MEETING REPORT

5th International Kloster Seeon Meeting "Angiogenesis: Molecular Mechanisms and Functional Interactions"

Chair: Christer Betsholtz, Stockholm; Co-Chair: Lena Claesson-Welsh, Uppsala

Hosted by the Verein für Wissenschaftliche Fachtagungen in der Biomedizin e.V.

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Session 1: REGULATION OF VASCULAR STABILITY, PERMEABILITY AND TRANSPORT I

"Signaling through cadherins and beta catenin in vascular development"
Elisabetta Dejana (Milan) introduced the broad field of intercellular junctions between endothelial cells that mediate adhesion and communication. In endothelial cells, adherens junctions are mostly composed of vascular endothelial cadherin (VE-cadherin), an endothelium-specific member of the cadherin family of adhesion proteins that binds to several protein partners, including p120, beta-catenin and plakoglobin. VE-cadherin null endothelial cells strongly disconnect from each other and detach from the basement membrane. Conditional inactivation of beta-catenin in the endothelium using the Cre/loxP system results in vascular defects and loss of vessel integrity, causing early embryo lethality at day E10.5-11. Interestingly, some similarities between beta-catenin null embryos and the knockout of the Wnt receptor frizzled 5 suggest that some of the features of null embryos are due to inhibition of Wnt-signaling. Finally, Elisabetta Dejana described a crosstalk between adherens and tight junctions. It was demonstrated that endothelial VE-cadherin expressed at adherens junctions upregulates the gene encoding the tight junction adhesive protein claudin-5.

"Role of VEGF-B in the regulation of endothelial fatty acid transport"
Ulf Eriksson (Stockholm) investigated a novel function of vascular endothelial growth factor B (VEGF-B) which is a member of the VEGF family of growth factors. Microarray analysis revealed that VEGF-B expression was tightly co-regulated with genes encoding proteins in the respiratory chain of mitochondria and with genes involved in mitochondria biogenesis. In contrast, the expression pattern of closely related genes such VEGF-A, VEGF-C, PIGF did not show a correlation with genes involved in mitochondria functions. Additionally, VEGF-B expression is highly upregulated in high energy consuming organs and tissues such as heart and white adipose fatty tissue. VEGF-B-/- mice phenotype had slower fatty acid clearance from plasma and increased lipid uptake into white adipose tissue, resulting in increased body weight. VEGF-B specifically controlled accumulation of dietary fatty acids to some tissues via regulation of two fatty acid transporters expressed on endothelial cells. Isolation of heart endothelial cells from wild type and VEGF-B-/- mice shows that VEGF-B induces fatty acid uptake by endothelial cells via FATP3 and FATP4. Altered lipid metabolism and fatty acid uptake is involved in several human disorders including diabetes, obesity and cardiovascular diseases. Taken together, VEGF-B might have the potential to be a therapeutic target in the future.

"S1P signaling in the vascular system"
Timothy Hla (Farmington) presented Sphingosine 1-phosphate (S1P), a bioactive sphingolipid metabolite involved in several cellular processes such cell growth and survival, cytoskeletal remodeling and cell motility. Extracellular signaling of S1P is transduced through G protein-coupled receptors (GPCR) on the cell plasma membrane. Downstream effectors of S1P signaling are cytosolic Ca^{++}, cAMP and phosphorylation of protein kinases. S1P is highly upregulated during development of the vasculature and its receptors S1PR1 and S1PR2 can transduce distinct intracellular pathways. S1PR2 can antagonize S1PR1 which results in disruption of endothelial adherent junctions through downstream signaling via Rho, ROCK and PTEN. Retinopathy of prematurity (ROP) was examined using S1PR2-deficient mice. Hyperoxia-induced retinal neovascularization was observed in wild type mice. However homozygous null mice for S1PR2 developed a normalized vasculature in response to hyperoxia. In his point of view S1PR1 is involved in normal vascular development, whereas S1PR2 acts in pathological angiogenesis.

Session 2: REGULATION OF VASCULAR STABILITY, PERMEABILITY AND TRANSPORT II

"Role of HIF prolyl hydroxylases in angiogenesis"
Georg Breier (Dresden) presented data on the role of PHD-2 and PHD-4 in physiological and pathological angiogenesis by loss-of-function and by gain-of-function experiments. HIF prolyl hydroxylases (PHD) act as cellular oxygen sensors by regulating HIF stability. Mutation of the genes encoding PHD-2 or PHD-4 in mice led to embryonic or postnatal lethality, respectively. In order to elucidate the function of PHD-2 and PHD-4 in tumor progression and metastasis, his group manipulated the expression of these genes in experimental LM-8 osteosarcoma. PHD-2 overexpression in tumor cells markedly reduced tumor vasculature, growth, and metastasis. In contrast, vessel density was increased in tumors overexpressing PHD-4, as a result of enhanced vessel branching. Paradoxically, vessels in PHD-4 overexpressing tumors had poor functionality – as shown by reduced perfusion of the tumor tissue, and decreased tumor growth.

