MEETING REPORT

6th International Kloster Seeon Meeting
"Angiogenesis: Molecular Mechanisms and Functional Interactions"

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

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Session 1: THE QUIESCENCE SWITCH

"Anti-angiogenic vessel pruning versus vessel normalization strategies"
Peter Carmeliet (Leuven) - content embargoed.

"VEGF signaling regulates centrosome duplication in endothelial cells of developing blood vessels"
Victoria Bautch (Chapel Hill) focused on the correlation of vascular dysfunction in tumor angiogenesis and aneuploidy. Flt-1 negative zebrafish show vessel overgrowth *in vivo* and these endothelial cells display excess centrosomes. This is also true *in vitro* in HUVECs stimulated with a high concentration of VEGF. The mechanism of developing aneuploidy may be found in cell cycle regulation. Stimulation with VEGF leads to hyperactivity of CyclinE/Cdk2, mediated through either MEK/ERK or AKT and is associated with centrosome over-duplication. Conversely, blockade of components in these pathways can rescue the centrosome over-duplication defect. Endothelial cells with excess centrosomes show no apoptosis and an aberrant spindle formation at mitosis. Time dependent stimulation with VEGF leads to an increase in centrosome number. *In vivo* studies in Flt1 negative mice showed a correlation between excess centrosome number and aneuploidy. Also, endothelial cells with increased centrosome number show aberrant migration patterns. In conclusion, endothelial cell exposed to increased VEGF concentration show a centrosome over-duplication which leads to aberrant angiogensis. This phenotype may contribute to the abnormalities in tumor vessels.

"Social cell behaviors in the angiogenic sprout"
Holger Gerhardt (London) presented data which demonstrated that endothelial cells compete for the tip cell position through relative levels of VEGFR1 and VEGFR2. The selection of tip- and stalk-cells is regulated by the DLL4-NOTCH signaling. The levels of VEGFRs are influenced by DLL4-NOTCH signaling and in turn VEGFR activity affects the expression of DLL4. By using computational modeling and genetic mosaic sprouting assays *in vitro* and *in vivo,* he was able to demonstrate that the endothelial cells dynamically compete with each other for the tip cell position. High levels of VEGFR2 and low levels of VEGFR1 in the cell induces a tip cell phenotype and so enables cells from the stalk region to get into the leading position. In conclusion, endothelial cells dynamically compete for the tip cell position through VEGFR-DLL4-Notch feedback.

Session 2: NOVEL FUNCTIONS

"Tumor angiogenesis is reduced in the Tc1 mouse model of Down syndrome"

Kairbaan Hodivala-Dilke (London) presented data on the correlation between gene dosis and tumor angiogenesis by using the Tc1 mouse model of Down syndrome (DS). The solid tumor incidence in DS patients was reported to be decreased and recent work using the Ts65Dn model of DS has suggested that three copies of the ETS2 or DSCR1 genes (angiogenic suppressor) are sufficient to inhibit tumor growth. Hodivala-Dilke used the Tc1 transchromosomic mouse model of DS to dissect the contribution of extra copies of genes on human chromosome 21 (Hsa21) to tumor angiogenesis. These mice express ca. 81% of Hsa21 genes, but lack the human DSCR1 region. Tc1 mice transplanted with B16F0 and Lewis Lung Carcinoma (LLC) tumor cells showed reduced tumor growth and angiogenesis compared to wild-type mice. She further demonstrated by *in vitro* (aortic ring assay) and *in vivo* approaches that the angiogenic responses to VEGF were inhibited in Tc1 mice. Examination of genes on Hsa21segment in Tc1 mice identified putative anti-angiogenic genes (ADAMTS and ERG) and novel endothelial cell-specific genes (JAM-B and PTTG1IP) that, when overexpressed, were responsible for the inhibition of angiogenic responses to VEGF. Three copies of these genes within the stromal compartment reduced tumor angiogenesis providing an explanation for the reduced tumor growth in DS. Upon reducing the copy number of ERG, ADAMTS and JAM-B from 3 to 2 (human specific siRNA) the angiogenic response was increased in the aortic ring assay. Further reduction of the copy numbers of ADAMTS, JAM-B, PTTG1IP, but not ERG to 1 (mouse specific siRNA) resulted in an enhanced angiogenic response of, demonstrating the close correlation between gene dosis and phenotype. Studies on JAM-$B^{+/-}$ mice and anti-JAM-B treatment of mice showed that reduced JAM-B availability increased tumor growth and angiogenesis.
In conclusion, Hodivala-Dilke showed that the Tc1 mouse model is an interesting system to identify novel regulators of tumor angiogenesis in a gene dose dependent manner.

"Lipoprotein levels regulate angiogenesis by modulating expression of VEGFR1"

Karina Yaniv (Rehovot) presented her study on the regulation of angiogenesis by lipoprotein levels through modulation of VEGFR1 expression. Whereas endothelial-lipoprotein interactions have been described in several diseases, little is known about the mechanisms controlling the endothelial response to circulating lipoproteins. By using two novel zebrafish *in vivo* models of lipidstarvation and lipid-overload, Yaniv showed that circulating lipoproteins can affect angiogenesis. The zebrafish stalactite mutant ($Stl^{-/-}$), affecting the MTP gene, was defective in lipoprotein assembly. $Stl^{-/-}$ fish develop long ectopic sprouts in the intestinal vessels due to increased endothelial cell proliferation. ApoC2 morphants, which bear large amounts of lipoproteins in the circulation, showed an impaired angiogenesis and were embryonic lethal (E4). Furthermore, transplantation of apoB secreting COS cells into the periderm resulted in short sprout formation in the presence of MTP. Additional mechanistic studies revealed that VEGFR1 level were up-regulated in ApoC2 morphants and down regulated in $Stl^{-/-}$ fish. VEGFR1 expression in $stl^{-/-}$ zebrafish could rescue the angiogenic phenotype. These *in vivo* approaches were complemented by *in vitro* models, showing that treatment of HUVEC cells with LDL resulted in VEGFR1 upregulation and activation. By using apolipodemia mouse models ($ApoE^{-/-}$ & $LDLR^{-/-}$), Yaniv further demonstrated that VEGFR1 expression was increased in endothelial cells in response to increased lipid protein level. In conclusion, Yaniv could show that VEGFR1 is up-regulated in endothelial cells in response to circulating lipoproteins, resulting in impaired angiogenesis.

