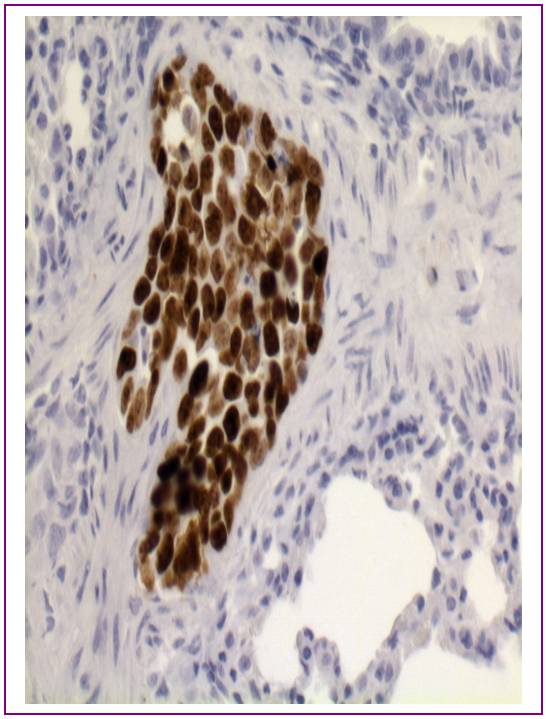
The Belgian Society for Cellular and Developmental Biology

Spring Meeting 2010

Tumor Microenvironment & Metastases

Abstract Book



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University of Liège - March 27, 2010

Organizers : Agnès Noël – Christine Gilles

Spring Meeting of the Belgian Society for Cell and Developmental Biology

FNRS-Ecole Doctorale Thématique en Cancérologie Expérimentale

"Tumor Microenvironment & Metastases"

Liège, Roskam auditorium, March 27, 2010

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CELL-CELL AND CELL-MATRIX INTERACTION

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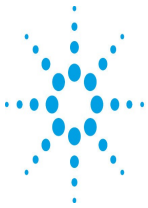

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PROGRAM

8H30 - 9H15	Registration and display of posters
9H15 - 9H30	Welcome and introduction : **Agnès Noël** (Liège, Belgium) ***Coordinator of FP7 project (MICROENVIMET)***
MORNING SESSION - Chairpersons : Agnès Noël & Frans Van Roy	
9H30 – 10H15	**David C. Lyden** (New York, USA) "Priming the Soil for the Pre-Metastatic Niche"
10H15 – 11H00	**Achim Krüger** (Munich, Germany) ***Partner of FP7 project (MICROENVIMET)*** "Tumor microenvironment determined by long-range communication via the proteolytic internet"
11H00 – 11H30	Coffee break
11H30 – 12H15	**Jonathan Sleeman** (Mannheim, Germany) ***Coordinator of FP7 project (TuMIC)*** "Lymphangiogenesis and Metastasis"
12H15 – 12H45	**An Hendrix** (UGent) : "Rab27B-mediated vesicle exocytosis regulates invasive tumor growth and metastasis" **Kiavash Movahedi** (VUB) : "Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(High) monocytes"
12H45 – 14H15	Lunch – Poster viewing
AFTERNOON SESSION - Chairpersons : Christine Gilles & Vincent Castronovo	
14H15 – 14H45	**Amber Van Dongen** (KUL) : "Tumor growth is accelerated in low-density lipoprotein receptor related protein 1 knock-in mice" **Charlotte Selvais** (UCL) : "Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1"
14H45 – 15H30	**Sandra McAllister** (Boston, USA) "Acquisition of Malignant Traits as a Result of Systemic Instigation"
15H30 – 16H15	**Vincent Castronovo** (Liège, Belgium) ***Partner of FP7 project (ADAMANT)*** "Tumor stroma: source of biomarker for targeted therapy and imaging"
16H15	Poster Awards and end of the meeting

INVITED SPEAKERS

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Priming the Soil for the Pre-metastatic Niche

Rosandra N. Kaplan and **David Lyden**

Pediatrics, and Cell and Developmental Biology, Weill Cornell Medical College and Memorial Sloan-Kettering Cancer Center, New York, USA

The importance of both "seed" and "soil" for cancer metastasis has long been recognized. Much attention has focused on understanding the molecular and genetic factors that govern the metastatic potential intrinsic to certain tumor cells. Meanwhile, the early molecular and cellular events occurring within distant tissues that "prime the soil" for tumor cell invasion have only recently begun to be unraveled. We have demonstrated that bone marrow-derived myeloid progenitor cells initiate key changes in sites designated for future metastasis, creating a receptive microenvironment for tumor growth termed the "Pre-metastatic Niche". Here, secretory factors of the primary tumor induced alterations within pre-metastatic lymph nodes and distant tissues and mobilize hematopoietic progenitor cells (HPCs) prior to the arrival of tumor cells and endothelial progenitor cells. At the pre-metastatic sites, HPCs maintained their progenitor cell status, expressing hematopoietic markers such as CD34, VEGFR1, CXCR4, CD11b, c-Kit and Sca-1.

Notably, prior to the arrival of HPCs at the pre-metastatic niche, focal upregulation of fibronectin occurred. VLA-4 expression allowed preferential adhesion of HPCs to the enriched fibronectin regions, contributing to site-specificity for tumor metastasis. Blocking myeloid cell establishment was seen to abrogate cluster development and consequent metastasis. Examining biopsy samples in patients with breast carcinoma revealed that $VEGFR1^+$ HPCs were always found in association with primary tumor lesions and in metastatic lymph nodes. Moreover, $VEGFR1^+$ HPC clusters were even more numerous in lymph nodes without any evidence of micrometastasis supporting our findings that these cells at the pre-metastatic niche preconditions the microenvironment for the promotion of tumor metastases. In addition to detection of these cells in pre-metastatic and metastatic tissues, we can monitor HPCs in the circulation of patients at diagnosis with local, locally invasive and metastatic breast, colon and lung carcinomas. These increased circulating cells expressed CD34, VEGFR1 and CXCR4 and can predict metastatic progression. Along with elevated HPCs, these phenotypically marked cells have the capacity to generate granulocyte-monocyte colony forming units (CFU-GM) corroborating the their functional proliferative capacity and CFU progenitor cell potential.

Investigations of the cross-talk between "seed" and "soil" at the pre-metastatic niche may further our understanding of the determinants that enable metastatic progression.

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Tumor microenvironment determined by long-range communication via the Proteolytic Internet

Achim Krüger

Klinikum rechts der Isar der Technischen Universität München, Institut für Experimentelle Onkologie und Therapieforschung der Technischen Universität München, Ismaninger Str. 22, D-81675 München, Germany

Proteases are the essential players in organ homeostasis in health and disease: they do not only exhibit the degradative role in the turn-over or break-down of the extracellular matrix but are recently appreciated as specific modulators of cell signaling molecules and their underlying pathways. In every tissue proteases and their natural inhibitors form dynamically interconnected cascades and regulatory circuits, forming a local proteolytic network. Primary tumor growth and local tumor cell invasion are often associated with overexpression of Matrix metalloproteinases (MMPs) as well as their natural inhibitor TIMP-1. A shift-of-balance in the regional proteolytic network towards net proteolysis, transforms the afflicted tissue to a possible source for mobile metastasizing tumor cells, the prerequisite for malignant cancer disease. In mouse models we found evidence for a Proteolytic Internet where alterations in one local proteolytic network are communicated to the local proteolytic network of a distant tissue with metastasis-promoting molecular and cellular consequences: In one example, elevated systemic levels of TIMP-1 induce uPA-dependent Met-signaling in the liver, rendering this organ –before tumor cell arrival- more susceptible for metastasis. This explains the paradox of TIMP-1 being a bad prognostic marker and indicates that TIMP-1 is one important player in the creation of a Pre-Metastatic Niche. In another example, we found that knock-out of metastasis-associated MMP-9 in the mouse host renders its bone marrow to produce elevated systemic levels of Il-6. Elevated Il-6 in the circulation induces MMP-9 expression in inoculated circulating tumor cells thereby increasing their metastatic potential. This counterintuitive result revealed that protease ablation triggers complex adaptations in the Proteolytic Internet of the host which cautions us in the drug-target search with knock out mice. Still, undesired information (*spam*) in the Proteolytic Internet needs to be understood and defined in order to design specific metastasis therapies employing protease inhibition, likely in combination with *spam*-filtering agents.

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Lymphangiogenesis and Metastasis

Jonathan P. Sleeman[1,2], Luca Quagliata[2], Anja Schmaus[1], Melanie Rothley[1,2], Jochen Bauer[1], Diana Plaumann,[1,2] and Wilko Thiele[1,2]

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Pro-lymphangiogenic factors produced by tumors can induce lymphangiogenesis within and / or at the periphery of tumors, promoting metastasis to regional lymph nodes. This tumor-induced lymphangiogenesis is thought to act by promoting the entry of tumor cells into the lymphatic vasculature, and / or by increasing interstitial fluid flow to the draining lymph nodes, thereby facilitating their access to and entry into the regional nodes. In animal models, tumor-induced lymphangiogenesis frequently induces metastasis not only to lymph nodes but also to other organs. Concordantly, many studies on tumor material from human cancer patients have correlated expression of pro-lymphangiogenic factors with lymph node metastasis formation and poor prognosis. Although pro-lymphangiogenic factors are produced in the primary tumor environment, there is mounting evidence to suggest that they may act systemically in addition to their local effects on the tumor-associated lymphatics. For example, tumors can remotely induce lymphangiogenesis in their draining lymph nodes, even before tumor cells colonise the nodes. Furthermore, a number of studies have reported increased levels of pro-lymphangiogenic factors in the blood of cancer patients that correlates with poor prognosis. Thus, tumor-derived pro-lymphangiogenesis factors have in principle the potential to act even more distantly. In this presentation our current progress in dissecting at what level pro-lymphangiogenic factors act to promote metastasis will be discussed and placed in the context of understanding the role of lymph nodes in metastatic dissemination.

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Acquisition of Malignant Traits as a Result of Systemic Instigation

Sandra S. McAllister

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Recent advances have revealed that tumor-host interactions extend well beyond the local tissue microenvironment and that tumors not only respond to, but also actively perturb host organs atdistant anatomical sites, and that these perturbations are a driving force in tumor progression.
Such results indicate that many aspects of tumor biology can only be explained by a detailedunderstanding of both local and systemic interactions, yet we currently have only a fragmentaryunderstanding of these processes.

We previously demonstrated that certain carcinomas ("instigators") stimulate the growth ofotherwise-indolent carcinoma cells ("responders") implanted at distant anatomical sites – aprocess we termed "systemic instigation". This process operates via endocrine signals, including osteopontin, sent from the instigating tumor to the bone marrow, resulting in activation and mobilization of bone marrow cells (BMCs) into the circulation. These mobilized BMCs subsequently contribute to the responding tumor stroma, thereby fostering its growth; however, the identity of the BMCs and their contribution to tumor growth was unknown. We now demonstrate that Sca1+/cKit– hematopoietic BMCs of hosts bearing instigating tumors promote the growth of responding tumors that proceed to form a myofibroblast-rich desmoplastic stroma. Antigenically identical Sca1+/cKit– BMCs prepared from control hosts do not have this tumorsupportive activity. We identified granulin (GRN) as the most highly upregulated gene in the instigating Sca1+/cKit- BMCs, when compared with control Sca1+/cKit- BMCs. Indeed, localized treatment with GRN mediated desmoplastic responding tumor growth *in vivo*. The activated GRN+ BMCs that are recruited into the responding tumor stroma neither give rise directly to the stromal myofibroblasts nor directly stimulate responding tumor cell proliferation. Instead, we found that GRN treatment induces tissue fibroblasts to express matrix remodeling and inflammation genes that are known to mediate tumor progression.

Thus, it is clear that tumors that would otherwise remain indolent can acquire malignant traits as a consequence of systemic process that are initiated and driven at distant anatomical sites. But can the process of systemic instigation also explain some of the observations that have been made in the oncology clinic? It is known that patients with one malignant neoplasm are at an increased risk of presenting with multiple primary cancers within a relatively short time period of the initial diagnosis, depending on the anatomical site of origin. Moreover, it was recently reported that surgical resection of primary tumors improved the survival of women who presented with metastatic breast cancer at the time of diagnosis. While there are a number of possible explanations for these observations, we suggest that one of these must now be entertained as a potentially important one that explains previously mysterious physiologic phenomena: that certain tumors or metastatic foci (e.g., "instigators") can support the growth of other tumors or disseminated metastatic cells (e.g., "responders") that co-exist in human cancer patients.