"Syk-deficiency in leukocytes leads to abnormal vascular morphogenesis"
Friedemann Kiefer (Münster) presented his group's recent data regarding the function of the non-receptor tyrosine kinase Syk, which is a well characterized signal transducer in leukocytes, in vessel development. In the mouse, genetic ablation of Syk leads to vascular malformations and most prominently to aberrant blood-lymphatic shunting. This results in embryonic lethality at midgestation. However, Syk expression has never been demonstrated in mature endothelium *in vivo*. To test the role of Syk in vessel development, his group performed lineage tracing experiments in mice. They expressed Cre recombinase under the control of the endogenous Syk promoter and subsequently visualized Cre expression using various R26-driven reporter strains with enzymatic and fluorescent readouts. The data did not provide any evidence for Syk expression in the endothelium. However, he was able to demonstrate an accumulation of Syk-deficient leukocytes, primarily in those areas of the embryo showing the most pronounced vascular malformations. Transcriptional and expression profiling of this expanded myeloid population revealed the production of chemokines as well as highly vasoactive growth factors. His group, therefore, proposes a model in which the elevated production of chemotactically, pro-proliferative and morphogenetically highly active cytokines by a derailed Syk-deficient myeloid population causes massive vascular malformations and ultimately results in lethal edema and hemorrhage during mouse embryogenesis.

"VE-PTP as regulator of endothelial cell contact integrity and vascular remodeling"
Dietmar Vestweber (Münster) - content embargoed.

"Robo4 promotes vascular stability"
Dean Li (Salt Lake City) presented data on the role of Roundabouts (Robos), which are guidance receptors with well-established functions in the nervous system. Robo4 is an endothelial-specific Robo. However, Robo4 knock out mice display a normal vascular patterning. He showed that Robo4 maintains vascular integrity and that Robo4 is expressed in the stalk cell of endothelial tubes. Activation of Robo4 by Slit2 inhibits vascular endothelial growth factor $(VEGF)_{165}$-induced migration, tube formation and permeability *in vitro* and $VEGF_{165}$-stimulated vascular leakage *in vivo*. Surprisingly, Robo4 signaling does not affect the phosphorylation of VEGFR-2. It probably inhibits the signal transduction from VEGFR-2 to Src. In murine models of retinal and choroidal vascular disease, Slit2 inhibits angiogenesis and vascular leakage, while deletion of Robo4 enhances these pathologic processes. These results suggest a novel function for Slit-Robo signaling in stabilizing the vasculature.

Session 3: LYMPHANGIOGENESIS IN HEALTH AND DISEASE

"Mechanisms mediating lymphatic vessel function and growth"
Michael Detmar (Zürich) presented data regarding the promotion of lymph node metastasis. Tumors exhibit a lymphatic vasculature and can actively induce lymphatic vessel growth which leads to the promotion of lymph node metastasis. It was demonstrated that the extent of the lymphatic tumor vasculature is a sufficient parameter to determine whether primary cutaneous tumors have already metastasized or not. Thereby, VEGF-A plays an important role in the formation of tumor lymph vessels which was shown by increased tumor lymphangiogenesis of VEGF-A transgenic mice. He also showed that metastatic tumor cells can induce lymphangiogenesis within lymph nodes and, surprisingly, VEGF-A expressing tumors can induce lymphangiogenesis by draining lymph nodes even before they have metastasized (adapting the "seed and soil" hypothesis). He also showed in VEGF-C transgenic mice that lymphangiogenesis in sentinel lymph nodes promotes lymphatic vessel growth in distant lymph nodes which again is a promoter of metastasis. Further, Detmar's group did great efforts

on identifying and targeting factors of lymphangiogenesis. Thereby, they identified different miRNAs with enhanced expression in lymphatic or blood vascular endothelium. Amongst others, Prox1 was dramatically downregulated in the blood vasculature by one of these miRNAs (miR-31). Further gain- and loss-of-function studies with miR-31 showed dramatic effects on lymph vascular development and lymphangiogenesis; for example, miR-31 overexpression impairs lymphatic vascular sprouting in Xenopus laevis. Data were also presented on chemical genetics and small molecule library screens in Xenopus laevis tadpoles and found different phenotypes with edema and late lethality. Using in situ hybridization, the group identified 34 compounds affecting embryonic formation of lymphatics as well as blood vessels. In summary, the Detmar group presented several novel pathways promoting lymphatic formation and function and different new factors affecting lymphangiogenesis.

"The relationship between tumors and the lymphatics: Implications for metastasis"
Jonathan Sleeman (Mannheim) presented new data concerning the role of hyaluronic acid on lymphangiogenesis and metastasis. Tumor-induced lymphangiogenesis is hypothesized to be a mechanism that promotes metastasis. The data presented showed that cleavage products of extracellular matrix glycosaminoglucan hyaluronic acid (HA) are bioactive. His group identified Hyaluronidase-1 as the only hyaluronidase with the appropriate enzymatic activity to cleave the active short hyaluronic acid fragments (sHAs). The sHAs are inducing capillary outgrowth from thoracic duct rings in the so-called aortic ring assay. By analyzing these phenomena Sleeman's group detected LYVE-1 as a mediator of the proliferation-inducing effects of sHA. Furthermore they showed that CCL21 is upregulated by sHA but not by high molecular weight HA in lymphatic endothelial cells. Moreover, Sleeman presented data of natural products with effects on lymphangiogenesis like the herbal molecule Hyperforin and its derivate Aristoforin. Aristoforin for example inhibits strongly the proliferation of primary lymphatic endothelial cells by inhibiting cell cycle progression at low concentrations and inducing apoptosis in high concentrations. Also the anthocyanidin delphinidin showed effects on endothelial cells by inhibiting the ligand induced VEGFR-2 and VEGFR-3 autophosphorylation. Taken this data together, Sleeman showed a lot of interesting novel approaches of molecules effecting lymphangiogenesis with a big therapeutic capability.

