4TH INTERNATIONAL KLOSTER SEEON MEETING „ANGIOGENESIS" (16 -19 SEPTEMBER 2006): A MEETING REPORT

SESSION 1 : BLOOD VESSEL MORPHOGENESIS

Christer Betsholtz ("Role for Dll4/Notch1 in the selection of single endothelial tip cells during sprouting angiogenesis") addressed the question of how single tip cells are selected following VEGF-A stimulation. In order to investigate whether the number of tip cells is fixed through genetic predetermination within the endothelial cell population, Christer Betsholtz and colleagues performed *in vivo* experiments involving the application of gamma-secretase inhibitor (GSI), Delta-like 4 (DII4)/Notch loss of function studies, analysis of endothelial Notch signalling, and Notch1 gain of function. Inhibition of Notch signalling by GSI led to an increase in the number of endothelial tip cells and excessive sprout branching and fusion. Similar observations were made in DII4+/- mice, in which increased tip cell number and capillary plexus density was noticeable. Taken together, the results showed that a balanced action of VEGF-A and DII4/Notch1 is required to induce, select and guide single endothelial tip cells. Moreover, Notch signalling between endothelial cells at the sprouting front serves to limit the number of tips, as inhibition of Notch signalling in endothelial cells leads to excess tip-cell formation and vessel hyper-sprouting.

Ralf Adams ("Regulation of blood vessel morphogenesis through cell-cell and cell-matrix interactions"). In the first part of his presentation, Ralf Adams emphasized the importance of Ephrin-B2 for vascular smooth muscle cell (vSMC) and pericyte function during embryonic development, as observed in mouse embryos lacking functional Ephrin-B2 in mural cells. The second part of his presentation was devoted to the role of the integrin β-1 (*Itgb1*) subunit in mural cells. Inactivation of *Itgb1* gene in mural cells was achieved by cross-breeding *Itgb1*-lox/lox and *Pdgfrb-Cre* mice and caused vascular abnormalities in mutant embryos, leading to death at birth. vSMC defects were also observed in *Itgb1* mutants. Although vSMC were present in mutant embryos, they were unable to support the formation of veins. Further analyses revealed that *Itgb1* is required for vSMC attachment and spreading as well as normal motility and cytoskeletal organisation. *Itgb1* is also important for the organisation, but not the formation of focal adhesions. *Itgb1* mutant pericytes showed reduced proliferation, spreading and attachment.

Holger Gerhardt ("Loss of endothelial guidance by MMP-dependent cleavage of VEGF-A causes retinal vasculopathy"). Dr. Gerhard analysed the role of matrix metalloproteinases (MMP) in the development of retinopathy. He showed that principal characteristics of pathological vascular pattering in a mouse retinopathy model are loss of endothelial guidance and proliferation control, correlating with disturbed extracellular VEGF-A distribution. Several analyses demonstrated that the heparin-binding VEGF-A was locally produced in the ischemic area, but the protein became re-distributed, suggesting loss of cell-surface binding capabilities. Almost 80% of retinal VEGF-A protein was proteolytically cleaved, removing the heparin-binding C-terminus. The MMP inhibitor GM6001 blocked VEGF-A cleavage, and restored VEGF-A protein localization and guided sprouting. MMP characterization revealed upregulation of macrophage-specific MMP-12. Consistently, MMP-12 deficient mice were protected from retinal vasculopathy. These data confirm previous

ideas that MMPs are of importance in retinal vasculopathies, yet suggest a previously unanticipated mechanism.

Dieder Y.R. Stainier ("A common progenitor for hemapoietic and endothelial lineages in the zebrafish gastrula"). It is commonly assumed that hematopoietic and endothelial cells arise from a common precursor, the hemangioblast. Didier Stainier reported pioneering work that provides the first clear-cut *in vivo* evidence for the existence of the hemangioblast and reveals distinct features of this cell population. Through the construction of single cell resolution fate maps, his group showed that single mesodermal cells in the early zebrafish gastrula can give rise to both (and only) endothelial and hematopoietic cells. Moreover, only a subset of hematopioetic and endothelial cells appear to differentiate from the hemangioblast. Didier Stainier also presented data on the role of the cIAP (inhibitor of apoptosis) protein family. cIAP is expressed in endothelial cells during development, and caspase inhibition can rescue endothelial cell apoptosis in cIAP mutants. Moreover, NF-kappaB appears to be required for vascular integrity during zebrafish development.

SESSION 2: CELL-CELL SIGNALING IN ANGIOGENESIS

Jan Kitajewski ("Notch in development and tumor angiogenesis") reported a mechanism by which the interaction between tumor cells and endothelial cells promotes angiogenesis through MAPK and Notch signalling pathways. He showed that the Notch ligand Jagged1, induced in tumor cells by growth factors via MAPK, triggers Notch activation in neighbouring endothelial cells (EC) and promotes capillary-like sprout formation. Thus, Jagged1-expressing tumor cells significantly enhanced neovascularization and tumor growth *in vivo*. Adenovirus-mediated delivery of a Notch decoy (consisting of Notch1 extracellular domain) to tumors inhibited VEGF-induced angiogenesis and lead to tumor suppression. These results and the observation that high-level coexpression of Jagged1 and Notch1 in human breast cancer is associated with poor overall survival, suggest that Notch is a potent target for therapeutic intervention of cancer progression.

