de Duve
INSTITUTE

Scientific Report

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2014

DE DUVE INSTITUTE

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de Duve Institute

Ludwig Institute for Cancer Research

The de Duve Institute

The de Duve Institute • An international biomedical research institute

Emile Van Schaftingen

Originally named *International Institute of Cellular and Molecular Pathology* (abbreviated ICP), the de Duve Institute was founded in 1974 by Professor Christian de Duve († 4th of May 2013) to develop basic biomedical research with potential medical applications. *Excellence and freedom* of the researchers to choose their own line of investigation are perfectly illustrated by de Duve's dramatic reorientation of his research from biochemical problems linked to insulin's action towards an exploration of the cell leading him to the lysosomes and the peroxisomes. This freedom has a corollary, which is the responsibility to help make these discoveries translate into *medical progress*. Having worked as a team leader at the border between biochemistry and cell biology, de Duve insisted also on the importance of *collaborative work* and valued *interdisciplinary* research.

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

Benoît Van den Eynde

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions has been pursued ever since. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is

extremely close and the sharing of resources is considerable. The Director of the Brussels Branch of the Ludwig Institute is also a member of the de Duve Institute Directorate.

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

Miikka Vikkula

Frédéric Lemaigre

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Frédéric Lemaigre

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Miikka Vikkula

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Acknowledgements

In 2013, 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 2013 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

The «Haas-Teichen» fellowship was attributed to Audrey de Rocca Sera and Thoueiba Saandi,

the «Michel de Visscher» fellowship to Anubha Saxena,

the «Maurange» fellowship to Harakleia Episkopou,

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

and other fellowships have been awarded by the Institute to Anubha Saxena and Mylah Villacorte

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

The de Duve Memorial Lecture 2013

The Institute has decided to launch a biennial Memorial Lecture in honor of its founder, the late Professor Christian de Duve. The first of this Distinguished Lectures series was held on November 13, 2013. On this occasion, the Institute was honored to host Michael Hall, from the Biozentrum of the University of Basel. Professor Hall is well known for his discovery of the mTOR pathway, an important cellular signaling pathway sensing the presence of nutrients and growth factors, and favoring cell growth. The work of Michael Hall has been honored by numerous scientific prizes, including the prestigious Louis Jeantet prize in 2009.

Scientific prizes and Awards in 2013 - 2014

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

To Laurent Knoops • 2013
Prix Lambertine-Lacroix

To Jean-François Collet • 2014
Prix Henri Fauconnier 2011-2013

Doctoral theses (PhD) in 2013-2014

5 • 9 • 2013
Catheline PLAIDEAU, PHOS
Effect of modulating adenine nucleotide levels on AMPK activation by targeting AMP-metabolizing enzymes
Promoter: M. Rider

9 • 9 • 2013
Yang LIU, PHOS
Control of glucose uptake and protein synthesis during contraction in skeletal muscle
Promoter: M. Rider

14 • 11 • 2013
Teofila SEREMET, GECE
The mechanism whereby activated human T cells trigger inflammatory cytokine production by monocytes
Promoter: P. Coulie

18 • 11 • 2013
Emilie GAUTHY, GECE
GARP: how it controls TGF-b1 production in human regulatory T cells, and how its expression is regulated at transcriptional and post-transcriptional levels
Promoter: S. Lucas

2 • 12 • 2013
Amandine VAN BENEDEN, GEPI
Regulation of telomeric repeat-containing RNA expression by telomere length and heterochromatin in human cells
Promoter: A. Decottignies

6 • 12 • 2013
Mickael QUENTRIC, GEHU
Identification and characterization of predisposing genetic factors in patients with oral cleft and/or dental anomalies
Promoter: M. Vikkula

13 • 12 • 2013
Adrien GRIMONT, LPAD
Rôles des facteurs de transcriptions HNF6 et Sox9 dans l'initiation de lésions métaplasiques et néoplasiques du pancréas
Promoter: P. Jacquemin

7 • 2 • 2014
Pascale HERMANT, VIRO
Non-conventional interferons : Characterization of interferon epsilon (IFN-) and specificities of the interferon lambda (IFN-) response in the liver
Promoter: T. Michiels

27 • 2 • 2014
Antoine COMINELLI, CELL
La MMP-27, une MMP intracellulaire exprimée par des macrophages M2 dans l'endomètre et l'endométriose
Promoter: P. Henriet

23 • 4 • 2014
Valérie NICOLAES, BCHM-GRM
Characterization of new proteins maintaining the integrity of the *Escherichia coli* envelope: a step towards the development of new antibiotics
Promoter: J.-F. Collet

23 • 5 • 2014
Rachid AMSAILALE, BCHM-GRM
New insights into the regulation of deoxycytidine kinase activity via Ser-74 phosphorylation: toward improved activation of anticancer nucleoside analogs
Promoter: F. Bontemps

24 • 6 • 2014
Laure-Alix CLERBAUX, BCHM-GRM
Role of microRNA-33 family in lipid metabolism in mammals and in *Drosphila melanogaster*
Promoter: G. Bommer

The J.-F. Heremans Lecture

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

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

1978 Henry KUNKEL • Rockefeller University, New York, USA

1980 Michael SELA • Weizmann Institute of Science, Israel

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

1984 Avrion MITCHISON • University College of London, UK

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

1988 Zanvil COHN • Rockefeller University, New York, USA

1990 André CAPRON • Institut Pasteur de Lille, France

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

1994 Fritz MELCHERS • Basel Institute for Immunology, Switzerland

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

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

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

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

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

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

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

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

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

Plenary lectures 2013

July

Kathleen REYSKENS
Department of Physiological Sciences, Stellenbosch University, South Africa.
The maladaptive effects of the protease inhibitor Lopinavir/Ritonavir on the rat heart

September

Michael SAUNDERS and Hans de HAARD
arGEN-X, Gent, Belgium
arGEN-X, a therapeutic antibody biotechnology company encouraging target collaborations with academic groups

October

Mihai G. NETEA
Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
A systems approach to antifungal immunity

Virginie FAURE
MCF - Equipe 10: cancer, stress et dynamique de l'organisation du génome, Institut Albert Bonniot, Centre de recherche-INSERM/UJF -U823, Grenoble, France
The secret message of heterochromatin

November

Juan IOVANNA
Centre de Recherche en Cancérologie de Marseille (CRCM), Institut Paoli-Calmettes, Université d'Aix-Marseille, France
The stress protein Nupr1/p8 in development and progression of pancreatic cancer

Yves DE CLERCK
Children's Hospital Los Angeles and The University of Southern California, Los Angeles, CA, USA
Beyond the genes, the tumor microenvironment in cancer progression

Natacha ROCKS
Université de Liège, Liège, Belgium
Roles of polarized neutrophils on lung tumour development in an orthotopic lung tumour mouse model

1st de Duve Memorial Lecture
Michael HALL
Biozentrum - University of Basel, Switzerland
Tor signaling in growth and metabolism

December

Eduardo GROISSMAN
Howard Hughes Medical Institute, Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, USA
The Control of Stress-Responsive Pathways in Bacteria

Alessio PERACCHI
Department of Life Sciences Laboratory of Biochemistry, Molecular Biology and Bioinformatics, University of Parma, Italy
Pyridoxal phosphate-dependent enzymes: seeking functions for genes and vice versa

Plenary lectures 2014

January

Richard RANDALL
University of St Andrews, UK
Interferon induction by, and antiviral activities against, paramyxoviruses and influenza viruses

Tom LENAERTS
ULB Brussels & VUB Brussels
Proteins as communication units

February

Rym AGREBI
Aix-Marseille University
Emergence of an integrated system in prokaryotes: motility systems and their coordination in *M. Xanthus*

Jean-Marc TAYMANS
Research Group for Neurobiology and Gene Therapy, KUL, Leuven
Progress in understanding the cellular signaling of LRRK2, a proposed therapeutic target for Parkinson's disease

Salvatore VALITUTTI
INSERM U1043, CHU Purpan, Toulouse, FRANCE
Immunological Synapses in Human Health and Disease

Cherifa AYARI
Centre de recherche en cancérologie, Centre Hospitalier Universitaire de Québec, Université Laval
Optimisation de l'immunothérapie non spécifique du cancer superficiel de la vessie

Emile VAN SCHAFTINGEN
de Duve Institute, BCHM-GRM
Orphan enzymes in intermediary metabolism: molecular identification and function

March

Frédéric LEMAIGRE
de Duve Institute, LPAD
Unravelling the mechanisms of liver development. Importance for understanding disease.

Sarantis KORNIOTIS
Université Paris Descartes, Hôpital Necker, Paris
Tolerogenic B cell progenitors for cell therapy in experimental models of autoimmune diseases

Thomas BERNARDT
Harvard Medical School, MA, USA
How beta-lactam antibiotics tear down the (cell) wall

April

Frans SCHUIT
Dept Cellular and Molecular Medicine, KU Leuven
Lineage specific gene loss in birds: a natural gene knockout experiment?

Jo VAN GINDERACHTER
Myeloid Cell Immunology Lab, VIB,
Cellular and Molecular Immunology, Vrije Universiteit Brussel
Regulation, targeting and function of distinct tumor-associated macrophage subsets

May

Fabrice GOUILLEUX
Université de Tours, France
Stat5 signalling in hematopoietic neoplasms

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

June

B. DOMON
Luxembourg Clinical Proteomics Center, Centre de Recherche Public de la Santé (CRP-Santé)
Development of Proteomic Clinical Assays based on High-Resolution / Accurate Mass Spectrometry

Laszlo RADVANYI
Department of Melanoma Medical Oncology, MD Anderson Cancer Center, Houston, Texas, USA &
Chief Scientific Officer, Lion Biotechnologies, Tampa, Florida, USA
TIL Adoptive Cell Therapy for Melanoma: Recent Advances and Prospects for Commercialization

Pierre VAN DER BRUGGEN
Brussels Branch of the Ludwig Institute for Cancer Research
Are you using sugar to boost exhausted T-lymphocytes?

Research Groups

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

Miikka Vikkula

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

Venous malformations and glomuvenous malformations ("glomangiomas")

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

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

We previously discovered that rare, hereditary venous malformations are caused by an activating mutation in the endothelial cell receptor tyrosine kinase TIE2/TEK (Vikkula et al, 1996) (Fig 1). 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 inherited 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 in the affected tissue only, causing a local loss of its ability to function [1]. We have further shown that at least 50% of the far more common sporadic VMs are also caused by somatic mutations in TIE2 [1] (Soblet et al, 2013).

To understand how TIE2 mutations cause VMs, we carried out functional analyses of the effects of VM-causative mutations on endothelial cells, in vitro and in vivo. We found that the aberrant activation of AKT by VM-causative mutations inhibits the transcription factor FOXO1, resulting in a lack of the major smooth muscle cell recruitment factor PDGFB [2]. This would account for the sparse, irregular mural layer characteristic of VM. The importance of the AKT pathway in VM pathogenesis is borne out by the ability of the mTORC inhibitor, rapamycin, to control the expansion of lesions in a mouse model of the disease (Boscolo et al, submitted). It also ameliorated symptoms in a preliminary trial of patients with VMs recalcitrant to conventional therapies. In an effort to identify the cause of the 50% of sporadic VMs in which no TIE2 mutations are detected, we are using Next Generation Sequencing Technology to carry out deep-sequencing of DNA from a series of VM tissues.

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 (Brouillard et al, 2002). So far, we have identified GLMN mutations in 162

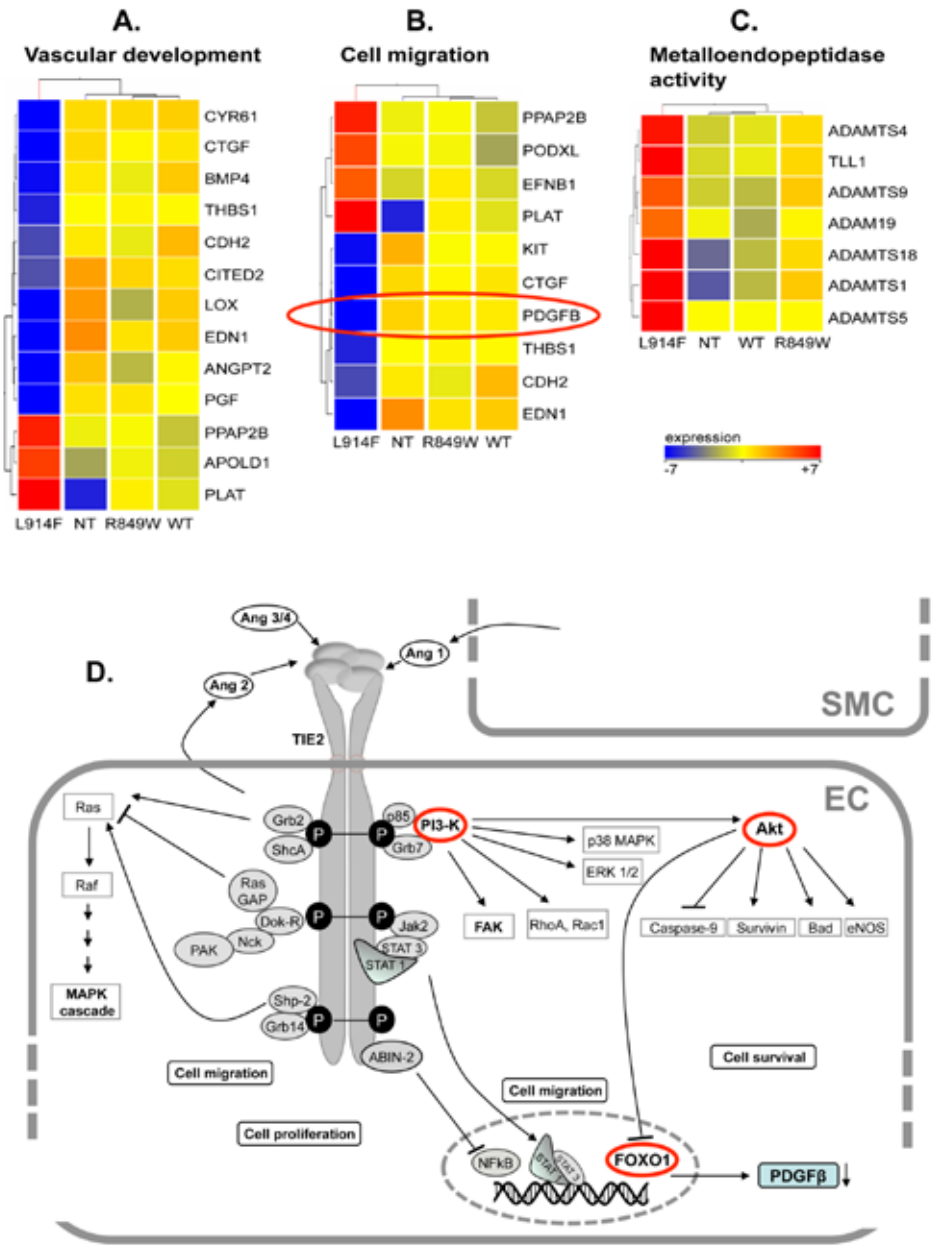

Figure 1. Effects of TIE2 mutations on endothelial cell transcription. The most frequent TIE2 mutation, L914F, dysregulates genes that participate in angiogenesis (A), cell migration (B), and extracellular matrix turnover (C). In particular, it causes an AKT-dependent deficiency of PDGFB, a major smooth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

families, the most frequent being present in 44% (Brouillard et al, 2013). We have also discovered that the double-hit mechanism holds true for most if not all GVMs, predominantly in the form of acquired uniparental isodisomy (aUPID) of chromosome 1p [3]. This renders the inherited glomulin mutation homozygous in affected tissues, without loss of genetic material. To better understand the role of glomulin, we generated glomulin-deficient mice, which are embryonic-lethal early in development (Brouillard et al, unpublished). While also lethal, conditional knock-down of glmn (using RNAi technology) during midgestation allows embryos to develop further. These mice exhibit multifocal vascular defects (Nguyen et al, unpublished), and therefore serve as a model in which to study the role of glomulin in vascular development and in GVM.

Lymphedema

P. Brouillard, M. Schlögel, E. Fastré, 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; A. Dompmartin, CHU, Caen, France.

Lymphatic anomalies include a variety of developmental and/or functional defects affecting lymphatic vessels: sporadic and familial forms of primary lymphedema, secondary lymphedema, chylothorax and chylous ascites, lymphatic malformations, and overgrowth syndromes with a lymphatic component. Lymphedema leads to disabling swelling that occurs mostly on the extremities, and is extremely difficult to treat. Inherited mutations have been identified in at least 20 genes encoding proteins that participate in signaling, mainly by VEGFR-3, but also by other tyrosine kinase receptors (Fig 2) [4]. These mutations exert their effects via the RAS/MAPK and the PI3K/AKT pathways and explain about a quarter of the incidence of primary lymphedema, mostly the inherited forms. We are now using Next Generation Sequencing in order to identify additional players, which likely interact with these pathways and cause lymphedema. Most murine knock-outs of lymphedema-causative genes are homozygous lethal, while heterozygotes are healthy, pointing to differences in human and murine physiology, and the influence of other factors (Brouillard et al, 2014). We have found that mutations in SOX18 not only cause the Hypotrichosis-Lymphedema-Telangiectasia syndrome, but a specific Stop codon leads to severe glomerulonephritis that requires renal transplantation in the short term (Moalem et al, 2014).

Vascular anomalies affecting capillaries

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

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as "portwine" stain), a localized defect of capillary-like vessels. Hemangiomas have a frequency of up to 12% in 1-year-old children, and typically undergo a period of rapid expansion followed by spontaneous regression. We have an extensive collection of tissue and blood samples of hemangiomas, and are using Next Generation Sequencing in an effort to identify causative genomic variants. These likely perturb VEGF signaling, which -work done with collaborators- has demonstrated is key in the pathogenesis of hemangioma [5].

CMs occur in 0.3% of newborns. Unlike hemangiomas, they persist throughout life if left untreated. Certain capillary malformations affect specific organs, such as the brain in the case of cerebral capillary malformations (CCMs). We discovered that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with CCM are caused by a muta-

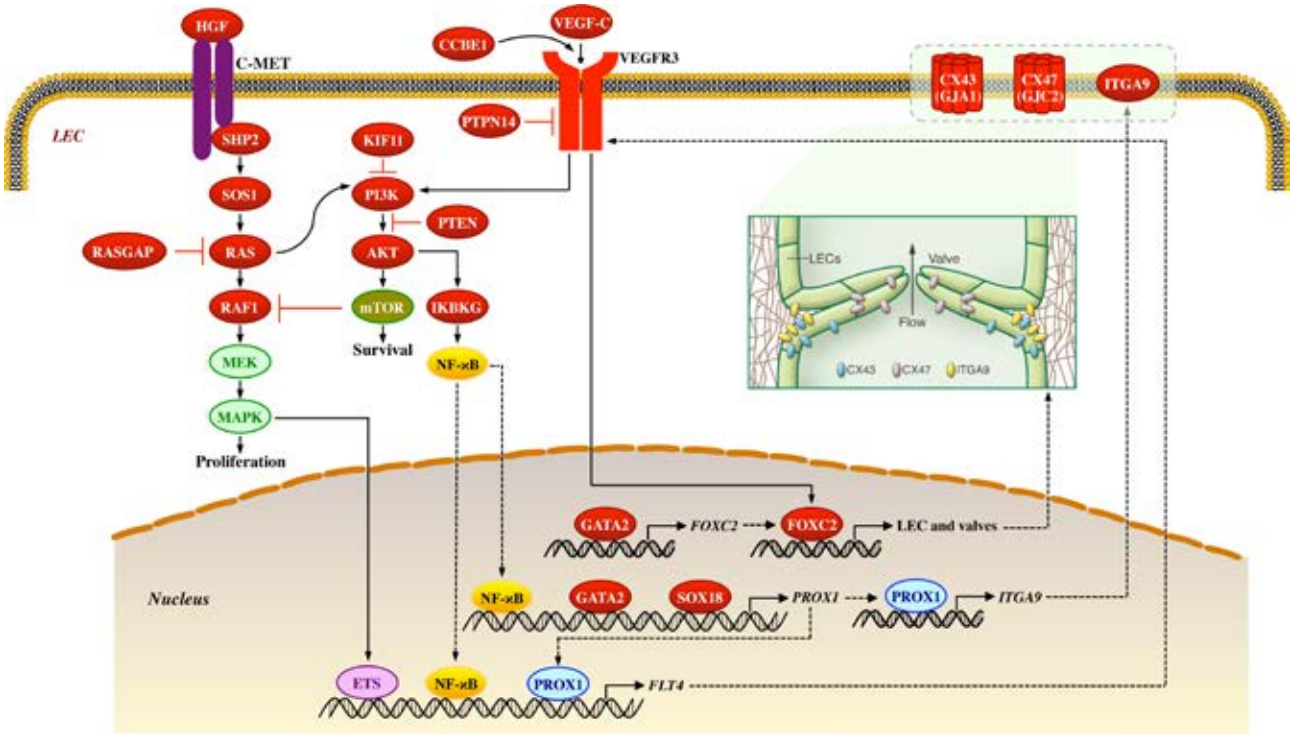

Figure 2 - Schematic view of a lymphatic endothelial cell (LEC) and valve.

Lymphatic anomaly-associated proteins are frequently associated with the VEGF-C/VEGFR-3 signaling pathway. Proteins mutated in lymphedema or other lymphatic disorders are shown in red.

tion 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 112 index patients. This has allowed for a more precise clinical description of the clinical signs and symptoms associated with this newly recognized disorder that we have named Capillary malformation-arteriovenous malformation (CM-AVM) [6, 7]. 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 syndrome, and Vein-of-Galen aneurysmal malformations, which warrant careful clinical management. Subsequently we tested RASA1 in overlapping phenotypes, such as sporadic CM with limb overgrowth and Klippel-Trenaunay syndrome. We did not identify any clear pathogenic change in these patients (Revencu et al, 2013).

Cleft lip and palate

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

Cleft lip and palate (CLP) is a congenital anomaly of complex etiology. Predisposition is governed by numerous genetic loci, in combination with environmental factors. Clefts have an incidence of 1/700 births. We have 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 [8]. This gene is associated with clefts across populations. Zebrafish studies confirmed its role during embryonic development and jaw formation. We have begun to use Next Generation Sequencing (NGS) in an effort to uncover additional genes that play a role in non-syndromic orofacial clefts.

Cerebral tumors

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

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymomas. To better understand the molecular alterations leading to ependymomal oncogenesis, we performed microarray-based expression profiling on a series of 34 frozen ependymomas. Results of our profiling study are in concordance with the "oncology recapitulates ontology" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype. Using array-CGH, we recognized a subgroup of supratentorial ependymomas affecting young adults, which are characterized by trisomy of chromosome 19.
Within the posterior fossa compartment, ependymomas cluster into three sub-groups. The first corresponds to ependymomas that are histologically of WHO grade II, the second to those of WHO grade III, and the third to 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

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

Pheochromocytomas and head and neck paragangliomas are neuroendocrine tumours derived from the neural crest. Paragangliomas are associated with parasympathetic ganglia and are usually non-secreting. By contrast, pheochromocytomas are derived from paraganglia associated with the 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 >200 patients with pheochromocytoma and paraganglioma, and more recently >50 patients suffering from thyroid tumours has been obtained.
The SDH genes code for the subunits of succinate deshydrogenase, at the crossroad of the mitochondrial respiratory chain and Krebs cycle. The four subunits of succinate deshydrogenase, i.e. SDHD, SDHB, and more rarely SDHC and SDHA, have

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

We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine dissemination. No evidence of mutations was found in the known predisposing genes by dHPLC and/or SSCP. An in depth search for the genetic abnormality underlying this unusual form of paraganglioma disclosed a large deletion encompassing SDHB and part of TIMM8B, a gene encoding a mitochondrial chaperone, which may account for the severe phenotype observed in this family.

Our group was also involved in an international collaborative effort to look for the clinical spectrum associated with mutations of TMEM127, a recently described pheochromocytoma susceptibility gene.

Essential hypertension

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

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

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

Hematological malignancies and tumors of soft tissue and bone

H. Antoine-Poirel, 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, P. Brouillard, R. Helaers and M. Vikkula

We host the UCL microarray platform (Affymetrix), used by several groups in the de Duve Institute, other institutes of the medical faculty of UCL and Cliniques universitaires Saint-Luc for expression profiling as well as genotyping. We also collaborate with several groups from around the world on whole genome mapping studies. In work done with Dr Jüppner (Harvard Medical School, Boston), we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes (Lorenz-Depiereux et al, 2006). In collaboration with G. Matthijs (KULeuven), we have used autozygosity mapping along with expression profile analysis

to identify a new gene for congenital glycosylation disorder [9]. In collaboration with Dr Vermeesch (KULeuven), we established that chromosome instability is common during early human embryogenesis in study of 23 pre-implantation embryos from 9 fertile couples. Additionally, rearrangements such as segmental imbalances were observed in 70% of the 23 embryos tested. This explains low human fecundity and identifies post-zygotic chromosome instability as a leading cause of constitutional chromosomal disorders [10].

In an exciting development, this platform is now complemented by a High Throughput Sequencing platform. Funded by the Fondation Contre le Cancer, it consists of a Solid 5500XL sequencer (Life technologies), a Personal Genome Machine (Ion Torrent, Life technologies) and a computing cluster for bioinformatics processing. This equipment allows us to perform Exome-seq, Genome-seq, RNA-seq, Small RNA profiling, ChIP-seq and methylation studies. Data analysis is performed using Lifescope software (Life technologies), and a combination of open source packages (BWA, GATK, snpEff). Downstream evaluation and prioritization of variants is performed using "Highlander", a package that integrates several in-silico analysis programs and utilities a user-friendly graphical interface (developed in-house by Raphaël Helaers, Ph.D., Bioinformatician). This enhances our ability to identify and explore the genetic and epigenetic bases of disease.

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Liver and pancreas development

Frédéric Lemaigre
Patrick Jacquemin

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

Liver development

Y. Achouri, J.-B. Beaudry, C. Demarez, A. Poncy, T. Saandi

The main cell types of the liver are the hepatocytes, which exert the metabolic functions of the organ, and the biliary cells (cholangiocytes) which delineate the bile ducts. We study how the hepatocytes and biliary cells differentiate and how bile ducts are formed in the embryo. Our preferred model organism to investigate liver development is the mouse. This includes generation and analysis of transgenic mouse lines with help of the Transgene Technology platform which is managed by our research group.

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

We have identified molecular markers that enabled us to revisit the morphogenesis of the intrahepatic bile ducts, and to uncover that biliary morphogenesis occurs according to a new mode of tubulogenesis [2]. Starting from this new knowledge, we analysed several mouse models and samples from human liver fetuses affected with biliary anomalies. Our findings led us to propose a new pathophysiological classification of biliary malformations [3]. This work is being pursued with a focus on polycystic liver disease.

In search for new molecular determinants of biliary differentiation, we further investigated the transcription factor network that drives cholangiocyte differentiation and bile duct formation. Using liver-specific gene inactivation strategies we found that Sox9 controls the timing of bile duct development [reviewed in 2], and are currently finalising the work on the role of another Sox factor family member. The latter is a new key regulator that is predominantly expressed in biliary cells and which is essential for cholangiocyte differentiation and polarization.

Transcription factors determining how hepatoblasts differentiate toward the cholangiocyte or hepatocyte lineages have also been studied in collaboration with Dr. Beatriz Sosa-Pineda (St. Jude Children's Research Hospital, Memphis, USA). Our earlier work and that of others had defined a gene regulatory network promoting cholangiocyte differentiation. This network has now been extended by integrating the function of the Prox1 transcription factor (Figure 1) [4].

MicroRNAs stand out as essential regulators of cell differentiation. We have now analysed the microRNA expression profile of developing biliary cells purified from embryonic liver. This

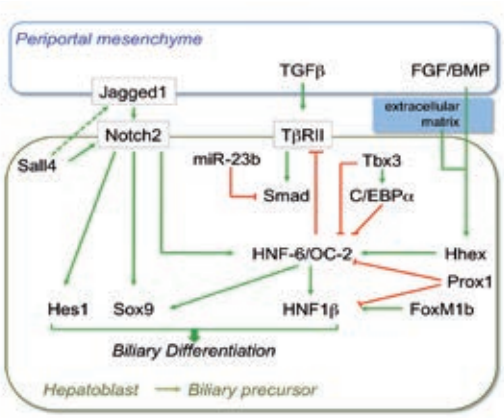

Fig. 1. Molecular mechanisms of biliary differentiation. The periportal mesenchyme signals to the hepatoblasts and controls their differentiation to the biliary lineage. A transcriptional network regulates differentiation within the hepatoblasts (adapted from Lemaigre FP. Progress in Molecular Biology and Translational Science, Elsevier, 2010).

enabled us to select microRNAs that are candidate regulators of cholangiocyte differentiation, and we are currently investigating how these microRNAs control cell differentiation in normal developing liver as well as in cholangiocarcinoma.