"A zebrafish mutant defective in lymphangiogenesis identifies a novel protein as an essential element in thoracic duct formation"
Stefan Schulte-Merker (Utrecht) showed the role of the gene *full-of-fluid* (*fof*) for lymphatic development by investigating a zebrafish mutant completely lacking lymphatic vasculature. Schulte-Merker's group did different forward genetic screens with zebrafish mutants defective in thoracic duct development. Thereby they found the fof phenotype, a zebrafish with normal vasculature but completely lacking the thoracic duct and other lymphatic vessels. Optically, the phenotype is marked by a big round abdomen, which is a consequence of lymphedema caused by the lymphatic deficiency. Schulte-Merker's group showed that the loss of *fof* function can be rescued by wild type *fof* mRNA injection. Interestingly, *fof* has so far not been associated with lymphangiogenesis. It is expressed in cells neighboring the lymphatics but not in lymphatic endothelial cells themselves. In cell transplantation experiments the group showed that wild type cells are able to induce the formation of a thoracic duct from mutant cells. This affirmed the assumption that *fof* functions non cell autonomously. Schulte-Merker used a novel double-transgenic zebrafish mutant with specifically highlighted lymphatic cells to distinguish between blood vessel cells and lymphatic cells. Thereby, he found that the gene precedes lymphatic endothelial cell migration. The group also identified the necessity of *fof* for lymphangioblast and venous sprouting and showed that *fof* acts in the same time frame as VEGF-C. To sum the data up, Schulte-Merker's group identified a completely new gene for the development of lymphatics in zebrafish with an elementary role for vertebrate lymphangiogenesis.

"Essential regulation of angiogenesis and lymphangiogenesis by the microRNA miR-126"
Frank Kuhnert (Stanford) gave a talk highlighting the essential regulation of angiogenesis by the microRNA miR-126. MiR-126 is an endothelium specific miRNA, which is embedded in Egfl7 and coordinately expressed with this gene. Kuhnert showed that selective deletion of miR-126 in miR-126Δ/Δ mice recapitulated the previously described Egfl7Δ/Δ mutant with edema and delayed retinal angiogenesis. Surprisingly, Egfl7Δ/Δ showed no abnormal phenotype. He also found that miR-126 functions cell-autonomously in endothelial cells and regulates vascular integrity, endothelium cell migration and PI3 kinase and MAP kinase signaling in HUVECs. Kuhnert identified p85α and -β encoding inhibitory/regulatory subunits of PI3K (encoded by PI3KR1 and PI3KR1/2) as the target of miR-126. The expression of both was increased in miR-126Δ/Δ mice. Overexpression of p85α or -β was sufficient to inhibit VEGF dependent activation of Akt. The data led to the model that PI3K is a regulator of Akt. The expression of PI3KR1/2 in miR-126Δ/Δ mice results in the observed angiogenic

phenotype. With this data, Kuhnert showed the essential role of secondary regulation of different host genes involved in angiogenesis.

"VEGFR-3 pathway as a target to inhibit angiogenesis, lymphangiogenesis and tumor metastasis"

Kari Alitalo (Helsinki) first gave a colorful overview of VEGF signaling in lymphangiogenesis. VEGF signaling via the VEGF receptors VEGFR-1 and VEGFR-2 is important for the regulation of angiogenesis and the permeability of blood vessels. In comparison, VEGFR-3 does not bind VEGF but the ligands VEGF-C and VEGF-D. Furthermore, VEGFR-3, intially expressed by all entothelia, becomes exclusively expressed by lymphatic tissue during development. Studies with transgenic mice showed the role of VEGFR-3 for vascular and lymphatic development: VEGFR-3 targeted mice die around midgestation due to failure of cardiovascular development. Furthermore, transgenic mice expressing VEGF-C show evidence of lymphangiogenesis whereas VEGF-C knockout mice show defective lymphatics. Gene therapy with viral VEGF-C overexpression was successful to treat lymphedema even in mice with a defective VEGFR-3. VEGF-C and the closely related VEGF-D appear to be lymphangiogenic factors. Furthermore, both ligands can be proteolytically processed and also bind to VEGFR-2 to stimulate angiogenesis. Dr. Alitalo showed that VEGF-C overexpression induces lymphangiogenesis and the growth of the draining lymphatic vessels, intralymphatic tumor growth and lymph node metastasis in several tumor models, including transgenic models. The inhibition of VEGF-C/-D signaling by blocking VEGFR-3 with a tyrosine kinase inhibitor or neutralizing VEGF-C/-D with a soluble VEGFR-3 led to a reduction of early steps of metastasis in breast and lung cancer models. Unexpectedly, inhibition of VEGFR-3 with a neutralizing antibody showed small but significant inhibition of tumor growth in several xenograft models. More detailed studies showed that VEGFR-3 is induced by VEGF in blood vessels, expressed by endothelial sprouts and its inhibition leads to suppression of angiogenic sprouting. Notch suppresses VEGFR-3 expression, while blocking of Notch leads to an upregulation of VEGFR-3 expression, again leading to increased sprouting. Blocking VEGFR-3 in addition to VEGFR-2 improved tumor growth inhibition in several tumor models. The role of VEGFR-3 as a positive regulator of angiogenesis shows that the receptor is a promising target for anti-angiogenic treatment.

