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ABSTRACT

Meeting abstracts of the 4th international meeting on angiogenesis

Amsterdam, The Netherlands. 2–4 March 2011

Welcome

The Organising Committee welcomes you to the fourth International Meeting on Angiogenesis in Amsterdam, the Netherlands.

The first meeting was held in the Spring of 2000 in Amsterdam to mark the beginning of a joint venture in Vascular Medicine between Leiden University Medical Center (LUMC), the VU University Medical Center (VUmc), and the Gaubius Laboratory TNO Prevention and Health, in Leiden, the Netherlands. Because of the enthusiasm shown by the participants at that meeting as well as during the second meeting in Leiden (2003) and the third meeting in Amsterdam (2007), we have decided to organise the fourth meeting in a row.

We have tried to create an opportunity to bring together investigators in cardiovascular medicine, oncology, and tissue engineering where issues on angiogenesis are closely linked. There is a great need for addressing these links between basic investigators in pathophysiology and clinical investigations and treatment. The meeting is divided into five sessions respectively dealing with:

I. Development and physiological angiogenesis
II. Angiogenesis and regenerative edicine
III. Progenitor cells/stem cells: applications in vascular regenerative medicine
IV. Tumour angiogenesis: from endogenous inhibitors to clinical experience
V. Angiogenesis and arteriogenesis in cardiovascular medicine.

We are pleased that internationally recognised scientists have accepted the invitation to give lectures. Furthermore, we have selected nineteen of the submitted abstracts to be delivered as oral presentations at the appropriate sessions. The other abstracts are displayed during the meeting and will receive special attention during two poster viewings.
Wishing you a pleasant and interesting stay in Amsterdam,
The Organising Committee

Organising Committee
P. Koolwijk
V.W.M. van Hinsbergh
A.W. Griffioen

Scientific Committee
J.W. Jukema
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V.W.M. van Hinsbergh
A.W. Griffioen
A.J.G. Horrevoets

Scientific Program

Wednesday Afternoon, March 2, 2011

12.30–14.00 Registration

14.00–14.15 Opening by **Pieter Koolwijk (Amsterdam)**

Session I: Development and physiological angiogenesis
Chair: Victor van Hinsbergh and Victor Thijssen

14.15–14.45 **Stefan Schulte-Merker (Utrecht)**
Building the lymphatic vascular network from embryonic endothelium

14.45–15.15 **Peter ten Dijke (Leiden)**
TGF-β receptor signaling and tumor angiogenesis

15.15–15.30 Sarah Garrida-Urbani (Geneva)
Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPARα-mediated mechanism

15.30–15.45 Esther A. Kleibeuker (Oxford)
RhoQ as a novel target of Notch has a distinctive role in angiogenesis

15.45–16.15 **Tea break + Poster Viewing**

16.15–16.45 **Hellmut G. Augustin (Heidelberg)**
VEGF- and Angiopoietin-mediated signaling pathways in endothelial cells

16.45–17.00 Roy Heusschen (Amsterdam)
Identification and characterization of novel galectin-9 splice variants in endothelial cells

17.00–18.00 **Pieter Brakman Lecture by Eli Keshet (Jerusalem)**

18.00–20.00 **Welcome Reception and poster viewing**

Thursday Morning, March 3, 2011

Session II: Angiogenesis and regenerative medicine

Chair: Marco Harmsen and Pieter Koolwijk

09.00–09.30 **Roeland Merks (Amsterdam)**
Cell-based, computational modelling of angiogenesis

09.30–10.00 **Jeff Hubbell (Lausanne)**
Engineering matrix binding of angiogenic growth factors

10.00–10.15 Ester M. Weijers (Amsterdam)
Molecular weight fibrinogen variants alter endothelial cell characteristics

10.15–10.30 Shulamit Levenberg (Haifa)
The degree of vascularization in vitro required to enhance in vivo integration of engineered skeletal muscle graft

10.30–11.00 **Coffee break + Poster viewing**

11.00–11.30 **Ralf H Adams (Muenster)**
Angiogenesis: growth of new blood vessels and beyond

11.30–11.45 Christian Freund (Leiden)
Induced pluripotent (9iPS) cell lines from patients as a disease model to study hereditary hemorrhagic telangiectasia (HHT)

11.45–12.00 Evangelia Papadimitriou (Patras)
Cell surface expression of nucleolin is stimulated by $\alpha_v\beta_3$ integrin activation and is required for pleiotrophin-induced cell migration

12.00–12.30 **Frank B.T. Baaijens (Eindhoven)**
Engineering vascularized aligned muscle constructs using mechanical strain

12.30–13.45 **Lunch and poster viewing**

Thursday afternoon, March 3, 2011

Session III: Progenitor cells/stem cells: applications in vascular regenerative medicine

Chair: Wouter Jukema and Anton Horrevoets

13.45–14.15 **Jamie Case (Indianapolis)**
The role of human circulating hematopoietic stem and progenitor cells (CHSPCs) in promoting tumor growth, angiogenesis, and vascular repair

14.15–14.45 **Marianne Verhaar (Utrecht)**
Stem and progenitor cell based therapies for cardiovascular and kidney disease

14.45–15.00 Karoline Lipnik (Vienna)
Human FOXF1 induces sprouting angiogenesis by Notch-2 Dll1 signalling

15.00–15.15 Urszula Florczyk/Jozef Dulak (Krakow)
Nrf2 and heme oxygenase-1 affect endothelial progenitor cells

15.15–15.45 **Tea break + Poster viewing**

15.45–16.15 **Joyce Bischoff (Boston)**
Building vascular networks with human endothelial and mesenchymal progenitor cells.

16.15–16.30 Julie Halkein/Sebastien Tabruyn (Liege)
MiR-146a: a new angiostatic miRNA with tumor-suppressive properties.

16.30–16.45 Guido Krenning (Groningen)
MiR-15b inhibits endothelial-mesenchymal transdifferentiation and cardiac fibrosis by targeting Ras signaling.

16.45–17.15 **Victor van Hinsbergh (Amsterdam)**
Contribution of chronic hypoxia and endothelial colony-forming cells to angiogenesis

17.15–21.30 **Poster presentation**

18.30–21.30 **Congress diner: Indonesian buffet**

Friday Morning, March 4, 2011

Session IV: Tumor angiogenesis: from endogenous inhibitors to clinical experience

Chair: Henk Verheul and Arjan Griffioen

09.00–09.30 **Arjan Griffioen (Amsterdam)**
Novel mechanisms of angiogenesis inhibition and targeting of tumor endothelium

09.30–10.00 **Dave O. Bates (Bristol)**
Manipulating VEGF splicing to be anti-angiogenic

10.00–10.15 Peter Timmerman (Lelystad)
Synthetic VEGF-mimic: application for anti-angiogenic vaccine therapy

10.15–10.30 Elisabeth JM Huibers (Uppsala)
Vaccination against the extra domain-B of fibronectin as a novel tumor therapy

10.30–11.00 **Coffee break + Poster viewing**

11.00–11.30 **Napoleone Ferrara (San Francisco)**
To be announced

11.30–11.45 Ji-Liang Li (Oxford)
DLL4-Notch signaling mediates tumour resistance to anti-VEGF therapy in vivo

11.45–12.00 Patrycja Nowak-Sliwinska (Lausanne)
Photodynamic angio-occlusion combined with anti-angiogenic tyrosine kinase inhibitors in the treatment of wet age-related macular degeneration

12.00–12.30 **Reinier Schlingemann (Amsterdam)**
What can we learn from the success of anti-angiogenesis therapy in eye diseases?

12.30–13.45 **Lunch and poster presentation**

Friday Afternoon, March 4, 2011

Session V **Angiogenesis and arteriogenesis in cardiovascular medicine**

Chair: Niels van Royen and Paul Quax

13.45–14.15 **Jean-Sébastian Silvestre (Paris)**

Post-ischemic angiogenesis: molecular and cellular mechanisms and therapeutic perspectives

14.15–14.30 Elisabeth Deindl (Munich)

New tasks for platelet receptors GPIbα and GPIIb in arteriogenesis

14.30–14.45 Sofie Struyf (Leuven)

CXCR3 mediates the angiostatic and chemotactic activities of the CXC chemokine CXCL4L1

14.45–15.15 **James E. Faber (Chapel Hill)**

The native collateral circulation: genetic and environmental determinants

15.15–15.45 **Tea break + Poster viewing**

15.45–16.00 Anja van der Laan

Galectin-2 expression is dependent on the rs7291467 polymorphism and acts as an inhibitor of arteriogenesis

16.00–16.15 Richard D. Starke

Endothelial Von Willebrand Factor regulates angiogenesis

16.15–16.45 **Paul Quax (Leiden)**

Inflammation and immune modulation in arteriogenesis

16.45–17.00 **Closure by Pieter Koolwijk (Amsterdam)**

Abstracts invited speakers
(in alphabetic order of presenting author)

O-1 (Invited speaker)

Angiogenesis: growth of new blood vessels and beyond

Ralf H. Adams[1] and colleagues

[1]Max Planck Institute for Molecular Biomedicine, Department Tissue Morphogenesis, and University of Muenster, Faculty of Medicine, Muenster, Germany

Angiogenesis is the main process mediating the expansion of the blood vessel network during development, tissue regeneration, or in pathological conditions such as cancer. The formation of new endothelial sprouts, a key step in the angiogenic growth program, involves the selection of endothelial tip cells, which lack a lumen, are highly motile, extend numerous filopodia, and lead new sprouts. Angiogenic sprouting is induced by tissue-derived, pro-angiogenic signals such as vascular endothelial growth factor (VEGF), which activates and triggers signaling by cognate receptor tyrosine kinases in the endothelium. However, this response is strongly modulated by intrinsic signaling interactions between endothelial cells (ECs). For example, expression of the ligand Delta-like 4 (Dll4) in tip cells activates Notch receptors in adjacent (stalk) ECs and is thought to downregulate VEGF receptor expression in these cells. Thus, the tip cell phenotype is suppressed in stalk cells and a balance between sprouting and the necessary preservation of existing endothelial tubes is established.

Our work has shown that sprouting is also controlled by a second Notch ligand, Jagged1, which is a potent pro-angiogenic regulator with the opposite role as Dll4, which suggests that the balance between two Notch ligands with opposing roles controls physiological and perhaps also pathological angiogenesis. Our present work addresses the critical link between VEGF and Notch signaling. We discovered that, very much in contrast to current models, Notch controls VEGFR2 only moderately, whereas VEGFR3 is strongly regulated. Moreover, blocking of Notch enables angiogenic growth even in mutant animals lacking endothelial VEGFR2 expression.

We also found that endothelial sprouting and proliferation extension depend on VEGF receptor endocytosis. Ephrin-B2, a ligand for Eph family receptor tyrosine kinases, is required for endothelial cell motility, VEGF receptor endocytosis, and the activation of downstream signal transduction cascades. More recently, we have identified Disabled 2, a clathrin-associated sorting protein, and the cell polarity protein PAR-3 as novel interaction partners of ephrin-B2 and VEGF receptors. We show that VEGFR internalization and the activation of downstream signal transduction cascades are compromised in the absence of Dab2 or PAR-3. These results establish that regional VEGF receptor endocytosis, which is controlled by a complex containing Dab2, PAR-3, and ephrin-B2, plays a key role in the spatial organization of angiogenic growth.

Acknowledgement. This study is supported by the Max Planck Society and the German Research Foundation.

O-2 (Invited speaker)

VEGF- and angiopoietin-mediated signaling pathways in endothelial cells

Hellmut G. Augustin

Vascular Biology, Medical Faculty Mannheim, Heidelberg University (CBTM), and German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Germany

The VEGFs and the angiopoietins act coordinately to control sprouting angiogenesis (VEGF) and vascular differentiation and maturation (angiopoietins). Their signaling pathways have been analyzed in great detail. Yet, key unanswered questions remain unresolved. For example, VEGF exerts distinctly different functions (permeability, mitogenicity, and survival factor function), but it has not been fully clarified how these signals are differentially transduced in endothelial cells. Clearly, signaling through the two VEGF receptors is not sufficient to account for the complexity of VEGF functions. Likewise, genetic loss-of-function experiments have unambiguously established the neuropilins as co-receptors of VEGF signaling. Yet, a detailed functional analysis of neuropilin function on the cellular level is largely missing. VEGF signaling acts coordinately with angiopoietin signaling. While this is believed to contribute to controlling the therapeutic window and efficacy of anti-VEGF therapies, angiopoietin signaling in endothelial cells in relation to cellular function continues to be poorly understood. Angiopoietin-1 (Ang-1) is expressed by multiple cell types and acts in a paracrine manner as agonistic ligand of Tie2, thereby controlling vascular differentiation, pericyte recruitment, and endothelial quiescence. In turn, genetic experiments have identified Ang-2 as endothelial cell produced, autocrine-acting antagonistic ligand of Tie2 that interferes negatively with Ang-1/Tie2 signaling to control vessel destabilization and responsiveness to exogenous cytokines. Yet, Ang-2 has under certain conditions also been shown to exert direct pro-angiogenic functions, and Ang-2 is heavily explored as a candidate molecule for anti-angiogenic drug development. Mechanistically, the analysis of agonistic vs. antagonistic functions of Ang-2 is completely unresolved. This presentation will discuss recent experiments aimed at analyzing VEGF and angiopoietin signaling in relation to endothelial cell function.

O-3 (Invited speaker)

Engineering vascularized aligned muscle constructs using mechanical strain

Van der Schaft DWJ, van Spreeuwel ACC, van Assen HC, Duits R, Baaijens FPT

Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

Introduction. Skeletal muscle tissue engineering holds promise for the treatment of patients with traumatic injury. We are currently able to make small three-dimensional (3D) muscle tissues with aligned muscle fibers. In order to advance toward in vivo application, which requires the growth of larger tissue constructs, vascularization is required. We hypothesize that by using co-cultures of muscle and endothelial cells, merely mechanical cues are sufficient to generate a properly aligned vascularized muscle.
Methods. C2C12 myoblasts and H5V endothelial cells were cultured in a 3D hydrogel culture system with or without anchoring points. After one week, the constructs were fixated, and a whole mount staining was done with antibodies against CD31 and alpha-actinin. The constructs were analyzed using confocal microscopy, and alignment of muscle fibers and vascular structures was investigated using Mathematica (Wolfram Research, Champaign, IL). VEGFR-2 blocking antibody was used in some experiments.
Results. Endothelial cells in 3D-anchored constructs align in the direction of strain, but without the presence of muscle fibers do not form tubular structures. When muscle cells are co-cultured with endothelial cells, both the muscle fibers and endothelial cells align in the direction of strain induced by the anchoring points. When cultured without anchoring points, no alignment was observed. Moreover, in the co-cultured constructs, the endothelial cells form vascular structures dependent on the presence of the muscle cells. In a pilot experiment, we tested whether VEGF, produced by the muscle cells, was the factor essential for the organization of endothelial cells in tubular structures, which indeed seemed to be the case.
Conclusions. Strain induced by anchoring of 3D tissue constructs induced an alignment of both muscle fibers and endothelial cells. Moreover, in the constrained co-culture tissue samples, the endothelial cells organized into tubular structures, whereas this was not the case in the unconstrained situation.

O-4 (Invited speaker)

Manipulating VEGF splicing to be anti-angiogenic

David O. Bates

Microvascular Research Laboratories, School of Physiology and Pharmacology, Preclinical Veterinary Sciences Building, University of Bristol, Southwell Street, Bristol BS2 8EJ

Anti-angiogenic VEGF isoforms, generated from differential splicing of exon 8, are widely expressed in normal human tissues but down-regulated in cancers and other pathologies associated with abnormal angiogenesis (cancer, diabetic retinopathy, retinal vein occlusion, Denys Drash syndrome, (DDS) and pre-eclampsia). Therapeutically, administration of recombinant $VEGF_{165}b$ inhibits mouse models of prostate and colorectal carcinoma, neuroblastoma and metastatic melanoma. It has recently been demonstrated that the reduction in $VEGF_{165}b$ expression in DDS is a result of the reduced repressor activity of WT1 on the SRPK1 promoter, resulting in over-expression of SRPK1 and increased proximal splicing by nuclear localization of ASF/SF2 after its phosphorylation by SRPK1. These splicing factors and their regulatory molecules alter the splice site selection, such that cells can switch from the anti-angiogenic $VEGF_{xxx}b$ isoforms to the pro-angiogenic $VEGF_{xxx}$ isoforms. Inhibitors of these molecules can inhibit angiogenesis in the eye, and splice site selection in cancer cells, and SRPK1 downregulation blocks tumour growth. Other regulators of VEGF splicing, including polyketides such as borrelidin derivatives, and the T-cell intracellular antigen 1 (TIA-1) are also potential targets in oncology, and we have shown that both these compounds and their targets regulate angiogenesis through modulating splicing, not only of VEGF, but also of other components of the VEGF pathway such as VEGF receptors. In summary, alternative splicing is a key component of VEGF biology, overlooked by the vast majority of publications in the field, and these findings require a radical revision of our understanding of VEGF contribution to angiogenesis in health and disease.

O-5 (Invited speaker)

Building Vascular Networks with Human Endothelial and Mesenchymal Progenitor Cells

Joyce Bischoff

Vascular Biology Program and Department of Surgery, Children's Hospital, Boston, MA, USA

The fields of regenerative medicine and tissue engineering face the challenge of rapidly implementing a blood vessel network within damaged tissues or bio-engineered implants to facilitate oxygen, nutrient, biochemical, and waste exchange. We and others have built vascular networks in various in vivo models using human endothelial and mesenchymal progenitor cells (EPCs and MPCs, respectively) suspended as single cells in an extracellular matrix (ECM) and injected or implanted subcutaneously in immune-deficient mice. Specifically, we demonstrated the de novo formation of microvascular networks within 7 days after implantation of human EPCs from adult blood or umbilical cord blood with human smooth muscle cells (1) or with adult bone marrow MPCs (2). This showed that a functional vessel network can be formed rapidly, from injectable constituents, using clinically accessible adult cells that have been expanded ex vivo (2). In all the studies described above, blood vessels were organized with perivascular cells closely surrounding EPC-lined lumens. These studies employed Matrigel, a proprietary substance whose complete composition, including trace angiogenic factors, is variable and not publicly known (3), and since it is derived from a murine tumor, its translational potential is limited. Therefore, we investigated vascularization in ECMs whose composition is fully defined, which are mechanically and chemically tunable, and therefore may have potential for clinical translation. We showed that EPCs and MPCs formed perfused blood vessels when suspended and injected in the ECM proteins type I collagen and fibrin, as well as PuraMatrix, a hydrogel-forming synthetic peptide (4). Our on-going studies are focused on 1) onset of perfusion when using these different matrices, 2) direct visualization of anastomoses between the nascent vessels built from human EPCs and MPCs and host vessels, and 3) building vessels with human EPCs and MPCs in ischemic hind limb.

1. Melero-Martin, J.M., Khan, Z.A., Picard, A., Wu, X., Paruchuri, S., and Bischoff, J. 2007. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. Blood 109:4761–4768.

2. Melero-Martin, J.M., De Obaldia, M.E., Kang, S.Y., Khan, Z.A., Yuan, L., Oettgen, P., and Bischoff, J. 2008. Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. Circ Res 103:194–202.

3. BD-Biosciences. 2008. Guidelines for Use: BD Matrigel Basement Membrane Matrix Phenol Red Free.

4. Allen, P., Melero-Martin, J., and Bischoff, J. 2011. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med.

O-6 (Invited speaker)

The role of human circulating hematopoietic stem and progenitor cells (CHSPCs) in promoting tumor growth, angiogenesis, and vascular repair

Julie Mund[1] and Jamie Case[1]

[1]Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University Melvin and Bren Simon Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana, USA

Angiogenesis and neovascularization are increasingly recognized as having an important role in a wide array of diseases. Angiogenesis has been shown to be crucial for tumor growth, and novel anti-angiogenic therapies are currently being tested as a means of slowing or preventing growth of tumors. In the past decade, researchers have gained important insights on the role of bone marrow (BM)-derived cells in adult neovascularization.

Recently, flow cytometry is being utilized to characterize and detect circulating blood cell subsets that comprise the hematopoietic and endothelial progenitor cell (EPC) pool in human peripheral blood (PB), the presence of which is increasingly being used as a biomarker for tumor angiogenesis. EPCs and other BM-derived circulating angiogenic cells have been proposed as possible targets to restrict vessel growth in tumor pathology or as a possible mechanism for vascular regeneration and repair.

Utilizing a novel protocol for polychromatic flow cytometry (PFC) data acquisition and analysis, we have identified a distinct population of circulating hematopoietic stem and progenitor cells (CHSPCs, $CD14^{-}glyA^{-}ViViD^{-}CD31^{+}CD34^{bright}CD45^{dim}AC133^{+}$ cells), which dramatically facilitates tumor growth in a melanoma (i.e. C32) xenograft model. Intravenous injection of CHSPCs in mice harboring melanoma xenografts results in a statistically significant increase in tumor growth overtime when compared to controls (i.e. PBS, $CD34^{+}$ cells, $p<0.001$). In addition, excised tumors from the mice injected with the CHSPCs were significantly larger than tumors from the mice that received vehicle control (PBS) or $CD34^{+}$ cells ($p<0.001$). Consistent with their biological function, it is not surprising then that increased concentrations of CHSPCs correlate with risk for tumor recurrence and patient responsiveness to anti-angiogenic therapies.

Further studies are now required to enable us to determine the specific function of these CHSPCs in tumor angiogenesis and vascular repair to demonstrate that they either home to sites of new blood vessel formation, and facilitate either arteriogenesis or angiogenesis by direct integration into the emerging endothelium or paracrine stimulation of existing vessel wall-derived cells. By gaining a detailed understanding of CHSPC function, the validity of the CHSPC as a new therapeutic target, or tool for vascular regeneration, can be established.

Acknowledgement. This study is supported by the American Cancer Society (IRG-84-002-25) and the Showalter Trust Foundation, USA (ERA31948).

O-7 (Invited speaker)

Homeostatic functions of VEGF in adult organs

Eli Keshet

Dept of Developmental Biology & Cancer Research, the Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Apart from its indispensible roles in developmental vasculogenesis and angiogenesis and its function as a vascular permeability factor, VEGF also plays multiple homeostatic functions in adult organs. The latter, we show, includes adjusting microvascular densities to constant changes in oxygen supply and demand and in maintaining endothelial cell fenestrations.

To uncover additional VEGF functions, we employ conditional transgenic systems designed for inducing a reversible VEGF-loss-of-function in selected organs. In the brain, VEGF was shown to act, not only as an angiogenic- and neurogenic factor, but also as a modulator of neuronal plasticity and cognitive function. VEGF-induced expansion of the hippocampus microvasculature on its own was sufficient to augment basal level of adult neurogenesis, thus providing further support to the general concept of a vascular stem cell niche. Similarly, VEGF induction in the adult liver induced full-blown liver haematopoiesis through re-call and entrapment of adult BM hematopoietic stem cells and creating a proper niche for extra-medullary haematopoiesis.

Switchable VEGF systems are also being used for gaining further insights on VEGF-promoted cell-assisted neovascularisation. We have previously shown that VEGF acts to recruit circulating monocytes and retains them around activated vessels, thereby allowing their functioning as paracrine angiogenic accessory cells. We now show that VEGF also instructs the re-programming of recruited monocytes in a way enhancing their elaborated pro-angiogenic and pro-arteriogenic activities.

O-8 (Invited speaker)

The native collateral circulation: Genetic and environmental determinants

James Faber, Hua Zhang, Jennifer Lucitti, Scott Moore, Robert Sealock, Shiliang Wang, Dan Chalothorn[2], Xuming Dai[3], William McFadden, Pranay Prabhakar, Jeffrey Morrison, Sadana Rangarao and Matthew Waters

[1]Departments of Physiology and [3]Medicine, University of North Carolina, Chapel Hill, NC, USA; [2]Regeneron Pharmaceuticals Inc, Tarrytown, NY

Native (pre-existing) collaterals are rare arteriole anastomoses that interconnect two adjacent arterial trees, thus serving as endogenous bypass vessels. Despite their importance, nothing is known about when or how these vessels form. Furthermore, recent evidence indicates that the density and diameter of collaterals in tissues of healthy mice and humans evidence remarkable variation from as yet unknown mechanisms. This presentation will summarize evidence in mice for the following: The collateral circulation develops late embryonically and undergoes maturation early postnatally. Genetic polymorphisms exert a profound effect on both processes, and on collateral remodeling in obstructive disease. This variation results in equally wide variation in the severity of tissue injury in models of stroke and peripheral artery disease. Several genes impacting collateral formation and remodeling have been identified.

In addition, genome-wide mapping studies have identified novel genetic loci and candidate genes linked to variation in collateral abundance. Besides genetic variation, environmental factors, e.g, aging and endothelial dysfunction and possibly other cardiovascular risk factors and disease, cause collateral rarefaction. It is anticipated that identification of genetic polymorphisms and environmental factors affecting the native collateral circulation will lead to an understanding of the pathways controlling its formation and persistence, identify mechanisms underlying its wide variation, and possibly provide a means to predict risk-severity for ischemic injury should obstructive disease develop. Such knowledge may also assist in clinical decision making and stratification of patients in clinical trials of collaterogenic treatments.

This study was supported by National Institutes of Health grants HL-062584 and HL-090655.

O-9 (Invited speaker)

To be announced

Napoleone Ferrara

O-10 (Invited speaker)

Novel mechanisms of angiogenesis inhibition and targeting of tumor endothelium

Arjan W. Griffioen and Judy R. van Beijnum

Angiogenesis Laboratory, Dept. of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.

Most current clinically used angiostatic drugs are targeting tumor-derived growth factors or their receptors. As this approach will induce resistance, it seems to be more critical to target tumor endothelial cells directly, because endothelial cells are genetically stable and are not expected to mutate into resistant variants. Key to the design of such strategies is the discovery of target molecules on tumor endothelium. Since angiogenesis is not limited to pathologies, careful evaluation of putative therapeutic targets is warranted to prevent side effects associated with impaired physiological angiogenesis. To identify tumor-specific angiogenesis markers, we compared transcriptional profiles of angiogenic endothelial cells isolated from both malignant and non-malignant tissues with that of resting endothelial cells. We identified 17 genes that show specific overexpression in tumor endothelium but not in angiogenic endothelium of normal tissues, creating a therapeutic window for tumor vasculature specific targeting. Antibody targeting of four cell-surface expressed or secreted products inhibited angiogenesis in vitro and in vivo. Therapeutic application of ligands or drugs targeting these markers of tumor angiogenesis is of immediate interest. In addition, such ligands can be used for diagnostic PET or MRI imaging of angiogenesis for early detection, monitoring of therapy response or prediction of responsiveness. Our results demonstrate the utility of the identification and subsequent targeting of specific tumor endothelial markers for anticancer therapy.

O-11 (Invited speaker)

Engineering matrix binding of angiogenic growth factors

Mikaël Martino, Mayumi Mochizuki, Melody A. Swartz, and Jeffrey A. Hubbell

Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

In natural situations, angiogenic growth factors such as vascular endothelial growth factor (VEGF) are present in a matrix-bound form, yet therapeutic use of such growth factors has focused on application in soluble form. To explore matrix immobilization of angiogenic growth factors, we have explored two approaches, both in the context of fibrin as a surgically relevant matrix: enzymatic conjugation of variant forms of the growth factors, and complexation with recombinant variants of fibronectin.

Angiogenic growth factors such as VEGF were engineered so as to contain a substrate domain for the coagulation transglutaminase factor XIIIa, modelled after the N terminus of alpha2 plasmin inhibitor. To provide a release mechanism, an enzymatic substrate was included in the growth factor variant between the transglutaminase substrate and the growth factor domain, taken as either a plasmin substrate domain or a matrix metalloproteinase domain. Thus, we have explored the activity of tripartite fusion proteins for inducing angiogenesis. We have most thoroughly studied the variant of VEGF-A, and we have demonstrated that the variant form of VEGF-A induces more angiogenesis than the wild type and induces a less hyperpermeable phenotype, both in the chick chorioallantoic membrane and in mouse skin.

To explore noncovalent immobilization upon matrices, we have engineered a fibrin-binding domain of fibronectin, containing the 12th–14th type III repeat (which was known to bind VEGF-A). In studies of the 12th–14th type III repeat, we determined that the growth factor-binding activity of this domain was rather promiscuous, binding to VEGF-A, VEGF-C, PDGF-AA, PDGF-BB and PDGF-AB, for example, in addition to a wide number of other growth factors. Incorporation of this domain into fibrin, also through transglutaminase activity, provides a powerful and generalizable method to retain such growth factors into surgical matrices.

Acknowledgement. This study is supported by the FP7 Large Scale Integrated Project AngioScaff.

O-12 (Invited speaker)

Cell-based computer simulations help unravel the mechanisms of angiogenesis

Roeland M. H. Merks[1,2].

[1]Life Science Group, CWI, Science Park 123, 1098 XG Amsterdam. [2]Netherlands Consortium for Systems Biology/Netherlands Institute for Systems Biology (NCSB-NISB), Amsterdam.

Angiogenesis is a topic of intensive experimental investigation, so its phenomenology and the molecular signals contributing to it have been well characterized. To find the "controlling handles" of angiogenesis, a typical experimental approach knocks out or knocks in genes involved in various aspects of angiogenesis (e.g. signalling, ECM proteolysis, cell migration) and observes the consequences on angiogenesis and on the activities of other cellular components. Such genetic dissection is an invaluable approach, but we believe it is only a first step because it only maps out the components *involved* in angiogenesis.