Thomas Gridley ("Notch signalling during vascular development in mice") presented a targeted mutation in the gene encoding the Notch ligand Dll4. His group observed that mouse embryos heterozygous for the DII4 mutation exhibit haplo-insufficient lethality due to defects in vascular remodelling, indicating that vascular remodelling in the mouse is sensitive to Dll4 gene dosage. These results demonstrate that, as was suggested by gene expression studies, the DII4 gene encodes the prominent Notch ligand during early vascular development in mice.

Manfred Gessler ("Dll4-Notch-Hey signalling in endothelial progenitor cells") discussed the role of hypoxia in Dll4-Notch-Hey signalling in embryonic endothelial progenitor cells (eEPC). eEPC expressed high amounts of Coup-TFII, a regulator of vein identity, while levels of the arterial markers DII4 and Hey2 were low. Hypoxia-mediated induction of DII4 and Hey2 led to repression of COUP-TFII in eEPC, suggesting that hypoxia-induced Notch signalling may also determine endothelial identity. Furthermore he showed that Hey factors are capable of repressing HIF-1alpha mediated gene expression, which represents a negative feedback loop preventing excessive hypoxic gene induction. Thus, low oxygen

levels lead to activation of a Dll4-Notch-Hey signalling cascade and subsequent repression of COUP-TFII in eEPC.

SESSION 3: ANGIOGENESIS IN PATHOLOGICAL SITUATION AND ITS MODULATION IN THERAPY

Donald McDonald ("Cessation of VEGF inhibition is followed by rapid regrowth of blood vessels in tumors") presented data on the effects of VEGF inhibitors on tumor vascularity and consequences to the vasculature after drug withdrawal. He reported that in spontaneous pancreatic islet tumors in RIP-Tag2 transgenic mice and in implanted Lewis lung carcinomas in wild-type mice, treatment with VEGF inhibitor caused a 50-60% reduction in tumor vasculature and led to the occurence of empty sleeves of basement membrane. Treatment of normal mouse trachea with VEGF inhibitor led to the regression of some normal capillaries, but only little formation of empty basement membrane sleeves. After the end of the treatment, endothelial sprouts grew into empty sleeves of basement membrane and tumors were fully revascularized at day 7 in RIP-Tag2 transgenic mice. In normal mouse trachea, only 70% of the former EC regrew after drug withdrawal. These results indicate that empty sleeves of basement membrane and the accompanying pericytes provide a scaffold for rapid revascularization of tumors after removal of anti-VEGF therapy and may be potential targets in cancer therapy.

Valentin Djonov („Escape mechanisms involving intussusceptive angiogenesis and the massive production of smooth-muscle actin after short-term anti-angiogenic therapy and treatment with ionizing radiation in mice carrying MMTV/c-neu xenografts") proposed that resistance to treatment after short-term angiogenic therapy or treatment with ionising radiation can be caused by the occurrence of a switch from angiogenic sprouting to intussusception. He reported that mice carrying MMTV/c-neu mammary xenografts which underwent either treatment with VEGF receptor tyrosine kinase inhibitors or radiotherapy showed different phenomena in the post-treatment period, which act together as an escape mechanism: i) intussusceptive vascular growth; ii) up-regulation of smooth muscle actin and iii) a dramatic decrease in intratumoral microvascular density, but with only a minimal alteration in the vascular exchange surface. Based on these findings, Valentin Djonov postulated that the experimental tumors used escaped anti-angiogenic therapy and treatment with ionizing radiation because of the occurrence of intussusceptive angiogenesis, suggesting that this process plays an important role in cancer therapy.

Lieve Moons ("Therapeutic potential of VEGF family members in pro- and anti-angiogenic strategies") discussed the role of the VEGF family members, PlGF and VEGF-B, as potential therapeutics in pro- and anti-angiogenic treatment. PlGF and VEGF-B, known to affect only pathological angiogenesis and thereby not causing the side effects of VEGF-A, might be novel candidates for the treatment of ischemic tissues. She showed that in a murine myocardial infarction model, delivery of PlGF amplified local VEGF levels and led to revascularization. In hind limb ischemia models in mice and rabbits, PlGF stimulated collateral vessel growth and the functional recovery of the ischemic hind limb. VEGF-B administration also stimulated revascularization of ischemic myocardium and had a direct protective effect on cultured mouse cardiomyocytes.