Session 4: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS I

"FoxC2 and the control of collecting lymphatic vessel formation"

Tatjana Petrova (Lausanne) gave an introduction into the lymphatic system. Her presentation focused on Lymphedema-distichiasis, an autosomal disease with early onset. The transcription factor FoxC2 from the forkhead family of proteins is involved in the reorganization of vessels when the stable adult lymphatic network is formed; its knockout leads to early lethality in mice. The molecular characterization of the lymphatic maturation process begins at E14.5: VEGFR-3 expression is downregulated while Prox1 expression is uniform; FoxC2 is weakly expressed. When the patterning of the vessels starts at E15.5, FoxC2 expression is increased, but decreases again after E16.5, similar to Lyve-1 expression. When the lymphatic cells normally turn quiescent, they remain active when FoxC2 is knocked out. Cells of FoxC2-/- mice fail to downregulate VEGFR-3 and Lyve-1. Petrova found out that the NFAT/Calcineurin pathway was involved. NFATc1 mediates VEGF-induced proliferation. It is not expressed in blood vessels but co expressed in developing lymphatic vessels with Prox1, which together with VEGFR-3 regulates expression and localization of NFATc1. Cyclosporin A-treatment *in vivo,* blocking the NFAT pathway, leads to an early death at E17.5. The phenotype displayed normal blood vasculature, enlarged lymphatic vessels and overexpression of VEGFR-3. Applying this treatment with FoxC2+/- mice, the phenotype was even more drastic, which implies a crosstalk of these pathways. Examination of the FoxC2 transcriptional network with ChIP-chip analysis revealed highly conserved FoxC2 sequences, which are enriched in NFAT and AP-1 sites. FoxC2 cooperates with NFATc1 on ChIP target enhancers, but antagonizes classic NFAT promoters. It was also found that NFATc1 and FoxC2 co-precipitate.

"How endothelial cells integrate spatial cues to make blood vessels"

Victoria Bautch (Chapel Hill) explained how to get from vascular tubes to the vascular network. Endothelial cells have to organize according to spatial signals from their surroundings to form a blood vessel. An important part of this is the action of VEGF receptors in organizing oriented cell divisions and sprouting migration. Flt-1 (VEGFR-1) modulates vascular development via effects on VEGF/Flk-1 (VEGFR-2). Loss of Flt-1 leads to vessel overgrowth and disproportion, confirmed by an increased mitotic index. Her working model is vessel development *in vitro* from mouse stem cells. Her group inserted GFP-reporters into this system, which enabled them to look at sprout formation and fusion.

Flt-1-/- cells show increased proliferation, but decreased branching and migration. Flt-1 encodes both the membrane-anchored mFlt-1 and the soluble sFlt-1, which is secreted and can act as a decoy receptor to modulate VEGF signaling through Flk-1. mFlt-1 and sFlt-1 transgenic rescue experiments were both able to partially rescue the proliferation, while sFlt-1 is much more successful in rescuing the branching. Wild type and sFlt-1 rescued vessels showed heterogeneous phosphorylation of tyrosine 1175 on the Flk-1 receptor, which implies that modulation of VEGF/Flk-1 signaling is important in the regulation of branching. Bautch's group also found that γ-secretase inhibition rescues the branching in Flt-1-/- mice; this might be a potential link between the VEGF and Notch pathway. Flt-1 expression in endothelial cells guide cell migration, as these cells show significantly reduced speed and forward motion. The Flt-1-/- cells conversely showed blunted, misguided and more randomized filopodia. Flt-1-/- sprouts form closer to other vessels and form more acute angles. The hypothesis is that VEGF signaling leads to crosstalk between endothelial cells leading to local sprout guidance and increased Flt-1 expression in lateral areas next to the sprout.

"Notch signaling dynamics and the control of angiogenesis: Studies in zebrafish"
Jonathan Leslie (London) presented data on how the motile endothelial cell type is switched on and off in zebrafish. He used a fli-EGFP transgenic to take a closer look at the tip/stalk cell differentiation. In the developing zebrafish embryos, after 20 hours of development, the intersegmental vessels start to sprout. At the age of 48 hours, the T-shape of the vessels joining to form the dorsal longitudinal anastomotic vessel is completed. Dll4 is expressed in arteries but not in venous endothelial cells in early angiogenesis in zebrafish. Loss of Dll4 or Notch signaling causes overproduction of endothelial cells. Morpholino-induced loss of Dll4 shows no phenotype in the early stages, but when the angiogenesis is supposed to stop after 48 hours as endothelial cells become quiescent, there is still sprouting going on. Blocking of Notch leads to the same result. The same phenotype is also displayed in Dll4-mutant zebrafish. When VEGF signaling is blocked in addition to Dll4- or Notch-knockdown, the endothelial cells remain quiescent. Therefore, Dll4-Notch signaling acts as a switch to turn off responsiveness to VEGF, thereby reducing endothelial migration and proliferation. These data support a model in which the crosstalk of Notch and VEGF pathway enables the response to VEGF of few endothelial cells while inhibiting the neighboring cells. According to this model, during the formation of the dorsal longitudinal anastomotic vessel, two tip cells migrate towards each other until they start to inhibit each other's response to VEGF and become quiescent. Blocking the Dll4/Notch signaling did not inhibit the sprouting of endothelial cells.