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Tumor Stroma : Source of Biomarkers for Targeted Therapies and Imaging

Vincent Castronovo, M.D., Ph.D.

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The selective delivery of bioactive agents at the tumor site, while sparing normal tissues, represents one of the most promising avenues for the development of anti-cancer therapies with unprecedented efficacy and tolerability. Markers of angiogenesis and of the tumor stroma represent particularly attractive targets for the antibody-based delivery of therapeutic agents, in view of their selective, abundant and accessible expression in aggressive solid tumors. Several experimental approaches have been proposed in order to achieve a preferential accumulation of bioactive agents in tumors. However, no molecular strategies have so far been able to compete with monoclonal antibodies and their derivatives in terms of targeting efficiency, selectivity and broad therapeutic applicability. Human monoclonal antibodies specific to suitable tumor-associated antigens can display tumor : (non tumor) ratios as large as 100 : 1 after intravenous administration. Eligibility of being a targetable marker for therapeutic use implies ideally three criteria: (i) accessibility from the bloodstream, (ii) expression at sufficient level and (iii) no (or much lower) expression in normal tissues. Most current discovery strategies (such as biomarker searching into body fluids) provide no clue as to whether proteins of interest are accessible, in human tissues, to suitable high-affinity ligands, such as systemically delivered monoclonal antibodies. To address this limitation, we have developed innovative methodologies, based on chemical proteomic modifications, enabling the discovery of proteins accessible from the bloodstream and the extracellular space, in human pathological tissues. This approach has led to the discovery of a repertoire of potentially accessible biomarkers in human glioblastoma, pancreas carcinomas and lymphomas. This project is part of ADAMANT, a FP7 European project.

SHORT PRESENTATIONS

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Rab27B-mediated vesicle exocytosis regulates invasive tumor growth and metastasis

An Hendrix[1,2], Geert Braems[3], Hannelore Denys[2], Wendy Westbroek[4], Veronique Cocquyt[2], Marc Bracke[1] and Olivier De Wever[1]

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Background: Vesicle exocytosis, controlled by secretory GTPases such as Rab27B, delivers critical pro-invasive growth regulators into the tumor microenvironment. The biological role and expression status of Rab27B in breast cancer was unknown.

Methods: Rab27B was studied in estrogen receptor (ER)-positive human breast cancer cell lines (MCF-7, T47D, ZR75.1) using GFP-fusion constructs, including wild type Rab3D, Rab27A, Rab27B and Rab27B point mutants defective in GTP/GDP-binding or geranylgeranylation, and transient siRNA targeting. In cell culture, cell-cycle progression was evaluated by flow cytometry, Western blotting and measurement of cell proliferation rates, invasion was assessed using Matrigel and native collagen type I substrates. Orthotopic tumor growth, local invasion and metastasis were analyzed in mouse xenograft models. Mass spectrometry was performed to identify Rab27B-secreted pro-invasive growth regulators. In clinical breast cancer, Rab3D, Rab27A and Rab27B mRNA levels were analyzed by quantitative RT-PCR (n=20) and Rab27B protein level was evaluated by immunohistochemistry (n=60).

Results: Rab27B-upregulation promoted G1/S phase cell cycle transition and increased proliferation, F-actin reorganization and invasion in cell culture, and invasive tumor growth and haemorrhagic ascites in a xenograft mouse model (at 10 weeks, survival of MCF-7 GFP vs GFP-Rab27B injected mice was 100% vs 62.5%, P=0.0307). Proteomic analysis of purified Rab27B-secretory vesicles and the secretome of exogenous Rab27B-expressing breast cancer cells identified heat shock protein (HSP)90 alpha as key pro-invasive growth regulator. HSP90 alpha secretion occurred in a Rab27B-dependent manner and was required for matrix metalloproteinase(MMP)-2 activation. All Rab27B-mediated functional responses were GTP- and geranylgeranyl-dependent. Endogenous Rab27B mRNA and protein, but not Rab3D and Rab27A mRNA, associated with lymph node metastasis (P=0.0002) and differentiation grade (P=0.0014) in ER-positive human breast tumors.

Conclusion: Rab27B regulates invasive growth and metastasis in ER-positive breast cancer.

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Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(High) monocytes

Kiavash Movahedi[1,2], Damya Laoui[1,2], Conny Gysemans[3], Martijn Baeten[1,2], Geert Stangé[4], Jan Van den Bossche[1,2], Matthias Mack[5], Peter In't Veld[4], Patrick De Baetselier[1,2] and Jo A. Van Ginderachter[1,2]

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[4] *Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium*
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Tumor-Associated Macrophages (TAMs) form a major component of the tumor stroma. However, important concepts such as TAM heterogeneity and the nature of the monocytic TAM precursors remain speculative. Here, we demonstrate for the first time that mouse mammary tumors contained functionally distinct subsets of TAMs and provide markers for their identification. Furthermore, in search for the TAM progenitors, we show that the tumor-monocyte pool almost exclusively consisted of Ly6ChiCX3CR1low monocytes, which continuously seeded tumors and renewed all TAM subsets. Interestingly, gene and protein profiling indicated that the distinct TAM populations differed at the molecular level and could be classified based on the classical (M1) versus alternative (M2) macrophage activation paradigm. Importantly, the more M2-like TAMs were enriched in hypoxic tumor areas, had a superior pro-angiogenic activity in vivo and increased in numbers as tumors progressed. Finally, it was shown that the TAM subsets were poor antigen-presenters, but could suppress T-cell activation, albeit by employing different suppressive mechanisms. Together, our data help to unravel the complexities of the tumor-infiltrating myeloid cell compartment and provide a rationale for targeting specialized TAM subsets, thereby optimally "re-educating" the TAM compartment.

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Tumor growth is accelerated in low-density lipoprotein receptor related protein 1 knock-in mice

Amber Van Dongen[1], Philip Gordts[1], Leen Verbeek[1], Guido David[2] and Anton Roebroek[1]

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Objectives: The low-density lipoprotein receptor-related protein 1 (LRP1) is a multifunctional receptor involved in receptor-mediated endocytosis and cell signalling. The intracellular domain of LRP1 (LRP1-ICD) contains a number of signalling motifs, which are relevant for regulation of its function. Analysis of a murine model having knock-in mutations into endogenous LRP1 allows us to evaluate how LRP1 contributes to the regulation of tumor growth and subsequently indentify the motifs and signalling pathways involved.

Methods: An LRP1 knock-in mouse (with a C57BL6 genetic background) carrying an inactivating mutation in the NPxYxxL (NPVYATL -> AAVAATL) motif in the LRP1-ICD was generated by recombinase mediated cassette exchange (RMCE) (Roebroek et al., MCB, 2006). The NPxYxxL domain contains motifs for endocytosis and tyrosine phosphorylation. To evaluate the impact of the LRP1 mutation (present only in the host tissue) on tumor growth in vivo B16-F10 melanoma cells were subcutaneously injected into the flanks of this syngenic mouse model. In a first experimental approach subcutaneous tumors were surgically removed when they reached a diameter of 1 cm. In a second approach tumors were removed after a period of 3 weeks.

Results: Homozygous LRP1 knock-in mice have normal levels of LRP1 gene expression and show no obvious phenotype. Upon subcutaneous injection of melanoma cells tumors developed more rapidly in knock-in animals than in C57BL6 controls. In the first experimental approach 100% of the LRP1 knock-in mice had developed tumors with end point size of 1 cm diameter by day 21, in contrast to only 66% of the control animals. In the second approach, collecting all tumors after 3 weeks, LRP1 knock-in mice developed much larger tumors in comparison with wild-type mice (respectively 0.41 cm3 ± 0.15 vs. 0.03 cm3 ± 0.01; $p<0.05$). Western blot analysis revealed a significant, 1.7-fold increase of VEGF expression in tumors which developed in LRP1 knock-in mice. This observation was confirmed by immunohistochemical evaluation of the tumors, and suggests a possible role for LRP1 in angiogenesis.

Conclusion: Reduced LRP1 functionality due to inactivation of the NPxYxxL motif resulted in increased tumor growth, possibly via regulating angiogenesis. The mechanism or signalling pathway responsible for this accelerated growth needs to be further investigated.

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Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1

Charlotte Selvais[‡], Ludovic D'auria[‡], Pascale Lemoine[‡], Linda Troeberg[§], Stéphane Dedieu[¶], Agnès Noël[**], Hideaki Nagase[§], Patrick Henriet[‡], Pierre J. Courtoy[‡], Etienne Marbaix[‡] and Hervé Emonard[‡]

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[**] *Laboratory of Tumor and Developmental Biology, GIGA-Cancer, University of Liege, B-4000 Liège, Belgium*

The low-density lipoprotein receptor-related protein-1 (LRP-1) is a membrane receptor involved in both endocytic scavenging and signaling functions. LRP-1 cell surface level is controlled by proteolytic shedding which releases its ectodomain. Here we identified two membrane-associated metalloproteinases that shed LRP-1 from human fibrosarcoma HT1080 cells: a disintegrin and metalloproteinase (ADAM)-12 and membrane-type 1 matrix metalloproteinase (MT1-MMP). We further compared the shedding potential of classical fibroblastoid HT1080 cells with a variant featuring an epithelioid phenotype. Although both fibroblastoid and epithelioid variants expressed similar levels of ADAM-12 and MT1-MMP and of their specific tissue inhibitor of metalloproteinases (TIMPs), epithelioid cells, which contained twice more cholesterol than fibroblastoid cells (17.5 ± 3.6 versus 8.4 ± 2.8 µg cholesterol/mg cell protein), shed 4-fold less LRP-1 ectodomain. Moreover, LRP-1 ectodomain shedding was increased by cholesterol depletion in epithelioid cells and impaired by cholesterol overload in fibroblastoid cells. Our data demonstrate that cholesterol may regulate LRP-1 level at the plasma membrane, possibly by controlling encounter with its sheddases.

POSTERS

POSTER PRIZES

At the end of the Spring Meeting of 2010, poster prizes were awarded to

- **Julie LECOMTE (ULG)**
  for the posters:

CHARACTERIZATION OF BONE MARROW DERIVED-CELLS RECRUITED INTO MOUSE TUMORS.
Julie Lecomte (1), Maud Jost (1), Sandrine Bekaert (1), Silvia Blacher(1), Françoise Bruyère (1), Martin Illeman (2), Gunilla Hoyer-Hansen (2), Fabrice Olivier (1), Jean-Michel Foidart (1) and Agnès Noel (1).
1: Laboratory of Tumor and Development Biology (GIGA-Cancer), Ulg, B-4000 Liège;
2: Finsen Laboratory, Righospitalet, Copenhagen, Denmark.
and
BONE MARROW-DERIVED MESENCHYMAL CELLS AND MMP13 CONTRIBUTE TO EXPERIMENTAL CHOROIDAL NEOVASCULARIZATION.
Julie Lecomte (1), Krystel Louis (1), Benoit Detry (1), Silvia Blacher (1), Sandrine Bekaert (1), Jenny Paupert (1), Vincent Lambert (1,2), Pierre Blaise (2), Jean-Michel Foidart (1), Jean-Marie Rakic (2), Stephen M. Krane (3) and Agnès Noel (1).
1: Laboratory of Tumor and Developmental Biology (GIGA-Cancer), University of Liege, B-4000 Liège;
2: Department of Ophthalmology, CHU, B-4000 Liège;
3: Center for Immunology and Inflammatory Disease, Harvard Medical School and Massachusetts General Hospital, Boston, MA 02129, USA.

and

- **Lionel FLAMANT (FUNDP)**
  for the poster:

BREAST CANCER CELLS UNDER HYPOXIA.
Lionel Flamant, Annick Notte, Noelle Ninane, Martine Raes and Carine Michiels.
Laboratory of Biochemistry and cellular Biology (URBC), University of Namur-FUNDP, 61 rue de Bruxelles, 5000 Namur.

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Hyaluronidase Hyal2: a tumor suppressor in invasive breast cancer?