Along the same lines, cell differentiation is tightly controlled by intercellular signaling mechanisms. Several signaling pathways stimulate differentiation of developing biliary cells. Our group identified TGFβ signaling as a key driver of this process [1], and contributed to the understanding of the role of the Notch pathway [5]. We pursue this work by investigating the function of the Wnt signaling.

Beyond the mechanisms of cell differentiation, the fate of differentiated cells needs to be considered. The availability of methods allowing to trace the cells in developing and adult organisms enabled us to analyse in detail the fate of biliary cells in the embryo and in adult liver. The classical model of liver development predicted that hepatoblasts give rise to hepatocyte precursors, which mature to hepatocytes, and to ductal plate cells, which generate the cholangiocytes. However, only a fraction of the ductal plate cells contribute to bile ducts, while the remaining part was considered to involute by apoptosis. We have now used a lineage tracing approach in the mouse, which consisted in genetically labeling the ductal plate cells in the embryo, followed by the analysis of their progeny after birth. This uncovered a new fate map of the hepatic cells: the ductal plate cells were shown to generate bile ducts, but also a subset of periportal hepatocytes, as well as cells lining the canals of Hering which harbor the adult liver progenitor cells (Figure 2) [6].

To investigate the fate of biliary cells and adult liver progenitor cells in normal homeostatic liver and in disease (liver regeneration) we generated a new mouse line. This line expresses tamoxifen-inducible Cre recombinase specifically in the cholangiocytes and adult liver progenitors, under control of the Osteopontin gene (OPN-CreER mice). Similar to our previous work on genetic marking of ductal plate cells in the embryo, the OPN-CreER mice enable us to genetically label biliary cells and progenitors in adult liver and to follow their fate. In collaboration with the Laboratory of Hepato-Gastroenterology, Université catholique de Louvain (Prof. I. Leclercq), we provided the first in vivo demonstration that biliary cells or liver

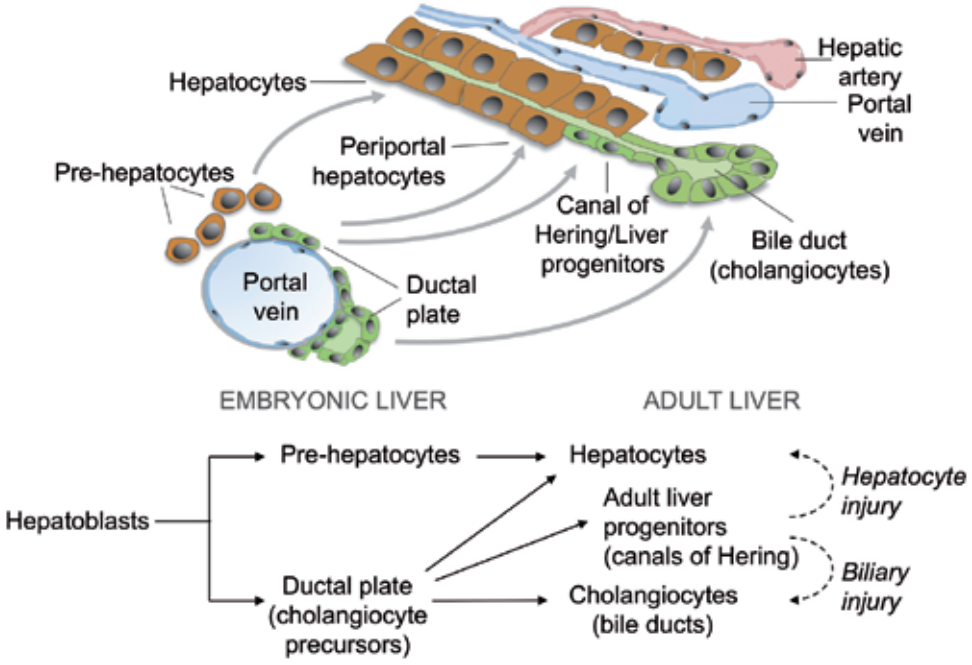

Fig. 2. Fate map of the hepatic cells in embryonic and adult liver (adapted from Lemaigre, Hepatology 2014; 59:726-728).

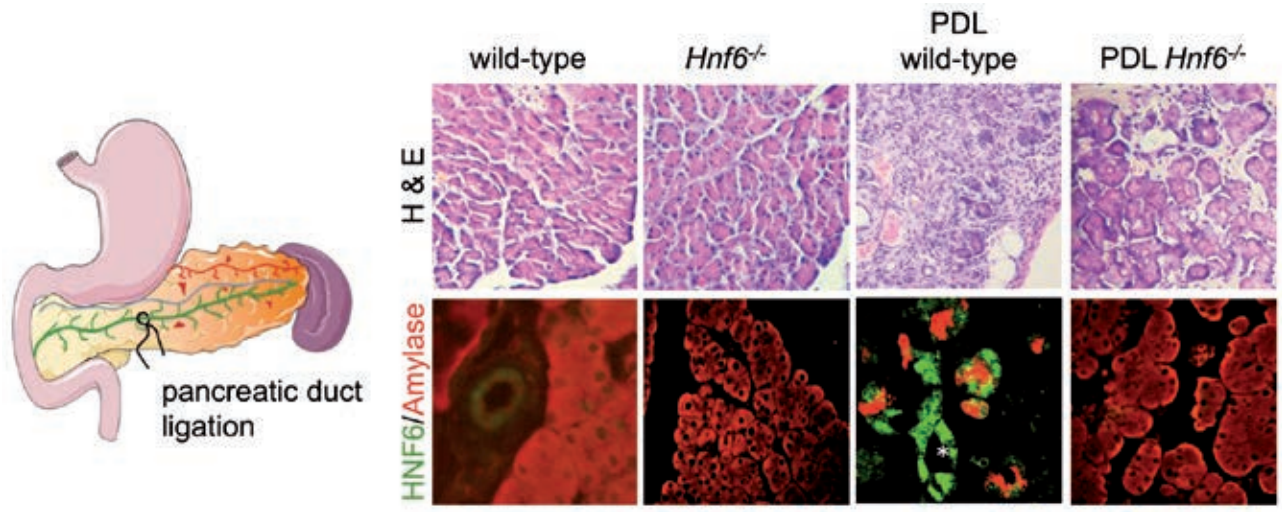

Fig. 3. When the pancreatic duct is ligated, inflammation induces acinar-to-ductal metaplasia (H&E, hematoxylin-eosin staining). HNF6 (green) is induced in acinar cells while the acinar cells loose expression of the acinar-specific gene amylase (red). In the absence of HNF6, pancreatic duct ligation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

progenitor cells differentiate to functional hepatocytes after liver injury [7]. Therefore, our work provides an updated cell fate map of embryonic and adult liver progenitor cells (Figure 2). This work is being pursued in collaboration with Prof. Leclercq and we investigate the fate of cells in various disease conditions.

Our work also addresses the mechanisms of hepatocyte differentiation. HNF6 and OC2 are transcription factors that control a number of genes in hepatocytes. In addition, HNF6 and OC2 are critical for normal differentiation of hepatoblasts to hepatocytes or cholangiocytes : in the absence of HNF6 and OC2, the hepatoblasts generate hybrid hepato-biliary cells instead of distinct hepatocyte and cholangiocyte populations [1]. HNF6 also fine-tunes gene expression during hepatocyte differentiation. This involves a feedback loop with microRNAs, in which the liver-specific miR-122 and HNF6 stimulate each other's expression while promoting hepatocyte maturation [8]. Further investigation of this network suggests that miR-122 indirectly stimulates HNF6 expression by repressing a microRNA that functions as a HNF6 repressor.

Pancreatic cell differentiation

Y. Achouri, C. Augereau, C. Gérard, A. Grimont, P.-P. Prévot, T. Saandi

In the embryo, the pancreas develops as an outgrowth of the endoderm, the cell layer that delineates the primitive gut. Pancreatic progenitors derived from the endoderm give rise, through a stepwise process, to endocrine, acinar and duct cells. Our group investigates how pancreatic cell types differentiate in the embryo and how differentiation is perturbed in adult pancreas in the context of tumorigenesis of pancreatic ductal adenocarcinoma. Similar to our approach in liver-related research, we address differentiation in the pancreas by focusing on the role of transcription factors, microRNAs and signal transduction pathways.

The role of the transcription factor HNF-6 in pancreas development is being studied since several years. We showed that HNF6 is required for development of endocrine cells and pancreatic ducts [9]. After birth, HNF6 is expressed exclusively in the duct cells where it most likely maintains duct cell identity. Interestingly, pancreatic ductal adenocarcinoma may derive from acinar cells which switch their phenotype from acinar to ductal during progression to cancer. This process is called acinar-to-ductal metaplasia, and constitutes a preneoplastic state. We hypothesized that the switch in cell identity depends on the ectopic expression of ductal transcription factors and tested if HNF6 is ectopically induced in acinar cells undergoing metaplasia. This was the case in human pancreas. In addition, we also collected evidence from mouse models that induction of a HNF6 – Sox9 cascade in acinar cells promotes acinar-to-ductal metaplasia, suggesting that these factors are key inducer of preneoplastic lesions (Figure 3) [10].

Our results on the HNF6 – Sox9 cascade raised the question of the targets of Sox9 in acinar-to-ductal metaplasia and during tumor progression. In collaboration with the Department of Pathology of the Université catholique de Louvain (Prof. Christine Sempoux) and with the teams of Ilse Rooman and Andrew Biankin (Garvan Institite of Medical Research, Sydney, Australia) we currently investigate how Sox9 controls Epidermal Growth Factor signaling. An additional question relates to the requirement of Sox9 expression in tumor progression. Using a mouse model where pancreatic adenocarcinoma is induced by inflammation and expression of the oncogenic mutant K-RasG12D protein, we and others found that inhibition of Sox9 protects against tumor development (Figure 4).

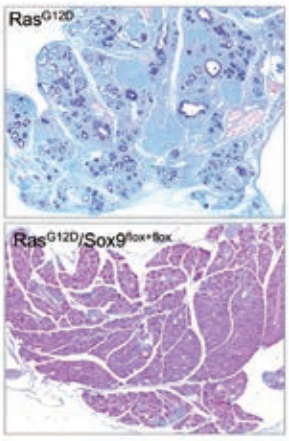

Fig. 4. Chronic inflammation combined with expression of oncogenic K-RasG12D (Elastase-CreER x lox-stop-lox- K-RasG12D) in mouse acinar cells induces lesions called Pancreatic Intraepithelial Neoplasia, which are detected by dark blue staining using alcyan blue (top). In the absence of Sox9 (Elastase-CreER x lox-stop-lox- K-RasG12D x Sox9flox/flox), such lesions fail to develop (bottom). Both sections are stained with Alcyan blue/Hematoxylin/Eosin.

MicroRNAs were also found to play a role in establishment and maintenance of acinar cell differentiation. We discovered that microRNAs are required in normal pancreas to repress HNF6 expression in acinar cells; in the absence of microRNAs, and more specifically when miR-495 and let-7b are inhibited, HNF6 becomes ectopically induced in acinar cells. This is sufficient to initiate conversion of acinar cells toward a ductal phenotype: acinar cells loose acinar marker expression, while adopting a duct-like morphology [10]. We currently pursue this work by searching for new microRNAs controlling acinar-to-ductal metaplasia and tumor progression. We have also initiated new research on mathematical modeling of the gene network driving metaplasia.

Conclusions

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

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Signaling Crosstalk in Skin Stem Cells

Wen-Hui Lien

The epidermis is in dynamic equilibrium and is constantly renewed throughout life. Skin epithelial stem cells that can self-renew and differentiate provide the unlimited source of cells required for long-term tissue morphogenesis, homeostasis, and injury repair. Skin epithelial stem cells encompass epidermal stem cells and hair follicle stem cells. The behavior of stem cell is fine-tuned by the microenvironmental cues and their responsive intracellular signaling regulation. The main interest of our group is to understand how one signaling pathway integrates to another and how their downstream regulators crosstalk to each other in stem cells and/or with neighboring cells in molecular and cellular levels. Our studies will help us better understand how stem cells function in the defined adult organs, and how to use stem cells to treat diseases and cancer.

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

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

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

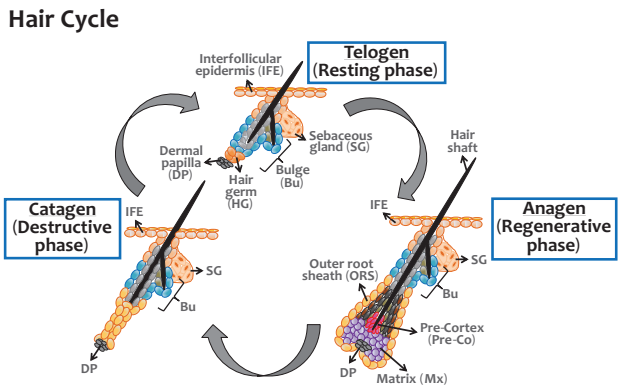

Fig 1. Hair cycle. During the resting phase (telogen), HFSCs residing in the bulge (Bu) remain in quiescence as the terminally differentiated inner bulge cells express high levels of inhibitory signals. At the onset of the regenerative phase (anagen), activated HFSC progeny located in hair germ (HG) proliferate and initiate HF regeneration in response to the activating cues produced from crosstalk with the underlying mesenchymal stimulus, referred to as the dermal papilla (DP). Soon after, HFSCs in the bulge are also activated. Some activated HFSCs move downward from the bulge along the outer layer of HFs (ORS), creating an inverse gradient of proliferative cells that fuel the continued

production of most proliferative, transient amplifying matrix cells (Mx) at the base of the full anagen HFs. In response to high levels of Wnt signaling, matrix progenitors in the pre-cortex region (Pre-co) terminally differentiate to form the hair shaft (HS). At the end of anagen, HFs enter a destructive phase (catagen) and the matrix and much of the lower part of the HF undergo apoptosis. As the epithelial strand regresses, DP is drawn upward towards the bulge/HG and the HF re-enters telogen. IFE, interfollicular epidermis; SG, sebaceous gland.

Tissue homeostasis and regeneration require a precise balance between quiescence and activation of adult stem cells. What keeps stem cells in their bulge niche during times of quiescence and what prompts them or their immediate progeny to regenerate the follicle during hair growth or repair the epidermis upon wounding? The behavior of stem cell is fine-tuned by the microenvironmental cues and their responsive intracellular signaling regulation. Transcriptional profiling of purified HFSCs in the adult bulge has provided a molecular signature which offers some insights into the possible mechanisms. Coupled with gene profiling, subsequent functional studies have revealed several signaling pathways, including BMP, Wnt and TGFβ signaling pathways, essential for maintaining stem cells in either a quiescent or activated state. Thus, studying the mechanism underlying signaling-mediated regulation on stem cell function becomes important for regenerative medicine and also for treatment of pathological conditions such as cancer.

In our laboratory, we use mouse skin as a model system to address this important biological question. Our research mainly focuses on understanding how one signaling pathway integrates to another and how their downstream regulators cross-talk to each other in hair follicle stem cells and/or with their neighboring cells in molecular and cellular levels.

Regulation of hair follicle stem cells by Wnt signaling

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

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

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

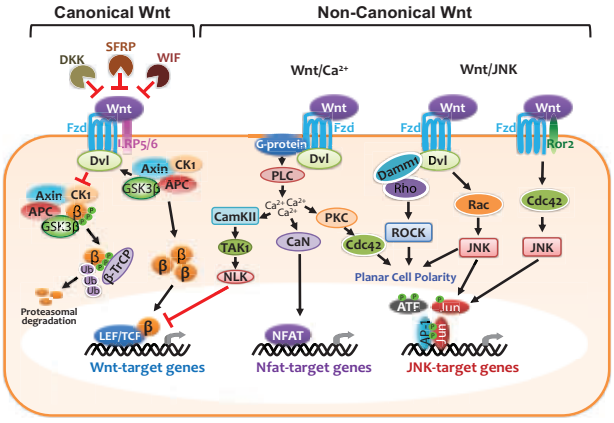

Fig 2. Wnt signaling pathways. This schematic diagram displays simplified canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) Wnt signaling pathways. In the absence of Wnt, β-catenin is targeted by a destructive complex that phosphorylates β-catenin for its degradation. Binding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

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

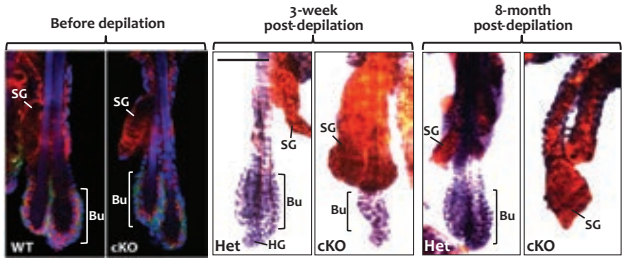

Fig 3. β-catenin is essential for HFSCs to make hair follicle fate choice. Immunofluorescence staining (left panel) shows that β-catenin-deficient HFSCs (cKO) could be maintained in a quiescent state without losing stemness (Red, β-catenin; Green, stem cell marker CD34); however, upon depilation-induced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

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

While Wnt/β-catenin pathway has been extensively studied in stem cells, non-canonical Wnt pathways are underappreciated and remain elusive. In the quiescent hair follicle stem cell niche, perceived Wnt signaling is low, corresponding to the high levels of TCF3, TCF4 and TLEs as well as BMP and calcium signaling, which leads to high levels of nuclear NFATc1, typically viewed as a non-canonical Wnt effector. In one of our recent collaborative studies, we revealed Nfatc1-bound target genes in quiescent HFSCs (3). Interestingly, by comparing gene targets of HFSC key regulators, we found that TCF3, TCF4 and NFATc1 share a cohort of target genes that are usually highly expressed in quiescent HFSCs and many of which involve in regulation of HFSC maintenance. This intriguing finding leads to one important question: whether Wnt/β-catenin signaling and non-canonical Wnt pathways crosstalk to each other to maintain HFSC function and to modulate stem cell behavior.

In order to address this important question, our group will couple with multiple technologies, such as transgenic mouse model, fluorescent activated cell sorting (FACS), chromatin-immunoprecipitation sequencing (ChIP-seq), primary cell culture, and proteomic approaches to better understand how Wnt signaling pathways integrate to each other and how their downstream regulators cross-interact in stem cells and/or with neighboring cells in molecular and cellular levels. Our long-term goal is to appreciate how stem cells function in the defined adult organs and how to use stem cells to treat diseases and cancer.

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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, cladribine and fludarabine, which have revolutionized the treatment of indolent lymphoproliferative disorders. Despite their efficacy, clinical resistance to these drugs is frequently observed. The main objectives of our present studies are to unravel the mechanisms leading to resistance to nucleoside analogues and to find novel therapeutic strategies to counteract them, particularly in chronic lymphocytic leukaemia.

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

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

Mechanisms of resistance

To improve our understanding of the mechanisms of resistance to nucleoside analogues in leukaemic cells, we investigated the EHEB cell line, a continuous cell line derived from a patient with CLL, which was found to be less sensitive (about 10-fold) to CdA than sensitive primary CLL cells. Resistance of EHEB cells to CdA could be partly explained by a lower intracellular accumulation of CdATP, its active metabolite, due to reduced dCK activity. In addition, analysis of the cell cycle showed that CdA induced accumulation of cells in S phase due to acceleration of the G1/S phase transition (2). This cell response was surprizing because nucleoside analogues are known to activate the transcription factor p53, which typically results in the accumulation of p21, an inhibitor of the cyclin-dependent kinase 2 (Cdk2), and in arrest of cell cycle. This unexpected 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, although p53 was upregulated (3). This p21 depletion resulted from increased proteasomal degradation, which had already been reported after UV-irradiation, but never after nucleoside analogue treatment. In addition, depletion of p21 was accompanied by Cdk2 activation, which could explain the activation of the cell cycle by CdA, and by monoubiquitination of PCNA (proliferating cell nuclear antigen). This post-translational modification is a process known to promote translesion DNA synthesis and favour DNA repair and cell survival. Further work is needed to determine whether PCNA monoubiquitination could play a role in the clinical resistance to nucleoside analogues.

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

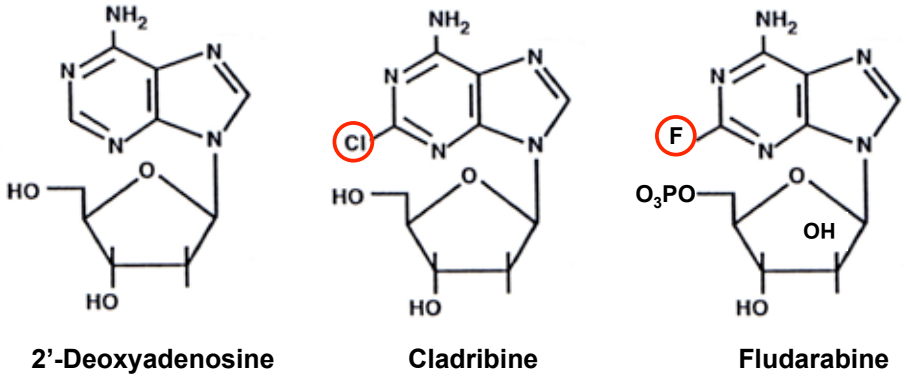

Fig. 1. Structures of 2'-deoxyadenosine and antileukaemic nucleoside analogues.

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

Potentiation of their action

Aiming to improve the efficacy of nucleoside analogues, we investigated their combination with DNA-damaging agents, such as cyclophosphamide derivatives (6) or UV-light. We showed that combination of CdA with these agents resulted in synergistic cytotoxicity in CLL lymphocytes, which could be explained by inhibition of DNA repair. The *in vitro* synergy between CdA and cyclophosphamide 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, known as a survival pathway. We observed that CdA, at concentrations close to the IC_{50}, activated the ERK pathway in EHEB cells. Because this activation is assumed to produce anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway, which were found to enhance CdA-induced apoptosis. Therefore, the efficacy of CdA could be strengthened by combining it with inhibitors of the ERK pathway (7).

Activation by deoxycytidine kinase

Deoxycytidine kinase (dCK) is the limiting enzyme in the activation of CdA, fludarabine and several other nucleoside analogues used in anticancer and antiviral therapy (Fig. 2). Study of the mechanisms that control the activity of this enzyme is thus of particular interest. We demonstrated that dCK is a phosphoprotein, containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74. Moreover, we showed by site-directed mutagenesis that Ser-74 phosphorylation only plays a role in the control of dCK activity (8). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from CLL patients in which variability in dCK activity could be related to variations in basal Ser-74 phosphorylation level. Treatment of these cells with genotoxic agents (CdA, UV light, etoposide, genistein and aphidicolin…) was found to increase dCK phosphorylation on Ser-74 in close parallel with dCK activity, suggesting that all these agents in-

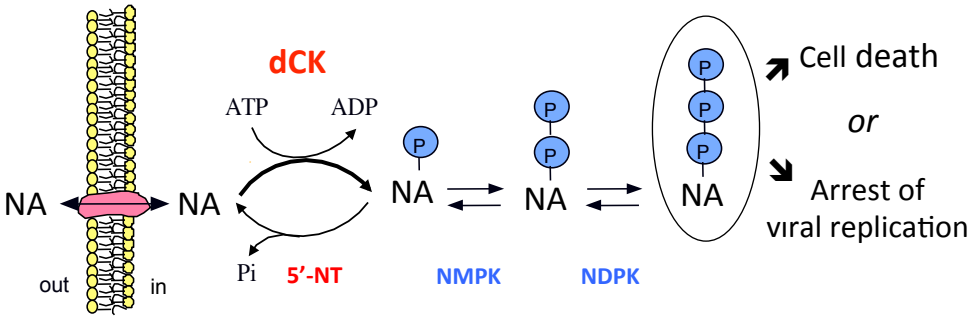

Fig. 2. Activation of nucleoside analogues.

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 often the rate limiting-step in the activation of NA. 5'-NT, 5'-nucleotidase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase.

crease dCK activity *via* Ser-74 phosphorylation. Conversely, Ser-74 phosphorylation was decreased by osmotic stress, which reduced dCK activity. 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 in basal conditions as well as after DNA-damaging treatment. We showed that casein kinase 1 δ can phosphorylate dCK on Ser-74 and increase its activity *in vitro*, but not *in vivo*, while another group recently reported that ATM, a sensor of DNA damage, can phosphorylate Ser-74 *in vitro* and *in vivo* and so activate dCK. As for Ser-74 dephosphorylation, we showed that protein phosphatase 2A (PP2A) was involved in Ser-74 dephosphorylation in intact cells and was therefore a negative regulator of dCK activity (9).

On the other hand, we investigated whether an increase of Ser-74 phosphorylation could enhance dCK activity towards nucleoside analogues. Studies with recombinant dCK showed that mimicking Ser-74 phosphorylation by a S74E mutation increases the catalytic rate of dCK toward CdA and clofarabine, but not fludarabine, indicating that the effect of Ser-74 phosphorylation on dCK activity depends on the nucleoside substrate. Moreover, the catalytic efficiencies (k_{cat}/K_m) were not, or slightly, increased. Importantly, we did not observe an increase of endogenous dCK activity towards fludarabine and CdA after *in vivo*-induced increase of Ser-74 phosphorylation. Accordingly, treatment of CLL cells with aphidicolin, which enhances dCK activity through Ser-74 phosphorylation, did not modify the conversion of CdA or fludarabine into their active triphosphate form. Nevertheless, the same treatment enhanced activation of gemcitabine, a pyrimidine nucleoside 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 analogues, like gemcitabine, but not of CdA or fludarabine (10).

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Genetic and epigenetic alterations in genomes

Anabelle Decottignies
Charles De Smet

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

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

S. Lenglez, A. Decottignies

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

Regulation of mammalian telomeric transcription

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

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

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

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

in ATRX, an histone chaperone acting at telomeres, have been correlated with the ALT phenotype in pediatric tumors. In the laboratory, we are interested in understanding the nature of defects that drive telomeres towards recombination. To address this issue, we set up an experimental system that allows reliable comparisons between telomeres from ALT and telomerase-positive cells with similar background, and this includes the analysis of cellular hybrids. Our results point towards reduced nucleosome density at ALT telomeres (2). We now wish

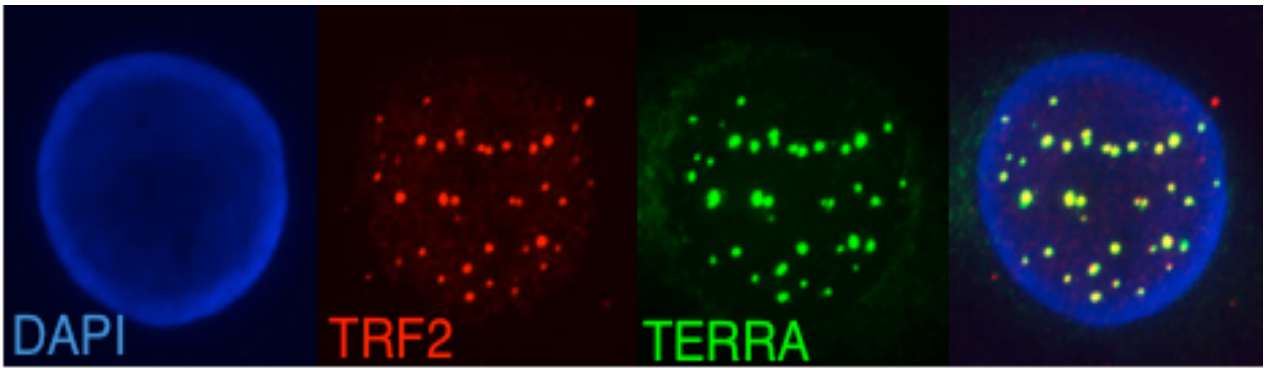

Fig. 1. Detection of TERRA telomeric transcripts at human telomeres. Telomeres are detected by immunofluorescence against TRF2 telomeric protein (red), TERRAs are detected by RNA-FISH using a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue)

Telomeres defects in cells with an alternative mechanism of telomere lengthening

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

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

In normal cells, the binding of very specialized telomeric proteins acts together with a proper telomere structure to represses recombination. The reasons why telomeres undergo so frequent recombinations in ALT cells are still not elucidated. Understanding this may help developing anti-cancer drugs targeting ALT tumors and possibly offer a means to target telomere maintenance in survivor cells that may arise from anti-telomerase treatments that are currently being tested in clinical trials to treat cancer.
Recently, evidences were provided in favor of a distinct heterochromatin structure at ALT telomeres. Notably, mutations

to confirm these data and to investigate the genetic defects underlying these distinct heterochromatin features of ALT telomeres, as well as the consequences this has on recombination-based telomere maintenance pathway.