During the next step, we need to find out how these components fit together to build a blood vessel. This insight is required to rationally select new targets for stimulating or inhibiting angiogenesis. Because of the many counteracting influences and feedback loops, and the biophysics involved, this problem is far too complex for intuition and pen-and-paper diagrams alone. Cell-based computer simulation models help. Such models of angiogenesis take as input a set experimentally identified components and their interactions, e.g. the endothelial cell's chemotactic response to VEGF and its response to cell–cell contact. From this input data, the model predicts the (collective) behaviour of a cluster of endothelial cells, e.g. whether a sprout will form or not, how rapidly and with what morphology.

Thus, reconstructing angiogenesis in a computer model suggests plausible mechanistic roles for each of the components and identifies the "missing links" in our experimental understanding of angiogenesis suggesting next experimental steps. Parameter studies rationally identify the key controlling factors of the system that further experimental research can be focused on.

I will illustrate this systems biology approach to angiogenesis with examples. Our simulation models have shown that the elongated shape of endothelial cells is key to correct spatiotemporal in silico replication of vascular network growth. We also identified a new potential mechanism for angiogenic sprouting driven by random cell motility. Finally, I will discuss more recent work focusing on the extracellular matrix.

Acknowledgement: This work was financed by the Netherlands Consortium for Systems Biology (NCSB) which is part of the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research

O-13 (Invited speaker)

Inflammation and immune modulation in arteriogenesis

Paul Quax, Alwine Hellingman, Leonard Seghers, Teun Bastiaansen, Jacco Karper, Mark Ewing, Erna Peters, Margreet de Vries, Sacha Geutskens, Yael Nossent, Wouter Jukema, Jaap Hamming and Hajo van Bockel

Dept of Surgery, Leiden University Medical Center and Einthoven Laboratory for Experimental Vascular Medicine, LUMC, Leiden, The Netherlands

Angiogenesis and arteriogenesis are the two major processes contributing to post-ischemic neovascularisation. After the initial studies demonstrating a prominent role for monocytes in the regulation of arteriogenesis/collateral formation, many groups have focussed on the regulation of the inflammatory cells and processes in arteriogenesis. We and others have studied how attraction and activation of monocytes, e.g. by MCP-1, contributes to the induction of arteriogenesis. Furthermore, we demonstrate how collateral formation is hampered in situations where monocyte activation is disturbed. This includes situations in which monocytes do not receive the proper pre-treatment in a cell therapeutic approach, in which monocytes can not be stimulated properly as observed in PAR2 -/- mice, or in which monocytes are overstimulated as observed in mice deficient for RP105, a prominent accessory molecule for TLR4.

Next to inflammatory processes and factors leading to monocyte activation, other immune cells like NK cells, T cells, and regulatory T cells, play a crucial role in arteriogenesis. We previously showed a prominent role for NK cells and now demonstrate a key role for the NKC locus containing a range of NK cell activating and inhibitory receptors in the arteriogenic response. Furthermore, we demonstrate that for arteriogenesis, a proper balance in T-cell activation, including the T-Regs the activation, is required.

In conclusion, a delicate regulation of the inflammatory and immune modulatory processes is essential for the induction of the post-ischemic neovascularisation processes of angiogenesis and arteriogenesis.

O-14 (Invited speaker)

What can we learn from the success of anti-angiogenesis therapy in eye diseases?

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Angiogenesis and vascular leakage in the eye are the main pathogenic causes of visual loss and blindness in the Western world. Antagonists of vascular endothelial growth factor-A (VEGF) have recently been shown to have a strikingly beneficial effect in ocular conditions associated with these processes and have therefore created a landslide in their treatment and clinical outcome. Angiogenesis can occur as a final common pathway in the course of a large variety of pathological conditions in all anatomical compartments of the eye. However, angiogenesis and the associated haemorrhages are usually the visible signs of wound-healing-like responses that will ultimately cause fibrosis and scarring and obscure the normally avascular transparent tissues such as the cornea and vitreous.

There are two main forms of ocular angiogenesis: Pre-retinal angiogenesis occurs as the final common pathway in proliferative retinopathies, such as diabetic retinopathy, that are associated with capillary non-perfusion and ischemia of the neuroretina. Hypoxia-induced VEGF produced in ischemic retinal areas incites growth of new vessels along the interface of the vitreous and the optic disc and retina, finally forming large contractile fibrovascular membranes within the vitreous cavity. Sub-retinal (or choroidal) neovascularization (CNV) also occurs as a final common pathway of various pathological conditions, among which age-related macular degeneration, which affect the layers of the eye beneath the neuroretina: the retinal pigment epithelium (RPE), Bruch's membrane and the choroid. Typically, CNV is a wound-healing response that occurs only when an anatomical discontinuation (break) of Bruch's membrane is present, in combination with a driving force such as inflammation, hypoxia and oxidative stress.

Studies in ocular angiogenesis have shown that human angiogenesis can be effectively inhibited by anti-VEGF agents and have identified the angio-fibrotic switch, the transition from angiogenesis to fibrosis in wound-healing responses, which appears to be driven by a shift in the balance of VEGF and connective tissue growth factor in the vitreous. Furthermore, studies in the normal eye have first identified the physiological role of VEGF in the maintenance of fenestrated vascular beds adjacent to epithelial cells.

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O-15 (Invited speaker)

Building the lymphatic vascular network from embryonic endothelium

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The lymphatic vasculature serves key functions in fluid homeostasis within the body. It absorbs fluids and macromolecules from the interstitium, it takes up dietary lipids from the small intestine, and it provides a trafficking route for cells of the immune system. The architecture of the lymphatic vessel system is quite different from the one of the blood vasculature: it is blind ending with uni-directional flow, and its endothelium is specialized to allow uptake of macromolecules and other substances. Perturbations in the function of the lymphatic system can be primary (hereditary) or secondary (acquired through parasite infection or surgery) and lead to disfigurating lymphedema formation.

We have carried out genetic screens to isolate mutants affecting the zebrafish lymphatic vasculature and identified a number of genes that affect this system to varying degrees. One gene that we are particularly interested in is *ccbe1*, a secreted factor that contains a collagen-repeat domain as well as EGF-motifs. Absence of Ccbe1 leads to defects in venous sprouting and to a complete lack of embryonic lymphatics. In collaboration with R Hennekam and M Alders (Amsterdam), we have been able to show that lack of CCBE1 function in humans causes Hennekam Syndrome, a severe congenital disease presenting with lymphedema, mental retardation, and lymphangioectasias. To better understand how Ccbe1 affects lymphangiogenesis, we have also generated a mouse model for this gene and are now in a position to compare the phenotypic effects of *ccbe1*/*Ccbe1* mutants in zebrafish, mouse, and humans. This sheds light not only on the molecular function of Ccbe1 and its effect on endothelial cell behavior, but also allows an evolutionary comparison of lymphatic vasculature formation between mammals and teleosts.

O-16 (Invited speaker)

Post-ischemic angiogenesis: molecular and cellular mechanisms and therapeutic perspectives

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The four principal processes, vasculogenesis, angiogenesis, arteriogenesis, and collateral growth, contribute to tissue repair and remodeling during acute and chronic ischemic vascular diseases and represent the final targets of therapeutic angiogenesis aimed at providing an alternative treatment strategy for patients with lower limb ischemia and coronary artery disease. Inflammation and, in particular, monocyte recruitment might be one of the most important stimuli for initiation of vessel growth in the setting of ischemia. We have recently shown that in a model of murine hindlimb ischemia, both Ly6C(hi) and Ly6C(lo) monocyte circulating levels were increased after femoral artery ligation. CCL2/CCR2 activation enhanced blood Ly6C(hi) and Ly6C(lo) monocyte counts, although the opposite effect was seen in mice with CCL2 or CCR2 deficiency. CX3CL1/CX3CR1 strongly impacted Ly6C(lo) monocyte levels, whereas CCL5/CCR5 had no role. Only CCL2/CCR2 signaling influenced neovascularization. Moreover, adoptive transfer of Ly6C(hi)-but not Ly6C(lo)-monocytes enhanced vessel growth and blood flow recovery. Regulatory T cells (Treg) activation may also modulate the immunoinflammatory response to ischemic injury. Hence, disruption of CD28 pathways or B7-1/2 signaling or anti-CD25 treatment and subsequent Treg deletion significantly enhanced post-ischemic neovascularization. These effects were associated with enhanced accumulation of CD3-positive T cells and Mac-3-positive macrophages in the ischemic leg. Conversely, coadministration of Treg cells and CD28(-/-) splenocytes in Rag1(-/-) mice with hindlimb ischemia abrogated the CD28(-/-) splenocyte-induced activation of the inflammatory response and neovascularization. Finally, therapeutic strategies include transplantation of angiogenic bone marrow-derived mononuclear cells (BMMNC) or gene transfer for systemic or local upregulation of pro-angiogenic proteins. In this line, we have shown that inhibition of prolyl hydroxylase using in vivo administration of SiRNA or BMMNC-based eNOS gene therapy promoted therapeutic neovascularization.

O-17 (Invited speaker)

TGF-β receptor signaling and tumor angiogenesis

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Genetic studies in mice and humans have revealed an important role for TGF-β in vascular development and maintenance. Mice deficient for various TGF-β signaling components develops an embryonic lethality due to vascular defects. In patients, mutations in the TGF-β type I receptor ALK1 or in the accessory TGF-β receptor endoglin are linked to a vascular dysplasia termed Hereditary Hemorrhagic Telangiectasia (HHT).

TGF-β has potent direct effects on endothelial cells and smooth muscle cells. TGF-β produced by endothelial cells can be activated from its latent form upon contact between pericytes or smooth muscle cells with endothelial cells. We have proposed a model in which TGF-β regulates the activation state of the endothelium via two opposing type I receptor/Smad pathways: ALK1 induces Smad1/5 phosphorylation, leading to an increase in endothelial cell (EC) proliferation, migration, and invasion, while ALK5 promotes Smad2/3 activation and inhibits these processes. Consistent with the notion that TGF-β/ALK5 signaling inhibits angiogenes, we observed that an ALK5 kinase inhibitor strongly promoted VEGF-induced angiogenesis. Conversely, we found that targeting ALK1 impairs angiogenesis. Endoglin is predominantly expressed on proliferating ECs in culture and on angiogenic blood vessels in vivo. We found that endoglin is required for efficient TGF-β/ALK1 signaling and potently inhibits TGF-β/ALK5 signaling. Interfering with endoglin function in endothelial cells potently inhibited VEGF-induced angiogenesis. Targeting strategies to inhibit endoglin/ALK1 axis in tumor angiogenesis will be discussed.

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O-18 (Invited speaker)

Contribution of chronic hypoxia and endothelial colony-forming cells to angiogenesis

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Poor tissue perfusion by acute or chronic obstruction of the proximal arteries can cause a tissue response to restore tissue perfusion. The response includes an angiogenic response of vascular cells, in particular the endothelium in the hypoxic tissue; recruitment of circulating bone-marrow-derived cells, which facilitate the angiogenic response; and widening of the proximal vessels to ensure an increase in blood flow. In disease, this response is often inadequate. Analysis of gene expression data of endothelial cells cultured under various oxygen regimens provides information regarding the induction of genes that facilitate angiogenesis and maintenance of the tissue. Major regulators in hypoxia are the transcription factors HIF-1 and HIF-2, which are mainly regulated by the availability and nuclear accumulation of their HIF-1 and HIF-2 subunits, respectively. Both HIF-1 and HIF-2 can contribute to neovascularization in vitro and in vivo. We compared the effects of acute and chronic hypoxia on endothelial cell behavior and gene expression and looked for differences in expression of angiogenesis factors, such as VEGF-A, receptors, and chemokines. Furthermore, hypoxia affects the recruitment of endothelial colony-forming cells and so-called endothelial progenitor cells, which include cells with myeloid characteristics. They are thought to be attracted to the sites of neovascularization and to accelerate the angiogenic response, and their number is reduced in diabetes and myocardial patients. The growth and angiogenic behavior of these cells was further evaluated. The obtained insights may help to improve neovascularization of ischemic tissues and tissue grafts.

O-19 (Invited speaker)

Stem and progenitor cell-based therapies for cardiovascular and kidney disease

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Cardiovascular and kidney diseases are major causes of morbidity and mortality worldwide. Injury to the vascular endothelium and an impaired endothelial repair response are important events in the initiation and progression of both cardiovascular and kidney disease. Therapeutic vascular regeneration may offer potential for reducing disease burden. Vascular repair or regeneration not only relies on resident endothelial cells but also involves BM-derived endothelial progenitor cells. Endothelial progenitor cell-based therapies hold promise for atherosclerotic and kidney disease. Recent and ongoing clinical trials on stem- and progenitor cell therapy in cardiac and peripheral vascular disease will be discussed as well as preclinical studies in renal disease models. Focus will be on the development of optimized progenitor cell-based therapies, which will require important research questions to be addressed, in clinical as well as in preclinical studies.

Abstracts short oral presentations
(in alphabetic order of presenting author)

O-21

RhoQ as a novel target of Notch has a distinctive role in angiogenesis

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Delta Like 4 (Dll4)/Notch signalling plays an important role in the regulation of tumour angiogenesis. However, little is still known about the mechanisms and genes involved in the downstream responses following Dll4/Notch signalling. Cap-analysis gene expression (CAGE) array on Human Umblical Vein Endothelial Cells (HUVEC) was performed to identify novel downstream targets of Dll4/Notch signalling. Among these, RhoQ was selected to investigate its role in angiogenesis. RhoQ is a member of the family of small Rho GTPases and known to be involved in cytoskeleton rearrangements and neurite elongation during both development and regeneration

We examined the expression profile of RhoQ in endothelial cells (HUVECs and Human Microvascular Endothelial Cells (HMEC-1)) and glioblastoma cells (U87), stimulated with Dll4. To inhibit the Notch pathway, we treated the cells with DBZ. We also silenced RhoQ expression with siRNAs to study the role of RhoQ in various models of angiogenesis and observed any phenotypic changes.

We confirmed that RhoQ expression is induced by Dll4/Notch signalling in HUVECs and HMEC-1 cells, and we demonstrated that its expression was significantly reduced when the cells were treated with DBZ. In contrast, we showed that RhoQ expression was not induced by Notch in U87 cells. In HUVECs, silencing RhoQ resulted in disruption of the vascular network formed on top of matrigel. Additionally, loss of RhoQ resulted in slower migration, integrin adhesion, and proliferation. In a more complex angiogenic model, the hangingdrop assay, loss of RhoQ, resulted in excessive sprouting. We observed the same phenotype when the Notch pathway was inhibited. In situ expression profiles in Zebrafish further confirmed that RhoQ was affiliated with blood vessels.

Concluding, RhoQ is a novel downstream target of Notch in HUVECs and potentially plays an important role in the regulation of angiogenesis in vitro and vivo. This information points to RhoQ as a novel potential target for angiogenesis inhibition in cancer therapy.

We would like to thank the FANTOM consortium for assisting us with the CAGE data, CRUK, and MRC for funding.

O-22

New tasks for platelet receptors GPIbα and GPIIb in arteriogenesis

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The role of leukocytes, especially monocytes in collateral artery growth (arteriogenesis), has been well characterized. The role of platelets and their receptors still remain to be evaluated, which was the aim of our study.

Six male C57BL/6 mice underwent right femoral artery ligation (fal) to induce arteriogenesis under generated thrombocytopenia (platelet reduction: 95%) by treatment with anti-GPIbα depleting antibody (50μg/mouse). A different experimental group received a GPIbα inhibiting antibody in the same dosage. Controls were treated with 0.9% saline. Seven male GPIIb ($_{\alpha IIb}$integrin)-deficient mice and their correspondent wild-type controls underwent fal in order to examine the function of the GPIIb/IIIa receptor.

Arteriogenesis was monitored in all groups by Laser Doppler Imaging and quantified as relative perfusion recovery (right/left leg) before, immediately after surgery, until day 7. Poor recovery on day 7 was observed under platelet depletion (0.49±0.04 vs. 0.79±0.04,

p<0.005) as well as GPIbα inhibition only (0.51±0.06 vs. 0.79±0.04, p<0.005). Via intravital microscopy, platelets transiently adherent at the endothelium of collateral arteries were quantified on day 3 using in GPIbα-/- and control mice. Controls showed a significantly increased platelet endothelial interaction on the occluded side (21491±779 vs. sham 13931±1389, p<0.05), whereas in GPIbα-/- mice, this effect was blunted (13435±2378 vs. sham 10095±3070).

Relative perfusion recovery of GPIIb ($_{\alpha IIb}$integrin)-deficient mice was significant better than controls on day 7 (0.75±0.19 vs. 0.53±0.14 p<0.05), day 14 (0.99±0.15 vs. 0.83±0.1 p<0.05), and day 21 (1.12±0.19 vs. 0.97±0.05 p<0.05).

Conclusions: Platelets promote arteriogenesis through interaction with the endothelium via their GPIbα receptor and may initiate important pathways like endothelial cell and leukocyte activation. Inhibiting GPIIb/IIIa, however, promotes arterial growth and might serve as a therapeutic target in patients.

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O-23

Nrf2 and heme oxygenase-1 affect endothelial progenitor cells

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Nrf2 transcription factor regulates expression of anti-oxidant genes, some of which, such as heme oxygenase-1 (HO-1), are also important for blood vessel formation (*Dulak* et al*., Circulation, 2008*). Recently, we showed that inhibition of Nrf2 by HIF-1 attenuates the production of IL-8 in endothelial cells (*Loboda* et al*., Antioxid Redox Signal 2009*). Here, we aimed to examine the role of Nrf2 and HO-1 in angiogenic potential of endothelial progenitor cells (EPCs) and endothelial cells.

In human microvascular endothelial cells (HMEC-1), treatment with VEGF-A, SDF-1α, or IL-8 increases Nrf2 protein level and enhances HO-1 expression. Activation of Nrf2 by sulforaphane stimulates tube formation of HMEC-1 in vitro. Accordingly, inhibition of Nrf2 by siRNA augmented HMEC-1 sensitivity to oxidative stress and attenuated their differentiation on Matrigel. Moreover, EPCs (Sca-1+, VEGFR-2+, $CD45^+$, Ac-LDL-, and *Griffonia simplicifolia*-binding adherent fraction of bone marrow mononuclear cells) isolated from Nrf2 knockout ($Nrf2^{-/-}$) mice were more sensitive to oxidative stress and produced more reactive oxygen species in comparison to $Nrf2^{+/+}$ EPCs. Lack of Nrf2 inhibited proliferation and migration of EPCs upon VEGF or SDF-1α stimulation, respectively. Accordingly, $Nrf2^{-/-}$ EPCs and endothelial cells have decreased angiogenic potential as evidenced by tube formation on Matrigel, spheroid assay, and aortic ring assay. Similar effect has been observed in EPCs derived from HO-1 KO mice. Comparison of the angiogenic transcriptome of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ or $HO\text{-}1^{+/+}$ and $HO\text{-}1^{-/-}$ EPCs under normoxia and hypoxia revealed different regulation of numerous angiogenic genes. Finally, mobilization of EPCs from bone marrow to peripheral blood after hind limb ischemia was attenuated in Nrf2 knockout mice and lack of HO-1 significantly impaired limb revascularization.

In summary, Nrf2 and HO-1 mediate response of endothelial cells and EPCs to angiogenic stimuli. Hence, Nrf2 and HO-1 can affect revascularization under ischemic conditions.

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O-24

Induced pluripotent (iPS) cell lines from patients as a disease model to study hereditary hemorrhagic telangiectasia (HHT)

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Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder caused by mutations in genes of the TGF-β signaling pathway, notably Endoglin (HHT type 1) or ALK-1 (HHT2). HHT is characterized by multiple telangiectasias in the gastrointestinal tract, skin, oral, and nasal mucosa. Patients suffer from profuse nosebleeds leading to chronic anemia. In addition, telangiectasias in the brain, liver, or lungs can be potentially fatal. Affected vessels appear dilated, tortuous, with abnormal direct connections between arteries and veins without connecting capillary beds. The observed phenotype results from severe disruption of vessel wall maturation, as a consequence of loss of pericyte and smooth muscle cell (SMC) coverage. Despite that mouse models of HHT are available, the human model system has not been developed yet. We have recently derived induced pluripotent stem (iPS) cells from patients with HHT. iPS cells closely resemble embryonic stem cells (ESC) and can differentiate into derivatives of the three germ layers in vitro and in vivo. Vascular differentiation of iPS cells can be enhanced in the presence of serum and VEGF. Notably, differentiated EBs readily give rise to the two major vascular cell types, namely vascular smooth muscle cells and endothelial cells. The development of methods for enrichment of both cell types, functional assays for vascular differentiation, heterotypic endothelial-smooth muscle cell interaction is currently ongoing. This will allow us to study the mechanism of HHT disease and to develop novel drugs by utilizing vascular cells derived from iPS cells from HHT patient or healthy individuals.

O-25

Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPARa-mediated mechanism

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Reactive oxygen species, ROS, are regulators of endothelial cell migration, proliferation, and survival events critically involved in angiogenesis. Different isoforms of ROS-generating NOX enzymes

are expressed in the vasculature and provide distinct signaling cues through differential localization and activation. We show that mice deficient in NOX1, but not NOX2 or NOX4, has impaired angiogenesis. NOX1 expression and activity is increased in primary mouse and human endothelial cells upon angiogenic stimulation. NOX1 silencing decreases endothelial cell migration and tube-like structure formation, through the inhibition of PPARα, a regulator of NF-κB. Administration of a novel NOX-specific inhibitor reduced angiogenesis and tumor growth in vivo in a PPARα-dependent manner. In conclusion, vascular NOX1 is a critical mediator of angiogenesis and an attractive target for anti-angiogenic therapies.

O-26

MiR-146a: a new angiostatic miRNA with tumor-suppressive properties

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MiRNAs have emerged as important players in tumor progression and angiogenesis. In the present study, using the 16-kDa N-terminal fragment of human prolactin (16K hPRL) as a model of endogenous angiostatic factor, we indentified miR-146a as a new mediator of angiostatic response. In vitro, 16K hPRL induced up-regulation of miR-146a via a NF-κB-dependent pathway. Functional assays revealed that miR-146a overexpression reduced proliferation and induced apoptosis of endothelial cells without affecting migration and tubulogenesis. Transcriptomic analysis revealed that expression of 389 genes mainly involved in regulation of immune response, proliferation, and apoptosis was significantly affected in endothelial cells overexpressing miR-146a. NRAS was further identified as a new target that could contribute to miR-146a anti-proliferative action. In an ex vivo aortic ring assay, miR-146a overexpression reduced sprout formation. Furthermore, in vivo in a mouse model of age-related macular degeneration, we showed that overexpression of miR-146a decreased pathological neovascularization. Since miRNAs have been shown to be present in blood inside exosomes, we then explored whether miR-146a can be used as circulating biomarker to monitor 16K hPRL angiostatic response. Blood from B16F10 melanoma-bearing mice treated with 16K PRL displayed a significant higher level of circulating miR-146a than the control animals. In vitro, endothelial cells treated with 16K hPRL released exosomes loaded with a higher content of miR-146a. In addition, exosomes from endothelial cells overexpressing miR-146a interfered with B16-F10 melanoma cell proliferation. These results suggest that, in response to angiostatic factors, endothelial cells release miRNA-containing exosomes that affect tumor cell properties. In conclusion, our data present miR-146a as a new angiostatic miRNA produced by endothelial cells with intrinsic effect on the endothelium and acting at distance as a tumor-suppressor.

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O-27

Identification and characterization of novel galectin-9 splice variants in endothelial cells

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Evidence is accumulating that endothelial galectins play an important role in tumor angiogenesis. Recently, we reported altered galectin-9 expression during endothelial cell activation. Here, we further assessed the expression and function of galectin-9 in endothelial cells.

Using cDNA flanking PCR and subsequent cloning, we identified 5 different galectin-9 splice variants in endothelial cells, 2 of which result in truncation of the C-terminal carbohydrate recognition domain, and one of which has not been described previously i.e. galectin-9Δ5/6/10. By real-time PCR analysis, we confirmed the expression of these 5 splice variants in endothelial cells and found that galectin-9Δ5 is the dominant splice variant in endothelial cells, as has been described for most other cell types. Endothelial cell activation resulted in a decreased expression of total galectin-9 levels which was mainly due to a decrease in galectin-9Δ5 expression. The expression of 2 other splice variants, galectin-9Δ5/6 and galectin-9Δ5/6/10, was retained after cell activation. To study the functional consequences of alternative splicing patterns on endothelial cell function, we cloned the 5 endothelial galectin-9 splice variants into expression constructs. We were able to demonstrate efficient transcription/translation of galectin-9 splice variants by real-time PCR and Western blot, respectively, following transfection of cell lines from non-endothelial (HEK293T) and endothelial (HMEC) origin. These data confirmed that exclusion of exon 10 causes a frameshift and premature stopcodon, resulting in the generation of protein that lacks the C-terminal CRD. Next, we assessed the function of gal-9 splice variants in endothelial cells by determining the effects on endothelial cell proliferation, migration, and sprouting. Finally, we explored whether these splice variants have diverging roles in regulating the interaction between endothelial cells and immune cells.

In conclusion, we report that endothelial cells express 5 splice variants of gal-9, one of which has not been described previously, and we show that these splice variants have diverging roles in endothelial cell biology. We hypothesize that interfering with the function of endothelial gal-9 splice variants might be an interesting approach for novel anti-cancer therapies.

O-28

Vaccination against the extra domain-B of fibronectin as a novel tumor therapy

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During physiological angiogenesis (*e.g.* wound healing and proliferation of the endometrium), embryonic development and tumor

angiogenesis certain extra domains of the extracellular matrix molecules fibronectin and tenascin-C are inserted into the parent molecule by alternative splicing leading to the formation of different isoforms. Splice variants of both fibronectin and tenascin-C are often co-expressed specifically around neovasculature and tumor vasculature but have low or no expression in normal adult tissue, rendering them specific targets for anti-tumor therapy.

In our approach, we have targeted the extra domain-B (ED-B), which is a 91 amino acid domain alternatively spliced into fibronectin and highly conserved between species. It is identical in mouse, human, rabbit, dog, monkey, and several other species. To evoke an immune response against the self-antigen ED-B, we used a recombinant fusion protein consisting of a bacterial thioredoxin (TRX) part fused with the extra domain-B (ED-B), termed TRX-EDB. This fusion protein has been injected together with Freund's adjuvant, a strong immunostimulator, into eight-week-old female wild-type C57bl6 mice. Mice were boostered twice in a period of five weeks before they were inoculated subcutaneously with T241 fibrosarcoma cells, a tumor type known to express ED-B. After a tumor growth period of three weeks, animals were killed, and blood and tumors were removed. Nineteen of 20 vaccinated mice responded with production of anti-ED-B antibodies and showed a 70% reduction in tumor size compared to control animals injected with Freund's adjuvant and vehicle, lacking anti-ED-B antibodies. Staining of murine grade III glioma tissue, to see whether the serum from TRX-EDB mice could detect native ED-B, showed an extensive vascular staining pattern compared to normal brain tissue, which is devoid of ED-B. This shows that the anti-ED-B antibodies are able to detect native tissue ED-B. Quantification of tumor necrotic area revealed a tendency toward a greater necrotic area in TRX-EDB injected compared to control animals. Further investigation of the tumors with electron microscopy revealed morphological changes of the tumor vasculature of animals with anti-ED-B antibodies, which was consistent with an immune response toward the tumor vasculature expressing ED-B. Moreover, in tumors from animals with anti-ED-B antibodies, an increased number of infiltrating neutrophils were observed, compared to controls, confirming an immune attack of the vasculature. Furthermore, we detected an increased amount of extravasated fibrinogen, indicative of vascular leakage, in tumors of animals with anti-ED-B antibodies compared to controls.

Therefore, ED-B and possibly other tumor vascular antigens such as the extra domain A of fibronectin or the extra domain C of tenascin-C alone or in combination are interesting candidate targets for treatment of solid tumors.

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O-29

The degree of vascularization in vitro required to enhance in vivo integration of engineered skeletal muscle graft

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Severe traumatic events such as open fractures, burns, and cancer therapy often involve a significant loss of muscle tissue, requiring surgical reconstruction by means of autologous flaps. However, the availability of quality flaps and donor site morbidity often limit their use. Engineered skeletal muscle may provide a relevant alternative for this need. This work describes a first-time analysis, of the degree of in vitro vasculature and muscle organization required to enhance the pace and efficacy of vascularized skeletal muscle graft integration in vivo. While a short culture in vitro was sufficient for graft integration, longer culture periods yielding semi-organized structures significantly improved grafting efficacy. The grafted vessel networks were gradually replaced by host blood vessels with enhanced perfusion. Additionally, upregulation of key angiogenic factors suggests that the graft actively promotes angiogenesis. Transition from satellite cells to mature fibers was indicated by increased gene expression, increased capillary density, and a morphology similar to normal muscle tissue. Due to these findings, we suggest a "relay" mechanism where extended in vitro incubation, which enables the formation of a more structured muscle graft, allows for faster graft integration and more advanced muscle maturation within the host.

