de Duve
INSTITUTE

Scientific Report

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2016

DE DUVE INSTITUTE

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de Duve Institute

Ludwig Institute for Cancer Research

The de Duve Institute

The de Duve Institute • An international biomedical research institute

Originally named *International Institute of Cellular and Molecular Pathology* (abbreviated ICP), the de Duve Institute was founded in 1974 by Professor Christian de Duve († 4th of May 2013) to develop basic biomedical research with potential medical applications. *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.

Emile Van Schaftingen

Benoît
Van den Eynde

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.

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.

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. Researchers also benefit from technology platforms that offer state-of-the-art servicing in innovative technologies.

Miikka Vikkula

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.

Frédéric Lemaigre

Directorate

Emile Van Schaftingen, Director

Frédéric Lemaigre

Benoît Van den Eynde

Miikka Vikkula

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Supporting Organizations

Belgian Ministry of Health, Plan Cancer

Belgian Science Policy Office

Centre du Cancer, Cliniques universitaires Saint-Luc

Cystinosis Research Foundation

European Commission

European Research Council

Fédération Wallonie-Bruxelles

Fondation Charcot

Fondation contre le Cancer

Fondation Louvain

Fondation Roi Baudouin

Fondation Salus Sanguinis

Fonds Jacques Goor

Fonds Joseph Maisin

Fonds National de la Recherche Scientifique

Ligue Nationale Belge de la Sclérose en Plaques

Loterie Nationale

MPN Research Foundation, USA

National Institutes of Health, USA

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WELBIO Laboratories

The following laboratories are supported by WELBIO (**W**alloon **E**xcellence in **L**ife Sciences and **Bio**technology)

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Acknowledgements

In 2015, 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 2015 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Leila Varghese,

the «Maurange» fellowship to Rym Agrebi,

the «Pierre M» fellowship to Ha-Long Phuoc Nguyen,

and an ICP fellowship has been awarded to 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.

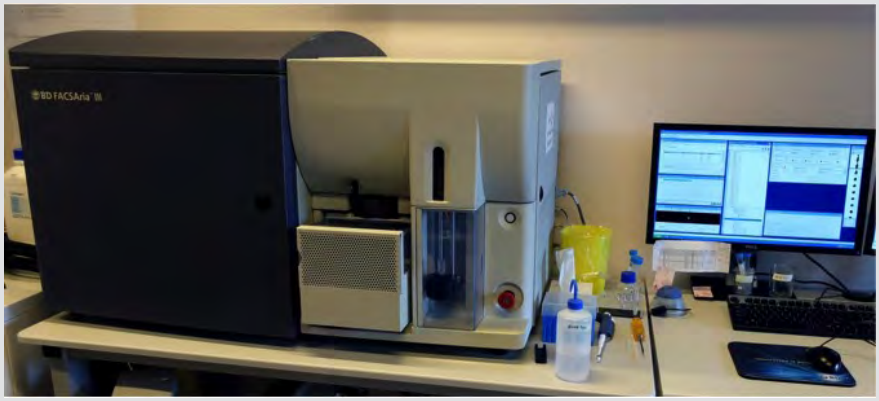
Luc Bertrand
President of the Development and Expansion Council

Technology platforms

Flow Cytometry and Cell Sorting

Flow cytometry technology allows simultaneous multiparametric analysis of thousands of cells per second, enabling trained users to rapidly analyze complex cell populations based on phenotypic and functional features. High-speed assisted cell sorting services provide researchers with physical separation of identified cell populations, for any downstream characterizations. The platform is managed by Prof. P. Coulie and is run by Dr. N. Dauguet.

[W] http://www.deduveinstitute.be/flow-cytometry-and-cell-sorting



BD FACSAria III cell sorter.

Genomics

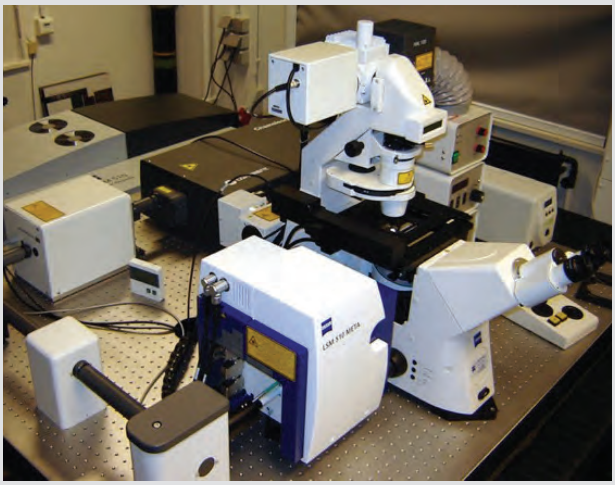
The genomics platform provides the scientific community with the latest technologies, such as Next Generation Sequencing (Massive Parallel Sequencing). Theses techniques facilitate and speed up data acquisition, which is beneficial for many different fields, such as biology, medicine, agronomy, ... Their use in clinical diagnosis also broadens the spectrum of molecular diagnosis and opens new ways for personalized medicine. The platform is managed by Prof. M. Vikkula and is run by Drs. P. Brouillard, M. Amyere and R. Helaers.

[W] http://www.deduveinstitute.be/genomics-platform

Ion Proton sequencer (left) and the Ion Chef (right) robot for template preparation and chip loading.

Imaging

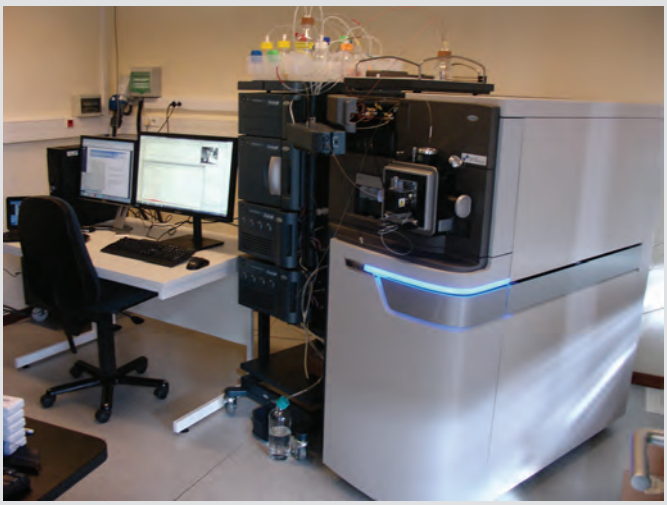
The imaging platform trains and provides the scientific community with various confocal and electronic microscopes and a wide range of sophisticated methods of vital confocal microscopy and immunolabeling. It is also a source of advices, collaborations and a «school of morphology» for users, providing them with the necessary expertise at all stages of the experiment, from sample preparation to analysis and interpretation of data. The platform is managed by Prof. D. Tyteca and is run by Dr. P. Van Der Smissen.
[W] http://www.deduveinstitute.be/pict-platform-imaging-cells-and-tissues



Confocal/multiphoton microscope Zeiss LSM 510. Equipped with a thermostatic chamber and CO_2 control. Wide range of applications: (i) *in vivo* imaging by multiphoton microscopy, (ii) tissue and cell immunolabeling, (iii) cell vital imaging, and (iv) advanced applications including dynamics of molecular movement by FRAP (Fluorescence Recovery After Photobleaching), molecule:molecule interaction by FRET (Fluorescence Resonance Energy Transfer) and protein dimerization by BiFC (Bimolecular Fluorescence Complementation).

Mass Spectrometry

The platform provides proteomics services principally through gel-free approaches coupled to mass spectrometry. It specializes in the identification and quantification of proteins from complex samples, and can also provide data on the location of post-translational modifications, even in complex samples. The platform is managed by Prof. M. Rider and is run by Dr. D. Vertommen, together with Prof. P. Morsomme and Dr. H. Degand at the Institute of Life Sciences (University of Louvain).
[W] http://www.uclouvain.be/en-proteomics



High Resolution / Accurate Mass mass spectrometer for LC-MS/MS applications (Synapt G2-Si from Waters).

Transgenesis

The transgenesis platform offers transgene technology tools to research teams of the University of Louvain and other Belgian universities at the lowest possible cost. It also enables the sharing of expertise in designing and creating transgenic mouse lines and offers training opportunities to PhD students and post-doctoral researchers. The platform is managed by Profs. P. Jacquemin and F. Lemaigre, and is run by Dr. Y. Achouri.

[W] http://www.deduveinstitute.be/transgenesis

Workstation for generation of genetically modified animals using pronuclear micro-injection (A1 inverted microscope, 2 TransferMan NK2, FemtoJet 4i microinjector, 1 CellTram Oil).

Cryopreservation room

The de Duve Institute and the UCL have gathered in a single place the freezers of all laboratories on the Woluwe campus. This new cryopreservation room allows researchers to store and freeze biological samples in 20 tanks supplied with liquid nitrogen (-190°C) and 32 freezers (-80°C). In total, the room can hold 360,000 samples in the tanks and 1.8 million samples in the freezers. In case of failure, the safety of these samples is ensured by a generator and an automatic supply of liquid nitrogen from an external tank of 5,250 liters.

Scientific Prizes and Awards in 2015 - 2016

To Jean-Baptiste Demoulin • 2015
Prix Nutricia sur la recherche translationnelle ou clinique en pédiatrie

To Violaine Havelange • 2015
Prix de la Fondation Bekales (leukemia)

To Miikka Vikkula & Laurence Boon • 2014-2015
Prix du concours ordinaire de la 1ère section de l'Académie royale de Médecine de Belgique

Doctoral Theses (PhD) - August 2015 to July 2016

5 • 10 • 2015
Lorraine SPRINGUEL, MEXP
Loss of MLH1 expression promotes the acquisition of oncogenic JAK1 and JAK3 mutants that cooperatively increase resistance to JAK inhibitors
Promoter: J.-C. Renauld

23 • 11 • 2015
Sarah LEGRAIN, MEXP
Role of macrophages from LDV-infected mice in the onset of blood autoimmune diseases
Promoter: J.-P. Coutelier

30 • 11 • 2015
Julie CANNUYER, GEPI
Mechanisms involved in DNA demethylation and activation of cancer-germline genes in human tumors
Promoter: C. De Smet

16 • 12 • 2015
Nathalie LANNOY, GEHU
Molecular analysis of simple and complex genetic variants in a cohort of patients with hemophilia A: Mechanisms and diagnostic implications
Promoter: C. Hermans

22 • 12 • 2015
Gaëlle TAHAY, BCHM
Characterization of aspartate-N-acetyltransferase and search of the catalytic activity of other proteins form the same family
Promoter: E. Van Schaftingen

12 • 1 • 2016
Jean-Philippe DEFOUR, SIGN
Thrombopoietin receptor: regulation, dimerization, oncogenic activation and bimodal signaling
Promoter: S. Constantinescu

21 • 1 • 2016
Christophe BOURDEAUX, LICR
Local immunostimulation leading to rejection of accepted male skin grafts by female mice as a model for cancer immunotherapy
Promoter: T. Boon

27 • 1 • 2016
Jennifer BOLSEE, BCHM
Mitochondrial metabolism in intestinal epithelial cells - Role of the NMES1/miR-147b locus and hydrogen sulfide detoxification
Promoter: G. Bommer

15 • 2 • 2016
Anne-Sophie DELMARCELLE, CELL
Endothelial cells and BMP signaling control thyroid folliculogenesis via basement membrane assembly
Promoter: C. Pierreux

21 • 3 • 2016
Cécile LARDINOIS, VIRO
Neuroinvasion by Theiloviruses
Promoter: T. Michiels

11 • 4 • 2016
Aurélie DE COCK, VIRO
RNA interference: impact on RNA virus replication and use to study virus-host interaction
Promoter: T. Michiels

15 • 4 • 2016
Alexandra GENNARIS, BCHM
Discovering a new pathway involved in methionine repair in the bacterial envelope
Promoter: J.-F. Collet

27 • 5 • 2016
Cécile AUGEREAU, LPAD
Identification of mechanisms leading to preneoplastic lesions in the pancreas
Promoter: P. Jacquemin

2 • 6 • 2016
Céline DEMAREZ, LPAD
Identification of a microRNA controlling hepatic cell differentiation
Promoter: F. Lemaigre

24 • 6 • 2016
Mélanie CARQUIN, CELL
Recent progress on lipid lateral heterogeneity in plasma membranes: From red blood cell to yeast
Promoter: D. Tyteca

27 • 6 • 2016
Monika LAMBA SAINI, CELL
Molecular characterization of papillary thyroid carcinoma
Promoter: E. Marbaix

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 & Eliza Hall Institute of Medical Research, Melbourne, Australia

1978 Henry KUNKEL • Rockefeller University, New York, NY, USA

1980 Michael SELA • Weizmann Institute of Science, Rehovot, Israel

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

1984 Avrion MITCHISON • University College London, UK

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

1988 Zanvil COHN • Rockefeller University, New York, NY, USA

1990 André CAPRON • Institut Pasteur de Lille, France

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

1994 Fritz MELCHERS • Basel Institute for Immunology, Switzerland

1996 Lorenzo MORETTA • National Institute for Cancer Research, University of Genova, Italy

1998 Charles WEISSMANN • Institut für Molekularbiologie, Universität Zürich, 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, Université de Strasbourg, France

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

2014 George STARK • Case Western Reserve University, Cleveland, OH, USA

The de Duve Memorial Lecture

This biennial Distinguished Lectures series was created in 2013 in honor of the founder of our Institute, the late Professor Christian de Duve († May 4, 2013). It is to be given every other year by a prominent international scientist.

2013 Michael HALL • Biozentrum, University of Basel, Switzerland

2015 Andrea BALLABIO • Telethon Institute of Genetics and Medicine, Naples, Italy

Plenary Lectures - August 2015 to July 2016

August

Nan-Shan CHANG
Institute of Molecular Medicine, National Cheng Kung University Medical College, Taiwan; Dept of Neuroscience & Physiology, Upstate Medical University, Syracuse, NY, USA
Tumor suppressor WWOX in immune regulation and neurodegeneration

September

Claude HAAN
Signal Transduction Laboratory, Life Sciences Research Unit, University of Luxembourg, Luxembourg
Characterisation of pharmacological inhibitors of Janus kinases and the influence of the Jak-hierarchy in cytokine receptor complexes on the efficiency of these pharmacological inhibitors

Martin GUILLIAMS
VIB - Inflammation Research Center, Ghent University, Belgium
Cellular origin and functional specialization of tissue-resident macrophages

Mehmet BERKMEN
New England Biolabs (NEB), Ipswich, MA, USA
Going to rabbit heaven – Expression and engineering of antibodies in *E. coli*

Natividad RUIZ
Dept of Microbiology, Ohio State University, Columbus, OH, USA
Translocation of lipid-linked peptidoglycan precursors across membranes

October

Claus AZZALIN
Dept of Biology, Institute of Biochemistry, ETH, Zurich, Switzerland
Transcription of telomeres in cancer

An ZWIJSEN
VIB - Center for the Biology of Disease, KU Leuven, Belgium
BMPs going vascular

Catherine PARADIS-BLEAU
Dept of Microbiology, Infectiology & Immunology, Faculty of Medicine, University of Montréal, Canada
Peptidoglycan assembly, oxidative stress and protein folding

Ted HUPP
Institute of Genetics & Molecular Medicine, University of Edinburgh, UK
Defining the oncogenic landscape in oesophageal adenocarcinoma using proteogenomics platforms

Mirna PEREZ-MORENO
Epithelial Cell Biology Group, Spanish National Cancer Research Center (CNIO), Madrid, Spain
Contributions of immune cells to the skin stem niche

Jean-Christophe MARINE
VIB - Laboratory for Molecular Cancer Biology, KU Leuven, Belgium
Integrated genomic and transcriptomic analysis identifies novel therapeutic targets in melanoma

Fabian GEISLER
Dept of Internal Medicine II, Technical University Munich, Germany
Ductular reactions in the liver: liver progenitors or lame ducts?

Eli GILBOA
Dept of Microbiology & Immunology, Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, FL, USA
Cancer immunotherapy with aptamer-targeted immune modulatory agents

November

Iris BEHRMANN
Signal Transduction Laboratory, Life Sciences Research Unit, University of Luxembourg, Luxembourg
How does cytokine signaling affect miRNAs and metabolism?

January

Charles DE SMET
de Duve Institute, GEPI
DNA hypomethylation and activation of germline-specific transcripts in cancer: causes and consequences

Nisha LIMAYE
de Duve Institute, GEHU
The genetic bases of venous malformation: TIE2 and beyond

Theresa AHRENS
Institute for Surgical Pathology, University of Freiburg, Germany
Epigenetic targeting of esophageal cancer

Anita ROY
INSERM UMR 1170, Institut Gustave Roussy, Université Paris-Saclay, Villejuif, France
Regulation of megakaryocyte differentiation and polyploidy - Role of RhoGTPase mediated signaling pathways

Maria Carla SALEH
CNRS URM 3569, Viruses and RNA interference, Institut Pasteur, Paris, France
RNAi-mediated antiviral immunity in insects

Jean-François COLLET
de Duve Institute, BCHM
How bacteria protect their envelope

February

Victor DILLARD
Desktop Genetics, London, UK
Making the cut: Designing CRISPR/Cas9 experiments

Corine BOUSQUET
Cancer Research Center, Université de Toulouse, France
Deciphering the antitumoral potential of somatostatin receptor signal

Angela QUEISSER
Institute of Pathology, University Hospital Bonn, Germany
Ecotropic viral integration site 1, a novel oncogene in prostate cancer

Christophe PIERREUX
de Duve Institute, CELL
Building epithelial organs: tube or not tube

March

Jens SIVEKE
Division of Translational Solid Tumor Oncology, German Cancer Research Center, University Hospital Essen, Germany
Cellular plasticity in pancreatic cancer

Sarah ADES
Dept of Biochemistry & Molecular Biology, Pennsylvania State University, University Park, PA, USA
Pushing the envelope: Role, regulation, and inhibition of the *E. coli* SigmaE-dependent extracytoplasmic stress response

Mark RIDER
de Duve Institute, PHOS
Drug targeting the AMP-activated protein kinase (AMPK) system for treating metabolic disorders

Alan GROSSMAN
Dept of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA
Horizontal gene transfer: Visualization and control of bacterial sex

Eugénie ANSSEAU
Laboratory of Molecular Biology, Research Institute for Health Sciences and Technology, University of Mons, Belgium
Homologous DUX transcription factors, involved in Facioscapulohumeral muscular dystrophy, interact with cytoplasmic proteins during muscle differentiation

April

Mircea IVAN
Dept of Microbiology & Immunology, Indiana University School of Medicine, Indianapolis, IN, USA
The expanding universe of hypoxia sensing: From HIF-prolyl hydroxylases to noncoding RNAs

Laure DUMOUTIER
de Duve Institute, MEXP
The dark side of IL-22 receptor in skin diseases

François-Xavier MAHON
Dept of Hematology, Institut Bergonié, Bordeaux, France
Curing chronic myeloïd leukemia: Clinical and biological aspect

Pedro BAPTISTA
Aragon Health Sciences Institute, Zaragoza, Spain
Self-assembled liver organoids recapitulate hepato-biliary organogenesis in vitro

Julie GUILLERMET
Centre de Recherche en Cancérologie de Toulouse, Université de Toulouse, France
Targeting the PI3K pathway in cancer: When PI3K isoform matters

May

2nd de Duve Memorial Lecture

Andrea BALLABIO
Telethon Institute of Genetics and Medicine, Naples, Italy
The lysosome: From trash can to control center of cell metabolism
(podcast: www.youtube.com/watch?v=pm4CLYxhZE8)

Toshihide KOBAYASHI
Lipid Biology Laboratory, RIKEN Institute, Tokyo, Japan; INSERM U1060, Université Lyon 1, France
Imaging lipids and lipid domains

Sophie LUCAS
de Duve Institute, GECE
Targeting GARP on Tregs: A new approach for cancer immunotherapy?

Yves POUMAY
URPhyM-NARILIS, University of Namur, Belgium
Existence of specialized lipid microdomains in keratinocytes: Reality in membranes and impacts on epidermal physiology and pathology

James DI SANTO
Innate Immunity Unit, Immunology Dept, Institut Pasteur, Paris, France
Identity and plasticity of innate lymphoid cells

June

Frédéric DE SAUVAGE
Dept of Molecular Oncology, Genentech, Inc., San Francisco, CA, USA
Targeting developmental pathways in cancer

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

Ignacio MORAGA
College of Life Sciences, University of Dundee, UK
Structural and biophysical parameters determining cytokine signal activation

July

Alan BENARD
Dept of Tuberculosis & Infection Biology, Institute of Pharmacology & Structural Biology, University of Toulouse, France
B cells modulate inflammation during *Mycobacterium tuberculosis* infection in a cytokine-dependent manner

Marc VELDHOEN
Laboratory of Lymphocyte Signalling & Development, Babraham Institute, Cambridge, UK
Epithelial barriers: «maintaining and directing T cell responses»

Research Groups

Human genetics

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 multidisciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques universitaires Saint-Luc, UCL; Vascular Anomalies Center, Children's Hospital, Boston, USA; Consultation des Angiomes, CHU de Caen; and Centre labiopalatin, Cliniques universitaires Saint-Luc, UCL).

Venous malformations and glomuvenous malformations ("glomangiomas")

J. Soblet, P. Brouillard, M. Amyere, H.-L. Nguyen, A. Mendola, N. Limaye, N. Revencu, L.M. Boon, 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 de Caen, France), A. Irvine (Dublin, Ireland)

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.

We previously discovered that rare, hereditary venous malformations are caused by activating mutations in the endothelial cell receptor tyrosine kinase *TIE2/TEK*. Despite the ubiquitous presence of these germline mutations, the lesions they cause are localized. We hypothesized that lesion-development requires a somatic second hit in *TIE2*, and obtained proof for this from lesions from two different patients [1]. At least 50% of common sporadic VMs are also caused by somatic mutations in *TIE2* [1], as are lesions of Blue Rubber Bleb Nevus syndrome, a rare congenital disorder whose etiology was unknown [2]. To understand how *TIE2* mutations cause VMs, we carried out functional analyses of the cellular and molecular effects of VM-causative mutations on endothelial cells. We found that PI3K/AKT is the major activated pathogenic signaling pathway [3] (Fig. 1A). It is associated with increased survival of mutant endothelial cells, accompanied by defects in endothelial cell morphology and function including sprouting, vascular smooth muscle cell recruitment and extracellular matrix breakdown, and increased colony-formation and invasiveness. The importance of the PI3K/AKT pathway in VM is borne out by our recent discovery of somatic, activating mutations in *PIK3CA,* encoding the PI3K p85α catalytic subunit, in 20% of sporadic VMs [4]. The mTORC inhibitor rapamycin acts within this pathway to effectively control the expansion of lesions in a mouse model of the disease [5]. Rapamycin also ameliorated symptoms in a preliminary trial of six patients with VMs recalcitrant to conventional therapies (Fig. 1B). This demonstrates, for the first time, the feasibility of a molecular approach to therapy in this developmental disease [5], and provides great hope for all patients affected with vascular anomalies.

Glomuvenous malformations (GVM) are mostly, if not always, inherited. We discovered that GVMs are caused by loss-of-function mutations in a gene we named glomulin. So far, we have identified GLMN mutations in 162 families, the most frequent being present in 44%. 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 [6]. This renders the inherited glomulin mutation homozygous in affected tissues, without loss of genetic material. To better understand the role of

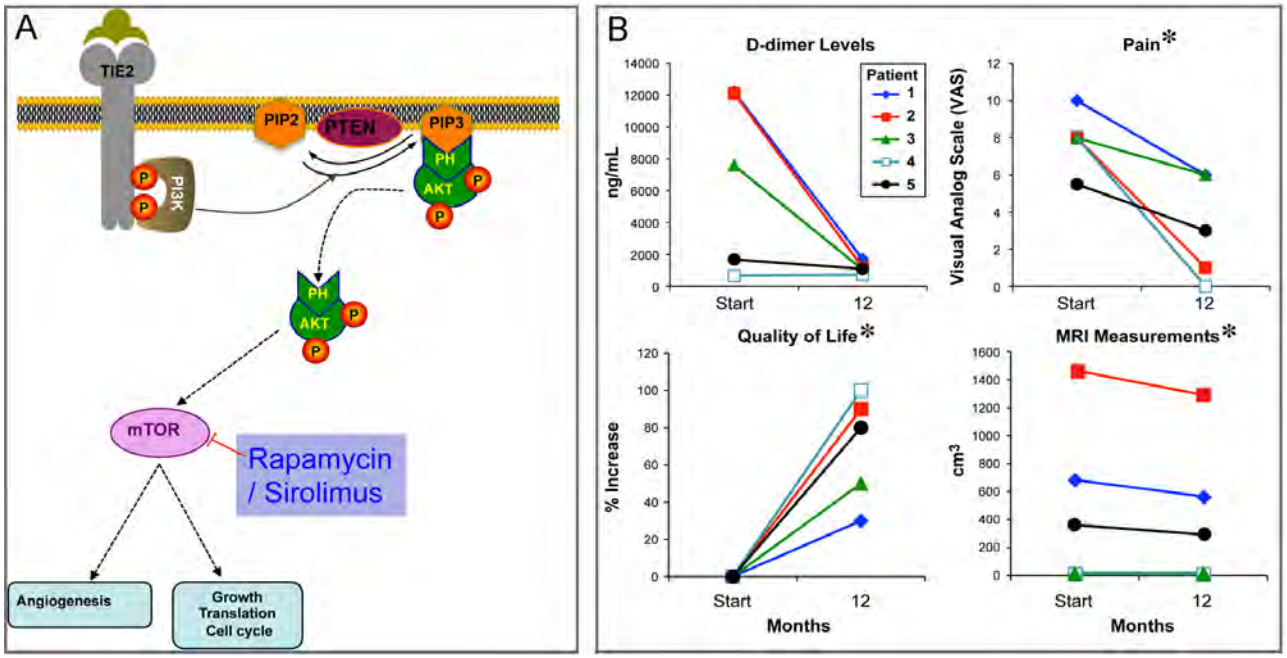


Fig. 1. (A) Rapamycin acts on mTOR, downstream of PI3K/AKT activation by VM-causative TIE2 mutations; (B) Evolution of quantitative parameters from start to 12 months after rapamycin treatment. Analysis of D-dimer levels, pain, Quality of Life, and MRI measurements in five patients at start and after 12 months of rapamycin treatment [5].

glomulin, we generated glomulin-deficient mice, which are embryonic-lethal early in development. While also lethal, conditional knock-down of *glmn* (using RNAi technology) during midgestation allows embryos to develop further. These mice exhibit multifocal vascular defects, and therefore serve as a model in which to study the role of glomulin in vascular development and in GVM.

Vascular anomalies affecting capillaries

E. Khoury, A. Mendola, M. Amyere, N. Revencu, N. Limaye, L.M. Boon, M. Vikkula, in collaboration with J.B. Mulliken and J. Bischoff (Children's Hospital, Boston, USA), S. Watanabe (Showa University School of Medicine, Tokyo, Japan), A. Dompmartin (CHU de Caen, France) & an international network of clinicians

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: (i) hemangioma, a benign, localized overgrowth of capillary-like vessels; (ii) capillary malformation (CM; commonly known as port-wine 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 tissue and blood samples of resected hemangiomas, and are using Next Generation Sequencing in an effort to identify causative genomic variants. These likely perturb VEGF signaling, which our work done with collaborators has demonstrated is key in the pathogenesis of hemangioma [7].

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 cavernous 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. 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 [8]. 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 named Capillary Malformation-ArterioVenous Malformation (CM-AVM) [9]. Importantly, capillary lesions can be associated with deeper, more dangerous anomalies about 20% of the time; these include arteriovenous malformation and fistula (AVM/AVF), Parkes Weber syndrome, and Vein-of-Galen aneurysmal malformation, 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.

Lymphedema

P. Brouillard, M. Schlögel, E. Fastré, A. Mendola, L.M. Boon, N. Revencu, M. Vikkula, in collaboration with G. Giacalone, (Geel, Belgium), I. Quere (Montpellier, France), D. Chitayat (Toronto, Canada) & an international network of clinicians

Lymphatic anomalies include a variety of developmental and/or functional defects affecting lymphatic vessels: sporadic and familial forms of primary lymphedema, secondary lymphedema, chylothorax and chylous ascites, lymphatic malformations, and overgrowth syndromes with a lymphatic component. Lymphedema leads to disabling swelling that occurs mostly on the extremities, and is extremely difficult to treat. Mutations have been identified in at least 20 genes encoding proteins that participate in signaling, mainly by VEGFR-3 but also by other tyrosine kinase receptors (Fig. 2) [10]. These mutations explain about a quarter of the incidence of primary lymphedema, mostly the inherited forms. Using Next Generation Sequencing, we have identified mutations in at least 5 novel genes that interact with these pathways, and are now expressing the mutant proteins in cellular models in order to delineate their functional effects. This is done in collaboration with several research groups worldwide. These studies are supported by an advanced research grant from the WELBIO.

Cleft lip and palate

M. Basha, B. Demeer, N. Revencu, M. Vikkula, in collaboration with B. Bayet, G. François, N. Deggouj (Centre Labiopalatin, Cliniques universitaires Saint-Luc, UCL) & an international network of clinicians

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 have collected and continue to collect DNA samples from a large number of patients. These include a series 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. This study 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. 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 lip anomalies. In parallel, we identified a new gene, *FAF1*, responsible for cleft palate only and Pierre Robin sequence [11]. 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 and syndromic orofacial clefts.

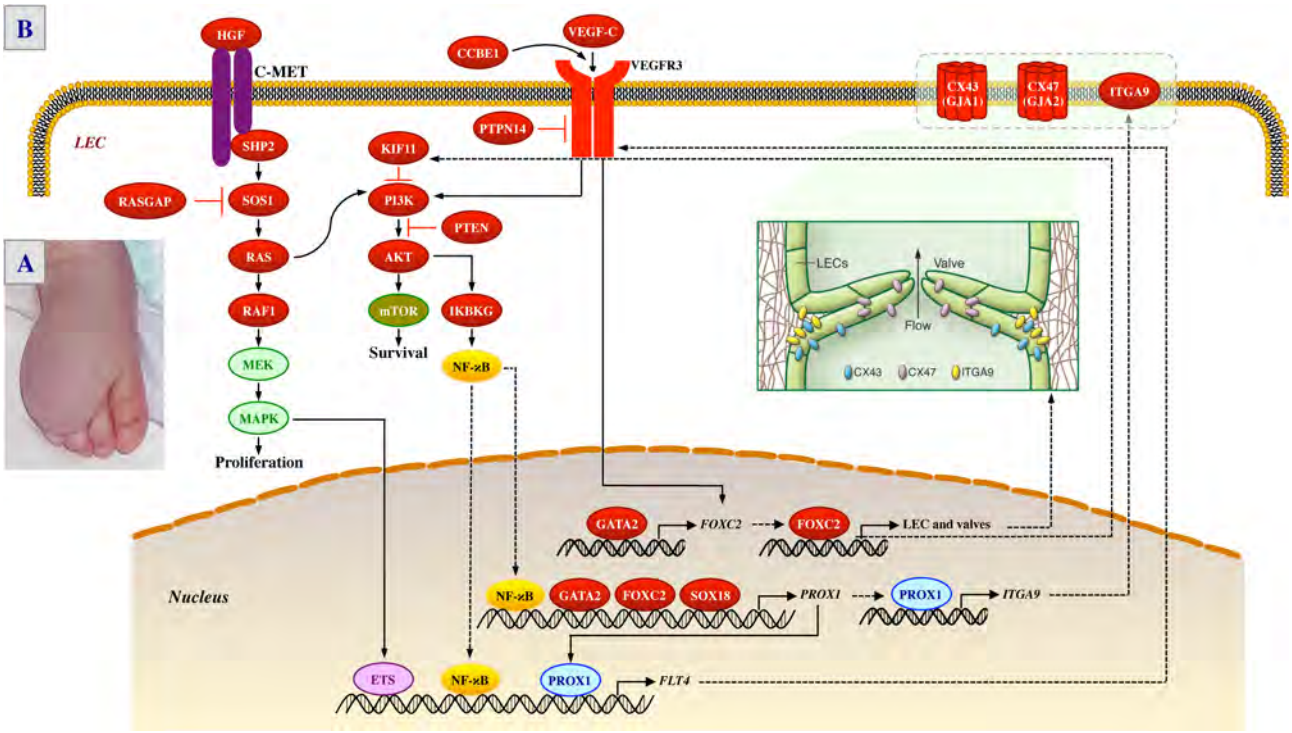


Fig. 2. (A) Primary lymphedema of the foot; (B) Schematic view of a lymphatic endothelial cell (LEC) and conducting lymphatic vessel valves. Lymphatic anomaly-associated proteins are frequently associated with the VEGF-C/VEGFR-3 signaling pathway. Proteins mutated in lymphedema or other lymphatic disorders are shown in red.

Essential hypertension

L. Evenepoel, A. Mendola, M. Vikkula, in collaboration with A. Persu (Division of Cardiology, Cliniques universitaires Saint-Luc, UCL)

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. Association was identified with STK39 and WNK1, suggesting that variants in these genes may predispose to hypertension [12].

Hypermobility

D. Manicourt, P. Brouillard, M. Vikkula

Using whole exome sequencing on DNA from familial cases, we try to identify the gene(s) responsible for the Ehlers-Danlos syndrome (EDS), hypermobility type, an under-recognized and yet relatively frequent condition. The syndrome has no cure and no distinctive biochemical collagen findings. Because of their excessive range of movement, joints are prone to joint dislocation and subluxations. The syndrome is a common cause of severe, early and multifocal osteoarthritis. Other clinical manifestations include chronic pain, easy bruising, functional bowel disorders, autonomic dysfunction and aortic root dilatation.

Selected cancers

Cancer is a genetic disease, the characterization of the underlying alterations of which is the key for understanding disease mechanisms, for improving diagnostics and for developing novel therapeutic concepts. Next Generation Sequencing (NGS) is a novel high-throughput technology, which allows unbiased characterization of complete tumoral genomes, transcriptomes and epigenomes. The technology has progressed quickly during the past years, and has now proven its capabilities. The manipulations have become simpler and standardized, sample costs have diminished, and the output has increased. That is why we have started to use it in several collaborative cancer projects, in addition to our projects on vascular anomalies, lymphedema and cleft lip & palate.

Breast Cancer

C. van Marcke, M. Amyere, M. Vikkula, in collaboration with F. Duhoux and J.-P. Machiels (Cliniques universitaires Saint-Luc, UCL)

Approximately 10% of all breast cancer cases are due to genetic predisposition. Half of them are due to alterations in two tumor suppressor genes involved in double strand break repair, *BRCA1* and *BRCA2*. Their constitutional alterations also confer an elevated lifetime risk for ovarian cancer. Inherited inactivating mutations or sequence variants in other genes, mainly tumor suppressor genes, often playing a role in various DNA repair pathways, have been reported in families with multiple cases of breast cancer (among others *TP53, ATM, CHEK2, RAD51C, PALB2, BARD1, BRIP1, RAD50, BAP1, XRCC2* and *XRCC3*, …). Until now, due to technical limitations in DNA sequencing, only a restricted number of candidate genes could be tested for mutations. Indeed, routine laboratory testing currently investigates only high-penetrance *BRCA1* and *BRCA2* alterations. While detecting germline mutations in genes involved in DNA repair currently has a huge impact on the screening and preventive measures offered to patients carrying these mutations, the therapeutic impact is rather limited. This will certainly change in the near future, with the advent of agents that require repair of double strand breaks by homologous recombination, such as poly ADP ribose polymerase (PARP) inhibitors. Our project aims to unravel the predisposing genetic variations in patients with family history of breast cancer without a BRCA1/2 mutation by using next generation sequencing.

Hematological malignancies and tumors of soft tissue and bone

H. Antoine-Poirel, N. Limaye, M. Vikkula, in collaboration with A. Ferster (Huderf, Jette), G. Ameye, J. Luciani, G. Tilman (Center for Human Genetics, Cliniques universitaires Saint-Luc, UCL)

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 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 (FISH and molecular karyotyping) to identify partner genes in reciprocal translocations, in non-classifiable cases of mesenchymal tu-

mors to help understanding their physiopathogeny as well as their diagnosis.

We have a special interest to better characterize pediatric aggressive lymphomas. We identified prognostic chromosomal aberrations of childhood mature B-cell lymphoma in the frame of an international therapeutic trial. The MYC deregulation through chromosomal translocation is the genetic hallmark of Burkitt lymphoma (BL). This genomic event is not sufficient to BL development. Several cooperating genomic/epigenetic changes have been characterized. We dissected the pattern of genomic aberrations in a series of 34 lymphomas with *MYC* rearrangement for: (i) genome-wide copy number variations and copy-neutral loss of heterozygosity; (ii) *TP53, P14ARF, ID3 CCND3* and *TCF3* sequencing. Our results suggest heterogeneity in Burkitt lymphomagenesis depending on the age of onset [13, 14].

Infant (≤ 1 yr-old) acute leukemias are rare (5-10% of childhood leukemias) and exhibit clinical and genetic features distinct from those occurring in older children, especially poor outcome, and high incidence (60-80%) of translocation involving the MLL gene (11q23). We are characterizing a series of 13 cases of infant acute leukemia already collected during the past 10 years in the Cliniques universitaires Saint-Luc by a panel of eight genes with a kinase activity, which has been recently identified by whole exome sequencing in infant acute leukemias. This study will be extended to other Belgian cases included in the International Interfant therapeutic trial, coordinated by Alice Ferster in Belgium. The aim is to detect additional genetic events, which may cooperate with MLL rearrangement, even at a very low proportion, especially those who are expanded at relapse and which are targetable.

Familial inherited malignant disorders

H. Antoine-Poirel, N. Limaye, M. Vikkula, in collaboration with C. Besson (CHU Kremlin-Bicêtre, France), E. Wiame, E. Van Schaftingen (de Duve Institute)

While 5 to 10% of malignant solid tumors are inherited, familial aggregation of hematological malignancies is much more rarely reported. Some presentations are suggestive of an autosomal dominant transmission and thus to be monogenic, mainly in myeloid malignancies. Mutations in three genes encoding transcription factors important for hematopoiesis predispose to myeloid malignancies: *RUNX1*, *CEBPA* and recently *GATA2*. However, the majority of familial forms of nonsyndromic hematologic malignancies associated or not with solid tumors remain unexplained. Hodgkin lymphoma (HL) is one of the most common forms of lymphoma occurring in young adults in developed countries. It differs from other lymphomas for different characteristics. Both genetic and environmental factors (EBV infection) are thought to be involved.

In collaboration with Dr Caroline Besson, we are analyzing by whole exome sequencing a series of 10 families with at least two cases of Hodgkin lymphoma in first or second-degree relatives, which is suggestive of an autosomal dominant trait. The first screening of seven different families allowed identifying one gene mutated in two different families. Expression studies of the gene variants are under process.

Neuroendocrine tumors

L. Evenepoel, A. Mendola, M. Vikkula, in collaboration with A. Persu (Division of Cardiology, Cliniques universitaires Saint-Luc, UCL)

Pheochromocytomas, and head and neck paragangliomas are neuroendocrine tumors 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 orthosympathetic 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 tumors 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 tumors 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 tumors 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.