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

J. Boros, N. Arnoult, A. Decottignies

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

Arnoult N*, Boros J*, Stroobant V, Collet JF, Decottignies A. Mol Cell. Biol., in press

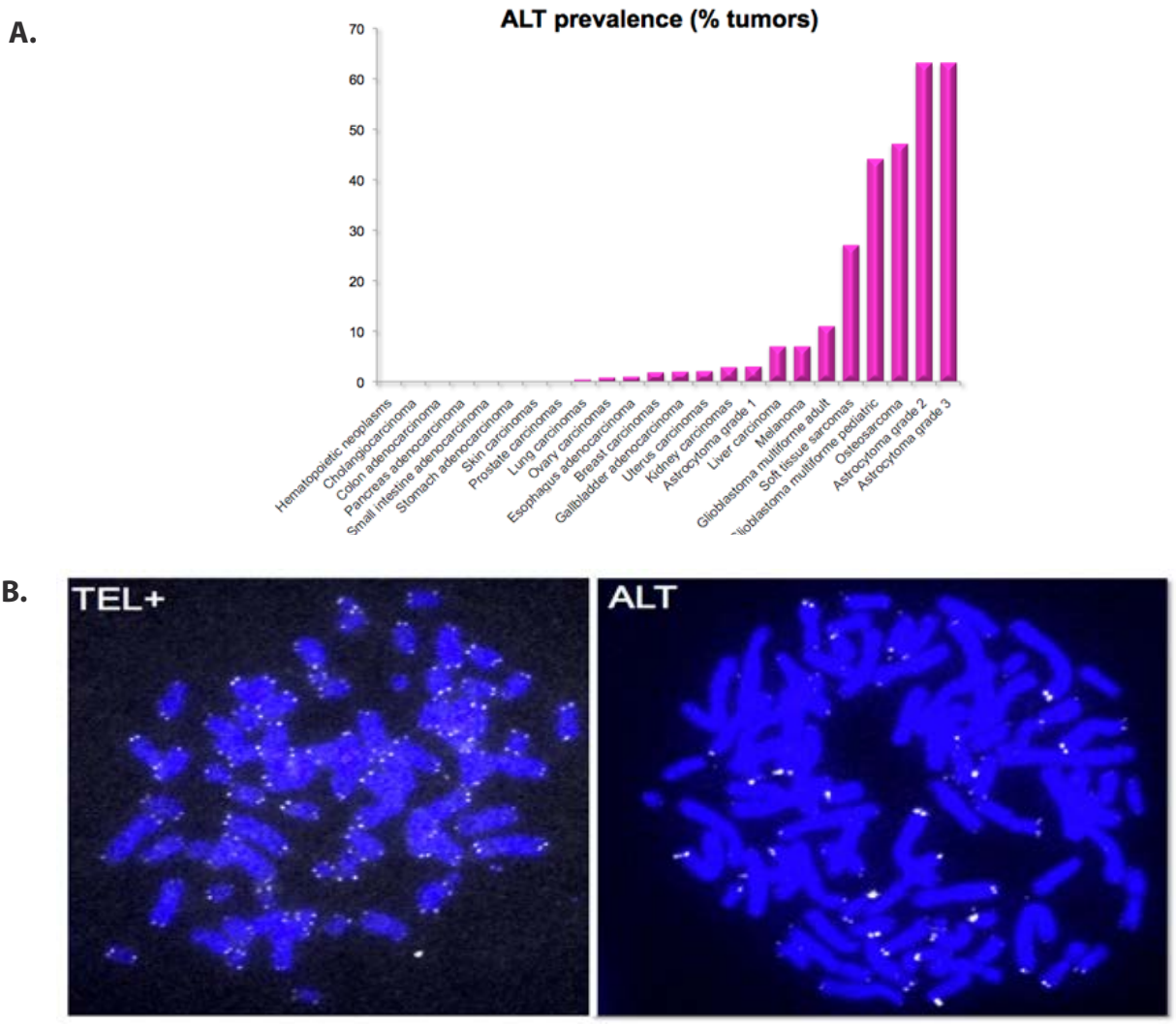

Fig. 2. Alternative mechanism(s) of telomere maintenance.

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

DNA hypomethylation and aberrant gene activation in cancer

A. Loriot, C. De Smet

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

DNA methylation patterns often become altered in cancer cells. Alterations include hypermethylation of selected promoters, leading to silencing of critical genes such as tumor suppressor genes, and hypomethylation of numerous other DNA sequences. We have shown that genome hypomethylation in tumors results in the activation of a group of germline-specific genes, which use primarily DNA methylation for repression in somatic tissues (3). These genes, which were originally discovered because their activation in tumors leads to the expression of tumor-specific antigens, were named cancer-germline (CG) genes. To date, ~50 CG genes or gene families have been identified. Several of these were isolated in our group (3).

The process leading to hypomethylation of DNA sequences in tumors remains obscure. We undertook to address this issue by using MAGEA1, the founding member of the CG group of genes, as a model. Detailed methylation analyses of the MAGEA1 genomic locus in expressing tumor cells, revealed preferential hypomethylation within the 5′ region of the gene. Furthermore, transfection experiments with in vitro methylat-

ed 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 CG gene promoters unmethylated remain to be identified.

Processes associated with CG gene demethylation in tumors

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

Histone modifications have been shown in some cases to dictate DNA methylation states, for instance by regulating access of DNA methyltransferases. We therefore searched to determine if MAGEA1 demethylation and activation in tumor cells is associated with changes in histone marks. Chromatin immunoprecipitation experiments revealed that DNA demethylation and transcriptional activation of MAGEA1 is accompanied by increases in histone H3 acetylation (H3ac) and H3 lysine 4 methylation (H3K4me), and by a decrease in H3 lysine 9 dimethylation (H3K9me2). However, our experiments demonstrate that changes at the histone level within the MAGEA1 promoter are a consequence, not a cause, of DNA demethylation. Consistently, epigenetic drugs that target histone modifications were unable to induce DNA demethylation and stable activation of the MAGEA1 gene. Altogether, these observations confirm that DNA methylation has a dominant role in the epigenetic hierarchy that governs MAGEA1 silencing (5).
A search for gene expression changes associated with the activation of CG genes was performed by analyzing microarray datasets deriving from a series of melanoma cell lines. This led to the identification of a group of genes that were consistently downregulated in the cell lines showing activation of multiple CG genes. A similar gene expression signature was observed in vivo in melanoma tissue samples. Most of the genes that were downregulated in association with CG gene activation appeared to exert functions related to cellular proliferation. Intriguingly, a previous study identified a similar gene expression signature in cells that had been depleted of DNMT1, the enzyme involved in maintenance of DNA methylation marks. By using several cellular models, we demonstrated that transient depletion of DNMT1 leads to both activation of CG-genes and long-term repression of proliferation genes. For one of these genes (CDCA7L), we showed that the mechanism of repression involves deposition of a repressive histone mark (H3K27me3), and is dependent on the pRB transcriptional repressor. Our observations point therefore towards DNMT1 depletion as a causal factor in the activation of CG-genes in melanoma.

DNA hypomethylation and activation of CG-type miRNAs in tumors

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

The role of DNA hypomethylation and CG gene activation on tumor development is only partially understood. To further explore the impact of DNA hypomethylation on tumorigenesis, we decided to find out if this epigenetic alteration also leads to the activation of CG-type microRNAs (miRNAs). It has indeed become clear that this type of small non-coding RNAs exerts important regulatory functions, by controlling the expression of target genes at the post-transcriptional level. Dysregulated expression of miRNAs is a hallmark of many cancers, where it appears to contribute to several important steps of tumor development. In a recent study, we reported identification of a novel cancer-germline transcript (CT-GABRA3) displaying DNA hypomethylation-dependent activation in various tumors, including melanoma and lung carcinoma. Importantly, CT-GABRA3 harbors a microRNA (miR-105), which was recently identified as a promoter of cancer metastasis by its ability to weaken vascular endothelial barriers following exosomal secretion. CT-GABRA3 also carries a microRNA (miR-767) with predicted target sites in TET1 and TET3, two members of the ten-eleven-translocation family of tumor suppressor genes, which are involved in the conversion of 5-methylcytosines to 5-hydroxymethylcytosines (5hmC) in DNA. Decreased TET activity is a hallmark of cancer, and we provided evidence that aberrant activation of miR-767 contributes to this phenomenon. We demonstrated that miR-767 represses TET1/3 mRNA and protein expression, and regulates genomic 5hmC levels. Additionally, we showed that high CT-GABRA3 transcription correlates with reduced TET1 mRNA levels in vivo in lung tumors. Together our studies reveal the first example of a cancer-germline gene that produces microRNAs with oncogenic potential (Loriot, Van Tongelen, et al, in press). Moreover, our data indicate that DNA hypomethylation in tumors can contribute to reduced 5hmC levels via activation of a TET-targeting microRNA.

Tumorigenesis-associated DNA hypomethylation within heterochromatic regions of chromosomes

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

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

telomeric chromatin are associated with telomere length deregulation in mouse cells and a decreased subtelomeric DNA methylation level was reported to up-regulate homologous recombination between mouse telomeric sequences (T-SCE for Telomeric Sister Chromatid Exchange), a hallmark of human ALT cells. This prompted us to evaluate the subtelomeric DNA methylation level of human TEL+ and ALT cancer cell lines (5). We detected a significant hypomethylation of subtelomeric DNA in ALT cancer cell lines when compared to TEL+ cell lines. However, subtelomeric DNA was not hypomethylated in ALT cell lines derived from in vitro immortalization of human fibroblasts with SV40 T antigen, although T-SCE frequencies in the latter cells were similar to those in ALT cancer cells (6). 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 (7).

We also investigated the causes and consequences of pericentromeric Satellite 2 DNA (Sat2) in tumor cells. We showed that, although Sat2 locus is frequently hypomethylated in cancer cells, hyperactivation of Sat2 RNA transcription is not observed. Similarly to what we described before for telomeres, pericentromeric Satellite 2 DNA is indeed transcribed to yield non-coding RNA molecules that appear to remain associated with the locus they are transcribed from where they may play important roles in heterochromatin formation. We showed that Sat2 RNA is mainly induced by heat shock pathway activation, both in vitro and in vivo. Besides, we found that hyperactivation of the heat shock pathway, through either hyperthermia or RasV12 oncogene overexpression, is able to induce local demethylation of Sat2, leaving a demethylation signature that was also detected in tumor cell lines displaying moderate levels of genome-wide hypomethylation (8).

Epigenetic repression of CG genes in human embryonic stem cells

A. Loriot, C. De Smet

The stage at which CG genes become methylated during human embryo development has not been determined. We found previously that human CG genes are repressed and methylated in human blastocyst-derived embryonic stem cells and in comparable embryonal carcinoma cells (9). By performing transfection experiments, we demonstrated that human embryonal carcinoma cells target active de novo methylation towards MAGEA1, as the gene became methylated and silenced following integration into these cells. Consistently, silencing of MAGEA1 in embryonal carcinoma cells depended on the presence of both DNMT3A and DNMT3B de novo DNA methyltransferases. Moreover, by analyzing transcription profiling datasets from human preimplantation embryos, we found that transcripts of CG genes increase up to the morula stage, and then decrease dramatically in blastocysts (10). Altogether our data indicate that human CG genes are programmed for repression in the blastocyst, and suggest that de novo DNA methylation is a primary event in this process.

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Metabolite repair and inborn errors of metabolism

Emile Van Schaftingen
Maria Veiga-da-Cunha

The study of L-2-hydroxyglutaric aciduria, a neurometabolic disease characterized by the accumulation of L-2-hydroxyglutarate and the identification of the enzymes that make and destroy L-2-hydroxyglutarate in mammals led us to realize the importance of a novel type of repair mechanism : metabolite repair [4, 8]. This research theme is connected with our other main research interest, the elucidation of inborn errors of metabolism.

Metabolite repair

A. Marbaix, F. Baldin, N. Chevalier, F. Collard, E. Wiame, M. Veiga-da-Cunha, E. Van Schaftingen, in collaboration with C.L. Linster (Luxembourg), Y. Achouri, I. Gerin, G. Bommer and D. Vertommen

L-2-hydroxyglutaric aciduria is due to a defect in a FAD-linked enzyme that catalyses the irreversible conversion of L-2-hydroxyglutarate to α-ketoglutarate, a Krebs cycle intermediate. L-2-hydroxyglutarate does not belong to any classical metabolic pathway, but is formed by a (minor) side-activity of mitochondrial L-malate dehydrogenase, the enzyme that normally interconverts oxaloacetate and L-malate [8]. As L-2-hydroxyglutarate does not play any physiological role but is toxic if it accumulates, L-2-hydroxyglutarate dehydrogenase has to be considered as a repair enzyme, playing a role akin to the proofreading activities associated with DNA polymerases and aminoacyl-tRNA synthases. Hence the designation 'metabolite proofreading enzymes' for enzymes serving to repair 'wrong' reaction products made by other enzymes of intermediary metabolism [7]. Because the specificity of metabolic enzymes is far from absolute, it is likely that metabolite proofreading enzymes abound, but that most of them are still unknown. One of our aims is to identify 'new' metabolite proofreading enzymes, not only because this will allow to attribute a function to putative enzymes encoded by mammalian and other genomes, but also because it may help understand the pathophysiology of some inborn errors of metabolism. Examples of newly identified metabolite repair enzymes are ethylmalonyl-CoA decarboxylase (which corrects a mistake made by acetyl-CoA carboxylase (see previous scienfic report), NAD(P)HX dehydratase, NAD(P)HX epimerase and ß-alanyl-lysine dipeptidase (see below). TIGAR, a phosphatase acting on 2,3-bisphosphoglyce-rate and other phosphate esters [2], may also be involved in metabolite repair (see report of G. Bommer).

Repair of damaged NAD(P)H

As initially shown by the group of Edwin Krebs in the 1950's, NADH is slowly converted to a hydrated form by glyceraldehyde-3-phosphate dehydrogenase. This hydrated form of NADH (called NADHX) and a similar hydrated form of NADPH (NADPHX) also spontaneously arise at high temperatures or acidic pH. An ATP-dependent dehydratase that reconverts NADHX and NADPHX to NAD(P)H was also described by Krebs' group, but its sequence had not been identified. To fill this gap, we purified yeast NAD(P)HX dehydratase and identified it to a highly conserved and nearly ubiquitous protein, named YKL150c in *Saccharomyces cerevisiae* and CarkD (carbohydrate kinase domain) in mammals [5]. We showed that both the yeast and mammalian proteins catalyze the dehydration of the (S) form of NADHX and NADPHX, while converting ATP to ADP. Surprisingly, the *Escherichia coli* homolog, YjeF, a bi-domain protein, catalyzes a similar reaction, but uses ADP instead of ATP (Fig. 1). This represents an unprecedented example of orthologous enzymes using either ADP or ATP as phosphoryl donor.

The dehydration reaction is ascribable to the C-terminal domain of YjeF. Its N-terminal domain is also highly conserved in the living world, corresponding to a separate protein named apolipoprotein A-1-binding protein (AIBP) in mammals and YNL200C in yeast. We showed that these proteins catalyze the epimerization of the (S) and (R) forms of NAD(P)HX, thereby allowing, in conjunction with the energy-dependent dehydratase, the repair of both epimers of NAD(P)HX (Fig. 1). Both

enzymes are very widespread in eukaryotes, prokaryotes, and archaea. This wide distribution and the ADP-dependence of the dehydratase in some species indicate the ancient origin of this repair system [5].

Recent work [6] indicates that mammalian NAD(P)HX dehydratase and NAD(P)HX epimerase are present in the cytosol, in the mitochondria and, in the case of the former enzyme, also in the endoplasmic reticulum. This is in accordance with the presence of NAD(P)H pools in these compartments. The Carkd gene encodes proteins with a predicted mitochondrial propeptide (mCARKD), a signal peptide (spCARKD) or neither of them (cCARKD). Confocal microscopy analysis of transfected CHO (Chinese-hamster ovary) cells indicated that cCARKD remains in the cytosol, whereas mCARKD and spCARKD are targeted to the mitochondria and the endoplasmic reticulum, respectively. Unlike the other two forms, spCARKD is N-glycosylated, supporting its targeting to the endoplasmic reticulum. The *Aibp* gene encodes two different proteins, which we showed to be targeted to the mitochondria (mAIBP) and the cytosol (cAIBP). Quantification of the NAD(P)HX dehydratase and epimerase activities in rat tissues, performed after partial purification, indicated that both enzymes are widely distributed, with total activities of ≈3-10 nmol/min/g of tissue. Liver fractionation by differential centrifugation confirmed the presence of the dehydratase and the epimerase in the cytosol and in mitochondria. These data support the notion that NAD(P)HX repair is virtually ubiquitous.

Repair in the synthesis of carnosine [10]

Carnosine (β-alanyl-histidine) is an abundant dipeptide present in skeletal muscle of many vertebrates where it serves as a pH buffer and maybe also as a radical scavenger. The related dipeptide homocarnosine (γ-aminobutyryl-histidine) is present in brain, where its function is still unknown. Carnosine synthase is the ATP-dependent ligase responsible for carnosine and homocarnosine synthesis in skeletal muscle and brain, respectively (Fig. 2). This enzyme uses also at substantial rates lysine, ornithine and arginine instead of histidine, yet the resulting dipeptides are virtually absent from muscle or brain, suggesting that they are removed by a "metabolite repair" enzyme.

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

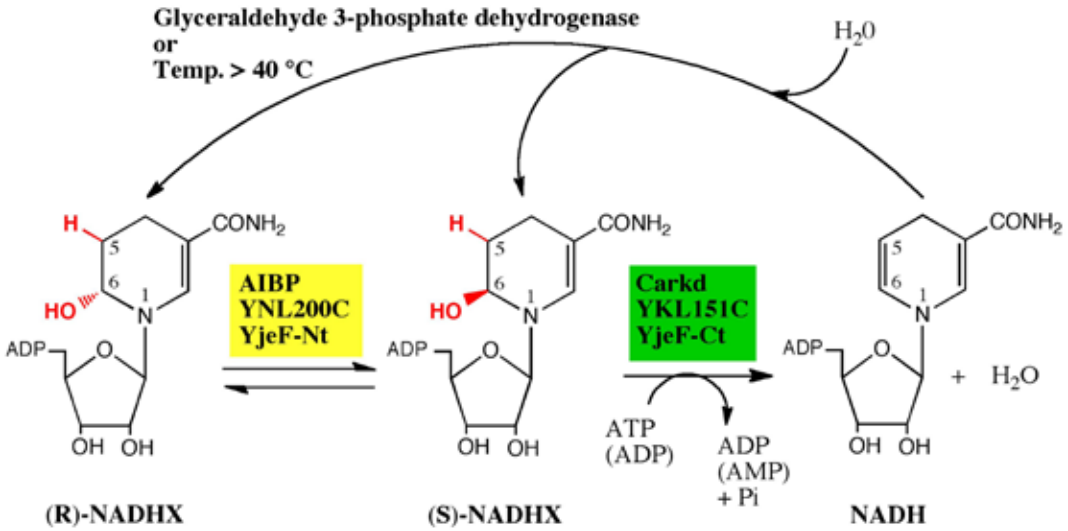

Fig. 1. Formation and repair of hydrated NAD(P)H. Modified from Marbaix *et al*. [5]

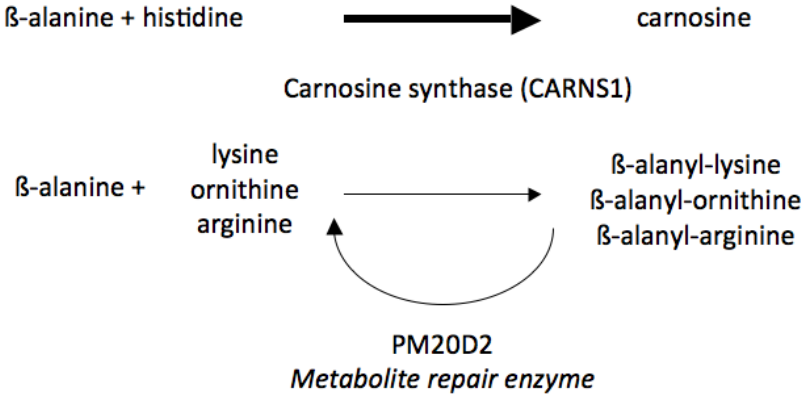

Fig. 2. Synthesis of carnosine and other dipeptides by carnosine synthase and hydrolysis of the 'wrong' dipeptides by PM20D2. Carnosine synthase also uses GABA instead of ß-alanine to make homocarnosine and related dipeptides. PM20D2 also hydrolyses γ-aminobutyryl-lysine, γ-aminobutyryl-ornithine and γ-aminobutyryl-arginine (not shown).

Molecular identification of enzymes

J. Drozak, A. Grahn, S. Marlaire, G. Noël, G. Tahay, E. Wiame M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with V. Stroobant

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.

Metabolism of hydroxylysine and phosphoethanolamine

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. We recently identified the ammoniophospholyases that deaminate and dephosphorylate phosphohydroxylysine and ethanolamine-phosphate as the products of the genes AGXT2L2 and AGXT2L1. We also identified the gene encoding hydroxylysine kinase (see report of 2012).

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

Synthesis of glutaryl-CoA [7]

Glutarate, a side-product in the metabolism of tryptophan and lysine, is metabolized by conversion to glutaryl-CoA by a transferase using succinyl-CoA as a coenzyme donor. The enzyme catalyzing this conversion has not been formally identified. However, a benign form of glutaric aciduria (glutaric aciduria type III) is due to mutations in C7orf10, a putative member of the coenzyme A transferase class III family. We showed that recombinant human C7orf10 catalyzes the succinyl-CoA-dependent conversion of glutarate to glutaryl-CoA. C7orf10 could use many dicarboxylic acids as CoA acceptors, the best ones being glutarate, succinate, adipate, and 3-hydroxymethylglutarate. Confocal microscopy analysis of CHO cells transfected with a C7orf10-GFP fusion protein indicated that C7orf10 is a mitochondrial protein, in agreement with the presence of a predicted mitochondrial propeptide at its N-terminus. The effect of a missense mutation (p.Arg336Trp) found in the homozygous state in several patients with glutaric aciduria type III and present in the general population at a low frequency was also investigated. The p.Arg336Trp mutation led to the production of an insoluble and inactive protein both in *Escherichia coli* and in HEK293T cells. These findings indicate that C7orf10 is implicated in the metabolism of glutarate, but possibly also of longer dicarboxylic acids. Homologues of this enzyme are found in numerous bacterial operons comprising also a putative glutaryl-CoA dehydrogenase, indicating that an enzyme with similar specificity exists in prokaryotes.

A ß-alanine activating enzyme [1]

Microorganisms are well-known producers of a large number of chemically diverse secondary metabolites such as an-

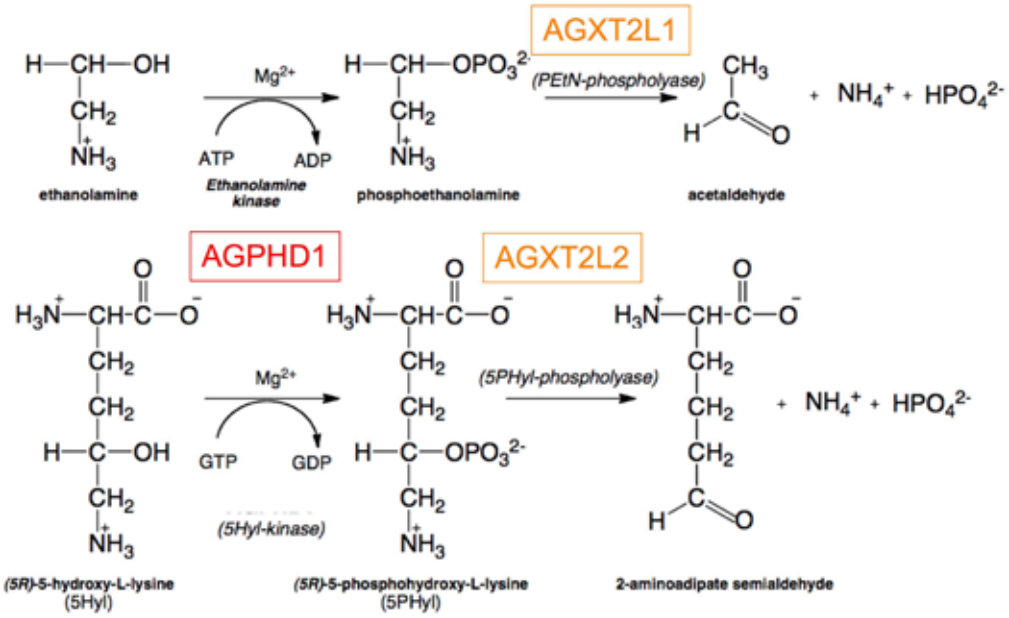

Fig. 3. Metabolism of hydroxylysine and phosphoethanolamine. From [9].

tibiotics, immunosuppressants or cytostatics. Many of these compounds are peptide-like and are formed via nonribosomal pathways, utilizing large multifunctional enzymes, called nonribosomal peptide synthetases (NRPSs). The only mammalian homologue of these enzymes is ACSF4 (Acyl-CoA Synthetase Family Member 4), a protein of unknown function, which comprises a putative adenylation-domain (AMP-binding domain) similar to those of bacterial non-ribosomal peptide synthetases, a putative phosphopantetheine attachment site and a C-terminal PQQDH (pyrroloquinoline quinone dehydrogenase)-related domain. Orthologues comprising these three domains are present in many eukaryotes including plants. Remarkably, the adenylation domain of plant ACSF4 shares more identity with Ebony, the insect enzyme that ligates β-alanine to several amines, than with vertebrate or insect ACSF4 and prediction of its specificity suggests that it also serves to activate β-alanine. In the presence of ATP, purified mouse recombinant ACSF4 progressively formed a covalent bond with radiolabeled β-alanine. The bond was alkali labile, suggesting a (thio)ester, and was not formed in a point-mutant devoid of the phosphopantetheine attachment site. Competition experiments with various amino acids indicated that the reaction was nearly specific for β-alanine, for which a KM of ≈ 5 μM was found. The loaded enzyme was used to study the formation of a potential end-product. Among the twenty standard amino acids, only cysteine was able to cause unloading of the enzyme. This effect was mimicked by cysteamine and by dithiothreitol, and was unaffected by the absence of the PQQDH-related domain, suggesting that the β-alanine transfer onto thiols is catalyzed by the ACSF4 adenylation domain, but is physiologically irrelevant. We conclude that ACSF4 is a β-alanine activating enzyme, which presumably participates in an infrequent, but quite conserved modification of a protein or an RNA species.

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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 (3,4,10).
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 proofDsbA is a very powerful oxidant that apparently lacks proofreading activity. If the native disulfide bond pattern involves cysteine residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect disulfides. The correction of these non-native disulfides is the role of a disulfide isomerization system. This system involves a soluble periplasmic protein disulfide isomerase, DsbC (Figure 2). Like DsbA, DsbC presents a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC is kept reduced in the periplasm. This allows DsbC to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC reduced is the membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions.