Session 5: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS II

"Selection and function of endothelial tip and stalk cells in sprouting angiogenesis"
Holger Gerhardt (London) demonstrated in his talk the mechanism of tip cell selection during angiogenesis. Dll4 is highly expressed by tip cells and in turn inhibits Notch signaling of adjacent cells to form stalk cells. Using genetic and pharmacological inhibition of Notch signaling in endothelial cells these results in excessive filopodia formation and tip cell formation under VEGF-A stimulation. These studies illustrate that the formation of tip cells is the default EC phenotype following VEGF-A stimulation, whereas the stalk cell phenotype is an acquired function dependent on Notch activity.

"Exploring the link between endothelial differentiation and vascular morphogenesis"
Nathan D. Lawson (Worcester) presented the mechanisms controlling aorta formation in zebrafish. It is well known that KDR and EphrinB2 are markers for arteries and Flt-4 and EphB4 are expressed in veins in zebrafish. He used engineered Zinc finger nucleases to silence target genes in zebrafish and found out that ets related protein (etsrp) functions at top of the hierarchy of vessel morphogenesis signaling. Loss of etsrp preferentially affects truncated blood vessels. Etsrp induces many endothelial specific genes such as Hey2 and scl through Notch to establish the proper patterning and function of the circulatory system.

Session 6: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS III

"Endothelial origin of hematopoietic stem cells"
Luisa Iruela-Arispe (Los Angeles) addressed the question, which cell types were responsible for definitive hematopoiesis and whether an intra-embryonic population could autonomously give rise to adult lineages? By employing a temporally restricted genetic tracing strategy using VE-cadherin CRE to selectively label the endothelium, she found that the VE-cadherin lineage of the aorta-gonad-mesonephros region (AGM) is capable of long-term, multi-lineage adult hematopoiesis. Furthermore,

using SM22-CRE lines, she presented data revealing that the mesoderm contributes to the endothelial floor of the aorta, but not to other endothelium, and gives rise to both endothelial and hematopoietic cells, labeling both early and late mesodermal populations. Myocardin-CRE labels the underlying mesenchyme during AGM development and reveals a later permanent population that neither contributes to the endothelium nor gives rise to hematopoietic cells. Thus, only the early mesenchymal population, which contributes to the aortic floor endothelium, has the capacity for hematopoiesis and does so via an endothelial intermediate.

"VEGFR levels regulate endothelial competition for the tip cell position"
Lars Jakobsson (London) - content embargoed

"Oxygen sensors as regulators of tumor vessel morphogenesis"
Peter Carmeliet (Leuwen) - content embargoed.

"EphrinB2 regulates VEGFR trafficking to mediate tip cell filopodial extension during angiogenesis"
Amparo Acker-Palmer (Frankfurt) focused on neuronal cues by looking at reverse signaling by ephrin ligands. Interactions of ephrinB2 and the postsynaptic density-95/Discs large/zona occludens-1 (PDZ) domain are required for developmental and pathological angiogenesis. She showed that ephrinB2 induces a repulsive phenotype in ECs and that this repulsive phenotype is PDZ-dependent. *In vivo*, ephrinB2-PDZ signaling deficient mice show reduced numbers of total tip cells and reduced numbers of filopodia extensions per tip cell. EphrinB2 is required for VEGF-induced VEGFR-2 internalization and phosphorylation of VEGFR-2 in endothelial cells, thus regulating the extension of filopodia.

Session 7: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS IV

"Vascular lumen formation *in vitro* and *in vivo*"
Eckhard Lammert (Dresden) started with an overview of pancreatic islets that are formed by beta cells in vertebrates. He pointed out that EphA and ephrin-A are co expressed in beta cells and that forward and reverse signaling contribute to glucose homeostasis in pancreatic islets. Additionally, he presented evidence for a new function of the well-known term "vascular niche"-responsible for many cellular and developmental processes- in providing a basement membrane to cells that are unable to form their own, e.g. beta cells. Afterwards, he focused on vascular lumen formation of the dorsal aorta and introduced an *ex vivo* model of cultured mouse embryos. This model enables in an elegant manner the monitoring of somite stages from 1S-15S after 20h, respectively. Lammert and colleagues observed between the 3S and 5S stage an intercellular lumen formation with only a few observed putative vacuoles. Cross-sections through these vacuoles reveal that they have distinct membrane restricted areas therefore this putative vacuoles are indeed small vesicles. During intercellular lumen formation they showed in early somite stages a remodeling of tight junctions and gaps between endothelial cells which results in vascular leakage. With this observation, Lammert proposes a new theory that lumen formation occurs not only due to vacuole coalescence but rather due to an intercellular *de novo* lumen formation.

"Extracellular matrix remodeling events control endothelial cell lumenogenesis and pericyte-directed endothelial tube stabilization"
George E. Davis (Columbia) investigated an extracellular matrix (ECM) dependent mechanism of endothelial cell (EC) tube formation. EC tube formation is regulated by the cell surface proteinase MT1-MMP through its ability to create proteolysed spaces within collagenous ECM. MT1-MMP acts in conjunction with the Rho GTPases, Cdc42 and Rac1, downstream of integrin signaling to control EC lumenogenesis. He presented an *in vitro* 3D collagen matrix model with 5 times more EC than pericytes and presented that the newly formed ducts serve as vascular guidance tunnels allowing EC motility, tube remodeling and a marked pericyte recruitment. The interaction between EC and pericytes leads to a remarkable remodeling of the basement membrane components like collagen type IV, laminin, nidogen1/2 and perlecan along with fibronectin assembly which is not apparent when EC are cultured alone. In addition, also an upregulation of different integrins (α5β1, α3β1, α6β1, α2β1) occurs during co culture of EC with pericytes. Finally, pericyte TIMP-3 is required for stabilization of the newly formed and remodeled basement membrane as siRNA suppression of TIMP-3 results in marked loss of collagen type IV around EC tubes leading to vessel instability.