O-30

Cell surface expression of nucleolin is stimulated by $\alpha_v\beta_3$ integrin activation and is required for pleiotrophin-induced cell migration

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Pleiotrophin (PTN) is a secreted heparin-binding growth factor with roles in many different processes, such as cell growth and survival, neurite outgrowth, tumour growth and metastasis, as well as endothelial cell migration and angiogenesis. We have previously shown that PTN induces tumour and endothelial cell migration through its receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) that forms a functional complex with $\alpha_v\beta_3$ integrin on the cell surface. Besides RPTPβ/ζ and $\alpha_v\beta_3$, the multifunctional protein nucleolin (NCL) has been also mentioned to be a low affinity cell surface receptor for PTN. NCL acts as a shuffle between cytoplasm and nucleolus, is increased on the surface of angiogenic endothelial cells and binds a variety of ligands that play critical role(s) in tumorigenesis and angiogenesis. We have previously shown that NCL interacts with PTN in endothelial cells and participates in the nuclear translocation of PTN. In the present work, we studied whether cell surface NCL plays a role in PTN-induced cell migration. Down-regulation of NCL by siRNA or blockage of cell surface NCL by its ligand 5(KPR)TASP in human endothelial cells completely abolished PTN-induced cell migration. NCL was found to directly interact not only with PTN, but also with both RPTPβ/ζ and $\alpha_v\beta_3$ on the membrane of human endothelial and cancer cells. Interestingly, NCL was detected on the surface of cells that express $\alpha_v\beta_3$, while in cells that do not express $\alpha_v\beta_3$ or where β_3 is down-regulated or inactivated, NCL expression was restricted to the cell nucleus. The signalling pathway that is involved in $\alpha_v\beta_3$-mediated cell surface expression of NCL and the role of the latter in the angiogenic activities of other growth factors is being studied. Our data suggest that $\alpha_v\beta_3$ activation is important for the cell surface expression of NCL and support a significant role of cell surface NCL in PTN and VEGF-induced endothelial cell migration.

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O-31

miR-15b inhibits endothelial-mesenchymal transdifferention and cardiac fibrosis by targeting Ras signalling

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Fibrosis is a leading cause of cardiac morbidity. Cardiac fibrosis is characterized by blood vessel loss, an increase in the number of fibroblast and excessive extracellular matrix (e.g. collagens) production, leading to the formation of scar tissue. Endothelial cells have been implicated in the progression of cardiac fibrosis. Through endothelial-mesenchymal transdifferentiation (EndMT), cardiac endothelial cells form fibroblast-like cells, which produce collagens and thereby add to the pool of pro-fibrotic cells. MicroRNAs are small (20-23 bases) nucleotides that regulate gene expression through base pairing with the 3′UTR of their target genes. Hence, microRNAs are in principle able to modulate cell function and possibly transdifferentiation.

We used an array-based approach to identify microRNAs that are involved in EndMT and identified miR-15b as a potential regulator of endothelial transdifferentiation. Also, in a pressure overload murine model of cardiac fibrosis, miR-15b was progressively lost. Target gene analysis showed that the Ras-activating proteins Grb2 and SOS1/2 are miR-15b targets and exogenous miR-15b reduced Ras activation in cardiac fibroblasts. Furthermore, EndMT of cardiac endothelial cells was inhibited by exogenous miR-15b.

In conclusion, miR-15b inhibits endothelial-mesenchymal transdifferentiation by inhibition of Ras-activating proteins Grb2 and SOS1/2. Hence, miR-15b may inhibit fibroblast formation during cardiac fibrosis and is a potential candidate for anti-fibrosis therapy.

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O-32

Galectin-2 expression is dependent on the rs7291467 polymorphism and acts as an inhibitor of arteriogenesis

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In patients with obstructive coronary artery disease (CAD), arteriogenesis is an important process that preserves myocardial tissue perfusion. Monocytes are critical regulators of arteriogenesis. The aim of the present study is to identify targets in monocytes that are important in arteriogenesis.

A total of 50 patients with a chronic total coronary occlusion were dichotomized according to their pressure-derived collateral flow index (CFI_p), which indicates the capacity of the collateral circulation. From each patient, RNA was isolated from unstimulated peripheral blood monocytes, monocytes stimulated by lipopolysaccharide (LPS) or interleukin (IL)-4, and from macrophages. Increased mRNA expression of galectin-2 was found in all 4 monocytic cell types of patients with a low capacity of the collateral circulation (P=0.03 for unstimulated monocytes, P=0.02 for LPS stimulated monocytes, P=0.20 for IL-4 stimulated monocytes, and P=0.02 for macrophages). Additionally, galectin-2 mRNA expression was significantly associated with the rs7291467 polymorphism in *LGALS2* encoding galectin-2 in all 4 monocytic cell types. Patients with the rs7291467 CC genotype displayed highest galectin-2 expression, and these patients also tended to have a lower arteriogenic response. To evaluate the effect of galectin-2 on arteriogenesis in vivo, we used a murine hindlimb model. Treatment with galectin-2 markedly impaired the perfusion restoration at day 7 (0.55 ± 0.11 in the galectin-2 group versus 0.75 ± 0.13 in the control group, P=0.002).

Collectively, these results identify galectin-2 as a novel inhibitor of arteriogenesis. Modulation of galectin-2 may constitute a new therapeutic strategy for the stimulation of arteriogenesis in patients with CAD.

O-33

DLL4-Notch signalling mediates tumour resistance to anti-VEGF therapy in vivo

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Anti-VEGF therapy has been used for the treatment of numerous cancers, but tumour resistance to VEGF inhibitors is a major clinical problem. Understanding the resistance mechanisms may enhance therapeutic efficacy by developing new therapeutic approaches or rational drug combinations. We show that Delta-like ligand 4 (DLL4)-Notch signalling mediates tumour resistance to bevacizumab. The large functional vessels induced by DLL4 increased tumour blood supply but were insensitive to bevacizumab. VEGFR2 expression was decreased in these large vessels with a concomitant increase in VEGFR1, and hypoxia-induced VEGF levels were also decreased due to better perfusion. These changes may be responsible for tumour insensitivity to bevacizumab by reducing their dependence on VEGF/VEGFR2 signalling. Dibenzazepine, a Notch inhibitor, disrupted these large vessels and enhanced the tumour response to bevacizumab. Activation of other pathways including FGF2-FGFR and EphB4-EprinB2 contributed to the resistance. These findings suggest that combination therapy by blocking both DLL4 and VEGF pathways may increase the anti-angiogenic efficacy, particularly in anti-VEGF resistant tumours in the clinic.

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O-34

Human FOXF1 induces sprouting angiogenesis by Notch-2 Dll1 signalling

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Searching for genes preferentially expressed in circulating endothelial progenitor cells (CEPs), we detected that FOXF1, a member of the human forkhead winged helix transcription factor family, displays strongly increased expression in CEPs derived from human umbilical cord blood when compared to HUVECs. In this context, previous studies have revealed that the forkhead proteins play pivotal roles in cell differentiation, some being involved in embryonic vessel formation. In particular, FOXF1 deficiency in mouse embryos results in misexpression of vascular and hematopoietic markers as well as in disturbed yolk sac vasculogenesis.

To further decipher the role of FOXF1 in CEPs and endothelial cells, we evaluated the transcriptional regulation and biological function of FOXF1 by modulating its expression. A distinct impact of FOXF1 levels on sprouting angiogenesis was detected in vitro. FOXF1 overexpression increased, whereas shRNA-mediated reduction of FOXF1 diminished sprouting activity. Investigating the underlying mechanism, we detected that FOXF1 regulates Notch-2 receptor expression as well as the subcellular localization of the Notch ligand Dll1. It was intriguing that Inhibition of Notch-2 expression by shRNA caused a reduction of sprouting comparable to diminished FOXF1 expression, suggesting that FOXF1 regulates sprouting activity via Notch-2.

Hence, our data support a crucial importance of FOXF1 for the sprouting ability of CEPs and endothelial cells and implicate the Notch-signalling pathway as a downstream mediator of FOXF1 function.

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O-35

Photodynamic angio-occlusion combined with anti-angiogenic tyrosine kinase inhibitors in the treatment of wet age-related macular degeneration

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There is currently a substantial and wide-spread interest in targeting specific signaling pathways in order to inhibit unwanted angiogenesis, especially for the treatment of wet age-related macular degeneration (AMD). Photodynamic therapy (PDT) is an effective clinical therapy for treating wet AMD, and polypoidal choroidal vasculopathy. Since PDT can cause a combination of hypoxia and inflammation leading to the release of HIF and VEGFs, it evokes an angiogenic response. This has led to the suggestion that the combination of PDT with anti-angiogenic compounds will lead to improved therapy.

In this study, we applied photodynamic angio-occlusion, the vasculature of the chicken embryo chorioallantoic membrane (CAM), and we tested the combination of this treatment modality with 3 tyrosine kinase inhibitors (TKIs): Sunitinib®, Erlotinib® and Sorafenib®. Topical administration of these compounds in mono-therapy on the CAM at embryo development day (EDD) 6 inhibited developmental angiogenesis, as measured on day 9. In combination therapy, Visudyne®-PDT was first applied in a circular area of 2 mm diameter in the CAM, a procedure that results in immediate angio-occlusion of all vessels <100 μm. This treatment was then combined with the subsequent treatment with the aforementioned TKIs. With all compounds, a significant inhibition of revascularization was demonstrated, noting a prolonged vaso-occlusion. A significant decrease of the micro-vessel density, the number of branching points/mm^2, the number of vascular segments/mm^2, as well as a significant increase in the vascular mesh size, were also observed. The latter is closely related to a decrease in the potential oxygenation. Differences observed in the relative efficiencies of the 3 TKIs both in the developmental angiogenesis of the CAM, as well as in the matured CAM after PDT will be discussed.

These results suggest the therapeutic potential of these compounds for application in combination with PDT for treating wet AMD, and possibly other diseases where angiogenesis plays an important role.

O-36

Endothelial Von Willebrand Factor regulates angiogenesis

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Angiogenesis is essential for normal development and for physiological processes in the adult. Dysregulation of angiogenesis is implicated in several diseases including cardiovascular disease. Von Willebrand factor (VWF), a large plasma glycoprotein essential for normal haemostasis, is synthesized by endothelial cells (EC) and megakaryocytes. A qualitative or quantitative deficiency of VWF causes Von Willebrand disease (VWD), the most common congenital bleeding disorder in man. Several clues suggest that VWF may be involved in the formation of blood vessels: 1) VWD can be associated with angiodysplasia, a degenerative lesion of blood vessels which causes intractable gastrointestinal bleeding; 2) VWF is essential for the formation of Weibel-Palade bodies (WPB), storage organelles that also contain angiogenesis regulators e.g. angiopoietin (Ang)-2; 3) VWF binds to the angiogenic regulator $\alpha v\beta 3$ on EC. Therefore, we hypothesised that VWF is involved in angiogenesis. To test this hypothesis, we used siRNA to inhibit VWF expression in human umbilical vein EC (HUVEC) and found that loss of VWF caused increased capillary tube formation on Matrigel. VWF-deficient HUVEC also showed a constitutive increase in vascular endothelial growth factor receptor-2 (VEGFR-2)-dependent migration and proliferation. Interestingly addition of purified VWF corrected some, but not all, the in vitro angiogenic phenotypes, suggesting that VWF mediates angiogenesis both via extracellular and intracellular pathways. The best characterised receptor for VWF on EC is the integrin $\alpha v\beta 3$. In VWF-deficient HUVEC, $\beta 3$ integrin expression was decreased both at the RNA and protein level, and adhesion to $\alpha v\beta 3$-specific substrates was reduced compared to control cells, confirming the functional relevance of this finding. VWF-deficient cells also showed increased secretion of Ang-2, which has recently been shown to regulate $\beta 3$ integrin expression (Thomas et al., JBC 2010). To validate our findings in vivo, we studied the VWF-deficient mouse. In vivo Matrigel angiogenesis and imaging of blood vessels in the ear showed increased angiogenesis and vascular network compared to

littermate controls. We studied the relevance of these findings in VWD patients by isolating blood-derived late-outgrowth endothelial progenitor cells (BOEC). BOEC from VWD patients showed decreased VWF release, consistent with the patients' clinical data. In line with the findings from siRNA-treated HUVEC, capillary tube formation, migration and proliferation were all increased in VWD BOEC. Thus, we have identified a novel mechanism for the regulation of angiogenesis, involving the haemostatic protein VWF, the integrin $\alpha v\beta 3$ and the angiogenesis regulator Ang-2. These results confirm VWF as a multifunctional protein and could have clinical implications for the management of VWD. Moreover, the use of BOEC represents a novel approach in the study of primary endothelial defects, applicable to many other diseases.

O-37

CXCR3 mediates the angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 (platelet factor-4 variant)

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The chemokine platelet factor 4 variant (PF-4var/CXCL4L1) is a non-allelic variant of PF-4/CXCL4 with lower affinity for heparin and higher angiostatic activity. We investigated possible cellular receptors for this human chemokine variant. CXCL4L1 was found to bind heparin and chondroitin sulfate-E with lower affinity than CXCL4. Labeled CXCL4L1 bound to CXCR3A- and CXCR3B-transfectants and was displaced by CXCL4L1, CXCL4, and CXCL10. Also on microvascular endothelial cells, CXCL10 and CXCL4L1 could displace each other. The CXCL4L1 anti-angiogenic activity was blocked by anti-CXCR3 Abs in the Matrigel and cornea micropocket assays. CXCL4L1 application in $CXCR3^{-/-}$ or in wild-type mice treated with neutralizing anti-CXCR3 Abs resulted in reduced inhibitory activity of CXCL4L1 on tumor growth and vascularization of Lewis lung carcinoma. Furthermore, CXCL4L1 and CXCL4 chemoattracted activated T cells, human NK cells, and human immature dendritic cells (DC). Pertussis toxin inhibited migration of DC toward CXCL4 and CXCL4L1, indicating the involvement of a $Galpha_i$ protein-coupled receptor. CXCL4L1- and CXCL4-induced chemotaxis of DC was desensitized by pre-incubation of the cells with the CXCR3 ligands CXCL10 and CXCL11 and was neutralized by anti-CXCR3 antibodies. Chemotaxis of T cells, NK cells, and DC is likely to contribute to the anti-tumoral action. However, the in vivo data indicate that the angiostatic property of CXCL4L1 is equally important in retarding tumor growth. Thus, both CXCR3A and CXCR3B are implicated in the chemotactic and vascular effects of CXCL4L1.

O-38

Synthetic VEGF-mimic: application for anti-angiogenic vaccine therapy

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Immunotherapy through antibodies in combination with classical therapies is nowadays regarded as one of the most promising therapeutic approaches to treat cancer. Immunotherapy can be based on either passive (using externally produced antibodies) or active immunization (generation of antibodies through vaccination). At present, the major thrust of immunotherapy is based on passive immunization, often against 'self' antigens. Active immunization for therapeutic purposes is still in its infancy, but offers a number of advantages (cost-effectiveness, ease-of-application, tumor-penetrability, etc.). Nonetheless, it is to be expected that passive and active immunization shall complement each other in the future.

This lecture will present several examples of peptide-based protein mimics that can be used in vivo for generating (neutralizing) antibodies against the target protein. We developed several technologies (a.o. CLIPS™ technology: **C**hemical **LI**nkage of **P**eptides onto **S**caffolds) for chemical fixation of peptides into the correct 3D-orientation to properly mimic the target protein [1,2]. One particular example involves a protein mimic of VEGF that covers only ~25% of the full protein, while covering the complete discontinuous and conformational binding site of Avastin™, the anti-VEGF therapeutic moAb that was FDA-approved in February 2004 for treatment of metastatic colorectal cancer. The antisera generated in rats showed very high potency in reducing tumor growth. Data from a recent study in a xenograft mouse model (Swiss nu/nu mice) clearly demonstrated the strong anti-tumor activity of antibodies generated by immunization with this VEGF-mimic [3] which turned out to be even stronger than treatment with Avastin™.

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Unpublished results.

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O-39

Molecular weight fibrinogen variants alter endothelial cell characteristics

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Fibrin provides a temporary matrix structure for invading cells during angiogenesis in wound healing and tumor growth. After the conversion of fibrinogen into fibrin, a biological, degradable matrix with cell-binding properties is formed. Two naturally occurring fibrinogen variants, high molecular weight (HMW) and low molecular weight (LMW) fibrinogen, display different properties in supporting angiogenesis. HMW-fibrinogen represents the native fibrinogen form, whereas in LMW-fibrinogen, one alpha-chain is partially degraded at the C-terminus. Since vascularization of tissue engineered scaffolds is

a problem to be conquered, this study aims to investigate human endothelial cells (HMVEC) and mesenchymal stem cell (ASC) characteristics during culture on unfractionated, HMW, and LMW-fibrin matrices.

HMVEC on HMW-fibrin matrices showed an increased proliferation, migration, and tube formation, when compared to their counterparts on unfractionated-fibrin. In contrast, LMW-fibrin decreased the proliferation and tube formation by HMVEC. The degradation of HMW-fibrin was markedly enhanced in the presence of HMVEC, that of LMW-fibrin only slightly. Gene array analysis revealed that the expression of 377 genes was significantly differentially regulated in HMVEC on HMW and LMW-fibrin matrices. Among these genes DLL4 and the DLL4-Notch downstream targets, Hey 1, Hey 2, and Hes 1 were significantly increased in HMVEC cultured on LMW-fibrin. DLL4-Notch alterations could not explain the enhanced tube formation in HMW-fibrin.

In contrast to HMVEC, the stem cellness, proliferation, and differentiation of ASC did not change upon culture on HMW- and LMW-fibrin. This indicates that fibrin variants can be used to influence angiogenesis, with maintenance of stem cell characteristics. In conclusion, small differences in the fibrin matrix lead to altered functional characteristics and gene expressions of endothelial cells, but not of mesenchymal stem cells. This study provides new perspectives for influencing angiogenesis for therapeutic and tissue engineering applications.

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Abstracts poster presentations
(in alphabetic order of first author)

P-1

Chloride intracellular channel protein 4 (CLIC4) in the regulation of angiogenic responses in human pulmonary endothelial cells

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New blood vessel formation (angiogenesis) has been postulated to play a key role in the development of plexiform lesions in pulmonary arterial hypertension (PAH). These lesions represent sites of endothelial cell proliferation and disorganised angiogenesis, which results in the obstruction of pulmonary arteries. The mechanisms responsible for angiogenesis in plexiform lesions are not well understood.

We have found that the expression of CLIC4, a multifunctional protein implicated in angiogenesis, is markedly increased in the lungs of patients with PAH, with CLIC4 immunostaining being prominent in the endothelium of remodelled pulmonary arteries and plexiform lesions (1). CLIC4 expression is also elevated in the lungs of rats with monocrotaline- or hypoxia-induced pulmonary hypertension. Overexpression of CLIC4 in human pulmonary artery endothelial cells increases endothelial permeability, enhances cell proliferation/survival, and promotes pro-angiogenic responses. CLIC4 expression also increases hypoxia-inducible factor activity under both normoxic and hypoxic conditions, stimulates the release of vascular endothelial growth factor and increases filopodia formation.

In summary, CLIC4 promotes angiogenic responses in human pulmonary artery endothelial cells in vitro and may play a role in pulmonary vascular remodelling in PAH.

(1) Vahitha B. Abdul-Salam et al. Proteomic Analysis of Lung Tissues from Patients with Pulmonary Arterial Hypertension, Circulation 2010, in press.

P-2

The shear stress-induced transcription factor KLF2 affects dynamics and angiopoietin-2 content of Weibel-Palade bodies

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Endothelial cells contain rod-shaped organelles designated Weibel-Palade bodies (WPBs) that release their content into the vascular lumen following stimulation with agonists such as thrombin or epinephrine. In this study, we investigated the effect of the shear stress-induced transcription factor KLF2 on clustering and composition of WPBs using peripheral blood-derived endothelial cells. Lentiviral over-expression of KLF2 resulted in a 4.5-fold increase in number of WPBs per cell when compared to mock-transduced endothelial cells. Unexpectedly, the average length of WPBs was significantly reduced. In mock transduced endothelial cells, WPBs had an average length of 1.8 μm, whereas in KLF2 overexpressing cells, WPBs had an average length of 1.4 μm. Overexpression of KLF2 abolished the perinuclear clustering of WPBs observed following stimulation with cAMP-raising agonists such as epinephrine. We previously hypothesized that perinuclear clustering of WPBs provides a means to limit excessive release of bioactive components from these organelles. We subsequently explored whether storage of P-selectin and angiopoietin-2 (Ang-2) in WPBs is affected by KLF2. P-selecting contributes to the transmigration of leukocytes, whereas Ang-2 has been shown to promote vascular leakage and endothelial cell migration thereby contributing to vascular remodeling. P-selectin was readily visualized in both KLF2 and mock-transduced endothelial cells. In contrast, confocal microscopy revealed that WPBs in KLF2-transduced cells did not contain Ang-2. Together, our findings suggest that KLF2 not only regulates the size and dynamics of WPBs but also regulates the contents of this highly versatile storage pool in endothelial cells.

P-3

Small artery remodeling depends on the redox state of transglutaminase

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Background. In our group, we study the regulation of small artery structure and function. Small arteries are the main site of vascular resistance and regulate blood flow. The adaptation of arterial caliber, i.e. vascular remodeling, remains poorly understood. Yet, inward remodeling is a strong predictor of future cardiovascular events. The

transglutaminase family of enzymes cross-links extracellular matrix proteins and could therefore play an important role in arterial remodeling. Indeed, transglutaminase activity has recently been related to age-related stiffening of large arteries. We previously showed that transglutaminases play a crucial role in the inward remodeling of small arteries in response to low blood flow and hypertension, using type 2 transglutaminase (TG2) knockout mice. Here, we studied the regulation of TG2 activity, its (sub) cellular localization, substrates, and its specific mode of action during small artery inward remodeling.

Methods and Results. Isolated mouse mesenteric arteries that were exposed to exogenous TG2 required the presence of a reducing agent to induce inward remodeling. The effect of TG2 depended on its cross-linking activity, as indicated by the lack of effect of mutant TG2. Exposure of arteries to the cell-impermeable reducing agent TCEP did not induce remodeling. However, the cell-permeable reducing agent DTT induced translocation of endogenous TG2 and high transglutaminase activity at the smooth muscle membrane. This resulted in inward remodeling, characterized by a stiffening of the artery. The remodeling could be inhibited by a TG2 inhibitor or the nitric oxide donor SNAP. From smooth muscle cells, a panel of 21 proteins was identified as cross-linking substrates, using a specific labeling assay and mass spectrometry. Finally, inward remodeling induced by low blood flow was associated with the upregulation of several reducing enzymes in vivo.

Conclusions: These results show that inward remodeling depends on the cross-linking function of TG2. A reduced state induces TG2 activity at the interface of smooth muscle cells with the extracellular matrix and results in inward remodeling. A number of proteins were identified as TG2 substrates, including collagen, fibronectin, and nidogen. Of (patho)physiological relevance, nitric oxide is able to fully counteract the actions of TG2.

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P-4

TLR accessory molecule RP105 (CD180) moderates arteriogenesis

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RP105 (CD180) is a toll-like receptor 4 (TLR4) homolog and physiological negative regulator specific of TLR4-signaling. TLR4 is involved in vascular remodeling during restenosis, vein graft disease, and atherosclerosis. Also in arteriogenesis, TLR4 plays an important role. The role of RP105 in cardiovascular disease and in arteriogenesis in particular is still unknown. Since mRNA expression pattern of RP105 mirrors that of TLR4 in adductor muscle after hindlimb ischemia in the mouse, we hypothesize that RP105 is involved in the regulation of arteriogenesis and that RP105 deficiency, and therefore the lack of TLR4 inhibition, results in an enhanced arteriogenesis.

RP105 deficiency does result in a proinflammatory cytokine response. In vitro whole blood stimulation with LPS (as TLR4 ligand) showed a dose-dependent exaggerated TNFα response in $RP105^{-/-}$ mice compared to wild-type (WT) mice, measured by ELISA. Also in vivo, $RP105^{-/-}$ mice produced significantly more TNFα in response to ip LPS injection (1ug). Hindlimb ischemia was induced by coagulation of the proximal and distal femoral artery of WT and $RP105^{-/-}$ mice. Unexpectedly, blood flow recovery, assessed by laser Doppler perfusion imaging, was significantly impaired in $RP105^{-/-}$ mice up to four weeks. FACS analysis demonstrates equal numbers of circulating monocytes in both WT and $RP105^{-/-}$ mice. In vitro whole blood stimulation by LPS equally activates monocytes from WT en $RP105^{-/-}$ mice, measured by mean fluorescence intensity of CD11b. However, the (pro-inflammatory) Ly6Chigh monocyte population is significantly more activated in the $RP105^{-/-}$ mice at baseline and after LPS stimulation. These data suggest a premature or overactivated immune system in $RP105^{-/-}$ mice. To test this, we investigated whether the beneficial effect of LPS on arteriogenesis is absent in $RP105^{-/-}$ mice. Hindlimb ischemia was induced in both WT and $RP105^{-/-}$ mice, and mice were ip injected with saline or LPS (1ug) at day 3 after hindlimb ischemia. LPS injection enhanced blood flow recovery in WT mice but showed no additional effect in $RP105^{-/-}$ mice.

We conclude that RP105 plays an important role in collateral flow recovery after hindlimb ischemia and that the hampered blood flow recovery in $RP105^{-/-}$ mice is a result of premature activated monocytes.

P-5

Dentin Matrix Protein 1 inhibits VEGF-induced angiogenesis and impairs tumor growth

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Dentin matrix protein 1 (DMP1) is a member of the Small Integrin-Binding LIgand N-linked Glycoproteins (SIBLINGs) family, a group of proteins initially described as mineralized extracellular matrices components. More recently, SIBLINGs have been implicated in several key steps of cancer progression, including angiogenesis. Although pro-angiogenic activities have been demonstrated for two SIBLINGs, the role of DMP1 in angiogenesis has not been addressed yet.

We demonstrated that this secreted protein induced the expression of VE-cadherin, a key regulator of intercellular junctions and contact inhibition of growth of endothelial cells that is also known to modulate VEGFR-2 activity, the major receptor for VEGF. DMP1 induced VE-cadherin and $p27^{Kip1}$ expression followed by cell cycle arrest in human umbilical endothelial vein cells (HUVEC) in a CD44-dependent manner. VEGF-induced proliferation, migration, and tubulogenesis responses were specifically blocked upon DMP1 pre-treatment of HUVEC. Indeed, subsequently to VE-cadherin induction, DMP1 inhibited VEGFR-2 phosphorylation and Src-mediated signaling. In vivo, DMP1 significantly reduced laser-induced choroidal neovascularization lesions and tumor-associated angiogenesis.

These data enable us to put DMP1 on the angiogenic chessboard for the first time and to identify this protein as a new specific inhibitor of VEGF-induced angiogenesis.

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P-6

A genomic screen for angiosuppressor genes identifies BRD7 as an inhibitor of tumor angiogenesis and a promoter of anti-tumor immunity

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Tumor angiogenesis is generally acknowledged to mediate cancer progression and metastasis, and strategies to inhibit this process are subject of intense investigation. To meet the functional demands associated with new vessel formation, tumor endothelial cells (EC) are characterized by a gene expression signature that contributes to enhanced proliferation, tube formation, and matrix remodeling. While studies until now have focused predominantly on genes overexpressed in tumor endothelium, we undertook a search for genes that are repressed in tumor endothelium and thus have a potential suppressive effect on angiogenesis.

To maximize clinical relevance, we used EC isolated from resected human colorectal tumors and patient-matched normal colon tissue. We queried for transcripts specifically downregulated in tumor endothelium by including placenta EC to exclude targets representative of physiological angiogenesis. One candidate gene, bromomdomain 7 (BRD7), has putative tumor suppressor functions and was therefore analyzed in more detail to reveal its role in tumor angiogenesis.

BRD7 expression was markedly reduced in colon tumor tissues, notably in the vasculature. Furthermore, BRD7 expression was inversely related to EC activation and was induced following anti-angiogenic therapy. Ectopic expression of BRD7 in EC resulted in a reduction of proliferation and MEK/ERK signaling. In parallel, NF-κB-activity and NF-κB-dependent gene expression were induced, including ICAM1 that mediates leukocyte-endothelial interactions. These effects were largely dependent on the presence of the bromodomain in BRD7 as bromodomain deletion attenuated these events. As such, therapeutic restoration of BRD7 expression in tumor endothelium may not only inhibit EC proliferation, but in addition may contribute to improved immune cell infiltration by enhancing adhesion molecule expression.

P-7

The transcription factor Erg regulates angiogenesis and endothelial cell migration

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Angiogenesis is a tightly regulated process that requires the integration of signals from growth factors, adhesion molecules and other cellular pathways. Specialised endothelial cells (EC) known as "tip cells" emerge from the parent vessel and lead the growth of the developing sprout. Tip cells are highly motile, polarized cells, projecting lamellipodia and filopodia which allow the cell to sense the microenvironment for migration cues and guide the sprouting vessel towards an angiogenic stimulus. Erg, the ETS family member most highly expressed in resting EC, drives the expression of genes involved in cell adhesion, survival and angiogenesis. We have previously shown that Erg is required for angiogenesis and endothelial survival, using in vitro and in vivo models. Here, we demonstrate that Erg is involved in regulating EC migration: inhibition of Erg expression in EC results in decreased migration in an in vitro wound assay; whilst Erg over-expression caused an increase in cell migration. A microarray experiment comparing gene expression profiles of Erg-deficient EC to control identified >50 genes involved in cell motility and migration. Candidate hits included regulators of the actin and microtubule cytoskeleton and of the small GTPase pathways. One of the most significantly down-regulated genes in the microarray was the cytosolic histone deacetylase (HDAC)-6. Inhibition of HDAC6 causes hyperacetylation of its target proteins, which include tubulin, cortactin and Hsp90. We show that Erg regulates endothelial HDAC6 expression and that inhibition of Erg leads to a dramatic increase in tubulin acetylation throughout the entire cell body. To study cytoskeletal dynamics, we performed live-cell imaging of Erg-deficient EC by transfecting cells with actin-RFP and recording images by time-lapse microscopy. Control cells exhibit a characteristic asymmetrical shape with lamellae orientated towards the direction of migration; Erg-deficient cells exhibit a more angular shape with cortical actin and markedly reduced lamellipodia. Quantification using kymographs showed that Erg inhibition results in loss of lamellipodia formation. Our results indicate that Erg is required for endothelial cell migration, regulating HDAC6 expression and tubulin acetylation, and for the dynamic movement of actin-rich lamellipodia. Studies are ongoing to define the role of Erg in regulating these pathways.