Andrea Banfi ("Angiogenic potentiation, vascular normalization and hind-limb ischemia improvement by matched-level co-delivery of VEGF164 and PDGF-BB") discussed the role of PDGF-BB as a modulator of VEGF-induced angiogenesis. By implanting retrovirally transduced primary mouse myoblasts that deliver VEGF164 to skeletal muscle, Andreas Banfi´s group previously found that a switch between the induction of stable capillaries and progressive angioma growth occurs at a certain threshold level in microenvironmental VEGF concentration. Aberrant vessels growing above this threshold lacked normal pericytes. As pericytes are known to be recruited by PDGF-BB to stabilize vessels at angiogenic sites, myoblasts were transduced with either VEGF164 or PDGF-BB and implanted independently or together in mice. PDGF-BB alone did not stimulate angiogenesis, however, in conjugation with VEGF164 increased vascular density. Low VEGF levels induced aberrant vessels when PDGF-BB was blocked by soluble PDGFRbeta, whereas high VEGF levels induced normal vessels if co-delivered with PDGF-BB. These results showed that manipulation of the PDGF-BB signalling can shift the threshold between normal and aberrant angiogenesis by VEGF.

SESSION 4: REGULATION AND MODULATION OF ENDOTHELIAL CELL FUNCTION

Elena Pasquale („The EphB4 receptor as a target to inhibit angiogenesis") talked about the observation that EphB4 on the tumor cell surface promotes tumor growth by stimulating ephrinB2 reverse signalling in tumor blood vessels. In this light EphB4 antagonists show promise as inhibitors of tumor angiogenesis and tumor growth. To identify small EphB4-ephrin B2 inhibitors her group identified by a phage display a TNYL peptide which binds preferentially to the RAW motif of EphB4 and antagonizes Ephrin B2 binding to EphB4. She showed that the TNYL-RAW peptide is selective for EphB4 and is active in low concentrations. The TNYL-RAW peptide inhibited EphB4 activation in MCF7 breast cancer cells.

Hellmut Augustin („Regulation of vascular homeostasis by angiogenic cytokines") discussed Eph/Ephrin adhesive interactions between tumor cells and endothelial cells and their role in the dissemination of metastatic tumor cells. He showed that EphB4 overexpression leads to an increase in the number of tumor cells homing to the lung whereas untransfected tumor cells metastasize almost exclusively to the bone. In the second part of his presentation, Hellmut Augustin discussed the function of Ang-2 which is selectively produced and stored in endothelial cells. Ang-2 null mice can not elicit a rapid inflammatory response but exogenous Ang-2 rescued the normal inflammatory response of Ang-2 null mice. Cell adhesion experiments and corresponding *in vivo* experiments indicated that the firm adhesion but not leukocyte rolling is perturbed during inflammatory cell recruitment in Ang-2 null mice. It was also proposed that Ang-2 affects early tumor growth but is dispensable during later stages of tumor growth.

Jan Schnitzer („Proteomic imaging of microenvironment-dependent expression in endothelium and its caveolae for organ- and tumor-specific delivery *in vivo*") talked about the molecular complexity of tissues and the inaccessibility of most cells within a tissue which limits the discovery of key targets for tissue-specific delivery of therapeutic and imaging agents *in vivo*. Yet luminal endothelial surfaces directly in contact with circulating blood provide accessible interfaces for targeting. By isolation of luminal endothelial plasma

membranes and their caveolae from major organs and solid tumors using a nanoparticle coating method, his group identified 83 known endothelial cell marker proteins. They also identified distinct molecular signatures ("fingerprints") for the luminal endothelial cell surface in tumors and each organ and then validated tissue–induced endothelial targets accessible to intravenously applied antibodies.

SESSION 5: HYPOXIA SIGNALLING

Jacques Pouysségur ("Hypoxia signalling from survival to tumor cell death") summarized the current knowledge about the key players involved in the cellular response to hypoxia. His group found that of the 3 known HIF prolyl hydroxylases (PHD), PHD-2 is the most prominent one earmarking HIF for degradation. In addition, FIH-1 (Factor inhibiting HIF) is known to reduce the HIF-1 transcriptional activity by hydroxylation on an asparagine residue. Silencing of both PHD2 and FIH-1 during normoxia induced a complete hypoxia response, indicating that these two enzymes act synergistically. Because HIF-1 alpha possesses two transactivation domains (N- and C-TAD), Jacques Pouysségur hypothesized that HIF-1alpha shows a bi-functional activity depending on the available oxygen levels and acidic environment. Hypoxia induces the expression of carbonic anhydrase IX (CA IX), which helps to retain a relatively neutral intracellular pH, while the expression of the pro-apoptotic protein BNIP-3 is induced under moderately hypoxic conditions, but requires acidosis to promote cell death. Thus, under the extreme conditions of low pO2 and acidosis, necrotic areas often develop.