In our lab, we study the Dsb proteins of E. coli and of other Gram-Negative bacteria. Over the past few years, we have characterized the disulfide cascade within DsbD, we have identified the proteins that depend on DsbA and DsbC for folding and we have engineered a new periplasmic oxidizing system (13). We have also proposed a revised model for

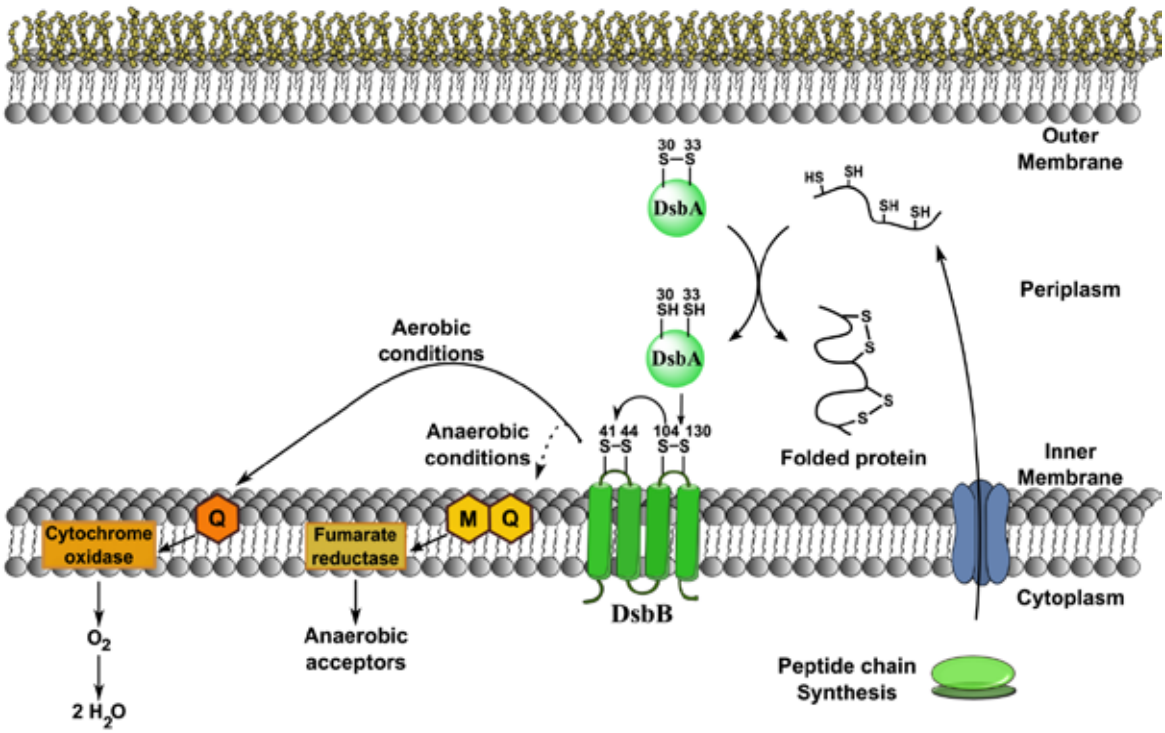

Fig. 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to DsbB. DsbB transfers electrons to the respiratory chain (adapted from (1)).

the pathways of disulfide bond formation in the periplasm by showing that DsbC may be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively. We have also identified two new substrates of the protein disulfide isomerase DsbC. Interestingly, these two substrates, LptD and RcsF, are located in the outer membrane and play important roles in the maintain of envelope integrity (8,9). LptD is an essential ß-barrel protein that inserts lipopolysacharrides in the outer membrane. We also found that LptD assembly depends on the periplasmic chaperone SurA (7,12). RcsF is an outer membrane lipoprotein sensor that detects defects in envelope integrity and transduces the signal to the Rcs phosphorelay, a signaling system that allows bacteria to react to a range of envelope stresses by modulating the expression of specific genes (8).

In collaboration with Prof. J. Beckwith (Harvard Medical School), we identified a third distinct class of DsbD-like homologues (5), which is found in proteobacteria and Chlamydia. The prototype of this new class is Salmonella typhimurium ScsB. Using Caulobacter crescentus as a model organism, we searched for the substrates of ScsB (5). We discovered that ScsB provides electrons to the first peroxide reduction pathway identified in the bacterial cell envelope. The reduction pathway comprises a thioredoxin-like protein, TlpA, and a peroxiredoxin, PprX. We showed that PprX is a thiol-dependent peroxidase that efficiently reduces both hydrogen peroxide and organic peroxides. Our results reveal that the array of proteins involved in reductive pathways in the oxidative cell envelope is significantly broader than has been understood.

Recently, we also characterized the machinery that catalyzes disulfide bond formation in the pathogenic bacterium *Pseudomonas aeruginosa*, an opportunistic human pathogen responsible for nosocomial infections and chronic colonization of cystic fibrosis patients. We found that the genome of *P. aeruginosa* uniquely encodes two DsbA proteins and two DsbB proteins and we studied the respective importance of these various Dsb proteins in bacterial virulence (1). Furthermore, using a high-throughput proteomic approach, we identified more than 20 new substrates of the disulfide bond formation machinery, including virulence factors. We also solved the structure of PaDsbA2, a *P. aeruginosa* oxidoreductase (Fig. 3). By fully dissecting the machinery that introduces disulfide bonds in P. aeruginosa, **our work opens the way to the design of novel antibacterial molecules able to disarm this pathogen by preventing the proper assembly of its arsenal of virulence factors**.

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

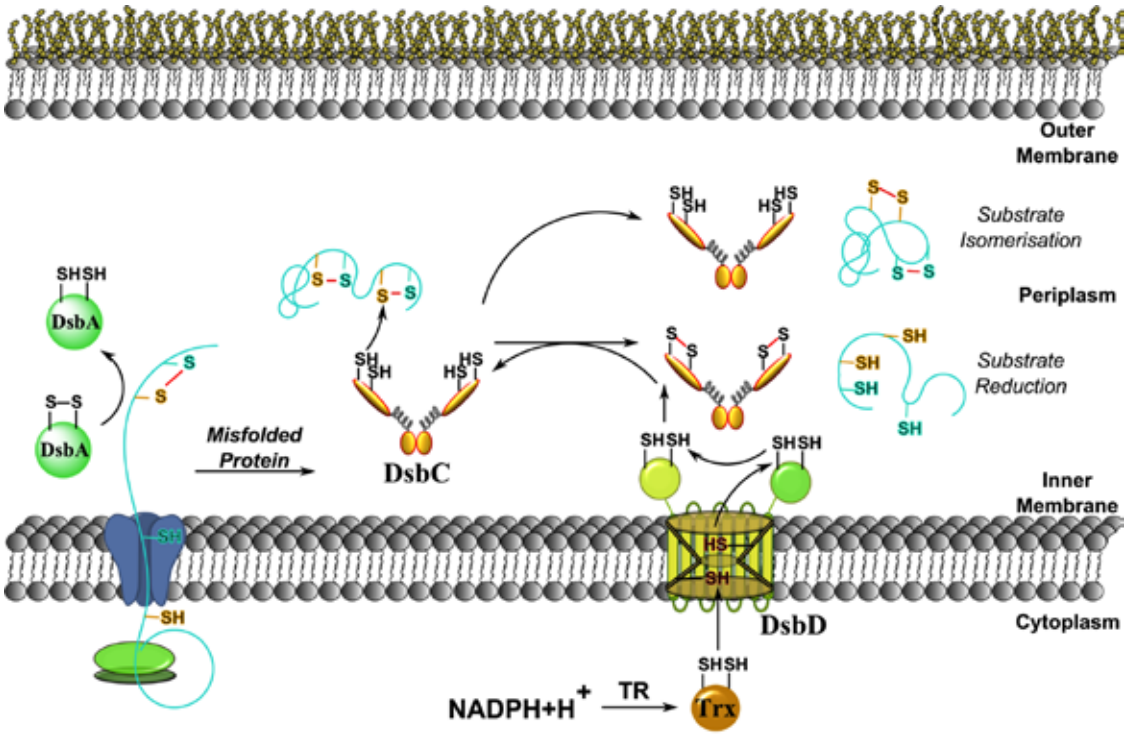

Fig. 2. Disulfide bond reduction/isomerization in the periplasm. DsbA can introduce non-native disulfides when they need to be formed beween non-consecutive cysteines. Incorrect disulfides are either isomerized or reduced by DsbC. Oxidized DsbC is regenerated by the membrane protein DsbD that transfers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

DsbC system might be utilized in organisms lacking the DsbA/DsbB machinery to introduce disulfides into secreted proteins. This work, which results from a collaboration with the group of J. Beckwith, has been published recently (6).

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 (11).

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. We recently found that DsbC serves as a backup for DsbG and has its own subset of favorite sulfenic acid modified substrates to reduce (2). 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 sequenced so far, making it tempting to speculate that some of these thioredoxin superfamily members, particularly those that are closely related to DsbC and DsbG, play similar roles in controlling the global sulfenic acid content of eukaryotic cellular compartments (11).

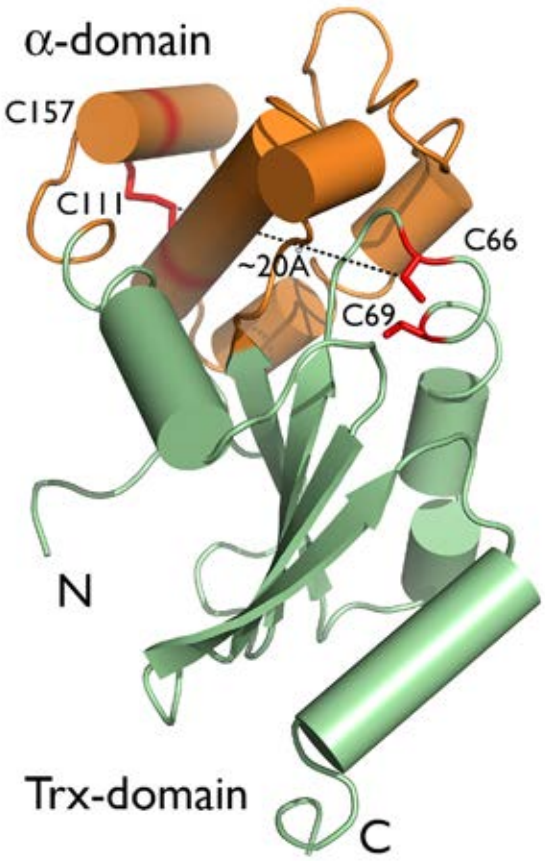

Fig. 3. The structure of PaDsbA2, an oxidoreductase from *P. aeruginosa* The structure of PaDsbA2 was solved using single anomalous dispersion and refined to 1.3 Å. It consists in a thioredoxin fold (in green) in which α helical domain is inserted (in orange). The catalytic cysteines (C66 and C69) and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

Selected Publications

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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. In addition, we are also investigating the regulation of metabolic processes by conserved signaling pathways. Most recently this concerns the function of the p53 target gene TIGAR.

Role of miRNAs in intestinal differentiation

The intestine is required for the digestion and absorption of essential nutrients and water. In this process, its surface epithelium is exposed to one of the most toxic milieus of the whole body. It has to resist aggressive digestive juices, large pH changes, anaerobic bacteria and numerous toxic compounds. To resist this, its surface epithelium is completely renewed in less than 2 weeks. All intestinal epithelial cells are derived from stem-cells located at the base of the crypt. An intricate network of signaling pathways controls proliferation and differentiation of these cells.
These pathways are crucial for tissue homeostasis and intestinal function.

In addition, several of the pathways that are required for stem cell maintenance are activated during colorectal carcinogenesis. For example the Wnt signaling pathway is essential for the maintenance of stem cells in the normal intestine. In colorectal cancers, mutations in different components of this pathway, lead to its activation in more than 80% of colorectal cancers.

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

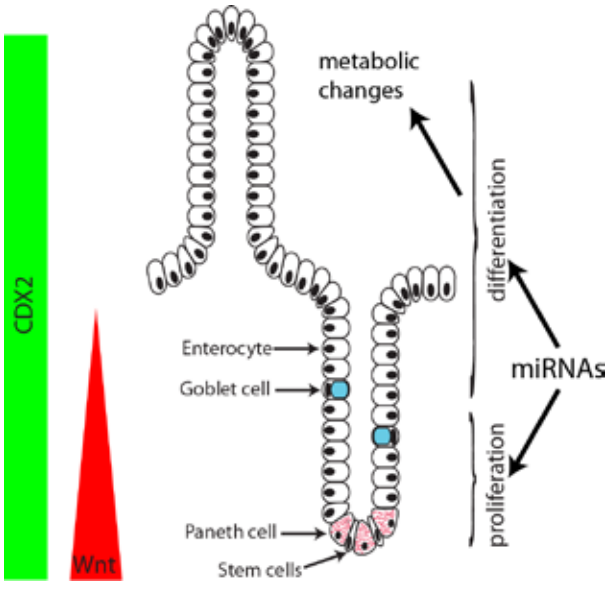

Fig. 1. Intestinal architecture is maintained by the interplay of many signaling pathways. The intestinal architecture is maintained by the interplay of signaling patways that ensure complete renewal of intestinal surface epithelia within 7 to 14 days. New cells are generated from a stem cell compartment at the base of the crypts and successively migrate up, where they are eventually shed in the lumen. In this process, different cell types with distinct functions are generated (= cell differentiation). We are interested in miRNAs that regulate this process and that play a role in the metabolic changes required for normal intestinal cell function.

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

Regulation of cholesterol metabolism by the bifunctional locus SREBF2-miR33

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

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

We are continuing to investigate the physiological role of miR-33 family members in different experimental systems throughout evolution. Currently, we are studying the *in vivo* effects of miR-33 in *Drosophila melanogaster* (in collaboration with the laboratory of Jennifer Kennel, Vassar College, NY, USA). Surprisingly, the effect of miR-33 overexpression in the fly is extremely dependent on the developmental stage and nutritional status. We are currently trying to understand whether these observations have implications for miR-33 as a therapeutic target in humans.

Metabolic effects of the tumor suppressor gene p53 : identification of the primary substrate of the enzyme TIGAR

(in collaboration with Emile Van Schaftingen)

Glucose is metabolized in glycolysis and in citric acid cycle (Figure 3). This leads to the production of ATP and reducing

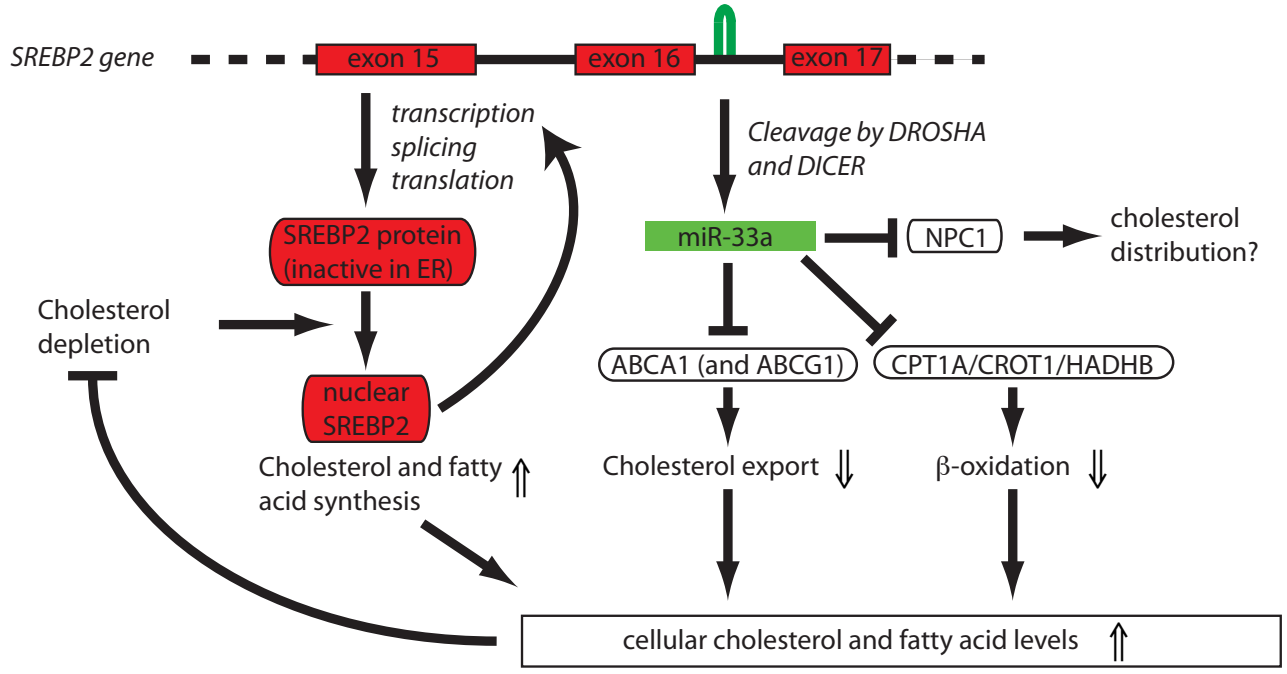

Fig. 2. The bifunctional locus of SREBF2-miR33 regulates cholesterol and fatty acid metabolism
After processing from an intron of SREBF2, miR-33a reduces cellular cholesterol export by inhibiting expression of ABCA1 (and in the mouse ABCG1). In addition, miR-33a reduces mitochondrial fatty acid b-oxidation via inhibition of HADHB, CROT, and CPT1A to increase intracellular lipid levels. Thus the SREBF2 locus uses two distinct mechanisms to maintain lipid homeostasis: regulated transcriptional activity of SREBP-2 and translational repression by miR-33a

equivalents that can be used to drive thermodynamically unfavorable reactions. In addition, these metabolic pathways serve as sources of building blocks required for cellular proliferation (indicated by black fields). Many cancer cells show increased glycolytic flux to account for the constant need for building block and the regulation of glycolytic flux in cancer cells has therefore received significant attention.

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

Most prominently, it had been suggested that the p53 target TIGAR is an important regulator of glycolytic flux by removing a phosphate group from the metabolic regulator fructose 2,6-bisphosphate (Bensaad et al in Cell 2005). Surprisingly, the sole kinetic investigation on recombinant TIGAR (Li & Jogl, 2009) has shown that its fructose-2,6-bisphosphatase activity was much weaker than that of authentic fructose-2,6-bisphosphatases. In collaboration with the group of Emile Van Schaftingen, who together with Louis Hue and Géry Hers had first described and characterized fructose 2,6-bisphosphate in the 1980s, we re-evaluated the substrate spectrum of TIGAR. Remarkably, we found that 2,3-bisphosphoglycerate was an about 400x better substrate for TIGAR than fructose 2,6-bisphosphate (Gerin et al 2014). Using knockout and shRNA-based techniques, we were able to demonstrate that 2,3-bisphosphoglycerate is likely a physiological substrate of TIGAR.

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

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

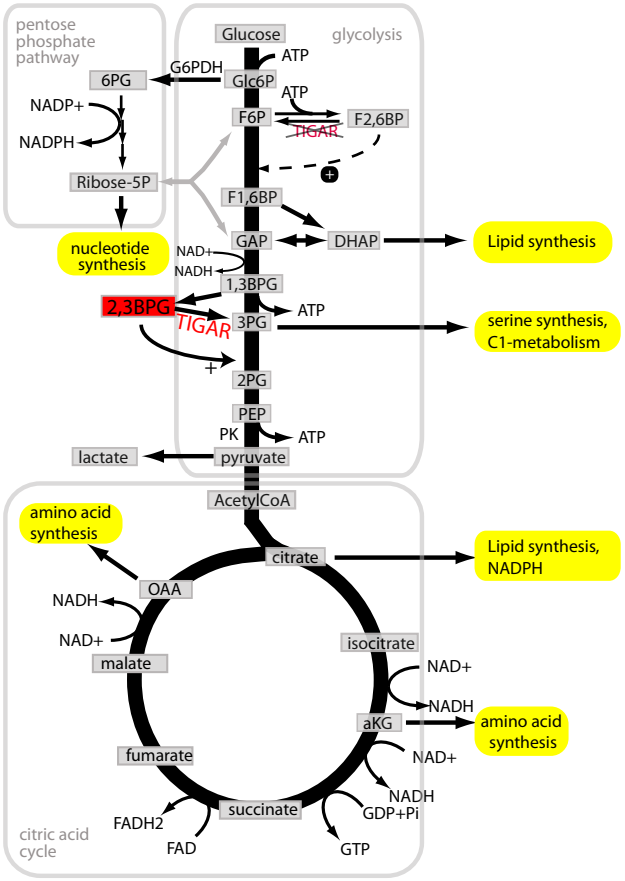

Fig. 3. TIGAR is a 2,3-bisphosphoglycerate phosphatase. Glycolysis and citric acid cycle lead to the production of ATP, reducing equivalents and synthetic building blocks. It had previously been suggested that the p53 target gene TIGAR would regulate cellular metabolism by dephosphorylation of the glycolytic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

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Signal transduction and protein phosphorylation

Mark Rider

Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. We started out in this field many years ago by studying 6-phosphofructo-2-kinase (PFK2)/fructose-2,6-bisphosphatase (FBPase-2), the bifunctional enzyme which synthesizes and degrades fructose-2,6-bisphosphate (Fru-2,6-P_2), and the control of activity by protein kinases (reviewed in ref. 1). This prompted us to investigate the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are our main research interests.

Insulin signalling

N. Hussain, M.H. Rider

Insulin-stimulated lipogenesis in white adipose tissue

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

AMP-activated protein kinase

Y. Liu, C. Plaideau, Y.-C. Lai, D. Vertommen, R. Jacobs, S. Pyr dit Ruys, M. Johanns, A. Houddane, N. Hussain, L. Hue, M.H. Rider, in collaboration with D. Tyteca and P. Courtoy, UCL, J. Tavaré, Bristol, P. Shepherd, Auckland, C. Erneux, ULB, F. Dequiedt, ULg, J. Jenssen, Oslo, B. Viollet, Paris, P. Gailly, UCL, S. Hallén, AstraZeneca

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 muscle contraction/exercise. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells and is converted to ZMP, an analogue of AMP. AMPK can also be activated by incubating cells with the small-molecule activator, A769662, also known as the Abbott compound. Full AMPK activation requires phosphorylation of the α-catalytic subunits at Thr172 by upstream kinases, either LKB1 (the Peutz-Jeghers protein) or calmodulin-dependent protein kinase kinase-β (CaMKKβ). The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways (Fig. 1). We have made significant contributions to the field by discovering new substrates of AMPK. Protein synthesis inhibition in response to AMPK activation during anoxia can partly be explained by a rise in eEF2 (eukaryotic elongation factor-2) phosphorylation leading to its inactivation (ref. 3). Regulation of the upstream eEF2 kinase (eEF2K) is complex involving phosphorylation-induced activation and inactivation by kinases from various signalling pathways. Indeed we, and others, reported that AMPK phosphorylates and activates eEF2K. However, phosphorylation at the previously proposed

Ser398 site by AMPK is probably incorrect and our recent findings indicate that AMPK phosphorylates other sites responsible for eEF2K activation. AMPK activation also activates eEF2K indirectly by decreasing mTORC1 signalling.

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

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

Control of PIKfyve by AMPK

Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), the lipid kinase that phosphorylates PtdIns3P to PtdIns(3,5)P_2, has been implicated in insulin-stimulated glucose uptake. We investigated whether PIKfyve could also be involved in contraction/AMPK-stimulated glucose uptake in skeletal muscle. Incubation of rat epitrochlearis muscles with YM201636, a selective PIKfyve inhibitor, reduced contraction- and AICA riboside-stimulated glucose uptake. Consistently, PIKfyve knockdown in C2C12 myotubes reduced AICA riboside-stimulated glucose transport. Electrical stimulation of muscles increased PtdIns(3,5)P_2 levels and PIKfyve phosphorylation. AMPK phosphorylated PIKfyve at Ser307 both in vitro and in intact cells. Following subcellular fractionation, PIKfyve recovery in a crude intracellular membrane fraction was increased in contracting versus resting muscles. Also in opossum kidney cells, wild-type, but not S307A mutant, PIKfyve was recruited to endosomal vesicles in response to AMPK activation. We propose that PIKfyve activity is required for the stimulation of skeletal muscle glucose uptake in response to contraction/AMPK activation. PIKfyve is a thus new AMPK substrate whose phosphorylation at Ser307 could promote PIKfyve translocation to endosomes for PtdIns(3,5)P_2 synthesis to facilitate GLUT4 translocation (ref. 5).

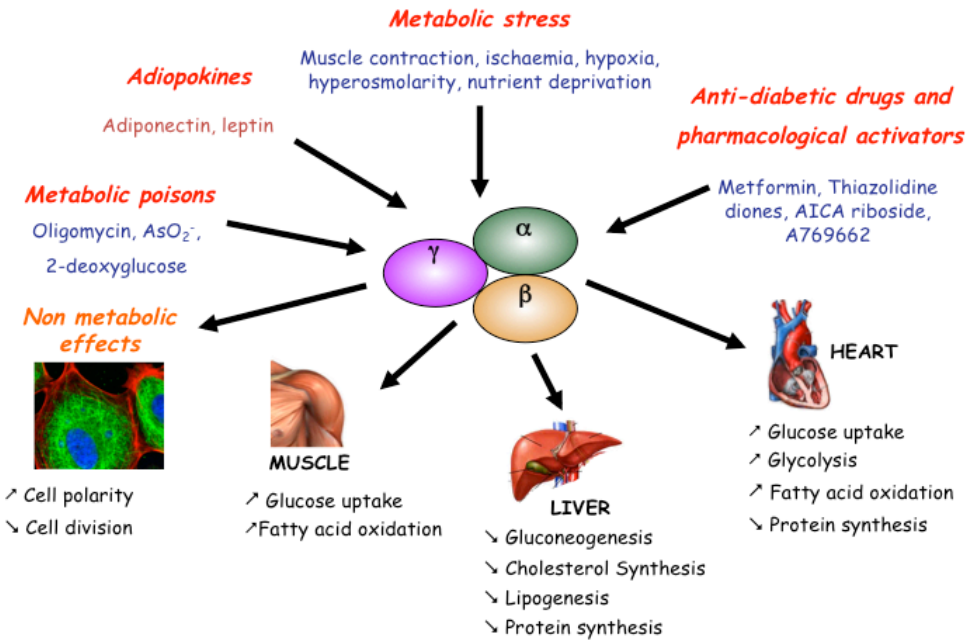

Fig. 1. Conditions leading to AMPK activation in higher eukaryotes and some of its consequences.

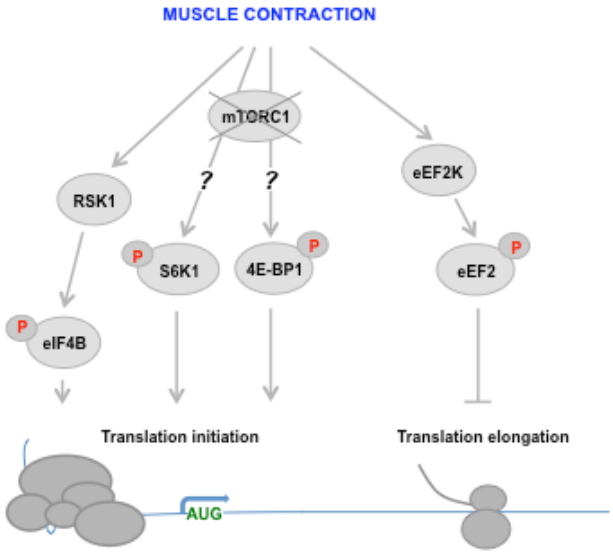

Fig. 2. Signalling during contraction maintains S6K1, eIF4B and 4E-BP1 phosphorylation, while blocking translation elongation via eEF2 phosphorylation. Contraction increases the phosphorylation of S6K1 and 4E-BP1 (at non-conventional sites different from those phosphorylated by mTORC1) in an mTORC1-independent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

AMPK as a drug target for type 2 diabetes

AMPK is an attractive therapeutic drug target for treating metabolic disorders such as type 2 diabetes and cancer. In collaboration with the pharmaceutical company AstraZeneca (Mölndal, Sweden), we have investigated whether inhibition of AMP metabolizing enzymes (Fig. 3) could be a means of achieving or potentiating AMPK activation. The effects of pharmacological AMP-deaminase (AMPD) inhibition on purine nucleotide levels and AMPK activation in contracting skeletal muscle were studied. Pre-incubation of rat epitrochlearis muscles with AMPD inhibitors potentiated rises in AMP, AMP:ATP ratio, AMPK Thr172 and ACC-2 Ser218 phosphorylation induced by electrical stimulation. However, in spite of potentiation of AMPK activation by contraction, glucose transport was not enhanced. Mice harbouring whole body AMPD1 deletion were generated at AstraZeneca. In incubated extensor digitorum longus and soleus muscles from *Ampd1* KO mice, increases in AMP levels and AMP:ATP ratio by electrical stimulation were potentiated considerably compared with muscles from wild-type mice, whereas enhanced AMPK activation was moderate and was significantly increased only in soleus. This suggests control of AMPK by factors other than changes in adenine nucleotides. We conclude from our studies (ref. 6) that the principle of indirect AMPK activation via inhibition of AMPD is not a viable approach to treat metabolic disease. However, the pharmacological AMPD inhibitors that have been developed would be useful tools for enhancing AMPK activation in muscle and other tissues and cells during ATP-depletion.