"Coordinated branching morphogenesis of the airways and vasculature in the developing lung"

In his presentation, **Eli Keshet (Jerusalem)** addressed the questions whether the vasculature influences epithelial branching and if so, whether in that case it is proceeding in a perfusive manner and vessels are required for the branching process per se. To answer these questions, he presented first an *in vivo* murine Tet-on model with an inducible expression of soluble VEGFR-1, which leads to ablation of the vasculature. Ablation of the vasculature resulted in inhibition of airway branching at day E12.5. Knockdown of the soluble VEGFR-1 expression resulted in the rescue of airway branching arrest after E12.5. In his second *ex vivo* lung explant model he treated the embryonic mouse lung with U7, an inhibitor of VEGFR-2, which resulted in a decrease of vessel density after 2h. There were no side effects of U7 observed on epithelial cell proliferation, however an abnormal branching phenotype of the airways was detectible. Loss of the vasculature leads to an upregulation of negative branching regulators and to an inhibition of FGFR signaling.

Session 8: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS V

"Guidance of vascular development"

Anne Eichmann (Paris) gave a talk about the vertebrate vascular system. She highlighted the anatomical similarities between blood vessels and nerves. The two networks are often aligned in peripheral tissues, with nerve fibers and blood vessels following parallel routes. Neuropilins (NRP) bind to different molecules due to their expression profile. On nerves Neuropilin binds to Semaphorin and interacts with Plexin and on blood vessels Neuropilin binds to VEGF and interacts with VEGF Receptors. NRP2-/- mice display reduced lymphatic coverage and sprouting of lymphatic vessels compared to the WT mice. In addition, blocking of NRP2 functions inhibits tumor cell metastasis. She demonstrated that VEGFR-2 expression is located to lymphatic vessel sprouts. Her group generated the following double-heterozygote mice. NRP2+/- VEGFR-2+/- mice are normal and show no alteration in vessel patterning, however, NRP2+/- VEGFR-3+/- mice display abnormal lymphatic sprouting, blunted tips and reduced branch points. Blocking the binding of VEGF-C to NRP2 reduces lymphatic sprouting.

"Guiding blood vessels: Exploring neuropilin's role in development and disease"

Ryan Watts (South San Francisco) presented data on the role of Neuropilin-1 (NRP1), which is both a receptor for several members of the class 3 Semaphorins, known for their axon chemorepellent activity, and VEGF, a major regulator of physiological and pathological angiogenesis. He is searching for monoclonal antibodies against the axon guidance molecule Neuropilin as a vascular and tumor target. More than 40 unique antibodies were screened in neuronal and endothelial assays. VEGF binding to NRP1 is blocked by anti-$NRP1^{B}$. Anti-$NRP1^{A}$ but not anti-$NRP1^{B}$ blocks Sema3A-induced growth cone collapse. Anti-$NRP1^{B}$ reduces endothelial cell migration and endothelial bead outgrowth *in vitro* and angiogenesis *in vivo*. His data demonstrates that Sema3A and $VEGF_{165}$ do not compete for binding to NRP1, instead the two molecules exhibit additive binding. NRP1 binds $VEGF_{121}$, but it is not sufficient to bring NRP1 and VEGFR-2 together, which only $VEGF_{165}$ does. Consistent with the genetic deletion of NRP1 he showed that inhibiting NRP1 with antibodies disrupts vascular remodeling during development. In addition, anti-NRP1 in combination with anti-VEGF therapy exhibits an additive effect in reducing vascular density in both the developing retina and in tumors.

"Role of neuropilin-1 and its ligands in neuronal and vascular patterning"

Christiana Ruhrberg (London) gave a talk about the role of SEMA3A. She demonstrated that SEMA3A does not control vascular development, as SEMA3A knock out mice display normal vessel remodeling when compared to wild type mice. She showed that there is no genetic interaction between SEMA3A and $VEGF_{164}$ and no competition of $VEGF_{164}$ and SEMA3A in endothelial cells *in vivo*. She demonstrated that SEMA3A and semaphorin-signaling through NRP1 are not required to modify the migration of endothelial cells, which is instead controlled by $VEGF_{164}$. NRP1 and SEMA3A knock out mice display an abnormal sympathetic nervous system. She showed that SEMA3A/NRP1 signaling contributes to cardiovascular development by guiding the precursors of the sympathetic nervous system to appropriate destinations.