Genomics platform: DNA-Chip, next generation sequencing and bioinformatics

P. Brouillard, M. Amyere, R. Helaers, M. Vikkula

Our laboratory hosts the Genomics Platform of UCL, with Dr Pascal Brouillard as its Senior Platform Manager. The platform includes microarray equipment (Affymetrix; managed by Dr Mustapha Amyere), used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. It is also used in the diagnostic setting, by the Center for Human Genetics, Cliniques universitaires Saint-Luc. High Throughput Sequencing systems (funded by the Fondation Contre le Cancer) include a Personal Genome Machine (Ion Torrent) for targeted genetic studies, an Ion Proton with Ion Chef robot for larger scale targeted studies (all from Life Technologies), and a Solid 5500XL sequencer for whole genome/exome approaches. A computing cluster with 80 Terabytes of storage capacity is dedicated to data storage and processing, and bioinformatics analysis of Exome-seq, Genome-seq, and RNA-seq. Dr Raphaël Helaers (Bioinformatician) has developed "Highlander" (http://sites.uclouvain.be/highlander/), a package for in-silico annotation and filtering of sequence variants. Highlander is currently also used for diagnostics in medical centers at ULB and VUB, as well as in the IB2 institute. This platform greatly enhances our ability to identify and explore the genetic and epigenetic bases of disease.

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Hepatic and pancreatic cell differentiation in health and disease

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 our investigation of abnormal differentiation of liver and pancreatic cancer cells.

Liver development and disease

Y. Achouri, S. Cordi, V. De Greef, C. Demarez, M. Di-Luoffo, C. Gérard, A. Poncy, T. Saandi, J. Tys

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. Our preferred model organism to investigate liver development is the mouse. This includes analysis of transgenic mouse lines generated with help of the Transgene Technology platform which is managed by our research group. When disease mechanisms are investigated, findings from mouse models are validated using human tissue samples obtained from collaborating clinical research centers.

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 led to the identification of the first transcriptional network regulating cholangiocyte differentiation. Subsequent research on bile duct development led to the finding that duct formation occurs via a unique morphogenic process [1, 2]. Also, the use of lineage tracing approaches enabled us to demonstrate that hepatoblasts give rise to embryonic hepatocyte and cholangiocyte precursors, but that a fraction of the latter differentiate to adult periportal hepatocytes and adult liver progenitor cells. This work drew a new fate map of the hepatic cells during development [3] (Fig. 1A). Current efforts are devoted to the identification of the transcription factors, microRNAs and signal transduction pathways that control bile duct development in health and disease. Mathematical modeling is implemented to determine the temporal and quantitative dynamics of gene regulatory networks during hepatic cell differentiation.

Discovery of new regulators of normal liver development

In search for new molecular determinants of biliary differentiation, we investigated the transcription factor network that drives cholangiocyte differentiation and bile duct formation. Using liver-specific gene inactivation strategies we found that Sox9 controls the timing of bile duct development [2]. Sox9 is only transiently required for bile duct development, suggesting that other Sox family members are involved in biliary morphogenesis. Liver-specific inactivation of Sox4 in the mouse inhibited cholangiocyte differentiation and bile duct development. In cooperation with Sox9, Sox4 controlled several processes that are known to promote biliary development. These include Transforming Growth Factor-β (TGF-β), Notch, and Hippo-Yap signaling, as well primary cilia formation and secretion of laminin α5. Therefore, Sox4 is a new regulator of biliary development which exerts pleiotropic functions in cooperation with Sox9 [4] (Fig. 1B-D).

MicroRNAs stand out as essential regulators of hepatic cell differentiation. Our earlier work has shown how miR-122 fine-tunes the expression of liver-specific transcription factors that are key drivers of hepatocyte development [5]. Using cultured cells and transgenic mice, we have now identified a new microRNA which controls hepatic cell differentiation during development. When hepatoblasts differentiate toward the hepatocyte

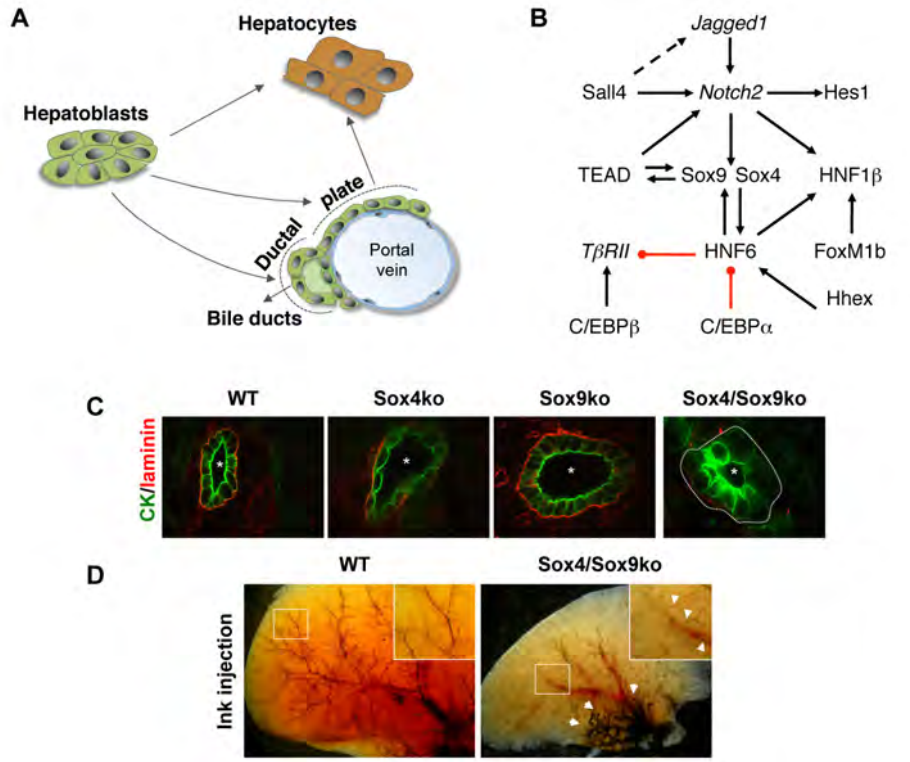

Fig. 1. Sox4 cooperates with Sox9 to control bile duct development. (A) Cell fate map in developing liver: hepatoblasts give rise to hepatocytes and to cholangiocyte precursors; the latter mature to adult cholangiocytes lining bile ducts or revert to a periportal hepatocyte phenotype. (B) Gene regulatory network driving differentiation of hepatoblasts to the cholangiocyte lineage. (C) In the absence of Sox4 (Sox4ko), mouse livers at postnatal day 6 show cobblestone-like cholangiocytes lining ducts that are irregularly delineated by laminin. In the combined absence of Sox4 and Sox9 (Sox4/Sox9ko) no laminin is detected, cholangiocytes have irregular shape and abnormal cytokeratin expression. (D) Staining of bile ducts in adult mouse liver by retrograde injection of ink shows that the combined absence of Sox4 and Sox9 prevents development of intrahepatic ducts and is associated with dilated hilar ducts. WT, wild-type; dotted line delineates duct; *, duct lumen; arrowheads, ink-labeled bile ducts (from Ref. 4).

or cholangiocyte lineage, this microRNA stimulates expression of cholangiocyte genes while repressing hepatocyte genes. After lineage segregation, it controls the transcriptional network dynamics of developing hepatocytes. Consistent with a mathematical model, it exerts a biphasic control on a key transcriptional regulator by means of an incoherent feed-forward loop motif. Our work highlights that control of hepatic cell differentiation depends on microRNAs, and that slight changes in microRNA concentration can very significantly affect cell differentiation dynamics (manuscript in preparation). Further integration of experimental and mathematical approaches is ongoing to unravel hepatic cell differentiation. This work is in collaboration with Prof. Emmanuel Hanert, Université catholique de Louvain, for the modeling aspects.

Along the same lines, cell differentiation is tightly controlled by intercellular signaling mechanisms. Several signaling pathways stimulate differentiation of developing biliary cells. Our group identified TGF-β signaling as a key driver of this process [1], and contributed to the understanding of the role of the Notch pathway. We pursued this work by investigating the function of the Wnt signaling. In the latter context, we revisited the role of β-catenin, a mediator of canonical Wnt signaling, in bile duct development. Using gain- and loss-of-function experiments in transgenic mice, we found that β-catenin expression is dispensable for differentiation of developing cholangiocytes and bile duct morphogenesis, but that upregulation of its expression significantly perturbs bile duct structure [6]. Like for our work on microRNAs, our findings on β-catenin indicate that expression of developmental regulators must be kept within very tight limits for normal liver differentiation and morphogenesis (Fig. 2).

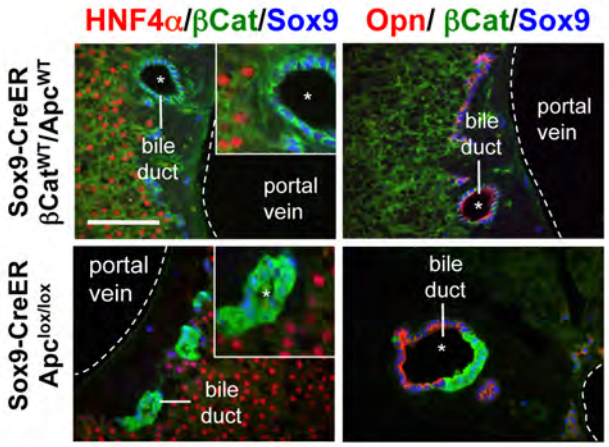

Fig. 2. Overexpression of β-catenin perturbs bile duct morphogenesis. β-catenin was induced by inactivation of Adenomatous Polyposis Coli (APC) in developing bile ducts using tamoxifen-inducible Sox9-CreER. Tamoxifen was injected at E14.5 and embryos were collected at E18.5, prior immunostaining analysis with the indicated markers. Scale bar, 100 μm (from Ref. 6).

Pathogenesis of biliary malformations and liver cancer

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, 7]. Starting from this new knowledge, we analyzed several mouse models and samples from human liver fetuses affected with biliary anomalies.

The work on biliary malformations has now focused on polycystic liver disease. Indeed, it has been shown that biliary cysts in adult patients affected by polycystic liver disease are lined by cholangiocytes that proliferate, suggesting that initiation of cyst formation depends on proliferation. We have challenged this view by analyzing cyst-lining cell proliferation and differentiation in livers from human fetuses affected by Autosomal Recessive Polycystic Kidney Disease (ARPKD), and in *Cpk* mouse embryos (a mouse model of ARPKD), at early stages of cyst formation. Our analyses provide evidence that the polycystic livers exhibit increased and accelerated differentiation of hepatoblasts into cholangiocyte precursors, eventually forming large biliary cysts. Therefore, contrary to current belief, cyst formation in polycystic liver disease does not necessarily depend on overproliferation. Combining our prenatal data with available data from adult livers, we propose that polycystic liver can be initiated by proliferation-independent mechanisms at a fetal stage, followed by postnatal proliferation-dependent cyst expansion [8].

Cholangiocarcinoma is the second most common primary liver tumor, and histological differentiation is usually considered a prognostic criterion, with well-differentiated tumors being associated with higher survival rate. In an effort to address the role of microRNAs in cholangiocarcinoma cell differentiation, we have measured the expression of cholangiocyte-enriched microRNAs in several samples of human cholangiocarcinoma and attempted to correlate the expression of microRNAs, cholangiocyte differentiation markers and histological criteria of cholangiocarcinoma cell differentiation. To our surprise, no correlation was found between cholangiocyte differentiation marker expression and histological criteria of cholangiocarcinoma cell differentiation (Fig. 3). This indicates that classification of intrahepatic cholangiocarcinoma and prognostic criteria need to be revisited [9]. This work was performed in collaboration with Profs. C. Sempoux (Centre Hospitalo-Universitaire Vaudois, Lausanne) and Catherine Hubert (Cliniques universitaires Saint-Luc). In parallel, to functionally address differentiation of cholangiocarcinoma cells, we have started to set up a novel mouse model of cholangiocarcinoma, in collaboration with Dr. M. Komuta (Department of Pathology, Cliniques universitaires Saint-Luc).

Chronic liver injury in humans is often associated with proliferation of cells that derive from the bile ducts. Using lineage tracing tools that were developed in the laboratory (Osteopontin-CreER mice), we showed in collaboration with Prof. Isabelle Leclercq (Laboratory of Hepato-Gastroenterology, Université catholique de Louvain) that these proliferating cells can give rise to functional hepatocytes, albeit in small proportion [10]. Further work in collaboration with Prof. Robert Schwabe (Columbia University, New York) showed that these biliary-derived cells are unlikely to be the source of hepatocellular carcinoma, even when the cancer cells express genes typical for progenitor cells and bile duct cells [11]. Therefore, hepatocellular carcinoma originate from hepatocytes and can undergo a switch toward a progenitor or biliary phenotype.

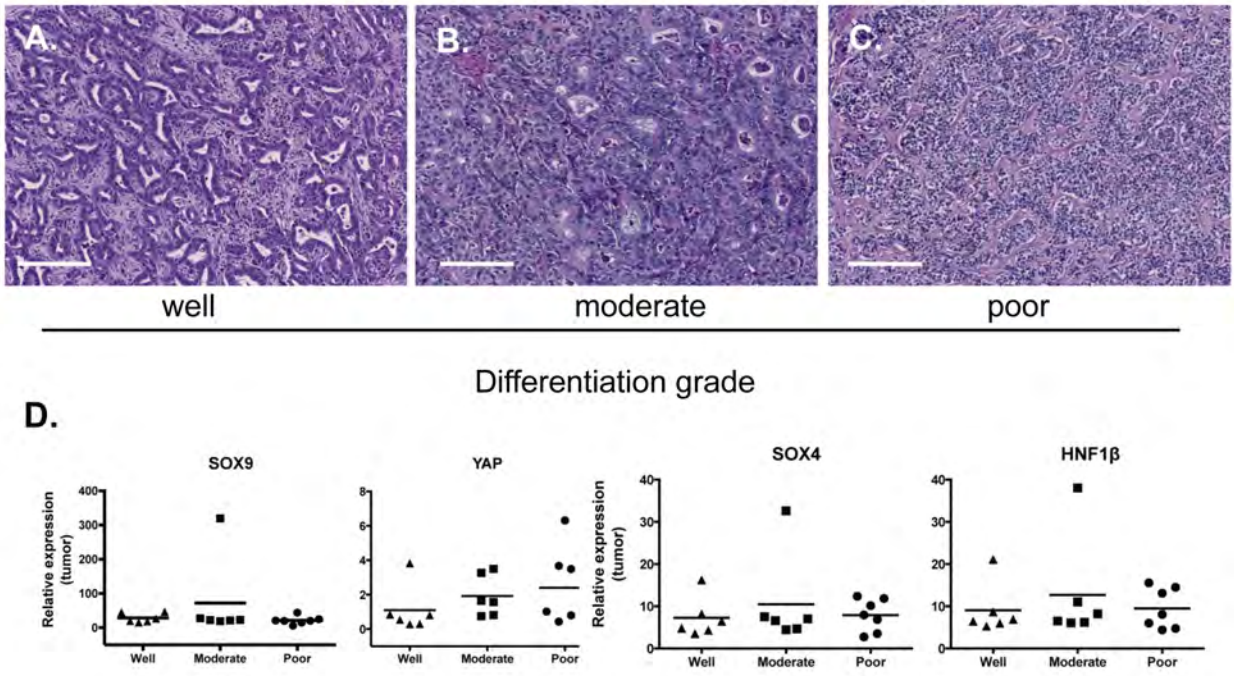

Fig. 3. Histological differentiation criteria do not correlate with differentiation marker expression in intrahepatic cholangiocarcinoma. Tumors are classified as well (A), moderately (B) or poorly (C) differentiated after hematoxylin/eosin staining. (D) Expression of biliary differentiation markers does not correlate with histological differentiation grade.

Pancreatic cell differentiation

Y. Achouri, C. Augereau, L. Collet, S. Cordi, V. De Greef, C. Gérard, E. Ghurburrun, N. Meyers, T. Saandi

In the embryo, pancreatic progenitors derive from the endoderm and give rise to endocrine, acinar and duct cells. Our group investigates how pancreatic cell types differentiate in the embryo and how differentiation is perturbed in adult pancreas in the context of tumorigenesis of pancreatic ductal adenocarcinoma. Similar to our approach in liver-related research, we address differentiation of pancreatic cells by focusing on the role of transcription factors, microRNAs and signal transduction pathways, during pancreas development and during initiation of pancreatic cancer.

Discovery of a gene network determining pancreatic acinar cell differentiation

The role of the 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 [12, 13]. After birth, HNF6 is expressed exclusively in the duct cells where it contributes most likely to maintain duct cell identity.

In acinar cells HNF6 expression must be actively kept low to maintain normal acinar differentiation. Indeed, overexpression of HNF6 in acinar cells repressed acinar-specific transcription factors (RBPjl, Mist1) while inducing the ductal transcription factor Sox9 as well as a number of ductal markers and duct-like morphology [14]. Repression of HNF6 in normal acinar cells critically depends on miRNAs. Indeed, when miRNA synthesis was inactivated by knocking out expression of Dicer, HNF6 was aberrantly induced in acinar cells. Systematical screening of candidate miRNAs identified miR-495 and let-7b as key repressors of HNF6. Together, this work identified a gene network determining normal acinar cell identity (Fig. 4). Further work investigates the role of other candidate miRNAs in acinar cell differentiation.

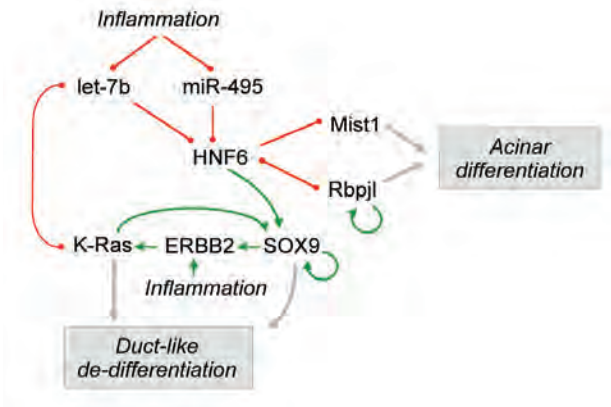

Fig. 4. Gene regulatory network controlling acinar differentiation and acinar-to-ductal metaplasia.

Pathogenesis of pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) may derive from acinar cells which switch their phenotype from acinar to ductal during progression to cancer. Therefore, similar to cholangiocarcinoma, transdifferentiation events may initiate tumorigenesis in the pancreas. Pathologists have described the conversion of acinar cells to a ductal phenotype, a process called *acinar-to-ductal metaplasia* which constitutes a preneoplastic state. We hypothesized that such switch in cell identity would depend on perturbation of the acinar gene network involving HNF6 and on the ectopic expression of ductal transcription factors. This was the case since HNF6 was found to be ectopically induced in human acinar cells undergoing metaplasia. In addition, we collected evidence from mouse models that induction of a HNF6 – Sox9 cascade in acinar cells promotes acinar-to-ductal metaplasia. This indicated that HNF6 and Sox9 are key inducer of preneoplastic lesions [15].

Acinar-to-ductal metaplasia can progress to a more advanced pre-neoplastic stage called *Pancreatic Intraepithelial Neoplasia* (PanIN). This raised the question about the role of Sox9 in tumor progression. Using a mouse model where PDAC is induced by inflammation and expression of the oncogenic mutant K-Ras^{G12D} protein, we and others found that inhibition of Sox9 protects against tumor development [16]. These results raised questions on the targets of Sox9 in acinar-to-ductal metaplasia and during tumor progression to PanIN and PDAC. In collaboration with Prof. C. Sempoux (Centre Hospitalo-Universitaire Vaudois, Lausanne) and with the teams of Ilse Rooman and Andrew Biankin (Garvan Institute of Medical Research, Sydney, Australia) we analyzed genomic and transcriptomic data from surgically resected PDAC, from xenografts from PDAC samples and from PDAC cell lines. SOX9 gene aberrations were found in about 15% of patient tumours. Most PDAC samples strongly expressed SOX9 protein and high expression of SOX9 correlated with expression of genes belonging to the ERBB pathway (Fig. 5). In particular, ERBB2 expression in human PDAC cell lines was stimulated by SOX9, and work using mouse models with pancreas-specific inactivation of Sox9 confirmed its role in PDAC initiation and stimulation of ERBB signaling activity. Therefore, by integrating data from patient samples and mouse models, we conclude that SOX9 regulates the ERBB pathway throughout pancreatic tumorigenesis.

This work is now pursued in collaboration with Prof. Ivan Borbath (Laboratory of Hepato-Gastroenterology, Université catholique de Louvain) and is extended by investigating the function of miRNAs, oncogenes and ERBB signaling in PDAC initiation from acinar cells and from duct-derived cystic preneoplastic lesions. Along the same lines, the relationship between genetic lesions initiated in the pancreatic duct cells and subsequent inflammation and phenotypic switches affecting the acinar cells are currently being investigated. Our initial results, which were obtained with help of improved RNA purification techniques [17], suggest that there is a tight association between defects in primary cilia function occurring in duct cells, and pancreatitis leading to perturbed acinar homeosta-

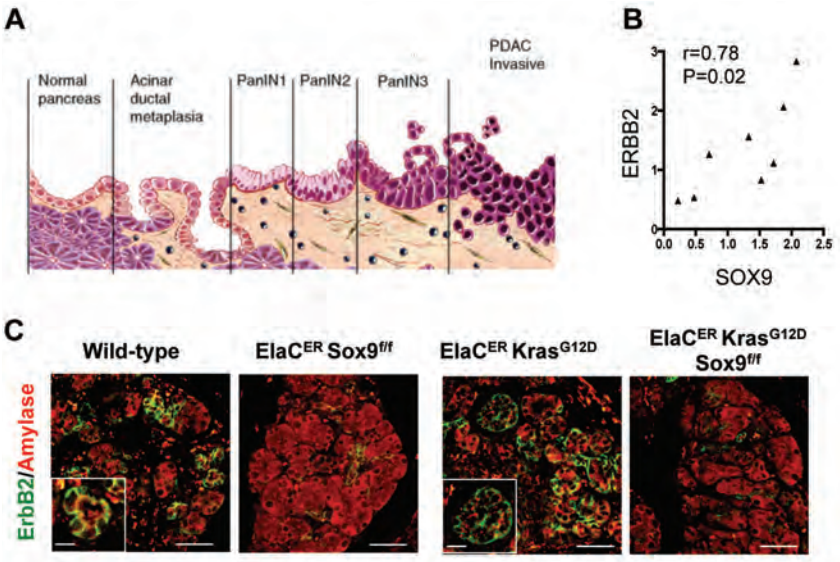

Fig. 5. SOX9 regulates ERBB2 expression during pancreatic tumorigenesis. (A) Schematic representation of pancreatic tumorigenesis resulting from neoplastic transformation of acini into acinar-to-ductal metaplasia, PanIN and PDAC (adapted from: Molecular Relationships Between Chronic Pancreatitis and Cancer, Logsdon C.D. *et al.*, *in* Pancreatic Cancer, Neoptolemos J.P. *et al.* (Eds), Springer). (B) Correlation plot of SOX9 and ERBB2 expression in a panel of PDAC cell lines. (C) During acute cerulein-induced pancreatitis in mice, ERBB2 is induced in metaplastic acini; this induction is increased in the presence of oncogenic K-Ras^{G12D} and requires Sox9 (from Ref. 16).

sis. These observations can account for the increased risk to develop pancreatic cancer in families with high predisposition to develop such tumor types.

Finally, we also resort to mathematical modeling which helps in identifying the dynamics of tumor progression and in predicting the role of specific components of gene networks during cell transformation [18]. Modeling of the network illustrated in Figure 3 has progressed and is focusing on the development of a quantitative model based on gene and protein expression data from cultured cells and transgenic mice.

Conclusions

Our findings on the mechanisms of hepatic and pancreatic cell differentiation contributed to a better understanding of the diseases affecting these organs. In liver, our work opens perspectives for characterizing the pathophysiology of congenital diseases of the liver, for directed differentiation of hepatocytes in culture for cell therapy of liver deficiencies, and for unraveling the mechanisms of tumor initiation. In pancreas, our observations on the gene regulatory networks in preneoplastic lesions are expected to accelerate diagnosis and to help preventing progression towards pancreatic ductal adenocarcinoma.

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Signaling crosstalk in skin epidermal cells: From tissue homeostasis to tumorigenesis

Wen-Hui Lien

Skin epidermis is constantly renewed, and mature hair follicles (HFs) undergo cyclical bouts of regeneration throughout life. Skin epidermal stem cells that can self-renew and differentiate provide the unlimited source of cells required for long-term tissue morphogenesis, homeostasis, and injury repair. The behavior of stem cells is fine-tuned by the microenvironmental cues and their responsive intracellular signaling regulation. Disruption of such regulation could have contributed to the development of tumors that originate from dysregulated stem cells. The main interest of our group is to understand how one signaling pathways cooperate and integrate to dynamically regulate stem cell activation, differentiation and fate determination, as well as tumor formation when stem cells go wrong. Our studies will provide an integrative view of signaling regulation on stem cell function and tumorigenesis, and extend our understanding for regenerative medicine and treatment of pathological conditions.

The behavior of stem cell is fine-tuned by the microenvironmental cues and their responsive intracellular signaling regulation

The skin is the largest organ in the body. It is composed of two major compartments: an outer epithelial compartment (epidermis) and an inner mesenchymal compartment (dermis). Epithelial skin is made of epidermis and its appendages: hair follicles and sebaceous glands. Mesenchymal skin is largely made by fibroblast cells dispersed within extracellular matrix. Throughout life, skin epithelium undergoes constant turnover and regeneration, and the unlimited source for this regeneration is fueled by skin epidermal stem cells that can self-renew and differentiate. Skin epidermal stem cells encompass epidermal stem cells (IFE-SCs), which are localized in the basal layer of epidermis, and hair follicle stem cells (HFSCs), which reside in a region of the hair follicle (HF) outer root sheath, known as the bulge.

In adult skins, hair follicles undergo cyclical bouts of active hair follicle regeneration and hair growth (anagen), followed by degeneration (catagen) and then rest (telogen) (Fig. 1). HFSCs are located at the base of the telogen phase HF and maintained in a quiescent state during the resting phase of hair cycle. During hair growth, they then get activated and replace the cells in the lower hair follicle, or move upward and differentiate into the epidermis and sebaceous gland during wound healing. HFSCs are surrounded by their niche cells, including inner layer of keratin-6 (K6)-expressing cells and the outer layer of basement membrane. A small group of primed HFSCs, called hair germ (HG), reside at the base of bulge and will be the first progenitors activated at the onset of each new hair cycle. In addition, a group of the specialized mesenchymal cells, called dermal papilla (DP), underlies the HG and provides essential mesenchymal signaling for HFSCs.

Tissue homeostasis and regeneration require a precise balance between quiescence and activation of adult stem cells. What keeps stem cells in their bulge niche during times of quiescence and what prompts them or their immediate progeny to regenerate the follicle during hair growth or repair the epidermis upon wounding? The behavior of stem cell is fine-tuned by the microenvironmental cues and their responsive intracellular signaling regulation. Transcriptional profiling of purified HFSCs in the adult bulge has provided a molecular signature which offers some insights into the possible mechanisms. Coupled with gene profiling, subsequent functional studies have revealed several signaling pathways, including BMP, Wnt and TGFβ signaling pathways, essential for maintaining stem cells in either a quiescent or activated state. While the role of individual signaling pathway regulating stemness and differentiation of stem cells has been well documented, understanding how these pathways cooperate is critically missing. Thus, studying the mechanism underlying signaling-mediated regulation on stem cell function and how signaling pathways integrate and cooperate to dynamically regulate

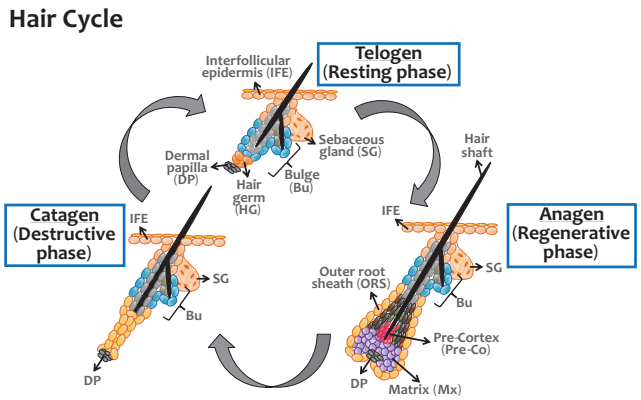

Fig. 1. Hair cycle. During the resting phase (telogen), HFSCs residing in the bulge (Bu) remain in quiescence as the terminally differentiated inner bulge cells express high levels of inhibitory signals. At the onset of the regenerative phase (anagen), activated HFSCs located in hair germ (HG) proliferate and initiate HF regeneration in response to the activating cues produced from crosstalk with the underlying mesenchymal stimulus, referred to as the dermal papilla (DP). Soon after, HFSCs in the bulge are also activated. Some activated HFSCs move downward from the bulge along the outer layer of HFs (ORS), creating an inverse gradient of proliferative cells that fuel the continued prodution of most proliferative, transient amplifying matrix cells (Mx) at the base of the full anagen HFs. In response to high levels of Wnt signaling, matrix progenitors in the pre-cortex region (Pre-co) terminally differentiate to form the hair shaft (HS). At the end of anagen, HFs enter a destructive phase (catagen) and the matrix and much of the lower part of the HF undergo apoptosis. As the epithelial strand regresses, DP is drawn upward towards the bulge/HG and the HF re-enters telogen. IFE, interfollicular epidermis; SG, sebaceous gland.

context-dependent cell fate changes becomes important for regenerative medicine and also for treatment of pathological conditions such as cancer.

In our laboratory, we use mouse skin as a model system, as well as human skin tumor tissues, to address these important biological questions. Our research mainly focuses on understanding how one signaling pathway integrates to another and how their downstream regulators crosstalk to each other in HFSCs and/or with their neighboring cells during tissue homeostasis and tumorigenesis.

Wnt signaling pathways in hair follicle stem cells and skin tumors

In mammals, Wnt signaling functions in tissue morphogenesis, wound repair and cancers. Wnt signaling is known to play a role in many adult stem cells, but exactly how it functions and for what purpose has been a matter of much debate. Wnt proteins are secreted glycoproteins that can stimulate multiple intracellular signaling pathways. They act as growth factors that regulate diverse processes, including cell proliferation, differentiation, migration and polarity. In addition, deregulation of Wnt signaling has been linked to several human diseases and cancers.

The interaction between Wnt ligands and receptors results in an activation of various intracellular signaling cascades that might be cross-connected or act independently. In general, Wnt signaling pathways can be divided into two categories: canonical and non-canonical Wnt pathways. Canonical Wnt signaling is often referred to as Wnt/β-catenin pathway, in which Wnt-stimulated signals trigger a β-catenin-dependent transcriptional activation, whereas non-canonical Wnt pathways are β-catenin-independent and usually trigger a variety of different intracellular signaling cascades [1].

Among Wnt-stimulated pathways, canonical Wnt signaling is known as an important regulatory pathway that governs developmental process and regulates maintenance and differentiation of adult stem cells. The downstream effector of canonical Wnt signaling is β-catenin that plays dual roles in cell-cell adhesion and transcriptional regulation. In the absence of Wnt signals, β-catenin levels are regulated by a destructive complex that phosphorylates β-catenin and targets for its degradation. Upon the interaction of Wnt ligands to its receptors, the formation of the degradation complex is inhibited, thereby stabilizing β-catenin. Stabilized β-catenin is accumulated in the cytoplasm and then enters to the nucleus where it acts as a transcriptional co-activator for the lymphoid enhancer-factor/T-cell factor (LEF/TCF) transcription factors (Fig. 2). It is also known that in the absence of nuclear β-catenin, TCFs interact with transducin-like Enhancer of split (TLE) proteins that in turn recruit histone deacetylases (HDACs) to prevent the inappropriate activation of TCF target genes.

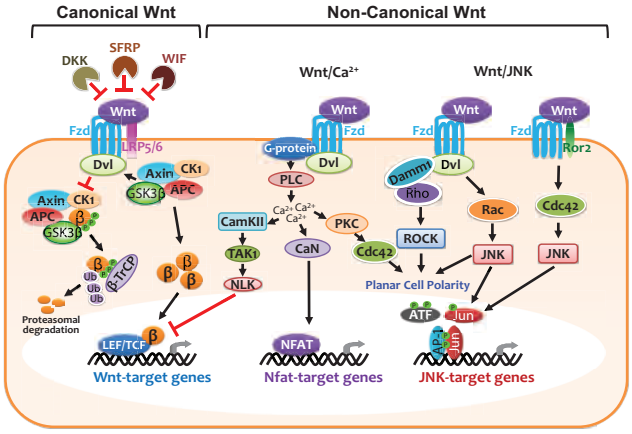

Fig. 2. Wnt signaling pathways. This schematic diagram displays simplified canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) Wnt signaling pathways. In the absence of Wnt, β-catenin is targeted by a destructive complex that phosphorylates β-catenin for its degradation. Binding of Wnt to Frizzled (Fzd) and lipoprotein receptor-related protein 5/6 (LRP5/6) activates the cytosolic protein Dishevelled (Dvl), leading to the inhibition of the complex. Accumulation of stabilized β-catenin in the presence of LEF/TCF transcription factors results in their translocation into the nucleus to activate Wnt-responsive genes. This activation can be suppressed by TAK1-NLN, which is activated through non-canonical Wnt pathways. Delineated here are also Wnt/Ca2+ and Wnt/JNK pathways, both of which are β-catenin-independent. Binding of Wnt isoforms to either Fzd or other tyrosine kinase-like receptors, *e.g.* Ror2, can trigger multiple signaling cascades. Some of them result in activation of small GTPase Rho, Rac, and Cdc42 that regulate cytoskeleton rearrangement and planar cell polarity (PCP); some of cascades trigger transcriptional events by activating transcription factors, *e.g.* NFAT or AP-1.

In adult HFs, Wnt/β-catenin signaling is required for HFSC activation and hair cycle progression. Our recent study revealed that β-catenin is essential to activate genes that launch HF fate and suppress sebocyte fate determination (Fig. 3). We further uncovered that TCF3, TCF4 and TLEs bind coordinately and transcriptionally repress Wnt target genes during HFSC quiescence. This TCF-TLE interaction recruits HDAC and acts as a repressive rheostat, whose action can be relieved by Wnt/β-catenin signaling. When TCF3/4 and TLE levels are high, HFSCs can maintain stemness, but remain quiescent. When these levels drop or when Wnt/β-catenin levels rise, this balance is shifted and hair regeneration initiates [2].

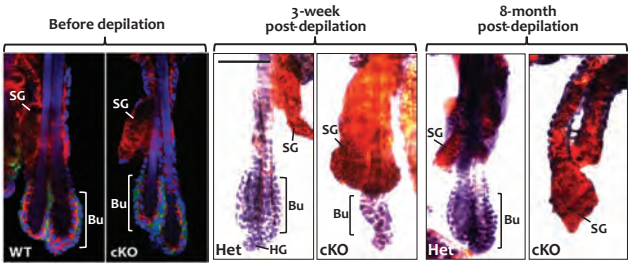

Fig. 3. β-catenin is essential for HFSCs to make hair follicle fate choice. Immunofluorescence staining (left panel) shows that β-catenin-deficient HFSCs (cKO) could be maintained in a quiescent state without losing stemness (Red, β-catenin; Green, stem cell marker CD34); however, upon depilation-induced activation (middle and right panels), β-catenin-deficient HFSCs differentiate into sebocytes progressively and eventually deplete the niche of its stem cells. Oil Red O stainings of sebocytes on whole mount skins are shown.

Unlike Wnt/β-catenin pathway, non-canonical Wnt pathways are more diverse and less well-studied, and most of their attention comes from their ability to interfere with canonical Wnt/β-catenin signaling. Based upon the intracellular mediators used, the non-canonical Wnt pathways can be subdivided into two general categories: Wnt/calcium (Ca2+) and Wnt/ c-Jun N-terminal kinase (JNK) pathways (Fig. 2). Some of downstream Wnt/Ca2+ pathways, such as TGFβ-activated kinase 1-activated Nemo-like kinase (TAK1/NLK) and calcium-calmodulin dependent kinase II (CamKII), can block β-catenin-induced transcriptional activity; others, such as calcineurin, can activate nuclear factor of activated T cell (NFAT)-mediated transcriptional regulation. In parallel, protein kinase C (PKC) members can activate the small GTPase Cdc42 which can in turn funnel into the planar cell polarity pathway (PCP). PCP can also be co-regulated by Rho and Rac GTPases, which are activated in Wnt/JNK non-canonical signaling. In contrast to calcium-regulated non-canonical signaling, Wnt/JNK signaling uses Ror2-dependent circuitry to activate downstream effectors of the activating protein-1 (AP-1) family of transcription factors (Fig. 2).

While Wnt/β-catenin pathway has been extensively studied in stem cells, non-canonical Wnt pathways are underappreciated and remain elusive. In the quiescent hair follicle stem cell niche, perceived Wnt signaling is low, corresponding to the high levels of TCF3, TCF4 and TLEs as well as BMP and calcium signaling, which leads to high levels of nuclear NFATc1, typically viewed as a non-canonical Wnt effector. In one of our recent collaborative studies, we revealed Nfatc1-bound target genes in HFSCs [3]. Interestingly, by comparing gene targets of HFSC key regulators, we found that TCF3, TCF4 and NFATc1 share a cohort of target genes that are usually highly expressed in quiescent HFSCs and many of which involve in regulation of HFSC maintenance. This intriguing finding leads to one important question: whether TCF3/4 and NFAT cross-interact with each other to maintain HFSC function. To tackle this question, our group generated a mouse line to disrupt Wnt/calcium signaling pathway specifically in HFSCs. By coupling multiple technologies, such as fluorescent activated cell sorting (FACS) and primary cell culture, we are examining the crosstalk of downstream effectors of Wnt signaling pathways in HFSCs during skin homeostasis.

In parallel, our group also conducted loss-of-function approaches using transgenic mouse models to determine the role of Ror2-dependent Wnt signaling pathway in HFSCs during HF regeneration, as well as in carcinogen- or oncogene-induced skin tumors, *e.g.* squamous cell carcinoma (SCC). By collaborating with Dr. Benoît Lengele, surgeon at the Cliniques universitaires Saint-Luc, we have collected and analyzed human SCC tissues (Fig. 4). Using these human specimens, we have successfully purified and grown human SCC keratinocytes in culture for further analyses. Currently, we are investigating how non-canonical Wnt signaling pathways regulate stem cell function and stem-cell-originated tumorigenesis. The ultimate goal of our research from mouse models and human tissues is to identify clinical relevance of main regulators involved in non-canonical Wnt signaling pathways and to use them as therapeutic targets to treat diseases and cancer.

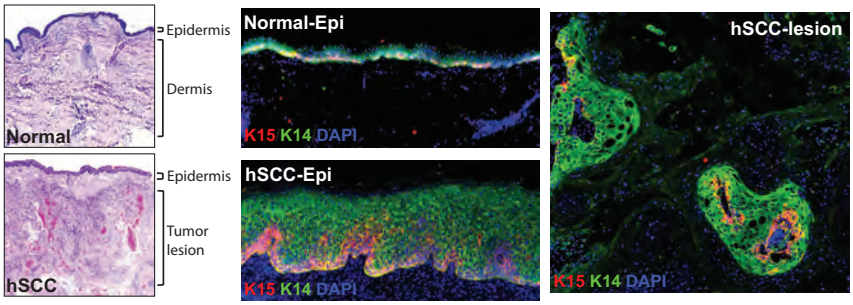

Fig. 4. Human squamous cell carcinoma. H&E staining (left panel) and immunofluorescence staining (right panel) of human normal skin (Normal) and squamous cell carcinoma (hSCC) show that patient's tumor samples display a significant expansion of interfollicular epidermis (hSCC-Epi vs Normal-Epi) and a massive outgrowth of tumor lesion (hSCC-lesion) that are positive for epidermal basal cell markers, Keratin 15 (K15, red) and Keratin 14 (K14, green).