We turned to studying the effects of an AMPK activator developed by Merck (ex229 from patent application WO2010036613), comparing chemical activation with contraction and those of A769662 in incubated rat epitrochlearis muscles. Ex229 dose-dependently increased AMPK activity of α1-, α2-, β1- and β2-containing complexes with significant increases in AMPK activity seen at a concentration of 5 μM. At a concentration of 100 μM, AMPK activation was similar to that observed after contraction and importantly led to an ~2-fold increase in glucose uptake. In AMPK α1-/α2-subunit catalytic subunit double KO myotubes incubated with ex229, the increases in glucose uptake and ACC phosphorylation seen in control cells were completely abolished, suggesting that the effects of the compound were AMPK-dependent. When muscle glycogen levels were reduced by ~50% after starvation, ex229-induced AMPK activation and glucose uptake were amplified in a wortmannin-independent manner, suggesting antagonism between glycogen and ex229 which bind at distinct sites on the β-subunit. In L6 myotubes incubated with ex229, fatty acid oxidation was increased. In summary, ex229 efficiently activated skeletal muscle AMPK and elicited metabolic effects in muscle appropriate for treating type 2 diabetes by stimulating glucose uptake and increasing fatty acid oxidation (ref. 7).

Mass spectrometry

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

The development of mass spectrometry (MS) facilities within our laboratory, and for our Institute and University, has been an enormous asset (see http://www.uclouvain.be/en-proteomics.html). Since the acquisition of an electrospray mass

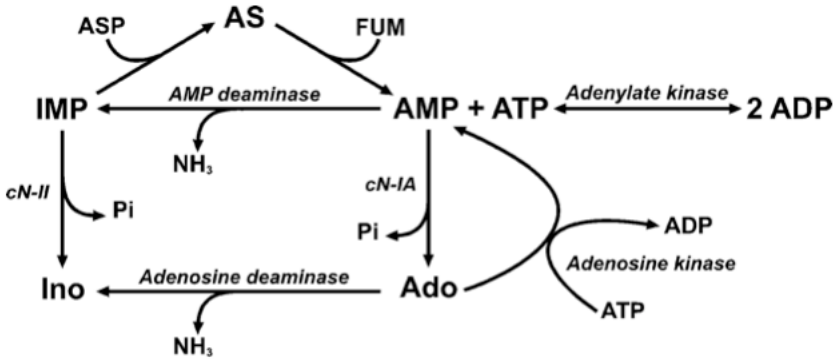

Fig. 3. Scheme showing AMP metabolizing pathways. The enzymes implicated are indicated in italics. ASP: aspartate; AS: adenylosuccinate; FUM: fumarate; Ado: adenosine; Ino: inosine.

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 80 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. 2).

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

We use label-free multidimensional LC-MS to study differential protein expression. We also use phosphoproteomics strategies (in collaboration with E. Waelkens, see ref. 10) to identify new targets downstream of different signalling pathways under various conditions. We have developed 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) protein extraction in SDS, by the filter-assisted sample preparation (FASP) procedure, followed by hydrophilic liquid chromatography (HILIC) and metal oxide affinity capture (MoAC) on TiO2 to enrich and concentrate phosphopeptides. Analysis of brown adipose tissue from hibernating ground squirrels indicated that protein levels of the mitochondrial respiratory chain, fatty acid oxidation, fatty acid and cholesterol synthesis and glycogen breakdown were decreased compared with tissue from euthermic animals whereas the expression of enzymes of gluconeogenesis and triacylglycerol synthesis were increased. Also, perilipin and hormone-sensitive lipase phosphorylation at pro-lipolytic, cyclic AMP-dependent protein kinase sites increased. Thus, while oxidative catabolism would be suppressed in brown fat during hibernation, gluconeogenesis and triacylglycerol synthesis would be maintained with the lipolytic system primed in an active state, presumably for thermogenesis on arousal.

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Membrane organization / dynamics and epithelial differentiation

Pierre J. Courtoy
Christophe E. Pierreux
Donatienne Tyteca

This group focuses on intertwined topics related to the control of membrane organization/dynamics at the subcellular level and its significance for cell physiology and disease; and of epithelial polarity during tissue differentiation and disease. By high-resolution vital imaging of the erythrocyte plasma membrane after insertion of lipid analogs or decoration of endogenous lipids by toxin fragments, we uncovered labeling of distinct submicrometric domains. These data force to revise concepts on scale (submicrometric) and stability (minutes) of lateral lipid organization at the living cell surface. We currently address whether submicrometric domains could play a role in erythrocyte deformation and membrane fragility diseases. Using polarized kidney tubular cells and transgenic mice as models, we identified the transcription factor, ZONAB, as essential component of the switch between epithelial proliferation and apical differentiation. Studying endocytosis in kidney tubules, we found that endosome to lysosome route is important for lysosomal biogenesis and that endosome recycling to the apical membrane is controlled by class III PI3-kinase/VPS34 and dynamin. At the tissue level, we study epithelial tubulogenesis and differentiation, using developing pancreas and thyroid glands as complementary models to study conversion of non-polarized endoderm-derived cell masses into polarized monolayers, with emphasis on paracrine and transcriptional control. Combined expertises help us addressing physiopathology and adaptation mechanisms in kidney and thyroid of cystinosis, a paradigmatic lysosomal storage disorder due to inherited defective lysosomal cystine exporter. Besides integration of cell and developmental biology, two strong assets of our group are decades of expertise in structural biology and advanced imaging thanks to a versatile Platform for Imaging Cells and Tissues.

Plasma membrane lipids segregate into distinct submicrometric domains

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

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

As we observed by vital confocal microscopy, trace insertion of exogenous fluorescent analogs (BODIPY) of sphingomyelin (SM) (< 1% of endogenous) labels submicrometric domains at the plasma membrane of freshly isolated red blood cells (RBCs) partially spread onto coverslips and of cultured cells. In CHO cells, intracellular conversion of BODIPY-ceramide into -SM produced similar plasma membrane submicrometric domains as direct BODIPY-SM insertion into the cell surface. Inhibition of endogenous SM synthesis or surface SM depletion by sphingomyelinase erased BODIPY-SM domains. Both controls suggested that domains labelled by exogenous BODIPY-SM reflect endogenous SM compartmentation (Ref. 1). Double labelling with BODIPY-phosphatidylcholine and -GM1 (a ganglioside) revealed co-existence of distinct classes of submicrometric lipid

domains, differing in composition, cohesion and cytoskeleton interaction.

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

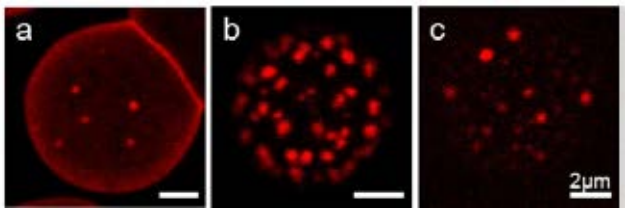

Fig. 1. Labelling of endogenous SM, cholesterol and ganglioside GM1 by toxin (fragments) show submicrometric domains on RBCs spread onto coverslips. Erythrocytes labelled by fluorescent lysenin (SM, a), theta toxin (cholesterol, b) or cholera toxin B subunit (ganglioside GM1, c). Adapted from Refs. 2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

Regulation and significance of submicrometric lipid domains

M. Carquin, P.J. Courtoy and D. Tyteca

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

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

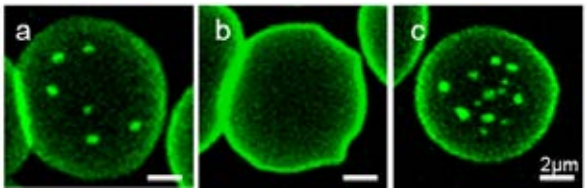

Fig. 2. Control of submicrometric domains labelled on spread erythrocytes upon insertion of fluorescent sphingomyelin (BODIPY-SM). (a) control erythrocytes; (b) cholesterol depletion (-25%); (c) familial spherocytosis. Adapted from Ref. 2.

Two opposite roles for submicrometric domains may be considered: they could (i) promote lipid resilience necessary to membrane deformability, by providing stretchable membrane reservoirs for RBC squeezing into the narrow pores of spleen sinusoids; or (ii) reflect high-tension fragility sites, i.e. propensity to fragmentation and hemolysis during splenic filtration of senescent or diseased RBCs (spherocytosis). Testing these hypotheses will require biophysical studies.

Apical endocytosis and diseases

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

Due to extraordinary efficiency of apical receptor-mediated endocytosis, kidney proximal tubular cells (PTCs) are a unique system to study machineries of apical endocytic trafficking and their involvement in kidney diseases. Using KO mice for the chloride channel, ClC-5, as model of Dent's disease (familial predisposition to kidney stones), we demonstrated that circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, thus providing a major source of enzymes to PTCs. These observations extend the significance of megalin in PTCs and have several physiopathological and clinical implications (Ref 4).

Recently, we identified a key role of class III PI3-kinase/VPS34 in apical recycling of endocytic receptors. In vitro, VPS34 inhibition with LY294002 induced selective apical endosome swelling and sequestration of the endocytic receptor, megalin. This effect was reversible: removal of the inhibitor induced a spectacular burst of recycling tubules and restored the megalin surface pool. In mousepups PTCs, conditional Vps34 inactivation also led to vacuolation and intracellular megalin redistribution. We anticipate that these KO mice and reversible PI3K inhibition will help further identify rate-limiting actors of apical endocytosis, of both fundamental and clinical importance (Ref 5).

Current investigations are also addressing the pathophysiology of cystinosis, a multisystemic lysosomal disease due to defective lysosomal membrane cystine/H+ antiporter, cystinosin. This disease first manifests itself in kidney as a generalized PTC dysfunction, referred to as kidney Fanconi syndrome. Endocytosis of ultrafiltrated plasma proteins rich in disulfide bridge rich

must be the main source of lysosomal cystine in PTCs. Analysis of cystinosin KO mice helped us understand how cystine accumulation causes apical PTC dedifferention and eventual atrophy (Fig. 3) and to identify adaptation mechanisms. We are now focusing onhow cystinosis can be corrected by grafting of hematopoietic stem cells (Ref 6).

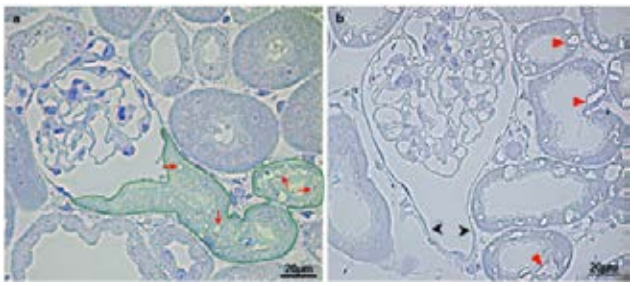

Fig. 3. Progression of PTC lesions in cystinosin KO mice. At 6 months (a), only PTCs immediately following glomeruli (overlaid in pale green) show extensive apical vacuolation (arrows), indicating osmotic swelling of lysosomes by accumulating cystine, contrasting with integrity of kidney elsewhere, including more distal PTCs. (b) At 12 months, proximal PTCs are now completely atrophic (arrowheads) and more distal PTCs harbour numerous crystals (appearing as empty spaces with characteristic geometric shape; red triangles) (From Ref. 6)

ZONAB/YBX3 as regulator in the proliferation/differentiation switch

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

Epithelial polarization depends, and impacts, on gene expression. The transcription factor, ZONAB/YBX3, can shuttle between tight junctions and the nucleus to promote expression of cyclin D, and thus participate in the control of proliferation. During mouse kidney ontogeny and polarization of PTC monolayers in vitro, we confirmed that high ZONAB expression was associated with epithelial proliferation but not with differentiation (Fig. 4). Conversely, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium markers. In PTC, we further showed that ZONAB can simultaneously repress the expression of differentiation markers and that its expression was regulated by polarity. These in vitro and in vivo data suggest that ZONAB is a sensor of epithelial density, involved in their switch from proliferation to differentiation (Ref 7).

Recently, we studied ZONAB/YBX3 expression in developing and diseased epithelial organs. In the embryonic pancreas and thyroid glands, ZONAB was only expressed in cells localized at the periphery of the expanding epithelia, known as proliferating progenitors.

We also re-assessed the expression level of ZONAB/YBX3 in clear-cell renal cell carcinomas as compared to their normal tissues. Paying special attention to the housekeeping genes used for normalization, we found that ZONAB expression depends on the tumour grade and may serve as an early-stage biomarker (Ref 8).

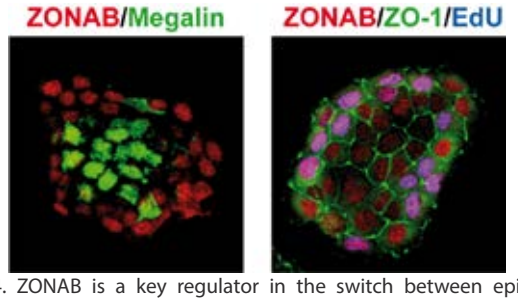

Fig. 4. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right) (From Ref 7).

Epithelial differentiation

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

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

Epithelial monolayer formation requires a coordinate and dynamic interaction with the close environment, composed of mesenchymal and endothelial cells. By three-dimensional analysis of developing pancreatic and thyroid epithelial buds, we uncovered a dense, closely apposed endothelial network (Fig. 5). Our in vivo and in vitro data show that endothelial cell recruitment is dependent on VEGF produced by the epithelium and that, in turn, endothelial cells, control pancreatic acinar differentiation (Ref 9), and promote thyroid follicle formation by releasing soluble and sedimentable factors (Ref 10). These data demonstrate that paracrine epithelial:mesenchyme and epithelial: endothelial interactions are crucial for organ differentiation.

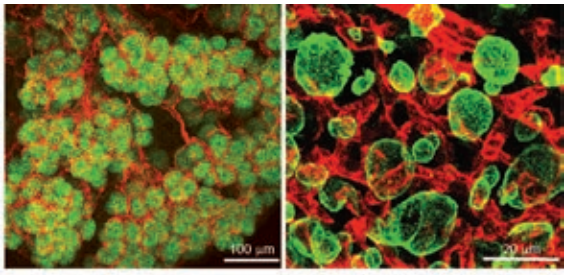

Fig. 5. Epithelial:endothelial interactions in the pancreas and thyroid. Projections of 40 to 50 confocal images showing the dense and close association of endothelial cells, labelled for PECAM (red) with pancreatic epithelial cells, labelled for E-cadherin (green, left) or with thyroid follicles, labelled for Ezrin (green, right) (From Ref 9 & 10).

Brief report on the Platform for Imaging Cells and Tissues

P. Van Der Smissen, T. Lac, J. Daubie, A. Errachid, 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; Cominelli et al., 2014, Traffic 15:401-17 see their report p 73), we have pursued our long-term commitment to promote collaborations by sharing expertise in cellular imaging. For the recent years, see our contribution to subcellular trafficking of the thrombopoietin receptor (Pecquet et al., 2012, Blood; see report by S. Constantinescu, p 121) 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., 2010, J. Biol. Chem. 285:18888-98; Tahay et al., Biochem J. 2012,441:105-12); subcellular distribution of the NAD(P)HX repair system (Marbaix et al., 2014, Biochem J. 460:49-58 see report by E. van Schaftingen, p 50); 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); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., 2005, Genes Dev 19:1849-54; see report by F. Lemaigre, p 30) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., Biochim. Biophys Acta Mol Cell Res 2007, 1773:521-35), 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

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

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4. 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.

5. Carpentier S, N'kuli F, Grieco G, Van Der Smissen P, Janssens V, Emonard H, Bilanges B, Vanhaesebroeck B, Gaide Chevronnay HP, Pierreux CE, Tyteca D, Courtoy PJ. Class III phosphoinositide 3-kinase/VPS34 and dynamin are critical for apical endocytic recycling. Traffic 2013; 14:933-48.

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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, 6, 7). This seminal observation led us to use this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes. We recently focused on the control by individual cells of local MMP activity, including induction by hypoxia and down-regulation by receptor-mediated endocytosis and degradation. We also investigate whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding (2) and endometriosis.

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

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

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

We noticed that MMP-27 was produced by various cell lines but was not secreted. Because sequence comparison with other MMPs suggested that MMP-27 was prolonged by a unique C-terminal extension (CTE) partially hydrophobic but shorter than the transmembrane domain of well-known membrane-type-MMPs (MT-MMPs), we investigated the effects of the CTE on MMP-27 intracellular retention (9). Subcellular fractionation and/or confocal microscopy highlighted retention of endogenous MMP-27 (Fig. 2) and of tagged recombinant rMMP-27 in the endoplasmic reticulum (ER). In striking contrast, a truncated form of rMMP-27 without CTE was secreted. Moreover, addition of the MMP-27 CTE to rMMP-10 (a classical secreted MMP) blocked its secretion and resulted in a subcellular localization similar to that of rMMP-27. In the same publication, we further demonstrated, by proteinase K protection assay and surface biotinylation, that MMP-27 is not a transmembrane protein. MMP-27 is rather a peripheral membrane protein since endogenous or recombinant MMP-27 was found exclusively in the aqueous phase after Triton X-114 extraction.

In parallel, expression of MMP-27 was measured by quantitative PCR in endometrial samples representative of the different phases of the menstrual cycle (10). The levels of MMP-27 mRNA steadily increased during the secretory phase to culminate at the menstrual phase and decreased during the proliferative phase. MMP-27 mRNA was also detected by in-situ hybridization, in isolated cells from various organs during mouse development, suggesting strict control of cellular origin. In agreement, MMP-27 was immunostained in the human

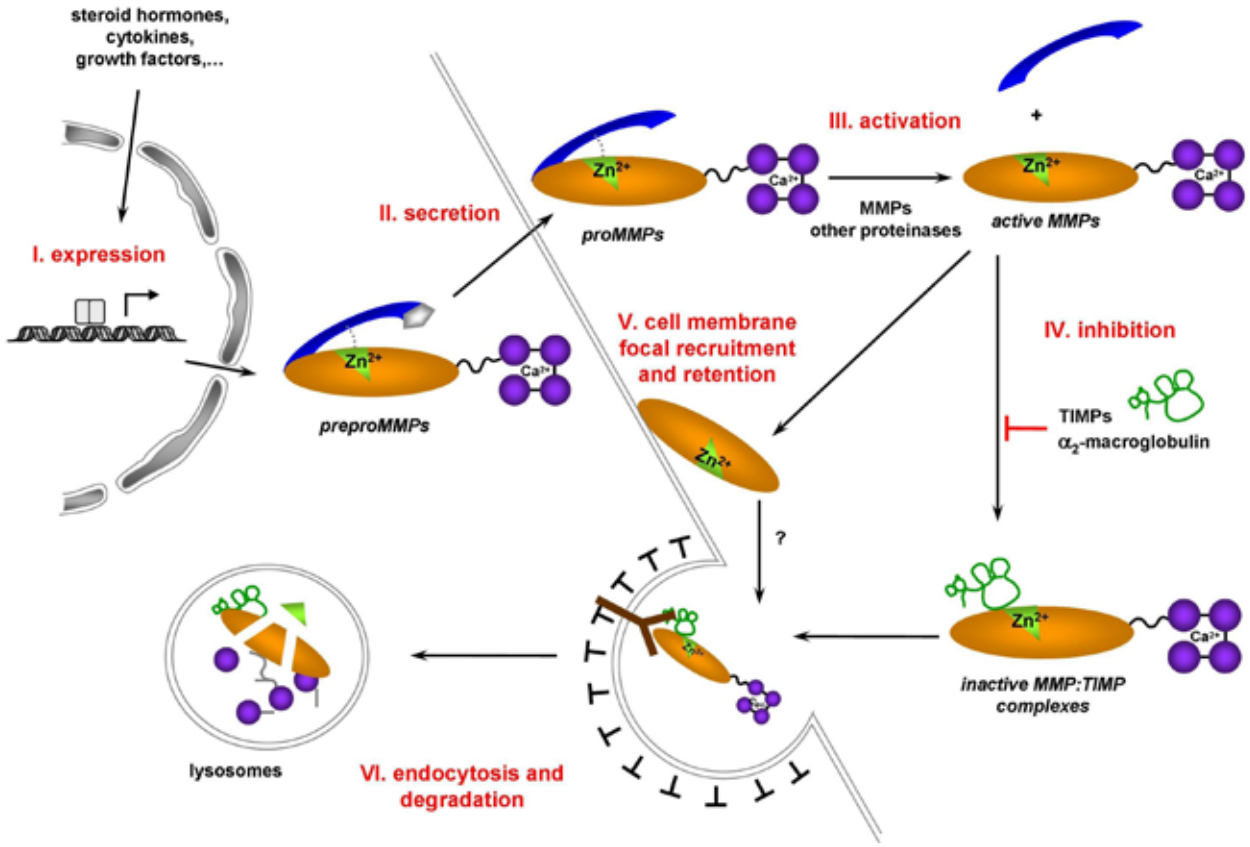

Fig. 1. Regulation of soluble MMP activity in the human endometrium: current model.MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn2+-dependent catalytic site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

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

endometrium in large cells expressing CD163 and CD206, two specific markers of M2 macrophages. In the same publication, we also reported that MMP-27 was abundant in superficial endometriotic lesions (ovary, peritoneum) but not in deep endometriosis lesion (recto-vaginal wall).

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 (3). Using human fibrosarcoma HT1080 cells, we recently identified two membrane-associated metalloproteinases, ADAM-12 and MT1-MMP that shed LRP-1 ectodomain (5). 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

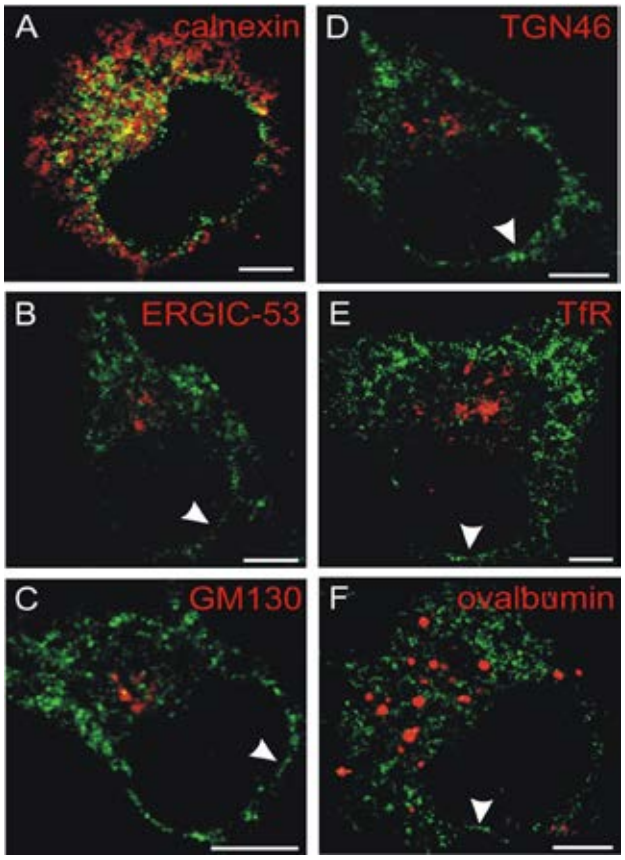

Fig. 2. MMP-27 partially colocalizes with an endoplasmic reticulum marker but dissociates from other cell compartments. COS cells were cultured on coverslips, fixed, permeabilized and double-immunolabeled for MMP-27 (in green) and marker antibodies (in red) identifying the ER (calnexin, A), the ER-Golgi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

shedding from epithelioid cells was ~4-fold lower than from fibroblastoid cells. Release of the ectodomain was triggered by cholesterol depletion in epithelioid cells and impaired by cholesterol overload in fibroblastoid cells. Modulation of LRP-1 shedding on clearance was reflected by accumulation of gelatinases (MMP-2 and -9) in the medium. We conclude that cholesterol exerts an important control on LRP-1 level and function at the plasma membrane by modulating shedding of its ectodomain, and therefore represents a novel regulator of extracellular proteolytic activities (Fig. 3).

Endometrial xenografts

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

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

Menstrual endometrial breakdown induced by estradiol and progesterone withdrawal is regularly attributed to vasospasm of spiral arteries causing ischemia and hypoxia. We investigated whether hypoxia actually occurred in our xenograft model (8). Three complementary approaches were used to look for signs of hypoxia in fragments of human functionalis xenografted to ovariectomized immunodeficient mice bearing pellets-releasing estradiol and progesterone, and then deprived of ovarian steroids. Hormone withdrawal 21 days after grafting induced menstrual breakdown and MMP expression within 4 days. Local partial oxygen pressure (pO2) was measured by electron paramagnetic resonance using implanted lithium phtalocyanine crystals. In mice with hormone maintenance until sacrifice, pO2 was low one week after grafting

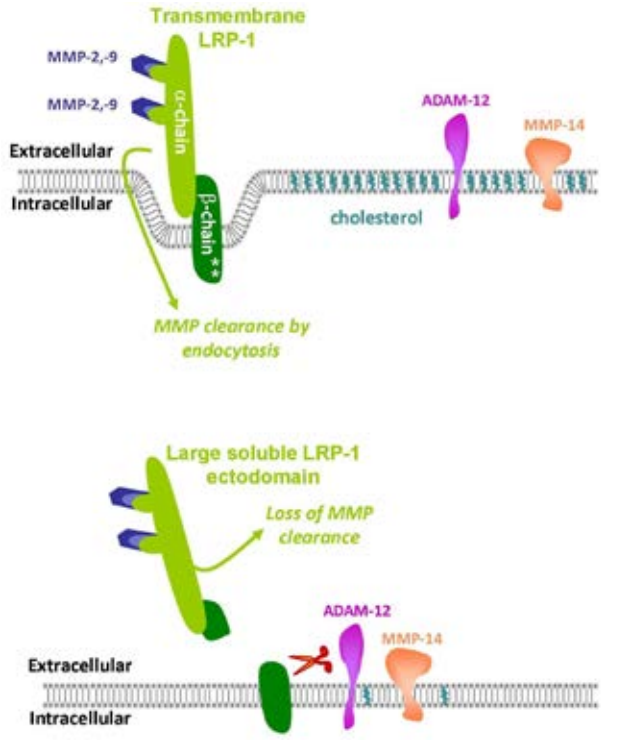

Fig. 3. A model for regulation of gelatinase activity by LRP-1. Upper panel: Binding of gelatinases (MMP-2 and -9) to LRP-1 triggers avid receptor-mediated endocytosis thanks to its two NPxY motifs (indicated by *). Sheddase activity of ADAM-12 and MT1-MMP is prevented by cholesterol-induced membrane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

but increased twofold from the second week when tissue was largely revascularized. After 3 weeks, pO2 was not modified by hormone withdrawal but was slightly increased on hormone reimpregnation 4 days after removal by comparison with hormone maintenance. These results were confirmed using fluorescence quenching-based OxyLite measurements. In a further search for signs of hypoxia, we did not find significant HIF1-α immunostaining, nor pimonidazole adducts after hormone withdrawal. This study allowed us to conclude that hypoxia is not needed to trigger menstrual-like tissue breakdown or repair in human endometrial xenograft.

Selected publications

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10. Cominelli A, Gaide Chevronnay HP, Lemoine P, Courtoy PJ, Marbaix E, Henriet P. Matrix metalloproteinase-27 is expressed in CD163+/CD206+ M2 macrophages in the cycling human endometrium and in superficial endometriotic lesions. Mol Hum Reprod 2014; in press.

