Scientific Report

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2012

DE DUVE INSTITUTE

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The de Duve Institute

The de Duve Institute • An international biomedical research institute

Emile Van Schaftingen

In 1974, when Christian de Duve founded the Institute of Cellular Pathology (ICP), now renamed the de Duve Institute, he was acutely aware of the contrast between the enormous progress in biological sciences that had occurred in the 20 preceding years and the modesty of the medical advances that had followed. He therefore created a research institute based on the principle that basic research in biology would be pursued by the investigators with complete freedom, but that special attention would be paid to the exploitation of basic advances for medical progress. It was therefore highly appropriate for the Institute to be located on the campus of the Faculty of Medicine of the University of Louvain (Université catholique de Louvain) in Brussels. The University hospital (Cliniques Universitaires St Luc) is located within walking distance of the Institute.

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 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.

Benoît Van den Eynde

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions has been pursued ever since. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is extremely close and the sharing of resources is considerable. This strong integration was valued as an asset in a recent evaluation of the Ludwig Institute. 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 of three scientists, presently composed of Emile Van Schaftingen, Benoît Van den Eynde, and Miikka Vikkula. 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. Despite this relatively small size, 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. 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.

Miikka Vikkula

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Benoît Van den Eynde

Miikka Vikkula

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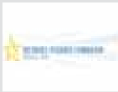
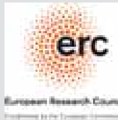


Cystinosis Research Foundation



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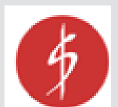

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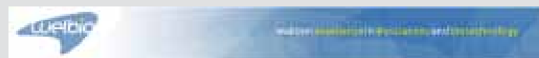

The following laboratories are supported by WELBIO (**W**alloon **E**xcellence in **L**ife Sciences and **Bio**technology)

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Acknowledgements

In 2011, 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 2011 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Julia Cuende Villasur,

the «Pierre Lacroix» fellowship to Vitalina Gryshkova,

the «Maurange» fellowship to Seima Charni,

and other fellowships have been awarded by the Institute to Monica Gordon-Alonzo and Susanna Infantes.

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

Luc Bertrand
President of the Development and Expansion Council

Doctoral theses (PhD) in 2011 - 2012

Benoît Guillaume - Ludwig Institute for Cancer Research
Identification de nouveaux types de protéasome et leur implication dans l'apprêtement de peptides antigéniques présentés par les molécules CMH de classe I

Alexandre Dalet - Ludwig Institute for Cancer Research
L'épissage de peptides antigéniques par le protéasome

Olga Kholmanskikh - Ludwig Institute for Cancer Research
Mechanisms of gene regulation in melanoma: Interleukin-1 strongly reduces expresses of MITF, and BORIS is not required for MAGE1 activation

Ilaria Laudadio - Liver and Pancreas Development Pole
Contrôle de la différenciation des hépatocytes par les facteurs de transcription et un micro ARN hépato-spécificiques

Julie Stockis - Human Tumor Immunology Pole
Activation of the latent form of transforming growht factor-beta occurs at the surface of human regulatory T cells

Aline Antoniou - Liver and Pancreas Development Pole
Les facteurs de transcription Sox contrôlent le développement des canaux biliaires.

Ludovic D'Auria - Membrane Organization - dynamics and epithelial differentiation Pole
Plasma membrane lipids form structurally and kinetically distinct micrometric domains, regulated by membrane tension and altered in spherocytosis

Carmen Montano - Growth Factor Receptors Pole
Transduced CD34+ human hematopoietic progenitors as a model for myeloid neoplasms associated with PDFG receptor fusion genes

Dan Su - Experimental Medecine Pole
Modulation by LDV infection of macrophage receptors involved in endotoxin shock and blood autoimmune disease

Mélanie Uebelhoer - Human Genetics Pole
Pathogenic Effects of Mutations causing Venous Malformation

Laurent Bultot - Phosphorylation Pole
AMPK phosphorylates and inactivates liver glycogen synthase but does not phosphorylate myosin regulatory light chains

Frédéric Sorgeloos - Virology Pole
Evasion of antiviral innate immunity by Theiler’s virus L* protein

Muriel Lemaire - Experimental Medicine Pole
Roles of chitinases and Th17/Th1 cytokines in mouse experimental asthma

Sarah Carpentier - Cell Biology Pole
Phosphoinositide 3 kinase type III and dynamin are critical for apical endocytic recycling in kidney proximal tubular cells

Melisa Gualdron-Lopez - Tropical Medecine Pole

Study of the molecular mechanism involved in recycling of matrix protein receptor, PEX5, during glycosome biogenesis in Trypanosoma brucei

Nicolas Parmentier - Ludwig Institute for Cancer Research

Production d'un peptide antigénique par l'insulin-degrading enzyme

Emeline de Viron - Biochemistry Pole

Predicting resistance to chemotherapy in chronic lymphocytic leukemia - towards a role of PLK2 and miR-27 in oncogenesis

Ana Brennand - Tropical Medecine Pole

Characterization of proteins involved in turnover of glycosomes in *Trypanosoma brucei*

Laurent Detalle - Experimental Medecine Pole

Experimental model of autoimmune thrombocytopenia in the mouse: analysis of autoreactive antibodies and of T lymphocyte-dependent pathogenic mechanisms

Scientific prizes and Awards in 2011 - 2012

To Frédéric Lemaigre

Fonds Alphonse et Jean Forton

To Stefan Constantinescu

Prix Scientifique Madame Veuve André Matthys-Bove

To Guido Bommer

Prix Léopold et Marthe Delsaux-Champy

To Jean-Baptiste Demoulin

Prix Jean-Oscar Maes

To Bernard Lauwerys

Prix Suzanne et Liliane Chermanne

To Emile Van Schaftingen

Chaire Francqui au Titre belge aux Facultés Universitaires de Namur

To Jean-Christophe Renauld

Prix Scientifique Wivine et Jacques Allard - Janssen 2012

To Benoît Van den Eynde

Chaire Francqui au Titre belge à l'Université de Liège

The J.-F. Heremans Lecture

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

1976	Sir Gustav NOSSAL • The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
1978	Henry KUNKEL • Rockefeller University, New York, USA
1980	Michael SELA • Weizmann Institute of Science, 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 of London, UK
1986	Jan KLEIN • Max-Planck-Institut für Biologie, Tübingen, Germany
1988	Zanvil COHN • Rockefeller University, New York, USA
1990	André CAPRON • Institut Pasteur de Lille, France
1992	Jean-Charles CEROTTINI • Lausanne Branch Ludwig Institute for Cancer Research, Switzerland
1994	Fritz MELCHERS • Basel Institute for Immunology, Switzerland
1996	Lorenzo MORETTA • University of Genova, National Institute for Cancer Research (Immunopathology laboratories), Italian Society of Immunology and Immunopathology, Italy
1998	Charles WEISSMANN • Universität Zürich, Institut für Molecularbiologie, Switzerland
2000	Antonio LANZAVECCHIA • Institute for Research in Biomedicine, Bellinzona, Switzerland
2002	Salvador MONCADA • The Wolfson Institute for Biomedical Research, University College London, UK
2004	Alain FISCHER • Groupe Hospitalier Necker-Enfants Malades, Paris, France
2006	Harvey LODISH • Whitehead Institute for Biomedical Research and Massachussetts Institute of Technology, Cambridge, MA, USA
2008	Jean-Laurent CASANOVA • Hôpital Necker-Enfants Malades, Paris, France
2010	Jules HOFFMANN • Nobel Laureate • Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France

Plenary Lectures 2011

January

Salvador MONCADA
The Wolfson Institute for Biomedical Research, London, UK
Discovery of the Mechanism that Enables the Provision of Nutrients to Proliferating Cells

Philippe PIERRE
Centre d'Immunologie Marseille-Luminy, France
Regulating autophagy for controling endogenous antigens presentation: an aggregated story

Guido BOMMER
de Duve Institute, BCHM-GRM, Brussels
Collaborating miRNA-protein pairs: roles in metabolism and cancer

February

Lutz KONRAD
Justus Liebig University, Giessen, Germany
Connection of the TGF-betas with Non-Smad-Pathways

Miikka VIKKULA
de Duve Institute, GEHU, Brussels
Venous anomalies: from identification of causes towards mouse models

March

Jessica ZUCMAN-ROSSI
INSERM U674, Université Paris Descartes, Paris, France
Molecular classification of the benign liver tumors: identification of new tumorigenesis pathways

Cédric BLANPAIN
Interdisciplinary Research Institute (IRIBHM) ULB, Brussels
Skin stem cells during DNA damage and cancer initiation.

Thomas DECKER
University of Vienna, Austria
Signaling crosstalk in infected cells through cooperative assembly of a transcription initiation complex.

Thomas MICHIELS
de Duve Institute, VIRO, Brussels
Theiler's virus accessory proteins: playing with cell biology and IFN responses

Michel TOLEDANO
CEA, Saclay, France
Cellular Compartmentation of eukaryotic thiol-redox homeostasis

April

Ana P. COSTA-PEREIRA
Imperial College London & Hammersmith Hospital, London, UK
JAKs and STATs in Drug Resistance Mechanisms

Julien MARIE
Cancer Research Center, Centre Léon Bérard, Lyon, France
TGF-ß signaling: a key signal for T lymphocyte homeostasis and activation

Pierre COULIE
de Duve Institute, GECE, Brussels
T lymphocytes in human melanoma tumors

May

Carolyn SEVIER
Cornell University, Ithaca, USA
Pathways for oxidative protein folding and redox homeostasis in the endoplasmic reticulum (ER)

Sabine COLNOT
Institut Cochin, Paris, France
Wnt signaling and liver zonation: genome-wide analysis of beta-catenin/Tcf4 DNA-binding

Paul MICHELS
de Duve Institute, TROP, Brussels
30 years of Research on Neglected Tropical Diseases. The TROP unit at the centre of a multidisciplinary, international endeavour

Anne GRAPIN-BOTTON
Swiss Institute for Experimental Cancer Research and Ecole Polytechnique Fédérale de Lausanne, Switzerland
Role of canonical and planar cell polarity Wnt pathways in renal cysts and diabetes syndrome (RCAD)

Nathalie VIGNERON
Ludwig Institute Brussels, LICR, Brussels
Processing and presentation of antigenic peptides recognized by cytolytic T lymphocytes on tumors

June

Paulina ALEKSANDROWICZ
Institute of Physiology - Technical University Dresden, Germany
The entry of Ebolavirus into host cells: coated pits versus macropinocytosis ?

Géraldine CANNY
Université de Lausanne, Suisse
A novel role for Lipoxin A4 in endometrial immuno-endocrine signaling

August

Ha-Long NGUYEN
Department of Physiology and Functional Genomics, University of Florida, Gainesville, USA
In vivo roles of endothelial TGF-beta receptors in vascular development and malformation

September

Michael PACK
University of Pennsylvania School of Medicine, Philadelphia, USA
Invasive transformation of tissue architecture arising from genetically encoded changes in mechanical signaling

Felix GONI
Biophysical Institute, Leioa, Spain
How ceramides behave in a lipid bilayer

Francis H. GRAND
Wessex Regional Genetics Laboratory, University of Southampton, Salisbury, UK
Myeloproliferative neoplasms: new insights into pathogenesis

October

Florence APPARAILLY
INSERM, CHU Saint Eloi, INM, Montpellier, France
Targeting the inflammatory LyC6high monocyte subset in vivo for efficient intervention in immune-mediated inflammatory disorders

Erwin VAN MEIR
Emory University, Atlanta, GA, USA
Galectin-3 mediates a p53-induced bystander anti-cancer effect

Philippe SOUBEYRAN
INSERM, Marseilles, France
Deregulation of signal transduction in pancreatic cancer: scaffolds and modifiers

Pete PEDERSEN
The Johns Hopkins University, School of Medicine, Baltimore, USA
3-bromopyruvate and cancer, mechanisms and clinical prospects

November

Stefania CANE
Department of Microbiology and Immunology at the University of Arkansas for Medical Sciences, Fayetteville, USA
Regulatory role of HSP90 in Immune Senescence

Chieko SAITO
Osaka University Medical School, Japan
Protective strategies against acetaminophen induced hepatotoxicity

Julia BANDOW
Microbial Antibiotic Research, Department for Biology and Biotechnology, Ruhr University, Bochum, Germany
Let the cells speak - a proteomic view on antibacterial mechanisms

December

Shozo IZUI
Department of Pathology and Immunology, C.M.U., Geneva, Switzerland
TLR7, Retrovirus and SLE

Jacques PIETTE
Laboratory of Virology & Immunology, GIGA, University of Liège, Belgium
Phototherapy-induced cell death mechanisms in glioblastoma

Carole LINSTER
de Duve Institute, BCHM-GRM, Brussels
Metabolite proofreading or how to prevent 'messiness' in intermediary metabolism

Plenary lectures 2012

January

Prof. Jan LERUT
Cliniques Universitaires St-Luc, Brussels
Liver transplantation or Thomas Starzl's legacy: our experience with a look backwards and forwards

Kei SAKAMOTO
MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK
Molecular control of glycogen metabolism in muscle and liver

February

Michel BRAUN
Institute for Medical Immunology, Faculty of Medicine, ULB, Brussels
T cell adaptation to persistent antigenic stimulation

Lauri EKLUND
University of Oulu, Finland
Angiopoietin-regulated cellular trafficking of endothelial tyrosine kinase receptor Tie2

Ester ZITO
University of Cambridge, UK
Exploring the redundancy and function of ERO1 pathway in mammals

Antonella DE MATTEIS
Telethon, Naples, Italy
Deciphering Lowe disease reveals a link between phosphatidylinositol> metabolism and actin remodelling in endosomal trafficking

Herman FAVOREEL
University of Ghent, Belgium
The conserved US3 protein kinase of alphaherpesviruses, and its effect on actin and actin-controlling signaling pathways

March

Patrick JACQUEMIN
de Duve Institute, LPAD, Brussels
Transcription factors in pancreatic cell plasticity and cancer

Jean-Baptiste DEMOULIN
de Duve Institute, MEXP, Brussels
PDGF receptor activation in cancer

April

Emmanuel DONNADIEU
Institut Cochin, Paris, France
Imaging T cell migration in human tumors

Nicolas VAN BAREN
Ludwig Institute Brussels, LICR, Brussels
A multifaceted view at inflammatory and immune events in melanoma metastases

Robert H SILVERMAN
Lerner Research Institute, Cleveland Clinic, USA
The Many Lives of RNase L: An Antiviral Nuclease Activated by 2',5'-Oligoadenylates

Donatienne TYTECA
de Duve Institute, CELL, Brussels
Spontaneous organization of fluorescent membrane lipids into micrometric domains: discovery and implications

May

Patrick HENRIET
de Duve Institute, CELL, Brussels
Focal control of coupling between tissue degradation and regeneration: the paradigm of the human endometrium

Jean-Christophe RENAULD
Ludwig Institute Brussels, MEXP, Brussels
Combined acquisition of growth factor independence and drug resistance during tumoral transformation

Ramiro GARZON
Division of Hematology, The Ohio State University, Columbus, Ohio, USA
MicroRNAs in AML: from the bench to the clinic

Nassos TYPAS
EMBL, Heidelberg, Germany
Dissecting bacterial lifestyle with systems-based approaches

Tracy PUTOCZKI
Ludwig Institute for Cancer Research - Melbourne Branch, Melbourne, Australia
Can we therapeutically target cytokines in cancer?

June

Anabelle DECOTTIGNIES
de Duve Institute, GEPI, Brussels
Regulation of telomeric non-coding RNAs and heterochromatin at human telomeres

François FUKS
Laboratory of Cancer Epigenetics - Faculté de Médecine - ULB, Brussels
Epigenetics and epigenomics in health and disease

Jean-Paul COUTELIER
de Duve Institute, MEXP, Brussels
Immune microenvironment and pathogenesis: protective effect of virally-induced type I interferons against macrophage-mediated diseases

Audrey de ROCCA SERRA
INSERM, Toulouse, France
Conséquences physiopathologiques des mutations de la tyrosine phosphatase SHP2 dans les voies de signalisation cellulaire associées aux syndromes de Noonan et L.E.O.P.A.R.D.

Peter VAN ENDERT
Hôpital Necker, Paris, France
The cell biology of phagosome maturation and antigen presentation in dendritic cells

Xin LU
Ludwig Institute for Cancer Research, Oxford, UK
Location, location, location: Restoring p53's tumour suppressive function in human melanoma cells

Research Groups

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

Miikka Vikkula

The aim of our research is to understand the molecular mechanisms underlying a variety of disorders of the cardiovascular and skeletal systems, as well as certain cancers. We are especially interested in evaluating the contribution of genetic variation to human disease. The precise cause of many disorders remains 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 "curative" and specific. As this research is based on human DNA extracted from blood and tissue samples from patients, the group works closely with several clinicians and multidiciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques universitaires St-Luc; Vascular Anomalies Center, Children's Hospital, Boston, USA; Consultation des Angiomes, CHU de Caen, and Centre labiopalatin, Cliniques Universitaires St-Luc).

Venous malformations and glomuvenous malformations ("glomangiomas")

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

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

We previously discovered that rare, hereditary venous malformations are caused by an activating mutation in the endothelial cell receptor tyrosine kinase TIE2/TEK (Vikkula et al, 1996) (Fig1). The use of high-throughput screening technologies was instrumental in the identification of several novel mutations amongst affected families. Despite the ubiquitous presence of these germline mutations, the lesions they cause are localized. We therefore hypothesized that lesion-development requires a somatic second hit to locally disrupt the normal allele of TIE2. We obtained proof for this from one lesion, in which the ligand-binding region of the wild-type allele was deleted somatically, causing a local loss of its ability to function [1]. At least 50% of the far more common sporadic VMs are also caused by somatic mutations in TIE2 [2] (Soblet et al, Submitted), as are lesions of Blue Rubber Bleb Nevus syndrome, a rare congenital disorder whose etiology had been unknown (Soblet et al, In Preparation). All of the VM-causative TIE2 mutations are intracellular and cause receptor hyperphosphorylation in vitro, although much remains to be learnt as to precisely why this causes lesions. Towards this end, we are currently carrying out functional analyses of the role of TIE2 in VM-pathogenesis, using a variety of in vitro and in vivo methods. These include the use of Affymetrix expression profiling to compare the effects of the wild-type receptor with those of different mutant forms; the generation of mouse models of the anomaly, by "knock-in" substitution of the normal TIE2 allele with mutant forms, is also underway.

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 [2]. So far, we have identified GLMN mutations in 162 families, the most frequent being present in 44% of them. We have also discov-

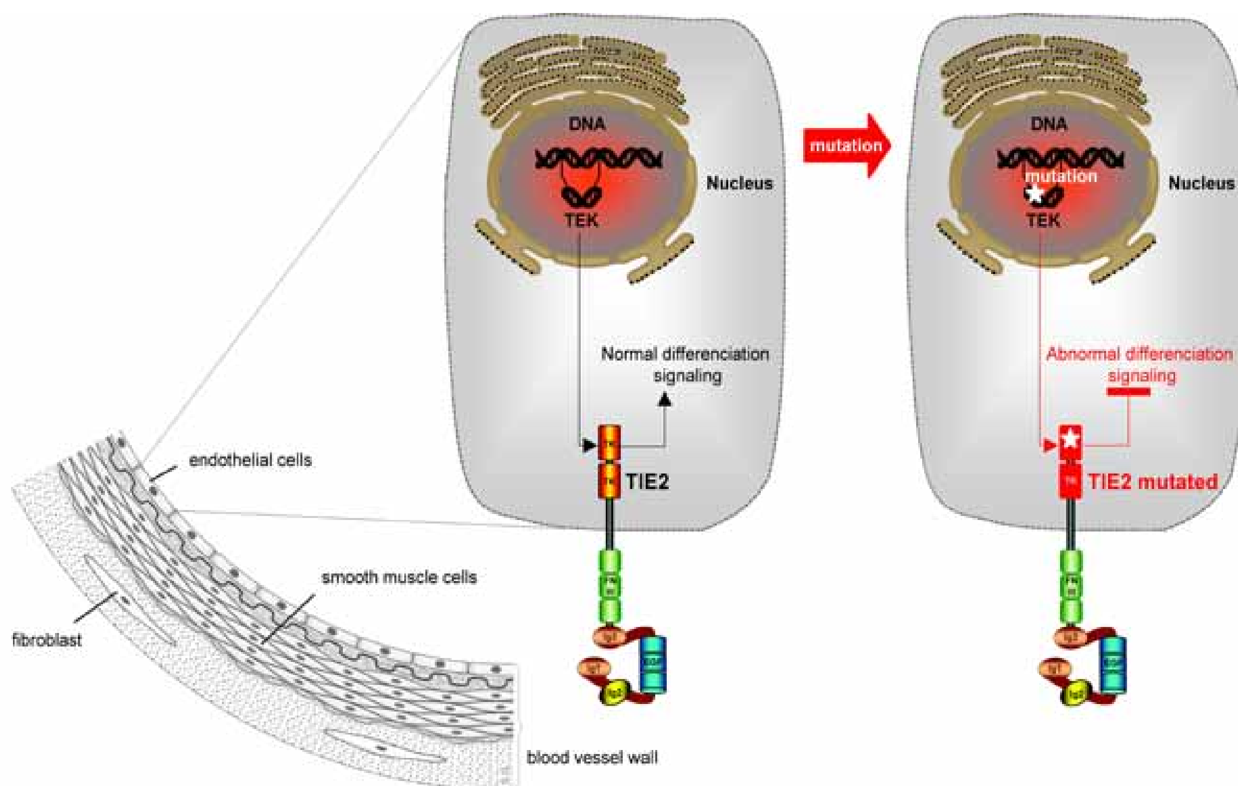

Fig. 1. Mutations in TIE2/TEK cause inherited and sporadic venous malformations. All of the mutations identified cause hyperphosphorylation of this tyrosine kinase receptor, resulting in abnormalities in signaling pathways known to be critical to endothelial cell function (Limaye et al, Nat Genet 2009).

ered that the double-hit mechanism holds true for most if not all GVM [2] (Amyere et al, Submitted). To better understand the role of glomulin, we generated glomulin-deficient mice, which are embryonic-lethal. Mice in which glomulin inactivation is conditional (RNAi knockdown) develop further and present defects in the vasculature (Brouillard et al, Unpublished). These mice are being used to locally inactivate Glmn in order to produce a GVM model. In parallel, we have pursued in vitro methods to clarify the function of glomulin, identifying potential interacting partners that are now being characterized.

Lymphedema

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

Lymphedema is an external manifestation of lymphatic failure. It may be categorized as primary (idiopathic) or secondary (acquired) lymphedema. Primary hereditary lymphedema can occur at birth (Nonne-Milroy disease) or at puberty (Meige's disease). Lymphedema is extremely difficult to treat. Patients have a lifelong problem with progressive swelling of extremities. Both syndromic and non-syndromic cases have been linked by us and other to mutations in eight genes with major role in lymphangiogenesis. FLT4 (encoding VEGFR3) and GJC2 (Cx47) are mutated in patients with autosomal dominant, and rarely autosomal recessive, primary congenital lymphedema, and in hydrops fetalis. Mutations in the latter were also recently shown to predispose to secondary lymphedema following breast cancer therapy. Mutations in FOXC2 and SOX18 cause lymphedema-distichiasis and hypotrichosis-lymphedema-telangiectasia syndromes, respectively, which can be inherited or sporadic, and can also be expressed as hydrops fetalis. Mutations in the CCBE1 were identified only in patients with Hennekam syndrome [3], and PTPN14 was found mutated in rare cases of lymphedema associated with choanal atresia. A few months ago, mutations in GATA2 were shown to cause Emberger syndrome (lymphedema associated with myelodysplasia), and early this year, we were involved in the discovery of KIF11 mutations in patients with autosomal dominant or sporadic lymphedema associated with microcephaly and/or chorioretinopathy [4]. We have now screened these genes in a large series of sporadic and familial index patients with primary lymphedema (n=270), and available family members. We discovered mutations in about 20% of the patients, and more specifically 40% of the familial cases (Mendola et al, In Preparation). Using massive parallel sequencing we aim to identify novel genes explaining the large proportion of unexplained lymphedema.

Vascular anomalies affecting capillaries

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

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a localized maldevelopment of capillary like vessels. Hemangiomas have a frequency of up to 12% in 1-year-old children, and typically undergo a period of rapid expansion, followed by spontaneous regression. We have an extensive collection of samples from sporadic as well as rare familial forms of hemangioma, and are using Affymetrix high-density whole genome SNP arrays in order to carry out linkage, loss of heterozygosity and copy number analyses on them in an effort to identify causative genomic variants. Work done with collaborators has demonstrated that perturbations of the vascular endothelial growth factor (VEGF) signaling pathway can cause hemangioma pathogenesis [5].
CMs occur in 0.3% of newborns. Unlike hemangiomas, they persist throughout life if not treated. Certain capillary malformations affect other organs, such as the brain in the case of cerebral capillary malformations or 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 cutaneous vasculature (Eerola et al, 2000). In addition, genome-wide linkage mapping on families with inherited capillary malformations led us to identify a linked locus CMC1. Screening of positional functional candidate genes resulted in the identification of mutations in the RASA1 gene, a modifier of the Ras signaling pathway (Eerola et al 2003). This implies that RAS pathway modulators may serve as a novel therapy for these patients in the future. Ongoing studies have led to the identification of RASA1 mutations in 90 index patients. This has allowed for a more precise clinical description of the clinical signs and symptoms associated with this newly recognized disorder that we have named Capillary malformation-arteriovenous malformation (CM-AVM) [6]. Importantly, capillary lesions can be associated with deeper, more dangerous anomalies about 20% of the time; these include arteriovenous malformations and fistulas (AVM/AVF), Parkes Weber, and Vein-of-Galen aneurysmal malformations, which warrant careful clinical management.

Cleft lip and palate

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

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

We collected DNA samples from a large number of patients affected with popliteal pterygium syndrome, as well as van der Woude syndrome, the most common cleft syndrome. We showed that IRF6 is the major causative gene in our Belgian cohort (Ghassibe et al, 2005). This study in turn led to several collaborations that allowed us to carry out a genotype-phenotype correlation on hundreds of patients from different ethnic backgrounds. Results showed that IRF6 is mutated in 69% of VWS patients and 97% of PPS patients. Interestingly, mutation-distribution is non-random: 80% are localized in IRF6 exons 3, 4, 7 and 9 for VWS, and 72% in exon 4 for PPS patients (de Lima et al, 2009). These findings are of great importance for clinical diagnosis, mutational screens and genetic counseling. We also demonstrated that IRF6 predisposes to non-syndromic clefts in Europe and that it is mutated in familial clefts with minor lip anomalies (Desmyter et al, 2010). In parallel, we identified a new gene, FAF1, responsible for cleft palate only and Pierre Robin sequence [7]. This gene is associated with clefts across populations. Zebrafish studies confirmed its role during embryonic development and jaw formation (Fig 2). We have begun to use Next Generation Sequencing (NGS) in an effort to uncover additional genes that play a role in non-syndromic orofacial clefts.

Cerebral tumors

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

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymomas, as well as ocular melanomas. To better understand the molecular alterations leading to ependymomal oncogenesis, we performed microarray-based expression profiling on a series of 34 frozen ependymomas. Results of our profiling study are in concordance with the "oncology recapitulates ontology" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype. Using array-CGH, we recognized a subgroup of supratentorial ependymomas affecting young adults, which are characterized by trisomy of chromosome 19 (Rousseau et al, 2007).

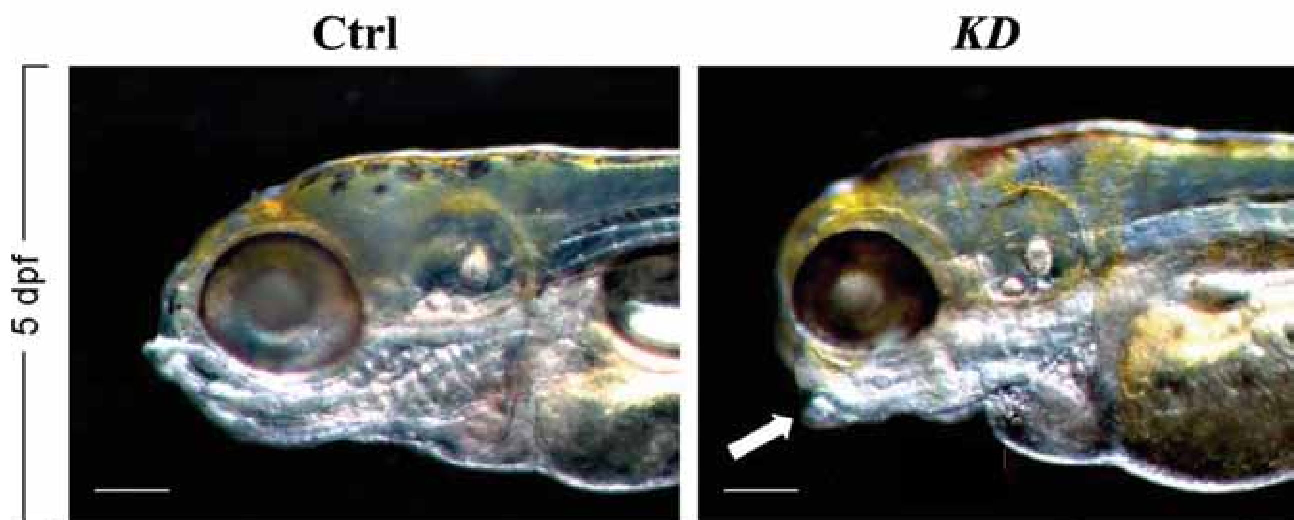

Figure 2 – FAF1 mutations cause abnormalities in jaw development across species. Morpholino-induced knockdown of the protein in zebrafish causes an open-mouthed appearance due to retrognathia, similar to the phenotype in human lacking FAF1. (Ghassibé-Sabbagh et al, Am J Hum Genet 2011)

Within the posterior fossa compartment, ependymomas cluster into three sub-groups. The first corresponds with ependymomas that are histologically of WHO grade II, the second with those of WHO grade III, and the third with a group of ependymomas of a bi-phasic appearance, combining regions of both grades. This sub-group shares gene-sets with tumors of both other groups, and in addition has a glycogen metabolism signature of its own. Whether these groups correspond to three distinct tumoral entities, or demonstrate multifocal tumor progression remains to be investigated.

Neuroendocrine tumors

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

Pheochromocytomas, and head and neck paragangliomas are neuroendocrine tumours derived from the neural crest. Paragangliomas are associated with parasympathetic ganglia and are usually non-secreting. By contrast, pheochromocytomas are derived from paraganglia associated with the 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 tumours in Belgium and to detect possible genotype-phenotype correlations.
A multicentric collaboration including more than 20 hospitals from Belgium has therefore been established. Clinical information and DNA from >160 patients with pheochromocytoma and paraganglioma, and more recently 50 patients suffering from thyroid tumours has been obtained.
The SDH genes code for the subunits of succinate deshydrogenase, at the crossroad of the mitochondrial respiratory chain and Krebs cycle. The four subunits of succinate deshydrogenase, i.e. SDHD, SDHB, and more rarely SDHC and SDHA, have been associated with paraganglioma and pheochromocytoma. Furthermore, SDHB mutations have been associated with an increased risk of recurrence and malignancy in several European series.

In our study, the prevalence of SDHx mutations in head and neck paraganglioma was high (in the range of 40%). In sporadic cases, the prevalence of SDHB mutations (20 %), was similar to that of SDHD (18 %). Surprisingly, SDHB-associated tumours were mostly late-onset unilateral head and neck paraganglioma without evidence of recurrence or malignancy.

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 (Yao et al, 2010).

Essential hypertension

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

High blood pressure - commonly called hypertension - is found in almost 20 % 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 genome wide association study (HYPERGEN) with the support of the Belgian Hypertension Committee. Thousand hypertensive patients and an equal number of normotensive subjects have been recruited. Detailed phenotyping including renin and aldosterone dosages was obtained, and more than 20 candidate SNPs distributed over 12 candidate genes have been genotyped. The analysis of the results of this large association study is currently under way.