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Purine analogs in leukemia

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 analogs, cladribine 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 purine analogs and to find novel therapeutic strategies to counteract them, particularly in chronic lymphocytic leukemia.

In 1997, a collaborative study of the antileukemic purine analogs cladribine (CdA) and fludarabine (Fig. 1) was started with the Department of Hematology of the University Hospital Saint-Luc. These two analogs of 2'-deoxyadenosine display remarkable therapeutic properties in indolent lymphoid malignancies, especially chronic lymphocytic leukemia (CLL). Nevertheless, resistance is also observed, and these drugs 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 purine analogs, and to improve their therapeutic efficacy by searching for synergisms with other compounds.

CdA and fludarabine are prodrugs. To exert their antileukemic effect, they have to be phosphorylated by deoxycytidine kinase (dCK) into purine analog monophosphate, which is then converted into di- and triphosphate derivatives. The latter are the active metabolites. Their intracellular accumulation causes inhibition of various reactions involved in DNA and RNA synthesis. Moreover, they can be incorporated into newly synthesized DNA during DNA replication or repair. Together, these actions induce DNA damage and lead to apoptosis by mechanisms that are not yet entirely clear [1, for a review].

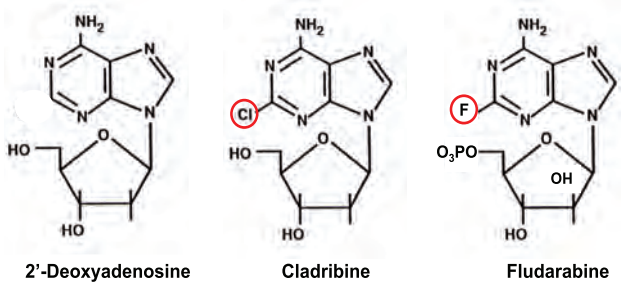

Fig. 1. Structures of 2'-deoxyadenosine and antileukemic purine analogs.

Mechanisms of resistance

In collaboration with L. Knoops from the Ludwig Institute for Cancer Research (Brussels), we performed microarray analyses to compare the genes induced or repressed by purine analogs in sensitive and refractory CLL patients. We found that CdA and fludarabine predominantly increased the expression of p53-dependent genes in chemosensitive samples, 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 CdA and fludarabine 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 CdA and fludarabine correlated well with PLK2 mRNA induction. In conclusion, we proposed that testing PLK2 activation after a 24-h incubation with CdA or fludarabine could be used to investigate the functional integrity of the p53/DNA damage pathway in CLL cells [2], and predict clinical sensitivity to these drugs [3, for a review of p53 functional analysis in CLL]. The following step was to investigate the role of PLK2 during CdA- or fludarabine-induced apoptosis. However, the protein PLK2 could not be detected in CLL cells, even after treatment with purine analogs, precluding a role of PLK2 in induction of apoptosis by these compounds. 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

Aiming to improve the efficacy of purine analogs, we investigated their combination with some other DNA-damaging agents, such as cyclophosphamide derivatives [4]. We showed that combination of CdA with these agents resulted in synergistic cytotoxicity in CLL lymphocytes, due to inhibition by CdA of DNA repair activated in response to these compounds. The *in vitro* synergy between CdA and cyclophosphamide has provided the rationale for a clinical trial of this combination, which gave encouraging results.

Later, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway, known as a survival pathway. We observed that CdA, at concentrations close to the IC50, activated the ERK pathway in EHEB cells. Because this activation is assumed to produce anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway, which were found to enhance CdA-induced apoptosis. Therefore, combining CdA with inhibitors of the ERK pathway could enhance its efficacy [5].

Finally, we recently demonstrated that aphidicolin, a DNA polymerase inhibitor, markedly synergizes with fludarabine and cladribine in primary CLL cells, decreasing their IC_{50} by 4.5- and 2.8-fold, respectively [6]. We found that this potentiating effect of aphidicolin was associated with an increase of purine analog-induced DNA damage due to inhibition of DNA repair. We also obtained experimental evidences that the DNA repair pathway targeted by aphidicolin was the NER (nucleotide excision repair) pathway, providing a rationale for combining purine analogs with NER inhibitors.

Activation by deoxycytidine kinase

Deoxycytidine kinase (dCK) is the limiting enzyme in the activation of CdA, fludarabine and several other nucleoside analogs used in anticancer and antiviral therapy (Fig. 2). Study of the mechanisms that control the activity of this enzyme is thus of particular interest. We demonstrated that dCK is a phosphoprotein, containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylation site and the only one to play a role in the regulation of dCK activity [7]. Phosphorylation of dCK at Ser-74 was observed in several cell lines and in lymphocytes from CLL patients in which variability in dCK activity could be related to variations in basal Ser-74 phosphorylation level [8]. Treatment of these cells with genotoxic agents (CdA, UV light, etoposide, genistein and aphidicolin, …) was 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. Next step was the identification of the protein kinase(s) and phosphatase(s) that control Ser-74 phosphorylation and dCK activity. We showed that casein kinase 1 δ can phosphorylate dCK on Ser-74 and increase its activity *in vitro*, but not *in vivo*, while another group thereafter reported that ATM, a DNA damage-activated kinase, can phosphorylate Ser-74 *in vitro* and *in vivo* and so activate dCK after γ irradiation. We recently demonstrated that ATR, besides ATM, plays a crucial role in the control of dCK activity. Indeed, we found that ATR is the kinase that controls basal dCK activity and activates dCK in response to replication stress (UV, aphidicolin) [9]. As for the dephosphorylation of Ser-74, we showed that protein phosphatase 2A (PP2A) is involved in Ser-74 dephosphorylation in intact cells and is therefore a negative regulator of dCK activity [10].

On the other hand, we investigated whether increase of Ser-74 phosphorylation enhances dCK activity toward nucleoside analogs as it does for deoxycytidine. 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 (*k*cat/*K*m) were not, or only slightly, increased. Importantly, we did not observe an increase of endogenous dCK activity toward fludarabine and CdA 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 analog, 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 CdA or fludarabine [11].

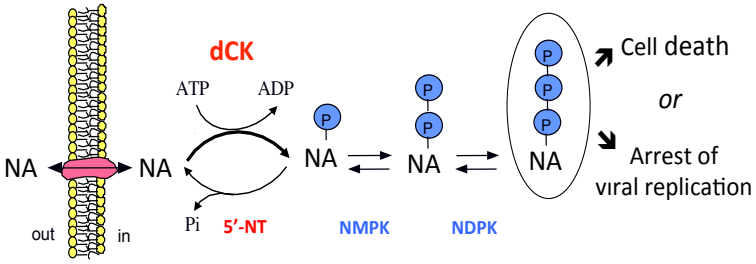

Fig. 2. Activation of nucleoside analogs. Nucleoside analogs (NA), including purine and pyrimidine analogs, are transported across cell membrane via nucleoside-specific membrane transporters and phosphorylated by cellular kinases to their triphosphate form responsible for their pharmacological activity. The first phosphorylation is catalyzed by deoxycytidine kinase (dCK) and is the rate limiting-step in NA activation. 5'-NT, 5'-nucleotidase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase.

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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.

Regulation of mammalian telomeric transcription

A. Diman, F. Poulain, 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 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 positively 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, 2]. 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. In a separate study, we described the role of H3K27me3 mark in H3K9me3-dependent anchorage of HP1α at chromatin [3].

We are continuing our investigation of telomere transcription regulation. Notably, we screened human subtelomeric promoters for the presence of predicted transcription factor bind-

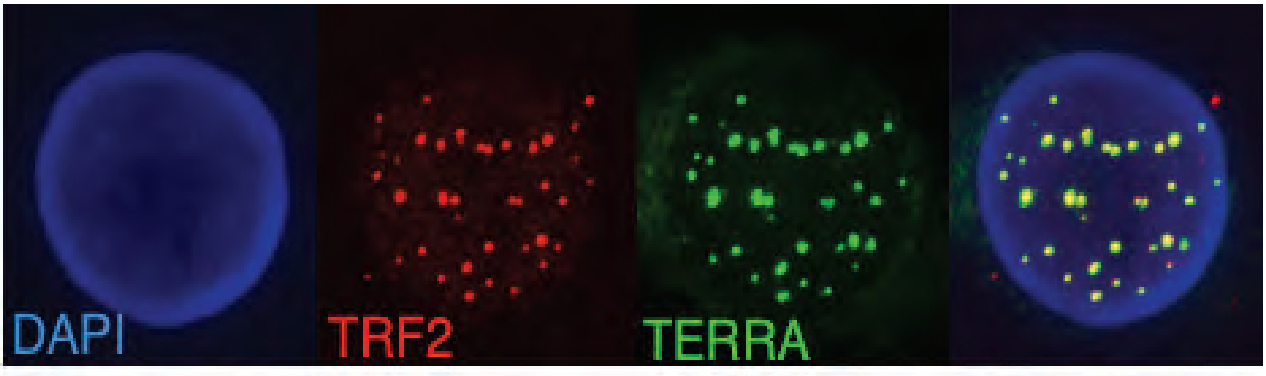

Fig. 1. Detection of TERRA telomeric transcripts at human telomeres. Telomeres are detected by immunofluorescence against TRF2 telomeric protein (red), TERRAs are detected by RNA-FISH using a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue)

ing sites. We identified Nuclear Respiratory Factor 1 (NRF1), an AMPK-regulated transcription factor, as a possible candidate. We found that NRF1 indeed regulates human telomere transcription and that transcription is co-activated by PGC-1α, a master regulator of cell metabolism. In collaboration with the team of Marc Francaux (Institute of NeuroScience, Université catholique de Louvain), we tested the impact of endurance exercise on telomere transcription in muscle biopsies and found that exercise-induced PGC-1α activation up-regulates TERRA [4]. Altogether, our data suggest a new link between metabolism and human telomere biology and fits with the emerging idea that physical exercise protects against telomere erosion. Recently, we also started a collaboration with the group of Patrick Révy (Institut Imagine, INSERM, Paris) to investigate the impact of mutations in poly(A)-specific ribonuclease PARN gene on TERRA regulation. Patients with PARN mutations suffer from telomeropathies that are characterized by, among others, a premature ageing phenotype and a severely compromised immune response.

We also started to investigate mouse TERRA regulation. We are currently characterizing mouse TERRA molecules at the level of their sequence, length and intracellular localization. In collaboration with the team of Frédéric Lemaigre, we are studying TERRA regulation during mouse embryogenesis.

Telomeres defects in cells with an alternative mechanism of telomere lengthening

H. Episkopou, E. Majerova, A. Decottignies

Cancer cell immortalization is achieved through acquisition of a telomere maintenance mechanism (TMM). Most of the time, this is achieved through telomerase gene reactivation. However, some tumors, like sarcomas and central nervous system tumors, frequently activate an alternative mechanism of telomere maintenance, called ALT, that relies on homologous recombinations between telomeric sequences. Pediatric tumors, that mostly comprise tumors from the CNS (neuroblastomas, ...) and sarcomas, are characterized by high frequency of ALT. These two pathways of telomere maintenance are very distinct phenotypically. In telomerase-expressing cells (TEL+), telomere length is homogenous. However, in ALT cells, telomeres are very heterogeneous in length and co-localize with PML bodies where they recombine (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 ALT telomeres undergo so frequent recombinations 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. 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]. Recently, new evidences were provided in favor of a distinct heterochromatin structure at ALT telomeres. Notably, mutations in ATRX, a histone chaperone acting at telomeres, have been correlated with the ALT phenotype in pediatric tumors. To analyze telomeric chromatin in ALT and TEL$^+$ cells, we set up an experimental system that allows reliable comparisons between cells with similar background. To this end, we engineered cellular hybrids between two IMR90-derived cell lines, one ALT and one TEL$^+$. After random elimination of parental chromosomes, followed by natural selection events, hybrids display either the TEL$^+$ or the ALT phenotype, segregating with the corresponding genotype. Using our ALT and TEL$^+$ hybrids, we showed that nucleosome density is systematically reduced at ALT telomeres and this is associated with increased telomere transcription activity [6]. We now wish 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. We performed microarray screens on ALT and TEL$^+$ hybrids and identified ALT-specific candidate genes that we are currently studying.

Human telomerase: regulation and non-canonical functions

H. Episkopou, E. Majerova, A. Decottignies

It has been proposed for a long time that ALT cells may be able to repress telomerase activity. The nature of the inhibitor(s) is however still unknown. Unraveling telomerase repressor(s) represents an interesting research objective, not only to understand ALT cells, but also in a clinical perspective as telomerase has now entered the list of targets in anti-cancer therapy. Relying on the cellular hybrids and the microarray analyses that we described above, we started looking for these putative repressors as all ALT hybrids were found to repress telomerase activity. The work is in progress and we are currently testing putative candidates.

On the other hand, our microarray analyses further revealed distinct gene expression signature between TEL$^+$ and ALT hybrids that was reminiscent to the so-called non-canonical functions of telomerase. Indeed, a decade ago, the group of Robert Weinberg reported for the first time that telomerase may contribute to tumorigenesis by a telomere-independent mechanism. Several studies, including ours [7], confirmed these observations and the non-canonical functions of telom-

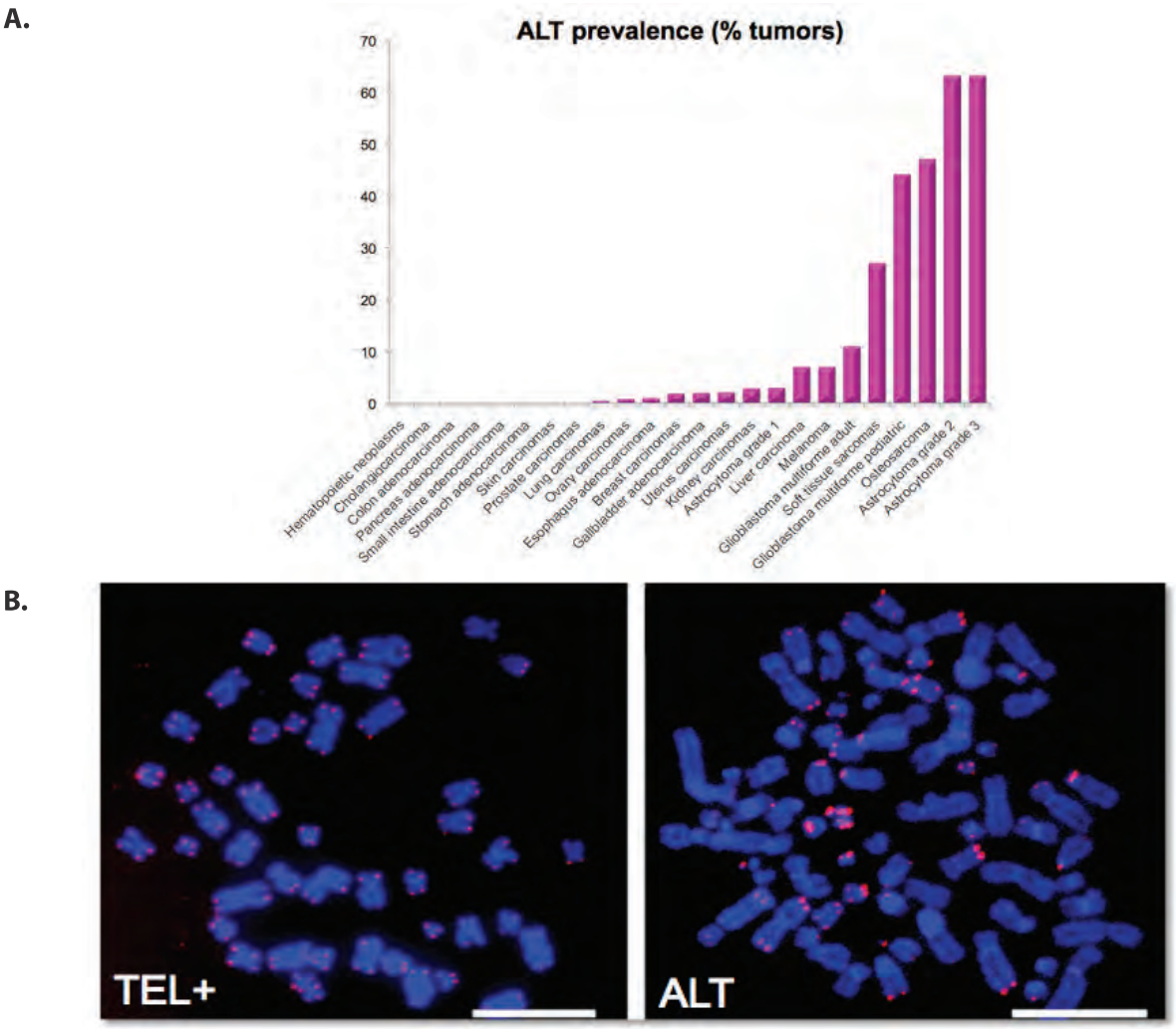

Fig. 2. Alternative mechanism(s) of telomere maintenance. (A) ALT prevalence in human tumors (adapted from Heaphy *et al.*, Am J Pathol 2011). (B) Telomere-specific fluorescence *in situ* hybridization (FISH) on metaphase chromosomes of telomerase-positive (TEL$^+$) and ALT cancer cells (ALT). Telomeres 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.

erase are now actively studied. Our microarray screen revealed new putative non-canonical targets of telomerase that are likely to contribute to tumorigenesis by promoting epithelial-to-mesenchymal transition and angiogenesis. We are currently investigating this.

Telomere maintenance mechanims in melanoma

N. Viceconte, A. Decottignies

Melanoma is a multi-stage genetic disease with progressive acquisition of mutations and high propensity to metastasize. The current model of melanoma progression implies activation of a telomere maintenance mechanism as an obligate step towards metastasis formation. Agreeing with this model, activating point mutations in the promoter of telomerase-encoding gene were first reported in melanoma three years ago and turned out to be present in about 80% of metastatic melanoma. These mutations up-regulate gene transcription. In collaboration with the team of Nicolas van Baren, we are currently screening primary melanoma cell lines and their matching metastatic tumors for telomerase activity or ALT markers. We also aim at answering the question of whether metastasis formation absolutely requires the activation of any telomere maintenance mechanism. This, we believe, is an important question to address knowing that telomere maintenance has become a new target in anti-cancer therapy. Frequency of ALT in melanoma is also quite significant but, to date, ALT-driving mutations have not been studied in that cancer. Identifying new ALT melanoma cell lines from this collection is likely to help understanding how this mechanism is activated and whether it may represent new future targets in anti-cancer therapy.

DNA hypomethylation and aberrant gene activation in cancer

A. Loriot, J. Fain, 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 [8]. 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 [8].

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 [8]. The factors that are responsible for the initial DNA demethylation process and for maintaining CG gene promoters unmethylated remain to be identified.

Processes involved in 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 dimethylation (H3K9me2). 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 [9].

A bioinformatic 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. A similar gene expression signature was observed *in vivo* in melanoma tissue samples. Most of the genes that were downregulated in association with CG gene activation appeared to exert functions related to cellular division/mitosis. Intriguingly, a previous study identified a similar gene expression signature in cells that had been depleted of DNMT1, the enzyme involved in maintenance of DNA methylation marks. By using several cellular models, we demonstrated that transient depletion of DNMT1 leads to both activation of CG-genes and long-term repression of proliferation genes. For one of these genes (*CDCA7L*), we showed that the mechanism of repression involves deposition of a repressive histone mark (H3K27me3), and is dependent on the pRB transcriptional repressor. Our observations point therefore towards DNMT1 depletion as a causal factor in the activation of CG-genes in melanoma [10].

DNA hypomethylation and activation of CG-type miRNAs in tumors

A. Van Tongelen, A. Loriot, A. Diacofotakis, 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 target 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. In a recent study, we reported identification of a novel cancer-germline transcript (*CT-GABRA3*) displaying DNA hypomethylation-dependent activation in various tumors, including melanoma and lung carcinoma. Importantly, *CT-GABRA3* harbors a microRNA (miR-105), which was recently identified as a promoter of cancer metastasis by its ability to weaken vascular endothelial barriers following exosomal secretion. *CT-GABRA3* also carries a microRNA (miR-767) with predicted target sites in *TET1* and *TET3*, two members of the ten-eleven-translocation family of tumor suppressor genes, which are involved in the conversion of 5-methylcytosines to 5-hydroxymethylcytosines (5hmC) in DNA. Decreased TET activity is a hallmark of cancer, and we provided evidence that

aberrant activation of miR-767 contributes to this phenomenon. We demonstrated that miR-767 represses *TET1/3* mRNA and protein expression, and regulates genomic 5hmC levels. Additionally, we showed that high *CT-GABRA3*transcription correlates with reduced *TET1* mRNA levels *in vivo* in lung tumors. Together our studies reveal the first example of a cancer-germline gene that produces micoRNAs with oncogenic potential [11] (Fig. 3).

Bioinformatic approaches, combining prediction algorithms and RNA-seq data, are being conducted to identify other targets of miR-105 and miR-767. Moreover, using CRISPR/Cas-directed homologous recombination, we were able to generate a tumor cell line in which the MIR105/767 locus can be conditionally deleted. Together, this will help us to get a better understanding of the oncogenic roles of these microRNAs. Importantly, we found that, like miR-105, miR-767 is also secreted *via* exosomes. It is therefore likely that both microRNAs can contribute to tumor progression by acting not only on cancer cells from which they are produced, but also on non-cancerous neighboring cells.

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. 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 [12]. 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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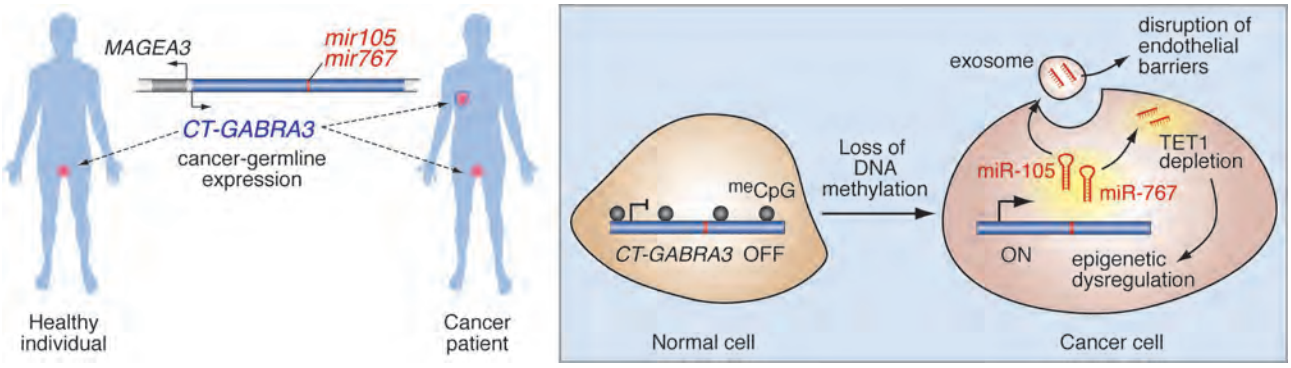

Fig. 3. Pattern of expression of the newly identified cancer-germline gene *CT-GABRA3*. DNA hypomethylation in tumor cells induces aberrant expression of the gene, and of the two miRNAs (miR-105 and miR-767) it harbors. These two miRNAs display oncogenic potential.

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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 [1, 2]. This research theme is connected with our other main research interest, the elucidation of inborn errors of metabolism.

Metabolite repair

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L-2-hydroxyglutaric aciduria is due to a defect in a FAD-linked enzyme that catalyses the irreversible conversion of L-2-hydroxyglutarate to α-ketoglutarate [2], a Krebs 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 [3]. 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 [4]. 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. Examples of newly identified metabolite repair enzymes are ethylmalonyl-CoA decarboxylase (which corrects a mistake made by acetyl-CoA carboxylase; see previous scientific report) [1], NAD(P)HX dehydratase [5], NAD(P)HX epimerase [5] and β-alanyl-lysine dipeptidase (see below). TIGAR, a phosphatase acting on 2,3-bisphosphoglycerate and other phosphate esters [6], may also be involved in metabolite repair.

A mouse model of L-2-hydroxyglutaric aciduria [7]

To progress in our understanding of the pathophysiology of L-2-hydroxyglutaric aciduria, we created and studied a mouse model of L-2-hydroxyglutarate dehydrogenase deficiency. Knockout (l2hgdh-/-) mice accumulated L-2-hydroxyglutarate in tissues, most particularly in brain and testis, where the concentration reached ≈3.5 µmol/g. Male mice showed a 30% higher excretion of L-2-hydroxyglutarate compared to female mice, supporting that this dicarboxylic acid is partially made in males by lactate dehydrogenase C, a poorly specific form of this enzyme exclusively expressed in testes. Involvement of mitochondrial malate dehydrogenase in the formation of L-2-hydroxyglutarate was supported by the commensurate decrease in the formation of this dicarboxylic acid when down-regulating this enzyme in mouse l2hgdh-/- embryonic fibroblasts.

The concentration of lysine and arginine was markedly increased in the brain of l2hgdh-/- adult mice. Saccharopine was depleted and glutamine was decreased by ≈40%. Lysine-α-ketoglutarate reductase, which converts lysine to saccharopine, was inhibited by L-2-hydroxyglutarate with a Ki of ≈0.8 mM. As low but significant activities of the bifunctional enzyme lysine-α-ketoglutarate reductase/saccharopine dehydrogenase were found in brain, these findings suggest that the classical lysine degradation pathway also operates in brain and is inhibited by the high concentrations of L-2-hydroxyglutarate found in l2hgdh-/- mice.

Pathological analysis of the brain showed significant spongiosis. The vacuolar lesions mostly affected oligodendrocytes and myelin sheats, as in other dicarboxylic acidurias, suggesting that the pathophysiology of this model of leukodystrophy may involve irreversible pumping of a dicarboxylate in oligo-

dendrocytes. Neurobehavioral testing indicated that the mice mostly suffered from a deficit in learning capacity. In conclusion, the findings support the concept that L-2-hydroxyglutaric aciduria is a disorder of metabolite repair. The accumulation of L-2-hydroxyglutarate exerts toxic effects through various means including enzyme inhibition and glial cell swelling.

Repair of damaged NAD(P)H

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 [5]. We showed that both the yeast and mammalian 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 [8] 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. The *CARKD* gene encodes proteins with a predicted mitochondrial propeptide (mCARKD), a signal peptide (spCARKD) or neither of them (cCARKD). Confocal microscopy analysis of transfected CHO (Chinese-hamster ovary) cells indicated that cCARKD remains in the cytosol, whereas mCARKD and spCARKD are targeted to the mitochondria and the endoplasmic reticulum, respectively. Unlike the other two forms, spCARKD is N-glycosylated, supporting its targeting to the endoplasmic reticulum. The *AIBP* gene encodes two different proteins, which we showed to be targeted to the mitochondria (mAIBP) and the cytosol (cAIBP). Quantification of the NAD(P)HX dehydratase and epimerase activities in rat tissues, performed after partial purification, indicated that both enzymes are widely distributed, with total activities of ≈3-10 nmol/min per g of tissue. Liver fractionation by differential centrifugation confirmed the presence of the dehydratase and the epimerase in the cytosol and in mitochondria. These data support the notion that NAD(P)HX repair is virtually ubiquitous.

Repair in the synthesis of carnosine [9]

Carnosine (β-alanyl-histidine) is an abundant dipeptide present in skeletal muscle of many vertebrates where it serves as a pH buffer and maybe also as a radical scavenger. The related dipeptide homocarnosine (γ-aminobutyryl-histidine) is present in the brain, where its function is still unknown. Carnosine synthase is the ATP-dependent ligase responsible for carnosine and homocarnosine synthesis in skeletal muscle and brain, respectively. This enzyme uses also at substantial rates lysine, ornithine and arginine instead of histidine, yet the resulting dipeptides are virtually absent from muscle or brain, suggesting

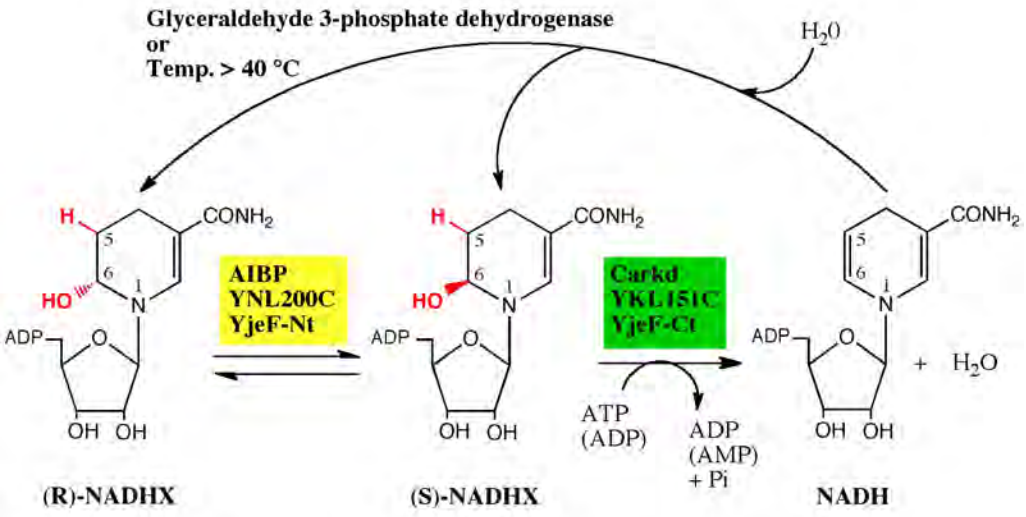

Fig. 1. Formation and repair of hydrated NAD(P)H [modified from Ref. 5]

that they are removed by a 'metabolite repair' enzyme.

We have indeed identified such an enzyme. Using a radiolabeled substrate, we found that rat skeletal muscle, heart and brain contained a cytosolic β-alanyl-lysine dipeptidase activity. This enzyme, which has the characteristics of a metalloenzyme, was purified from rat skeletal muscle and identified with the help of mass spectrometry as the product of the gene PM20D2, a peptidase of unknown function belonging to the metallopeptidase 20 family. Recombinant mouse PM20D2 hydrolysed β-alanyl-lysine, β-alanyl-ornithine, γ-aminobutyryl-lysine and γ-aminobutyryl-ornithine as its best substrates. It acted also, at lower rates, on β-alanyl-arginine and γ-aminobutyryl-arginine, but virtually not on carnosine or homocarnosine. Though acting preferentially on basic dipeptides derived from β-alanine or γ-aminobutyrate, PM20D2 also acted, at lower rates on some 'classical dipeptides' like α-alanyl-lysine and α-lysyl-lysine. The same activity profile was observed with human PM20D2, yet this enzyme was about 100 to 200-fold less active on all substrates tested than the mouse enzyme. Cotransfection in HEK293T cells of mouse or human PM20D2 together with carnosine synthase prevented the accumulation of abnormal dipeptides (β-alanyl-lysine, β-alanyl-ornithine, γ-aminobutyryl-lysine), thus favoring the synthesis of carnosine and homocarnosine and confirming the metabolite repair role of PM20D2.

Repair in glycolysis and the pentose phosphate pathway [10] (Fig. 2)

In our quest to identify metabolite repair enzymes, we are interested by enzyme side activities leading to the formation of compounds with predictable toxicity, because the cells must have a way of protecting themselves against such toxic molecules. This is the case for a side activity of glyceraldehyde-3-phosphate dehydrogenase, a central enzyme of glycolysis. This enzyme is indeed known to act slowly on erythrose-4-P and to convert it to 4-P-erythronate, a very potent inhibitor (Ki < 1 µM) of 6-P-gluconate dehydrogenase, an enzyme of the pentose phosphate pathway. We reasoned that cells must have an enzyme, most likely a phosphatase, that destroys 4-P-erythronate.

We found indeed that mammalian tissues contain a phosphatase acting very well on 4-P-erythronate. We purified this enzyme and identified it as a phosphatase known to act on 2-P-glycolate and therefore named PGP (phosphoglycolate phosphatase). Analysis of the specificity of this enzyme indicated that it acts best on 4-P-erythronate, 2-P-glycolate and 2-P-L-lactate, and that it barely acts, if at all, on physiological phosphate esters belonging to glycolysis or the pentose-phosphate pathway.

As expected, inactivation of the PGP gene in HTC116 cells with the CrispR/Cas9 technique led to marked (>10-fold) increases in the concentrations of 4-P-erythronate and 6-P-gluconate. It did not lead to detectable accumulation of 2-P-glycolate, but to a dramatic increase in the concentration 2-P-L-lactate, a phosphate ester whose presence had never been reported

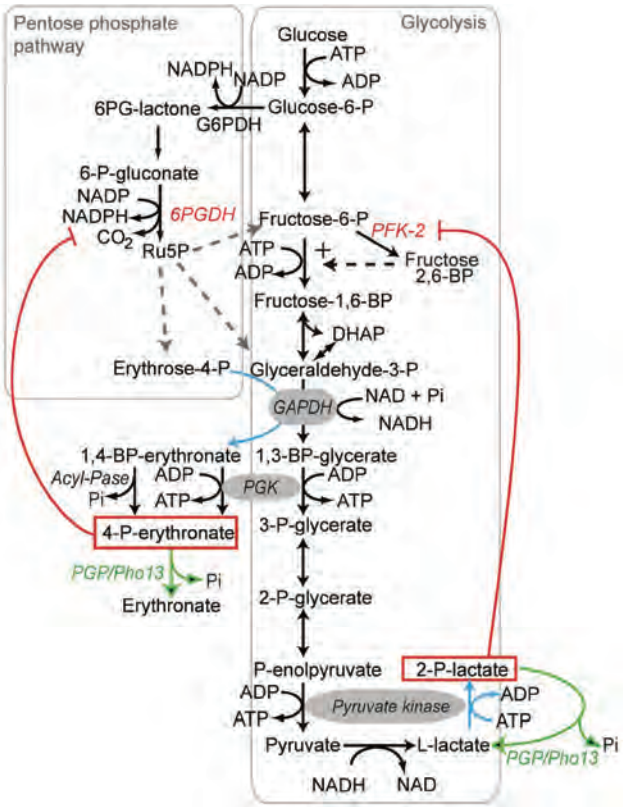

Fig. 2. The enzyme PGP/Pho13 eliminates glycolytic side products and allows parallel action of glycolysis and the pentose phosphate pathway. Side activities of glycolytic enzymes are shown in blue; new metabolite repair activities in green and inhibitory interferences discovered or characterized in the paper of Collard and colleagues in red. 6PGDH, 6-P-gluconate dehydrogenase; PGK, phosphoglycerate kinase; 6PG-lactone, 6-P-gluconolactone; Ru5P, ribulose-5-P; BP, bisphospho; PFK-2, phosphofructokinase-2 [modified from Ref. 10].

before in living cells. We obtained evidence for the formation of 2-P-L-lactate by a side activity of pyruvate kinase, which slowly phosphorylates L-lactate in an ATP-dependent reaction. Among the other metabolic changes that we noticed were an ≈50% decrease in the glycolytic flux and an ≈80% decrease in the concentration of the glycolytic activator fructose 2,6-bisphosphate, presumably due to inhibition of 6-phosphofructo-2-kinase, the enzyme that forms fructose 2,6-bisphosphate, by 2-P-L-lactate.

These findings led us to the conclusion that PGP serves to eliminate two glycolytic side products that are formed by two major enzymes of glycolysis. One of these side products – 4-P-erythronate – inhibits a step in the pentose phosphate pathway, while the other indirectly inhibits glycolysis via a depletion of its major activator fructose 2,6-bisphosphate. Interestingly, we found that the homologous enzyme in *Saccharomyces cerevisiae*, called Pho13, has a comparable, though not identical function: it also dephosphorylates 4-P-erythronate, but its other physiological substrate is 2-P-glycolate rather than 2-P-L-lactate. Contrary to mammalian cells, yeasts do not contain L-lactate, but substantial amounts of glycolate, which is also a weak substrate for pyruvate kinase.

Molecular identification of enzymes

A. Bersweiler, G. Tahay, E. Van Schaftingen, M. Veiga-da-Cunha, E. Wiame, in collaboration with G. Bommer and I. Gerin (de Duve Institute)

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 [10]. Thus in collaboration with Guido Bommer, and his team, we identified the function of three proteins involved in the synthesis of dystroglycan [11] (see report of G. Bommer). We also contributed to the identification of the function of enzymes involved in bacterial metabolism.

Elucidation of the pathway of erythritol catabolism in bacteria [12]

In collaboration with T. Barbier, J.-J. Letesson (Namur)

Erythritol is an important nutrient for several α-2 Proteobacteria, including N2-fixing plant endosymbionts and *Brucella*, a worldwide pathogen that finds this four-carbon polyol in genital tissues. Erythritol metabolism involves phosphorylation to L-erythritol-4-phosphate by the kinase EryA and oxidation of the latter to L-3-tetrulose 4-phosphate by the dehydrogenase EryB. It is accepted that further steps involve oxidation by the putative dehydrogenase EryC and subsequent decarboxylation to yield triose-phosphates (upper pathway of Fig. 3).

Accordingly, growth on erythritol as the sole carbon source should require aldolase and fructose-1,6-bisphosphatase to produce hexose-6-monophosphate, which is essential for producing many biomolecules. However, we observed that a mutant devoid of fructose-1,6-bisphosphatases grew normally on erythritol and that EryC, which was assumed to be a dehydrogenase, actually belongs to the xylose isomerase superfamily. Moreover, we found that TpiA2 and RpiB, distant homologs of triose phosphate isomerase and ribose 5-phosphate isomerase B, were necessary, as previously shown for *Rhizobium*.

By using purified recombinant enzymes, we demonstrated that L-3-tetrulose-4-phosphate was converted to D-erythrose 4-phosphate through three previously unknown isomerization reactions catalyzed by EryC (tetrulose-4-phosphate racemase), TpiA2 (D-3-tetrulose-4-phosphate isomerase; renamed EryH), and RpiB (D-erythrose-4-phosphate isomerase; renamed EryI), a pathway fully consistent with the isotopomer distribution of the erythrose-4-phosphate-derived amino acids phenylalanine and tyrosine obtained from bacteria grown on (^{13})C-labeled erythritol. D-erythrose 4-phosphate is then converted by enzymes of the pentose phosphate pathway to glyceraldehyde 3-phosphate and fructose 6-phosphate, thus bypassing fructose-1,6-bisphosphatase. This is the first description of a route feeding carbohydrate metabolism exclusively via D-erythrose 4-phosphate, a pathway that may provide clues to the preferential metabolism of erythritol by *Brucella* and its role in pathogenicity.