"VEGF-B regulates endothelial fatty acid transport"

Ulf Eriksson (Stockholm) demonstrated his study on the role of VEGFB on blood vessel function. In a bioinformatic screen, he found that Vefb is co-expressed with nuclear-encoded mitochondrial genes across a large variety of physiological conditions in mice, pointing at a role for VEGFB in metabolism. By using $VEGFB^{-/-}$ mice and performing Ad-hVEGFB rescue experiment, he could show that VEGF-B specifically controls endothelial uptake of C_{12}-C_{22} fatty acids via regulation of the vascular fatty acid transport proteins FATP3 and -4. The regulation of fatty acid uptake was further mediated by VEGFR1 and neuropilin 1 expressed by the endothelium. VEGFB promoter studies showed that VEGFB expression is regulated by ERRα and PGC-1α, which are both known to be involved in energy metabolism. $VEGFB^{-/-}$ mice showed a reduced uptake and accumulation of lipids in muscle, heart and brown adipose tissue and instead shunted lipids to white adipose tissue. The cardiac lipid uptake could be restored in $VEGFB^{-/-}$ mice by Ad-VEGFB administration. The redistribution of lipids from heart to white adipose tissue resulted in a net shift of the metabolism in $VEGFB^{-/-}$ mice to increased glucose consumption, as measured by ^{18}FJ-deoxyglucose uptake into the heart. In conclusion, Eriksson presented a novel VEGFB dependent mechanism in which endothelial lipid uptake and mitochondrial lipid use are tightly coordinated.

"Identification of novel genes involved in tumor angiogenesis"

Roy Bicknell (Birmingham) presented novel tumor endothelial markers which were identified in a genome wide screen. CLEC14A, a novel C-type lectin that is over-expressed in endothelial cells of the tumor vasculature, was shown to be induced in the absence of shear stress. Bicknell suggested that reduced blood flow in irregular tumor vessels leads to low shear stress and thus up-regulation of CLEC14A expression. Functionally, CLEC14A mediates filopodia formation, endothelial cell migration as assessed by CLEC14A antiserum treatment and CLEC14A silencing approaches of cells by scratch assay. Furthermore, CLEC14A was shown to be important for vascular development in zebrafish. The tumor specificity of CLEC14A was increased compared to the known tumor endothelial marker ROBO4, concluding that CLEC14A is a novel gene that offers therapeutic potential for cancer. Bicknell further presented data on Rhombiod like proteins 1/2/3 (RHBDL1/2/3), which are seven transmembrane serine proteases that cleave EGF precursors in the Golgi to produce mature EGF. The closely related Rhomboid family members (RHBDF1/2) show a similar structure but are catalytically inactive and carry an elongated N-terminus. RHBDF2 was shown to be up-regulated in tumors and silencing of RHBDF2 inhibited TNFα response, scratch wound closure and tube formation. In zebrafish RHBDF2 was shown to be expressed, although not exclusively, in endothelial cells. RHBDF2 morpholinos inhibited intersomitic vessel formation and showed aberrant arteriogenesis, whereas no effects were observed in the venous system. Bicknell further showed by co-immunoprecipitation studies that CLEC14A forms a complex with RHBDL2 and is subsequently cleaved by RHBDL2 in the transmembrane region, resulting in inactivation of the protein. RHBDL2 itself is regulated by RHBDF2, which specifically associates with RHBDL2 to inhibit its activity. In conclusion, Bicknell identified a new signaling pathway in which RHBDF2, RHBDL2 and CLEC14A regulate tumor angiogenesis.

<u>Session 3: ENDOTHELIAL TIES</u>

"Regulatory mechanisms at endothelial cell contacts"

Dietmar Vestweber (Muenster) - content embargoed.

"Acetylation dependent regulation of endothelial Notch signaling by the SIRT1 deacetylase"

Michael Potente (Frankfurt) presented data on the role of metabolic and redox sensing deacetylase SIRT1 as regulator of Notch signaling in endothelial cells. He demonstrated that SIRT1 overexpression leads to downregulation of Notch signaling and that the catalytic domain (NICD) is crucial for the negative modulator activity. NICD is acetylated at several evolutionary conserved lysine residues and he could show that an acetylation defect (K > R) makes it refractory to SIRT siRNA induced increase in Notch activity. Moreover, endothelial cells lacking SIRT1 were shown to be sensitized to Notch signaling and displayed impaired growth, sprout elongation and enhanced Notch target gene expression in response to Dll4 stimulation. *In vivo*, SIRT1mutant mice retina were shown to have decreased vascular density and reduced branching apparently due to an enhanced level of Notch signaling.