Hematological malignancies and tumors of soft tissue and bone

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

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

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

SNP-Chip & next generation sequencing platforms

M. Amyere, R. Helaers and M. Vikkula

We host the UCL microarray platform (Affymetrix), used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. We also collaborate with several groups from around the world on whole genome mapping studies. In work done with Dr Jüppner (Harvard Medical School, Boston), we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes [8]. In collaboration with G. Matthijs (KULeuven), we have used autozygosity mapping along with expression profile analysis to identify a new gene for congenital glycosylation disorder [9]. In collaboration with Dr Vermeesch (KULeuven), we genotyped a large family with autosomal-dominant microtia. Copy number analysis led to the identification of five tandem copies of a copy number-variable region at chromosome 4p16, linked to the disease. With the same group, we established that chromosome instability is common during early human embryogenesis in a study of 23 pre-implantation embryos from 9 fertile couples. Additionally, rearrangements such as segmental imbalances were observed in 70% of the 23 embryos tested. This explains low human fecundity and identifies post-zygotic chromosome instability as a leading cause of constitutional chromosomal disorders [10].
In an exciting development, this platform is now complemented by a High Throughput Sequencing platform. Funded by the Fondation Contre le Cancer, it consists of a Solid 5500XL sequencer (Life technologies), a Personal Genome Machine (Ion Torrent, Life technologies) and a computing cluster for bioinformatics processing. This equipment allows us to perform Exome-seq, Genome-seq, RNA-seq, Small RNA profiling, ChIP-seq and methylation studies. Data analysis is performed using Lifescope (software from Life technologies), and a combination of open source packages (BWA, GATK, snpEff) as well as home-made filtering algorithms (by Raphaël Helaers, Ph.D., Bioinformatician). This enhances our ability to identify and explore the genetic and epigenetic bases of disease.

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Liver and pancreas development

Frédéric Lemaigre
Patrick Jacquemin

The group studies the molecular and cellular mechanisms that govern development of the liver and pancreas, two organs which play essential metabolic roles and which derive from the endoderm (primitive gut of the embryo). The fundamental knowledge gained by this work is essential for improving cell therapy of liver and pancreas diseases (metabolic disease, diabetes), and for understanding the pathophysiology of organ malformations (e.g. polycystic liver diseases). Identifying developmental mechanisms also impacts on understanding abnormal differentiation of liver and pancreatic cancer cells.

Liver development

A. Antoniou, J.-B. Beaudry, R. Carpentier, C. Demarez, I. Laudadio, A. Poncy

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

The biliary tract consists of intrahepatic bile ducts which collect bile produced by the hepatocytes, and of extrahepatic ducts which drain bile from the liver to the intestine. Biliary cells, also called cholangiocytes, delineate the lumen of the bile ducts and modify the composition of bile. These cells, like hepatocytes, derive from liver progenitor cells called hepatoblasts. Our discovery of the Onecut transcription factors Onecut-1 (OC-1/HNF-6), OC-2 and OC-3, and the subsequent phenotypic characterization of HNF-6 and OC-2 knockout mice led to the identification of the first transcriptional network regulating bile duct development [1, 2]. Current efforts are devoted to the characterization of the transcription factors and signal transduction pathways that control bile duct development in health and disease.

We have recently identified novel molecular markers that enabled us to revisit the morphogenesis of the intrahepatic bile ducts. We found that biliary morphogenesis occurs according to a new mode of tubulogenesis [3, 4]. Biliary tubulogenesis starts with formation of asymmetrical ductal structures, lined on one side (adjacent to the portal vein) by cholangiocytes and on the other side (adjacent to the liver parenchyma) by hepatoblasts. When the ducts grow from the hilum to the periphery of the liver, the hepatoblasts lining the asymmetrical structures differentiate to cholangiocytes, thereby allowing formation of symmetrical ducts lined only by cholangiocytes. This mode of tubulogenesis is unique as it is to our knowledge the only one characterized by transient asymmetry (Figure 1). We further investigated how this new knowledge impacts on the interpretation of congenital malformations of the bile ducts. To this end we studied several mouse models and samples from human liver fetuses. This work allowed us to propose a new pathogenic classification of biliary malformations (Figure 1) [5].

The transcription factor network that drives cholangiocyte morphogenesis and bile duct formation has been further investigated. By means of a liver-specific gene inactivation strategy we found that Sox9 controls the timing of bile duct development [3]. We pursue this research by evaluating the role of other members of the Sox family: the hepatic expression of several Sox factors has been determined and their function is currently being characterized.

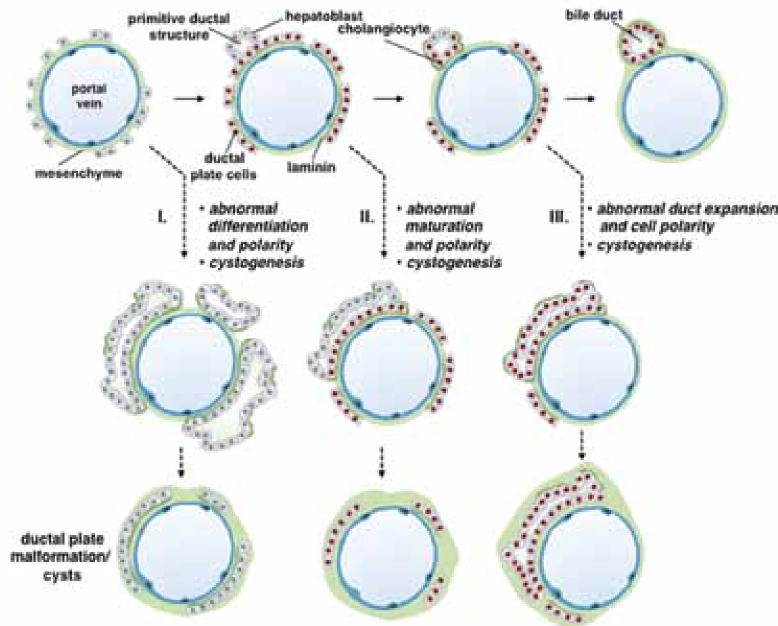

Fig. 1. Classification of biliary malformations based on distinct pathogenic mechanisms. Normal bile duct morphogenesis is illustrated at the top. Malformations can arise by three mechanisms: (I) differentiation of hepatoblasts to ductal plate cells is abnormal and associated with perturbed polarization and cyst formation; (II) asymmetrical ducts are formed but fail to mature to bile ducts; this is associated with formation of cysts and abnormal polarity; (III) differentiation of ductal plate cells and maturation of asymmetrical ducts proceeds but duct expansion is perturbed; this is associated with defects in cell polarity.

Our work also addresses the mechanisms of hepatocyte differentiation. HNF-6 and OC-2 are critical for normal differentiation of hepatic precursor cells to hepatocytes or cholangiocytes : in the absence of HNF6 and OC2, the precursor cells generate hybrid hepato-biliary cells instead of distinct hepatocyte and biliary cell populations [2]. HNF6 also fine-tunes gene expression during hepatocyte differentiation. This involves a feedback loop with microRNAs (Figure 2), in which the liver-specific miR-122 and HNF6 stimulate each other's expression while promoting hepatocyte maturation [6]. In collaboration with the Katholieke Universiteit Leuven, we found that the expression of enzymes synthesizing ketone bodies rises during development, while that of an enzyme catabolising ketone bodies (2-oxoacid CoA transferase) decreases. We showed that the decrease in 2-oxoacid CoA transferase is in part due to repression mediated by miR-122 [7]. Therefore, this work has identified molecular feedback loops and some of their targets to stimulate hepatocyte differentiation.

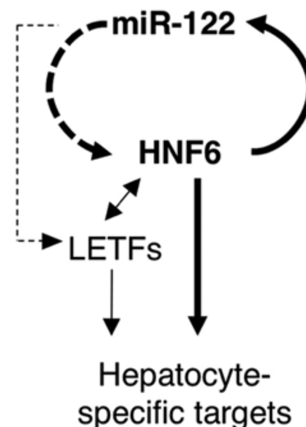

Fig. 2. A positive feedback loop between HNF6 and miR-122 drives hepatocyte differentiation. HNF6 and miR-122 stimulate each other while promoting hepatocyte differentiation and expression of liver-specific transcription factors (LETF) [6].

Also, the current model of liver development predicts that embryonic liver progenitors (hepatoblasts) give rise to hepatocyte precursors, which mature to hepatocytes, and to ductal plate cells, which generate the bile ducts (see Figure 1). However, only a fraction of the ductal plate cells contribute to bile ducts, while the remaining part was considered to involute by apoptosis. We have now used a lineage tracing approach, which consists in genetically labeling ductal plate cells in the embryo, and then analyzing their progeny after birth. This uncovered a new fate map of the hepatic cells: the ductal plate cells were shown to generate bile ducts, but also a subset of periportal hepatocytes, and cells lining the canals of Hering which harbor the adult liver progenitor cells (Figure 3) [8].

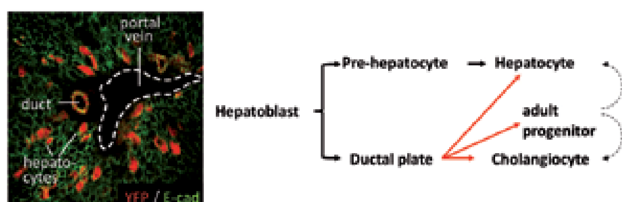

Fig. 3. Left: Ductal plate cells were induced to express Yellow Fluorescent Protein (YFP) at the embryonic stage, by tamoxifen injection of females bearing embryos that harbor inducible Cre recombinase (Sox9-CreER) and a Cre-dependent YFP reporter (Rosa26R-YFP). The liver was analyzed in the postnatal period and showed YFP-labeling of the bile ducts and periportal hepatocytes. Right: Fate map of the hepatc cells in the embryo.

Pancreas Development

C. Augereau, M. Colletti, A. Grimont, P.-P. Prévot

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

The role of the Onecut transcription factor HNF-6 in pancreas development is being studied since several years. We showed that HNF6 is required for development of endocrine cells and pancreatic ducts [9,10].

After birth, HNF6 is expressed exclusively in the duct cells where it most likely maintains duct cell identity. Interestingly, there is evidence from other laboratories that pancreatic ductal adenocarcinoma may derive from acinar cells which switch their phenotype from acinar to ductal during progression to cancer. This process is called acinar-to-ductal metaplasia, and constitutes a preneoplastic state. We hypothesized that the switch in cell identity depends on the ectopic expression of ductal transcription factors and tested if HNF6 is ectopically induced in acinar cells undergoing metaplasia. This was the case in human pancreas. In addition, we also collected evidence from mouse models that induction of a HNF6 – Sox9 cascade in acinar cells promotes acinar-to-ductal metaplasia, suggesting that these factors are key inducer of preneoplastic lesions [11]. This work is currently being pursued by investigating the mechanisms that activate HNF6 and Sox9 expression in acinar cells.

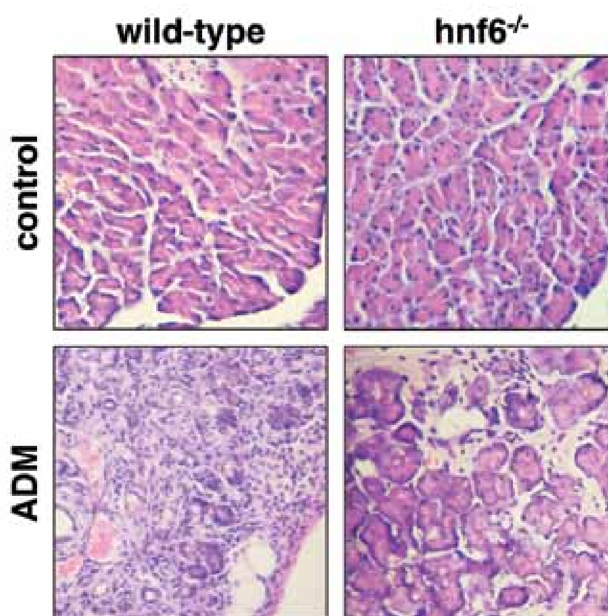

Fig. 4. When the pancreatic duct is ligated in the pancreas, acinar-to-ductal plate metaplasia (ADM) is induced. Development of ADM is impaired in HNF6-knockout mice.

Conclusions

Our findings on the role of transcription factors that regulate liver and pancreas development contribute to a better understanding of the diseases affecting these organs. In liver, our work opens perspectives for understanding the pathophysiology of congenital diseases of the liver. Applying our findings to the programmed differentiation of cultured stem cells should also help in developing cell therapy of hepatic deficiencies. In pancreas, our observations on the expression and function of HNF6 in preneoplastic lesions are expected to improve diagnosis and to help preventing progression towards pancreatic ductal adenocarcinoma.

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Genetic and epigenetic alterations in genomes

Anabelle Decottignies
Charles Desmet

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 tumour cells maintain their telomeres to acquire immortality. We have demonstrated that epigenetic alterations in tumours, involving loss of DNA methylation marks, can lead to the aberrant activation of a particular group of genes. We are currently investigating how epigenetic marks are established on these genes in embryonic cells and how they become altered in tumour cells.

Asymmetrical segregation of oxidized proteins in Schizosaccharomyces pombe fission yeast

S. Lenglez, A. Decottignies

We use *S. pombe* fission yeast as model organism to study the cellular mechanisms of asymmetrical segregation of carbonylated proteins. It is now well established that protein carbonylation levels increase with age in various species and in age-related degenerative diseases. The reasons underlying this were proposed to be as diverse as: 1) a decline in the cell antioxidant defense system, 2) an increased production of reactive oxygen species, 3) a diminished capacity to remove carbonylated proteins or, 4) an increased susceptibility of proteins to oxidative attack in aging cells. Some of the proteins that were reported to be more sensitive to carbonylation in *E. coli* were also shown to undergo carbonylation in yeast subjected to oxidative stress, in aging flies, in plants and in Alzheimer's diseased brain. Carbonylation is probably not reversible and cells must therefore degrade oxidized proteins. However, carbonylated proteins can escape degradation by the proteasome and form high-molecular-weight aggregates that accumulate with age. A decade ago, it was found that *Saccharomyces cerevisiae* carbonylated proteins are not inherited by the daughter cell during cytokinesis. Asymmetrical cytokinesis allows the production of a small and young progeny from a large and old precursor cell and oxidatively damaged proteins were found to accumulate in the mother bud prior to division. Although asymmetrical cytokinesis –like the one occurring in budding yeast- offers a convenient way to segregate cellular damage, it has been recently discovered that damage partitioning exists in systems dividing by binary fission (the new daughter cell being of the same size as the mother cell at the time cytokinesis occurs), like *Schizosaccharomyces pombe* or *Escherichia coli*. The discovery of an aging phenomenon in organisms dividing by binary fission, like *S. pombe*, is of great importance for the understanding of human aging and stem cell biology and provides excellent tools to identify a series of aging agents. During the past few months, we started to set up the tools required to investigate the mechanisms of asymmetrical segregation of carbonylated proteins in fission yeast. In particular, we wish to focus on the role of the cytoskeleton.

Impact of telomerase on NK-κB signaling and cellular responses to TNF-α in human fibroblasts

M. Mattiussi, G. Tilman and A. Decottignies

Telomeres are specialized protein-DNA structures, which prevent chromosome ends from being recognized as DSBs. Syn-

thesis of telomeric DNA sequences in replicating cells requires telomerase. Cancer cells often show an increased level of telomerase, and this contributes to their unlimited proliferation potential. In some cancers, however, telomeres are maintained in the absence of telomerase activity by one or more mechanisms that are known as alternative lengthening of telomeres (ALT). These two pathways of telomere maintenance are very distinct phenotypically. In telomerase-expressing cells (TEL+), telomere length is very homogenous and telomeres are found at the end of all chromosomes. However, in ALT cells, telomeres are very heterogeneous in length and some chromatids lack telomeres (Fig. 1).

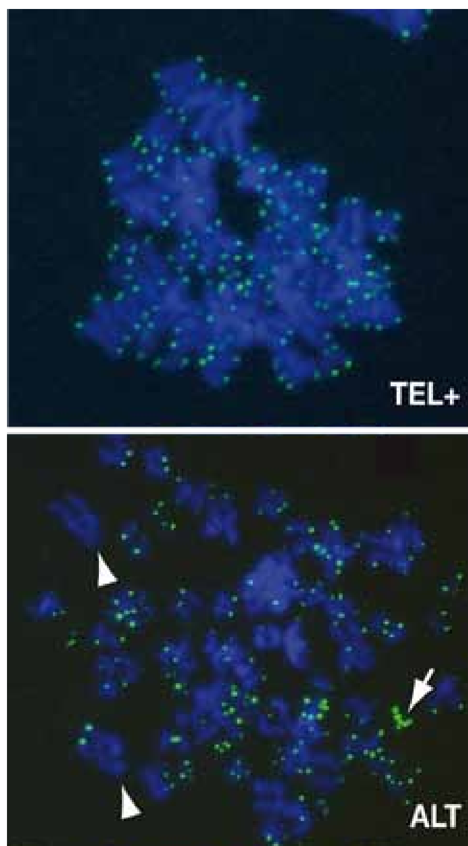

Figure 1. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive (TEL+) and ALT cancer cells (ALT). Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous, and even absent at some chromosome ends (arrowheads). ALT cells are further characterized by the presence of extrachromosomal telomeric DNA (arrow).

In addition to its well-established role in telomere synthesis, telomerase exerts non-canonical functions that may promote cancer and stem cell survival, notably as transcriptional cofactor in Wnt-β-catenin signaling pathway. The previously reported physical interaction between telomerase and NF-κBp65 suggested that telomerase may similarly modulate NF-κB pathway, another master regulator of cell proliferation and survival. We investigated telomerase impact on NF-κB signaling in normal human fibroblasts (1). Strikingly, telomerase overexpression induced constitutive nuclear accumulation of NF-κBp65 that, however, lacked activating Ser-536 phosphorylation. Although NF-κBp65 nuclear accumulation constitutively up-regulated *IL*-6, basal expression levels of most NF-κB target genes were unaffected, arguing against a general hyperactivation of the pathway. Conversely, prolonged culture of telomerase-expressing fibroblasts down-regulated *TNF-α* target gene, due to progressive promoter hypermethylation. Telomerase did not either alter NF-κB pathway activation by tumor necrosis factor-a (TNF-α). Interestingly however, owing to its ability to reduce reactive oxygen species (ROS), telomerase impaired *SOD*2 transcriptional activation as ROS contribute to TNF-α-dependent *SOD*2 induction. Accordingly, other ROS-dependent TNF-α-induced processes, including Mitogen Activated Protein Kinase (MAPK) activation and cell death, were reduced by telomerase. Our results demonstrate a new way by which telomerase, through a reduction of ROS, modulates intracellular signaling and gene expression in response to cytokines.

Regulation of telomeric transcripts in human cells

N. Arnoult, A. Van Beneden, E. Schultz, A. Decottignies

Recent studies indicated that telomeres of eukaryotic cells are frequently transcribed, yielding non-coding RNAs dubbed "TERRAs" (TElomeric Repeat-containing RNAs). Human TERRA molecules mostly consist of $(UUAGGG)_n$ repeats that, at least partially, remain bound to telomeres where they may impact on diverse aspects of telomere biology, including regulation of telomerase access to telomeres and DNA damage checkpoint. We are interested in understanding how TERRAs are regulated in human cells, and what impact they have on telomeres, both in TEL+ and ALT cells.

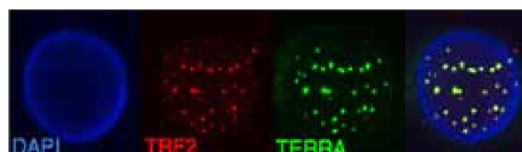

Figure 2. Detection of TERRA telomeric transcripts by RNA-FISH at human telomeres. Telomeres are detected by immunofluorescence against TRF2 telomeric protein (red), TERRAs are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue).

We showed that telomere length-dependent modifications of telomeric heterochromatin marks, namely H3K9me3 and Heterochromatin Protein 1a (HP1α), impact on TERRA expression levels in human cells. We propose the existence of a negative feedback mechanism for the regulation of TERRA expression as our data provided further evidences that TERRA is itself involved in cell cycle-regulated telomeric heterochromatin formation. 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 (Arnoult et al., submitted).

DNA hypomethylation and aberrant gene activation in cancer

A. Loriot, C. De Smet

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

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

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 cancer-germline 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 (3). 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 (4). The factors that are responsible for the initial DNA demethylation process and for maintaining cancer-germline gene promoters unmethylated remain to be identified.

Search for factors that dictate MAGEA1 methylation states

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

Histone modifications have been shown in some cases to dictate DNA methylation states, for instance by regulating access of DNA methyltransferases. We therefore searched to determine if *MAGEA1* demethylation and activation in tumor cells is associated with changes in histone marks. Chromatin immunoprecipitation experiments revealed that DNA demethylation and transcriptional activation of *MAGEA1* is accompanied by increases in histone H3 acetylation (H3ac) and H3 lysine 4 methylation (H3K4me), and by a decrease in H3 lysine 9 methylation (H3K9me). However, our experiments demonstrate that changes at the histone level within the *MAGEA1* promoter are a consequence, not a cause, of DNA demethylation. Consistently, epigenetic drugs that target histone modifications were unable to induce DNA demethylation and stable activation of the *MAGEA1* gene. Altogether, these observations confirm that DNA methylation has a dominant role in the epigenetic hierarchy that governs *MAGEA1* silencing (Cannuyer et al., manuscript in preparation).

We also decided to perform an unbiased search for factors that participate in maintaining methylation within the promoter of *MAGEA1*. To this end we have transduced a lentiviral shRNA library into a human melanoma cell line containing a methylated transgene comprising the *MAGEA1* promoter followed by the sequence encoding the green fluorescent protein (GFP). Transduction of shRNAs directed against factors that contribute to methylation maintenance should lead to de-repression of the transgene and emergence of GFP positive clones. GFP positive cell clones, which emerged at different time points after transduction, have been isolated by cell sorting. The shRNA sequences they contain are currently being identified. Single shRNAs directed against candidate genes will be tested individually to confirm activation and demethylation of the *MAGEA1* transgene.

Tumorigenesis-associated DNA hypomethylation within heterochromatic regions of chromosomes

G. Tilman, N. Arnoult, , S. Lenglez, A. Van Beneden, A. Loriot, C. De Smet, A. Decottignies

Constitutive heterochromatin is mainly located at centromeric, pericentromeric, and (sub)telomeric regions of chromosomes. Heterochromatin is usually characterized by a high DNA methylation content, an increased enrichment in repressive histone marks like H3K9me3 and H4K20me3 and the binding of HP1 isoforms. Increasing evidence, mainly obtained from studies in mouse cells, indicates that chromatin modifications at chromosome ends are important regulators of mammalian telomeres. In particular, alterations of histone modifications in telomeric

chromatin are associated with telomere length deregulation in mouse cells and a decreased subtelomeric DNA methylation level was reported to up-regulate homologous recombination between mouse telomeric sequences (T-SCE for Telomeric Sister Chromatid Exchange), a hallmark of human ALT cells. This prompted us to evaluate the subtelomeric DNA methylation level of human TEL+ and ALT cancer cell lines (5). We detected a significant hypomethylation of subtelomeric DNA in ALT cancer cell lines when compared to TEL+ cell lines. However, subtelomeric DNA was not hypomethylated in ALT cell lines derived from in vitro immortalization of human fibroblasts with SV40 T antigen, although T-SCE frequencies in the latter cells were similar to those in ALT cancer cells (5). Strikingly, subtelomeric DNA hypomethylation in ALT cancer cells was also associated with lower global DNA methylation, raising the interesting possibility that DNA demethylation in tumor cells may be linked to the process that cells use to escape from senescence and/or crisis, two anti-proliferative barriers thought to require bypass during tumorigenesis. Indeed, evidence accumulated during the past decade that senescent and cancer cells share similarly altered global epigenetic profiles that includes changes in DNA methylation, is in agreement with the hypothesis that senescence, whether induced by ageing or by oncogene activation, may be a common step in the tumorigenesis process (6). We are currently investigating whether deep DNA hypomethylation may have favored the emergence of ALT cells during tumorigenesis, notably by allowing transcriptional induction of *MAGE* cancer-testis genes that encode antagonists of p53 function. We showed that *MAGE-A* knock-down induces cellular senescence of ALT cells through p53 activation and we are now studying the mechanisms behind this observation.

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

Epigenetic repression of cancer-germline genes in human embryonic stem cells

A. Loriot, C. De Smet

The stage at which cancer-germline genes become methylated during human embryo development has not been determined. We found previously that human cancer-germline genes are repressed and methylated in human blastocyst-derived embryonic stem cells and in comparable embryonal carcinoma cells (7). By performing transfection experiments, we now 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 cancer-germline genes increase up to the morula stage, and then decrease dramatically in blastocysts. Altogether our data indicate that human cancer-germline genes are programmed for repression in the blastocyst, and suggest that *de novo* DNA methylation is a primary event in this process (8).

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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. This research theme is connected with our other main research interest, the elucidation of inborn errors of metabolism. Many metabolic diseases are due to a defect in an enzyme that is not easily measured or that is expressed in a tissue that is not 'accessible'. In such cases, the defect can be easily established through the search of mutations in the gene encoding the relevant enzyme, provided this gene is known. Quite a number of enzymes are still 'orphan', i.e., the gene that encodes them is not known for any species. One of our aims is to carry out the molecular identification of 'orphan' enzymes that are potentially deficient in metabolic disorders.

Metabolite repair

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

Repair of damaged NAD(P)H

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

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

YNL200C in yeast. We showed that these proteins catalyze the epimerization of the (S) and (R) forms of NAD(P)HX, thereby allowing, in conjunction with the energy-dependent dehydratase, the repair of both epimers of NAD(P)HX (Fig. 1). Both enzymes are very widespread in eukaryotes, prokaryotes, and archaea. This wide distribution and the ADP-dependence of the dehydratase in some species indicate the ancient origin of this repair system [5].

EthylmalonylCoA decarboxylase

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

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

Identification of enzymes potentially implicated in neurometabolic diseases

Characterization of the enzyme that synthesizes N-acetylaspartate

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

We reported in 2009 the identification of the enzyme that catalyses the synthesis of N-acetylaspartate, a most abundant compound present in brain. We also reported that the gene (NAT8L) encoding this enzyme is mutated in the only case of N-acetylaspartate deficiency that is known worldwide. NAT8L is membrane-bound and is at least partially associated with the endoplasmic reticulum (ER). We recently determined which regions of the protein are important for its catalytic activity and its subcellular localization. Transfection of truncated forms of NAT8L into HEK (human embryonic kidney)-293T cells indicated that the 68 amino-terminal residues have no importance for the catalytic activity and the subcellular localization of this

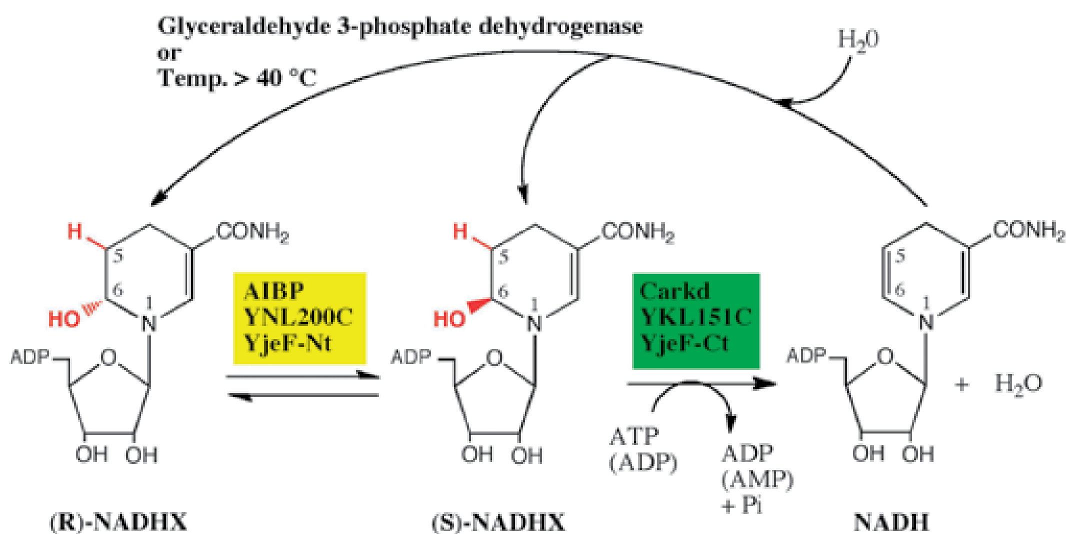

Figure 1. Formation and repair of hydrated NAD(P)H. Modified from Marbaix *et al*. [5]

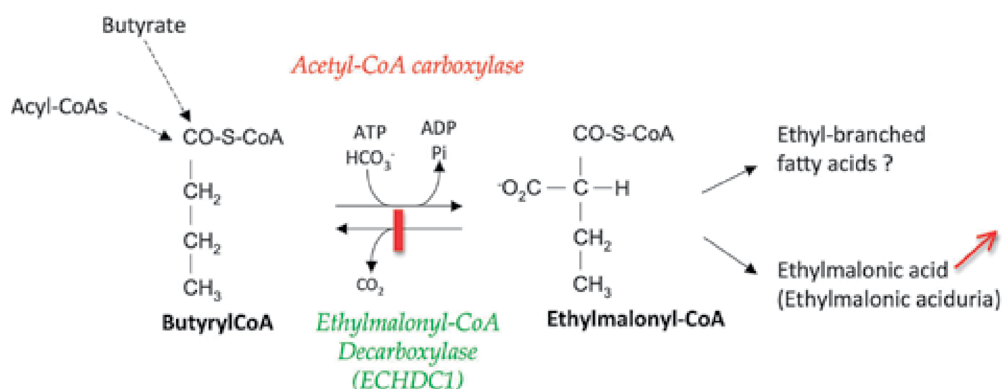

Figure 2. Ethylmalonyl-CoA formation and repair. See text. [4]

enzyme, which was exclusively associated with the ER. Mutation of conserved residues that precede (Arg81 and Glu101) or follow (Asp168 and Arg220) the putative membrane region (a stretch of ≈ 30 hydrophobic residues) markedly affected the kinetic properties, suggesting that the catalytic site involves residues that precede and follow the membrane region in the sequence and that this membrane region has a loop structure. Transfection of chimaeric proteins in which GFP (green fluorescent protein) was fused to different regions of NAT8L indicated that the membrane region is necessary and sufficient to target NAT8L to the ER. Thus NAT8L is targeted to the ER membrane by a hydrophobic loop that connects two regions of the catalytic domain. This structure accounts for the high sensitivity of this enzyme to detergents. Other experiments showed that the catalytic site of NAT8L faces the cytosol. Thus, NAT8L's activity is controlled by the cytosolic concentrations of its substrates and products [6].

Synthesis and breakdown of N-acetylaspartylglutamate and beta-citrylglutamate

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

N-acetylaspartylglutamate (NAAG) is the most abundant dipeptide present in vertebrate central nervous system. β-citrylglutamate, a structural analogue of NAAG is present in testis and immature brain. The function of both compounds is still mysterious, and until recently only little was known on the enzymes involved in their metabolism, except for glutamate carboxypeptidase 2 (GCP2), a membrane-bound ectoenzyme, which is responsible for the hydrolysis of NAAG. We reported in 2010 the molecular identification of the two enzymes (RIMKLA and RIMKLB) that synthesize NAAG and ß-citrylglutamate [1]. More recently we reported the identification of the enzyme that hydrolyses ß-citryl-glutamate [2].
To this end, this enzyme was partially purified from mouse testis and characterized. Interestingly, in the presence of Ca^{2+}, the purified enzyme specifically hydrolyzed β-citrylglutamate and did not act on NAAG. However, both compounds were hydrolyzed in the presence of Mn2+. This behaviour and the fact that the enzyme was glycosylated and membrane-bound suggested that β-citrylglutamate hydrolase belonged to the same family of protein as glutamate carboxypeptidase 2 (GCP2), the enzyme that catalyzes the hydrolysis of NAAG. The mouse tissue distribution of β-citrylglutamate hydrolase was strikingly similar to that of the glutamate carboxypeptidase 3 (GCP3) mRNA, but not that of the GCP2 mRNA. Furthermore, similarly to β-citrylglutamate hydrolase purified from testis, recombinant GCP3 specifically hydrolyzed β-citrylglutamate in the presence of Ca^{2+}, and acted on both NAAG and β-citrylglutamate in the presence of Mn^{2+}, whereas recombinant GCP2 only hydrolyzed NAAG and this, in a metal-independent manner. A comparison of the structures of the catalytic sites of GCP2 and GCP3, as well as mutagenesis experiments revealed that a single amino acid substitution (Asn-519 in GCP2, Ser-509 in GCP3) was largely responsible for GCP3 being able to hydrolyze β-citrylglutamate. Based on the crystal structure of GCP3 and kinetic analysis, we propose that GCP3 forms a labile catalytic Zn-Ca cluster that is critical for its β-citrylglutamate hydrolase activity [2].