Session 9: ENDOTHELIAL PHENOTYPES AND VASCULAR MORPHOGENESIS VI

"Regulation of arterial specification"

Michael Simons (New Haven) presented an overview over the fate specification of the vasculature, which implicated a key role for the scaffold protein synectin in arterial specification. Synectin-/- mice

have smaller arteries, in addition, the arteries of synectin knockdown zebrafish are completely absent. Synectin was found to be involved in endocytosis, as it binds myosin VI, the only motor protein that performs retrograde transport of endosomes. This endocytosis is important for the synectin-binding proteins NRP1 and TrkA. Synectin-/- cells show abnormal VEGFR-2 trafficking: the receptor is endocytosed, but not transported to the sorting endosomes as in wild type cells. Myosin VI knockdown in zebrafish reduces the lumen size of the dorsal aorta and impairs intersegmental vessel formation. The same phenotype was observed in myosin VI-/- mice. The synectin knockout leads to different VEGF signaling: Upon stimulation with VEGF Erk phosphorylation was decreased instead of increased. This was rescued with synectin. In synectin-/- cells, increased Erk induction can be achieved by partial PI3K inhibition, which rescues VEGF-induced migration and branching morphogenesis. Knockdown of Akt1 but not of Akt2 restores Erk activation, which reinduces EphrinB2 and Dll4 expression and rescues the synectin-/- phenotype. *In vivo*, PI3K inhibition rescues angiogenesis and arteriogenesis in synectin-/- mice and in synectin knockdown zebrafish.

"Regulation of the angiogenic endothelial phenotype"
Ralf Adams (Münster) - content embargoed.

"Glioma tumor stem cells are defined by a characteristic gene signature and occupy perivascular and hypoxic niches"
Till Acker (Giessen) presented his group's recent data about the relationship of tumor growth and stem cell hierarchy. According to the stochastic model, tumor generating cells are random cells, on the other hand, according to the hierarchy model, only specialized cells like stem cells have the capacity to form tumors. By fluorescence-assisted cell sorting, Acker's group found a side-population of tumor cells which have typical stem cell characteristics. They have a larger capacity to form tumors and are characterized by a different expression profile of more than 800 genes, especially NFATc2 is highly expressed on these cells. These side population signature genes regulate tumor stem cell maintenance. The tumor stem cells are found around vessels and in other stem-cell specific niches. They are regulated by endothelial cell signals and tumor hypoxia. There may be a vascular niche and a hypoxic niche which may interact with each other, which suggests a niche specific targeted therapy. There are indications for niche specific control of the $CD133^{+}$-phenotype. ASAID2 could serve as a prognostic marker for tumor stem cells.

Session 10: ANGIOGENESIS IN INFLAMMATION; CANCER AND OTHER PATHOLOGICAL PROCESSES I

"The cystatin type 3 HRG in regulation of tumor growth and dissemination"
Lena Claesson-Welsh (Uppsala) presented data regarding the different roles of Histidine-rich glycoprotein (HRG). HRG is a 75 kDa heparin-binding multidomain plasma protein. It is a type 3 protein of the cystatin family and contains an anti-angiogenic central histidine/proline rich domain. The release of this domain is necessary for the inhibition of endothelial cell chemotaxis. By creating a truncated version of the protein consisting of 35 amino acids the relevant part of this his/pro-domain for anti-angiogenic activity was determined. Claesson-Welsh also showed that this minimal-anti-angiogenic domain reduces tumor angiogenesis and tumor growth, whereas in HRG knock out mice tumor size was increased. Further gain- and loss-of-function studies revealed that HRG is a negative regulator of tumor expansion and dissemination. Claesson-Welsh's group used T241 fibrosarcoma cells expressing HRG and showed that tumors derived from these transfected cells display a decrease in tumor growth, vascular density and in hypoxia as well as a decrease in pericyte attachment in comparison to wild type tumors. Inflammatory cells exposed to HRG showed reduced expression of PlGF indicating that PlGF is a transducer of the HRG effect. In addition, PlGF-/- mice showed no effects to HRG expression. In summary, these data suggests that HRG acts to suppress tumor vascularization and growth by the regulation of PlGF expression in inflammatory cells.

"Multiple actions of angiogenesis inhibitors on blood vessels in tumors"
Donald Mc Donald (San Francisco) gave a broad overview of the effects of different anti-angiogenic inhibitors. The formation of blood vessels is dependent on VEGF or other growth factors. A well-known treatment of colorectal cancer is the treatment with the VEGF neutralizing antibody Bevacizumab. Normally Bevacizumab is given in combination with a chemotherapeutic treatment. Besides Bevacizumab there are other possible anti-angiogenic targets for inhibitors like PDGF/PDGFR and HGF/HGFR. Each individual inhibitor leads to multiple actions on endothelial cells. In addition to the wanted vascular effects inhibitors also show multiple other effects which can also be non angiogenic. Another effect of inhibitor treatment is the normalization of surviving tumor vessels, which then are

adapted to the low growth factor levels. By treating Lewis Lung Carcinoma with an inhibitor against PDGF-B accelerated tumor growth with normalized pericytes on surviving tumor vessels was found. Inhibition of VEGF/VEGFR leads to a rapid regression and normalization of endothelial cells. Pericytes are found sparsely and empty sleeves of the basal membrane remain. Inhibition of PDGF/PDGFR leads to a slower regression and normalization compared to VEGF/VEGFR inhibition, but secondary effects occur. Blockage of VEGF and HGFR results in rapid regression and less normalization of endothelial cells. Pericytes are extensively lost and fewer empty sleeves of the basal membrane remain.

Session 11: ANGIOGENESIS IN INFLAMMATION; CANCER AND OTHER PATHOLOGICAL PROCESSES II

"Stimulation of tumor growth and VEGF-mediated angiogenesis by low concentrations of anti-angiogenic αvβ3/αvβ5-integrin inhibitors"
Kairbaan M. Hodivala-Dilke (London) - content embargoed.