"miR-221 contributes to endothelial cell morphogenesis in developing zebrafish intersegmental arteries"

Stefania Nicoli (Worcester) presented a new molecule involved in primary sprouting of the intersegmental artery (ISA) in zebrafish. By deep sequencing small RNAs from endothelial cells during ISA sprouting, she identified miR-221 as a pro-angiogenic miRNA *in vivo*. Loss-of-function experiments showed reduced sprouting and cell proliferation of ISAs at 30 hpf. Mosaic analysis demonstrated that miR221-deficient cells fail to contribute to the tip cell position. Two major targets of miR221 were identified and confirmed by an endothelial cell autonomous sensor assay: PIK3R1, the small inhibitory subunit of PI3K signaling and CDKN1B, a member of the Cip/Kip family of Cdk inhibitor proteins. Down regulation of these proteins rescued ISA proliferation and migration in miR221 deficient embryos and inhibition of their pathways recapitulated the miR221 phenotype. Additionally, a role of miR221 in modulating Flt4 signaling was suggested, pinpointing miR221 as a novel player in vascular network regulation.

"Role of Angiopoietin-Tie2 system in retinal angiogenesis"

Gou Young Koh (Daejeon) presented data providing evidence for important therapeutic implications of targeting Ang-Tie system in mouse retinopathy of prematurity (ROP) model. His talk focused primarily on Ang-1 as a potential therapeutic molecule in ROP model. He could show that targeting VEGF using VEGF Trap suppressed the formation of neovascular tufts and also induced an avascualr area in the retina. In addition, intraocular administration of Ang-1 reduced both tuft formation and the retinal avascular area. Ang-1 treatment also resulted in improved endothelial-pericyte interaction in superficial and deep plexus of the retina. The vessels were better perfused and showed decreased apoptosis. Koh also focused on the role of endogenous Ang-2 in comparison to exogenous Ang-2. Furthermore his data suggested that DAAP, a double decoy receptor for VEGF-A and angiopoietin was even more effective than anti-VEGF or therapy alone in the ROP model.

"Angiopoietin-1/Tie-2 signal-mediated vascular maturation and quiescence are regulated by Dll4-Notch signaling"

Naoki Mochizuki (Osaka) talked about the role of Dll4 in Ang1/Tie2 regulated vascular maturation. Microarray data obtained by comparing sparse and confluent endothelial cells in culture allowed him to identify potential players in Ang1/Tie2 mediated vascular quiescence. Dll4 expression was induced in confluent cells after Ang1 stimulated, but not in cells missing cell-cell contacts. This up-regulation

could be blocked by treating the cells with a PI3-kinase or Akt inhibitor, targets that are both downstream of Ang1/Tie2 signaling. Another link between both pathways was established by showing an increase of NICD upon Ang1 stimulation, which leads to Dll4 expression. A model to confirm Dll4-regulated vascular quiescence a transgenic zebrafish expressing a vessel-specific cell cycle indicator was generated. Using this *in vivo* approach, he could show a proliferative effect in Dll4 morphants, whereas uninjected embryos expressed lower amounts of the S/G2/M-indicator.

Session 4: ENDOTHELIAL BARRIERS

"The role of Wnt signaling in vascular development"

Elisabetta Dejana (Milan) presented data regarding the role of Wnt signaling in vascular development. In canonical Wnt signaling, β-catenin is released from its multi-protein destruction complex upon Wnt ligand binding to Fzd/LRP receptor-complexes, translocates into the nucleus and forms a transcriptional complex with Tcf/Lef transcription factors for activation of target gene expression. Dejana demonstrated that endothelial specific deletion of β-catenin in mice alters the development of embryo vasculature and induces early lethality *in utero*. The vessels showed changes in vascular lumen, lack of vascular remodeling and diffuse hemorrhages in different regions of the vascular tree. Furthermore, loss of β-catenin caused defective endocardial cushion and cardiac valve development due to altered endothelial mesenchymal transformation. β-catenin transcriptional activity was also required for the specification of endothelial cells in the brain microvasculature to acquire blood brain barrier characteristics. Mutant mouse embryos which express a stabilized mutant of β-catenin die *in utero* with major defects in vascular development. Vessels were unable to sprout and branch correctly and alterations in vessel lumen as well as arterial venous shunt formation were observed. Moreover, venous specification was strongly affected and hyperperfusion of head and tail were detected. Furthermore, crosstalk between Notch and canonical Wnt signaling was identified, namely through the binding of β-catenin to the Dll4 promoter and subsequent up-regulation of NICD signaling. In conclusion, Dejana demonstrated that canonical Wnt signaling is important at different stages of development and in different regions of the vascular tree, most likely via intensive crosstalk with the Notch signaling pathway.

"Role of pericytes at the blood brain barrier"

Christer Betsholtz (Stockholm) addressed the role of pericytes in controlling the "blood-brain barrier" (BBB), using a pericyte impaired (PDGF-$B^{ret/ret}$) mouse model in combination with a one (R26P+/0) or two (R26P+/+) allele rescue mutant. PDGF-$B^{ret/ret}$ mice showed increased permeability of the BBB to water and unselectively to a wide range of low and high molecular weight tracers (e.g. Evans blue, Dextran & IgG). The leakage phenotype was still observed in R26P+/0 mice, but could be rescued in R26P+/+ mice. The increased permeability in PDGF-$B^{ret/ret}$ mice occurred by endothelial transcytosis, a reversible process, which could be blocked by Imatinib. Endothelial cell junctions electron microscopically showed a high overlap, whereas the endothelial cell identity was upheld at the BBB. Regulatory mechanisms of pericytes at the BBB included induction of astrocyte end-foot polarization through the dystrophin associated glycoprotein complex with abnormal aquaporin 4 distribution. Betsholtz concluded that pericytes play a critical role in the integration of endothelial and astrocyte functions at the neurovascular unit and that they regulate the BBB through an effect on endothelial transcytosis.