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P-8

A computational cell-based model of sprouting and lumen formation during angiogenesis

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Angiogenesis is the formation of new blood vessels from existing vessels. Angiogenesis is not only important during embryogenesis, but also in wound healing and even tumor growth. Many experimental models have been developed to examine angiogenesis. Koolwijk et al. have developed an in vitro model, in which a mono-layer of endothelial cells is seeded on a three-dimensional fibrin matrix. Upon stimulation, sprouts grow into the fibrin matrix and form capillary-like tubular structures. Although much can be learned from experimental models, it is often difficult to reveal the mechanisms underlying the observations. Therefore, we have developed a computational model, based on this experimental set-up, to test the relevance of different mechanisms in sprouting.

The computational model is cell-based, representing individual cells and the fibrin matrix as computational entities. Several processes, like cell adhesion and matrix degradation, are implemented, and their relative importance can be examined. Besides the mechanisms of sprouting, we focus on lumen formation. Multiple theories explain lumen formation in sprouts. Vacuoles can be created in the cells, and these vacuoles grow and fuse. Eventually, the vacuoles of neighboring cells fuse to create a tube in the middle of the sprout. Alternatively, space can be created between cells by the lack of cell–cell adhesions at the luminal side of the cells. The computational model for sprouting can be extended with lumen formation and yields experimental predictions that can be tested in the laboratory. Hereby, an attempt is made to complement experimental with computational models to unravel the mechanisms that drive sprouting as well as lumen formation.

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P-9

Anti-angiogenic treatment reverses pulmonary vascular remodeling in experimental pulmonary hypertension but worsens failure of the pressure-overloaded right ventricle

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Rationale. Pulmonary arterial hypertension (PAH) is characterized by focal lesions of proliferating, apoptosis-resistant (cancer-like) pulmonary endothelial cells. In contrast, PAH-associated maladaptive right ventricular (RV) remodeling is characterized by a loss of capillaries and fibrosis. We hypothesized that anti-angiogenic treatment strategies in PAH will decrease the obliterative pulmonary angiopathy but will also worsen RV failure with continued pressure overload.
Methods. PAH was induced in rats by the combined exposure to the VEGF receptor blocker SU5416 and hypoxia (SuHx). In rats of similar age and strain, isolated RV pressure overload was induced by pulmonary arterial banding (PAB). Angiogenesis was inhibited by altering copper metabolism (either dietary copper restriction, DCR, or copper chelation using tetrathiomolybdate, TTM) or by induction of hypothyroidism (either with propylthiouracil, PTU, or by performing thyroidectomy).
Results. Anti-angiogenic therapy in the SuHx model was associated with a reduction in the number of occluded small pulmonary vessels, a decrease in mean pulmonary artery pressure, a decrease in RV hypertrophy, and an improved RV function on cardiac ultrasound. However, in the PAB model, both treatment modalities induced severe RV failure, as evidenced by RV dilatation, a reduced TAPSE and a reduced cardiac output. Whereas PAB per se was not associated with capillary rarefaction and fibrogenesis, PAB animals treated with DCR or PTU showed a loss of RV capillaries and fibrosis. The nature of the maladaptive RV remodeling in these animals closely resembled the RV remodeling that we previously described in the SuHx model, both in terms of histology and gene expression.
Conclusions. Anti-angiogenic therapy in PAH may decrease pulmonary vascular resistance, but in those instances where it is not successful in re-opening the pulmonary vessels, it may actually worsen RV failure.

P-10

Common angiogenic signalling pathways induced by monomeric- CRP and FGF-2 through PI3K in vascular endothelial cells

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Angiogenesis is the process of new blood vessel growth from the pre-existing vascular structures. The new vessels from atherosclerotic lesions may be a focus of instability, since they facilitate the infiltration of inflammatory cells and due to their tendency to leak, they may produce haemorrhagic complications. Pentameric C-reactive protein (CRP), a strong marker of inflammation, is a risk factor for cardiovascular diseases with a direct role in the development of atherosclerotic lesions. Under abnormal conditions, CRP dissociates irreversibly into monomeric CRP (mCRP), which was previously demonstrated to be pro-angiogenic on bovine aortic endothelial cells (BAEC). Our main study was to examine the vessel forming capability of CRP in the presence of other angiogenic factors known to be present in the microenvironment of unstable plaques with immature vasculature.

Here, we studied the effects of mCRP in the presence or absence of FGF-2 on BAEC proliferation, migration, tube formation in Matrigel and on the vascular remodelling using spheroids, a tridimensional system of endothelial cell culture embedded in collagen gel. For a better understanding of the molecular mechanisms involved, the signalling pathways were investigated by Western blotting, and all the essays were performed in the presence or absence of pharmacological inhibitors of MAPK (PD98059), γ-secretase (DAPT inhibitor) and PI3K pathways (LY294002).

We showed that mCRP-induced endothelial cell proliferation, migration and tube formation required activation of the PI3K pathway. MAPK activation was essential in mCRP-induced cell differentiation (tube formation and sprouting from the core of spheroids), and γ-secretase activity was required for mCRP-induced tube formation only. For its pro-angiogenic activity, FGF-2 required all of these key pathways with the exception that γ-secretase activity was not associated with FGF-2-induced cell migration. In all assays including the over-expression of phospho-ERK, the synergistic pro-angiogenic effect of mCRP added to FGF-2 was completely inhibited by LY294002.

Therefore, mCRP and FGF-2 have a common signalling pathway through PI3K and an eventual deregulation of their pro-angiogenic effects due to an excessive inflammation inducing a hyper-vascularisation which could contribute to formation of unstable plaques with haemorrhagic risk, and therefore, might be prevented by targeting the key proteins of the PI3K pathway.

P-11

Effect of the anti-diabetic drug rosiglitazone in pleiotrophin-induced endothelial cell activation

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Thiazolidinediones are a class of peroxisome proliferator-activated receptor γ (PPARγ) agonists that reduce insulin resistance in type 2 diabetic patients. In recent years, there has been increasing appreciation of the fact that PPARγ agonists might be involved in the molecular mechanisms that regulate angiogenesis by either affecting the expression levels of growth factors, such as vascular endothelial growth factor, or by inhibiting the stimulatory activities of growth factors on endothelial cell proliferation, survival, or migration. Pleiotrophin (PTN) is an 18-kDa secreted growth factor that has high affinity for heparin. PTN is expressed in various cancer cell lines and affects many different processes, such as cell growth, survival, migration, and angiogenesis. In the present study, we examined the effect of the PPARγ agonist rosiglitazone (RSG) on the expression and secretion of PTN, as well as on the stimulatory effect of PTN in human umbilical vein endothelial cell (HUVEC) migration and reactive oxygen species (ROS) production. RSG inhibited secretion of PTN into the cell culture medium of HUVEC but had a biphasic effect on the intracellular levels of PTN, as well as on *ptn* gene transcription, with the lowest concentrations causing increase and the highest

concentrations causing a decrease in PTN amounts. RSG significantly decreased levels of ROS, as well as unstimulated endothelial cell migration. However, it did not affect PTN-induced HUVEC migration, in contrast to its effect on VEGF-induced endothelial cell migration. In order to find out whether the actions of RGS on HUVEC migration and ROS production are PPARγ-dependent, we used a selective antagonist of PPARγ, GW9662. It was found that GW9662 also inhibited both HUVEC migration and ROS production to a similar degree compared with RSG, suggesting that these effects of RSG are PPARγ-independent. These data also suggest that GW9662 should be treated cautiously as a PPARγ antagonist. In conclusion, although RSG was found to affect endogenous ROS levels and migration of unstimulated HUVEC in a PPARγ-independent manner, it did not affect the corresponding PTN-induced HUVEC activities.

P-12

Sulfatase1, a novel downstream target of Dll4/Notch signalling in endothelial cells, modulates sprouting angiogenesis

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The Notch ligand Delta-like 4 (Dll4) functions as a negative regulator of tumour angiogenesis Blocking Dll4/Notch signalling overcame resistance to anti-VEGF treatment (Ji-Liang *et. al. paper in press* 2010). However, less is known about the mechanisms/genes involved in the downstream responses following Dll4/Notch signalling and their subsequent role in tumour vascular biology.

Deep sequence analysis (CAGE array) of HUVECs stimulated with human recombinant tethered Dll4 for 16h, identified several novel downstream targets that may potentially regulate angiogenesis. One was sulfatase1 (SULF1), a extracellular 6-O-sulfatase enzyme that alters the sulfation patterns on heparin sulphate proteoglycans, and therefore the growth factor affinity on the cell surface. The role of SULF1 in modulating angiogenesis by the promotion of pro-angiogenic signalling was investigated.

SULF1 was validated as a novel downstream target of Dll4/Notch signalling by QPCR and western blotting in HUVECs. SULF1 was highly expressed in HUVECs compared to a variety of cancer cell lines. Suppressing expression of SULF1 by siRNA or following treatment with inhibitors (e.g. Suntinib and Notch inhibitor DBZ) affected the response to VEGF and Dll4/Notch signalling, shown by modulation of downstream targets (for example phosphorylation of AKT, ERK, Dll4, HEY1, VEGFR2, VEGFR1 expression) by both QPCR and western blotting. Changes in the heparin sulfate state and adherence of growth factors (e.g. VEGF) to the cell surface following suppression of SULF1 expression were examined by both immunofluorescence and flow cytometry. The changes in the level of growth factors (e.g. VEGF) in the culture media following HUVEC stimulation by Dll4 were examined by ELISA. The promotion of various pro-angiogenic signalling by SULF1 was also examined in zebrafish. The effect of modifying expression of SULF1 by siRNA was also examined in various models of angiogenesis, including the hanging drop assay. Loss of SULF1 did not affect the ability of HUVEC to form networks on top of matrigel however, in the hanging drop assay, excessive sprouting was observed. Loss of SULF1 also effected the migration of cells in response to environmental cues.

SULF1 is a novel downstream target of Dll4/Notch signalling pathway. The ability of SULF1 to influence cellular signalling and thereby modulate angiogenesis identifies SULF1 as a novel target of anti-angiogenic therapy. The effect of modulating this pathway in zebrafish embryos and in xenografts is on going.

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P-13

Inactivation of glomulin causes glomuvenous malformations in man and results in early embryonic lethality in mouse

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Glomuvenous malformations (GVM, MIM #13800) are characterized by aberrantly differentiated vSMC-like "glomus cells" around distended vein-like channels. We showed that the lesions result from complete lack of glomulin due to the combination of a germline and a somatic second-hit loss-of-function mutation in *glomulin*. To generate a model in which to test potential therapies, and to understand the developmental role of glomulin, we inactivated the murine gene by inserting the *LacZ* reporter gene at the start codon. Glmn-heterozygotes are healthy for more than one-year-old and show wide vascular expression of LacZ. In contrast, no live knockouts are obtained, embryonic lethality occurring around E8.5, with severe mesodermal defects.

To enable studies beyond the lethality time point, we generated transgenics with conditionally inducible expression of two glomulin-specific RNAi. When down-regulation of glomulin is induced ubiquitously, embryonic lethality ensues. However, knockdown embryos develop further than the knockouts and show vascular abnormalities, such as absent yolk-sac vasculature and hemorrhages. The early lethality in absence of glomulin suggests a crucial role in embryogenesis, even before vascular development. Spatio-temporally controlled induction is thus needed to elucidate the role(s) of glomulin during development and to mimic the double-hit mechanism underlying GVM, so as to obtain a model to study novel GVM therapies.

P-14

Angiogenic response in patients with MPM after treatment with cisplatin, pemetrexed and axitinib: results of a feasibility study

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The vasculature in malignant pleural mesothelioma (MPM) is considered an important treatment target. Since standard treatment with platinum and antifolate does not lead to long survival, we aimed at additionally targeting one of the major regulators of blood vessel formation, vascular endothelial growth factor receptor 2 (VEGFR2) with the small tyrosine kinase inhibitor axitinib (Pfizer).

M and M: We performed a feasibility study in patients who received cisplatin 75 mg/m^2, pemetrexed 500 mg/m^2 (q3) and daily 2x 5 mg axitinib orally. Before treatment and after 3 courses, a

thoracoscopy was performed to collect tissue and evaluate the intrathoracic response. Material was collected for assessment of microvessel density (CD31/34 staining); number of proliferating endothelial cells. Furthermore, biopsies were analysed for VEGFR2, phosphorylated VEGFR2 and VEGF by Western blotting. Immunohistochemistry was used to detect expression levels of VEGFR2 in the respective cells types. VEGF-A concentration in frozen patient plasma samples was determined.

Results: Six male patients were included of which 4 had both 3 courses of chemotherapy and evaluable thoracoscopies. Epithelial type MPM was observed in four patients who all showed either stable disease (SD) or partial response (PR). One patient with sarcomatoid type MPM progressed during treatment. One patient with mixed type had SD, he allowed only true cut biopsies after chemotherapy. Median age: yrs 57 (range 56-61); WHO 0-1: 5pts, WHO 2: 1pt. Hematological toxicity: neutropenia grade 3: 60%, grade 4: 20%, no febrile neutropenia/infection occurred. All patients experienced grade 2 hypertension; no other cardiovascular toxicity was seen.

Assessment of angiogenesis parameters in the tumour tissues before and after therapy revealed a decrease of microvessel density by factor of 2.72. Moreover, the number of proliferating endothelial cells decreased by approximately 4-fold. Patients with PR or SD displayed decreased or constant expression and activity of VEGFR2. VEGF expression levels followed a similar pattern. In biopsies of the patient with tumour progression, higher VEGFR2 levels and activity and higher VEGF protein expression after treatment were measured. VEGFR2 immunohistochemistry showed strong staining of tumour cells and blood vessels before treatment. VEGFR2 positive areas and staining intensity were clearly reduced in some of the patients. There was no clear pattern in VEGF-A levels during axitinib treatment in patient plasma; however, in 2 patients, VEGF was downregulated after start of treatment and upregulated between axitinib treatment courses.

Conclusions: The combination of cis/pem/axitinib was well tolerated. There was an apparent decrease in microvessel density and proliferating endothelial cells after treatment. VEGFR2 protein expression and activity and VEGF protein levels correlated with treatment response. We will further extend our findings in a randomised phase 2 study and investigate additional effects of this treatment approach.

P-15

Molecular imaging of the endothelin-a-receptor expression as a benchmark of angiogenesis in murine thyroid cancer xenografts using small animal PET and optical imaging

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Introduction. Angiogenesis is essential for tumor growth and metastasis, making it an important target for cancer imaging and therapy. Noninvasive imaging technologies like PET or optical imaging, capable of visualizing tumor angiogenesis and evaluating disease progression and therapy response, are therefore gaining increasing importance. A potential target for molecular imaging of angiogenesis in cancer is the endothelin-A-receptor (ETAR). Dysregulation of this receptor has been described in a number of pathophysiological processes, including cancer. A number of human cancer cell lines, e.g. thyroid cancer cells (1), exhibit an upregulated density of ETARs, influencing tumor growth and aggressiveness. In this study, a radiofluorinated and a fluorescent biomarker, targeted to the ETAR, were used for the evaluation of target expression in papillary thyroid carcinoma xenografts using small animal PET and optical imaging techniques.

Methods. Subcutaneous and orthotopic xenograft models of papillary thyroid carcinoma cells (K1) were established in CD-1 nude mice. RT-PCR analysis was used to evaluate ETAR expression of the tumors. A radiofluorinated analog of the known ETAR ligand PD 156707 was designed and applied in small animal PET experiments. In addition, a fluorescently labeled analog of PD 156707 was designed and used for optical imaging techniques such as (FRI) and (FMT).

By microautoradiography using ^{125}I-ET-1, the binding of the fluorinated and the fluorescently labeled analogs to ETARs on tumor sections was evaluated.

Results. Using RT-PCR, the expression of the ETAR on human papillary thyroid carcinoma was confirmed. Small animal PET experiments showed accumulation of the ETAR-targeted radiotracer in the tumor. In optical imaging experiments, a high fluorescence signal was visible in the tumor region. Significant reduction in signal intensity was observed after predosing with lead compound PD 156707. Biodistribution studies after 48 h confirmed a significantly lower fluorescent signal in the tumor when predosed. In vitro autoradiography proved specific binding of the tracers to the ETAR in tumor sections.

Conclusion. Papillary thyroid carcinoma xenografts are a feasible model for the detection of ETAR expression as a benchmark of tumor angiogenesis and progression. PET or optical imaging in combination with radioisotope- or fluorescently labeled ET receptor ligands can be useful tools for the evaluation of the endothelin axis in tumoral lesions.

Literature:
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P-16

The adult mouse lung contains a niche for endothelial progenitor cells with high capacity to form blood and lymphatic vessels in vivo

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Postnatal endothelial progenitor cells (EPCs) have been successfully isolated from whole bone marrow, blood, and the vessel walls. They can, therefore, be classified into circulating and resident progenitor cells. The differentiation capacity of resident lung endothelial progenitor cells from mouse has not been evaluated.

In an attempt to isolate differentiated mature endothelial cells from mouse lung, we found that the lung contains EPCs with a high vasculogenic capacity and capability of de novo vasculogenesis for blood and lymph vessels.

Mouse lung microvascular endothelial cells were isolated by selection of $CD31^+$ cells. Whereas the majority of the $CD31^+$ cells did not divide, some scattered cells started to proliferate giving rise to large colonies. These highly dividing cells possess the capacity to integrate into various types of vessels including blood and lymphatic vessels unveiling the existence of local microvascular endothelial progenitor cells in adult mouse lung. EPCs could be amplified >passage 30 and still expressed panendothelial markers as well as progenitor cell antigens, but not antigens for immune cells and hematopoietic stem cells. A high percentage of these cells are also positive for Lyve1, Prox1, podoplanin, and VEGFR-3 indicating that

a considerabe fraction of the cells are committed to develop lymphatic endothelium. Clonogenic highly proliferating cells from limiting dilution assays were also bipotent. Combined in vitro and in vivo spheroid and matrigel assays revealed that these EPCs exhibit vasculogenic capacity by forming functional blood and lymphatic vessels. The secretome of the cells was studied by nanoflow LC–MS. For analysis, three-day conditioned serum-free media were used. We found 133 proteins belonging to the categories of membrane-bound or secreted proteins. Thirty-four proteins from this group are well known as endothelial cell- or angiogenesis-related proteins. The MS analysis of the secretome was supplemented and confirmed by FACS analysis, ELISA measurements, and immuno-cytological studies of selected proteins.

In summary, mouse lung contains large numbers of EPCs that display commitment for both types of vessels, suggesting that lung blood and lymphatic endothelial cells are derived from a common progenitor cell. The secretome data may provide a platform for further studies of progenitor cells and to discover new cellular markers and signalling pathways.

P-17

Post-translational processing of Vascular Endothelial (VE)-cadherin generates candidate biomarkers of tumoral vasculature in human glioma

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Background. Human glioma are highly vascularized tumors that exhibit elevated levels of vascular endothelial growth factor (VEGF). Vascular endothelial (VE)-cadherin is an endothelial specific adhesion molecule of vital importance in angiogenesis, endothelium integrity, and VEGF signaling. We hypothesized that alterations in VE-cadherin post-translational processing in glioma could provide a signature of tumoral vasculature.

Methods. We studied VE-cadherin expression and phosphorylation in glioma from human malignant glioma specimen. We studied the effect of VEGF and glioma-conditioned medium on VE-cadherin shedding on human umbilical vein endothelial cells. We analyzed VE-cadherin in peripheral blood collected from newly diagnosed glioma patients prior to therapy that were retrospectively classified as responders or non-responders to three-months temozolomide therapy.

Results. VE-cadherin was highly expressed in the glioma capillary network and was among the most strongly tyrosine phosphorylated proteins in addition to Src kinase. We demonstrate that VEGF and glioma-conditioned medium induced VE-cadherin cleavage leading to the release of its full-length extracellular domain (sVE). We detected a soluble form of VE-cadherin in glioma patient's serum that corresponded to sVE. Importantly, sVE-cadherin in serum was found to be carried by lipoproteins, and we established that it could be dissociated by using a detergent. We designed an enzyme-linked immunosorbent assay supplemented with detergent for further sVE quantification. We found that sVE levels could discriminate between non-responder vs responder patients.

Conclusions. Our findings provide evidence that VE-cadherin alterations appeared of particular interest as candidate biomarkers for glioma to detect activated endothelial cells and to assess agressiveness of glioma.

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Enhanced platelet activation mediates the accelerated angiogenic switch in mice lacking histidine-rich glycoprotein

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Histidine-rich glycoprotein (HRG; alternatively, HRGP/HPRG) is a heparin-binding plasma protein capable of suppressing tumor angiogenesis and growth in vitro and in vivo. To study the effect of HRG on tumor development, we have crossed HRG-deficient mice with the RIP1-Tag2 mouse, an orthotopic model of multistage carcinogenesis. RIP1-Tag2 HRG-/- mice displayed an enhanced angiogenic switch and significantly larger tumor volumes compared to their RIP1-Tag2 HRG+/+ littermates (see abstract from Ringvall et al.). In addition, platelet activation was elevated in HRG-deficient mice, compared to their wild-type littermates. Since increased coagulation and platelet activation are known to stimulate tumor angiogenesis, we aimed to elucidate whether the elevated platelet activation was contributing to the increased pathological angiogenesis in HRG-deficient mice. For this purpose, mice were rendered thrombocytopenic before the onset of the angiogenic switch by injection of the anti-platelet antibody GP1bα. Interestingly, this treatment suppressed the increase in angiogenic neoplasias seen in HRG knockout mice, suggesting that increased platelet activation mediates the accelerated angiogenic switch in HRG-deficient mice. No such effect could be detected if mice were treated with GP1bα at a later stage, i.e. after onset of the angiogenic switch, indicating that the platelets are of minor important for tumor growth during later phases of tumor development.

We are currently addressing the mechanism behind the increased platelet activation in HRG-deficient mice and how they contribute to the enhanced pathological angiogenesis in this model. To determine whether the enhanced platelet activation is due to altered signaling capacity of the platelets *per se*, expression levels of activation markers (GPIIbIIIa and P-selectin) on the platelet surface were analyzed by FACS. However, these results revealed no such differences, indicating that the effect is not due to altered activation capacity of the platelets in HRG ko mice, but rather depends on factors in the surrounding plasma microenvironment. Different factors interacting with HRG, known to also affect platelet aggregation, will be analyzed for their potential involvement. Moreover, since platelets are known to facilitate

metastatic spread, we aim to determine whether lack of HRG is affecting the metastatic potential in RIP1-Tag2 mice.

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Lymphatic microvascular density (LMVD) correlates with tryptase-positive mast cell density in molecular types of breast cancer

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Lymphangiogenesis in breast cancer was extensively investigated in last years, and it was shown that LMVD, VEGF-C, and VEGFR-3 are good predictors of lymph node metastasis. The basic mechanisms of new lymphatic vessels' (LVs) formation in the tumor area are still controversial, and the particularities of lymphangiogenesis in the molecular types of breast cancer are virtually unknown. Moreover, the contribution of the microenvironment to this process was less investigated. In the present study, we investigated the relationships between LMVD, mast cell density (MCD), and the molecular profile of breast cancer.

There were investigated 55 patients, aged between 26 and 81 years, admitted with breast cancer. There were selected only ductal invasive carcinomas with tumor stage T2-T4, and from these, 26 (47.27%) showed lymph node metastasis at the routine examination. To classify specimens of breast cancer according to the molecular profile, there were performed immunohistochemical stainings to detect the expression of hormone receptors (estrogens and progesterone), HER-2 protein, cytokeratin 5/6, epidermal growth factor receptor (EGFR), p53, and Bcl-2. Mast cells (MCs) and LVs were detected using two murine monoclonal antibodies against the lymphatic endothelial cell marker D2-40 and mast cell tryptase. MCs and LVs were simultaneously counted in the tumoral and peritumoral areas, and results were compared with the molecular type, grade, lymphovascular invasion, and lymph node status.

We found basal-like carcinoma in 8 cases, luminal A in 26 (47.27%), luminal B in 7 (12.72%), HER2 in 10 (18.18%), and unclassified in 4 (7.27%). In cases with luminal type A, LVs were found in all cases in the peritumoral area and in 19 from 26 in the intratumoral area. MCs were identified in all cases in both peri- and intratumoral area. In the luminal B type, MCD was not significantly different from values found in luminal A, but an increase in the number of intratumoral LVs was noticed. LMVD value was more close to the HER2 type than to the luminal A. In the HER2 type, MCD was significantly higher in the intratumoral area than in the peritumoral tissue. LVs were found in 7 from 10 cases in the intratumoral area and in all cases in the peritumoral tissue. Basal-like carcinoma was the only type that showed a higher MCD in the peritumoral than in the intratumoral area. LVs were not found in one case in the intratumoral area and in another in the peritumoral tissue.

A strong correlation was found between both intratumoral/peritumoral LMVD and MCD in HER2 and luminal B breast cancers, and both correlated with lymph node metastasis.

P-20

Assembly and pharmacological validation of a fully kinetic 96-well in vitro vascular tube formation assay

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In vitro models of vascular tube formation are valuable tools for probing the complex signalling pathways of angiogenesis. Donovan et al. (2001) first described a co-culture system of stromal human dermal fibroblasts and umbilical vein endothelial cells (HUVECs), in which the key phases of endothelial cell proliferation, migration, differentiation, and, ultimately, micro-vascular tube formation are recapitulated. Here, we have evolved this model to allow fully kinetic, rather than end point, measures.

HUVECs were initially transfected with a lentiviral GFP construct to produce highly homogeneous and stable fluorescence for >15 days in culture. These cells were then mixed with dermal fibroblasts and seeded on to 96-well microtitre plates, in proprietary media. Using IncuCyte, a compact imaging system that resides within the stable environment of a cell incubator, phase contrast and fluorescence images (10x) were gathered every 12 h for 14 days (with re-feeding every 2nd–3rd day). An imaging algorithm was applied to quantify the extent of vascular tube formation (length, area, branch points) in each well and at each time point. During the first few days of the co-culture, the HUVECs proliferated and migrated to form (green) clusters. At day 4, addition of the exogenous growth factors VEGF, bFGF or EGF evoked time- and concentration-dependent promotion of tube formation with mean EC_{50} values of 27pm, 109pM and 38pM, respectively. The pro-angiogenic effects of VEGF could be inhibited in a concentration-dependent manner by suramin (1–30μM). Withholding additional growth factors to later time points delayed the onset of tube formation. Established tubes could be de-stabilised by removal of growth factors. The pharmacological agents wortmannin (non-specific PI3kinase inhibitor), CCT018159 (Hsp90) and KU0063794 (mTOR) each attenuated tube formation in a characteristic and time-dependent manner. Soluble DLL-4 (1 μg ml^{-1}) exerted a small but significant inhibition of late (>day 8) branching, whereas the γ-secretase inhibitor L-685,458, (1-4μM) greatly enhanced (>2-fold) late branching.

We conclude that the addition of full temporal measures to this co-culture system, and potentially other in vitro tube formation models, not only provides greater precision and sensitivity, but also affords greater resolution and understanding of mechanistic events (e.g. tube retraction). This approach should prove valuable in dissecting the pathways of angiogenesis and in the search for novel therapeutics.

Donovan D., et al. 2001, Angiogenesis 4, 113-121.

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Melanomas differentially regulate directionally persistent EC migration through TIMP-3 expression

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Introduction: Cutaneous melanoma is a complex genetic disease and the most aggressive form of skin cancer. Although early detection and

surgical resection of the primary lesion could significantly improve survival (>90% survival in Stage I melanoma patients), metastatic melanoma is by large refractory to existing therapies and predicts poor prognosis. Melanoma progression and metastatic dissemination fundamentally relies on the process of angiogenesis. Physiologically, the angiogenic process itself is comprised of a stringent cascade of events, ultimately resulting in a new vessel. Pathological (tumor) angiogenesis, however, is fundamentally mediated by an array of angiogenic modulators produced by the tumor itself. Such tumor-associated modulators arbitrate the enhanced and awry proliferative, survival, and migratory responses exhibited by endothelial cells, culminating in unregulated and unimpeded angiogenesis.

Project Description: In the current study, we evaluated the angiogenic potential of human melanoma cell lines, based on their ability to regulate directionally persistent endothelial cell (EC) migration. Melanoma conditioned medium (CM) from three human melanoma cell lines (BLM, Mel57, 1F6), differing in their degree of aggressiveness and clinical staging, was used to assess the migratory responses of ECs in a 2D migration assay. Cell migration of ECs in response to the tested CMs was monitored real-time, and various parameters like total and effective distance of migration, migration velocity, and migration efficiency were determined. Additionally, CM-size fractions were tested to delineate the differences observed in migratory responses of the ECs to the panel of tested CMs.

