importance of the AKT pathway in VM pathogenesis is borne out by the ability of the mTORC inhibitor, rapamycin, to reduce the expansion of lesions in a mouse model of the disease (Boscolo et al, submitted). In an effort to identify the cause of the 50% of sporadic VMs in which no TIE2 mutations are detected, we are using Next Generation Sequencing Technology to carry out deep exome-sequencing of DNA from a series of VM tissues.

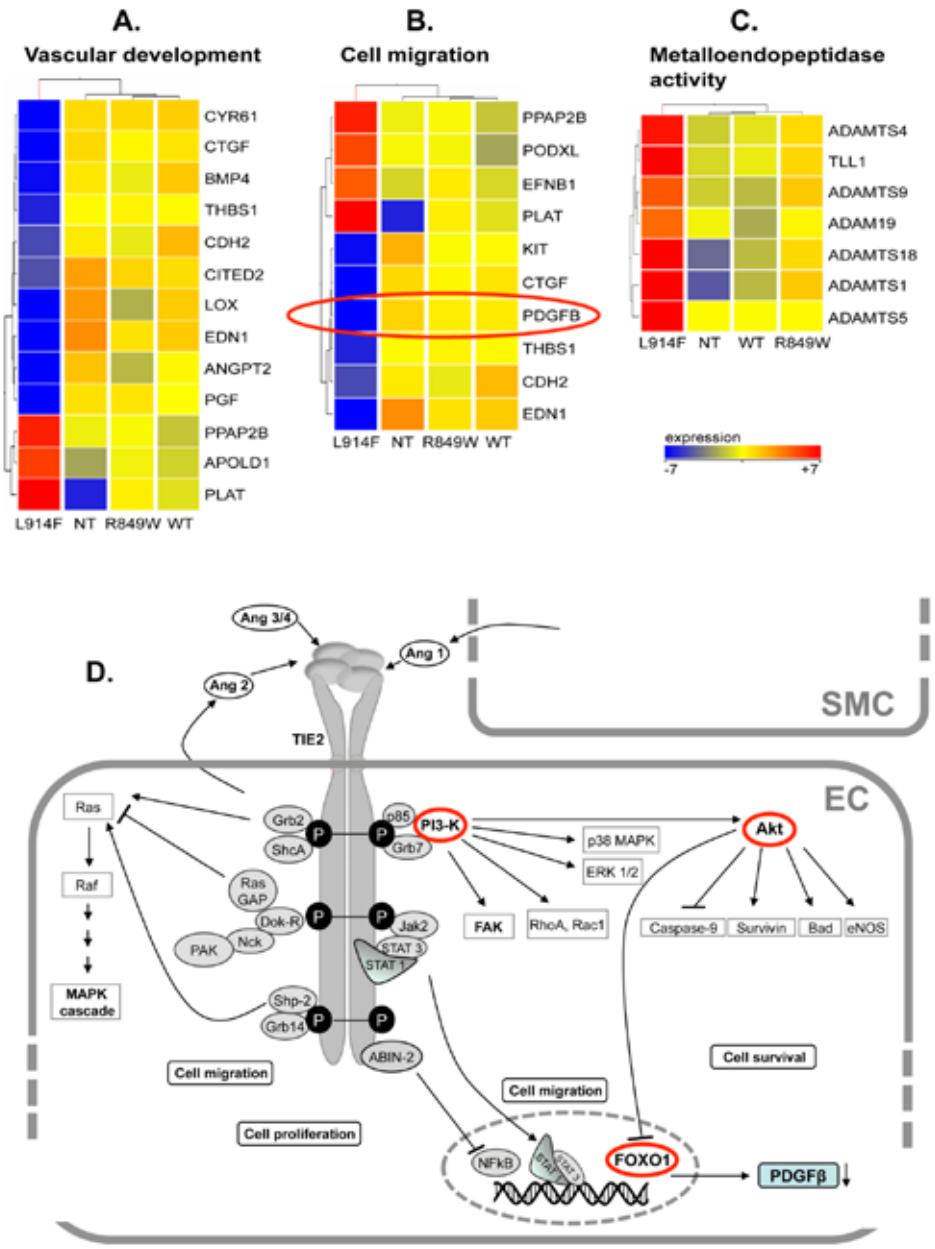

Fig. 1. Effects of TIE2 mutations on endothelial cell transcription. The most frequent TIE2 mutation, L914F, dysregulates genes that participate in angiogenesis (A), cell migration (B), and extracellular matrix turnover (C). In particular, it causes an AKT-dependent deficiency of PDGFB, a major smooth muscle cell attractant produced by normal endothelial cells via its transcription factor FOXO1 (D).

Glomuvenous malformations (GVM) are mostly, if not always, inherited. We discovered that GVM are caused by loss-of-function mutations in a gene we named glomulin (Brouillard et al, 2002). So far, we have identified GLMN mutations in 162 families, the most frequent being present in 44% of them (Brouillard et al, 2013). We have also discovered that the double-hit mechanism holds true for most if not all GVM, predominantly in the form of acquired uniparental isodisomy (aUPID) of chromosome 1p [3]. This renders the inherited glomulin mutation homozygous in affected tissues, without loss of genetic material. To better understand the role of glomulin, we generated glomulin-deficient mice, which are embryonic-lethal. Mice in which glomulin inactivation is conditional (RNAi knockdown) develop further and present defects in the vasculature (Brouillard & Nguyen et al, unpublished). These mice are being used to locally inactivate Glmn in order to produce a GVM model. In parallel, we have pursued in vitro methods to clarify the function of glomulin, identifying potential interacting partners that are now being characterized.

Lymphedema

P. Brouillard, M. Schlögel, HL. Nguyen, A. Mendola, L.M. Boon and M. Vikkula in collaboration with K. Devriendt, KUL; D. Chitayat, Hospital for Sick Children, Toronto, Canada; I. Quere, Montpellier, France.

Lymphedema is caused by a dysfunction of lymphatic vessels, leading to disabling swelling that occurs mostly on the extremities and is extremely difficult to treat. Lymphedema can be either primary or secondary (acquired). Familial primary lymphedema commonly segregates in an autosomal dominant or recessive manner. It can occur in combination with other clinical features. Ten mutated genes have been identified so far in different isolated or syndromic forms of lymphedema: FLT4, FOXC2, SOX18, GJC2, GATA2, CCBE1 [4], PTPN14, KIF11 [5] (Schlogel et al, In Preparation), VEGFC and GJA1. As the prevalence of mutations in these genes was unknown, we investigated seven of them in 78 index patients with familial lymphedema, and five genes in 149 sporadic cases. 28 mutations were found in families and 12 in sporadic patients. Altogether, 17.6% of patients have mutations in one of the known genes, leaving 64% of families and 92% of the sporadic cases unexplained. Interestingly, the proteins encoded by the 10 primary lymphedema genes seem to act primarily within a single functional pathway around VEGFR3 signaling (Mendola & Schlogel et al, in press) (Fig 2). This underscores the importance of this pathway in lymphatic development and function. It suggests that the remaining causative genes, which we have set out to identify using Next Generation Sequencing techniques, are also likely to interact with it.

Vascular anomalies affecting capillaries

M. Amyere, N. Revencu, N. Limaye, L.M. Boon and M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA; S. Watanabe, Showa University School of Medicine, Tokyo, Japan; A. Dompmartin, CHU de Caen, France; Virginia Sybert, Washington University, Seattle, USA

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a localized defect of capillary-like vessels. Hemangiomas have a frequency of up to 12% in 1-year-old children, and typically undergo a period of rapid expansion followed by spontaneous regression. We have an extensive collection of samples from sporadic as well as rare familial forms of hemangioma, and are using Affymetrix high-density whole genome SNP arrays in order to carry out linkage, loss of heterozygosity and copy number analyses on them in an effort to identify causative genomic variants. Moreover, Next Generation Sequencing is being used to screen few smaller coding region alterations. Work done with collaborators has demonstrated that perturbation of the vascular endothelial growth factor (VEGF) signaling pathway can cause hemangioma [6].

CMs occur in 0.3% of newborns. Unlike hemangiomas, they persist throughout life if left untreated. Certain capillary malformations affect specific organs, such as the brain in the case of cerebral capillary malformations (CCMs). We discovered that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with CCM are caused by a mutation in the KRIT1 (Krev interaction trapped 1) gene, suggesting it is important not only for cerebral but also for the cutaneous vasculature (Eerola et al, 2000). In addition, genome-wide linkage mapping on families with inherited capillary malformations led us to identify a linked locus CMC1. Screening of positional functional candidate genes resulted in the identification of mutations in the RASA1 gene, a modifier of the Ras signaling pathway (Eerola et al 2003). This implies that RAS pathway modulators may serve as a novel therapy for these patients in the future. Ongoing studies have led to the identification of RASA1 mutations in 112 index patients. This has allowed for a more precise clinical description of the clinical signs and symptoms associated with this newly recognized disorder that we have named Capillary malformation-arteriovenous malformation (CM-AVM) [7] (Revencu et al, Submitted). Importantly, capillary lesions can be associated with deeper, more dangerous anomalies about 20% of the time; these include arteriovenous malformations and fistulas (AVM/AVF), Parkes Weber, and Vein-of-Galen aneurysmal malformations, which warrant careful clinical management. Subsequently we tested RASA1 in overlapping phenotypes, such as sporadic CM with limb overgrowth and Klippel-Trenaunay syndrome. We did not identify any clear pathogenic change in these patients (Revencu et al, 2013).

Cleft lip and palate

L. Desmyter, M. Quentric, M. Basha, N. Revencu, M. Vikkula, in collaboration with B. Bayet, G. Francois, N. Deggouj, St-Luc, UCL

Cleft lip and palate (CLP) is a congenital anomaly of complex etiology. Predisposition is governed by numerous genetic loci, in combination with environmental factors. Clefts have an incidence of 1/700 births.

We collected DNA samples from a large number of patients affected with popliteal pterygium syndrome, as well as van der Woude syndrome, the most common cleft syndrome. We showed that IRF6 is the major causative gene in our Belgian cohort (Ghassibe et al, 2005). This study in turn led to several collaborations that allowed us to carry out a genotype-phenotype correlation on hundreds of patients from different ethnic backgrounds. Results showed that IRF6 is mutated in 69% of VWS patients and 97% of PPS patients. Interestingly, mutation-distribution is non-random: 80% are localized in IRF6 exons 3, 4, 7 and 9 for VWS, and 72% in exon 4 for PPS patients (de Lima et al, 2009). These findings are of great importance for clinical diagnosis, mutational screens and genetic counseling. We also demonstrated that IRF6 predisposes to non-syndromic clefts in Europe and that it is mutated in familial clefts with minor

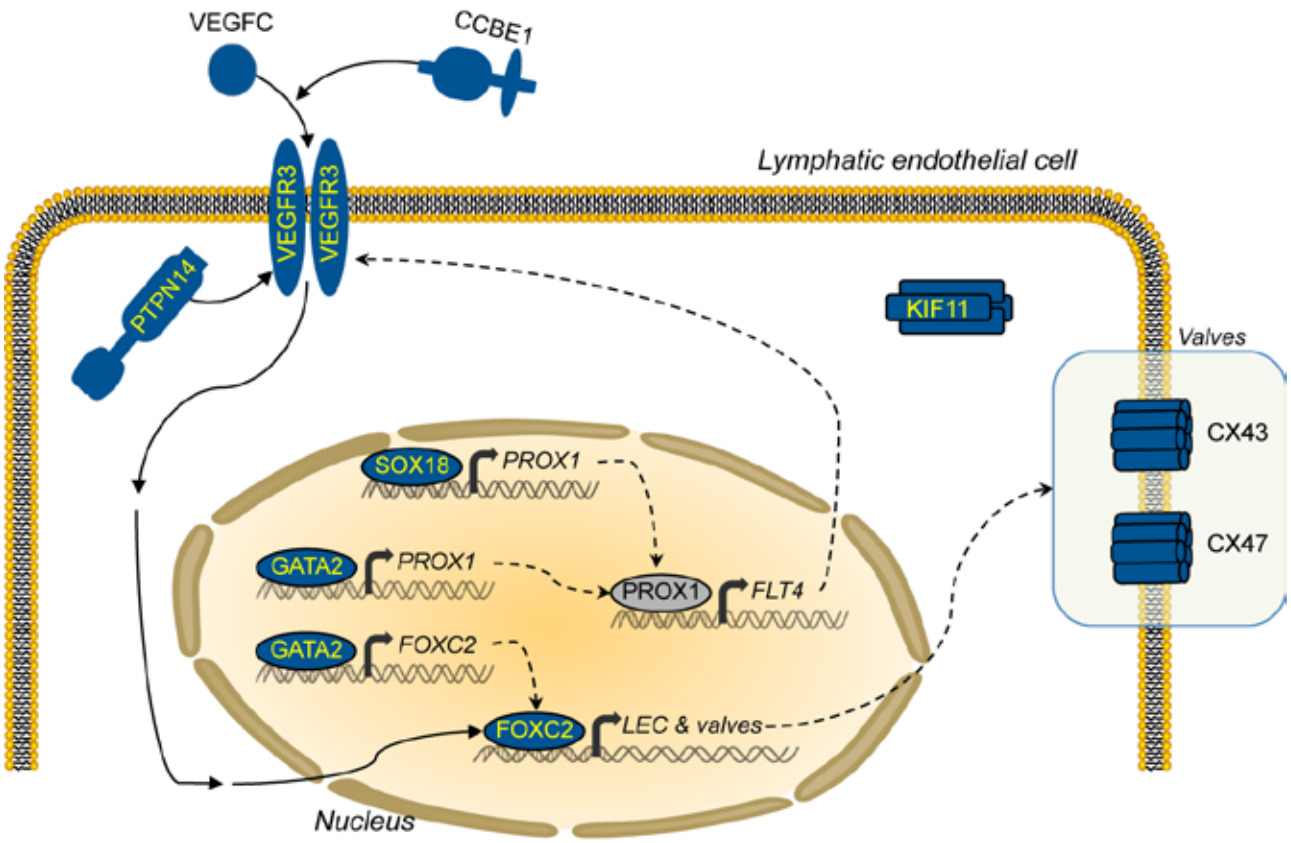

Fig. 2. The function of the proteins encoded by genes mutated in primary lymphedema cluster around a central molecular pathway: VEGFR3 signaling.

lip anomalies (Desmyter et al, 2010). In parallel, we identified a new gene, FAF1, responsible for cleft palate only and Pierre Robin sequence [8]. This gene is associated with clefts across populations. Zebrafish studies confirmed its role during embryonic development and jaw formation. We have begun to use Next Generation Sequencing (NGS) in an effort to uncover additional genes that play a role in non-syndromic orofacial clefts.

Cerebral tumors

M. Vikkula, in collaboration with C. Godfraind, Laboratory of Neuropathology, St-Luc, UCL

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymomas. To better understand the molecular alterations leading to ependymomal oncogenesis, we performed microarray-based expression profiling on a series of 34 frozen ependymomas. Results of our profiling study are in concordance with the "oncology recapitulates ontology" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype. Using array-CGH, we recognized a subgroup of supratentorial ependymomas affecting young adults, which are characterized by trisomy of chromosome 19.
Within the posterior fossa compartment, ependymomas cluster into three sub-groups. The first corresponds with ependymomas that are histologically of WHO grade II, the second with those of WHO grade III, and the third with a group of ependymomas of a bi-phasic appearance, combining regions of both grades. This sub-group shares gene-sets with tumors of both other groups, and in addition has a glycogen metabolism signature of its own. Whether these groups correspond to three distinct tumoral entities, or demonstrate multifocal tumor progression remains to be investigated.

Neuroendocrine tumors

A. Persu, Division of Cardiology, Saint-Luc, UCL; A. Mendola with M. Vikkula.

Pheochromocytomas and head and neck paragangliomas are neuroendocrine tumours derived from the neural crest. Paragangliomas are associated with parasympathetic ganglia and are usually non-secreting. By contrast, pheochromocytomas are derived from paraganglia associated with the orthosym-

pathetic system and are characterized by increased secretion of catecholamines and paroxystic hypertension.
The current project aims to look at the nature and frequency of mutations in the known predisposing genes in pheochromocytoma, paraganglioma and other tumours in Belgium and to detect possible genotype-phenotype correlations.
A multicentric collaboration including more than 20 hospitals from Belgium has therefore been established. Clinical information and DNA from >200 patients with pheochromocytoma and paraganglioma, and more recently >50 patients suffering from thyroid tumours has been obtained.
The SDH genes code for the subunits of succinate deshydrogenase, at the crossroad of the mitochondrial respiratory chain and Krebs cycle. The four subunits of succinate deshydrogenase, i.e. SDHD, SDHB, and more rarely SDHC and SDHA, have been associated with paraganglioma and pheochromocytoma. Furthermore, SDHB mutations have been associated with an increased risk of recurrence and malignancy in several European series.
In our study, the prevalence of SDHx mutations in head and neck paraganglioma was high (in the range of 40%). In sporadic cases, the prevalence of SDHB mutations (20 %), was similar to that of SDHD (18 %). Surprisingly, SDHB-associated tumours were mostly late-onset unilateral head and neck paraganglioma without evidence of recurrence or malignancy.
We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine dissemination. No evidence of mutations was found in the known predisposing genes by dHPLC and/or SSCP. An in depth search for the genetic abnormality underlying this unusual form of paraganglioma disclosed a large deletion encompassing SDHB and part of TIMM8B, a gene encoding a mitochondrial chaperone, which may account for the severe phenotype observed in this family.
Our group was also involved in an international collaborative effort to look for the clinical spectrum associated with mutations of TMEM127, a recently described pheochromocytoma susceptibility gene.

Essential hypertension

A. Persu, Division of Cardiology, Saint-Luc, UCL; A. Mendola; with M. Vikkula.

High blood pressure - commonly called hypertension - is found in almost >25% of the adult population worldwide and affects 2 million Belgians. Despite the wide range of antihypertensive therapies available, blood pressure is adequately controlled in only 30-40% of hypertensive patients. In a large majority of cases, no specific cause is found ("essential hypertension") and high blood pressure reflects the interplay of lifestyle (obesity, high salt intake) and genetic factors; 30-50 % of blood pressure level is thought to be genetically determined. Despite this, conventional linkage and small, underpowered association studies have failed to establish the role of genetic variants in susceptibility.

In an effort to find genetic variations that account for a significant proportion of blood pressure heritability, and to study the interactions between known variants with mild to moderate effects, we set up a multicentric national association study (HYPERGEN) with the support of the Belgian Hypertension Committee. Thousand hypertensive patients and an equal number of normotensive subjects have been recruited. Detailed phenotyping including renin and aldosterone dosages was obtained, and more than 20 candidate SNPs distributed over 12 candidate genes have been genotyped. The analysis of the results of this large association study is currently under way.

Hematological malignancies and tumors of soft tissue and bone

H. Antoine-Poirel, V. Havelange, F. Duhoux, J. Bodart, G. Ameyre, Human Genetics Center, St.Luc, UCL; with M. Vikkula

The explosion in the identification of genetic biomarkers of hematological malignancies and tumors of soft tissue and bone over the past 20 years has had significant impact on diagnosis, prognosis and treatment, as well as our understanding of the genetic and epigenetic processes that lead to tumorigenesis. Our aim is to characterize genomic alterations in both types of malignancy because their oncogenic mechanisms exhibit significant similarities. Towards this end, we use a variety of techniques including conventional and molecular cytogenetics or FISH, molecular biology, and microarrays to identify partner genes in reciprocal translocations and study their functional consequences. We characterized new partner genes of known multipartner genes MLL/11q23, ABL/9q34, PDGFRβ/5q33, and USP6/17p13, as well as unknown partner genes. We demonstrated that the functional consequences may be more complex than transcriptional dysregulation by promoter-swap of fusion genes.

In an international therapeutic trial of children with mature B-cell lymphomas, we showed the adverse prognostic impact of chromosomal alterations of 13q and 7q, detected by cytogenetics. Using genome-wide SNP array technology, we found that most 13q alterations lead to an amplification of the microRNA 17-92 cluster, known to interact with the MYC oncogene, a finding confirmed with mature miRNA expression profiling. In addition, we detected numerous cryptic genomic alterations including partial uniparental disomies. Their prognostic value is currently under study in collaborations within different therapeutic trials across Europe.

SNP-Chip & next generation sequencing platforms

M. Amyere, R. Helaers and M. Vikkula

We host the UCL microarray platform (Affymetrix), used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. We also collaborate with several groups from around the world on whole genome mapping studies. In work done with Dr Jüppner (Harvard Medical School, Boston), we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes (Lorenz-Depiereux et al, 2006). In collaboration with G. Matthijs (KULeuven), we have used autozygosity mapping along with expression profile analysis to identify a new gene for congenital glycosylation disorder [9]. In collaboration with Dr Vermeesch (KULeuven), we genotyped a large family with autosomal-dominant microtia. Copy number analysis led to the identification of five tandem copies of a copy number-variable region at chromosome 4p16, linked to the disease. With the same group, we established that chromosome instability is common during early human embryogenesis in study of 23 pre-implantation embryos from 9 fertile couples. Additionally, rearrangements such as segmental imbalances were observed in 70% of the 23 embryos tested. This explains low human fecundity and identifies post-zygotic chromosome instability as a leading cause of constitutional chromosomal disorders [10].
In an exciting development, this platform is now complemented by a High Throughput Sequencing platform. Funded by the Fondation Contre le Cancer, it consists of a Solid 5500XL sequencer (Life technologies), a Personal Genome Machine (Ion Torrent, Life technologies) and a computing cluster for bioinformatics processing. This equipment allows us to perform Exome-seq, Genome-seq, RNA-seq, Small RNA profiling, ChIP-seq and methylation studies. Data analysis is performed using Lifescope software (Life technologies), and a combination of open source packages (BWA, GATK, snpEff). Downstream evaluation and prioritization of variants is performed using "Highlander", a package that integrates several in-silico analysis programs and utilities with a user-friendly graphical interface (developed in-house by Raphaël Helaers, Ph.D., Bioinformatician). This enhances our ability to identify and explore the genetic and epigenetic bases of disease.

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Liver and pancreas development

Frédéric Lemaigre
Patrick Jacquemin

The group studies the molecular and cellular mechanisms that govern development of the liver and pancreas. The fundamental knowledge gained by this work is essential for improving cell therapy of metabolic diseases of the liver and pancreas (enzyme deficiencies, diabetes), and for understanding the pathophysiology of organ malformations (e.g. polycystic liver diseases, biliary anomalies). Cell differentiation mechanisms which are operating in normal development and which are identified by the research group, are the basis for investigation of abnormal differentiation of liver and pancreatic cancer cells.

Liver development

Jean-Bernard Beaudry, Céline Demarez, Alexis Poncy, Thoueiba Saandi

The main cell types of the liver are the hepatocytes, which exert the metabolic functions of the organ, and the biliary cells (cholangiocytes) which delineate the bile ducts. We study how the hepatocytes and biliary cells differentiate and how bile ducts are formed in the embryo. Our preferred model organism to investigate liver development is the mouse, and this includes generation and analysis of transgenic mouse lines. In collaboration, we also use the Zebrafish as a model organism.

The biliary tract consists of intrahepatic bile ducts which collect bile produced by the hepatocytes, and of extrahepatic ducts which drain bile from the liver to the intestine. Biliary cells delineate the lumen of the bile ducts and modify the composition of bile. These cells, like hepatocytes, derive from embryonic liver progenitor cells called hepatoblasts. Our discovery of the Onecut transcription factors Onecut-1 (OC1/HNF6), OC2 and OC3, and the subsequent phenotypic characterization of HNF6 and OC2 knockout mice led to the identification of the first transcriptional network regulating bile duct development [1]. Current efforts are devoted to the characterization of the transcription factors, microRNAs and signal transduction pathways that control bile duct development in health and disease.

We have identified molecular markers that enabled us to revisit the morphogenesis of the intrahepatic bile ducts, and to uncover that biliary morphogenesis occurs according to a new mode of tubulogenesis [2]. We further investigated how this new knowledge impacts on the interpretation of congenital malformations of the bile ducts. To this end we studied several mouse models and samples from human liver fetuses. Our findings led us to propose a new pathogenic classification of biliary malformations [3].

The transcription factor network that drives cholangiocyte morphogenesis and bile duct formation has been further investigated. By means of a liver-specific gene inactivation strategy we found that Sox9 controls the timing of bile duct development [2]. We pursue this research and have identified another member of the Sox factor family that is essential for cholangiocyte differentiation and development of the bile ducts.

Also, the classical model of liver development predicts that hepatoblasts give rise to hepatocyte precursors, which mature to hepatocytes, and to ductal plate cells, which generate the cholangiocytes. However, only a fraction of the ductal plate cells contribute to bile ducts, while the remaining part was considered to involute by apoptosis. We have now used a lineage tracing approach, which consisted in genetically labeling the ductal plate cells in the embryo, followed by the analysis of their progeny after birth. This uncovered a new fate map of the hepatic cells: the ductal plate cells were shown to generate bile ducts, but also a subset of periportal hepatocytes, as well as cells lining the canals of Hering which harbor the adult liver progenitor cells (Figure 1) [4].

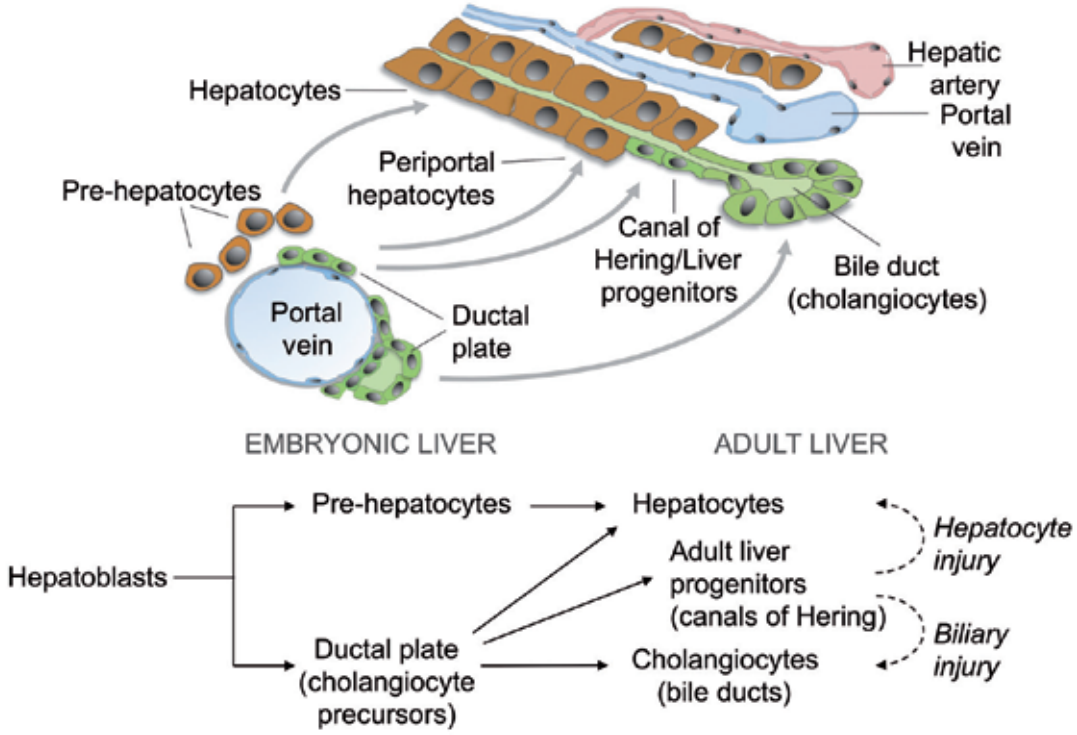

Fig. 1. Fate map of the hepatic cells in embryonic and adult liver

Our work also addresses the mechanisms of hepatocyte differentiation. HNF6 and OC2 are transcription factors that control a number of genes in hepatocytes. Recent findings revealed that lack of binding of HNF6 and OC2 to the gene coding for Coagulation Factor IX causes a common form of hemophilia [6]. In addition, HNF6 and OC2 are critical for normal differentiation of hepatoblasts to hepatocytes or cholangiocytes : in the absence of HNF6 and OC2, the hepatoblasts generate hybrid hepato-biliary cells instead of distinct hepatocyte and cholangiocyte populations [1]. HNF6 also fine-tunes gene expression during hepatocyte differentiation. This involves a feedback loop with microRNAs, in which the liver-specific miR-122 and HNF6 stimulate each other's expression while promoting hepatocyte maturation [7]. In collaboration with the Katholieke Universiteit Leuven, we found that the expression of enzymes synthesizing ketone bodies rises during development, while that of an enzyme catabolising ketone bodies (2-oxoacid CoA transferase) decreases. We showed that the necessary decrease in 2-oxoacid CoA transferase is in part due to repression mediated by miR-122. This illustrates how genes that must be repressed (disallowed genes) become silent during differentiation. Therefore, this work has identified molecular feed-back loops that control gene expression during hepatocyte differentiation (Figure 2).

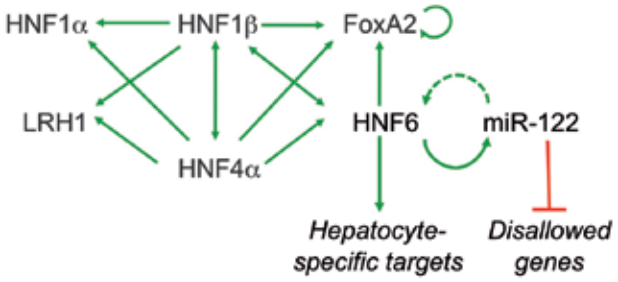

Fig. 2. A positive feedback loop between HNF6 and miR-122 drives hepatocyte differentiation. HNF6 and miR-122 cross-regulate each other while stimulating expression of liver-specific transcription factors and liver-specific functions.

Simultaneously, miR-122 represses genes whose expression in hepatocytes is detrimental to hepatocyte metabolism (disallowed genes).

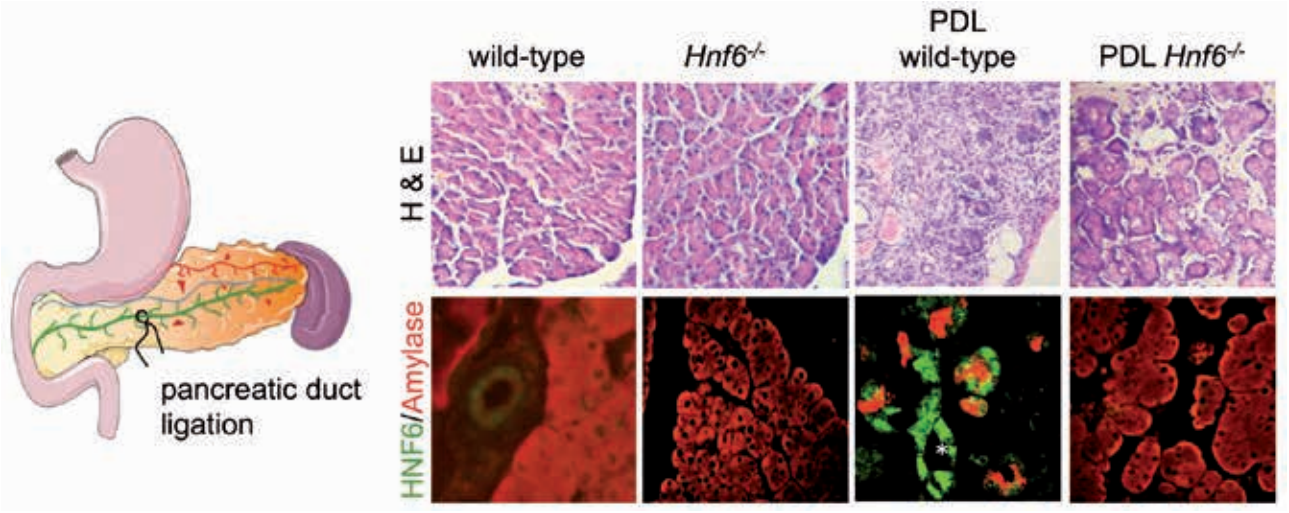

Fig. 3. When the pancreatic duct is ligated, inflammation induces acinar-to-ductal metaplasia (H&E, hematoxylin-eosin staining). HNF6 (green) is induced in acinar cells while the acinar cells loose expression of the acinar-specific gene amylase (red). In the absence of HNF6, pancreatic duct ligation (PDL) induces a less pronounced metaplastic conversion of acinar cells, and acinar-specific genes are less well repressed, indicating that the lack of HNF6 protects against metaplasia.

Pancreatic cell differentiation

Cécile Augereau, Floriane Ausloos, Adrien Grimont, Pierre-Paul Prévot, Thoueiba Saandi

In the embryo, the pancreas develops as an outgrowth of the endoderm, the cell layer that delineates the primitive gut. Pancreatic progenitors derived from the endoderm give rise, through a stepwise process, to endocrine, acinar and duct cells. Our group investigates the molecular mechanisms that control development of the various pancreatic cell types.

The role of the Onecut transcription factor HNF-6 in pancreas development is being studied since several years. We showed that HNF6 is required for development of endocrine cells and pancreatic ducts [8].

After birth, HNF6 is expressed exclusively in the duct cells where it most likely maintains duct cell identity. Interestingly, there is evidence that pancreatic ductal adenocarcinoma may derive from acinar cells which switch their phenotype from acinar to ductal during progression to cancer. This process is called acinar-to-ductal metaplasia, and constitutes a preneoplastic state. We hypothesized that the switch in cell identity depends on the ectopic expression of ductal transcription factors and tested if HNF6 is ectopically induced in acinar cells undergoing metaplasia. This was the case in human pancreas. In addition, we also collected evidence from mouse models that induction of a HNF6 – Sox9 cascade in acinar cells promotes acinar-to-ductal metaplasia, suggesting that these factors are key inducer of preneoplastic lesions (Figure 3) [9].

We then investigated why HNF6 is ectopically expressed in acinar cells when acinar-to-ductal metaplasia is initiated. We found that microRNAs are required in normal pancreas to repress HNF6 expression in acinar cells; in the absence of microRNAs, and more specifically when miR-495 and let-7b are inhibited, HNF6 becomes ectopically induced in acinar cells. This is sufficient to initiate conversion of acinar cells toward a ductal phenotype: acinar cells loose acinar marker expression, while adopting a duct-like morphology. In addition, ectopic liver gene expression is detected. In this process, HNF6 represses transcription factors (Mist1 and RBP-jl) that are essential to maintain acinar differentiation and stimulates hepatic genes. Therefore, our work has uncovered a transcription factor/microRNA network that is essential to maintain acinar cell identity, and whose perturbation contributes to acinar-to-ductal metaplasia, an initial event in cancerogenesis (Figure 4) [10].

Conclusions

Our findings on the role of transcription factors that regulate liver and pancreas development contribute to a better understanding of the diseases affecting these organs. In liver, our work opens perspectives for understanding the pathophysiology of congenital diseases of the liver and for directed differentiation of hepatocytes in culture for cell therapy of liver deficiencies. In pancreas, our observations on the gene regulatory networks in preneoplastic lesions are expected to improve diagnosis and to help preventing progression towards pancreatic ductal adenocarcinoma.

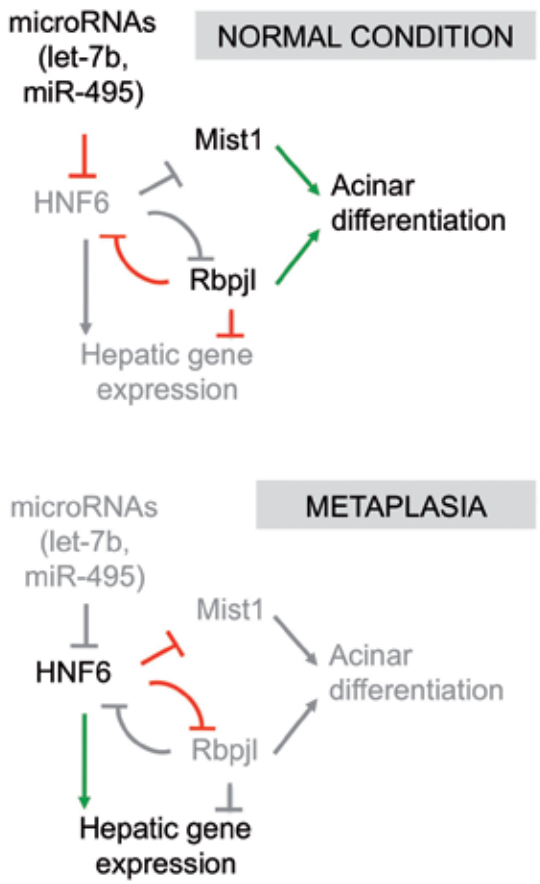

Fig. 4.
Top: transcription factor network maintaining normal acinar cell identity.

Bottom: when miRNA expression is inhibited, like in metaplasia, HNF6 is induced, leading to repression of acinar genes and induction of hepatic genes.