Randall S. Johnson ("Physiology of angiogenic response during hypoxia") described a keratinocyte specific VHL knockout mouse (K14-VHL mice). These mutant mice are significantly smaller than wild type mice and show redness of the skin accompanied by augmented expression levels of hypoxia related genes like PGK, Glut-1 and VEGF, and a slightly elevated vessel density in skin. Furthermore, the mice were hypothermic (34°C) leading to death. This phenomenon could be reversed when the mice were fed with a calory rich diet. K14-VHL/HIF or K14-VHL/VEGF double mutants however had normal size but redness and some other phenotypes remained different (elevated hematocrit, hypothermia). In these mice also higher levels of EPO were found in the circulation, however, the source was not the skin but the liver and kidney. This was due to the up-regulation of NO in the skin. Treatment of the animals with L-NAME (a NOS inhibitor) restored the blood pressure and reduced the EPO level to normal levels within 4 days.

Lorenz Poellinger ("Mechanisms of signal transduction and gene regulation in hypoxic cells") showed that in neuroblastoma cells kept in 1% O_2, HIF1alpha is immediately up-regulated to a maximum after 4 hours. HIF-2alpha expression on the other hand is induced much slower although there is a long term expression (maximum at 72h). In 5% oxygen, HIF-1alpha was not detected. Under these conditions, HIF2alpha is only detectable at later time points but is augmented more rapidly to levels comparable to those detected in 1% oxygen conditions. Lorenz Poellingers group also showed that HIF1alpha interacts with Notch on the Hey2 promotor and that FIH-1 represses the Notch function by hydroxylation of Notch1ICD, showing that FIH can indeed influence both the HIF and Notch pathway.

SESSION 6: LYMPHATIC DEVELOPMENT, LYMPHANGIOGENESIS AND TUMOR METASTASIS

Kari Alitalo ("Lymphangiogenesis in development and disease") gave a broad overview over the progress that has been made in the field of lymphangiogenesis in development and human disease during the last few years. VEGF-C and VEGF-D are growth factors that bind to VEGFR-3 and VEGFR-2 and appear to be both lymphangiogenic as well as angiogenic. VEGF-C knockout mice have defective lymphatic vessels while VEGF-C overexpression leads to lymphangiogenesis, growth of draining lymphatic vessels, intralymphatic tumor growth and lymphatic metastasis in several tumor models. Therefore treatment with VEGF-C was suggested to improve reconstitution of draining lymph nodes after lymph node extraction. Soluble VEGFR-3 blocked lymphangiogenesis and lymphatic metastasis in VEGF-C/D overexpressing tumors. Yet this VEGF-C/D trap had only an effect on growing vessels and did not affect normal lymph vessels. The monotherapy of cancer with an antibody against VEGFR-3 using a xenograft tumor model caused a 30-40% reduction of tumor growth. Altogether Kari Alitalo demonstrated the important role of VEGF-C and -D in lymphangiogenesis.

Brant M. Weinstein ("Live imaging of lymphatic development using the zebrafish") outlined the advantages of zebrafish as a model for visualization and characterisation of lymphatic vessels. His studies showed that the zebrafish possesses a lymphatic system that shares many of the characteristic features of lymphatic vessels found in vertebrates. In addition his group focuses on defining the origin of lymphatic endothelial cells. Their multiphoton time-lapse imaging techniques allowed *in vivo* cell tracking and provided conclusive new evidence supporting a venous origin for primitive lymphatic endothelial cells.

Peter Carmeliet ("Genetic analysis of lymph/angiogenesis and the neurovascular link: therapeutic implications") reported in the first part of his presentation that a neutralizing anti-mPlGF monoclonal antibody efficiently blocks tumor growth and angiogenesis in different mouse tumor models. This antibody inhibited PlGF binding to its receptor Flt1 (VEGFR-1). By inhibiting lymphangiogenesis this antibody also repressed lymphatic metastasis. The use of anti-PlGF antibody in combination with chemotherapy or anti-VEGFR-2 antibody appears promising because it showed a chemo-sensitizing activity and may interfere with the upregulation of PlGF observed after anti-VEGFR-2 treatment. In the second part of his presentation, Peter Carmeliet demonstrated the role of multi FGFR antagonists as inhibitors of the development of lymphatic and blood vessels. Treatment with FGFR antagonists induced lymphedema in Xenopus tadpoles and inhibited vessel regeneration after tail fin amputations in adult zebrafish. In a pancreatic tumor model, mice treated with FGFR antagonists showed reduced tumor growth and arthritis.

Gerhard Christofori ("Angiogenesis, lymphangiogenesis, and tumor metastasis") discussed the mechanisms of lymphogenic and hematogenic metastatic tumor cell dissemination, based on the analysis of Rip1/Tag2 transgenic mice which develop carcinoma in pancreatic islet beta-cells and undergo an angiogenic switch during tumor progression. Intercross of these mice with transgenic mice expressing different angiogenic factors in pancreatic beta-cells revealed that VEGF-A leads to an early angiogenic switch, and that VEGF-C as well as VEGF-D are important for lymphangiogenesis and the formation of lymphatic metastasis. Furthermore it was shown in cultured human breast cancer cells and in

the Rip1/Tag2 transgenic mouse model that podoplanin induces a novel molecular pathway of collective tumor cell migration and invasion that does not involve the loss of E-cadherin function or epithelial-mesenchymal transition. Podoplanin rather induced rearrangements of the actin cytoskeleton and the formation of filopodia.