Session 5: ENDOTHELIAL REGULATION

"Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction"

Christiana Ruhrberg (London) presented her new data about the interaction of macrophages and vessels at endothelial tip cells. Blood vessel networks are known to expand in a two-step process that begins with vessel sprouting and is followed by vessel fusion. Vessel sprouting is induced by chemotactic gradients of VEGF, which stimulates tip cell protrusion. Using different mouse mutants that are defective in macrophage development or VEGF signalling, it could be shown that macrophages did not influence vessel sprouting, but promoted tip cell fusion. Macrophage-deficient mice presented reduced vascular complexity and capillary remodelling. Moreover, striking molecular similarities were found between the pro-angiogenic tissue macrophages essential for vascular development and those that promote the angiogenic switch in cancer, including the expression of the cell surface proteins TIE2 and Neuropilin1 (NRP1). Ruhrberg further presented that deletion of the NRP1 cytoplasmic domain did not impair angiogenesis, but conferred permeability resistance, as assessed by a choroidal neovascularisation assay.

"VEGF and Angiopoietin mediated signaling in endothelial cells"

Hellmut Augustin (Heidelberg) addressed the role of VEGF as permeability factor and Ang-2 as pro-angiogenic molecule. Previous studies have shown that NRP-1 acts as VEGFR-2 co-receptor and NRP-1/VEGFR-2 complex formation is induced by VEGF. However, Augustin presented data that this extracellular VEGF bridging model is not sufficient to explain NRP-1/VEGFR-2 complex formation. He showed that the intracellular domain of NRP-1 is important for NRP-1/VEGFR-2 complex formation, most likely via a synectin-dependent intracellular bridging of the receptors, as complex formation was reduced in synectin$^{-/-}$ endothelial cells (EC). Based on this work, his group set out experiments aimed at identifying distinct functions mediated by the intracellular domain of NRP-1. Cellular experiments and experiments in mice lacking the cytoplasmic domain of NRP1 (NRP1^{CYTOKO}) (collaboration with Christiana Ruhrberg) indicated distinct roles of NRP-1 in controlling VEGF angiogenesis vs. permeability regulating effects. In the second part of the presentation, Augustin his talk, Augustin discussed the role of Ang-2 as proangiogenic molecule and its role in anti-angiogenic therapy by controlling the therapeutic window and efficiency of anti-VEGF therapies. Depending on the presence of VEGF, Ang-2 acts as a context dependent antagonist on Ang-1/Tie2 signaling which induces quiescence in ECs. Ang-2 blockade in post natal retina resulted in reduced vessel density, altered maturation and reduced vessel outgrowth, presenting a tip cell phenotype. However, only Ang-2 but not Tie2 is expressed in tip cells, arguing for a Tie2 independent mechanism of Ang-2 signaling in tip cells. Augustin showed that Ang-2 can activate ERK, AKT, FAK signaling and induces EC migration independent of Tie2. Collectively, the data argue for a direct, pro-angiogenic role of Ang-2 in Tie2-negative EC and for a vessel maturation modulating role of Ang-2 in Tie2-expressing EC.

Session 6: VESSELS AND NERVES

"Axon guidance receptors in vascular development"

Anne Eichmann (Paris) discussed the function of Robo4 and Unc5B, ligand and receptor in neural signalling, to stabilize vessels by counteracting VEGFR2 signaling. The interaction of Robo4 and Unc5B was determined by protein tagged to alkaline phosphatase. Unc5b is required in endothelium to bind Robo4. Coculture experiments showed repulsion of Unc5b expressing endothelial cells when bound

to Robo4. Upon stimulation of Unc5b with Robo4, Src and Erk are phosphorylated, however Akt and Src do not colocalize with Unc5b. Blocking of Src activation impairs repulsion of endothelial cells upon Robo4 stimulation. Immunoprecipitation experiments showed less Src localized to VEGFR2 when cells were stimulated with Robo4, activation blocking antibody for Unc5b reversed this effect. This could also be shown *in vivo* in Unc5b retina. Taken together, Robo4 is a ligand for Unc5b in endothelial cells and triggers the signaling cascade leading to Src localization to Unc5b and activation of Erk. This data reveals a mechanism of Unc5b counteracting VEGFR2.

"The cerebral cavernous malformation protein CCM1 keeps the endothelium quiescent"

Andreas Fischer (Mannheim) investigated a novel function of cerebral cavernous malformation protein CCM1 as an anti-angiogenic protein. Cerebral cavernous malformations (CCM) are frequent vascular abnormalities caused by mutations in one of the three known CCM genes (CCM1, CCM2, CCM3). The CCM1 protein is part of a large protein complex together with CCM2, CCM3, HEG1 and ICAP1. He showed that CCM1 and also ICAP1 inhibit endothelial proliferation, apoptosis, migration, lumen and network formation and sprouting angiogenesis. A global gene analysis revealed that CCM1 and ICAP1 activate DLL4-NOTCH signaling, a central regulator of angiogenesis. Conversely, blocking NOTCH activity was able to rescue the gain-of-function CCM1 and ICAP1 phenotype. Furthermore, CCM1 and also ICAP1 reduce phosphorylation of the mitogen-activated protein kinase ERK but increases AKT phosphorylation. This induces NOTCH signaling which shifts the endothelium from proliferation to quiescence. Conversely, human CCM lesions showed highly elevated levels of phosphorylated ERK. In an *in vivo* model CCM1-silenced human endothelial cells were transplanted into SCID mice, this enabled to mimic the CCM pathology. Treatment with Sorafenib a multi-kinase inhibitor was able to prevent excessive angiogenesis and so normalize the disturbed vasculature. These data suggested that the origin of CCM lesions is caused by perturbed NOTCH signaling-induced excessive capillary sprouting and that anti-angiogenic treatment could be beneficial to prevent progression of CCM.