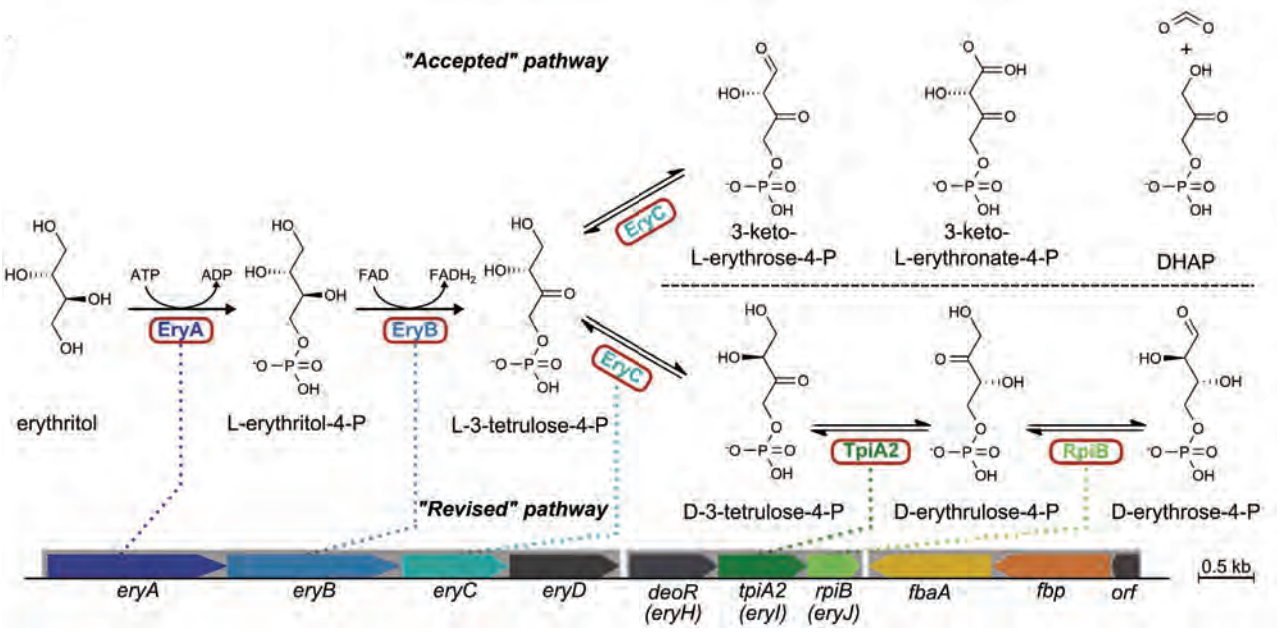

Fig. 3. Erythritol catabolism by *Brucella*. The scheme shows the previously accepted pathway and the one that we have now established by characterization of recombinant enzymes. The two first reactions of the 'accepted' and the 'revised' pathways are similar. While it was previously admitted that L-3-tetrulose-4-P is afterwards oxidized to dihydroxyacetone-phosphate (DHAP) and CO2, we showed that this intermediate is converted to D-erythrose-4-P *via* three isomerisation reactions catalyzed by EryC, TpiA2 and RpiB. The relevant enzyme-encoding genes are organized as illustrated in two operons comprising also two regulators (EryD and DeoR) [from Ref. 12].

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Bacterial stress responses

Jean-François Collet

The overuse of antibiotics to treat bacterial infections in human and veterinary medicine has created a global resistance crisis that could lead to a surge in infection-related mortality. A recent report predicted that multi-drug resistant bacteria will kill more than cancer by 2050. A particularly serious threat is the emergence of a new wave of multidrug-resistant Gram-negative bacteria, including *Pseudomonas aeruginosa* and enterobacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. It is therefore urgent to develop new antibiotics against resistant bacteria, which requires a deep understanding of the biology of these microorganisms. Our laboratory wants to contribute to the global effort aiming to prevent the return of untreatable epidemics by better understanding how bacteria respond to the different types of stress to which they are exposed. In particular, we want to understand how bacteria defend themselves against oxidative stress and how they maintain the integrity of their cell envelope despite always changing environmental conditions.

Defense mechanisms against oxidative stress

Exposure of cells to elevated levels of reactive oxygen species (ROS) damages DNA, membrane lipids and proteins, which can potentially lead to cell death. In proteins, the sulfur-containing residues cysteine and methionine are particularly sensitive to oxidation, forming sulfenic acids and methionine sulfoxides (Met-O), respectively [1]. The presence of protection mechanisms to scavenge ROS and repair damaged cellular components is therefore essential for cell survival. The bacterial cell envelope, which constitutes the first protection barrier from the extracellular environment, is particularly exposed to the oxidizing molecules generated by the host cells to kill invading microorganisms. Therefore, the presence of oxidative stress defense mechanisms in that compartment is crucial for cell survival. One of the goals of our laboratory is to contribute to a **better understanding of the mechanisms that repair envelope proteins with oxidized cysteine and methionine residues**.

An important contribution of our group has been the identification of a new periplasmic reducing system that protects single cysteine residues from oxidation by reactive oxygen species (ROS) (Fig. 1). We discovered this system by trapping DsbG, a protein from the thioredoxin (Trx) superfamily whose function had remained elusive, with three of its substrates [2]. These three periplasmic proteins (YbiS, ErfK and YnhG) found in complex with DsbG were homologous proteins and belonged to the same family of L,-D transpeptidases. Unexpectedly, all three enzymes contained 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 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, CA, USA), 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 [2]. 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 [2]. On the basis of these results, we proposed the following model (Fig. 1). In the oxidizing periplasm, most proteins contain an even number of cysteine residues. These residues form disulfide bonds [3-7] and are therefore protected from further cysteine oxidation.

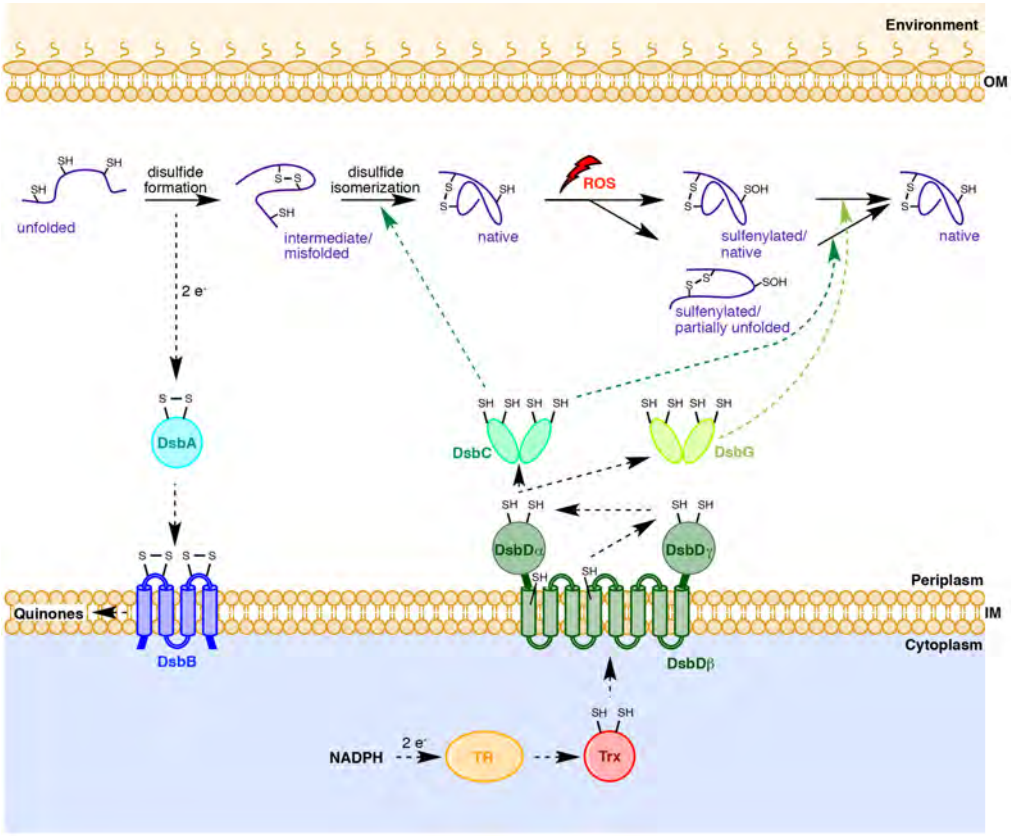

Fig. 1. DsbG and DsbC protect proteins with single cysteine residues from irreversible oxidation. Disulfide bonds are introduced into newly translocated, unfolded proteins in the periplasm by DsbA. Electrons are then transferred to the IM protein DsbB and finally to quinones. DsbC corrects DsbA mistakes and is maintained reduced by DsbD, an IM protein which receives electrons from the cytoplasmic thioredoxin (Trx) system. DsbG, which rescues sulfenylated proteins in the periplasm, is also reduced by DsbD. In addition to its role as protein disulfide isomerase, DsbC cooperates with DsbG in the rescue of certain sulfenylated periplasmic proteins. We propose that DsbC preferentially interacts with (partially) unfolded sulfenylated proteins. In contrast, DsbG seems to be better designed to react with globular proteins presenting oxidized cysteine residues. Dashed arrows represent electron flows. TR = thioredoxin reductase, OM = outer membrane, IM = inner membrane (adapted from Ref. 1).

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.**

We recently found that DsbC serves as a backup for DsbG and has its own subset of favorite sulfenic acid modified substrates to reduce [8]. The activity of DsbC and DsbG depends on the IM protein DsbD, which provides reducing equivalents to a variety of reducing pathways functioning in the cell envelope [4, 9] (Fig. 1). DsbD is composed of three domains, an N-terminal periplasmic Ig-like α domain, a central transmembrane β domain and a C-terminal periplasmic Trx-like γ domain. Each domain of DsbD possesses a pair of redox-active cysteines that are essential for activity. These cysteines form a relay that shuttle electrons from the cytoplasmic Trx system to periplasmic oxidoreductases *via* a cascade of thiol-disulfide exchange reactions. In this mechanism, electrons are first transferred from the reduced CXXC residues of Trx to the cysteines of DsbDβ and then successively to the cysteines of DsbDγ and DsbDα, which finally reduces substrate proteins. A particularly intriguing question regarding DsbD is how DsbDβ transports electrons across the membrane. We recently obtained, in collaboration with two groups at Harvard Medical School, insights into this mechanism by solving the structure of a reduced-state mimic of an archaeal CcdA using NMR [10]. CcdA is a stripped-down version of DsbD that does not possess the two periplasmic domains and only comprises six TM segments instead of the eight found in *E. coli* DsbD. The data support the existence of multiple conformational states and suggest a four-state model to explain how the cysteine residues of the membranous domain are able to alternatively interact with oxidoreductases in the cytoplasm and in the periplasm [10].

Another major contribution of our lab, in collaboration with the group of F. Barras (CNRS, Marseille), has been the discovery of a system protecting methionine residues from oxidation in the cell envelope. This system, called MsrPQ, is widely conserved in Gram-negative bacteria [11]. In MsrPQ, MsrP functions as a soluble periplasmic methionine sulfoxide reductase (Msr) reducing Met-O residues back to methionine, while MsrQ is a membrane-bound cytochrome b functioning both as a membrane anchor and a redox partner for MsrP. The MsrPQ system is very different from all the Msrs that had been

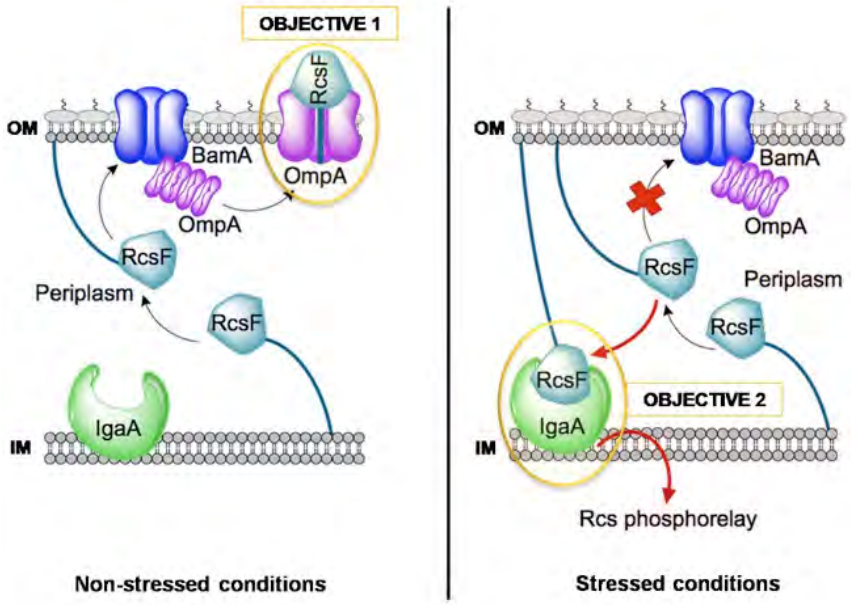

Fig. 2. Stress conditions prevent the export of RcsF to the cell surface. The sensor RcsF is exported to the cell surface by the Bam machinery. In the absence of stress (left), RcsF interacts with BamA, the key component of the β-barrel assembly machinery. BamA assembles a complex between RcsF and OmpA, an abundant β-barrel protein, in such a way that RcsF is displayed on the cell surface. Once engaged in interactions with BamA and/or OmpA, RcsF is occluded from IgaA, the downstream component of the Rcs signaling cascade located in the IM. Upon OM or PG-related stress (right), RcsF fails to bind BamA and remains exposed to the periplasm where it binds to IgaA and triggers the Rcs system.

identified previously. First, unlike other enzymes able to repair Met-O residues, MsrP is non-stereospecific, being able to reduce both the *S* and *R* isoforms of Met-O. Second, whereas all the other identified Msrs use a thiol-based reaction mechanism, MsrP uniquely involves a molybdopterin-based chemical mechanism to reduce Met-O residues in proteins. Third, MsrQ uses electrons from the quinone pool to recycle MsrP, which makes the MsrPQ system independent of Trx (the other Msrs depend on Trx for reduction). **Several lines of evidence indicate that MsrPQ plays a particularly important role in the defense mechanisms against bleach stress and could be an attractive target for the design of new antibiotics.**

Defense mechanism against envelope stress

Gram-negative bacteria are surrounded by a complex cell envelope, which consists of two concentric membranes separated by the periplasm, a viscous compartment containing a single layer of peptidoglycan (PG). This envelope, a permeability and structural barrier, is essential for cell shape and growth [12]. It is therefore a matter of life and death for bacteria to detect when their envelope is perturbed and to respond to the inflicted damage in a fast and adequate manner. To this end, bacteria have evolved several signaling systems that enable them to sense envelope perturbations and to mount a repair and/or a preventive response to minimize the damage. A second major objective of our group is to **study these signal transduction systems enabling bacteria to sense when their envelope is perturbed and to respond to the inflicted damage in a fast and adequate manner.**

A recent major development is the discovery of the elegant mechanism used by the *E. coli* lipoprotein RcsF to sense peptidoglycan and outer membrane (OM) damages and to turn on the Rcs signaling system. The Rcs phosphorelay is a particularly complex signal transduction system present in *E. coli* and other enterobacteria where it controls the expression of genes involved in motility, biofilm formation, virulence and periplasmic quality control. The Rcs consists of an IM-localized sensor histidine kinase (HK), which autophosphorylates in response to environmental signals, and of a cytoplasmic response regulator (RR), which binds to target promoters on the chromosome upon phosphorylation by the HK (Fig. 2). Unlike typical two-component systems, the Rcs has at least 4 additional components. In addition to RcsC (HK) and RcsB (RR), the system contains an intermediate IM phosphorelay protein, RcsD, an auxiliary transcription factor RcsA, and two proteins that act upstream of the phosphorelay cascade and are associated with signal sensing, IgaA and RcsF (Fig. 2). IgaA is an essential IM protein that down-regulates the Rcs pathway by an unknown mechanism. RcsF is a lipoprotein anchored to the OM where it functions as a sensor for damages occurring in the outer part of the envelope.

In 2011, we reported that RcsF depended on the protein disulfide isomerase DsbC for folding and solved its structure, in collaboration with JP Declercq [13]. Furthermore, we recently discovered, in collaboration with N. Typas (EMBL, Germany), how RcsF controls the activation of the Rcs phosphorelay [14]. First, we found that RcsF interacts with BamA, the major component of the β-barrel assembly machinery, and that BamA continuously assembles complexes between RcsF and the β-barrel OmpA (Fig. 2). RcsF interacts with OmpA in such a way that at least portions of the protein are displayed on the cell surface, which represents a novel mechanism of lipoprotein export. This process spatially separates RcsF from IgaA, the downstream Rcs component, keeping the system off. Second, we found that stress conditions prevent BamA from binding to RcsF and assembling the RcsF-OmpA complexes. This keeps RcsF facing the periplasm, where it can reach IgaA and activate the cascade (Fig. 2). Thus, we found that **RcsF detects envelope stress by monitoring the activity of the machinery that assembles β-barrel proteins in the OM**.

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miRNAs and p53 at the crossroad of cancer and metabolism

Guido Bommer

Cellular metabolism needs to be tightly regulated. We are interested in understanding how miRNAs and conserved transcription factors regulate cancer metabolism. 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, and we are only beginning to understand their effects on cellular metabolism. We are also interested in specific aspects cancer metabolism, and how it is regulated by factors like the tumor suppressor gene p53. This has led us to revise the notion of the molecular function of the p53 target gene TIGAR [1], and, more recently, allowed us to discover a metabolic clean-up system, that prevents the accumulation of undesirable side-products generated in glycolysis (in a collaboration with Emile Van Schaftingen) [2]. As a side-product of these studies, we recently found a novel post-translational modification, which is deficient in a group of neuromuscular diseases [3].

Role of miRNAs in intestinal differentiation and metabolism

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 two 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 (Fig. 1). 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 [4-8].

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

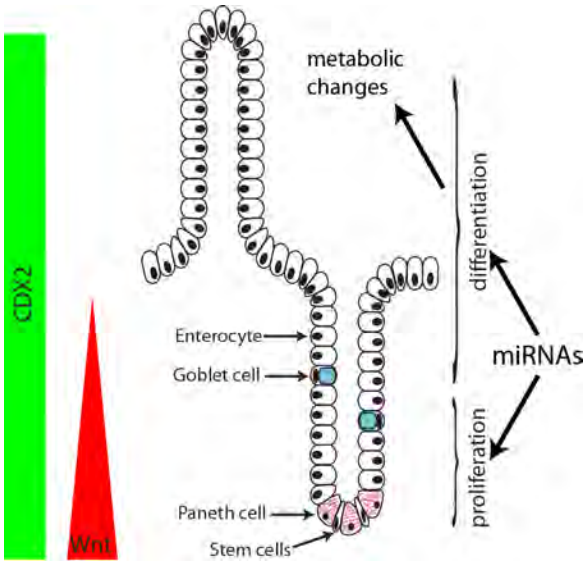

Fig. 1. Intestinal architecture is maintained by the interplay of many signaling pathways. The intestinal architecture is maintained by the interplay of signaling pathways that ensure complete renewal of intestinal surface epithelia within 7 to 14 days. New cells are generated from a stem cell compartment 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 that play a role in the metabolic changes required for normal intestinal cell function.

In our laboratory, we are investigating the contribution of miRNAs to intestinal cell differentiation and the development of colorectal cancer [9]. Our work in this area is focused on the contribution of miRNAs to the metabolic changes that are required for normal intestinal cell function. Interestingly, some miRNAs seem to be tightly embedded in a functional network that alters the composition of the mitochondrial respiratory chain *(i.e.* the major source of "energy production" in most cells). This indicates that miRNAs might play an important role in the decision how intestinal cells utilize nutrients to make energy (*i.e.* ATP) that can be used for other processes. Currently, we are trying to understand the relevance of these observations for normal intestinal function and colorectal cancer.

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 [10-12] (Fig. 2). 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 with the laboratory of Jennifer Kennel, Vassar College, NY, USA). Surprisingly, the effect of miR-33 overexpression in the fly is extremely dependent on the developmental stage and nutritional status. We are currently trying to understand whether these observations have implications for miR-33 as a therapeutic target in humans.

Cancer metabolism: Identification of the primary substrate of the enzyme TIGAR, a transcriptional target of the tumor suppressor p53

In collaboration with E. Van Schaftingen (de Duve Institute)

Glucose is metabolized in glycolysis and in citric acid cycle (Fig. 3). This leads to the production of ATP and reducing equivalents that can be used to drive thermodynamically unfavorable reactions. In addition, these metabolic pathways serve as sources of building blocks required for cellular proliferation (indicated by black fields). Many cancer cells show increased glycolytic flux to account for the constant need for building block and the regulation of glycolytic flux in cancer cells has therefore received significant attention.

The p53 tumor-suppressor gene is inactivated in the majority of cancers. It codes for a transcriptional factor that acts in the center of a cellular stress response. When cells are exposed to a large range of stresses, p53 accumulates and activates transcription of many different target genes. Collectively, these target genes lead to the induction of cell cycle arrest, apoptosis and cellular senescence. In addition to this, p53 also has metabolic effects.

Most prominently, it had been suggested that the p53 target TIGAR is an important regulator of glycolytic flux by removing a phosphate group from the metabolic regulator fructose 2,6-bisphosphate [Bensaad, Cell 2006]. Surprisingly, the sole kinetic investigation on recombinant TIGAR [Li & Jogl, J Biol Chem 2009] has shown that its fructose-2,6-bisphosphatase activity was much weaker than that of authentic fructose-2,6-bisphosphatases. In collaboration with the group of Emile Van Schaftingen, who together with Louis Hue and Géry Hers had

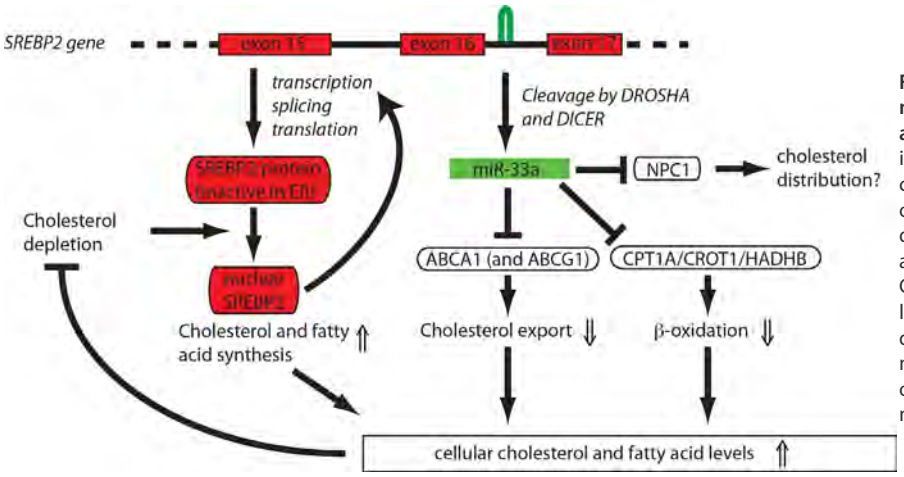

Fig. 2. The bifunctional locus of SREBF2-miR33 regulates cholesterol and fatty acid metabolism. After processing from an intron of SREBF2, miR-33a reduces cellular cholesterol export by inhibiting expression of ABCA1 (and in the mouse ABCG1). In addition, miR-33a reduces mitochondrial fatty acid b-oxidation 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.

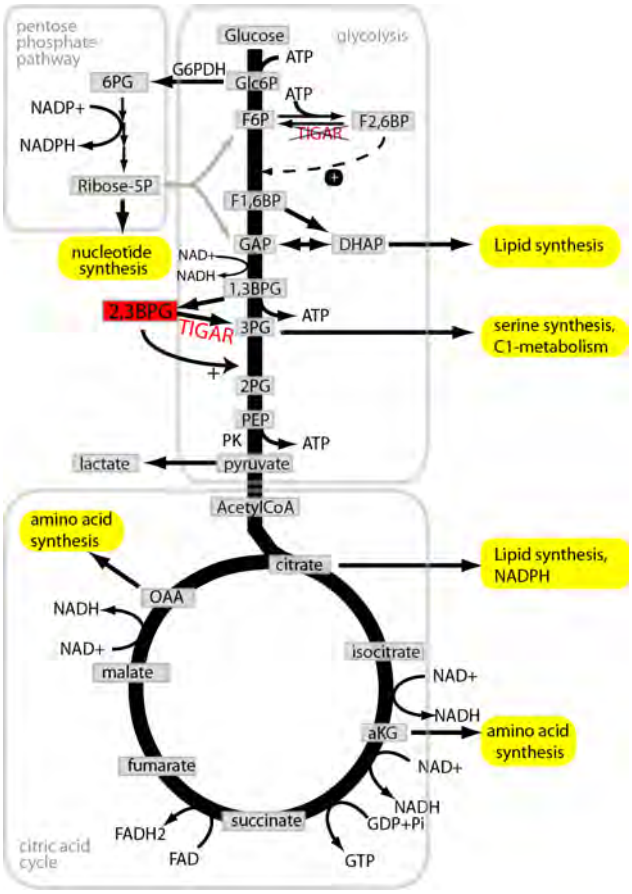

Fig. 3. TIGAR is a 2,3-bisphosphoglycerate phosphatase. Glycolysis and citric acid cycle lead to the production of ATP, reducing equivalents and synthetic building blocks. It had previously been suggested that the p53 target gene TIGAR would regulate cellular metabolism by dephosphorylation of the glycolytic regulator fructose 2,6-bisphosphate. An in-depth analysis of TIGAR substrates showed that 2,3-bisphosphoglycerate is an about 400x better substrate for TIGAR than fructose 2,6-bisphosphate. This indicates that the current concepts of the metabolic changes downstream of p53 need to be revised, and that the role of 2,3-bisphophoglycerate in cells (outside of red blood cells) needs to be further investigated.

first described and characterized fructose 2,6-bisphosphate in the 1980s, we re-evaluated the substrate spectrum of TIGAR. Remarkably, we found that 2,3-bisphosphoglycerate was an about 400x better substrate for TIGAR than fructose 2,6-bisphosphate [1]. Using knockout and shRNA-based techniques, we were able to demonstrate that 2,3-bisphosphoglycerate is likely a physiological substrate of TIGAR.

This observation is surprising, since 2,3-bisphosphoglycerate is mainly known as a cofactor for phosphoglycerate mutase, a glycolytic enzyme catalyzing a near-equilibrium reaction, and as a regulator of hemoglobin's oxygen affinity in red blood cells, where its concentration reaches up to 10 mM. Why cells different from erythrocytes do have a mechanism for regulating the 2,3-bisphosphoglycerate concentrations is still mysterious and an open field for future investigations.

Given the fact that knockout of TIGAR in mice does not result in any obvious phenotype, but offers some protection in mouse models of colorectal cancer and myocardial infarction, TIGAR might present a target for therapeutic intervention. Understanding the role of 2,3-bisphosphoglycerate in these outcomes might open up novel insights in the intricacies of cellular metabolic regulation, and lead to future therapeutic approaches.

Cancer metabolism: Identification of an enzyme eliminating metabolic side products generated in glycolysis

In collaboration with E. Van Schaftingen (de Duve Institute); see his report and Ref. 2.

Identification of a novel post-translational modification deficient in a group of neuromuscular diseases

In collaboration with E. Van Schaftingen (de Duve Institute), N. Seta (Paris), I. Breloy (Cologne)

The protein α-dystroglycan plays an important role in linking the intracellular cytoskeleton to the extracellular matrix. For this function, it needs to be glycosylated in a process that involves at least 12 enzymes. Mutations in any of these enzymes lead to muscle and brain diseases of varying severity, for which currently no therapy exists. At present, the precise structure of the glycan and the function for some enzymes involved in its assembly are still incompletely understood.

We have recently discovered the function of three enzymes required for α-dystroglycan glycosylation - ISPD, FKTN and FKRP [3]. The three enzymes are working together to attach a sugar derivative (ribitol) together with a phosphate group onto the glycan of α-dystroglycan (Fig. 4A). Specifically, we demonstrate that isoprenoid synthase domain-containing protein (ISPD) synthesizes CDP-ribitol, present in muscle, and that both recombinant fukutin (FKTN) and fukutin-related protein (FKRP) can transfer a ribitol phosphate group from CDP-ribitol to α-dystroglycan (Fig. 4A). Similar structures are often encountered in the capsules or cell walls of bacteria. However, up to now these structures were not known to exist in eukaryotes before. We find that ribitolphosphorylation represents an essential step in the glycosylation of α-dystroglycan.

Notably, cells from some patients with ISPD mutations partially or completely restore α-dystroglycan glycosylation when ribitol is added to the medium (Fig. 4B). This indicates that dietary supplementation with ribitol should be evaluated as therapeutic approach for a subset of patients with ISPD mutations.

Currently, we are attempting to identify the remaining steps in the biogenesis of ribitolphosphorylation, and whether proteins other than α-dystroglycan also contain this modification.

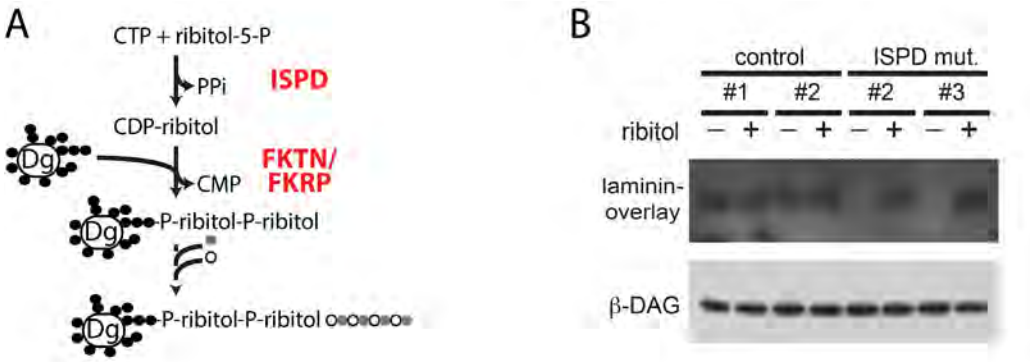

Fig. 4. Ribitolphosphorylation of α-dystroglycan requires ISPD, FKTN and FKRP. (A) Schematic representation of the enzymatic repertoire required for ribitolphosphorylation. ISPD generates CDP-ribitol as an activated form of ribitol by using CTP and ribitol-5-P as substrates. In turn, FKTN and FKRP can use CDP-ribitol to transfer a ribitol 5-phosphate group onto the glycan of α-dystroglycan. Introduction of this modification is a prerequisite for the final steps of glycosylation to occur (indicated by grey and white circles). (B) Ribitol can restore deficient α-dystroglycan glycosylation in patient fibroblasts carrying ISPD mutations. Fibroblasts from patients carrying ISPD mutations or from normal control patients were incubated in the presence (+) or absence (-) of ribitol (added to the tissue culture medium). Cellular proteins were separated by gel electrophoresis and transferred to membranes. Functionality of α-dystroglycan glycosylation was assessing whether laminin (one of its ligands) binds to it (so-called laminin overlay assay). Binding of laminin was revealed using an anti-laminin antibody and can be seen by the appearance of a dark band in the corresponding lane.

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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-kinase (PFK2)/fructose-2,6-bisphosphatase (FBPase-2), the bifunctional enzyme which synthesizes and degrades fructose-2,6-bisphosphate (Fru-2,6-P2), and the control of its activity by protein kinases [reviewed in Ref. 1]. This led to our investigation of the insulin and AMP-activated protein kinase (AMPK) signaling cascades, which are now our main research interests.

Insulin signaling

N. Hussain, M.H. Rider

Insulin-stimulated lipogenesis in white adipose tissue

A key mediator of the short-term effects of insulin downstream of phosphatidylinositol 3-kinase signaling is protein kinase B (PKB). A well-known metabolic effect of insulin is the stimulation of adipose tissue lipogenesis, mediated by increased glucose transport, pyruvate dehydrogenase (PDH) activation via dephosphorylation, and acetyl-CoA carboxylase (ACC) activation. We studied the effects of Akti-1/2 and the next generation MK-2206 PKB inhibitor on insulin-stimulated lipogenesis in rat epididymal adipocytes. Insulin-stimulated rates of lipogenesis were completely blocked dose-dependently by both Akti-1/2 and MK-2206. PDH dephosphorylation, ATP-citrate lyase (ACL) and serum- and glucocorticoid-induced protein kinase-3 (SGK3) activation loop Thr320 phosphorylation by insulin, however, were unaffected by PKB inhibition. By contrast, ACC Ser79 dephosphorylation by insulin was completely reversed by Akti-1/2 and MK-2206. PKB thus plays an important role in the control of the lipogenic pathway by insulin and dephosphorylation of ACC mediated by PKB would be one of the mechanisms (manuscript in preparation). Indeed, we showed previously that PKB antagonizes activation of AMPK [2], the protein kinase responsible for ACC inactivation via Ser79 phosphorylation.

AMPK

S. Kviklyte, D. Vertommen, R. Jacobs, M. Johanns, S.-J. Chuang, A. Houddane, N. Hussain, L. Hue, M.H. Rider, in collaboration with B. Viollet and M. Foretz (Paris), D. Carling and A. Woods (London), S. Hallén (AstraZeneca), J. Van Sande and J.E. Dumont (ULB, Brussels), K. Sakamoto (Lausanne)

AMPK acts as a sensor of cellular energy status activated by an increase in the AMP/ATP ratio as occurs during hypoxia or muscle contraction/exercise. 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). In certain cells, AMPK can be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells and is converted to ZMP, an analogue of AMP. AMPK can also be activated by incubating cells with the small-molecule activator, A769662, also known as the Abbott compound. Another more potent direct AMPK activator called compound "991" (also known as "ex229") was recently discovered, that bins to the same site as the Abbott compound in a cleft between the AMPKα and AMPKβ subunits. We recently showed that ex229 efficiently activated skeletal muscle AMPK and elicited metabolic effects in muscle appropriate for treating type 2 diabetes by stimulating glucose uptake (Fig. 2) and increasing fatty acid oxidation [3]. In addition, we have made significant contributions to the field by discovering new substrates of AMPK. Protein synthesis inhibition in response to AMPK activation during anoxia can partly be explained by a

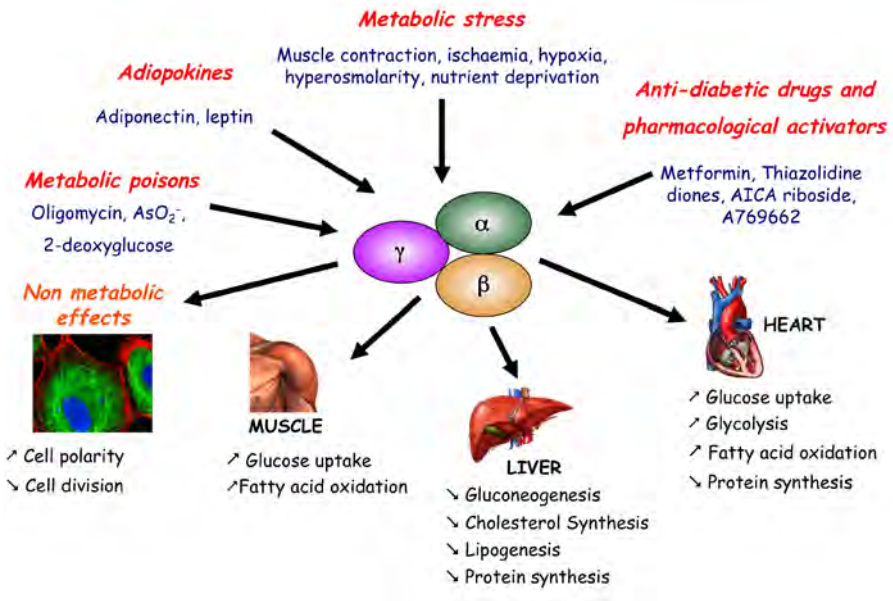

Fig. 1. Conditions leading to AMPK activation in higher eukaryotes and some of its consequences.

rise in eEF2 (eukaryotic elongation factor-2) phosphorylation leading to its inactivation [4]. Regulation of the upstream eEF2 kinase (eEF2K) is complex involving phosphorylation-induced activation and inactivation by kinases from various signaling pathways, including AMPK. However, phosphorylation at the previously proposed Ser398 site by AMPK is probably incorrect and our recent findings indicate that AMPK phosphorylates other sites responsible for eEF2K activation. AMPK activation also activates eEF2K indirectly by reduced mTORC1 signaling (manuscript in preparation).

AMPK as a drug target for the treatment of type 2 diabetes

Effects of pharmacological AMP deaminase inhibition and genetic Ampd1 deletion on nucleotide levels and AMPK activation in contracting skeletal muscles

AMPK is an attractive therapeutic drug target for treating metabolic disorders. In collaboration with the pharmaceutical company AstraZeneca (Mölndal, Sweden), we have investigated whether inhibition of AMP metabolizing enzymes could be a means of achieving or potentiating AMPK activation (Fig. 2). AMP levels are maintained by deamination by AMP-deaminase to IMP, which is further hydrolyzed by cytosolic 5′-nucleotidase-II (NT5C2). Otherwise, AMP can be hydrolyzed to adenosine by cytosolic 5′-nucleotidase-IA (NT5C1A). Using whole body genetic knockout mouse models, we showed that NT5C1A and NT5C2 deletions in extensor digitorum longus (EDL) and soleus muscles did not enhance the increases in AMP:ATP or ADP:ATP ratios to potentiate AMPK activation by electrical stimulation. Taken together with our previous work on pharmacological AMPD1 inhibition and AMPD1 genetic deletion [5], and the fact that administration of AMPD inhibitors in insulin-resistant or diabetic rodent disease models did not improve glucose control, we conclude that pharmacological

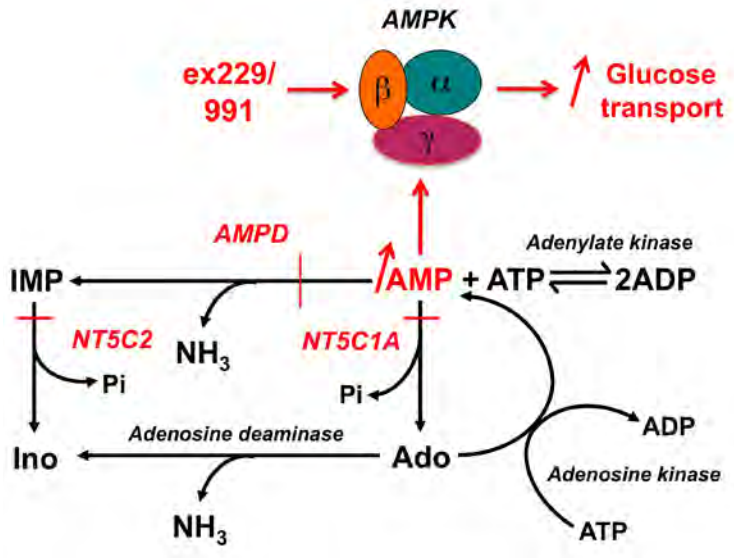

Fig. 2. Scheme showing AMP metabolizing enzymes for pharmacological inhibition together with direct small-molecule AMPK activation by ex229/991 as a strategy for increasing glucose transport in skeletal muscle for the treatment of type 2 diabetes. AMPD: AMP-deaminase; NT-5C1A: soluble 5′-nucleotidase-1A; NT5C2: soluble 5′-nucleotidase-II; Ado: adenosine; Ino: inosine.

inhibition of AMP-metabolizing enzymes would not be a viable strategy for increasing AMPK activity and glucose uptake for the treatment of type 2 diabetes. It is likely that the fluxes through NT5C1A and NT5C2 in muscle are too low for pharmacological inhibition to be exploited.

AMPK antagonizes hepatic glucagon-stimulated cyclic AMP signaling via phosphorylation-induced activation of cyclic nucleotide phosphodiesterase 4B

Biguanides such as metformin have previously been shown to antagonize hepatic glucagon-stimulated cyclic AMP (cAMP) signaling, independently of AMPK, via direct inhibition of adenylate cyclase by AMP. Here, we show that incubation of hepatocytes with the small-molecule AMPK activator 991 decreases glucagon-stimulated cAMP accumulation, cyclic AMP-dependent protein kinase (PKA) activity and downstream PKA target phosphorylation. Moreover, incubation of hepatocytes with 991 increases the V_{max} of cyclic nucleotide phosphodiesterase 4B (PDE4B) without affecting intracellular adenine nucleotide concentrations. The effects of 991 to decrease glucagon-stimulated cAMP concentrations and activate PDE4B are lost in hepatocytes deleted for both catalytic subunits of AMPK. Purified PDE4B is phosphorylated *in vitro* by AMPK at three sites, and by site-directed mutagenesis, Ser304 phosphorylation is important for activation. In conclusion, we provide a new mechanism by which AMPK antagonizes hepatic glucagon signaling via phosphorylation-induced PDE4B activation (Fig. 3) [6].