Michael Detmar ("Lymphangiogenesis and cancer metastasis – new insights") presented a recent analysis of human melanoma suggesting that tumor lymphangiogenesis may serve as a prognostic marker for the metastatic risk of human cancers. These findings reveal that tumor cells, via VEGF-A and -C, can induce lymph node lymphangiogenesis before they have metastasized. This suggested that tumor cells can prepare their future arrival in the lymph node. In the second part of his talk, Michael Detmar reported the establishment of a low-density microvascular differentiation assay that allows quantitative determination of endothelial cell lineage and (lymph-) angiogenesis *in vivo*. Using this assay, his group identified a number of new lymphangiogenic factors including hepatocyte growth factor (HGF) which induces proliferation of lymphatic endothelial cells independent of VEGFR-3.

SESSION 7: VEGF SIGNALING AND FUNCTION

Lena Claesson-Welsh ("VEGF co-receptors in regulation of angiogenesis") talked about the role of Neuropilin-1 (Np1) and heparan sulfate proteoglycans (HS) in VEGF signaling. To determine the contribution of Np1, the signalling properties of the Pox/Orf virus-derived VEGF-E were compared to those of VEGF-A165 and VEGF-A121. VEGF-E bound to Np1 but not to HS whereas VEGF-A165 bound both. Both forms of VEGF induced VEGFR-2 phosphorylation. Using binding studies with PAEC, she showed that in the presence of Np1, VEGF-E, VEGF-A165 and VEGF-A121 bound to VEGF-R2, and VEGF-E competed better with VEGF-A165 than VEGF-A121. However, VEGF-E bound poorly in the absence of Np1 whereas VEGF-A121 bound better. The binding site of VEGF-E on Np1 overlaps with the binding site of Sema3A and therefore, VEGF-E binding to Np1 can be competed for with Sema3A. Both HS and Np1 were necessary for efficient cell migration. Taken together she could show that HS and Np1 are not required for the acute activation of VEGFR-2 but modulate the VEGFR-2 response. Furthermore she demonstrated in the embryoid body model that Np1 is necessary for the organization of a three-dimensional capillary plexus. p38 and the MAP kinase pathways were activated by VEGF-A165 and VEGF-E. The blocking of p38 led to dysfunctional vessels in the center of the embryoid body and an arrest of endothelial cell organization.

Gera Neufeld ("The molecular mechanisms by which neuropilins potentiate VEGF signaling") showed in his talk that the VEGF-induced phosporylation of VEGFR-2 is enhanced by neuropilins even in the absence of VEGFR/Np complex formation, Thus, the commonly used "bridge model" for VEGF binding to VEGF-R2 and Np1 might not be correct. To demonstrate this, he used VEGF-A165KF, a VEGF mutant which binds to Np1 and Np2 but only weakly to VEGFR-1 and -2. In PAEC expressing VEGFR-2 and Np1, this form also induced weak phosphorylation of VEGFR-2 and activation of ERK1/2. Moreover, the combination of VEGF-A165KF and VEGF-A165 did not reduce receptor phosphorylation. In HUVEC, high levels of VEGF-A165KF did not block the VEGF-A165 induced sprouting, and no formation of VEGF-R2/NP1 complexes could be detected.

Christiana Ruhrberg **("Role of the neuropilin ligands $VEGF_{164}$ and Sema3A in neuronal and vascular patterning")** showed that Sema3A and C bind to neurons and vessels in the brain of mouse embryos, whereas Sema3F binds only to neurons, and Sema3E like VEGF-A164 binds strongly to vessels. She proposed that Sema3A is required for neuronal guidance but not for vessel patterning, which is also implicated by the neural phenotype of Sema3A knockout mice. In neuropilin loss-of-function experiments, in which the binding site of neuropilins is mutated so that VEGF, but not semaphorins can bind, semaphorin signaling through NP1 was not required for vessel patterning. In the hind limb model, VEGF-A164 and Sema3A pattern distinct cell types: VEGF is required for soma migration and Sema3A for axon pathfinding. Thus these two molecules act more in cooperation than in competition.