Bénédicte André and Bruno Flamion

Laboratory of Physiology and Pharmacology, URPHYM, University of Namur, Belgium

Many tumors including breast cancers contain a high amount of hyaluronan (HA) compared to the normal tissues. The level of HA is usually linked to an aggressive phenotype. HA is synthesized by 3 different HA synthases (HAS) and degraded by at least 3 active hyaluronidases (Hyal) but the respective functions of these proteins in cancer cells are not yet clear. Hyal1 and Hyal2 have been shown to inhibit tumor growth in vivo but not in vitro. We found that the breast cancer cell line MDA-MB231 expresses a high level of Hyal2 and decided to probe its functions using small RNA interference.

Immunoreactive Hyal2 was present exclusively in the membrane fraction of MDA-MB231 cells. The amounts of Hyal2 mRNA and protein were decreased by ~80% following transfection with Hyal2-specific siRNA compared with a scrambled small RNA. Hyal2 knockdown caused 30-40% decreases in HAS2 and HAS3 mRNA expression evaluated using real-time RT-PCR (HAS1 mRNA was not detected) as well as a large decrease in Hyal1 mRNA amounts. These secondary modifications suggest that HA metabolism is based on an intricate balance.

The level of expression of Hyal2 did not affect the adhesion of MDA-MB231 cells on different substrates (HA, collagen, and fibronectin) but lowering Hyal2 expression increased cell proliferation (+17%; p=0.0012, paired t-test) and motility (+39%; p=0.0015, t-test). However, the phosphorylation of proteins involved in major signaling pathways (Rac, AKT, ERK, and ERM) was not modified in siRNA transfected cells. Apoptosis was then studied in paclitaxel-treated cells. The activity of caspase3 and the cleavage of PARP were clearly decreased in Hyal2-siRNA transfected cells. Therefore, Hyal2 promotes chemotherapy-induced apoptosis of MDA-MB231 cells.

Hyal2 is known to reduce the size of the pericellular HA-rich coat, or glycocalyx, which can be measured using red blood cells sedimentation. However, no coat was detected using this technique in MDA-MB231 cells ± Hyal2-siRNA. The amount of HA was then assayed in the medium, the trypsinate, and a cellular homogenate. A change in the amount of HA following Hyal2-siRNA transfection was found only in the trypsinate, where it increased by 73% (p=0.0006, t-test). Neither the binding of fluorescent HA nor the expression of the main HA receptors, CD44 and RHAMM, was affected by Hyal2 knockdown.

In summary, Hyal2 acts as a functional tumor suppressor and proapoptotic agent in MDA-MB231 breast cancer cells. The mechanisms of this potentially important control of malignant behavior remain unknown but do not seem to depend on the major signaling pathways of cell proliferation and motility. On the other hand, Hyal2 keeps pericellular HA (detected in the "trypsinate") at a low level. We suggest that cancer cells require a certain level of pericellular HA, even if not organized in a full glycocalyx, to sustain their intrinsic neoplastic behavior.

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The hyaluronan (HA) coat of breast cancer stem-like cells (CSC) in culture

Bénédicte André and Bruno Flamion

Laboratory of Physiology and Pharmacology, URPHYM, University of Namur, Belgium

Tumors contain a small population of cells called cancer stem-like cells (CSC) that are responsible for their development and maintenance. A better understanding of their properties and molecular mechanisms will help eradicate cancers. CSC can be isolated from primary breast tumors and established cell lines using different methods such as a high dilution cloning technique or, more commonly, fluorescence-activated cell sorting (FACS) based on the overexpression of surface markers. Among those is the adhesion molecule CD44. Its major ligand, hyaluronan (HA), is implicated in various cellular behaviors including adhesion, migration and invasion.

HA, hyaluronidases, HA synthases (HAS), and CD44 are all implicated in tumorigenic processes but their roles in the malignant phenotype remain unclear. We decided to study the expression of the different actors of HA metabolism in CSC in the breast and prostate cancer cell lines MCF-7 and DU145. Cells were plated at low density to develop large colonies. Under these conditions, they generate different types of clones, classified morpho¬logically as holoclones, meroclones, and paraclones, containing a majority of stem, early-, and late-amplifying cells, respectively. The morphology of these clones was observed at 4, 7 and 14 days by contrast microscopy. The expression of CD44 was studied by immunofluorescence and FACS. CD44 was over-expressed in holoclones. The size of the pericellular HA-rich coat, aka glycocalyx, was measured using sedimented red blood cells. It was significantly thicker in holoclone cells than in the total population. Moreover, holoclone cells displayed considerable plasma membrane protrusions (visible in scanning electron microscopy) which may be related to the expression of HAS.

In summary, breast and prostate CSC identified functionally in the MCF-7 and DU145 cell lines present an overexpression of CD44, as expected based on published data, but also a thick HA-rich coat and numerous plasma membrane protrusions. These features may play a role in their stemness and/or their capacity to metastasize and resist chemotherapies.

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A constitutional translocation t(1;17)(p36.2;q11.2) in a neuroblastoma patient disrupts NBPF1, a novel putative tumor suppressor gene

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Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by a number of recurrent genetic alterations: gain of chromosome 17q, amplification of MYCN, and deletion of 1p36. We found that a constitutional translocation t(1;17)(p36.2;q11.2) in a neuroblastoma patient resulted in the disruption of a novel gene, NBPF1 (Neuroblastoma Breakpoint Family, member 1). This gene is built of repetitive elements and is subject of structural variation in the human population. Thorough analysis of genomic sequences revealed that NBPF1 is a member of a recently expanded gene family, with gene copies located on segmental duplications of chromosome 1. Both in silico and in vitro analysis failed to identify any rodent orthologs for the human NBPF genes. The members of the NBPF gene family are widely expressed, both in normal and cancerous tissues, including neuroblastoma cells expression profiling of NBPF1 in a panel of neuroblastoma cell lines showed that expression of NBPF1 is significantly lower in cell lines with 1p deletion than in cell lines with a normal 1p chromosome, making NBPF1 a viable candidate as a tumor suppressor gene in neuroblastoma. Transfection experiments revealed a cytoplasmic localization of the different NBPF proteins. Constitutive overexpression of different NBPF paralogs resulted in cell death in a variety of cell lines, including MCF7/AZ, HEK293T, and the neuroblastoma cell line IMR-32. We use now a conditional expression system to circumvent the detrimental properties of the overexpressed NBPF proteins and to investigate the process leading to NBPF1-induced cell death. In a preliminary in vivo experiment, expression of NBPF1 decreased proliferation of xenograft tumors. Additional experiments will be performed to validate this finding and to establish the molecular basis of the observed results.

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The role of PHD in vascular development

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Angiogenesis is a key process in embryonic development and tumorigenesis (cancer). In both, physiological and pathological processes, it is triggered by tissue hypoxia and mediated by hypoxia-inducible factors (HIFs). Prolyl-Hydroxylases (PHD) are oxygen sensors that regulate the stability of HIFs in an oxygen-dependent manner. As it has been previously showed in the host lab, haplodeficiency of PHD2 did not affect tumor vessel density or lumen size, but normalized the endothelial lining and vessel maturation (Mazzone et al, 2009). This leads to an improved perfusion and oxygenation. Thus, modulating angiogenesis by impairing with PHD activity may offer alternative therapeutic opportunities in the oncology but also, in the cardiovascular or ophthalmologic fields.

Haplodeficiency of PHD2 redirects the specification of endothelial tip cells to a more quiescent cell type, lacking filopodia and arrayed in a phalanx formation. We tested this observation in a normal developing retina and in retinas were tip cell formation was enhanced by NOTCH inhibition. The impact of PHD2 haplodeficiency on rescuing the migrating phenotype of the endothelial cells was analyzed.

Furthermore, we hypothesized that inhibiting HIF degradation, by impairing PHD activity, would also reduce vascular malformations described in the Retinopathy Of the Premature (ROP). ROP is an ischemic retinopathy dependent on oxygen-induced vascular obliteration. In this study, we analyzed retinal vascular development in PHD1, 2 and 3 pups, knock out and wild-type, while triggered or not with a high oxygen level (75%) modeling the ROP disease. Stimulating hypoxia genetically by stabilizing HIF activity during the causative ischemia phase (hyperoxia) results in prevention of the progression to the proliferative stage of the disease in PHD1 knockout mice.

In conclusion, it has been demonstrated that PHDs are potential target to improve the outcome from tumor conditions. Here we present that PHD1 inhibition could have implication onto the normal development of retinal vessels and on ophthalmologic diseases.

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Implication of Zonula Occludens-1 (ZO-1) in the metastatic progression of breast tumor cells

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Increasing data suggest that the acquisition of migratory and invasive properties by tumor cells is associated with epithelial-to-mesenchymal transition (EMT) processes. At the molecular level, EMT phenomena involve, among other mechanisms, a reorganisation of cell-cell adhesion complexes as well as an increased expression of proteolytic enzymes including matrix metalloproteases (MMPs).
ZO-1 is classically known as a submembranous cytoplasmic molecule contributing to the structural organization of tight junctions (TJs). A concept has recently emerged that, in addition to its structural role in TJ organization, ZO-1 could be involved in signalling pathways favouring tumor progression once delocalized from TJs.
Here, we first analyzed the subcellular localization of ZO-1 in relationship with the invasive potential of tumor cells. By comparing different cell lines in vitro by immunostaining, we found ZO-1 mainly localized at the membrane of non-invasive cell lines (MCF-7), whereas a predominantly diffuse cytoplasmic staining was observed in invasive tumor cells (BT-549). We also examined ZO-1 distribution in breast tumor biopsies. In in situ carcinomas, ZO-1 was expressed mostly at the cell membrane whereas a cytoplasmic/nuclear staining was observed in invasive breast cancers.
These data prompted us to examine the potential pro-metastatic role of ZO-1. We thus employed shRNAs to downregulate ZO-1 in human breast tumor cell lines: MDA-MB-231 LUC D3H2LN and MDA-MB-435 LUC, largely described as highly metastatic cell lines once injected subcutaneously in mice. Using an in vivo imaging system (IVIS 200, Xenogen) allowing the visualisation of luciferase expressing cells in living animals, we obtained preliminary results showing that transfection of ZO-1 shRNA can inhibit the invasive properties of this cells.
So far, our data suggest a pro-metastatic of ZO-1. Experiments are ongoing to determine the potential target genes of ZO-1 signalling in our models.

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Generation and characterization of Mdm2 and Mdmx condtitional transgenic mice

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The two structurally related proteins Mdm2 and Mdmx function in a non-redundant manner to restrain the growth-suppressive activity of p53. A large body of evidence indicates that aberrant expression of either Mdm2 or Mdmx impair p53 tumor suppression function and favour tumor formation.
To test this hypothesis further, we generated conditional Mdm2 and Mdmx transgenic mouse lines. Expression of the transgenes is under the control of the artificial pCAGG promoter for robust expression and a single copy of both transgenes was targeted into the ROSA26 locus to allow reliable and comparable expression levels (Nyabi et al., 2009).
The levels of pCAGG driven transgene expression were analysed after intercrossing the conditional transgenic mice with a general deleter strain (Sox2-cre). Both Sox2-cre;Rosa26pCAGG-Mdmx/+ and Sox2-cre;Rosa26pCAGG-Mdm2/+ mice are viable and fertile. In these mice, a robust and comparable increase in Mdmx and Mdm2 expression was observed at the transcriptional level in all organs analyzed. At the protein level, Mdmx was clearly overexpressed whereas Mdm2 exogenous expression was only marginally increased (two fold at best) compared to endogenous Mdm2. Experiments performed in Mouse Embryonic Fibroblasts (MEFs) established that the Mdm2 transgenic protein is extremely unstable and undergo constitutive proteasomal-dependent degradation.
Importantly the Mdmx transgene is functional as it rescues the Mdmx null phenotype. Surprisingly, however, Sox2-cre;Rosa26pCAGG-Mdmx/pCAGG-Mdmx homozygous mice die during embryogenesis, likely due to a vascular defect. Experiments are ongoing to further characterize the nature of this defect.
As expected, Sox2-cre;Rosa26pCAGG-Mdmx/+ MEFs are immortal (3T3 and colony assays) suggesting the Mdmx overexpression is sufficient to bypass p53-mediated cell cycle arrest. Surprisingly, however, overexpression of Mdmx in Sox2-cre;Rosa26pCAGG-Mdmx/+ mice did not accelerate Myc-induced B-cell lymphomagenesis.
These observations suggest that we may need to revisit the simplistic model by which elevated Mdmx expression will systematically accelerate tumor formation in tumors that retain wild-type p53. Clearly more attention should be paid to the level of Mdmx expression. Our conditional Mdmx transgenic line should help us to address this issue and further dissect Mdmx oncogenic properties in vivo.