Results and Conclusions: ECs showed varied migratory responses with reference to the melanoma CMs tested. There was also a clear correlation between in vitro EC migration and the degree of aggressiveness of the melanoma cell line used to generate the CM. While the CM of the highly aggressive BLM tumor cells induced a high migratory response in ECs, the CM of the non-metastatic, less aggressive 1F6 cells had an inhibitory effect. The 1F6 CM was further size fractionated to demarcate the fraction responsible for the observed inhibitory effect. It was observed that the factor/s responsible for this inhibition lay in the 10- to 30-kD range. Gene and protein expression verification helped identify a varied expression profile of the 24-kD molecule tissue inhibitor of metallo-proteinase-3 (TIMP-3), which is secreted into the CM. TIMP-3 expression inversely correlated with aggressiveness of the melanoma cell line and ability of the respective CMs to induce EC migration. CM of 1F6 cells where TIMP-3 was silenced with shRNA induced EC migration, while both recombinant human TIMP-3 and the CM of BLM cells transfected with TIMP-3-GFP plasmid mitigated EC migration. Taken together, these results demonstrate that TIMP-3 inhibits the inherent propensity of endothelial cells to undergo directionally persistent migration and that TIMP-3 expression can impede EC migration during melanoma-induced angiogenesis.

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Melanoma provides a proangiogenic milieu which promotes endothelial cell survival

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Introduction: Malignant melanoma is a complex genetic disease and the most aggressive form of skin cancer. Although early detection and surgical resection of the primary lesion could significantly improve survival (>90% survival in Stage I melanoma patients), metastatic melanoma is by large refractory to existing therapies and predicts poor prognosis. Melanoma progression and metastatic dissemination fundamentally relies on the process of angiogenesis. Solid tumors produce an array of angiogenic modulators that mediate pathological angiogenesis. Such tumor-associated modulators arbitrate the enhanced proliferative, survival, and migratory responses exhibited by endothelial cells, in the hypoxic tumor environment.

Project Description: The current study focuses on melanoma-induced survival of endothelial cells (ECs) under hypoxic conditions. The angiogenic potential of four human melanoma cell lines (BLM, Mel57, 1F6, 530), differing in their degree of aggressiveness and clinical staging, was determined by monitoring survival of ECs upon exposure to melanoma-conditioned medium (MCM), under hypoxic conditions (1% O_2). The MCM was further size fractionated and subjected to an array of treatments such as heat inactivation and enzymatic degradation in order to further characterize the survival factor. Mass spectrometry was undertaken in order to determine the active component in the MCM. Additionally, key components of the survival pathway were analyzed in order to determine the signaling pathway adopted by the survival factor/s, specific to melanomas.

Results and Conclusions: ECs showed survival under hypoxic conditions when treated with conditioned medium of all melanoma cell lines tested. No such survival effect was observed with the conditioned medium of the melanocytes. The conditioned medium of the pancreatic and breast tumors showed short-term (24 h) but no long-term survival effect, suggesting that the survival factor is specific to the melanoma cells. Further, all the size fractions (<50 kD, <30 kD, <10 kD, <5 kD <3 kD, <1 kD) of the MCM induced long-term survival (up to 72 h) of the ECs. The survival effect observed by the <1-kD fraction eliminates known pro-angiogenic factors. Heat inactivation of this fraction up to 100°C showed retention of the survival effect. Moreover, enzymatic digestion of the MCM with trypsin and chymotrypsin did not inactivate the survival factor. The analysis of the phosphorylation status of central components of the survival pathway suggests that this effect is mediated via the AKT signaling pathway. Taken together, these data indicate the production of (a) survival factor/s (<1 kD) by melanoma cell lines, which enable long-term survival of the ECs and promote melanoma-induced angiogenesis.

Our present study thus reports the presence of a survival factor/s <1 kD which is specific to melanomas. As the <1-kD size range eliminates previously known angiogenic factors, validation and elucidation of the survival molecule/s could assist in more specific treatment regimes to target the aggressive attributes of malignant melanomas.

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Onset and early angiogenesis in tumor development: tip cells and interacting partners

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Introduction: The vertebrate vasculature is the most prevalent and predominant tubular organ system. Expansion and remodeling of the primitive plexus entails sprouting, splitting, branch regression, vessel stabilization, and maturation, ultimately culminating in a mature quiescent functional vasculature. The organization of a sprouting tube can be characterized by the three distinct endothelial cell phenotypes observed in a growing sprout; tip cells, stalk cells, and phalanx cells. Although physiologically this cascade is controlled by a tightly regulated spatial and temporal neovascular response, pathological angiogenesis is characterized by deregulation in multiple steps of this highly conserved cascade. This results in a chaotic and unstructured vascular architecture that impairs perfusion, oxygenation, and drug delivery. In the present study, we evaluated discrete steps in melanoma-mediated angiogenic induction and sprouting.

Project description: Onset of angiogenesis and vascular sprouting in melanoma was studied using in vitro and in vivo models in order to identify and characterize tumor associated tip cells. Endothelial cells, coated on Cytodex beads, were embedded in MatriGel to observe sprout formation and tubule formation in vitro. Melanoma-associated angiogenic sprouting in vivo was visualized real-time using confocal microscopy, in the dorsal skin-flap window mouse model. The expression of key regulators of angiogenesis was visualized using immunofluorescence stainings and in situ hybridizations.
Results and Conclusions: Sprouting angiogenesis in melanoma was clearly visible using the in vitro and in vivo models tested. In vitro, the ECs coated on cytodex beads were able to form structures resembling vascular sprouts, with a single motile non-lumenized cell at the edge of the sprout. The use of the dorsal skin-flap window model assisted in the visualization and following of angiogenic induction in melanoma tumors, real-time. Angiogenic sprouting started at around day 7 of tumor implantation, and rapidly progressed to supply the entire tumor mass. Tumor-associated vessels showed marked structural disorganization when compared with the patent host vessels. The angiogenic sprouts showed the presence of a single highly polarized, motile, lumenless cell, with numerous filopodial extensions. In situ hybridizations, and immunofluorescence stainings on frozen sections and whole mount tumors, assisted in the visualization of both presence and distribution of angiogenesis specific markers (e.g.; VEGF, VEGFR2) during tumor-mediated angiogenic sprouting. Taken together, our studies report novel insights into the paradigm of physiological versus pathological angiogenesis. Validation and expounding of melanoma pathophysiology could therefore assist in more specific treatment regimes to target the aggressive attributes of malignant melanomas.

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Genotype–phenotype variation in retina vasculature using a quantitative synthesis of systems biology and morphological analysis

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Phenotypic variation and diversity in behavioral response within a single genotype are ubiquitous. Sources, mechanisms, and innovations in variation within a genotype are hardly understood. The problem of the genotype–phenotype relationship in the retina is notoriously difficult due to the many intermediate layers that control attributes in development and interactions within the neurovascular systems. We study neuroangiogenesis in developing mice retinas and the relationship between genotype and phenotype at myriad levels of diversity and detail.

In this preliminary report, we discuss progress in design of novel methods to quantify variations on retinal branch structures as they occur naturally or due to genomic perturbations, such as gene knockout, or due to disease, as in diabetic retinopathy. Ultimately, we will be offering a suite of software applications that provide opportunities to biomedical researchers to study different levels of detail in vasculature structures in retina images and relate the morphological variation to variation of dynamic patterns in systems biology molecular networks. Our objective is to endow sufficient accuracy and practicality to enable clinical applications such as distinguishing between diseased and healthy retina through non-invasive imaging and common genomic-metabolomic data collected from patients.

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P-25

VEGF111, a diffusible and resistant-to-degradation variant of VEGF-A, induces the formation of a dense and characteristic network of small functional capillaries in vivo.

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VEGF111 is a newly described variant of VEGFA resulting from an alternative splicing mechanism retaining exons 1–4 and 8a in the mature transcript. It contains the domains interacting with VEGF-R1 and VEGF-R2 and possesses the amino acid residues encoded by exon 8a, which has been suggested to mediate some interaction with neuropilin 1. VEGF111 is remarkably resistant to proteolytic degradation and is expected to be highly "diffusible" due to the absence of sequences of exons 6–7. VEGF111 or its rodent counterpart (VEGF110, analogous to human VEGF111 but lacking one codon) was not found so far in normal human and mice cells and tissues in physiological conditions. It is produced however by dog leukocytes, illustrating some specific regulation of alternative splicing. Its expression can be strongly induced in vitro, ex vivo (hair follicles and surgical tumor specimens in culture) and, to some extent, in vivo by genotoxic stresses as UV irradiation and chemotherapeutic drugs. Recombinant VEGF111 and VEGF165 are being characterized by surface plasmon resonance. Preliminary data demonstrate that both variants bind to VEGF-R2 with a similar efficiency but that only VEGF165 has affinity for neuropilin 1.

In endothelial cells in culture, VEGF111 induces the phosphorylation of VEGFR2 and of two main downstream signaling molecules: ERK1/2 (responsible for the endothelial cell proliferation and angiogenesis) and AKT (regulating cells survival and vascular permeability). However, the levels of induction seem to be lower than the induction observed with VEGF165. When subcutaneously injected in mice, VEGF111 has an increased half-life in the peripheral blood when compared to VEG165 (105 min versus 50 min). Locally, it induces also vascular permeability (Miles and Miles assay) in a larger area than VEGF165, likely due to its highest diffusion from the site of injection. In a model of chronic subcutaneous injection, VEGF111 promotes the formation of a dense and extended network of functional capillaries while VEGF165 induces the formation of a large and localized blood lacuna.

Our data demonstrate that VEGF111 has specific biological properties that could be useful for the treatment or the prevention of ischemic diseases. These results allow us also to hypothesize that the complex formed by VEGF, NRP1, and VEGFR2 is not necessary to induce vascular permeability but might determine the size of the newly formed blood vessels.

P-26

Novel calixarene-based angiostatic and anti-tumor agents

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Galectin-1 (gal-1), which binds ß-galactoside groups on various cell surface receptors, is crucial to cell adhesion and migration and is found to be highly elevated in tumor stroma in several cancers including breast, colon, prostate, and ovarian. Previously, we reported on the designed peptide anginex as a potent antiangiogenic and anti-tumor agent that targets gal-1.

Here, we report on the development of new agents that target gal-1 and thereby inhibit tumor growth in mice. Since small molecules have pharmacological advantages over therapeutic peptides, our partial peptide and non-peptide mimetics were designed to approximate the molecular dimensions, amphipathicity, and cationic topology of the β-sheet-folded anginex peptide. Chemical modification of the hydrophobic and hydrophilic faces of these mimetics resulted in the discovery of new lead compounds that possess in vitro and in vivo activities similar to and better than that of anginex. These studies reveal that small alkyl hydrophobic groups on these compounds are particularly important for biological activity. NMR structural studies indicate that the binding site of these agents is the same and is comprised of hydrophobic groups surrounded by cationic groups on the surface of gal-1 near the ß-galactoside-binding domain.

Overall, this research contributes to our ability to design pharmaceutical agents that target gal-1 and thereby inhibit angiogenesis and tumor growth, for potential use in the clinical setting.

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VEGF induces human endothelial progenitor cells to proliferate by eliciting oscillations in intracellular ca^{2+} concentration

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Circulating endothelial progenitor cells (EPCs) home from the bone marrow to the site of tissue regeneration and sustain neo-vascularization after acute vascular injury and upon the angiogenic switch in solid tumors. Therefore, they represent a suitable tool for cell-based therapy in regenerative medicine and provide a novel promising target in the fight against cancer. Intracellular Ca^{2+} signals regulate numerous endothelial functions, such as proliferation and migration. We have recently shown that EPC growth is governed by a store-dependent Ca^{2+} entry (SOCE) pathway. The present study aimed at investigating the nature and the role of VEGF-elicited Ca^{2+} signals in EPCs. All the putative SOCE mediators (i.e. TRPC1, TRPC4, Orai1 and Stim1) were present in EPCs. VEGF induced long lasting Ca^{2+} oscillations; however, removal of external Ca^{2+} ($0Ca^{2+}$) and SOCE inhibition with BTP-2 reduced the number of Ca^{2+} spikes. Blockade of phospholipase C-γ (PLC-γ) with U73122 and emptying the inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} pools with cyclopiazonic acid (CPA) prevented the Ca^{2+} response to VEGF. Accordingly, the Ca^{2+} response to VEGF was inhibited by superfusing CPA during the ongoing oscillations. Notably, VEGF-induced EPC was abrogated by SOCE inhibition with BTP-2. Similarly, VEGF promoted NF-kB translocation into the nucleus in a BTP-2-sensitive manner. Thus, VEGF causes an initial $InsP_3$-dependent Ca^{2+} discharge followed by SOCE-mediated Ca^{2+} entry in cEPCs. SOCE, in turn, controls store refilling and induces cell proliferation by recruiting NF-kB.

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Angiogenic properties of aged adipose derived mesenchymal stem cells after hypoxic conditioning

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Mesenchymal stem cells derived from adipose tissue (ADSC) are multipotent stem cells, originating from the vascular-stromal compartment of fat tissue. ADSC are used as an alternative cell source for many different cell therapies; however, in ischemic cardiovascular diseases, the therapeutic benefit was modest. One of the reasons could be the use of autologous-aged ADSC, which recently were found to have impaired functions. We therefore analysed the effects of age on age markers and angiogenic properties of ADSC. Hypoxic conditioning was investigated as a form of angiogenic stimulation.
Methods: ADSC were harvested from young (1–3 month), adult (12 month), aged (18 month) and old (24 month) mice and cultured under normoxic (20%) and hypoxic (1%) conditions for 48h. Differences in proliferation, viability, telomere length and oxidative stress were assessed in addition to angiogenic properties of ADSC.
Results: Proliferation potential and telomere length were decreased in aged ADSC compared with young ADSC. Frequency of apoptotic cells was higher in aged ADSC. ROS levels were significantly increased in aged ADSC. Gene expression of pro-angiogenic factors including vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) was down-regulated, and hepatic growth factor (HGF) was up-regulated in adult and aged ADSC. Hypoxia stimulated expression of angiogenic factors, but this effect declined with increasing age. Transforming growth factor (TGF-β) increased in the old ADSC and was reduced by hypoxia. Expression of anti-angiogenic factors including thrombospondin-1 (TBS1) and plasminogen activator inhibitor-1 (PAI-1) was increased in old ADSC, but could be reduced by hypoxic stimulation. We noted higher gene expression of proteases system factors like urokinase-type plasminogen activator receptor (uPAR), matrix metalloproteinases (MMP2 and MMP9) and PAI-1 in aged ADSC compared with young ADSC, but they decreased in old ADSC. Tube formation on matrigel was higher in the presence of conditioned medium from young ADSC in comparison to aged ADSC. Young ADSC stimulated vascularization of Matrigel plugs injected subcutaneously to syngenic mice better than aged ADSC.
Conclusions: ADSC isolated from older animals show changes, including impaired proliferation and ability to stimulate blood vessels growth. Angiogenic gene expression can be partially be improved by hypoxic preconditioning; however, the effect is age-dependent. This supports the hypothesis that autologous ADSC from aged subjects might have an impaired therapeutic potential.

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Lack of primary cilia primes shear-induced Endothelial-to-Mesenchymal Transformation

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Primary cilia are cellular protrusions that serve as mechanosensors for fluid flow in many organs. In endothelial cells (EC), they function in the transduction of information on local blood flow into functional responses, like the production of nitric oxide and the initiation of shear related gene expression. Cilia are present on endothelial cells in areas of low or disturbed flow and absent in areas of high flow. In the embryonic heart, a high flow regime applies to the endocardial cushion area, and the absence of endothelial cilia coincides in time and space with endothelial to mesenchymal transition (endoMT). During this transdifferentiation process, EC loose their endothelial characteristics, gain a mesenchymal phenotype, and migrate into the cardiac jelly to form the primordia of the cardiac valves. In this study, we demonstrate the role of the primary cilium in defining the morphological and functional response of EC to fluid flow and concomitant shear stress, and in EndoMT. Non-ciliated transgenic mouse embryonic EC, mutated in the gene for Tg737/Ift88, were used to compare the response to fluid shear stress and TGFβ ligand stimulation to that of wild-type ciliated EC, in vitro. Non-ciliated EC undergo flow-induced EndoMT that is accompanied by induction of TGFβ/Alk5 signaling and downregulation of the transcription factor Klf4. This process can be prevented by (1) blocking TGFβ receptor kinase activity, (2) induction of Klf4 expression, and (3) by rescue of the ciliated phenotype. Recent in vitro and in vivo data suggest that cilium-dependent intracellular signaling lies at the basis of the predisposition of non-ciliated EC to shear stress-mediated EndoMT. This study provides a functional link between primary cilia and flow-related endothelial performance.

P-30

Identification of a potent, non-toxic adjuvant for use in therapeutic vaccines

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Vaccination has made a crucial contribution to control infectious diseases. Therefore, intense research has been focused on the possibility to use vaccination also as a treatment strategy for non-infectious diseases, such as most types of cancer. Since the targeted antigen is not of foreign origin, but a self-antigen, the self-tolerance of the immune system must be circumvented. This requires an effective vaccination technology as well as a potent adjuvant. In our group, a therapeutic vaccine has been developed, which successfully breaks self-tolerance against the tumour vascular marker extra-domain-B (ED-B) of fibronectin using the recombinant protein TRX-EDB and Freund's adjuvant. However, introduction of therapeutic vaccines to the clinic has been limited by the lack of potent, but non-toxic adjuvants. We previously identified the biodegradable squalene-based Montanide ISA720 combined with phosphorothioate-stabilized CpG oligo 1826 (Montanide/CpG) as an adjuvant able to break tolerance against a self-molecule.

To compare the immunostimulatory properties of Freund's adjuvant (the "golden standard" within vaccine technology) and Montanide/CpG, we have vaccinated two groups of C57BL/6 mice with TRX-EDB in combination with either Montanide/CpG or Freund's adjuvant. Blood samples were collected regularly, and anti-ED-B antibody titres as well as antibody characteristics were analysed with ELISA and a Biosensor system. Comparing the ability of Montanide/CpG and Freund's adjuvant to stimulate an antibody response against ED-B revealed that Montanide/CpG induced anti-ED-B antibody titres with less variation between individual animals than Freund's. Analysis of the IgG isotypes showed that IgG1 was the main isotype in both treatment groups, indicating a TH2-response. However, Montanide/CpG induced antibodies with higher affinity to ED-B. Furthermore, anti-ED-B antibodies in the group treated with Montanide/CpG are not only detectable earlier, but also persist for a longer time than in mice treated with Freund's adjuvant.

With Montanide/CpG, we have identified a non-toxic alternative to Freund's adjuvant, which is at least as potent with respect to inducing an immune response against a self-antigen. This will facilitate introduction of therapeutic vaccines to the clinic.

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P-31

Human mesenchymal stromal cells as pro-angiogenic releasing systems

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The multilineage potential of mesenchymal stromal cells (MSCs) is one of the key reasons for their broad application in tissue engineering (TE). Generally, MSCs are differentiated in vitro prior to implantation using mostly diffusible molecules. Given the current number of available molecules to test, it is conceivable that key molecules will never be tested. Moreover, the complexity of the cocktails used during in vitro differentiation (frequently consisting of recombinant proteins) as well as the costs-associated hamper the current protocols. Upon in vivo implantation, new challenges need to be overcome. One of the key challenges comprises cell survival. Poor vascularization of cell-based constructs has dramatic consequences for large, clinically relevant, cell-based implants. Enhancing vascularization of these constructs will certainly boost the field of TE.

Here, we describe an efficient system to screen for new molecules capable of controlling the angiogenic secretome of MSCs by impinging on the hypoxia inducible factor (HIF) pathway.

A human immortalized MSCs line containing green fluorescence protein (GFP) under control of a hypoxia responsive element (HRE) promoter was generated. Small molecules (1280 molecules) were screened for their capacity to induce HRE activity. In addition, compounds previously described as HRE activators in different cell lines were taken along to analyse cell-specific mechanisms controlling HRE activity. Four compounds were found to enhance HRE activity in MSCs and were further tested in secondary assays to show their pro-angiogenic potential. Of the previously described HRE activators, only two resulted in an increase of HRE activity in MSCs. Our secondary assays showed that the selected molecules increased the expression of angiogenic genes in MSCs. Moreover, using conditioned media from MSCs treated with these small compounds, we showed increase tube formation by HUVECs. We are currently studying the mechanism of action of the selected compounds.

Our system allows us to efficiently screen molecules with pro and/or anti-angiogenic capacity which can later find their way in several tissue-engineering applications as well as in cancer therapies.

P-32

Targeting SRPK1 as a novel approach to suppressing VEGF-mediated ocular neovascularisation and pathological tumour angiogenesis in melanoma cells

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Serine-rich protein kinase 1 (SRPK1) has been identified as a target in controlling the splicing of vascular endothelial growth factor (VEGF). VEGF, a key regulator of tumour angiogenesis and ocular neovascularisation, is alternatively spliced to produce two families of isoforms, proangiogenic $VEGF_{xxx}$ during proximal splice site (PSS) selection and anti-angiogenic $VEGF_{xxx}b$ during distal splice site (DSS) selection. SRPK1 results in the phosphorylation and nuclear localization of alternative splice factor/splice factor 2 (ASF/SF2), a promoter of PSS selection, thus upregulating pro-angiogenic VEGF production. We wished to determine SRPK1 expression, and the effect of SRPK1 inhibition on VEGF splicing and choroidal neovascularisation (CNV).

Primary retinal-pigmented epithelial (RPE) and metastatic melanoma (A375) cells were treated with SRPIN340, an inhibitor of SRPK1 and 2, at 5 and 10 μM. SRPK1 expression and VEGF splice isoform balance were then determined by PCR. Additionally, SRPIN340 was tested in a laser-induced CNV model in Norway Brown rats. Following laser photocoagulation, rats received two intraocular injections of 25 ng SRPIN340 in the ipsilateral eye and saline in the contralateral eye.

SRPK1 was highly expressed in both primary RPE and A375 cells. SRPK1 inhibition, by SRPIN340, altered the balance of VEGF isoforms to favour anti-angiogenic $VEGF_{165}b$ in A375 and attenuate the splicing of pro-angiogenic $VEGF_{165}$ in both A375 and primary RPE. Furthermore, we saw a significant reduction in CNV ($p<0.05$) in our rat model. Targeting SRPK1 has the potential to prevent VEGF-mediated CNV in vivo, and we postulate that it may prevent tumour-associated angiogenic VEGF production in melanomas.

P-33

A simple and robust assay to study endothelial cell function in vivo

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Tumor growth depends on the ability to recruit blood vessels from the host tissue. Correspondingly, angiogenesis has become a major target for antitumor therapy. However, the complexity of the angiogenic cascade limits cellular approaches to studying angiogenic endothelial cells (EC). To overcome this limitation, we developed a reliable and robust in vivo angiogenesis assay that is based on the xenotransplantation of human EC in immunocompromized mice. Implantation of spheroidal endothelial aggregates in a Matrigel/fibrin matrix resulted in the formation of a complex three-dimensional network of human neovessels. Implants were dissected after 20 days and analyzed for microvessel density (MVD), mural cell recruitment, and perfusion. The assay provides unique opportunities to perform studies in the field of vascular research. First, EC can be manipulated ex vivo prior to implantation for GOF and LOF studies to investigate vascular function. Downregulation of Ang-2 or PDGF-BB in HUVEC led to a significant reduction of MVD. Loss of Ang-2 or PDGF-BB in HUVEC resulted in the reduction of α-smooth muscle actin (α-SMA) coverage. However, Desmin coverage was only increased in Ang-2 silenced HUVECs. Second, EC can be manipulated to mimic pathological conditions. Toward this end, we generated HUVECs lentivirally silenced for CCM1, one of the three genes causally involved in the formation of Cerebral Cavernous Malformations. CCM1 LOF led to the formation of hypervascularized vascular networks mimicking human CCM lesions. Third, HUVEC can be co-grafted with other cell types to explore intercellular communication during vessel formation. Co-implantation of HUVEC with pericytes led to an increase in vessel density and perfusion. α-SMA and Desmin coverage were also increased. Fourth, implantation of lymphatic endothelial cells allows the investigation of the lymphangiogenic potential of different LEC populations and the mechanism of LEC differentiation. Implantation of LEC spheroids led to the formation of CD31-positive vascular structures. Electron microscopy revealed typical lymphatic structures with intercellular gaps and numerous endocytotic vesicles. In conclusion, the EC spheroid transplantation assay offers numerous applications for the field of vascular research and beyond. The simplicity and robustness of the assay promise to make it a versatile tool to study EC functions and to improve the development of anti-angiogenic therapies.

P-34

Radiation-amplified drug delivery to the angiogenic tumor vasculature via Galectin-1

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In collaboration with the radiochemistry facility at our institution, we developed a method to label the anti-angiogenic peptide anginex with [18]-F in order to assess its binding and distribution in the body via its receptor on endothelial cells, Galectin-1. Initial work with this tracer in our model of focal myeloma for radiation and anti-angiogenesis research showed a vibrant and specific targeting of the tumor/bone graft. Further work to define the receptor profiles of myeloma and other tumor types is ongoing. The development of the [18]-F-labeled anginex tracer for PET-imaging has allowed us to assess changes of in vivo tumor uptake after a typical clinical radiation dose of 2 Gy. While in vitro work demonstrated that Galectin-1 expression was indeed upregulated in endothelial cells after radiation, we also found an induction of anginex uptake after radiation in SCK tumors in vivo using the microPET imaging strategy. Radiation exposure increased tumor uptake by an average of 141 ± 49% which would be considered a substantial increase clinically (significance is typically regarded as a 30% change in standardized uptake value in solid tumors).

We subsequently developed a liposomal formulation including fluorescently tagged lipids and a covalent linkage using maleimide of anginex on the surface to determine the potential of guiding drugs to the tumor via radiation-amplified Galectin-1 target expression. Our initial studies with 'anginex-tagged' liposomes indicate significant binding and uptake of the carrier by endothelial cells in culture and an

increase in the binding of the anginex-tagged liposomes specifically to endothelial cells after exposure of the cells to 2–4 Gy, supporting the observations made with our PET-imaging results before and after tumor irradiation. Non-tagged liposomes exhibit little to no binding to either cell type before or after radiation. We also observed that adding either free anginex or anti-Galectin-1 antibody to the cells along with the targeted liposomes blocked the uptake by nearly 50% in general and specifically blocked the radiation-induced uptake observed in endothelial cells, suggesting a Galectin-1-mediated pathway for liposome binding.

Answering the question of why and how anginex uptake selectively increases in endothelial cells after irradiation and determining what connection this has to the radiation-sensitivity of endothelial cells vs. tumor cells exposed to anginex is our current focus. We plan to expand our studies to incorporate doxorubicin chemotherapy into these liposomes and fully characterize the in vivo targeting and therapy potential in our models in combination with radiation exposure.

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P-35

A critical requirement for heparan sulphate activation of vascular endothelial growth factor to mediate angiogenesis

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Angiogenesis is a fundamental process of blood vessel growth. Two key factors involved in angiogenesis are vascular endothelial growth factor (VEGF) and heparan sulphate (HS) which is a ubiquitous pericellular polysaccharide component of proteoglycans. The interaction of these via the specific sulphation patterns of HS is critical for the spatial distribution of VEGF and to regulate VEGF-mediated activation of endothelial cells. Normal regulation of angiogenesis appears to be lost in a number of diseases such as cardiac ischemia and cancer. It is hypothesised that a possible therapy for the treatment of these diseases could be the addition of short HS oligosaccharides that would interact with VEGF, affect receptor signalling and thus modify angiogenesis.

To determine the likely success of this therapy, the requirement for HS:VEGF interaction in angiogenesis in vivo is under investigation. $VEGF_{165}$ DNA plasmids coding for either wild-type human $VEGF_{165}$ or $VEGF_{165}$ that contained several mutations within its HS binding domain were injected into single-cell zebrafish (*Danio rerio*) embryos. The subintestinal baskets of the embryos were used as an easily measurable assessment of angiogenesis. By 72 h, native $VEGF_{165}$ had induced a 5-fold increase in total ectopic vessel length (One-way Anova; $p < 0.001$), and a 50% increase in number of ectopic vessels in the baskets ($p < 0.05$). This effect was not seen in HS-mutant-injected embryos or controls which suggested that the interaction between HS and VEGF is vital for angiogenesis in vivo. We are currently investigating the angiogenic capacity of several other $VEGF_{165}$ mutants that have differing affinities for HS.

The specific sulphation patterns of HS confer its ability to bind to proteins such as VEGF. In order to manipulate this interaction in vivo, a more complete understanding of this specific structure is required. We investigated the VEGF binding capacity of a series of synthetic HS mimetics using surface plasmon resonance (Biacore). We determined the structure of HS that binds strongly to VEGF consists of a relatively unsulphated hexa/octa-saccharide domain flanked by two highly sulphated octasaccharide domains. Further characterisation of HS from a VEGF affinity assay suggested that these highly sulphated octasaccharide domains are rich in 6-O sulphation and contain less 2-O and N-sulphation. We are currently investigating the ability of these strongly VEGF binding HS mimetic to affect angiogenesis in vivo.