Ferdinand Le Noble **("A novel role for VEGFR2-Neuropilin-1 signaling in hypoxia-induced cardiomyopathy: rescue with semaphorin 3A")** presented data about the effects of prenatal hypoxia on cardiac development. Hypoxic chicken embryos had dilated left ventricles, showed an increased number of blood vessels in the heart, suffered from cardiac dysfunctions and cardiomyopathy, and increased expression levels of VEGF165 and VEGFR-2. In *ex vivo* experiments with left ventricle muscle bundles he demonstrated that treatment with VEGF165 led to loss of cardiac contractility. This effect could be rescued with soluble VEGFR-1 (sFlt1), VEGFR-2 tyrosine kinase inhibitor SU5416, or Sema3A, a ligand for Np1. Also *in vivo*, prenatal treatment of hypoxic embryos with sFlt1 or Sema3A could prevent cardiac dysfunction and increased survival of the embryos. This data showed a novel role for VEGF-R2/NP1 signaling in hypoxia induced cardiomyopathy.

David O. Bates **("The distal spliced isoforms of VEGF, $VEGF_{xxx}b$, in pathology and physiology")** discussed the role of VEGF splice variants. He presented the $VEGF_{xxx}b$ variants of the commonly known VEGF isoforms which are differentially spliced in exon 8. In most normal tissues the predominant form is $VEGF_{xxx}b$ (50% to 96% of total VEGF) whereas in angiogenic tissue like placenta or tumors, these isoforms are downregulated. $VEGF_{165}b$ was capable to block $VEGF_{165}$-mediated angiogenesis and acted as a competitive inhibitor of $VEGF_{165}$. Thus $VEGF_{xxx}b$ appears to be antiangiogenic, and the balance of the two forms may determine the angiogenic activity. A possible mechanism could be the incomplete phosphorylation of VEGF-R2 by $VEGF_{165}b$ which leads to internalization and degradation of the receptor. Cytokines like IGF and TGFβ can alter the regulation of VEGF isoform splicing.

SESSION 8: ANGIOGENIC SIGNALING: VEGF AND BEYOND

Johannes Waltenberger **(„Monocytes as biosensors for the assessment of vascular integrity")** reminded that endothelial dysfunction is associated with vascular pathologies. VEGF, which is crucial for maintaining endothelial integrity, seems to counteract endothelial dysfunction by stimulation and production of NO and by its anti-apoptotic properties. Johannes Waltenberger claimed that monocytes are good surrogate markers for analysing the VEGF response in patients because, like endothelial cells, they express VEGFR-1 and have functional downstream signal transduction machinery. Moreover, monocytes contribute to vascular repair and collateral growth. His group had previously found that monocyte function is severely impaired in the presence of cardiovascular risk factors and that VEGF-induced migration of monocytes is significantly decreased in patients suffering from diabetes

mellitus, hypercholesterolemia and in smokers. This he explained by defects in signalling downstream from VEGFR, since the phosphorylation of VEGFR was intact but migration was impaired. He also reported that monocyte dysfunction can be reverted by statins, which improve VEGF-induced monocyte migration. In smokers, monocyte dysfunction can be reverted by high doses of vitamin C. He concluded by stating that monocytes may be used to monitor the individual cardiovascular risk burden.

Erhard Hofer ("Genes preferentially induced by VEGF/VEGFR2"). Using Affymetrix microarray analysis and real-time RT-PCR, his group identified a set of genes induced by VEGF/VEGFR2 in comparison with EGF induced genes. The data obtained show that VEGF/VEGFR2 preferentially triggers signals through PLC-gamma, PKC and Ca^{2+}. Transcription factors such as EGR-1 (downstream from PKC) and NFAT (downstream from Ca^{2+}) appear to be important regulators of VEGF induced genes. This provides VEGF with a potency to induce genes that are normally regulated by inflammatory cytokines, probably mediated through binding sites for NFAT and NFkappaB in these genes. Another set of genes upregulated by VEGF only included transcription factors such as Nurr1, Egr3, Hlx1 and MEF2C. He concluded by stating that the ability to induce a large number of genes common with genes induced by inflammatory mediators, and a small number of inflammation independent genes is important for strong angiogenic properties of VEGF.

Veronique Orian-Rousseau ("The co-receptor function of CD44v6 for c-Met and for VEGFR2 in angiogenesis") presented a new concept demonstrating a direct function of the cytoskeleton as an inducer of growth factor receptor signaling. She proposed that receptor tyrosine kinases require cell adhesion molecules as co-receptors to function. For example CD44v6 serves as a co-receptor for c-Met and plays a dual function: the extracellular part of CD44v6 is necessary for receptor tyrosine kinase activation, whereas the intracellular part recruits the cytoskeleton to the membrane. Pancreatic carcinoma cells transfected with CD44v6 formed metastasis to lymph nodes and lungs, which was blocked by the co-transfection of a CD44v6-specific peptide. The peptide as well as anti-CD44v6 antibody were able to block cell migration, spheroid sprouting and capillary formation in HUVEC cells. HGF/c-Met induced activation of endothelial cells as well as VEGFR-2 activation and VEGF-induced migration of endothelial cells was dependent on CD44v6, suggesting that CD44v6 is a co-receptor for VEGFR-2.