"G protein-coupled receptor regulation of central nervous system angiogenesis"

Calvin Kuo (Stanford) gave a talk about the function of GPCR 124 (tumor endothelial marker 5/TEM5) in vasculature of CNS. Knock out mice for GPR124 show hemorrhages in CNS and are nonviable. Also there are severe defects in developing CNS angiogenesis and vascular migration. However, this phenotype is restricted to the central nervous system, other organs are not involved. This phenotype was validated by the generation of a tissue specific knock down of GPR124 which phenocopies the finding of the global knock out. In GPR124 the expression of the blood-brain barrier marker Glut-1 is impaired but this effect is independent of the Wnt/β-Catenin signalling which could be shown in β-Catenin deficient mice. *In vivo* models overexpressing GPR124 show vascular hyperplasia and CNS vascular malformation, though the vascular pattern in non CNS organs appears normal. In an *in vitro* model using a shear-free gradient generator the GPR124 dependent migration to forebrain conditioned medium but not hindbrain conditioned medium has been shown. This is mediated by Cdc42 activity. So, GPR124 knock down leads to defective cell polarization and orientation. Combined knock down of GPR124 with the 40% similar GPR125 showed enhanced angiogenic defects, whereas the single knock down of GPR125 revealed no vascular defects. This data indicates the essential regulatory role of GPR124 in CNS angiogenesis most notably in the Cdc42 directed migration.

Session 7: VESSEL FUSION, LUMEN FORMATION

"Post-transcriptional control of vascular development by microRNAs"

Nathan Lawson (Worcester) gave an overview about his project to identify microRNAs involved in the developing zebrafish embryo. Screening was performed by deep-sequencing having the advantage to identify both endothelial-expressed ECs and the co-expressed 3'UTRs. At the same time, immunoprecipitation of the RNA-induced silencing complex (RISC) was done to identify microRNA:target interactions at a particular time during development or in a specific cell type. By combining these two tools, a microRNA:target network can be generated for different time points in development. Despite the high-throughput potential a number of challenges remain, as targeting underlies complex pairing rules, 3'UTR sequences are still poorly defined. In the last part of the talk, he showed recent progress in the identification of EC-expressed microRNAs, focusing on miR-126. This microRNA is regulated by the flow-induced transcription factor klf2a and is involved in the regulation of VEGFR2 signaling via spred1. (Nicoli et al., Nature 2010)

"Molecular mechanism of vascular lumen formation"

Eckhard Lammert (Düssledorf) presented a talk focussing on molecular mechanisms and principles of blood vessel lumen formation. Using a mouse embryo model and monitoring EC cord to tube transition, he could show that aortic lumen formation starts extracellularly between the 1S and 3S stages of mouse embryonic development. Furthermore, he showed that CD34-Sialomucins, Moesin, F-Actin, and Non-Muscle Myosin II localize to the endothelial cell-cell contact at the onset of aortic lumen formation. He emphasized on the importance of sialomucin for cell-cell contact and used Podx1 as an apical marker. Dwelling deeper in molecular mechanisms he described de-adhesion as separation of EC by glycocalyx precursor for lumen formation. He showed that removal of the glycocalyx (sialic acid) leads to loss of negative charge, which made a simulation of de-adhesion more energy intensive using atomic force microscopy. He also mentioned that sialic acid clusters at the site of lumen formation in the dorsal aorta, which is therefore negatively charged.

In vivo, he could show that neuraminidase treatment led to a decrease in lumen formation owing desialation. Interestingly, he also demonstrated that lumen formation could be rescued by dextran sulphate, while dextran alone had no effect.

In conclusion, Lammert provided convincing evidence that negative charge on apical surface is required for lumen formation.

"In vivo analysis of vessel fusion during DLAV formation in the zebrafish embryo"

Markus Affolter (Basel) presented a talk about molecular control and regulation of vessel fusion in zebrafish. His talk focused on the development of intersegmental vessels and the formation of dorsal longitudinal anastomotic vessels (DLAV) by sprout fusion. In a transgenic zebrafish expressing an eGFP-fused version of the AJ/TJ protein ZO1 he could show how tip cells first interact and become a part of a lumenized tube. EGFP-ZO1 co-localized with endogenous ZO1 and VE-Cad and served as a marker for formation of AJ/TJ and the outline of endothelial cells. He further showed that during DLAV formation, tip cells fusion occurs via filopodial extension and eGFP-ZO1 and VE-cad localize to these contact sites. The sites extend into ring-like structures, suggesting of a compartment between two fused tip cells. Luminal growth was reported to extend from proximal to distal side of fusion driven by apparently luminal pressure.

"Tu-be or not tu-be: tubologenesis during cardiovascular development"

Ondine Cleaver (Dallas) presented data regarding Rasip1, a highly endothelial cell specific Ras binding protein. Initial *in vitro* experiments proved that Rasip1 is required for migration and tube formation. The newly generated Rasip null mouse shows embryonic lethality at E10.5 and start to display failures of vascular remodeling at E9.5. This includes loss of arterial and venous markers, defective hemodynamics, abnormally thin aorta and no lumen formation, as angioblasts remain adhered to each other and their surrounding tissue. A CoIP/Mass spectrometry was performed and identified NMHCIIA and Arhgap29 as Rasip1 binding partners in angioblasts. Both proteins are implicated in focal adhesion maturation and their regulation by Rasip1 could explain the missing adhesion to the ECM and dominant clustering of angioblasts, which lead to failures in lumen formation.

Session 8: VESSEL MORPHOGENESIS I

"Targeting the VEGF-C/VEGFR-3 pathway of inhibition of angiogenesis and lymphathic metastasis"

Kari Alitalo (Helsinki) talked about VEGFR3 blocking antibodies and their application in treatment of metastasis. Blocking of VEGFR3 in tumor models decreased sprouting of blood vessels. VEGFR3 deficient endothelium displays reduced level of NOTCH signalling. This indicates an important role of VEGFR3 in angiogenesis. In VEGFC heterozygous deficient mice disrupted vascular branching in the retina occurred, which verifies the previous findings. Combined inhibition of VEGFR2 and VEGFR3 resulted in additive inhibition of tumor angiogenesis and growth. It could be shown that the VEGFR3 blocking antibody does not bind to the ligand binding site but inhibits growth. The maximal inhibition of the endothelial network formation was achieved by the combination of blocking ligand binding and receptor dimerisation antibody.