P-36

Tumor angiogenesis betrays breast cancer to near-infrared photoacoustic imaging

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The angiogenic phenomenon associated with tumor growth has implications for detection and diagnosis of tumors using near-infrared light. Enhanced blood content in tumor vascularization provides an optical absorption contrast compared with non-pathological tissue [i]. While research into purely optical breast imaging has shown that detection of tumors based on optical absorption is possible, the techniques suffer from poor resolution due to high light scattering [ii]. Photoacoustic imaging is a hybrid technique based on ultrasound generated by short light pulses. It brings together advantages of optical contrast in optical imaging on the one hand, and lower scattering experienced by ultrasound in tissue on the other hand.

A first pilot study with the Twente Photoacoustic Mammoscope (PAM), which was performed in 2006, showed the feasibility of this technique. Of the 5 technically acceptable patient measurements, regions of higher optical absorption were clearly identified in 4 cases. Regions as deep as 26 mm were visible, and the results correlated well with conventional radiological images and with histopathological results [iii].

We recently started a new clinical study using PAM. In this study, patients with symptomatic breasts are examined using conventional X-ray mammography and breast sonography. Before a biopsy is performed, the region-of-interest in the breast is imaged in a craniocaudal view using PAM. Pulsed light of wavelength 1064 nm and pulse duration 5 ns is used. Reconstructed images are compared with the conventional radiological images. The results are analyzed in the light of pathology of resected tumors in order to define photoacoustic markers that are indicative for the presence of an increased tumor vascularization. Moreover, the results are used to guide developments in PAM in order to pave the way toward an optimal technique for early diagnosis of breast cancer. We review the results of our first study and discuss details of the new study.

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S. Manohar, et al., Initial results of in vivo non invasive cancer imaging in the human breast using near-infrared photoacoustics, Optics Express 2007, 15(19), pp.12277:12285

P-37

Loss of mitochondrial thioredoxin reductase limits tumor growth

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Hypoxia is a key driver of tumor angiogenesis. While the roles of HIF1α and prolylhydroxylases (PHDs) in oxygen sensing and tumor angiogenesis have been well established, little is known regarding a possible involvement of mitochondrial reactive oxygen species (ROS) in tumor progression. The mitochondrial thioredoxin system consists of thioredoxin, thioredoxin reductase (Txnrd2), and peroxiredoxins and is one of the primary antioxidant defense systems controlling mitochondrial ROS. Since ROS are considered to be implicated in multiple steps during tumorigenesis, we examined whether loss of Txnrd2 may affect tumor growth and tumor angiogenesis. Here, we show that in vitro cultured *c-myc* and *ha-ras* transformed Txnrd2 null fibroblasts have a strongly impaired clonogenic potential compared to wild-type control cells. When transplanted subcutaneously into mice, tumor growth was reduced by as much as 50%. Txnrd2 knockout tumors displayed a delayed angiogenic switch, a strongly diminished tumor vascularization, and impaired tumor perfusion. Knockout tumors revealed decreased HIF1α and vascular endothelial growth factor (VEGF) levels. Transformed Txnrd2 knockout cells failed to stabilise HIF1α in response to starvation in vitro, pointing to an important role of Txnrd2 in HIF1α regulation. When tumor-bearing mice were deprived from intracellular glutathione by pharmacological inhibition of glutathione synthesis, Txnrd2 knockout tumors showed a further reduction in tumor growth. In summary, these studies reveal that mitochondrial ROS metabolism has a major influence in tumor growth and tumor angiogenesis, providing a potential therapeutic rationale to hinder tumor growth.

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P-38

A limited role for regulatory T cells in post-ischemic neovascularisation

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Background. Recently, it was demonstrated that arteriogenesis is enhanced in mice deficient for regulatory T cells ($CD4^+CD25^+FoxP3^+$ T cell), which can suppress effector T-cell responses. The present study investigates the effects of these regulatory T cells on arteriogenesis in more detail by either specific expanding or depleting regulatory T cells.
Methods and materials. Hind limb ischemia was induced by electrocoagulation of the femoral artery in mice. Regulatory T cells were either expanded by injecting mice with a complex of interleukin (IL)-2 with the IL-2 monoclonal antibody JES6-1, or depleted by anti-CD25 antibody or diphtheria toxin (DT)-injections in DEREG mice (DEpletion of REGulatory T cells). Blood flow restoration was monitored using Laser Doppler Perfusion Imaging. Collateral arteries were visualised by immuno-histochemistry.
Results. Regulatory T-cell expansion led to a moderate though significant suppression of blood flow restoration after ischemia induction. Surprisingly, depletion of regulatory T cells resulted in minor increase on blood flow recovery. However, collateral and capillary densities in the post-ischemic skeletal muscle were significantly increased in DEREG mice depleted for regulatory T cells. The presence of regulatory T cells after ischemia induction when analyzed in non-depleted DEREG mice could be demonstrated by GFP staining only in lymph nodes in the ischemic area, and not in the ischemic muscle tissue.
Conclusion. The current study demonstrates that, even under conditions of major changes in regulatory T cell content, the contribution of regulatory T cells to the regulation of the arteriogenic response is only moderate.

P-39

A limited role for regulatory T cells in post-ischemic neovascularization

AA Hellingman[1], LEPM van der Vlugt[2], MA Lijkwan[1], AJNM Bastiaansen[1], T Sparwasser[3], HH Smits[2], JF Hamming[1], PHA Quax[1,4]

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Conclusion. The current study demonstrates that, even under conditions of major changes in regulatory T-cell content, the contribution of regulatory T cells to the regulation of the arteriogenic response is only moderate.

P-40

Variations in surgical procedures for hind limb ischemia mouse models result in differences in collateral formation

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Objective. To identify the optimal mouse model for hind limb ischemia which offers a therapeutic window that is large enough to detect improvements of blood flow recovery e.g. using cell therapies.
Materials and Methods. Different surgical approaches were performed: single coagulation of femoral and iliac artery, total excision of femoral artery, and double coagulation of femoral and iliac artery. Blood flow restoration was analyzed with Laser Doppler Perfusion Imaging (LDPI). Immuno-histochemical stainings, angiography, and micro-CT-scans were performed for visualisation of collaterals in the mouse.
Results. Significant differences in flow restoration were observed depending on the surgical procedure. After single coagulation, blood flow already restored 100% in 7 days, in contrast to a significant delayed flow restoration after double coagulation (54% after 28 days, $P < 0.001$). After total excision, blood flow was 100% recovered within 28 days. Compared to total excision, double coagulation displayed more pronounced corkscrew phenotype of the vessels typical for collateral arteries on angiographs.
Conclusion. The extent of the arterial injury is associated with different patterns of perfusion restoration. The double coagulation mouse model is in our hands the best model for studying new therapeutic approaches since it offers a therapeutic window in which improvements can be monitored efficiently.

P-41

T-cell-pre-stimulated monocytes promote neovascularization in a murine hind limb ischemia model

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Aim. Monocytes play a significant role in neovascularization. The stimuli that differentiate monocytes along a pro-angio/arteriogenic-supporting pathway are currently unclear. We investigated whether pre-stimulation of human monocytes with soluble T-cell-derived factors improves re-vascularization in murine hind limb ischemia as a new option for therapeutic angio- and arteriogenesis.
Design. Human monocytes were cultured with or without soluble T-cell-derived factors. Unstimulated and pre-stimulated monocytes were transfused after induction of hind limb ischemia in nude mice.
Methods. Blood flow was measured with Laser Doppler Perfusion Imaging. Collaterals were visualized by immunohistochemistry and angiography. Monocytes were characterized by flowcytometry and Bio-plex assays.
Results. Transfusion of T-cell-pre-stimulated monocytes significantly improved blood flow recovery after hind limb ischemia and increased collateral size and collateral and capillary number in the post-ischemic paw. Pre-stimulated monocytes produced a wide variety of factors that support neo-vascularization such as platelet-derived growth factor-BB, vascular-endothelial growth factor, interleukin-4, and tumor necrosis factor-α. Few transfused human cells were detected in the muscle tissue, suggesting that paracrine rather than direct effects appears responsible for the enhanced recovery of bloodflow observed.
Conclusion. These results show a beneficial role for T-cell-pre-stimulated monocytes in neovascularization, rendering the monocyte a potential candidate for regenerative cell therapy that promotes revascularization in peripheral arterial disease patients.

P-42

Network formation of endothelial precursor cells in a fibrin matrix

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Vascularization of engineered tissues is one of the current challenges in tissue engineering. Several strategies aim to generate a prevascularized scaffold which fulfils the requirements for being implanted at sites of injury or trauma. Endothelial precursor cells (EPCs) and outgrowth endothelial cells (OECs) display promising features for the prerequisites of vascularization, like proliferating capacity, capability to migratory stimuli and the ability to form mature vessels. In this study, we investigated several characteristics of OECs embedded in fibrin gels. Using flow cytometry and immunofluorescence, we show that OECs derived from peripheral blood displayed molecular markers typical for endothelial cells, like CD34, KDR, VE-Cadherin and Tie-2. Furthermore, these cells lack monocytic and hematopoietic markers CD14 and CD45. Matrigel assays revealed the tube forming ability of these cells. As a model, we attached OECs to a channel in fibrin gels and found ingrowth into the surrounding matrix. Furthermore, using Cytodex-bead-coated OECs, we found migration through

low concentration fibrin gels and the formation of vessel-like structures. This effect is even enhanced in co-cultures with human foreskin fibroblasts. Our studies should lead to a better understanding of endothelial tube formation in fibrin gels.

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P-43

The soluble epoxide hydrolase is required for normal retinal angiogenesis

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Epoxyeicosatrienoic acids (EETs), which are generated by the cytochrome P450 (CYP) from arachidonic acid and mainly metabolized by the soluble epoxide hydroylase (sEH) to their corresponding diols, are important anti-hypertensive and anti-inflammatory mediators in the cardiovascular system. Recent studies have shown that the EETs are also involved in the endothelial cell proliferation and migration. We investigated the role of CYP/sEH pathway in the regulation of vascularization in the retina.

The retinal angiogenesis in the $sEH^{-/-}$ mice and their wild-type littermates (WT) was investigated at different time points by using immunofluorescence and confocal laser scanning microscopy. Immunohistochemistry was used for the localization of the sEH. In the retina, sEH protein expression was detected after birth and increased in the first postnatal month and also demonstrated a high level of activity compared to kidney and lung. Retinal vascularisation was markedly delayed in $sEH^{-/-}$ mice at postnatal day (P) 2 and P5 and associated with reduced tip cell numbers and filopoida extension at the angiogenic front (P5). Moreover, endothelial cell proliferation assessed using BrdU incorporation was also attenuated in $sEH^{-/-}$ mice. Superficial vessel network complexity and deeper vessel formation were also significantly suppressed in the $sEH^{-/-}$ pups at P9. This phenotype could be reproduced in WT mice by giving them an sEH inhibitor. We found that the sEH is highly expressed in Müller cells and astrocytes that are the two types of glia cell in the retina. Therefore, we used cell-specific knockout mice to investigate in detail the function of sEH in different glia cells. The astrocyte-specific sEH knockout mice exhibited a significant reduction in vessel density at P5 and P7. Whereas the Müller cell-specific sEH knockout mice displayed a reduced radial expansion of retinal vasculature at P2 and P5 as compared to controls. Moreover, conditioned medium from wild-type Müller cells was able to stimulate significantly more endothelial cell proliferation compared to $sEH^{-/-}$; the proliferation was abolished by a VEGF neutralizing antibody. qRT-PCR analysis of retina extracts showed that a consistent induction of Notch-dependent transcription factors Hes1 and Hey1 in $sEH^{-/-}$ mice.

Taken together, these data indicate that the CYP/sEH cascade is required for normal retinal vascularisation, and this involvement seems to depend on the sEH-regulated angiogenic factors from glia cells which result in an induction of Notch activity.

P-44

Local regulation of the VE-cadherin complex through Vinculin recruitment

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Endothelial cell–cell adhesion is tightly regulated during processes such as inflammation, transendothelial migration, and angiogenesis. To regulate cell–cell adhesion, the VE-cadherin complex, a central component of endothelial junctions, is targeted by many angiogenic growth factors and inflammatory cytokines. It is likely that molecular changes in the VE-cadherin complex underly the remodeling or stabilization of cell–cell junctions. We find that vinculin specifically associates with VE-cadherin at a subset of cell–cell junctions that are directly connected to radial actin bundles. Live imaging experiments further demonstrate that the radial actin bundle-connected junctions are remodeled and subject to cytoskeletal-derived pulling forces. The connection of VE-cadherin to radial actin bundles and vinculin recruitment strictly depends on actomyosin contractility. Vice versa, thrombin-induced signaling, which raises actomyosin contractility, rapidly promotes the recruitment of vinculin to VE-cadherin-based junctions. Finally, by measuring transendothelial resistance, we find that vinculin is important for maintaining the barrier function of endothelial monolayers. Our data strongly imply that forces derived from the actomyosin cytoskeleton regulate vinculin recruitment to the VE-cadherin complex, which is important for the remodeling of cell–cell junctions by endothelial permeability factors and angiogenic growth factors.

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P-45

Inhibition of notch signalling induces intussusceptive angiogenesis by recruitment of mononuclear cells

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The formation of blood vessels during angiogenesis is a result of tight coordination of cell proliferation, differentiation, migration, matrix adhesion and cell–cell cross talk. Notch-signalling is an intercellular pathway, which plays a central role in the establishment of patterns of gene expression, defining cell fate during development and angiogenesis. Because of ubiquitous role of the Notch receptor, we assumed that it interferes with the intussusceptive mechanism of angiogenesis. Here, we have studied the effects of inhibiting Notch by the highly potent γ-secretase inhibitor (GSI) on vascular development using the chick area vasculosa (CAV), a model with rapidly growing vasculature.

Our results indicated that Notch inhibition leads to formation of an immature capillary network by i) activating intussusceptive angiogenesis through robust induction of pillar formation which results in increased capillary density by more than 50%; ii) detachment of pericytes from endothelium followed by extravasation of mononuclear cells. The latter are recruited most likely to the growing transluminaly tissue pillars, i.e. contribute to intussusceptive angiogenesis; iii) relatively retardation in arterial tree formation corresponding to reduction of arterial length and diameter in a range of 30–40%. The observed effects were dependant on developmental stage, applied dosage and the treatment protocol. The morphologic alterations were associated with marked downregulation of EphrinB2 transcriptional and αSMA protein levels 24 h after GSI treatment. On transcription levels, VEGFR1 demonstrated significant elevation in spite of the expression profiles of VEGFR2, bFGF and PCNA, which increased of about 2 times on average but without reaching statistically significant level.

Notch inhibition caused dramatically extravasation of mononuclear cells into the perivascular space. In order to investigate the recruitment pattern, injection of fluorescently traced mouse bone-marrow-derived cells after Notch inhibition revealed spectacular induction of intussusception 4h after injection by utilisation of traced cells.

All our observations assumed that Notch inhibition destroys vessel stability by disturbing maturity and alignment of pericytes. This effect is followed by invasion of progenitor cells in perivascular space, participating actively in the process of intussusceptive vascular growth.

Our study is considerable progress in understanding of cellular and molecular events in intussusceptive angiogenesis. It also provides with data for molecular modulation of angiogenesis with medical relevance.

P-46

Expression of placental *FLT1* transcript variants relates to both gestational hypertensive disease and fetal growth

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Hypertension-related disorders are a major cause of pregnancy complications. In particular, preeclampsia, defined as new-onset hypertension in combination with proteinuria after 20 weeks' gestation, is a leading cause of both maternal and fetal mortality and morbidity. The vascular endothelial growth factor (VEGF) ligands/receptors play an essential role in both normal and pathological functioning of the endothelium and have been implicated in the development of preeclampsia. In particular, the soluble truncated version of VEGF receptor 1 (sFLT1) has been shown to be markedly elevated in women that suffer from preeclampsia.

The recent discovery of additional alternative spliced FLT1 transcripts that encode novel soluble (s)FLT1 protein isoforms complicates both the predictive value and functional implications of sFLT1 in preeclampsia. We investigated the FLT1 expression level and splicing pattern in placentas of normotensive and preeclamptic women, and we established the tissue specificity of all FLT1 transcript variants. For that purpose, mRNA levels of sFLT1 splice variants were determined by real-time PCR in 21 normal human tissues and placental biopsies from 91 normotensive and 55 preeclamptic women. The cellular localization of placental FLT1 expression was established by RNA in situ hybridization.

Of all tissues investigated, placenta has by far the highest FLT1 mRNA expression level, mainly localized in the syncytiotrophoblast layer. Over 80% of placental transcripts correspond to the sFLT1_v2 variant. Compared to normotensive placenta, preeclamptic placenta has about 3-fold higher expression of all FLT1 transcript variants ($P<0.001$), with a slight shift in favor of the sFLT1-v1 variant. Transcript levels are also increased in placenta from normotensive women that deliver a small for gestational age neonate.

From the data, we conclude that the use of sFLT_v2-specific assays could potentially improve the accuracy of the current sFLT1 assays for the prediction of preeclampsia. However, placental FLT1 transcript levels are increased not only in preeclampsia but also in normotensive pregnancy with a small for gestational age fetus. This may indicate a common pathway involved in the development of both conditions, but complicates the use of circulating sFLT1 protein levels for the prediction or diagnosis of preeclampsia.

P-47

Influence of macrophage subtypes on angiogenesis

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Background. Angiogenesis, the formation of new blood vessels from preexisting vessels, is not only an essential process in embryonic development and wound healing but also in pathological conditions like atherosclerosis. Newly formed vessels in the atherosclerotic plaque can serve as an entry site for macrophages that promote the progression of atherosclerosis and affect plaque stability. Activated macrophages play a key role in generating signals for induction of angiogenesis in the atherosclerotic plaque.

Specific macrophage subsets have been identified, and based on their characteristics, they can generally be subdivided into M1 and M2 macrophages. Whereas M1 macrophages are activated by microbial agents and IFNγ (classically activated, type I response), M2 macrophages are activated by cytokines such as IL-4, IL-10, and IL-13 (alternative activation, type II response). As the anti-inflammatory M2 macrophage expresses angiogenic growth factors, we hypothesize that this subset enhances angiogenesis.

Methods. To study the effect of macrophage subtypes on angiogenesis, we used the in vivo Matrigel plug assay. C57Bl/6 mice ($n = 48$) were s.c. injected with Matrigel ($\pm$bFGF). Bone marrow was isolated from donor mice and cultured for 8 days into differentiated macrophages, followed by 24-h polarization with IFNγ, IL-4, or IL-10. Prior to injection, the different macrophage subtypes were mixed with the Matrigel. Plugs were recovered from mice after 14 days. We performed stainings for CD31 (PECAM-1) and Moma-2 to quantify endothelial cell and macrophage content.

Results. Adding bFGF resulted in an increase of endothelial cells in the plug. There was no additional effect of M0 and M1 macrophages on endothelial cell influx. However, more endothelial cells migrated into the plug under the influence of M2a and M2c macrophages and formed tube-like structures. Also, more macrophages were present in the plugs when supplemented with bFGF. Overall, there was no difference in the amount of macrophages between the different groups.

Conclusions. In vivo, M2 macrophages are more pro-angiogenic compared to other macrophage subtypes. This is probably an effect of functional differences between macrophage subtypes.

P-48

Diaphanous-related formin 2 induces firm adhesion of endothelial cells, thereby attenuating cell migration and increasing resistance to shear flow

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Absence of shear flow at arterial bends and bifurcations induces atherosclerotic lesions. Krüppel-like factor 2 (KLF2) is a major effector of beneficial effects of shear stress on endothelial cells by

suppressing AP1 and NFkB signalling and was shown to induce an anti-angiogenic phenotype. KLF2 expression also induces the formation of distinct actomyosin structures in endothelial cells important for cell function. Small GTPases such as Rho, Rac, Cdc42 and the diaphanous-related formin (DRF) family of proteins are important molecules in actin dynamics. In this study, we set out to explore the role of the DRF family of proteins in the formation of KLF2-induced cytoskeletal rearrangements.

Human umbilical vein endothelial cells (HUVECs) grown on fibronectin-coated coverslips were transduced with KLF2-expressing lentiviral vectors or exposed to pulsatile laminar shear flow. Consequently, we induced a DRF2 knockdown in these cells by transduction with lentiviral vectors expressing a shRNA against DRF2. The cells were fluorescently stained for focal adhesion proteins and analysed by 3-colour immunofluorescence microscopy. Effects on signalling and transcriptome were determined by quantitative Western blotting and real-time PCR. Effects on cell motility were assessed by life cell imaging.

Endothelial cells grown under shear conditions or overexpressing KLF2 show distinct actin shear fibres, which are thick actin cables connected at both ends to focal adhesions running across the basal membrane of the cells. Inclusion of a shRNA against the well-known DRF family members DRF1 or DRF3 did not affect KLF2-induced expression of shear fibre structures. In contrast, specific knockdown of DRF2 completely abrogated KLF2-induced and shear-induced actin fibre structures.

Endothelial cells grown under shear conditions align in the direction of the flow in a KLF2-dependent manner. Endothelial cells expressing a shRNA against DRF2 align better to the direction flow when compared to control endothelial cells, suggesting a less firm adhesion of endothelial cells lacking shear fibre structures. To further explore the effects of shear fibre structures on cell–matrix adhesion, cell motility analysis was performed on statically grown endothelial cells overexpressing KLF2. Endothelial cells overexpressing KLF2 show an almost absence of cell motility when compared to control cells, which was rescued by silencing DRF2.

This study gives new insights in the role of actin cytoskeletal rearrangement in flow-exposed or KLF2-expressing endothelial cells as cellular 'anchors'. Our results show DRF2 to be the cellular mediator of these effects, suggesting a novel role of DRF2 as anti-angiogenic protein.

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P-49

Neovascularization in the intracoronary thrombi of patients with ST-segment elevation myocardial infarction (STEMI)

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Aim. ST-segment elevation myocardial infarction (STEMI) is usually caused by occlusive intracoronary thrombus, of which the majority is preceded by repeated apparently unsuccessfully healed plaque disruptions. We investigated neovascularization as a potential contributor to the process of thrombus organization/healing in coronary thrombectomy materials of STEMI patients.

Material and methods. Thrombectomy materials obtained from STEMI patients were histologically classified according to thrombus age in three groups: fresh (<1 day), lytic (1–5 days) or organized (>5 days) thrombi. Forty thrombi of each group were randomly collected. Neovascularization in thrombi was evaluated with use of immunodouble stains: CD31/CD34, CD105/CD34, Ki67/CD34, C-kit/CD34 and C133/CD34.

Results. Morphologically, endothelium in the intracoronary thrombi manifested as: single ECs, clusters or distinct microvessels. Ninety-eight percentage of all the thrombi contained $CD31^+/CD34^+$ endothelial cells. Significantly, more ECs (representing single cells, cluster and microvessels) were observed in organized thrombi compared with fresh thrombi ($P<0.05$). $CD105^+$, $Ki67^+$ or C-kit^+ ECs (active, proliferating cells) were present in all the stages, but significantly more in organized thrombi and mainly as clusters ($P\leq0.05$ for all). In contrast, $CD133^+$ ECs (primitive cells/EPCs) were found only sporadically in all groups.

Conclusion. Endothelial cells contribute to initiation and progression of thrombus organization after plaque rupture. The role of stem cells appears to be limited in this process.

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P-50

Critical role of adventitial macrophages and their cytokine product TNF in the ex vivo angiogenic response of aortic explants

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The rat/mouse aortic ring model is one of the most commonly used methods to study angiogenesis and its mechanisms. Aortic angiogenesis mimics many stages of the angiogenic response to injury in an ex vivo setting which can be easily monitored and analyzed. We used microarray analysis and qRT-PCR to identify early events occurring in the aortic wall prior to angiogenic sprouting in a collagen matrix. Microarray analysis of aortic cultures demonstrated upregulated expression of inflammatory chemokines and cytokines in 24-hour-old cultures. Time course qRT-PCR studies identified the macrophage-derived cytokine TNF among the genes upregulated within 10 min after embedding the aortic rings in collagen gel. Rapid overexpression of TNF was followed by progressive increase in VEGF expression during the first 24 h and by endothelial sprouting after 48–72 h. Treatment with exogenous TNF or VEGF markedly enhanced the angiogenic response. The proangiogenic effect of TNF was associated with increased outgrowth of CD45+ CD68+ resident macrophages from the aortic adventitia. Experiments with anti-VEGF blocking antibody showed that the TNF stimulatory effect was in part mediated by increased production of VEGF. Pharmacologic ablation of macrophages from aortic explants markedly inhibited VEGF production and angiogenesis. Angiogenesis and VEGF production in macrophage-depleted aortic cultures were restored by exogenous macrophages. Experiments with aortic rings from genetically modified mice showed that aortic angiogenesis was markedly impaired by disruption of the TNF or TNFR1 genes. These studies indicate that aortic angiogenesis is preceded by widespread activation of the resident immune system and suggest that adventitial macrophages and their cytokine product TNF play a critical role in the early mechanisms responsible for the initiation of angiogenesis following injury of the vessel wall.

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P-51

Semaphorin 3A blocks tumor invasiveness and prevents resistance to anti-angiogenic therapies by overcoming cancer hypoxia

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Class 3 Semaphorins (Sema3) are implicated in the regulation of both physiological and tumor angiogenesis. We have recently shown that endothelial Sema3A is an endogenous inhibitor that is lost during tumor progression and its reintroduction into a mouse model of pancreatic islet b-cell carcinogenesis (RipTag2), resulted in reduced vascular density, blood vessel normalization, restoration of tumor normoxia, and inhibition of tumor growth (1).

Here, we show that the treatment of tumor-bearing RipTag2 with exogenous Sema3A induced a dramatic reduction of tumor invasiveness, the reappearance of E-cadherin, and a down-modulation of vimentin, two known targets of cancer hypoxia that are also regulated during epithelial-mesenchymal transition (EMT). Then, we sought to investigate whether the preventive administration of exogenous Sema3A was able to overcome the evasive resistance observed in RipTag2 upon treatment with Sunitinib, an anti-angiogenic agent that inhibits several tyrosine kinase receptors (2). Notably, we observed a dramatic reduction of tumor volume, cancer invasiveness, liver and peripancreatic lymph node metastases in RipTag2 treated for 2 weeks with Sema3A followed by 2 weeks of Sunitinib treatment, compared to Sunitinib-treated controls. Moreover, while Sunitinib-treated tumors were highly hypoxic and displayed few pericyte-covered vessels, the combinatorial regimen of Sema3A and Sunitinib normalized the vasculature and restored tumor normoxia. Finally, real-time RT-PCR and confocal microscopy analysis revealed a strong increase of E-cadherin expression and a complete inhibition of vimentin in tumors treated with Sema3A and Sunitinib when compared to Sunitinib alone.

Therefore, re-expression of Sema3A in tumors may safely harness the therapeutic potential of anti-angiogenic drugs, by normalizing the vasculature, inhibiting tumor hypoxia, and modulating the expression of EMT markers and other hypoxic-induced genes activated by anti-angiogenic treatments.

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P-52

Therapeutic angiogenesis in skeletal muscle: combining gene and cell therapy for gene delivery

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Induction of angiogenesis in ischemic tissue is an important therapeutic goal. It can be achieved by gene transfer of angiogenic growth factor genes or by cell therapy using wide variety of cell types. In most cases, such therapies show high efficacy in animal studies yet lacking long-term tissue protection in clinical trials thus indicating the need for development of new approaches in therapeutic angiogenesis.

The first emerging solution is application of new vectors. In our study, we have tested a panel of constructs based on a novel pC4W plasmid that is characterized by higher in vitro protein output compared to pcDNA3 used as reference.

Next step, we tried to use gene transfer of several therapeutic genes to enhance angiogenic response in tissue. We cloned cDNAs of human VEGF165, HGF, angiopoietin-1, and mouse urokinase to pC4W and tested efficacy of sole genes or their pair combinations in murine hind limb ischemia model. We found that gene transfer of VEGF+HGF, VEGF+ANG1, or ANG1+HGF amplified limb perfusion compared to sole vectors yet uPA combinations failed to show synergistic interaction. Our findings were confirmed by histological studies and showed reduced necrosis, higher CD31+ and SMA+ density in combined gene transfer groups.

Next widely used approach is cell therapy using adipose-derived stromal cells due to their feasibility and well-characterized tissue-protecting properties. In our study, we tried to use gene-modified ADSC overexpressing human VEGF165. Overexpression was induced by rAAV2-mediated transduction of human ADSC taken from different donors during surgery. In vitro tests confirmed stable and potent secretion of VEGF165 for at least 30 days after transduction.

Further in vivo studies revealed that VEGF-expressing ADSC induce higher vascularization of Matrigel plug in nude mice compared to unmanipulated ADSC or GFP-transduced cells. Vessel density (identified by CD31 and SMA staining) was higher in Matrigel plugs from VEGF-ADSC animals indicating VEGF-driven formation of mature arterioles.

Hind limb ischemia model test showed increased limb perfusion after injection of VEGF-ADSC compared to controls, and furthermore, we found that VEGF-ADSC-treated mice had higher perfusion than animals that received VEGF-carrying plasmid.

Thus, we can conclude that combined gene transfer and gene-modified cell therapy can be used to enhance angiogenic response in ischemic tissue and can present an attractive target for further translation after appropriate safety tests.

P-53

Human endothelial Cell lines with an In Vivo physiology

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Cells reflecting in vivo properties are of high interest for modern life sciences. Existing cellular models are either lacking biological relevance or are not available in sufficient amounts.