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Genetic and epigenetic alterations in genomes

Anabelle Decottignies
Charles De Smet

Preservation and regulation of genetic information is essential for proper cell function. Consequently, cells have evolved mechanisms of DNA repair, telomere maintenance, and epigenetic regulation of gene expression patterns. Deregulation of these processes contributes to the appearance and progression of cancer cells, which are characterized by genomic rearrangements and dysregulated gene expression patterns. Studies in our group explore the cellular events leading to genomic instability and the mechanisms by which tumor cells maintain their telomeres to acquire immortality. They also investigate the causes and consequences of epigenetic alterations in tumors, such as those involving loss of DNA methylation marks.

Impact of oxidative stress on *Schizosaccharomyces pombe* fission yeast telomeres

S. Lenglez, A. Decottignies

It has long been postulated that telomeres may be especially sensitive to cellular oxidative stress levels, because of their high content in guanine residues, which can be easily oxidized into 8-oxoguanine residues. In this view, oxidized telomeres may possibly be the first triggers of cellular senescence induced by reactive oxygen species (ROS). However, until now, it has been extremely difficult to firmly demonstrate this hypothesis in cells from higher eukaryotes. In the laboratory, we chose to use *S. pombe* fission yeast as model organism to investigate this hypothesis as, first, we had all the tools to construct an isogenic set of mutants defective for oxidative stress response and, second, fission yeast mutants can be obtained that do not have telomeric repeats anymore, because of the circularization of their three chromosomes. Hence, combining these mutations offers great tools to investigate our hypothesis.
During the past few months, we constructed a series of isogenic mutant strains and analyzed their responses to oxidative stress. We also started sequencing fission telomeres in various mutant backgrounds and made some preliminary observations suggesting the existence of ROS-induced recombination events at telomeres.

Regulation of mammalian telomeric transcription

A. Van Beneden, J. Boros, A. Decottignies

Telomeres are specialized protein-DNA structures, which prevent chromosome ends from being recognized as DSBs. Because of their heterochromatic structure, telomeres have been long believed to be silent. However, studies carried out during the last decade indicated that telomeres are frequently transcribed, yielding non-coding RNAs dubbed "TERRAs" (TElomeric Repeat-containing RNAs). Human TERRA molecules mostly consist of (UUAGGG)n repeats that, at least partially, remain bound to telomeres (Fig. 1) where they may impact on diverse aspects of telomere biology, including regulation of telomerase access to telomeres, heterochromatin formation and telomeric loop formation. We are interested in understanding how TERRAs are regulated in mammalian cells.

We showed that telomere length-dependent modifications of telomeric heterochromatin marks, namely H3K9me3 and Heterochromatin Protein 1α (HP1α), impact on TERRA expression levels in human cells. Our data led us to postulate the existence of a negative feedback mechanism for the regulation of TERRA expression and a possible role for TERRA in cell cycle-regulated telomeric heterochromatin formation (1). In addition to providing data about TERRA regulation, our study demonstrated,

for the first time, the existence of an endogenous "telomere position effect" in human cells.
We are continuing our investigation of telomere transcription regulation, both in human and in mouse cells. Notably, we screened TERRA promoters for the presence of predicted transcription factor binding sites. We identified some candidates that we are currently testing. We also started a collaboration with the group of Alban de Kerchove at ULB to study TERRA regulation in mouse brain.

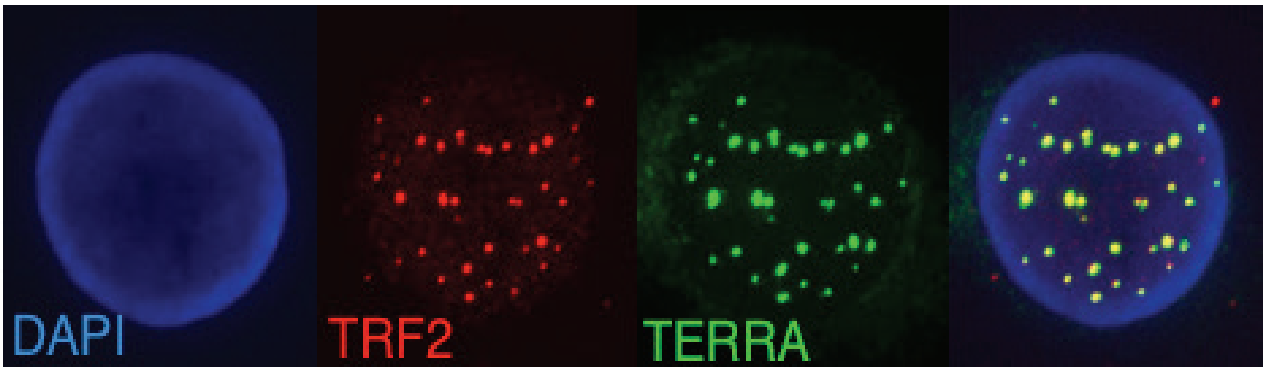

Fig. 1. Detection of TERRA telomeric transcripts at human telomeres. Telomeres are detected by immunofluorescence against TRF2 telomeric protein (red), TERRAs are detected by RNA-FISH using a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue)

Telomeres defects in cells with an alternative mechanism of telomere lengthening

A. Van Beneden, H. Episkopou, A. Decottignies

Cancer cells need an active telomere maintenance mechanism for indefinite proliferation. In about 90% of tumors, this is achieved through reactivation of telomerase. In the remaining 10%, that mostly comprise sarcomas and central nervous system tumors, an alternative mechanism of telomere maintenance, called ALT, is set up that relies on homologous recombinations between telomeric sequences. These two pathways of telomere maintenance are very distinct phenotypically. In telomerase-expressing cells (TEL+), telomere length is very homogenous. However, in ALT cells, telomeres are very heterogeneous in length and some chromatids lack telomeres (Fig. 2).

In normal cells, the binding of very specialized telomeric proteins acts together with a proper telomere structure to represses recombination. The reasons why telomeres undergo so frequent recombinations in ALT cells are still not elucidated. Understanding this may help developing anti-cancer drugs targeting ALT tumors and possibly offer a means to target telomere maintenance in survivor cells that may arise from anti-telomerase treatments that are currently being tested in clinical trials to treat cancer.
Recently, evidences were provided in favor of a distinct heterochromatin structure at ALT telomeres. Notably, mutations in ATRX, an histone chaperone acting at telomeres, have been correlated with the ALT phenotype in pediatric tumors. In the laboratory, we are interested in understanding the nature of defects that drive telomeres towards recombination. To address this issue, we set up an experimental system that allows reliable comparisons between telomeres from ALT and telomerase-positive cells with similar background, and this includes the analysis of cellular hybrids. Our results point towards distinct heterochromatin at ALT telomeres. We now wish to confirm these data and to investigate the genetic defects underlying these distinct heterochromatin features of ALT telomeres, as well as the consequences this has on recombination-based telomere maintenance pathway.

Regulation of Heterochromatin Protein 1 stability at chromatin by PRC2 and H3K27me3

J. Boros, N. Arnoult, A. Decottignies

Heterochromatin Protein 1, or HP1, is a major component of heterochromatin playing crucial roles in chromatin compaction. In 2001, three independent studies reported that HP1 requires the presence of H3K9me2/3 repressive mark on histone H3 to be anchored at chromatin. In the laboratory, we discovered that H3K27me3, a repressive mark that has long been thought to be a marker of facultative heterochromatin, plays an additional and very important role in ensuring the stability of H3K9me3-bound HP1 molecules. Our data revealed that H3K27me3-dependent stabilization of HP1 requires the Polycomb Repressive Complex 2, providing a new important piece of information in the field of HP1 regulation. This work has been performed in collaboration with V. Stroobant and J.F. Collet and is currently under review.

Arnoult N*, Boros J*, Stroobant V, Collet JF, Decottignies A. H3K27me3 cooperates with H3K9 methylation for HP1 binding to chromatin, submitted to Mol Cell.

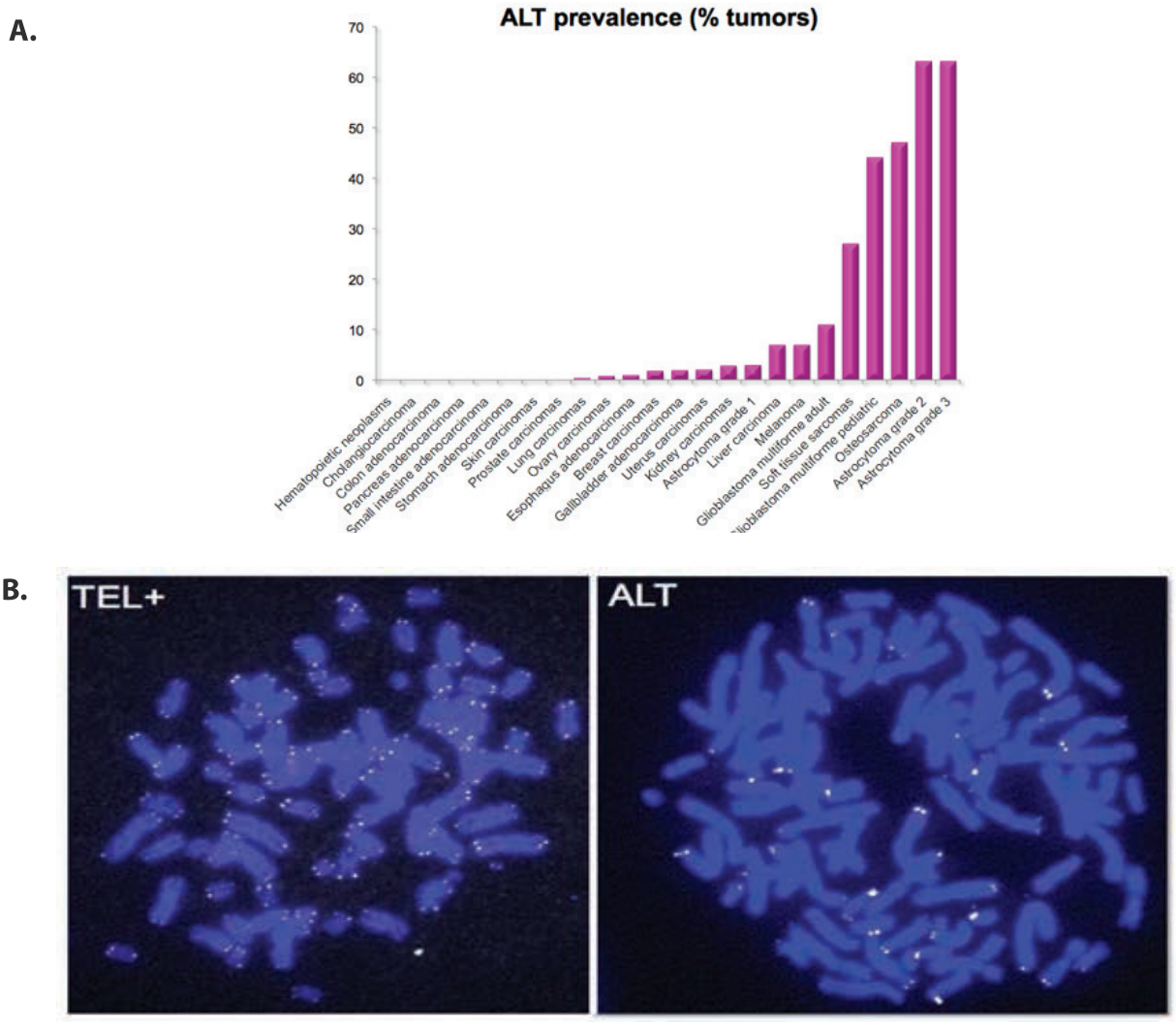

Fig. 2. Alternative mechanism(s) of telomere maintenance. A) ALT prevalence in human tumors. Adapted from Heaphy et al., Am. J. Pathol 2011. B) Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive (TEL+) and ALT cancer cells (ALT). Telomeres are hybridized with a fluorescent telomeric probe (white) and DNA is stained with DAPI (blue). In ALT cells, telomeres display very heterogeneous lengths and, in some instances, are not even detected by the FISH probe.

DNA hypomethylation and aberrant gene activation in cancer

A. Loriot, C. De Smet

Genomic DNA in multiple species is modified by the addition of a methyl group to cytosines in CpG dinucleotides. This heritable epigenetic modification is associated with transcriptional repression. Cell-type specific DNA methylation patterns are established during embryonic development, and are usually maintained in adult somatic cells.

DNA methylation patterns often become altered in cancer cells. Alterations include hypermethylation of selected promoters, leading to silencing of critical genes such as tumor suppressor genes, and hypomethylation of numerous other DNA sequences. We have shown that genome hypomethylation in tumors results in the activation of a group of germline-specific genes, which use primarily DNA methylation for repression in somatic tissues (2). These genes, which were originally discovered because their activation in tumors leads to the expression of tumor-specific antigens, were named cancer-germline (CG) genes. To date, ~50 CG genes or gene families have been identified. Several of these were isolated in our group (2).

The process leading to hypomethylation of DNA sequences in tumors remains obscure. We undertook to address this issue by using MAGEA1, the founding member of the CG group of genes, as a model. Detailed methylation analyses of the MAGEA1 genomic locus in expressing tumor cells, revealed

preferential hypomethylation within the 5' region of the gene. Furthermore, transfection experiments with in vitro methylated MAGEA1 constructs, indicated that this site-specific hypomethylation relies on a historical event of DNA demethylation, and on the presence of appropriate transcription factors to protect the region against subsequent remethylation (3). The factors that are responsible for the initial DNA demethylation process and for maintaining CG gene promoters unmethylated remain to be identified.

Processes associated with CG gene demethylation in tumors

J. Cannuyer, A. Loriot, C. De Smet

Histone modifications have been shown in some cases to dictate DNA methylation states, for instance by regulating access of DNA methyltransferases. We therefore searched to determine if MAGEA1 demethylation and activation in tumor cells is associated with changes in histone marks. Chromatin immunoprecipitation experiments revealed that DNA demethylation and transcriptional activation of MAGEA1 is accompanied by increases in histone H3 acetylation (H3ac) and H3 lysine 4 methylation (H3K4me), and by a decrease in H3 lysine 9 methylation (H3K9me). However, our experiments demonstrate that changes at the histone level within the MAGEA1 promoter are a consequence, not a cause, of DNA demethylation. Consistently, epigenetic drugs that target histone modifications were unable to induce DNA demethylation and stable activation of the MAGEA1 gene. Altogether, these observations confirm that DNA methylation has a dominant role in the epigenetic hierarchy that governs MAGEA1 silencing (4).
A search for gene expression changes associated with the activation of CG genes was performed by analyzing microarray datasets deriving from a series of melanoma cell lines. This led to the identification of a group of genes that were consistently downregulated in the cell lines showing activation of multiple CG genes. Most of these downregulated genes appeared to exert functions associated with cellular proliferation. Intriguingly, this same group of genes was found in another study to be downregulated in cells undergoing senescence, and we showed that their transcription is indeed regulated by pRB, a master regulator of senescence. These observations point therefore towards a possible connection between DNA hypomethylation and cellular senescence in tumor development.

DNA hypomethylation and activation of CG-type miRNAs in tumors

A. Van Tongelen, A. Loriot, J. Blanco, C. De Smet

The role of DNA hypomethylation and CG gene activation on tumor development is only partially understood. To further explore the impact of DNA hypomethylation on tumorigenesis, we decided to find out if this epigenetic alteration also leads to the activation of CG-type microRNAs (miRNAs). It has indeed become clear that this type of small non-coding RNAs exerts important regulatory functions, by controlling the expression of targeted protein-coding genes at the post-transcriptional level. Dysregulated expression of miRNAs is a hallmark of many cancers, where it appears to contribute to several important steps of tumor development. To identify CG-type miRNAs that would become activated in hypomethylated tumors, we initially conducted an in silico selection of miRNAs displaying specific expression in the germline, and determined among these miRNAs those that are susceptible to induction upon treatment with a DNA demethylating agent. This led to the identification of several CG-type miRNAs, which we found to be aberrantly activated in a significant proportion of tumors. Studies aiming at understanding the cellular functions of these miRNAs and their role in tumor development are currently being pursued.

Tumorigenesis-associated DNA hypomethylation within heterochromatic regions of chromosomes

A. Loriot, C. De Smet, A. Decottignies

Constitutive heterochromatin is mainly located at centromeric, pericentromeric, and (sub)telomeric regions of chromosomes. Heterochromatin is usually characterized by a high DNA methylation content, an increased enrichment in repressive histone marks like H3K9me3 and H4K20me3 and the binding of HP1 isoforms. Increasing evidence, mainly obtained from studies in mouse cells, indicates that chromatin modifications at chromosome ends are important regulators of mammalian telomeres. In particular, alterations of histone modifications in telomeric chromatin are associated with telomere length deregulation in mouse cells and a decreased subtelomeric DNA methylation level was reported to up-regulate homologous recombination between mouse telomeric sequences (T-SCE for Telomeric Sister Chromatid Exchange), a hallmark of human ALT cells. This prompted us to evaluate the subtelomeric DNA methylation level of human TEL+ and ALT cancer cell lines (5). We detected a significant hypomethylation of subtelomeric DNA in ALT cancer cell lines when compared to TEL+ cell lines. However, subtelomeric DNA was not hypomethylated in ALT cell lines derived from in vitro immortalization of human fibroblasts with SV40 T antigen, although T-SCE frequencies in the latter cells were similar to those in ALT cancer cells (5). Strikingly, subtelomeric DNA hypomethylation in ALT cancer cells was also associated with lower global DNA methylation, raising the interesting possibility that DNA demethylation in tumor cells may be linked to the process that cells use to escape from senescence and/or crisis, two anti-proliferative barriers thought to require bypass during tumorigenesis. Indeed, evidence accumulated during the past decade that senescent and cancer cells share similarly altered global epigenetic profiles that includes changes in DNA methylation, is in agree-

ment with the hypothesis that senescence, whether induced by ageing or by oncogene activation, may be a common step in the tumorigenesis process (6).

We also investigated the causes and consequences of pericentromeric Satellite 2 DNA (Sat2) in tumor cells. We showed that, although Sat2 locus is frequently hypomethylated in cancer cells, hyperactivation of Sat2 RNA transcription is not observed. Similarly to what we described before for telomeres, pericentromeric Satellite 2 DNA is indeed transcribed to yield non-coding RNA molecules that appear to remain associated with the locus they are transcribed from where they may play important roles in heterochromatin formation. We showed that Sat2 RNA is mainly induced by heat shock pathway activation, both in vitro and in vivo. Besides, we found that hyperactivation of the heat shock pathway, through either hyperthermia or RasV12 oncogene overexpression, is able to induce local demethylation of Sat2, leaving a demethylation signature that was also detected in tumor cell lines displaying moderate levels of genome-wide hypomethylation (7).

Epigenetic repression of CG genes in human embryonic stem cells

A. Loriot, C. De Smet

The stage at which CG genes become methylated during human embryo development has not been determined. We found previously that human CG genes are repressed and methylated in human blastocyst-derived embryonic stem cells and in comparable embryonal carcinoma cells (7). By performing transfection experiments, we demonstrated that human embryonal carcinoma cells target active de novo methylation towards MAGEA1, as the gene became methylated and silenced following integration into these cells. Consistently, silencing of MAGEA1 in embryonal carcinoma cells depended on the presence of both DNMT3A and DNMT3B de novo DNA methyltransferases. Moreover, by analyzing transcription profiling datasets from human preimplantation embryos, we found that transcripts of CG genes increase up to the morula stage, and then decrease dramatically in blastocysts (8). Altogether our data indicate that human CG genes are programmed for repression in the blastocyst, and suggest that de novo DNA methylation is a primary event in this process.

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Metabolite repair and inborn errors of metabolism

Emile Van Schaftingen
Maria Veiga-da-Cunha

The study of L-2-hydroxyglutaric aciduria, a neurometabolic disease characterized by the accumulation of L-2-hydroxyglutarate and the identification of the enzymes that make and destroy L-2-hydroxyglutarate in mammals led us to realize the importance of a novel type of repair mechanism : metabolite repair [4, 7]. This research theme is connected with our other main research interest, the elucidation of inborn errors of metabolism. Many metabolic diseases are due to a defect in an enzyme that is not easily measured or that is expressed in a tissue that is not 'accessible'. In such cases, the defect can be easily established through the search of mutations in the gene encoding the relevant enzyme, provided this gene is known. Quite a number of enzymes are still 'orphan', i.e., the gene that encodes them is not known for any species. One of our aims is to carry out the molecular identification of 'orphan' enzymes that are potentially deficient in metabolic disorders.

Metabolite repair

L-2-hydroxyglutaric aciduria is due to a defect in a FAD-linked enzyme that catalyses the irreversible conversion of L-2-hydroxyglutarate to alpha-ketoglutarate, a tricarboxylic acid cycle intermediate. L-2-hydroxyglutarate does not belong to any classical metabolic pathway, but is formed by a (minor) side-activity of mitochondrial L-malate dehydrogenase, the enzyme that normally interconverts oxaloacetate and L-malate [7]. As L-2-hydroxyglutarate does not play any physiological role but is toxic if it accumulates, L-2-hydroxyglutarate dehydrogenase has to be considered as a repair enzyme, playing a role akin to the proofreading activities associated with DNA polymerases and aminoacyl-tRNA synthases. Hence the designation 'metabolite proofreading enzymes' for enzymes serving to repair 'wrong' reaction products made by other enzymes of intermediary metabolism [7]. Because the specificity of metabolic enzymes is far from absolute, it is likely that metabolite proofreading enzymes abound, but that most of them are still unknown. One of our aims is to identify 'new' metabolite proofreading enzymes, not only because this will allow to attribute a function to putative enzymes encoded by mammalian and other genomes, but also because it may help understand the pathophysiology of some inborn errors of metabolism.

Repair of damaged NAD(P)H

A.Y Marbaix, G. Noël, E. Van Schaftingen , C.L. Linster in collaboration with D. Vertommen

As initially shown by the group of Edwin Krebs in the 1950's, NADH is slowly converted to a hydrated form by glyceraldehyde-3-phosphate dehydrogenase. This hydrated form of NADH (called NADHX) and a similar hydrated form of NADPH (NADPHX) also spontaneously arise at high temperatures or acidic pH. An ATP-dependent dehydratase that reconverts NADHX and NADPHX to NAD(P)H was also described by Krebs' group, but its sequence had not been identified. To fill this gap, we purified yeast NAD(P)HX dehydratase and identified it to a highly conserved and nearly ubiquitous protein, named YKL150c in Saccharomyces cerevisiae and CarkD (carbohydrate kinase domain) in mammals. We showed that both the yeast and human proteins catalyze the dehydration of the (S) form of NADHX and NADPHX, while converting ATP to ADP. Surprisingly, the Escherichia coli homolog, YjeF, a bi-domain protein, catalyzes a similar reaction, but uses ADP instead of ATP (Fig. 1). This represents an unprecedented example of orthologous enzymes using either ADP or ATP as phosphoryl donor.

The dehydration reaction is ascribable to the C-terminal domain of YjeF. Its N-terminal domain is also highly conserved in the living world, corresponding to a separate protein named

apolipoprotein A-1-binding protein (AIBP) in mammals and YNL200C in yeast. We showed that these proteins catalyze the epimerization of the (S) and (R) forms of NAD(P)HX, thereby allowing, in conjunction with the energy-dependent dehydratase, the repair of both epimers of NAD(P)HX (Fig. 1). Both enzymes are very widespread in eukaryotes, prokaryotes, and archaea. This wide distribution and the ADP-dependence of the dehydratase in some species indicate the ancient origin of this repair system [5]. Recent work indicates that mammalian NAD(P)HX dehydratase and NAD(P)HX epimerase are present in the cytosol, in the mitochondria and, in the case of the former enzyme, also in the endoplasmic reticulum. This is in accordance with the presence of NAD(P)H pools in these compartments.

EthylmalonylCoA decarboxylase

C.L. Linster, G. Noël, M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with V. Stroobant, D. Vertommen, M.F. Vincent and G.T. Bommer

The identification of ethylmalonyl-CoA decarboxylase is the result of a search for an enzyme that would correct a known side-activity of acetyl-CoA carboxylase and propionyl-CoA carboxylase. This side activity is the carboxylation of butyryl-CoA to ethylmalonyl-CoA, a product without any known function in animals. We found that mammalian tissues contain a previously unknown enzyme that decarboxylates ethylmalonyl-CoA (Fig. 2) and, at lower rates, methylmalonyl-CoA, but does not act on malonyl-CoA. Ethylmalonyl-CoA decarboxylase is particularly abundant in brown adipose tissue, liver, and kidney in mice, and is essentially cytosolic. Because *Escherichia coli* methylmalonyl-CoA decarboxylase belongs to the family of enoyl-CoA hydratase (ECH), we searched mammalian databases for proteins of uncharacterized function belonging to the ECH family. Combining this database search approach with mass spectrometry data obtained on a partially purified enzyme preparation, we identified ethylmalonyl-CoA decarboxylase as ECHDC1 (ECH Domain containing 1). We confirmed this identification by showing that recombinant mouse ECHDC1 has a substantial ethylmalonyl-CoA decarboxylase activity and a lower methylmalonyl-CoA decarboxylase activity but no malonyl-CoA decarboxylase or enoyl-CoA hydratase activity. Furthermore, ECHDC1-specific siRNAs decreased the ethylmalonyl-CoA decarboxylase activity in human cells and increased the formation of ethylmalonate, most particularly in cells incubated with butyrate. These findings indicate that ethylmalonyl-CoA decarboxylase may correct a side activity of acetyl-CoA carboxylase and suggest that its mutation may be involved in the development of certain forms of ethylmalonic aciduria [3,4].

Identification of enzymes potentially implicated in neurometabolic diseases

Characterization of the enzyme that synthesizes N-acetylaspartate

G. Tahay, E. Wiame, E. Van Schaftingen in collaboration with D. Tyteca and P.J. Courtoy

We reported in 2009 the identification of the enzyme that catalyses the synthesis of N-acetylaspartate, a most abundant compound present in brain. We also reported that the gene (NAT8L) encoding this enzyme is mutated in the only case of N-acetylaspartate deficiency that is known worldwide. NAT8L is membrane-bound and is at least partially associated with the

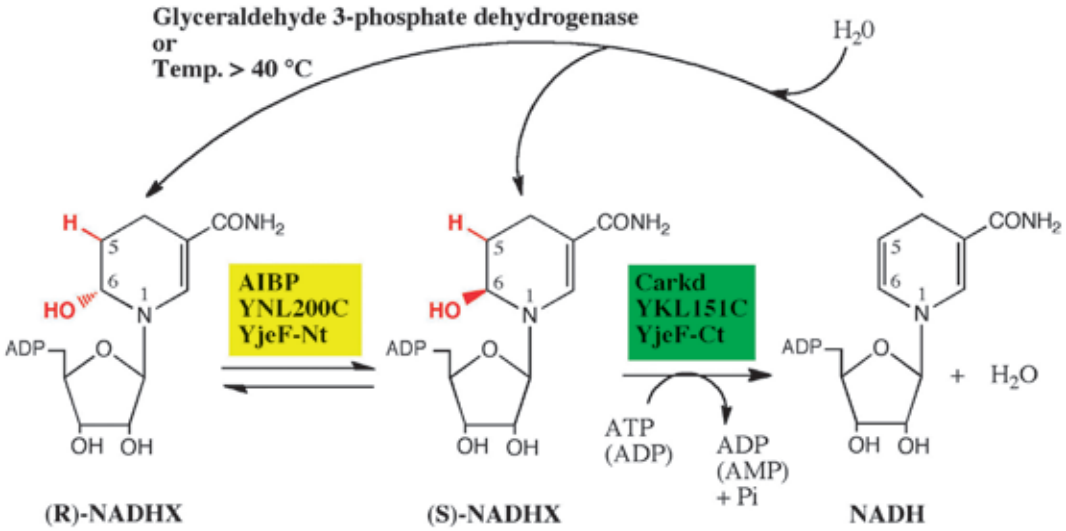

Fig. 1. Formation and repair of hydrated NAD(P)H. Modified from Marbaix *et al*. [5]

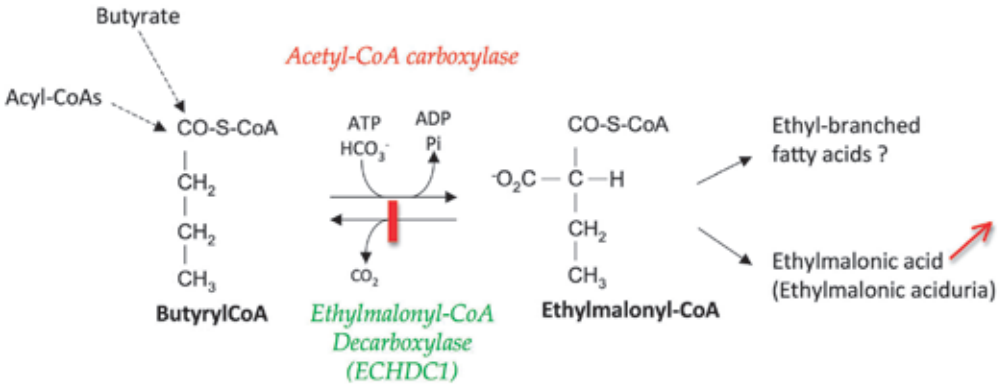

Fig. 2. Ethylmalonyl-CoA formation and repair. See text. [4]

endoplasmic reticulum (ER). We recently determined which regions of the protein are important for its catalytic activity and its subcellular localization. Transfection of truncated forms of NAT8L into HEK (human embryonic kidney)-293T cells indicated that the 68 amino-terminal residues have no importance for the catalytic activity and the subcellular localization of this enzyme, which was exclusively associated with the ER. Mutation of conserved residues that precede (Arg81 and Glu101) or follow (Asp168 and Arg220) the putative membrane region (a stretch of ≈ 30 hydrophobic residues) markedly affected the kinetic properties, suggesting that the catalytic site involves residues that precede and follow the membrane region in the sequence and that this membrane region has a loop structure. Transfection of chimaeric proteins in which GFP (green fluorescent protein) was fused to different regions of NAT8L indicated that the membrane region is necessary and sufficient to target NAT8L to the ER. Thus NAT8L is targeted to the ER membrane by a hydrophobic loop that connects two regions of the catalytic domain. This structure accounts for the high sensitivity of this enzyme to detergents. Other experiments showed that the catalytic site of NAT8L faces the cytosol. Thus, NAT8L's activity is controlled by the cytosolic concentrations of its substrates and products [6].

Synthesis and breakdown of N-acetylaspartylglutamate and beta-citrylglutamate

F. Collard, E. Van Schaftingen in collaboration with D. Vertommen and S. Constantinescu

N-acetylaspartylglutamate (NAAG) is the most abundant dipeptide present in vertebrate central nervous system. β-citrylglutamate, a structural analogue of NAAG is present in testis and immature brain. The function of both compounds is still mysterious, and until recently only little was known on the enzymes involved in their metabolism, except for glutamate carboxypeptidase 2 (GCP2), a membrane-bound ectoenzyme, which is responsible for the hydrolysis of NAAG. We reported in 2010 the molecular identification of the two enzymes (RIMKLA and RIMKLB) that synthesize NAAG and ß-citrylglutamate [1]. More recently we reported the identification of the enzyme that hydrolyses ß-citryl-glutamate [2].

To this end, this enzyme was partially purified from mouse testis and characterized. Interestingly, in the presence of Ca2+, the purified enzyme specifically hydrolyzed β-citrylglutamate and did not act on NAAG. However, both compounds were hydrolyzed in the presence of Mn2+. This behaviour and the fact that the enzyme was glycosylated and membrane-bound suggested that β-citrylglutamate hydrolase belonged to the same family of protein as glutamate carboxypeptidase 2 (GCP2), the enzyme that catalyzes the hydrolysis of NAAG. The mouse tissue distribution of β-citrylglutamate hydrolase was strikingly similar to that of the glutamate carboxypeptidase 3 (GCP3) mRNA, but not that of the GCP2 mRNA. Furthermore, similarly to β-citrylglutamate hydrolase purified from testis, recombinant GCP3 specifically hydrolyzed β-citrylglutamate in the presence of Ca2+, and acted on both NAAG and β-citrylglutamate in the presence of Mn2+, whereas recombinant GCP2 only hydrolyzed NAAG and this, in a metal-independent manner. A comparison of the structures of the catalytic sites of GCP2 and GCP3, as well as mutagenesis experiments revealed that a single amino acid substitution (Asn-519 in GCP2, Ser-509 in GCP3) was largely responsible for GCP3 being able to hydrolyze β-citrylglutamate. Based on the crystal structure of GCP3 and kinetic analysis, we propose that GCP3 forms a labile catalytic Zn-Ca cluster that is critical for its β-citrylglutamate hydrolase activity [2].

Metabolism of hydroxylysine and phosphoethanolamine

M. Veiga-da-Cunha, F. Hadi, T. Balligand, E. Van Schaftingen in collaboration with V. Stroobant

Hydroxylysine, an amino acid found in collagen, is known to be metabolized by phosphorylation of its hydroxyl group, followed by ammonia and phosphate elimination to yield aminoadipate semialdehyde (Fig. 3). The latter reaction is similar to the conversion of ethanolamine-phosphate to acetaldehyde,

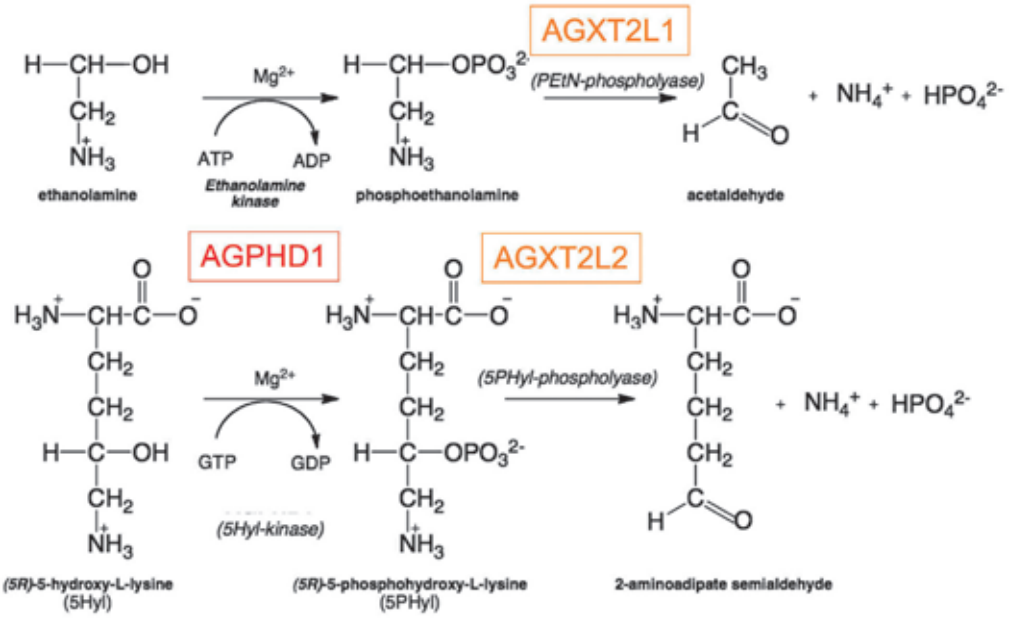

Fig. 3. Metabolism of hydroxylysine and phosphoethanolamine. From [9].

inorganic phosphate and ammonia. None of the three enzymes involved in these reactions was molecularly identified. We recently identified them through an approach that was largely based on bioinformatics.

Database searches drew our attention on AGXT2L1 and AGXT2L2, two vertebrate genes encoding closely related pyridoxal-phosphate-dependent enzymes of unknown function. Interestingly, bacterial homologues of AGXT2L1 and AGXT2L2 often form bi- or tri-functional proteins with a putative kinase belonging to the family of aminoglycoside phosphotransferases. Because of the frequent functional relationship between the different domains found in multifunctional proteins, these observations suggested that AGXT2L1 and AGXT2L2 acted on phosphorylated, aminated compounds. Furthermore, the kinase domain of these bacterial proteins is homologous to a vertebrate protein of unknown function, designated AGPHD1, which was therefore likely to phosphorylate a compound bearing both an amine and a hydroxyl.