"Guidance and patterning of developing lymphatic vessels"

Brant Weinstein (Bethesda) presented data regarding the development of lymphatics in zebrafish trunk. Secondary sprouts arise from posterior cardinal vein to horizontal myoseptum to form the parachordal vessel. From there sprouts emerge along the arterial intersomitic vessels to form ventrally the thoracic duct between dorsal aorta and posterior cardinal vein and dorsally the dorsal lymphatic line. This mechanism is directed by chemokines. Cxcr4a is a receptor for Cxcl12a and b. By cloning the Cxcr4a promoter to RFP the expression in developing lymphatic endothelium was detected. The same technique showed that Cxcl12a is expressed in superficial horizontal myoseptum and later in the posterior cardinal vein, whereas Cxcl12b is expressed in arterial intersomitic vessels and dorsal aorta. Morpholino knock down verified this finding. Furthermore Cxcr4a antagonist treatment showed the involvement of the receptor during multiple stages of lymphatic endothelium development. Treatment with angiogenic sprouting inhibiting morpholino showed impaired intersomitic vessels formation but normal sprouting of lymphatic endothelial cells. Combined treatment with Cxcr12a morpholino showed sprouting but no parachordal vessel formation. In conclusion chemokines are important in two distinct steps of lymphatic network formation but not for initial sprouting from posterior cardinal vein. Summarized, chemokines direct alignment and migration of lymphatic progenitors.

"Lymphangioblast formation and migration in zebrafish"

Stefan Schulte-Merker (Utrecht) showed in vivo imaging of the zebrafish trunk lymphatic vasculature, using a number of different transgenic lines that allow distinguishing arterial, venous and lymphatic endothelial cells within the same specimen. Venous cells (secondary sprouts) migrate from the cardinal vein to the region of the horizontal myoseptum, where they constitute a pool of parachordal lymphangioblasts. These cells use arteries to either migrate ventrally to form the thoracic duct, or dorsally to define a set of intersegmental lymphatic vessels. Genetic screens and the identification of a number of mutants show that the orthologues of mammalian FLT4 and VEGF-C are required for venous sprouting in zebrafish. Another mutant affecting the ccbe1 gene exhibits the same phenotype, and (possibly hypomorphic) mutant forms of the human CCBE1 gene cause Hennekam Syndrome, a rare congenital disease presenting with lymphedema, lymphangiectasias and mental retardation.

Session 9: VESSEL MORPHOGENESIS II

"Mechanisms of lymphatic remodeling"

Taija Makinen (London) - content embargoed.

"Amotl2 controls endothelial cell polarity and aortic lumen formation"

Lars Holmgren (Stockholm) - content embargoed.

"Regulation of angiogenesis by the Rac1 GEF Dock4"

Sabu Abraham (London) gave a blitz talk about the function of Dock4 as a GEF for Rac1 downstream of VEGFR2. Defects in tube formation were detected in a siRNA mediated knock down screening of Rho GTPases. Knock down of Rac1 lead to the lowest branch point index, representing the most severe phenotype. To analyse the activators for Rac1 in these pathway, the same experiment was performed with siRNA mediated knock down of GEFs for Rac1. Here, knock down of Dock4 showed the same branching phenotype with suppressed cluster formation. Filopodia formation was impaired at the lateral site of the tubes, however, at the tip of the cell filopodia still occur. Pull down experiments of Rac1 upon VEGF stimulation in control and Dock4 deficient cells showed no activation. In a mice tumor angiogenesis model impaired lumen formation could be observed. Hence, Dock4 is necessary for cell cell adhesion, sprouting and regulates lumen formation downstream of VEGFR2.

"Calcineurin/NFAT signaling in lymphatic vascular development and maintenance"

Tatiana Petrova (Epalinges) - content embargoed.

Session 10: ENDOTHELIAL – MONOCYTE CROSSTALK

"Neuropilin-1 Expressing Mononuclear cells (NEMs), a novel population of bone marrow cells recruited by Sema3a, contributes to vessel normalization and inhibits tumor growth"

Mauro Giacca (Trieste) presented data on the recruitment of bone-marrow derived Nrp1-expressing mononuclear cells (NEMs) to tissues that express VEGF-A165 and Sema3A over a prolonged period time, caused by Adeno-associated Virus (AAV) vectors. He could show that NEMs exert a strong anti-tumoral effect by promoting vessel normalization leading to reduced hypoxia in melanoma and fibrosarcoma in immunocompromised mice. Tumors treated with NEMs or Sema3a showed a

reduction in size and better perfusion in comparison to untreated controls. NEMs were phenotypically characterized as $CD11b^{+}/Nrp^{+}/Gr1^{-}/Tie2^{-}$ in mice, but are not yet identified in humans as the marker Gr1 is not present. As a final remark, he presented a study in human patients, in which colorectal tumors that express high Sema3A levels were less invasive and less prone to metastasize.