Metabolism of hydroxylysine and phosphoethanolamine

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

Hydroxylysine, an amino acid found in collagen, is known to be metabolized by phosphorylation of its hydroxyl group, followed by ammonia and phosphate elimination to yield aminoadipate semialdehyde (Fig. 3). The latter reaction is similar to the conversion of ethanolamine-phosphate to acetaldehyde, inorganic phosphate and ammonia. None of the three enzymes involved in these reactions was molecularly identified. We recently identified them through an approach that was largely based on bioinformatics.
Database searches drew our attention on AGXT2L1 and AGXT2L2, two vertebrate genes encoding closely related pyri-

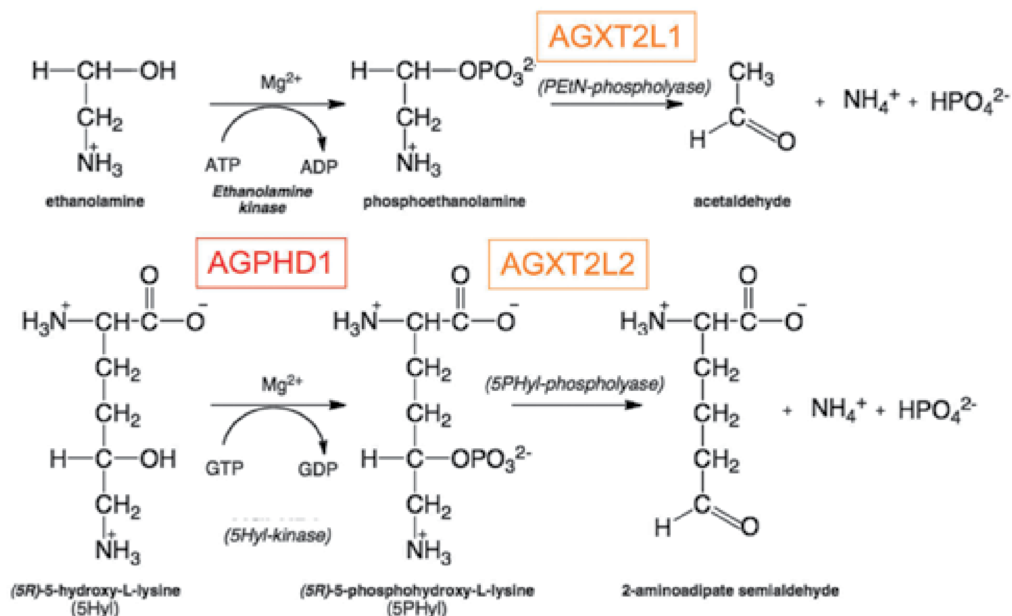

Figure 3. Metabolism of hydroxylysine and phosphoethanolamine. From [9].

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

These and other considerations led us to hypothesize that AGPHD1 corresponded to 5-hydroxy-L-lysine kinase and that AGXT2L1 and AGXT2L2 catalyzed the pyridoxal-phosphate-dependent breakdown of phosphoethanolamine and 5-phosphohydroxy-L-lysine. The three recombinant human proteins were produced and purified to homogeneity. AGPHD1 was indeed found to catalyze the GTP-dependent phosphorylation of 5-hydroxy-L-lysine (Fig. 3). The phosphorylation product made by this enzyme was metabolized by AGXT2L2, which converted it to ammonia, inorganic phosphate, and 2-aminoadipate semialdehyde. AGXT2L1 catalyzed a similar reaction on phosphoethanolamine, converting it to ammonia, inorganic phosphate, and acetaldehyde. AGPHD1 and AGXT2L2 are likely to be the mutated enzymes in 5-hydroxylysinuria and 5-phosphohydroxylysinuria, respectively. The high level of expression of AGXT2L1 in human brain, as well as data in the literature linking AGXT2L1 to schizophrenia and bipolar disorders, suggest that these diseases may involve a perturbation of brain phosphoethanolamine metabolism. AGXT2L1 and AGXT2L2, the first ammoniophospholyases to be identified, belong to a family of aminotransferases acting on ω-amines [9].

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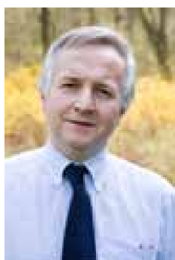

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Nucleoside analogues in leukaemia

Françoise Bontemps
Eric Van Den Neste

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

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

Mechanisms of action

F. Bontemps, C. Smal, L. Bastin-Coyette, E. de Viron, A. Arts, E. Van Den Neste

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

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

In collaboration with L. Knoops from the Ludwig Institute for Cancer Research (Brussels), we performed microarray analyses to identify survival or death pathways that are activated in

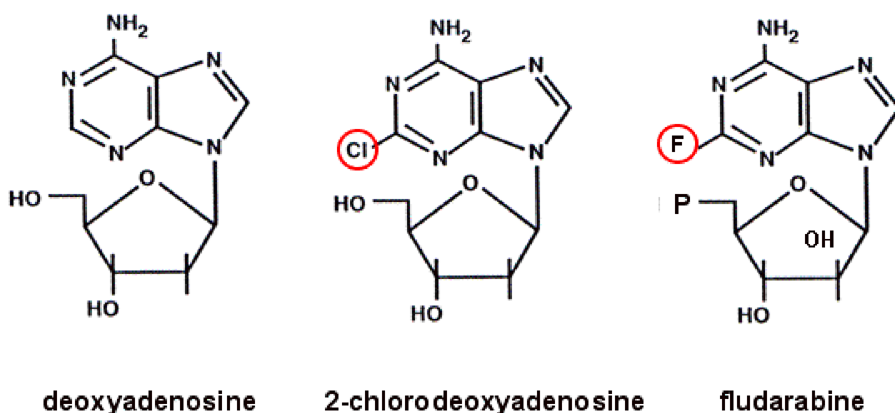

Figure 1. Structures of deoxyadenosine and purine nucleoside analogues.

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

Potentiation of their action

E. Van Den Neste, C. Smal, F. Bontemps

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

Lately, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. We have shown that CdA, at concentrations close to the IC_{50}, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (7).

Activation by deoxycytidine kinase

C. Smal, R. Amsailale, A. Arts, E. Starczewska, E. Van Den Neste, F. Bontemps

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

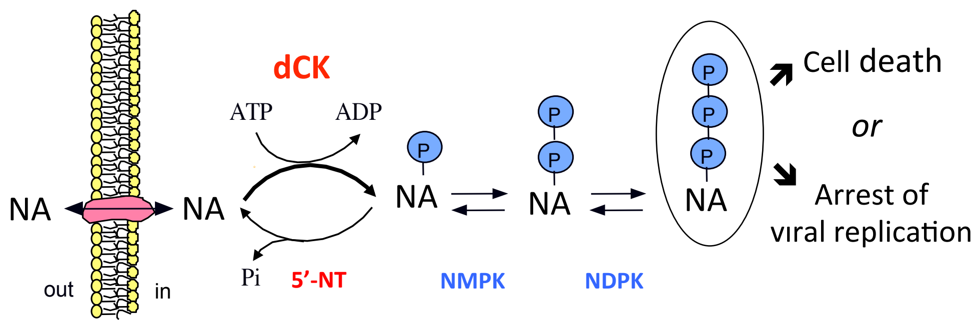

Fig. 2. Activation of nucleoside analogues.

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

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

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

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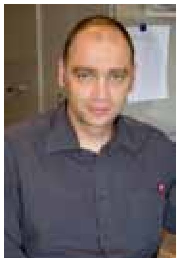

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Redox biology

Jean-François Collet

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

Disulfide bond formation in the periplasm

In *E. coli*, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) protein family (3, 7).
The primary oxidant is the soluble protein DsbA. DsbA has a CXXC catalytic site motif present within a thioredoxin fold. The cysteine residues of this motif are found oxidized in vivo. The disulfide bond of DsbA is very unstable and is rapidly transferred to secreted unfolded proteins. DsbA is then re-oxidized by the inner-membrane protein DsbB that transfers electrons from DsbA to the electron transport chain (Figure 1).

DsbA is a very powerful oxidant that apparently lacks proofreading activity. If the native disulfide bond pattern involves cysteine residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect disulfides. The correction of these non-native disulfides is the role of a disulfide isomerization system. This system involves a soluble periplasmic protein disulfide isomerase, DsbC (Figure 2). Like DsbA, DsbC presents a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC is kept reduced in the periplasm. This allows DsbC to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC reduced is the membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions.
In our lab, we are studying the Dsb proteins of *E. coli* and of other Gram-Negative bacteria. Over the past few years, we have characterized the disulfide cascade within DsbD, we have identified four proline residues that play an important role in DsbD mechanism and we have identified the proteins that depend on DsbA and DsbC for folding. Moreover, we have engineered a new periplasmic oxidizing system (10) and have characterized

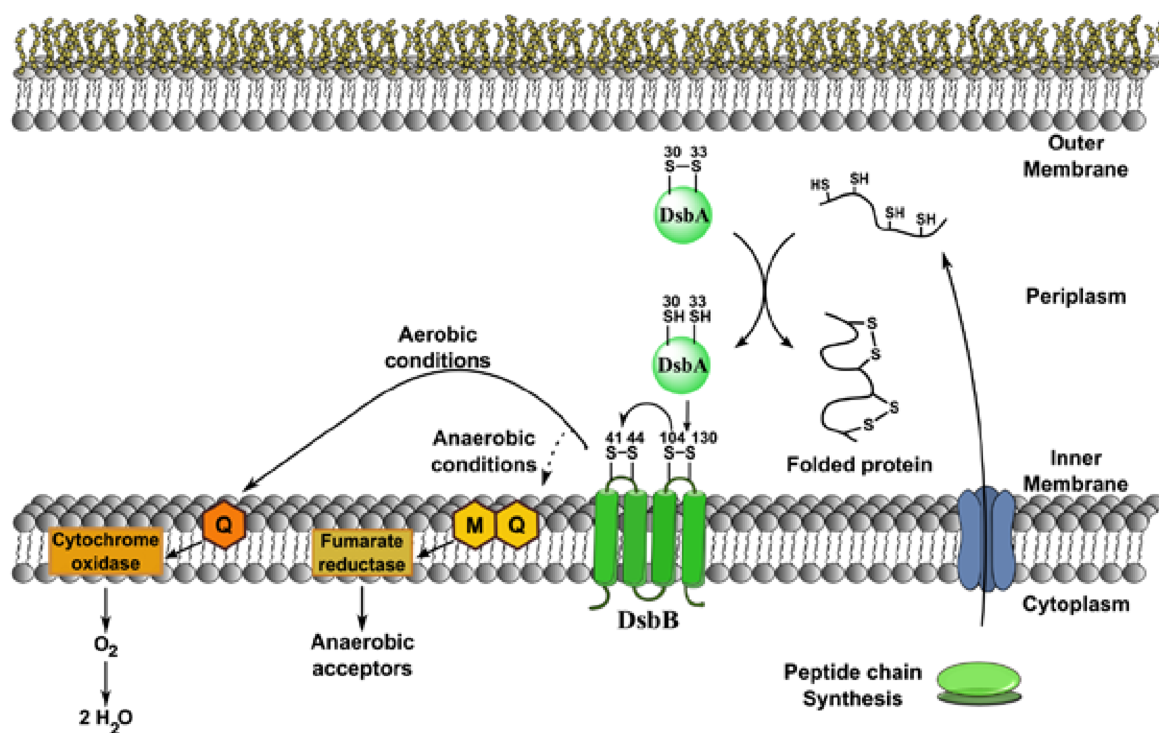

Figure 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbB transfers electrons to the respiratory chain (adapted from (1)).

the sensitivity of dsbC mutant to copper stress. We have also proposed a revised model for the pathways of disulfide bond formation in the periplasm by showing that DsbC may be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively.

Moreover, we have also identified two new substrates of the protein disulfide isomerase DsbC. Interestingly, these two substrates, LptD and RcsF, are located in the outer membrane and play important roles in the maintain of envelope integrity. LptD is an essential ß-barrel protein that inserts lipopolysacharrides in the outer membrane and whose folding depends on the periplasmic chaperone SurA (4). We found that deletion of dsbC in strains lacking surA increases E. coli sensitivity to hydrophobic antibiotics due to an impaired assembly of LptD. The definitive evidence that DsbC plays a role in the folding of LptD came from the identification of a LptD-DsbC mixed-disulfide complex (6). RcsF is an outer membrane lipoprotein sensor that detects defects in envelope integrity and transduces the signal to the Rcs phosphorelay. The Rcs phosphorelay is a signaling system that allows bacteria to react to a range of envelope stresses by modulating the expression of specific genes. The Rcs system is required for normal biofilm formation, contributes to antibiotic resistance and regulates virulence-associated structures involved in motility and host recognition. In collaboration with Prof. Jean-Paul Declercq (UCL), we solved the structure of RcsF (5).

Recently, we also initiated the characterization of the machineries that catalyze disulfide bond formation in bacteria other than E. coli. For instance, we are currently studying the proteins that introduce disulfides in the periplasm of the pathogenic bacteria Pseudomonas aeruginosa. Moreover, in a recent study in collaboration with Prof. J. Beckwith (Harvard Medical School), we identified a third distinct class of DsbD-like homologues (1), which is found in proteobacteria and Chlamydia. The prototype of this new class is Salmonella typhimurium ScsB. The ScsB class has a domain organization comparable to that of DsbD. However, the N-terminal domain (ScsBα), which is the final electron donor for substrate proteins, differs significantly from DsbDα, which suggested that it acts on a different array of substrate proteins from those already known for DsbD. Using Caulobacter crescentus as a model organism, we searched for the substrates of ScsB (1). We discovered that ScsB provides electrons to the first peroxide reduction pathway identified in the bacterial cell envelope. The reduction pathway comprises a thioredoxin-like protein, TlpA, and a peroxiredoxin, PprX. We showed that PprX is a thiol-dependent peroxidase that efficiently reduces both hydrogen peroxide and organic peroxides. Our results reveal that the array of proteins involved in reductive pathways in the oxidative cell envelope is significantly broader than has been understood. Moreover, the identification of a new periplasmic peroxiredoxin indicates that in some bacteria, it is important to directly scavenge peroxides in the cell envelope even before they reach the cytoplasm.

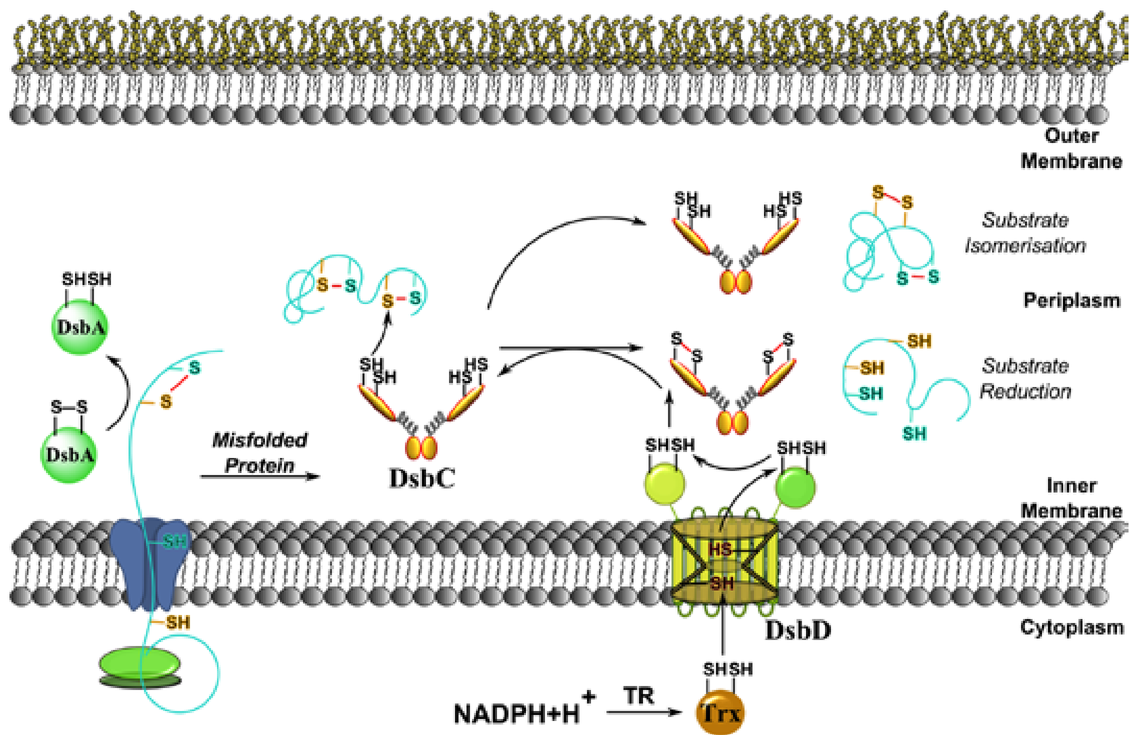

Figure 2. Disulfide bond reduction/isomerization in the periplasm. DsbA can introduce non-native disulfides when they need to be formed beween non-consecutive cysteines. Incorrect disulfides are either isomerized or reduced by DsbC. Oxidized DsbC is regenerated by the membrane protein DsbD that transfers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

Control of cysteine sulfenylation in the periplasm

The periplasm possesses another soluble Dsb protein, DsbG, but the function of this protein has remained elusive. We sought to find the function of DsbG in the periplasm by trapping it with its substrates. We identified three periplasmic proteins, in complex with DsbG. The interaction between DsbG and those proteins was confirmed in vitro and in vivo (8).

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

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

Proteins from the thioredoxin superfamily are very widespread and have been identified in the majority of the genomes se-

quenced so far, making it tempting to speculate that some of these thioredoxin superfamily members, particularly those that are closely related to DsbC and DsbG, play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments (8).

Recently, in another collaboration with the group of Jon Beckwith (2), we found that sulfenic acid formation may also lead to the formation of disulfide bonds in secreted proteins. We found indeed that overexpressing PspE, a periplasmic rhodanese containing a single cysteine residue, partially restores substantial disulfide bond formation to a dsbA strain. This activity depends on DsbC, the bacterial disulfide bond isomerase, but not on DsbB. We showed that overexpressed PspE is oxidized to the sulfenic acid form and reacts with substrate proteins to form mixed disulfide adducts. DsbC either prevents the formation of these mixed disulfides or resolves these adducts subsequently. In the process, DsbC itself gets oxidized and proceeds to catalyze disulfide bond formation. Our results suggest that the DsbC/PspE system might be utilized in organisms lacking the DsbA/DsbB system.

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miRNAs in physiology and disease

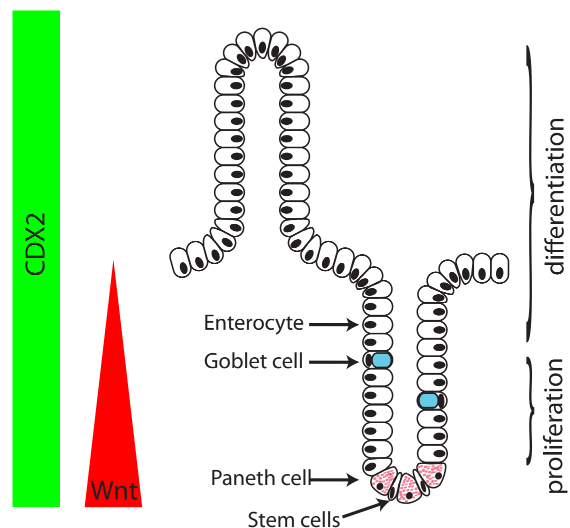
Guido Bommer

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

The focus of our group is on the role of miRNAs in conserved signaling pathway at the crossroad of cancer and metabolism.

Role of miRNAs in intestinal differentiation

The intestine is required for the digestion and absorption of essential nutrients and water. In this process, its surface epithelium is exposed to one of the most toxic milieus of the whole body. It has to resist aggressive digestive juices, large pH changes, anaerobic bacteria and numerous toxic compounds. To resist this, its surface epithelium is completely renewed in less than 2 weeks. An intricate network of signaling pathways controls the proliferation and differentiation from intestinal stem cells to the mature cell types. We are studying the role of miRNAs in this differentiation process and how they contribute to intestinal differentiation as well as the development of colorectal cancer.



Intestinal architecture is maintained by the interplay of many signaling pathways

The intestinal architecture is maintained by the interplay of signaling patways that ensure complete renewal of intestinal surface epithelia within 7 to 14 days. New cells are generated from a stem cell compartment at the base of the crypts and successively migrate up, where they are eventually shed in the lumen. We are focusing our interest on miRNAs that regulate this process.

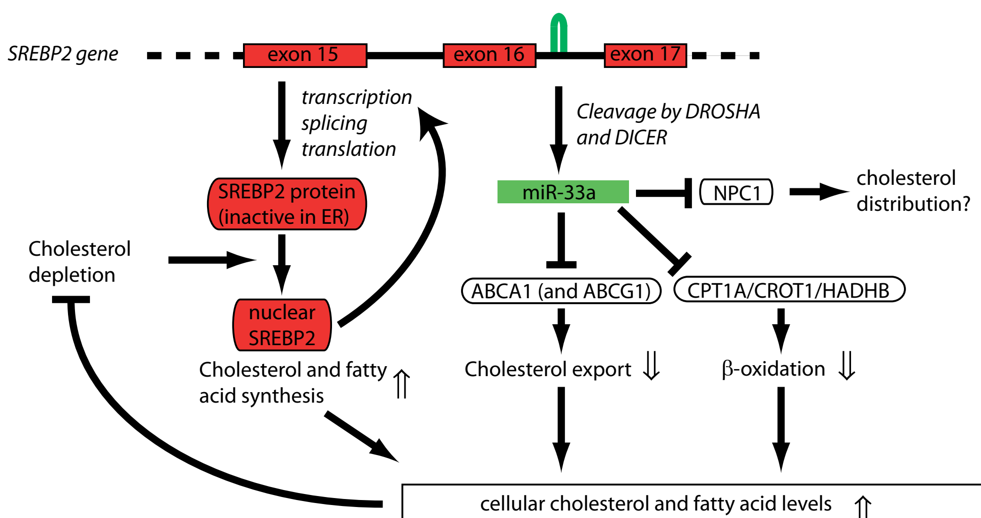
Regulation of cholesterol metabolism by the bifunctional locus SREBF2-miR33

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 tran-

scriptional regulator of genes involved in cholesterol and fatty acid biosynthesis, and miR-33a. We and others have recently shown that miR-33a can reduce the expression of several proteins involved in the cellular export of cholesterol and β-oxidation of fatty acids, thus adding an unexpected layer of complexity and fine-tuning to regulation of lipid homeostasis. In fact, work of other groups has demonstrated that this mechanism might represent a therapeutic target in the treatment of hypercholesterolemia.

We are continuing to investigate the physiological role of miR-33 family members in different experimental systems throughout evolution.



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

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

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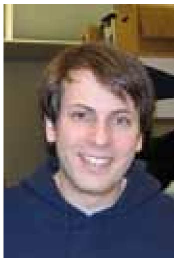

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Signal transduction and protein phosphorylation

Mark Rider

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

Insulin signalling

N. Hussain, Y.-C. Lai, R. Jacobs, L. Hue, M.H. Rider, with help from D. Alessi, Dundee and B. Hemmings, Basel

Metabolic effects of insulin in heart, skeletal muscle and adipose tissue

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate (1). Recombinant heart PFK-2 isozyme is a substrate of protein kinases of the insulin signalling pathways, such as protein kinase B (PKB), also known as Akt, which is believed to mediate most metabolic effects of insulin. Our previous studies suggested that PFK-2 activation by insulin was dependent on PDK1, a protein kinase upstream of insulin-stimulated protein kinases. We have studied the role of SGK3 in insulin action in perfused rat hearts and in HEK 293T cells. SGK3 is activated by insulin in heart and cardiac PFK-2 can be phosphorylated by SGK3 in vitro leading to its activation (2). However, co-transfection of HEK 293T cells with SGK3 siRNA did not affect insulin-induced PFK-2 activation. We reinvestigated the role of PKB in heart PFK-2 activation by insulin in different models. In rat hearts perfused with the Akti-1/2 selective PKB inhibitor, insulin-induced PFK-2 activation was abrogated. Results from PKBβ-knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin. PKBα knockdown by siRNA transfection suggested that this isoform likely mediates insulin-induced heart PFK-2 activation (2).

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

Using PKB inhibitors, we also studied the role of PKB in the insulin-induced stimulation of glucose transport, glycogen synthesis and protein synthesis in incubated rat soleus muscles. In parallel we measured of the phosphorylation state of signalling proteins, PKB downstream targets and enzyme activities. Our data point to the central role of PKB in metabolic control by insulin in skeletal muscle. The results also argue for both allosteric and phosphorylation mechanisms of control of GS as important determinants of insulin-stimulated glycogen synthesis in muscle (submitted).

AMP-activated protein kinase

L. Bultot, Y. Liu, C. Plaideau, Y.-C. Lai, D. Vertommen, R. Jacobs, S. Pyr dit Ruys, L. Hue, M.H. Rider, in collaboration with S. Carpentier and P. Courtoy, de Duve Institute, K. Sakamoto, Dundee, J. Tavaré, Bristol, J. Jenssen, Oslo, K. Storey, Ottawa and B. Viollet, Paris, AstraZeneca, Mölndal

AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as occurs during hypoxia or after ATP depletion with oligomycin. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phosphorylated to ZMP, an analogue of AMP, or by the A769662 Abbott compound. Full AMPK activation requires phosphorylation of the α-catalytic subunits at Thr172 by upstream kinases, either LKB1 (the Peutz-Jeghers protein) or calmodulin-dependent protein kinase kinase-β (CaMKKβ). The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways (Fig. 1). We contributed to the field by discovering new substrates of AMPK and mechanisms of upstream regulation. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia. We also showed that AMPK activation is associated with protein synthesis inhibition in anoxic rat hepatocytes (4) and in ischaemic rat hearts. Protein synthesis inhibition in response to AMPK activation can partly be explained by a rise in eEF2 (eukaryotic elongation factor-2) phosphorylation leading to its inactivation. Regulation of the upstream eEF2 kinase (eEF2K) is complex involving phosphorylation-induced activation and inactivation by kinases from various signalling pathways. Indeed we, and others, reported that AMPK phosphorylates and activates eEF2K. Recently, we identified nine autophosphorylation sites in eEF2K by mass spectrometry, some of which are highly conserved amongst eukaryotes and are required for eEF2K activity (5). Finally, we demonstrated that PKB-induced phosphorylation of the AMPK catalytic α/β subunits at Ser485/491 in response to insulin antagonizes AMPK activation by LKB1 (3).

AMPK activation in diapausing insects as a means for surviving energy stress

Winter survival for many insects depends on cold hardiness adaptations as well as entry into a hypometabolic diapause state that minimizes energy expenditure. We investigated whether AMPK could be involved in this adaptation in larvae of two cold hardy insects, Eurosta solidaginis that is freeze tolerant and Epiblema scudderiana that uses a freeze avoidance strategy. AMPK activity was almost 2-fold higher in midwinter larvae (February) compared with animals collected in September and accordingly phosphorylation of ACC increased more than 3-fold in both species during midwinter which would strongly suppress lipogenesis. Overall, our study suggests a role for AMPK in minimizing anabolic processes to decrease energy expenditure during insect diapause (6).

Control of liver glycogen synthesis by AMPK

We found that liver glycogen synthase isoenzyme-2 (GYS2) is a new AMPK target (Fig. 2). Recombinant muscle glycogen synthase-1 (GYS1) and recombinant GYS2 were phosphorylated by recombinant AMP-activated protein kinase (AMPK) in

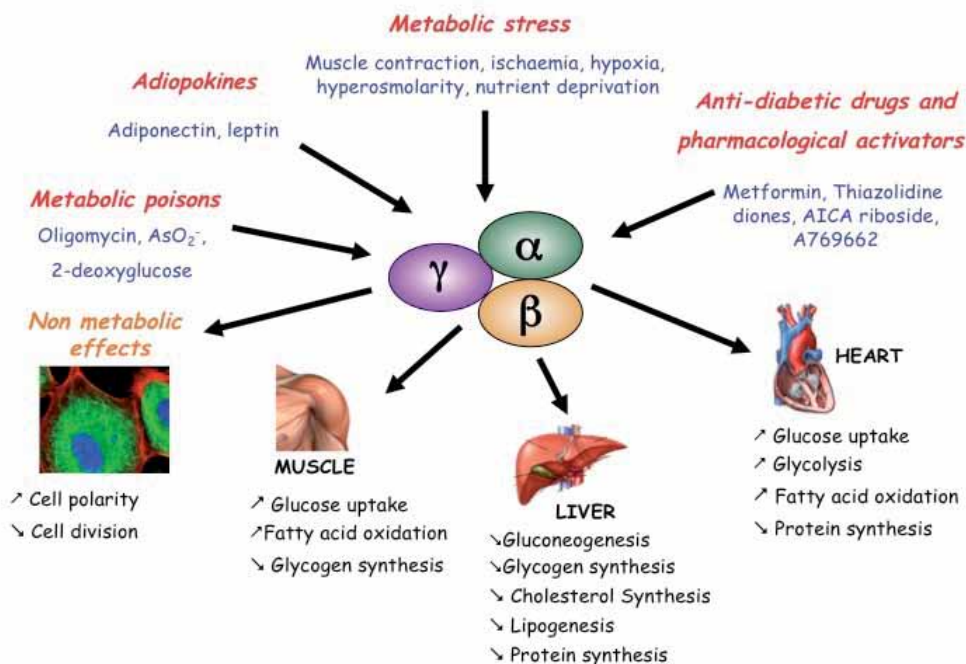

Fig. 1 Conditions leading to AMPK activation in higher eukaryotes and some of its consequences.

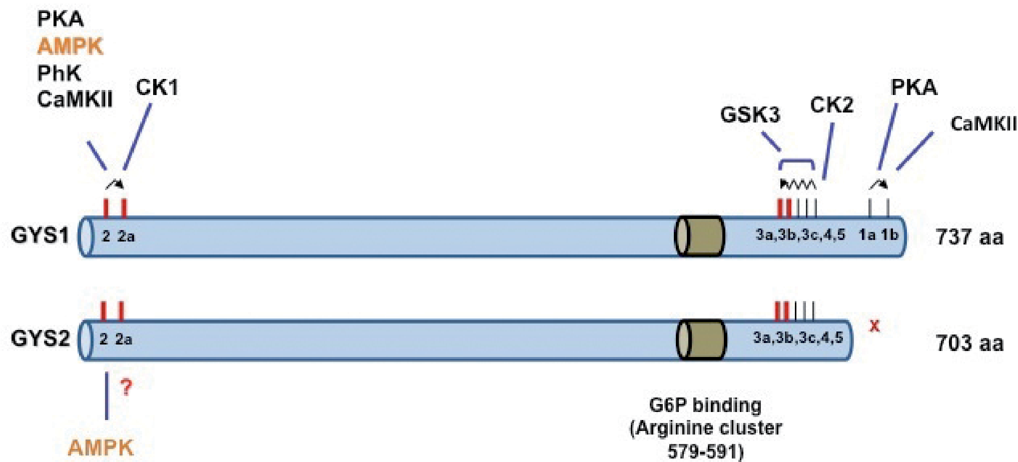

Fig. 2 Schematic representation of the two GYS isoenzymes showing the AMPK and other phosphorylation sites. The sites responsible for GYS inactivation are conserved and highlighted in red.

a time-dependent manner and to a similar stoichiometry. The phosphorylation site in GYS2 was identified as Ser7, which lies in a favorable consensus for phosphorylation by AMPK. Phosphorylation of GYS1 or GYS2 by AMPK led to enzyme inactivation by decreasing the affinity for both UDP-Glc (assayed in the absence of Glc-6-P) and Glc-6-P (assayed at low UDP-Glc concentrations). Incubation of freshly isolated rat hepatocytes with pharmacological AMPK activators 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICA riboside) or A769662 led to persistent GYS inactivation and Ser7 phosphorylation, whereas inactivation by glucagon treatment was transient. In hepatocytes from mice harboring a liver-specific deletion of the AMPK catalytic α1/α2 subunits, GYS2 inactivation by AICA riboside and A769662 was blunted whereas inactivation by glucagon was unaffected. The results suggest that GYS inactivation by AMPK activators in hepatocytes is due to GYS2 Ser7 phosphorylation (7).