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Shifting the equilibrium between RhoA and RhoC towards RhoA inhibits prostate cancer cells tumorigenesis

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The small GTPases of the Rho family are key signaling molecules regulating various cellular functions including cell migration, proliferation, survival and gene expression. RhoGTPases act downstream of cell surface receptors such as growth factor receptors, integrins and cadherins and, in their activated state, interact with a wide-range of effectors mediating their cellular functions. Within the RhoA-related sub-class, both RhoA and RhoC, in contrast to RhoB, are reported to contribute to cancer progression. Moreover, regulation of their expression by miRNA can lead to metastasis or epithelial-to-mesenchymal transition. To gain insight into the individual role of RhoC and RhoA in prostate cancer cells (PC-3) phenotype, we used both loss and gain-of-function strategies. The silencing of RhoC, but not that of RhoA, significantly decreased the anchorage-independent growth of PC-3 and increased the expression of several genes encoding tumor suppressors such as NAG-1, p21Cip1 and p8. In contrast, the expression of SPARC, a matricellular protein involved in tumorigenesis, was inhibited. Interestingly, the transfection of RhoC-silenced PC-3 with a siRNA targeting NAG-1 reversed most of these gene regulations and restored the anchorage-independent growth of PC-3. Tumor growth of PC-3 in nude mice was significantly delayed by intratumoral injection of siRNA targeting RhoC, an inhibitory effect relieved by co-injecting siRNA targeting NAG-1. To further validate our observations, we generated PC-3 clones overexpressing in an inducible way RhoA or RhoC. The overexpression of RhoC did not affect the anchorage-independent growth of PC-3 cells and the expression of NAG-1, p21Cip1 or SPARC were not modified. However, overexpression of RhoA reduced the anchorage-independent growth of PC-3 cells, increased the expression of NAG-1 and p21Cip1 while SPARC expression was repressed. These seemingly contrasting results can be explained by the fact that the expression of RhoA and RhoC are interdependent. We indeed demonstrated that silencing one of them up-regulates the expression of the other while overexpressing one of them decreases the expression of the other. Due to this compensatory mechanism, silencing RhoC is similar to overexpressing RhoA, while silencing RhoA is equivalent to overexpressing RhoC. Hence, our results suggest that shifting the equilibrium between RhoA and RhoC towards RhoA decreased the tumorigenic potential of PC-3 cells by a mechanism involving NAG-1.

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Lactate activates HIF-1 in tumor cells: paracrine and autocrine effects

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Hypoxia is a hallmark of cancer. In cells, it is inextricably coupled to a switch to a glycolytic metabolism and, consequently, high lactate production. The transcription factor hypoxia inducible factor-1 (HIF-1) plays a major role in the response to hypoxia. HIF-1 indeed triggers the expression of a series of hypoxia-induced genes, such as vascular endothelial growth factor (VEGF). HIF-1 is a dimer and its activity is mainly regulated by the stability of its alpha subunit. In normoxic cells, HIF-1alpha is hydroxylated by the 2-oxoglutarate-dependent dioxygenase prolylhydroxylase 2 (PHD2), addressing it for proteolytic degradation. Under hypoxia, the PHD2 reaction does not occur, the HIF-1alpha protein is stabilized, and HIF-1 activity increases. The posttranslational regulation of HIF-1 is more complex, though. In normoxic conditions, Lu et al. (J Biol Chem 2005;280:41928-39) have indeed reported that pyruvate can promote HIF-activation, whereas a similar response to nitric oxide and reactive oxygen species is well characterized. Considering that lactate, not pyruvate, is the end-product of glycolysis, we tested the existence of a lactate signaling pathway in tumor cells.

In SiHa human cervix carcinoma cells cultured under normoxic conditions, we found that exogenous lactate stabilizes HIF-1alpha protein expression. The response was dose-dependent, with a plateau corresponding to a 3-fold increase in HIF-1alpha protein expression reached after a 24h exposure to lactate concentrations X05; 10 mM. It was also time-dependent with a maximal induction of HIF-1apha 12h after the treatment with 10 mM lactate. Using a dual luciferase reporter assay in SiHa cells, we consistently detected a 3-fold increase in HIF-1 activity 24h after the addition of 10 mM lactate. We further showed that lactate stimulates VEGF transcription. Similar to the response to hypoxia, lactate-induced HIF-1 activation was prevented by echinomycin, a pharmacological inhibitor of HIF-1-dependent transcription. Next, we found that lactate competes with 2-oxoglutarate to promote HIF-1alpha protein expression in normoxic SiHa cells based on these data, we tested the contribution of glycolysis to the response to hypoxia. In SiHa cells exposed to short term hypoxia (1% O2, 1h), glycolysis inhibition (either with the GAPDH inhibitor iodoacetate, glucose starvation and/or the hexokinase competitor 2-deoxyglucose) prevented the stabilization of the HIF-1alpha protein. We thus propose that metabolism, through lactate production, is an oxygen sensor in the HIF-1 system. This hypothesis is currently tested using other tumor cell types.

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Membrane Proteomic Analysis Identifies Proteins Differentialy Expressed in Breast Cancer and Associated Bone Metastasis

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One of the typical consequences of the metastasizing breast cancer cells is their colonialization of the bone tissue. In this context bone metastasis are not only responsible for mortality but are also accompanied with severe and prolonged pain symptoms. Up to date there is no effective specific treatment to reduce the suffering and to provide a potential cure. Targeted immunotherapy is increasingly becoming an alley of opportunity to specifically deliver high concentrations of cytotoxic agents directly to the malignant lesions. Therefore the identification of targetable systemically reachable tumor antigens is a major step towards the development of novel and effective antibody-based targeted therapies. In this study, we had the unique opportunity to examine bone metastasis and the corresponding breast cancer primary lesion obtained simultaneously from a fresh autopsy performed on a patient who died from disseminated breast cancer. The tissues samples were processed for proteomic analysis. We were particularly interested in identifying accessible protein biomarkers using a procedure that we recently developed and which consists in the soaking of the tissue in the EZ-link Sulfo-NHS-SS-biotin solution. The biotinylated proteins are captured by streptavidin affinity chromatography and the peptides derived from tryptic digestion are analyzed using the 2D-HPLC-MS/MS technique. In the present study 519 proteins were identified in the primary breast cancer and 768 proteins were found in the bone metastasis lesion. The comparison of the differential expression (unique presence or overexpression) of the primary breast tumor and the bone metastasis yielded 234 biomarker proteins of which 78 were found to be located in the extracellular matrix and/or in the plasma membrane. The latter characteristics made these biomarkers particularly interesting to serve as potential candidates for antibody targets. 29 of these potential biomarkers were found uniquely expressed in the primary breast cancer while 27 proteins were detected only in the bone metastasis lesions alone. In particular several proteins belonging to small leucine rich proteo-glycans, thrombospondin and integrin families were found up-regulated in the primary breast tumor in comparison to the bone metastasis. Next, we were able to confirm the differential expression of a selected group of the identified biomarkers by immunohistochemistry performed on a collection of primary breast cancers and bone metastases. Our study has identified potential valuable biomarkers for the selective antibody-based targeted eradication of bone metastases and the results particularly emphasize that the accessible biomarkers for bone metastases are significantly different than the ones of the corresponding primary breast tumor.

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Anti-apoptotic role of HIF-1 and AP-1 in paclitaxel exposed breast cancer cells under hypoxia

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Background : Hypoxia is a hallmark of solid tumors and is associated with metastases, therapeutic resistance and poor patient survival.

Results : In this study, we showed that hypoxia protected MDA-MB-231 breast cancer cells against paclitaxel- but not epirubicin-induced apoptosis. The possible implication of HIF-1 and AP-1 in the hypoxia-induced anti-apoptotic pathway was investigated by the use of specific siRNA. Specific inhibition of the expression of these two transcription factors was shown to increase apoptosis induced by chemotherapeutic agents under hypoxia indicating an involvement of HIF-1 and AP-1 in the anti-apoptotic effect of hypoxia. After HIF-1 specific inhibition and using TaqMan Human Apoptosis Array, 8 potential HIF-1 target genes were identified which could take part in this protection. Furthermore, Mcl-1 was shown to be a potential AP-1 target gene which could also participate to the hypoxia-induced chemoresistance.

Conclusions: Altogether, these data highlight two mechanisms by which hypoxia could mediate its protective role via the activation of two transcription factors and, consecutively, changes in gene expression encoding different anti- and pro-apoptotic proteins.

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Study of keratinocyte responses after exposure to sensitizers in a reconstructed human epidermis

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Keratinocytes represent 95% of epidermal cells in human skin and play a crucial role in events underlying irritation and sensitization. Indeed, a variety of identified cytokines are constitutively produced by keratinocytes upon induction by various environmental stimuli. For this reason, keratinocytes embedded in reconstructed human epidermis (RHE) are adequate models for in vitro toxicological studies of irritation and sensitization.

Using ELISA assays, we confirm, that irritant (benzalkonium chloride) and sensitizers (dinitrofluorobenzene (DNFB), oxazolone and cinnamaldehyde) induce different profiles of interleukin-1 alpha and interleukin-8 expression and release from the RHE. Indeed, the ratio of released IL-8/IL-1 alpha measured after 22 h of treatment at a concentration of the chemicals that preserves 50% of cell viability is a criterion proposed to easily differentiate irritant compounds from sensitizers.

The objective of this study was to identify cellular mechanisms induced in such circumstances that are potentially involved in the specific expression and release of cytokines.

Intracellular signalling pathways were analysed by Western blot analysis in order to investigate potential involvement of signalling in the differential release of interleukins. Our current data illustrate important differences in EGFR, p38 and ERK1/2 MAPKs activation sustained with sensitizing treatments. Exposures of RHE to specific inhibitors of EGFR, p38 and ERK1/2 kinase activities suggest that the activation of EGF receptor only is involved in the induction of IL-8 release after treatment with sensitizers.

The potential implication of H2O2 as an intermediate involved in responses of RHE towards sensitizers was also investigated. Using the anti-oxidant agent N-acetyl cysteine as well as performing intracellular measurement of ROS, results were obtained that reveal strong evidences of oxidative stress in events triggering IL-8 release after the contact of the epidermis with the DNFB sensitizer.

These investigations suggest that accurate cell biological studies of keratinocyte responses towards chemicals may refine in vitro investigation of epidermal toxicity.

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Basal and induced release of sVEGFR-2 by endothelial cells

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Angiogenesis is essential for successful placentation and for tumor growth. Among the various angiogenic factors, we are particularly interested in the VEGF family and their receptors. The two main VEGF receptors implicated in angiogenesis are VEGFR-1 and VEGFR-2. A variant transcript of VEGFR-1 leads to a soluble form: sVEGFR-1. It is detected in plasma of preeclamptic women, during ischemia and in some cancer cases. A soluble form of VEGFR-2, named sVEGFR-2, is detected in plasma of healthy people, in leukaemia and in systemic lupus erythematosus cases. However, the mode of generation of this soluble receptor remains controversial and its physiological and pathological implications are still poorly characterized.
In this context, the aim of this study was to identify the mechanisms leading to sVEGFR-2 release by endothelial cells (EC). Additionally, we characterized how phorbol 12-meristate 13-acetate (PMA) promotes sVEGFR-2 release. Indeed, PMA is described to promote ectodomain shedding by increasing ADAM17 activity.
The ectodomain shedding process was assessed by evaluating the impact of various protease inhibitors on sVEGFR-2 release. Among them, BB94 and TIMP-3 induced a partial inhibition of sVEGFR-2 release. Additionally, PMA promoted sVEGFR-2 release. Altogether, these observations demonstrated a partial implication of the ectodomain shedding process in the release of sVEGFR-2 by EC. An RT-PCR strategy was developed to identify the generation of a variant transcript of VEGFR-2. This strategy conducted us to amplify a splice variant from HUVEC-derived RNA. Otherwise, regarding the promoting effect of PMA on sVEGFR-2 release, we observed that PMA upregulated the transcription of the full-length VEGFR-2 but did not significantly modulate the spliced form. Furthermore, PMA is known to induce a signal transduction pathway involving the protein kinase C (PKC). The PKC inhibitor, GF109203x, did not affect the basal release of sVEGFR-2, however, it inhibited the PMA-related increase of VEGFR-2 expression and sVEGFR-2 release. These results indicate that the PMA-dependent increase of sVEGFR-2 release is not only related to an increase of ADAM17 activity, but results from a PKC-dependent increase of full-length VEGFR-2 transcription.
In conclusion, our results demonstrate that the release of sVEGFR-2 by EC relies at least on two different cellular mechanisms: ectodomain shedding and alternative splicing. A combination of these two mechanisms is observed under basal conditions. However, if EC undergo an increase of PKC activity, the full-length VEGFR-2 is predominantly produced and the ectodomain shedding process prevails. Altogether, our results suggest that the way by which sVEGFR-2 is generated by EC is closely related to the cell microenvironment context.