These and other considerations led us to hypothesize that AGPHD1 corresponded to 5-hydroxy-L-lysine kinase and that AGXT2L1 and AGXT2L2 catalyzed the pyridoxal-phosphate-dependent breakdown of phosphoethanolamine and 5-phosphohydroxy-L-lysine. The three recombinant human proteins were produced and purified to homogeneity. AGPHD1 was indeed found to catalyze the GTP-dependent phosphorylation of 5-hydroxy-L-lysine (Fig. 3). The phosphorylation product made by this enzyme was metabolized by AGXT2L2, which converted it to ammonia, inorganic phosphate, and 2-aminoadipate semialdehyde. AGXT2L1 catalyzed a similar reaction on phosphoethanolamine, converting it to ammonia, inorganic phosphate, and acetaldehyde. AGPHD1 and AGXT2L2 are likely to be the mutated enzymes in 5-hydroxylysinuria and 5-phosphohydroxylysinuria, respectively. The high level of expression of AGXT2L1 in human brain, as well as data in the literature linking AGXT2L1 to schizophrenia and bipolar disorders, suggest that these diseases may involve a perturbation of brain phosphoethanolamine metabolism. AGXT2L1 and AGXT2L2, the first ammoniophospholyases to be identified, belong to a family of aminotransferases acting on ω-amines [8].

We recently showed that phosphohydroxylysinuria is indeed due to mutations in the AGXT2L2 gene. Two allelic mutations, p. Gly240Arg and p.Glu437Val, were found in a patient with this condition. Both mutations replace conserved residues and cause major folding problems, as assessed by expression of recombinant proteins in Escherichia coli and HEK293T cells. The finding that the mutations p.Gly240Arg and p.Glu437Val are present at low frequencies in the European and/or North American population suggests that this phosphohydroxylysinuri is more common than previously thought. The diversity of the clinical symptoms described in three patients with phosphohydroxylysinuria indicates that this is most likely not a neurometabolic disease [9].

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Nucleoside analogues in leukaemia

Françoise Bontemps
Eric Van Den Neste

Our group was initially interested in purine metabolism, particularly adenine nucleotide metabolism, and its genetic defects. Thereafter, we expended our investigations on two therapeutic purine nucleoside analogues, 2-chlorodeoxyadenosine and fludarabine, which have revolutionized the treatment of indolent lymphoproliferative disorders. Despite their efficacy, clinical resistance to these drugs is frequently observed. The main objectives of our present studies are to unravel the mechanisms leading to resistance to nucleoside analogues and to find novel therapeutic strategies to counteract them, particularly in chronic lymphocytic leukaemia.

In 1997, a collaborative study of the antileukaemic purine nucleoside analogues (PNA), 2-chlorodeoxyadenosine (CdA) and fludarabine (Fig. 1), was started with the Department of Haematology of the University Hospital Saint-Luc. These two deoxyadenosine analogues display remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and chronic lymphocytic leukaemia (CLL). Nevertheless, resistance is also observed, and PNA do not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of our studies are to understand the mechanisms that lead to resistance to nucleoside analogues, and to improve their therapeutic efficacy by searching for synergisms with other compounds.

CdA and fludarabine are prodrugs. To exert their antileukaemic effect, they have to be phosphorylated by deoxycytidine kinase (dCK) into nucleoside analogue monophosphates, followed by conversion into di- and triphosphate derivatives. The latter are the active metabolites. They have been shown to inhibit various processes involved in DNA and RNA synthesis. Moreover, they can be incorporated into newly synthesised DNA, causing chain termination. Together, these actions result in arrest of DNA synthesis (replication and repair) and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms that are not yet entirely clear (1, for a review).

Mechanisms of action

To improve our understanding of the mechanisms by which CdA induces apoptosis in CLL cells, we studied its effects in EHEB cells, a continuous cell line derived from a patient with CLL. The EHEB cell line was found to be less sensitive (about 10-fold) to CdA than primary CLL cells and other human lymphoblastic cell lines. This could be partly explained by a lower intracellular accumulation of CdATP, the active metabolite of CdA, due to a reduced dCK activity. In addition, analysis of the cell cycle showed that CdA accelerated the progression from G1 to S phase before inducing cell death (2). This cell response was unexpected because PNA are known to induce accumulation of p53, which typically results in the accumulation of its target p21, inhibition of cyclin-dependent kinase 2 (Cdk2) and G1/S phase arrest. This paradoxical result led us to examine the effects of CdA on the p53-p21 axis. We clearly demonstrated that CdA, but also fludarabine and pyrimidine analogues induced p21 depletion in EHEB cells, while p53 was upregulated (3). This p21 depletion resulted from an increased proteasomal degradation, which had already been reported after UV-irradiation, but never after nucleoside analogue treatment. In addition, we found that p21 depletion was associated with Cdk2 activation, which could explain the activation of the cell cycle by CdA in this cell line, and also by PCNA monoubiquitination, which promotes translesion DNA synthesis and favours DNA repair and cell survival. Further work is needed to determine whether PCNA monoubiquitination could play a role in the clinical resistance to PNA.

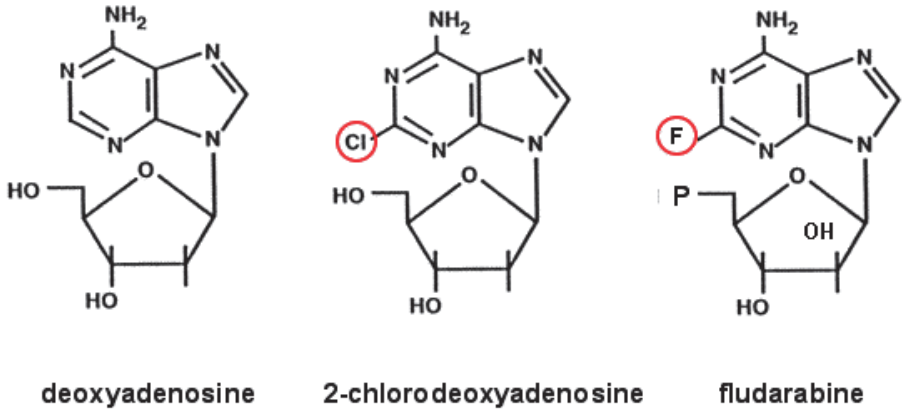

Fig. 1. Structures of deoxyadenosine and purine nucleoside analogues.

In collaboration with L. Knoops from the Ludwig Institute for Cancer Research (Brussels), we performed microarray analyses to identify survival or death pathways that are activated in response to CdA and fludarabine. We intended to compare genes induced or repressed by these PNA in sensitive and refractory CLL patients. We found that, in chemosensitive samples, PNA predominantly increased the expression of p53-dependent genes, among which PLK2 (polo-like kinase 2) was the most highly activated at early time points. Conversely, in chemoresistant samples, p53-dependent and PLK2 responses were abolished. Using qPCR, we confirmed that PNA dose-and time-dependently increased PLK2 expression in chemosensitive, but not chemoresistant CLL samples. Analysis of a larger cohort of CLL patients showed that the cytotoxicity induced by PNA correlated well with PLK2 mRNA induction. In conclusion, we propose that testing PLK2 activation after a 24-h incubation with PNA could be used to investigate the functional integrity of the p53/DNA damage pathway in CLL cells (4), and predict clinical sensitivity to these drugs (5, for a review of p53 functional analysis in CLL). The following step was to investigate the role of PLK2 during PNA-induced apoptosis. However, the protein PLK2 could not be detected in CLL cells, even after treatment with PNA, precluding a role of PLK2 in PNA-induced apoptosis. In collaboration with G. Bommer, we investigated whether PLK2 expression could be regulated by microRNAs in CLL. But so far, this hypothesis could not be confirmed.

Potentiation of their action

Some years ago, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (6) or UV-light, resulted in synergistic cytotoxicity in CLL lymphocytes, due to inhibition of DNA repair. The *in vitro* synergy between CdA and CP derivatives has provided the rationale for a clinical trial of this combination, which gives encouraging results.

Lately, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. We have shown that CdA, at concentrations close to the IC50, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (7).

Activation by deoxycytidine kinase

Deoxycytidine kinase (dCK) is the limiting enzyme in the activation of CdA and fludarabine as well as of several other nucleoside analogues used in anticancer and antiviral therapy (Fig. 2). Studies of the mechanism(s) that control the activity of this enzyme are thus of particular interest. As literature data suggested that dCK activity could be increased through reversible phosphorylation, we decided to investigate this hypothesis. We overexpressed dCK in HEK 293T cells and observed that the enzyme was labelled after incubation with [^{32}P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by D. Vertommen and M. Rider from the Horm-Phos unit (de Duve Institute) allowed the identification of four in vivo phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74. Site-directed mutagenesis demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK

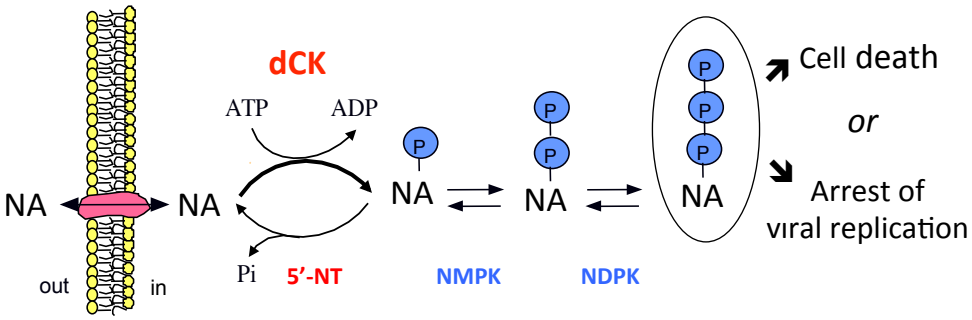

Fig. 2. Activation of nucleoside analogues.

Nucleoside analogues (NA) are transported across cell membrane via nucleoside-specific membrane transporters and phosphorylated by cellular kinases to their triphosphate form responsible for their pharmacological activity. The first phosphorylation is catalysed by deoxycytidine kinase (dCK) and is the rate limiting-step in the activation of NA. 5'-NT, 5'-nucleotidase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase.

293T cells, whereas phosphorylation of other identified sites did not seem essential (8). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from CLL patients. Moreover, treatment of these cells with genotoxic agents (CdA, UV-C irradiation, etoposide, genistein and aphidicolin…) were found to increase dCK phosphorylation on Ser-74, in close parallel with dCK activity, suggesting that all these agents increase dCK activity via Ser-74 phosphorylation. Conversely, Ser-74 phosphorylation was decreased by osmotic stress, which reduced dCK activity. Moreover, the high variability in dCK activity among CLL patients could be related to dCK phosphorylation level on Ser-74 (9). To conclude, our work has demonstrated that dCK activity in leukaemic cells largely depends on the phosphorylation state of Ser-74. We are now attempting to identify the protein kinase(s) and the protein phosphatase(s) that control Ser-74 phosphorylation and the signalling pathways that lead to dCK activation following treatment with DNA damaging agents. We showed that casein kinase 1 δ can phosphorylate dCK on Ser-74 and increase its activity *in vitro*, but not *in vivo*. Recent studies from another group indicate that ATM, a sensor of DNA damage, can phosphorylate Ser-74 *in vitro* and *in vivo* and so activate dCK.

We also investigated whether an increase of Ser-74 phosphorylation could enhance dCK activity toward PNA. Studies with recombinant dCK showed that mimicking Ser-74 phosphorylation by a S74E mutation increases the catalytic rate of dCK toward CdA and clofarabine, but not fludarabine, indicating that the effect of Ser-74 phosphorylation on dCK activity depends on the nucleoside substrate. Moreover, the catalytic efficiencies (kcat/Km) were not, or slightly, increased. Importantly, we did not observe an increase of endogenous dCK activity toward PNA after *in vivo*-induced increase of Ser-74 phosphorylation. Accordingly, treatment of CLL cells with aphidicolin, which enhances dCK activity through Ser-74 phosphorylation, did not modify the conversion of CdA or fludarabine into their active triphosphate form. Nevertheless, the same treatment enhanced activation of gemcitabine, a pyrimidine nucleoside analogue, in CLL as well as in HCT-116 cells and produced synergistic cytotoxicity. We conclude that increasing phosphorylation of dCK on Ser-74 might constitute a valuable strategy to enhance the clinical efficacy of some nucleoside analogs, like gemcitabine, but not of CdA or fludarabine (10).

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Redox biology

Jean-François Collet

The thiol group of the amino acid cysteine is found in vivo under various oxidation states. This versatility allows cysteine residues to be at the heart of numerous cellular processes by stabilizing protein structures or by fine-tuning protein activity. Some cysteine residues play an essential role in catalysis and in thiol-disulfide exchange reactions by cycling between the thiol state and the disulfide bonded state, such as in ribonucleotide reductase. Another class of cysteine residues is found in many extracellular proteins, which do not benefit from the surveillance of the cellular chaperones and other protection mechanisms. Here, cysteines form disulfide bonds that stabilize protein structure and protect the thiol group from over-oxidation. More recently, some cysteine residues have been shown to play a regulatory role and mediate cellular signaling. For instance, oxidation of cysteine residues to sulfenic acid (-SOH) turns off the activity of protein tyrosine phosphatases. It is now clear that complex enzymatic systems control the oxidation state of cysteine residues, either by reducing or oxidizing them depending on the identity of the protein target, the subcellular compartment and the redox properties of the environment. In our group, we study the mechanisms that control the oxidation state of cysteine residues, mostly using the bacterium Escherichia coli as experimental model. We have two main areas of investigation. First, we study the pathways of disulfide bond formation in the bacterial periplasm. Second, we study the mechanisms that regulate the formation of sulfenic acids by reactive cysteine residues.

Disulfide bond formation in the periplasm

In *E. coli*, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) protein family (1,2,8).
The primary oxidant is the soluble protein DsbA. DsbA has a CXXC catalytic site motif present within a thioredoxin fold. The cysteine residues of this motif are found oxidized in vivo. The disulfide bond of DsbA is very unstable and is rapidly transferred to secreted unfolded proteins. DsbA is then re-oxidized by the inner-membrane protein DsbB that transfers electrons from DsbA to the electron transport chain (Figure 1).

DsbA is a very powerful oxidant that apparently lacks proofreading activity. If the native disulfide bond pattern involves cysteine residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect disulfides. The correction of these non-native disulfides is the role of a disulfide isomerization system. This system involves a soluble periplasmic protein disulfide isomerase, DsbC (Figure 2). Like DsbA, DsbC presents a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC is kept reduced in the periplasm. This allows DsbC to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC reduced is the membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions.
In our lab, we study the Dsb proteins of *E. coli* and of other Gram-Negative bacteria. Over the past few years, we have characterized the disulfide cascade within DsbD, we have identified the proteins that depend on DsbA and DsbC for folding and we have engineered a new periplasmic oxidizing system (11). We have also proposed a revised model for

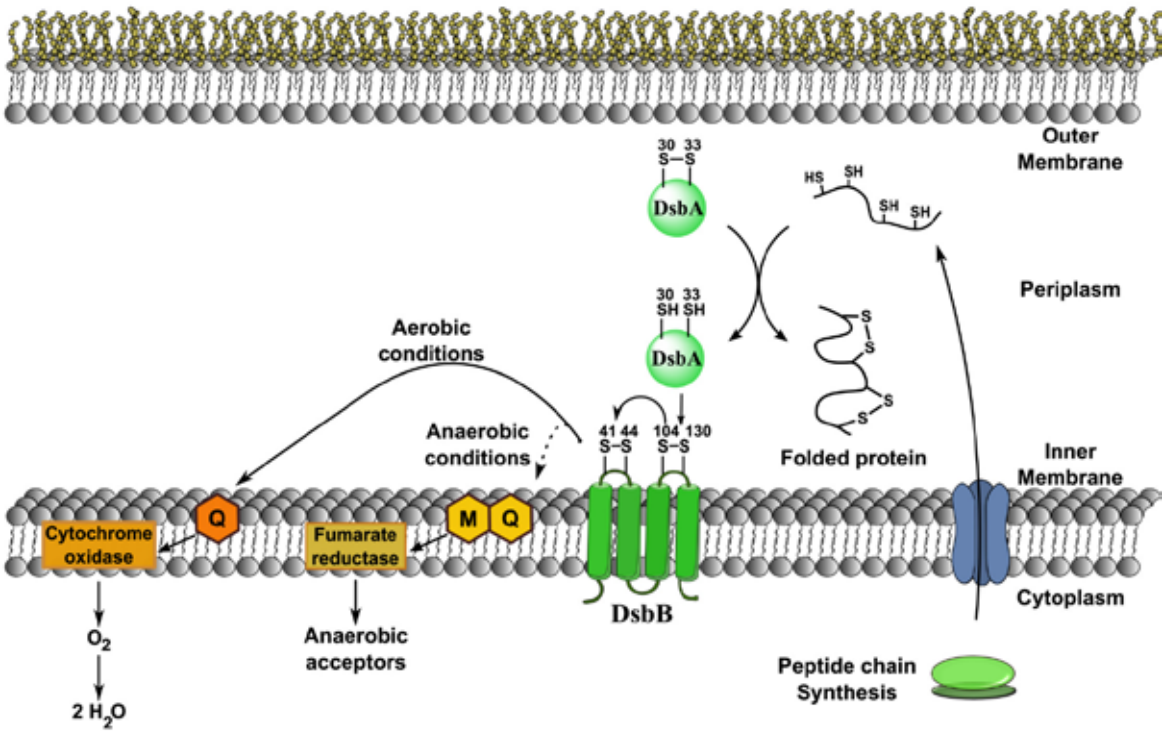

Fig. 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbB transfers electrons to the respiratory chain (adapted from (1)).

the pathways of disulfide bond formation in the periplasm by showing that DsbC may be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively. We have also identified two new substrates of the protein disulfide isomerase DsbC. Interestingly, these two substrates, LptD and RcsF, are located in the outer membrane and play important roles in the maintain of envelope integrity (6,7). LptD is an essential ß-barrel protein that inserts lipopolysacharrides in the outer membrane. We also found that LptD assembly depends on the periplasmic chaperone SurA (5,10). RcsF is an outer membrane lipoprotein sensor that detects defects in envelope integrity and transduces the signal to the Rcs phosphorelay, a signaling system that allows bacteria to react to a range of envelope stresses by modulating the expression of specific genes (6).

In collaboration with Prof. J. Beckwith (Harvard Medical School), we identified a third distinct class of DsbD-like homologues (3), which is found in proteobacteria and *Chlamydia*. The prototype of this new class is *Salmonella typhimurium* ScsB. Using *Caulobacter crescentus* as a model organism, we searched for the substrates of ScsB (3). We discovered that ScsB provides electrons to the first peroxide reduction pathway identified in the bacterial cell envelope. The reduction pathway comprises a thioredoxin-like protein, TlpA, and a peroxiredoxin, PprX. We showed that PprX is a thiol-dependent peroxidase that efficiently reduces both hydrogen peroxide and organic peroxides. Our results reveal that the array of proteins involved in reductive pathways in the oxidative cell envelope is significantly broader than has been understood.

Recently, we also characterized the machinery that catalyzes disulfide bond formation in the pathogenic bacterium *Pseudomonas aeruginosa*, an opportunistic human pathogen responsible for nosocomial infections and chronic colonization of cystic fibrosis patients. We found that the genome of *P. aeruginosa* uniquely encodes two DsbA proteins and two DsbB proteins and we studied the respective importance of these various Dsb proteins in bacterial virulence. Furthermore, using a high-throughput proteomic approach, we identified more than 20 new substrates of the disulfide bond formation machinery, including virulence factors. We also solved the structure of PaDsbA2, a *P. aeruginosa* oxidoreductase (Fig. 3). By fully dissecting the machinery that introduces disulfide bonds in *P. aeruginosa*, **our work opens the way to the design of novel antibacterial molecules able to disarm this pathogen by preventing the proper assembly of its arsenal of virulence factors.**

In a parallel study, we found the rhodanese PspE to accumulate in the sulfenic acid form when overexpressed in the periplasm. The sulfenic acid serves as a source of oxidizing equivalents allowing PspE to restore disulfide bond formation to an E. coli strain lacking DsbA. The disulfide generating activity of PspE is dependent on DsbC. Our data suggest that the PspE/

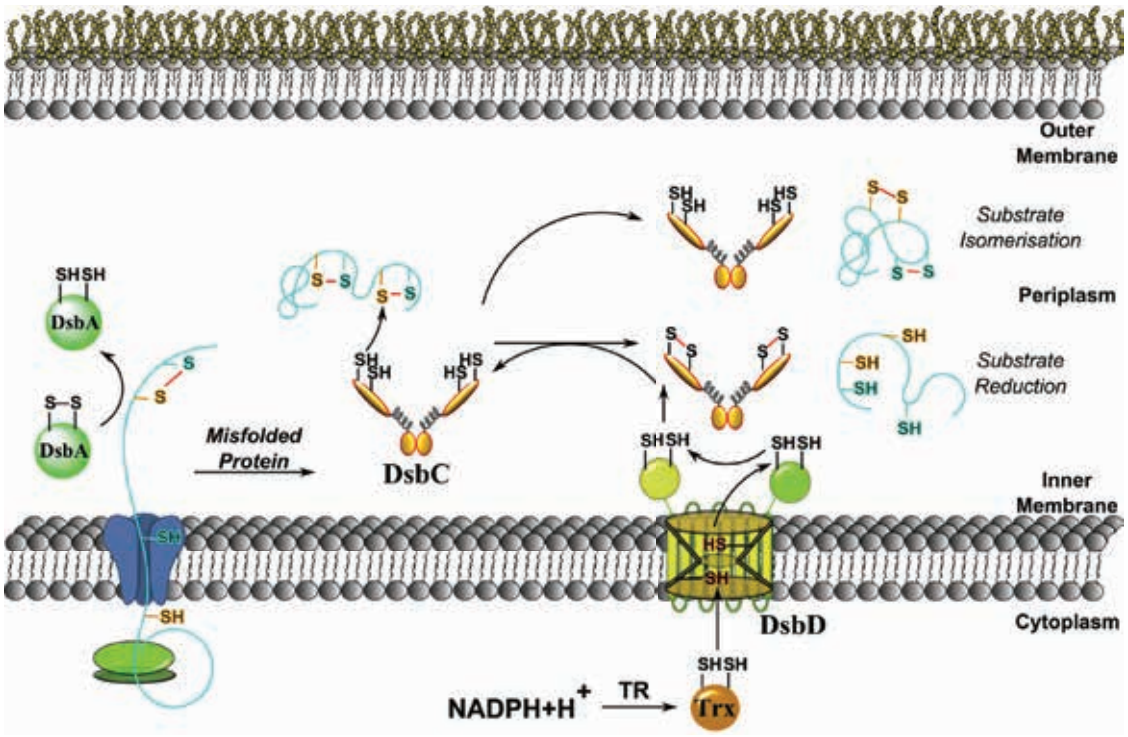

Fig. 2. Disulfide bond reduction/isomerization in the periplasm. DsbA can introduce non-native disulfides when they need to be formed beween non-consecutive cysteines. Incorrect disulfides are either isomerized or reduced by DsbC. Oxidized DsbC is regenerated by the membrane protein DsbD that transfers electrons from the cytoplasmic thioredoxin reductase (TR) / thioredoxin (Trx) system to the periplasm (from reference 1).

DsbC system might be utilized in organisms lacking the DsbA/DsbB machinery to introduce disulfides into secreted proteins. This work, which results from a collaboration with the group of J. Beckwith, has been published recently (4).

Control of cysteine sulfenylation in the periplasm

The periplasm possesses another soluble Dsb protein, DsbG, but the function of this protein has remained elusive. We sought to find the function of DsbG in the periplasm by trapping it with its substrates. We identified three periplasmic proteins, in complex with DsbG. The interaction between DsbG and those proteins was confirmed *in vitro* and *in vivo* (9).

The three periplasmic proteins (YbiS, ErfK and YnhG) are homologous proteins and belong to the same family of L,-D transpeptidases. Unexpectedly, all three enzymes contain only a single cysteine residue. An intriguing question pertained therefore to the nature of the oxidation that affects the single cysteine residue of DsbG substrates. We considered the possibility that the cysteine residue might be oxidized to a sulfenic acid (Cys-SOH) by biological oxidants present in the periplasm. Sulfenic acids are highly reactive groups that tend to either rapidly react with other cysteine residues present in the vicinity to form a disulfide bond or to be further oxidized by reactive oxygen species (ROS) to irreversible sulfinic or sulfonic acids.

To test whether the cysteine residue of one of those proteins, YbiS, is indeed able to form a stable sulfenic acid, we used the dimedone-based DAz-1 probe (in collaboration with K. Carroll's lab, The Scripps Institute), which is chemically selective for sulfenic acids. We found that the cysteine residue of YbiS can form a sulfenic acid *in vitro* and *in vivo* and that the reduction of this cysteine depends on the presence of DsbG. In the course of our experiments, we observed that, in addition to YbiS, several other periplasmic proteins were also labeled by the probe and that the level of sulfenylation in this compartment is controlled by DsbG and DsbC. On the basis of these results, we proposed the following model. In the oxidizing periplasm, most proteins contain an even number of cysteine residues. These residues form disulfide bonds and are therefore protected from further cysteine oxidation. However, there is a significant number of proteins that contain a single cysteine residue. Because they are not involved in disulfide bonds, these cysteines are vulnerable to oxidation and form sulfenic acids which are susceptible to reaction with small molecule thiols present in the periplasm to form mixed disulfides or to further oxidation to sulfinic and sulfonic acids. DsbG appears to be a key player in a reducing system that protects those single cysteine residues from oxidation. DsbC could serve as a backup for DsbG and could even have its own subset of favorite sulfenic acid modified substrates to reduce. Both DsbC and DsbG are kept reduced in the periplasm by DsbD, which transfers reducing equivalents from the thioredoxin system across the inner membrane. Thus, the electron flux originating from the cytoplasmic pool of NADPH provides the reduc-

ing equivalents required for both the correction of incorrect disulfides and the rescue of sulfenylated orphan cysteines.
Proteins from the thioredoxin superfamily are very widespread and have been identified in the majority of the genomes sequenced so far, making it tempting to speculate that some of these thioredoxin superfamily members, particularly those that are closely related to DsbC and DsbG, play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments (9).

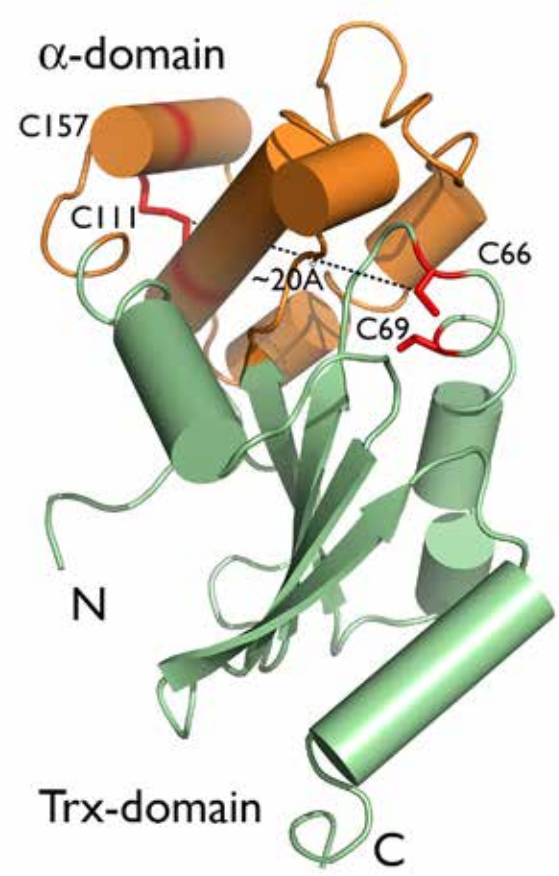

Fig. 3. The structure of PaDsbA2, an oxidoreductase from *P. aeruginosa* The structure of PaDsbA2 was solved using single anomalous dispersion and refined to 1.3 Å. It consists in a thioredoxin fold (in green) in which α helical domain is inserted (in orange). The catalytic cysteines (C66 and C69) and two cysteines involved in a structural disulfide (C111 and C157) are shown in red (the figure was kindly prepared by J. Messens)

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miRNAs in physiology and disease

Guido Bommer

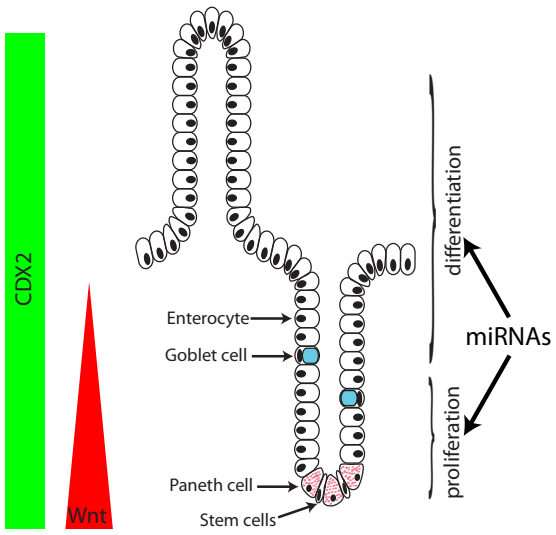
miRNAs are small non-protein-coding RNAs that can bind to mRNA transcripts of protein coding genes. Upon binding to these mRNAs, they inhibit their translation into proteins. However, each miRNA does not only recognize one target transcript, but rather numerous – in some cases several hundreds – of target transcripts. In addition, for many miRNAs, multiple different genes exist, that encode highly similar or identical mature miRNAs. The potential for combinatorial complexity and functional redundancy is therefore enormous.
The focus of our group is on the role of miRNAs in conserved signaling pathway at the crossroad of cancer and metabolism.

Role of miRNAs in intestinal differentiation

The intestine is required for the digestion and absorption of essential nutrients and water. In this process, its surface epithelium is exposed to one of the most toxic milieus of the whole body. It has to resist aggressive digestive juices, large pH changes, anaerobic bacteria and numerous toxic compounds. To resist this, its surface epithelium is completely renewed in less than 2 weeks. All intestinal epithelial cells are derived from stem-cells located at the base of the crypt. An intricate network of signaling pathways controls proliferation and differentiation of these cells. These pathways are crucial for issue homeostasis and intestinal function. In addition, several of the pathways that are required for stem cell maintenance are activated during colorectal carcinogenesis. For example the Wnt signaling pathway is essential for the maintenance of stem cells in the normal intestine. In colorectal cancers, mutations in different components of this pathway, lead to its activation in more than 80% of colorectal cancers.

Much is known about the protein coding genes that mediating the effects of these signaling pathways. Much less is known about the role of noncoding RNAs

In our laboratory, we are investigating the contribution of miRNAs to intestinal cell differentiation and the development of colorectal cancer. Currently, our work in this area is centered around the interplay between miRNAs and the metabolic changes that are required for normal intestinal cell function.



Intestinal architecture is maintained by the interplay of many signaling pathways

The intestinal architecture is maintained by the interplay of signaling patways that ensure complete renewal of intestinal surface epithelia within 7 to 14 days. New cells are generated from a stem cell compartment at the base of the crypts and successively migrate up, where they are eventually shed in the lumen. In this process, different cell types with distinct functions are generated (= cell differentiation). We are interested in miRNAs that regulate this process and the metabolic changes required for normal intestinal cell function.

Regulation of cholesterol metabolism by the bifunctional locus SREBF2-miR33

Fatty acids, cholesterol, and their lipid derivatives play essential roles in normal cellular function and serve as structural components, signaling molecules, and/or as storage forms of energy. In multicellular organisms, cellular lipid metabolism is regulated to match the needs both of individual cells and of the entire organism.

The *sterol regulatory element-binding factor-2 (SREBF2)* gene is a bifunctional locus encoding SREBP-2, a well-known transcriptional regulator of genes involved in cholesterol and fatty acid biosynthesis, and miR-33a. We and others have recently shown that miR-33a can reduce the expression of several proteins involved in the cellular export of cholesterol and β-oxidation of fatty acids, thus adding an unexpected layer of complexity and fine-tuning to regulation of lipid homeostasis. In fact, work of other groups has demonstrated that this mechanism might represent a therapeutic target in the treatment of hypercholesterolemia.

We are continuing to investigate the physiological role of miR-33 family members in different experimental systems throughout evolution. Currently, we are pursuing the in vivo effects of miR-33 in *Drosophila melanogaster* in collaboration.

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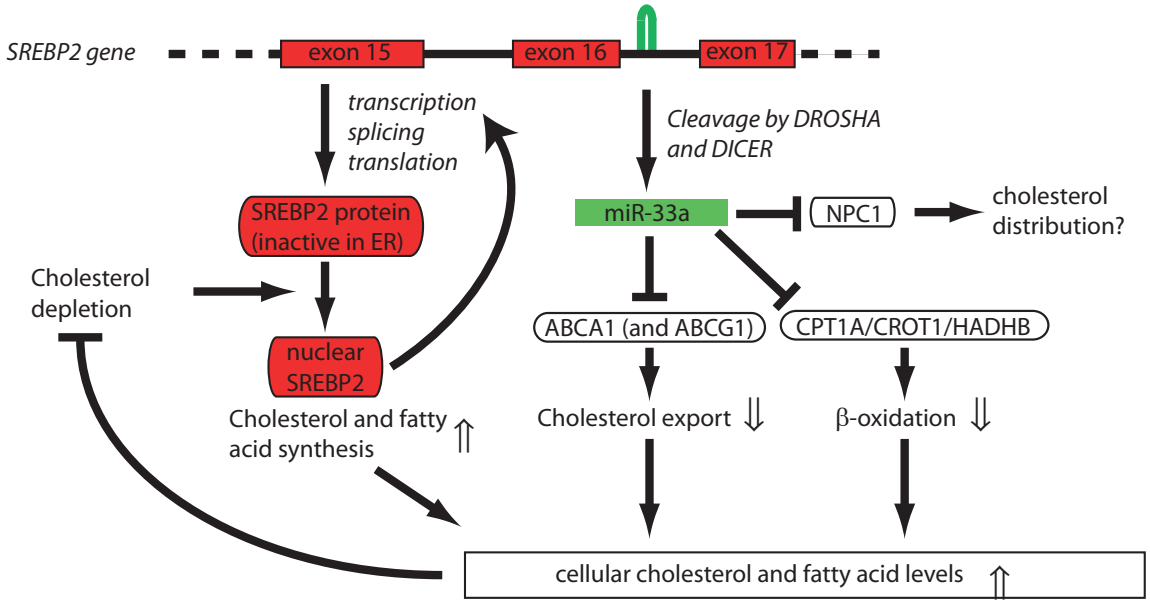

The bifunctional locus of SREBF2-miR33 regulates cholesterol and fatty acid metabolism.

After processing from an intron of SREBF2, miR-33a reduces cellular cholesterol export by inhibiting expression of ABCA1 (and in the mouse ABCG1). In addition, miR-33a reduces mitochondrial fatty acid b-oxidation via inhibition of HADHB, CROT, and CPT1A to increase intracellular lipid levels. Thus the SREBF2 locus uses two distinct mechanisms to maintain lipid homeostasis: regulated transcriptional activity of SREBP-2 and translational repression by miR-33a.

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Signal transduction and protein phosphorylation

Mark Rider

Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. We started out in this field many years ago by studying 6-phosphofructo-2-kianse (PFK2)/fructose-2,6-bisphosphatase (FBPase-2) and the control of its activity by protein kinases (reviewed in ref. 1). This prompted us to investigate the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are now our main research interests.

Insulin signalling

Y.-C. Lai, Y. Liu, R. Jacobs, N. Hussain, M.H. Rider, with help from D. Alessi, Dundee

Metabolic effects of insulin in skeletal muscle and adipose tissue

We compared the effects of the new generation allosteric PKB inhibitor, MK-2206, with those of Akti-1/2 on the insulin-induced stimulation of glucose transport, glycogen synthesis and protein synthesis in incubated rat soleus muscles. In parallel we measured of the phosphorylation state of signalling proteins, PKB downstream targets and enzyme activities. Our data point to the central role of PKB in metabolic control by insulin in skeletal muscle and indicate that MK-2206 would be the tool of choice for studying insulin action in this tissue. The results also argue for both allosteric and phosphorylation mechanisms of control of glycogen synthase as important determinants of insulin-stimulated glycogen synthesis in muscle (ref. 2).

A well-known effect of insulin is the stimulation of adipose tissue lipogenesis, mediated by pyruvate dehydrogenase (PDH) activation via dephosphorylation, and acetyl-CoA carboxylase (ACC) activation. Using Akti-1/2 and the next generation MK-2206 PKB inhibitor, we studied the mechanism by which insulin stimulates lipogenesis in rat epididymal adipocytes. PDH dephosphorylation by insulin was unaffected by PKB inhibitors. By contrast, ACC Ser79 dephosphorylation by insulin was completely reversed by Akti-1/2 and MK-2206. Therefore, a key point of control of the lipogenic pathway by insulin is the dephosphorylation of ACC mediated by PKB, which we showed previously to antagonize AMPK (ref. 3) responsible for ACC inactivation via Ser79 phosphorylation (manuscript in preparation).