AMPK as a drug target for type 2 diabetes

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

Mass spectrometry

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

The development of mass spectrometry (MS) facilities within our laboratory, and for our Institute and University, has been an enormous asset (lien vers site "Massprot": http://www.uclouvain.be/en-proteomics.html). Since the acquisition of an electrospray mass spectrometer in 1997, the application of MS techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to over 40 joint publications. In our own research, the use of MS enabled us to identify new AMPK targets. We also discovered new phosphorylation sites in the AMPK complex itself and demonstrated that in heart, insulin antagonized AMPK activation during ischaemia via PKB-induced phosphorylation of the AMPK catalytic α-subunits at Ser495/491 (ref 3).
We collaborated with the group of J.-F. Collet by using prot-

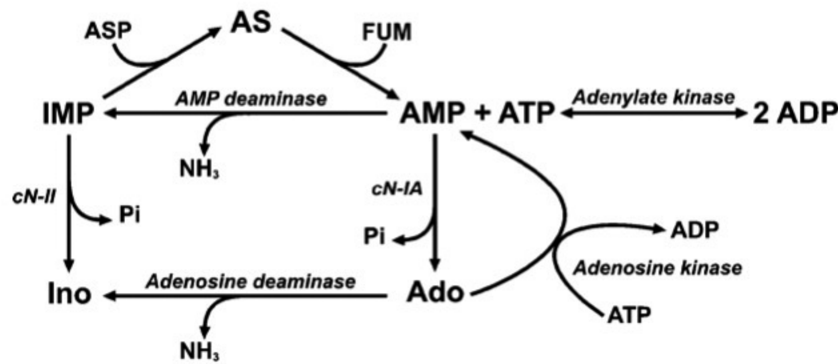

Fig. 3 Scheme showing AMP metabolizing pathways. The enzymes implicated are indicated in italics. ASP: aspartate; AS: adenylosuccinate; FUM: fumarate; Ado: adenosine; Ino: inosine.

eomics to study the biogenesis of bacterial outer membranes (9). We are now involved in developing new proteomics strategies to study sulfenic acid formation in vivo. A new concept is that sulfenylation of specific cysteine residues modulates signal transduction pathways by altering the activity and function of cellular proteins, just as phosphorylation and dephosphorylation cycles regulate enzyme activities and cellular pathways. We are using MS to clearly establish the role of reversible sulfenic acid formation in living cells and to identify new proteins and pathways regulated by sulfenylation.

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

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

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Metabolism of trypanosomatid parasites and drug discovery

Paul Michels

Trypanosomatidae are parasitic protists that cause sleeping sickness in Africa, Chagas' disease in Latin America and various manifestations of leishmaniasis in man, in many tropical and subtropical parts of the world. These diseases threaten a few hundreds of millions of people worldwide, affect millions and cause many thousands of deaths each year. The parasites responsible for these diseases are transmitted between humans by insects: tsetse flies, triatomine bugs and sand flies, respectively. There is an urgent need for new, adequate, affordable drugs to treat these diseases, because those currently in use are often toxic and not efficacious, sometimes difficult to administer or too expensive. Moreover, increasing resistance has been reported against some of these currently used drugs. By a molecular and cell biological investigation of these parasites we intend to provide a basis for the development of such new drugs. African trypanosomes rely entirely on glycolysis for their ATP supply. Interestingly, trypanosomatids are characterized by a unique form of glycolytic compartmentalization by which the majority of the enzymes of this pathway are sequestered inside peroxisome-like organelles called glycosomes. In the past we have validated many of the glycolytic enzymes as potential drug targets. Their structural information is used for the discovery of potent and selective inhibitors. Glycosome assembly and degradation, taking place during cell differentiation, are being studied as well. Many so-called peroxins, proteins involved in glycosome biogenesis, have been identified and also validated as excellent drug targets. Moreover, the availability of the genome sequences of different species of the trypanosomatid parasites causing the three groups of diseases, in combination with various proteomic approaches, has allowed to make a comprehensive inventory of the metabolic capacities of these organisms and to identify other essential differences between the respective parasites and their human host. Other potential drug targets that have been identified are the pentose-phosphate pathway, lipid metabolism and the biosynthesis of biopterin and reduced folate.

Metabolic pathways, enzymes and drug discovery

Glycolytic enzymes – characterization and drug discovery

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Glycolytic enzymes are very important for all trypanosomatids. For *Trypanosoma brucei*, the parasite that causes sleeping sickness when infecting people in sub-Saharan Africa, glycolysis is even the sole free-energy source when the parasite resides in the human blood. Therefore the glycolytic enzymes of these parasites are considered as promising drug targets. Over the years, we have expressed and kinetically characterized trypanosomatid enzymes for all 10 successive steps of the pathway for breakdown of glucose into pyruvate and two additional enzymes present in the glycosome, glycerol-

3-phosphate dehydrogenase and glycerol kinase, which also play a crucial role in this process. Structures of most of these enzymes have become available through our collaboration with protein crystallographers elsewhere. All these analyses have revealed important differences between the enzymes of the parasites and the corresponding ones from the human host, offering prospects for developing parasite-enzyme specific potent inhibitors that may be used as lead drugs.

Our collaborative work with the University of Edinburgh has focused on a few selected glycolytic enzymes: phosphofructokinase (PFK), phosphoglycerate mutase (PGAM) and pyruvate kinase (PYK) from different trypanosomatid parasites. The structures of the latter four enzymes have been determined both without and with various ligands. This has provided insight into mechanisms of catalysis and in the conformational changes required for catalysis by these enzymes. Inhibitors for these enzymes are searched by using them in high-throughput screens of available large libraries of drug-like compounds – mainly at the NIH Chemical Genomics Center, Rockville, USA – and designed by structure-based approaches followed by their synthesis. To date, compounds with potent inhibitory activity, even down to nanomolar concentrations, on trypanosomatid PFK and PYK but not on the corresponding mammalian enzymes have already been obtained. Some of them inhibit growth of *in vitro* cultured bloodstream-form trypanosomes at concentrations in the low micromolar range with no or less effect on cultured human fibroblasts. Detailed analysis of the structure-activity relationship of these compounds is now being used for improving the inhibitors by following medicinal chemistry principles to arrive at leads for anti-parasite drugs.

Research on PGAM was performed in collaboration with colleagues in Brazil and Edinburgh. The trypanosomatid PGAM is a 2,3-bisphosphoglycerate independent metalloenzyme, non-homologous with the cofactor dependent, metal independent mammalian enzyme, thus offering great perspectives for drug discovery. Previously, the structure of PGAM with its substrate bound in the active site was determined for *Leishmania mexicana*, the trypanosomatid responsible for leishmaniasis. Now also the structure of the *T. brucei* enzyme without bound ligand has been obtained. Comparison of these structures showed a major domain movement resulting from active site occupation. Mutagenesis studies showed the critical involvement of a specific residue in metal binding and the role of the metal in the conformation of the enzyme. The physiologically relevant metal of these PGAMs remains to be established. It was shown that the *T. brucei* enzyme requires Co^{2+} to reach maximal activity *in vitro*. However, cobalt ions are present in the cell below detection limit and therefore unable to support maximal activity *in vivo*. It is likely that one of abundant metal ion species, Mg^{2+} or Zn^{2+}, assumes the role of metal cofactor in the PGAMs in trypanosomatids.

In the collaboration with Venezuelan colleagues the glycolytic enzyme enolase (ENO) was also detected at the surface of *Trypanosoma cruzi*, the parasite that causes Chagas' disease in Latin America and *Leishmania* species. In these parasites, which live mainly intracellularly in the human host, this isoform of ENO seems to function as plasminogen receptor, playing a role in the parasites' invasiveness and virulence. This additional location and probable function of ENO offer perspectives to use the enzyme not only as a drug target, but also for vaccination.

A small focused library of naphtoquinone-carrying compounds that showed anti-parasite effects in *in vitro* growth assays with different parasites was synthesized by colleagues at the University of Bologna. Several compounds exhibited potency in the nanomolar range, with up to 80-fold less activity on human cells. By means of a chemical proteomics approach several *T. brucei* molecular targets of the most promising compound, 2-phenoxy-1,4-naphtoquinone, were identified. Amongst these targets were the glycosomal enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol kinase. Subsequently, these two targets were expressed in bacteria and purified, and then used for inhibition assays. The selected compound was able to inhibit both enzymes with IC_{50} values in the low micromolar range. Supporting the notion that GAPDH is indeed an *in situ* target was the observation that glucose-grown procyclic trypanosomes, for which the GAPDH activity is crucial, were 28-fold more susceptible to the inhibitor than cells relying much less on GAPDH when they use proline as energy and carbon source. Moreover, it was observed that the compound was able to generate oxygen radicals in mitochondrial fractions of bloodstream-form trypanosomes, but much less so in human mitochondrial preparations, probably related to the highly different respiratory systems of both organisms. This mechanism of inducing the production of oxygen radicals could be additionally responsible for the compound's trypanocidal activity. Indeed, we could show that growth of trypanosomes in which glucose-6-phosphate dehydrogenase was depleted by RNA interference (RNAi), causing them to be susceptible to oxidative stress, was inhibited at about 100-fold lower concentration of the compound than growth of wild-type trypanosomes. Overall, the molecule showed a multitarget mechanism of action, likely forming the basis of its promising anti-trypanosomatid profile.

T. brucei contains two very similar isoenzymes of hexokinase (HXK), essentially only differing in a 15 residue stretch near the C-terminus. Both isoenzymes have a type 2 peroxisomal-targeting signal (PTS2) near their N-terminus for routing the proteins, after their synthesis in the cytosol, to the glycosomes. Collaborating with colleagues in Clemson (USA), we surprisingly found that one of the isoenzymes (HXK2) has also an extraglycosomal localization; in bloodstream-form parasites the protein localizes to both glycosomes and the flagellum, whereas in procyclic forms it is localized to glycosomes and proximal to the basal bodies. HXK1 is always fully compartmentalized inside glycosomes. The function of this extraglycosomal HXK remains to be determined. The location suggests that it may have a role in locomotion.

Folate metabolism

V. Hannaert, P. Michels, in collaboration with D. Guerrieri, S. Ferrari, M.-P. Costi (Università di Modena e Reggio Emilia, Italy), S. Henrich, R. Wade (Heidelberg Institute for Theoretical Studies, Germany)

Enzymes involved in the provision of reduced folate cofactors, such as dihydrofolate reductase (DHFR) and enzymes that use these cofactors, like thymidylate synthase (TS) have been validated as drug targets for cancer and certain bacterial infections and parasitic diseases such as malaria. When DHFR is inhibited, DNA replication is impaired, resulting in cell death. Trypanosomes are auxotrophic for folates and pterins, and inhibition of the enzymes involved in the salvage pathways like the bifunctional DHFR-TS used by trypanosomatids should provide effective treatment. However, antifolates are currently not employed in therapy of trypanosomatid infections, mainly because of the presence of a pteridine reductase (PTR1) – absent from human cells – that is able to carry out successive reductions of both conjugated (folate) and unconjugated (biopterin) pterins and therefore might provide a bypass for DHFR. While DHFR can only reduce folic acid, PTR1 can act on a broader range of substrates. Under physiological conditions, PTR1 is responsible for 10% of the folic acid required by the cell, but inhibition of DHFR often leads to overexpression of PTR1. It was anticipated that folate analogue inhibitors of *Leishmania* PTR1 are potential drugs for combined therapy with DHFR inhibitors. Based on a virtual screening of the Available Chemicals Directory (ACD) database and the known crystal structure of *L. major* PTR1, followed by two rounds of structure-based design to optimize the compounds, 18 drug-like molecules were identified that displayed low micromolar affinities for *L. major* PTR1 and high *in vitro* specificity when compared to *Leishmania* and human DHFR. The compounds were tested as growth inhibitors of cultured *L. mexicana* and *L. major* promastigotes and human fibroblasts, without and in combination with the DHFR inhibitor pyrimethamine (PYR). Six compounds showed efficacy in combination with PYR, one was active alone and several compounds showed low toxicity for human cells. Interestingly, one compound, Riluzole, a known drug approved for pathologies of the central nervous system, was active in combination and is suitable for early preclinical evaluation of its potential as an antiparasitic drug. Riluzole is notably effective on proliferating parasites. Moreover, the compound was shown to also increase the parasite’s susceptibility to oxidative stress.

Thiolase

P. Michels, in collaboration with R. Wierenga (University of Oulu, Finland), M. Mazet, F. Bringaud (Université Bordeaux Segalen, France)

Thiolases are enzymes that remove an acetyl-CoA group from acyl-CoA in the catabolic β-oxidation of fatty acids, or catalyze the reverse condensation reaction for anabolic processes such as the biosynthesis of sterols and ketone bodies. In humans, six homologous isoforms of thiolase have been described, differing from each other in sequence, oligomeric state, substrate specificity and subcellular localization. Using the sequences of these six isoforms as queries in searches of databases of African trypanosomes, *T. cruzi* and *Leishmania* species, one of these isoforms (called SCP2-thiolase) was found in each of them, another one (TFE -thiolase) in *T. cruzi* and *Leishmania* and a third one (AB-thiolase) only in *T. cruzi*. The single *T. brucei* SCP2-thiolase has been expressed as recombinant enzyme in *Escherichia coli*, purified and its activity determined. Interestingly, it shows activity in both the degradative and synthetic reaction, with the latter one being 3-fold higher, whereas the synthetic activity of SCP2 from other organisms studied is negligible. mRNA of the thiolase was detected in both bloodstream form and procyclic trypanosomes, but translation of the transcript into protein only occurred in procyclics as revealed by western blot analysis. The encoded protein has both a predicted N-terminal signal peptide for routing to the mitochondrion and a C-terminal candidate type 1 peroxisomal-targeting signal (PTS1) for sorting it to glycosomes. However, both fluorescence analysis of the thiolase with the Green Fluorescent Protein (GFP) fused to either its N- or C-terminus and cell fractionation by differential centrifugation followed by western blot analysis showed only a mitochondrial localization for procyclic cells, irrespective whether the cells were grown with glucose or amino acids as carbon and energy source. When the expression of the thiolase in procyclic cells was knocked down by RNAi, no significant change in growth rate occurred, weather the cells were grown with or without glucose. This absence of a growth phenotype indicates that the metabolic pathway involving this enzyme is not essential for the parasite under either of these growth conditions. Further research is currently being performed to determine the role of this enzyme in the metabolism of the trypanosomes.

Translocation of solutes across the glycosomal membrane

M. Gualdrón-López, P. Michels, in collaboration with V. Antonenkov (University of Oulu, Finland)

The glycosomal membrane of *T. brucei* possesses three half-size ABC transporters, designated GAT1-3. GAT1 and GAT3 are expressed in both bloodstream and procyclic-form trypanosomes, whereas GAT2 was only found in bloodstream-form cells. Expression knockdown of GAT1 and GAT3 by RNAi resulted in a growth phenotype that is dependent on the nutritional conditions of the trypanosomes. In the presence of glucose, growth is not affected. When, however, for the GAT1 RNAi cell line, no glucose is available and proline forms the predominant source of free energy, the growth rate is reduced and eventually the trypanosomes die. Glucose-grown cells depleted of GAT1 show a modification of the total cellular fatty-acid composition; no or only minor changes were observed in the levels of most fatty acids, including oleate (C18:1), but the linoleate (C18:2) abundance was significantly increased. We hypothesized that GAT1 is a fatty-acid transporter, like some of its homologues in the peroxisomal membrane of yeasts and mammalian cells. Fatty-acid uptake

into glycosomes may be important either for the synthesis of ether-lipids, a process that is crucial for cells, and/or for β-oxidation. Cell fractionation in conjunction with enzyme activity assays, indeed confirmed the association of enzymes of both processes with glycosomes. Glycosomes purified from procyclic wild-type trypanosomes incorporate the activated fatty acid oleoyl-CoA in a temperature-, concentration- and ATP-dependent manner, whilst this incorporation was severely reduced in glycosomes from cells in which GAT1 levels had been decreased. This result strongly suggested that GAT1 is an oleate transporter. The increase in linoleate rather than oleate levels upon GAT1 depletion is probably due to the conversion of the latter into the former fatty acid by the high activity of oleate desaturase found in trypanosomes.

In addition, the possible presence of channel-forming activities in glycosomal membrane preparations of *T. brucei* bloodstream forms was investigated. When glycosomal membrane proteins were reconstituted in planar lipid bilayers, the formation of three main kinds of channels with different conductances were revealed by electrophysiological techniques. The channels were in a fully open state over the membrane potential range +150 to -150 mV and showed no sub-conductance transitions. One of the channels has a three-fold selectivity of anions over cations while the other two types are slightly selective for cations. These findings strongly indicate that small solutes (< 500 Da) may cross the glycosomal membrane by permeation through the channel, whereas larger solutes (cofactors, acyl-CoAs) may require carrier molecules such as ABC transporters or other still to be identified transporters (*e.g.* molecules of the class of MCF transporters), similar to what has been found in peroxisomes of yeasts, plants and mammalian cells.

The apparent paradox of on one hand the presence of non-selective channels in the glycosomal membrane and on the other hand the previously experimentally proved low exchange of metabolites between the glycosomal matrix and cytosol may be explained in different ways: (i) existence of an intraglycosomal multienzyme complex and metabolite channelling; (ii) accumulation of negative metabolites in the organelles as a result of a Donnan potential; (iii) regulation of the opening of the channels by association with other proteins. Experimental data supporting each of these explanations, which are not mutually exclusive, is available. Importantly, the discovery of these non-selective channels in the glycosomal membrane of *T. brucei* may set new criteria for the size and physicochemical properties of inhibitors targeted to glycolytic and other enzymes inside glycosomes that will be designed as potential drugs for sleeping sickness.

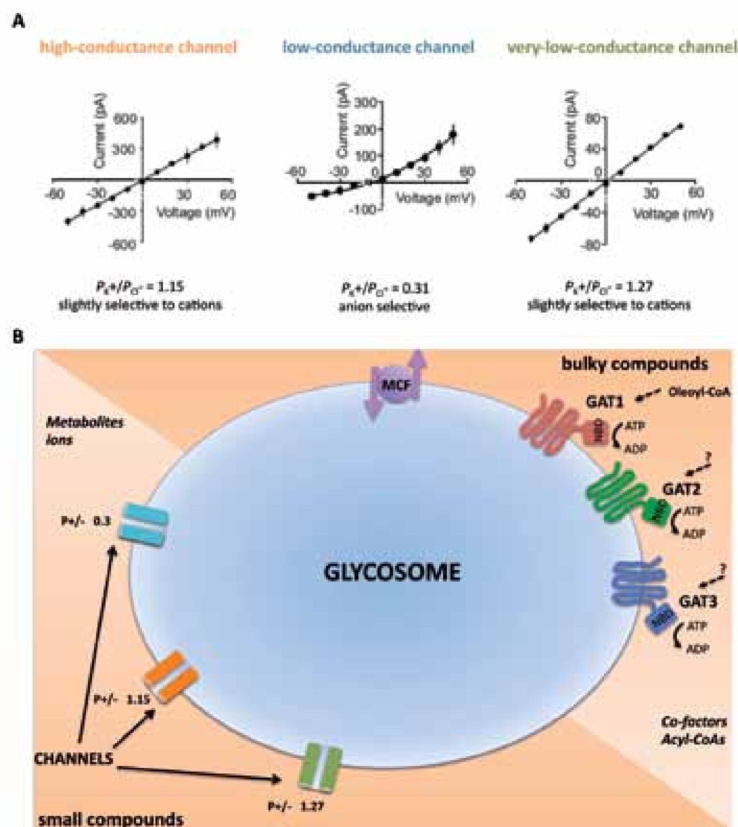

Fig. 1. Translocation of solutes through the glycosomal membrane. A. Channel-forming activities in glycosomal membrane protein preparations. Channels were reconstituted in artificial lipid bilayers by insertion of solubilized membrane proteins from ultrapure glycosomes. Electrophysiological measurements allowed the identification of three main channel-forming activities with differences in conductance and ion selectivity. B. Current model for the translocation of solutes through the glycosomal membrane. Mitochondrial carrier family (MCF) proteins and glycosomal ABC transporters (GAT) are presented as being involved in the translocation of bulky compounds as co-factors and acyl-CoAs. Channels involved in the translocation of small solutes such as metabolites and inorganic ions are also presented.

Phytomonas metabolism

F. Opperdoes, in collaboration with M. Dollet (Université de Montpellier, France)

Phytomonads are trypanosomatids parasitizing on plants and are transmitted by insects. They are responsible for important economic losses in tropical and subtropical regions. The sequencing of the genomes of two phytomonads, one isolated from a palm tree and the other from an *Euphorbia* plant in France, has been completed in collaboration with Genoscope (France). Their annotation is now in progress. *Phytomonas* has a highly streamlined genome as compared to the other trypanosomatids already sequenced. With its 6400 gene models the size of its predicted proteome is only 70% of that of *T. brucei*. This streamlining probably reflects the fact that there is little or no need for adaptation to strikingly different host temperatures (25 and 37°C) and to strikingly different environments as is the case for the mammalian trypanosomatids that shuttle between insects and warm-blooded animals. Typical examples of this streamlining in *Phytomonas* are (1) the virtual absence of tandemly-linked gene duplications in its genome, (2) the complete loss of mitochondrial cytochromes and (3) the loss of the possibility to oxidize fatty acids and long-chain and branched-chain amino acids by β-oxidation. Carbohydrate metabolism is characterized by an incomplete aerobic oxidation of glucose, which partly takes place inside glycosomes. One of the mitochondrial TCA cycle enzymes is absent and therefore this cycle is not operational but its enzymes can be used for the interconversion of building blocks for the cell. Although *Phytomonas* is able to synthesize its own pyrimidines it depends on the presence of external purines. It lacks the capacity to oxidize aromatic amino acids and requires an external supply of most of the essential amino acids and of cofactors and/or vitamins for its growth.

Glycosome turnover

Glycosome biogenesis in Trypanosoma brucei

M. Gualdrón-López, A. Brennand, P. Michels in collaboration with D. Rigden (University of Liverpool, UK)

So far, 12 candidate peroxins (acronym PEX), proteins involved in glycosome biogenesis in *T. brucei* have been identified; for the majority of them, their role in glycosome biogenesis has been experimentally confirmed and, using RNAi, their essentiality for the viability of both cultured bloodstream- and procyclic-form trypanosomes has been demonstrated. Most of these peroxins are involved in the transport of newly synthesized glycosomal proteins from the cytosol to the organellar matrix.

The *T. brucei* PEX proteins display only a low to very low degree of sequence identity with their homologues from yeasts, plants and mammals: typically between <15 and 35%. Guided by the knowledge of yeast peroxisome biogenesis we have unravelled the overall process of glycosomal matrix-protein import as well as mechanistic details of some steps. PEX5 and PEX7 are cytosolic receptors for proteins to be transported to the glycosomal matrix with a C-terminal or N-terminal peroxisomal-targeting signal (PTS), type 1 (PTS1) and type 2 (PTS2), respectively. PTS-bearing proteins associate with these receptors in the cytosol followed by interaction of the loaded receptor with a membrane-bound docking complex comprising PEX13 and PEX14. PEX5 associates with the membrane, and – as has been shown by others for yeast peroxisomes, not yet for glycosomes – forms a transient pore through which the PTS proteins are delivered. The mechanism of PEX7-dependent PTS2-protein delivery remains to be determined. The PEX5 is subsequently removed from the organelle's membrane. The trigger for its retrieval is its ubiquitination by the activity of an ubiquitin-conjugating enzyme (PEX4 that is anchored at the cytosolic face of the glycosomal membrane by PEX22) and ubiquitin-ligating enzymes (PEX2, 10 and 12, a complex of RING-finger, integral membrane proteins). The ubiquitinated PEX5 is then retrieved by a complex of two cytosolic AAA$^+$-ATPases, PEX1 and 6, recruited to the membrane through a still unknown protein. Monoubiquitinated PEX5 (or PEX5 with only a few ubiquitin moieties) is deubiquitinated in the cytosol and so becomes available for further rounds of import, while polyubiquitination serves as a signal to route damaged or redundant PEX5 for degradation to proteasomes. The different possible fates of PEX5 appear to involve the activity of distinct RING-finger PEX proteins.

Although the overall process of matrix-protein import in trypanosomes is similar to that of peroxisomes in yeasts and mammalian cells, considerable differences have been found in many details, such as regions of peroxins that interact with each other, residues responsible for critical interactions or activities, etc. These differences, together with the very low overall sequence conservation and the fact that RNAi-dependent knockdown of the expression of all peroxins attempted (PEX2, 5, 6, 7, 10, 12, 13, 14) except PEX4 leads to death of the parasites, validate these proteins as promising drug targets. In the case of PEX4, we observed that creation of a null-mutant did not lead to cell death, but only to growth retardation and important changes in morphology indicative of a cytokinesis defect. Our analysis indicated that deletion of PEX4 was rescued by up-regulation of another ubiquitin-conjugating enzyme that probably caused the aberrations observed.

Previously, *T. brucei* PEX13 has been characterized; a glycosomal membrane protein with a Tyr-Gly rich N-terminal region of low sequence complexity, two transmembrane segments, a SH3 domain in its C-terminal half and, uniquely for the trypanosomatid PEX13, terminating with a PTS1-like sequence. Recently, another, very different candidate PEX13 has been identified in the trypanosomatid databases. Although this new candidate lacks the SH3 domain of PEX13s, it has a higher overall sequence identity with PEX13s of other organisms and possesses the typical PEX13 N-terminal half with Tyr-Gly motifs and transmembrane segments. It does not contain a PTS1. We showed that this PEX13.2, like the PEX13.1 previ-

ously described, is associated with glycosomes and that its depletion by RNAi affects the biogenesis of the organelles and viability of trypanosomes. Trypanosomatids are the first organisms in which two distinct PEX13 isoforms have been detected. The reason for this is unclear, but it is interesting to note that these two very different *T. brucei* isoforms of PEX13 appear to interact with each other, as shown in yeast two-hybrid assays and are both important for the correct association of PEX14 with the glycosomal membrane. Moreover, they are not redundant; decreasing the cellular level of one of them, either PEX13.1 or PEX13.2, appears to affect glycosome biogenesis, as shown by a relocation of glycosomal matrix enzymes to the cytosol, and is detrimental for growth of the trypanosomes.

Glycosome degradation in Trypanosoma brucei

A. Brennand, P. Michels

Trypanosomes encounter highly different environments during the successive stages of their life cycle and have to adapt their metabolism accordingly. Previously we have provided strong indications that, when the trypanosomes develop from the bloodstream into the procyclic form, the adaptation involves a drastic degradation of glycosomes by a selective form of autophagy called pexophagy and the synthesis of new glycosomes with a different repertoire of metabolic enzymes. Furthermore, we performed a bioinformatics analysis that allowed us to identify in the trypanosomatid databases orthologues of about 20 of the 40 known yeast proteins known to be involved in autophagy (ATGs = AuTophaGy-related proteins). Also orthologues specifically required for pexophagy were recognized in trypanosomatids. Recently, this bioinformatics analysis was extended to a taxonomically diverse range of other protists. This analysis, together with similar analyses performed by others for plants, animals and fungi confirmed autophagy as an ancient eukaryotic invention, utilizing a conserved core machinery but also with lineage-specific moderation (specific losses of ATGs) and elaboration (expansion of a paralogous repertoire of some ATGs). This was indeed also observed in trypanosomatids. Some protists seem to have undergone a secondary loss of macroautophagy, the best understood of the autophagy pathways. This is possibly due to adaptation to a very constant niche, two of the three examples found being parasites with a very simple life cycle and one free-living organism adapted to an extreme environment. Furthermore, although pexophagy is a conserved process in all organisms having peroxisomes, it seems to involve at least some proteins that are not widely conserved. With regard to the process in parasitic protists, this may offer opportunities for drug design targeting autophagy proteins in these organisms.

Experimental support for degradation of glycosomes in trypanosomes by autophagy, both under conditions of nutrient deprivation and during differentiation – from long-slender bloodstream forms to short stumpy forms and from short stumpy to procyclic forms – has previously been obtained by immuno-electron and -fluorescence microscopy.

Several of the *T. brucei* homologues of yeast proteins involved in autophagy, notably that of peroxisomes, have been cloned, sequenced and expressed as recombinant proteins for functional studies: VPS34, ATG7, ATG8, ATG24 and VAC8. Most of our recent research was focused on TbATG24 and TbATG8. We identified genes for three different ATG8 forms in *T. brucei*: ATG8.1, ATG8.2 and ATG8.3. ATG8 is generally considered as the marker for autophagosomes. It is recruited to the membrane of these organelles which are formed in order to sequester cytoplasmic constituents (cytosol and organelles) to be degraded by autophagy. Subsequently, the autophagosomes deliver their contents to lysosomes (or vacuoles in yeasts).

We identified and functionally characterized the *T. brucei* orthologue of yeast ATG24 that participates in specific autophagy. This protein is part of a putative complex that is involved in autophagy induction and the switch between general autophagy and various specific autophagy processes, including pexophagy. TbATG24 seems to be required for maintenance of the lysosomal morphology in bloodstream and procyclic cells. However, we observed that it also participates in endocytosis, as indicated by its RNAi-dependent partial depletion on transferrin import in a bloodstream-form cell line. This latter function is evolutionary conserved, as it is also found for its orthologues in mammalian cells and yeasts. Moreover the interaction of TbATG24 with endosomal membranes has been shown to be dependent on TbVPS34 and to be sensitive to wortmannin treatment, in agreement with observations reported for its yeast counterpart. Interestingly, its down-regulation leads to an increase in autophagosome numbers in autophagy-inducing conditions and an increase of the rate of *in vitro* differentiation of monomorphic *T. brucei* cells.

Using GFP-tagged constructs, we observed that two differ-

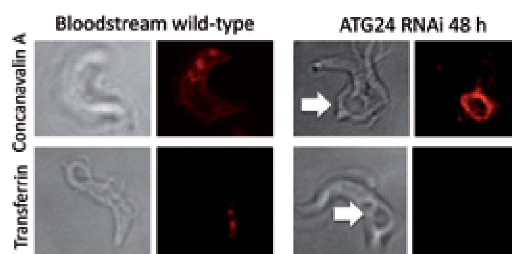

Fig. 2. The protein ATG24 of trypanosomes is involved in receptor-mediated endocytosis. Fluorescence of wild-type bloodstream form trypanosomes and mutant cells, 48 hour after induction of a decreased production of ATG24 by RNA interference. The trypanosomes were incubated with AlexaFluor (red fluorescent)-conjugated concanavalin A, that is imported via bulk endocytosis or incubated with AlexaFluor-conjugated transferrin, imported via receptor-mediated endocytosis. Note the enlarged lysosome, indicated by the arrow, and the defect in the import of transferrin, but not concanavalin A in the ATG24 depleted cells, as indicated by the absence of a fluorescent signal in the former cells.

ent isoforms of ATG8, TbATG8.1 and TbATG8.2, both locate to puncta under different autophagy-inducing conditions and therefore can both be considered as autophagosomal markers. Moreover, both isoforms of the protein colocalise with glycosomal markers during induction of *in vitro* differentiation, indicating that both are involved in pexophagy for glycosome turnover. Intriguingly, we observed a faster rate of differentiation of cells expressing the GFP-tagged ATG8.2 than cells with GFP-ATG8.1.