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HB-EGF synthesis and release is induced by extracellular ATP after lipid rafts disruption in human epidermal keratinocytes

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The skin is the most outer organ of the mammalian body and so its major function is to provide at the level of the epidermis a protective barrier against insults from the environment and against dehydration. The epidermis is mainly composed of keratinocytes that undergo a complex differentiation program leading to keratinization. The maintenance of the barrier is regulated by keratinocyte proliferation and differentiation, thus when injuries appear in the barrier it is crucial to repair and restore rapidly the epidermal tissue in order to maintain the epidermal superficial barrier. The Heparin-Binding EGF-like Growth Factor (HB-EGF) is an autocrine/paracrine keratinocyte growth factor which binds to the EGF (Epidermal Growth Factor) receptor family and plays a critical role during the re-epithelization of cutaneous wound by stimulating the keratinocytes proliferation and migration. Like other members of the EGF family of growth factors, HB-EGF is synthesized as a transmembrane protein (proHB-EGF) that can be shed enzymatically by metalloproteases to release the soluble growth factor. Previous studies have shown that extracellular ATP regulates proliferation and differentiation in keratinocytes by his interaction with P2 purinergic receptor and that ATP released in epithelial cell upon scratch wound induces the HB-EGF shedding. In this study, cellular stressing condition in autocrine cultures of human keratinocytes was induced by cholesterol depletion with the molecule methyl-beta-cyclodextrin (MbCD). MbCD treatment induces the expression and the release of HB-EGF. By analysis of the culture media, large amounts of cellular ATP were measured particularly after 1h of MbCD treatment. To investigate whether ATP contributes to the expression of HB-EGF, the nonhydrolyzable ATP analogue, ATP-gamma-S, was used to mimic the extracellular ATP released. Keratinocytes stimulated with ATP-gamma-S induce HB-EGF expression and activate EGFR and ERK1/2. Using an antagonist of P2 purinergic receptors, we demonstrate that HB-EGF synthesis induced by lipid rafts disruption is dependent on ATP interaction with P2 purinergic receptors. These finding provide new insight into the signalling pathway by which HB-EGF is expressed after lipid rafts disruption. In conclusion, after lipid raft disruption, keratinocytes release large amount of extracellular ATP. This release, by interacting with the P2 purinergic receptor, induces HB-EGF synthesis and release, a growth factor considered as a marker of the keratinocyte's response to a challenging environment. We suggest that the release of extracellular ATP act as an early stress response in keratinocytes.

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How molecular and cellular events direct mesenchymal stem cells to a neural fate ?

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Mesenchymal stem cells (MSC) are widely considered as a potential cell-based therapeutic tool for a diverse range of clinical purposes. They can be found in several organs and tissues like adipose tissue, dental pulp, umbilical cord, placenta and especially in the adult bone marrow. MSC normally give rise to cells of mesodermal origin like adipocytes, chondrocytes or osteocytes, but under appropriate experimental conditions they are also able to undergo differentiation into representative lineages of the three germ layers as a result of their great plasticity. These multipotent cells appear to differentiate for instance into neurons and glial cells.

As it has been recently shown that adult bone marrow contains neural crest-derived cells, we used a protocol to select MSC with neural crest origin under clonal culture conditions. Using Wnt-1 and BMP2, two factors known to maintain and stimulate the proliferation of neural crest stem cells, we selected five clones. Those clones were immunologically and functionally characterized. Interestingly, two of them showed opposite differentiating abilities, as clone 1 was more gliogenic while clone 5 was more neurogenic. In order to understand the differentiating abilities and the identity of those clones, we performed several transcriptomic and proteomic comparisons using RT-PCR Array, microarray, qRT-PCR, 2D-DIGE and western blotting techniques. Our preliminary results demonstrated up-regulation of some glycolytic enzymes such as: aldolase, TPI, phosphoglycerate mutase by the gliogenic clone 1 which could be a consequence of the different identities of those neural crest-derived clones. Moreover clone 1 seems to possess stem cell-like characteristics and differentiates easily into adipocytes, melanocytes and smooth muscles compare to the neurogenic clone 5 which could be more progenitor-like cell type.

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Analysis of the membrane proteome in minute quantities of human cells

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Recent developments in the field of biomarker and cancer research have raised the requirements on the analytic tools and methods. Relevant samples such as tumor biopsies or precious primary cell lines and stem cells are all available in minute quantities. Therefore identification of proteins biomarkers from such scarce samples is a challenging task that is the focus of the present work. Due to the dynamic range limitations encountered in the current proteomic analytical methods it is essential to address a specific group of proteins bearing the highest relevance. In this work we have focused our method development to target specifically membrane associated and extracellular proteins. They have the greatest potential to be systemically reached in-vivo for diagnostic and therapeutic purposes. To this extend we have applied the technique of chemical linkage of biotin to free lysine and N-terminal amino-acids of accessible proteins. The method development was performed by decreasing quantities of MDA-MB-231 cells which were biotinylated. Following this step the cells were lysed and the biotinylated proteins were selectively captured using streptavidin resin. Subsequently, the proteins were eluted and analyzed in an ion trap mass spectrometer coupled with the nano-HPLC. The method optimization was performed through adaptation of several key parameters including volume of the lysis buffer, biotin concentration and incubation period as well as consideration of different steps where potential sample loss might occur. Preliminary results show that the optimal amount of lysis buffer depends significantly on the quantity of the cells used. This observation is especially critical for cell numbers below 25000. The concentration of biotin has been optimized as well. For example it has been shown that 160 µM biotin reagent is an optimal amount necessary to label 500000 cells. The future work will further optimize key parameters like the biotin incubation temperature, streptavidin beads quantity and involve quantitative mass spectrometry techniques in order to give more informative and conclusive picture of the potential modulations of the membrane and extracellular proteome.

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Neurotensin receptor 1 and trophoblast glycoprotein are expressed in gastrointestinal stromal tumors but not in interstitial cells of cajal

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Gastrointestinal stromal tumors (GIST) are thought to derive from the interstitial cells of Cajal (ICC) or an ICC precursor. Oncogenic mutations of the KIT or PDGFRA receptor tyrosine kinases are present in the majority of GIST, leading to ligand-independent activation of the intracellular signal transduction pathways. A mouse model harboring a germline Kit K641E mutant was created to model familial GIST (Rubin et al. 2005). We have investigated the gene expression profile in the gastric antrum of the KitK641E murine GIST model by microarray and quantitative PCR. By immunofluorescence, we have investigated localization of selected candidates in KitK641E mouse model and human GIST using tissue microarray.

Most upregulated genes in this mouse model belong to the normal profile of Kit-ir ICC (Chen et al. 2007). Conversely the proteins encoded by Neurotensin receptor 1 (Ntsr1) and Trophoblast glycoprotein (Tpbg/5T4) were detected in Kit-ir cells only in mutant KitK641E, but not in Kit-ir ICC of WT animals (Gromova et al.2009/unpublished data).

Similarly, in the normal human gut, NTSR1and TPBG/5T4 immunoreactivity was detected in myenteric neurons but not in KIT-ir ICC. Using tissue microarray, NTSR1 and TPBR/5T4 expression were characterized in human GIST, including 40 cases with KIT and 9 with PDGFRA mutation. NTSR1 immunoreactivity was observed in all GIST, including the 4 KIT negative cases with PDGFRA mutation. TPBG/5T4 was present in 36/49 human GIST and strong TPBG/5T4 staining associate with tumor malignancy.

It is possible to target NTSR1 with radio-labeled NTS analogues for whole body tumor imaging and for therapeutic interventions. TPBG/5T4 is a tumor-associated antigen and a promising target for anti-tumor vaccine development. The induction of NTSR1 and TPBG/5T4 expression in the murine KitK641E GIST model and in human GIST raises perspectives for innovative diagnostic and therapeutic approaches in GIST.

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Rab27B-mediated vesicle exocytosis regulates invasive tumor growth and metastasis

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Background: Vesicle exocytosis, controlled by secretory GTPases such as Rab27B, delivers critical pro-invasive growth regulators into the tumor microenvironment. The biological role and expression status of Rab27B in breast cancer was unknown.

Methods: Rab27B was studied in estrogen receptor (ER)-positive human breast cancer cell lines (MCF-7, T47D, ZR75.1) using GFP-fusion constructs, including wild type Rab3D, Rab27A, Rab27B and Rab27B point mutants defective in GTP/GDP-binding or geranylgeranylation, and transient siRNA targeting. In cell culture, cell-cycle progression was evaluated by flow cytometry, Western blotting and measurement of cell proliferation rates, invasion was assessed using Matrigel and native collagen type I substrates. Orthotopic tumor growth, local invasion and metastasis were analyzed in mouse xenograft models. Mass spectrometry was performed to identify Rab27B-secreted pro-invasive growth regulators. In clinical breast cancer, Rab3D, Rab27A and Rab27B mRNA levels were analyzed by quantitative RT-PCR (n=20) and Rab27B protein level was evaluated by immunohistochemistry (n=60).

Results: Rab27B-upregulation promoted G1/S phase cell cycle transition and increased proliferation, F-actin reorganization and invasion in cell culture, and invasive tumor growth and haemorrhagic ascites in a xenograft mouse model (at 10 weeks, survival of MCF-7 GFP vs GFP-Rab27B injected mice was 100% vs 62.5%, P=0.0307). Proteomic analysis of purified Rab27B-secretory vesicles and the secretome of exogenous Rab27B-expressing breast cancer cells identified heat shock protein (HSP)90 alpha as key pro-invasive growth regulator. HSP90 alpha secretion occurred in a Rab27B-dependent manner and was required for matrix metalloproteinase(MMP)-2 activation. All Rab27B-mediated functional responses were GTP- and geranylgeranyl-dependent. Endogenous Rab27B mRNA and protein, but not Rab3D and Rab27A mRNA, associated with lymph node metastasis (P=0.0002) and differentiation grade (P=0.0014) in ER-positive human breast tumors.

Conclusion: Rab27B regulates invasive growth and metastasis in ER-positive breast cancer.

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Membrane-anchored protease regulation during tumoral growth

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Tumor growth and metastatic dissemination are associated with an important tissue remodelling that requires different proteases. The matrix metalloprotease (MMP) are involves in cell migration, cell proliferation, invasion, apoptosis and also in physiological and pathological remodelling of the extracellular matrix. In addition to their classical tissue-remodeling functions, MMPs act as processing enzymes that perform highly limited cleavage of specific substrates including growth factor, cell surface molecules, cytokines, chemokines and angiogenic factor. Among 24 MMPs known, 6 are anchored to the plasma membrane (Membrane type-MMP, MT-MMP). Previous works of our laboratory have shown that MT4-MMP overexpression confers to cancer cell lines an increase of tumoral growth and metastatic spreading. These works also suggest that MT4-MMP is not playing a redundant role with the other MMPs and that their molecular mechanisms are different. Nevertheless, whether MT4-MMP displays pro-tumorigenic and pro-metastatic effects through its catalytic activity remains to be determined. Furthermore, in vivo substrates of MT4-MMP are not known.