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Growth factor receptors: From signal transduction to human diseases

Jean-Baptiste Demoulin

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

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

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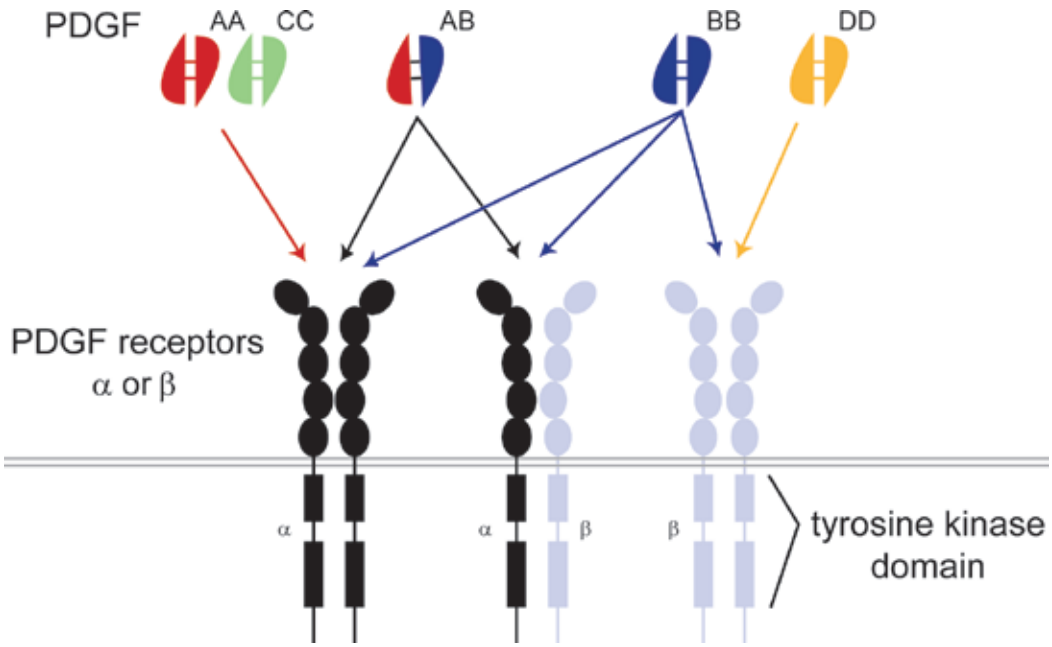

Fig. 1. PDGF receptors and ligands

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

A. Coomans de Brachène, A. de Rocca Serra, E. Bollaert, A. Essaghir 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 (1). 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 (2). We also analyzed gene expression in neural stem cells, glioma, carcinoid tumors and leukemic cells.

One key transcription factor family that is regulated by growth factors is FOXO. These factors induce a cell cycle arrest, increase resistance toward oxidative stress and regulate metabolism. They are inactivated by growth factors via AKT, which phosphorylates three conserved sites within FOXO proteins. Phosphorylated FOXO is excluded from the nucleus and targeted for degradation by proteasomes (Fig. 2). We observed that FOXO mRNA expression is also decreased upon stimulation with growth factors. We showed that the promoter of the FOXO1 gene is stimulated by FOXOs themselves, a process that is disrupted by growth factors, most likely via AKT, and regulates cell growth. We are now analyzing whether this mechanism could play a role in the proliferation of tumor cells. We also identified several mediators of the effects of FOXO and growth factors on the cell cycle, such as HBP1 (3).

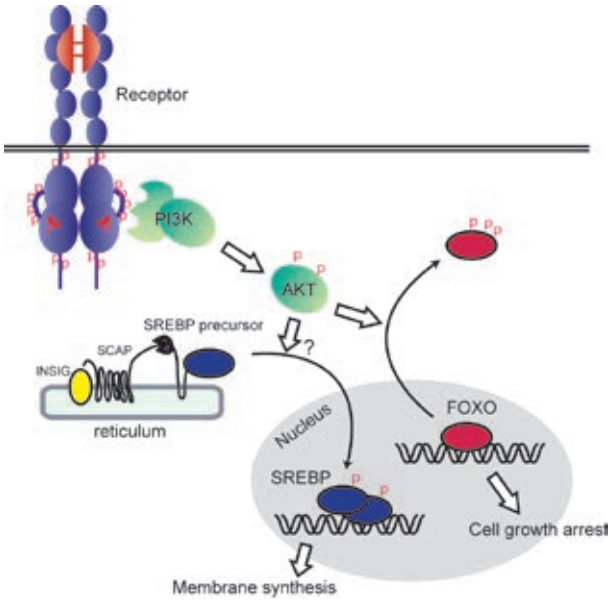

Fig. 2. Activation of SREBP and inactivation of FOXO by PDGF

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

Rearrangements of receptor tyrosine kinase genes associated with leukemia

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

Although PDGF receptors are expressed on platelets and macrophages, PDGF receptor-deficient mice show no primary hematopoietic or immune defect. In vitro, PDGF is a poor mitogen for hematopoietic cells. However, alterations of PDGF receptor genes, as a result of chromosomal translocation or deletion, are found in chronic myeloid neoplasms associated with hypereosinophilia (4). These chromosomal alterations create fusion genes that produce hybrid proteins comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. In most cases, they also retain the receptor transmembrane domain, which plays a particular role in the activation of these oncoproteins (4).

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

TPβ and FPα do not induce eosinophilia in mice. In order to develop a model that is more relevant for the human disease, we introduced TPβ and FPα in human CD34+ cells, which were purified from umbilical cord blood and are enriched in hematopoietic stem cells. These cells are able to differentiate

normally in vitro into various blood cell types, depending on the cytokine cocktail that is added in the culture medium. We observed that TPβ and FPα induce the proliferation of these cells and their differentiation into eosinophils in the absence of cytokine. We showed that this process is dependent on the activation of the NF-κB pathway via PI3K (6). We also showed that the SHP2 tyrosine phosphatase plays an important role in cell transformation by these oncogenes (7).
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 5). We are now looking for other mutations in tyrosine kinase genes.

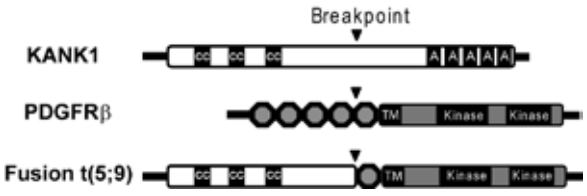

Fig. 3. Structure of the KANK1-PDGFRβ fusion protein created by the t(5;9) translocation.
CC: coiled-coil domain; A: ankyrin repeat; TM: transmembrane domain

Functional characterization of novel PDGF receptor mutations in cancer

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

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

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 (2). We generated a catalogue containing 352 transcription factors associated with 2,721 target genes and 6,422 regulations. When it was available, a distinction between transcriptional activation and inhibition was included for each regulation. Next, we built a tool (www.TFactS.org) that compares new submitted gene lists with target genes in the catalogue to detect regulated transcription factors. We validated TFactS with our own microarray experiments and with published lists of regulated genes in various models and compared it to tools based on in silico promoter analysis. Our results show that changes in the expression level of transcription factor target genes constitute a robust signature for transcription factor regulation, and can be efficiently used for microarray data mining. We are now introducing new features into this tool and we are using it to analyze cancer genome data. This tool was also used in a number of transcriptomics projects in our laboratory (see for instance references 3 and 6) and in collaboration with other groups. We are now integrating micro-RNA targets into TFactS to take into account these important regulators.

Roles of microRNAs in the pathogenesis of myeloid neoplasms

V. Havelange, V. Vandewalle

MicroRNAs (miRNAs) are evolutionary conserved tiny noncoding RNAs (~18-25 nucleotides) that regulate negatively gene expression. MiRNAs have been involved in critical cell processes such as proliferation, apoptosis, differentiation and

tumorigenesis. Recent data indicated that miRNAs are deregulated in hematological malignancies, particularly in acute myeloid leukemia (AML). The functions of most of these miRNAs in AML are currently unknown.

In collaboration with Carlo Croce and Ramiro Garzon, we focused our work on elucidating the roles of miRNAs in AML. We first integrated messenger RNA and miRNA expression profilings from AML patients to identify functional links between the whole transcriptome and microRNome involved in myeloid leukemogenesis (9). We could identify correlations between miRNAs and HOX-related genes, genes involved in immunity and inflammation (e.g., IRF7, TLR4), proapoptotic genes (e.g., BIM, PTEN), genes involved in hematopoiesis (eg, CEBPB, JAK2) and in chromatin remodeling (e.g., PCAF, HDAC9). These correlations suggested a central role for miRNAs in regulating these pivotal pathways in AML.

Furthermore, we hypothesized that miR-29b could act as a tumor suppressor in AML as miR-29b was down-regulated in many subtypes of AML. Restoration of miR-29b expression in AML cell lines and in primary samples reduced cell growth, induced apoptosis and dramatically reduced tumorigenicity in a xenograft leukemia model. Transcriptome analysis indicated that miR-29b targeted genes involved in apoptosis, cell cycle and proliferation pathways such as MCL-1 or CDK6. We also found that miR-29b was involved in DNA methylation in AML. MiR-29 targeted and down-regulated DNA methyltransferases (DNMT3A and 3B) and indirectly DNMT1 by targeting Sp1. Enforced expression of miR-29b in AML cell lines resulted in a decrease of 30% in global DNA methylation and the reexpression of p15INK4B and ER via promoter DNA hypomethylation. Together, these data provided a rationale for the use of synthetic miR-29b oligonucleotides as a novel strategy to improve treatment response in AML.

We investigated whether miRNAs could predict chemotherapy response. In a phase II clinical trial with single hypomethylating agent decitabine in older de novo AML patients, higher levels of pretreatment miR-29b were associated with clinical response ($P = 0.02$). More recently, we found that high baseline miR-10 family expression in untreated AML patients was associated with achieving complete response (10). There was a significant interaction effect between miR-10a-5p expression and nucleophosmin (NPM1) mutation status. Loss and gain of function experiments using miR-10a-5p in cell lines and primary blasts did not demonstrate any effect in apoptosis or cell proliferation in baseline conditions nor after chemotherapy. These data support a bystander role for miR-10 family in NPM1-mutated-AML.

We are currently investigating the roles of miRNAs and target genes in chemoresistance in AML patients.

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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, as well as their consequences on unrelated diseases that develop concomitantly in the infected host.

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. We found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. The modulations of antibody responses was analysed with more details by using a model of infection with lactate dehydrogenase-elevating virus (LDV), a common mouse nidovirus that induces strong and early immune responses (1). We could demonstrate that a dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias. IgG2a anti-LDV antibodies were found to be more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (2). However, the modification of the isotype of antibodies reacting with self antigens could potentially lead to more deleterious autoimmune reactions.

T helper lymphocyte differentiation

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

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

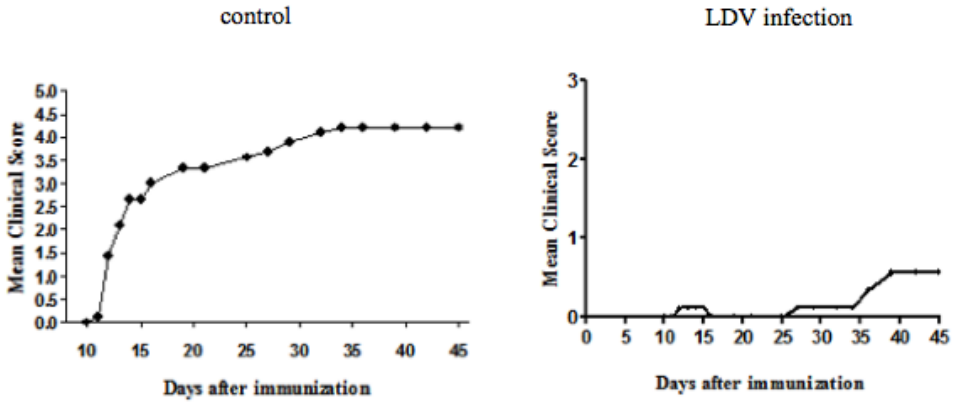

Fig. 1. Encephalitis progression in control mice and in animals infected with LDV.

immune system that are able to secrete this cytokine, namely the natural killer (NK) cells. Within a few days after infection, a strong and transient NK cell activation, characterized by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Two pathways of IFN-γ production have been observed that both involve NK cells. The first pathway, found in normal mice, is independent from type I IFN and from interleukin-12. The second pathway involves interleukin-12, but is suppressed by type I IFN. 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. 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 (3). Therefore, different pathways of NK cell activation, leading to various phenotypes and, probably various functions, may be observed.

Because cancer development is controled by immunosurveillance, including by NK cells, we analysed the effect of LDV infection on plasmacytoma growth (4). Acutely infected animals were significantly protected against tumor development. This protection was mediated by natural killer cell activation and by interferon-γ production. It might also be related to activation of NK/T cells, although this remains to be formally proven.

Dendritic cells and antigen presentation

In collaboration with J Van Snick and C Uyttenhove, acute LDV infection was found to prevent also graft-versus-host disease. This protective effect was correlated with the functional impairment, followed by the disappearance of a subset of dendritic cells involved in allogeneic reactions. The mechanisms leading to this effect of LDV on dendritic cells and antigen presentation are under investigation.

Activation of macrophages and enhanced susceptibility to endotoxin shock

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

Blood autoimmune diseases

Virally-induced macrophage activation leads also to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. LDV infection resulted in moderate thrombocytopenia in normal animals through enhanced spontaneaous platelet phagocytosis (7). Our analysis was then focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti- platelet response was developed in the mouse. Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s) shared by rat and mouse platelets. 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, 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. The increase of thrombocytopenia triggered by LDV after administration of anti-platelet antibodies was largely suppressed in animals deficient for IFN-γ receptor. Moreover, LDV infection resulted in an increased expression of receptors recognizing the Fc portion of antibodies, which may at least partially leads towards the enhanced phagocytic activity of macrophages. Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia and anemia by activating macrophages through IFN-γ production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. Regulation of macrophage activation results in modulation of autoantibody-mediated cell destruction and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism.

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Viral persistence and interferon response

Thomas Michiels

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

1. Theiler's virus

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

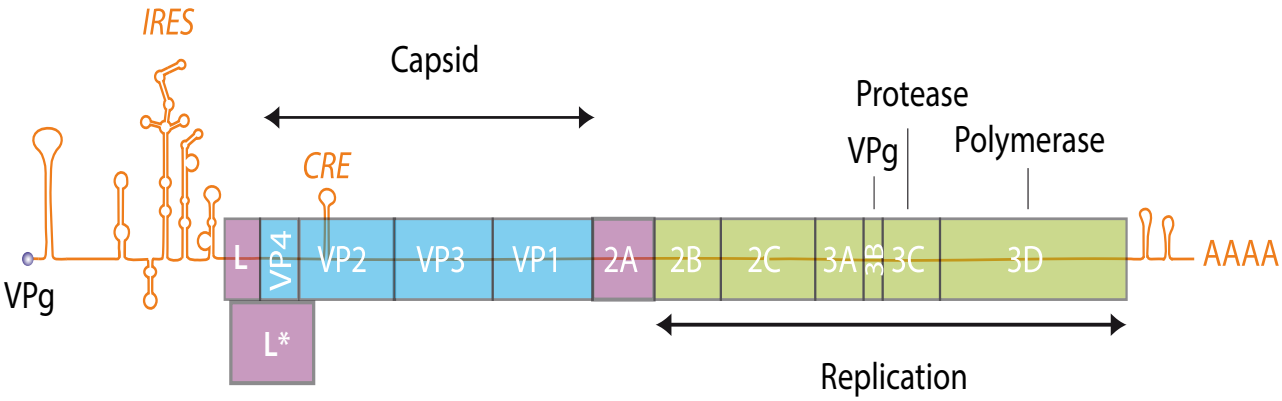
Analysis of viral proteins involved in Theiler's virus escape of the host immune response.

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

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

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

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



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

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

adapted from www.virologie-uclouvain.be

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

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

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

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

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

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

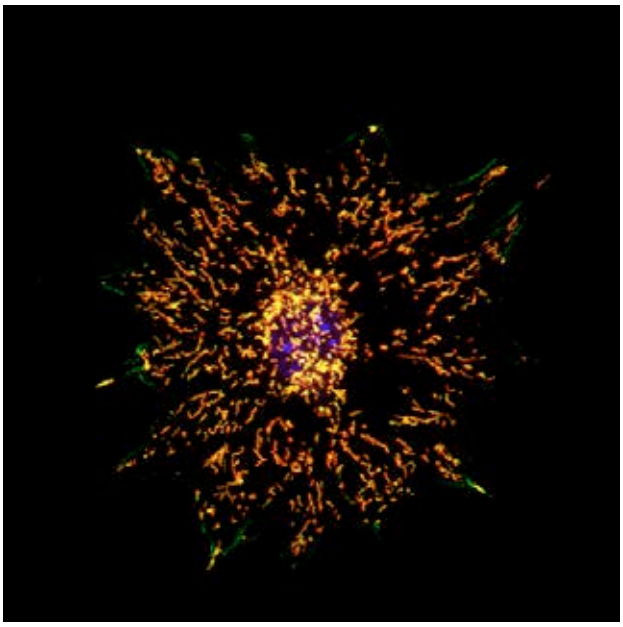


Fig. 2. Mitochondrial localization of L*
Mouse embryonic fibroblast expressing L* from a lentiviral vector. L* was immunolabeled in green and mitochondria were labeled in red with mitotracker. Yellow indicates co-localization.

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

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

2. Type-I and Type-III Interferons

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

Type I IFNs

P. Hermant, C. Lardinois and T. Michiels

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

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

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

Type-III Interferons (IFN-λ)

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

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

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

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

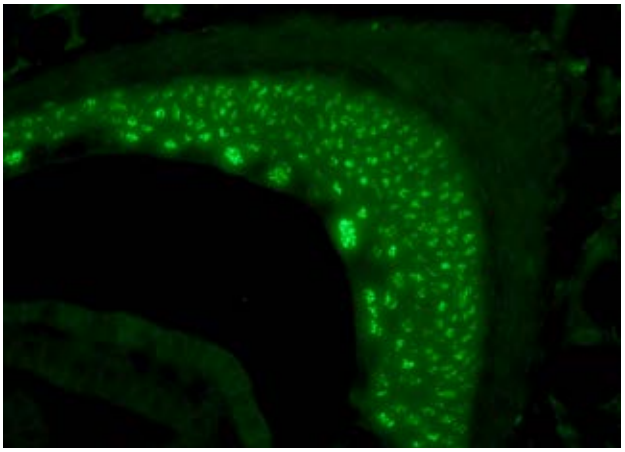


Fig. 3. Response to IFN-I. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-I. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Neurons and IFN-α/β

M. Kreit and T. Michiels

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

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

Recent work from our laboratory shows that neurons are also restricted in their response to IFN. After IFN treatment, primary neurons respond to IFN through the transcriptional upregulation of many IFN-stimulated genes. However, in contrast to other cell types, IFN-treated neurons are not efficiently protected against viral infection. We identified a series of 15 genes that were upregulated by IFN in fibroblasts but that were very weekly or not expressed in neurons. Among these genes is the gene encoding apolipoprotein L9, a protein that proved to have antiviral activity against the neurotropic Theiler's murine encephalomyelitis virus. Our data suggest important functional differences in the IFN response mounted by specific cell populations (9).

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Human tumor immunology

Pierre Coulie
Sophie Lucas

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

Immune responses to cancer vaccine antigens

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

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

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

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

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

other modalities of cancer immunotherapy such as adoptive transfer of antitumor T lymphocytes or immunostimulatory antibodies targeting the CTLA-4 or PD-1 pathways.

Inflammatory cytokines and tumor-infiltrating lymphocytes

T. Seremet, N. Remy, J. Vanacker, C. Muller, P.G. Coulie.

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

Analysis of lymphocytes infiltrating human breast carcinomas

D. Schröder, G. Hames, N. Dauguet, P.G. Coulie in collaboration with D. Godelaine, Brussels branch of the Ludwig Institute for Cancer Research, , J. Carrasco, Grand Hôpital de Charleroi, and J.-P. Machiels, M. Berlière and C. Galant, Cliniques universitaires St Luc.

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

Human regulatory T cells and TGF-ß

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

Regulatory T cells, or Tregs, are a subset of CD4+ lymphocytes specialized in the suppression of immune responses. They prevent the development of auto-immune diseases, but in mice they also contribute to cancer progression by inhibiting anti-tumor immune responses. Whether Tregs play a negative role in cancer patients has remained difficult to verify due to the lack of a Treg-specific marker in humans, as well as to an incomplete understanding of the mechanisms underlying their suppressive function.
Our objective is twofold: develop tools to quantify Tregs in human tissues, and identify mechanisms important for their suppressive function, which could be specifically targeted to improve the efficiency of cancer vaccines.
Our previous work lead to the obtention of stable human Treg clones, representing long-term cultures of pure lymphocyte populations available for repeated analysis (5). A stable epigenetic mark unambiguously distinguished human Treg clones from non regulatory T cells: a conserved non-coding region in gene *FOXP3*, encoding a transcription factor indispensable for the development and function of Tregs, was found demethylated in Treg clones only. We set up a methylation-specific real-time PCR assay to quantify demethylated *FOXP3* sequences, indicative of the presence of Treg cells. We used this assay to

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

Functional analysis of our Treg clones revealed that a hallmark of stimulated human Tregs is to produce the active form of TGF-ß, a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF-ß.
Many cell types produce the latent, inactive form of TGF-ß. In latent TGF-ß, the mature TGF-ß protein (in green in Fig. 1) is bound to the Latency Associated Peptide, or LAP (in grey in Fig. 1), and is thereby prevented from binding to the TGF-ß receptor. We recently showed that latent TGF-ß binds to GARP, a transmembrane protein which is present on the surface of stimulated Tregs but not on other T cells (9). We hypothesise that membrane localization of latent TGF-ß through binding to GARP is required for activation of the cytokine by Tregs. We are currently trying to obtain antibodies against GARP that inhibit active TGF-ß production by Tregs. We also observed that Tregs secrete a new form of soluble latent TGF-ß, in which it is disulfide-linked to GARP (Fig. 1). Secretion of soluble GARP/TGF-ß complexes, possibly through shedding form the cell surface, appears to be specific of the T cell lineage (10). We will try to understand what function these new types of complexes could exert.

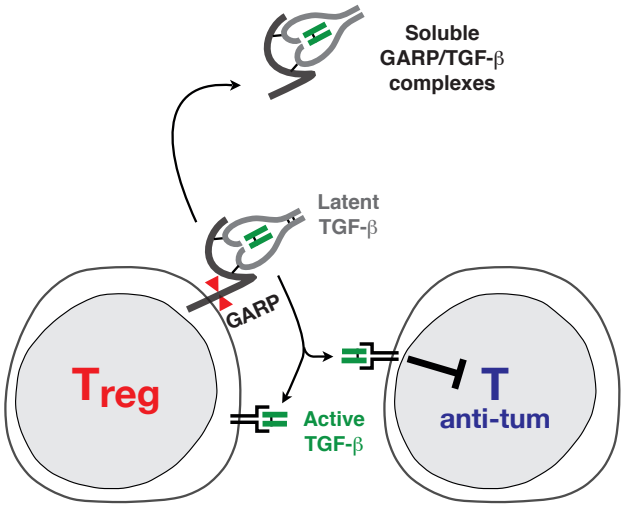

Fig. 1. Human Tregs produce active TGF-ß by a mechanism that appears to require GARP, and secrete soluble GARP/TGF-ß disulfide-linked complexes.

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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

In recent years, cancer immunotherapy made tremendous progresses and reached the clinical arena by showing its ability to prolong the survival of advanced cancer patients. This was largely based on the discovery of tumor antigens by our groups in Brussels in the nineties, a finding that demonstrated that the immune system has the capacity to recognize cancer cells as foreign bodies. These recent clinical results have generated enormous interest in the oncology field and the pharmacological industry. Yet, clinical benefits remain limited to a subset of patients, and further research is needed to understand the reason for this. Our current research focuses on two aspects that are relevant to this question. The first is the processing of tumor antigens, i.e. the intracellular mechanisms responsible for the expression of antigens at the surface of tumor cells. The second is the tumor microenvironment, whose immunosuppressive properties emerge as a major reason why many patients currently do not benefit from cancer immunotherapy.

Processing of tumor antigens

Peptide splicing by the proteasome

N. Vigneron, V. Stroobant, A. Michaux

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They result from degradation of these proteins, mainly exerted by the proteasome. We have identified a new mode of production of antigenic peptides, which involves the splicing of peptide fragments by the proteasome (1). Peptide splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate (Figure 1). Splicing of peptide fragments can occur in the forward or reverse order to that in which they appear in the parental protein (2). We have now described five spliced peptides, three of which are spliced in the reverse order (3, 4) One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines into aspartic acids, through a process a N-glycosylation/deglycosylation (3). We also showed that the splicing reaction required a minimal size of 3 amino acids for the fragments to splice (4). Both the standard proteasome and the immunoproteasome have the ability to splice peptides. However, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice.

Intermediate proteasome types

J. Abi Habib, E. De Plaen, B. Guillaume, N. Vigneron, A. Michaux

The proteasome exists in two forms: the standard proteasome, which is constitutively present in most cells, and the immunoproteasome, which is constitutive in many immune cells and can be induced by interferon-gamma in most other cells. They differ by the three catalytic subunits they use: ß1, ß2 and ß5 for the standard proteasome; ß1i, ß2i and ß5i for the immunoproteasome. We have described two new proteasome subtypes that are intermediate between the standard proteasome and the immunoproteasome (5). They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30 to 54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. They uniquely process several tumor antigens (5, 6). We are currently studying the function of these intermediate proteasomes, not only in terms of processing of antigenic peptides, but also for other functional aspects

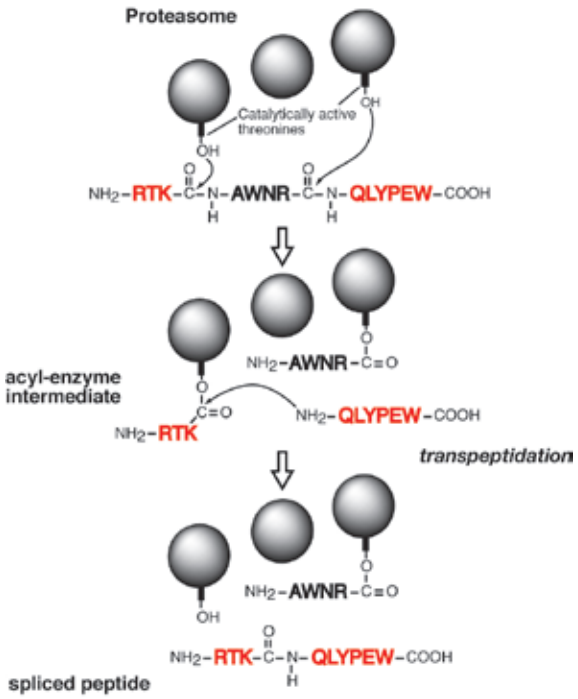

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme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

in which the proteasome plays a crucial role, such as the regulation of the cell cycle, the activation of transcription factors and the regulation of inflammation and immune responses.

Other proteases involved in antigen processing

N. Vigneron, A. Michaux, V. Stroobant

We are interested in characterizing the processing of human antigenic peptides that are not produced by the proteasome. We studied a proteasome-independent peptide derived from tumor protein MAGE-A3, and identified insulin-degrading enzyme as the protease producing this peptide (7). Insulin-degrading enzyme is a cytosolic metallopeptidase not previously known to play a role in the antigen processing pathway. The parental protein MAGE-A3 appears to be degraded along two parallel pathways involving insulin-degrading enzyme or the proteasome, each pathway producing a distinct set of antigenic peptides presented by MHC class I molecules. We are currently studying the processing of other proteasome-independent peptides and aiming to identify the protease(s) involved.

TAP-independent antigenic peptides

N. Vigneron, V. Stroobant, L. Pilotte

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

Cross-presentation

W. Ma, N. Vigneron, in collaboration with P. Courtoy and P. Van Der Smissen

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

Mechanisms of tumoral immune resistance

Indoleamine 2,3-dioxygenase

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

We previously discovered that tumors often resist immune rejection by expressing Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme that is profoundly immunosuppressive (8). We showed that immune rejection was restored by administration of a pharmacological inhibitor of IDO. In collaboration with medicinal chemists in Namur and Lausanne, we identified several families of new IDO inhibitors that will be further optimized to develop drug candidates. We currently pursue functional studies on the mechanisms of IDO-induced immunosuppression, and on the signaling pathway responsible for IDO expression in tumors.