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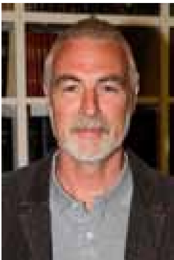

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Membrane organization/dynamics and epithelial differentiation

Pierre Courtoy
Christophe Pierreux

Endocytosis, a central activity of all eukaryotes, allows for cell nutrition and regulates their surface. For decades, Courtoy's team has been defining endocytic pathways and their contribution to numerous physiopathological processes. These investigations now focus on apical endocytosis in polarized epithelia such as kidney proximal tubular cells (PTC). We surmise that this most active endocytic surface will help defining rate-limiting components of the different subcellular steps. These should also emerge from the dissection of various kidney genetic defects causing low-molecular weight proteinuria, mimicked by transgenic mice. Our work led to : (i) deciphering the signalling cascade whereby the paradigmatic oncogene, v-Src, and the master catalyst, Rab5a, impact on the apical actin cytocortex, so as to induce macropinocytosis, like enteropathogens (2); (ii) unraveling an apical endocytotic recycling defect in ClCn5 KO mice, a model of Dent's disease (familial predisposition to kidney stones) (1); (iii) the serendipitous discovery that recapture of ultrafiltrated lysosomal proteases is a central mechanism for PTC lysosomes biogenesis (3); and (iv) the demonstration that the transcription factor, ZONAB, is a key component for the switch between PTC proliferation and apical differentiation (6). By high-resolution vital imaging of the plasma membrane after labelling by fluorescent lipid analogs, Tyteca and colleagues recently discovered the spontaneous formation of three types of micrometric lipid domains differing by composition, cohesion and preference for cytoskeleton anchorage. These novel observations favor a revised model of the scale (micrometric) and stability (minutes) of lateral lipid organization at the plasma membrane in living cells (7, 9). The group of Pierreux studies epithelial tubulogenesis and differentiation, using developing pancreas, salivary 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 (5, 8). Besides integration of cell and developmental biology, two strong assets of our group are decades of expertise in structural biology and intertwining with a versatile Platform for Imaging Cells and Tissues (4).

Plasma membrane lipids form distinct micrometric domains: discovery

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

Our project addresses a fundamental property of plasma membrane (PM) polar lipids - self-assembly into stable micro-domains - and its significance for cell physiology and disease. Focus is placed on red blood cells (RBCs), which offer the best characterized membrane model, a featureless surface, and no lipid turnover nor vesicular trafficking. RBCs exhibit remarkable deformability and stability, allowing them to squeeze multiple times into the narrow pores of spleen sinusoids.
Long viewed as homogenous solvent for membrane proteins, the lipid bilayer shows heterogeneity at two different scales: transient nanometric "lipid rafts" versus stable micrometric/

mesoscale assemblies. These are well-characterized on artificial vesicles but relevance for living cells remains controversial. We first examined whether micrometric lipid domains occur at the PM, using a fluorescent analog (BODIPY: *) of sphingomyelin (SM*). Both in RBCs and CHO cells, SM* insertion labelled (sub)micrometric fluorescent PM domains (Fig 1a). In CHO cells, direct PM insertion of SM* and intracellular enzymatic conversion of ceramide* into SM* produced similar PM micrometric domains. Depletion of endogenous SM by sphingomyelinase or biosynthetic inhibition erased (sub)micrometric SM* domains, suggesting these reflect endogenous SM compartmentation (ref. 7).
Using fluorescent analogs of other lipids at the outer PM leaflet, we next show the co-existence of three segregated micrometric phases. Indeed, SM* differed from glycosphingolipids* and phosphatidylcholine (PC*) domains in temperature dependence, association with a glycosylphosphatidylinositol (GPI)-anchored fluorescent protein reporter, and lateral diffusion by FRAP, thus demonstrating different lipid phases and boundaries. This was further supported by double labelling experiments. Specific alterations of fluorescent micrometric domains by manipulation of corresponding endogenous sphingolipids suggest that distinct fluorescent lipid partition might reflect differential intrinsic propensity of endogenous membrane lipids to form large assemblies (ref. 9).

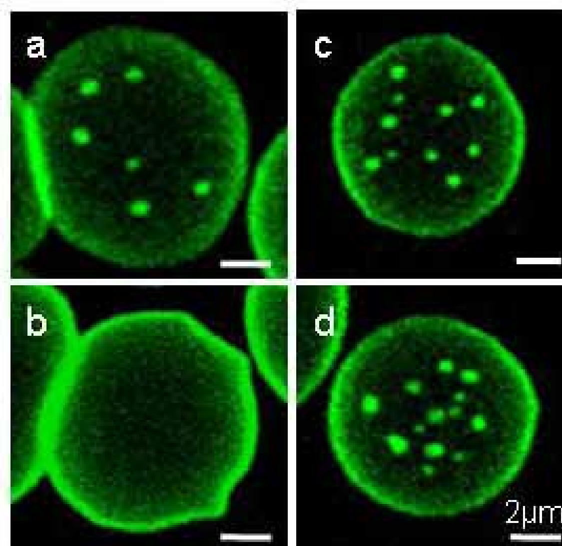

Fig. 1. Modulation of fluorescent sphingomyelin micrometric domains on erythrocytes by pharmacological (b,c) or genetic (d) perturbation of membrane architecture. (a) control erythrocyte at 37°C; (b) + methyl-beta-cyclodextrin (- 25% cholesterol); (c) + calyculin A + PMA (hyperphosphorylation of 4.1R complex); (d) patient with spherocytosis and splenectomized.

Biogenesis and implications of plasma membrane micrometric domains

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

We further dissected the mechanism(s) of the formation and maintenance of micrometric lipid domains in living RBCs. Experimental modulation of RBC stretching and cholesterol content (by methyl-beta-cyclodextrin; Fig. 1b) and suppression of membrane-cytoskeleton anchorage via 4.1R complexes (hyperphosphorylation by PKC activation; Fig. 1c) and ankyrin complexes (splenectomized patients with spherocytosis; Fig. 1d) differentially affected SM*, glycosphingolipids* and PC* micrometric domains. Thus, membrane tension is a key parameter for micrometric lipid domain maintenance and formation. These novel observations prompt us to propose a revised model of the scale (micrometric) and stability (minutes) of lateral lipid organization at the PM in living cells. Our current projects aim at (i) directly looking at endogenous lipid enrichment in corresponding fluorescent micrometric lipid domains and (ii) addressing possible physiological roles for domains (e.g. RBC stability; endocytosis, migration, fission and fusion of muscle cells). Measuring parameters governing fluorescent lipid domain biogenesis/maintenance and evaluating implications for two genetic membrane fragility diseases (spherocytosis and Duchenne myopathy) are among our future challenges.

Contribution of apical endocytosis to biogenesis and storage (cystinosis) in lysosomes of kidney proximal tubular cells

P.J. Courtoy, W.R. Lima, V. Janssens and H.P. Gaide Chevronnay, in collaboration with O. Devuyst (NEFR, UCL), E.I. Christensen (Aarhus, DK), C. Antignac (Paris) and S. Cherqui (Scripps)

Recruitment of acid hydrolases to lysosomes generally occurs by intracellular sorting based on recognition of a common mannose 6-phosphate signal in the trans-Golgi network and selective transport to late endosomes/lysosomes. We have obtained direct evidence for an alternative, efficient secretion-recapture pathway mediated by megalin, exemplified by cathepsin B in kidney proximal tubular cells (PTC). We found that in mouse kidneys with defective megalin expression [megalin knockout (KO)] or apical PTC trafficking (ClC-5 KO, see below), the (pro)cathepsin B mRNA level was essentially preserved, but the protein content was greatly decreased and the enzyme was excreted in the urine as mannose 6-phosphate-devoid species.
In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane; uptake was abolished by the "receptor-associated protein", a megalin competitor. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Circulating

procathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (ref. 3). Current investigations are addressing the pathophysiology of cystinosis, a multisytemic lysosomal disease due to a defective lysosomal membrane cystine/H+ antiporter. This disease first manifests itself in kidney as a generalized PTC dysfunction, referred to as kidney Fanconi syndrome. Endocytosis of ultrafiltrated plasma proteins rich in disulfide bridges must be the main source of lysosomal cystine in PTC. We expect that analysis of Ctns-/- mice (Nevo et al, 2010) will help understanding how cystine accumulation causes apical PTC atrophy and how cystinosis can be corrected by grafting hematopoetic stem cells (Yeagy et al, 2011).

The transcription factor, ZONAB, is controlled during epithelial polarization and is a key regulator in the proliferation/ differentiation switch

W.R. Lima, S. Dupasquier, A.-S. Delmarcelle, C.E. Pierreux and P.J. Courtoy, in collaboration with K. Parreira and O. Devuyst (NEFR)

Epithelial polarization depends, and impacts on, gene expression. The transcription factor, ZONAB, can shuttle between tight junctions and the nucleus to promote expression of cyclin D, thus participate in the control of proliferation. We have examined whether ZONAB simultaneously represses differentiation, using renal proximal tubular cells (PTC) as a model. During mouse kidney ontogeny and polarization of PTC monolayers in vitro, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium markers. Conversely, ZONAB was reexpressed in dedifferentiated renal carcinomas. Sparsely plated PTC formed small islands : peripheral cells, necessarily lacking external tight junctions, strongly expressed nuclear ZONAB, proliferated and failed to differentiate; central cells, able to form continuous junctional belts, lost nuclear ZONAB, stopped proliferating and engaged in apical differentiation (Fig. 2). In confluent PTC monolayers, stable ZONAB transfection repressed expression and function of the endocytic receptors and impaired brush border and primary cilium maturation. Reporter and chromatin immunoprecipitation assays demonstrated that megalin and cubilin are ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranscriptional levels (proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear ZONAB to central cells, which reversed their choice from differentiation to proliferation.

Thus, ZONAB is down-regulated by epithelial polarity at both mRNA and protein levels and acts simultaneously to promote proliferation and repress differentiation. In vitro and in vivo data suggest that ZONAB is a sensor of epithelial density, involved in their switch from proliferation to differentiation (ref. 6).

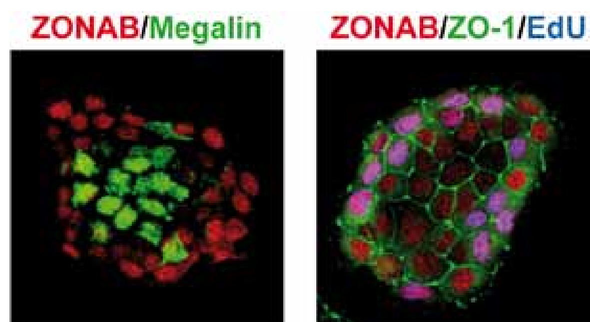

Fig. 2. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right) (From Ref 6).

Epithelial differentiation

A.-C. Hick, A.-S. Delmarcelle, S. Dupasquier, P.J. Courtoy and C.E. Pierreux, in collaboration with F.P. Lemaigre (LPAD)

Several endoderm-derived organs, such as the pancreas, salivary and thyroid glands, are composed of polarized epithelial monolayers. The pancreas and salivary glands are made of open branched tubules specialized in external secretion (exocrine), while the thyroid glands is organized as closed follicles for internal secretion (endocrine). During embryogenesis, the epithelial cells of these developing organs first form a proliferating mass before reorganizing in specialized monolayers (Fig. 3). Once fully differentiated, epithelial monolayers possess three distinct membrane domains, each able to transmit signals to the nucleus. First, the basal domain ensures adhesion to the extracellular matrix. Next, the lateral domain allows direct interactions with adjacent cells via junctional complexes. Finally, the free apical domain is now facing a lumen and bears a mecano-sensory cilium.

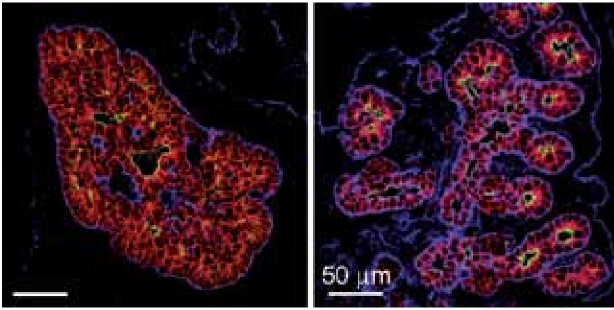

Fig. 3. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left, red) into polarized monolayers with distinct apical domains (mucin, green) and their merging to create tubules (right). Laminin (blue) delineates basement membranes (From Ref 5).

Epithelial monolayers formation requires a coordinate and dynamic interaction with their environment, composed of mesenchymal and endothelial cells. This process is gland-autonomous, as it is faithfully reproduced in explant cultures. We have shown that embryonic salivary and pancreatic epithelial cells highly express receptors for SDF-1 (Stromal cell-Derived Factor-1), produced by mesenchymal cells immediately adjacent to the epithelial buds. Pharmacological inhibition of SDF-1/receptor interaction in explants of the pancreas or salivary glands fully abrogated the reorganization of the epithelial masses into monolayers (5). By three-dimensional analysis of the developing pancreas, we also uncovered a dense and close association of the epithelium with the endothelium (Figure 4). Our in vivo and in vitro data show that endothelial cell recruitment is dependent on VEGF production by the pancreatic epithelium and that endothelial cells, in turn, limit acinar differentiation (8). These data demonstrate that paracrine epithelial:mesenchyme as well as epithelial:endothelial interactions are crucial for organ differentiation and bring a proof-of-principle that molecular components of this interaction can be readily dissected in explant cultures (5,9).

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

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

Besides sharing the same laboratory and continuing a two-decade fruitfull collaboration with the group of E. Marbaix and P. Henriet (Selvais et al., 2011, FASEB J. 25:2770-81; see their report p 72), we have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, see our contribution to the study of the endocytic trafficking of syndecans and its

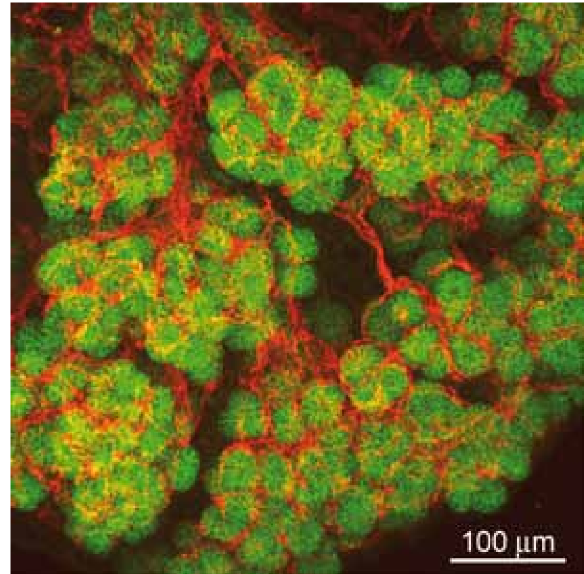

Fig. 4. Epithelial:endothelial interactions in the pancreas. Projections of 40 confocal images showing the dense and close association of pancreatic epithelial cells, labelled for E-cadherin (green), with endothelial cells, labelled for PECAM (red), in the embryonic pancreas.

role in signalling by its heparin-sulfate ligands (Zimmermann et al., 2005, Dev Cell 9:377-88); the subcellular trafficking of the thrombopoietin receptor (Pecquet et al., 2012, Blood; see report by S. Constantinescu, p 118) and the amyloid precursor protein, APP (Ben Khalifa*, Tyteca* et al., 2012, FASEB J. 26:855-67); elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 (Wiame et al., 2010, Biochem J 425:127-36; Veiga da Cunha et al., J Biol Chem 2010, 285:18888-98; Tahay et al., Biochem J. 2012,441:105-12 see report by E. van Schaftingen, p 38); subcellular localization of reactive oxygen species (Denamur*, Tyteca* et al., 2011,Free Radic Biol Med. 251:1656-65); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Miranda et al, 2010, Biochem Biophys. Res. Comm, 396:656-661; see report by M. Rider, p 54); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., 2005, Genes Dev 19:1849-54; see report by F. Lemaigre, p 29) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., Biochim. Biophys Acta Mol Cell Res 2007, 1773:521-35; see report by P. Michels, p 59), or the morphological evidence by FRET of tight interaction between key players of CTL, that is interrupted during their anergy in cancer but can be reversed by galectins (Demotte et al., Immunity 2008; 28:414-24; Cancer Res 2010, 70:7476-88 see report by P. Van der Bruggen, p 101).

Selected publications of the past ten years

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2. Mettlen M, Platek A, Van Der Smissen P, Carpentier S, Amyere M, Lanzetti L, de Diesbach Ph, Tyteca D, Courtoy PJ. Src triggers circular ruffling and macropinocytosis at the apical surface of polarized MDCK cells. Traffic 2006;7:589-603.

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

4. Caplanusi A, Parreira KS, Lima WR, Marien B, Van Der Smissen P, de Diesbach Ph, Devuyst O, Courtoy PJ. Intravital multi-photon microscopy reveals several levels of heterogeneity in endocytic uptake by mouse renal proximal tubules. J Cell Mol Med 2008;12:351-4.

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

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

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

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

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

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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, 9, 10). This seminal observation led us to : (i) use this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes; and (ii) explore whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding, a major health problem (2). We also investigate the control by individual cells of local MMP activity, which can be either increased by recruitment and retention to the plasma membrane (3), or down-regulated by receptor-mediated endocytosis and degradation.

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

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

The general aim of this study was to further elucidate the mechanisms ensuring the spatio-temporal control of menstrual endometrial remodeling in response to the global regulation by estradiol and progesterone. The experimental strategy relied on two advanced methodologies : (i) to separate, by laser capture microdissection, stromal and glandular cells from degraded or preserved areas of the human endometrium after ultrafast immunolabelling and (ii) to compare their global transcriptome by non-supervised microarray analysis.

First, we compared the transcriptomes of stromal and glandular cells microdissected from (i) the basalis as well as from (ii) degraded and (iii) preserved areas of the functionalis in menstrual endometria (6). Algorithms for sample clustering (PCA) segregated biological samples according to cell type and tissue depth, indicating distinct gene expression profiles (Fig. 2). Strikingly, in addition to genes products associated with tissue degradation (MMP and plasmin systems) and apoptosis, lysed areas in the superficial stroma were enriched in gene products associated with ECM biosynthesis (collagens and their processing enzymes). The presence of new synthesized collagens and increased integrin production was confirmed at the protein level. Overexpression of ECM components and adhesion molecules by lysed menstrual fragments could participate in post-menses endometrial reconstruction but also facilitate implantation of endometriotic lesions.

In the second part of the study, stromal and glandular areas were microdissected from explants cultured without or with estradiol and progesterone (7). The microarray datasets were also compared to other published endometrial transcriptomes. Moreover, the contribution of proteolysis, hypoxia and mitogen-activated protein kinases (MAPKs) to the regulation of selected genes was further investigated in explant culture. Like in the menstrual endometrium, this analysis identified distinct gene expression profiles in stroma and glands but functional clustering underlined convergence in biological processes, further supporting cooperative interactions between cell types. Only partial overlaps were observed between lists of genes involved in different occurrences of endometrial remodeling, pointing to a limited number of potentially crucial regulators but also to the requirement for additional mechanisms control-

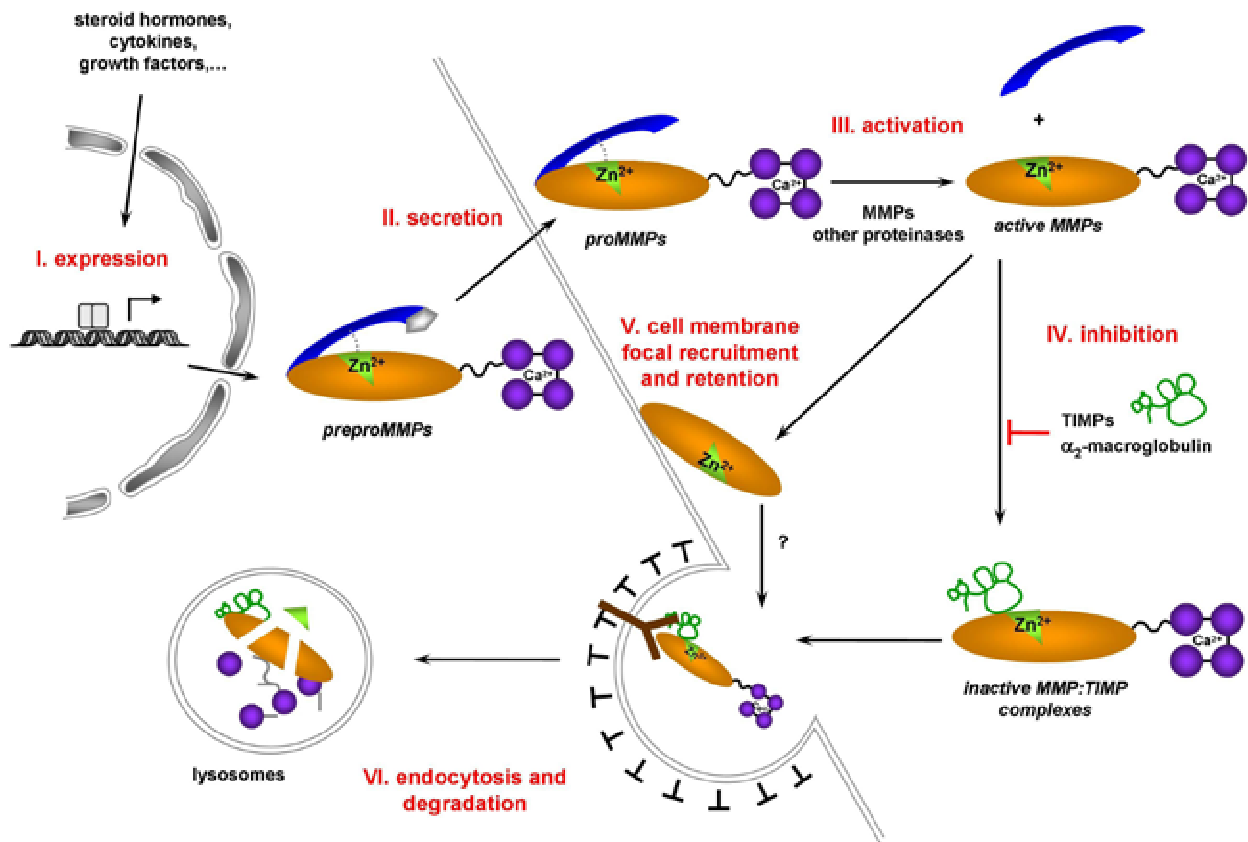

Figure 1. Regulation of soluble MMP activity in the human endometrium: current model. MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn2+-dependent catalytic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

In the cycling human endometrium, MMPs activity is tightly regulated to remodel the extracellular matrix both for blastocyst implantation and, in its absence, for menstrual breakdown of an irreversibly specialized tissue. At menses, the rise of active MMP-1 in the functionalis can exceed one-million-fold as compared with mid-phase tissue. 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). For reviews, see (9) and (10).

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

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

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

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

We have previously shown that the efficient LRP-1-mediated clearance of MMP-2 and -9 activity in non-bleeding endometrium was abrogated upon hormone withdrawal, due to shedding of LRP-1 ectodomain by a metalloproteinase, presumably ADAM-12, itself regulated by estradiol and progesterone (5). Using human fibrosarcoma HT1080 cells, we recently identified

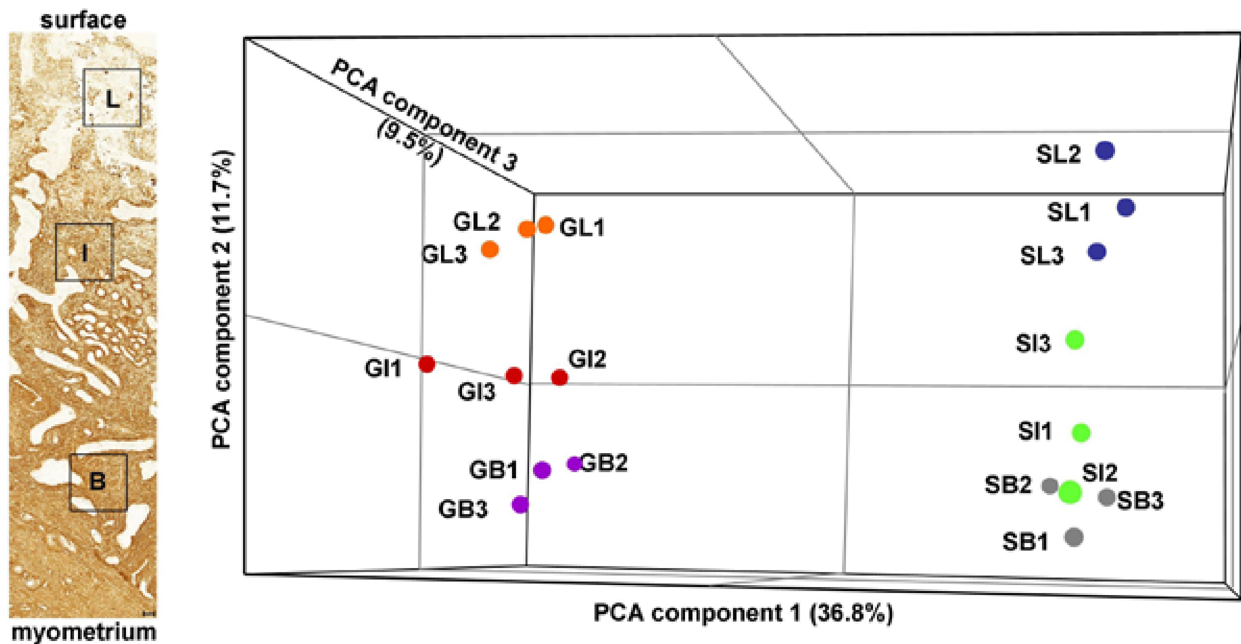

Figure 2. Comparison of transcriptomes from microdissected areas identifies remarkable cell type- and layer-specific signatures. Tissue samples containing either stroma or glands were microdissected from the three layers of 3 menstrual endometria : lysed superficial functionalis (L), preserved intermediate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

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 its ectodomain, and therefore represents a novel regulator of extracellular proteolytic activities (Fig. 3).

Endometrial xenografts

C. Galant, H. Gaide Chevronnay, P.J. Courtoy, P. Henriet, E. Marbaix
(in collaboration with J.M. Foidart, M. Nisolle and A. Béliard at the University of Liège, Belgium)

MMPs are thought to induce menstruation as well as dysfunctional endometrial bleeding, a benign pathology characterized by spontaneous and irregular bleeding associated with menstrual-like stromal breakdown (2). Because menstruation only occurs in few species, in vivo exploration of the physiopathological regulation and role of MMPs is limited. In collaboration with the laboratory of Dr. J.M. Foidart (ULg), we have developed a new experimental model of endometrial xenografts in immunodeficient mice (4). The model allowed us to investigate the alterations of endometrial ECM remodelling upon levonorgestrel treatment and will be used to directly address the role of MMPs in physiological and abnormal endometrial bleeding, endometrial angiogenesis and vessel maturation, as well as in tissue regeneration after menstrual shedding.
Menstrual-like tissue degradation was shown to occur after progesterone withdrawal in a deciduoma induced in the mouse uterus, but involvement of MMPs in this model was not clear. We therefore investigated by immunohistochemistry and quantitative RT-PCR the expression of MMPs in human endometrium xenografted subcutaneously for 3 weeks to immunodeficient mice treated with estradiol- and progesterone-releasing pellets, and compared them to the mouse menstruation model and the uterus of the recipient mice.
The decidualized xenografted endometrium showed focal tissue breakdown and bleeding 3 to 4 days after hormonal withdrawal. Human MMP-1, -3, -8 and -9 expression and MMP-2 immunostaining were strongly increased and TIMP-3 expression decreased. MMP-7 immunostaining was increased but not consistently its mRNA level. In the mouse menstruation model, most murine Mmps had high mRNA level in both the deciduoma and the control horn, essentially not affected by hormones withdrawal, whereas increased expression of Mmp-2, -3 and -10

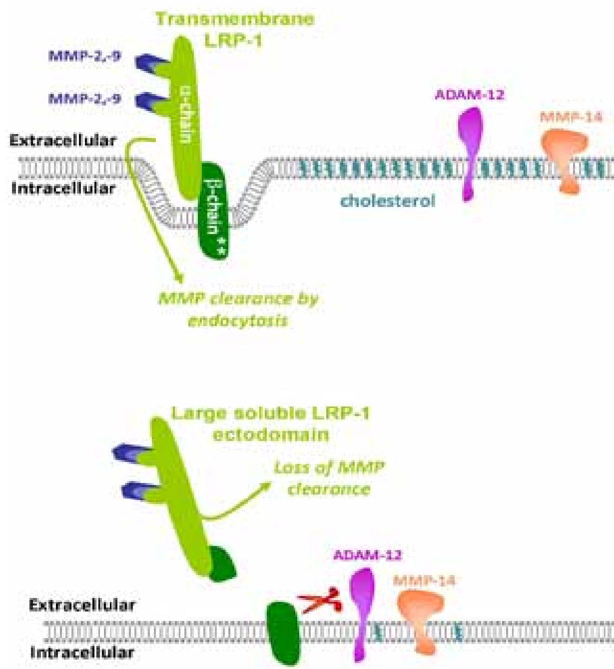

Figure 3. A model for regulation of gelatinase activity by LRP-1. Upper panel: Binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). Sheddase activity of ADAM-12 and MT1-MMP is prevented by cholesterol-induced membrane rigidity. Lower panel: Shedding of LRP-1 ectodomain is enhanced by membrane fluidity due to cholesterol depletion. For details, see (5 and 8).

and decreased expression of Mmp-7 were observed in the uterus of the grafted mice.

In conclusion, hormonal withdrawal induces a menstrual-like pattern of expression of most MMPs and TIMPs in human endometrial xenografts but not in the mouse uterus. The xenograft model seems thus appropriate to study the induction of menstruation, in particular changes in the vasculature and infiltration by leukocytes, as well as of its related pathologies.

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Growth factor receptors From signal transduction to human diseases

Jean-Baptiste Demoulin

Growth factors such as platelet-derived growth factors (PDGF) are secreted proteins that stimulate cell proliferation via transmembrane receptors. PDGF binds to a receptor-tyrosine kinase which signals by phosphorylating various intracellular proteins on tyrosine residues, leading to the regulation of multiple transcription factors and profound changes in genes expression. Understanding how this network of signaling cascades and transcriptional regulations controls cell growth is the first goal of our team.
The uncontrolled activation of PDGF receptors has been linked to several diseases, such as cancer, leukemia and fibrosis. Our second objective is to clarify the role of PDGF receptors in these diseases and to identify new groups of patients who could benefit from a therapy based on PDGF receptors inhibitors.

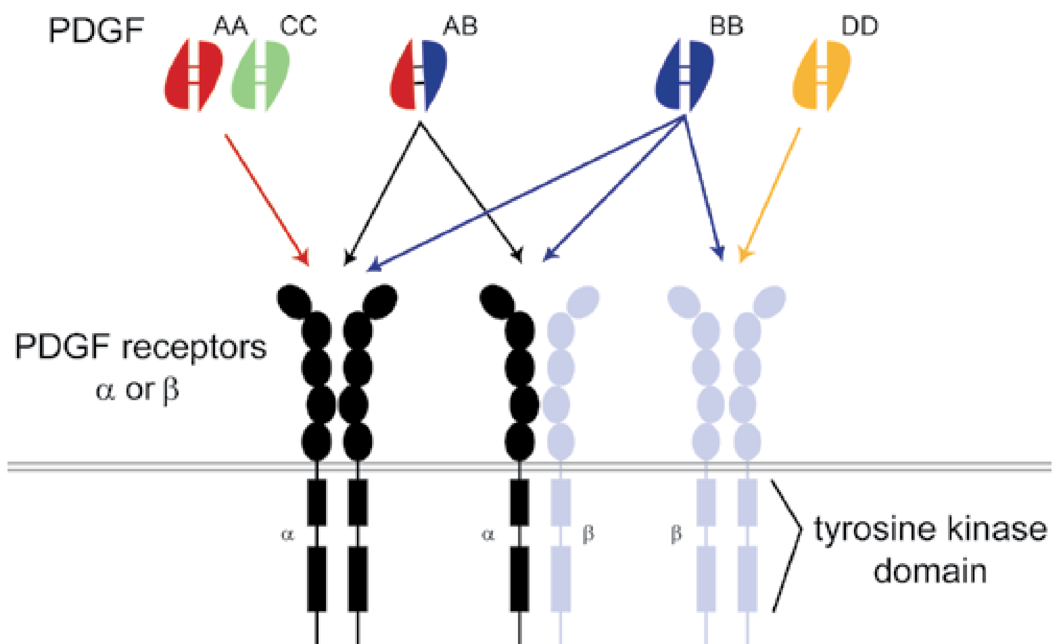

Figure 1. PDGF receptors and ligands

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

A. Coomans de Brachène, A. Essaghir and J.B. Demoulin.

Most of the cellular effects of growth factors are mediated by reprogramming gene expression within the cell nucleus. Each signal transduction cascade controls a number a transcription factors, which activate or repress the expression of many genes. We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with growth factors (PDGF or FGF) using microarrays. In several successive analyses, we identified hundreds of regulated transcripts that had not previously been linked to PDGF signaling (1-3). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells.