AMP-activated protein kinase

Y. Liu, C. Plaideau, Y.-C. Lai, D. Vertommen, R. Jacobs, S. Pyr dit Ruys, M. Johanns, A. Houddane, L. Hue, M.H. Rider, in collaboration with D. Tyteca and P. Courtoy, de Duve Institute, J. Tavaré, Bristol, Peter Shepherd, Auckland, Christophe Erneux, ULB, Franck Dequiedt, ULg, J. Jenssen, Oslo, B. Viollet, Paris, Stefan Hallén, AstraZeneca

AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as occurs during hypoxia or after ATP depletion with oligomycin. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phosphorylated to ZMP, an analogue of AMP, or by the A769662 Abbott compound. Full AMPK activation requires phosphorylation of the α-catalytic subunits at Thr172 by upstream kinases, either LKB1 (the Peutz-Jeghers protein) or calmodulin-dependent protein kinase kinase-β (CaMKKβ). The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways (Fig. 1). We have made significant contributions to the field by discovering new substrates of AMPK and mechanisms of upstream regulation. For example, we showed that AMPK activation is associated with protein synthesis inhibition in anoxic rat hepatocytes (ref. 4). Protein synthesis inhibition in response to AMPK activation can partly be explained by a rise in eEF2 (eukaryotic elongation factor-2) phosphorylation leading to its inactivation. Regulation of the

upstream eEF2 kinase (eEF2K) is complex involving phosphorylation-induced activation and inactivation by kinases from various signalling pathways. Indeed we, and others, reported that AMPK phosphorylates and activates eEF2K. However, phosphorylation at the previously proposed Ser398 site by AMPK is probably incorrect and our recent findings indicate that the effect of AMPK activation to increase eEF2 phosphorylation is indirect and partly due to eEF2K activation due to reduced mTORC1 signalling. In fact, we identified nine autophosphorylation sites in eEF2K by mass spectrometry, some of which are also sites for protein kinases from different signaling pathways and are required for eEF2K activity (ref. 5).

Mammalian target of rapamycin-independent S6K1 and 4E-BP1 phosphorylation during contraction in rat skeletal muscle

Muscle protein synthesis rates decrease during contraction/exercise, largely due to the rise in eEF2 phosphorylation downstream of AMPK, but rapidly increase post-exercise. Previous studies have mainly focused on signalling pathways that control protein synthesis during post-exercise recovery, such as mTOR and its downstream targets S6K1 and 4E-BP1. We investigated the effect of high-frequency electrical stimulation on the phosphorylation state of signalling components controlling protein synthesis in rat skeletal muscle. Electrical stimulation increased S6K1 Thr389 phosphorylation, which was mTOR-independent. Phosphorylation of eIF4B Ser422 was also increased during electrical stimulation, and abrogated by inhibition of MEK/ERK/RSK1 activation. Although phosphorylation of conventional mTOR sites in 4E-BP1 decreased during contraction, mTOR-independent phosphorylation was also apparent, which was associated with the release of 4E-BP1 from eIF4E. The results indicate mTOR-independent phosphorylation of S6K1 and 4E-BP1 and suggest MEK/ERK/RSK1-dependent phosphorylation of eIF4B during skeletal muscle contraction. Thus, while translation elongation is paused during muscle contraction via eEF2 phosphorylation, parts of the translation initiation machinery would be maintained in a "primed" active state (Fig. 2). This would allow translation to resume quickly post-exercise when conditions would be favourable for increasing protein synthesis (ref. 6).

Control of PIKfyve by AMPK

Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), the lipid kinase that phosphorylates PtdIns3P to PtdIns(3,5)P2, has been implicated in insulin-stimulated glucose uptake. We investigated whether PIKfyve could also be involved in contraction/AMPK-stimulated glucose uptake in skeletal muscle. Incubation of rat epitrochlearis muscles with YM201636, a selective PIKfyve inhibitor, reduced contraction- and AICA riboside-stimulated glucose uptake. Consistently, PIKfyve knockdown in C2C12 myotubes reduced AICA riboside-stimulated glucose transport. Electrical stimulation of muscles increased PtdIns(3,5)P2 levels and PIKfyve phosphorylation. AMPK phosphorylated PIKfyve at Ser307 both in vitro and in intact cells. Following subcellular fractionation, PIKfyve recovery in a

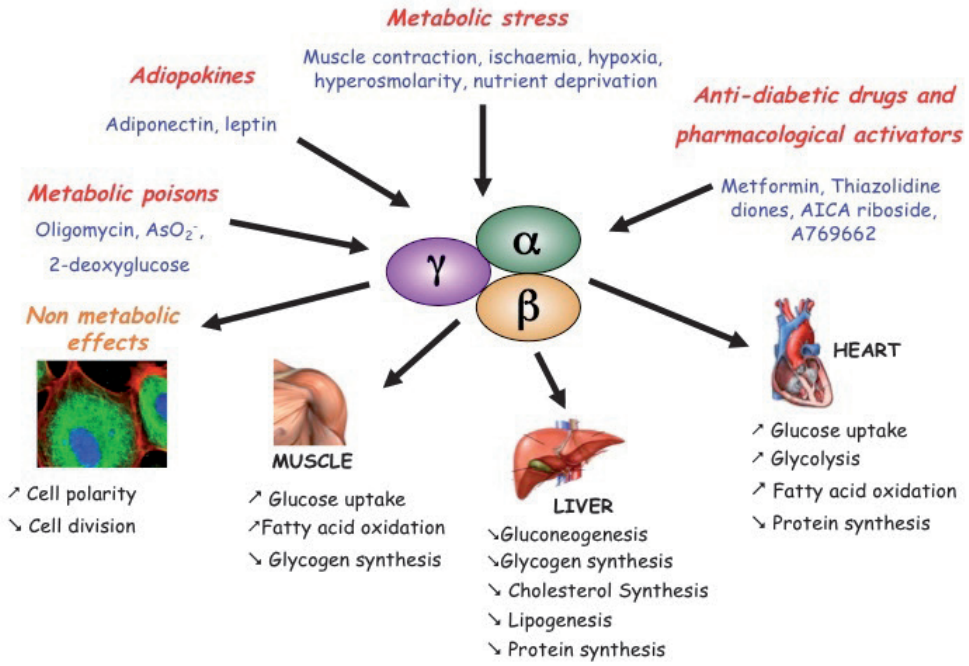

Fig. 1. Conditions leading to AMPK activation in higher eukaryotes and some of its consequences.

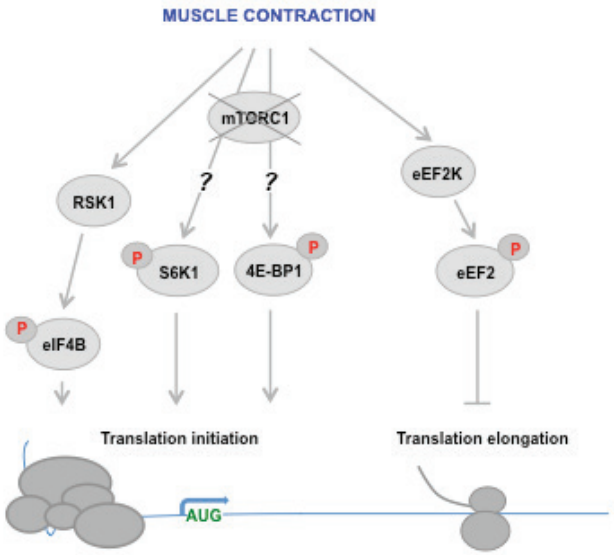

Fig. 2. Signalling during contraction maintains S6K1, eIF4B and 4E-BP1 phosphorylation, while blocking translation elongation via eEF2 phosphorylation. Contraction increases the phosphorylation of S6K1 and 4E-BP1 (at non-conventional sites different from those phosphorylated by mTORC1) in an mTORC1-independent manner. Furthermore, contraction increases eIF4B phosphorylation through MEK/ERK/RSK rather than S6K1 signalling. This would maintain parts of the translation initiation machinery in a "primed" active state while elongation is paused during contraction via eEF2 phosphorylation.

crude intracellular membrane fraction was increased in contracting versus resting muscles. Also in opossum kidney cells, wild-type, but not S307A mutant, PIKfyve was recruited to endosomal vesicles in response to AMPK activation. We propose that PIKfyve activity is required for the stimulation of skeletal muscle glucose uptake in response to contraction/AMPK activation. PIKfyve is a thus new AMPK substrate whose phosphorylation at Ser307 could promote PIKfyve translocation to endosomes for PtdIns(3,5)P2 synthesis to facilitate GLUT4 translocation (manuscript submitted).

AMPK as a drug target for type 2 diabetes

In collaboration with the pharmaceutical company AstraZeneca (Mölndal, Sweden), we investigated whether overexpression of AMP-metabolizing enzymes (Fig. 3) in cells would modulate oligomycin-induced AMPK activation (ref. 7). HEK (human embryonic kidney) 293T cells were transiently transfected with increasing amounts of plasmid vectors to obtain a graded increase in overexpression of AMP-deaminase (AMPD)-1, AMPD2 and soluble 5'-nucleotidase IA (cN-IA), (see Fig. 3), for measurements of AMPK activation and total intracellular adenine nucleotide levels induced by oligomycin treatment. Overexpression of AMPD1 and AMPD2 only slightly decreased AMP levels and oligomycin-induced AMPK activation. Increased overexpression of cN-IA, on the other hand, led to reductions in the oligomycin-induced increases in AMP and ADP concentrations by about 70 and 50%, respectively, concomitant with a 50% decrease in AMPK activation. The control coefficient of cN-IA on AMP was 0.3-0.7, whereas the values for AMPD1 and AMPD2 were less than 0.1, suggesting that in this model cN-IA exerts a large proportion of control over intracellular AMP. The results also support the view that a rise in ADP as well as AMP is important for activation of AMPK, which can thus be considered as an adenylate energy charge-regulated protein kinase. Importantly, our results support the notion that small molecule inhibition of cN-IA could be a strategy for achieving AMPK activation in muscle for the treatment of type 2 diabetes. Further studies are in progress on small molecule pharmacological AMPD inhibition and effects of deletions of AMPD1, cN-1A and cN-II in mice on AMPK activation by contraction in muscle.

Mass spectrometry

D. Vertommen, G. Herinckx, M.H. Rider in collaboration with C. Sindic, UCL and J.-F. Collet, UCL, E. Waelkens, KULeuven, Joris Messens, VIB-VUB

The development of mass spectrometry (MS) facilities within our laboratory, and for our Institute and University, has been an enormous asset (lien vers site "Massprot": http://www.uclouvain.be/en-proteomics.html). Since the acquisition of an electrospray mass spectrometer in 1997, the application of MS techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to over 50 joint publications. In our own research, the use of MS enabled us to identify new AMPK targets. We also discovered new phosphorylation sites in the AMPK complex itself and demonstrated that in heart, insulin antagonized AMPK activation during ischaemia via PKB-induced phosphorylation of the AMPK catalytic α-subunits at Ser495/491 (ref 3).

We collaborated with the group of J.-F. Collet by using prot-

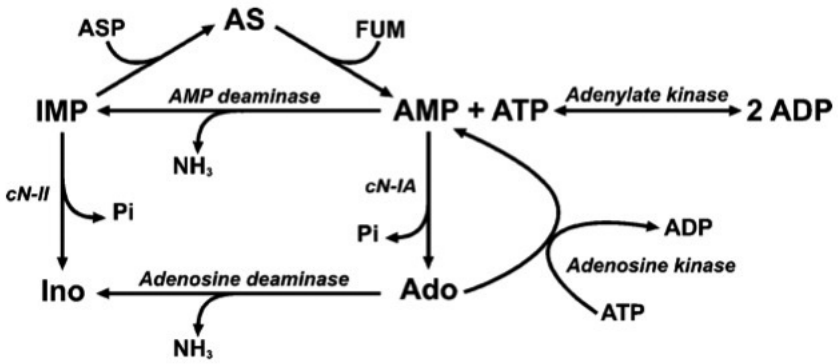

Fig. 3. Scheme showing AMP metabolizing pathways. The enzymes implicated are indicated in italics. ASP: aspartate; AS: adenylosuccinate; FUM: fumarate; Ado: adenosine; Ino: inosine.

eomics to study the biogenesis of bacterial outer membranes (ref. 8). Along with the Brussels Center For Redox Biology (J.-F Collet and J. Messens) we are developing new proteomics strategies to investigate proteins that are involved in redox regulation, thiol-based catalysis mechanisms and oxidative protein folding. Recently, we discovered new redox pathways involved in the oxidative stress defense of Mycobacterium tuberculosis. Mycothiol is used by this bacterium as a redox buffer against oxidative stress. We identified Mycoredoxin-1, a new mycothiol-dependent reducatase, to be S-mycothiolated on its N-terminal nucleophilic cysteine (ref. 9).

We also study differential protein expression by label-free multidimensional LC-MS. One application is the screening of proteins and neuropeptides from cerebro-spinal fluid of patients with neurodegenerative diseases to discover biomarkers (collaboration with C. Sindic). This research falls within the framework of the project DIANE on neurodegenerative diseases, particularly Alzheimer's disease, Parkinson's disease and multiple sclerosis.

Lastly, we use phosphoproteomics strategies (in collaboration with E. Waelkens, see ref. 10) to identify new targets downstream of different signalling pathways under normal and pathological conditions. We are developping inovative approaches based on two strategies: 1) the use of natural phosphoprotein binders such as 14-3-3 proteins to pull-down phosphoproteins from cell extracts 2) the use of a combination of hydrophilic liquid chromatography (HILIC) followed by metal oxide affinity capture (MoAC) to enrich and concentrate phosphopeptides.

Selected publications

1. Rider MH, Bertrand L, Vertommen D, Michels PA, Rousseau GG, Hue L. 6-Phosphofructo-2-kinase/fructose-2,6-bispphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem J. 2004; 381:561-79.

2. Lai YC, Liu Y, Jacobs R, Rider MH. A novel PKB/Akt inhibitor, MK-2206, effectively inhibits insulin-stimulated glucose metabolism and protein synthesis in isolated rat skeletal muscle. Biochem J. 2012; 447:137-47.

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5. Pyr dit Ruys S, Wang X, Smith EM, Herinckx G, Hussain N, Rider MH, Vertommen D, Proud CG. Identification of autophosphorylation sites in eukaryotic elongation factor-2 kinase. Biochem J. 2012; 442:681-92.

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7. Plaideau C, Liu J, Hartlieb-Geschwindner J, Bastin-Coyette L, Bontemps F, Oscarsson J, Hue, L, Rider MH. Overexpression of AMP-metabolizing enzymes controls adenine nucleotide levels and AMPK activation in HEK293T cells. FASEB J. 2012; 26: 2685-94.

8. Denoncin K, Schwalm J, Vertommen D, Silhavy TJ, Collet JF Dissecting the Escherichia coli periplasmic chaperone network using differential proteomics. Proteomics. 2012; 12:1391-401.

9. Van Laer K, Buts L, Foloppe N, Vertommen D, Van Belle K, Wahni K, Roos G, Nilsson L, Mateos LM, Rawat M, van Nuland NA, Messens J.Mycoredoxin-1 is one of the missing links in the oxidative stress defence mechanism of Mycobacteria. Mol Microbiol. 2012; 86:787-804.

10. Rider MH, Waelkens E, Derua R, Vertommen D. Fulfilling the Krebs and Beavo criteria for studying protein phosphorylation in the era of mass spectrometry-driven kinome research. Arch Physiol Biochem. 2009; 15:298-310.

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Membrane organization / dynamics and epithelial differentiation

Pierre J. Courtoy
Christophe E. Pierreux

This group focuses on intertwined topics related to the control of membrane organization/dynamics at the subcellular level; and of epithelial polarity during tissue differentiation and disease. By high-resolution vital imaging of the erythrocyte plasma membrane after insertion of lipid analogs, we uncovered labeling of three types of micrometric domains differing by lipid composition, cohesion and preference for cytoskeleton anchorage. Lipid analogs reflect organization of endogenous lipids, which can now be decorated by toxin fragments. These data force to revise concepts on scale (micrometric) and stability (minutes) of lateral lipid organization at the living cell surface (1-3). Using polarized kidney tubular cells and transgenic mice as models, we identified the transcription factor, ZONAB, as essential component of the switch between epithelial proliferation and apical differentiation (4). Looking at recycling as an essential arm for apical membrane homeostasy, we found it defective in ClCn5 KO mice, a model of Dent's disease (familial predisposition to kidney stones) (5,6) and demonstrated its control by class III PI3-kinase/VPS34 and dynamin (7). At the tissue level, we study epithelial tubulogenesis and differentiation, using developing pancreas and thyroid glands as complementary models to study conversion of non-polarized endoderm-derived cell masses into polarized monolayers, with emphasis on paracrine and transcriptional control (8-10). Besides integration of cell and developmental biology, two strong assets of our group are decades of expertise in structural biology and advanced imaging thanks to a versatile Platform for Imaging Cells and Tissues. Combined expertises help us currently address physiopathology in kidney and thyroid of cystinosis, a paradigmatic lysosomal storage disorder due to inherited defective lysosomal cystine exporter.

Plasma membrane lipids segregate into distinct micrometric domains

L. D'Auria, M. Carquin, P. Aleksandrowicz, P. Van Der Smissen, P.J. Courtoy and D. Tyteca

This project addresses a fundamental property of plasma membrane (PM) lipids - self-assembly into stable micrometric domains - and its significance for cell physiology and disease. Long viewed as homogenous solvent for membrane proteins, the lipid bilayer shows heterogeneity at two different scales: transient nanometric "lipid rafts" versus stable micrometric assemblies. These are well-known on artificial vesicles but relevance in vivo is controversial.

As we observed by vital confocal microscopy, trace insertion of exogenous fluorescent analogs (BODIPY, indicated as *) of sphingomyelin (SM) (< 1% of endogenous) labels micrometric domains at the PM of freshly isolated red blood cells (RBCs) and cultured cells. In CHO cells, intracellular conversion of ceramide* into SM* produced similar PM micrometric domains as direct SM* insertion into the cell surface. Inhibition of endogenous SM synthesis or surface SM depletion by sphingomyelinase erased SM* domains. Both controls suggested that domains labelled by exogenous SM* reflect endogenous SM compart-mentation (Ref 1). Double labelling with phosphatidylcholine* and GM1* (a ganglioside) revealed co-existence of three classes of micrometric lipid domains, differing in composition, cohesion and cytoskeleton interaction (Ref 2).

We next extended these studies to the endogenous SM, GM1 and cholesterol, using fluorescent toxin fragments. These labelled undistinguishable micrometric domains (Fig. 1), with almost perfect co-localization between exogenous tracer insertion and labelling of endogenous GM1 and SM by fluorescent toxins. We conclude that fluorescent micrometric domains reflect a genuine organization of endogenous lipids.

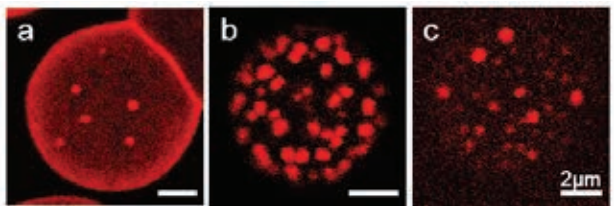

Fig. 1. Labelling of endogenous SM, cholesterol and ganglioside GM1 by toxin fragments show micrometric domains. Erythrocytes labelled by fluorescent lysenin (SM, a), theta toxin (cholesterol, b) or cholera toxin B subunit (ganglioside GM1, c).

Regulation and significance of micrometric lipid domains

M. Carquin, L. D'Auria, P.J. Courtoy and D. Tyteca

To address the mechanism(s) of biogenesis and maintenance of micrometric lipid domains, we focus on living RBCs. These offer the best characterized biological membrane, a featureless surface, and no lipid turnover nor vesicular trafficking. RBCs exhibit remarkable deformability and stability, allowing them to squeeze into the narrow pores of spleen sinusoids for ~12.000 times during their 120 days lifetime.
Controlled changes of RBC stretching and cholesterol content (moderate extraction; Fig. 2b) and suppression of membrane:cytoskeleton anchorage via ankyrin complexes (spherocytosis; Fig. 2c) differentially affected SM* micrometric domains. Thus, membrane tension is a key parameter controlling micrometric lipid domains (Ref 3).
These observations support a revised model of the scale (micrometric) and stability (minutes) of lateral lipid organization at the PM in living cells.

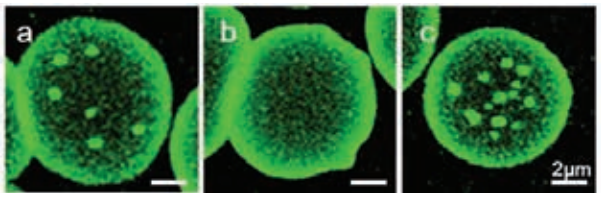

Fig. 2. Control of micrometric domains labelled on erythrocytes upon insertion of fluorescent sphingomyelin (BODIPY-SM). (a) control erythrocyte; (b) cholesterol depletion (- 25%); (c) familial spherocytosis (adapted from Ref 3).

Apical endocytosis and diseases

H.P. Gaide Chevronnay, S. Carpentier, G. Grieco, V. Janssens, C.E. Pierreux and P.J. Courtoy, in collaboration with C. Antignac (Paris) and S. Cherqui (Scripps)

Due to extraordinary efficiency of apical receptor-mediated endocytosis, kidney proximal tubular cells (PTCs) are a unique system to study machineries of apical endocytic trafficking and their involvement in kidney diseases. Using KO mice for the chloride channel, ClC-5, as model of Dent's disease (familial predisposition to kidney stones), we found that this channel is essential for apical endocytic recycling (Ref 5). Using similar systems, we demonstrated that circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, thus providing a major source of enzymes to PTCs. These observations extend the significance of megalin in PTCs and have several physiopathological and clinical implications (Ref 6).
Recently, we identified a key role of class III PI3-kinase/VPS34 in apical recycling of endocytic receptors. In vitro, VPS34 inhibition with LY294002 induced selective apical endosome swelling and sequestration of the endocytic receptor, megalin. This effect was reversible: removal of the inhibitor induced a spectacular burst of recycling tubules and restored the megalin surface pool (Fig. 3). In mouse pups PTCs, conditional Vps34 inactivation also led to vacuolation and intracellular megalin redistribution. We anticipate that these KO mice and reversible PI3K inhibition will help further identify rate-limiting actors of apical endocytosis, of both fundamental and clinical importance (Ref 7).

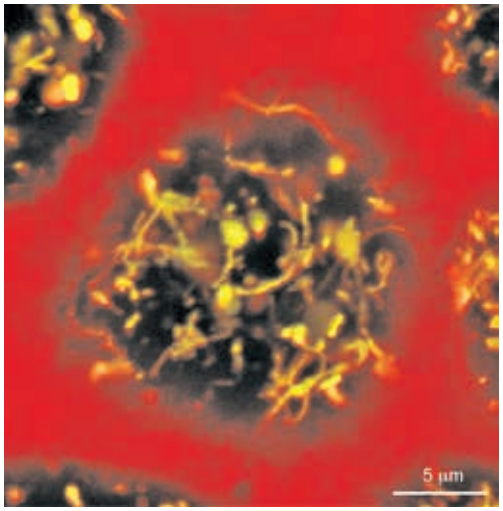

Fig. 3. Reversible PI3K inhibition as a tool to synchronize apical endocytic recycling. Apical endosomes of polarized PTCs were loaded by endocytosis of fluorescent tracers, then PI3K was inhibited by LY294002 to induce endosome vacuolation. PI3K restoration by LY294002 chase reverses vacuolation and triggers a spectacular burst of recycling tubules (From Ref 7).

Current investigations are also addressing the pathophysiology of cystinosis, a multisytemic lysosomal disease due to defective lysosomal membrane cystine/H+ antiporter, cystinosin. This disease first manifests itself in kidney as a generalized PTC dysfunction, referred to as kidney Fanconi syndrome. Endocytosis of ultrafiltrated plasma proteins rich in disulfide bridges must be the main source of lysosomal cystine in PTCs. Current analysis of cystinosin KO mice helps us understanding how cystine accumulation causes apical PTC vacuolation, dedifferention and eventual atrophy (Fig. 4), and how cystinosis can be corrected by grafting of hematopoietic stem cells.

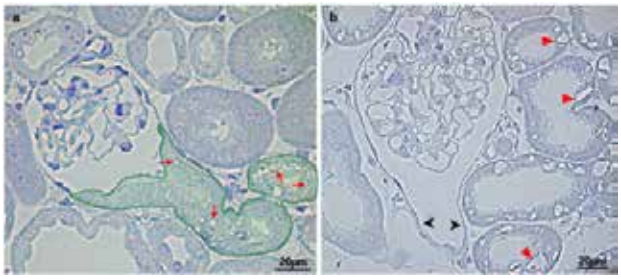

Fig. 4. Progression of PTC lesions in cystinosin KO mice. At 6 months (a), only PTCs immediately following glomeruli (overlaid in pale green) show extensive apical vacuolation (arrows), indicating osmotic swelling of lysosomes by accumulating cystine, contrasting with integrity of kidney elsewhere, including more distal PTCs. (b) At 12 months, proximal PTCS are now completely atrophic (arrowheads) and more distal PTCs harbour numerous crystals (appearing as empty spaces with characteristic geometric shape; red triangles).

ZONAB as regulator in the proliferation/ differentiation switch

A.-S. Delmarcelle, S. Dupasquier, W.R. Lima, C.E. Pierreux and P.J. Courtoy

Epithelial polarization depends, and impacts, on gene expression. The transcription factor, ZONAB, can shuttle between tight junctions and the nucleus to promote expression of Cyclin D1, and thus participate in the control of proliferation. During mouse kidney ontogeny and polarization of PTC monolayers in vitro, we confirmed that high ZONAB expression was associated with epithelial proliferation but not with differentiation (Fig. 5). Conversely, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium markers. In PTC, we further showed that ZONAB can simultaneously repress the expression of differentiation markers and that its expression was regulated by polarity. These in vitro and in vivo data suggest that ZONAB is a sensor of epithelial density, involved in their switch from proliferation to differentiation (Ref 4).

Recently, we studied ZONAB expression in developing and diseased epithelial organs. In the embryonic pancreas and thyroid glands, ZONAB was only expressed in cells localized at the

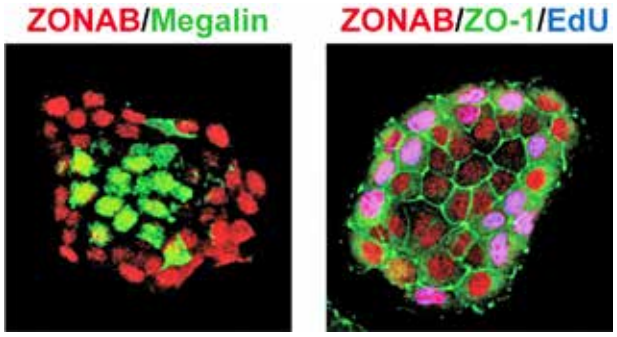

Fig. 5. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right) (From Ref 4).

periphery of the expanding epithelia, known as proliferating progenitors.

We also re-assessed the expression level of ZONAB in clear-cell renal cell carcinomas as compared to their normal tissues. Paying special attention to the housekeeping genes used for normalization, we found that ZONAB expression depends on the tumour grade and may serve as an early-stage biomarker.

Epithelial differentiation

A.-C. Hick, A.-S. Delmarcelle, M. Villacorte, P. Van Der Smissen, P.J. Courtoy and C.E. Pierreux

The endoderm-derived pancreas, salivary and thyroid glands are mainly composed of polarized epithelial monolayers. The pancreas and salivary glands are organized as open branched tubulo-acinar structures for exocrine secretion (digestive fluids), while the thyroid gland is a collection of closed follicles for endocrine secretion (thyroid hormones). During embryogenesis, pancreas, salivary and thyroid buds first expand as proliferating non-polarized epithelial masses, and then reorganize in specialized monolayers (Fig. 6). This process is

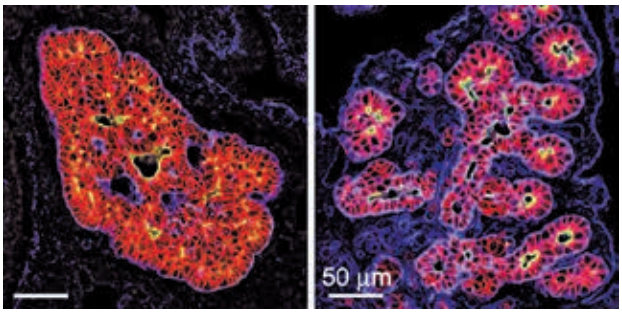

Fig. 6. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left, red) into polarized monolayers with distinct apical domains (mucin, green) and their merging to create tubules (right). Laminin (blue) delineates basement membranes (From Ref 8).

gland-autonomous (reproduced in explant culture) (Ref 8, 10).

Epithelial monolayer formation requires a coordinate and dynamic interaction with the close environment, composed of mesenchymal and endothelial cells. We have shown that the mesenchymal factor, SDF-1 (Stromal cell-Derived Factor-1), binds to its receptor on pancreatic and salivary epithelial cells and controls reorganization of the epithelial masses into monolayers (Ref 8). By three-dimensional analysis of developing pancreatic and thyroid epithelial buds, we uncovered a dense, closely apposed endothelial network (Fig. 7). Our in vivo and in vitro data show that endothelial cell recruitment is dependent on VEGF produced by the epithelium and that, in turn, endothelial cells, control pancreatic acinar differentiation (Ref 9), and promote thyroid follicles formation (Ref 10). These data demonstrate that paracrine epithelial:mesenchyme and epithelial: endothelial interactions are crucial for organ differentiation.

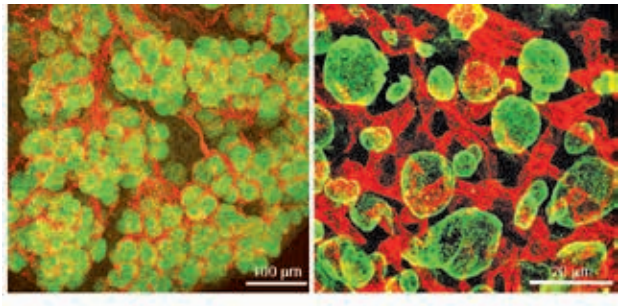

Fig. 7. Epithelial:endothelial interactions in the pancreas and thyroid. Projections of 40 to 50 confocal images showing the dense and close association of endothelial cells, labelled for PECAM (red) with pancreatic epithelial cells, labelled for E-cadherin (green, left) or with thyroid follicles, labelled for Ezrin (green, right).

Collaborations on membrane trafficking and brief report on the Platform for Imaging Cells and Tissues

P. Van Der Smissen, D. Tyteca and P.J. Courtoy

Besides sharing the same laboratory and continuing a two-decade fruitfull collaboration with the group of E. Marbaix and P. Henriet (Selvais et al., 2011, FASEB J. 25:2770-81; see their report p 69), we have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, see our contribution to the study of the endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., 2005, Dev Cell 9:377-88); subcellular trafficking of the thrombopoietin receptor (Pecquet et al., 2012, Blood; see report by S. Constantinescu, p 115) and the amyloid precursor protein, APP (Ben Khalifa*, Tyteca* et al., 2012, FASEB J. 26:855-67; elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 (Wiame et al., 2010, Biochem J 425:127-36; Veiga da Cunha et al., J Biol Chem 2010, 285:18888-98; Tahay et al., Biochem J. 2012,441:105-12 see report by E. van Schaftingen, p 42); subcellular localization of reactive oxygen species (Denamur*, Tyteca* et al., 2011, Free Radic Biol Med. 251:1656-65); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Miranda et al, 2010, Biochem Biophys. Res. Comm, 396:656-661; see report by M. Rider, p 59); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., 2005, Genes Dev 19:1849-54; see report by F. Lemaigre, p 31), or the morphological evidence by FRET of tight interaction between key players of CTL, that is interrupted during their anergy in cancer but can be reversed by galectins (Demotte et al., Immunity 2008; 28:414-24; Cancer Res 2010, 70:7476-88 see report by P. Van der Bruggen, p 98).

Selected publications of the past ten years

1. Tyteca D, D'Auria L, Van Der Smissen P, Medts T, Carpentier S, , Monbaliu JC, de Diesbach P, Courtoy PJ. Three unrelated sphingomyelin analogs spontaneously cluster into plasma membrane micrometric domains. Biochim Biophys Acta Biomembranes 2010;1798:909-27.

2. D'Auria L, Van Der Smissen P, Bruyneel F, Courtoy PJ*, Tyteca D*. Segregation of fluorescent membrane lipids into distinct submicrometric domains: evidence for large-scale phase compartmentation of natural lipids. PlosOne 2011;6 e17021.

3. D'Auria L, Fenaux M, Aleksandrowicz P, Van Der Smissen P, Chantrain C, Vermylen C, Vikkula M, Courtoy PJ, Tyteca D. Micrometric segregation of fluorescent membrane lipids: relevance for endogenous lipids and biogenesis in erythrocytes. J. Lipid Res. 2013;54:1066-1076.

4. Lima WR, Parreira KS, Devuyst O, Caplanusi A, N'Kuli F, Van Der Smissen P, Alves PM, Verroust P, Christensen EI, Terzi F, Matter K, Balda MS, Pierreux CE*, Courtoy PJ*. ZONAB is controlled during epithelial cell polarization and is a key regulator in the switch between proliferation and differentiation (*, equal senior authors). J Am Soc Nephrol, 2010;21:478-88.

5. Christensen EI, Devuyst O, Dom G, Nielsen R, Van Der Smissen P, Verroust P, Leruth M, Guggino WB, Courtoy PJ. Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. Proc Natl Acad Sci USA 2003;100:8472-7.

6. Nielsen R*, Courtoy PJ*, Jacobsen C, Dom G, Rezende Lima W, Jadot M, Willnow TE, Devuyst O, Christensen EI. Endocytosis provides a major alternative pathway for lysosomal biogenesis in kidney proximal tubular cells (*, equal first authors). Proc Natl Acad Sci USA 2007;104:5407-12.

7. Carpentier S, N'kuli F, Grieco G, Van Der Smissen P, Janssens V, Emonard H, Bilanges B, Vanhaesebroeck B, Gaide Chevronnay HP, Pierreux CE, Tyteca D, Courtoy PJ. Class III phosphoinositide 3-kinase/VPS34 and dynamin are critical for apical endocytic recycling. Traffic 2013, in press.

8. Hick A-C, van Eyll JM, Cordi S, Forez C, Passante L, Kohara H, Nagasawa T, Vanderhaeghen P, Courtoy PJ, Rousseau GG, Lemaigre FP, Pierreux CE. Mechanism of primitive duct formation in the pancreas and submandibular glands : a role for SDF-1. BMC Dev Biol 2009; 9:66

9. Pierreux CE, Cordi S, Hick A-C, Achouri Y, Ruiz de Almodovar C, Prévot P-P, Courtoy PJ, Carmeliet P, Lemaigre FP. Epithelial:Endothelial cross-talk regulates exocrine differentiation in developing pancreas. Dev Biol 2010; 347:216-227

10. Hick AC, Delmarcelle AS, Bouquet M, Klotz S, Copetti T, Forez C, Van Der Smissen P, Sonveaux P, Feron O, Courtoy PJ, Pierreux CE. Reciprocal epithelial : endothelial paracrine interactions during thyroid development govern follicular organization and C-cells differentiation. Dev. Biol. In press

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Extracellular matrix remodeling

Etienne Marbaix
Patrick Henriet

The extracellular matrix (ECM) plays a central role in the structural and functional organization of tissues and organs. ECM constituents, in particular fibrillar collagens, are the most abundant proteins of the human body. Physiological and pathological breakdown of ECM is predominantly achieved by a family of enzymes called matrix metalloproteinases (MMPs; see Fig. 1). Our laboratory was the first to demonstrate that menstrual tissue breakdown is due to a dramatic change in the focal expression and/or activation of MMPs (1, 8, 9). This seminal observation led us to : (i) use this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes; and (ii) explore whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding, a major health problem (2). We also investigate the control by individual cells of local MMP activity, which can be either increased by recruitment and retention to the plasma membrane (3), or down-regulated by receptor-mediated endocytosis and degradation.