"The role of semaphorins in tumor progression and metastatic dissemination"
Charlotte Rolny (Uppsala) shows that Semaphorin 3B plays an unexpected role in tumor progression. Sema3B is widely expressed by a large number of human tumor cell lines. It binds to plexins and neuropilins. Sema3B repulses endothelial cells *in vitro*. By investigating experimental tumor model, they found that Sema3B expression can inhibit tumor growth, whereas tumor metastatic dissemination was surprisingly increased. Microarray data indicates that the pro-angiogenic cytokine IL-8 is one of the few genes that are regulated by Sema3B. The increased IL-8 level in tumors recruits tumor-associated macrophages and elevates metastatic dissemination. Metastasis and tumor-associated macrophage recruitment caused by Sema3B can be all abolished by the treatment with IL-8 neutralizing antibody. Rolny further showed that this effect is the consequence of IL-8 induced by semaphorin 3B through Neuropilin-1 and p38 MAPK dependent pathways.

"Role of semaphorin 3E/plexin D1 in vascular and tumor biology"
Alexander W. Koch (South San Francisco) reported that Sema3E can bind directly to PlexinD1 with high affinity, and efficiently inhibits endothelial cell migration and sprouting in a PlexinD1 dependent and Neuropilin independent manner. PlexinD1 is expressed in endothelial cells during vasculature development. He had found out that PlexinD1 expression is highly upregulated in tumor vasculature. Sema3E is also expressed during vessel development to provide guidance information. Koch developed a short form of Sema3E protein, indicated as $Sema3E^{SD}$. $Sema3E^{SD}$ is sufficient to bind to PlexinD1 and inhibits endothelial cell migration and VEGF induced endothelial sprouting. He further analyzed Sema3E/PlexinD1 function in a mouse xenograft model, indicating that the tumor growth can be significantly reduced by a Sema3E Fc-fusion protein. These results imply that Sema3E/PlexinD1 are potential targets for anti-angiogenic therapy.

"A novel human-specific soluble VEGF receptor-1"
Shay Sela (Jerusalem) presented a novel human-specific splice variant of VEGF receptor-1 (Flt1) that is generated cell type-specifically and functions as a potent VEGF inhibitor. Soluble Flt1 has a protective role against excessive VEGF signaling in adult animals and is ubiquitously expressed in the Vascular Smooth Muscle Cells of many organs. Preeclampsia (PE) in pregnant women is characterized by hypertension and proteinuria, which is known to be caused by soluble Flt1 secreted by the placenta. Sela showed that the human specific soluble Flt1 (sFlt1-14) is the dominant placental soluble VEGFR-1 isoform during preeclampsia.
Soluble Flt1-14 is translated to a protein in the human placenta and can be found in maternal circulation. He could also reveal that syncytial knots in the placenta are the major producers of soluble Flt1-14.

"Immune cells as regulators of tumor angiogenesis"
Lisa Coussens (San Francisco) introduced the concept that immune cells are components of malignant tumors and are functionally involved as promoting forces of tumor progression, mediating chronic inflammation in human cancers and promoting cancer growth in experimental models (skin, mammary, lung models). Leukocytes are recruited to neoplastic tissue and once activated, they can play a tumor-promoting role in cancer progression as mature leukocytes. Utilizing a transgenic mouse model of squamous carcinoogenesis, Coussens reported that activation of humoral immunity (B cells) regulates recruitment and activation of myeloid cells (including mast cells, granulocytes and monocytes) into premalignant tissues through deposition of immunoglobulins (Ig) and increased

presence of circulating immune complexes (CICs). Thereby, prominent Ig and CIC deposits in neoplastic skin are indicative of peripheral adaptive immune responses, which then directly interact with and activate Fc receptors on resident and recruited innate immune cells: for example, circulating immune complex (CIC)-FcγR interaction regulates mast cell cytokine expression and angiogenic factors needed for inducing angiogenic activation of endothelial cells. Overall it was suggested that cancer development can be functionally regulated by affecting B and/or T lymphocyte function, depending on the organ microenvironment, and activation of pro-angiogenic programs in neoplastic tissues.

"Ang-2 and Tie1 as dynamic regulators of constitutive Ang-1/Tie2 signaling in the vascular system"

Hellmut Augustin (Heidelberg/Mannheim) presented an overview of the present state-of-the-art of Angiopoietin/Tie function in the cardiovascular system. Ang-2 is the functional antagonist of Ang-1/Tie2 signaling but may also act as an agonist of Tie2, protecting endothelial cells from apoptosis in an autocrine manner. Dr. Augustin showed that endothelial cell-derived Ang-2 antagonizes Tie2 activation *in vivo*. VEGF stimulation leads to shedding of the Tie1 ectodomain, which was revealed to lead to an Ang-2-independent endoTie1 and Tie2 phosphorylation. Also, silencing of Tie1 reduces VEGF-mediated Tie2 phosphorylation. Furthermore, a dynamic autocrine role of Ang-2 to control vascular reactivity was defined, as Ang-2 stimulation leads to the recruitment of Tie receptors and FAK (focal adhesion kinase) to $\alpha_v\beta_3$ integrin and results in FAK phosphorylation at Ser910. Increased pericyte coverage in Ang-2 deficient tumor vessels was observed. Data showing that endothelial cells and tumor cells are sources of Ang-2 in human melanomas indicate that human melanoma cells entertain an autocrine Ang-2/Tie2 loop.

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