Mass spectrometry

D. Vertommen, G. Herinckx, M.H. Rider, in collaboration with J.-F. Collet (UCL), E. Waelkens (KULeuven), Joris Messens (VIB-VUB), K. Storey (Ottawa)

The development of mass spectrometry (MS) facilities within the laboratory has been an important asset for our Institute and the University (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 well over 90 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 [2].

We collaborated with the group of J.-F. Collet by using proteomics to study how bacteria rescue proteins that get damaged by oxidation. We discovered a new enzymatic system, MsrPQ, repairing proteins containing methionine sulfoxide in the bacterial cell envelope, a compartment particularly exposed to reactive oxygen species (ROS) and chlorine generated by the host defense mechanisms [7]. 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 catalytic mechanisms and oxidative protein folding. Recently, to gain insight into cysteine thiol-dependent ROS signaling in *Corynebacterium diphteriae*, we have unraveled the redox relay mechanisms of methionine sulfoxide reductase A and shown that this enzyme is coupled to two independent redox relay pathways involving either thioredoxin 1 or mycoredoxin 1 [8].

We currently use label-free multidimensional LC-MS to study differential protein expression. We also use phosphoproteomics strategies to identify new targets downstream of different signaling pathways under various conditions. We have developed an approach based on protein extraction in SDS, by the filter-assisted sample preparation (FASP) procedure, followed by hydrophilic liquid chromatography (HILIC) and metal oxide affinity capture (MoAC) on TiO_2 to enrich and concentrate phosphopeptides. We applied the protocol to analyze protein expression and perform phosphoproteomics on brown adipose tissue (BAT) from hibernating versus euthermic ground squirrels. Surprisingly, expression of mitochondrial membrane proteins, mitochondrial matrix proteins, and proteins involved in both glucose and lipid metabolism in BAT remained largely constant between active euthermic squirrels and their hibernating counterparts. Indeed, further validation with immunoblotting confirmed that the protein levels of mitochondrial respiratory chain complexes were largely unchanged. Through phosphoproteomics, we were able to identify increased levels

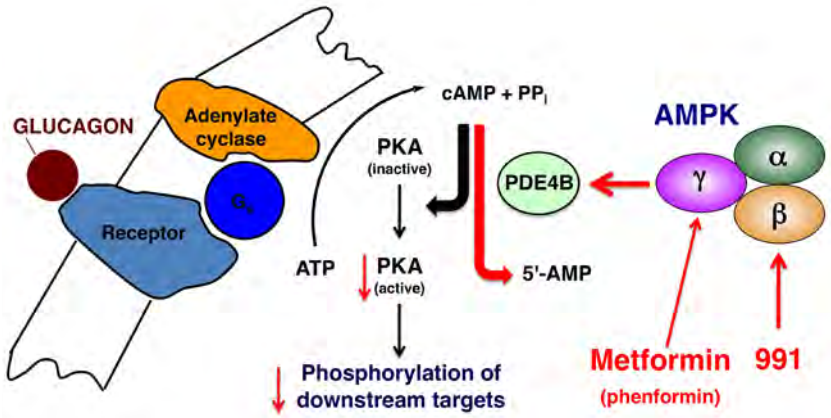

Fig. 3. Mechanism by which metformin and compound 991 antagonize glucagon signaling. Unlike biguanides, treatment with 991 activates AMPK without increasing cellular AMP levels. Both biguanides and 991 activate the major PDE isoenzyme 4B in hepatocytes in an AMPK-dependent manner. Metformin and phenformin can activate hepatic AMPK via a rise in AMP. Phosphorylation-induced activation of PDE4B by AMPK reduces glucagon-stimulated cAMP accumulation. As a consequence, PKA activation by glucagon and downstream signaling are decreased in hepatocytes incubated with 991, the effect being AMPK-dependent.

of PDH phosphorylation at Ser293 and Ser295 in BAT during ground squirrel hibernation, as confirmed by immunoblotting with phospho-specific antibodies. Phosphorylation at these sites leads to PDH inactivation, which suggests that carbohydrate oxidation would be inhibited in BAT from hibernating squirrels. We also identified increases, confirmed by immunoblotting, in hormone-sensitive lipase (HSL) Ser554, Thr648 and Ser649 phosphorylation in BAT during hibernation, suggesting that HSL would be in a partly activated state in BAT during hibernation for producing fatty acids required for thermogenesis during arousal [9].

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PRESS RELEASE

"Une nouvelle arme anti-diabète dévoilée par l'UCL. La découverte des chercheurs de l'Institut de Duve pourrait mener à sélectionner de nouvelles molécules" (Le Soir, 9/03/2016)

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Membrane organization / dynamics and epithelial differentiation

Pierre J. Courtoy
Christophe E. Pierreux
Donatienne Tyteca

Our group focuses on intertwined topics related to the control of membrane organization/dynamics at the subcellular level and its significance for cell physiology and disease; and of epithelial polarity during tissue differentiation and disease. By high-resolution vital imaging of the erythrocyte plasma membrane after insertion of lipid analogs or decoration of endogenous lipids by toxin fragments, we provided evidence for submicrometric lipid domains. These data force to revise concepts on scale (submicrometric) and stability (minutes) of lateral lipid organization at the living cell surface. We currently address how membrane lipid domains are regulated and whether they could play a role in erythrocyte deformability and membrane fragility diseases. Investigating endocytosis in kidney tubules, we found that recapture of ultrafiltrated enzymes is a key process in lysosomal biogenesis and that endosome recycling to the apical membrane, thus apical membrane composition, is selectively controlled by class III PI3-kinase/Vps34. At the tissue level, we analyze 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. Combined expertises help us addressing physiopathology and adaptation mechanisms in kidney and thyroid of cystinosis, a paradigmatic lysosomal storage disorder due to inherited defective lysosomal membrane cystine exporter. 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.

Segregation of plasma membrane lipids into submicrometric domains

M. Carquin, L. Conrard, P. Van Der Smissen, P.J. Courtoy, D. Tyteca

This project addresses a fundamental property of plasma membrane lipids - self-assembly into stable submicrometric 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 submicrometric 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) of sphingomyelin (< 1% of endogenous) labels submicrometric domains at the plasma membrane of freshly isolated red blood cells (RBCs) partially spread onto coverslips and of cultured cells. In CHO cells, intracellular conversion of BODIPY-ceramide into -sphingomyelin produced similar plasma membrane submicrometric domains as direct BODIPY-sphingomyelin insertion into the cell surface. Inhibition of endogenous sphingomyelin synthesis or surface sphingomyelin depletion by sphingomyelinase erased BODIPY-sphingomyelin domains. Both controls suggested that domains labelled by exogenous BODIPY-sphingomyelin reflect endogenous sphingomyelin compartmentation [1]. Labelling with BODIPY-phosphatidylcholine and -GM1 (a ganglioside) also revealed submicrometric lipid domains, exhibiting differential temperature dependence and cytoskeleton interaction.

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

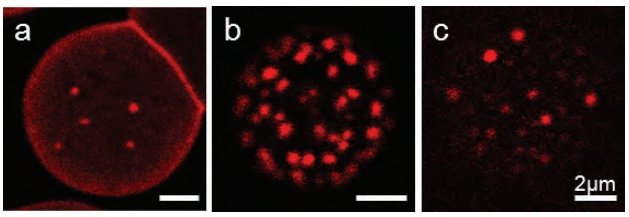

Fig. 1. Labelling of endogenous sphingomyelin, cholesterol and ganglioside GM1 by toxin fragments show submicrometric domains on RBCs spread onto coverslips. Erythrocytes labelled by fluorescent lysenin (sphingomyelin, a), theta toxin (cholesterol, b) or cholera toxin B subunit (ganglioside GM1, c) (adapted from Refs. 2, 3 & 4).

Using multiple confocal vital imaging and atomic force microscopy (collaboration with D. Alsteens, UCL), combined with modulation of membrane lipid composition and membrane:cytoskeleton interaction, we currently address whether distinct lipid domains coexist at the RBC plasma membrane.

Regulation and biophysical properties of lipid domains

C. Léonard, M. Carquin, H. Pollet, P. Van Der Smissen, P.J. Courtoy, D. Tyteca

To address the mechanism(s) of biogenesis and maintenance of submicrometric lipid domains, we focus on living RBCs. These offer the best characterized biological membrane, a featureless surface and no 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 ankyrin-dependent membrane:cytoskeleton anchorage (spherocytosis; Fig. 2c) differentially affected BODIPY-sphingomyelin submicrometric domains. This indicates that membrane tension is a key parameter controlling submicrometric lipid domains [2].

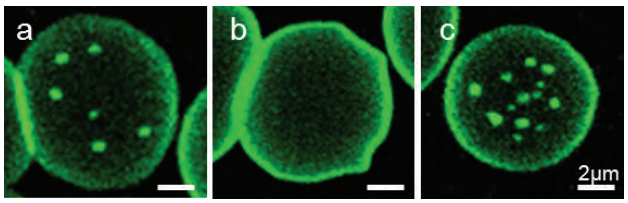

Fig. 2. Control of submicrometric domains labelled on spread erythrocytes upon insertion of fluorescent sphingomyelin (BODIPY-sphingomyelin). (a) control erythrocyte; (b) cholesterol depletion (-25%); (c) familial spherocytosis (adapted from Ref. 2).

The above observations have been obtained using RBCs spread onto coverslips, up to almost a flat, two-dimensional rigid system, which is far from the biconcave shape and plasticity of RBCs in the circulation. However, imaging of living RBCs gently suspended in a 3D-gel, thus without artificial stretching, confirmed the existence of submicrometric domains for endogenous sphingomyelin and cholesterol. This suggests that submicrometric compartmentation of endogenous lipids is a genuine feature of erythrocytes *in vivo* [3-4].

Altogether, our observations support a revised model of the scale (submicrometric) and stability (minutes) of lateral lipid organization at the plasma membrane in living cells [5].

We currently address whether and how lipid domains are stabilized/restricted by intrinsic lipid packing and/or protein:lipid interactions. Depending on lipid and protein composition of lipid domains, their biophysical properties can strongly vary. We therefore specifically ask whether lipid domains exhibit differential membrane fluidity and curvature on living RBCs. To analyze protein:lipid interactions, we use an alternative model system to RBC, the budding yeast *S. cerevisiae*, which offers a large collection of mutants and displays plasma membrane submicrometric protein domains, termed eisosomes (collaboration with B. André, ULB). A fluorescent analog of ceramide (BODIPY-ceramide), the precursor of complex sphingolipids, revealed that sphingolipids form submicrometric domains at the yeast plasma membrane. Deletion of Pil1, the major eisosome protein, decreased domain abundance, suggesting that eisosome proteins promote sphingolipid domain biogenesis and/or maintenance.

Physiopathological significance of lipid domains

H. Pollet, L. Conrard, C. Léonard, P.J. Courtoy, D. Tyteca

To address the physiopathological roles of lipid domains, we focus on RBCs. Two opposite roles may be considered: they could (i) promote lipid resilience necessary to membrane deformability, by providing stretchable membrane reservoirs for RBC squeezing into the narrow pores of spleen sinusoids (Fig. 3, hypothesis #1A) and/or platforms for recruitment of proteins involved in RBC deformation (#1B); or (ii) reflect high-tension fragility sites, *i.e.* propensity to fragmentation and hemolysis during splenic filtration of senescent or diseased RBCs (#2).

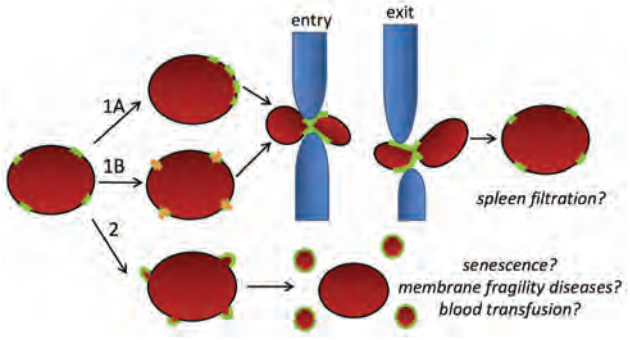

Fig. 3. Hypothetical roles for submicrometric lipid domains on RBC membrane stability and deformability. #1A, domain clustering into a membrane reservoir; **#1B**, domains as platforms for recruitment of proteins involved in RBC deformation; **#2**, domains as fragility sites allowing membrane rupture. Red, RBC; green, lipid domains; orange, recruited proteins; blue, narrow pores of spleen sinusoids.

The membrane reservoir hypothesis (#1A) is currently tested by biophysical approaches. Our data reveal that cholesterol-enriched domains gather into high curvature areas during RBC deformation. To examine whether lipid domains could control the recruitment and/or activation of proteins at the RBC membrane (#1B), we focus on calcium exchanges since these are triggered by mechanical deformations.

To investigate the fragility site hypothesis (#2), we analyze lipid domain organization in RBCs from patients with hereditary spherocytosis, a genetic membrane fragility disease that impairs resistance to shear stress and causes hemolytic anemia, which eventually requires spleen removal. This long-term study included so far six patients, splenectomized or not, from three families (collaboration with C. Vermylen, Cliniques universitaires Saint-Luc). Our data evidence a specific, splenectomy-dependent, increase of sphingomyelin-enriched domains, suggesting these domains could represent fragility sites susceptible to vesiculation. Current investigations aim at testing the consequences of lipid domain alterations for RBC deformability by biophysical approaches.

Apical endocytosis and diseases

H.P. Gaide Chevronnay, G. Grieco, V. Janssens, P. Van Der Smissen, C.E. Pierreux, P.J. Courtoy

Due to the extraordinary efficiency of apical receptor-mediated endocytosis, kidney proximal tubular cells (PTCs) offer a unique system to study rate-limiting machineries of apical endocytic trafficking and their involvement in kidney diseases.

We identified a key role of class III PI3-kinase/Vps34 in apical recycling of endocytic receptors. *In vitro*, acute 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. In mouse pups PTCs, conditional Vps34 inactivation also led to vacuolation and intracellular redistribution of apical endocytic receptors and solutes carriers, causing a global functional defect known as renal Fanconi syndrome. These data together underline the importance of apical endocytic trafficking in epithelial biology [6].

Current investigations also address the pathophysiology of cystinosis, a multisystemic 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, then in thyroid. Our calculations predict that endocytosis of ultrafiltrated plasma proteins rich in disulfide bridges is the main source of lysosomal cystine in PTCs. Analysis of cystinosin KO mice helped us understand how cystine accumulation in PTC causes apical dedifferentiation and eventual atrophy (Fig. 4) as well as to identify natural adaptation mechanisms slowing down disease progression [7]. In the thyroid, we uncovered sustained endoplasmic reticulum stress and impaired lysosomal function [8], and demonstrated that grafting of hematopoietic stem cells effectively correct cystinosis in the cystinosin KO mouse model [9].

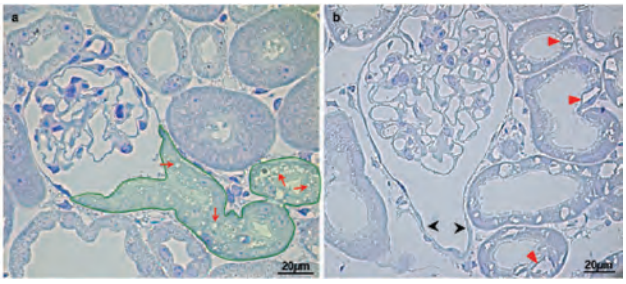

Fig. 4. Longitudinal extension of PTC lesions in cystinosin KO mice. At 6 months (a), only PTCs immediately following glomeruli (overlaid in pale green) show extensive apical vacuolation (arrows), indicating swelling of lysosomes by accumulating non-degraded protein, contrasting with integrity of kidney 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) (from Ref. 7).

Epithelial differentiation

M. Villacorte, A.-S. Delmarcelle, J. Degosserie, C. Heymans, C. Spourquet, P.J. Courtoy, C.E. Pierreux

The endoderm-derived pancreas and thyroid glands are mainly composed of polarized epithelial monolayers. The pancreas is 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 and thyroid buds first expand as a proliferating non-polarized epithelial mass, and then reorganize in specialized monolayers. This process is gland-autonomous (reproduced in explant culture).

Epithelial monolayer formation requires a coordinate and dynamic interaction with the close environment, composed of mesenchymal and endothelial cells. By three-dimensional analysis of developing pancreatic and thyroid epithelial buds, we uncovered a dense, closely apposed endothelial network (Fig. 5). Our *in vivo* and *in vitro* data show that in the pancreas, endothelial cell recruitment is dependent on VEGFa produced

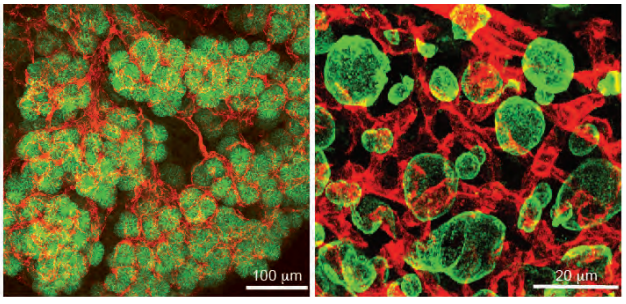

Fig. 5. Epithelial:endothelial interactions in the pancreas and thyroid. Projections of 40 to 50 confocal images showing the dense and close association of endothelial cells, labelled for PECAM (red) with pancreatic epithelial cells, labelled for E-cadherin (green, left) or with thyroid follicles, labelled for ezrin (green, right) (from Refs. 11 & 12).

by the epithelium. Interestingly, only epithelial cells from the trunk and branches of the developing pancreas produce VEGFa; this latter is absent from the peripheral tip cells of the pancreas. Localized VEGFa expression in the pancreatic epithelium generates a regionalized recruitment of endothelial cell, which prevents acinar differentiation from trunk and branches, only allowing acinar differentiation from the tip cells at a distance from endothelial vessels [10].

Such regionalized VEGFa expression was not observed in the thyroid anlage where all the epithelial cells express high level of the angiogenic factor. Recruitment of endothelial cells is thus abundant and homogeneous. Using in vivo and in vitro approaches, we demonstrated that VEGFa is required for endothelial cells recruitment and expansion in the thyroid, and that in turn endothelial cells promote thyroid epithelial cell organization into follicles independently of blood supply, by releasing soluble and sedimentable paracrine instructive factors [11]. In addition, we found that the BMP/Smad signalling pathway also controls thyroid epithelial cell organization in follicles. BMP not only controls epithelial expression of VEGFa, thus sustaining endothelial cell recruitment, but also production of basement membrane components. Deposition and assembly of a basal lamina around the epithelial cells orient apico-basal polarization and stimulate lumen enlargement [12]. These data demonstrate that paracrine epithelial:mesenchyme and epithelial: endothelial interactions are crucial for organ differentiation.

Brief report on the Platform for Imaging Cells and Tissues

P. Van Der Smissen, T. Lac, D. Tyteca, P.J. Courtoy

Besides sharing the same laboratory and continuing a two-decade fruitful collaboration with the group of E. Marbaix and P. Henriet [Selvais et al., FASEB J 2011;25:2770-81; Cominelli et al., Traffic 2014;15:401-17; see their report], we have pursued our long-term commitment to promote collaborations by sharing expertise in cellular imaging. For the recent years, see our contribution to the evidence for the reprogrammation of human pancreatic duct-derived cells into insulin-secreting cells by MAFA smRNA [Corritore et al., Stem Cells Transl Med, Epub 2016 Jul 12]; subcellular trafficking of the thrombopoietin receptor [Pecquet et al., Blood 2012;119:4625-35; see report by S. Constantinescu] and the amyloid precursor protein, APP [Ben Khalifa*, Tyteca* et al., FASEB J 2012;26:855-67]; elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 [Wiame et al., Biochem J 2010;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]; subcellular distribution of the NAD(P)HX repair system [Marbaix et al., Biochem J 2014;460:49-58; see report by E. Van Schaftingen]; differential subcellular localization of reactive oxygen species in mitochondria or lysosomes [Denamur*, Tyteca* et al., Free Radic Biol Med 2011;251:1656-65]; first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase [Miranda et al., Biochem Biophys Res Comm 2010;396:656-61; see report by M. Rider]; 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].

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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, 2]. This seminal observation led us to 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. We recently focused on the control by individual cells of local MMP activity, including induction by hypoxia and down-regulation by receptor-mediated endocytosis and degradation. We also investigate whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding [3] and endometriosis.

Matrix metalloproteinase (MMP)-27 is retained in the endoplasmic reticulum and is expressed by M2 macrophages in the human endometrium and in endometriotic lesions

A. Cominelli, H. Gaide Chevronnay, P.J. Courtoy, D. Tyteca, E. Marbaix, P. Henriet

Our recent comparative analysis of the transcriptomes in different tissue compartments of the human endometrium [4] suggested that, during menstruation, MMP-27 was expressed in degrading areas within the stroma of the superficial layer. Because knowledge on MMP-27 is extremely limited, we decided to investigate the molecular properties of this protease as well as its expression in the human endometrium and in related diseases.

We noticed that MMP-27 was produced by various cell lines but was not secreted. Because sequence comparison with other MMPs suggested that MMP-27 was prolonged by a unique C-terminal extension (CTE) partially hydrophobic but shorter than the transmembrane domain of well-known membrane-type-MMPs (MT-MMPs), we investigated the effects of the CTE on MMP-27 intracellular retention [5]. Subcellular fractionation and/or confocal microscopy highlighted retention of endogenous MMP-27 (Fig. 2) and of tagged recombinant rMMP-27 in the endoplasmic reticulum (ER). In striking contrast, a truncated form of rMMP-27 without CTE was secreted. Moreover, addition of the MMP-27 CTE to rMMP-10 (a classical secreted MMP) blocked its secretion and resulted in a subcellular localization similar to that of rMMP-27. In the same publication, we further demonstrated, by proteinase K protection assay and surface biotinylation, that MMP-27 is not a transmembrane protein. MMP-27 is rather a peripheral membrane protein since endogenous or recombinant MMP-27 was found exclusively in the aqueous phase after Triton X-114 extraction.

In parallel, expression of MMP-27 was measured by quantitative PCR in endometrial samples representative of the different phases of the menstrual cycle [6]. The levels of MMP-27 mRNA steadily increased during the secretory phase to culminate at the menstrual phase and decreased during the proliferative phase. MMP-27 mRNA was also detected by *in-situ* hybridization, in isolated cells from various organs during mouse development, suggesting strict control of cellular origin. In agreement, MMP-27 was immunostained in the human endometrium in large cells expressing CD163 and CD206, two specific markers of M2 macrophages. In the same publication, we also reported that MMP-27 was abundant in superficial endometriotic lesions (ovary, peritoneum) but not in deep endometriosis lesion (recto-vaginal wall).

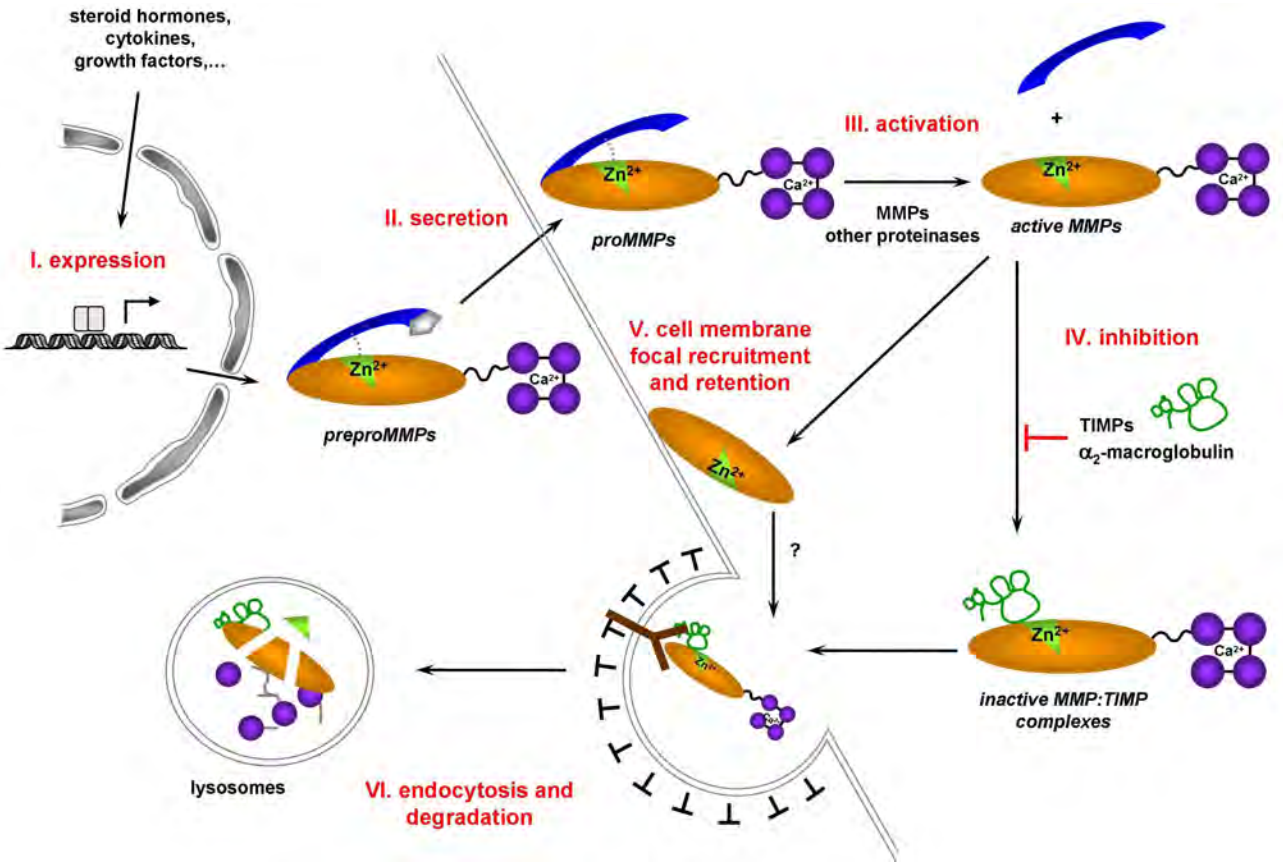

Fig. 1. Regulation of soluble MMP activity in the human endometrium: current model. MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn^{2+}-dependent catalytic 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 α_2-macroglobulin; (v) secondary membrane recruitment increasing pericellular activity; and (vi) down-regulation by endocytosis.
In the cycling human endometrium, MMP 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-secretory 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).

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 (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 [7]. Using human fibrosarcoma HT1080 cells, we recently identified two membrane-associated metalloproteinases, ADAM-12 and MT1-MMP, that shed LRP-1 ectodomain [8]. 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

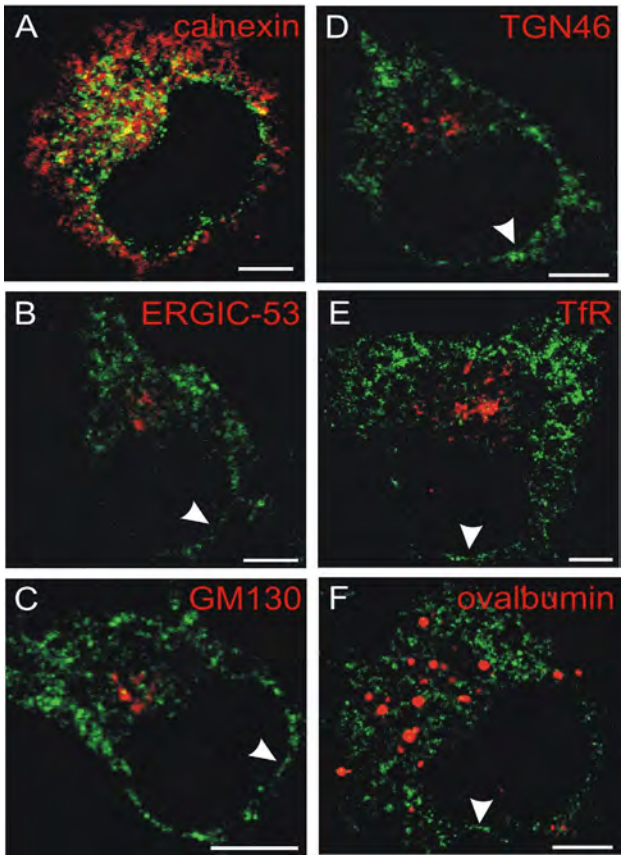

Fig. 2. MMP-27 partially colocalizes with an endoplasmic reticulum marker but dissociates from other cell compartments. COS cells were cultured on coverslips, fixed, permeabilized and double-immunolabeled for MMP-27 (in green) and marker antibodies (in red) identifying the ER (calnexin, A), the ER-Golgi intermediate compartment (ERGIC-53, B), the Golgi stacks (GM130, C), the trans Golgi network (TGN46, D), the endocytic/recycling apparatus (transferrin receptor TfR, E) or the lysosomes (Ovalbumin-texas red pulse-chase, F) Scale bars, 5 µm. Arrowheads point to MMP-27 labeling at the nuclear envelope.

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, A. Béliard (University of Liège, Belgium) and with B.F. Jordan and B. Gallez (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 [3]. 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 is 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 [9]. 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. However, pO_2 was not modified by hormone withdrawal and we did not find significant HIF1-α immunostaining, nor pimonidazole adducts in grafts. This study allowed us to conclude that hypoxia is not needed to trigger menstrual-like tissue breakdown or repair in human endometrial xenograft.

Using the same model, we also showed that hormone withdrawal induces a rapid decrease in graft volume mainly attributable to stroma condensation and breakdown, concomitant with an increase of proliferation markers [10]. Reinsertion of estradiol pellets after induced menstruation blocked volume

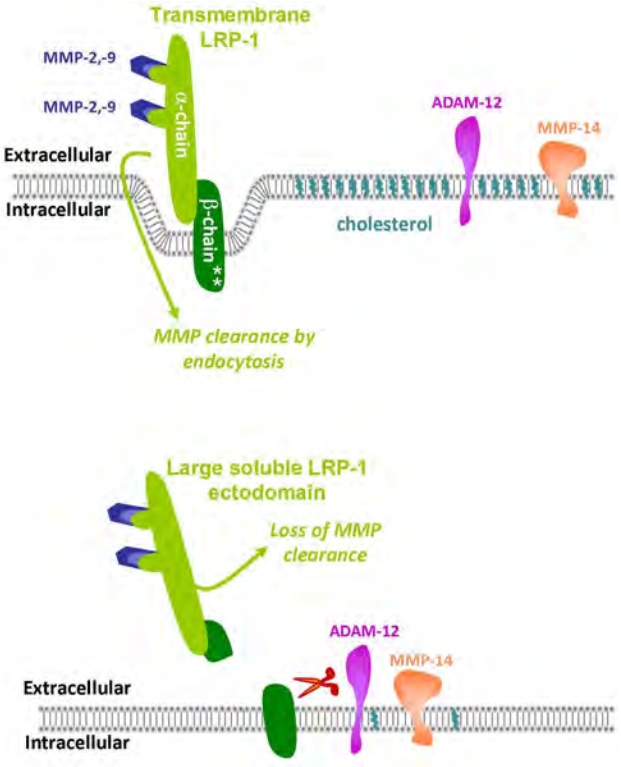

Fig. 3. A model for regulation of gelatinase activity by LRP-1. Upper panel: Binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). Sheddase activity of ADAM-12 and MT1-MMP is prevented by cholesterol-induced membrane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

decrease and stimulated epithelial and stromal growth, but, surprisingly, did not induce graft enlargement. Reinsertion of both estradiol and progesterone pellets blocked apoptosis. This was the first evidence that endometrial fragments that are not shed after menstrual tissue breakdown can support endometrial regeneration. Our study provides support to the hypothesis suggesting that endometriosis frequently results from retrograde migration of menstrual fragments of the degraded *functionalis* into the peritoneal cavity.

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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 (Fig. 1). 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. Recently, we also started to analyze how small inhibitory RNA molecules interfere with this process. 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 receptor inhibitors.

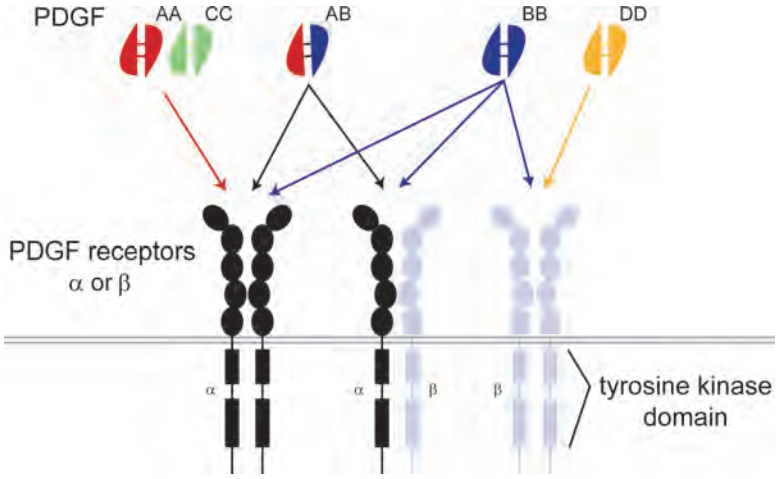

Fig. 1. PDGF receptors and ligands. Four different PDGF polypeptides (named A, B, C and D) form dimeric ligands that are binding to two receptors, α and β, encoded by two genes, *PDGFRA* and *PDGFRB*.

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

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 of 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). In several successive analyses, we identified hundreds of regulated transcripts that had not previously been linked to PDGF signaling [1-2].

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. 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 and growth factors on the cell cycle, such as HBP1 [3].

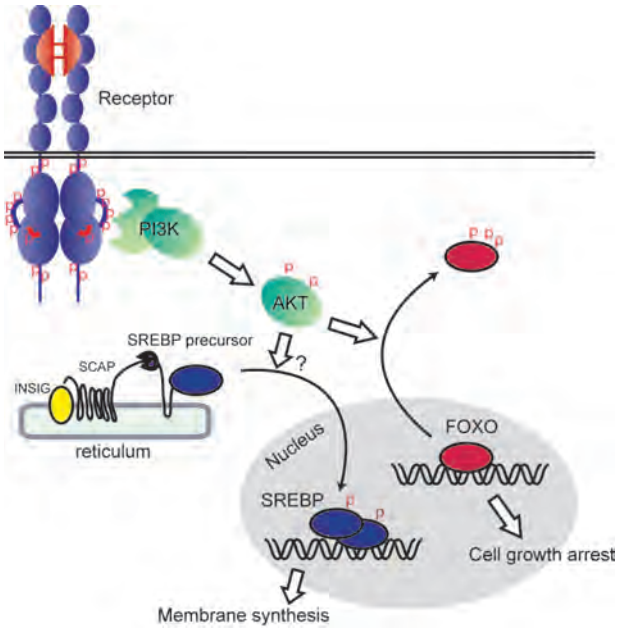

Fig. 2. Activation of SREBP and inactivation of FOXO by PDGF

In our transcriptome 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. In conclusion, our results suggest that PDGF induces membrane lipid synthesis *via* PI3K and the activation of SREBP (Fig. 2).

Rearrangements of receptor tyrosine kinase genes associated with leukemia

F. Arts, V. Havelange, 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 [4]. These chromosomal alterations create fusion genes that produce hybrid proteins 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 [4].

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 [4]. 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 [4]. This was confirmed in leukocytes from patients. 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 [4]. This is a new mechanism that contributes to cell transformation by fusion kinases.

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 [5]. We also showed that the SHP2 tyrosine phosphatase plays an important role in cell transformation by these oncogenes [6].

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 *PDGFRB* with the *KANK1* gene in a leukemia patient harboring a t(5;9) translocation [7] (Fig. 3). We are now looking for other mutations in tyrosine kinase genes.

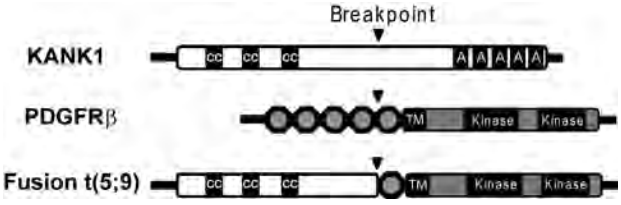

Fig. 3. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation. CC: coiled-coil domain; A: ankyrin repeat; TM: transmembrane domain

Functional characterization of novel PDGF receptor mutations in cancer and Fahr disease

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

Activating mutations in *PDGFRA* have been described in patients with gastrointestinal stromal tumors (GIST), myeloid neoplams associated with hypereosinophilia and inflammatory fibroid polyps. Some of these patients respond well to imatinib mesylate, raising the question as to whether patients with a PDGF receptor mutation in other tumor types should receive a tyrosine kinase inhibitor treatment. In this respect, novel PDGF receptor mutations have been reported in isolated cases of glioblastoma, melanoma, acute myeloid leukemia, lymphoma, peripheral nerve sheath tumors, neuroendocrine carcinoma and familial infantile myofibromatosis. However, whether these mutations are drivers or passengers in the tumorigenesis process remains an open issue since they have not been characterized functionally. This is the purpose of the present project. PDGFRα transmembrane domain mutation V536E, which stimulated cell growth and signaling *via* ERK and STAT5 in the absence of ligand. This mutant, identified in glioblastoma, was strongly inhibited by imatinib [8]. Modeling suggested that the mutation increased the packing efficiency of the transmembrane domain helices in the receptor dimer. By contrast, a number of mutations in highly conserved residues affected the receptor folding, traffic to the cell surface or kinase activity, thereby preventing the response to PDGF. Other mutations had no significant impact on the receptor activity. Altogether, several newly identified *PDGFRA* mutations do not activate the receptor and may therefore be passenger mutations. Our results also underline the importance of characterizing novel kinase alterations in cancer patients. This is a key issue in the development of personalized cancer treatments.

So far, somatic *PDGFRB* point mutations have not been reported in cancers, by contrast to *PDGFRA*. Recently, germline mutations were described in infantile myofibromatosis, a disease characterized by benign soft tissue tumors. We showed that these mutations are activating the receptor in the absence of ligand and induce fibroblast proliferation *in vitro* [9]. We also demonstrated that these mutants are sensitive to the tyrosine kinase inhibitor imatinib, suggesting a treatment for severe cases.

PDGFRB mutations have also been reported in patients diagnosed with idiopathic basal ganglia calcification (IBGC or Fahr disease), a rare inherited dominant neurological disorder. We analyzed the impact of these mutations on the PDGFRβ activity [10]. We showed that the L658P mutation results in a complete loss of kinase activity. The R987W mutant activated Akt and MAP kinases but did not induce the phosphorylation of signal transducer and activator of transcription 3 (STAT3) after PDGF stimulation. This mutant was also more rapidly degraded upon PDGF binding compared to wild-type PDGFRβ. Altogether, our data show that *PDGFRB* mutations associated with IBGC impair the receptor signaling [10]. How this defect results in basal ganglia calcification in patients remains to be established.

TFactS and ExpressGene: bioinformatics tools for transcriptome analysis

A. Essaghir, J.-B. Demoulin

Deciphering transcription factor networks from transcriptome data remains difficult. We have developed a simple method to infer the regulation of transcription factors from microarray or RNAseq data based on well-characterized target genes [2]. 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. This tool was used in a number of transcriptomics projects in our laboratory (see for instance references [2, 5]) and in collaboration with other groups.

A second bioinformatics tool was developed in collaboration with Nicolas van Baren to provide an easy access to gene expression data in normal and cancer human tissues. This tool, named ExpressGene, integrates data from gTEX and TCGA.