Klaus Preissner („Extracellular RNA induces endothelial cell permeability via vascular endothelial growth factor") reported that RNA is associated with thrombus. Pre-treatment with RNase or heparin, but not DNase, decreased thrombus formation and vessel occlusion rate. Vessel permeability induced with natural or artificial (poly:IC) RNA correlated with topographic alterations of tight junction proteins and VE-cadherin and was mediated through VEGF. This hyperpermeability could be decreased by pre-treatment with RNase. Anti-VEGFR-2, but not anti-VEGFR-1 antisense oligos decreased hyperpermeability induced by RNA or poly:IC, suggesting the involvement of VEGFR-2. Klaus Preissner concluded that RNA/ poly:IC induce mobilisation and stabilization, but not expression of VEGF.

Georgia Mavria ("Control of sprouting and vessel quiescence by MAP-kinase and Rho GTPase signalling") used a co-culture assay of endothelial cells (HUVEC) and human dermal fibroblasts (HDF) that models three major stages of angiogenesis: endothelial cell proliferation, migration and tube formation. She showed that the selective inhibition of ERK in

the migratory phase disrupts tube formation, results in loss of cell bipolarity and detachment of isolated endothelial cells as well as retraction of sprouting vessels. It also led to upregulated Rho-kinase (ROCK) activity. Inhibition of ROCK rescued the anti-angiogenic effect of ERK inhibition both *in vivo* and *in vitro*, and promoted angiogenesis by increasing branching and vessel length. She also showed that inhibition of ROCK led to destabilisation of VE-cadherin from cell junctions, increased cell motility and sprouting that depended on the activity of small GTPase Rac. She concluded that ROCK promotes vessel quiescence by antagonising the pro-migratory effects of Rac.

Marina Schorpp-Kistner („Transregulatory and cell-autonomous function for JunB in angiogenesis"). Her group has previously shown that loss of JunB affects vascular development and placentation, and that JunB regulates VEGF expression. She reported that JunB is induced by hypoxia via NFkappaB, independent from the HIF pathway. She also showed that JunB is required for tumor angiogenesis, because JunB deficiency leads to reduced VEGF expression and recruitment of host-derived cells in tumors. Next, she talked about the requirement of JunB for endothelial cell morphogenesis. Her group identified core-binding factor-beta (CBF-beta) as a direct JunB target gene. CBF-beta together with Runx protein forms the CBF that binds to JunB promotor under hypoxic conditions. She also showed that tube formation failure in JunB knockout mice can be rescued by addition of either JunB or CBF-beta. Moreover, she pointed out that expression of MMP-13 (a target gene of CBF) is impaired in the $JunB^{-/-}$ endothelial cells and that these cells had decreased invasive capacity. She concluded that JunB has regulatory and cell-autonomous functions in angiogenesis.

SESSION 9: ENDOTHELIAL CELL CONTACTS IN BLOOD VESSEL FORMATION

Elisabetta Dejana ("Role of SOX18 in the regulation of lymphatic differentiation") talked about spontaneous mutations in the Sox18 gene, represented in the *ragged* mouse mutant. She described the *opossum* allele of the *ragged* mouse (Ra^{op}). Heterozygote $Ra^{op/+}$ mice are characterized by ragged hair, growth retardation of late hair follicles and lymphedema. Lymphatic vessels of these mice are increased, but thinner. In humans, Hypotrichosis-Lymphedema, characterized by defects in the lymphatic system, is caused by Sox18 mutation. Ra^{op}/Ra^{op} shows reduced expression of Prox1 in the skin. She proposed that SOX18 is an upstream regulator of Prox1, because SOX18 is expressed earlier than Prox1. However, VEGF-C and SOX18 together regulate the expression of Prox1, resulting in the differentiation of lymphatics, whereby a VEGF-C gradient leads to regulated formation of lymphatic vessels.

Andrea Horst ("CEA-related cell adhesion molecule 1 (CEACAM1) modulates vascular remodelling *in vitro* and *in vivo*") talked about the role of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in vascular remodeling. CEACAM1 is known to enhance endothelial sprouting and migration, acting synergistically with VEGF *in vitro*. Here she focused on the *in vivo* functions of CEACAM1. $CEACAM1^{-/-}$ mice show chaotic assembly of vessels, whereas CEACAM1endo+ mice which overexpress CEACAM1 show extensive vascularization. After induction of hindlimb ischemia by femoral artery ligation, $CEACAM1^{-/-}$ mice exhibited significantly reduced growth of arterioles and collateral blood flow whereas CEACAM1endo+ mice reveal an increase in re-vascularization and collateral blood flow.

Thus, CEACAM1 expression is important for the establishment of newly formed vessels and might be a target for therapeutic manipulation of angiogenesis in disease.