With the aim to investigate the mechanism of action of MT4-MMP (catalytic dependent or not), we have generated a mutated form of MT4-MMP by site-directed mutagenesis. The mutation (in the highly conserved domain, HExxHxxGxxH U94; HAxxHxxGxxH) was performed to inactivate the catalytic domain of this enzyme and the construction has been transfected into MDA-MB-231. After transfected cells screening, the in vitro phenotyping of cells did not reveal any differences in the proliferation between the inactive form of the MT4-MMP (deltaMT4-pop) versus the active enzyme (MT4-pop) and the control conditions (CTR-pop). In sharp contrast, after injection of these cells in RAG -/- mice, MT4-MMP overexpression resulted in a significant increased of tumor growth compared to control condition. Interestingly, the overexpression of the mutated form showed a significant decrease of the tumoral volume, even when compared to control condition.

We extend actually our research to other kinds of tumors. Different pulmonary epithelial cancer cell lines (BZR and BZR-T33) who are express endogenous MT4-MMP were transfected with shRNA directed against transcripts of this protease. These cells will be studied in vitro and in vivo to check if the inhibition of MT4-MMP expression had an effect on the different process involve in metastatic spreading (proliferation, migration, apoptosis, invasion and angiogenesis).

These results suggest that the pro-tumoral effect of the MT4-MMP is catalytic-dependent and also indicate unexpectedly that the inactive form has a "dominant negative" effect.

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Generation of novel cancer mouse models for Protocadherin-10 and Protocadherin-11

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Protocadherins are transmembrane proteins that differ in various aspects from classic cadherins, and whose functions are largely unexplored. We are especially interested in two smaller protocadherin subfamilies (delta1- and delta2-protocadherins) featuring two or three conserved motifs (CM) in their cytoplasmic domains. In this study we are focusing on protocadherin-10 (PCDH10) and protocadherin-11Y (PCDH11Y), which were recently found to act either as candidate tumor suppressor or as proto-oncogene product.

The human PCDH10 gene is frequently silenced in several carcinomas, and its ectopic expression strongly suppresses tumor cell growth, migration and invasion. Recently, a germline Pcdh10 knockout mouse has been reported on. This mouse has a severe brain abnormality leading to death within three weeks after birth. To avoid this lethality problem we aim at ablating Pcdh10 in a tissue- and time-specific manner. First, we are establishing a model in which all isoforms of Pcdh10 can be conditionally knocked out. This mouse will then be crossed with different Cre mice as well as with various tumor mouse models to elucidate the role of Pcdh10 in important cellular processes, such as control of proliferation, migration, differentiation and programmed cell death. Second, we are generating mice for conditional knockout only of the long isoforms, in this way deleting also the conserved CM1 and CM2 sequences. The latter model will be used to explore the role of these conserved domains in various intracellular signaling pathways, including oncogenic and tumor progression pathways.

A cytoplasmic form of PCDH11Y has been implicated in Wnt signaling and in acquisition of hormone resistance by progressed prostate tumors. We are generating transgenic mice with conditional overexpression of selected PCDH11X and PCDH11Y isoforms. Of particular interest to us is the generation of a transgenic mouse conditionally expressing the cytoplasmic, human-specific PCDH11Y variant, which has been proposed to be causally related to prostate cancer progression. All mouse models will be analyzed in detail to confirm and extend the hypothesis that these two delta-protocadherins play key roles in either stimulating or repressing tumorigenesis or tumor progression.

Research supported by the Research Foundation (FWO) – Flanders, the Foundation against Cancer, Belgium, and the Queen Elisabeth Medical Foundation (GSKE), Belgium.

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16K human prolactin is an anti-lymphangiogenic factor in vitro and in vivo

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Development of metastasis is one of the most fearsome aspects of cancer. The contribution of the lymphatic system during tumor cell dissemination has been established in various murin and human cancer studies. In certain types of cancer, such as breast and melanoma cancer, the predominant route of tumor spread is via the lymphatic system. Hence the presence of metastasis in the regional lymph nodes is one of the first signs of cancer spread. Results obtained previously in our lab show that 16K hPRL is a very potent protein for antitumor therapy directed against the expansion of new blood vessels necessary for the tumor development. In addition, Nguyen et al, have recently showed that 16K hPRL can reduce the establishment of B16F10 metastasis in an experimental lung metastasis model [1]. However, the implication of 16K hPRL in tumor lymphangiogenesis has not yet been investigated. It is therefore interesting to determine if 16K hPRL besides diminishing angiogenesis can also inhibit lymphangiogenesis. In this study, we show that 16K hPRL induces apoptosis and reduces proliferation, migration and tube formation of primary lymphatic endothelial cells in vitro. We also show that 16K hPRL administered trough adenovirus-mediated gene transfer inhibits angiogenesis as well as lymphangiogenesis in a subcutaneous B16F10 mouse melanoma model in the primary tumor and in the sentinel lymph nodes. These results show that the antitumoral activity of the factor 16K hPRL is mediated by an effect on blood and lymphatic vasculature.

This work is supported by the FRIA, the FNRS, the TELEVIE and the program NEOANGIO from the "region wallonne".

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Targeting PHD2 oxygen senor: novel and promising avenue to treat cancer

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Oxygen supply by vessels is important during development and progression of ischemic diseases and cancer. A number of recent studies highlighted the importance of normalizing the tumor vessel architecture in improving response to chemo/radiotherapy. In a recent study, the host laboratory demonstrated that haplodeficiency of PHD2 (prolyl hydroxylase 2) restores tumor oxygenation and inhibits metastasis via endothelial normalization (Mazzone et al, Cell 2009). Heterozygous deficiency of PHD2 in tumor stroma resulted in "phalanx" cells formation, which participate in the normalization of the endothelial lining to a smooth, continuous cell layer, leading to decreased tumor cell intravasation and metastasis, and allowing vessels to readjust their shape, not their numbers, and to optimize oxygenation.

Directing endothelial cells towards a phalanx cells phenotype provides a conceptual novel strategy to combat cancer by increasing the efficacy of chemo/radiotherapy. Genetically engineered spontaneous murine tumor models are more reliably mimicking human oncology than syngeneic models. Therefore, the spontaneously arising tumor model MMTV-PyMT intercrossed with PHD2+/- mice was used to dissect in depth the effect of PHD2 inhibition on both, tumorigenic and stromal, compartments. As a confirmation of the hypothesis, metastatic index was reduced in PHD2+/- mice compared to the control WT.

To prove the importance of PHD2 inhibition as an adjuvant therapy to a clinically validated anticancer treatment strategy, we performed two independent experiments by injecting subcutaneously Lewis Lung Carcinoma tumor cells combined with the anti-VEGFR2 treatment or with irradiation (15 Gy). Herein, we tested whether PHD2 haplodeficiency by its phenotypic vessel normalization, improves oxygenation and reduce metastasis, effecting in the extended survival.

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Pharmacological correction of aberrant splicing of VEGF induced by chemotherapy in vitro

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We previously detected a new splice variant of VEGF-A (VEGF111, GeneBank ID: DQ229900) induced by chemotherapy in vitro, in hair follicles and surgical specimens ex vivo, and in human MCF-7 cells injected into nude mice. VEGF111 is the only human VEGF variant lacking the sequence encoded by exon 5. Its biological activity is demonstrated by induction of human umbilical vein cells proliferation and by phosphorylation of VEGF receptor-2 and activation of downstream signalling cascades. VEGF111 is remarkably resistant to proteolysis degradation by plasmin and fluids collected from chronic ulcers, suggesting potential benefits in the treatment of ischemic diseases. HEK293 cells expressing VEGF111, VEGF121 or VEGF165, and control cells have been injected in nude mice. Tumours were analyzed after three weeks. VEGF111 induces the formation of a peri-tumoural vascular network of small blood vessels and enlargement of the lateral thoracic vein. By contrast VEGF121 and VEGF165 induce the formation of blood vessels in the tumour. Together these data suggest that its potential induction by chemotherapy in Man may result in higher levels of VEGF in the tumour environment, and increased angiogenesis, metastasis and cancer cell survival. The effect of several agents used as dietary supplements as epigallocathechin gallate and resveratrol, and curcumin and silybin derivatives inhibit the expression of VEGF111 induced by doxorubicin in vitro. Works to analyze their potential benefit as adjuvant to chemotherapy in vitro and in animals are in progress.

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Osteopontin expression is regulated in human glioma cancer stem cells

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Glioblastoma, the most aggressive cerebral tumors, are invariably lethal. Glioblastoma tumors have been shown to contain a subpopulation of stem cell-like tumor cells called cancer stem cells (CSCs). In this study, we used a well characterized human glioblastoma cell line (U87-MG) that has been recently shown to contain a small fraction of CSCs that grow as floating tumor spheres (neurospheres) when the cells are cultured in a defined serum-free medium containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Osteopontin (OPN) is a member of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) family of multifunctional extracellular matrix protein that is involved in tumor growth, angiogenesis and metastasis. OPN is expressed in human glioma tumors and the extent of its expression has been correlated with the malignancy grade. We took advantage of the U87-MG neurospheres model to study the potential association of OPN expression with the stem cell phenotype of glioma cancer cells. First, we confirmed the stemness properties of the spheroids by evaluating the expression of known neural stem-cell markers such as SOX2, Klf4 and nestin. Next, we used immunofluorescence and western blot techniques to evaluate OPN expression in floating neurospheres when compared to the fraction of U87-MG cells remaining adherent when cultured in bFGF/EGF medium. Interestingly, we found a high level of OPN in neurospheres when compared to attached U87-MG cells. To evaluate further whether OPN is associated with the stem cells phenotype, we evaluated OPN and SOX2 expression upon differentiation of CSCs. For this purpose, neurospheres were induced to differentiate in presence of serum for 1, 2, 5, 8 and 10 days and the expression of both SOX2 and OPN was evaluated. SOX2 was mainly expressed in undifferentiated stem cells and disappeared at day 5 when these cells began to differentiate in serum-containing medium. In contrast, OPN was highly detectable in CSCs, decreased at day 3 of differentiation and then was re-expressed in day 8 differentiated cells. These preliminary results urged us to investigate the role of OPN in CSCs. Ongoing experiments will help demonstrating whether OPN inhibition using specific lentiviral shRNA constructs has an impact on neurospheres formation in vitro and glioblastoma tumor development in vivo.

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Characterisation of the effects of hydrolyzed pectin in HepG2 and A549 cells

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Several studies have shown that modified forms of citrus pectin induce tumoral cell death. However, the nature of the active fragments and the mechanisms by which they act remain unclear.

In this work, HepG2 and A549 cancer cells were incubated in the presence of 3 mg/ml of hydrolyzed pectin (HP). HP was generated by hydrolyzing citrus pectin from Sigma by autoclaving at 123°C during 60 min. HP induced HepG2 and A549 cell death, as measured by LDH release and MTT staining, while whole pectin did not have any cytotoxic effect. However, caspase 3 activity was not increased, indicating that HP rather induced necrosis than apoptosis. Dialysis of HP with a membrane with a cut-off of 3500Da but not of 500Da, induced the lost of the active fragments. We then tried to identify which components of the hydrolyzed pectin displayed the toxic effect. With HPLC, we compared HP and dialyzed HP profiles. It appears that the active HP fraction is found in small fragments of pectin that include low degrees of polymerization polygalacturonic acid and neutral sugars. Once identified, the HP active fraction could be used in complement to conventional chemotherapeutics in cancer treatment.

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Bone marrow-derived mesenchymal cells and MMP13 contribute to experimental choroidal neovascularization

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In this study, we evaluate the potential involvement of collagenase-3 (MMP13), a matrix metalloproteinase (MMP) family member, in the exudative form of age-related macular degeneration (AMD) characterized by a neovascularisation into the choroid. The contribution of MMP13 in choroidal neovascularization (CNV) formation was explored by using a murine model of laser-induced CNV and applying it to wild type mice (WT) and MMP13-deficient mice (MMP13-/- mice).

Angiogenic and inflammatory reactions were explored by immunohistochemistry. Implication of bone marrow (BM)-derived cells was determined by BM engraftment into irradiated mice and by injecting mesenchymal stem cells (MSC) isolated from WT BM. The deficiency of MMP13 impaired CNV formation which was fully restored by WT BM engraftment and partially rescued by several injections of WT MSC. The present study sheds light on a novel function of MMP13 during BM-dependent choroidal vascularization and provides evidence for a role for MSC in the pathogenesis of CNV.