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

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

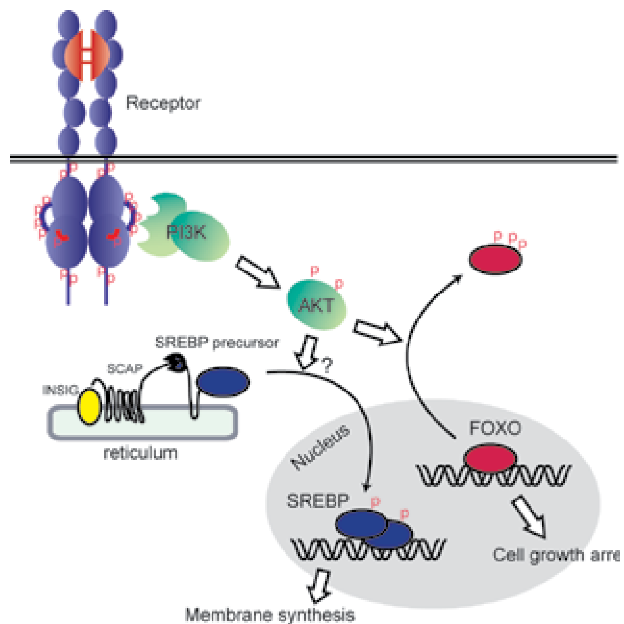

Figure 2. Activation of SREBP and inactivation of FOXO by PDGF

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

A. Essaghir and J.B. Demoulin.

Deciphering transcription factor networks from microarray data remains difficult. We have developed a simple method to infer the regulation of transcription factors from microarray data based on well-characterized target genes (1). We generated a catalogue containing 352 transcription factors associated with 2,721 target genes and 6,422 regulations. When it was available, a distinction between transcriptional activation and inhibition was included for each regulation. Next, we built a tool (www.TFactS.org) that compares new submitted gene lists with target genes in the catalogue to detect regulated transcription factors. We validated TFactS with our own microarray experiments and with published lists of regulated genes in various models and compared it to tools based on in silico promoter analysis. We also analyzed the NCI60 cancer microarray dataset and showed the regulation of SOX10, MITF and JUN in melanomas. Our results show that the expression level of transcription factor target genes constitute a robust signature for transcription factor regulation, and can be efficiently used for microarray data mining. We are now introducing new features into this tool and we are using it to analyze cancer genome data. This tool was also used in a number of transcrip-

tomics projects (see for instance reference 4).

Rearrangements of receptor tyrosine kinase genes associated with leukemia

L. Noël, V. Havelange, A. Velghe and J.B. Demoulin, in collaboration with H. Antoine-Poirel (Cliniques Universitaires Saint-Luc, UCL).

Although PDGF receptors are expressed on platelets and macrophages, PDGF receptor-deficient mice show no primary hematopoietic or immune defect. In vitro, PDGF is a poor mitogen for hematopoietic cells. However, alterations of PDGF receptor genes, as a result of chromosomal translocation or deletion, are found in chronic myeloid neoplasms associated with hypereosinophilia, a disease which affects mostly male patients (5). In all cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. In most cases, they also retain the receptor transmembrane domain, which plays a particular role in the activation of these oncoproteins (5, 8). Similar hybrid oncogenes derive from FGF receptors.
TEL-PDGFRβ (TPβ, also called ETV6-PDGFRB) is a hybrid protein produced by the t(5;12) translocation. FIP1L1-PDGFRα (FPα) results from a deletion on chromosome 4q12 (5). These oncogenes were studied in Ba/F3 cells, a mouse hematopoietic cell line that is easy to culture and transfect. In contrast to wild-type PDGF receptors α and β, which are quickly degraded upon activation, we observed that TPβ and FPα escaped down-regulation resulting in the accumulation of these oncoproteins in cells (9). This was confirmed in leukocytes from patients. Similar data were obtained in cells expressing ZNF198-FGFR1, another fusion protein associated with the 8p11 myeloproliferative syndrome. Ubiquitination of TPβ and FPα was much reduced compared to wild-type receptors. We showed that the accumulation of TPβ is required to activate STAT5 efficiently and transform Ba/F3 cells. Thus, chimeric receptor tyrosine kinases escape efficient ubiquitination and degradation through lysosomes and proteasomes (9). This is a new mechanism that contributes to cell transformation by fusion kinase.
TPβ and FPα do not induce eosinophilia in mice. In order to develop a model that is more relevant for the human disease, we introduced TPβ and FPα in human CD34+ cells, which were purified from cord blood and are enriched in hematopoietic stem cells. These cells are able to differentiate normally in vitro into various blood cell types, depending on the cytokine cocktail that is added in the culture medium. We observed that TPβ and FPα induce the proliferation of these cells and their differentiation into eosinophils in the absence of cytokine. We showed that this process is dependent on the activation of the NF-κB pathway via PI3K (4).
It is particularly important to identify PDGF receptor alterations in cancer patients, as they can benefit from tyrosine kinase inhibitor therapy. Imatinib mesylate, for instance, is very efficient in patients with leukemia that present a PDGF receptor fusion. In collaboration with the hematology unit of the Saint-Luc university hospital, we identified a novel fusion of the PDGF receptor β with the KANK1 gene in a leukemia patient harboring a t(5;9) translocation (Fig. 3 and reference 6). The mechanism of activation of this new fusion gene was analyzed in details (7). We are now looking for other mutations in tyrosine kinase genes.

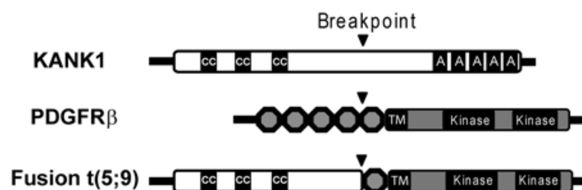

Figure 3. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation.
CC: coiled-coil domain; A: ankyrin repeat; TM: transmembrane domain

Activation of PDGF receptors in systemic sclerosis

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

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

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Viral immunity and pathogenesis

Jean-Paul Coutelier

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

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

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

T helper lymphocyte differentiation

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

Activation of natural killer cells

Many of the influences that viruses may have on diverse immune responses can be explained by the production of pro-inflammatory cytokines, including IFN-γ. Therefore, our analysis

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

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

Activation of macrophages and enhanced susceptibility to endotoxin shock

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

Blood autoimmune diseases

Virally-induced macrophage activation leads also to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. Our analysis has been focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti- platelet response was developed in the mouse (6). Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s) shared by rat and mouse platelets. Two IgM anti-platelet monoclonal autoantibodies were further analyzed. They recognized mouse platelet antigens and could induce both platelet destruction and impairment of their function (7). This response was found to depend on CD4+ T helper lymphocytes reacting with rat, but not with mouse platelets. These anti-rat platelet T helper cells were mainly of the Th1 phenotype. When transferred into naive mice, they enhanced the anti-mouse platelet antibody response induced by subsequent immunization with rat platelets. In addition, depletion of CD25+ cells enhanced the thrombocytopenia induced by immunization with rat platelets whereas adoptive transfer of CD4+CD25+ cells from immunized mice suppressed it (8). Our results suggest therefore that activation of anti-rat platelet T helper cells can bypass the mechanism of tolerance and result in the secretion of autoreactive antibodies, but this response is still controlled by regulatory T cells that progressively develop after immunization.

We have analysed whether a viral infection could modulate such an autoantibody-mediated autoimmune disease. In mice treated with anti-platelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with LDV or mouse hepatitis virus was followed by severe thrombocytopenia (9), whereas infection alone, without autoantibody administration led to a moderate disease. Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose in vitro autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (10).

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

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

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Viral persistence and interferon response

Thomas Michiels

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

1. Theiler's virus

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine picornavirus showing a striking ability to persist in the central nervous system of the host in spite of a specific cellular and humoral immune response. Persistence of the virus is associated with a strong inflammatory response and with lesions of primary demyelination reminiscent of those found in human multiple sclerosis. Our work aims at understanding how a virus can persist in the central nervous system (CNS) of an immuno-competent host, thus evading the immune response (for review, see ref 4, 7).

Recently, a novel human virus closely related to Theiler's virus was discovered and named Saffold virus. This virus is highly prevalent in the human population. Further work is needed to evaluate its virulence potential and its impact on global health.

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

F. Sorgeloos, F. Borghese, Aurélie De Cock and T. Michiels

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

Inhibition of type-i ifn production and alteration of nucleo-cytoplasmic trafficking by the l protein

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. We observed that this protein exerts pleiotropic activities in infected cells. The first activity that was detected for the L protein is its ability to inhibit the transcription of type I IFN and of chemokine genes (4, 7). This activity likely results from the fact that the L protein inhibits the dimerization of IRF-3, the main transcriptional activator of these genes.

Infection of mice deficient for the type-I interferon receptor (IFNAR-/-) indicates that IFN production is critical for resistance against virus infection and that inhibition of IFN production by the L protein also occurs in vivo. However, in vivo, the inhibition of IFN production is not absolute. If it were, the DA virus would cause a fatal encephalomyelitis in wild-type mice analogous to the disease it causes in IFNAR–/– mice.

The L protein also interferes with nucleo-cytoplasmic trafficking of host proteins and mRNA. It promotes the subcellular redistribution of host nuclear and cytoplasmic proteins (2). It also represses the exportation of mRNA from the nucleus to the cytoplasm, thereby shutting-off the expression of host cell proteins. This might explain the highly toxic nature of this protein. These traffic perturbating activities of the L protein correlate with L-mediated hyperphosphorylation of the Nup proteins that make up the nuclear pore complex (7).

Recently, we observed that the L protein of Theiler's virus also inhibited stess granule assembly. Stress granules are stalled

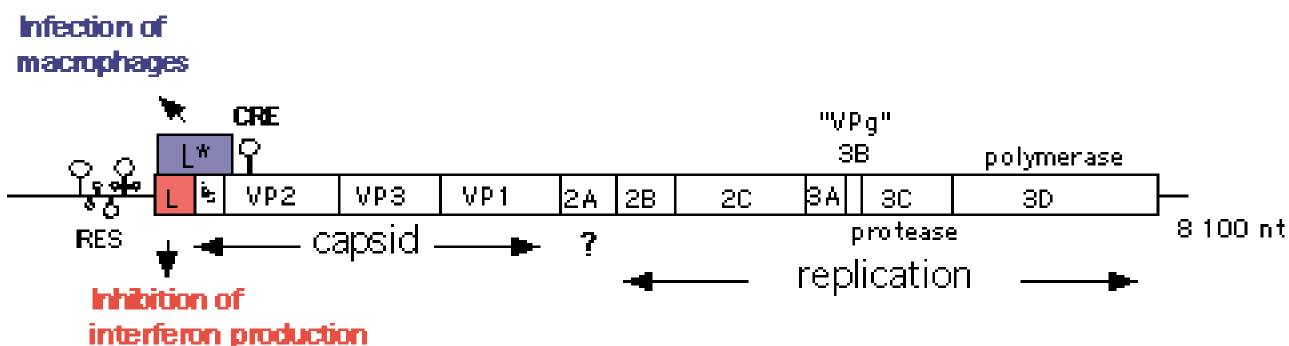

Fig. 1. Genome of Theiler's virus.
A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, to yield the viral mature proteins. Our analysis focuses on 2 proteins L and L* that interfere with the host innate immune responses and therefore facilitate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

translation initiation complexes forming in cells after a translation blockade consecutive to a cellular stress. Cells infected with a L-mutant virus but not with the wild-type virus produce stress granules. These granules contain typical stress granule-associated proteins as well as proteins like PTB which are not found in all types of stress granules. However, we failed to detect viral RNA or replicative forms of the viral genome in stress granules (10).

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

Mutations of either the Zn-finger or of the Theilodomain abolished all reported activities of the L protein, suggesting that the various activities of the protein are linked. Current efforts are devoted to finding the master L interactor in infected cells.

Influence of the l* protein on macrophage infection and viral persistence

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

We showed that L* protein is partitioned between the cytoplasm and the mitochondria (Fig. 2). In mitochondria, L* is anchored in the outer membrane, facing the cytosol (8). Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection. Our recent data suggest that the L* protein antagonizes an important effector pathway of the interferon response. Thus Theiler's virus would interfere both with the production of IFN by infected cells and with the response of cells to this cytokine. This outlines the major importance of the IFN system in the defense of the host against viral infections.

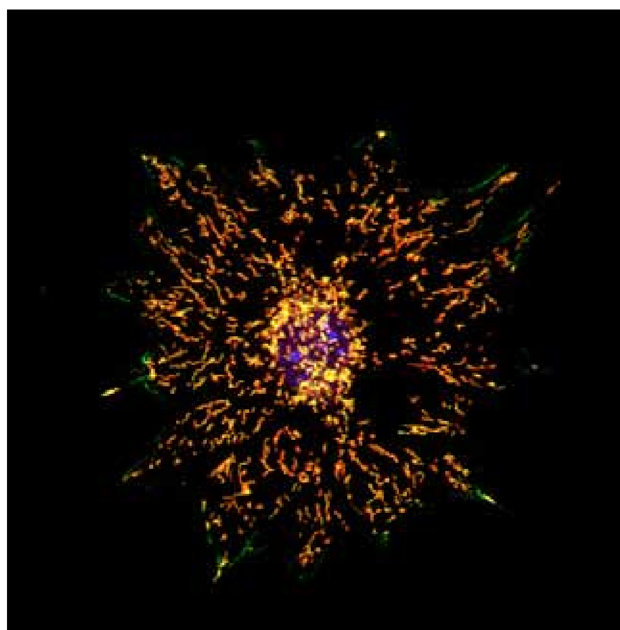

Fig. 2. Mitochondrial localization of L*
Mouse embryonic fibroblast expressing L* from a lentiviral vector. L* was immunolabeled in green and mitochondria were labeled in red with mitotracker. Yellow indicates co-localization.

2. Type-I and Type-III Interferons

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

Type I IFNs

P. Hermant, C. Lardinois and T. Michiels

Mouse and human genomes carry more than a dozen of genes coding for closely related interferon-alpha (IFN-α) subtypes. These interferons and other interferons, like IFN-β, IFN-κ, IFN-ε, IFN-ω, and limitin, form the type-I IFN family. In spite of important sequence divergences, all these IFNs bind the same receptor, raising the question of whether they possess specific functions.
We previously characterized the murine IFN-α family. 14 IFN-α genes were detected in the mouse genome, two of which, IFN-α13 and IFN-α14, were previously undescribed. In addition the mouse genome contains three IFN-α pseudogenes (3).
Ongoing work aims at understanding the specificities of the various type-I IFN subtypes.

Type-III Interferons (IFN-λ)

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

More recently, another IFN family was described and called "type-III IFN" or "IFN-λ". Although type III IFNs bind a receptor distinct from the type-I IFN receptor, they activate the same signal transduction pathway and upregulate the same set of genes. Thus, they are expected to exert very similar biological activities as type I IFNs. Our work addresses the question of the redundancy of these two IFN systems in vivo.
We observed that the relative expression of IFN-λ over that of IFN-α/β exhibited some extent of tissue specificity and was low in the brain (6). We also used a strategy based on in vivo expression of cloned IFN genes to compare the responses of different tissues to IFN-α and IFN-λ. Interestingly, response to IFN-λ appeared to be **specific to epithelial cells** (Fig. 3), unlike response to IFN-α which occurred in most cell types and was particularly prominent in endothelial cells. Accordingly, tissues with a high epithelial content such as intestine, skin or lungs were the most responsive to IFN-λ and expressed the higher amounts of IFN-λ receptor (6). Work performed in collaboration with the groups of P. Stäheli (Univ. of Freiburg, Germany) and M. Hornef (Univ. of Hannover, Germany) confirmed that IFN-λ participates to the protection of lung and intestine epitheliums (where the response to IFN-λ is prominent) against infection with several viruses such as influenza virus respiratory syncytial virus or SARS coronavirus. Interestingly, IFN-λ turned out to be the major player in the defense against rotaviruses, common enteric pathogens causing diarrhea (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.

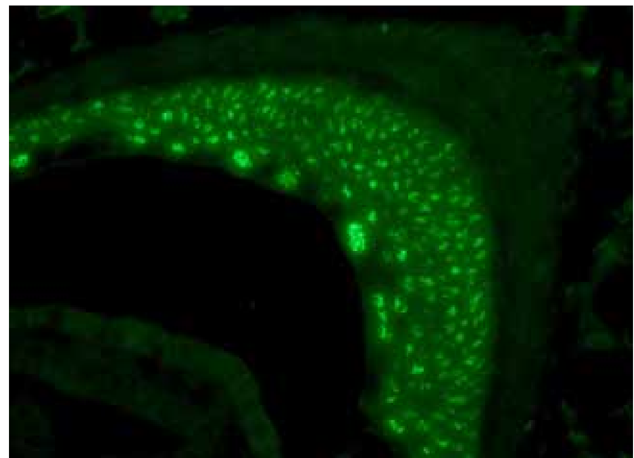

Fig. 3. Response to IFN-λ.
Section of the urinary epithelium showing a strong response of epithelial cells to IFN-λ. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Neurons and IFN-α/β

M. Kreit and T. Michiels

In collaboration with the teams of Peter Stäheli and Friedemann Weber (Univ. Freiburg, Germany) we analyzed the cells that are responsible for type I IFN production in the central nervous system, during acute encephalitis caused by two neurotropic viruses: Theiler's virus (picornavirus) or La Crosse virus (bunyavirus).
IFN-α/β turned out to be produced by many scattered parenchymal cells and less by cells of the inflammatory foci. Interestingly, besides some macrophages and few ependymal cells, neurons turned out to be important producers of both IFN-α and IFN-β. However, not more than 3% of infected neurons expressed IFN, suggesting that some restriction to IFN production might occur in these cells (5).

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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 tumors but appear to be quiescent. Third to understand the mechanism of immunosuppression by human regulatory T cells and its importance in human antitumor immunity. The main objective of this work is to better understand the mechanisms and limitations of human antitumor immunity in order to improve the clinical efficacy of cancer immunotherapy.

Immune responses to cancer vaccine antigens

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

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

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

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

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

In situ analysis of melanoma-infiltrating lymphocytes

A. Cipponi, N. Remy, P.G. Coulie, in collaboration with N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

Our detailed analyses of melanoma patients vaccinated with tumor-specific antigens indicated that anti-tumor T lymphocytes were already present prior to vaccination, in blood and in tumors. It is obvious that there is a seemingly pacific coexistence between tumor cells and tumor-specific T lymphocytes that occurs in many of these cancer patients (5). The reasons for this coexistence may well be the key towards improving the clinical efficacy of cancer vaccines. We are gaining information about human tumor-infiltrating or tumor-associated T cells through an *in situ* analysis. Human tumor samples are processed simultaneously for immunochemical detection of immune cells, for complete gene profiling on a fragment of the tumor, and for laser microdissection on frozen material. Whenever possible a small piece is put into culture to derive a melanoma cell line.

We compared the gene expression profiles of pre-vaccine cutaneous metastases from melanoma patients who showed either complete tumor regression or no regression following vaccination with tumor antigens. We observed no relevant difference between the two groups. But we noticed the presence of a specific inflammatory signature, quite variable between samples, and independent of the clinical evolution of the patients. It comprises T cell and macrophage markers. The T cell signature includes activation markers, IFNg target genes, and the *IFNG* transcript itself. Using immunohistology on adjacent tumor sections, we established that this inflammatory signature correlates with the degree of immune cell infiltration in these tumors. Thus melanoma metastases host various degrees of active Th1 inflammation, and we conclude that the immunosuppressive environment in these tumors does not result in a complete inhibition of T cell activation.

In about 25% of cutaneous melanoma metastases, we observed ectopic lymphoid structures, defined as lymphoid follicles comprising clusters of B lymphocytes and follicular dendritic cells, associated with high endothelial venules and clusters of T cells and mature dendritic cells. Some follicles contained germinal centers. Analysis of the repertoire of rearranged immunoglobulin genes in the B cells of microdissected follicles revealed clonal amplification, somatic mutation and isotype switching, indicating a local antigen-driven B cell response (6). These results demonstrate the existence of lymphoid neogenesis in melanoma and suggest that the presence of functional ectopic lymphoid structures in direct contact with the tumor makes the local development of anti-melanoma B and T cell responses possible.

Human regulatory T cells and TGF-ß

S. Lucas, J. Stockis, C. Huygens, E. Gauthy, J. Cuende, N. Remy, M. Panagiotakopoulos, and P.G. Coulie.

Regulatory T cells, or Tregs, are a subset of $CD4^+$ lymphocytes specialized in the suppression of immune responses. They are required to prevent the development of auto-immune diseases, but in mice they were also shown to contribute to cancer progression by inhibiting anti-tumor immune responses. Tregs could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a Treg-specific marker in humans, as well as to an incomplete understanding of the mechanisms underlying their suppressive function.

Our objective is twofold: develop tools to quantify Tregs in human tissues, and identify mechanisms important for their suppressive function which could be specifically targeted to improve the efficiency of cancer vaccines.

Our previous work lead to the obtention of stable human Treg clones, representing long-term cultures of pure lymphocyte populations available for repeated analysis (7). A stable epigenetic mark unambiguously distinguished human Treg clones from non regulatory $CD4^+$ (Thelper) or $CD8^+$ (cytolytic) clones: a conserved region in intron 1 of gene *FOXP3*, encoding a transcription factor indispensable for the development and function of Tregs, was found demethylated in Treg clones only. We set up a methylation-specific real-time PCR assay to quantify demethylated *FOXP3* sequences, indicative of the presence of Treg cells. In collaboration with laboratories from Italy, The Netherlands and Germany, we used this assay to measure Treg frequencies in the blood of patients who received tumor vaccines in combination with different potentially Treg depleting strategies (8). None of the strategies tested up to now (i.e. low dose Cyclophosphamide, Ontak or Daclizumab) induced a significant decrease in Treg frequencies in a majority of patients. We attempted to use our assay to measure Treg frequencies directly inside tumor samples. However, we observed that melanoma cells themselves could harbor demethylated *FOXP3* sequences, probably as a consequence of aberrant methylation patterns that frequently occur in human tumors. This observation precludes the use of *FOXP3* demethylation as a marker of Treg cells in tumors, unless tumor-infiltrating T cells are separated from tumor prior to analysis (9).

T cell receptor (TCR) stimulation is required for the suppressive function of Tregs. We used expression microarrays to identify functional features that are unique to stimulated Treg clones, by comparison to stimulated Thelper clones. This analysis revealed that a hallmark of stimulated human Treg clones is to produce the active form of TGF-ß, a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF-ß.

Many cell types, including Treg and Thelper clones, produce the latent, inactive form of TGF-ß. In latent TGF-ß, the mature TGF-ß protein is bound to the Latency Associated Peptide, LAP,

and is thereby prevented from binding to the TGF-ß receptor. We recently showed that latent TGF-ß, i.e. both LAP and mature TGF-ß, binds to GARP, a transmembrane protein containing leucine rich repeats which is present on the surface of stimulated Treg clones but not on Th clones (10). Membrane localization of latent TGF-ß mediated by binding to GARP may be necessary for the ability of Tregs to activate TGF-ß upon TCR stimulation. As illustrated in the figure below, a model by which activated Tregs would accumulate latent TGF-ß on their surface and release its active form in close proximity to their target represents an interesting intermediate between the release of a soluble active TGF-ß in the environment, and that of a Treg acting by direct contact with its target. If this model proves to be relevant, it will be important to elucidate the precise mechanism which produces active TGF-ß at the surface of Tregs. Our results imply that binding to the GARP receptor is not sufficient, as lentiviral mediated expression of GARP in human Th cells induces binding of latent TGF-ß to the cell surface, but does not result in the production of active TGF-ß upon stimulation of these Th cells. We are currently trying to identify additional proteins that interact with GARP, and could represent the missing link for the activation of TGF-ß by human Tregs.

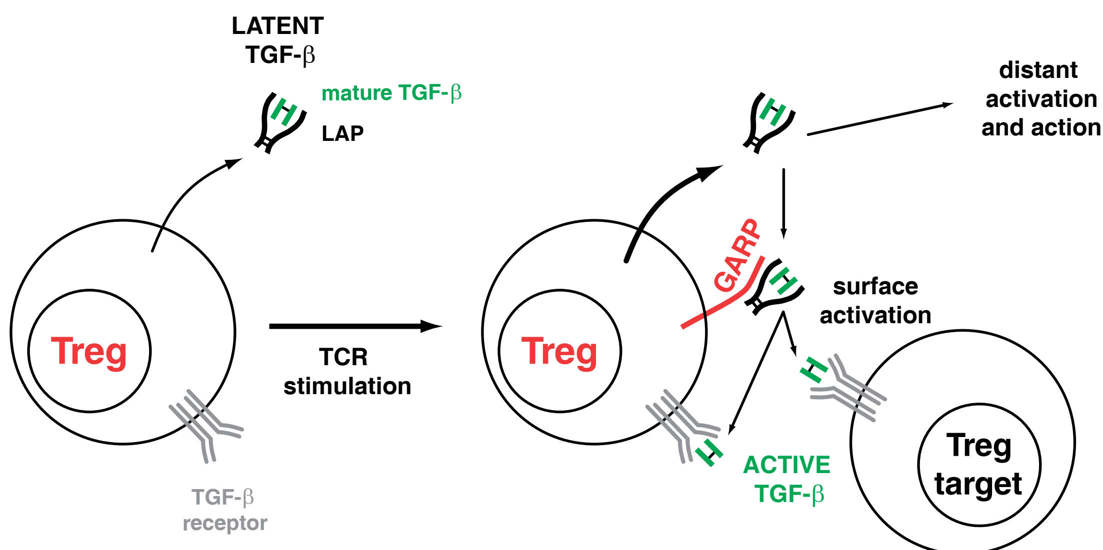

Figure 1. Possible model for TGFß production by human Treg clones.

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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 91 members and was headed by Thierry Boon until 2009. The Branch is now headed by Benoît Van den Eynde, the current Branch Director.

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Tumor immunology and antigen processing

Benoît Van den Eynde

The group follows three main lines of research. The first focuses on the processing of tumor antigens, studying the role of the proteasome and other proteases in the production of tumor antigenic peptides. The second studies mechanisms whereby tumors resist immune rejection. The third develops preclinical melanoma models for cancer immunotherapy. The long-term goal of these projects is to better understand the interaction of tumors with the immune system and devise strategies to improve the efficacy of cancer vaccines.

An antigenic peptide produced by reverse splicing in the proteasome and double asparagine deamidation

A. Dalet, V. Stroobant, N. Vigneron, in collaboration with P. Robbins, K.-I. Hanada and S. Rosenberg, NCI, NIH, Bethesda, USA

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They are recognized by cytotoxic T lymphocytes (CTL) and result from the degradation of these proteins, which is mainly exerted by the proteasome. We have described a new mode of production of antigenic peptides by the proteasome, which involves the splicing of peptide fragments, either in the normal or the reverse order [1, 2]. We showed that splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate. We have now described four spliced peptides, two of which are spliced in the reverse order. 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]. It is derived from tyrosinase and recognized by tumor-infiltrating lymphocytes isolated from a melanoma patient. The peptide is made of two noncontiguous tyrosinase fragments that are spliced together in the reverse order. In addition, it contains two aspartate residues that replace the asparagines encoded in the tyrosinase sequence. We confirmed that this peptide is naturally presented at the surface of melanoma cells, and we showed that its processing sequentially requires translation of tyrosinase into the endoplasmic reticulum and its retrotranslocation into the cytosol, where deglycosylation of the two asparagines by peptide-N-glycanase turns them into aspartates by deamidation. This process is followed by cleavage and splicing of the appropriate fragments by the standard proteasome and additional transport of the resulting peptide into the endoplasmic reticulum through the transporter associated with antigen processing (TAP) (Figure 1).

New proteasome types that are intermediate between the standard proteasome and the immunoproteasome

B. Guillaume, V. Stroobant , A. Busse, E. De Plaen

Using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation, we identified proteasomes that are intermediate between the standard proteasome and the immunoproteasome [4]. They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30-54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. We studied the processing of a series of antigenic peptides by these intermediate proteasomes, and identified two tumor antigens that are processed exclusively either by intermediate proteasome ß5i or by intermediate proteasome ß1i-ß5i. Other functional aspects of these intermediate proteasomes are currently evaluated.

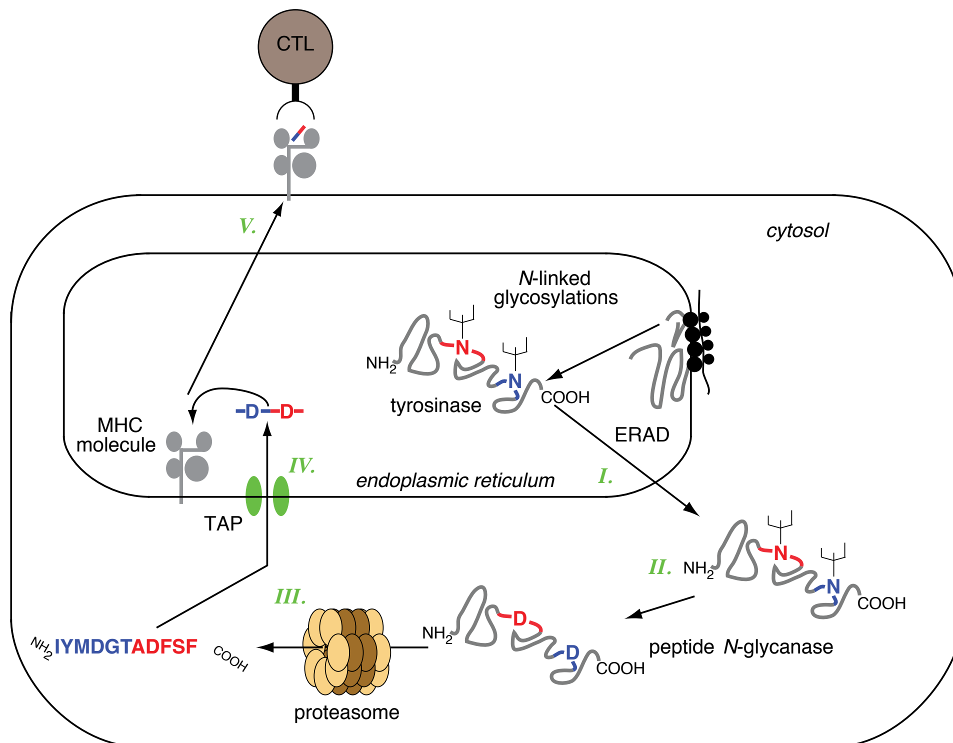

Figure 1. Production of the deamidated reverse-spliced antigenic peptide derived from tyrosinase. Misfolded N-glycosylated tyrosinase proteins are retrotranslocated from the endoplasmic reticulum (ER) into the cytosol (I), where peptide N-glycanase converts two asparagines into aspartates upon removal of the asparagine-associated sugars (II). This is followed by cleavage and reverse splicing of the deamidated protein by the proteasome (III), leading to production of the final antigenic peptide IYMDGTADFSF, which is then transported back into the ER by the TAP transporter (IV) (from reference 3).

Antigenic peptide production by insulin-degrading enzyme

N. Parmentier, V. Stroobant

We studied a proteasome-independent peptide derived form tumor protein MAGE-A3, and we identified insulin-degrading enzyme as the protease producing both the C-terminus and the N-terminus of this peptide [5]. This peptide, with sequence EVDPIGHLY, is presented by HLA-A1 and has been widely used in clinical trials of cancer vaccines. Insulin-degrading enzyme (IDE) is a cytosolic metallopeptidase not previously known to play a role in the class I processing pathway. Cytotoxic T lymphocyte recognition of tumor cells was reduced after metallopeptidase inhibition or IDE silencing. Separate inhibition of the metallopeptidase and the proteasome impaired degradation of MAGE-A3 proteins, and simultaneous inhibition of both further stabilized MAGE-A3 proteins. These results suggest that MAGE-A3 proteins are degraded along two parallel pathways that involve either the proteasome or IDE and produce different sets of antigenic peptides presented by MHC class I molecules.