On-site education of VEGF-recruited monocytes improves their performance as angiogenic- and arteriogenic accessory cells

Eli Keshet (Jerusalem) presented a talk about the role of monocytes in adult neovascularisation. His presentation focussed on the origin of pro-angiogenic monocytes and the reason for their plasticity. Using a genetic system for conditional and reversible induction of VEGF in selected adult organs (liver or heart) and genetically tagged monocyte populations, he could show that the pro-angiogenic monocytes are derived from a pre-existing pool of monocytes which are recruited in a VEGF-dependent manner. He could characterize these cells as Ly-$6C^{hi}$ monocytes which undergo ''education'' upon VEGF induction in particular organs, leading to phenotypic changes and rendering them pro-angiogenic. He showed higher retrieval of GFP tagged Ly-$6C^{hi}$ monocytes from specific organs upon VEGF induction and this resulted in higher sprouting angiogenesis in a hind limb ischemia model. He also showed that depleting circulating Ly-$6C^{hi}$ monocytes by CCR-2, led to decrease in endothelial cell proliferation. He further pointed out that VEGF recruited monocytes do not proliferate and are short lived.

Session 11: VESSEL PATTERNING

"Arterial specification signalling cascade"

Michael Simons (New Haven) presented his latest results in molecular determinants of arteriogenesis. In particular, the signalling events controlling arterial formation have not been established. Simons and his group showed increased ERK1/2 activity in mouse ECs with reduced VEGF responsiveness which was achieved *in vitro* and *in vivo* by downregulating PI3K activity, suppressing Akt1 but not Akt2 expression, or introducing a constitutively active ERK1/2 construct. Such restoration of ERK1/2 activation was sufficient to restore impaired arterial development and branching morphogenesis in synectin-deficient mice and synectin-knockdown zebrafish. The same approach effectively stimulated arterial growth in adult mice, restoring arteriogenesis in mice lacking synectin and in atherosclerotic (hyperlipdemic) mice lacking both LDL-R and ApoB48 using a hind limb ischemia model. He therefore concluded that PI3K-ERK1/2 crosstalk plays a key role in regulating arterial growth and that the augmentation of ERK signalling via suppression of the PI3K signalling pathway can effectively stimulate arteriogenesis. Furthermore, creating a dominant active and negative transgenic RAF SA 259 mutant mouse, he investigated induction of arterial genes (DLL4, Hey1, Hey2, Hee1, Jagged 1) but no changes in the venous genes and linked it to ERK signalling cascade via transcription factors Ets1 and SOX18.

"Pulsatile shear and Gja5 (Connexin-40) modulate arterial identity and remodeling events during flow driven arteriogenesis"

Ferdinand le Noble (Berlin) analyzed the influence of pulsatile shear on arterial identity gene expression. In chicken embryo yolk sac vasculature arterial identity gene expression requires the presence of arterial hemodynamics. Therefore, le Noble hypothesized that arterial flow must provide a unique signal relevant for supporting arterial identity gene expression, which is absent in veins. Le

Noble analyzed factors related to flow, pressure and oxygenation in the chicken embryo vitelline vasculature *in vivo* and the best discrimination between arteries and veins was obtained by calculating the relative pulse slope index (RPSI). He presented an increase in RPSI in arteries compared to veins during early developmental stages. *In vivo*, exposure of arteries to blood flow with low RPSI, hence venous characteristics, resulted in loss of arterial marker expression. Exposure of arterial endothelial cells to pulsatile shear *in vitro* resulted in augmented arterial marker expression compared to exposure to constant shear. Le Noble pointed out that oxygenation did not influence arterial development. However, exposure to hypoxia resulted in cardiac dysfunction, reduced vitellin lateral arterial network formation and was associated with increased expression of Gja5. In situ hybridization in normal and ligation embryos confirmed that Gja5 expression is confined to arteries and regulated by flow, not pressure. In the ligation model, the formation of the flow driven arterial network extension through the venous territory involved intussusceptive arborization at the distal front of the expanding arterial tree. He further showed that intussusceptive pillar formation and pillar fusion was increased at the capillary level, and immediately adjacent to the main arteriolar thoroughfare channels, resulting in separation of this arteriole from the surrounding capillaries and preventing AV shunt formation. In mice, Gja5 (Connexin-40) was also expressed in arteries. In the adult, increased flow drives arteriogenesis and the formation of collateral arterial networks in peripheral occlusive diseases. Genetic ablation of Gja5 function using Gja5 mutant or endothelial specific Gja5 conditional mutant mice, resulted in reduced arteriogenesis in two occlusion models. Gja5 mutant mice showed reduced arterial collateral blood flow upon femoral artery occlusion. By comparing wild-type and Gja5 mutants, a reduced number of arterial collaterals, reduced collateral arterial diameters, and impaired flow induced outward remodeling response in arteries was observed. Thus, le Noble concluded that pulsatile shear patterns may be central for supporting arterial identity, and that arterial Gja5 expression plays a functional role in flow driven arteriogenesis.

"Sprouting angiogenesis form the venous endothelial cells is regulated by BMP signaling"

Suk-Won Jin (Chapel Hill) talked about his results about sprouting angiogenesis of venous endothelial cells. During zebrafish development the axial artery (dorsal aorta) and the axial vein are spatially juxtaposed and the initial angiogenic sprouts from these vessels extend in opposite directions, suggesting that distinct cues may regulate angiogenesis of the axial vessels. Jin found that angiogenic sprouts from the dorsal aorta are dependent on Vegf signalling and do not respond to Bmp signals which are expressed within the axial vein. In contrast, sprouts from the axial vein are regulated by Bmp signalling independent of Vegf, suggesting that Bmp is a vein specific angiogenic cue during early development. These results support a novel paradigm of angiogenesis, whereby different signals regulate distinct programs of sprouting angiogenesis from the axial vein and artery, and suggest that signalling heterogeneity contributes to the complexity of vascular networks.