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Characterization of bone marrow derived-cells recruited into mouse tumors

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During tumor progression, the invasion of cancer cells into surrounding tissue is associated with considerable tissue remodeling called "cancer induced stromal reaction" or "desmoplastic reaction". The main constituents of cancer stroma are inflammatory cells, small blood and lymphatic vessels, fibroblastic and myofibroblastic cells, and extracellular matrix components. Although the inflammatory and endothelial cells have been involved in tumor immunity and neoangiogenesis, the functions of fibroblasts have not been fully elucidated yet.

Fibroblasts, which are widely distributed and play a key role in tissue fibrosis, represent a main source of interstitial collagens. This cell type is heterogeneous with respect to a number of phenotypic and functional features. This heterogeneity may arise not only from the cellular activation and differentiation processes but also from their different cellular origins. Fibroblasts and myofibroblasts, i.e. activated fibroblasts expressing alpha-smooth muscle actin, which produce collagen and extracellular matrix proteins contribute to the "desmoplastic reaction" and have been suggested to represent an important player in tumor invasion.

In the present study, we investigated in vivo the putative contribution of bone marrow-derived cells into malignant murine keratinocytes (PDVA). Mice were engrafted with bone marrow isolated from transgenic mice expressing green fluorescent protein (GFP), and the different types of cancer cells were subcutaneous injected. Bone marrow-derived cells positive for GFP were detected in each type of tumor xenografts. This was confirmed by immunohistochemistry stained against GFP. There is no doubt that bone marrow-derived cells (BMDC) are recruited into tumors and participate in cancer progression. The mechanisms through which they contribute to tumor development are numerous: inflammation, angiogenesis and also stromal reaction. Interestingly, bone-marrow derived cells were mostly localized in connective tissue bundles. These cells were fusiforms with a fibroblast-like morphology and were specifically associated with collagen deposition. The GFP+ bone marrow-derived cells express different fibroblastic/mesenchymal markers, such as alpha-SMA, Thy1, NG2. Our results suggested that bone marrow-derived cells (GFP+) are efficiently recruited into tumor, expressed several fibroblastic markers in vivo, and actively take part in the desmoplastic reaction.

We showed that alpha-SMA-expressing myofibroblasts can produce MMP13 and can participate in tissue remodelling. This protease plays a key role in the MMP activation cascade by activating MMP2 and MMP9 which are implicated in angiogenesis. MMP13 production by carcinoma-associated fibroblasts was demonstrated in many studies. Analyses of the impact of MMP13-producing bone marrow derived fibroblasts are under process.

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MT1-MMP activity protects against type I collagen-induced apoptosis

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During the neoplastic process, epithelial-derived cancer cells invade into the stromal tissue characterized by the presence of a dense three-dimensional (3D) matrix composed largely of type I collagen or crosslinked fibrin. This extracellular matrix represents a hostile environment for infiltrating tumour cells due to its capacity to induce apoptosis in epithelial cells. Matrix metalloproteinases (MMPs) are a family of secreted or membrane-anchored extracellular zinc-dependent neutral endopeptidases, which are collectively capable of degrading a large array of substrates including extracellular matrix components, growth factors, cell surface receptors…

Herein, we demonstrate that the membrane-anchored MMP, MT1-MMP, confers breast carcinoma cell lines with the capacity to survive in a 3D matrix of type I collagen. The collagen-induced apoptosis is characterized by chromatin condensation, nuclear segmentation, oligonucleosomal DNA fragmentation, PARP cleavage and is prevented by the broad-spectrum caspase inhibitor (Z-VAD-FMK). Transfection of MT1-MMP-negative MCF-7 adenocarcinoma cells with MT1-MMP cDNA does not significantly affect their sensitivity neither to cytotoxic agents including doxorubicin, etoposide, staurosporin and TNF-alpha, nor to apoptosis induced by growing the cells in 3D fibrin gels. In contrast, survival in type I collagen gels is strongly improved by this MMP. The MT1-MMP anti-apoptotic function is abrogated by treatment with a synthetic MMP inhibitor (BB-94) or upon transfection with a catalytically inactive MT1-MMP mutant, demonstrating the requirement for an intact catalytic activity of this proteinase. Comparison of the transcriptomes of MCF-7 cells expressing or not MT1-MMP reveals that MT1-MMP decreases the pro-apoptotic Bik mRNA levels, suggesting a potential contribution of these genes in the apoptosis-resistance conferred by this proteinase.

Collectively, these data demonstrate that the proteolytic activity of MT1-MMP confers apoptosis-resistance to cells growing in a 3D matrix of type I collagen.

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Involvement of proteases in metastatic dissemination related to mesenchymal stem cell

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Mesenchymal stem cells (MSC) are pluripotent progenitor cells that contribute to the maintenance and regeneration of a variety of connective tissues, including bone, adipose, cartilage and muscle. Although MSC reside predominantly in the bone marrow, they are also distributed throughout many other tissues, where they are thought to serve as local sources of dormant stem cells. Several reports proposed that the bone-marrow-derived MSC is a cell type that is recruited in large numbers in the stroma of developing tumours. In addition, bone marrow-derived stem cells have multiple roles in assisting or regulating cancer metastasis. Recently, Karnoub et al (Nature, 449, 2007) showed that weakly metastatic human breast cancer cells acquired an increased ability to disseminate to the lungs when they are mixed with MSC before being subcutaneously injected. However, mechanisms by which MSC increase metastasis are not well known. In another hand, matrix metalloproteases (MMP) are involved in several stages of metastasis process, including the escape of individual tumor cells from the primary tumor, their intravasation, survival in circulation, and extravasation at the secondary site.

Therefore, the purpose of this study is to determine the implication of proteases in metastasis development related to MSC. We established a model to study metastasis in C57BL/6J mice. Lewis Lung Carcinoma (LLC-Luc), cells express luciferase, were injected subcutaneously into C57BL/6J mice with or without MSC. Tumors were resected on day 14 after tumor implantation. Lung metastasis development was visualized by luciferase detection through IVIS® imaging technology. Preliminary results show that MSC enhance lung metastasis development in mice. Effectively, 35 days after injection, we observed a two-fold increase of mice bearing lung metastasis when MSC were present. In vitro, MSC increase the proliferation and migration of LLC. In addition, co-cultures in which LLC and MSC were separated by a permeable membrane were established. We observed an increase in PAI-1, MMP-3, -9, -13, -12, TIMP-1 and -3 RNA expression in LLC.

Altogether, these results suggest that these proteases could contribute to explain the increase of metastasis development in mice with MSC. Investigations regarding the elucidation of the biological mechanism regulated by MSC in cancer metastasis are currently under progress.

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Transcriptional profiling in cholesterol-depleted keratinocytes evokes characteristics of inflammatory skin diseases

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Lipid rafts are cholesterol- and sphingolipid-rich cell membrane signalling platforms that are crucial in epidermal keratinocytes. The cellular physiological role of these dynamic membrane microdomains can be explored by methyl-beta-cyclodextrin, a cholesterol-sequestrating molecule. In keratinocytes, it has been shown that lipid raft disorganization alters the expression of epidermal differentiation markers with induction of late differentiation marker involucrin and repression of keratin 10. Moreover, cholesterol depletion activates the p38 MAP kinase which is responsible for high mRNA and protein expression of heparin-binding EGF-like growth factor (HB-EGF). The importance of this autocrine growth factor has been shown in cutaneous wound healing as HB-EGF stimulates proliferation and migration of epidermal keratinocytes. Subsequent to experimental data showing that oxidative stress mechanisms are not involved in the keratinocyte's response to lipid raft disruption, we performed a microarray analysis to dress a global picture of transcriptional changes ongoing in this cell type after lipid raft disruption. Whole-genome expression profiling reveals that over 3000 genes are differentially regulated (criteria for differential expression:-2>FC>2 and p-value adjusted for multiple testing <0,05). In fact, many differentially up-regulated genes after cholesterol extraction are important in inflammatory processes, such as interleukin-8 (IL8), urokinase-like plasminogen activator receptor (PLAUR), suppressor of cytokine signalling 3 (SOCS3). Strengthening our earlier data, HB-EGF was also detected among major induced genes. qRT-PCR validated microarray results and measurements of extracellular IL8 and urokinase-like plasminogen activator illustrate the functional relevance of elevated mRNA levels for these genes. Major enriched gene ontology terms and functions of genes regulated after lipid raft disruption were determined with IPA (Ingenuity Pathway Analysis). Interestingly, the inflammatory skin disorder atopic dermatitis was identified as the closest disease associated with the expression profile of lipid raft-disrupted keratinocytes. This itchy and chronically relapsing skin disease affects 10-20% children in industrialized countries and lesional atopic skin presents a disturbed epidermal barrier function. Currently gene expression parallelisms in lipid raft-disrupted and atopic keratinocytes are being investigated. In summary, major transcriptional targets were identified and bioinformatic analysis shows that lipid raft disruption in keratinocytes evokes typical features of skin inflammatory diseases, suggesting thereby that lipid raft organization and signalling might be perturbed in atopic keratinocytes and could represent a mechanism involved in the etiology of the disease.

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Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(High) monocytes

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Tumor-Associated Macrophages (TAMs) form a major component of the tumor stroma. However, important concepts such as TAM heterogeneity and the nature of the monocytic TAM precursors remain speculative. Here, we demonstrate for the first time that mouse mammary tumors contained functionally distinct subsets of TAMs and provide markers for their identification. Furthermore, in search for the TAM progenitors, we show that the tumor-monocyte pool almost exclusively consisted of Ly6ChiCX3CR1low monocytes, which continuously seeded tumors and renewed all TAM subsets. Interestingly, gene and protein profiling indicated that the distinct TAM populations differed at the molecular level and could be classified based on the classical (M1) versus alternative (M2) macrophage activation paradigm. Importantly, the more M2-like TAMs were enriched in hypoxic tumor areas, had a superior pro-angiogenic activity in vivo and increased in numbers as tumors progressed. Finally, it was shown that the TAM subsets were poor antigen-presenters, but could suppress T-cell activation, albeit by employing different suppressive mechanisms. Together, our data help to unravel the complexities of the tumor-infiltrating myeloid cell compartment and provide a rationale for targeting specialized TAM subsets, thereby optimally "re-educating" the TAM compartment.

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Tumor - stroma interaction: a novel proteomic based method for the identification of stromal biomarker of human glioblastoma

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Background: High-grade gliomas are the most common and deadly brain tumors in adults. The development of novel more specific therapeutic possibilities, like the antibody-based therapy, would provide new hope for the affected patients. Monoclonal antibodies (MAb) that bind to particular antigens on the surface of tumour cells or within its surrounding stroma, offer an excellent opportunity to confer specificity to highly cytotoxic drugs. The bottleneck for developing such therapies consists of identifying suitable tumor specific antigens.

Purpose: To identify, characterize and validate new and differentially expressed stromal biomarkers accessible through the blood stream.

Material and methods: Glial tumors were obtained after orthotopic graft of T98G and U373 human cells into the brain of nude mice. A third group of mice was used as control. Brains were biotinylated allowing the labeling of accessible proteins. Labeled proteins were isolated using streptavidin affinity chromatography. Proteins were eluted and digested with trypsin and peptides were analyzed by 2D nano-LC MS/MS. The first identification of potentially accessible proteins was obtained using the Swissprot mouse database. The list of proteins was successively narrowed down by comparison with the Normal Mouse Brain (NMB) data in order to identify proteins that are differentially expressed. However, because of the genetic proximity between human and mouse it was not possible to distinguish mouse proteins exclusively on the basis of this database search. Therefore the following strategy was applied: 1) the protein list was narrowed down to the differentially expressed proteins; 2) MS data corresponding to these proteins were blasted using Swissprot database filtered for the taxonomy "mammals", allowing the identification of both human and mouse proteins at the same time; 3) each protein of interest was manually checked for its human or mouse origin on the basis of its sequence coverage or the presence of a mouse/human specific peptide. The best sequence coverage as well as the presence of a specific peptide was taken as critical information pointing at the possible protein origin. Validation of the target proteins was conducted using immunohistochemistry and western blot analysis.

Results: In total 67 and 116 proteins have been found exclusively expressed in the T98G and U373 conditions respectively. From those 23 proteins were shared by both tumors. Several potential biomarkers have been

identified to be uniquely present (or highly abundant) in the tumor condition. Further investigations are ongoing.