Roles of microRNAs in chemoresistance in myeloid neoplasms

V. Havelange, V. Vandewalle

MicroRNAs (miRNAs) are evolutionary conserved tiny noncoding RNAs (~18-25 nucleotides) that negatively regulate gene expression. MiRNAs have been involved in critical cell processes such as proliferation, apoptosis, differentiation and tumorigenesis. Recent data indicated that miRNAs are deregulated in hematological malignancies, particularly in acute myeloid leukemia (AML). The functions of most of these miRNAs in AML are currently unknown.

In collaboration with Carlo Croce and Ramiro Garzon, we focused our work on elucidating the roles of miRNAs in AML. We first integrated messenger RNA and miRNA expression profilings from AML patients to identify functional links between the whole transcriptome and microRNome involved in myeloid leukemogenesis [11]. Furthermore, we hypothesized that miR-29b could act as a tumor suppressor in AML as miR-29b was down-regulated in many subtypes of AML. Restoration of

miR-29b expression in AML cell lines and in primary samples reduced cell growth, induced apoptosis and dramatically reduced tumorigenicity in a xenograft leukemia model. Together, these data provided a rationale for the use of synthetic miR-29b oligonucleotides as a novel strategy to improve treatment response in AML.

We investigated whether miRNAs could predict chemotherapy response. In a phase II clinical trial with single hypomethylating agent decitabine in older de novo AML patients, higher levels of pretreatment miR-29b were associated with clinical response. More recently, we found that high baseline miR-10 family expression in untreated AML patients was associated with achieving complete response [12]. There was a significant interaction effect between miR-10a-5p expression and nucleophosmin (NPM1) mutation status. Loss and gain of function experiments using miR-10a-5p in cell lines and primary blasts did not demonstrate any effect in apoptosis or cell proliferation in baseline conditions nor after chemotherapy. These data support a bystander role for miR-10 family in NPM1-mutated-AML.

We will continue to investigate the roles of miRNAs in chemoresistance in AML patients.

Roles of microRNAs in tyrosine kinases alterations in myeloid neoplasms

M. Claus, J.-B. Demoulin, V. Havelange

The goal of our project is to identify miRNAs regulated by oncogenic tyrosine kinases in hematological malignancies and identify their functions, their target genes and the signaling pathways that they deregulate during leukemogenesis.

We are currently investigating miRNA expression profiles in myeloid malignancies with tyrosine kinase alterations before and after treatment with tyrosine kinase inhibitor (TKI). We will study the functional implications of miRNAs of interest in AML cell lines and identify their target genes.

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Viral immunity and pathogenesis

Jean-Paul Coutelier

The possibility for evoluted organisms to survive infections depends on the ability of their immune system to eliminate the pathogenic agent. Therefore, specialized responses, involving different subsets of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the molecules that allow those cells to communicate, and the products of those interactions, including antibodies, have been elaborated. Infections result therefore in a bias in the immune microenvironment of the host which often leads to alterations of responses elicited against non-infectious antigens and of concomitant diseases with an immune component. Our project is to analyse, in murine models, some aspects of these relations between a virus and the immune microenvironment, as well as their consequences on unrelated diseases that develop concomitantly in the infected host.

Adaptative immunity

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. We found that viral infections result in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies [1], but also in immunoglobulins with an antigenic target unrelated to viral proteins [2]. This was one of the first observations that particular infectious agents trigger distinct types of immune responses that might be more appropriate to host protection. The modulation 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. IgG2a anti-LDV antibodies were found to be more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus [3]. We could demonstrate that a dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias. This property of viruses to enhance selectively the production of one immunoglobulin isotype most probably depends on the preferential activation of a subset of T helper lymphocytes. We found indeed that LDV infection results in a suppression of Th2 responses elicited by immunization with an antigen unrelated to the virus. Moreover, we observed a dramatic prevention of diseases such as autoimmune encephalitis 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.

Innate immunity

Due to their early response after host invasion by a pathogen, innate immune cells are key players in the modulation of the immune microenvironment following infections. Their activation results in the production of a cocktail of cytokines and chemiokines that will recruit other cells and activate effector functions. The components of this cocktail strongly vary, depending on the nature of the microorganism involved, with major consequences on the course of concomitant pathologies, initially unrelated to the pathogen. LDV has proven to be a useful model to analyse some of these consequences, in the context of a viral infection.

Activation of natural killer cells and protection against cancer development

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 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-γ 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.

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.

Because cancer development is controled by immunosurveillance, including by NK cells, we analysed the effect of LDV infection on plasmacytoma growth [5]. Acutely infected animals were significantly protected against tumor development. This protection was mediated by natural killer cell activation and by interferon-γ production. It might also be related to activation of NK/T cells, although this remains to be formally proven. A similar protection by LDV infection was observed against a mesothelioma cell growth. Interestingly, *in vivo* administration of ligands of innate receptors of the TLR family, which mimicks early steps of innate immune system stimulation by infectious agents, similarly protects against early development of some cancers. This may suggest that common infections may enhance anti-tumoral immunosurveillance.

Dendritic cells and antigen presentation

In collaboration with J. Van Snick and C. Uyttenhove, acute LDV infection was found to prevent also graft-versus-host disease. This protective effect was correlated with the functional impairment, followed by the disappearance of a subset of dendritic cells involved in allogeneic reactions. Type I interferon production, resulting from TLR-7 ligation is involved in this suppressive effect.

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 exacerbated susceptibility to endotoxin shock [6]. 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 [7]. 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 spontaneaous platelet phagocytosis [8]. 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. 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 [9]. 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 analyzed whether a viral infection could modulate such an autoantibody-mediated autoimmune disease. In mice treated with anti-platelet antibodies, infection with LDV or mouse hepatitis virus was followed by severe thrombocytopenia [10], whereas infection alone, without autoantibody administration led to a moderate disease. This provide a new experimental model for human Immune Thrombocytopenic Purpura, a frequent child disease, based on an unexpected pathogenic mechanism (Fig. 1). Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was

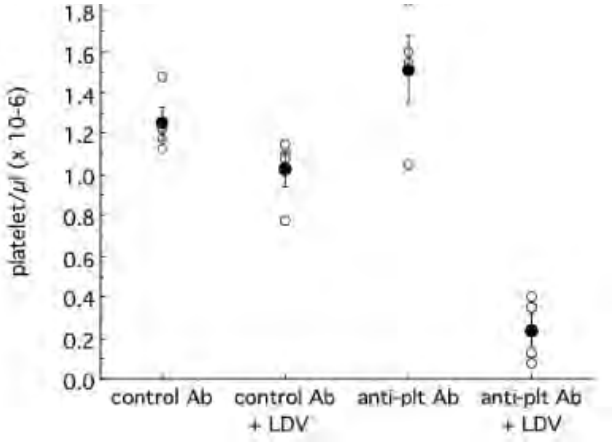

Fig. 1. Platelet counts in control mice and in animals infected with LDV and treated with control antibody or anti-platelet autoantibody.

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 enhance-

ment of erythrophagocytosis in the liver [11].

Treatment of thrombopenic or anemic mice with clodronate-containing liposomes and with total IgG indicated that opsonized platelets and erythrocytes were cleared by macrophages. 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 some, but not all of the receptors recognizing the Fc portion of antibodies, which may at least partially leads towards the enhanced phagocytic activity of macrophages.

We analyzed also the particular case of IgM autoantibodies [12]. LDV exacerbates the pathogenicity of IgM anti-platelet, but not anti-erythrocyte autoantibodies. To define the role of Fcα/μ receptor (Fcα/μR) in IgM-mediated thrombocytopenia and anemia, mice deficient for this receptor were generated. These animals were resistant to IgM autoantibody-mediated thrombocytopenia, but not anemia. However, LDV-induced exacerbation of thrombocytopenia was not associated with enhanced Fcα/μR expression on macrophages. These results indicate that Fcα/μR is required for the pathogenicity of IgM anti-platelet autoantibodies, but is not sufficient to explain the full extent of the disease in virally-infected animals.

Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia, and some but not all anemias, 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.

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Viral persistence and interferon response

Thomas Michiels

About viruses ... Viruses need to parasite human cells to replicate. Therefore they produce proteins that were shaped by evolution to target critical decision centers of the host cell. Thus, studying how viral proteins interfere with the host not only helps in the development of antiviral therapies but also contributes to decipher key pathways of the cell biology and of the host immune response.

We analyze the interplay between viral infections and the **innate immune response** of the host. On one hand, we study the model infection of the central nervous system by Theiler's virus. This virus can escape host immune defenses and provoke a persistent infection of the **central nervous system** of the mouse leading to chronic demyelinating disease reminiscent of human multiple sclerosis [1, 2]. Our analysis mostly focuses on two proteins encoded by **Theiler's virus, L and L*** (Fig. 1), that interfere with the host innate immune responses and therefore facilitate the establishment of a persistent infection. On the other hand, we study 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.

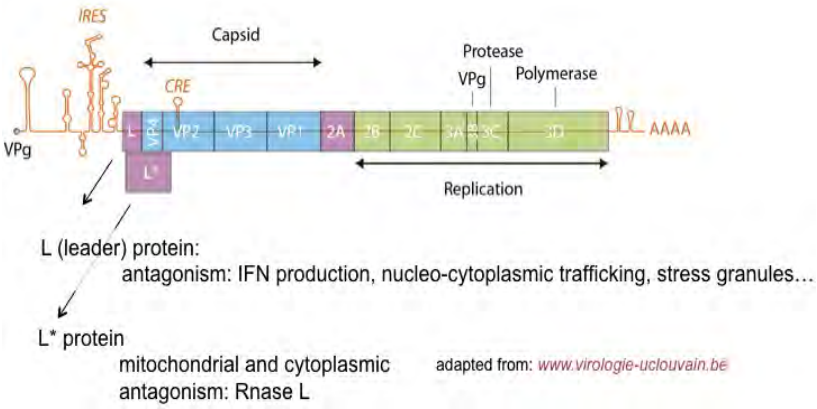

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2,000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Two proteins produced by Theiler's virus, namely L and L*, were found to be dispensable for viral replication in 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.

Theiler's virus and Saffold virus

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a small RNA virus of the family *Picornaviridae*, which has a striking ability to escape immune responses and to establish persistent infections of the central nervous system of the mouse.

Recently, a human virus was discovered, Saffold virus, that is closely related to Theiler's virus and was therefore classified with Theiler's virus in the species *Theilovirus*. Saffold virus is highly seroprevalent in the human population but has not been firmly associated with any pathology although some infection cases correlated with neurological symptoms. We examined Saffold virus propagation in tissues of experimentally infected mice. Interestingly, the virus was **neuroinvasive** as it could reach the central nervous system after primary replication in peripheral tissues (Fig. 2). On the other hand, Saffold virus also displayed a marked tropism for the pancreas, which warrants further studies about the possible involvement of SAFV in pancreatic diseases in humans [3].

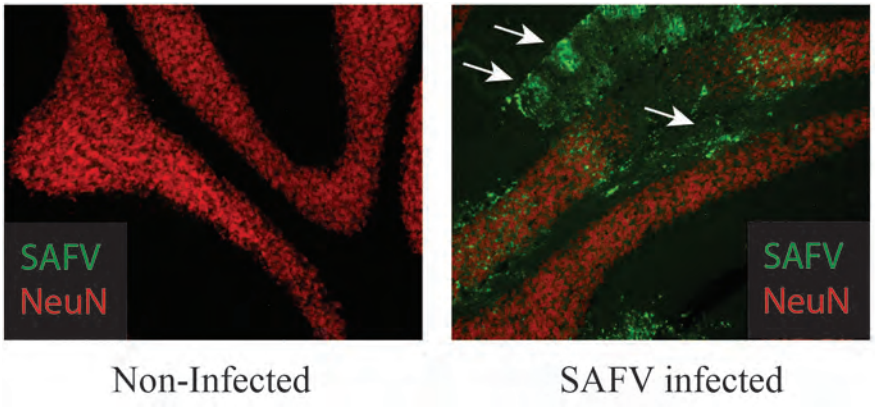

Fig. 2. Saffold virus detection in the cerebellum of infected mice. Saffold virus was detected by immunohistochemistry (green), using a chicken antibody raised against the virus capsid protein VP1. Neurons were detected with an anti-NeuN antibody. White arrows indicate heavily infected cerebellum areas.

Regarding Theiler's virus, our group focuses on **two proteins encoded by this virus, L and L*, that interfere with the host innate immune responses** and therefore facilitate the establishment of a persistent infection.

The L protein: a multifunctional peptide interfering with innate immunity.

The L ("leader") protein encoded by Theiler's and Saffold virus is a 76 amino acid-long peptide containing a zinc-binding motif. We observed that this protein exerts pleiotropic activities in infected cells:

- **L inhibits the transcription of type I IFN** and of chemokine genes. 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*.

- **L 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. These traffic-perturbing activities of the L protein correlate with L-mediated hyperphosphorylation of the Nup proteins that make up the nuclear pore complex.

- **L inhibits stress granule assembly**. Stress granules are stalled 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 [4]. Current analyses suggest that inhibition of stress granule formation may be the consequence of L-mediated inhibition of PKR, a cellular kinase upregulated by the IFN response. Our data suggest that **L interferes with the ability of PKR to be activated by double-stranded RNA**.

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).

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 (Fig. 3). As potential master L target, we recently identified a family of **cellular kinases, which can be activated by contact with the L protein**. We undertook the analysis of the mechanisms by which these kinases can be activated by L binding and the relevance of this activity in host-pathogen interaction.

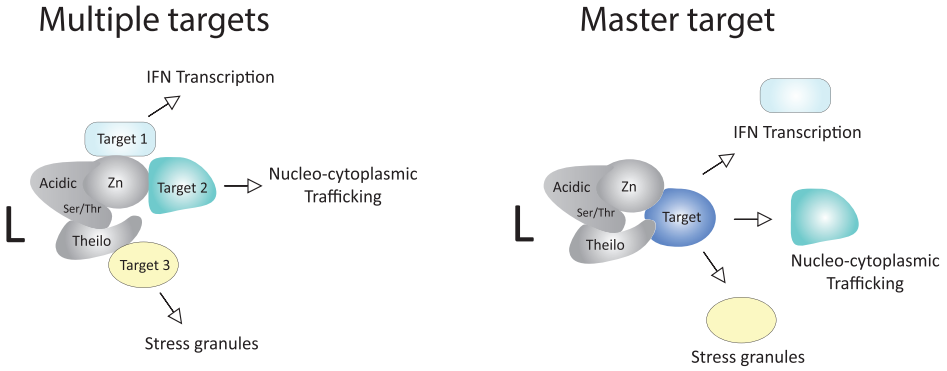

Fig. 3. L is a multifunctional protein that interferes with essential cellular functions. The L protein contains several domains: a Zinc finger, a central acidic domain, a Ser/Thr-rich domain and a c-terminal "Theilo"-domain, which is lacking in the L protein of the related EMCV virus. Two models can explain the multiple functions of L. In the first model, different L interactors mediate the different activities of L. In the second model, L interacts with one or few targets, which in turn mediate the various effects.

The L protein: Inhibition of the OAS/RNase L pathway*

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 Figure 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.

We showed that L* protein is **partitioned between the cytoplasm and the mitochondria** (Fig. 4). In mitochondria, L* is anchored in the outer membrane, facing the cytosol [5].

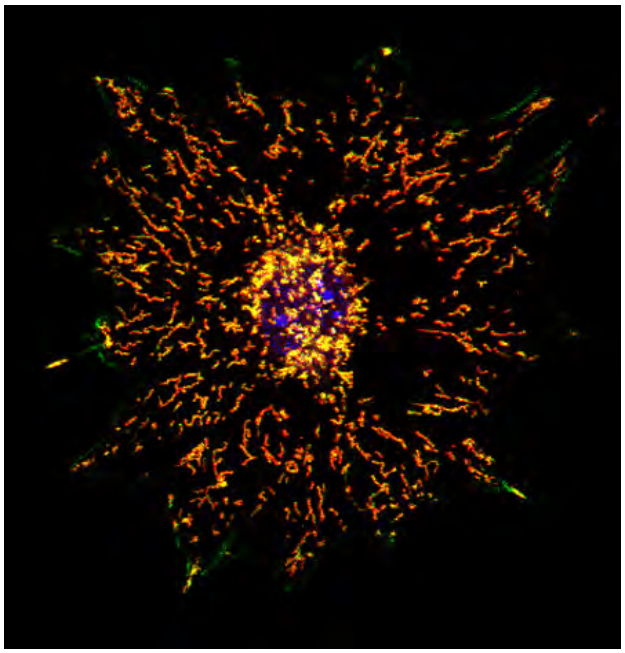

Fig. 4. Mitochondrial localization of L*. Mouse embryonic fibroblast expressing L* from a lentiviral vector. L* was immunolabeled in green and mitochondria were labeled in red with mitotracker. Yellow indicates co-localization.

Our recent data suggest that the cytosolic fraction of L* protein antagonizes the OAS/RNase L pathway [6]. 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 its mouse but not human RNase L. We took advantage of the species-specific activity of L* to map the interface between L* and mouse RNase L by testing the inhibition by L* of chimeric mouse/human RNase L. The L* footprint on the RNase L structure suggests that L* could act either by inhibiting 2-5A binding to RNase L or by inhibiting RNase L dimerization. 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 [7].

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 developed 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 neighboring 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 (IFN-α/β, IFN-ε)

Type I interferons (IFNs) are a family of cytokines that play a critical role in the defense of the organism against viral infection. In spite of their sequence divergences, all type I IFNs (IFN-α, IFN-β, IFN-κ, IFN-ε, IFN-ω, ...) bind the same heterodimeric receptor made of the IFNAR-1 and IFNAR-2c subunits. IFN binding to the receptor activates the JAK/STAT pathway and triggers the transcriptional upregulation of many genes called ISGs (Interferon Stimulated Genes). The proteins encoded by these genes (Mx, PKR, OAS, ...) are responsible for the antiviral, cytostatic and immunomodulatory activities of type I IFNs. We previously characterized the murine IFN-α family. Fourteen IFN-α genes were detected in the mouse genome, two of which, IFN-α13 and IFN-α14, were previously undescribed. We also showed that IFN-ε, another type I IFN subtype, was more specifically expressed by cells of reproductive organs. Importantly, dysregulation of IFN production can have adverse effects and induce diseases referred to as interferonopathies. In collaboration with Yanick Crow (University of Manchester and Institut Necker, Paris), we currently analyze gene mutations responsible for the **Aicardi-Goutières syndrome**, where excessive IFN production is responsible for a crippling developmental disease.

Type-III Interferons (IFN-λ)

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 [8]. 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. 5), unlike response to IFN-α, which occurred in most cell types and was particularly prominent in endothelial cells. Accordingly, tissues

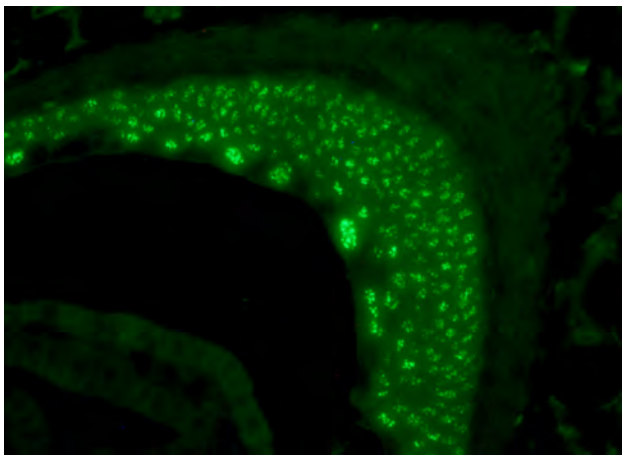

Fig. 5. Response to IFN-λ. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-λ. Shown is a fluorescent labeling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

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 [8].

Work performed in collaboration with the groups of P. Stäheli (University of Freiburg, Germany) and M. Hornef (University 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 [9].

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**.

Interferons, neurons, and neuroinvasion

In collaboration with the teams of P. Stäheli and F. Weber (University of Freiburg, Germany), we analyzed the IFN response in the central nervous system. We observed that the relative production of IFN-λ (over that of IFN-α/β) was low in the brain of mice infected with neurotropic viruses. Thus, the central nervous system appears to be both a poor producer of IFN-λ and a poor responder to this cytokine. In contrast, IFN-α/β is readily produced in the central nervous system and we showed that neurons were able to contribute to the production of these IFNs [10], although they appear to be much less potent than astrocytes at producing IFN [11]. Neurons also appear to have a restricted IFN response, **some ISGs, such as Apolipoprotein L9, being specifically not or little expressed in neurons** [12].

Finally, we study the implication of IFNs in the control of neuroinvasion by viruses. Viruses that infect the central nervous system, such as poliovirus or some herpes viruses, first infect the periphery. From their replication site in periphery, they can gain access to the central nervous system by different routes including the hematogenous route and the axonal transport. We currently analyze how IFN can restrict central nervous infections by these routes.

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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 is to better understand the mechanisms and limitations of T cell-mediated immunity to human tumors in order to improve the clinical efficacy of cancer immunotherapy.

From the discovery of human tumor-specific antigens recognized by cytolytic T lymphocytes to cancer immunotherapy

P.G. Coulie, G. Hames, C. Muller, N. Remy, T. Seremet, T. Gomard, O. Bricard, M.-S. Dheur, in collaboration with J.-F. Baurain (Department of Medical Oncology, Cliniques universitaires Saint-Luc), and N. van Baren, C. Lurquin, B. Lethé (Ludwig Institute for Cancer Research, Brussels branch)

Over the last five years immunotherapy has emerged as a new modality of cancer treatment, with remarkable results obtained in patients with advanced metastatic cancer, treated with immunostimulatory antibodies that enhance the activity of anti-tumor T lymphocytes. Many oncologists consider that cancer immunotherapy has positioned itself at the forefront of the oncology field, as it is clear that patients with a variety of cancers are achieving clinical benefit.

This success owes a lot to the pioneering work of T. Boon and colleagues at the de Duve Institute and Brussels branch of the Ludwig Institute [1]. They demonstrated that cancer cells bear tumor-specific antigens that can be recognized by cytolytic T lymphocytes, which then destroy the tumor cells. This work paved the way for clinical applications taking advantage of the tumor-specificity and memory of these cytolytic T lymphocytes to propose specific and harmless cancer treatments.

We have worked to develop immunizations to tumor antigens, thus therapeutic anti-cancer vaccines. Only 5-10% of cancer patients vaccinated with defined tumor antigens displayed 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 fluorescent formulations of antigens. We observed surprisingly low levels of anti-vaccine T cells 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 antigens against which a response is observed) of the immune responses and the clinical impact of the vaccinations. These results suggested that the main limitation to the clinical efficacy of these therapeutic anti-cancer vaccines was not the intensity of the anti-vaccine T cell responses.

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 [2]. 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 [3], 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 [4]. 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 an-

tigens. 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 or immunostimulatory antibodies targeting the CTLA-4 or PD-1 pathways.

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-1β 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 strengthen 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 T lymphocytes infiltrating human breast carcinomas

D. Schröder, O. Bricard, G. Hames, K. Missault, N. Dauguet, P.G. Coulie, in collaboration with J. Carrasco, J.-L. Canon (Grand Hôpital de Charleroi), and J.-P. Machiels, M. Berlière, C. Galant (Cliniques universitaires Saint-Luc)

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 from these tumors permanent 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 study T cell responses to human breast carcinomas in collaboration with clinical teams at the Cliniques universitaires Saint-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 in primary breast tumors contain $CD8^+$ T lymphocytes that recognize tumor-specific antigens encoded either by genes that are mutated in the tumor or by cancer-germline genes such as *MAGE*. We have established sets of about 100 $CD8^+$ T cell clones from fresh TILs, and screened them for recognition of candidate mutated antigenic peptides deduced from tumor exome sequencing, and of selected antigenic peptides encoded by *MAGE* genes.

We have analyzed the tumor-infiltrating CD8 T cells from six primary breast carcinomas, including 2 $ER^+/HER2^-$, 2 $ER^+/HER2^+$ and 2 $ER^-/HER2^-$ tumors. For five tumors, we screened for each tumor 60 to 140 different T cell clones for the recognition of 25 to 60 candidate mutant peptides, without any positive result. For the last tumor, 6 out of 57 CD8 T cell clones recognized 4 out of 109 candidate mutated peptides but not the corresponding wild-type peptides. This tumor contained more mutations than the other five, and displayed a microsatellite instability, an unusual feature (±1%) for breast carcinomas. This observation corroborates the association between high mutation burden and CTL response to mutated tumor antigens. We conclude that some human primary breast carcinomas are immunogenic, as one tumor contained at least 10% of tumor-specific cells among the $CD8^+$ TILs. The presence of tumor-specific $CD8^+$ suggests that the corresponding patient could benefit from the currently used immunostimulatory antibodies. However our analysis suggests also that most primary breast carcinomas are poorly immunogenic to T cells.

Human regulatory T cells and TGF-β

S. Lucas, J. Stockis, O. Dedobbeleer, S. Liénart, S. Lecomte, F. Lambert, A. Collignon, M. Panagiotakopoulos, C. Vanderaa, N. Huyghe, N. Dauguet, P.G. Coulie

Regulatory T cells (Tregs) are a subset of $CD4^+$ T lymphocytes specialized in the suppression of immune responses. They are essential for the maintenance of peripheral immunological tolerance. Treg development and function require transcription factor FOXP3, and in humans and mice *FOXP3* mutations result in lethal autoimmunity due to the lack of functional Tregs leading to uncontrolled activity of T cells directed against self-antigens. Excessive Treg function, on the other hand, is detrimental in cancer or chronic infections. Manipulation of Treg numbers or function is a therapeutic approach explored for several diseases, but it has faced very limited success thus far.

The mechanisms by which human Tregs suppress immune responses are still largely unknown. This is due to two major

hurdles in the field. First, we lack a Treg-specific protein marker to study these cells in humans. Whereas *FOXP3* expression is restricted to Tregs in mice, it is also found in many activated non-Treg human T cells, precluding the use of FOXP3 to accurately measure Treg proportions and numbers in human tissues. Second, a variety of Treg suppressive mechanisms have been identified in murine models but which, if any, plays a major role in humans is not known.

Our group studies the immunosuppressive mechanisms of human Tregs. We first set out to derive clones of human Tregs, to circumvent the difficulty of repeatedly isolating rare and difficult-to-identify Treg populations with poorly reproducible suppressive functions. Clones were defined as Tregs based on a stable epigenetic mark that is only found in fully differentiated Tregs in both mice and humans. This mark consists in the demethylation of a non-coding regulatory region of gene *FOXP3* that is required for the stability of FOXP3 expression upon cell divisions. Our Treg clones expressed *FOXP3*, were suppressive *in vitro* and turned out to represent uniquely pure and stable cell populations available to study human Treg function. Analyzing their transcriptional profiles, we demonstrated that activated human Tregs, but not other T cells, produced active TGF-β1. A possible contribution of soluble TGF-β1 to immunosuppression by Tregs was in line with the fatal autoimmune phenotype of *Tgfb1*$^{-/-}$ mice, but not with the contact dependency of T cell suppression by Tregs. But then we observed that TGF-β1-induced signaling in T cells co-cultured with Tregs was also contact-dependent, suggesting that active TGF-β1 was produced close to the Treg surface [5, 6]. This prompted us to study in detail the mechanisms of TGF-β1 activation by Tregs. Indeed, most cells produce inactive forms of TGF-β1 but very few are known to activate the cytokine, via tightly regulated mechanisms that are cell-type specific.

In all cells, signal peptide removal and homodimerisation of pre-pro-TGF-β1 yields pro-TGF-β1, further cleaved to produce latent TGF-β1. In latent TGF-β1, the C-terminal fragment, or mature TGF-β1, remains non-covalently bound to the N-terminal fragment known as the Latency Associated Peptide or LAP. All immune cells secrete latent TGF-β1, which is inactive because LAP prevents mature TGF-β1 from binding to its receptor. Further processing, referred to as 'TGF-β1 activation', is required to release mature TGF-β1 from LAP. A few proteins able to bind to LAP were shown to activate TGF-β1 *in vivo*. Mutant mice deficient in any one of these proteins display some of the phenotypic features of *Tgfb1*$^{-/-}$ mice, but notably none suffer from the lethal autoimmunity observed in *Tgfb1*$^{-/-}$ mice. Therefore none of these TGF-β1 activation mechanisms is required to maintain immune tolerance, and our human Treg clones appeared to use yet another, unknown mechanism.

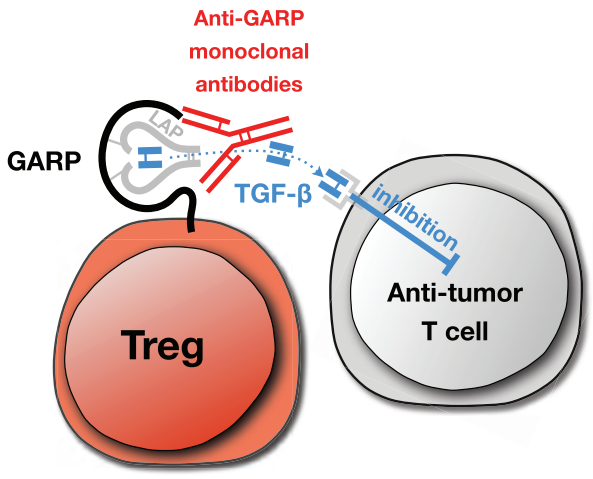

Fig. 1. Human Tregs release active TGF-β from LAP by a mechanism that requires transmembrane protein GARP. Active TGF-β produced by Tregs inhibits T cells located nearby (*e.g.* anti-tumor T cells in cancerous lesions). We derived monoclonal antibodies against GARP that are capable of inhibiting active TGF-β production, and thus immunosuppression by Tregs *in vivo*.

We then showed that human Tregs, but not other T cells, display latent TGF-β1 on their surface *via* disulfide-linkage of LAP to a transmembrane protein called GARP (Fig. 1). We thus hypothesized that GARP contributes to TGF-β1 activation at the Treg surface, and that TGF-β1 activation mediated by GARP is essential for Treg function.

Data supporting these hypotheses were difficult to obtain. We indeed first observed that transduction of non-Treg T cells with GARP constructs was sufficient to display latent TGF-β1 on their surface, but not to activate it [7, 8]. Also, four commercially available anti-GARP monoclonal antibodies (mAbs) did not block TGF-β1 activation by Tregs. But we then derived 31 additional anti-GARP mAbs, and obtained two that blocked TGF-β1 activation by human Tregs [9] (Fig. 1). Blocking anti-GARP mAbs recognize a conformational epitope that requires amino-acids $GARP_{137\text{-}139}$ within GARP/TGF-β1 complexes. The other mAbs bound other GARP epitopes and did not block TGF-β1 activation. Our two blocking anti-GARP mAbs inhibited Tregs *in vitro*, in suppression assays notoriously amenable to experimental artefacts. We wished to evidence their activity *in vivo*. Because our blocking mAbs do not recognize mouse GARP, we resorted to immunodeficient *NOD/Scid/Il2rg*$^{-/-}$ (NSG) mice grafted with human PBMCs. These mice develop clinical signs of graft-versus-host disease (GVHD) due to the activity of human T cells against murine tissues. Co-transfer of human Tregs attenuates GVHD. We transferred human PBMCs with or without autologous Tregs into NSG mice and treated the animals with blocking or non-blocking anti-hGARP mAbs. Tregs efficiently attenuated GVHD and blocking anti-hGARP mAbs completely abrogated this protection. The effect of blocking anti-hGARP mAbs was comparable to that of an anti-TGF-β1 mAb and was not observed with non-blocking anti-hGARP mAbs. The blocking anti-hGARP mAbs did not act by depleting human Tregs in NSG mice: human Treg numbers were not decreased in the treated mice, and a blocking anti-hGARP mAb carrying a mutation which precludes binding to Fc receptors retained full activity. Altogether, these results identified a new mechanism of TGF-β1 activation that requires GARP and is involved in immune suppression by human Tregs *in vivo*.

We are now trying to obtain proof-of-concept that anti-GARP mAbs could serve as a new approach for the immunotherapy

of cancer. We will derive antibodies that bind and block immunosuppression by mouse Tregs (our currently available antibodies only bind human Tregs), and we will test these in murine models of cancer to determine whether they improve immune responses against tumors and inhibit tumor growth. We are collaborating on this project with argen-x, a biotech company located in Ghent that is specialized in the development of therapeutic antibodies for the treatment of cancer or auto-immune diseases.

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Ludwig Institute for Cancer Research

Brussels Branch

Ludwig Institute for Cancer Research

Brussels Branch

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 90 members and was headed by Thierry Boon until 2009. The Branch is now headed by Benoît Van den Eynde.

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Tumor immunology and antigen processing

Benoît Van den Eynde

Cancer immunotherapy is a new approach of cancer treatment showing impressive clinical results that prolong dramatically the survival of advanced cancer patients. The concept of immunotherapy became realistic following the discovery by our groups in Brussels that the immune system has the capacity to recognize cancer cells as foreign bodies because they express tumor antigens at their surface. The recent clinical success of immunotherapy generates enormous interest in the oncology field and the pharmaceutical industry. Yet, clinical benefits remain limited to a subset of patients, and further research is needed to understand the reason for this. Our current research focuses on two aspects that are relevant to this question. The first is the processing of tumor antigens, *i.e.* the intracellular mechanisms responsible for the expression of antigens at the surface of tumor cells. The second is the tumor microenvironment, whose immunosuppressive properties emerge as a major reason why many patients currently do not benefit from cancer immunotherapy.

Processing of tumor antigens

Peptide splicing by the proteasome

N. Vigneron, V. Stroobant

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. Theses peptides result from the degradation of these proteins by a multicatalytic enzyme called the proteasome. A few years ago, we have identified a new mode of production of antigenic peptides, which involves the splicing by the proteasome of peptide fragments originally distant in the parental protein [1]. Peptide splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate (Fig. 1). Splicing of peptide fragments can occur in the forward or reverse order to that in which fragments appear in the parental protein [2]. We have now described five spliced peptides, three of which are spliced in the reverse order [3, 4]. 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]. More recently, we showed that the splicing reaction required a minimal size of three amino acids for the fragments to splice [4]. Although both the standard proteasome and the immunoproteasome have the ability to splice peptides, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice.

Intermediate proteasome types

N. Vigneron, J. Abi Habib, E. De Plaen

The proteasome exists in two forms: the standard proteasome, which is constitutively present in most cells, and the immunoproteasome, which is expressed in many immune cells and can be induced by interferon-gamma in most other cells. They differ by the three catalytic subunits they use: β1, β2 and β5 for the standard proteasome; β1i, β2i and β5i for the immunoproteasome. We have described two new proteasome subtypes that are intermediate between the standard proteasome and the immunoproteasome [5]. They contain only one (β5i) or two (β1i and β5i) 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 [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 degradation of oxidized proteins. Recently, we have developed a new assay to measure proteasome activity *in vitro* (CAPA for capture proteasome assay) [6], based on protea-

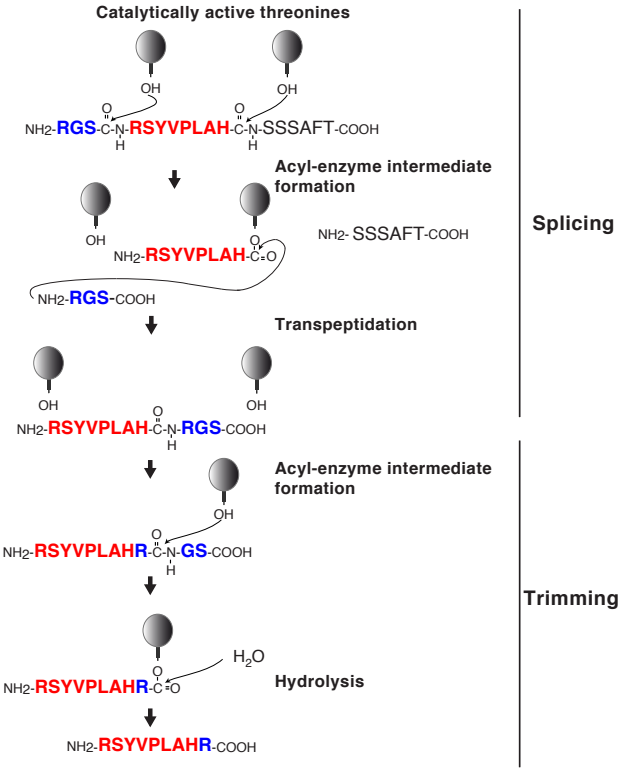

Fig. 1. Mechanism of reverse splicing by the proteasome. Model for the catalytic mechanism producing the reverse spliced peptide RSYVPLAHR. The hydroxyl group of a catalytic threonine of the proteasome produces a nucleophilic attack on the peptide bond after histidine 202, leading to the production of an acyl-enzyme intermediate comprising a peptide fragment (shown in red) attached to the catalytic subunit of proteasome by an ester link. A nucleophilic attack of this ester link by the N-terminus of another peptide fragment (shown in blue), which results from the cleavage of the same protein by another catalytic subunit of the same proteasome particle, leads to the production of a spliced peptide bearing an extended C-terminus. This peptide is further trimmed by the proteasome to release the antigenic peptide RSYVPLAHR.

some capture on an antibody-coated plate. When used with lysates originating from cells expressing standard proteasome, immunoproteasome or intermediate proteasomes β5i or β1i-β5i, this assay allows the monitoring of the chymotrypsin-like, trypsin-like and caspase-like activities of the corresponding proteasome subtype. It also represents a new tool to study the specificity of subtype-specific proteasome inhibitors.

Other proteases involved in antigen processing

N. Vigneron, V. Stroobant

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 [7]. 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 another proteasome-independent peptide, derived from MAGE-A4 and whose processing appears to involve at least two distinct proteases. The processing of a second antigenic derived from the transcription factor WT1 is also under study.

TAP-independent antigenic peptides

N. Vigneron, V. Ferrari, V. Stroobant

Presentation of most peptides depends on the TAP transporter, which transports peptides produced in the cytosol to the lumen endoplasmic reticulum for loading on MHC class I. A number of viruses and tumor cells tend to reduce their TAP expression to escape immune recognition. Therefore, antigenic peptides that are still presented by tumors in the absence of TAP represent clinical target with a great potential. We are studying several such tumor peptides derived from cytosolic proteins. We aim at characterizing their processing and more specifically define how these peptides can reach the MHC class I compartment in the absence of TAP transport.

Cross-presentation of long peptides

W. Ma, N. Vigneron, in collaboration with P. Courtoy and P. Van Der Smissen (de Duve Institute)

Class I and class II molecules of the Major Histocompatibility Complex (MHC) are responsible for the presentation of antigenic peptides derived from intracellular proteins or from engulfed exogenous proteins, respectively. As an exception to this rule, cross-presentation enables dendritic cells to present on their MHC class I molecules antigenic peptides derived from exogenous material, through a mechanism that remains unclear. Cross-presentation is essential to the activation of $CD8^+$ T lymphocytes against antigens derived from tumors and from viruses that do not infect dendritic cells. It is particularly efficient with long peptides, which are used in cancer vaccines. We are studying the mechanism involved in the cross-presentation of long peptides using human dendritic cells and specific CTL clones against melanoma antigens gp100 and Melan-A/MART1. We found that cross-presentation of long peptides does not depend on the proteasome nor on the TAP transporter, and therefore follows a vacuolar pathway. We also observed that it makes use of newly synthesized MHC class I molecules that are loaded with suboptimal peptides [8]. These nascent MHC-I molecules appear to diverge from the classical secretion pathway at an early stage and reach the late endosomes, where they exchange their suboptimal peptide cargo for the cross-presented peptide before reaching the cell surface in an endoH-sensitive form. These results indicate an alternative secretion pathway followed by HLA-I molecules that are used for cross-presentation, and may have implications for the development of vaccines based on long peptides.