Mechanisms of menstrual breakdown and regeneration: identification of new candidate genes by transcriptomic comparison of microdissected tissue areas

H. Gaide Chevronnay, P.J. Courtoy, E. Marbaix, P. Henriet

The general aim of this study was to further elucidate the mechanisms ensuring the spatio-temporal control of menstrual endometrial remodeling in response to the global regulation by estradiol and progesterone. The experimental strategy relied on two advanced methodologies : (i) to separate, by laser capture microdissection, stromal and glandular cells from degraded or preserved areas of the human endometrium after ultrafast immunolabelling and (ii) to compare their global transcriptome by non-supervised microarray analysis.
First, we compared the transcriptomes of stromal and glandular cells microdissected from (i) the *basalis* as well as from (ii) degraded and (iii) preserved areas of the *functionalis* in menstrual endometria (5). Algorithms for sample clustering (PCA) segregated biological samples according to cell type and tissue depth, indicating distinct gene expression profiles (Fig. 2). Strikingly, in addition to genes products associated with tissue degradation (MMP and plasmin systems) and apoptosis, lysed areas in the superficial stroma were enriched in gene products associated with ECM biosynthesis (collagens and their processing enzymes). The presence of new synthesized collagens and increased integrin production was confirmed at the protein level. Overexpression of ECM components and adhesion molecules by lysed menstrual fragments could participate in post-menses endometrial reconstruction but also facilitate implantation of endometriotic lesions.
In the second part of the study, stromal and glandular areas were microdissected from explants cultured without or with estradiol and progesterone (6). The microarray datasets were also compared to other published endometrial transcriptomes. Moreover, the contribution of proteolysis, hypoxia and mitogen-activated protein kinases (MAPKs) to the regulation of selected genes was further investigated in explant culture. Like in the menstrual endometrium, this analysis identified distinct gene expression profiles in stroma and glands but functional clustering underlined convergence in biological processes, further supporting cooperative interactions between cell types. Only partial overlaps were observed between lists of genes involved in different occurrences of endometrial remodeling, pointing to a limited number of potentially crucial regulators but also to the requirement for additional mechanisms

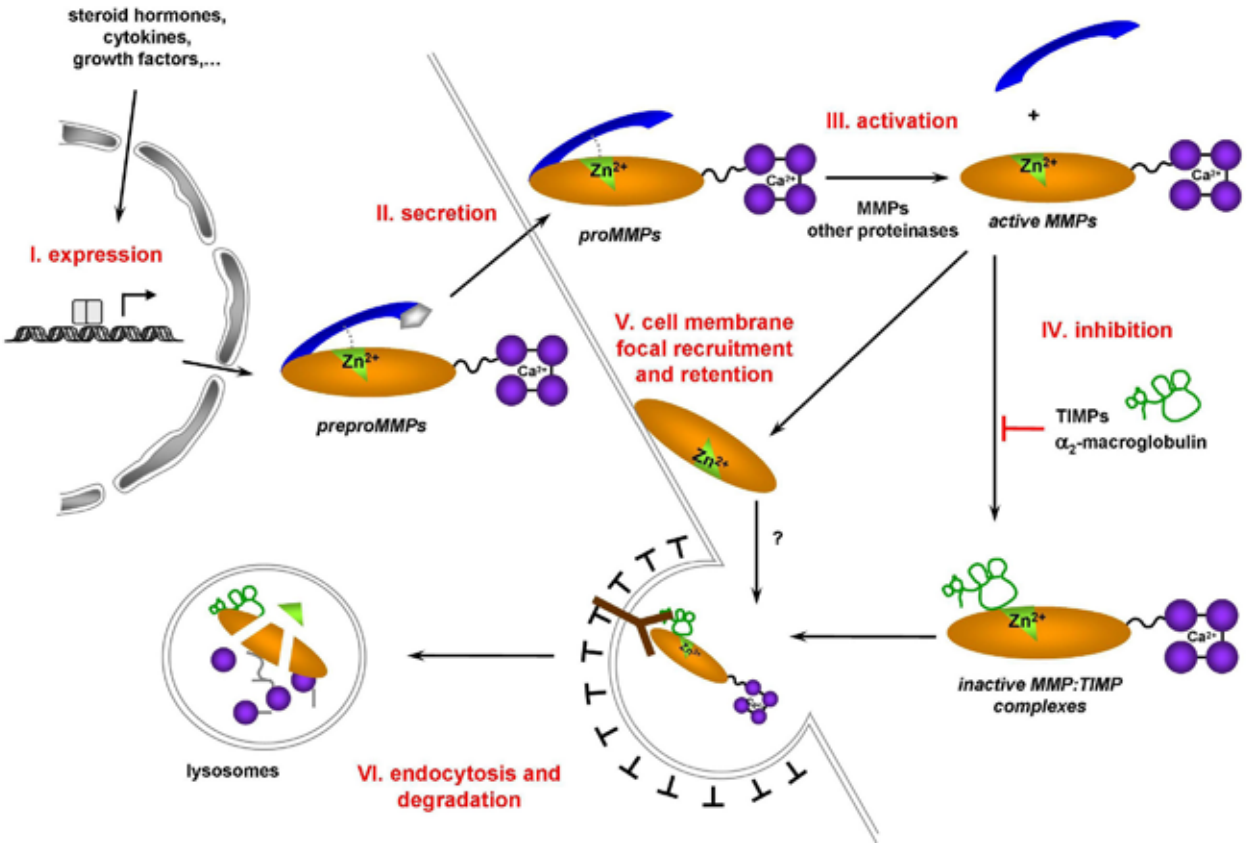

Fig. 1. Regulation of soluble MMP activity in the human endometrium: current model.MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn2+-dependent catalytic site (green) within the catalytic domain (orange). All but MMP-7 and -26 (matrilysins, the "mini-MMPs") are linked by a hinge domain (black) to a variable C-terminal hemopexin-like domain stabilized by calcium (mauve), responsible for substrate specificity,). The overall activity of MMPs can be controlled at six different levels: (I) expression; (II) secretion (regulated in a limited number of cell types such as neutrophils); (III) zymogen activation upon prodomain excision; (IV) inhibition of active forms by physiological inhibitors such as TIMPs (represented with their tertiary structure) and α2-macroglobulin; (V) secondary membrane recruitment increasing pericellular activity; and (VI) down-regulation by endocytosis.

In the cycling human endometrium, MMPs activity is tightly regulated to remodel the extracellular matrix both for blastocyst implantation and, in its absence, for menstrual breakdown of an irreversibly specialized tissue. At menses, the rise of active MMP-1 in the *functionalis* can exceed one-million-fold as compared with mid-phase tissue (1). Several levels of regulation can be evidenced : (I) ovarian steroids and their intracellular receptors as well as cytokines, growth factors and downstream signaling pathways interact to form an integrated system that differentially controls the focal expression of endometrial MMPs and TIMPs. (II) Neutrophils are numerous at menstruation and could contribute to an abundant secretion of MMPs. (III) MMPs can be activated by other MMPs, by plasmin, itself activated during menstruation, or by as yet unidentified proteinases. (IV) TIMPs are particularly abundant in the human endometrium; like MMPs, the level of TIMPs is regulated by ovarian steroids and cytokines. (V) MMP-7 binds to membrane receptors in cholesterol-rich domains, a mechanism which enhances pericellular MMP activity. (VI) Endometrial LRP-1 (brown) binds and internalizes MMP-2 and MMP-2: TIMP-2 complexes, leading to lysosomal degradation. Our research has unraveled (and is focused on) levels (I), (III), (IV), (V) and (VI).

controlling tissue remodeling. This feature was illustrated by a group of genes differentially regulated by ovarian steroids in stroma and glands and sensitive to MAPKs.

In conclusion, we have generated a reliable and useful database of genes differentially regulated in the human endometrium in the context of tissue remodeling. Their comparison suggests that fragments of the functionalis participate in endometrial regeneration during late menstruation, arguing against the classical straightforward model of regeneration from the basalis only. This study also indicates that MAPKs act in concert with hormone withdrawal to locally and specifically control expression of menstrual genes in the superficial layer of the human endometrium.

Cell cholesterol modulates LRP-1 ectodomain shedding as a mechanism to regulate MMP-2 and -9 endocytic clearance

C. Selvais, P.J. Courtoy, P. Henriet, E. Marbaix, H. Emonard (in collaboration with S. Dedieu at CNRS, Reims, France)

We have previously shown that the efficient LRP-1-mediated clearance of MMP-2 and -9 activity in non-bleeding endometrium was abrogated upon hormone withdrawal, due to shedding of LRP-1 ectodomain by a metalloproteinase, presumably ADAM-12, itself regulated by estradiol and progesterone (4). Using human fibrosarcoma HT1080 cells, we recently identi-

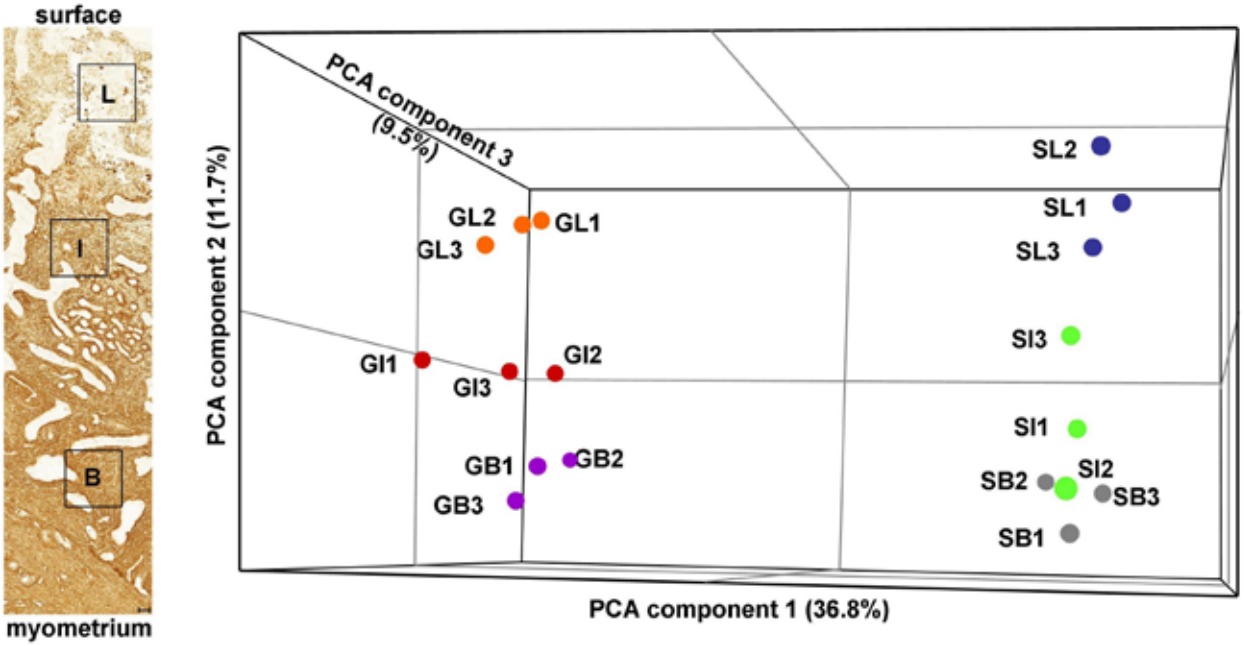

Fig. 2. Comparison of transcriptomes from microdissected areas identifies remarkable cell type- and layer-specific signatures. Tissue samples containing either stroma or glands were microdissected from the three layers of 3 menstrual endometria : lysed superficial *functionalis* (L), preserved intermediate *functionalis* (I) and *basalis* (B). The transcriptomes of the 18 samples were determined using whole genome microarrays. Principal component analysis of the datasets (PCA, at right) clearly indicates (i) the remarkable reproducibility of the biological triplicates (numbers and symbols colors); (ii) a major segregation between stromal (S) and glandular (G) gene expression profiles along axis 1; and (iii) a noticeable segregation between layers along axis 2. For details, see (6).

fied two membrane-associated metalloproteinases, ADAM-12 and MT1-MMP that shed LRP-1 ectodomain (7). We compared the shedding potential of classical fibroblastoid HT1080 cells with a spontaneous epithelioid variant, enriched ~2-fold in cholesterol. Although both fibroblastoid and epithelioid HT1080 cells expressed similar levels of LRP-1, ADAM-12, MT1-MMP and of their specific inhibitor TIMP-2, LRP-1 ectodomain shedding from epithelioid cells was ~4-fold lower than from fibroblastoid cells. Release of the ectodomain was triggered by cholesterol depletion in epithelioid cells and impaired by cholesterol overload in fibroblastoid cells. Modulation of LRP-1 shedding on clearance was reflected by accumulation of gelatinases (MMP-2 and -9) in the medium. We conclude that cholesterol exerts an important control on LRP-1 level and function at the plasma membrane by modulating shedding of its ectodomain, and therefore represents a novel regulator of extracellular proteolytic activities (Fig. 3).

Endometrial xenografts

P. Coudyzer, C. Galant, H. Gaide Chevronnay, P.J. Courtoy, P. Henriet, E. Marbaix (in collaboration with J.M. Foidart, M. Nisolle and A. Béliard at the University of Liège, Belgium and with B.F. Jordan and B. Gallez at the Biomedical Magnetic Resonance Research Group, Louvain Drug Research Institute, UCL)

MMPs are thought to induce menstruation as well as dysfunctional endometrial bleeding, a benign pathology characterized by spontaneous and irregular bleeding associated with menstrual-like stromal breakdown (2). Because menstruation only occurs in few species, in vivo exploration of the physiopathological regulation and role of MMPs is limited. In collaboration with the laboratory of Dr. J.M. Foidart (ULg), we have developed a new experimental model of endometrial xenografts in immunodeficient mice. The model allowed us to investigate the alterations of endometrial ECM remodelling upon levonorgestrel treatment and will be used to directly address the role of MMPs in physiological and abnormal endometrial bleeding, endometrial angiogenesis and vessel maturation, as well as in tissue regeneration after menstrual shedding.

Menstrual endometrial breakdown induced by estradiol and progesterone withdrawal is regularly attributed to vasospasm of spiral arteries causing ischemia and hypoxia. We investigated whether hypoxia actually occurred in our xenograft model (10). Three complementary approaches were used to look for signs of hypoxia in fragments of human functionalis xenografted to ovariectomized immunodeficient mice bearing pellets-releasing estradiol and progesterone, and then deprived of ovarian steroids. Hormone withdrawal 21 days after grafting induced menstrual breakdown and MMP expression within 4 days. Local partial oxygen pressure (pO2) was measured by electron paramagnetic resonance using implanted lithium phtalocyanine crystals. In mice with hormone maintenance until sacrifice, pO2 was low one week after grafting but increased twofold from the second week when tissue was largely revascularized. After 3 weeks, pO2 was not modified

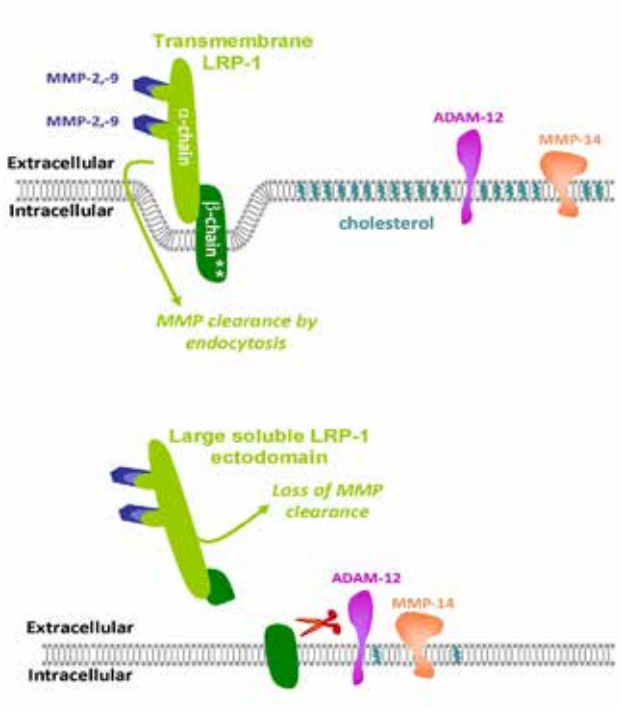

Fig. 3. A model for regulation of gelatinase activity by LRP-1. Upper panel: Binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). Sheddase activity of ADAM-12 and MT1-MMP is prevented by cholesterol-induced membrane rigidity. Lower panel: Shedding of LRP-1 ectodomain is enhanced by membrane fluidity due to cholesterol depletion. For details, see (4 and 7).

by hormone withdrawal but was slightly increased on hormone reimpregnation 4 days after removal by comparison with hormone maintenance. These results were confirmed using fluorescence quenching-based OxyLite measurements. In a further search for signs of hypoxia, we did not find significant HIF1-α immunostaining, nor pimonidazole adducts after hormone withdrawal. This study allowed us to conclude that hypoxia is not needed to trigger menstrual-like tissue breakdown or repair in human endometrial xenograft.

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Growth factor receptors: From signal transduction to human diseases

Jean-Baptiste Demoulin

Growth factors such as platelet-derived growth factors (**PDGF**) are secreted proteins that stimulate cell proliferation via transmembrane receptors. PDGF binds to a **receptor-tyrosine kinase** which signals by phosphorylating various intracellular proteins on tyrosine residues, leading to the regulation of multiple transcription factors and profound changes in **genes expression**. Understanding how this network of **signaling cascades** and transcriptional regulations controls cell growth is the first goal of our team.

The uncontrolled activation of PDGF receptors has been linked to several diseases, such as cancer, leukemia and fibrosis. Our second objective is to clarify the role of PDGF receptors in these diseases and to identify new groups of patients who could benefit from a therapy based on PDGF receptors inhibitors.

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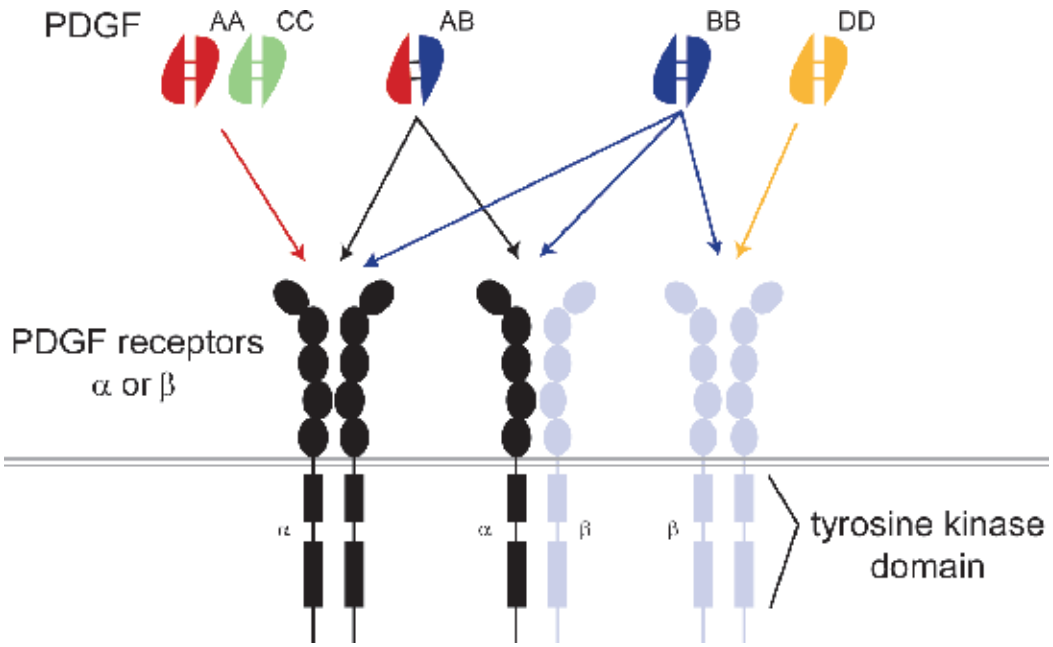

Fig. 1. PDGF receptors and ligands

Signal transduction and gene regulation by growth factors: role of the transcription factors FOXO, STAT and SREBP

A. Coomans de Brachène, A. de Rocca Serra, E. Bollaert, A. Essaghir, J.B. Demoulin.

Most of the cellular effects of growth factors are mediated by reprogramming gene expression within the cell nucleus. Each signal transduction cascade controls a number a transcription factors, which activate or repress the expression of many genes. We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with growth factors (PDGF or FGF) using microarrays. In several successive analyses, we identified hundreds of regulated transcripts that had not previously been linked to PDGF signaling (1-2). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells.

One key transcription factor family that is regulated by growth factors is FOXO. These factors induce a cell cycle arrest, increase resistance toward oxidative stress and regulate metabolism. They are inactivated by growth factors via AKT, which phosphorylates three conserved sites within FOXO proteins. Phosphorylated FOXO is excluded from the nucleus and targeted for degradation by proteasomes (Fig. 2). We observed that FOXO mRNA expression is also decreased upon stimulation with growth factors (2). We showed that the promoter of the FOXO1 gene is stimulated by FOXOs themselves, a process that is disrupted by growth factors, most likely via AKT, and regulates cell growth. We are now analyzing whether this mechanism could play a role in the proliferation of tumor cells. We also identified several mediators of the effects of FOXO on the cell cycle.

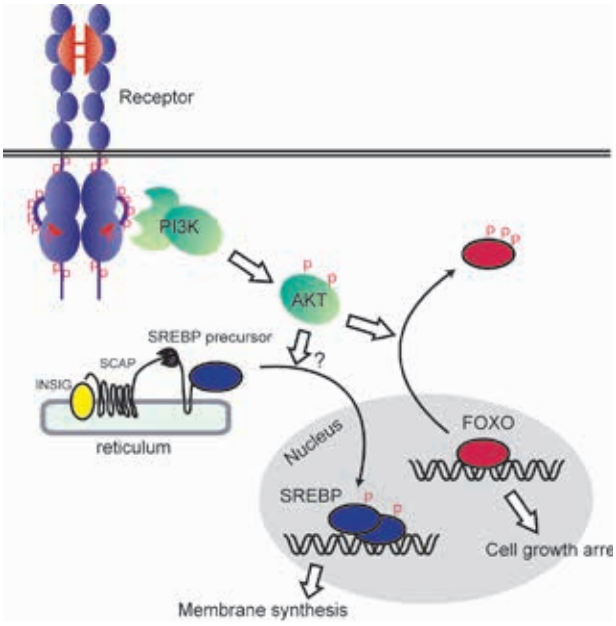

Fig. 2. Activation of SREBP and inactivation of FOXO by PDGF

In our microarray analysis, a cluster of genes involved in fatty acid and cholesterol biosynthesis, including stearoyl-CoA desaturase (SCD), fatty acid synthase and hydroxy-methylglutaryl-CoA synthase (HMGCS), was up-regulated by PDGF after 24 h of treatment. Their expression correlated with an increase in membrane lipid biosynthesis. All these genes are known to be controlled by sterol regulatory element-binding proteins (SREBP). PDGF increased the amount of mature SREBP-1, and regulated the promoters of SCD and HMGCS in a SREBP-dependent manner. In line with these results, blocking SREBP processing by addition of 25-hydroxycholesterol blunted the effects of PDGF on lipogenic enzymes and PDGF-driven proliferation. SREBP activation was dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, as judged from the effects of the inhibitor LY294002 and mutation of the PDGF receptor β tyrosine residues that bind the regulatory PI3K subunit p85. In conclusion, our results suggest that PDGF induces membrane lipid synthesis via PI3K and the activation of SREBP (Fig 2. and reference 3). The role of SREBP in PDGF and tumor development will be further analyzed. We went on by identifying novel target genes for the SREBP transcription factors. We found that these transcription factors regulate p55γ, a subunit of the PI3K complex and heme oxygenase, which plays an important role in stress responses. These results expand the list of genes regulated by SREBP to targets that are not directly involved in lipid metabolism. We are now trying to understand more precisely the role of the SREBP target genes in growth factor responses.

TFactS: a bioinformatics tool to predict transcription factor regulation from microarray data

A. Essaghir, J.B. Demoulin.

Deciphering transcription factor networks from microarray data remains difficult. We have developed a simple method to infer the regulation of transcription factors from microarray data based on well-characterized target genes (1). We generated a catalogue containing 352 transcription factors associated with 2,721 target genes and 6,422 regulations. When it was available, a distinction between transcriptional activation and inhibition was included for each regulation. Next, we built a tool (www.TFactS.org) that compares new submitted gene lists with target genes in the catalogue to detect regulated transcription factors. We validated TFactS with our own microarray experiments and with published lists of regulated genes in various models and compared it to tools based on in silico promoter analysis. Our results show that changes in the expression level of transcription factor target genes constitute a robust signature for transcription factor regulation, and can be efficiently used for microarray data mining. We are now introducing new features into this tool and we are using it to analyze cancer genome data. This tool was also used in a number of transcriptomics projects in our laboratory (see for instance reference 4) and in collaboration with other groups.

We are now integrating micro-RNA targets into TFactS to take into account these important regulators).

Rearrangements of receptor tyrosine kinase genes associated with leukemia

L. Noël, V. Havelange, F. Arts, D. Xhema, J.B. Demoulin.

Although PDGF receptors are expressed on platelets and macrophages, PDGF receptor-deficient mice show no primary hematopoietic or immune defect. In vitro, PDGF is a poor mitogen for hematopoietic cells. However, alterations of PDGF receptor genes, as a result of chromosomal translocation or deletion, are found in chronic myeloid neoplasms associated with hypereosinophilia, a disease which affects mostly male patients (5). In all cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. In most cases, they also retain the receptor transmembrane domain, which plays a particular role in the activation of these oncoproteins (5). Similar hybrid oncogenes derive from FGF receptors.

TEL-PDGFRβ (TPβ, also called ETV6-PDGFRB) is a hybrid protein produced by the t(5;12) translocation. FIP1L1-PDGFRα (FPα) results from a deletion on chromosome 4q12 (5). These oncogenes were studied in Ba/F3 cells, a mouse hematopoietic cell line that is easy to culture and transfect. In contrast to wild-type PDGF receptors α and β, which are quickly degraded upon activation, we observed that TPβ and FPα escaped down-regulation resulting in the accumulation of these oncoproteins in cells (8). This was confirmed in leukocytes from patients. Similar data were obtained in cells expressing ZNF198-FGFR1, another fusion protein associated with the 8p11 myeloproliferative syndrome. Ubiquitination of TPβ and FPα was much reduced compared to wild-type receptors. We showed that the accumulation of TPβ is required to activate STAT5 efficiently and transform Ba/F3 cells. Thus, chimeric receptor tyrosine kinases escape efficient ubiquitination and degradation through lysosomes and proteasomes (8). This is a new mechanism that contributes to cell transformation by fusion kinase.

TPβ and FPα do not induce eosinophilia in mice. In order to develop a model that is more relevant for the human disease, we introduced TPβ and FPα in human CD34+ cells, which were purified from umbilical cord blood and are enriched in hematopoietic stem cells. These cells are able to differentiate normally in vitro into various blood cell types, depending on the cytokine cocktail that is added in the culture medium. We observed that TPβ and FPα induce the proliferation of these cells and their differentiation into eosinophils in the absence of cytokine. We showed that this process is dependent on the activation of the NF-κB pathway via PI3K (4).

It is particularly important to identify PDGF receptor alterations in cancer patients, as they can benefit from tyrosine kinase inhibitor therapy. Imatinib mesylate, for instance, is very efficient in patients with leukemia that present a PDGF receptor fusion. In collaboration with the hematology unit of the Saint-Luc university hospital, we identified a novel fusion of the PDGF receptor β with the KANK1 gene in a leukemia patient harboring a t(5;9) translocation (Fig. 3 and reference 6). The mechanism of activation of this new fusion gene was analyzed in details (7). We are now looking for other mutations in tyrosine kinase genes.

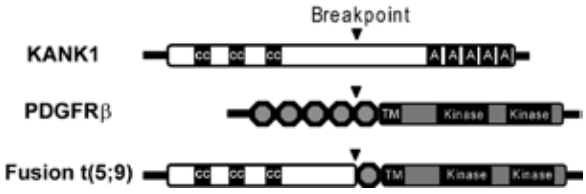

Fig. 3. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation.
CC: coiled-coil domain; A: ankyrin repeat; TM: transmembrane domain

Functional characterization of novel PDGF receptor mutations in cancer

J.B. Demoulin, A. Velghe, F. Arts.

Systemic sclerosis (also called scleroderma) is a severe connective tissue disease of unknown etiology characterized by vascular alterations, autoimmunity and fibrosis of the skin and multiple internal organs, which is potentially fatal. A recent report suggested that the disease is driven by stimulatory autoantibodies to the platelet-derived growth factor receptors (PDGFR), which stimulate the production of reactive oxygen species (ROS) and collagen by fibroblasts (Baroni et al, New Engl. J. Med 2006; 254:2667). These results opened novel research avenues for the diagnosis and treatment of systemic sclerosis. Several clinical trials using imatinib mesylate, a drug that inhibits PDGF receptors, were promptly initiated following this publication. In order to confirm this important observation, we purified immunoglobulins from 37 patients with systemic sclerosis by protein A/G chromatography. PDGFR activation was tested using four different sensitive bioassays, namely cell proliferation, ROS production, signal transduction and receptor phosphorylation. Purified IgG from patients with scleroderma comprised a panel of antinuclear autoantibodies, but did not specifically activate the PDGFR α or β in any of our tests, compared to controls. As positive control, cell stimulation with PDGF itself consistently produced a strong signal. Our results question the existence of agonistic autoantibodies to PDGFR in scleroderma (10). Four independent research centers have reported similar negative results. We are now trying to identify other factors that activate PDGF receptors in systemic sclerosis and other related fibrotic conditions, such as extensive chronic graft-versus-host disease.

Activation of PDGF receptors in systemic sclerosis

S. Charni and J.B. Demoulin, in collaboration with Bernard Lauwerys and Frédéric Houssiau (Cliniques Universitaires Saint-Luc, UCL).

Systemic sclerosis (also called scleroderma) is a severe connective tissue disease of unknown etiology characterized by vascular alterations, autoimmunity and fibrosis of the skin and multiple internal organs, which is potentially fatal. A recent report suggested that the disease is driven by stimulatory autoantibodies to the platelet-derived growth factor receptors (PDGFR), which stimulate the production of reactive oxygen species (ROS) and collagen by fibroblasts (Baroni et al, New Engl. J. Med 2006; 254:2667). These results opened novel research avenues for the diagnosis and treatment of systemic sclerosis. Several clinical trials using imatinib mesylate, a drug that inhibits PDGF receptors, were promptly initiated following this publication. In order to confirm this important observation, we purified immunoglobulins from 37 patients with systemic sclerosis by protein A/G chromatography. PDGFR activation was tested using four different sensitive bioassays, namely cell proliferation, ROS production, signal transduction and receptor phosphorylation. Purified IgG from patients with scleroderma comprised a panel of antinuclear autoantibodies, but did not specifically activate the PDGFR α or β in any of our tests, compared to controls. As positive control, cell stimulation with PDGF itself consistently produced a strong signal. Our results question the existence of agonistic autoantibodies to PDGFR in scleroderma (10). Four independent research centers have reported similar negative results. Although antibodies directed towards PDGF receptors may exist, they are not specific of scleroderma and they do not activate the receptor. We are now trying to identify other factors that activate PDGF receptors in systemic sclerosis and other related fibrotic conditions, such as extensive chronic graft-versus-host disease.

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Viral immunity and pathogenesis

Jean-Paul Coutelier

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the molecules that allow those cells to communicate, namely the lymphokines, and the products of those interactions, including antibodies, have been elaborated. On the other hand, viral infections strongly modulate the immune microenvironment of the host which often leads to alterations of responses elicited against non-viral antigens and of concomitant diseases with an immune component. Our project is to analyse, in murine models, some aspects of these relations between viruses and the immune system.

Viral infections result in a dramatic increase in the proportion of IgG2a

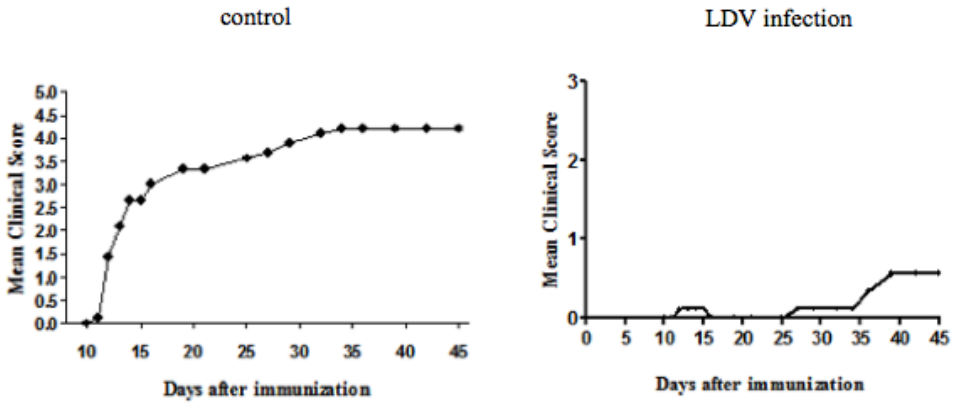
Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties, such as differential affinity for receptors expressed on phagocytes. During the last years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. The modulations of antibody responses was analysed with more details by using a model of infection with lactate dehydrogenase-elevating virus (LDV), a common mouse nidovirus that induces strong and early immune responses (1). We could demonstrate that a dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias. IgG2a anti-LDV antibodies were found to be more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (2). However, the modification of the isotype of antibodies reacting with self antigens could potentially lead to more deleterious autoimmune reactions.

T helper lymphocyte differentiation

This property of viruses to enhance selectively the production of one immunoglobulin isotype could depend on the preferential activation of a subset of T helper lymphocytes. Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively IFN-γ or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively. We have found that LDV infection results in a suppression of Th2 responses elicited by immunization with an antigen unrelated to the virus. More recently, other populations of Th lymphocytes, such as Th17 cells that are involved in some autoimmune responses, as well as T regulatory lymphocytes that inhibit ongoing responses have been described. Preliminary observations in our group show a dramatic prevention of diseases such as autoimmune encephalitis (Figure 1) and graft-versus-host disease in mice acutely infected with LDV. Whether this protective effect of the virus results from a modulation of T helper/ T regulatory cells remains to be determined.

Activation of natural killer cells

Many of the influences that viruses may have on diverse immune responses can be explained by the production of pro-inflammatory cytokines, including IFN-γ. Therefore, our analysis of the relationship between viruses and the immune system has focused on the activation, by LDV, of cells from the innate immune system that are able to secrete this cytokine, namely



Encephalitis progression in control mice and in animals infected with LDV.

the natural killer (NK) cells. Within a few days after infection, a strong and transient NK cell activation, characterized by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Two pathways of IFN-g production have been observed that both involve NK cells. The first pathway, found in normal mice, is independent from type I IFN and from interleukin-12. The second pathway involves interleukin-12, but is suppressed by type I IFN (3). Because NK cells and IFN-γ may participate in the defense against viral infection, we analyzed their possible role in the control of LDV titers, with a new agglutination assay. Our results indicate that neither the cytolytic activity of NK cells nor the IFN-γ secretion affect the early and rapid viral replication that follows LDV inoculation.

Interestingly, NK cell activation results in an increased expression of CD66a (CEACAM-1), an adhesion molecule that display immunoregulatory function on activated T lymphocytes. However, this enhanced expression, that is also found on immature NK cells, results from NK cell stimulation with IL-12 and IL-18, but not with LDV (4). Therefore, different pathways of NK cell activation, leading to various phenotypes and, probably various functions, may be observed.

Activation of macrophages and enhanced susceptibility to endotoxin shock

Activation of cells of the innate immune system by LDV includes also macrophages and leads to an enhanced response to lipopolysaccharide (LPS), and to an exacerbate susceptibility to endotoxin shock (5). A synergistic effect of LDV and LPS triggered dramatic production of tumor necrosis factor (TNF) and IFN-γ. Susceptibility to LPS shock was completely mediated by TNF, and partially by IFN-γ. This increased susceptibility of LDV-infected mice to endotoxin shock was not mediated by modulation of the expression of membrane receptors for LPS, but was correlated with increased levels of soluble LPS receptors (6). In this context, the production of type I IFNs may protect the host against exacerbated pathology by controling the production of IFN-γ.

Blood autoimmune diseases

Virally-induced macrophage activation leads also to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. LDV infection resulted in moderate thrombocytopenia in normal animals through enhanced spontaneous platelet phagocytosis (7). Our analysis was then focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti- platelet response was developed in the mouse. Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s) shared by rat and mouse platelets. Two IgM anti-platelet monoclonal autoantibodies were further analyzed. They recognized mouse platelet antigens and could induce both platelet destruction and impairment of their function. This response was found to depend on CD4+ T helper lymphocytes reacting with rat, but not with mouse platelets. These anti-rat platelet T helper cells were mainly of the Th1 phenotype. When transferred into naive mice, they enhanced the anti-mouse platelet antibody response induced by subsequent immunization with rat platelets. In addition, depletion of CD25+ cells enhanced the thrombocytopenia induced by immunization with rat platelets whereas adoptive transfer of CD4+CD25+ cells from immunized mice suppressed it (8). Our results suggest therefore that activation of anti-rat platelet T helper cells can bypass the mechanism of tolerance and result in the secretion of autoreactive antibodies, but this response is still controlled by regulatory T cells that progressively develop after immunization.

We have analysed whether a viral infection could modulate such an autoantibody-mediated autoimmune disease. In mice treated with anti-platelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with LDV or mouse hepatitis virus was followed by severe thrombocytope-

nia (9), whereas infection alone, without autoantibody administration led to a moderate disease. Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose in vitro autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (10).