Tryptophan-dioxygenase

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

Transgenic mice developing autochthonous melanomas expressing P1A

C. Powis de Tenbossche, S. Cané, J. Zhu, C. Uyttenhove, N. Arts, E. De Plaen in collaboration with J. Van Snick and C. Uyttenhove

We have created a mouse model of autochthonous inducible melanoma expressing a defined tumor antigen (TIRP10B) (10). In this model, melanomas are induced (70% incidence) with tamoxifen, which, by activating CreER in melanocytes, induces the expression of Ha-Ras, the deletion of INK4a/ARF and the expression the tumor antigen encoded by cancer/germline gene P1A. A unique feature of this model is that melanomas first develop as non-aggressive highly pigmented tumors (Mela), which later dedifferentiate into unpigmented highly aggressive inflammatory tumors (Amela). We found that TGF-ß was a key factor responsible for this switch to aggressive tumors, which is reminiscent of the epithelial-to-mesenchymal transition (EMT) described in other contexts. We developed antibodies able to neutralize TGFß1 and TGFß3, and found that the former were able to increase survival of mice in this melanoma model. These results support the use of TGFß neutralizing therapies in the treatment of human melanoma.
The loss of pigmentation in aggressive tumors appears to result from the strong inflammation, and we identified a microRNA that is induced by interleukin-1 and downregulates expression of MITF, a transcription factor acting as a master regulator of pigmentation.
In this model, both pigmented (Mela) and unpigmented (Amela) tumors express the tumor antigen encoded by P1A. Mela tumors are ignored by the immune system, while Amela tumors are infiltrated by T lymphocytes that are rendered ineffective. We are studying the mechanisms responsible for this ineffectiveness. Our current results indicate that the tumor microenvironment actively induces the apoptosis of tumor-specific T lymphocytes that infiltrate the tumor. We are studying the molecular mechanisms responsible for this apoptosis.

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Regulation of T lymphocyte function in tumors

Pierre van der Bruggen

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

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

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

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. 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 IFN-γ and other cytokines upon non-specific stimulation with anti-CD3 and anti-CD28 antibodies. 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 shown for CTL clones. 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 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.

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

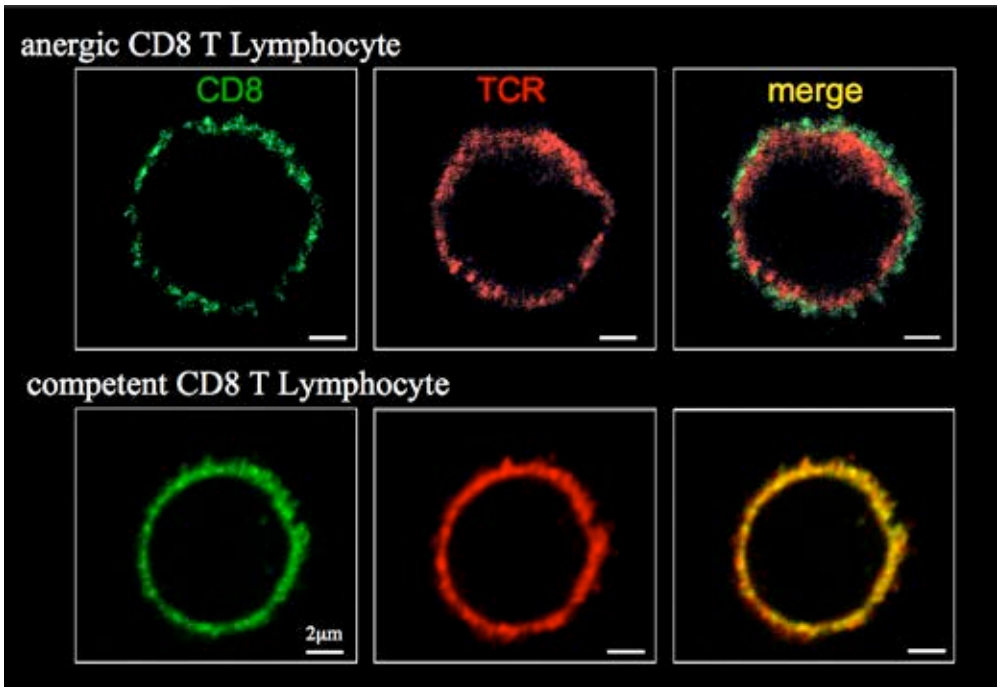

Fig. 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions

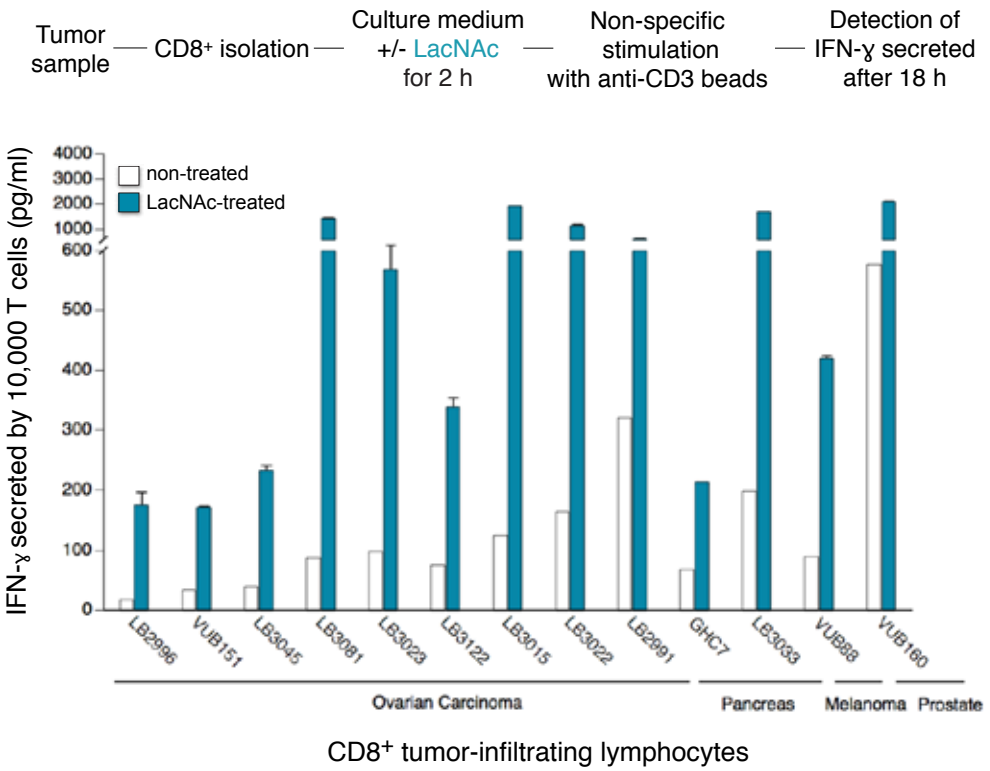

Fig. 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy

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.

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. The aim is to identify patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments. 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 centres. Blood samples are collected before and after surgery. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2- and A3-restricted HER2/Neu, hTERT, MUC1, and NY-ESO1 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 and will end in 2014. So far, 163 patients have been included. Blood frequencies of specific CD8 T lymphocytes before any oncological treatment were estimated on 48 patients. A small proportion (<20%) of patients seem to have a spontaneous anti-tumor response. Among screened antigens, detected responses are essentially directed against Her2Neu, hTERT and NY-ESO1. Frequencies are significantly higher than frequencies observed in the blood of healthy donors against the same antigen but remain very low (in the range of 1 anti-tumor CD8 among 1 million blood CD8). These low frequencies are difficult to detect and therefore their correlation with patient disease-free survival is tricky. In parallel to CD8 blood analysis, we try to perform a similar analysis on CD8 TIL extracted from the tumor at day of surgical removal. So far, seven tumors have been processed to this end. In

three of them, we detected responses directed against HER2/Neu, hTERT and NY-ESO1 at frequencies 10 times higher than the average frequency in the blood. Further genetic analysis of the clonal diversity is required to determine whether these responses are indicative of a clonal amplification of some anti-tumor CD8 T cells in the tumor.

Our current projects

- to examine T cell dysfunctions induced by chronic stimulation
- to examine the role of the different galectins in the impaired function of T cells
- to screen galectin ligands for their ability to restore T cell function
- to examine in different human pathologies if T cell functions are impaired and if it is possible to restore their function with galectin ligands
- to examine if the impaired secretion of IFN-γ by TIL is linked to defects in synapse formation and cytoskeleton remodelling
- to examine if galectins can sequester interleukins and chemokines
- to obtain ovarian carcinoma cell lines, generate tumor-specific T cell clones and identify the antigens recognized by these T cell clones.

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Immunotherapy analysis group

Thierry Boon

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

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

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

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

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

Female CBA mice do not reject male skin grafts, even though they are able to mount a cytolytic T cell response against HY, a male specific minor histocompatibility antigen encoded by the *Smcy* gene. To break this tolerance, we made repeated local injections of a low dose of IL-12, combined with IFN-α. This caused graft rejection in all the mice. Like IFN-α, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12. One finding of importance for the clinical application of this procedure is that several weekly cycles of cytokine treatments were 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 IFN-α (10^5 U) with TLR7 ligand gardiquimod (20 µg) caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. However, the same doses of IL-2 and IFN-α, without GM-CSF nor gardiquimod, were also effective when combined with both anti-TGF-β and anti-IL-10 antibodies, whereas anti-CTLA-4 antibody needed the additional presence of GM-CSF to show some effectiveness. IFN-γ could complement the local action of the IL-2 and gardiquimod combination as well as IFN-α. Repeated injections combining IL-2, INF-α and anti-PDL1 antibody had little effect but this effect was improved by adding anti-CTLA-4 antibody and GM-CSF in the drug mixture. We also determined that slow continuous release and long-term delivery formulations of IL-2 and IFN-α combined with gardiquimod provide the rejection effect obtained with the free cytokines. This makes it possible to reduce the frequency of injection.

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

To begin evaluating the action of the various immunostimulatory agents that proved capable of promoting graft rejection, we examined their ability to promote the presence of anti-HY CD8 T lymphocytes in the graft. We used CD8 T cells from spleens of T-cell receptor transgenic mice expressing a receptor specific for a HY peptide presented by H2-K^k. After i.p. adoptive transfer, the number of transgenic T cells present in any given location could be estimated by a quantitative PCR specific for this receptor sequence. No increase in the number of anti-HY transgenic T cells present in the graft was observed following treatment with either IFN-α or GM-CSF. In contrast, gardiquimod, IL-12, and IL-2 produced a very significant increase. We are unable to evaluate whether this increase is due mainly to increased influx, increased proliferation within the graft, or decreased efflux. The agents that cause a large increase in the number of anti-HY T cells found in the graft also cause a moderate increase of these cells in the blood and in the spleen. This could be due to some systemic action of these agents, or it could be a consequence of T-cell circulation between these compartments and the graft. We verified that the local cytokine treatment does not elicit a nonspecific influx of lymphocytes: no increase in lymphocytes was observed within female grafts [5].

Vaccinations with footpad injections of male lymphoblasts aimed at enhancing anti-H-Y T cell responses synergize effectively with the local cytokine treatment. We also observed that female CBA mice grafted with male skin not only failed to reject it without local treatment, but also invariably failed to reject male skin grafted later. In contrast, a majority of the mice that received a local treatment either with IL-12 and IFN-α or with IL-2, IFN-α and gardiquimod rejected such subsequent grafts, in the absence of further local treatment, indicating a degree of systemic increase of the anti-H-Y memory T cells in these mice [5].

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

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

Using our amine-reactive OVA multimers, as recently described [6], we have produced a series of monoclonal antibodies inhibiting many murine cytokines or proteins associated with cancer cell migration and metastasis.
We immunized mice against periostin/OSF-2, an extracellular matrix protein present in the stroma of many tumors, in mice and humans, that was recently implicated in metastasis development [7]. Several mAbs were produced that block periostin interaction with integrins αvß3 and αvß5, one of the mechanisms implicated in cancer cell migration and metastasis establishment (in collaboration with P. Jat (UCL, London) and S. Fields (LICR Oxford). They are currently tested in the 4T1 murine mammary adenocarcinoma model. They were tested in the 4T1 murine mammary adenocarcinoma. No effect on primary tumor size or survival advantage was observed in this model. TGFß3 was described as an inducer of periostin expression [8]. In addition, elevated TGFß3 levels were detected in the serum of patients suffering from various types of carcinomas [9]. We recently produced inhibitory mAbs that are strictly specific for TGFß3. They induced a slight reduction in primary tumor size, as previously observed by injecting our anti-TGFβ1 specific mAb, that did not however result in prolonged survival. These anti-TGFβ mAbs will now be combined in Balb/c mice implanted with 4T1 tumor cells transfected with the P1A tumor antigen to examine a pos-

sible synergy with an active anti-P1A immunization as well as in the inducible mouse model of melanoma developed in the laboratory [10].

Another cytokine that we wanted to inhibit *in vivo* is IL-27, a heterodimeric protein composed of p28 and EBI3 and that is one of the first cytokines produced, mainly by dendritic cells, in response to TLR stimulation and T cell activation. Applying our OVA conjugation procedure, in collaboration with Stanislas Goriely (*Institut d'Immunologie Médicale*, Campus de Gosselies, ULB), we successfully developed a mAb (MM27.7B1) that inhibits both human and mouse IL-27. This Ab prevented the development of mouse graft versus host disease in a parent to F1 model. Protection was associated with host cell survival and undiminished engraftment of donor cells, lack of host B-cell depletion, increased Th2-type immunoglobulin production, a decrease in serum IFN-γ, a drop in anti-H-2Dd cytotoxic T lymphocyte activity and an increase in Foxp3$^+$ T-reg cells. These observations raise fundamental questions about the role of IL-27 in the maintenance of tolerance as donor and host cells coexisted permanently in these animals. Besides pathogenic effects on the recipients, GVHD development is associated to a positive graft versus leukemia (GVL) effect. In the parent to F1 model of GVH, effects of IL-27 inhibition on GVL are currently investigated.

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Therapeutic vaccination and tumor expression profiling group

Nicolas van Baren

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

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

A. Clinical trials with cancer vaccines

Vaccination of melanoma patients with CyaA-Tyr

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc) and C. Leclerc (Institut Pasteur, Paris, France)

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

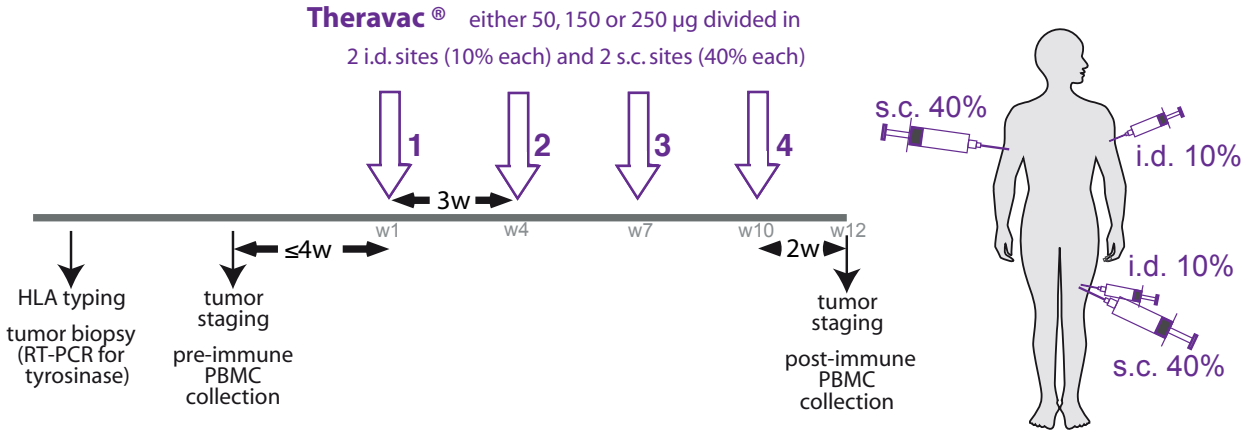

Fig. 1. Clinical trial : vaccination of melanoma patients with CyaA-Tyr

Tyr has a very potent capacity to activate Tyrosinase.A2-specific CTL. In our clinical trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of CyaA-Tyr, at increasing doses (Fig.1). If successful, this new vaccine modality could have a much broader application than melanoma vaccines.

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

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

In this phase 1/2 trial (EudraCT 2010-020435-40), patients with advanced melanoma comprising superficial lesions receive repeated ID and SC vaccinations with the MAGE-3.A1 or the NA17.A2 peptide, matching the antigens expressed by their tumor. Each of these peptides was previously tested in clinical vaccine trials, and was shown to be well tolerated and associated with tumor regression in some patients. In addition, a cocktail of immunostimulatory cytokines (IL-2, IFN-a and GM-CSF) at low doses is injected repeatedly in the periphery of a superficial tumor, which is also treated by ointment with Aldara® cream (imiquimod, a TLR7/8 ligand) (Fig. 2). It is hoped that this treatment will both induce new anti-tumoral CTL and modify the tumor environment in favor of immune rejection. The treatment was previously defined and optimized in a mouse model of skin graft rejection.

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 re-

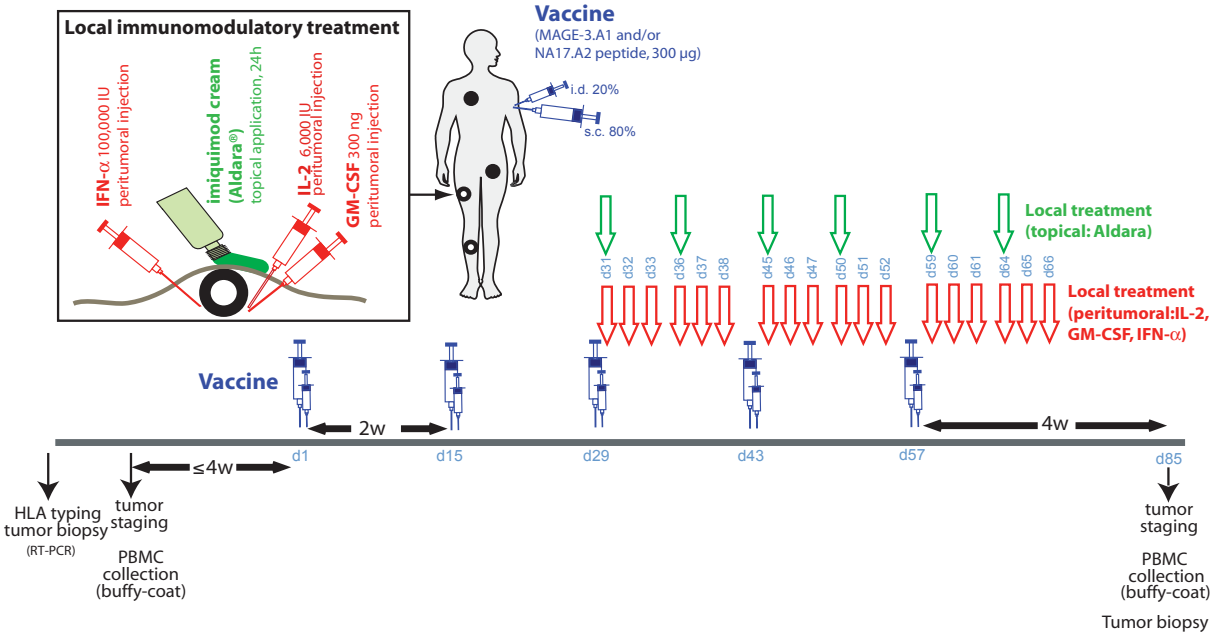

Fig. 2. Clinical trial : vaccination of melanoma patients with peptides associated with immunomodulation of the tumor environment

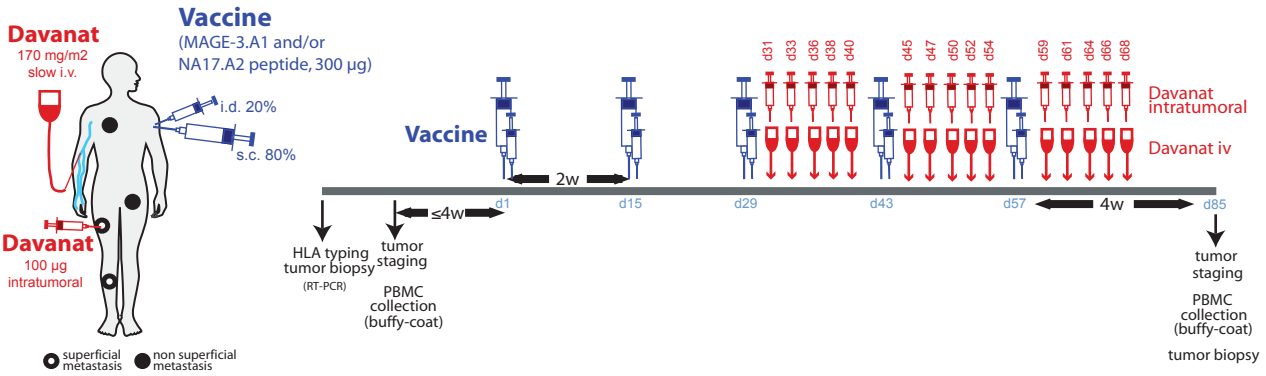

Fig. 3. Clinical trial: vaccination in melanoma patients with peptides associated with a galectin-3 inhibitor

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

B. Study of the inflammatory environment in melanoma metastases

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

We study freshly resected cutaneous metastases obtained from melanoma patients. Part of the resected tumors are put in culture, in order to attempt to derive immortalized melanoma cell lines, a precious tool for experimental tumor immunology. The remaining piece of tumor is kept frozen. Very thin tissue sections are cut from this material, and are used for RNA extraction followed by gene expression profiling, for immunohistochemistry and immunofluorescence stainings, for in situ hybridization and for laser capture microdissection of small cellular regions of interest (Fig.4). These complementary approaches help us to characterize the inflammatory events that take place inside the metastases, and to understand the interaction between the tumor cells and the inflammatory and immune cells at the tumor site.

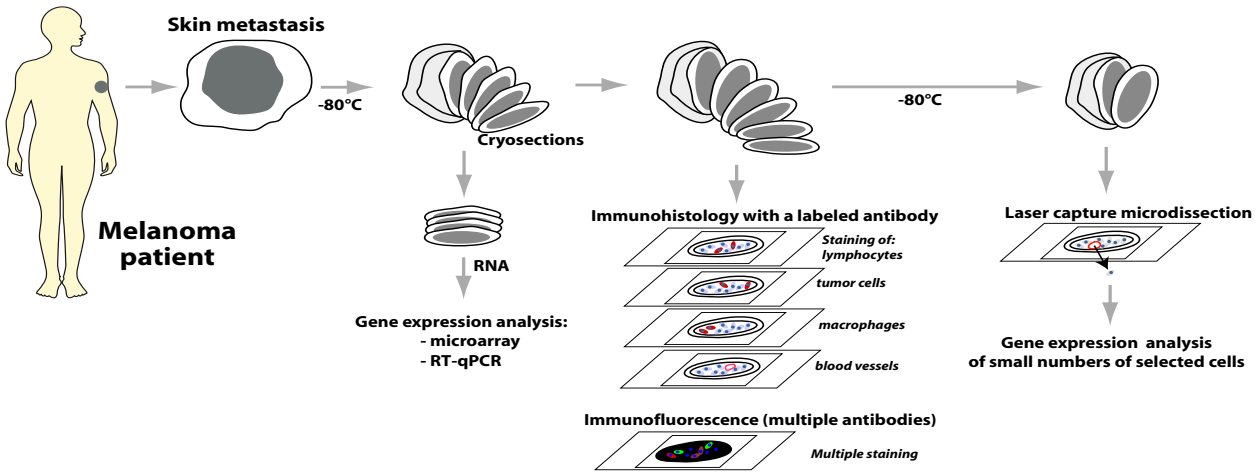

Fig. 4. Processing of tumor samples.

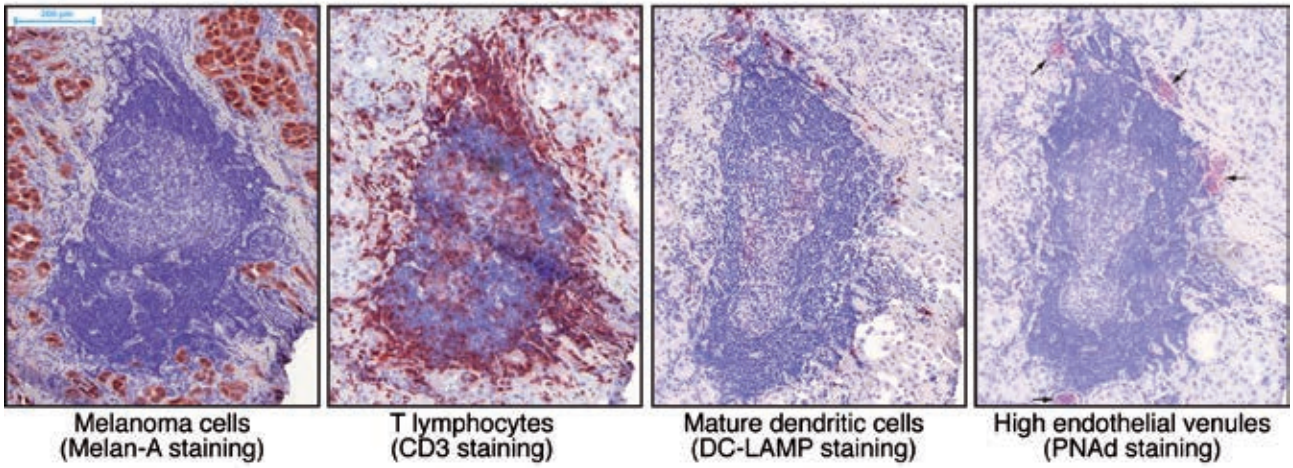

Fig. 5. An ectopic lymphoid structure in a melanoma metastasis.

We have also observed the presence of ectopic lymphoid structures, also called tertiary lymphoid organs, in melanoma metastases. These structures are organized in B cell follicles, adjacent T cell areas and neighbouring high endothelial venules, and thus contain the main components required to support local adaptive B and T cell responses. The presence of germinal centers and the occurrence of immunoglobulin affinity maturation in some follicles reveals ongoing B cell responses. The intimate association of mature dendritic cells and T lymphocytes in the T cell areas suggests that T cell responses also take place inside these structures (Fig. 5). This phenomenon, called lymphoid neogenesis, is frequently observed in many chronic inflammatory diseases. It has also been described in several types of tumors, including breast, lung and testis cancer. It is a consequence of sustained lymphocyte activation in the presence of persistent antigenic stimuli. We are currently investigating whether the B cell responses that take place into these structures are directed at melanoma antigens.