Mechanisms of tumoral immune resistance

Indoleamine 2,3-dioxygenase

M. Hennequart, J. Lamy, M. Solvay, E. De Plaen, L. Pilotte, V. Stroobant, D. Colau, N. van Baren

We previously discovered that tumors often resist immune rejection by expressing Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme that is profoundly immunosuppressive [9]. We showed that immune rejection was restored by administration of a pharmacological inhibitor of IDO. This opened the way to the development of IDO inhibitors for cancer therapy. To that end, we founded in 2012 a spin-off company, named iTeos Therapeutics. Within two years, iTeos has identified a clinical candidate, IDO inhibitor, which will be tested in clinical trials in 2016 in collaboration with Pfizer.

In parallel, we have refined current knowledge about IDO expression in normal and tumoral tissues: using a newly validated monoclonal antibody to human IDO1, we performed an extensive immunohistochemical analysis of IDO1 expression in normal and tumor tissues [10]. In normal tissues, IDO1 was expressed by endothelial cells in the placenta and lung and by epithelial cells in the female genital tract. In lymphoid tissues, IDO1 was expressed in mature dendritic cells with a phenotype distinct from plasmacytoid dendritic cells. Importantly, IDO1-expressing dendritic cells were not enriched in tumor-draining lymph nodes, in contrast with previously reported findings. IDO1-expressing cells were observed in a large fraction (505/866, 58%) of human tumors. They comprised tumor cells, endothelial cells, and stromal cells in proportions that varied depending on the tumor type (Fig. 2). Tumors showing the highest proportions of IDO1-immunolabeled samples were carcinomas of the endometrium and cervix, followed by kidney, lung, and colon. This hierarchy of IDO1 expression was confirmed by gene expression data mined from The Cancer Genome Atlas database. Expression of IDO1 may be used to select tumors likely to benefit from targeted therapy with IDO1 inhibitors.

We are currently studying the regulation of IDO expression in human tumors and the cellular response of T lymphocytes to tryptophan catabolism.

Tryptophan-dioxygenase

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

Besides IDO, we recently uncovered the role of tryptophan-dioxygenase (TDO) in tumoral immune resistance [11]. 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 [11]. 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. The development of TDO inhibitors is another program currently pursued by our spin-off iTeos Therapeutics.

We are currently studying the role of TDO in additional tumor models, and characterizing the regulation of TDO expression.

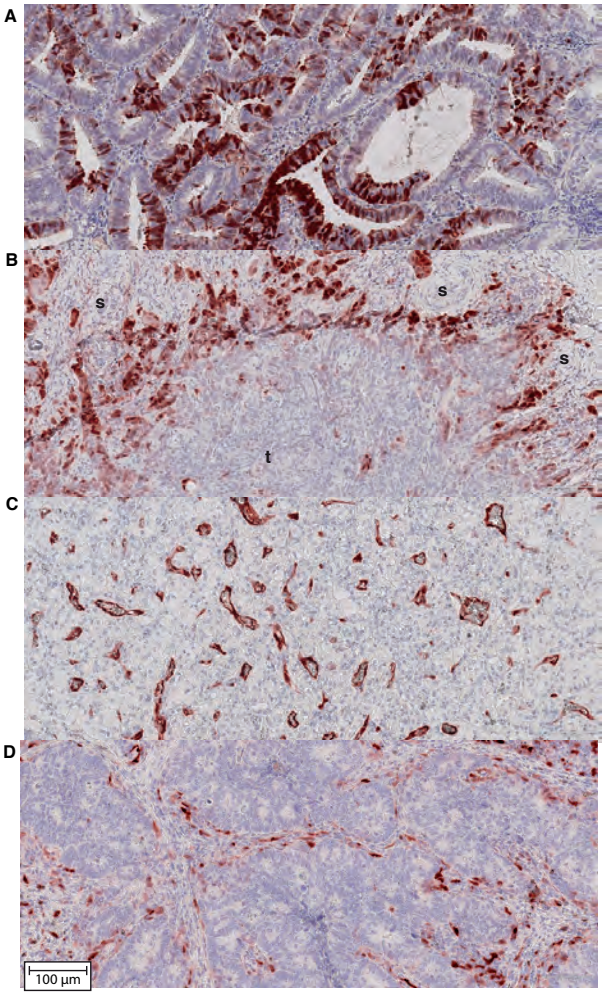

Fig. 2. IDO1 protein expression in human tumors assessed by IHC [from Ref. 10]. Illustrative images from FFPE tissue sections of an endometrial adenocarcinoma (A), a cervical squamous cell carcinoma (B), in which the tumor (t) and stromal (S) parts are indicated, a renal cell carcinoma (C), and a gastric adenocarcinoma (D) stained with the anti-IDO1 antibody 4.16H1. Immunolabeled cells are stained in dark red (AEC staining). They correspond to tumor cells (A and B), endothelial cells (C), and inflammatory stromal cells (D). Each image represents 3% of the surface of large, 5-mm-wide TMA sections.

Transgenic mice developing autochthonous melanomas expressing P1A

S. Cane, J. Zhu, C. Uyttenhove, C. Powis de Tenbossche, V. Finisguerra, M. Blackman, E. De Plaen, in collaboration with J. Van Snick

We have created a mouse model of autochthonous inducible melanoma expressing a defined tumor antigen (TIRP10B) [12]. 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). We found that TGFβ was a key factor responsible for this switch to aggressive tumors, which is reminiscent of the epithelial-to-mesenchymal transition (EMT) described in other contexts. We developed antibodies able to neutralize TGFβ1 and TGFβ3, and found that the former were able to increase survival of mice in this melanoma model. These results support the use of TGFβ neutralizing therapies in the treatment of human melanoma.

The loss of pigmentation in aggressive tumors appears to result from the strong inflammation, and we identified miR-155 as a microRNA that is induced by interleukin-1, overexpressed in unpigmented melanomas and able to downregulate expression of MITF, a transcription factor acting as a master regulator of pigmentation.

In this model, both pigmented (Mela) and unpigmented (Amela) 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. We are studying the mechanisms responsible for this ineffectiveness. Our current results indicate that the tumor microenvironment actively induces the apoptosis of tumor-specific T lymphocytes that infiltrate the tumor. We are studying the molecular mechanisms responsible for this apoptosis.

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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 the exhaustion of intratumoral lymphocytes. We believe therefore 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.

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 [1, 2].

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 [3]. It is therefore important to know which immunosuppressive mechanisms operate in human tumors.

Galectins and dysfunction of human tumor-infiltrating T lymphocytes

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 IFN-γ and other cytokines upon non-specific stimulation with anti-CD3 and anti-CD28 antibodies [4-7].

Galectin-3 seems to play a role in human TIL dysfunction. Galectin-3 belongs to a family of lectins, *i.e.* sugar-binding proteins, with pleiotropic functions both intracellularly and extracellularly (after secretion). Galectin-3 is mainly secreted by tumor cells and macrophages. By binding to glycoproteins at the TIL surface and forming glycoprotein-galectin lattices, galectin-3 restrain the mobility of surface molecules. We observed that extracellular galectin-3 blocks functions of human TILs, as treating TILs with an anti-galectin-3 antibody or galectin antagonists detached galectin-3 from the T cell surface and increased cytokine secretion and cytotoxicity of treated TILs [5-7] (Fig. 1).

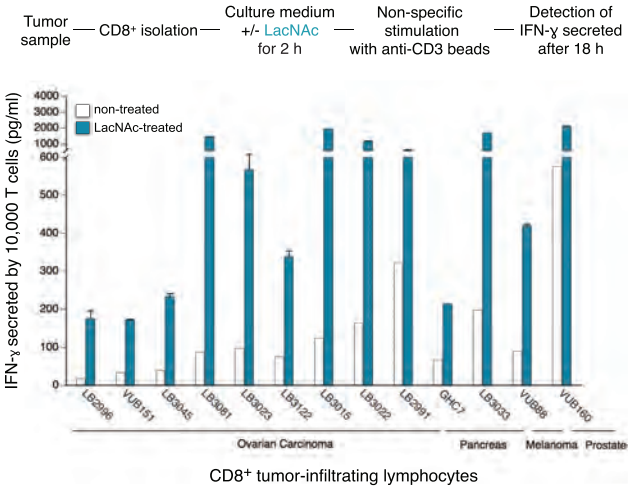

Fig. 1. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy.

One of these galectin antagonists [8] is GM-CT-01, a clinical grade galactomannan extracted from guar gum and reported to be safe in more than 50 cancer patients [7]. We have recently reported that treating TILs, which were isolated from patients with various cancers, with GM-CT-01 boosted their cytotoxicity and ability to secrete IFN-γ upon *ex vivo* stimulation. This was true for ~80% of the $CD8^+$ TIL samples.

Galectins block the secretion process in TILs

We made the unexpected discovery that a large fraction of TILs failed to secrete cytokines and lytic enzymes upon stimulation, although they were activated and expressed these effector molecules in intracellular vesicles. Thus, surprisingly, these effector molecules remained trapped inside the cell. The normal secretion process requires the formation of a secretory synapse allowing exocytosis of secretory granules. We found that this process is often blocked in TILs, due to impaired LFA-1 mobility and actin rearrangement at the secretory synapse. As a result, cytokines and lytic enzymes remain trapped inside TILs, thereby preventing their anti-tumor activity [9] (Fig. 2). This is the first observation of uncoupling between cytokine "intracellular expression" and secretion in TILs. This defect appears related to the presence of galectin-3 at the TIL surface, and can be relieved by agents that detach galectin-3 from the TIL surface, such as small glycans or antibodies to galectin-3. These results indicate that cancer immunotherapy regimens could be improved by blocking this additional mechanism of T cell dysfunction. They also provide further insight on the role of integrin LFA-1 in T cell secretion. We show that few LFA-1 molecules need to be triggered to support intracellular expression of cytokines, but that the formation of the secretory domain appears to be highly dependent on full recruitment of LFA-1. Entrapment of integrin LFA-1 in glycoprotein-galectin lattices is a sufficient explanation for the poor cytokine secretion by TILs. From a practical standpoint, this new mechanism of T cell dysfunction also indicates that evaluating T cell function by intracellular cytokine staining, a widely used immunomonitoring assay, can be highly misleading as it may wrongly suggest that T cells expressing intracellular cytokines are functional. According to our new mechanism of T cell dysfunction, some of these T cells may fail to secrete the cytokines.

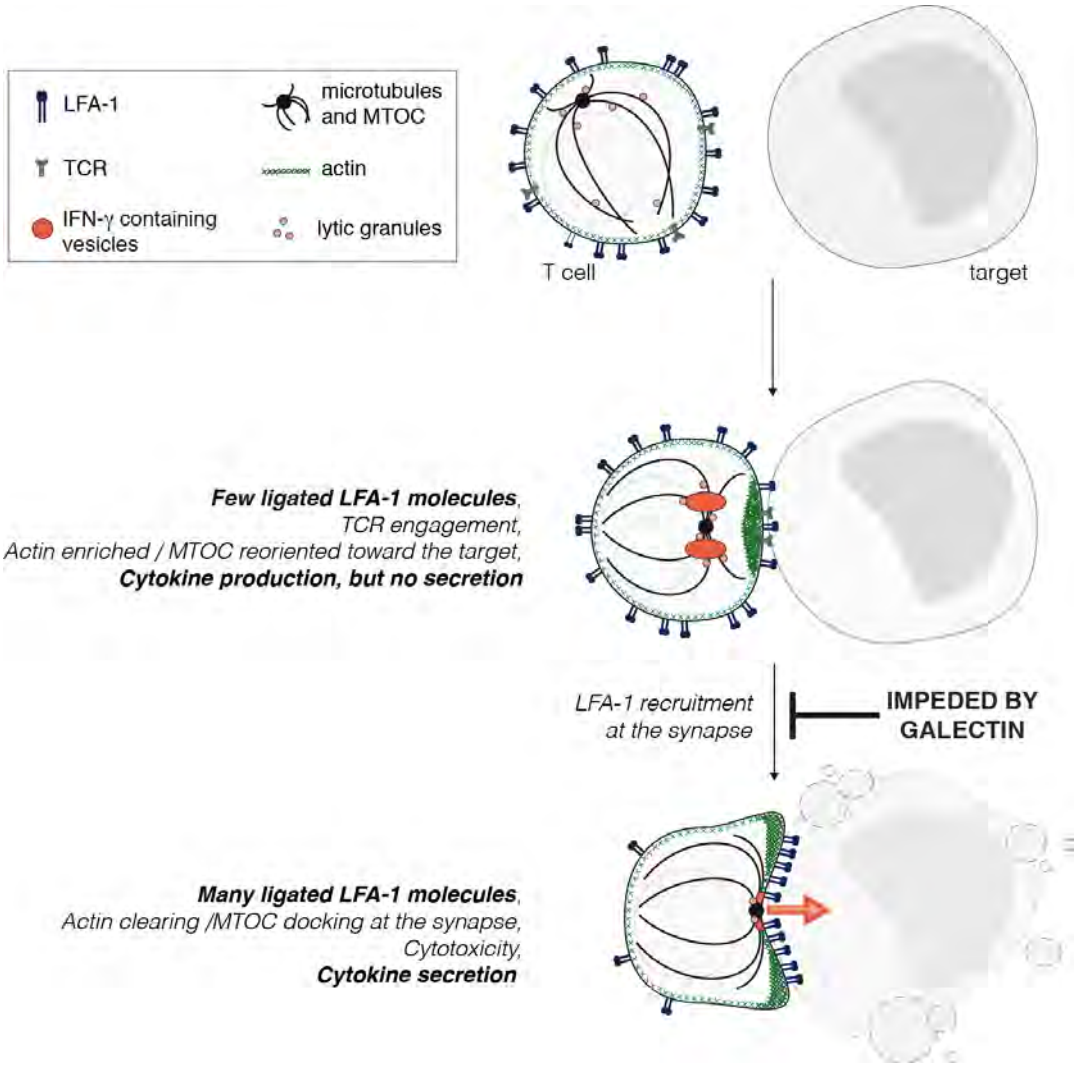

Fig. 2. Cytokines and lytic enzymes are produced normally by human tumor-infiltrating T lymphocytes but remain trapped inside the cells.

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 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 [10]. This was the first description of a gene, *MAGE-A1*, coding for a human tumor antigen recognized by T lymphocytes [1].

Genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in most 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 [2]. 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.

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/.

Our current projects:

- to examine T cell dysfunctions induced by chronic stimulation;

- to examine the different immune cells present in the tumor environment that could participate in T cell dysfunctions;

- to examine the role of the different galectins in the impaired function of T cells;

- to examine if galectins can sequester interleukins and chemokines.

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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 TGF-β, 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

C. Lurquin, T. Boon

Female CBA mice do not reject male skin grafts, even though they are able to mount a cytolytic T cell response against HY, a male-specific minor histocompatibility antigen encoded by the *Smcy* gene. 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 required to achieve 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 IFN-α (10^5 U) with TLR7 ligand gardiquimod (20 µg) caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod [5]. 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 required 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 IFN-α. Repeated injections combining IL-2,

IFN-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 also determined that 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 makes 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.

To begin evaluating the action of the various immunostimulatory agents that proved capable of promoting graft rejection, we examined their ability to promote the presence of anti-HY CD8 T lymphocytes in the graft. We used CD8 T cells from spleens of T-cell receptor transgenic mice expressing a receptor specific for a HY peptide presented by H2-K^k. After i.p. adoptive transfer, the number of transgenic T cells present in any given location could be estimated by a quantitative PCR specific for this receptor sequence. A few days after the adoptive transfer, we observed a natural invasion of the male skin graft by anti-HY T cells without immunostimulatory treatment. This invasion occurs sooner if the adoptive transfer is done 40 days after grafting rather than 5 days after. But in both cases, invasion kinetics is similar with an increase, a maximum and then a decrease of the number of anti-HY lymphocytes over a total period of about 30 days. It is difficult to evaluate whether this decrease is due to lymphocyte efflux outside the graft or apoptosis in the graft. When the anti-HY transgenic T cells are no longer detected in the graft, a second adoptive transfer of anti-HY lymphocytes can elicit a new infiltration of the graft with the same kinetics. From the beginning of the period of invasion of the graft, a steady decline in the number of anti-HY T cells is also observed in the spleen and in the blood. We observed a significant increase of anti-HY T cells in the graft following local treatment with either gardiquimod, IL-2 or IL-12 applied when the natural infiltration of lymphocytes was at its maximum. In contrast, no increase was observed if the cytokine treatments are applied later when anti-HY T cells were hardly detectable in the graft, except if the treatment combines gardiquimod, IL-2 and IFN-α and lasts two weeks. It is possible that the anti-HY cells increases observed in the grafts after immunostimulatory treatment are mainly due to increased proliferation rather than an increased influx. The agents that cause a large increase in the number of anti-HY T cells found in the graft also cause a moderate increase of these cells in the blood and in the spleen. This could be due to some systemic action of these agents, or it could be a consequence of T cell circulation between these compartments and the graft [5]. We verified that there was no natural invasion of anti-HY transgenic T lymphocytes into female skin grafts and no increase of these cells after local cytokine treatment.

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 that received a local treatment either with IL-12 and IFN-α or with IL-2, IFN-α and gardiquimod rejected subsequent grafts in the absence of further local treatment, indicating a degree of systemic increase of anti-H-Y memory T cells in these mice [5].

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

C. Uyttenhove, in collaboration with J. Van Snick

It has been extremely difficult to obtain specific antibodies against human periostin, a highly conserved secreted homodimeric protein that binds integrins and is over-expressed in human breast cancers, contributing to their metastatic potential.

Using the ovalbumin conjugation procedure that we previously described [6], and infection of mice with lactate dehydrogenase elevating virus (with the help of Dr. Jean-Paul Coutelier) to further disrupt immune tolerance, we produced a unique set of monoclonal antibodies specific for human periostin.

In collaboration with Dr. Parmjit S. Jat (University College London) and Dr. Vincent Stroobant from our laboratory, we identified a 10 amino acid linear sequence in the fascilin 1-1 domain of periostin that was recognized by our antibodies and involved in periostin-integrin interaction as well as in periostin-dependent migration of human endothelial colony forming cells. Staining of a panel of human tumor samples with one of these antibodies showed striking correlation between periostin expression and tumor aggressiveness. These antibodies provide new reagents for the analysis of periostin expression as a predictive tool for tumor severity and for its therapeutic inhibition [7].

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Therapeutic vaccination and tumor expression profiling group

Nicolas van Baren

Human tumors are antigenic and immunogenic. They express a wide diversity of tumor antigens that are not or poorly expressed on normal tissues and that can be recognized by cytolytic T lymphocytes (CTL) [1, 2]. They are often infiltrated by various numbers of immune cells including CTL [3]. We have previously shown that T cells present in melanoma metastases comprise CTL directed at tumor antigens [4], and that patients with advanced melanoma have a high frequency of circulating anti-tumor CTL [5, 6]. Altogether, these observations indicate that melanoma patients mount spontaneous immune responses against their tumor, but that these responses are not capable to control tumor growth in patients with advanced disease. It is therefore important to characterize these responses as well as the mechanisms of protection that the tumors select in order to counter them. The molecular identification of these mechanisms forms the basis of new therapeutic approaches to control tumor immunity in favor of efficient tumor rejection. Our team studies immune responses at the tumor sites in melanoma, non-hodgkin lymphoma and colorectal carcinoma, and develops new immunotherapy approaches to enhance these responses.

Characterization of T cell and B cell responses in melanoma metastases

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

We study the immune and inflammatory components and their interaction with tumor cells in freshly resected cutaneous metastases obtained from melanoma patients. Part of the resected tumors are put in culture, in order to attempt to derive immortalized melanoma cell lines, which are precious tools in experimental tumor immunology. The remaining piece of tumor is kept frozen. Thin tissue sections are cut from this material, and are used for RNA extraction followed by gene

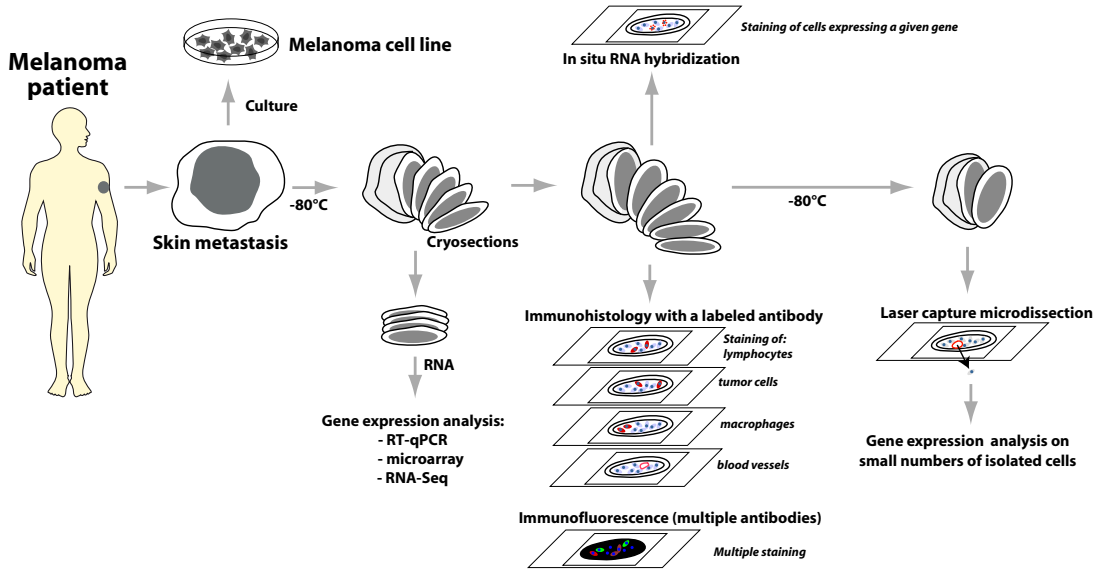

Fig. 1. Processing of tumor samples for the study of the inflammatory environment in melanoma metastases.

expression profiling, for immunohistochemistry and immunofluorescence stainings, for *in situ* RNA hybridization and for laser capture microdissection of small tissular regions of interest (Fig. 1). These complementary approaches are aimed at allowing us to characterize the mechanisms by which the tumors resist destruction by the anti-tumoral immune responses that they trigger.

We focus on the following elements to investigate intra-tumoral T cell responses:

. We study functional gene expression signatures observed in microarray and whole transcriptome profiling. We have identified a signature that comprises T cell activation genes, CTL genes and IFN-γ target genes, and reflects Th1-oriented T cell activation. We also assess the expression of phenotypic markers of T cell activation *in situ*.

. We study the abundance and pattern of T cell distribution in the tumors, and their spatial relationship with the tumor cells. This is illustrated in Figure 2.

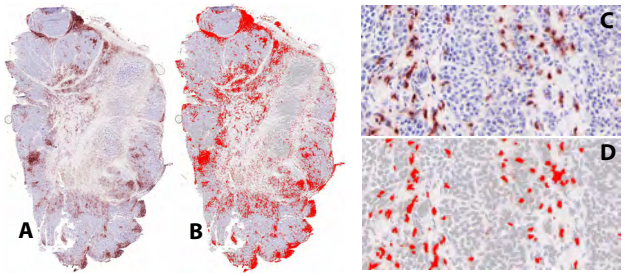

Fig. 2. The T lymphocytes present in a section from a frozen melanoma metastasis have been stained in dark red to visualize their distribution. The microscope image has been recorded by digital scanning. The image has been processed by a specialized software, which identifies and distinguishes the individual cell nuclei (grey) and the stained T cells (bright red). Automated counting of these elements gives 977,387 cells in the section, including 257,983 T lymphocytes (26%).

. As some tumors are known to resist immune rejection by loosing or down-modulating proteins required for antigen processing and presentation, we study the expression of the main components of the antigen presentation machinery (APM) in our tumor samples. An example is shown in Figure 3.

We assess whether these different markers are correlated with each other and with the clinical outcome of the patients.

We have observed the presence of ectopic lymphoid structures, also called tertiary lymphoid organs, in melanoma metastases [7]. 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. 4). This phenomenon, called lymphoid neogenesis, is frequently observed in various chronic inflammatory diseases. It has also been described in several types of

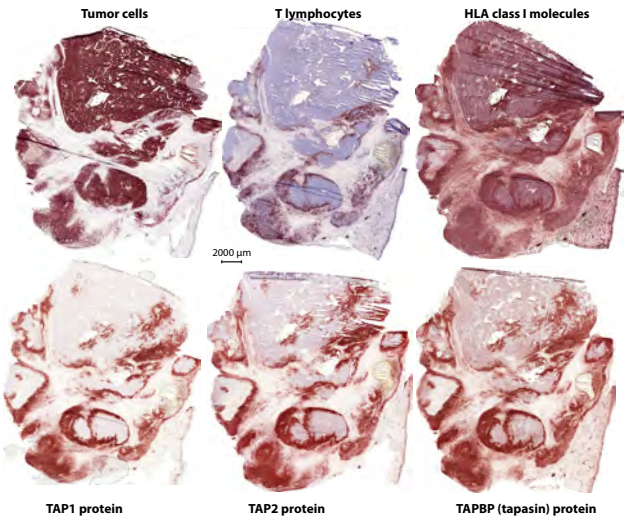

Fig. 3. Adjacent sections of a frozen melanoma metastasis have been stained for the indicated elements. The T lymphocytes are mainly found around the tumor nests. The tumor cells have a strongly reduced expression of the TAP1, TAP2 and tapasin proteins, and a reduced expression of the HLA class I molecules, which are all involved in the processing and presentation of antigenic peptides to the T cells. Presumably, a reduced expression of tumor antigens allows this tumor to resist immune recognition and destruction.

tumors, including breast, lung and testis cancer. It is a consequence of sustained lymphocyte activation in the presence of persistent antigenic stimuli.

Altogether, our observations suggest that the melanoma environment is the site of sustained immune activity rather than of a widespread immune suppression as is frequently proposed.

Characterization of tumor antigens and T cell responses in non-hodgkin lymphoma

In collaboration with the clinical and laboratory hematology teams at Cliniques universitaires Saint-Luc, Brussels

Diffuse large B cell lymphoma (DLBCL) is an aggressive malignancy that originates from germinal center B cells. As compared with other tumor types, it shows very frequent loss of HLA class I molecules, making it resistant to CTL-mediated attack. This suggests that DLBCL is originally a highly immunogenic tumor that needs to resist CTL in order to exist. Consistently, it is one of the few malignancies that arise in immunosuppressed patients. In addition, a minority of DLBCL express constitutively PD-L1, the ligand of PD-1 which upon binding inhibits T cell activation. The tumor antigens that would allow CTL to kill DLBCL cells are unknown. Using the approaches described above for melanoma, and our expertise in the identification of tumor antigens and study of anti-tumoral T cell responses, we will try to identify DLBCL antigens and study *in situ* T cell responses in DLBCL tumors in comparison with melanomas. We will also try to identify the putative resistance mechanisms that occur in antigen presentation-competent DLBCL.

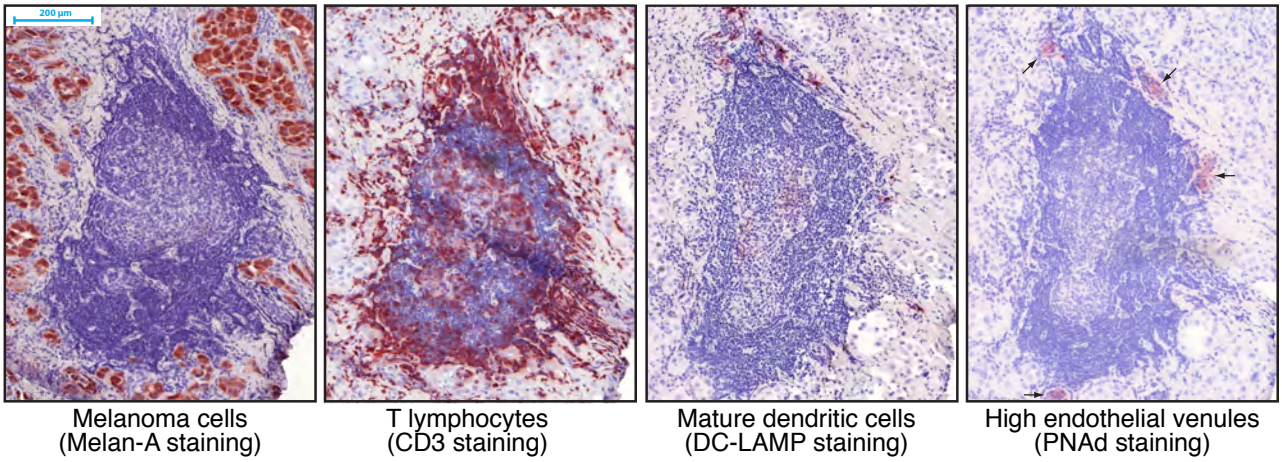

Fig. 4. An ectopic lymphoid structure in a melanoma skin metastasis. Adjacent cryosections have been stained for the indicated elements.

Study of IDO1 expression in human malignancies

In collaboration with the group of B. Van den Eynde, the Biobank and Department of Pathology (Cliniques universitaires Saint-Luc, Brussels), and iTeos Therapeutics

Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 (IDO1) plays an important role in tumor resistance to immune rejection (see the report of B. Van den Eynde). In humans, constitutive expression of IDO1 has been observed in several tumor types. The precise expression profile of IDO1 in human tissues has remained unclear, due to the use of different methodologies and antibodies used in immunostaining assays, including antibodies with doubtful specificity. A precise profiling is important to assess the risks and potential benefits of IDO1 inhibitors, which are currently in pre-clinical and early clinical development, and to identify the best tumor targets for this treatment.

We have performed an extensive immunohistochemical analysis of IDO1 expression in normal and tumor tissues [8, 9]. In normal tissues, IDO1 was expressed by endothelial cells in placenta and lung, and by epithelial cells in the female genital tract. It was also detected in lymphoid tissues, in interstitial cells corresponding to mature dendritic cells. IDO1-expressing cells were observed in a large fraction (505/866, 58%) of human tumors. Tumors showing the highest proportions of IDO1-immunolabelled samples included carcinomas of endometrium and cervix, followed by kidney, lung, and colon. Other tumors such as glioblastomas were often negative. This hierarchy was confirmed by gene expression data mined from the TCGA database. IDO1+ cells were tumor cells, endothelial cells and stromal cells, in proportions that varied depending on the tumor type (Fig. 5).

Based on these results, we now focus on selected tumor types with elevated IDO1 expression, which are analyzed in more details and on larger sample series. Next to IDO1 expression, we also analyze its spatial relationship with T cell distribution in tumors and with the clinical evolution of the patients.

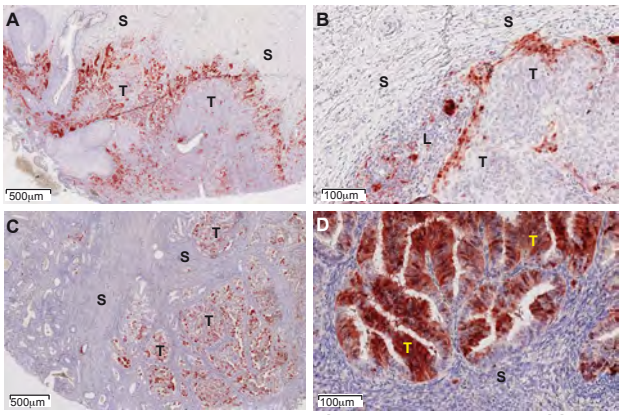

Fig. 5. IDO1 protein expression in human tumors assessed by immunohistochemistry. Illustrative images from formalin-fixed paraffin-embedded tissue microarray sections of cervical (A, B) and endometrial carcinomas (C, D) stained with an anti-IDO1 antibody. Tumoral (T), stromal (S), and lymphocyte-enriched (L) areas are indicated.

Clinical immunotherapy trials

In collaboration with J.-F. Baurain (Medical Oncology, King Albert II Cancer Institute, Brussels)

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. Since many years, 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 a recombinant poxvirus called ALVAC engineered to

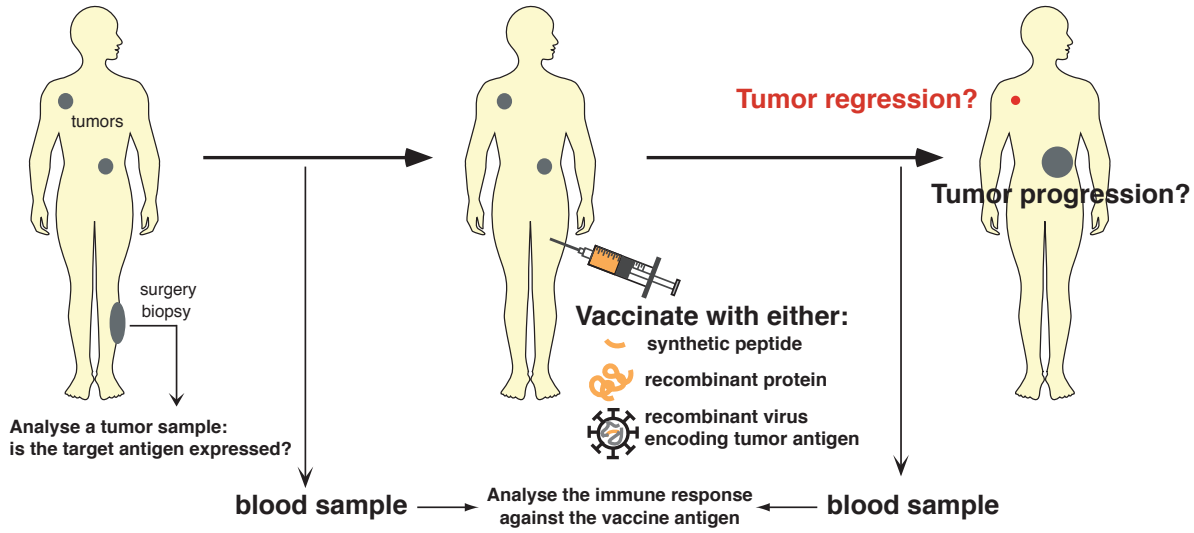

Fig. 6. Principles of clinical trials with cancer vaccines performed in our institution.

encode tumor antigens, have been tested in the clinic [10-12]. The principles of these treatments are illustrated in Figure 6. All these treatments were 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 about 5% of the patients experienced a true clinical benefit. Some of the remissions have lasted for many 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 so far, and the resistance against immune rejection that tumors select during their evolution in the face of spontaneous immune responses.

More recently, our efforts have been devoted to finding more immunogenic vaccines, and combining existing vaccine modalities with treatments that modify the tumor environment in favor of effective tumor rejection. A list of the clinical trials that have been carried out can be found in Table 1.

Table 1. List and main features of clinical trials involving cancer vaccines that we have developed

Study	Vaccine + adjuvant (+ immunomodulator)	Disease	Clinical Setting	Patients included	Tumor responses	Severe toxicity
Sponsor : Ludwig Institute, investigators : Marie Marchand, Nicolas van Baren, Jean-François Baurain						
Peptide trials						
LB 93-001	MAGE-1.A1 peptide sc	Melanoma + other cancers	measurable disease	10	none	none
LB 94-001	MAGE-3.A1 peptide id/sc	Melanoma + other cancers	measurable disease	53	3CR,1PR,3MxR	none
LUD 94-002	MAGE-1.Cw1601 peptide sc	Melanoma	measurable disease	3	1SD	none
LUD 95-002	MAGE-3.A1 peptide + QS21/MPL im	Melanoma + other cancers	measurable disease	10	1SD	none
LUD 95-004	MAGE-3.A2 peptide id/sc	Melanoma + other cancers	measurable disease	39	2SD,2MxR	none
LUD 96-007	MAGE-1.A1 & MAGE-3.A1 peptides id/sc	Melanoma + other cancers	measurable disease	28	1SD,2MxR	none
LUD 97-004	MAGE-3.A1 ± MAGE-3.DP4 peptide id/sc	Melanoma	measurable disease	45	2SD,3MxR	none
LUD 99-006	MAGE-4.A2 + MAGE-10.A2 peptides ± IL-12 id/sc	Melanoma	measurable disease	5	1SD	none
LUD 01-006	MAGE-3.A1 or MAGE-10.A2 peptide id/sc	Melanoma	disease-free	19	-	none
LUD 02-001	MAGE-3.A1 peptide + adjuvant CpG 7909 id/sc	Melanoma	measurable disease	1	none	none
LUD 03-007	8 HLA-A2 peptides + CpG or Montanide id/sc	Melanoma	measurable disease	23	1SD,4MxR	none
Non-peptide trials						
LUD 97-002	MAGE-3 protein + QS21/MPL im	Melanoma + other cancers	measurable disease	59	3PR,1SD,2MxR	none
LUD 99-003	MAGE-3 protein + CpG/QS21/MPL im	Melanoma	measurable disease	32	1PR,3SD,4MxR	none
LUD 02-002	MAGE-3 protein id/sc	Melanoma	measurable disease	11	3MxR	none
LUD 97-005	virus ALVAC miniMAGE-1/3 + MAGE-1.A1 & MAGE-3.A1 peptides id/sc	Melanoma + other cancers	measurable disease	40	1PR,2SD,4MxR	none
Sponsor : local university hospital (St-Luc, Centre du Cancer), investigator : Jean-François Baurain						
Peptide trials						
LUC 02-001	MAGE-3.A1 peptide id/sc + IFN-alpha sc	Melanoma	disease-free	6	-	none
LUC 03-001	MAGE-3.A1 peptide + CpG id/sc	Melanoma	disease-free	7	-	none
LUC 03-002	MAGE-3.A1 peptide + Montanide id/sc	Melanoma	disease-free	13	-	none
LUC 03-002	4 HLA-A2 peptides alone or + Montanide id/sc	Melanoma	disease-free	40	-	none
LUC 05-003	8 HLA-A2 peptides alone or + Montanide or + IMP321 id/sc	Melanoma	disease-free	16	-	none
LUC 10-001	MAGE-3.A1 + NA17.A2 peptides id/sc (+ galectin inhibitor GM-CT-01 iv)	Melanoma	measurable disease	6	2MxR	none
LUC 10-002	MAGE-3.A1 + NA17.A2 peptides id/sc (+ low dose IL-2, IFNa, GM-CSF and Aldara peritumorally)	Melanoma	measurable disease, superficial metastases	3	1CR,1MxR	none
Non-peptide trials						
LUC 09-003	CyaA-Tyr, a DC-targeting, recombinant, inactivated bacterial toxin combined with the TYR.A2 melanoma antigen	Melanoma	measurable disease	11	1MxR,1SD	none

CR: complete response, PR: partial response, SD: stable disease, MxR: mixed response

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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 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

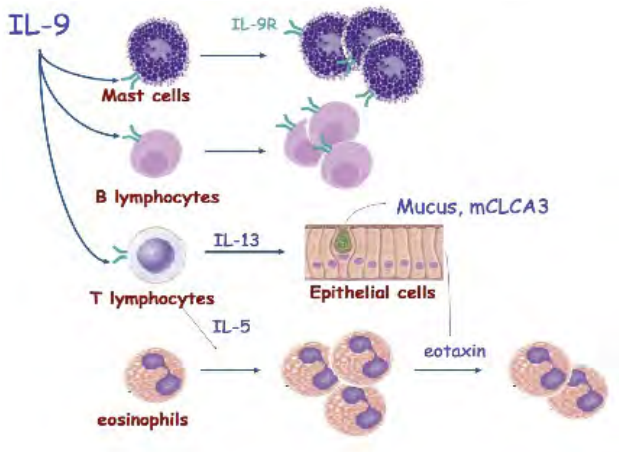

Fig. 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation of eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production 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].