Treatment of thrombopenic or anemic mice with clodronate-containing liposomes and with total IgG indicated that opsonized platelets and erythrocytes were cleared by macrophages. Administration of clodronate-containing liposomes decreased also the in vitro phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. The increase of thrombocytopenia triggered by LDV after administration of anti-platelet antibodies was largely suppressed in animals deficient for IFN-γ receptor. Moreover, LDV infection resulted in an increased expression of receptors recognizing the Fc portion of antibodies, which may at least partially leads towards the enhanced phagocytic activity of macrophages. Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia and anemia by activating macrophages through IFN-γ production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. Regulation of macrophage activation results in modulation of autoantibody-mediated cell destruction and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism.

Together, these two models may correspond to the development of some auto-immune diseases : a first stimulus triggers the production of autoantibodies, through molecular mimicry. A second stimulus, such as a viral infection, leads to the activation of macrophages and results in the destruction of opsonized target cells.

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Viral persistence and interferon response

Thomas Michiels

Our work focuses on the interplay between viral infections and the innate immune response of the host. On one hand, we analyze the model infection of the central nervous system by Theiler's virus. This virus can escape the immune defenses of the host and provoke a persistent infection of the central nervous system. Theiler's virus infection and the resulting inflammatory response can lead to a chronic demyelinating disease considered as a model of multiple sclerosis. On the other hand, we analyze the innate immune response against viral pathogens. We focus on the analysis of the type I (IFN-α/β) and type III (IFN-λ) interferon responses, which are critically important to control viral infections and to modulate the acquired immune responses.

1. Theiler's virus

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus showing a striking ability to persist in the central nervous system of the host in spite of a specific cellular and humoral immune response. Persistence of the virus is associated with a strong inflammatory response and with lesions of primary demyelination reminiscent of those found in human multiple sclerosis. Our work aims at understanding how a virus can persist in the central nervous system (CNS) of an immuno-competent host, thus evading the immune response (for review, see ref 1, 4).
Recently, a novel human virus closely related to Theiler's virus was discovered and named Saffold virus. This virus is highly prevalent in the human population. Further work is needed to evaluate its virulence potential and its impact on global health.

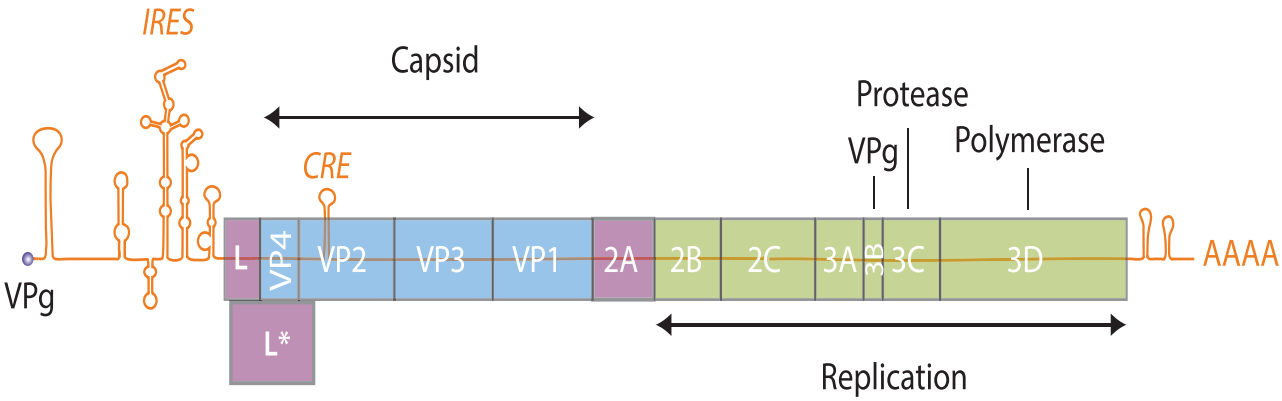
Analysis of viral proteins involved in Theiler's virus escape of the host immune response.

F. Sorgeloos, F. Borghese, A. De Cock, M. Drappier, M. Peeters and T. Michiels

Two proteins produced by Theiler's virus, namely L and L*, were found to be dispensable for viral replication in cell culture but to be crucial for the establishment of a persistent infection of the central nervous system. These proteins are targeting cellular pathways to counteract host immune defenses.

The leader (L) protein: A multifunctional peptide that interferes with cellular responses

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. We observed that this protein exerts pleiotropic activities in infected cells. The first activity that was detected for the L protein is its ability to inhibit the transcription of type I IFN and of chemokine genes (1, 4). This activity likely results from the fact that the L protein inhibits the dimerization of IRF-3, the main transcriptional activator of these genes.
Infection of mice deficient for the type-I interferon receptor (IFNAR-/-) indicates that IFN production is critical for resistance against virus infection and that inhibition of IFN production by the L protein also occurs in vivo. However, in vivo, the inhibition of IFN production is not absolute. If it were, the DA virus would cause a fatal encephalomyelitis in wild-type mice analogous to the disease it causes in IFNAR–/– mice.
The L protein also interferes with nucleo-cytoplasmic trafficking of host proteins and mRNA. It promotes the subcellular redistribution of host nuclear and cytoplasmic proteins. It also represses the exportation of mRNA from the nucleus to the cytoplasm, thereby shutting-off the expression of host cell proteins. This might explain the highly toxic nature of this protein. These traffic perturbating activities of the L protein correlate with L-mediated hyperphosphorylation of the Nup proteins that make up the nuclear pore complex (4).
Recently, we observed that the L protein of Theiler's virus also inhibited stess granule assembly. Stress granules are stalled



L: inhibition of IFN production
alteration of nucleo-cytoplasmic trafficking

L*: infection of macrophages
targeted to the mitochondrial outer-membrane
inhibition of RNase L activity

adapted from www.virologie-uclouvain.be

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Our analysis focuses on 2 proteins L and L* that interfere with the host innate immune responses and therefore facilitate the establishment of a persistent infection.

translation initiation complexes forming in cells after a translation blockade consecutive to a cellular stress. Cells infected with a L-mutant virus but not with the wild-type virus produce stress granules. These granules contain typical stress granule-associated proteins as well as proteins like PTB which are not found in all types of stress granules. However, we failed to detect viral RNA or replicative forms of the viral genome in stress granules (7).

In order to test whether the various activities of the L protein are linked, we subjected the L coding region to random mutagenesis and selected L mutants that lost toxicity. This study revealed that two domains of the L protein are critical for activity: the N-terminal zinc finger and a C-terminal domain that was named "Theilo-domain" since it is conserved in the L protein of Theiloviruses (i.e. Theiler's virus and Saffold virus) but not in the L protein of encephalomyocarditis virus (EMCV) (7). Mutations of either the Zn-finger or of the Theilodomain abolished all reported activities of the L protein, suggesting that the various activities of the protein are linked. Current efforts are devoted to finding the master L interactor in infected cells.

The L* protein : targeted to the mitochondrial outer-membrane and antagonist of RNase L activity

Persistent strains of Theiler's virus produce an 18 KDal protein called L*, which is encoded by an open reading frame (ORF) overlapping the ORF coding the viral polyprotein (see Fig. 1). This protein was shown to enhance the infection of macrophage cell lines and to be critical for persistence of the virus in the central nervous system of the mouse (1, 4).

We showed that L* protein is partitioned between the cytoplasm and the mitochondria (Fig. 2). In mitochondria, L* is anchored in the outer membrane, facing the cytosol (5). Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection.

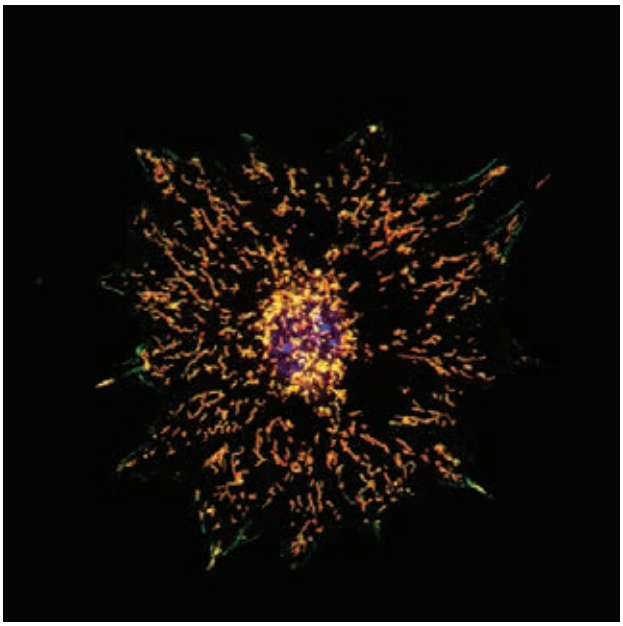


Fig. 2. Mitochondrial localization of L*
Mouse embryonic fibroblast expressing L* from a lentiviral vector. L* was immunolabeled in green and mitochondria were labeled in red with mitotracker. Yellow indicates co-localization.

Our recent data suggest that the cytosolic fraction of L* protein antagonizes the OAS/RNase L pathway (8). We showed that the L* protein interferes with RNase L activation by direct protein-protein interaction. Interestingly, RNase L antagonism by L* is host-specific as L* from the murine virus inhibits mouse but not human RNase L (8). Recently, a protein encoded by the mouse hepatitis virus (MHV - a Coronavirus) was shown to interfere with the same pathway, but in a different manner. Theiler's virus and MHV share a strong tropism for macrophages. We believe that the RNase L pathway is particularly active in these cells and that viruses infecting macrophages thus developed proteins to counteract this important cellular defense mechanism.

In conclusion, our data show that Theiler's virus interferes both with the production of IFN and with the response of cells to this cytokine. This outlines the major importance of the IFN system in the defense of the host against viral infections.

2. Type-I and Type-III Interferons

Interferons were the first cytokines to be identified. They were discovered, more than 50 years ago, by Isaacs and Lindemann who observed that chick chorio-allantoic membranes developped resistance to viral infection after exposure to heat-inactivated influenza virus. Interferons are typically secreted by cells that are infected by a virus. They alert neighbouring cells about the presence of a viral infection and allow these cells to develop an anti-viral state. The interferon system represents a critical protection mechanism of the body against viral infections. In addition, interferons have anti-cancer properties and modulate the acquired immune response of the host.

Type I IFNs

P. Hermant, C. Lardinois and T. Michiels

Mouse and human genomes carry more than a dozen of genes coding for closely related interferon-alpha (IFN-α) subtypes. These interferons and other interferons, like IFN-β, IFN-κ, IFN-ε, IFN-ω, and limitin, form the type-I IFN family. In spite of important sequence divergences, all these IFNs bind the same receptor, raising the question of whether they possess specific functions.

We previously characterized the murine IFN-α family. 14 IFN-α genes were detected in the mouse genome, two of which, IFN-α13 and IFN-α14, were previously undescribed. In addition the mouse genome contains three IFN-α pseudogenes.

Ongoing work aims at understanding the specificities of the various type-I IFN subtypes.

Type-III Interferons (IFN-λ)

M. Minet, P. Hermant, C. Lardinois and T. Michiels

More recently, another IFN family was described and called "type-III IFN" or " IFN-λ". Although type III IFNs bind a receptor distinct from the type-I IFN receptor, they activate the same signal transduction pathway and upregulate the same set of genes. Thus, they are expected to exert very similar biological activities as type I IFNs. Our work addresses the question of the redundancy of these two IFN systems in vivo.

We observed that the relative expression of IFN-λ over that of IFN-α/β exhibited some extent of tissue specificity and was low in the brain (3). We also used a strategy based on in vivo expression of cloned IFN genes to compare the responses of different tissues to IFN-α and IFN-λ. Interestingly, response to IFN-λ appeared to be specific to epithelial cells (Fig. 3), unlike response to IFN-α, which occurred in most cell types and was particularly prominent in endothelial cells. Accordingly, tissues with a high epithelial content such as intestine, skin or lungs were the most responsive to IFN-λ and expressed the higher amounts of IFN-λ receptor (3). Work performed in collaboration with the groups of P. Stäheli (Univ. of Freiburg, Germany) and M. Hornef (Univ. of Hannover, Germany) confirmed that IFN-λ participates to the protection of lung and intestine epitheliums (where the response to IFN-λ is prominent) against infection with several viruses such as influenza virus respiratory syncytial virus or SARS coronavirus. Interestingly, IFN-λ turned out to be the major player in the defense against rotaviruses, common enteric pathogens causing diarrhea (6).

Our data suggest that the IFN-λ system evolved as a specific protection of epithelia and that it might contribute to prevent viral invasion through skin and mucosal surfaces.

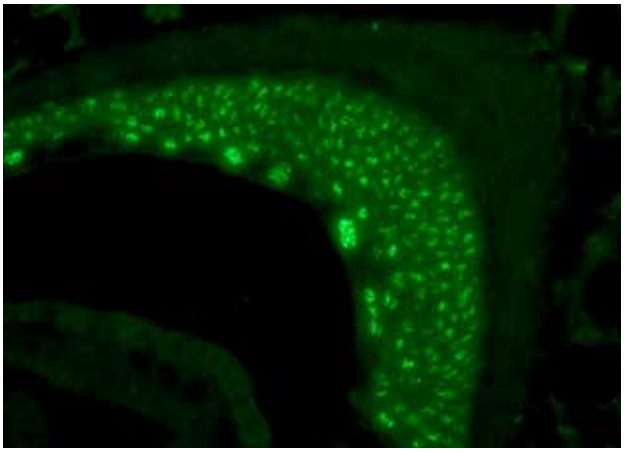


Fig. 3. Response to IFN-I. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-I. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Neurons and IFN-α/β

M. Kreit and T. Michiels

In collaboration with the teams of Peter Staeheli and Friedemann Weber (Univ. Freiburg, Germany) we analyzed the cells that are responsible for type I IFN production in the central nervous system, during acute encephalitis caused by two neurotropic viruses: Theiler's virus (picornavirus) or La Crosse virus (bunyavirus).

IFN-α/β turned out to be produced by many scattered parenchymal cells and less by cells of the inflammatory foci. Interestingly, besides some macrophages and few ependymal cells, neurons turned out to be important producers of both IFN-α and IFN-β. However, not more than 3% of infected neurons expressed IFN, suggesting that some restriction to IFN production might occur in these cells (2).

Recent work from our laboratory suggests that neurons are also restricted in their response to IFN. After IFN treatment, primary neurons seem to respond to IFN through the transcriptional upregulation of many IFN-stimulated genes. However, in contrast to other cell types, IFN-treated neurons are not efficiently protected against viral infection. We currently analyze the reason for this particular sensitivity of neurons to viral infection.

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Human tumor immunology

Pierre Coulie
Sophie Lucas

The group studies human T lymphocyte responses to tumors, with three lines of research. First trying to understand the mechanisms of the tumor regressions that occur in cancer patients vaccinated with tumor-specific antigens recognized by T lymphocytes. Second to examine the specificity and functional properties of the T lymphocytes that are present within human tumors, and in particular in melanomas and in breast carcinomas, but appear to be quiescent. Third to understand the mechanism of immunosuppression by human regulatory T cells and its importance in human antitumor immunity. The main objective of this work is to better understand the mechanisms and limitations of human antitumor immunity in order to improve the clinical efficacy of cancer immunotherapy.

Immune responses to cancer vaccine antigens

G. Hames, T. Gomard, O. Bricard, P.G. Coulie, in collaboration with J.-F. Baurain, Department of Medical Oncology, Cliniques universitaires St Luc, and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

Only 5-10% of cancer patients vaccinated with defined tumor antigens display an objective tumor regression. To measure blood T cell responses to the vaccine antigens we developed a sensitive approach based on *in vitro* restimulation of blood lymphocytes with the antigenic peptides over two weeks, followed by labeling with tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures were conducted under limiting dilution conditions. Cells that were labeled with the tetramer were cloned, the lytic specificity of the clones was verified, and their diversity was analyzed by T cell receptor (TCR) sequencing (1). We observed surprisingly low levels of anti-vaccine T cell in several of the vaccinated patients who displayed tumor regression. Moreover we did not observe the anticipated correlation between the intensities or breadth (number of peptides against which a response is observed) of the immune responses and the clinical impact of the vaccinations. These results suggest that the main limitation to the clinical efficacy of these therapeutic anti-cancer vaccines is not the intensity of the anti-vaccine T cell responses.

Tumor regressions observed after vaccination: the sparking role of tumor-specific cytolytic T lymphocytes that do not recognize the vaccine antigens

V. Corbière, C. Muller, P.G. Coulie, in collaboration with C. Lurquin and B. Lethé, Brussels branch of the Ludwig Institute for Cancer Research.

We identified a cancer patient whose tumors regressed following vaccination with tumor-specific antigens even though the anti-vaccine T-cell response was of very low magnitude. Moreover very few of these anti-vaccine T cells were present in the regressing tumors. But in these tumors we found T cells that were specific for non-vaccine tumor antigens (2), suggesting that antigen spreading was occurring in the regressing tumors. This was confirmed by the following observation. One T cell clone enriched in regressing metastases was retrieved and analyzed. It specifically lysed autologous tumor cells, and its target antigen was identified as the mitochondrial enzyme caseinolytic protease (3). The antigen gene was mutated in the tumor, resulting in production of a neoantigen. These results argue that tumor rejection effectors in the patient were indeed T cells responding to non-vaccine tumor-specific antigens (4). We propose that antigen spreading of an antitumor T-cell response to truly tumor-specific antigens contributes decisively to tumor regression. This process of antigen spreading appears to be sparked by the activity of the few anti-vaccine T cells that reach the tumor. This is likely to be important also for

other modalities of cancer immunotherapy such as adoptive transfer of antitumor T lymphocytes.

Inflammatory cytokines and tumor-infiltrating lymphocytes

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The nature of the 'spark' mentioned above, or how a few activated anti-vaccine T lymphocytes can trigger the activation of many other antitumor T cells, is unknown. We have explored the possibility that inflammatory cytokines participate in this process. Indeed, they are present at the early phases of all immune responses to pathogens, because the latter stimulate their production upon binding to various receptors such as the Toll-like receptors. But they are probably absent from most tumors, which are not known to trigger their production. We reasoned that a local production of inflammatory cytokines could be triggered by the activated anti-vaccine T cells, leading to a strictly local cytokine cascade that attracts other immune cells and therefore sustains and extends the anti-tumor T cell response. A likely source of inflammatory cytokines are monocytes/macrophages, which are always accompanying T cells in tumors.
We observed that human activated T cells cultured in the presence of monocytes stimulate the production by the latter of several inflammatory cytokines including IL-1ß and IL-6. T cell activation is mandatory in this process. Monocyte stimulation requires intercellular contacts with the activated T cells, through CD40L on the T cells and CD40 on the monocytes. It also requires cytokines produced by the T cells, namely TNF together with GM-CSF. The role of GM-CSF, unexpected in this context, appears to be to strenghten the TNF-induced NF-kB activation. Our results are compatible with a local production of inflammatory cytokines by monocytic cells in contact with activated anti-vaccine T cells that reach the tumor.

Analysis of lymphocytes infiltrating human breast carcinomas

D. Schröder, G. Hames, N. Dauguet, P.G. Coulie in collaboration with D. Godelaine, Brussels branch of the Ludwig Institute for Cancer Research.

Most of our current knowledge of the antigenicity and immunogenicity of human tumors is derived from the analysis of melanomas. The reason for this bias is not a higher antigenicity or immunogenicity of melanomas, though this is not to be excluded, but the possibility to obtain permanent cell lines from these tumors, cell lines with which tumor-specific T cell responses can be analyzed rigorously. Thus, while the genetic processes triggering the expression of tumor-specific antigens apply to all tumor types, we are still little closer to knowing to which extent non melanoma tumors are immunogenic.
This is especially true of breast cancer, by far the most common cancer diagnosed in women worldwide. Yet several results support the hypothesis that breast carcinoma cells bear antigens that can trigger tumor rejection T cell responses. We have initiated a project to study T cell responses to human breast carcinomas, in collaboration with clinical teams at the Cliniques universitaires St Luc (Profs. J.-P. Machiels, M. Berlière and C. Galant) and at the Grand Hôpital de Charleroi (Prof J.-L Canon and Dr J. Carrasco).
Our first objective is to demonstrate that tumor-infiltrating lymphocytes (TILs) in breast tumors contain CD8+ T lymphocytes that recognize truly tumor-specific antigens, thus encoded either by genes that are mutated in the tumor or by cancer-germline genes such as *MAGE*. We establish sets of CD8+ T cell clones from fresh TILs, and screen them for recognition of candidate mutated antigenic peptides deduced from tumor exome sequencing, and of selected antigenic peptides encoded by *MAGE* genes.
Thus far, we have obtained two sets of about 100 CD8+ clones derived from the TILs of two patients, and have analyzed the diversity of these T cells by sequencing the genes encoding their receptors to antigen. The repertoire of these receptors contains several receptors present in more than one clone. This suggests that T cells bearing these receptors had multiplied in the tumor, probably following a local contact with an antigen. Such a result is expected if tumor-specific T cells are active in the tumor, as we previously observed in human melanomas. We will now focus on these 'repeated' T cell clones and screen them for the recognition of candidate tumor-specific antigens.

Human regulatory T cells and TGF-ß

S. Lucas, J. Stockis, C. Huygens, E. Gauthy, J. Cuende, O. Dedobbeleer, S. Liénart, S. D'Hondt, M. Panagiotakopoulos, N. Dauguet and P.G. Coulie.

Regulatory T cells, or Tregs, are a subset of CD4+ lymphocytes specialized in the suppression of immune responses. They prevent the development of auto-immune diseases, but in mice they were also shown to contribute to cancer progression by inhibiting anti-tumor immune responses. Tregs could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a Treg-specific marker in humans, as well as to an incomplete understanding of the mechanisms underlying their suppressive function.
Our objective is twofold: develop tools to quantify Tregs in human tissues, and identify mechanisms important for their suppressive function, which could be specifically targeted to improve the efficiency of cancer vaccines.
Our previous work lead to the obtention of stable human Treg clones, representing long-term cultures of pure lymphocyte populations available for repeated analysis (5). A stable epigenetic mark unambiguously distinguished human Treg clones from non regulatory T cells: a conserved non-coding region in

gene *FOXP3*, encoding a transcription factor indispensable for the development and function of Tregs, was found demethylated in Treg clones only. We set up a methylation-specific real-time PCR assay to quantify demethylated *FOXP3* sequences, indicative of the presence of Treg cells. We used this assay to measure Treg frequencies in the blood of patients who received tumor vaccines in combination with different potentially Treg depleting agents (6). None of the agents tested induced a significant decrease in Treg frequencies in a majority of patients. We attempted to use our assay to measure Treg frequencies directly inside tumor samples. But we observed that melanoma cells themselves could harbor demethylated *FOXP3* sequences, probably as a consequence of aberrant methylation patterns that frequently occur in human tumors. This observation precludes the use of *FOXP3* demethylation as a marker of Treg cells in tumors, unless tumor-infiltrating T cells are separated from tumor prior to analysis (7). More recently, we used our assay in collaboration with the group of Frédéric Baron (GIGA, University of Liège), to measure proportions of Tregs in clinical-grade preparations of human Tregs infused in NSG mice to prevent xenogeneic Graft versus Host Disease (GvHD). Based on the results obtained in this pre-clinical model (8), Fréderic Baron is now launching a trial to test the therapeutic efficacy of donor Treg cell infusions to patients suffering from chronic GvHD. We will measure proportions of Tregs in the donor cell preparations prior to infusions.

Functional analysis of our Treg clones revealed that a hallmark of stimulated human Tregs is to produce the active form of TGF-ß, a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF-ß.

Many cell types produce the latent, inactive form of TGF-ß. In latent TGF-ß, the mature TGF-ß protein (in green in Fig. 1) is

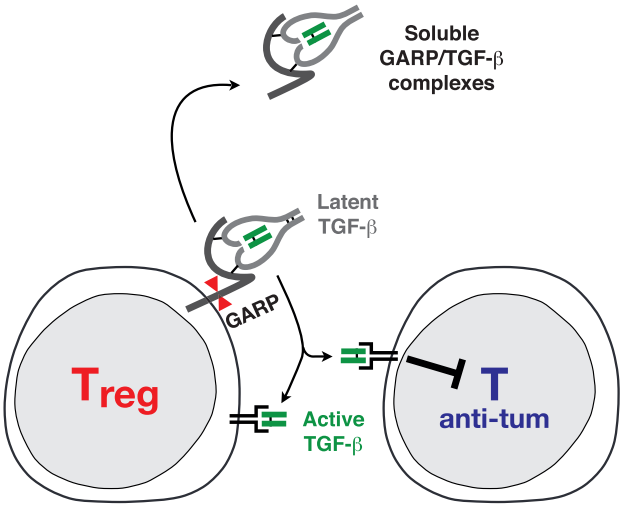

Fig. 1. Human Tregs produce active TGF-ß by a mechanism that appears to require GARP, and secrete soluble GARP/TGF-ß disulfide-linked complexes.

bound to the Latency Associated Peptide, or LAP (in grey in Fig. 1), and is thereby prevented from binding to the TGF-ß receptor. We recently showed that latent TGF-ß binds to GARP, a transmembrane protein which is present on the surface of stimulated Tregs but not on other T cells (9). We hypothesise that membrane localization of latent TGF-ß through binding to GARP is required for activation of the cytokine by Tregs. We are currently trying to obtain antibodies against GARP that inhibit active TGF-ß production by Tregs. We also observed that Tregs secrete a new form of soluble latent TGF-ß, in which it is disulfide-linked to GARP (Fig. 1). Secretion of soluble GARP/TGF-ß complexes, possibly through shedding form the cell surface, appears to be specific of the T cell lineage (10). We are currently trying to understand what function these new types of complexes could exert.

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Ludwig Institute for Cancer Research

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Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Many Ludwig investigators are leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.
Faithful to the organizing principles laid down by Mr. Ludwig, the Institute conducts its research through ten Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.
The biological properties of any given cancer cell constantly change, allowing tumors to spread and become more aggressive. To overcome these obstacles, the Ludwig Institute has developed a broad-based discovery program that seeks to understand the full complexity of cancer. Research is organized according to the four major programmatic themes that define the Institute: genetics, cell biology, cell signalling and immunology.

Branch staffs vary in size from 30 to over 90, and internationally the Institute employs some 800 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.
The Brussels Branch of the Institute was created in 1978. It is composed of 91 members and was headed by Thierry Boon until 2009. The Branch is now headed by Benoît Van den Eynde, the current Branch Director.

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Tumor immunology and antigen processing

Benoît Van den Eynde

Our group studies the immune responses to cancer, with the long-term goal of harnessing the immune system to fight cancer. We contributed to the molecular definition of human tumor antigens, which has opened a new era in cancer immunotherapy, recently showing its first clinical applications in the treatment of melanoma. Currently, we follow two lines of research. The first focuses on the processing of tumor antigens, studying the role of the proteasome and other proteases in the production of tumor antigenic peptides. The second studies innovative preclinical models for cancer immunotherapy to decipher the mechanisms whereby tumors resist immune rejection. The long-term objectives are to better understand the interaction of tumors with the immune system and devise strategies to improve the efficacy of cancer immunotherapy.

Processing of tumor antigens

Peptide splicing by the proteasome

N. Vigneron, V. Stroobant, A. Michaux

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They result from degradation of these proteins, mainly exerted by the proteasome. We have identified a new mode of production of antigenic peptides, which involves the splicing of peptide fragments by the proteasome (1). Peptide splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate (Figure 1). Splicing of peptide fragments can occur in the forward or reverse order to that in which they appear in the parental protein (2). We have currently described four spliced peptides, two of which are spliced in the reverse order (3). One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines into aspartic acids, through a process a N-glycosylation/deglycosylation (3). Both the standard proteasome and the immunoproteasome have the ability to splice peptides. However, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice. We are currently working on additional examples of spliced antigenic peptides.

Intermediate proteasome types

E. De Plaen, B. Guillaume, N. Vigneron, A. Michaux

The proteasome exists in two forms: the standard proteasome, which is constitutively present in most cells, and the immunoproteasome, which is constitutive in many immune cells and can be induced by interferon-gamma in most other cells. They differ by the three catalytic subunits they contain: ß1, ß2 and ß5 for the standard proteasome; ß1i, ß2i and ß5i for the immunoproteasome. We have described two new proteasome subtypes that are intermediate between the standard proteasome and the immunoproteasome (4). They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30 to 54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. They uniquely process several tumor antigens (4,5). We are currently studying the function of these intermediate proteasomes, not only in terms of processing of antigenic peptides, but also for other functional aspects in which the proteasome plays a crucial role, such as the regulation of the cell cycle, the activation of transcription factors and the regulation of inflammation and immune responses.

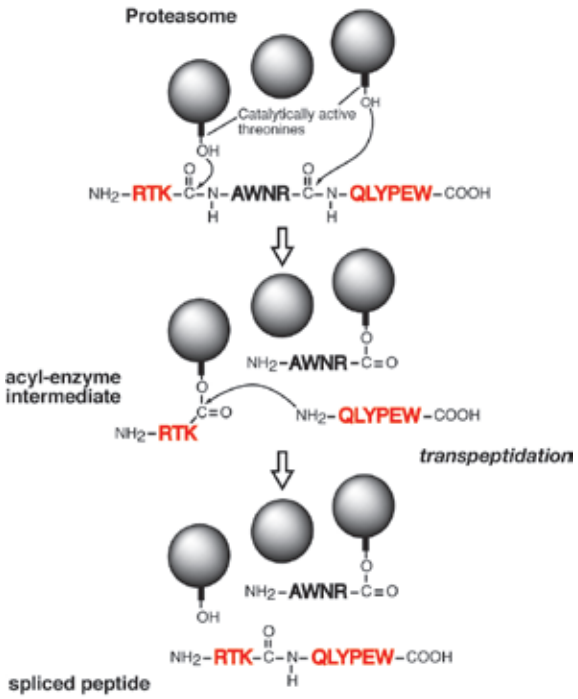

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme intermediate is formed, which is then liberated by hydrolysis. In the peptide-splicing reaction, a second peptide fragment appears to compete with water molecules for performing a nucleophilic attack on the acyl-enzyme intermediate, resulting in a transpeptidation reaction producing the spliced peptide. Experimental support for this model of reverse proteolysis includes evidence that the energy required to create the new peptide bond is recovered from the peptide bond that is cleaved at the amino-terminus of the excised fragment, and that the amino-terminus of the other fragment needs to be free for transpeptidation to occur.

Other proteases involved in antigen processing

N. Vigneron, A. Michaux, V. Stroobant, E. De Plaen

We are interested in characterizing the processing of human antigenic peptides that are not produced by the proteasome. We studied a proteasome-independent peptide derived from tumor protein MAGE-A3, and identified insulin-degrading enzyme as the protease producing this peptide (6). Insulin-degrading enzyme is a cytosolic metallopeptidase not previously known to play a role in the antigen processing pathway. The parental protein MAGE-A3 appears to be degraded along two parallel pathways involving insulin-degrading enzyme or the proteasome, each pathway producing a distinct set of antigenic peptides presented by MHC class I molecules. We are currently studying the processing of other proteasome-independent peptides and aiming to identify the protease(s) involved.

TAP-independent antigenic peptides

N. Vigneron, V. Stroobant, L. Pilotte

Presentation of most peptides depends on TAP, a dedicated transporter, which translocates peptides from the cytosol to the endoplasmic reticulum. A number of viruses and tumor cells tend to reduce their TAP expression to escape immune recognition. Therefore, there is great interest in the potential therapeutic use of peptides that are still presented in the absence of TAP. We are studying several such tumor peptides derived from cytosolic proteins. We aim at characterizing their processing and identifying the alternative transporter in charge of their transfer from the cytosol to the endoplasmic reticulum.

Cross-presentation

W. Ma, N. Vigneron

Cross-presentation is the pathway whereby endocytosed proteins can be presented on MHC class I molecules. This process, which is active in dendritic cells, has not been worked out in details. Several models have been proposed. One involves the transfer of the endocytosed antigen from the endosome to the cytosol, where it follows the classical MHC class I processing pathway involving the proteasome and TAP. Another model, called the vacuolar pathway involves processing of the antigen inside the endocytic compartment and binding of the peptides to MHC class I molecules recycled from the cell surface in recycling vesicles. We have set up a model system based on human dendritic cells to study *in vitro* cross-presentation of human tumor antigens.

Mechanisms of tumoral immune resistance

Indoleamine 2,3-dioxygenase

L. Pilotte, J. Lamy, M. Hennequart, E. De Plaen, V. Stroobant, D. Colau

We previously discovered that tumors often resist immune rejection by expressing Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme that is profoundly immunosuppressive (7). We showed that immune rejection was restored by administration of a pharmacological inhibitor of IDO. In collaboration with medicinal chemists in Namur and Lausanne, we identified several families of new IDO inhibitors that will be further optimized to develop drug candidates. We currently pursue functional studies on the mechanisms of IDO-induced immunosuppression, and on the signaling pathway responsible for IDO expression in tumors.

Tryptophan-dioxygenase

L. Pilotte, J. Lamy, E. De Plaen, V. Stroobant, F. Schramme, D. Colau

Besides IDO, we recently uncovered the role of tryptophan-dioxygenase (TDO) in tumoral immune resistance (8). TDO is an unrelated tryptophan-degrading enzyme, which is highly expressed in the liver to regulate systemic tryptophan levels. We found TDO to be expressed in a high proportion of human tumors. We showed that TDO-expressing mouse tumors are no longer rejected by immunized mice. Moreover, we developed a TDO inhibitor, which, upon systemic treatment, restored the ability of mice to reject tumors (8). These results describe a mechanism of tumoral immune resistance based on TDO expression and establish proof-of-concept for the use of TDO inhibitors in cancer therapy (9). In April 2012, we have launched an LICR spin-off company, iTeos Therapeutics, which will develop inhibitors of IDO and TDO.

Transgenic mice developing autochthonous melanomas expressing P1A

C. Powis de Tenbossche, S. Cané, F. Schramme, C. Uyttenhove, N. Arts

We have created a mouse model of autochthonous inducible melanoma expressing a defined tumor antigen (TIRP10B) (10). In this model, melanomas are induced (70% incidence) with tamoxifen, which, by activating CreER in melanocytes, induces the expression of Ha-Ras, the deletion of INK4a/ARF and the expression the tumor antigen encoded by cancer/germline gene P1A. A unique feature of this model is that melanomas first develop as non-aggressive highly pigmented tumors (Mela), which later dedifferentiate into unpigmented highly aggressive inflammatory tumors (Amela). The mechanisms that trigger this phenotype shift are unknown. Both tumors express the tumor antigen encoded by P1A. Mela tumors are ignored by the immune system, while Amela tumors are infiltrated by T lymphocytes that are rendered ineffective. This is accompanied with exacerbated systemic inflammation, involving disruption of secondary lymphoid organs, extramedullary hematopoiesis and accumulation of immature myeloid cells. Although more difficult to study than classical models based on transplantable tumors, we believe this model is more relevant to the human situation as it recapitulates the long-lasting tumor-host relationship that results in tumor tolerance. In addition, melanoma shows a similar biphasic evolution in humans, with dedifferentiated tumors becoming more aggressive. Therefore, we are currently studying in more details the mechanisms responsible for the phenotypic shift and for the immune suppression associated with the presence of inflammatory Amela tumors. In this model, inflammatory melanomas are associated with an enrichment in regulatory T cells and myeloid-derived suppressor cells (MDSC), with a TGFß and EMT-like signature, and with expression of arginase, pospho-STAT3 and COX2. We are combining vaccine approaches targeting tumor antigen P1A with strategies interfering with these immunosuppressive mechanisms.

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Regulation of T lymphocyte function in tumors

Pierre van der Bruggen

The identification of tumor-specific antigens recognized by T lymphocytes on human cancer cells has elicited numerous vaccination trials of cancer patients with defined tumor antigens. These treatments have induced T cell responses but have shown a low clinical efficacy in tumor-bearing melanoma patients. The analysis of the T cell responses of melanoma patients vaccinated against tumor antigens has led us to consider the possibility that the limiting factor for therapeutic success is not the intensity of the anti-vaccine response but the degree of anergy presented by intratumoral lymphocytes. We believe that progress depends on unraveling the different blockages for efficient tumor destruction. We aim at a better understanding of dysfunctions of the immune system in tumors and more precisely T lymphocyte dysfunctions.

Previous work in our group: Identification of tumor antigens recognized by T cells

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (2). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (3). A large set of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients. To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (4).

Human tumor antigens recognized by CD4+ or CD8+ T cells are being defined at a regular pace worldwide. Together with colleagues at the de Duve Institute, we read the new publications and incorporate the newly defined antigens in a database accessible at http://cancerimmunity.org/peptide/.

A mechanism causing anergy of CD8 and CD4 T lymphocytes

The identification of specific tumor antigens recognized by T lymphocytes on human cancer cells has elicited numerous clinical trials involving vaccination of tumor-bearing cancer patients with defined tumor antigens. These treatments have shown a low clinical efficacy. Among metastatic melanoma patients, about 5% show a complete or partial clinical response following vaccination, whereas an additional 10% show some evidence of tumor regression without clear clinical benefit. We believe that progress depends on unraveling the different blockages for efficient tumor destruction.