Inefficient exogenous loading of a tapasin-dependent peptide onto HLA-B*44:02 can be improved by acid treatment or fixation of target cells

N. Vigneron, V. Stroobant , W. Ma, A. Michaux, in collaboration with N. Demotte and P. van der Bruggen, LICR, Brussels, and with RM Leonhardt and P. Cresswell, Yale University, New Haven, CT, USA

We recently identified a new antigenic peptide, which is derived from the MAGE-A1-encoded protein and presented to cytotoxic T lymphocytes (CTLs) by HLA-B*44:02 [6]. Although this peptide is encoded by MAGE-A1, processed endogenously and presented by tumor cells, the corresponding synthetic peptide is hardly able to sensitize target cells to CTL recognition when pulsed exogenously. We observed that endogenous processing and presentation of this peptide is strictly dependent on the presence of tapasin, which is believed to help peptide loading by stabilizing a peptide-receptive form of HLA-B*44:02. In line with this, we showed that exogenous loading of the peptide can be dramatically improved by paraformaldehyde fixation of surface molecules or by peptide loading at acidic pH, two strategies presumably generating or stabilizing a peptide-receptive, empty conformation of the HLA. Altogether, our results indicate a potential drawback of

short peptide-based vaccination strategies and offer possible solutions regarding the use of such problematic epitopes.

A MAGE-C2 antigenic peptide processed by the immunoproteasome is recognized by cytolytic T cells isolated from a melanoma patient after successful immunotherapy

W. Ma, N. Vigneron, J. Chapiro, V. Stroobant, in collaboration with P. Coulie, de Duve Institute

We have pursued our analysis of a melanoma patient who showed almost complete tumor regression following vaccination with MAGE-A1 and MAGE-A3 antigens. We previously described high frequencies of tumor-specific CTL precursors in blood samples collected after but also before vaccination. A set of CTL clones were derived that recognized antigens different from those of the vaccine. Two of these antigens were peptides encoded by another MAGE gene, MAGE-C2. Here we describe the antigen recognized by another tumor-specific CTL clone [7]. It proved to be a third antigenic peptide encoded by gene MAGE-C2, ASSTLYLVF. It is presented by HLA-B57 molecules and proteasome-dependent. Tumor cells exposed to interferon-gamma (IFN-γ) were better recognized by this anti-MAGE-C2 CTL clone. This mainly resulted from a better processing of the peptide by the immunoproteasome as compared to the standard proteasome. Mass spectrometric analyses showed that the latter destroyed the antigenic peptide by cleaving between two internal hydrophobic residues. Despite its higher "chymotryptic-like" (posthydrophobic) activity, the immunoproteasome did not cleave at this position, in line with the suggestion that hydrophobic residues immediately downstream from a cleavage site impair cleavage by the immunoproteasome. We previously reported that one of the other MAGE-C2 peptides recognized by CTL from this patient was also better processed by the immunoproteasome. Together, these results support the notion that the tumor regression of this patient was mediated by an antitumor response shaped by IFN-γ and dominated by CTL directed against peptides that are better produced by the immunoproteasome, such as the MAGE-C2 peptides.

Modulation of tumor antigen expression by inflammatory cytokines

E. De Plaen, O. Kholmanskikh

We observed that treating some melanoma cell lines with the inflammatory cytokine IL-1ß leads to a 4- to 10-fold decrease in the level of Microphtalmia-associated transcription factor (MITF-M) (Kholmanskikh et al, 2010). This effect is NF-kB and JNK-dependent. MITF-M regulates the expression of melanocyte differentiation genes such as Melan-A, tyrosinase and gp100, which encode antigens recognized on melanoma cells by autologous cytolytic T lymphocytes (CTL). Accordingly, treating some melanoma cells with IL-1ß reduced by 40-100% their ability to activate such anti-melanoma CTL.

Minimal tolerance to a tumor antigen encoded by a cancer-germline gene

I. Huijbers, C. Uyttenhove, D. Colau, L. Pilotte, C. Powis de Tenbossche, in collaboration with AM Schmitt-Verhulst and S. Soudja, CIML, Marseille, France

Cancer-germline genes such as MAGE and NY-ESO1 encode the most relevant antigens for cancer immunotherapy. Some of these genes are expressed at low levels in the thymus, raising the possibility of central immune tolerance that might reduce the immunogenicity of such antigens. We created a mouse line knocked-out for cancer-germline gene P1A, and tested whether P1AKO mice develop stronger immune responses to P1A antigens as compared to wild type mice [8]. We found only slightly stronger P1A-specific immune responses in P1AKO mice, even though these responses were sufficient to induce tumor rejection in defined experimental settings. These results indicate only minimal immune tolerance to antigens encoded by cancer-germline genes and fully confirm their immunogenicity.

Tumoral immune resistance through tryptophan degradation by indoleamine 2,3-dioxygenase

L. Pilotte, P. Larrieu, V. Stroobant, D. Colau, C. Uyttenhove in collaboration with U. Rohrig, V. Zoete and O. Michielin, LICR, University of Lausanne, Switzerland

An important factor limiting the efficacy of immunotherapy is the development of mechanisms allowing tumors to resist or escape immune rejection. Immune resistance mechanisms often involve modulation of the tumoral microenvironment resulting in local immunosuppression. We described one such mechanism, based on the expression by tumor cells of indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme inducing a local tryptophan depletion that severely affects T lymphocyte proliferation [9]. Our data in a preclinical model indicate that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. In collaboration with the group of Olivier Michielin in Lausanne, we used computational structure-based methods to design new compounds able to inhibit IDO with a high potency (Röhrig et al, 2012). This approach yielded highly efficient low-molecular weight inhibitors, the most active being of nanomolar potency both in an enzymatic and in a cellular assay, while showing no cellular toxicity and a high selectivity for IDO1 over tryptophan 2,3-dioxygenase (TDO). These triazole compounds will be further optimized with the goal of developing drug candidates.

We have produced a monoclonal antibody against human IDO, which we used to characterize IDO expression in normal and tumoral tissues. Although others reported high expression of IDO in dendritic cells of murine tumor-draining lymph nodes, our results in humans indicate that a subset of mature human dendritic cells express IDO but these cells are present in normal lymph nodes and not enriched in tumor-draining lymph nodes. However, we observed expression of IDO in a high proportion of human tumors, confirming our initial observation.

Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase

L. Pilotte, P. Larrieu, V. Stroobant, D. Colau, E. De Plaen, C. Uyttenhove, in collaboration with E. Dolusic, R. Frédérick, J. Wouters and B. Masereel, NAMEDIC, University of Namur, Belgium

Besides IDO, we recently uncovered the role of another, unrelated, tryptophan-degrading enzyme named tryptophan-dioxygenase (TDO) in tumoral immune resistance [10]. TDO is highly expressed in the liver and regulates 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 new TDO inhibitor, which, upon systemic treatment, restored the ability of mice to reject tumors. 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. This concept will be further developed in the frame of a new spin-off company that we recently launched, named iTeos Therapeutics.

A preclinical melanoma model for cancer immunotherapy

C. Powis de Tenbossche, S. Cane, F. Schramme (in collaboration with C. Uyttenhove, de Duve Institute and A.-M. Schmitt-Verhulst, CIML, Marseille), France

We made transgenic mice developing melanomas with a 70-80% incidence after tamoxifen injection (Huijbers et al, 2006). These tumors express the tumor antigen encoded by cancer-germline gene P1A. Initially highly pigmented and indolent, they later dedifferentiate in unpigmented highly aggressive tumors. Mice bearing aggressive tumors show exacerbated systemic inflammation associated with disruption of secondary lymphoid organs, accumulation of immature myeloid cells and immunosuppression (Soudja et al, 2010). Current efforts aim at characterizing this immunosuppression and devising effective therapeutic approaches.

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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. We believe that progress depends on unraveling the different blockages for efficient tumor destruction. The analysis of the T cell responses of melanoma patients vaccinated against tumor antigens has led us to consider the possibility that the limiting factor for therapeutic success is not the intensity of the anti-vaccine response but the degree of anergy presented by intratumoral lymphocytes. We aim at a better understanding of dysfunctions of the immune system in tumors and more precisely T lymphocyte dysfunctions.

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

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (1). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (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 (3). To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (4).

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

A mechanism causing anergy of CD8 and CD4 T lymphocytes

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

blockages for efficient tumor destruction.

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

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

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

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

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

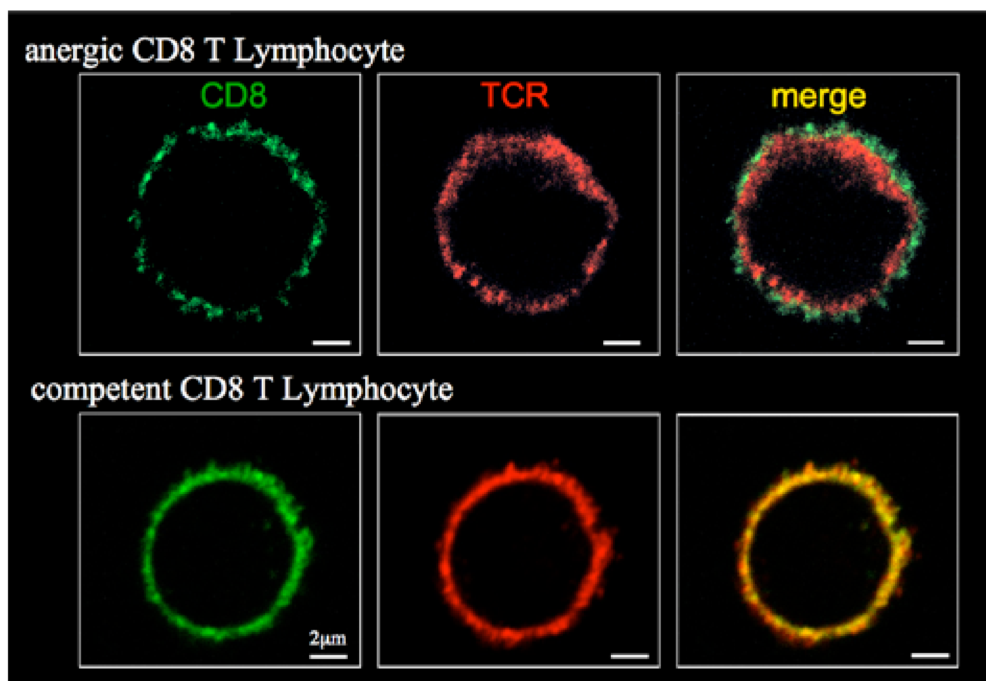

Figure 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions

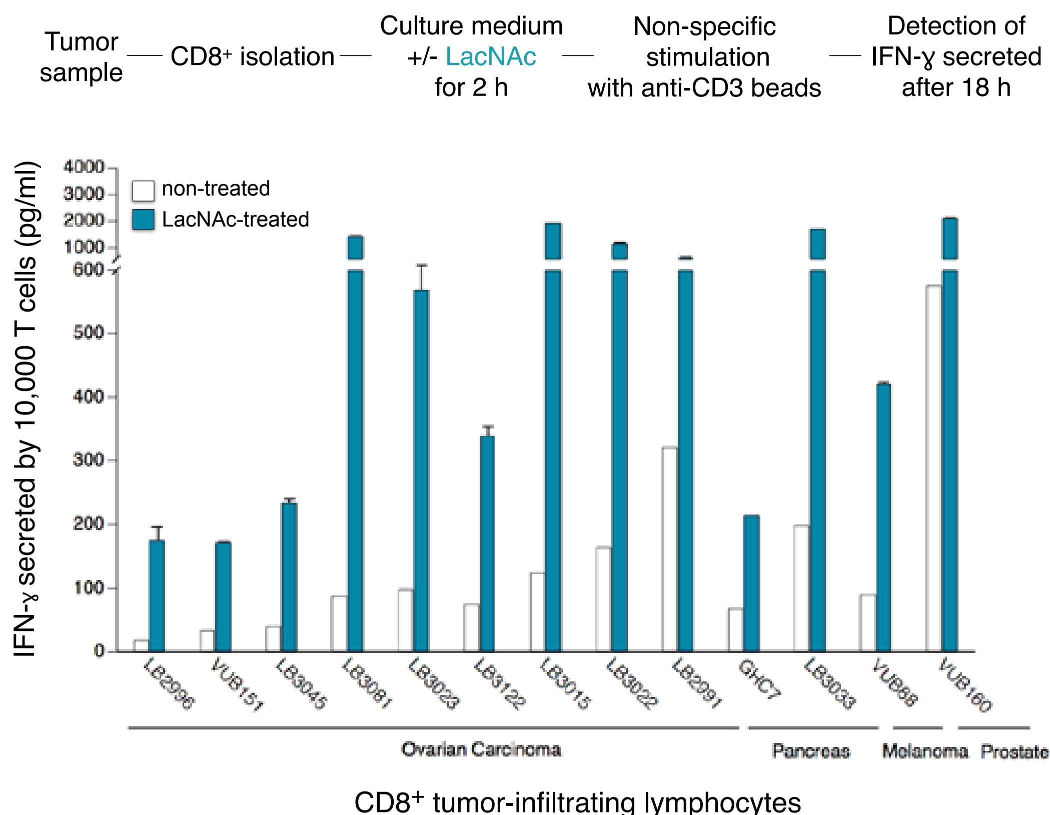

Figure 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

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

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

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

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

cells, and fragments are frozen for further genetic analysis of the T cell receptor repertoire. The prospective follow-up of 172 patients will extend over a 5-year-period. So far, 69 patients have been included and 33 have been screened for frequencies of specific CD8 T lymphocytes. Thirty percent of the screened patients have a frequency against the targeted antigens in the range of $3x10^{-6}$ among blood CD8 T cells, whereas the mean value in healthy donors is $3x10^{-7}$. We hope to identify patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments.

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Immunotherapy analysis group

Thierry Boon

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

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

Accordingly, our new strategy to improve anti-tumoral vaccination is to supplement it with a local treatment of the tumor with various cytokines and Toll-like receptor agonists, as well as antibodies directed against inhibitory cytokines such as TGF-ß, 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

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

A mouse model of skin grafts was developed that recapitulates what happens in cancer patients, where T lymphocytes often infiltrate the tumor without rejecting it. The group tests various approaches to overcome the anergy of such infiltrating T cells. These approaches involve cytokines and Toll-like receptor ligands, as well as antibodies directed against inhibitory cytokines such as TGF-β.

Female CBA mice do not reject male skin grafts, even though they are able to mount a cytolytic T cell response against H-Y, a male specific minor histocompatibility antigen. To break this tolerance, repeated local injections of a low dose of IL-12, combined with IFN-α, caused graft rejection in all 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 are necessary for complete rejection of the grafts.

We tested combinations of agents that are approved for clinical use. We observed that repeated local injections of a combination of low doses of IL-2 (300 ng), GM-CSF (300 ng) and IFNα (10^5 U) with TLR7 ligand gardiquimod (20 µg) caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. However, the same doses of IL-2 and IFN-α, without GM-CSF nor gardiquimod, were also effective when combined with both anti-TGF-β and anti-IL-10 antibodies, whereas anti-CTLA-4 antibody needed the additional presence of GM-CSF to be fully effective. Additional molecules in the process of clinical agreement, such as IFNγ, anti-PDL1 and anti-CD40 agonist antibodies, are presently under evaluation. Vaccinations with intra-peritoneal injections of male lymphoblasts aimed at enhancing anti-H-Y responses synergize effectively with the local cytokine treatment.

Nicolas van Baren presents elsewhere updated results of a clinical trial involving a local treatment composed of IL-2, GM-CSF, IFN-α and Aldara, a cream containing imiquimod, a TLR7 ligand of the same family as gardiquimod.

Amine-reactive OVA multimers for auto-vaccination against cytokines and other immune mediators

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

Using our amine-reactive OVA multimers, recently described, we have produced a series of monoclonal antibodies inhibiting murine GCP-2/CXCL6, cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, TGFβ-1 and matrix metalloproteinase-9 [5]. As these monoclonal antibodies were of mouse origin, they can be administered *in vivo* for long periods without inducing any immune reactions against a foreign protein as observed with rabbit or rat antibodies. The mAb against GCP-2 provided the first demonstration of the essential role played by this chemokine in rapid neutrophil mobilization after *Leishmania major* infection [5]. In collaboration with the group of Jo Van Damme (Rega Institute, KUL), we showed that neutralization of GCP-2 inhibited growth and metastasis of a melanoma cell line by decreasing angiogenesis [6]. Our mAbs against mouse IL-27, MM27.7B1, and against TGFβ1, 13A1, that potently inhibited the bioactivity of these cytokines *in vitro* are currently used to evaluate the contribution of IL-27 or TGFβ1 in anti-tumor activities, skin graft rejection and in a model of GVHD. We also succeded to isolate the first and unique monoclonal antibody directed against the IL-12p35 chain, that contrarily to the existing anti–p40 mAbs, the common chain to IL-12 and IL-23, selectively blocks the IL-12 bioactivity, allowing for the fine dissection of the respective roles of these two cytokines in various *in vivo* models.

Using the same OVA multimers, we immunized mice against periostin/OSF-2, an extracellular matrix protein present in the stroma of many tumors, in mice and humans, that was recently implicated in metastasis development [7]. The first mAbs we isolated are currently being tested (in collaboration with P. Jat and S. Fields, LICR Oxford) to evaluate their ability to block periostin interaction with integrins αvβ3 and αvβ5, one of the mechanisms implicated in cancer cell migration and metastasis establishment.

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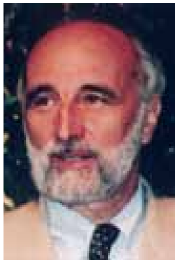

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Therapeutic vaccination and tumor expression profiling group

Nicolas van Baren

Cancer cells express tumor-specific antigens that can be targeted by cytolytic T lymphocytes (CTL). These antigens are small peptides derived from endogenous proteins presented at the surface of tumor cells by HLA molecules. In vitro, cytolytic T lymphocytes (CTL) lyse selectively tumor cell lines that express their cognate antigen. Our group has developed small scale clinical immunotherapy trials in which patients with advanced cancer, often metastatic melanoma, have been treated repeatedly with a vaccine containing one or several defined tumor antigens that are expressed by their tumor (Fig. 1). Different immunization modalities, such as vaccination with peptides like MAGE-3.A1 and NA17.A2, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with an ALVAC recombinant viral vector, have already been tested. They are all devoid of severe toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) showed regression of metastatic lesions (Fig. 2). This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. The most likely explanation for the poor effectiveness of cancer vaccines is the fact that tumors have acquired the ability to resist destruction by anti-tumoral T cells, following repetitive in vivo challenge with spontaneously occurring immune responses. The molecular mechanisms of tumor resistance remain largely unknown, despite the many candidates that have been proposed. Importantly, we have observed that tumor-infiltrating lymphocytes (TIL) purified from melanoma metastases can rapidly recognize and kill autologous tumor cells in vitro, indicating that tumor resistance is a local effect in the tumor environment. We are following two different approaches to try to improve these results: find more immunogenic vaccines, and combine vaccines with treatments that modify the tumor environment in favor of effective tumor rejection.

Vaccination of melanoma patients with Theravac, a new vaccine concept

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

In an ongoing phase I clinical trial, we are testing the safety, immunogenicity and anti-tumoral effect of a new promising vaccine called Theravac, developed at Institut Pasteur. Theravac is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DC) in vivo, and force them to express a Tyrosinase.A2 antigen, a peptide derived from the melanocyte and melanoma-specific tyrosinase protein. Theravac is derived from CyaA, a bacterial toxin that binds specifically to CD11b, an adhesion molecule expressed by dendritic cells and macrophages. Upon binding, a portion of the toxin is internalized and neutralizes its target cell, in order to turn off innate immunity at the infectious site. In the recombinant vaccine protein, the toxin activity has been inactivated by insertional mutagenesis, and coupled to the Tyrosinase.A2 peptide. Thus, the unique

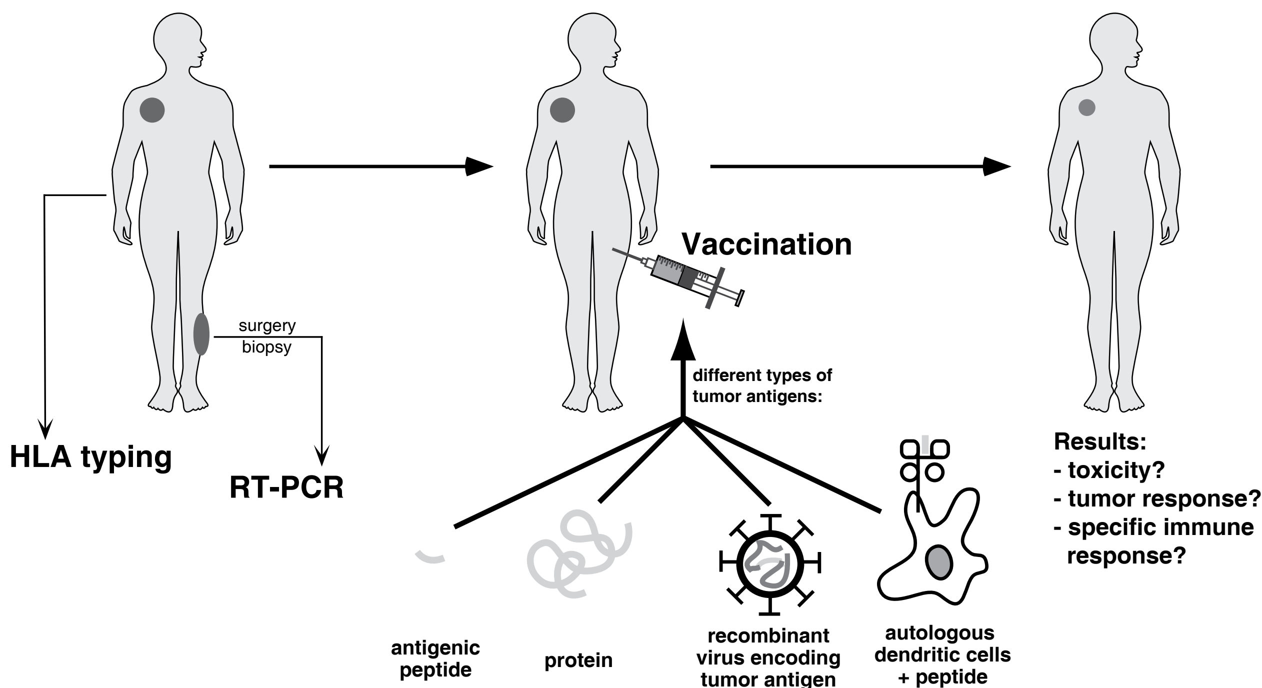

Figure 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients receive repeated injections of a vaccine with the antigen. Usually this vaccine is a synthetic peptide, a recombinant protein, a recombinant virus coding for the antigen, or dendritic cells derived from the patient's blood and forced to express this antigen. The effect of vaccinations on tumor progression is then assessed. Their immunogenicity is analyzed by comparing the frequency of anti-vaccine CTL in the pre and post-immune blood.

advantage of this vaccine is its ability to target dendritic cells in vivo, with an expected higher immunogenicity as a consequence. Preclinical experiments have shown that Theravac has a very potent capacity to activate Tyrosinase.A2-specific CTL. In our clinical trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of Theravac, at increasing doses, in a classical phase I clinical trial design. During the treatment, clinical signs of side effects of the vaccine, including depigmentation occurring as a consequence of anti-melanocyte immune activity, are assessed, and the size of the metastases is followed to detect anti-tumoral effect of the vaccine. Blood lymphocytes are collected before and after the vaccinations to measure the anti-Tyrosinase.A2 immune response. If successful, this new vaccine modality could have a much broader application than in melanoma vaccines.

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

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

In another ongoing clinical trial, melanoma patients with superficial metastases are being vaccinated with a peptide vaccine, either MAGE-3.A1 or NA17.A2, matching the antigenic profile of their tumor. Each of these peptides was previously tested in clinical vaccine trials, and was shown to be well tolerated and associated with tumor regression in some patients. In addition to the vaccine, the patients receive repeated peri-tumoral injections of a cocktail of pro-inflammatory cytokines and a TLR ligand, in one or two superficial metastases. This local treatment is aimed at inducing a "spark" effect in the tumor environment that could modify it in favor of effective tumor rejection. The same cocktail has been tested in the H-Y mouse model of skin graft rejection, in which it is able to induce effective tissue rejection (see the contribution of T. Boon in this report). As with the other clinical trials run by the group, great attention is given to the collection of biological material (tumor and blood samples), which will allow to study the effect of the treatment on the anti-tumoral immune responses.

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

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

Recent work in the laboratory has shown that the state of anergy that characterizes tumor-associated T cells can be reversed pharmacologically (see the contribution of P. van der Bruggen in this report). Inhibitors of galectin-3, a protein produced by cancer cells that is able to interfere with effective T cell activation, have been able to reactivate anergic T cells in vitro. In a recently started clinical trial, melanoma patients receive the same

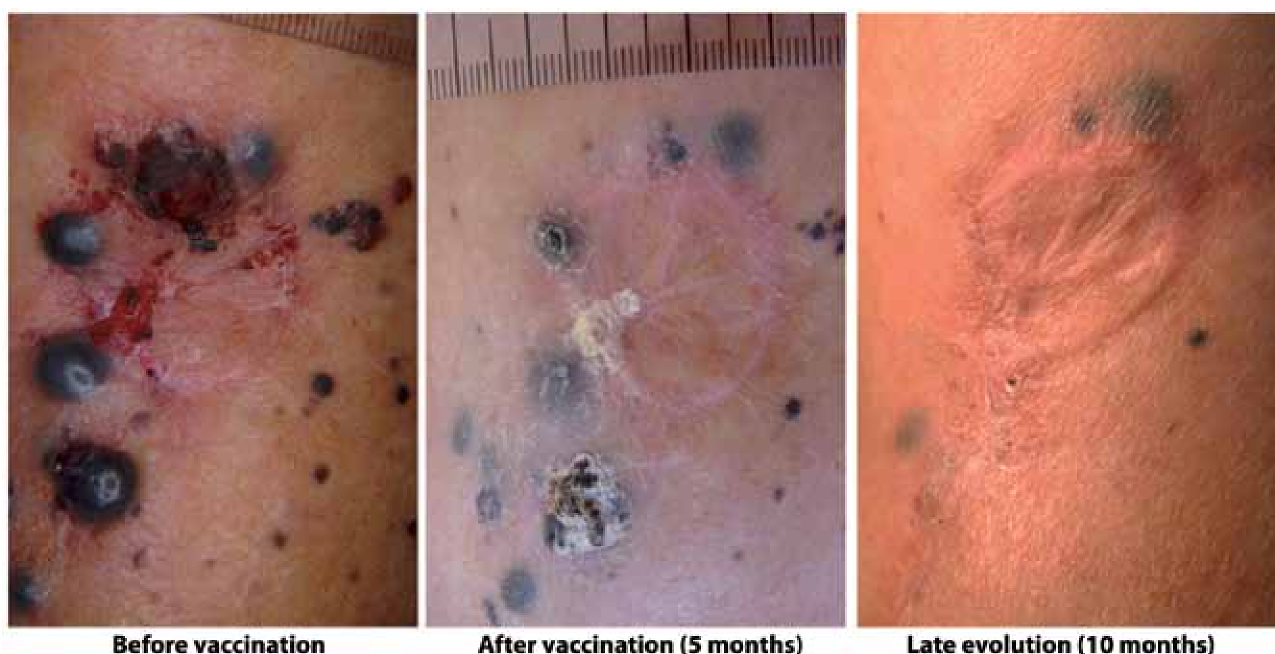

Figure 2. Example of a complete regression of cutaneous metastases in a melanoma patient after 4 priming vaccinations with an ALVAC recombinant virus expressing the MAGE-3.A1 and MAGE-1.A1 epitopes followed by 3 booster vaccinations with the corresponding peptides.

peptide vaccine as in the previous study, in association with repeated infusions of an experimental drug called Davanat®, a plant-extracted oligosaccharide that binds to and inhibits galectins. Galectin-3 is a protein produced by cancer cells that is able to inhibit T cell activation. The group of Pierre van der Bruggen has shown that the anergy that characterizes tumor-associated T cells can be reversed with galectin inhibitors including Davanat®. We hope that this combined treatment will favor a synergistic interaction between new anti-tumoral CTL responses induced by the vaccine and the inhibition of tumor resistance by the galectin inhibitor.

Study of the inflammatory environment in melanoma metastases

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

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients. This approach is combined with systematic immunohistological or immunofluorescence analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions (Fig. 3). In addition, adjacent cryosections are analyzed by performing laser capture microdissection of selected areas, e.g. T cell rich areas, followed by RT-qPCR analysis of T cell, macrophage, melanoma cell and inflammation associated genes. These complementary approaches help us to characterize the inflammatory events that take place inside the metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site. We are currently characterizing an inflammatory signature that is detected in most tumor samples, and that is associated with T cell activation. We also analyze lymphoid structures present in tumors in which B cell responses seem to occur. The informations gathered from these analyses help us to understand the immune pathways that are active or silent in the tumor environment.

Analysis of melanocyte-derived tumors by non-linear optics techniques.

Our group collaborates with several other European groups in a project aimed at developing innovative imaging microscopy and endoscopy approaches that might improve cancer diagnosis. These approaches are based on spectroscopical analysis of tissue sections or samples illuminated with one or several laser beams of selected frequencies, using so-called Raman and Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopes. The Raman and CARS effects involve light reflection that depends on the molecular bonds present in the illuminated sample. The objective is to identify spectral signatures associated with tumor cells, which would allow to detect and quantify these cells in conventional microscope preparations without staining. Eventually, this technique coupled to an endoscope might allow to detect the presence of cancer cells in vivo. The current project is focused on melanoma and benign naevus samples, and is at an early, proof-of-feasibility stage of development.

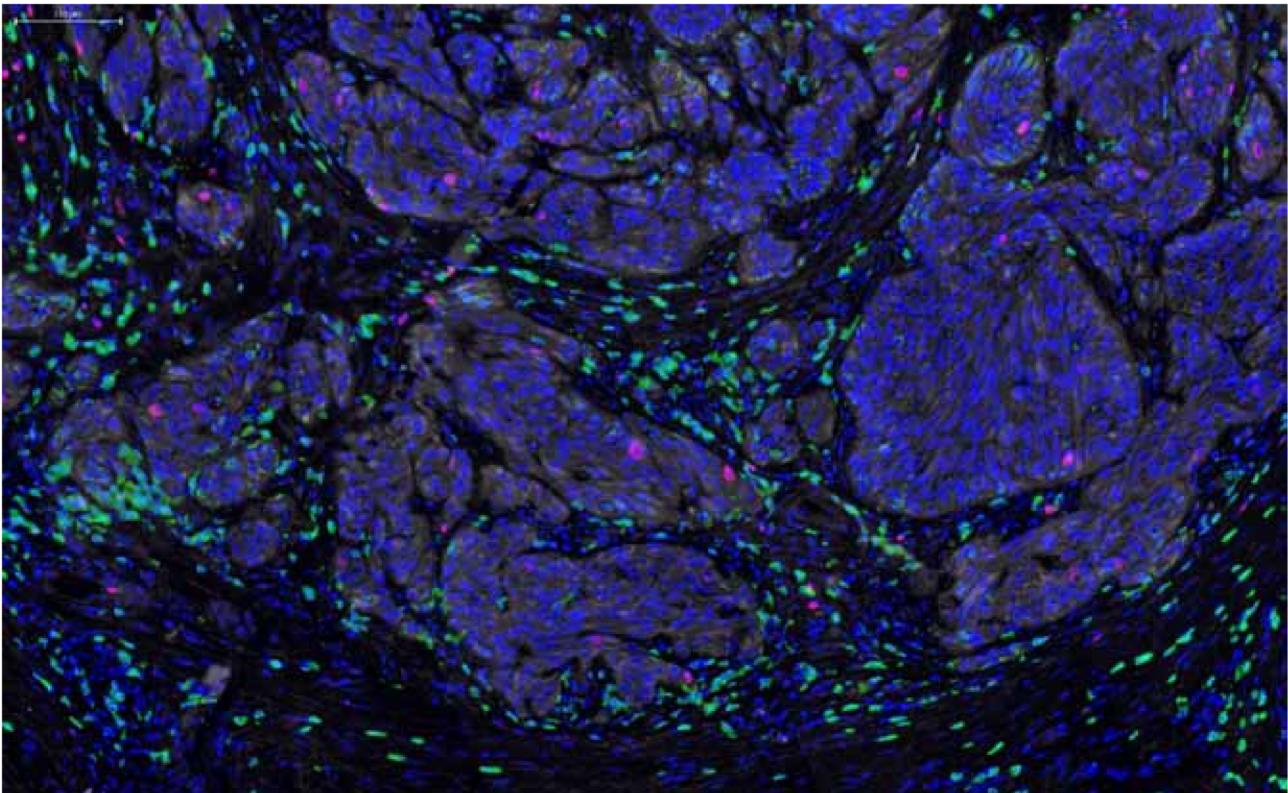

Figure 3. A melanoma metastatis infiltrated by cytolytic T lymphocytes. A tissue section of the tumor was stained with three antibodies coupled to a different fluorescent dye. Melanoma cells and T cells appear in brown and green, respectively. Some melanoma nuclei express the proliferation marker Ki67 (in red). All cell nuclei are stained with a blue fluorescent dye. This image shows that the tumor continues to grow in the presence of an abundant infiltration by T lymphocytes.