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].

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

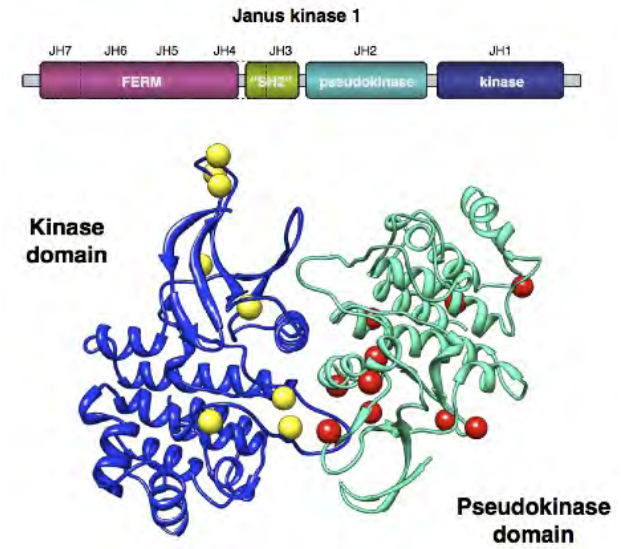

Fig. 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

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].

While most JAK1 mutants were sensitive to ATP-competitive JAK inhibitors, mutations targeting Phe958 and Pro960 in the hinge region of the kinase domain rendered JAK1 not only constitutively active, but also resistant to all tested JAK inhibitors. Furthermore, mutation of the homologous Tyr931 in JAK2 wild-type or JAK2 V617F mutant found in myeloproliferative neoplasms also conferred resistance to JAK inhibitors, including the clinically used INCB018424. These observations indicate that in JAK mutation positive patients, treatment with JAK inhibitors is likely to contribute to the selection of these mutations that combine increased oncogenicity and drug resistance [4].

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. To determine the biological activities of IL-22, we generated deficient mice for IL-22 and for its recepor. Analysis of these animals highlighted two major properties of IL-22: its activity on keratinocytes with consecutive implications in skin inflammatory disorders, and its protective role on intestinal epithelial cells with implication in colitis.

IL-22 receptor and signal transduction

Although IL-22 does not share any biological activity with IL-10, these two cytokines share a common component of their respective receptor complex, IL-10Rβ. Anti-IL-10Rβ 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-10Rβ 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-10Rβ, IL-22 signaling 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 signaling 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 STAT-3, and that STAT-3 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 [6].

Beside this cell membrane IL-22 receptor complex composed of IL-22R and IL-10Rβ, we identified a protein of 231 amino acids, 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 signaling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20Rβ, 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-20Rβ (see Ref. 7 for a review of this cytokine family).

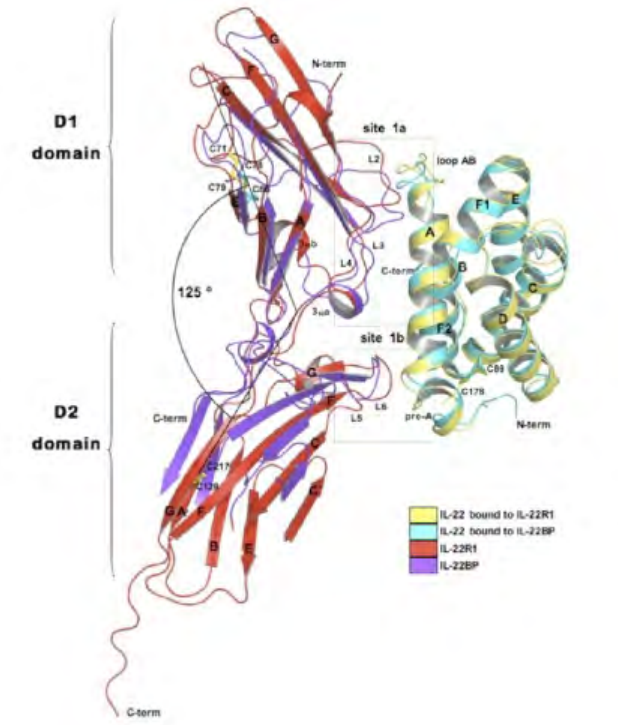

Fig. 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

Biological activities of IL-22

Biological activities of IL-22 include the induction of acute phase proteins in liver and protection against experimental hepatitis and colitis. In contrast, we showed that IL-22 plays a detrimental role in experimental arthritis.

More recently, 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 [8]. At the macroscopic level, scaly skin lesions induced by daily applications of imiquimod in wild-type mice were almost totally 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 (Fig. 4). 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.

Cellular source of IL-22

Originally, we show that the major source of IL-22 is activated T cells. Among the different T cell subsets, IL-22 was found to be preferentially produced by Th17 cells, and the aryl hydrocarbon receptor AhR turned out to be a major regulator of the expression of this cytokine in Th17 [9, 10]. Both 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 to 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 [11]. 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 $NKp46^+$ ROR γt^+ 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.

Anti-cytokine vaccination

Beside conventional gene targeting strategies, which 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

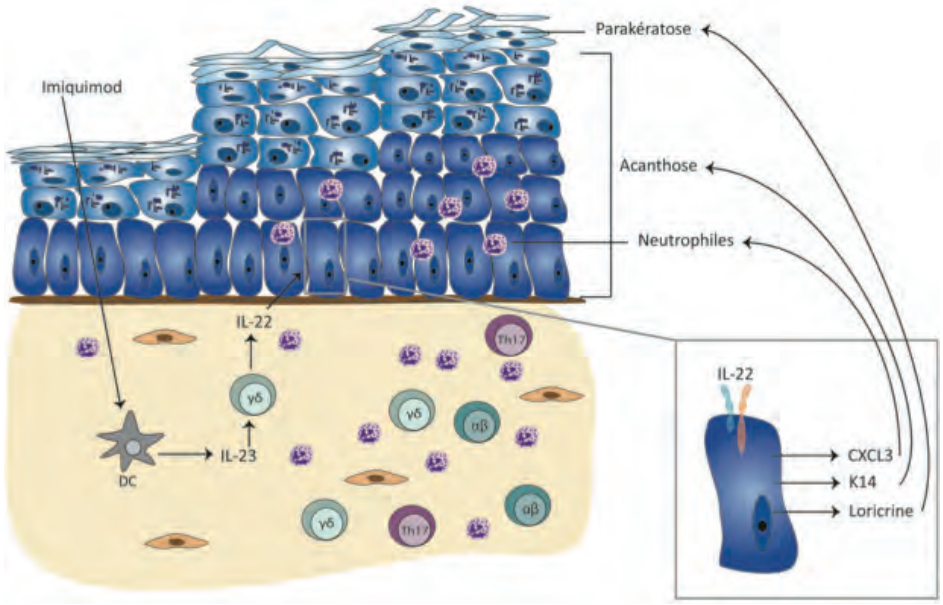

Fig. 4. Role of IL-22 in mouse model of psoriasis. Imiquimod acts on dendritic cells and induces the expression of IL-23, which is required for IL-22 production by γδ T cells in the skin. In turn, IL-22 acts on keratinocytes and induces the expression of several genes such as K14, Loricrine and CXCL3. K14 reflects the proliferation of keratinocytes and Loricrine the inhibition of their differentiation leading respectively to acanthosis and parakeratosis. CXCL3 is involved in neutrophils recruitment leading to strong inflammatory response.

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

Small proteins denoted cytokines, such as erythropoietin (Epo), interleukins, thrombopoietin (Tpo), colony stimulating factors or interferons are crucial for regulating blood formation or hematopoiesis and the function of the immune system. Cytokines act by binding to specific receptors localized at the surface of blood progenitors or mature cells. Cytokine receptors 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 30 cytokine receptors, for four JAKs (JAK1, JAK2, JAK3 and TYK2) and seven Signal Transducers and Activators of Transcription (STATs), which are signal transducers proteins that shuttle to the nucleus and regulate gene expression.

We aim to determine on a fundamental level: (i) how transmembrane and juxtamembrane sequences switch on or off cytokine receptor activity; (ii) what are the general rules by which hydrophobic transmembrane sequences 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.

On the translational level, in the past we identified constitutively active oncogenic mutants of three Janus kinases, namely JAK2 (V617F), JAK1 (V658F) and TYK2 (V658F) [1, 2]. These are involved in human myeloproliferative neoplasms- MPNs- (JAK2 V617F) and T cell leukemias (JAK1 V658F). JAK2 V617F proved to be very important for the BCR-ABL negative MPNs Polycythemia Vera, Essential Thrombocythemia and Primary Myelofibrosis. We also described the class of oncogenic activating mutants of TpoR at W515 and demonstrated the major role played by W515 in preventing TpoR self-activation [3]. Our aim is to harness our basic understanding of JAK-STAT signaling in order to propose specific ways to target mutant JAKs. Recently, we discovered that mutant chaperones, such as calreticulin mutant (CALR) proteins can activate pathologic signaling and induce myeloid cancers. The three classes of oncogenic drivers in MPNs, mutants of JAK2, TpoR and CALR induce persistent STAT5 activation, which leads to pathologic gene expression and contribute, along with p53 mutations and epigenetic mutations, to progression of MPNs to secondary acute myeloid leukemia. We collaborate with clinicians and clinical biologists at Saint-Luc Hospital and several international groups in order to study novel avenues for inhibiting pathologic signaling by JAK2 V617F, TpoR and CALR mutants.

The mechanisms of JAK2 V617F activation in human myeloproliferative neoplasms

E. Leroy, L. Varghese, J.-P. Defour, M. Swinarska, D. Colau

We have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation (Fig. 1a) in collaboration with William Vainchenker at the Institut Gustave Roussy in Paris [1]. JAK2 V617F is responsible for >98% of Polycythemia Vera (PV) and for >60% of Essential Thrombocythemia (ET) and Myelofibrosis (MF) cases [2]. This mutation in the pseudokinase domain (Fig. 1a) activates the kinase domain leading to constitutive signaling [1, 2] in complexes with cytokine receptors such as EpoR, TpoR and G-CSFR. PV, ET and MF are MPNs characterized by excessive production of mature red cells (in PV), platelets (in ET) and sometimes granulocytes. In MF, excessive myeloid cell proliferation leads to marrow scarring and fibrosis due to enzyme release and collagen secretion by bone marrow-derived fibroblasts.

The homologous V617F mutations in JAK1 and Tyk2 also en-

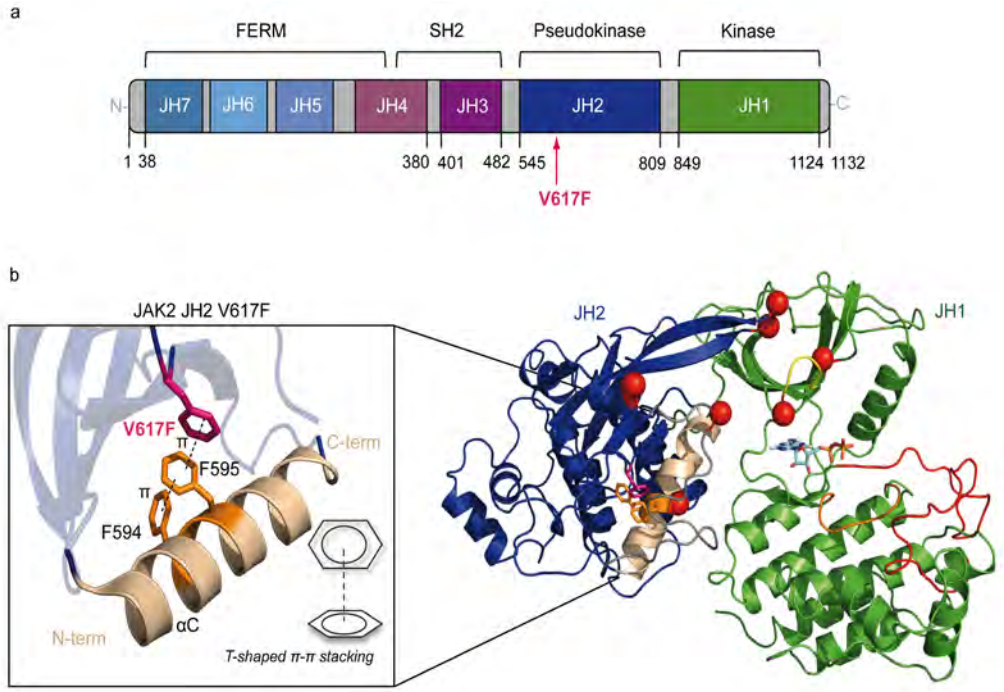

Fig. 1. Domain structure of JAK2/JAK2 V617F and conformational change induced by the V617F in the pseudokinase domain of JAK2. (a) Domain structure of JAK2. JH, JAK homology domains. The position of the V617F mutation in the pseudokinase domain is shown. This mutation leads to activation of the kinase domain. ATP-competitive inhibitors of JAK2 that are used in clinics bind to the kinase domain and do not discriminate between wild type and mutant JAK2. (b) Small square, left: Ribbon diagrams of the structure of the JH2 V617F of JAK2. Helix C of JH2 V617F is straight, long and stable when compared to JH2 WT helix. This conformational change is due to an aromatic stacking interaction between the F617 (pink) and F595 and F594 (orange); (based on Bandaranayake *et al.*, Nat Struct Mol Biol 2012;19:754 and Dusa *et al.*, PLoS ONE 2010;5:e11157). Right: Model of the pseudokinase domain (JH2) and kinase domain (JH1) interface (based on Shan *et al.* Nat Struct Mol Biol 2014; 21:579). A community of residues (red spheres) spreading from the JH2 helix C to JH1 active site are involved in the mechanism of activation of JAK2 V617F.

able these kinases to be activated without ligand-binding to cytokine receptors [2]. These findings contributed to the emergence of a very active research area around oncogenic mutations of the JAK-STAT pathway and cancer.

We aim to precisely define how a pseudokinase domain mutation (V617F and several others) can induce kinase domain activation in JAKs. This would be required for the isolation of small molecule-specific inhibitors of mutated JAK2 that would spare wild type JAK2, and thus would not induce adverse effects. We identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F [4]. 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 (Fig. 1b, left). A recent X-ray crystal structure from the Hubbard and Silvennoinen laboratories has visualized F595 in the conformation induced by V617F on helix C of JH2, with an aromatic stacking interaction between F617, F595 and F594, and prolongation by one turn of the helix C of JH2 (Fig. 1b, left). Expression of segments of JAKs and cytokine receptors is pursued in insect and bacterial cells by D. Colau and M. Swinarska. Via structure-guided mutagenesis and biochemical assays, we identified a community of residues from the JH2 helix αC, SH2-JH2 linker and JH1 kinase domain that mediate V617F-induced activation of JH1 (Fig. 1b, right) [5]. This circuit can be interrupted by introducing positive charges on the solvent-exposed face of the JH2 αC, which is predicted to interact with the SH2-JH2 linker and JH1 [6]. Such mutations that remove negative charges or add positive charges, as E596/R, do not alter the JH2 V617F fold, as shown by our crystal structure of JH2 V617F E596A (in collaboration with Dr Andrew Shiau, Ludwig San Diego) [5]. Instead, they prevent kinase domain activation via modulation of the C-terminal residues of the SH2-JH2 linker. These results suggest strategies for selective V617F JAK2 inhibition, with preservation of wild-type function.

The mechanisms of activation by JAK2 mutants in hereditary thrombocytosis and erythrocytosis

J.-P. Defour, E. Leroy, L. Varghese

In our initial description of JAK2 V617F we have explored by saturation mutagenesis the possibility that other V617 mutations could be also active [7]. We reported that V617W is strongly active, and V617I/L/M are also weakly active. Intriguingly, V617I for example could be obtained by one base pair change. The JAK2 V617I mutation was subsequently found in very rare MPN cases, and in one family with hereditary thrombocytosis. In addition, several other groups reported other JAK2 mutations in hereditary thrombocytosis. We found, for

all these kinds of mutants linked to hereditary thrombocytosis that in heterozygous conditions they mainly activate TpoR signaling via STAT1.

Furthermore, in collaboration with Vladimir Divoky of Palacky University, Olomuk, Czech Republic, we have studied the signaling by two JAK2 mutants associated with hereditary erythrocytosis [8]. A patient inherited two heterozygous JAK2 mutations, E846D from the mother and R1063H from the father, and exhibited erythrocytosis and megakaryocytic atypia, with normal platelet numbers. Culture of erythroid progenitors from the patient and his parents revealed in all three a hypersensitivity to erythropoietin (Epo). In cellular models, both E846D and R1063H led to constitutive signaling that was much weaker than JAK2 V617F [5]. Both mutants hyperactivated JAK2/STAT5 signaling only in the specific context of the EpoR, thus explaining the erythrocytosis phenotype. JAK2 E846D caused prolonged Epo-induced phosphorylation of JAK2/STAT5 via EpoR. Our data indicated that JAK2 E846D predominantly contributes to erythrocytosis, but requires JAK2 R1063H, in order to augment JAK2 activity caused by E846D above a threshold level. Both mutations were detected in the germ line of rare PV patients, as well of certain leukemia patients. Within this collaborative effort we are now studying the effect of these mutants in a large array of hematologic malignancies, with the hypothesis that weak JAK2 germ-line mutations might predispose to MPNs and leukemia.

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

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

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. Crystal structures of the extracellular domains exist for 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 with a coiled-coil dimer of α-helices [9]. Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), 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 receptor 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 cysteine is in the predicted interface [9]. This then allowed us to determine the active interfaces of TpoR and EpoR dimers. We apply this approach for determining the active interface for signaling of several membrane proteins, such as cytokine receptors and Amyloid Precursor Protein (APP).

Using this approach we have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the normal dimeric interface (cc-TpoR-I), that leads to formation of platelets, other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders [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. This orientation also appears to activate STAT2 and induce cross-talk with the type I interferon pathway (see below). 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 dimer orientation might be recapitulating the quiescence-inducing effects of TpoR in HSCs. 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.

Last but not least, in collaboration with the laboratory of Prof. K. Christopher Garcia at Stanford University we are beginning to explore the effect of surrogate ligands for cytokine receptors, such as diabodies targeting the extracellular domain of EpoR [10]. X-ray structures obtained in the Garcia lab showed that these diabodies maintain receptor monomers at larger distances than Epo, which impacts signaling. Of great interest, certain diabodies that are weak agonists for EpoR in complex with wild-type JAK2 exerted an inhibitory effect on cells expressing EpoR and JAK2 V617F [10]. This result indicates that the distance required for JAK2 activation differs between wild type and V617F in complexes with the same cytokine receptor, EpoR. Such surrogate ligands might be useful therapeutically in the future.

Involvement of pathologic TpoR signaling in myeloproliferative neoplasms

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

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. When we co-expressed TpoR with JAK2 V617F in bone marrow cells that were transplanted in lethally irradiated mice we observed a more severe phenotype than JAK2 V617F alone or JAK2 V617F and inactive TpoR mutants.

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitination, inhibition of recycling and degradation of TpoR [11]. We discovered that Tpo could induce a strong antiproliferative effect in cells that express high JAK2 levels [11]. This effect is physiologic and is detected in late megakaryocytes. Selection against the antiproliferative effect of Tpo occurs in JAK2 V617F cells, leading to TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells. Thus, TpoR can induce both proliferative and anti-proliferative signals, the latter being apparently erased in MPNs.

Recently, we identified one major mechanism by which Tpo can induce anti-proliferative effects. We showed that Tpo can activate STAT2 in hematopoietic progenitors that express high levels of JAK2 (Pecquet et al., submitted). This occurs in early CD34+ progenitors and in late megakaryocyte differentiation (Verteneoil et al., manuscript in preparation). STAT2 activation is mediated by the TpoR istself and does not require interferon receptors. Using biochemical and structural assays, we identified the key cytosolic tyrosine residues required for STAT2 activation, and the precise dimeric conformation of TpoR adopted in the presence of high JAK2 levels, which is cc-TpoR-IV, which also induces strong cell-to-cell adhesion. Engineering this conformation, using coiled coil fusions to the transmembrane-cytosolic domains of TpoR allows STAT2 activation in conditions where JAK2 is expressed at low levels, indicating that the role of high JAK2 levels is to stabilize a precise dimeric orientation of TpoR. The outcome of STAT2 activation is inhibition of cell proliferation and induction of Interferon-stimulated genes (ISGs). We thus uncovered a novel mechanism by which a cytokine receptor switches the type of STATs activation as a function of expression levels of JAK2.

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

A novel mechanism by which a tryptophan residue regulates TpoR activation

J.-P. Defour, T. Balligand, L. Varghese, I. Chachoua, E. Leroy, C. Pecquet

Several years ago we discovered that TpoR contained a unique motif at the junction between the transmembrane and cytosolic domains (RWQFP) [3] (Fig. 2a). 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 [3]. The question remained why would one tryptophan 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

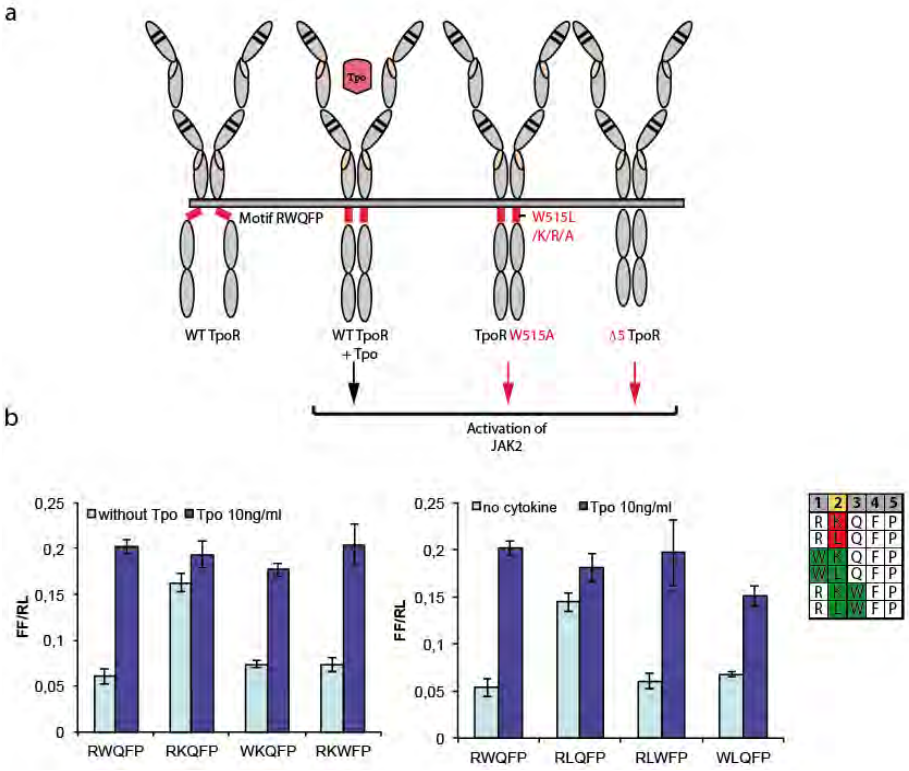

Fig. 2. Activating mutations in the thrombopoietin receptor (TpoR) that are associated with MPNs. (a) TpoR contains an amphipathic motif (RWQFP, in red) which maintains the receptor inactive physiologically in the absence of ligand Tpo. Binding of Tpo overcomes this inhibition leading to dimer formation in a productive conformation that leads to activation of JAK2. Mutation of W515 in this motif (W515L/K/A) or deletion of the entire motif leads to active receptor in the absence of Tpo. (b) Placement of a tryptophan residue before or after the W515K (left) or W515L (right) activating mutations reverts the active TpoR W515K/L to a wild type phenotype, namely receptors that are inactive in the absence of Tpo, and that are active in the presence of TpoR. Shown are STAT5 luciferase activities in JAK2-deficient gamma2A cells that were transfected with cDNAs coding for JAK2, STAT5 and TpoR wild type or the indicated mutants, along with pGRR5-luc (firefly luciferase) STAT5 reporter and pRLTK-renilla luciferase for transfection control. Luciferase signal was assessed 24h after transfection.

W515 to all other residues showed the unique role of this residue, in that even the closely related Y or F residues could not replace it without pathologic receptor activation [12]. Except for Trp, Cys and Pro, all other amino acid residues at position 515 activate TpoR, including residues that are shifted upstream at 515 by deletions in the juxtamembrane region [13]. Biophysical experiments represented by analytical ultracentrifugation, solid-state NMR, infrared spectroscopy performed by our collaborator, Prof. Steven O. Smith at SUNY Stony Brook (Long Island, NY), and fluorescence complementation studies performed in our laboratory by Vitalina Gryshkova showed that W515 actually regulates the orientation, tilt and dimerization of the upstream TpoR transmembrane helix, and prevents receptor activation [12]. Taken apart, the transmembrane-juxtamembrane sequence of TpoR can dimerize, as detected by analytical ultracentrifugation, but this was not the case when the RWQFP insert was added or when W515 in the insert was mutated to K [12]. Given that many proteins possess W residues at the cytosolic side of their transmembrane domains, we suggested a more general role for such W residues in preventing transmembrane domain dimerization and pathologic or premature activation of transmembrane protein signaling. Pathologic activation of TpoR by W515K and W515L mutations can be prevented by placing W residues at 514 or 516 positions (Fig. 2b). Such double mutant receptors respond normally to the ligand Tpo.

Structure and function of juxtamembrane and transmembrane sequences of membrane proteins

E. Leroy, F. Perrin, L. Varghese, J.-P. Defour

We study how sequences that are juxtaposed to the transmembrane domain (on the extracellular and intracellular regions) cytokine regulate receptor function. We are studying such sequences in TpoR (His499, site of small molecule agonist eltrombopag binding), EpoR and several other receptors including G-CSFR, IL-2 and IL-9 receptors, IL-7 receptor α (IL7R) or cytokine receptor-like factor 2 (CRLF2), which when mutated in such sequences induce pediatric acute lymphoblastic leukaemia.

Using a combination of functional and structural/biophysical assays (in collaboration with Steven O. Smith, SUNY Stony Brook, Long Island, NY), such as infrared spectroscopy and solid state NMR, we determined the structure of the extracellular JM region of TpoR. We showed that the human TpoR, which contains His499 at the outset of TM domain, exhibits a stretch of non-helical residues around His499 [6]. Activation of human TpoR by the small molecule agonist eltrombopag, or by placing an asparagine at position 505 in the transmembrane domain (S505N), leads to alpha-helix formation around His499, which promotes dimerization of membrane helices. Thus, the first step of human TpoR activation is represented by a secondary structure change (coil-to-helix) around His499 [6].

The constitutively active human TpoR S505N mutant is associated with rare acquired or familial MPNs. Asparagines are known to induce pathologic dimers via inter-helix H-bond formation in the membrane; this leads to activation in certain growth factor and cytokine receptors. We performed an asparagine scanning mutagenesis of the human and the murine TpoR transmembrane domains. For the human receptor, only S505N was activating, and several mutants in the downstream transmembrane region were not transported to the cell surface, and thus were also not responsive to Tpo. In stark contrast, at least fine different murine TpoR asparagine mutants were active, and all responded to Tpo [6]. Mutagenesis studies showed that the key residue responsible for the resistance of human TpoR to activation by asparagine substitutions in the transmembrane domain was, again, His499. Thus, human TpoR acquired in evolution a supplementary mechanisms to prevent safe-activation, the presence of His499 at the outset of its transmembrane domain.

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-Noël Octave and Pascal Kienlen-Campard in our university and Steven O. Smith at SUNY Stony Brook.

A novel oncogenic mechanism: activation of cytokine receptor intracellular and cell-surface signaling by calreticulin mutants

C. Pecquet, I. Chachoua, T. Balligand, G. Vertenoeil, A. Roy

Mutations in the calreticulin gene (CALR) represented by deletions and insertions in exon 9 always, leading to a +1 frameshift, are associated with one third of Essential Thrombocythemia and Myelofibrosis patients. These patients were previously denoted as double negative (absence of JAK2 and TpoR mutations). We demonstrated that the pathogenic CALR mutants specifically activate the thrombopoietin receptor (TpoR/MPL) [14]. No activation was detected for a battery of other type I and II cytokine receptors, except granulocyte colony-stimulating factor receptor (G-CSFR), which supported only transient and weak activation. CALR mutants induce ligand-independent activation of JAK2/STAT/phosphatydylinositol-3'-kinase (PI3-K) and mitogen-activated protein (MAP) kinase pathways via TpoR, and autonomous growth in Ba/F3 cells [14]. Expression of these mutants in primary human bone marrow cells induces Tpo-independent megakaryocyte colony forming unit formation. In transformed hematopoietic cells, no synergy was observed between JAK2 and PI3-K inhibitors in inhibiting cytokine-independent proliferation, indicating a major difference from JAK2 V617F cells where such synergy is strong.

The glycan binding site and the novel C-terminal tail of the mutant CALR proteins were required for TpoR activation, which was dependent on its extracellular domain and its N-glycosylation, especially at N117 (Fig. 3a) [14]. A soluble form

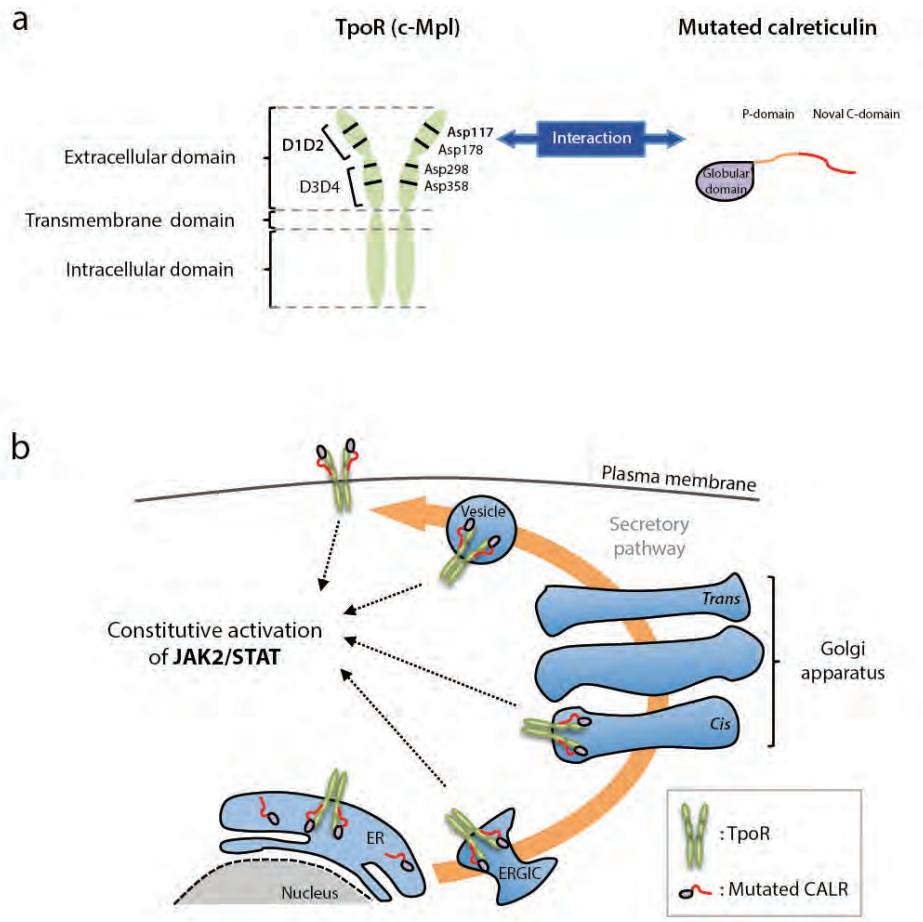

Fig. 3. Mechanism of action of CALR mutants. (a) The lectin binding protein calreticulin when mutated shows specific constitutive binding and activation of the N-glycosylated receptor TpoR through the first Asp 117 residue localized in the distal extracellular domain of TpoR, denoted D1D2. Next to the N-terminal globular domain and the P domain (proline rich), mutations of calreticulin created a new C-terminus possessing hydrophobic and positively charged amino acids that contribute to its oncogenic activity. (b) The binding of mutated CALR to the TpoR leads to the formation of a complex that will follow the secretory pathway (ER, ERGIC, Golgi apparatus, vesicule and plasma membrane) and activate constitutively downstream signaling pathways such as JAK2/STAT3-STAT5, MAP-kinase, PI-3'-kinase. ER : endoplasmic reticulum. ERGIC : ER-Golgi intermediate compartment.

of TpoR was able to prevent activation of full-length TpoR, provided that it was N-glycosylated. By confocal microscopy and subcellular fractionation, CALR mutants exhibit different intracellular localization from that of wild-type CALR, the mutants being found in ER-to-Golgi vesicles and on the cell-surface, while wild type CALR was confined to the ER (Fig. 3b). Importantly, in cells expressing CALR mutants, we detected immature TpoR at the cell surface. Taken together with studies from our collaborators William Vainchenker and Robert Kralovics, our study provides a novel oncogenic signaling paradigm, whereby a mutated chaperone constitutively activates cytokine receptor signaling.

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

I. Chachoua, C. Pecquet, G. Vertenoeil, J.-P. Defour

Using chromatin immunoprecipitation and sequencing, we have shown that STAT5 contacts a substantially different set of promoters in cells that exhibit constitutive STAT5 activation, versus cells that respond to cytokines by acute STAT5 tyrosine phosphorylation. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) [15], 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. We found miR-28 to be pathologically overexpressed in 30% of MPNs.

In transformed hematopoietic cells, STAT5 and p53 must be synergistically bound to chromatin (Fig. 4) for induction of LPP/miR-28 transcription [15]. Genome-wide association studies show that both STAT5 and p53 are co-localized on the chromatin at 463 genomic positions in proximal promoters. Binding of p53 to those promoters is dependent on STAT5 binding. We identified several novel STAT5-p53 target genes, namely LEP,

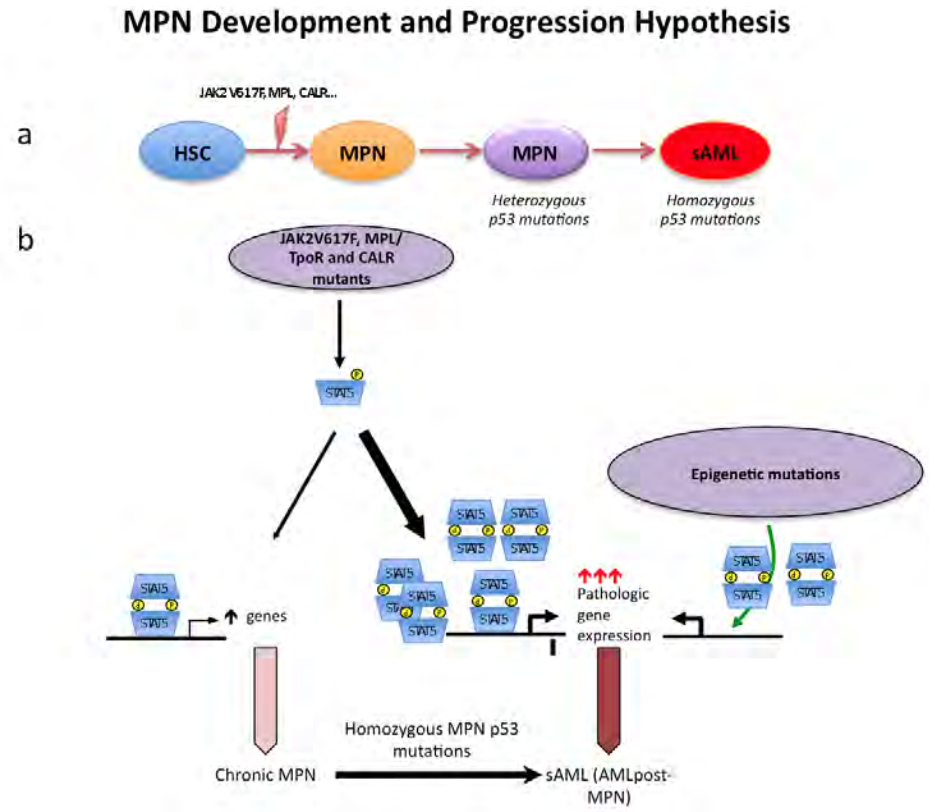

Fig. 4. Progression of MPNs to secondary acute leukemia. (a) Hematopoietic stem cells (HSC) acquire oncogenic driver mutations, JAK2 V617F, TpoR and calreticulin (CALR) mutations, which drive the phenotype of clonal disease. A fraction of MPN cases are associated with heterozygous p53 mutations, which can occur at the HSC level or downstream. Progression to sAML is associated with homozygous p53 mutations, or acquisition of a second p53 mutation. (b) The oncogenic driver mutations code for proteins that lead to persistent STAT5 activation. As a function of the chromatin state and p53 status, pathologic gene expression is induced [15] by complexes of STAT5 and p53. Mutations in epigenetic regulators also occur during the progression of chronic MPNs to sAML.

ATP5J, GTF2A2, VEGFC, NPY1R and NPY5R, which appear to be pathologically expressed in platelets of MPN patients. Furthermore we showed that a mutant p53 (M133K), expressed by HEL cells can also cooperate with constitutive active STAT5 on STAT5-p53 promoters. We concluded that persistently active STAT5 could recruit normal and mutated p53 to novel promoters leading to pathologic gene expression that differs from physiological STAT5 or p53 transcriptional programs. These data are relevant for progression of MPNs to sAML which requires in certain cases homozygous p53 mutations (Fig. 4). We also engineered several of the p53 mutants that are detected in secondary leukemia on MPNs, and use them for determining their effect on STAT5 signaling in cell lines and in murine bone marrow reconstitution, where progenitors from JAK2 V617F knock-in mice are transduced with wild type or mutant forms of p53 and used for reconstitution of lethally irradiated mice. We aim to determine the interplay between STAT5 and p53 in progression of MPNs to secondary myeloid leukemia.

We are studying the nature of genes targeted by STAT5 in chronic MPN and sAML cells from patients and these studies will be now extended to CALR mutant patients, where persistent STAT5 activation is also observed, but where p53 does not appear to be frequently associated with transformation to secondary leukemia.

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

J.-P. Defour, C. Pecquet, 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 Saint-Luc Hospital departments of Hematology (Profs. Cédric Hermans, Augustin Ferrant, Laurent Knoops and Violaine Havelange), Clinical Biology (Prof. Dominique Latinne, Drs. Jean-Philippe Defour and Pascale Saussoy) and groups of the de Duve Institute (Profs. Mark Rider and Jean-Baptiste Demoulin) a large study on the presence and signaling of JAK2, TpoR, and growth factor receptor mutations in patients with myeloproliferative neoplasms. The recently discovered calreticulin mutations (CalR), which are associated with 30% of JAK2 V617F/TpoR mutant negative ET and PMF are studied in a cohort of patients at Saint-Luc Hospital and CALR mutant testing was introduced in the routine clinical biology activity. Next generation sequencing will be employed for well-investigated triple negative (JAK2 V617F/TpoR/CALR mutant negative) patients in order to unravel novel molecular defects in MPNs and leukemias.

Selected publications

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Links

Group

Ludwig Cancer Research Highlights 2014:
http://www.ludwigcancerresearch.org/sites/default/files/media/news-pdf/Ludwig%20Annual%20Research%20Highlights%2C%202014%20%28screen%20friendly%29.pdf

Ludwig Institute for Cancer Research News January 2013:
http://www.eurekalert.org/pub_releases/2013-01/lifc-htt013113.php

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

European Hematology Association (EHA)
Scientific Program Committee EHA20 (Vienna)

Hematology (American Society of Hematology Education Book)
http://www.asheducationbook.org/

Learning:

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Bioinformatics:

Institute of Bioinformatics Bangalore, India
http://www.ibioinformatics.org/

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