The tumors of the patients about to receive the vaccine, already contain T cells directed against tumor antigens. Presumably these T cells are exhausted and this impaired function is maintained by immunosuppressive factors present in the tumor. The T cell response observed in some vaccinated patients reinforce an hypothesis proposed by Thierry Boon and Pierre Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction (2, 5). Accordingly, the difference between the responding and the non-responding vaccinated patients is not the intensity of their direct T cell response to the vaccine but the intensity of the immunosuppression inside the tumor. It is therefore important to know which immunosuppressive mechanisms operate in human tumors.

Human tumor-infiltrating T lymphocytes show impaired IFN-γ secretion

Both human CD8 and CD4 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. TIL secrete low levels of INF-γ and other cytokines upon non-specific stimulation with anti-CD3 and anti-CD28 antibodies (6-9). TCR were observed to be distant from the co-receptors on the cell surface of TIL, either CD8 or CD4, whereas TCR and the co-receptors co-localized on blood T lymphocytes (Figure 1).

Reversing the anergy of tumor-infiltrating T lymphocytes with galectin ligands

We have attributed the decreased IFN-γ secretion to a reduced mobility of T cell receptors upon trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Indeed, we have shown that treatment of TIL with N-acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen chronically, and that the resulting activation of T cells could modify the expression of enzymes of the N-glycosylation pathway, as shown for murine T cells. The chronically activated TIL, compared to resting T cells, could thus express surface glycoproteins decorated with a set of glycans that are either more numerous or better ligands for galectin-3, as we have recently shown for CTL clones (10). Galectin-3 is an abundant lectin in many solid tumors and carcinomatous ascites, and can thus bind to surface glycoproteins of TIL and form lattices that would thereby reduce TCR mobility. This could explain the impaired function of TIL. The release of galectin-3 by soluble competitor ligands would restore TCR mobility and boost IFN-γ secretion by TIL. We recently strengthened this hypothesis by showing that both CD4 and CD8 TIL that were treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN- secretion compared to untreated cells.

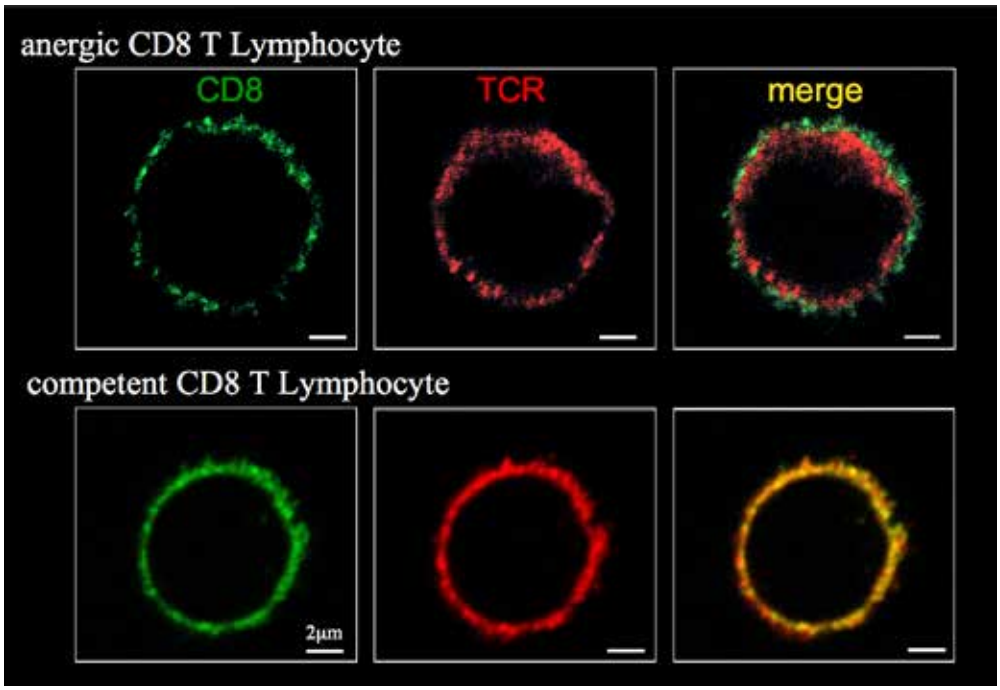

Fig. 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions

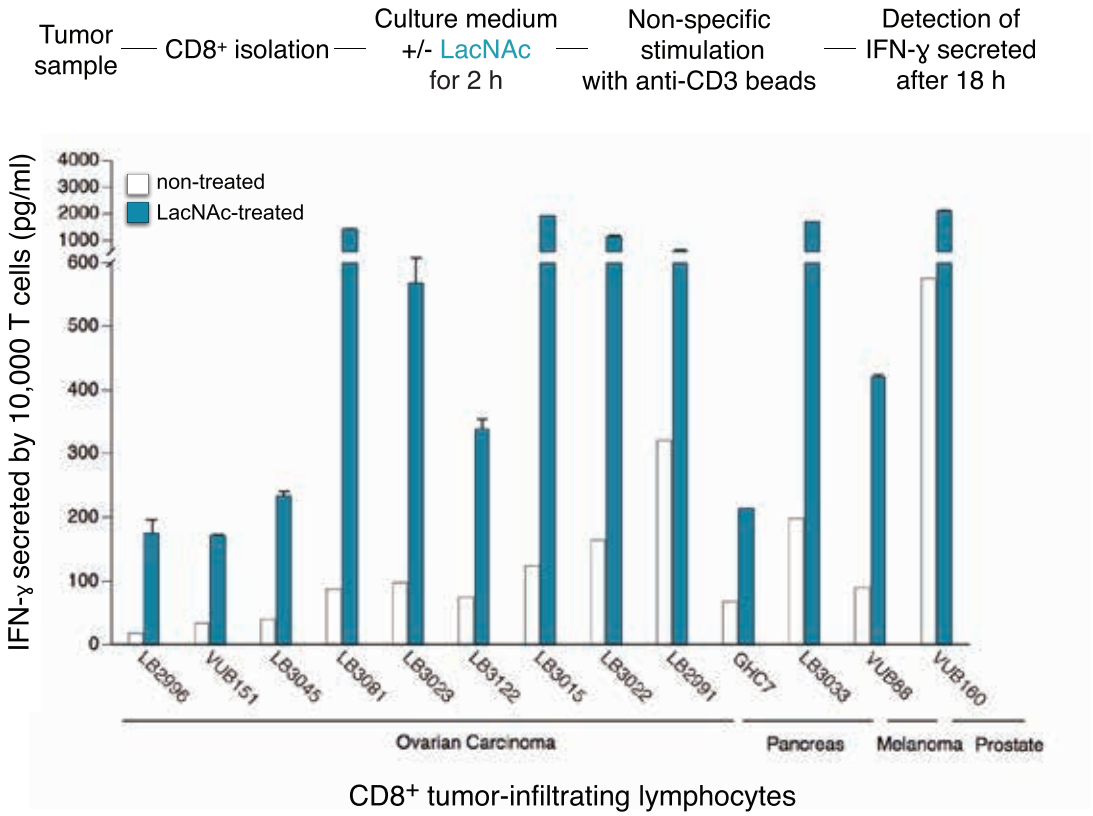

Fig. 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

Galectin competitor ligands, e.g. disaccharides LacNAc, are rapidly eliminated in urine, preventing their use *in vivo*. We recently found that a plant-derived polysaccharide, currently in clinical development, detached galectin-3 from TIL and boosted their IFN- secretion. Importantly, we observed that not only CD8+ TIL but also CD4+ TIL that were treated with this polysaccharide secreted more IFN- upon ex vivo re-stimulation. In tumor-bearing mice vaccinated with a tumor antigen, injections of this polysaccharide led to tumor rejection in half of the mice, whereas all control mice died. In non-vaccinated mice, the polysaccharide had no effect by itself. These results suggest that a combination of galectin-3 ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. Translation of these results to the clinic was unfortunately impossible because the company producing this polysaccharide got bankrupted. We recently identified another plant-derived polysaccharide that binds to galectins and was already used in combination with chemotherapy in phase II clinical trials in colorectal cancer patients. This compound was as effective as LacNAc in boosting the secretion of IFN- by treated TIL. A clinical trial with this new compound, in combination with anti-tumoral vaccination, was launched in 2012 in different clinical centers. We are currently trying to understand the very early activation events that are defective in TIL.

Is the spontaneous anti-tumor T cell response of breast carcinoma patients a clinical prognostic factor?

D. Godelaine and V. Ha Thi, in collaboration with Dr J. Carrasco (Grand Hôpital de Charleroi) and Dr J.P. Machiels (Cliniques Universitaires St-Luc)

Several retrospective studies suggest a correlation between the survival of patients with ovarian or colorectal carcinoma and infiltration of their tumors by immune cells. So far, prospective data validating these observations do not exist. We set out a prospective study aimed at looking for a correlation between the clinical outcome of patients with non-metastatic breast carcinoma and their spontaneous anti-tumor T cell response. Considering our experience in quantitative approaches to detect very weak T cell responses in the blood of melanoma patients, D. Godelaine set out to evaluate the frequencies of anti-tumor CD8 T lymphocytes in the blood of non-metastatic breast cancer patients prospectively recruited in several clinical centers. Blood samples are collected before and after surgery. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2- and A3-restricted HER2/neu, hTERT, MUC1 and NY-ESO-1 peptides, followed by detection of specific cells with HLA-peptide tetramers. Tumors removed at surgery are analyzed by immunohistochemistry for infiltration

by immune cells, and fragments are frozen for further genetic analysis of the T cell receptor repertoire. The prospective follow-up of 172 patients will extend over a 5-year-period. So far, 101 patients have been included . Blood frequencies of specific CD8 T lymphocytes before any oncological treatment were successfully estimated on 43 patients. For some of these patients, the same measure was repeated soon after tumor resection (n=26) and after 1 year of follow-up (n=35). Before and soon after tumor resection, 21% and 30% of the screened patients, respectively, have a CD8 T cell response against at least one of the targeted antigens. Frequencies were significantly higher than frequencies observed in healthy donors against the same antigen. This proportion falls to 6% in the same patients evaluated 1 year later, after surgical tumor resection and adjuvant oncological treatment. We hope to identify patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments.

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Immunotherapy analysis group

Thierry Boon

The identification in the early 1990's of human tumor-specific antigens that are recognized by T cells led to widespread attempts at vaccinating cancer patients with these antigens to induce tumor regression [1]. Vaccination of metastatic melanoma patients with MAGE peptides resulted in evidence of tumor regression in about 15% of the patients, with complete and partial clinical responses in only 7% of the patients [2]. Why did most patients fail to respond? A plausible hypothesis was that the anti-MAGE T cell response was too weak. However, none of the numerous attempts to boost the efficacy of the vaccines, for instance with adjuvants or by the use of dendritic cells, resulted in improvement of the clinical efficacy.

Our analysis of a few responding patients led us to a different hypothesis. Several groups reported a long time ago that human tumors contain tumor-infiltrating lymphocytes (TILs). These T lymphocytes could be extracted from the tumors and were capable of destroying tumor cells in vitro after short-term cultivation in the presence of IL-2. However, inside the tumor, they must have become inactive ("anergic") at one point, since the tumor is progressing. We made the paradoxical observation that, when vaccination causes complete tumor regression, the T lymphocytes directed against the vaccine antigen are present in the tumor in very small numbers, clearly insufficient to cause rejection. But they reactivate the "anergic" tumor infiltrating T lymphocytes that are present in the tumor in large numbers as a result of a past spontaneous immune response of the patient. It is these reactivated TILs which are capable of destroying the bulk of the tumor cells [3, 4]. Our new hypothesis is that what differentiates the non-regressing and the regressing patients is not their direct response to the vaccine but the severity of the anergy of their TILs.

Accordingly, our new strategy to improve anti-tumoral vaccination is to supplement it with a local treatment of the tumor with various cytokines and Toll receptor agonists effectors, as well as antibodies directed against inhibitory cytokines such as TGFß, to reduce the immunosuppression in the tumor. This should facilitate the action of the anti-vaccine T lymphocytes which provide the "spark" firing the regression response. This approach is proving to be effective in a mouse skin graft model. A small clinical trial involving tumor bearing melanoma patients is under way.

Inducing rejection of normally tolerated grafts in the H-Y mouse model

I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, T. Boon

Female CBA mice do not reject male skin grafts, even though they are able to mount a cytolytic T cell response against H-Y, a male specific minor histocompatibility antigen. To break this tolerance, we made repeated local injections of a low dose of IL-12, combined with IFN-α. This caused graft rejection in all the mice. Like IFN-α, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12. One finding of importance for the clinical application of this procedure is that several weekly cycles of cytokine treatments were necessary for complete rejection of the grafts.

We tested combinations of agents that are approved for clinical use. We observed that repeated local injections of a combination of low doses of IL-2 (300 ng), GM-CSF (300 ng) and IFNα (10^5 U) with TLR7 ligand gardiquimod (20 µg) caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. However, the same doses of IL-2 and IFN-α, without GM-CSF nor gardiquimod, were also effective when combined with both anti-TGF-β and anti-IL-10 antibodies, whereas anti-CTLA-4 antibody needed the additional presence of GM-CSF to show some effectiveness. IFN-γ could complement the local action of the IL-2 and gardiquimod combination as well as INF-α. Repeated injections combining IL-2, INF-α and anti-PDL1 antibody had little effect but this effect was improved by adding anti-CTLA-4 antibody and GM-CSF in the drug mixture. We will also determine whether slow continuous release and long-term delivery formulations of IL-2 and IFN-α combined with gardiquimod provide the rejection effect obtained with the free cytokines. This would make it possible to reduce the frequency of injection.

The observed rejections do not appear to be due to a non-specific local toxicity of the cytokines as female skin grafts were not rejected. Moreover, they seem to result principally from a local as opposed to a systemic effect of the cytokines since intraperitoneal injections of the same amounts of cytokines were ineffective.

Vaccinations with footpad injections of male lymphoblasts aimed at enhancing anti-H-Y T cell responses synergize effectively with the local cytokine treatment. We also observed that female CBA mice grafted with male skin not only failed to reject it without local treatment, but also invariably failed to reject male skin grafted later. In contrast, a majority of the mice which received a local treatment with either IL-12 and IFN-α or with IL-2, IFN-α and gardiquimod rejected such subsequent grafts, indicating some systemic increase of the anti-H-Y memory T cells in these mice.

On the basis of these results, we continue with a small clinical trial on melanoma patients presenting superficial lesions. They receive vaccinations with tumor antigens associated with a local treatment administered peritumorally and combining IL-2, GM-CSF, IFN-α and a TLR7 ligand.

Amine-reactive OVA multimers for auto-vaccination against cytokines and cancer metastasis associated proteins

C. Uyttenhove (in collaboration with R. Marillier and J. Van Snick)

Using our amine-reactive OVA multimers, as recently described [5], we have produced a series of monoclonal antibodies inhibiting many murine cytokines or proteins associated with cancer cell migration and metastasis.
We immunized mice against periostin/OSF-2, an extracellular matrix protein present in the stroma of many tumors, in mice and humans, that was recently implicated in metastasis development [6]. Several mAbs were produced that block periostin interaction with integrins αvß3 and αvß5, one of the mechanisms implicated in cancer cell migration and metastasis establishment. (in collaboration with P. Jat (UCL, London) and S. Fields (LICR Oxford). They are currently tested in the 4T1 murine mammary adenocarcinoma model. TGFß3 was described as an inducer of periostin expression [7]. In addition, elevated TGFß3 levels were detected in the serum of patients suffering from various types of carcinomas [8]. We recently produced inhibitory mAbs that are strictly specific for TGFß3 and that will be tested in the 4T1 model as well as in the inducible mouse model of melanoma developed in the lab [9].

As GM-CSF KO [10] mice were protected against EAE development, we tested two inhibitory anti-GM-CSF mAbs in the experimental autoimmune encephalomyelitis (EAE) model of B6 mice immunized against a peptide derived from the myelin oligodendrocyte glycoprotein (MOG). Both mAbs nearly completely blocked neurologic symptoms as well as weight loss, another hallmark of the disease. In the SJL model of EAE induced by a proteolipid protein (PLP) peptide immunization, anti-GM-CSF were injected at the time of EAE relapses. These were impressively decreased, whereas our potent MM17F3 anti-IL-17A mAb was totally inactive at that stage, even though it was completely protective against the first attack of the disease [11]. Cytokine and chemokine production by draining lymph nodes as well as cells infiltrating the central nervous system will be analyzed.

Another cytokine that we wanted to inhibit in vivo is IL-27, a heterodimeric protein composed of p28 and EBI3 and that is one of the first cytokines produced, mainly by dendritic cells, in response to TLR stimulation and T cell activation. Applying our OVA conjugation procedure, in collaboration with Stanislas Goriely (Institut d'Immunologie Médicale, Cam-

pus de Gosselies, ULB), we successfully developed a mAb (MM27.7B1) that inhibits both human and mouse IL-27. This Ab prevented the development of mouse graft versus host disease in a parent to F1 model. The mechanisms involved in this protection are currently investigated.

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Therapeutic vaccination and tumor expression profiling group

Nicolas van Baren

Cancer cells express tumor-specific antigens that can be targeted by cytolytic T lymphocytes (CTL). These antigens are small peptides derived from endogenous proteins presented at the surface of tumor cells by HLA molecules. In vitro, cytolytic T lymphocytes (CTL) lyse selectively tumor cell lines that express their cognate antigen. Our group has developed small scale clinical immunotherapy trials in which patients with advanced cancer, often metastatic melanoma, have been treated repeatedly with a vaccine containing one or several defined tumor antigens that are expressed by their tumor. Different immunization modalities, such as vaccination with peptides like MAGE-3. A1 and NA17.A2, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with an ALVAC recombinant viral vector, have already been tested in the clinic. They are all devoid of severe toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) showed regression of metastatic lesions. This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. The most likely explanations for the poor effectiveness of cancer vaccines are the weak immunogenicity of the vaccines tested up to now, and the resistance against immune rejection that tumors presumably select during their progression under the pressure of spontaneous immune responses.

We are following two different approaches to try to improve these results: find more immunogenic vaccines, and combine vaccines with treatments that modify the tumor environment in favor of effective tumor rejection. In addition, our group studies the immune and inflammatory events that occur in melanoma metastases, in order to identify mechanisms of tumor resistance to immune rejection.

A. Clinical trials with cancer vaccines

Vaccination of melanoma patients with CyaA-Tyr

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St-Luc), Cl. Leclerc (Institut Pasteur, Paris France), P. Coulie, and B. Van den Eynde

In an ongoing phase 1/2 clinical trial (EudraCT 2009-014651-77), we are testing the safety, immunogenicity and anti-tumoral effect of a new promising vaccine called CyaA-Tyr or Theravac, developed at Institut Pasteur. CyaA-Tyr is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DC) in vivo, and force them to express a Tyrosinase.A2 antigen, a peptide derived from the melanocyte and melanoma-specific tyrosinase protein. This vaccine is derived from CyaA, a bacterial toxin that binds specifically to CD11b, an adhesion molecule expressed by dendritic cells and macrophages. Upon binding, a portion of the toxin is internalized and neutralizes its target cell, in order to turn off innate immunity at the in-

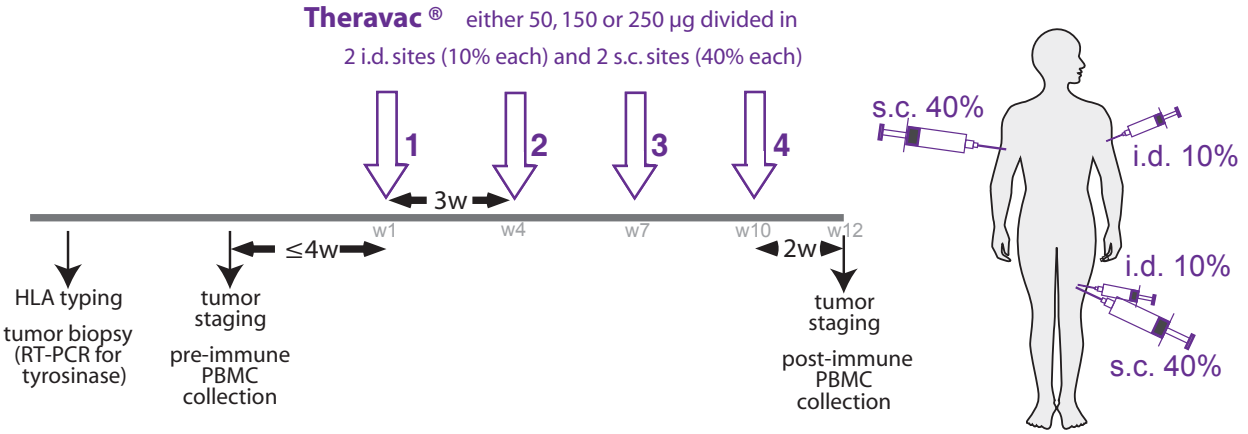

Fig. 1. Clinical trial : vaccination of melanoma patients with CyaA-Tyr

fectious site. In the recombinant vaccine protein, the toxin activity has been inactivated by insertional mutagenesis, and coupled to the Tyrosinase.A2 peptide. Thus, the unique advantage of this vaccine is its ability to target dendritic cells in vivo, with an expected higher immunogenicity as a consequence. Preclinical experiments have shown that CyaA-Tyr has a very potent capacity to activate Tyrosinase.A2-specific CTL. In our clinical trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of CyaA-Tyr, at increasing doses (Fig.1). If successful, this new vaccine modality could have a much broader application than melanoma vaccines.

Vaccination of melanoma patients with peptides associated with immunomodulation of the tumor environment

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St-Luc), P. Coulie and T. Boon.

In this phase 1/2 trial, patients with advanced melanoma comprising superficial lesions receive repeated ID and SC vaccinations with the MAGE-3.A1 or the NA17.A2 peptide, matching the antigens expressed by their tumor. Each of these peptides was previously tested in clinical vaccine trials, and was shown to be well tolerated and associated with tumor regression in some patients. In addition, a cocktail of immunostimulatory cytokines (IL-2, IFN-a and GM-CSF) at low doses is injected repeatedly in the periphery of a superficial tumor, which is also treated by ointment with Aldara® cream (imiquimod, a TLR7/8 ligand) (Fig. 2). It is hoped that this treatment will both induce

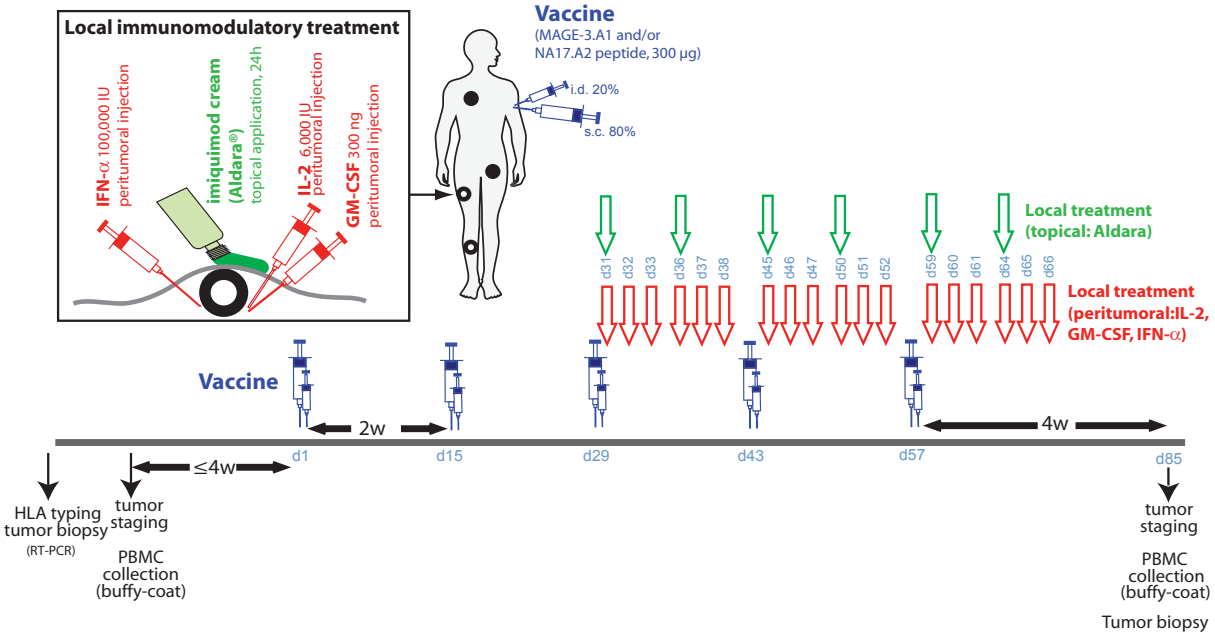

Fig. 2. Clinical trial : vaccination of melanoma patients with peptides associated with immunomodulation of the tumor environment

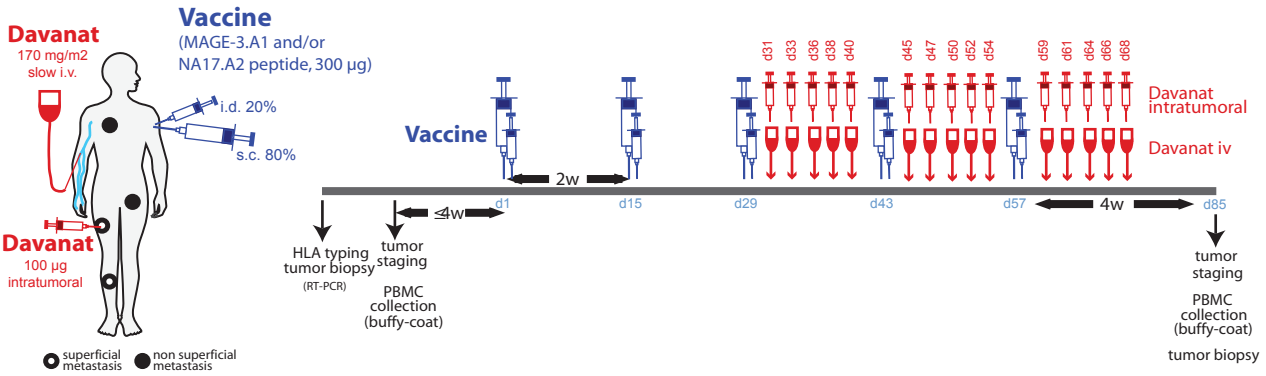

Fig. 3. Clinical trial: vaccination in melanoma patients with peptides associated with a galectin-3 inhibitor

new anti-tumoral CTL and modify the tumor environment in favor of immune rejection. The treatment was previously defined and optimized in a mouse model of skin graft rejection (EudraCT 2010-020435-40).

Vaccination of melanoma patients with peptides associated with a galectin-3 inhibitor

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie and P. van der Bruggen.

Recent work in the laboratory has shown that the state of anergy that characterizes tumor-associated T cells can be reversed pharmacologically (see the contribution of P. van der Bruggen in this report). In a phase 1/2 clinical trial (EudraCT 2010-018638-29), melanoma patients receive the same peptide vaccine as in the previous study, in association with repeated infusions of an experimental drug called GM-CT-01, a plant-extracted oligosaccharide that binds to and inhibits galectins (Fig.3). Galectin-3 is a protein produced by cancer cells that is able to inhibit T cell activation. The group of Pierre van der Bruggen has shown that the anergy that characterizes tumor-associated T cells can be reversed with galectin inhibitors including GM-CT-01. We hope that this combined treatment will favor a synergistic interaction between new anti-tumoral CTL responses induced by the vaccine and the inhibition of tumor resistance by the galectin inhibitor.

B. Study of the inflammatory environment in melanoma metastases

In collaboration with the groups of P. Coulie (Cellular Genetics Unit, de Duve Institute)

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients. This approach is combined with systematic immunohistological or immunofluorescence analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells,

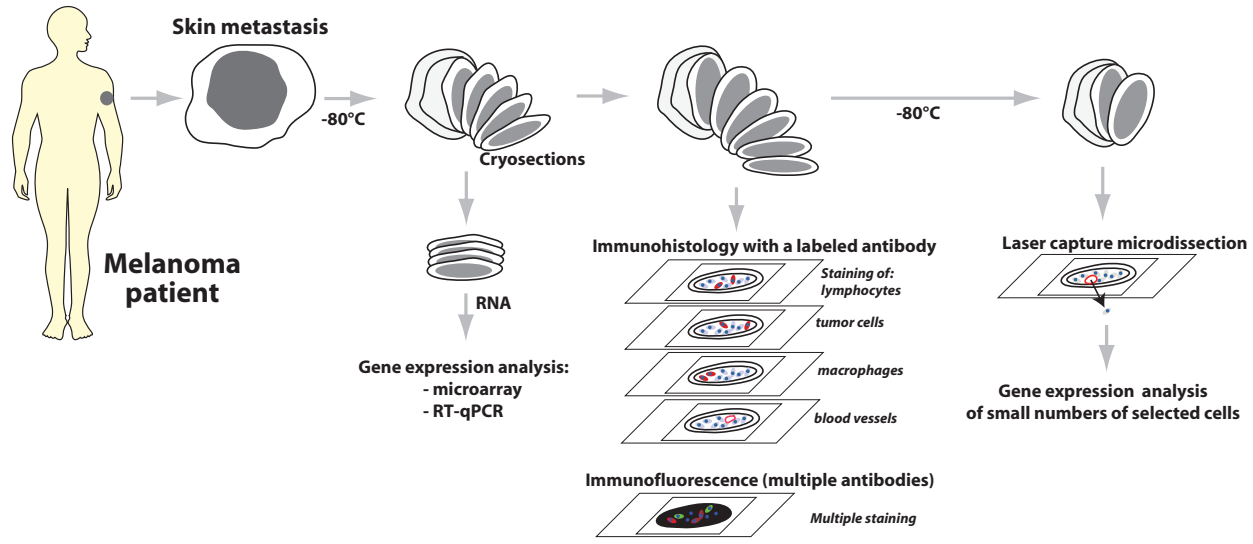

Fig. 4. Processing of tumor samples.

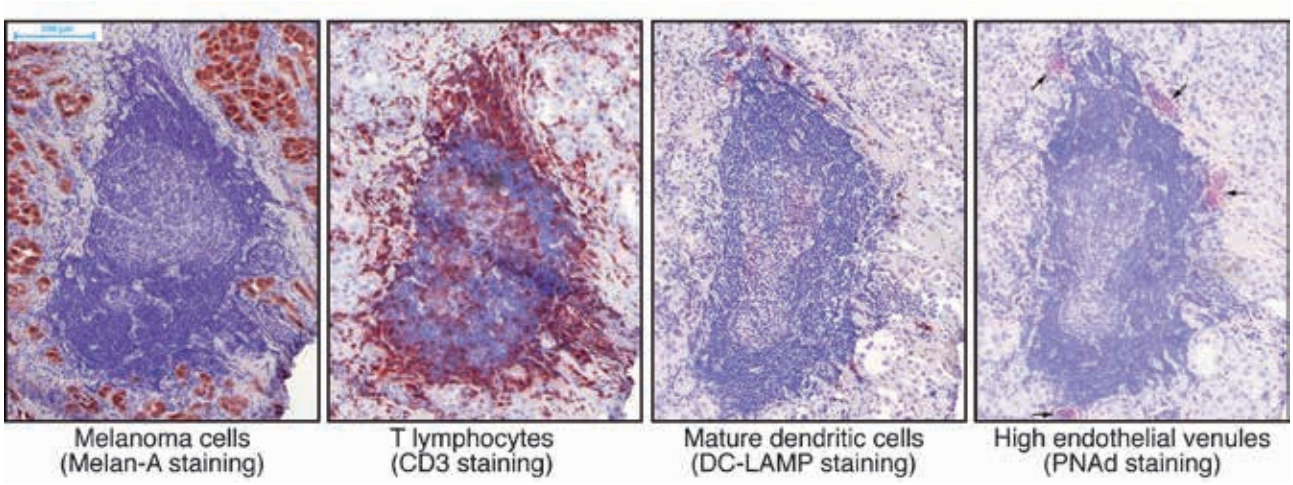

Fig. 5. An ectopic lymphoid structure in a melanoma metastasis.

macrophages, blood vessels, and various molecules involved in inflammatory reactions. In addition, adjacent cryosections are analyzed by performing laser capture microdissection of selected areas, e.g. T cell rich areas, followed by RT-qPCR analysis of T cell, macrophage, melanoma cell and inflammation-associated genes (Fig.4). These complementary approaches help us to characterize the inflammatory events that take place inside the metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site.

In this project, we have also observed the presence of ectopic lymphoid structures, also called tertiary lymphoid organs, in melanoma metastases. These structures are organized in B cell follicles, adjacent T cell areas and neighbouring high endothelial venules, and thus contain the main components required to support local adaptive B and T cell responses. The presence of germinal centers and the occurrence of immunoglobulin affinity maturation in some follicles reveals ongoing B cell responses. The intimate association of mature dendritic cells and T lymphocytes in the T cell areas suggests that T cell responses also take place inside these structures (Fig. 5). Altogether, these findings indicate that adaptive immune responses develop within the tumor environment in some melanoma metastases. At this stage, we do not know whether these responses are directed at melanoma antigens.
This phenomenon, called lymphoid neogenesis, is frequently observed in many chronic inflammatory diseases. It has also been described in several types of tumors, including breast, lung and testis cancer. It is a consequence of sustained lymphocyte activation in the presence of persistent antigenic stimuli. Therefore, our observations suggest that the melanoma environment is the site of immune activation rather than immune suppression as is frequently proposed.

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Cytokines in immunity and inflammation

Jean-Christophe Renauld
Laure Dumoutier

The cytokine group studies the biological activities of cytokines in inflammatory and tumoral processes, as well as the molecular mechanisms underlying these activities. Our work focuses on Interleukin-9 (IL-9) and IL-22, two cytokines discovered in our laboratory. IL-9 is produced by a particular T lymphocyte population, called TH9, and plays a role in immune responses against intestinal parasites and asthma. Dysregulation of IL-9 signalling is also implicated in tumoral transformation and this process has been studied in an in vitro tumorigenesis model, leading to the identification of oncogenic mutations of the JAK1 gene. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents and antibacterial proteins in the liver, the lung and intestinal mucosae, and in the skin. IL-22 appears to play a key role in wound healing and skin inflammation processes such as psoriasis. The role of these cytokines in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors, and by using an original strategy of anti-cytokine vaccination.

Interleukin 9

Interleukin-9 (IL-9) was discovered in our group, through its ability to sustain antigen-independent growth of certain murine T helper clones. Although IL-9 did not turn out to be a T cell growth factor for freshly isolated T cells, it was found particularly potent on T cell lymphomas, as an anti-apoptotic agent. To determine the biological activities of this factor, we generated transgenic mice overexpressing this cytokine. Analysis of these animals disclosed two essential properties of IL-9: its activity on mast cells and eosinophils with consecutive implications in asthma, and its tumorigenic potential in T lymphocytes.

IL-9-transgenic mice : parasite infections and asthma

Although IL-9 overproduction is viable and IL-9 transgenic mice did not show any major abnormality at the first look, they were found to harbor increased numbers of mast cells in the intestinal and respiratory epithelia, and were also characterized by a general hypereosinophilia. This phenotypic characteristic was found to increase the capacity of these animals to expel nematodes like Trichinella spiralis or Trichuris muris, suggesting that IL-9 administration could protect susceptible hosts against these parasites. Conversely, blocking IL-9 activity resulted in a failure to expel T. muris parasites and in decreased eosinophilic responses against the parasite. The other side of the coin was the discovery that IL-9 overexpression, such as that characterizing the IL-9 transgenic animals, resulted in bronchial hyperresponsiveness upon exposure to various allergens. Our observations showed that IL-9 promotes asthma through both IL-13-dependent and IL-13-independent pathways (1), as illustrated in figure 1. The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma. In addition, we found that asthma patients produce increased amounts of IL-9.