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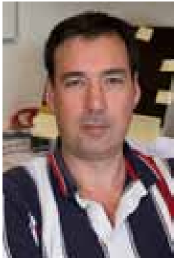

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Cytokines in immunity and inflammation

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 (1).

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 (2), 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%) when maintained in a conventional environment. 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

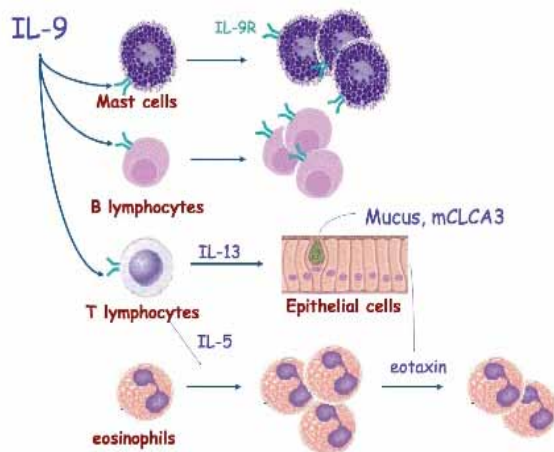

Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation of eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production 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 (2).

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 (3). The anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells generated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9 receptor and signal transduction

Analysis of the mode of action of IL-9 at the molecular level was initiated by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs (4). 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.

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. Some of our recent data 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 (5).

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 (figure 2).

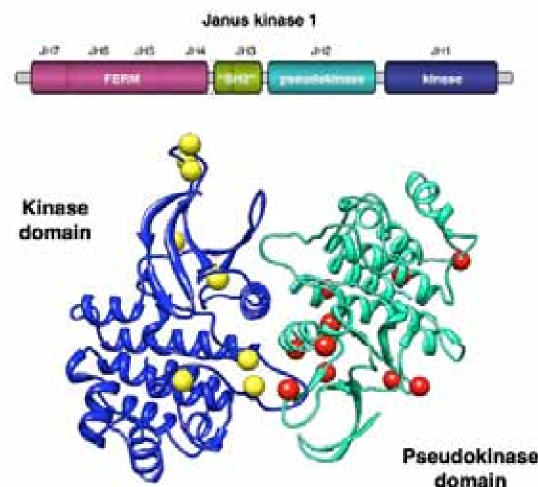

Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

In parallel to these observations, in collaboration with Prof. Marco Tartaglia (University of Rome), we identified activating mutations in JAK1 in 20% of T-cell acute lymphoblastic leukemia (T-ALL) and in 3% of B-ALL patients, confirming the relevance of our in vitro model-derived JAK1 mutations for human malignancies. Further analysis of human ALL samples showed that JAK1-mutated leukemias share a type I IFN transcriptional signature, suggesting that these mutants do not only activate growth-promoting pathways, but also antiviral pathways. Expression of these activating JAK1 mutants in murine hematopoietic cell lines recapitulated this signature in the absence of IFN, but also strongly potentiated the in vitro response to IFN. Finally, we also showed in an in vivo leukemia model that cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with JAK1 activating mutations (6).

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

Searching for genes specifically regulated by IL-9 in lymphomas, we identified a new gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver (7) and protection against experimental hepatitis and colitis (L. Dumoutier, unpublished results). Among the different T cell subset, IL-22 was found to be preferentially produced by TH17 cells, which are associated with several autoimmune and inflammatory processes. 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. 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 hyper- proliferative responses of keratinocytes. Contrasting with this proinflammatory effect of IL-22 in skin inflammation, asthma models showed that IL-22 can have a protective anti-inflammatory activity in lungs. This protective effect of IL-22 has been attributed to an inhibition of IL-13 activity on lung epithelial cells either for CCL17/TARC induction or for IL-25 production. Inhibiting IL-22 in vivo, through antibody treatment or by gene targeting, increased expression of these inflammatory mediators, infiltration by eosinophils and broncho-hyperrersponsiveness.

Both in the psoriasis and asthma models have challenged the dogma that IL-22 is mainly produced by TH17 lymphocytes, and γδ T cells as well as innate lymphoid cells turned out the be the major producers of this cytokine. We characterized the cells responsible for IL-22 production in response to TLR agonists such as LPS or flagelin. We identified a new innate lymphoid spleen cell population expressing CD25, CCR6 and IL-7R representing 1% of spleen cells from recombination activating gene (Rag2)-deficient mice. This population comprises 60–70% $CD4^+$ cells, which produce IL-22, and are still present in common γ chain-deficient mice; the $CD4^-$ subset coexpresses IL-22 and IL-17, and is common γ chain-dependent. These cells share a transcriptional program with NKp46+ RORγt+ cells found in intestinal mucosae and involved in antibacterial responses. The importance of IL-22 production for the LPS-triggered response is highlighted by the fact that IL-22-deficient mice are more resistant to LPS-induced mortality, pointing to the pro-inflammatory activity of this cytokine.
Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (7). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with JAK1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected feature of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (9).
Beside this cell membrane IL-22 receptor complex composed of IL-22R and IL-10Rß, we identified a protein of 231 amino acid, showing 33 % amino acid identity with the extracellular domains of IL-22R, respectively, but without any cytoplasmic or transmembrane domain. This soluble receptor has been named IL-22 binding protein (IL-22BP), because it binds IL-22 and blocks its activities in vitro, demonstrating that this protein can act as an IL-22 antagonist.
The crystal structure of IL-22, alone and bound to its cellular receptor IL-22R or to its soluble receptor IL-22BP has been characterized in collaboration with Prof. Igor Polikarpov (University of Sao Paulo) and is illustrated in figure 3.

In addition to its role in IL-22 binding and signalling, the IL-22R

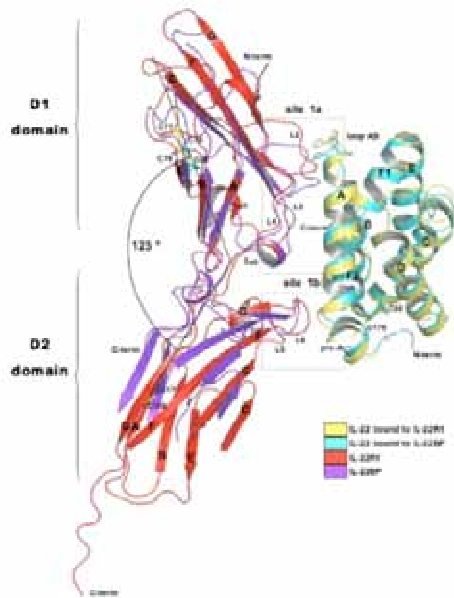

Figure 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

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

Anti-cytokine vaccination

Beside conventional gene targeting strategies, that were used in our lab to generate mice deficient in the IL-9R, in IL-22 or in IL-22R, we developed a new strategy of anti-cytokine vaccination leading to the production in vaccinated mice of anti-cytokine autoantibody that block the biological activities of endogenous cytokines. Neutralizing auto-antibodies against cytokines such as IL-9, IL-12 and IL-17 have been induced upon vaccination with the autologous cytokines chemically coupled with OVA (IL-9, IL-17) or with the Pan DR T helper epitope PADRE (IL-12). This strategy contributed to demonstrate the role of IL-9 in an intestinal helminth infection (1), of IL-12 in atherosclerosis and of IL-17 in experimental autoimmune encephalomyelitis. More recently, we developed a new procedure of anti-cytokine vaccination by taking advantage of tumor cells as a vaccine against peptides presented at their surface in fusion with a human transmembrane protein. These vaccination methods represent simple and convenient approaches to knock down the in vivo activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

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

Stefan Constantinescu

Cytokines and their receptors govern formation of blood cells and development and function of the immune system. Our broad interest is the understanding of the mechanisms that control assembly in the membrane of cell surface receptors that respond to extracellular cues. We study in detail the structure, function and orientation of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo), Granulocyte Colony Stimulating Factor (G-CSF), which function as homomeric complexes and of heteromeric receptors such as receptors for several interleukins.

Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which is transmitted via juxtamembrane and transmembrane domains to the cytosolic region and ultimately to members of the Janus family of tyrosine kinases (JAKs).
We aim to identify: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; ii) what are the general rules by which hydrophobic membrane-spanning domains interact in the membrane in a sequence-specific manner and how is this dimerization/oligomerization process regulated; and iii) the mechanisms of JAK attachment to receptors, and their subsequent activation, especially the role of pseudokinase domains in JAK kinase domain activation.

The laboratory identified constitutively active oncogenic mutants of JAK2, JAK1 and TYK2 and of cytokine receptors, with some being involved in human blood cancers. Specifically the mechanisms by which JAK2 V617F and TpoR W515 mutants induce, in humans, Myeloproliferative Neoplasms (MPNs) are actively pursued. The mutants we identified induce constitutive STAT activation, and we are interested in determining the gene targets of constitutive STAT5 activation, since we identified several novel target genes induced specifically by constitutively active STAT5, and that are involved in MPN pathogenesis. A close interaction structure has been created with clinicians and clinical biologists at St Luc Hospital for in-depth study of patient-derived cells.

The mechanisms by which the mutant JAK2 V617F induces Polycythemia Vera and other myeloproliferative neoplasms in humans

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

The JAK-STAT pathway mediates signaling by more than 25 cytokine receptors and is constitutively activated in many cancers. Several mutations in genes coding for JAKs have been identified in the past five years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudokinase domain, predicted to be inactive. Four Janus kinases are coded by the human genome JAK1, JAK2, JAK3 and TYK2. JAK2 is crucial for signaling by EpoR, TpoR, the G-CSFR, the interleukin 3 receptor and several others. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

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

We have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation in collaboration with William Vainchenker at the Institut Gustave Roussy in Paris. JAK2 V617F responsible for >98% of Polycythemia Vera and for >50% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (2). The mutation in the pseudokinase domain activates the kinase domain and constitutive signaling (3) in complexes between JAK2 V617F and cytokine receptors such as EpoR, TpoR and G-CSFR (Figure 1). The homologous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (3). These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1), with the already reported example of JAK1 mutations in T-lymphoblastic leukemia.

We are interested to understand how a pseudokinase domain mutation can induce kinase domain activation. The aim is to be able to specifically inhibit mutated JAK2 in myeloproliferative neoplasm patients, but not the wild type JAK2, which is crucial for red blood cell and platelet formation. We identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (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 (Figure 2). Expression of segments of JAKs is pursued in different cellular systems for structural studies. Furthermore, protein fragment complementation and protein ligation as-

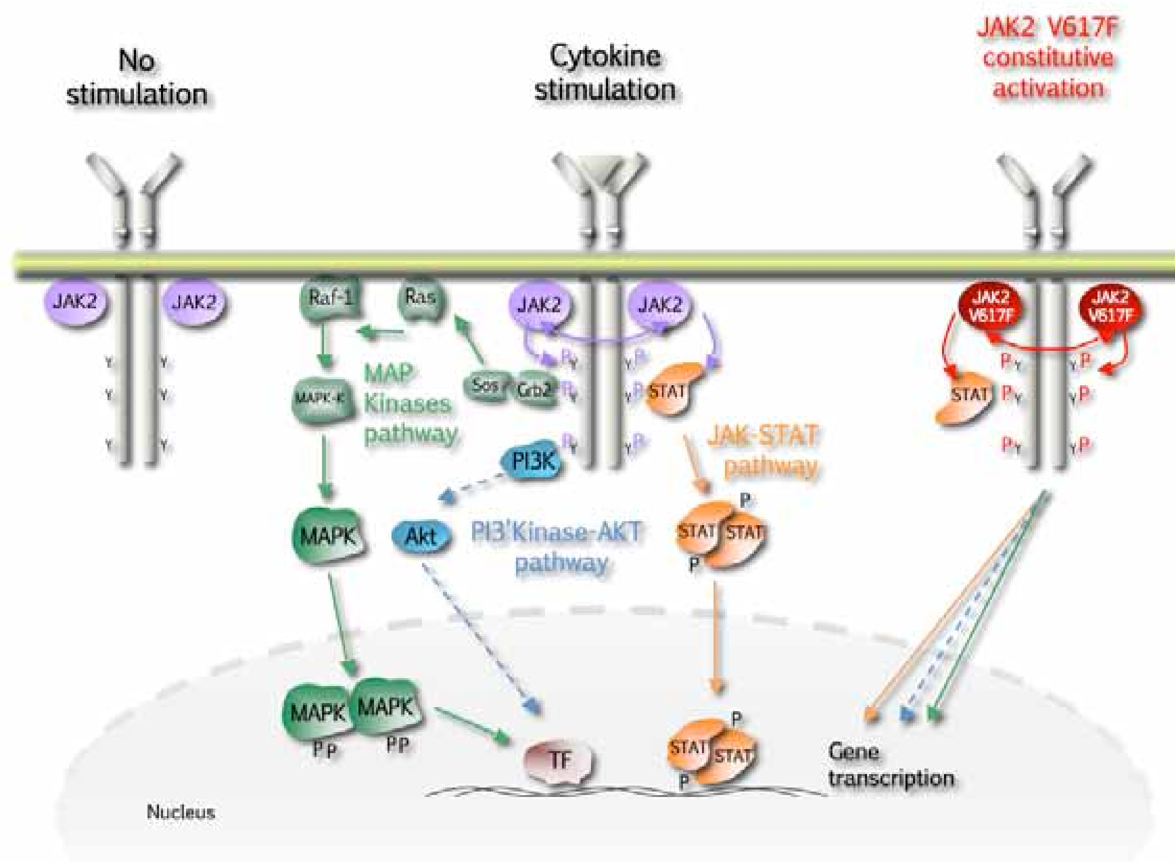

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs to cross-phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become themselves phosphorylated and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatydylinositol-3-kinase, PI3K, and Akt). The mutant JAK2 V617F binds to the cytosolic domains of receptors and can trigger signaling in the absence of any cytokine binding to the extracellular receptor domain (right complex). As a consequence, signaling is induced permanently and myeloid progenitors survive, proliferate and differentiate in an uncontrolled manner. (Jean-Michel Heine)

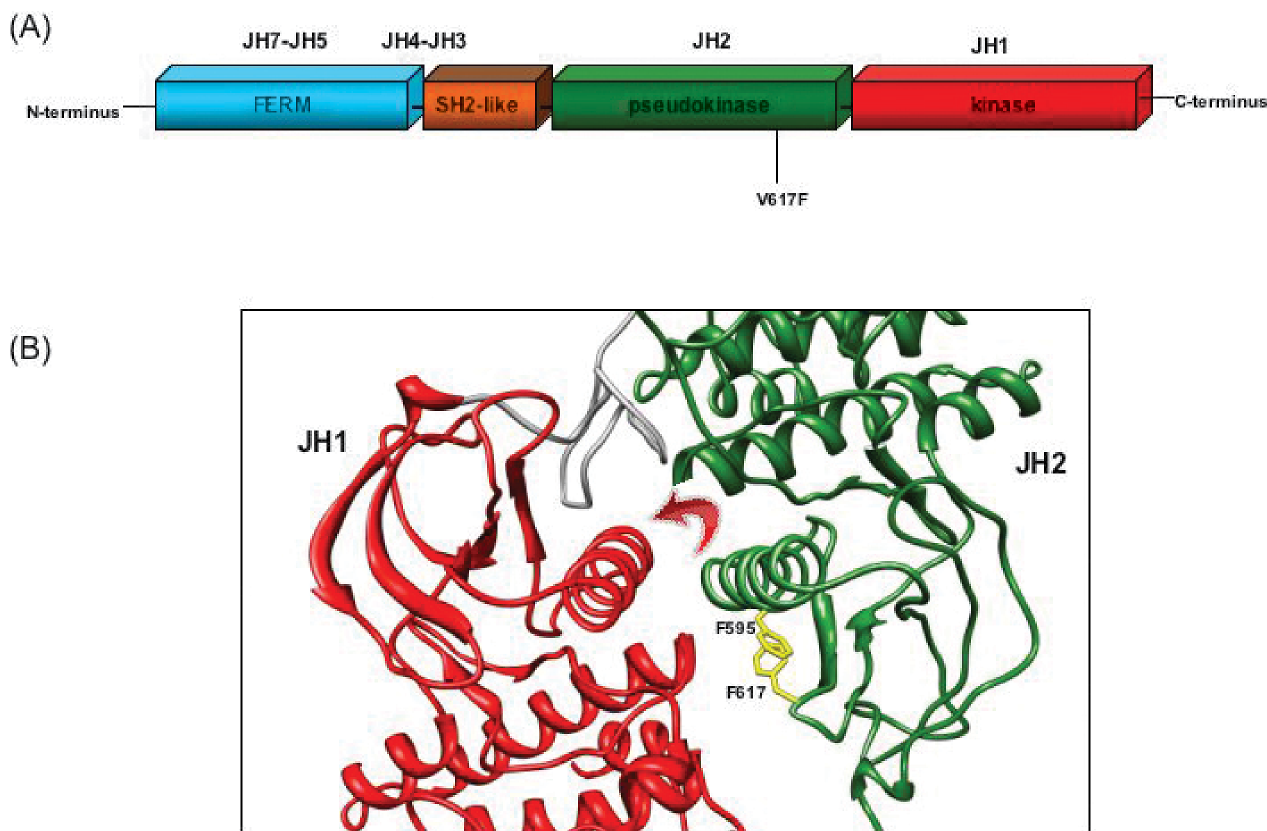

Figure 2. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pseudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients. (B) The pseudokinase (JH2) and kinase (JH1) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other and leading to JH1 inhibition. Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 (red arrow) and eventually in activating the kinase activity of JH1. The region around V617F and the middle of JH2 helix C surrounding F595 could become the target of inhibitors that might specifically decrease constitutive activation of JAK mutants.

says are employed in order to determine the dimerization state and proximity of interaction between mutated JAKs or wild type JAKs themselves and with cytokine receptors.

Involvement of pathologic TpoR signaling in myeloproliferative neoplasms

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

Thrombopoietin (Tpo) is a cytokine produced by the liver that is critical for regulation of the formation of platelet cells, the small enucleated blood cells that derive from the cytoplasm of megakaryocytes and that prevent bleeding upon injury. Tpo also regulates the numbers of hematopoietic stem cells and other myeloid cells.

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

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (5). In addition we discovered that Tpo can induce a strong antiproliferative effect in cells that express high JAK2 levels (5). This effect can be detected in postmitotic mega-

karyocytes (Plos Biol 2010, 8, e1000476). We showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F cells, and is partially responsible for TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells.
Biochemically, co-expression of TpoR and JAK2 V617F leads to strong ubiquitinylation of K540 and K544 of TpoR cytosolic domain and proteasome-mediated degradation of TpoR. In the same time, JAK2 itself is ubiquitinylated. Using the Ubi-Scan approach coupled to mass spectrometry we identified a number of proteins that acquire either ubiquitin or ISG15 or other ubiquitin-like molecules in a Tpo-dependent manner and we are establishing the roles of such post-translational modifications in Tpo-signaling and JAK2 V617F-mediated proliferation.

A novel mechanism by which a tryptophan residue regulates TpoR activation

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

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Mutagenesis of the RWQFP motif showed that W515 (W508 in the murine) is the key residue maintaining human TpoR normally inactive (6). Biophysical experiments performed in collaboration with Dr. Steve Smith at SUNY Stony Brook, and fluorescence complementation studies showed that W515 actually regulates the orientation, tilt and dimerization of the upstream transmembrane helix, and prevents receptor activation (Defour et al., manuscript in preparation). In vivo, in bone marrow reconstituted mice, the delta5TpoR and TpoR W515A mutant induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (Figure 3). We recently established that the myelofibrosis phenotype induced by TpoR W515 mutants depends on cytosolic Y112 (Y626) of TpoR, and appears to involve excessive STAT3 and MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 (Y626) might be useful in the treatment of myelofibrosis.

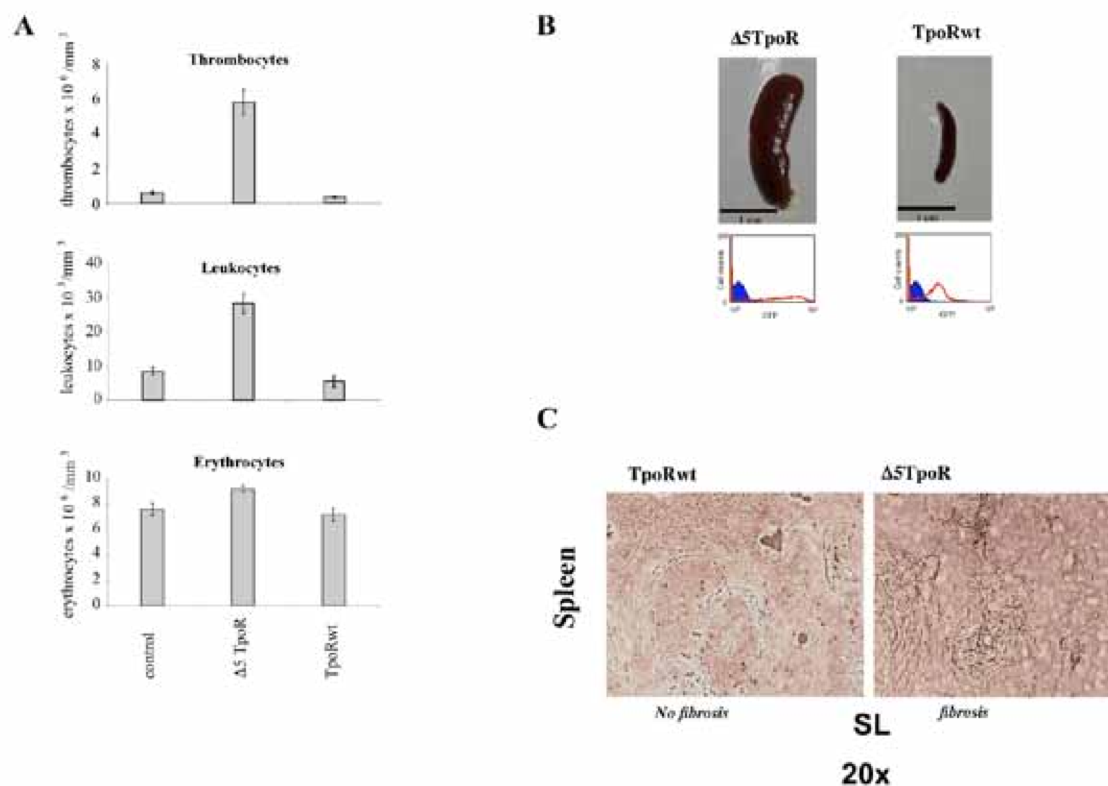

Figure 3. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ5TpoR is a mutant where the amphipathic RW515QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by Δ5TpoR. (B) Splenomegaly was induced by Δ5TpoR at day 45 post reconstitution. The spleen size in mice reconstituted with bone marrow transduced with wtTpoR was equivalent to that in control healthy mice. Green fluorescence protein (GFP) levels were equal after transduction, but enhanced migration to the spleen and proliferation explain the high GFP levels in Δ5TpoR spleens. (C) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates fibrosis of the spleen in the Δ5TpoR mice.

Determination of the interface and orientation of the activated cytokine receptors, such as EpoR and TpoR

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

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins. To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (8). 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.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins out of seven stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (8). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

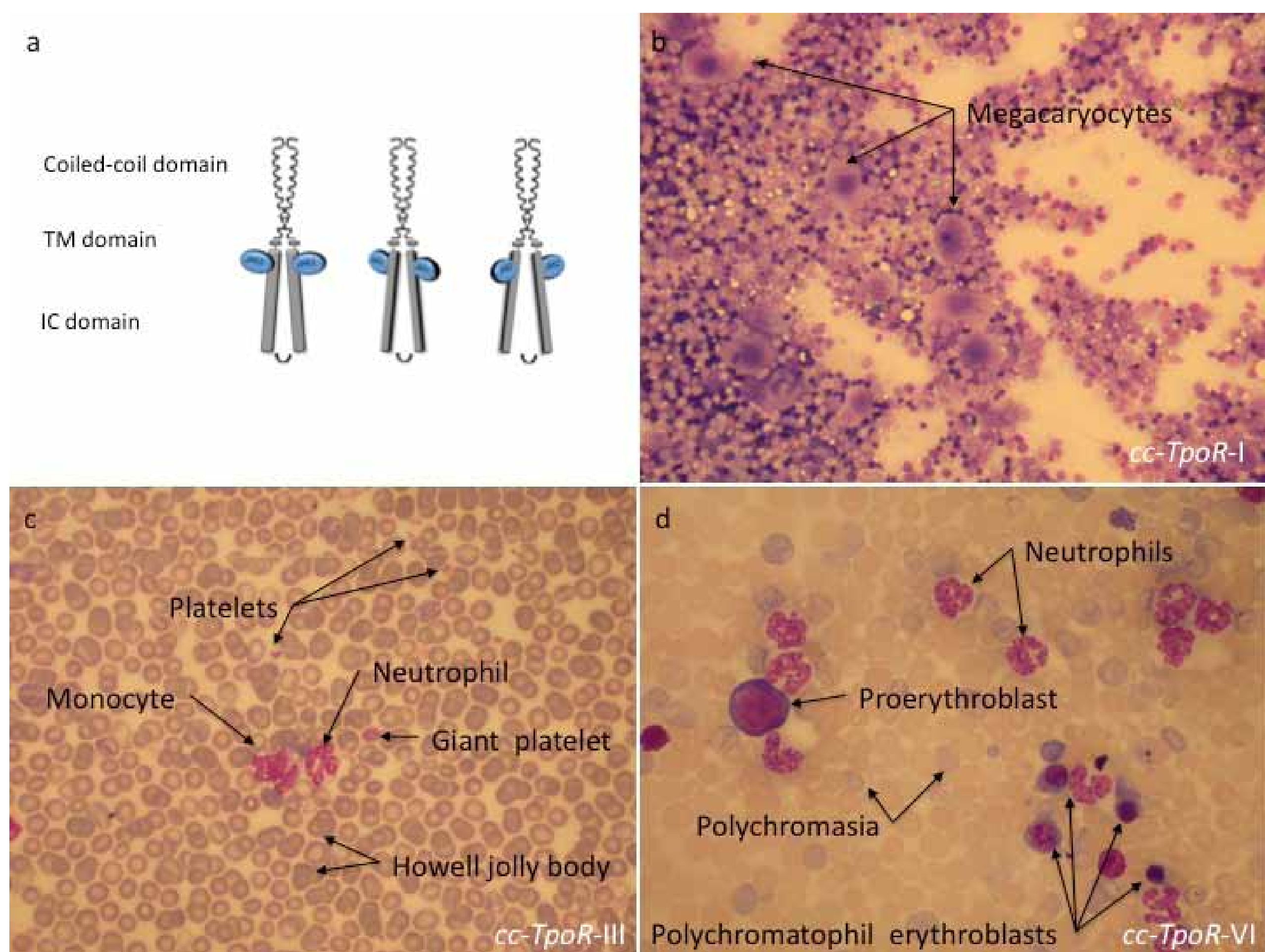

Figure 4. Different dimeric orientations of the thrombopoietin receptor lead to distinct phenotypes in the bone marrow and in the peripheral blood in bone marrow reconstituted mice. (A) The fusion of the coiled coil protein to differently engineered transmembrane domains of the thrombopoietin receptor (TpoR) imposes distinct dimeric orientations to the receptor. The different effects induced by three such chimeric receptors (cc-TpoR-I, cc-TpoR-III and cc-TpoR-VI) could be observed in the bone marrow and in the peripheral blood of lethally irradiated mice reconstituted with bone marrow cells retrovirally transduced with the indicated cc-TpoR fusion proteins. IC: intracellular, TM: transmembrane. (B) cc-TpoR-I induced a strong megakaryocytic response in the bone marrow with normal cellularity and a correct maturation of the myeloid lineages (May Grunwald stained bone marrow smear X 50, and normal platelet formation. (C) cc-TpoR-III induces granulocytosis, monocytosis and a very weak erythroblastosis in the peripheral blood (May Grunwald stained peripheral blood smear X 50) and weak dysplasia for the megakaryocytic and erythroid lineages in the marrow (not shown). (D) cc-TpoR-VI induced a stronger erythroblastosis and granulocytosis in the peripheral blood than cc-TpoR-III (May Grunwald stained peripheral blood smear X 50). (Jean-Philippe Defour).

Similar studies are undertaken for the related TpoR (Figure 4a). Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells. We have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the normal dimeric interface that leads to formation of platelets (Figure 4b), other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Figure 4c and d) (9). One orientation (cc-TpoR-II) corresponds to the inactive receptor state, and another (cc-TpoR-I) induces megakaryocyte differentiation and physiologic platelet formation (Figure 4b). The dimeric conformations represented by cc-TpoR-III and cc-TpoR-VI induce a combination of myeloproliferation and dysplasia of the erythroid lineage (9). 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 myeloid progenitors, as well as immortalization of myeloblasts. 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.

Structure and function of juxtamembrane and transmembrane sequences of transmembrane receptors

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We have previously shown that the EpoR as well as a fraction of IL2/IL9 receptors exist on the cell surface as a preformed ligand-independent inactive dimers (homomeric and heteromeric in the case of IL2/IL9 receptor complexes). For the EpoR, transmembrane domain interactions stabilize the inactive dimer at the surface and the EpoR TM sequence is an example of TM dimer based on purely hydrophobic sequences (Proc. Natl Acad. Sci USA 2001, 98, 4379-84; EMBO J., 1999, 18, 3334-47). We study potential transmembrane interactions in the context of other transmembrane proteins, such as TpoR, G-CSFR. We use cell surface immunofluorescence co-patching of differentially epitope tagged receptors along with protein ligation and protein fragment complementation assays in order to determine the ligand-independent state of cell surface complexes. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand. In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the groups of Profs. Jean-Noel Octave in our university and Steven O. Smith (SUNY Stony Brook, NY). We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these consecutive motifs promote transmembrane helix dimerization and amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733, Proc. Natl. Acad. Sci. USA 2009, 106, 1421).

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

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Cytokine stimulation of cytokine receptors induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1, 8). A similar picture has been noted in patient-derived leukemia cells. The critical question is which genes are specifically regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation (Chip) and sequencing of native promoters bound by STAT5 we noted that, in transformed cells, STAT5, and mainly STAT5B, can also bind to low affinity N4 (TTCNNNNGAA) DNA sites, not only to the high affinity N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) (10), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation (10). miR-28 is pathologically overexpressed in 30% of MPNs (10). Targets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders. Furthermore, miR-28 is specifically associated with megakaryocyte proliferation and induces a block in differentiation (10). We are studying the mechanisms of pathologic induction of LPP/miR-28 via constitutively active STAT5.

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

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

Selected publications

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2. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, Garçon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434:1144-8.

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Links

Group

Ludwig Institute for Cancer Research Ltd. NewsLink Sept 2005 of our group (http://www.licr.org/12124501528/newslink/0509/)

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

Research:

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

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

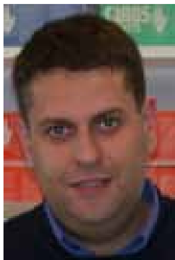
European Hematology Association (EHA)
Scientific Program Committee EHA16 (London), EHA17 (Amsterdam)
Hematology (American Society of Hematology Education Book)
http://www.asheducationbook.org/

Learning:

Biologie moléculaire de la cellule (Molecular Cell Biology H.F. Lodish) French Edition
Vainchenker W, Delhommeau F, Constantinescu SN, Bernard OA.
http://www.ncbi.nlm.nih.gov/pubmed/21653328"New mutations and pathogenesis of myeloproliferative neoplasms.
Blood. 2011 Aug 18;118(7):1723-35. Epub 2011 Jun 7. Review.

Bioinformatics:

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



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