We have developed a flexible cell expansion strategy that allows strict and reliable control of cell proliferation. The system is based on transcriptional control of expansion genes. Using this system, we established novel endothelial cell lines. The key feature of these cell lines is that their proliferation status can be strictly controlled. The system is flexible, and regulation is easily achieved which is the prerequisite for many applications.

Furthermore, these cell lines express endothelial specific markers, display properties of endothelial cells like eNos activity or tube formation on Matrigel. To further test the biological relevance of these cells, transplantation studies in mice were performed. We find that these cells form fully functional blood vessels: These newly formed vessels are interconnected with the host blood vessel system revealing that the cell lines fully retain their angiogenic potential.

We envision the controlled cell expansion strategy presented here to provide biological relevant cells in sufficient numbers needed e.g. for drug discovery, ADME/Tox testing and in the long term for regenerative medicine approaches.

P-54

Platelet-derived growth factor (PDGF) and PDGF receptor beta expression correlates with lymphatic vessels but not with blood vessels density in carcinoma of the uterine cervix

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Background. Uterine cervix carcinogenesis is driven by both phenotype of tumor cells and tumor microenvironment. Angiogenesis and lymphangiogenesis have been shown to contribute to the local growth and spread of tumor cells, and these processes are orchestrated by specific growth factors and their receptors. Although many studies evaluated mainly the contribution of VEGF family to tumor progression, the mechanisms that induce angiogenesis and lymphangiogenesis even in early stage of the uterine cervix neoplasia are less understood. As VEGF expression does not completely explain the behavior of these tumors, in the present study, we investigated the expression of PDGF/PDGFR axis in relation with microvessel density (MVD) and lymphatic microvessel density (LMVD) in 46 patients with neoplastic lesions of the uterine cervix.

Methods. PDGF and PDGFR alpha and beta expression was detected by immunohistochemistry and scored based on the intensity of the final product of reaction and percent of positive tumor cells, using a scale from 0 to 3. MVD was assessed on slides stained with anti-CD31 antibody, and LMVD was assessed on slides stained with anti-D2-40 antibody. Blood and lymphatic vessels were counted using Weidner's hot spot methods.

Results. Overall values of MVD were between 20 and 84 (average 41.78). LMVD values in the tumor area ranged between 0 and 4 (average 2.75) and in the peritumoral area ranged between 5 and 12.6 (average 7.42). PDGF, PDGFR alpha, and PDGFR beta expression in tumor cells was found in 41 (89.1%), 25 (54.35%), and 46 (100%), respectively. No correlation was found between PDGF and PDGFR beta expression and MVD. PDGFR alpha expression correlated with both MVD and LMVD ($p<0.036$ and 0.05, respectively). We found a significant correlation between PDGFR beta expression and both intratumoral and peritumoral LMVD ($p<0.045$, and 0.032, respectively).

Conclusion. Our preliminary results suggest that PDGF/PDGFRs expression by tumor cells could be involved in tumor progression by autocrine regulation of neoplastic growth and stimulates lymphatic vessel formation, but not angiogenesis.

P-55

Neuropilin-1 signaling *in trans* regulates VEGF signaling and angiogenesis

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Tumor growth is dependent on the formation of new blood vessels in and to the tumor. The inhibition of tumor-angiogenesis has therefore been hypothesized to be an effective anti-tumor therapy. Several signaling pathways have been shown to be essential for (tumor) angiogenesis and are therefore targeted by anti-angiogenesis drugs that have recently entered the clinic. Mostly targeted is vascular endothelial growth factor (VEGF) since it is a master regulator of vascular development and angiogenesis. VEGF binds the co-receptors heparan sulfate (HS) and neuropilin-1 (NRP1). We have shown that the co-receptor NRP1 is essential for the 3-dimensional (3D) organization of vessels in mouse embryoid bodies, in zebrafish and in subcutaneous matrigel plugs in mice (Kawamura et al., Blood, 2008).

We have now studied the role of NRP1-VEGF-receptor 2 (VEGFR2) complex formation *in trans* (i.e. complex formation between cells individually expressing either VEGFR2 or NRP1). Proximity ligation assays showed that complex formation *in trans* occurred at the interface between VEGFR2- and NRP1-expressing cells. The complexes *in trans* established slower and remained for a prolonged time at the cell–cell interface, whereas complexes *in cis* (i.e. between VEGFR2 and NRP1 co-expressed in the same cell) rapidly became internalized and degraded. Since internalization of VEGFR2 is needed for its efficient signaling, we hypothesized that VEGFR2-mediated signaling in *trans* complexes is altered. Indeed, PLCγ- and ERK2 activation was prolonged, whereas ERK1 activation was inhibited in the trans situation. We therefore suggest that PLCγ and ERK2 are activated by VEGFR2 at the plasma membrane, whereas activation of ERK1 is dependent on internalization of VEGFR2.

Complex formation *in cis* and *trans* was further studied in vivo by silencing NRP1 in the endothelium (using an inducible, endothelial-specific NRP1 knockout mouse model). Mice with or without endothelial NRP1 expression were subjected to tumor challenge, using tumor models expressing either NRP1 or not. In vitro growth of these tumor cells (T241 or B16) was independent of NRP1 expression. Remarkably, signaling *in trans* by tumor cells expressing NRP1 attenuated tumor growth in both models. A possible mechanism underlying the decreased tumor growth with NRP1 presentation *in trans* is the different kinetics and distinct VEGF signaling.

In conclusion, we suggest that VEGF-dependent complex formation *in trans* by NRP1 and VEGFR2 may be negative regulator of VEGF signaling.

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P-56

Visualization of the Endothelin (ET)-A-Receptor-Expression as a benchmark of tumor angiogenesis using ET-A-Receptor radioligands

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The endothelin (ET) axis is involved in tumor growth and progression. Activation of the ET_A receptor by the peptide ET-1 contributes to the different processes including angiogenesis. In papillary thyroid tumors, an increased expression of the ET axis has been reported (1). The visualization of the ET system by positron emission tomography (PET) in affected tissue would be of great clinical interest for clinical diagnosis and the prediction of therapy response. Recently, we published the synthesis of a ^{18}F-labeled derivative of the non-peptide ET_A receptor ligand PD 156707 (2). The aim of this study was the development of a subtype-selective ET_A radioligand with improved pharmacokinetics by introduction of hydrophilic building blocks.

We synthesized seven nonradioactive fluoro-substituted analogs of PD 156707 and investigated the receptor binding affinities. The tested compounds revealed high ET_A receptor affinity (1–7 nM) and displayed ET_A/ET_B selectivities of 70-350:1. Additionally, the radiosynthesis of three ^{18}F-labeled counterparts was successfully established with radiochemical yields of 5–25% (not corrected for decay). The radioligands showed excellent stability in human serum in vitro (37°C, 90 min). A glycosylated ET_A radioligand was applied to biodistribution studies using nude mice (CD1 Nu/Nu) bearing ET_A-receptor-positive papillary thyroid K1 tumors. In vivo μPET (Siemens Inveon) imaging studies confirmed specific tumor uptake and ET_A receptor-mediated binding of the glycosylated radioligand 55 min p.i., as demonstrated by coinjection experiments using the high-affinity ET_A-selective antagonist PD 156707.

Simple hydrophilic modifications of the lead structure PD 156707 improved the pharmacokinetics and offer access to promising radiotracers for the imaging of ET_A receptor expression as a benchmark of tumor angiogenesis.

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P-57

Imbalance of angiopoietin-1 and -2 in severe dengue; relationship with thrombocytopenia, endothelial activation, and vascular stability

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Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is the most feared complication of dengue virus infection. DHF/DSS is characterized by a transient hyperpermeability of the endothelial barrier, leading to plasma leakage and/or hemorrhages. The pathogenesis of DHF/DSS is still incompletely understood. An important role has been attributed to pro-inflammatory cytokines as well as to the angiogenic protein vascular endothelial growth factor, because of its profound permeability effects. Accumulating evidence, however, has shown that other angiogenic proteins are just as important in the regulation of vascular stability.

Angiopoietins (Ang) are central regulators of vascular integrity. Ang-1 is mainly present in platelets and maintains vascular integrity. In contrast, Ang-2 is an endothelium-derived Ang-1 antagonist, which is released upon endothelial activation and promotes vascular leakage. High Ang-2 levels along with low Ang-1 levels have previously been found in bacterial sepsis, malaria, and Hanta virus infection. DHF/DSS is characterized by thrombocytopenia and endothelial activation, thereby possibly affecting Ang-1 and Ang-2 plasma levels.

In this study, we showed that DHF/DSS is associated with reduced Ang-1 and increased Ang-2 levels in a cohort Indonesian children. Ang-1 levels correlated positively with platelet counts of the patients. Additionally, there was also a linear relationship between Ang-1 levels and the number of activated platelets in platelet rich plasma, suggesting that conditions with platelet activation can result in increased plasma Ang-1 levels.

In conclusion, we found high Ang-2 levels and low Ang-1 levels during DHF/DSS. We suggest that dengue-associated endothelial activation and thrombocytopenia can lead to this imbalance in Ang-2/Ang-1, which possibly contributes to the transient plasma leakage during severe dengue infections. This finding may create new input for hypothesis on the pathogenesis of endothelial leakage and the role of platelet count and function during DHF/DSS. Moreover, it may contribute to the development of dengue-specific treatment strategies, which are lacking thus far.

P-58

Fluid shear stress inhibits of TGF-β-induced Endothelial-to-Mesenchymal Transdifferentiation through activation of MAPkinases.

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Physiological stress such as hyperlipidemia, high blood pressure and inflammation cause a severe burden of cardiovascular disease. The endothelial lining is especially sensitive to inflammatory mediators such as tumor growth factor beta (TGF-β). In vitro TGF-β induces endothelial-to-mesenchymal transdifferentiation (EndoMT). During the initiation of EndoMT, TGF-β upregulates the expression of the Activin-like Kinase-5 (Alk5) that activates the canonical TGF-β signaling pathway *i.e.* phosphorylation of Smad2/3. During EndoMT, endothelial genes are repressed, while mesenchymal genes such as

SM22α, calponin and αSMA are activated. Structural genes like Collagens types I and III require longer for their activation. Of note, the EndoMT process results in an irreversible and stable transdifferentiated smooth muscle-like phenotype that does not depend on TGF-β. The EndoMT process occurs both in mature endothelial cells (EC) as well as in endothelial precursors cells (ECFC).

In arteries, areas of low-flow are considered atheroprone, which is in part likely due to EndoMT of the arterial EC. We hypothesized that the normal physiological conditions *i.e.* laminar flow and fluid shear stress would counteract the putative adverse process of EndoMT. Gene expression array comparisons of ECFC (n=3 donors) cultured under static and FSS (20Dyne/cm^2) conditions revealed that several downstream components of the canonical TGF-β pathway were downregulated. Conversely, FSS protected EC(FC) from TGFbeta-induced EndoMT.

Thus, the application of precursor cells, such as ECFC, for vascular repair is sensitive to local biochemical as well as biomechanical cues and may both compromise or augment the vascular integrity.c

P-59

Development of molecular imaging markers of breast tumors by targeting tumor angiogenesis

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Angiogenesis is a hallmark of cancer and can serve as an imaging biomarker for SPECT, PET, MRI, or optical imaging using probes that target angiogenesis-specific markers[1,2]. Identification of angiogenesis-specific markers remains challenging and depends on the identification of targets that are upregulated in tumor blood vessels when compared to quiescent blood vessels. Different genomic screening methods, such as in vivo phage display, yeast-2-hybrid, and gene expression profiling, have identified markers, e.g. galectin-1, on tumor endothelial cells[3,4]. Furthermore, ligands need to be designed that bind selectively and with high affinity to such targets.

Aim of the project is to develop new imaging tracers for nuclear imaging of breast cancer. Imaging tracers will be based on the ligand Anginex that specifically binds to galectin-1. These probes will be labeled with different isotopes, such as In-111 and Lu-177, for preclinical imaging studies to assess biodistribution and tumor site-specific targeting. The latter provides, next to visualization properties, an additive therapeutic effect on the tumor site. These new tracers will initially be evaluated in tumor-bearing animals, using SPECT/CT, PET, and optical imaging equipment. Dual isotope SPECT will be applied to image the specificity of the tracer when compared to a negative control or to fingerprint expression of two different targets. The tracers for these experiments, Anginex-based, positive, and negative control, are successfully synthesized and purified, and the first studies are planned.

Biodistribution and tumor uptake can be fine-tuned by chemical modification or introduction of multivalency and/or multi-avidity. Here, peptidomimetics can be employed to covalently conjugate multiple binding ligands and hereby improve PKPD and/or ADME.

To provide a valid platform to study in vitro qualities of the designed probes, target quantification will be performed employing real-time quantitative RT-PCR and flow cytometry. These methods offer a high sensibility and throughput capacity to affirm the obtained values from treatment studies. Cell studies with RF24 cells are being employed to obtain a representative in vitro model for angiogenesis.

In addition to in vivo assays, *in ovo* CAM assays will be employed to provide a rapid tool to assess visualization and therapeutic properties of the designed probes. Preliminary results show optimistic outcome with respect to SPECT/CT measurements.

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P-60

Targeting multiple angiogenic pathways in orthotopic glioma models

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Background. There is an ongoing debate on the efficacy of anti-angiogenic treatment for glioma. Despite an impressive radiological response accompanied with clinical improvement, there is no evidence showing prolonged overall survival as a result of bevacizumab treatment. Observed radiologic response and improved quality of life are probably related to the induction of vessel normalization and, concomitantly, a less leaky tumor vasculature.
Since platelet-derived growth factor (PDGF) receptor is involved in maturation of neovasculature, we argued that simultaneous targeting of VEGF and PDGF pathways may affect a larger proportion of the tumor vasculature and therefore improve anti-tumor effects.
Methods. Mice carrying orthotopic glioma xenografts (E98, E473) with geno- and phenotypic resemblance to human gliomas were treated with the VEGFR2 inhibitor vandetanib, the VEGFR/PDGFR inhibitor sunitinib, the monoclonal antibody bevacizumab or combinations thereof. Evaluation of treatment effects was done by Gd-DTPA-enhanced MRI and histopathologic analysis.
Results. Bevacizumab, vandetanib, and sunitinib all inhibited angiogenesis-dependent tumor growth. The diffuse infiltrative phenotype remained present and survival was not prolonged as the result of treatment. Additional targeting of PDGFR did not improve therapeutic efficacy. Vessel normalization by all (combinations of) compounds resulted in loss of detectability in Gd-DTPA enhanced MRI scans.

P-61

Combined integrin blockade reduces tumor growth in glioblastoma through antiangiogenic and direct antitumor activity

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Tumors may develop resistance to specific angiogenic inhibitors via activation of alternative pathways. Therefore, multiple pro-angiogenic

pathways should be targeted to achieve complete angiogenic blockade. Aim of the current study was to investigate the effects of combinations of the angiogenic inhibitors endostatin (ES), tumstatin (Tum), and soluble vascular endothelial growth factor receptor-2 (sKDR) in a model of human glioblastoma multiforme (GBM).

Effects of ES, Tum, and sKDR were studied in vitro in endothelial cells (ECs) and in G55 glioma cells alone or in combination (ES+Tum, ES+Tum+sKDR). Inhibitors released by porcine aortic endothelial cells (PAE) demonstrated antiangiogenic activity as shown by proliferation, tube formation, and wound assays. Interestingly, ES+Tum reduced also the proliferation of G55-cells. Moreover, the ES+Tum combination caused morphological changes and induced apoptosis in glioma cells in vitro.

Microencapsulated PAE-cells producing these inhibitors were applied for local therapy in a subcutaneous GBM model. In vivo tumor growth was inhibited by ES, Tum, or sKDR (58%, 50% and 33%, respectively). The ES+Tum combination further decreased tumor weight (83%), whereas additional application of sKDR abrogated the synergistic inhibitory effect. Furthermore, cDNA microarrays of treated tumors revealed a prolactin and prolactin receptor up-regulation after treatment with Tum alone and ES+Tum, respectively.

Integrin-targeting factors Tum and ES act synergistically by inhibiting GBM growth via reduction of vessel density but also directly affecting proliferation and viability of tumor cells. Future studies will investigate whether prolactin signaling pathway represents an additional target to improve therapeutic strategies in GBM.

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P-62

SRPK1 knock-down inhibits tumour angiogenesis through modulation of VEGF splicing

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Recent studies in our laboratories have shown that the balance of pro- versus anti-angiogenic vascular endothelial growth factor (VEGF) splicing isoforms is regulated by serine/arginine protein kinase 1 (SRPK1) through phosphorylation of the splicing factor SRSF1. To better understand the functional implications of this molecular pathway, we have undergone studies in cell culture and in vivo to characterize its importance in angiogenesis and tumour growth.

Stable knock-down (KD) of SRPK1 in LS174t colon carcinoma cells was obtained using a lentiviral system (Dharmacon). This induced a switch at the level of RNA (as assessed by RT-PCR) and protein (ELISA) from pro- towards anti-angiogenic VEGF in SRPK1 KD cells compared to control cells (transduced with lentiviral non-targeting shRNA).

As a test for angiogenic potential, an in vitro co-culture system has been used. HUVECs were plated on a layer of normal human dermal fibroblasts and treated for 6 days with conditioned media from either SRPK1 KD or control cells. Cells were stained with fluorescent-labelled CD31 antibody, imaged using a confocal microsope and CD31 positive areas quantified. This revealed a significant decrease in areas covered by HUVECs in wells incubated in contioned-media from SRPK1 KD compared to control cells.

SRPK1 KD and control cells were injected subcutaneously in nude mice. At 12 days post-inoculation, SRPK1 KD tumours were significantly smaller than control tumours. Excised tumours were sectioned and stained for VEGF-receptor 2 to visualize blood vessels. Quantification of blood vessel areas showed that there is a significant reduction in microvessel density in SRPK1 KD tumours compared with control tumours.

The in vitro and in vivo data suggest a role for SRPK1 as a major regulator of tumour angiogenesis through modulation of VEGF splicing.

P-63

Outgrowth endothelial cells isolated from patients with Loeys-Dietz syndrome display dysregulated expression of a number of angiogenesis-related genes

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The Loeys-Dietz syndrome (LDS) is an inherited connective tissue disorder with symptoms similar to those of Marfan syndrome and the vascular type of Ehlers-Danlos syndrome. Most patients with LDS develop severe aortic aneurysms resulting in early need of surgical intervention. Patients with LDS harbour a mutation in the TGF-β receptors TGFBR1 (also named ALK-5) or TGFBR2. Since the TGF-β pathway plays a crucial role in many processes in cell biology including angiogenesis, we wondered whether endothelial cells of LDS patients might show aberrant characteristics contributing to the progression of disease.

We were able to isolate circulating outgrowth endothelial cells (OEC) from the peripheral blood of two LDS patients (one female, 54 years; one male, 26 years old) both harbouring a mutation in the TGFBR2 gene. Gene expression profiles of OEC clones were performed using Affymetrix Human Genome U133 Plus 2.0 Arrays. OEC clones isolated from age- and sex-matched healthy controls served as reference subjects.

Data analysis revealed that in addition to genes belonging to the TGF-β family, a large number of angiogenesis-related genes showed altered expression in OECs isolated from LDS patients compared to healthy controls. These genes included members of the VEGF pathway such as VEGF-A and VEGF-C, semaphorin family members or angiopoietin-2. Furthermore, we observed a dysregulation of several genes encoding for structural proteins that are essential for the biology of endothelial cells and the vessel wall. Especially, the observed changes in expression of collagen family members (COL5A1 and COL5A2) seem to be relevant, since mutations in collagen genes are causative of the Ehlers-Danlos syndrome that demonstrates similar clinical features to LDS.

P-64

Cell shape and rigidity can be sufficient for vascular network formation

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Vascular networks form by migration of endothelial cells and their interaction with the ECM. Cell-based computational modeling of vascular network formation can provide insight into what cell properties and behavior are vital for pattern formation. The angiogenesis model by Merks et al. (Dev Bio, 2006) showed that cell elongation, combined with chemotaxis toward an autocrine chemoattractant, is a key property for vascular network formation. Although chemotaxis toward an autocrine chemoattractant is assumed in a multitude of computational angiogenesis models, experiments and simulations by Szabó et al. (PRL, 2007) suggest that networks may form in the absence of an autocrine chemoattractant. We present and alternative computational model to study network formation in absence of an autocrine chemoattractant.

For this, we use a cell-based computational model. That is, based on properties of single cells, our computational model predicts the tissue morphology. In the present model, the main parameters are cell shape, intercellular adhesion, and adhesion between cells and the ECM.

If we choose the parameters such that cells adhere, roundish cells form blobs as do long, flexible cells. Only long, rigid cells are able to form networks. This is because the rigidity prevents longs cells from aligning and without alignment long cells cannot form a blob.

Adding an autocrine chemoattractant to the model results in network formation that is less sensitive to parameters such as cell shape and rigidity. Moreover, the formed networks are much more coarse and stable.

Thus, our model suggests that an autocrine chemoattractant is not necessarily required for network formation; rigid, long, adhering cells can form networks without chemotaxis. However, chemoattraction toward an autocringe chemoattractant enables more stable network formation.

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P-65

CD44 expression in Ewing sarcomas is associated with tumor cell plasticity

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Vasculogenic mimicry (VM) describes the unique ability of highly aggressive tumor cells to dedifferentiate into multiple cellular phenotypes and to form vasculogenic-like matrix-embedded networks containing plasma and blood cells. The presence of VM-associated matrix-rich patterned networks in patient tumor tissues is related to increased risk of metastasis and therefore poor clinical outcome.

Microarray analysis of Ewing sarcoma and breast carcinoma cell lines demonstrated that various components of the CD44/c-Met signaling cascade are overexpressed in the aggressive tumor cells - i.e. VM positive cells - compared to poorly aggressive tumor cell lines. At the centre of this signaling cascade, and highly expressed in these aggressive tumors, was CD44. This protein is involved in various cellular processes such as proliferation, differentiation, and survival. Splice variant analysis validated the increased expression of CD44s, CD44v6, and CD44v10 at protein level in aggressive Ewing sarcoma cells. In human Ewing sarcoma tissue sections, the expression of CD44(v6) was found to be associated with the presence of VM-characteristic blood lakes. Interestingly, CD44 was specifically expressed at the cells surrounding these blood lakes. The presence of CD44 on aggressive Ewing sarcoma cells provided them increased adherence capacity on hyaluronic acid. Reducing the expression of CD44 by siRNA transfection in aggressive Ewing sarcoma cells resulted in a less-refined vascular network formation when grown in three-dimensional (3D) gel culture on Matrigel.

Together, our data demonstrate that CD44 is implicated in the process tumor progression through vasculogenic mimicry in aggressive Ewing sarcoma. These findings may provide a novel target for inhibition of vasculogenic mimicry in future.

P-66

T-cadherin attenuates insulin signaling and angiogenesis in vascular endothelial cells

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T-cadherin (T-cad), a GPI-anchored member of the cadherin family, is widely expressed in the cardiovascular system. Recent studies suggest important role for this molecule in pathogenesis of cardiovascular disorders. It is upregulated in atherosclerotic lesions, in restenosis and on tumor endothelial cells (EC). Release of T-cad from EC into circulation on the surface of apoptotic microparticles occurs at early stages of atherosclerosis and correlates with endothelial dysfunction. Genome-wide association studies show correlation between single nucleotide polymorphisms in T-cad gene and hypertension. Upregulation and homophilic ligation of T-cad on EC stimulates angiogenesis via activation of PI3-kinase/Akt signaling axis. T-cad acts as a receptor for adiponectin, a circulating adipocyte-derived secretory protein that regulates glucose utilization. Taken together, these data prompted the hypothesis that T-cad can modulate vascular effects of insulin, a hormone that controls glucose metabolism and has been shown to induce angiogenesis and NO-dependent vasorelaxation via PI3-kinase/Akt pathway. The goal of the study was to analyze whether T-cad modulates effects of insulin on angiogenesis and activity of endothelial nitric oxide synthase (eNOS) in EC.

Cultured microvascular EC line HMEC-1 was transduced with lentiviral vectors expressing T-cad gene, T-cad shRNA or respective control vectors. Angiogenic effects of insulin on transduced EC were analyzed using spheroid assay in fibrin gel. Activity of insulin signaling pathway and eNOS in insulin-treated EC was measured by Western blotting. As demonstrated earlier, T-cad overexpression in cultured EC *per se* stimulated outgrowth of EC sprouts into fibrin gel. Insulin caused prominent dose-dependent activation of angiogenesis in cells transduced with control vectors. In T-cad overexpressing EC angiogenic response to insulin was markedly attenuated, while T-cad silencing resulted in increase of insulin-induced EC sprout outgrowth. T-cad overexpression also attenuated insulin-dependent Akt and eNOS phosphorylation and promoted degradation of insulin receptor substrate IRS-1. Conclusions: T-cad attenuates insulin-induced angiogenesis and eNOS stimulation due to chronic activation of PI3-kinase/Akt signaling axis that in its turn induces negative

feedback loop in insulin signaling pathway making the cell insensitive to insulin stimulation. In vivo, T-cad upregulation in the vascular endothelium under stress condition may have not only beneficial (prosurvival) effects but also cause vascular insulin resistance and impair insulin-induced vasorelaxation and vascular growth.

P-67

Characterization of new low molecular weight-antiangiogenic compounds from natural and synthetic origins

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Angiogenesis is under stringent control in health and occurs only during embryonic development, growth, regeneration, wound healing and some physiological processes such as formation of the corpus luteum and endometrium. On the contrary, a persistent and deregulated angiogenesis is involved in a number of pathological processes such as tumour growth, metastasis, diabetic retinopathy, age-related macular degeneration, psoriasis or arthritis, among others. For this reason, angiogenesis inhibition has attracted broad attention in the field of pharmacological research [1,2]. Our group has been devoted for more than ten years to the search and characterization of new antiangiogenic compounds. By means of diverse experimental procedures, including in vitro assays that resemble different steps of the angiogenic process, and some assays to test the in vivo angiogenesis inhibitory activity, the antiangiogenic activity of a number of low molecular weight compounds has been identified and characterised. Some of them are derived from marine organisms [3,4] or medicinal plants [5-7], and others from synthetic origin [8,9]

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P-68

Cardiovascular differentiation potential of human mesenchymal stem cells strongly depends on developmental stage of donor tissue; implications for future vascular medicine

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Differentiation of mesenchymal stem cells (MSCs) into cardiac cells, especially vascular cells, may contribute to improved perfusion after transplantation into ischemic myocardium. Mostly adult, aged patients suffer from such diseases and are main candidates for autologous cell therapy. However, it is uncertain whether aging also affects the differentiation potential of MSCs. Here, we have compared the cardiovascular differentiation potential of human (h) MSCs derived from embryonic, fetal and adult sources to address this issue.

hMSCs derived from embryonic stem cells (hESC-MSCs), fetal umbilical cord, bone marrow, amniotic membrane, adult bone marrow, and adipose tissue were cultured under angiogenic conditions and with neonatal rat cardiomyocytes (nrCMCs) or cardiac fibroblasts (nrCFBs) for 10 days. Cardiovascular differentiation was assessed by immunocytochemistry, qRT-PCR and optical mapping.

In angiogenesis bioassays, only hESC-MSCs and fetal hMSCs were able to form capillary-like structures, which stained for both smooth muscle and endothelial cell markers. The same results were obtained after priming the cells in angiogenesis inducing medium for two weeks. After co-culture with nrCMCs but not nrCFBs, significantly more hESC-MSCs (7.2±0.4%) than fetal hMSCs (2.2±0.3%; $P<0.05$) stained positive for α-actinin including some in a striated, sarcomeric pattern, but no positive cells were detected among adult hMSCs. Of all MSC sources, hESC-MSCs expressed most cardiac-specific genes. hESC-MSCs and fetal hMSCs expressed significantly more basal levels of connexin43 than adult hMSCs and co-culture with nrCMCs increased connexin43 expression. Conduction velocities in co-cultures of nrCMCs (24.8±1.2 cm/s) and hESC-MSCs (25.9±0.9 cm/s) were significantly higher than co-cultures with fetal (22.0±1.8 cm/s, $P<0.001$) or adult MSCs (18.2±1.1 cm/s, $P<0.001$).

In this study, we show that human mesenchymal stem cells of embryonic stem cell or fetal, but not adult origin, can differentiate into cells that make up myocardial vasculature (smooth muscle and endothelial cells) but also cardiomyocytes.

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Enhanced angiogenic switch and tumor growth in Histidine-Rich Glycoprotein-Deficient Mice

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We have investigated how lack of histidine-rich glycoprotein (HRG) affects tumor angiogenesis and growth in vivo. To this end, we crossed HRG-deficient mice with the orthotopic RIP1-Tag2 mouse model for multistage carcinogenesis. We found that HRG-deficiency resulted in an enhanced angiogenic switch that is mediated by activated platelets and that lack of HRG also resulted in larger tumor volumes.

HRG is a multidomain plasma protein that has been identified as an angiogenesis inhibitor both in vitro and in vivo. Genetically, HRG-deficient mice have enhanced coagulation as the only obvious phenotype. In the RIP1-Tag2 model, the cells in the islets of Langerhans start to hyper-proliferate at around 5 weeks of age due to transgenic expression of the oncogenic SV40 T-antigens regulated by the rat

insulin promoter. A fraction of the hyperplastic islets go through the angiogenic switch around 6 weeks of age. Some of the angiogenic islets form benign tumors of which some eventually become invasive malignancies.