IL-9-transgenic mice : T cell lymphomas

IL-9 transgenic animals showed normal T cell development and T cell numbers but spontaneously developed thymic lymphomas at low frequency (5%). Two lines of evidence indicate that IL-9 is not a conventional oncogene but rather favors tumor development in response to exogenous stimuli. First, the tumor incidence was significantly lower when mice were maintained under pathogen-free conditions. Secondly, all IL-9

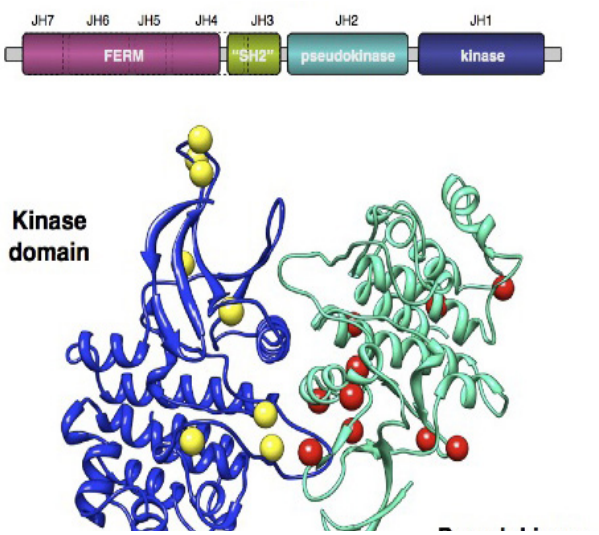

Fig. 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation of eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production by T cells mediates IL-9 activities on lung epithelial cells, including mucus production and secretion of eotaxin, which is required to recruit eosinophils into the lungs (1).

transgenic mice developed T cell lymphomas when exposed to subliminal doses of a chemical carcinogen or to irradiation, that were innocuous in wild type mice. The anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells generated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9 receptor and signal transduction

Analysis of the mode of action of IL-9 at the molecular level was initiated by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs. By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro. The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the activation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway (2).

Role of JAK1 overexpression and mutations in tumor cell transformation

Constitutive activation of the JAK-STAT pathway is frequent in cancer and contributes to oncogenesis. Our observations indicate that JAK overexpression plays a role in such processes. Using a murine proB cell line that strictly depends on IL-3 for growth in vitro, cytokine-independent and tumorigenic clones were derived from a two-step selection process. Cells transfected with a defective IL-9 receptor acquired IL-9 responsiveness during a first step of selection, and progressed after a second selection step to autonomously growing tumorigenic cells. Microarray analysis pointed to JAK1 overexpression as a key genetic event in this transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but most importantly allowed a second selection step towards cytokine-independent growth with constitutive STAT activation. This progression was dependent on a functional FERM and kinase JAK1 domain. Similar results were observed after JAK2, JAK3 and TYK2 overexpression. All autonomous cell lines showed an activation of STAT5, ERK1-2 and AKT. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway (3). Recently, we elucidated the mechanism responsible for the second step of this tumoral transformation process, as we found that the majority of the cytokine-independent tumorigenic clones acquired an activating mutation in the kinase or in the pseudokinase domain of JAK1 illustrated in figure 2 (4).

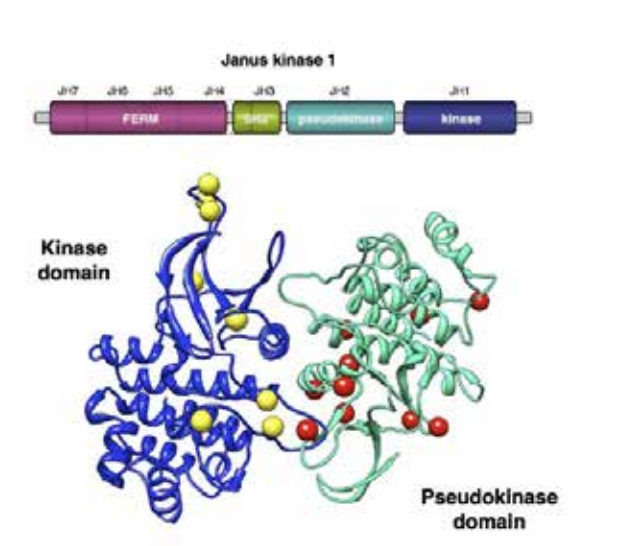

Fig. 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

In parallel to these observations, in collaboration with Prof. Marco Tartaglia (University of Rome), we identified activating mutations in JAK1 in 20% of T-cell acute lymphoblastic leukemia (T-ALL) and in 3% of B-ALL patients, confirming the relevance of our in vitro model-derived JAK1 mutations for human malignancies. Further analysis of human ALL samples showed that JAK1-mutated leukemias share a type I IFN transcriptional signature, suggesting that these mutants do not only activate growth-promoting pathways, but also antiviral pathways. Expression of these activating JAK1 mutants in murine hematopoietic cell lines recapitulated this signature in the absence of IFN, but also strongly potentiated the in vitro response to IFN. Finally, we also showed in an in vivo leukemia model that cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with JAK1 activating mutations (5).

IL-TIF/IL-22 : a new cytokine structurally related to IL-10

Searching for genes specifically regulated by IL-9 in lymphomas, we identified a new gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver and protection against experimental hepatitis and colitis. Among the different T cell subsets, IL-22 was found to be preferentially produced by TH17 cells, which are associated with several autoimmune and inflammatory processes, and the aryl hydrocarbon receptor AhR turned out to be a major regulator of the expression of this cytokine (6). We assessed the role of IL-22 in a mouse model where psoriasiform skin inflammation is triggered by topical application of the TLR7/8 agonist imiquimod (7). At the macroscopic level, scaly skin lesions induced by daily applications of imiquimod in wild-type mice were almost to- tally absent in IL-22–deficient mice or in mice treated with a blocking anti–IL-22 Ab. At the microscopic level, IL-22–deficient mice showed a dramatic decrease in the development of pustules and neutrophil infiltration and a partial decrease in acanthosis. At the molecular level, the absence or inhibition of IL-22 strongly decreased the expression of chemotactic factors such as CCL3 and CXCL3 and of biomarkers such as S100A8, S100A7, and keratin 14, which reflect the antimicrobial and hyperproliferative responses of keratinocytes. Contrasting with this proinflammatory effect of IL-22 in skin inflammation, asthma models showed that IL-22 can have a protective anti-inflammatory activity in lungs. This protective effect of IL-22 has been attributed to an inhibition of IL-13 activity on lung epithelial cells either for CCL17/TARC induction or for IL-25 production. Inhibiting IL-22 in vivo, through antibody treatment or by gene targeting, increased expression of these inflammatory mediators, infiltration by eosinophils and broncho-hyperrersponsiveness.

Both in the psoriasis and asthma models have challenged the dogma that IL-22 is mainly produced by TH17 lymphocytes, and γδ T cells as well as innate lymphoid cells turned out the be the major producers of this cytokine. We characterized the cells responsible for IL-22 production in response to TLR agonists such as LPS or flagelin (8). We identified a new innate lymphoid spleen cell population expressing CD25, CCR6 and IL-7R representing 1% of spleen cells from recombination activating gene (Rag2)-deficient mice. This population comprises 60–70% CD4+ cells, which produce IL-22, and are still present in common γ chain-deficient mice; the CD4- subset coexpresses IL-22 and IL-17, and is common γ chain-dependent. These cells share a transcriptional program with NKp46+ RORγt+ cells found in intestinal mucosae and involved in antibacterial responses. The importance of IL-22 production for the LPS-triggered response is highlighted by the fact that IL-22-deficient mice are more resistant to LPS-induced mortality, pointing to the pro-inflammatory activity of this cytokine.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells. All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with JAK1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected feature of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (9).

Beside this cell membrane IL-22 receptor complex composed of IL-22R and IL-10Rß, we identified a protein of 231 amino acid, showing 33 % amino acid identity with the extracellular domains of IL-22R, respectively, but without any cytoplasmic or transmembrane domain. This soluble receptor has been named IL-22 binding protein (IL-22BP), because it binds IL-22 and blocks its activities in vitro, demonstrating that this protein can act as an IL-22 antagonist.

The crystal structure of IL-22, alone and bound to its cellular receptor IL-22R or to its soluble receptor IL-22BP has been characterized in collaboration with Prof. Igor Polikarpov (University of Sao Paulo) and is illustrated in figure 3.

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex

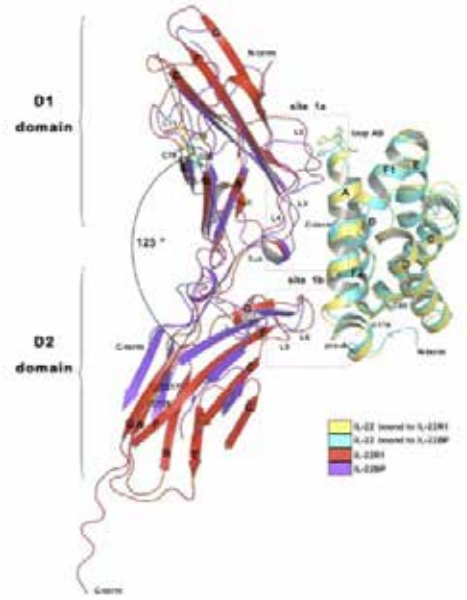

Fig. 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

by associating with IL-20Rß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22. In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß (see ref. 10 for a review of this cytokine family).

Anti-cytokine vaccination

Beside conventional gene targeting strategies, that were used in our lab to generate mice deficient in the IL-9R, in IL-22 or in IL-22R, we developed a new strategy of anti-cytokine vaccination leading to the production in vaccinated mice of anti-cytokine autoantibody that block the biological activities of endogenous cytokines. Neutralizing auto-antibodies against cytokines such as IL-9, IL-12 and IL-17 have been induced upon vaccination with the autologous cytokines chemically coupled with OVA (IL-9, IL-17) or with the Pan DR T helper epitope PADRE (IL-12). This strategy contributed to demonstrate the role of IL-9 in an intestinal helminth infection, of IL-12 in atherosclerosis and of IL-17 in experimental autoimmune encephalomyelitis. More recently, we developed a new procedure of anti-cytokine vaccination by taking advantage of tumor cells as a vaccine against peptides presented at their surface in fusion with a human transmembrane protein. These vaccination methods represent simple and convenient approaches to knock down the in vivo activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

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Signal transduction and molecular hematology group
Structure and function of cytokine receptors

Stefan Constantinescu

Blood formation and the functions of the immune system depend on cytokines and their specific receptors. The latter are transmembrane proteins that often form dimeric or oligomeric complexes and are coupled to one or several cytosolic tyrosine kinases belonging to the Janus kinase (JAK) family. The human genome codes for more than 25 cytokine receptors, four JAKs and seven Signal Transducer and Activator of Transcription (STATs) proteins that shuttle to the nucleus and regulate gene expression.

Our broad interest is the understanding of the mechanisms that control assembly in the membrane of cell surface receptors that respond to extracellular cues. We study in detail the structure, function and orientation of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo) and Granulocyte Colony Stimulating Factor (G-CSF), which function as homomeric complexes. We aim to identify: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; ii) what are the general rules by which hydrophobic transmembrane regions interact in the membrane in a sequence-specific manner; and iii) the mechanisms of JAK attachment to receptors, and their subsequent activation, especially the role of pseudokinase domains in JAK kinase domain activation.

The laboratory identified constitutively active oncogenic mutants of JAK2 (V617F), JAK1 (V658F) and TYK2 (V658F) and of erythropoietin and thrombopoietin receptors, with some being involved in human blood cancers. Specifically, the mechanisms by which JAK2 V617F and TpoR W515 mutants induce human Myeloproliferative Neoplasms (MPNs) and the role constitutive STAT5 activation play in these diseases are actively pursued directions. Close interactions with clinicians and clinical biologists at St Luc Hospital allow the in-depth study of patient-derived cells.

The mechanisms of JAK2 V617F activation in human myeloproliferative neoplasms

E. Leroy, V. Gryshkova, J.-P. Defour, M. Swinarska, D. Colau

Polycythemia Vera (PV), or the Vaquez-Osler disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Two other related diseases, Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are associated with excessive platelet, granulocyte production and fibrosis (scaring) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation (Figure 1A) in collaboration with William Vainchenker at the Institut Gustave Roussy in Paris (1, 2). JAK2 V617F is responsible for >98% of PV and for >50% of ET and PMF cases (2). The mutation in the pseudokinase domain (JH2, JAK homology 2 domain) activates the kinase domain and constitutive signaling (2, 3) in complexes between JAK2 V617F and cytokine receptors such as EpoR, TpoR and G-

CSFR (Figure 2). The homologous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors. JAK1 mutations have been described in adult acute T-lymphoblastic leukemia.

In addition to V617F we have described several other activating mutations on JAK2, V617I, L, M and W and modeled how such bulky residues could trigger the activation of JAK2 kinase domain (3). Interestingly, V617W is as strong as V617F, but requires a change in three base pairs to occur. In contrast, V617I is much weaker in inducing signaling than V617F/W. The V617I mutation can be obtained by one base pair change (3), has been detected in rare cases of myelofibrosis, and recently was identified in a family with hereditary thrombocytosis (4). JAK2 V617I promotes mainly STAT1 activation via TpoR, and very little EpoR activation, which may explain the limited thrombocytosis phenotype of this family (4). Primary cells from carriers of this heterozygous (germ line) mutation are being studied for signaling and function in the laboratory.

We aim to understand precisely how a pseudokinase domain mutation can induce kinase domain activation in a JAK. Besides the fundamental aspect, understanding of the mechanism of JAK activation via pseudokinase domain mutation would permit isolation of small molecule specific inhibitors of mutated JAK2 in myeloproliferative neoplasm patients, but not the wild type JAK2 (1), which is crucial for red blood cell and platelet formation. We identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (5). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinase domain might be a target for specific JAK2 V617F inhibition (Figure 1B and C). A recent X-ray crystal structure from the Hubbard and Silvennoinen laboratories has confirmed the major role of F595 in the conformation induced by V617F on helix C of JH2, with a complex three ring stacking interaction (F617-F595-F594) and prolongation by one turn of the helix C of JH2 (Figure 1C). Expression of segments of JAKs and cytokine receptors is pursued in insect and bacterial cells by D. Colau and M. Swinarska. Protein fragment complementation and ligation assays are used to determine the proximity and oligomerizationn state of receptors and JAKs.

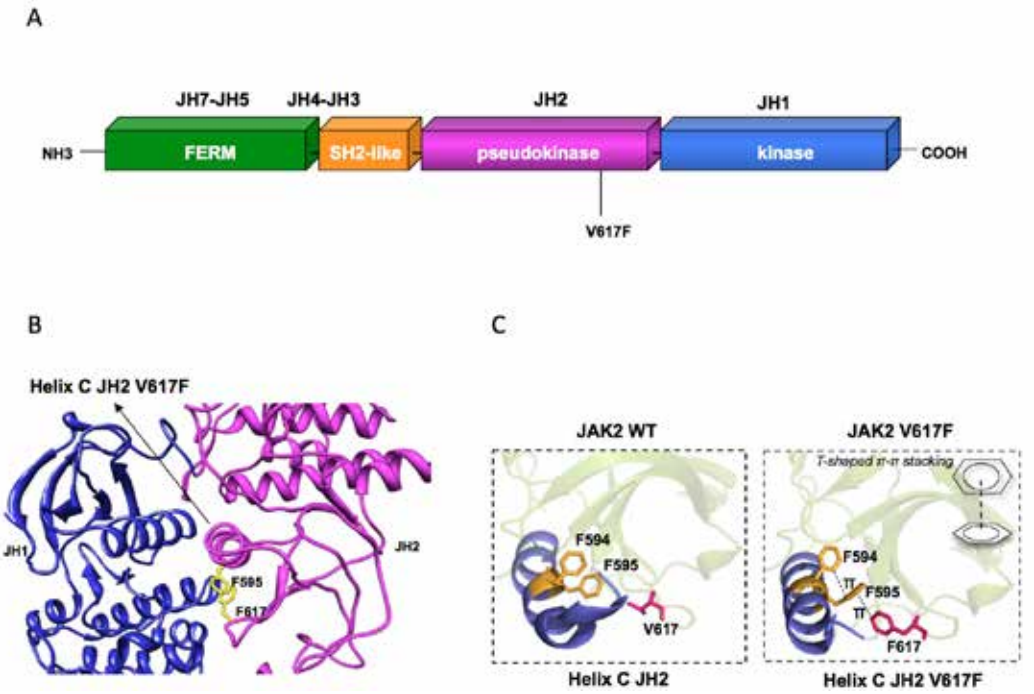

Fig. 1. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pesudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients.

(B) The pseudokinase (JH2) and kinase (JH1) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other and leading to JH1 inhibition. Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 (red arrow) and eventually in activating the kinase activity of JH1. The region around V617F and the middle of JH2 helix C surrounding F595 could become the target of inhibitors that might specifically decrease constitutive activation of JAK mutants.

(C) The X-ray crystal structures of JH2 and JH2 V617F solved by the Hubbard laboratory showed that the wild type helix C is short and contains a kink while in JH2 V617F an aromatic stacking interaction occurs between F617 and helix C F595 and F594, which stabilizes and prolonges helix C. This conformational change associated with activation of JAK2 JH1 suggests that interruption of the aromatic stacking by small molecules would inhibit JAK2 V617F activity, but would not inhibit the function of wild type JAK2.

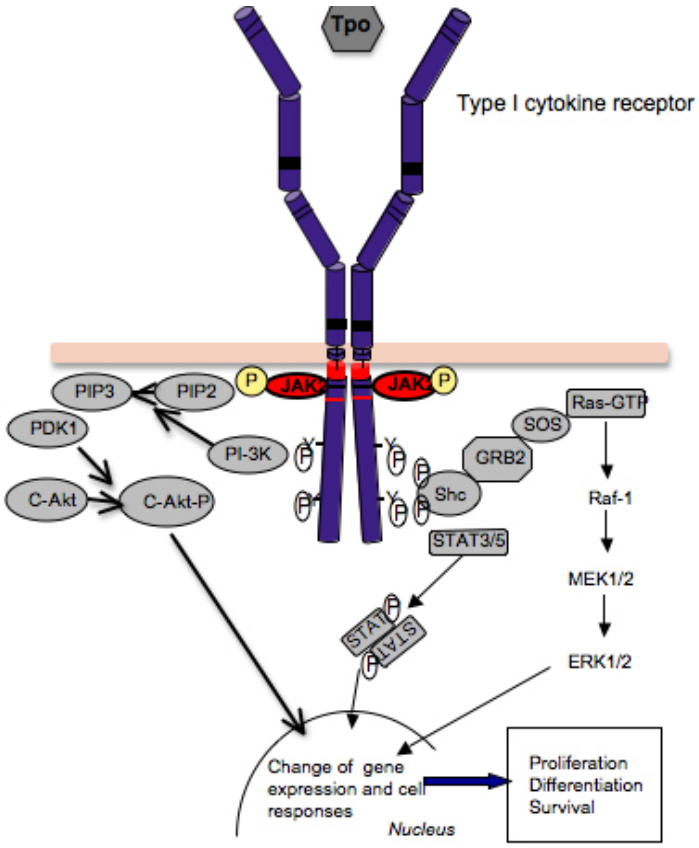

Fig. 2. Signaling by cytokine receptors. The thrombopoeitin receptor signals as a homodimer that activates JAK2, which is bound to the cytosolic juxtamembrane domain. Activation of JAK2 leads to phosphorylation of tyrosine residues in the TpoR cytosolic domain, which recruit several signaling proteins like STAT5 and STAT3, which become substrates of JAK2, and upon tyrosine phosphorylation they dimerize, detach from the receptor and migrate into the nucleus, where they regulate gene expression. Cytokine receptors also activate, via JAK2, other pathways, such as the phosphatydylinositol-3'-kinase (PI-3'K)/akt/mTOR and ras-MAP-kinase. The integration of these signals in the nucleus, at the level of gene expression, induces survival, proliferation and differentiation of myeloid progenitors.

Involvement of pathologic TpoR signaling in myeloproliferative neoplasms

C. Pecquet, J.-P. Defour, I. Chachoua, E. Leroy

Thrombopoietin (Tpo) is a cytokine produced by the liver that is critical for regulation of the formation of platelet cells. Tpo also regulates the numbers of hematopoietic stem cells and other myeloid cells.

TpoR appears to be central to MPNs. First, observations of Jerry Spivak from Johns Hopkins University indicated that most MPN patients strongly down-modulate TpoR levels in megakaryocytes and platelets. Second, mutations in the TpoR intracellular juxtamembrane motif W515 were shown by our group to lead to constitutive activation of the receptor, and by several groups to induce severe in vivo MPN with myelofibrosis. Third, asparagine mutations, which induce dimerization of the transmembrane domain of TpoR also activate TpoR and one such mutation has been shown to be associated with familial ET. Fourth, alterations of TpoR traffic to the cell surface can induce thrombocytosis due to insufficient clearance of Tpo and high sensitivity of early megakaryocytes to high Tpo.

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (6). We discovered that Tpo could induce a strong antiproliferative effect in cells that express high JAK2 levels (6). This effect can be detected in postmitotic megakaryocytes (Plos Biol 2010, 8, e1000476). We showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F cells, and is partially responsible for TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal megakaryocytes.

We employ a combination of Phospho-Scan (7) and Ubi-Scan approaches coupled to mass spectrometry in order to determine modifications in the profile of tyrosine phosphorylation and ubiquitination induced by expression of JAK2 V617F and TpoR W515 mutants.

A novel mechanism by which a tryptophan residue regulates TpoR activation

J.-P. Defour, V. Gryshkova, I. Chachoua, C. Pecquet

Several years ago we discovered that TpoR contained a unique motif (RWQFP) at the junction between the transmembrane and cytosolic domains. By deletion or mutation of individual residues in this motif, Judith Staerk determined that this amphipathic insert actually prevented self-activation of the receptor and identified W515 in the motif as the key residue for this inhibitory function (Blood 2006, 107(5), 1864-71). The question remained why would one W residue be so important in keeping a receptor inactive, and how could ligand addition defeat this inhibition. The question became even more relevant when several groups and ours detected TpoR W515L/K/A/R mutations in 5-8% of MPN patients that did not harbor JAK2 V617F. The answer came from two approaches. First, mutagenesis of W515 to all other residues showed the unique role played by this residue, in that even Y or F could not replace it (8). In W515L/A/K mutants, addition of W before or after the mutation could prevent activation. Second, biophysical experiments (analytical ultracentrifugation, solid-state NMR, infrared spectroscopy) performed by our collaborator, Prof. Steve O. Smith at SUNY Stony Brook, and fluorescence complementation studies performed in our laboratory by Vitalina Gryshkova showed that W515 actually regulates the orientation, tilt and dimerization of the upstream transmembrane helix, and prevents receptor activation (8). Taken apart, the transmembrane sequence of TpoR can dimerize, but this was not the case when the RWQFP motif was added or when W515 in the insert was mutated to K (8). Our data indicated that a W residue placed at the transmembrane-cytosolic junction could tilt the receptors so that the dimeric interface between transmembrane sequences can no longer be formed. Given that W residues were shown to promote transmembrane helix associations when present in the middle or on the extracellular side of membrane sequences, and given the many proteins that possess W residues at the cytosolic side of the transmembrane domain, we suggested a more general role for such W residues in preventing membrane domain self association. We pursue this direction by investigating the role of juxtamembrane W residues in several transmembrane proteins and we collaborate with Ahmed Essaghir in the group of Jean-Baptiste Demoulin at the de Duve Institute for bioinformatics of single and multi-span transmembrane proteins.

Mechanisms by which TpoR extracellular domain mutations induce severe hematological pathologies

R.-I. Albu, V. Gryshkova, J.-P. Defour, E. Leroy, C. Pecquet

TpoR contains in its extracellular domain two cytokine receptor modules, one distal that binds Tpo (D1-D2) and one proximal to the membrane (D3-D4), that appears to exert a negative role in receptor activation. Using domain deletions and swaps we showed that all 4 domains are required for physiologic traffic of the receptor to cell surface, and that deletion of D1-D2 significantly reduces traffic, while deletion of D3-D4 completely blocks traffic. Modeling and production of extracellular receptor segments indicated that the block in traffic was due to interactions with the membrane and the presence of transmembrane residues. The D1-D2 receptor resembles in its defective traffic to a point mutant receptor that cannot be displayed at the surface (R102P) and that causes in humans congenital amegakaryocytic thrombocytopenia (CAMT). Others (P106L) are expressed at lower than normal levels at the cell surface, but paradoxically induce a thrombocytosis phenotype. We study the intracellular localization of these receptors in collaboration with Pierre Courtoy. We aim to understand why and where is the intracellular traffic blocked, as well as how and where do these receptors signal, for example TpoR P106L, which seems to be resistant to Tpo-induced internalization. Preliminary evidence obtained recently, also in collaboration with William Vainchenker at Institut Gustave Roussy suggests that such receptors might take aberrant routes after cis-Golgi arrival and might be subject to a novel quality control step in the Golgi.

Determination of the interface and orientation of the activated dimeric cytokine receptors and downstream signaling pathways

J.-P. Defour, C. Pecquet, E. Leroy

While many X-ray crystal structures exist for G-protein coupled receptors and other membrane proteins with multiple transmembrane domains, no such structure could be obtained for single-span receptors. Extracellular domain structures exist for erythropoietin receptor (EpoR) or for G-CSFR, but it is not possible to relate those to transmembrane and cytosolic domains, and to their relative positioning in the inactive versus active states. To identify the residues that form the interface between the receptor monomers in an activated receptor dimer we have replaced the extracellular domain of the receptor (Figure 3a) with a coiled-coil dimer of α-helices (9 and Seubert et al. Mol Cell 2003, 12,1239). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one) and d (four), the register of the coiled-coil α-helices is imposed on the downstream TM α-helix and intracellular domain.

Each of the seven possible dimeric orientations will then be imposed by the coiled-coil on the fused TM and intracellular domain of receptors. We then express individually these dimers (seven for each receptor type) and test their function in cell lines, primary mouse and human cells and in vivo in mice. We expect that some dimer interfaces would be active and some inactive, reflecting requirements of the wild type recep-

tor for activation. To prove that our predictions are correct, i.e. rotation imposed at the outset of the transmembrane domain is transmitted to the end of the transmembrane domain, we employed cysteine-mediated cross-linking and showed that indeed covalent dimers are formed via the cross-linker only when cysteines are in the predicted interface (9). This then allowed us to determine the active interface of the EpoR dimer, where only one interface was active.

Using this approach we have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the dimeric interface (cc-TpoR-I), that leads to formation of platelets (Figure 3b), other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Figure 3c and d) (9). One orientation (cc-TpoR-II) corresponds to the inactive receptor state. Interestingly, the dimer orientation that induces the highest levels of JAK2 activation, cc-TpoR-IV, also induces strong cell-to-cell adhesion and expansion of early hematopoietic progenitors. Our hypothesis is that in this dimeric orientation the receptor signals to maintain progenitors and possibly hematopoietic stem cells in the niche, and that signals induced by this dimeric orientation might be recapitulating the quiescence-inducing effects of TpoR in hematopoietic stem cells. We will use this set of seven differently oriented TpoR dimers to dissect the signals induced by TpoR at the different stages of hematopoietic development and via the various downstream signaling proteins, JAK2, TYK2, STAT3, STAT5, MAP-kinase ERK1,2 and PI-3'-kinase/AKt/mTOR.

While all these pathways are activated by cytokines and mutant JAKs, we aimed to determine whether any of them could

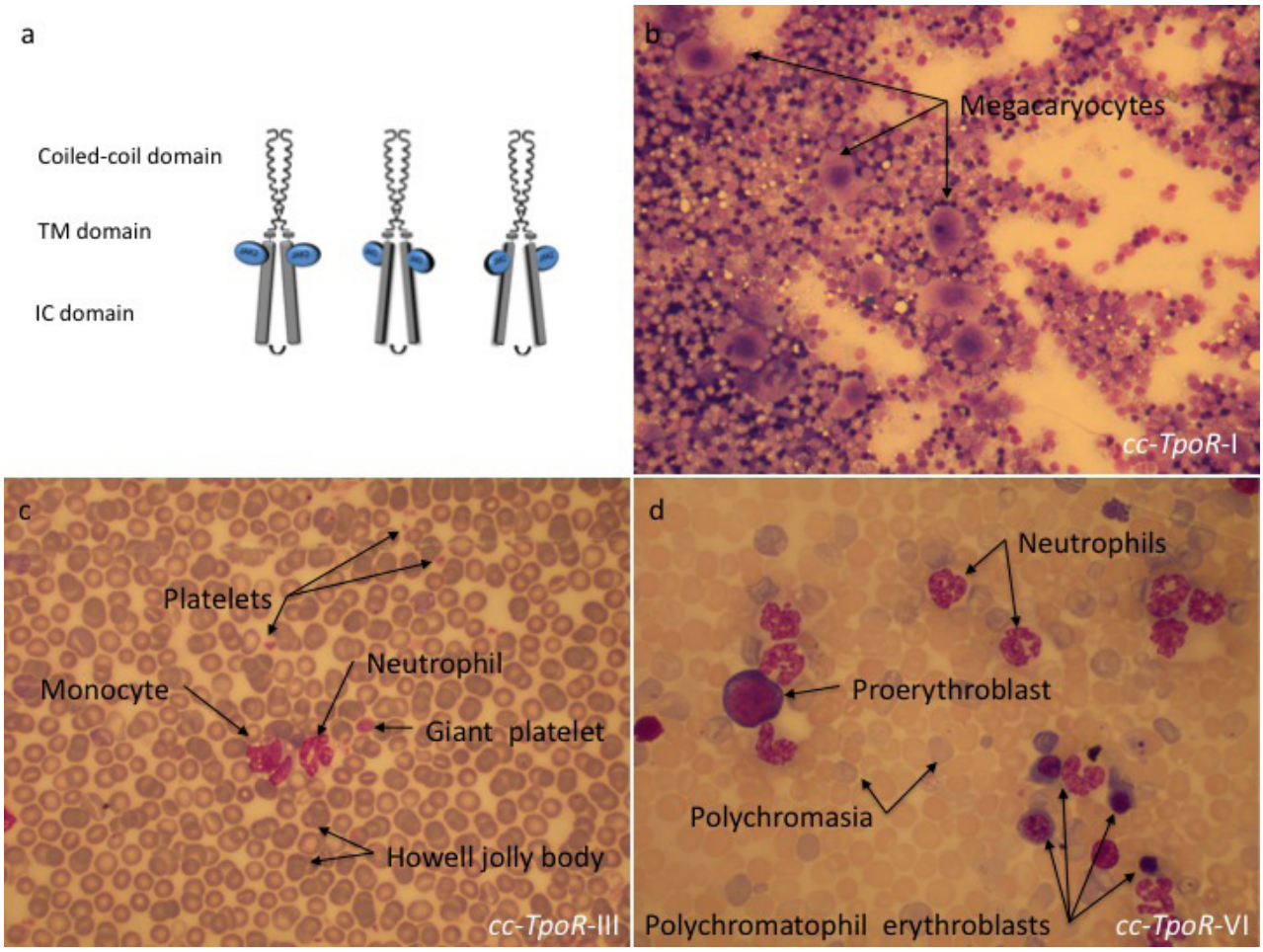

Fig. 3. Different dimeric orientations of the thrombopoietin receptor lead to distinct phenotypes in the bone marrow and in the peripheral blood in bone marrow reconstituted mice. (A) The fusion of the coiled coil protein to differently engineered transmembrane domains of the thrombopoietin receptor (TpoR) impose distinct dimeric orientations to the receptor. The different effects induced by three such chimeric receptors (cc-TpoR-I, cc-TpoR-III and cc-TpoR-VI) could be observed in the bone marrow and in the peripheral blood of lethally irradiated mice reconstituted with bone marrow cells retrovirally transduced with the indicated cc-TpoR fusion proteins. IC: intracellular , TM: transmembrane. (B) cc-TpoR-I induced a strong megakaryocytic response in the bone marrow with normal cellularity and a correct maturation of the myeloid lineages (May Grunwald stained bone marrow smear X 50, and normal platelet formation. (C) cc-TpoR-III induces granulocytosis, monocytosis and a very weak erythroblastosis in the peripheral blood (May Grunwald stained peripheral blood smear X 50) and weak dysplasia for the megakaryocytic and erythroid lineages in the marrow (not shown). (D) cc-TpoR-VI induced a stronger erythroblastosis and granulocytosis in the peripheral blood than cc-TpoR-III (May Grunwald stained peripheral blood smear X 50).

be essential for oncogenic proliferation. Within collaboration with Experimental Therapeutics Center in Singapore, we generated model cell lines that express JAK2 V617F or JAK2 with TpoR or EpoR, as well as cell lines that express TpoR W515L. These cells are screened for small molecule inhibitors at ETC. In collaboration with M. L. Choong, M. A. Lee and A. Matter at ETC we are showing that cells expressing JAK2 V617F are addicted to PI-3'-kinase signaling and that combinations of JAK2 inhibitors and pan type I PI-3'-kinase inhibitors are synergic in inhibiting proliferation of these transformed cells. Furthermore, the combinations are effective at inhibiting Epo-independent erythroid colony formation from JAK2 V617F knock-in mice or from MPN patient bone marrow cells.

Structure and function of juxtamembrane and transmembrane sequences of membrane proteins

E. Leroy, J.-P. Defour, J. Van Hees, C. Mouton

We have previously shown mutations in the extracellular juxtamembrane domain of EpoR can lead to constitutively active receptor mutants. Almost all cell surface EpoR, and a fraction of IL2/IL9/TpoR receptors exist on the cell surface as preformed ligand-independent complexes. Interactions between transmembrane domains are clearly maintaining EpoR dimers inactive. Introduction of asparagine residues in the transmembrane domain of TpoR defined two different interfaces that support TpoR activation and pointed to significant differences between mouse and human TpoR. Importantly, the human and not the mouse TpoR contains a His residue at the outset of the transmembrane domain, which is required for a small molecule agonist (Eltrombopag) to bind and activate the receptor. We study potential transmembrane interactions in the context of several transmembrane proteins, by cell surface immunofluorescence co-patching of differentially epitope tagged receptors along with protein ligation and protein fragment complementation. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand.
In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the groups of Profs. Jean-Noel Octave in our university and Steven O. Smith (SUNY Stony Brook, NY). We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these consecutive motifs promote transmembrane helix dimerization and amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733, Proc. Natl. Acad. Sci. USA 2009, 106, 1421).

Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells

I. Chachoua, C. Pecquet, J.-P. Defour, J. Van Hees

Using chromatin immunoprecipitation and sequencing, Virginie Moucadel in the lab had shown that STAT5 contacts a substantially different set of promoters in cells that exhibit constitutive STAT5 activation, versus cells that respond to cytokines by transient STAT5 tyrosine phosphorylation. Michael Girardot identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) (10), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, to inhibit translation of several proteins involved in megakaryocyte differentiation and to impair proplatelet formation (10). We found miR-28 to be pathologically overexpressed in 30% of MPNs (10). We identified the sites in LPP promoter that are required for induction by STAT5 and by CHIP on chip we identified several other genes that are regulated in the same way. Two of these genes appear to be associated with MPNs.

Interaction with St Luc Hospital clinicians and clinical biologists: Identification of the molecular bases of MPNs without known molecular cause

J.-P. Defour, J. Van Hees, C. Mouton

At present, our laboratory is performing under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Cédric Hermans, Prof. Augustin Ferrant, Prof. Laurent Knoops), Clinical Biology (Prof. Dominique Latinne, Prof. Hélène Antoine-Poirel) and groups of de Duve Institute (Prof. Mark Rider, Prof. Jean-Baptiste Demoulin) a large study on the presence and signaling of JAK2, TpoR, and growth factor receptor mutations in patients with myeloproliferative neoplasms. Next generation sequencing will be employed for well-investigated patients, using primary cells that are characterized for functional defects and that do not harbor known mutations in order to unravel novel molecular defects in MPNs and leukemias.

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Links

Group

Ludwig Institute for Cancer Research. NewsLink Sept 2005 of our group (http://www.licr.org/12124501528/newslink/0509/)
Ludwig Institute for Cancer Research - Brussels Branch
http://www.bru.licr.org/brussels/research/stg/stg.html
https://www2.licr.org/News/pdfs/April_2013_Newsletter.pdf

European Commission Marie Curie Research Training Network ReceptEUR (www.recepteur.org)

Research:

Whitehead Institute for Biomedical Research, MIT, Lodish Lab (http://www.wi.mit.edu/lodish/)

SUNY Stony Brook, Structural Biology, Smith Lab
http://sos.bio.sunysb.edu lab homepage
http://csb.sunysb.edu structural biology homepage
http://csb.sunysb.edu/bsb graduate program in biochemistry and structural biology

American Society of Hematology Educational Program 2012 (http://hematology.org/Meetings/Annual-Meeting/Program/3741.aspx)
The Spectrum of JAK2-Positive Myeloproliferative Neoplasms: Complications and Therapeutic Advances (http://hematology.org/Meetings/Annual-Meeting/Program/3741.aspx#ID0E5E)

Institut Gustave Roussy, Villejuif, France
(William Vaincheker's team)
http://www.gustaveroussy.fr/en/page/william-vainchenker_753#1

Belgian Medical Genomics Initiative IAP
http://www.bemgi.be/

Belgian Society of Cell Biology and Development
http://www.bscdb.ugent.be/

Learning:

Biologie moléculaire de la cellule (Molecular Cell Biology H.F. Lodish) French Edition
http://universite.deboeck.com/livre/?GCOI=28011100737460&fa=author&person_id=108&publishergcoicode=28011

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PMID: 22869151

Bioinformatics:

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