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

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Cytokines in immunity and inflammation

Jean-Christophe Renauld
Laure Dumoutier

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

Interleukin 9

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

IL-9-transgenic mice : parasite infections and asthma

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

IL-9-transgenic mice : T cell lymphomas

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

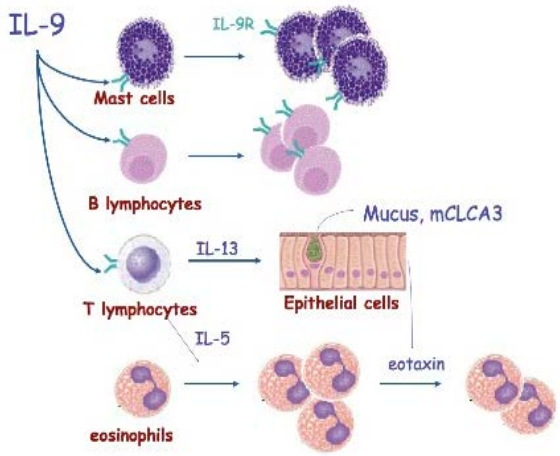

Fig. 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation of eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production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

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

IL-9 receptor and signal transduction

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

Role of JAK1 overexpression and mutations in tumor cell transformation

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

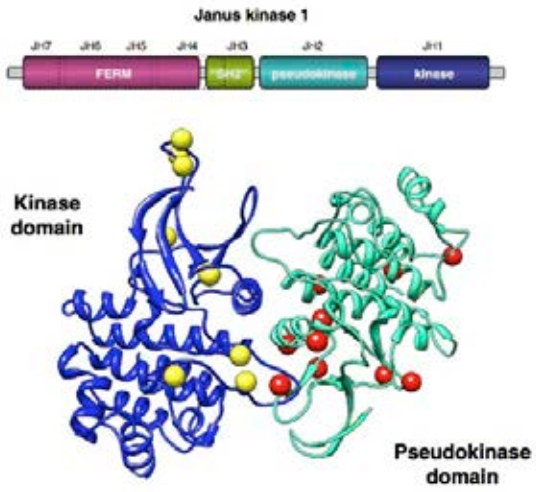

Fig. 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

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

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

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

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

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

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

Beside this cell membrane IL-22 receptor complex composed

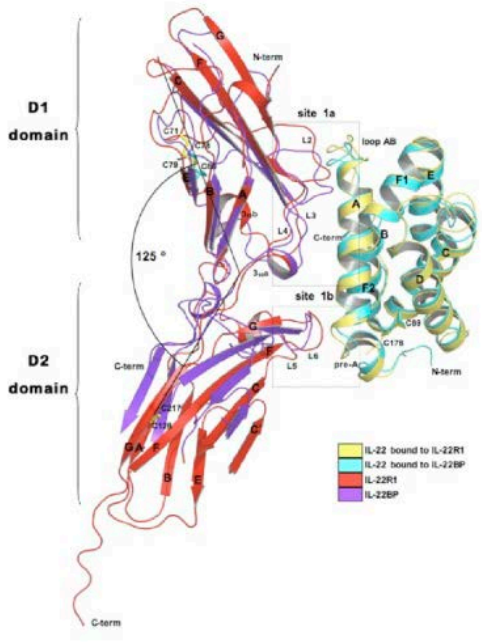

Fig. 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

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

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

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

Anti-cytokine vaccination

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

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

Stefan Constantinescu

Blood formation and the functions of the immune system depend on cytokines, such as Epo, interleukins, Tpo, colony stimulating factors or interferons. Those act by binding to specific receptors localized at the cell surface. We focus on signaling by cytokine receptors, such EpoR, TpoR, G-CSFR and interleukin receptors. These are transmembrane proteins that form dimeric or oligomeric complexes and are coupled to one or several cytosolic tyrosine kinases belonging to the Janus kinase (JAK) family. The human genome codes for more than 30 cytokine receptors, for four JAKs and seven Signal Transducers and Activators of Transcription (STATs), which are signal transducer proteins that shuttle to the nucleus and regulate gene expression.

Our broad interest is to understand the fundamental rules that govern protein-protein interaction in the cell membranes. We aim to identify: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch on or off cytokine receptor signaling; ii) what are the general rules by which hydrophobic transmembrane sequences interact in the membrane in a sequence-specific manner; and iii) the mechanisms of JAK attachment to receptors, and their subsequent activation, especially the role of pseudokinase domains in JAK kinase domain activation.

In the past we identified constitutively active oncogenic mutants of three Janus kinases, namely of JAK2 (V617F), JAK1 (V658F) and TYK2 (V658F). These are involved in human myeloproliferative neoplasms (JAK2 V617F) and T-cell leukemias (JAK1 V658F). We also described oncogenic mutants of several cytokine receptors. Mutants of TpoR W515, like JAK2 V617F, proved to be very important for Essential Thrombocythemia and Primary Myelofibrosis, two major myeloproliferative neoplasms. How JAK2 V617F and TpoR W515 mutants induce human myeloid cancers and how constitutive active STAT5 is mediating this oncogenic activation are actively pursued directions. We harness our basic understanding of JAK signaling in order to propose specific ways to target mutant JAKs. Recently, we investigated how mutant chaperones, such as mutant calreticulin proteins can activate pathologic signaling and induce myeloid cancers. These studies and those aiming to combine JAK inhibitors with other signaling inhibitors are performed in close interactions with clinicians and clinical biologists at St Luc Hospital and several international collaborators.

The mechanisms of JAK2 V617F activation in human myeloproliferative neoplasms

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

BCR-ABL negative myeloproliferative neoplasms (MPNs) consist of three related clonal diseases, Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). PV or the Vaquez-Osler disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. ET and PMF are associated with excessive platelet production (ET) and fibrosis/scarring (PMF) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation (Figure 1A) in collaboration with William Vainchenker at the Institut Gustave Roussy in Paris (1, 2). JAK2 V617F responsible for >98% of Polycythemia Vera and for >60% of ET and PMF cases (2). The mutation in the pseudokinase domain activates the kinase domain leading to constitutive signaling (1, 2) in complexes with cytokine receptors such as EpoR, TpoR and G-CSFR (Figure 2). The homologous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (2). JAK1 mutations have been described in adult acute T-lymphoblastic leukemia.

In addition to the V617F mutation we have described several other activating mutations on JAK2, V617I, L, M and W and modeled how such bulky residues could trigger the activation of JAK2 kinase domain (J. Biol. Chem. 283:12941-8.). The V617I mutation can be obtained by one base pair change and has been detected in rare cases of myelofibrosis, and recently was identified in a family with hereditary thrombocytosis (3). JAK2 V617I promotes mainly STAT1 activation via TpoR, and very little EpoR activation, which may explain the limited thrombocytosis phenotype of this family (3). This is supported by our finding that other activating germ line mutants of JAK2 also induce thrombocytosis and mainly STAT1 signaling in heterozygous conditions.

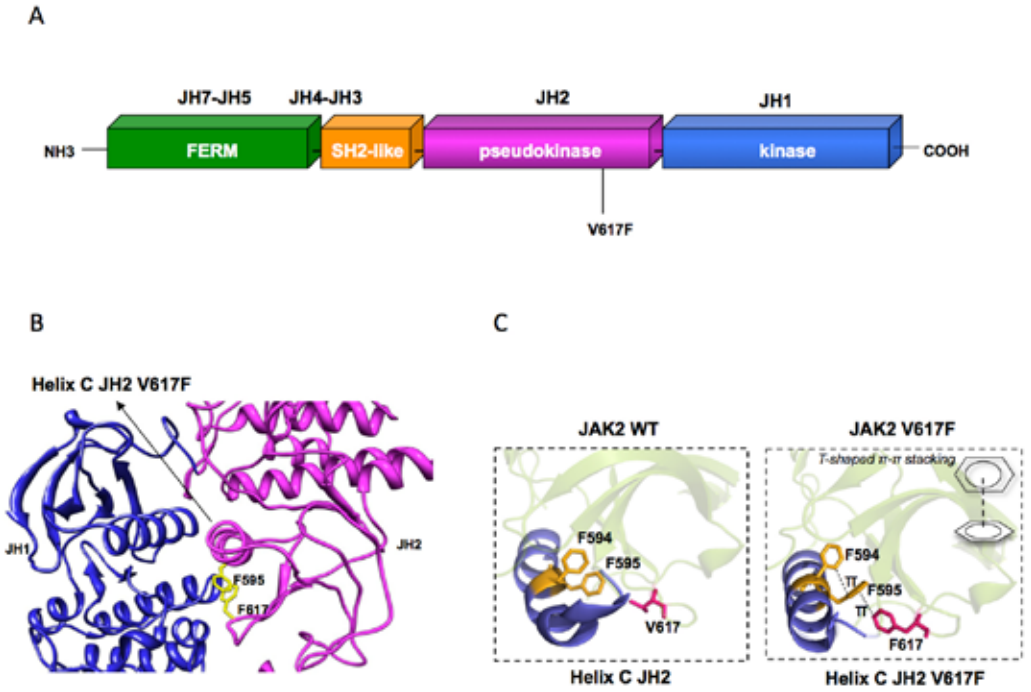

Fig. 1. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pesudokinase domain plays a major role in cytokine-dependent activation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

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

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

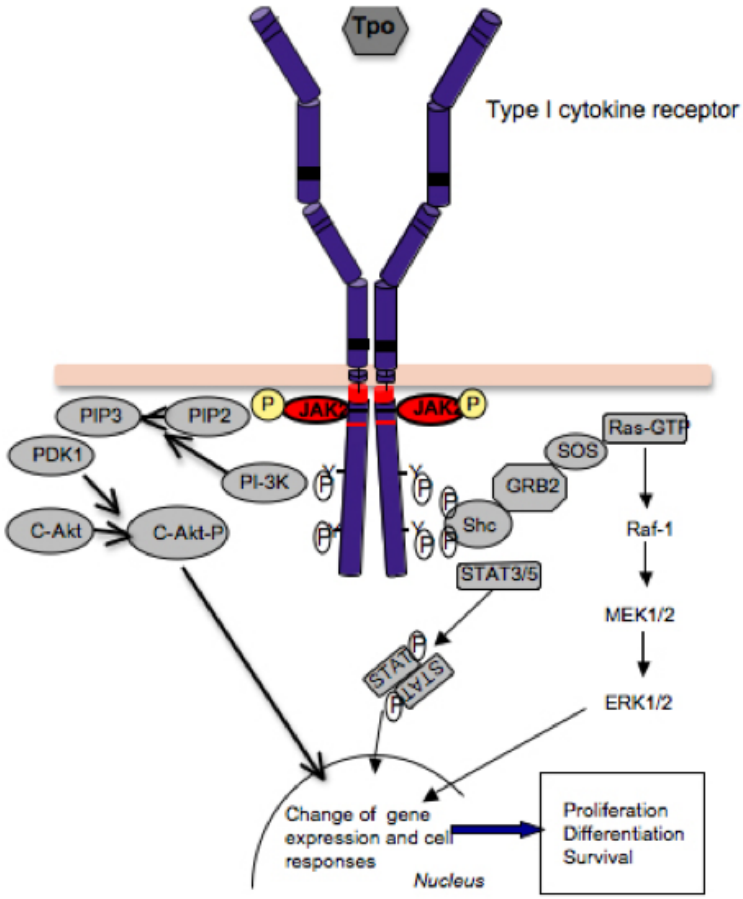

Fig. 2. Signaling by cytokine receptors. The thrombopoeitin receptor signals as a homodimer that activates JAK2, which is bound to the cytosolic juxtamembrane domain. Activation of JAK2 leads to phosphorylation of tyrosine residues in the TpoR cytosolic domain, which recruit several signaling proteins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

We aim to precisely understand how a pseudokinase domain mutation can induce kinase domain activation in JAKs. This would be required for the isolation of small molecule- specific inhibitors of mutated JAK2, that would spare wild type JAK2, and thus would not induce adverse effects. We identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (4). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinase domain might be a target for specific JAK2 V617F inhibition (Figure 1B and C). A recent X-ray crystal structure from the Hubbard and Silvennoinen laboratories has confirmed the major role of F595 in the conformation induced by V617F on helix C of JH2, with a complex three ring stacking interaction (F617-F595-F594) and prolongation by one turn of the helix C of JH2 (Figure 1C). Expression of segments of JAKs and cytokine receptors is pursued in insect and bacterial cells by D. Colau and M. Swinarska. Protein fragment Gaussia luciferase complementation assays are used to determine the proximity and oligomerization state of receptors and JAKs.

Involvement of pathologic TpoR signaling in myeloproliferative neoplasms

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

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

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

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (5). We discovered that Tpo could induce a strong antiproliferative effect in cells that express high JAK2 levels (5). This effect is physiologic and is detected in late megakaryocytes (Plos Biol 2010, 8, e1000476). Selection against the antiproliferative effect of Tpo occurs in JAK2 V617F cells, leading to TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells.
We employ a combination of Phospho-Scan and Ubi-Scan approaches coupled to mass spectrometry in order to determine modifications in the profile of tyrosine phosphorylation and ubiquitination induced by Tpo ligand or expression of JAK2 V617F and TpoR W515 mutants.

A novel mechanism by which a tryptophan residue regulates TpoR activation

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

Several years ago we discovered that TpoR contained a unique motif at the junction between the transmembrane and cytosolic domains (RWQFP). By deletion or mutation of individual residues in this motif, Judith Staerk determined that this amphipathic insert actually prevented self-activation of the receptor and identified W515 in the motif as the key residue for this inhibitory function (Blood 2006, 107(5), 1864-71). The question remained why would one W residue be so important in keeping a receptor inactive, and how could ligand addition defeat this inhibition. The question became even more relevant when several groups and ours detected TpoR W515L/K/A/R mutations in 5-8% of MPN patients that did not harbor JAK2 V617F. The answer came from two approaches. First, mutagenesis of W515 to all other residues showed the unique role of this residue, in that even the closely related Y or F residues could not replace it without pathologic receptor activation (6). Biophysical experiments represented by analytical ultracentrifugation, solid-state NMR, infrared spectroscopy performed by our collaborator, Prof. Steven O. Smith at SUNY Stony Brook, and fluorescence complementation studies performed in our laboratory by Vitalina Gryshkova showed that W515 actually regulates the orientation, tilt and dimerization of the upstream transmembrane helix, and prevents receptor activation (6). Taken apart, the transmembrane sequence of TpoR can dimerize as detected by analytical ultracentrifugation, but this was not the case when the RWQFP insert was added (6). Given that many proteins possess W residues at the cytosolic side of their transmembrane domains, we suggested a more general role for such W residues in preventing transmembrane domain dimerization and pathologic or premature activation of transmembrane protein signaling. We pursue this direction by investigating the role of juxtamembrane W residues in several transmembrane proteins and we collaborate with Ahmed Essaghir in the group of Jean-Baptiste Demoulin at the de Duve Institute for bioinformatics of single and multi-span transmembrane proteins.

Mechanisms by which TpoR extracellular domain mutations induce severe hematological pathologies

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

TpoR contains in its extracellular domain two cytokine receptor modules, one distal that binds Tpo (D1-D2) and one proximal to the membrane (D3-D4), that appears to exert a negative role in receptor activation. A mutant, TpoR R102P located in D1D2 causes in humans congenital amegakaryocytic thrombocytopenia (CAMT), while others (P106L), which are expressed at lower than normal levels at the cell surface, paradoxically induce a thrombocytosis phenotype. We study the intracellular localization of these receptors in collaboration with Pierre Courtoy and Donatienne Tyteca in the CELL Unit. We aim to understand why and where is the intracellular traffic blocked for these mutants, as well as how and where do these receptors signal. We investigate how chaperones of the ER interact with these defective receptors and why they appear to be blocked after cis-Golgi arrival, suggesting they might be subject to a novel quality control step in the Golgi.

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

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

While many X-ray crystal structures exist for G-protein coupled receptors and other membrane proteins with multiple transmembrane domains, no such structure could be obtained for single-span receptors. Crystal structures of the extracellular domains exist for erythropoietin receptor (EpoR) or for G-CSFR, but it is not possible to relate those to transmembrane and cytosolic domains, and to their relative positioning in the inactive versus active states. To identify the residues that form the interface between the receptor monomers in an activated receptor dimer, we have replaced the extracellular domain of the receptor (Figure 3A) with a coiled-coil dimer of α-helices (7 and Mol Cell 2003, 12, 1239). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil α-helices is imposed on the downstream TM α-helix and intracellular domain.

Each of the seven possible dimeric orientations will then be imposed by the coiled-coil on the fused TM and intracellular domain of receptors. We then express individually these dimers (seven for each receptor type) and test their function in cell lines, primary mouse and human cells and in vivo in mice.

We expect that some dimer interfaces would be active and some inactive, reflecting requirements of the wild type receptor for activation. To prove that our predictions are correct, i.e. rotation imposed at the outset of the transmembrane domain is transmitted to the end of the transmembrane domain, we employed cysteine-mediated cross-linking and showed that indeed covalent dimers are formed via the cross-linker only when cysteine is in the predicted interface (7). This then allowed us to determine the active interface of the EpoR dimer, where only one interface was active.

Using this approach we have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the physiological dimeric interface (cc-TpoR-I), that leads to formation of platelets (Figure 3b), other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Figure 3c and d) (7). One orientation (cc-TpoR-II) corresponds to the inactive receptor state. Interestingly, the dimer orientation that induces the highest levels of JAK2 activation, cc-TpoR-IV, also induces strong cell-to-cell adhesion and expansion of early hematopoietic progenitors. Our hypothesis is that in this dimeric orientation the receptor signals to maintain progenitors and possibly hematopoietic stem cells in the niche, and that signals induced by this dimer orientation might be recapitulating the quiescence-inducing effects of TpoR in HSCs. We will use this set of seven differently oriented TpoR dimers to dissect the signals induced by TpoR at the different stages of hematopoietic development and via the various downstream signaling proteins, JAK2, TYK2, STAT3, STAT5, MAP-kinase ERK1,2 and PI-3'-kinase/AKt/mTOR.

While all these pathways are activated by cytokines and mu-

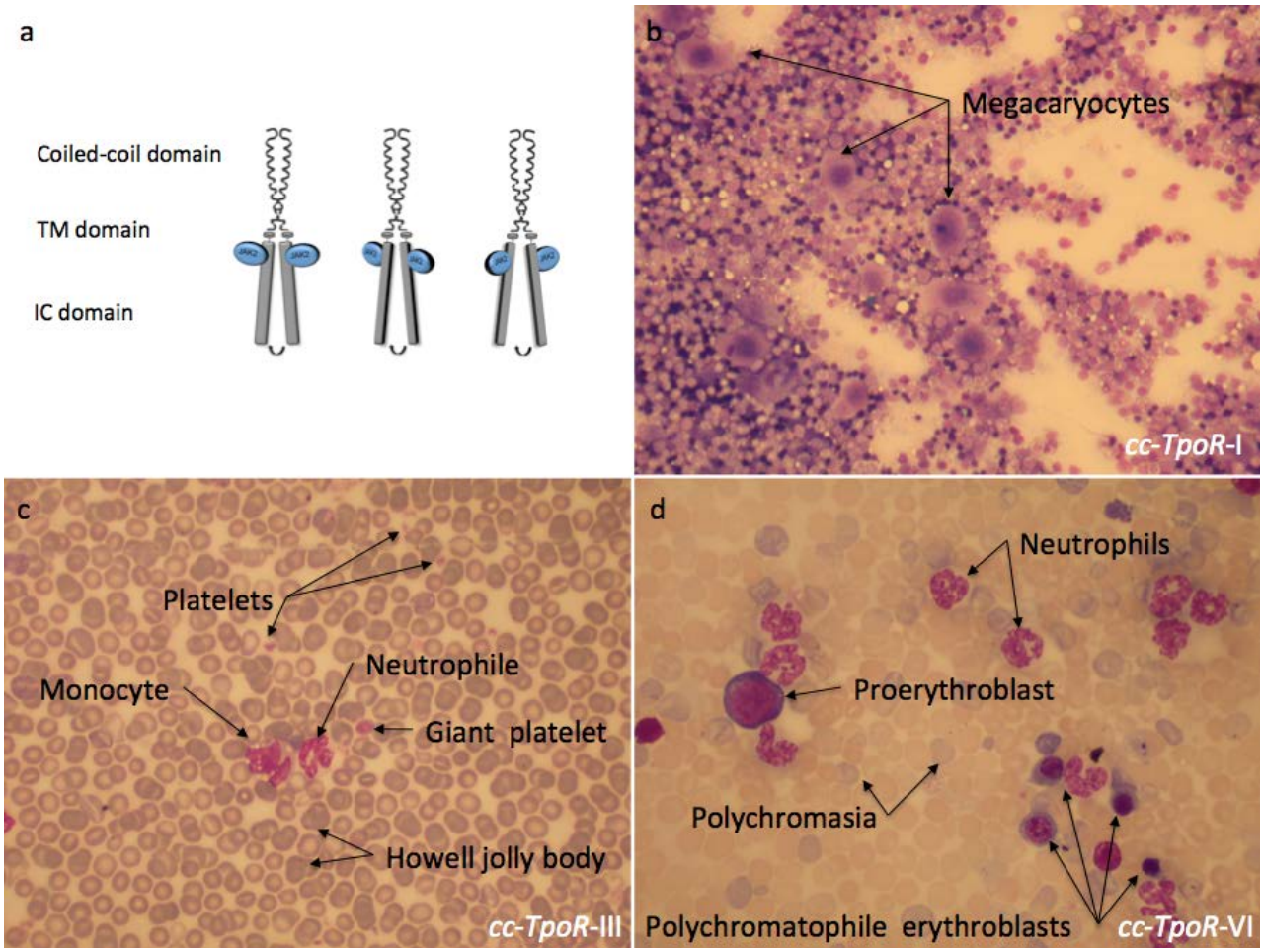

Fig. 3. Different dimeric orientations of the thrombopoietin receptor lead to distinct phenotypes in the bone marrow and in the peripheral blood in bone marrow reconstituted mice. (A) The fusion of the coiled coil protein to differently engineered transmembrane domains of the thrombopoietin receptor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

tant JAKs, we aimed to determine whether any of them could be essential for oncogenic proliferation. Within a collaboration with Experimental Therapeutics Center in Singapore, we generated model cell lines that express JAK2 V617F or JAK2 with TpoR or EpoR, as well as cell lines that express TpoR W515L. These cells are screened for small molecule inhibitors at ETC. In collaboration with M. L. Choong, M. A. Lee and A. Matter at ETC we reported that cells expressing JAK2 V617F are addicted to PI-3'-kinase signaling and that combinations of JAK2 inhibitors and pan type I PI-3'-kinase inhibitors are synergic in inhibiting proliferation of these transformed cells. These observations were confirmed on preclinical studies in immunodeficient mice inoculated with cells transformed by JAK2 V617F.

Structure and function of juxtamembrane and transmembrane sequences of membrane proteins

In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the groups of Profs. Jean-Noël Octave and Pascal Kienlen-Campard in our university and Steven O. Smith at SUNY Stony Brook, 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). More recently, we have shown that creation of a fourth in register Gly-X-X-X-Gly motif by the Flemish A21G mutation enhances amyloid processing by favoring dimerization via a beta-strand to alpha helix conformational change downstream A21G and helical interface represented by the glycines in the membrane (8). This enhanced processing explains the early onset of the Alzheimer's disease developed by these patients. We pursue a project of signaling by APP in neurons, glial cells and hematopoietic progenitors in collaboration with Profs. Jean-Noël Octave and Pascal Kienlen-Campard.

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

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

Using chromatin immunoprecipitation and sequencing, we have shown that STAT5 contacts a substantially different set of promoters in cells that exhibit constitutive STAT5 activation, versus cells that respond to cytokines by acute STAT5 tyrosine phosphorylation. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) (9), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, to inhibit translation of several proteins involved in megakaryocyte differentiation and to impair proplatelet formation (9). We found miR-28 to be pathologically overexpressed in 30% of MPNs (9).

In transformed hematopoietic cells, STAT5 and p53 must be synergistically bound to chromatin (Figure 4) for induction of LPP/miR-28 transcription (10). Genome-wide association studies show that both STAT5 and p53 are co-localized on the chromatin at 463 genomic positions in proximal promoters. Binding of p53 to those promoters is dependent on STAT5 binding. We identified several novel STAT5-p53 target genes, namely LEP, ATP5J, GTF2A2, VEGFC, NPY1R and NPY5R, which appear to be pathologically expressed in platelets of MPN patients (Figure 4). Furthermore we showed that also a mutant p53 (M133K), expressed by HEL cells can cooperate with constitutive active STAT5 on STAT5-p53 promoters. We concluded that persistently active STAT5 could recruit normal and mutated p53 to novel promoters leading to pathologic gene expression that differs from physiological STAT5 or p53 transcriptional programs.

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

J.-P. Defour, C. Pecquet, C. Mouton

At present, our laboratory is performing under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the 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. The recently discovered calreticulin mutations (CalR), which are associated with 30% of JAK2 V617F/TpoR mutant negative ET and PMF are studied in a cohort of patients at St Luc Hospital and CalR mutant testing was introduced in the routine clinical biology activity. Next generation sequencing will be employed for well-investigated triple negative (JAK2 V617F/ TpoR/CalR mutant negative) patients in order to unravel novel molecular defects in MPNs and leukemias.

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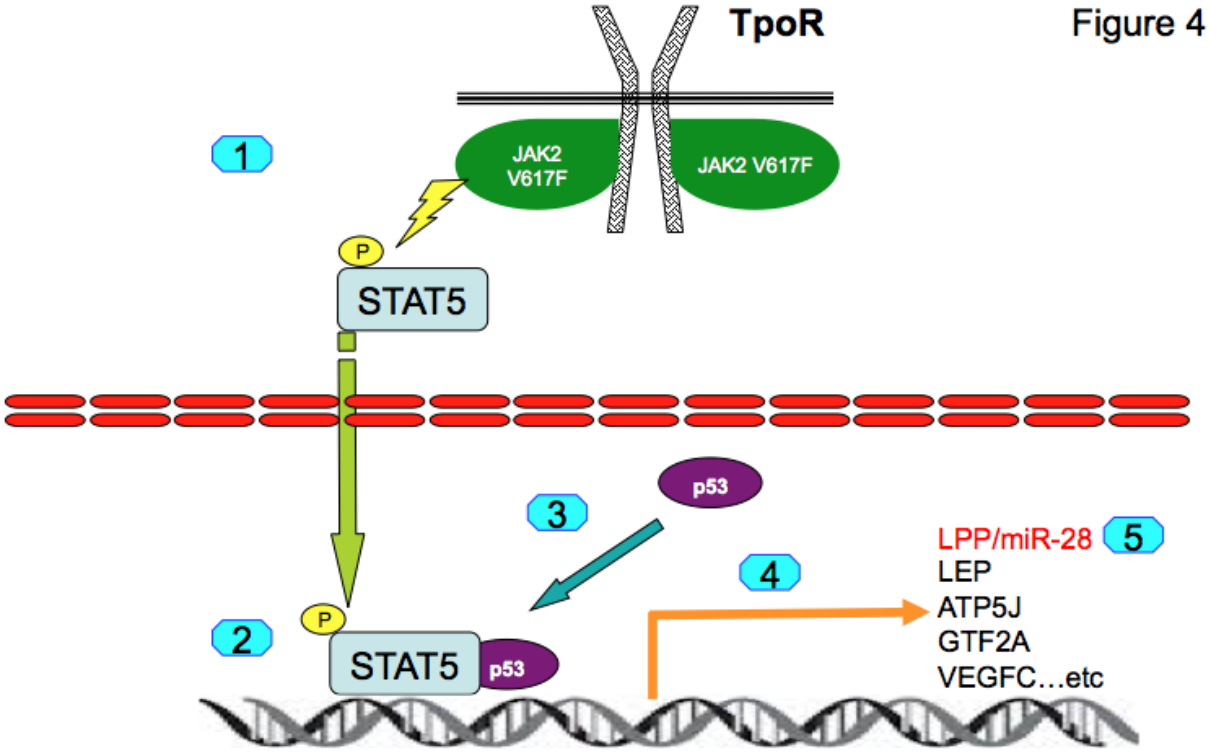

Fig. 4. Mechanism of pathologic induction of non-canonical target genes by constitutive active STAT5 and p53 in hematopoietic cells transformed by the oncogenic JAK2 V617F protein. 1) Mutated JAK2 V617F coupled to a cytokine receptor (TpoR) leads to constitutive tyrosine phosphorylation (yellow arrow) of STAT5. This process occurs in the absence of cytokines, in this case Tpo. 2) Phosphorylated STAT5 translocates into the nucleus and binds to promoters of canonical and non-canonical STAT5 target genes. 3) On certain non-canonical promoters, the persistent presence of tyrosine phosphorylated STAT5 is required for recruitment of p53 (wild type or mutants) to p53-binding sites in those promoters. 4) Both STAT5 and p53 are required for initiation of transcription of such regulated genes, which are non-canonical targets for both STAT5 and p53. 5) One such gene, which is pathologically induced in MPN megakaryocytic lineage, is LPP (Lipoma Preferred Partner), which hosts in one of its introns the microRNA-28 (miR-28). This miR-28 binds the 3'-UTR sequences of several mRNAs coding for proteins important for megakaryocyte differentiation, such as TpoR, inhibiting their translation.

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http://csb.sunysb.edu structural biology homepage
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American Society of Hematology Educational Program 2012 (http://hematology.org/Meetings/Annual-Meeting/Program/3741.aspx)
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Institut Gustave Roussy, Villejuif, France
(William Vaincheker's team)
http://www.gustaveroussy.fr/en/page/william-vainchenker_753#1

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

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Learning:

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