Pancreata from $HRG^{+/+}$ and $HRG^{-/-}$ RIP1-$Tag2^{pos}$ littermates were dissected at three different time points: 7, 12, and 15 weeks after birth. At seven weeks, we found that the number of islets that had gone through the angiogenic switch was twice as many in the $HRG^{-/-}$ group when compared with the $HRG^{+/+}$ group. At the later time points, tumor volumes were assessed. In 12-week-old mice, the tumor volumes were on average two times higher in the $HRG^{-/-}$ group than in the $HRG^{+/+}$ group. This difference was even more pronounciated three weeks later when the volume was more than tree times higher in the $HRG^{-/-}$ group than in the $HRG^{+/+}$ group. While we noticed higher proliferative activity in tumors from HRG-deficient mice, we did not find any significant difference in tumor vasculature parameters between the groups.

We have also found that activated platelets create an important change in the microenvironment for HRG function and observed increased platelet activation in HRG-deficient mice. Therefore, we rendered $HRG^{+/+}$ and $HRG^{-/-}$ RIP-$Tag2^{pos}$ mice thrombocytopenic by treatment with the Gp1bα antibody. We found that treatment of the mice before onset of the angiogenic switch reduced the number of angiogenic islets to wild-type levels in HRG-deficient mice while treatment at later stages had no obvious effect on tumor volumes. The mechanism behind this finding is still not known but is currently under investigation (for further information, see Cedervall et al.)

In conclusion, we have found that platelets are important for early angiogenic events in tumorigenesis while of less importance for later tumor angiogenesis and growth.

P-70

Inflammation and the development of late vascular damage after radiotherapy

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Microvascular damage in normal tissues is a serious late complication of cancer patients after radiotherapy. Vessel injury develops from months to years after irradiation and manifests as telangiectasia, which are characterized as dilated and thin-walled blood vessels. Problems arise when telangiectasia rupture, leading to excessive bleedings which may impair organ function and require surgical intervention. The vascular phenotype implies that an imbalance between vascular repair and homeostasis pathways causes blood vessel dilation; however, the mechanism is still unclear. We have identified the transforming growth factor-beta (TGF-β) co-receptor endoglin as being critically involved in the development of late normal tissue damage: mice with reduced endoglin levels (Eng±) display less vascular injury and fibrosis after kidney irradiation compared to Eng+/+ littermates. Endoglin is mainly expressed on endothelial cells, but also on monocytes/macrophages. Therefore, the observed differences in the repair capability after irradiation may be either due to changed endothelial cell function or due to an altered inflammatory response in Eng± mice.

We show that irradiation induces the infiltration of inflammatory cells, which mainly consist of macrophages. Macrophage numbers were only slightly decreased in irradiated Eng± mice; this was accompanied by reduced mRNA levels of the macrophage chemoattractant CCL2 (MCP-1). Analysis of the inflammatory cytokine profile revealed that irradiation induced an upregulation of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) mRNA in Eng+/+ mice which was significantly reduced in Eng± mice. We also show that these molecules are produced by macrophages in the irradiated tissue. In addition, isolation of bone marrow cells from Eng± mice showed aberrant/impaired cytokine expression in response to LPS stimulation compared to Eng+/+ mice.

Our studies suggest that endoglin mediates the inflammatory response in irradiated tissues by regulating the expression of IL-1β and IL-6 in macrophages. As these cytokines not only have pro-inflammatory, but also pro-angiogenic and pro-fibrotic properties, this strongly suggests that these cytokines contribute to the development of late normal tissue damage after irradiation.

P-71

Identification of galectin-1 interacting proteins

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Galectin-1, a member of the carbohydrate binding galectin family, is differentially expressed in tissues of various pathologies, including cancer. Moreover, galectin-1 contributes to different steps of angiogenesis in vitro and is essential in tumour growth and tumour angiogenesis in vivo. This makes galectin-1 a promising target for anti-angiogenesis therapy.

To gain insights into the binding characteristics of galectin-1 and to identify new targets for anti-angiogenesis treatment, we set out to identify galectin-1 interacting proteins using the yeast two-hybrid system.

To that end, the cDNA encoding human galectin-1 was cloned in-frame with the DNA-binding domain of the yeast Gal4 transcription factor. In-frame cloning was confirmed by sequence analysis and Western blotting. Additional analysis revealed no auto-activation of reporter gene expression or toxicity in yeast transformed with the gal-1/gal4 fusion protein. Next, gal-1/gal4 transformed yeast was mated with yeasts containing a normalized human cDNA library cloned in-frame with the activation domain of the yeast Gal4 transcription factor. Mated yeasts were analyzed on quadruple selection plates to select for strains that contain galectin-1 binding proteins. Positive strains were further validated on selection plates that allow blue/white screening and selected for antibiotic resistance. Finally, sequence analysis was performed to identify the galectin-1 binding proteins. Preliminary results revealed interaction of galectin-1 with proteins involved in chromatin remodelling and glucose metabolism. Studies are ongoing to confirm and characterize the interaction of the target proteins with galectin-1. In addition, additional screens are performed to identify additional galectin-1 binding proteins.

Altogether, yeast two-hybrid screening provides us with new insights into the binding characteristics of galectin-1 with endogenous proteins. This knowledge can be used to develop new anti-angiogenesis drugs for the treatment of cancer.

P-72

Tumor vessel abnormalization: opening up vessels to improve cancer therapy

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Poor drug uptake remains an important cause of failure in cancer therapy. To improve drug delivery, the tumor vascular bed can be

manipulated, and two opposing concepts can be identified: vascular normalization and vascular abnormalization. Normalization, as a consequence of antiangiogenic therapy, has been shown to change the tumor vasculature in a more clear-cut, healthy and mature looking vascular bed with improved blood flow and lower interstitial fluid pressure resulting in a more homogenous drug distribution. An alternative approach and quite opposite to this strategy is the concept of tumor vessel abnormalization. It has been shown that tumor vessels permit leakage of blood born elements. However, as not all vessels are leaky drug accumulation is very heterogeneous. We hypothesize that increasing vessel permeability using vasoactive agents, like tumor necrosis factor alpha (TNF), will result in a more efficient and homogenous drug distribution.

We show that the administration of low-dose TNF in combination with liposomal encapsulated doxorubicin (Doxil) increases the intratumoral doxorubicin concentration 5-fold significantly improving tumor response. Using intravital microscopy, we can study the drug distribution inside the tumor microenvironment in high detail. Especially, 100-nm liposomes extravasate from the blood vessels into the surrounding tumor tissue in the presence of TNF even in vessels that were in first instance not leaky. This suggests that TNF renders more vessels permeable leading to higher and more uniform drug distribution. However, not all tumor types respond to the addition of TNF. TNF-induced permeability acts predominantly through TNF-receptor 1, and we found that the receptor in the tumor vasculature is mainly expressed by pericytes. Using several tumor types with diverse pericyte coverage, we observe that tumors with a rich coverage benefited significantly from the addition of TNF suggesting that TNF-receptor 1 expressing pericytes is responsible for TNF-induced permeability.

In conclusion, tumor vessel abnormalization increases the bioavailability of chemotherapy leading to an improved tumor response in tumors with a rich pericyte covered vasculature. This outcome will ultimately lead to the selection of patients who will benefit from this therapy and also indicate pericytes as an alternative target for tumor vascular therapy.

P-73

The VEGF-induced transcription factor Myocyte Enhancing Factor 2 C (MEF2C) inhibits sprouting activity of endothelial cells

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VEGF-A_{165} via VEGF receptor-2 binding is the major physiologic initiator of angiogenesis. Despite intensive work on the signaling induced by VEGF in endothelial cells over the recent years, the transcription factors induced by VEGF-A and their specific roles for angiogenesis remain to a large extent uncharacterized.

In the course of a recent gene profiling experiment using VEGF-A_{165}-stimulated endothelial cells (1), we identified the MADS box transcription factor MEF2C to be preferentially induced by VEGF-A_{165}, but comparably not by other growth factors or cytokines. We therefore investigated the potential specific role of this transcription factor in the angiogenic response of endothelial cells mediated by VEGF.

Using real-time RT-PCR, we confirmed that MEF2C mRNA is upregulated by VEGF-A_{165} and to some degree also by the pro-angiogenic bFGF. However, it is not at all affected by the pro-inflammatory cytokine IL-1 or the more general growth factor EGF supporting a specific function within the response pattern towards angiogenic mediators. The VEGF-induction is clearly mediated by VEGF receptor-2 and not by VEGF receptor-1. This was discriminated by employing the viral VEGF-homologue VEGF-E, which selectively binds to VEGF receptor-2 and was at least as potent as VEGF-A in inducing MEF2C mRNA. In contrast, PlGF, which specifically binds to VEGF receptor-1 only, completely failed to induce MEF2C. Surprisingly, when we tested sprouting activity in in vitro angiogenesis assays, we found that adenoviral overexpression of MEF2C significantly inhibited sprout formation in the spheroid assay. This inhibition of sprout formation was observed with HUVEC as well as with circulating endothelial progenitor cells. In line with a primarily inhibitory response program mediated by MEF2C, overexpression of a dominant-negative MEF2 mutant enhanced sprouting. In contrast, MEF2C did not affect proliferation indicating a specific effect on the migration/sprouting process. These data suggest that MEF2C mediates, potentially in a negative feed-back loop, a secondary response to VEGF-A which could be important to establish a quiescent endothelial phenotype.

(1) Schweighofer et al. (2009), Thromb Haemost. 102, 544-554.

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P-74

Xenogenic and autologous ASCs stimulate angiogenesis in vivo via distinct mechanisms

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Previously, we and others have demonstrated that adipose-derived stromal cells (ASCs) stimulate angiogenesis as well as maturation of the newly formed blood vessels in a matrigel implant model. However, modes of participating in angiogenesis of ASCs from xenogenic and autologous sources are unclear.

Here, we injected PKH26-labeled ASCs from NUDE/SCID mice or human ASCs stably expressing DsRed in matrigel subcutaneously into NUDE/SCID mice (n=12). ASCs incorporation into growing blood vessels was analyzed 14 days after transplantation by confocal microscopy of frozen matrigel sections stained with CD31 antibody. Consistently with our previous observations, both autologous and xenogenic ASCs stimulated blood vessel formation when compared with matrigel without cells. However, we found that manner of labeled ASCs distribution in newly formed vascular network was strikingly different. Part of transplanted autologous ASCs directly incorporated in blood vessels. In matrigel plugs with mouse cells, we found PKH26-labeled capillaries as well as perivascularly localized ASCs. Xenogenic ASCs failed to incorporate into growing vasculature and were found as cell clusters within matrigel.

Taken together, our data indicate that autologous but not xenogenic ASCs is capable of tissue replacement during angiogenesis. We suggest that ASCs vascular differentiation and incorporation into growing vessels requires species-specific cell–cell interaction.

P-75

HIF-Isoforms have divergent roles in the angiogenesis of colorectal cancer

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The importance of angiogenesis in colorectal cancer (CRC) is evidenced by the success of anti-angiogenic therapies such as the anti-VEGF antibody bevacizumab. Hypoxia, one of the most potent drivers of angiogenesis through mediators such as VEGF, plays a pivotal role in CRC and is associated with increased metastasis and poorer survival. The transcription factor Hypoxia Inducible Factor (HIF) is the master regulator of hypoxia. Isoforms HIF-1α and HIF-2α were previously thought to be functionally redundant, but mounting evidence points to their divergent roles in many cancers. In CRC, their relative roles remain unclear. This study aims to elucidate the role of HIF-1α and HIF-2α in the regulation of angiogenesis in CRC.

Using small interfering RNA (siRNA), HIF-1α and HIF-2α genes were specifically knocked down in the CRC cell-line Caco-2. After exposure to hypoxia (1% O_2) for 24 h, expression of genes involved in angiogenesis (VEGF, BNIP-3) was quantified by quantitative polymerase chain reaction.

Successful mRNA knockdown (approximately 70%) markedly reduced the strong induction of HIF-1α and HIF-2α protein observed in hypoxia. Hypoxia-induced VEGF expression was reduced more markedly by siHIF-1α than siHIF-2α. BNIP3 expression was reduced by siHIF-1α only.

Prognosis, especially in late-stage CRC, remains poor despite anti-angiogenic therapy. These drugs have been shown to reduce HIF-1α activity, but their effect on HIF-2α is less well understood. The data presented here suggest that HIF-1α and HIF-2α have different roles in the regulation of CRC angiogenesis, implying that selective targeting of HIF isoforms may improve efficacy of anti-angiogenic therapy.

P-76

Abstract withdrawn

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RNA Interference-mediated Silencing of Rac1 Inhibits Angiogenesis and Tumor Growth

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Tumor angiogenesis is a well-established target for anti-cancer therapy. VEGF is arguably the most important pro-angiogenic factor. VEGF stimulates endothelial cells by signaling through VEGFR-1 and VEGFR-2 and subsequent activation of multiple intracellular effectors, including the Rho family of small GTPases. Rho GTPases function as molecular switches in the cell which cycle between an inactive GDP-bound state and an active GTP-bound state. Rac1 appears to be an essential GTPase responsible for VEGF-mediated angiogenesis and is therefore an attractive target for anti-cancer therapy. In the present study, we have used RNA interference to silence Rac1 expression. The main goal of this study was to inhibit tumor angiogenesis and growth by knockdown of Rac1.

HUVECs were transiently transfected with non-specific control siRNA (siNS) or Rac1 siRNA (siRac1) by electroporation. Functional assays with the transfected cells were performed to determine the effect of Rac1 knockdown on angiogenesis in vitro. Silencing of Rac1 reduced VEGF-mediated tube formation, cell migration, invasion, and proliferation. A Matrigel plug assay in mice was performed to determine the effects of Rac1 knockdown on angiogenesis in vivo. Mice bearing Matrigel plugs were injected locally with siNS or siRac1 followed by electroporation. Matrigel plugs isolated from si-Rac1 treated mice appeared less red-colored and contained 50% less hemoglobin when compared to plugs isolated from siNS-treated mice, indicating that knockdown of Rac1 inhibits angiogenesis. In addition, an allograft model was used to study the effects of Rac1 knockdown on tumor growth. Mice bearing subcutaneous Neuro2A neuroblastoma tumors were treated intratumorally with siNS or siRac1 followed by electroporation. Treatment with siRac1 almost completely inhibited tumor growth. Western Blot analysis on tumor lysates revealed efficient knockdown of Rac1. Furthermore, the expression of vasculature markers CD31 and VEGFR-2 was reduced in siRac1-treated tumors determined by Western Blot analysis. CD31 immunolabeling of tumor sections demonstrated much weaker staining in siRac1-treated tumors when compared to siNS-treated tumors indicating that the effect of Rac1 knockdown on tumor growth was likely caused by inhibition of tumor angiogenesis.

Taken together, these results demonstrate that Rac1 is an important regulator of VEGF-mediated angiogenesis and that silencing of Rac1 by RNA interference is an effective tool to inhibit tumor angiogenesis and growth.

P-78

Sunitinib reduces circulating angiopoietin-2 levels in patients with metastatic renal cell cancer

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Background. Vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors are effective agents in the treatment of metastatic renal cell cancer (mRCC). We here investigated whether inhibition of VEGFR signaling by sunitinib causes changes in plasma proteins associated with tumor endothelium.

Patients and methods. Forty-three patients with mRCC received sunitinib 50 mg/day in a 4-weeks on 2-weeks off schedule. Sequential plasma samples were obtained before treatment (C1D1), on C1D14, on C1D28, and on C2D1 before start of cycle 2. Plasma levels were assessed for VEGF, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular cell adhesion molecule-1 (sICAM-1), von Willebrand factor (vWF), circulating angiopoietin-2 (Ang-2), and the soluble receptor of Ang-2, sTie-2. Total tumor burden was calculated at baseline and at first evaluation. Progression-free survival (PFS) and overall survival (OS) were determined.

Results. Tumor burden was positively associated with baseline circulating Ang-2 (Spearman's rho [ρ] = 0.378, P = 0.028) and vWF (ρ = 0.417, P = 0.008). During sunitinib treatment, circulating Ang-2 and sTie-2 significantly decreased ($P < 0.001$ for both), levels of

sVCAM-1 and VEGF significantly increased ($P = 0.022$ and $P < 0.001$), whereas levels of vWF and sICAM-1 remained stable. The reduction in circulating Ang-2 levels was positively correlated with the percentage decrease in tumor burden on C1D28 ($\rho = 0.605$; $P = 0.002$). Baseline protein levels and protein changes during treatment were not associated with PFS or OS.

Conclusions. Circulating Ang-2 levels are associated with tumor burden in patients with mRCC. Reduction in circulating Ang-2 early predicts response to sunitinib. Our findings warrant further studies to clarify the role of Ang-2/Tie-2 signaling in sunitinib efficacy.

P-79

Primary breast and colon cancer: is hypertension/preeclampsia during pregnancy an independent marker for individual risk stratification?

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Introduction. Colon and breast cancer are leading causes of cancer-related death in the Netherlands. Recently, the stroma-percentage within the primary tumor is described as an independent parameter for survival for both cancer types. Patients with high stroma-percentage had a worse survival independent for tumor stage and tumor status compared to patients with a high carcinoma-percentage.

Pregnancy is also characterized by vaso-invasion. Abnormal function by angiogenesis, trophoblast, and stroma cells and their interaction play an important role in abnormal placentation, evident in pre-eclampsia, recurrent abortion, intra-uterine growth restriction, and abrutio placentae.

The main goal of this study is to gain insight in the pathophysiology of breast en colon cancer early in a women's life using pregnancy as a stress test: women who had a complicated pregnancy characterized by abnormal angiogenesis may also have abnormal angiogenesis related to cancer associated with worse survival in later life.

Methods. Patients included in the databases for breast ($n = 234$) and colon ($n = 337$) cancer have received an invitation to participate in this trial by filling out a validated questionnaire about their pregnancy outcomes. Stroma-percentage will be determined, and variables including tumor stage, tumor status, and smoking will be analyzed in a regression analysis.

Hypothesis. We hypothesize that preeclampsia during pregnancy is an independent, early clinical genetic marker of invasion for abnormal placental growth (decreased angiogenesis), and associated with cancer with low stroma formation (and thus better prognosis).

P-90

Expression of angiostatic PF-4var/CXCL4L1 counterbalances angiogenic impulses of VEGF, IL-8/CXCL8, and SDF-1/CXCL12 in esophageal and colorectal cancer

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Chemokines affect tumor progression through leukocyte attraction, tumor cell motility, and angiogenesis. In this study, the regulated expression of angiogenic (SDF-1/CXCL12 and IL-8/CXCL8) and angiostatic (PF-4var/CXCL4L1 and IP-10/CXCL10) chemokines was examined in human colorectal and esophageal cancer. In HCT 116 and HCT-8 colorectal adenocarcinoma cells, the production of IL-8 immunoreactivity was upregulated by IL-1β, TNF-α, the TLR ligands dsRNA, and peptidoglycan and phorbol ester. IL-8 and IP-10 were synergistically induced in these adenocarcinoma cell lines by IL-1β plus TNF-α or IL-1β plus IFN-γ, respectively. In HCT-8 cells, PF-4/CXCL4 and PF-4var production was also elicited after combinatorial treatment (IL-1β plus TNF-α) as demonstrated by ELISA, qRT-PCR and immunocytochemistry. The presence of PF-4var, SDF-1, and VEGF was evidenced by immunohistochemistry in surgical samples from 51 patients operated for colon adenocarcinoma (AC), esophageal AC, or esophageal squamous cell carcinoma (SCC). PF-4var was strongly detected in colorectal cancer, whereas its expression in esophageal cancer was rather weak. Staining for SDF-1 was almost negative in esophageal SCC, whereas a more intense and frequent staining was observed in AC of the esophagus and colon. Staining for VEGF was moderately to strongly positive in all three types of cancer, although less prominent in esophageal AC. The heterogenous expression of angiogenic as well as angiostatic chemokines within the tumor and between the different cases, but also between the different tumor cell types, may indicate a distinct role of the various chemokines in the complex process of tumor development.

P-81

High resolution imaging of coronary artery architecture with an imaging cryomicrotome

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Microvascular adaptation is an important determinant in heart failure progression. We investigated the feasibility of reproducing the three-dimensional microvascular arrangement in a healthy rabbit heart at microvessel resolution using our existing imaging cryomicrotome setup.

The imaging cryomicrotome, previously developed by the Department of Biomedical Engineering and Physics, was modified by the addition of a Plapo 1X Olympus microscope objective in line with the existing Nikkor zoom 70- to 180-mm lens. A rabbit heart was flushed with heparine containing modified saline solution. Subsequently, replica material (Mercox) containing Potomac yellow fluorescent was infused in the left anterior coronary artery. After polymerization, the heart was embedded and serially sliced at 2.4 micron thickness. The fluorescent was imaged with a 4096x4096 pixel camera (Alta Apogee U16). The structure and alignment of the capillary bed is apparent, and feeding arterioles can be identified as they branch into multiple parallel-arranged capillaries.

Using the cryomicrotome, the heart can be imaged from major vessels down to the capillary level. The setup can therefore be used to unravel the three-dimensional microvascular arrangement in rabbit hearts and establish its role in the development of heart failure.

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Investigating the importance of heparan sulphate proteoglycans as determinants of progenitor cell function in vascular ageing

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Cardiovascular repair mechanisms become impaired with age, resulting in an increased propensity towards vascular pathology. This decrease in vascular health may, in part, be due to a decline in the functional capabilities of endothelial progenitor cells (EPCs); a population of cells recognised as having a key role in vascular repair. Cell-surface heparan sulphate (HS) proteoglycans bind a plethora of factors that are essential for EPC function. These interactions are dependent upon specific sulphated domains within the HS chain. This study aims to investigate whether structural changes of HS on EPCs contribute to the functional deterioration of these cells with age.

Using umbilical cord blood and adult peripheral blood from young and old healthy subjects, we have successfully isolated a rare population of EPCs, known as late outgrowth endothelial cells (OECs). These cells i) exhibit a typical cobblestone appearance, ii) express a panel of endothelial markers, including CD31, vWF, CD105, and KDR, iii) lack expression of the hematopoietic markers, CD45 and CD14, iv) ingest Ac-LDL, v) are highly proliferative and vi) form capillary-like structures in Matrigel. High performance liquid chromatography (HPLC) and size exclusion chromatography are used to elucidate whether sulphation within the HS chain and/or HS chain length differs between cord and peripheral blood-derived OECs and with subject age. Our preliminary data suggest that decreased 6-O-sulphation of HS on OECs occurs in association with age. There is 19.2% 6-O-sulphation of HS chains on cord blood OECs compared to 11.8% and 6.1% on adult peripheral blood OECs from young (20–30 years) and older (>55 years) subjects, respectively. Moreover, this reduction in 6-O-sulphation correlates with a decrease in the proliferative and migratory capacities of these cells, using an MTT and in vitro scratch assay, respectively. It is now being investigated whether the impairment in function can be rescued by the addition of soluble heparin.

Understanding the role of HS structural modifications on EPC function will help redefine the molecular pathology of the age-associated decline in postnatal vasculogenic activity and repair. The findings may also have an impact on the development and/or rational design of therapeutic glycomimetics.

P-83

A mathematical model for cutaneous wound healing incorporating wound contraction, angiogenesis, and closure

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Wound healing is a complicated but crucial biological process, of which many of the underlying mechanisms are unclear, despite the long period of research devoted to these mechanisms. To get more insight into the fundamental biological processes, and to help physisians treat wounds after trauma, mathematical modeling can be a very helpful tool.

Cutaneous wound healing involves a whole score of processes, being signaling processes, wound contraction, angiogenesis, wound closure, and scar modeling as the most important ones. In most of the mathematical studies on wound healing, the emphasis is put on one biological process only, and the other processes are often assumed to proceed instantaneously. In the present work, we will combine several of the processes that are responsible for cutaneous wound healing. We will see how these processes influence each other and how this is incorporated in the mathematical models.

As angiogenesis is an indispensible process during wound healing, we will also review several mathematical models for this process. The models are mainly based on the continuum hypothesis, which gives a system of partial differential equations that are solved using the finite-element method. We will also make a short excursion to one of our cell-based semi-stochastic models. This model does have some similarities with the cellular Potts model; however, it also differs in a subtile way from to the cellular Potts models and other cellular automata models.

P-84

TIA-1 Tumour growth inhibition in murine xenograft colon cancer is VEGF dependent

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The TIA-1 RNA binding protein is alternatively spliced in adenocarcinoma colon and melanoma cells to form a truncated, short isoform lacking the last two zinc finger domains. This short isoform (shTIA-1) was expressed in 66% of colon cancer samples tested and correlates with high pro-angiogenic $VEGF_{xxx}$ expression. In colon cancer cells expressing the full-length TIA-1 protein (flTIA-1), TIA-1 bound to the angiogenic isoforms of VEGF, $VEGF_{xxx,}$ demonstrated by RNA immunoprecipitation followed by RT-PCR, but there was no TIA-1 binding in cells expressing shTIA-1. This effect was also demonstrated by RNA pull down assay using an MS2-Maltose binding protein assay. Over-expression of flTIA-1 also altered the transformed phenotype of LS174t colon cancer cells that normally express shTIA-1 and low or no $VEGF_{xxx}b$ expression. Transfected cells had suppressed ability to form colonies in soft agar in comparison to wild-type cells ($p < 0.05$). The growth was enhanced by treating cells with $rhVEGF_{165}$ or with neutralizing antibodies to $VEGF_{165}b$, while the untransfected cells were unaffected by either treatments. In tumour studies, flTIA-1 over-expressing cells injected in nude mice showed significantly reduced growth at 21 and 25 days post-injection compared to control tumours (both $p < 0.05$, one-way Anova) and reduced vascular density. To test whether this was due to the VEGF splicing switch, a $VEGF_{165}$ expression plasmid was co-expressed with flTIA-1 in LS174t cells, and these vector transfected and flTIA-1 transfected cells were implanted into nude mice. Tumour volumes were measured. The $VEGF_{165}$ expressing tumours grew significantly faster than the flTIA-1 parental line, and not significantly differently from the vector transfected line. **=$p < 0.01$ Anova, compared with flTIA-1. $N = 6$ per group. These results suggest that TIA-1 splicing to shTIA induces an angiogenic switch by preventing distal splicing of VEGF to anti-angiogenic isoforms, and that over-expression of flTIA-1 is anti-angiogenic.

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P-85

Thyroid hormone (T4) regulates endothelial progenitor cells function

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Recent studies have shown that thyroid hormone (L-thyroxine, T4) modulates endothelial progenitor cells (EPCs) number in subclinical hypothyroidism subjects, but the role of the hormone in the regenerative function of EPCs is incompletely understood. This study is to investigate the effects of T4 on functions of human EPCs in vitro and in vivo.

T4 up regulated $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin expression and mitogen-activated protein kinase (MAPK) phosphorylation in EPCs. In addition, T4 enhanced EPC proliferation, migration and adhesion and inhibited EPC apoptosis. These actions of T4 in EPCs were dependent on MAPK activation and could be prevented, at least in part, by anti-integrin $\alpha v\beta 3$. Furthermore, the hormone activated the endothelial nitric acid synthase (eNOS) system and increased expression of matrix metalloproteinase-2 (MMP2) gene and basic fibroblast growth factor (bFGF) gene expression and bFGF release. In the mouse ischemic hindlimb model, transplantation of T4-treated EPCs enhanced blood flow recovery to ischemic limbs compared with vehicle-treated EPCs. In integrin-αv small interfering RNA (siRNA)-treated mice, however, treatment with T4 did not enhance in vivo angiogenic potential. Administration of T4 increased hematopoietic stem cells in bone marrow and EPCs in peripheral blood; this was associated with incorporation of bone marrow-derived cells into new vessels in a wound healing neovascularization model and limb salvage with better blood flow in an ischemic hindlimb model.

The present study defines a novel action of T_4 that activates circulating EPCs and suggests this mechanism may be therapeutic approach to vessel regeneration following vascular injury.

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Alpha-Fetoprotein acts as enhancer of proangiogenic properties of human adipose tissue stromal cells

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The alpha-fetoprotein (AFP) is known as an oncodevelopmental protein with a growth-regulative and immunosuppressive activity. AFP is mainly synthesized in the developing fetus, and its expression has been found to be associated with highly vascularized tumors in the adult. The stromal microenvironment is well known to be a crucial regulator of tumor development due to malignant and stromal cells communication through cell–cell and cell–matrix interactions and secretion of soluble factors.

As far as human adipose-derived stromal cells (ASC) are mesenchymal stromal cells that play a role in the development of breast and prostate cancer, we designed a study to investigate proangiogenic properties of AFP in ASC.

We have shown that FITC-labeled AFP could be internalized into ASC within 1 h, and its uptake was inhibited by pretreatment of cells with chlorpromazine that suggests clathrin-mediated endocytosis. We also observed a weak proliferative effect of AFP in ASC accompanied by ERK1,2 phosphorylation that peaked at 30 min. Furthermore, we tested the influence of AFP *on* differentiation *of* ASC and found that it neither induces nor enhances endothelial, adipo- or osteogenic differentiation. The analysis of angiogenic factors produced by ASC revealed that AFP-treated cell showed increased VEGF-A expression that was observed at both protein and mRNA levels by real-time PCR and ELISA. Since VEGF-A is a well-known angiogenic factor, we suggest that AFP could promote tumor vascularization via ASC stimulation to proliferate and to secrete angiogenic factor(s).

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