"Stepwise arterial and venous differentiation in mammals"

Rong Wang (San Francisco) presented her study on arterial venous malformation (AVM), in which direct arterial venous shunts develop without intervening capillaries. AV shunts develop when vessels with high shear stress grow at the expense of other parallel vessels and then collapse into a single AV-shunt. The most severe form is brain AVM (BAVM), which is responsible for 50% of childhood hemorrhagic stroke cases that can only be treated by risky surgical interventions. The cellular and molecular pathways for this disease are still largely unknown. Wang showed that endothelial cell

specific expression of constitutive active Notch4 (Tie2-tTA:Cre-Notch4*(Notch4*)) resulted in an AVM phenotype in mice, which could be normalized by repression of Notch4*. 4D microscopy studies of vessel topology and blood flow allowed high resolution imaging of AV-shunts and demonstrated an increased blood flow in shunted vessels. AVM was observed to originate from capillaries without smooth muscle cell coverage. In Notch4* mice ephrinB2 was up-regulated, whereas EphB4 was repressed in shunts of BAVM, indicating arterialization of veins in these regions. In conclusion, Wang showed that Notch signaling is an essential regulator of AV-shunt formation.

"CD44v6 collaboration with VEGFR-2 and c-Met leads to angiogenesis and metastasis"

Veronique Orian-Rousseau (Karlsruhe) presented data about the collaboration between VEGFR2 and c-met with CD44v6. VEGFR-2, the most prominent receptor tyrosine kinase (RTK) involved in angiogenesis, recruits several partners for its function. CD44v6, a member of the CD44 family of transmembrane glycoproteins is required for VEGFR-2 activation. CD44v6 binds VEGF for presenting it to VEGFR-2 thereby inducing the activation process. In addition, the cytoplasmic domain of CD44v6 binds to ERM (ezrin-radixin-moeisin) proteins that in turn bind to the cytoskeleton, promoting signalling from VEGFR-2. VEGFR-2 and CD44v6 form a complex that might drive the angiogenic process in collaboration with neuropilin-1. CD44v6 is also a co-receptor for c-met, another RTK involved in angiogenesis and metastasis, providing the same functions as described for its interaction with VEGFR-2. Peptides from the CD44v6 protein were shown to block both receptors *in vitro* and also *in vivo*. Indeed, pancreatic carcinoma cells orthotopically injected into the pancreas did not metastasize in the presence of the CD44v6 peptides. In addition, microvessel density and consequently the pancreatic tumor growth were drastically decreased in animals treated with these peptides. In conclusion, CD44 proteins that are able to control several RTKs involved in the different steps of tumorigenesis seem to be promising target for therapy.

"Control of vascular guidance by neural cues"

Amparo Acker-Palmer (Frankfurt) presented data on the control mechanisms of vascular guidance by neuronal cues. Cell-to-cell communication during development and in the adult organism is controlled to a large extent by signalling events downstream of receptor tyrosine kinases (RTKs). Emerging evidence suggests that axonal growth cones and capillary tip cells use common repulsive and attractive signals in their environment that ultimately determine their directional guidance through the body. Eph receptors, the largest class of RTKs in the human genome, bind membrane-bound ephrin ligands expressed by neighboring cells and therefore mediate short-range cell-to-cell communication. EphrinB ligands possess intrinsic signalling capabilities that are required for the development and function of both nervous and vascular systems. However, the molecular mechanisms underlying reverse signalling and function of ephrinB ligands in coordinating proper development and function of these systems are currently unknown. Amaparo showed that ephrinB2 reverse signalling regulates the trafficking of AMPA receptors at the synapse thereby controlling synaptic transmission. Internalization is required for VEGF induced tip cell filopodia extension and VEGFR2 internalization as well as signalling is controlled by ephrinB2. In pathological settings, blockade of the ephrinB2 PDZ-signalling pathway decreases tumour growth and vascularisation and reduces angiogenic sprouting and branching of the tumour vasculature. In summary, her findings reveal an essential role for the axon guidance molecule ephrinB2 in the guidance and function of endothelial tip cells during both developmental and tumour sprouting angiogenesis.

"Angiogenic pathways control bone marrow architecture and homeostasis"

Ralf Adams (Muenster) presented new genetic cell labeling techniques for visualization of the bone marrow morphology, allowing high-resolution imaging of the hematopoietic bone marrow stem cell niche. Cdh5(pAC)-CRE^{ERT2} mice were crossed with ACTB-tdTomato-EGFP reporter mice to visualize bone sinusoidal vessels (EGFP) as well as non-endothelial cells (Tomato). Adams showed that the bone marrow has a defined architecture which undergoes substantial changes during adult life. Adult bone marrow remodeling includes angiogenic growth, which is not mediated by tip cells but rather involves the branching of blunt, fully lumenized endothelial protrusions near the growth plate. Adams further demonstrated that local delamination of the mesenchymal layer led to the formation of spherical, hematopoietic cell–containing structures, which were termed 'hemospheres'. Direct interaction between endothelial cells and mesenchymal cells as well as HSCs and uncoated ECs were important for the formation of these structures. The expansion and fusion of hemospheres was shown to generate a continuous lacunal structure, namely bone marrow. In Flk1$^{\Delta EC}$ and Jagged1$^{\Delta EC}$ mice destruction of vessels at the growth plate, reduced endothelial cell protrusions and hematopoietic cell numbers were detected, indicating the importance of VEGF and Notch signaling in regulating sinusoidal endothelial growth and hematopoietic stem cell numbers. By using Flk1$^{\Delta EC}$, Jagged1$^{\Delta EC}$, Jagged1$^{EC\text{-}GOF}$, Dll4$^{EC\text{-}GOF}$ he further pointed at the essential role of VEGF and Notch signaling in controlling hemosphere morphogenesis. Treatment with a short time VEGFR inhibitor further resulted in vessel disappearance and new distribution of HSCs. In conclusion, Adams demonstrated a first structural and functional framework for bone marrow formation and homeostasis.