Beat Imhof ("Junctional adhesion molecule C in tumor angiogenesis and monocyte transendothelial migration"). In tumors, monocytes are attracted to transmigrate from vessels into the abluminal compartment and fail to induce an immune response against the tumor. Instead, tumor-associated monocytes promote proliferation of tumor cells and tumor progression. Junctional adhesion molecule C (JAM-C), found in blood and lymphatic vessels, pericytes, intestinal epithelium and testis, is thought to play a role in the extravasation of monocytes within the tumor. JAM-C is a tight junction molecule that co-localizes with occludin. The application of antibodies against JAM-C leads to reduced numbers of blood vessels and a reduction of tumor size. Anti-JAM-C blocked the reverse transmigration of monocytes, resulting in a net decrease of monocytes in the abluminal compartment. Taken together, JAM-C plays a critical role in maintaining monocytes positioned in the abluminal compartment of blood vessels within a tumor.

Session 10: Cell-Cell Interactions during Angiogenesis

Dietmar Vestweber ("Endothelial cell contacts in inflammation and angiogenesis") demonstrated in his talk the role of two endothelial cell surface receptors in the control of junction integrity. Endothelial cell-selective adhesion molecule (ESAM) is a tight junction associated protein specifically expressed in endothelial cells and in platelets and mediates homophilic cell adhesion. Dietmar Vestweber reported that in different inflammation models in $ESAM^{-/-}$ mice, the lymphocyte migration into inflamed skin was not affected whereas the migration of neutrophils into the inflamed peritoneum was delayed. The impairment of leukocyte transmigration in combination with the reduced VEGF induced permeability of the endothelium leads to the conclusion that ESAM participates in transmigration of neutrophils by influencing the opening of EC contacts. Using a retinopathy of prematurity (ROP) model he demonstrated a role of ESAM in pathologic angiogenesis in adult mice. In the second part of his talk, Dietmar Vestweber demonstrated that the knock down of VE-PTP, an adherens junction associated transmembrane phosphatase, *in vitro* led to an increased permeability of the endothelial cell layer and a higher transendothelial migration that indicates a role of VE-PTP in leukocyte extravasation. The expression of a truncated form of VE-PTP in mice led to a dramatic developmental phenotype as well. The mutant embryos died at mid-gestation because of vascular malformations. In these mice, the vascular system developed but only as a flat and basic unstable network. These results suggest that VE-PTP is not required for the initial vessel formation but for their maintenance and remodelling.

Volkhard Lindner ("The novel gene Cthrc1 is essential for blood vessel formation") talked about the analysis of Collagen Triple Helix Repeat Containing-1 (Cthrc1) null mice regarding development of the vasculature and the marrow endothelial sinusoids. Cthrc1 was identified as gene induced in adventitial fibroblast after arterial injury, and overexpression of Cthrc1 has been shown to effect bone and collagen matrix formation. In the case of Cthrc1 loss of function they found out that embryos revealed bleeding primarily around mouth, eyes and paws what often resulted in a deformation of these organs and to early embryonic death of all Cthrc1 homozygous and some heterozygous mutant mice. Characterization of the viable $Cthrc1^{+/-}$ mice showed sudden quadriplegia along with bleeding into the CNS and axial

skeleton abnormalities in some animals. Mature red blood cell accumulations surrounded by endothelial cells could be detected in the bone marrow cavity, leading to an expansion of the marrow endothelial sinusoids. Abnormalities in bone and lung formation were also observed. Using luciferase reporter assays, Volkhard Lindner could show that Cthrc1 is an inhibitor of the TGF-beta signaling pathway, whose alteration could lead to the observed phenotypes.

Karl Plate ("Role of hypoxia in tumor vasculogenesis and tumor angiogenesis") examined the question if VEGFR-1 plays a role in the homing of bone marrow-derived endothelial progenitor cells to glioblastomas, one of the most vascularised cancers. For this, he transplanted GFP expressing VEGFR-1 deficient bone marrow cells in glioblastoma bearing, lethally irradiated mice. His results showed that VEGFR-1 is not necessary for the homing but affects tumor angiogenesis by reduction of tumor growth and glioma vascularisation. This phenotype could be rescued by overexpression of VEGF in tumor cells suggesting a paracrine effect.

Eli Keshet ("Recruitment by VEGF of angiogenic accessory cells") addressed the recruitment of accessory cells during adult neovascularisation. To study the role of VEGF in this recruitment process, double transgenic mice (MHCα-promotor-tTA/TET-promotor-VEGF or VEGF-Trap) were designed leading to a heart specific expression of VEGF or VEGF-Trap. These VEGF loss and gain of function experiments showed that VEGF leads to an organ specific homing of circulating monocytes as well as their perivascular positioning around angiogenic vessels through VEGF-mediated SDF1 expression. These recruited bone-marrow-derived circulating cells (RBCCs) on the other hand indicate pro-angiogenic activity what results in a proliferation of the endothelium. The question if these RBCCs are a pro-angiogenic subpopulation of circulating monocytes or monocytes that are re-programmed on-site to become more pro-angiogenic is still open.