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Soluble forms of VEGF receptor-1 and -2 can promote vascular maturation

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In the early steps of angiogenesis, endothelial cell (EC) proliferation leads to nascent vessel formation. These vessels are then stabilized by coverage with mural cells. Members of the VEGF family constitute key regulators of angiogenesis. sVEGFR-1 and sVEGFR-2 are soluble forms of the membrane-bound receptors of VEGF. They are overproduced in some pathology and they exhibit in vivo anti-angiogenic properties.

Development of anti-angiogenic therapies is an important challenge for the treatment of cancer and ocular or skin disorders characterized by excessive angiogenesis and abnormal vasculature. Besides triggering immature vessel pruning, anti-VEGF therapies are characterized by the induction of vessel normalization, particularly by increasing their pericyte coverage.

In this context, the aim of this study is to understand and characterize the role of sVEGFR-1 and sVEGFR-2 by identifying their potential implication in interactions between EC and mural cells. Through a multidisciplinary approach, we provide evidences that these soluble VEGF receptors promote mural cell migration through a paracrine mechanism involving interplay in EC between VEGF/VEGFR-2 and sphingosine-1-phosphate type-1 (S1P)/S1P1 pathways that leads to endothelial nitric oxyde synthase (eNOS) activation. This new paradigm is supported by the finding that sVEGFR-1 and -2: 1) induce an eNOS-dependent outgrowth of a mural cell network in an ex vivo model of angiogenesis, 2) increase the mural cell coverage of neovessels in vivo, 3) promote mural cell migration towards EC, 4) stimulate endothelial S1P1 overproduction and eNOS activation that promote the migration and the recruitment of neighboring mural cells.

These findings provide new insights into mechanisms regulating physiological and pathological angiogenesis and vessel stabilization.

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Growth factors are differentially expressed in rat mesenchymal and neural stem cells upon stimulation with brain and spinal cord extracts from amyotrophic lateral sclerosis-affected rats

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Introduction: Stem cells transplantation is an emerging therapeutical approach for amyotrophic lateral sclerosis (ALS). ALS is a fatal neurodegenerative disease characterized by loss of motoneurons and neuroinflammation. The challenge of cell therapy is to replace dying neurons and to provide support to remaining cells faced with a "bad neighborhood". This can be achieved, at least partially, through beneficial growth factors delivery by transplanted cells.

Aim: Here, we provide evidences that mesenchymal stem cells (MSC) and neural stem cells (NSC) are able to produce growth factors in response to protein extracts from rat ALS brain and spinal cord.

Material and Methods: Symptomatic mutant SOD1-linked ALS rats together with age-matched wild-type animals were euthanized. Brain cortex and lumbar spinal cord were removed and homogenized to prepare the protein extract supernatant. Rat MSC and NSC were incubated overnight (16 hours) with protein extracts and mRNA expression was measured by real-time PCR for the following growth factors: hepatic growth factor (HGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF).

Results: 1) Rat MSC cultured in supernatant derived from ALS extracts significantly increased BDNF, NGF and VEGF mRNA compared to supernatant from WT CNS while FGF-2 mRNA was drastically downregulated. 2) Rat NSC cultured in supernatant derived from ALS extracts significantly increased the production of BDNF, FGF-2, GDNF and NGF mRNA while no difference for VEGF or HGF mRNA could be found.

Conclusion: MSC and NSC exhibit different response to ALS nervous system derived protein extracts. Therefore, choosing of the most suitable cell population for particular application can improve efficiency of cell transplantation therapy.

We thank Emmanuel Hermans and Sabrina Schäfer for providing rat MSC.

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Survival fraction and gene expression pattern in human lung carcinoma A549 cells after X-rays and alpha particle irradiations

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Radiotherapy has been in constant progress for the past century but few studies have been performed concerning hadrontherapy. Hadrontherapy is a particular type of radiotherapy making use of beams of heavy particles called hadrons instead of X-rays. Hadrons are heavy fundamental particles such as protons or neutrons that participate in strong nuclear interactions. In this work, we compared the effects of irradiations using X-rays or heavy charged particles (alpha particles) on survival fraction and gene expression in a human lung carcinoma cell line (A549). The X-rays radiations were carried out using a cobalt irradiator (250 kV) whereas a particle accelerator was used to produce the alpha particle beam (100 keV/_6;m) necessary for the hadrontherapy. For the latter, cells were irradiated vertically in an irradiation chamber with a monoenergetic beam homogeneous over a ~0.5cm² surface. Quantitative reverse transcription-polymerase chain reaction results showed that mRNA overexpression of proinflammatory cytokines, such as Tumor Necrosis Factor=485;-alpha, Interleukin-6 and Interleukin-8, was higher when A549 cells were irradiated with alpha particles than with X-rays. This is consistent with a Relative Biological Efficiency (RBE) of alpha particles that can be 5 fold higher than X-rays RBE. Interestingly, p21 mRNA was dose dependently overexpressed by both types of irradiations. The overexpression was higher at short time (2h30) after X-rays irradiations compared to alpha particle irradiations while this profile was reversed 24h after irradiation. Further studies will be performed to complete these expression patterns as well as to obtain survival fraction curves.

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Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1

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The low-density lipoprotein receptor-related protein-1 (LRP-1) is a membrane receptor involved in both endocytic scavenging and signaling functions. LRP-1 cell surface level is controlled by proteolytic shedding which releases its ectodomain. Here we identified two membrane-associated metalloproteinases that shed LRP-1 from human fibrosarcoma HT1080 cells: a disintegrin and metalloproteinase (ADAM)-12 and membrane-type 1 matrix metalloproteinase (MT1-MMP). We further compared the shedding potential of classical fibroblastoid HT1080 cells with a variant featuring an epithelioid phenotype. Although both fibroblastoid and epithelioid variants expressed similar levels of ADAM-12 and MT1-MMP and of their specific tissue inhibitor of metalloproteinases (TIMPs), epithelioid cells, which contained twice more cholesterol than fibroblastoid cells (17.5 ± 3.6 versus 8.4 ± 2.8 µg cholesterol/mg cell protein), shed 4-fold less LRP-1 ectodomain. Moreover, LRP-1 ectodomain shedding was increased by cholesterol depletion in epithelioid cells and impaired by cholesterol overload in fibroblastoid cells. Our data demonstrate that cholesterol may regulate LRP-1 level at the plasma membrane, possibly by controlling encounter with its sheddases.

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Tumor growth is accelerated in low-density lipoprotein receptor related protein 1 knock-in mice

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Objectives: The low-density lipoprotein receptor-related protein 1 (LRP1) is a multifunctional receptor involved in receptor-mediated endocytosis and cell signalling. The intracellular domain of LRP1 (LRP1-ICD) contains a number of signalling motifs, which are relevant for regulation of its function. Analysis of a murine model having knock-in mutations into endogenous LRP1 allows us to evaluate how LRP1 contributes to the regulation of tumor growth and subsequently indentify the motifs and signalling pathways involved.

Methods: An LRP1 knock-in mouse (with a C57BL6 genetic background) carrying an inactivating mutation in the NPxYxxL (NPVYATL -> AAVAATL) motif in the LRP1-ICD was generated by recombinase mediated cassette exchange (RMCE) (Roebroek et al., MCB, 2006). The NPxYxxL domain contains motifs for endocytosis and tyrosine phosphorylation. To evaluate the impact of the LRP1 mutation (present only in the host tissue) on tumor growth in vivo B16-F10 melanoma cells were subcutaneously injected into the flanks of this syngenic mouse model. In a first experimental approach subcutaneous tumors were surgically removed when they reached a diameter of 1 cm. In a second approach tumors were removed after a period of 3 weeks.

Results: Homozygous LRP1 knock-in mice have normal levels of LRP1 gene expression and show no obvious phenotype. Upon subcutaneous injection of melanoma cells tumors developed more rapidly in knock-in animals than in C57BL6 controls. In the first experimental approach 100% of the LRP1 knock-in mice had developed tumors with end point size of 1 cm diameter by day 21, in contrast to only 66% of the control animals. In the second approach, collecting all tumors after 3 weeks, LRP1 knock-in mice developed much larger tumors in comparison with wild-type mice (respectively 0.41 cm3 ± 0.15 vs. 0.03 cm3 ± 0.01; $p<0.05$). Western blot analysis revealed a significant, 1.7-fold increase of VEGF expression in tumors which developed in LRP1 knock-in mice. This observation was confirmed by immunohistochemical evaluation of the tumors, and suggests a possible role for LRP1 in angiogenesis.

Conclusion: Reduced LRP1 functionality due to inactivation of the NPxYxxL motif resulted in increased tumor growth, possibly via regulating angiogenesis. The mechanism or signalling pathway responsible for this accelerated growth needs to be further investigated.

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Multiple myeloma induces myeloid-derived suppressor cell subpopulations with distinct T-cell suppressive activity in the bone marrow

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous mixture of myeloid cells in different maturation stages, induced by growing tumors. In cancer, MDSCs are able to suppress T-cell responses. Recently, we reported two MDSC subsets in mice: Ly6G+ granulocytic PMN-MDSC and Ly6G- monocytic MO-MDSC (Movahedi et al, 2008, Blood, 111:4233-4244). In multiple myeloma patients, the immune function is impaired, at least in part due to DC or T-cell dysfunction. However, the role of MDSCs in immune suppression by multiple myeloma has not been investigated. In this study, we assessed the presence, phenotype and immunosuppressive activity/mechanism of MDSC subsets in two syngeneic and immunocompetent 5TMM mouse models (5T2 and 5T33). In first instance, CD11b+Ly6G- and CD11b+Ly6G+ lineage-committed myeloid subsets were purified from the BM of naïve and 5TMM tumor-bearing mice, and analyzed for T-cell suppressive activity. Both MDSC subsets from 5TMM bone marrow, even at early stages of the disease, were able to suppress antigen-specific T-cell responses at a higher level compared to purified MDSC subsets from normal BM. Ly6G- MDSC-mediated suppression was partially reversed by iNOS inhibition. In contrast, superoxide dismutase and especially catalase enhance NO concentrations, resulting in enhanced T-cell suppression. None of these inhibitors has any impact on the Ly6G+ MDSC-mediated suppression. A further refinement of the BM Ly6G- MDSC population restricted suppressive activity to Ly6C(hi)SSC(lo) monocytes, but not Ly6C(int)SSC(lo) progenitors nor Ly6C(int)SSC(hi) eosinophils. Together, these data reveal MDSCs as a novel immune suppressive strategy employed by multiple myeloma in the bone marrow.

PROGRAM

8H30 - 9H15	Registration and display of posters
9H15 - 9H30	Welcome and introduction : **Agnès Noël** (Liège, Belgium) ***Coordinator of FP7 project (MICROENVIMET)***
MORNING SESSION - Chairpersons : Agnès Noël & Frans Van Roy	
9H30 – 10H15	**David C. Lyden** (New York, USA) "Priming the Soil for the Pre-Metastatic Niche"
10H15 – 11H00	**Achim Krüger** (Munich, Germany) ***Partner of FP7 project (MICROENVIMET)*** "Tumor microenvironment determined by long-range communication via the proteolytic internet"
11H00 – 11H30	Coffee break
11H30 – 12H15	**Jonathan Sleeman** (Mannheim, Germany) ***Coordinator of FP7 project (TuMIC)*** "Lymphangiogenesis and Metastasis"
12H15 – 12H45	**An Hendrix** (UGent) : "Rab27B-mediated vesicle exocytosis regulates invasive tumor growth and metastasis" **Kiavash Movahedi** (VUB) : "Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(High) monocytes"
12H45 – 14H15	Lunch – Poster viewing
AFTERNOON SESSION - Chaipersons : Christine Gilles & Vincent Castronovo	
14H15 – 14H45	**Amber Van Dongen** (KUL) : "Tumor growth is accelerated in low-density lipoprotein receptor related protein 1 knock-in mice" **Charlotte Selvais** (UCL) : "Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1"
14H45 – 15H30	**Sandra McAllister** (Boston, USA) "Acquisition of Malignant Traits as a Result of Systemic Instigation"
15H30 – 16H15	**Vincent Castronovo** (Liège, Belgium) ***Partner of FP7 project (ADAMANT)*** "Tumor stroma: source of biomarker for targeted therapy and imaging"
16H15	Poster Awards and end of the meeting