MEETING REPORT

2nd International Kloster Seeon Meeting
"Cellular and Molecular Mechanisms of Tumor Progression and Metastasis"

Hosted by the Nationwide DFG Priority Research Program 1190 "The Tumor-Vessel Interface"
with support from the Verein für Wissenschaftliche Fachtagungen in der Biomedizin e.V.

Kloster Seeon, September 19-22, 2009

Session 1: ANGIOGENESIS AND TUMOR PROGRESSION

"Reversing Angiogenesis in solid tumors"

Ruth Ganss (Perth) presented new data regarding regulator of G protein signaling (RGS) 5 as a master gene responsible for the abnormal morphology of tumor vessels. RGS5 is a marker for progenitor perivascular cells and regulates vessel stability in tumors. She found enriched expression of RGS5 in pericytes, whereas after tumor vessel normalization the signal was reduced. Several angiogenic tumors (astrocytomas, renal cell carcinoma and hepatocellular carcinoma) showed a correlation of high RGS5 expression and low vessel normalization. Loss of RGS5 altered intratumoral pericytes, and led to a shift to more mature pericytes within the vascular bed. Importantly, tumors in RGS5-deficient mice became susceptible to immune effector cell influx after adoptive transfer of *in vitro* activated, anti-Tag $CD4^+$ and $CD8^+$ T cells. In addition, RGS5 knockout mice were highly responsive to therapeutic vaccination. The tumors exhibited increased blood perfusion, reduced tumor hypoxia as well as reduced angiogenic activity. Ruth Ganss further showed that upon ligand binding to the G protein-coupled receptor (GPCR), RGS5 increased the rate of GTPα hydrolysis and therefore negatively regulated the signaling through the GPCR. Taken together, RGS5 regulates pericyte maturation and possibly the vascular composition in tumors with profound changes in the tumor environment. She proposed that a combination of vascular remodeling with anti-cancer immune strategies would hold great promise for the development of more effective anti-cancer therapies.

"The Role of Loxl2 in angiogenesis and tumor progression"

Gera Neufeld (Haifa) investigated the role of lysyl oxidase 2 in tumor angiogenesis. Loxl2 and Loxl3 mRNA expression was found in metastatic and non-metastatic breast cancer derived cell lines. He described a novel asymmetric 3D *in vitro* assay consisting of a tumor cell monolayer embedded between collagen layers. This assay permits a direct comparison of the effects of different tumor stroma compounds on tumor invasion. Inhibition of Loxl2 inhibited invasion of tumor cells and induced MET-like changes in several types of tumor cells, whereas Loxl2 gain-of-function resulted in increased tumor cell migration and invasion into vessels, nerves and muscles. Inhibition of Loxl2 by antibody treatment resulted in reduced tumor growth of MDA-MB-435 cells and reduced angiogenic factor expression such as SDF1 and VEGF in tumors. In addition, metastasis formation was also reduced. He introduced Platelet factor 4 (PF4) as a substrate for Loxl2 that inhibited VEGF function by a mechanism that does not interfere with VEGF binding to heparin sulfates. PF4 inhibited the enzymatic activity of Loxl2 and it is itself oxidized by Loxl2. External Loxl2 abrogated PF4 induced inhibition of bFGF induced proliferation of HUVECs. Inhibition of Loxl2 resulted in the inhibition of bFGF and VEGF induced proliferation and migration of HUVECs. Furthermore, inhibition of Loxl2 inhibited adhesion and tube formation on Matrigel and induced morphological changes in endothelial cells. In summary, Loxl2 plays a role in angiogenesis and tumor progression.

"Proteolytic processing controls pro-angiogenic and pro-tumorigenic effects of Semaphorin 3G"

Simone Kutschera (Heidelberg) presented data about a novel class 3 semaphorin, Semaphorin 3G (Sema3G) that is selectively expressed by sprouting endothelial cells upon stimulation with VEGF-A or FGF-2. Full length Sema3G (100 kDa) is a secreted molecule that is cleaved by pro-protein

convertases similar to the other class 3 semaphorins to yield 65 kDa and 35 kDa fragments. Cleavage enhanced the angiogenic activity of Sema3G in an *in vitro* 3D HUVEC sprouting assay. Antibody staining for Sema 3G revealed a peri-endothelial cell location suggesting an additional function on mural cells. To further unravel the role of Sema 3G on tumor progression, she generated Hek293 cells expressing different Sema3G constructs (full length wild type [WT-L], processed 65 kDa form [WT-S] and a full length cleavage-resistant form [MUT]) and implanted these cells subcutaneously into nude mice. WT-L tumors grew significantly faster compared to tumors growing from mock-transfected, WT-S or MUT Hek293 cells. Silencing Sema3G in MiaPaCa cells endogenously expressing Sema3G resulted in reduced tumor growth of MiaPaCa tumors. Taken together, Sema3G is a novel regulator of vascular function and tumor growth.

"Targeting tumor vessels by therapeutic vaccination"

Anna-Karin Olsson (Uppsala) - content embargoed.

Session 2: ANGIOGENESIS: EMERGING TARGETS

"miR-132 acts as an angiogenic switch by suppressing endothelial 120RasGAP"

David Cheresh (San Diego) presented the microRNA, miR-132, as a regulator of angiogenic signaling by targeting p120RasGAP in quiescent endothelium and thereby facilitating angiogenesis. His group identified miR-132 via a screen of human microRNAs that were upregulated in HUVECs upon growth factor treatment and in a human ES cell model of vasculogenesis. Expression of miR-132 in HUVECs resulted in increased proliferation as well as increased tube formation in a 3D assay, whereas down regulation of miR-132 resulted in decreased proliferation and tube formation, respectively. Injection of anti miR-132 in a Matrigel plug assay decreased FGF-mediated angiogenesis and intraoccular injection resulted in disruption of angiogenesis in the developing retina. Furthermore, using multiple prediction programs for interacting partners, he identified p120RasGAP as a direct target of miR-132. Expression of miR-132 in HUVECs results in decreased p120RasGAP levels and increased Ras activity. SiRNA of RasGAP phenocopied miR-132 mediated EC proliferation. RasGAP was associated with the development of human hemangiomas and was found in hyperproliferative hemangiomas. Further analysis revealed that p120RasGAP was expressed in normal vessels, whereas it was lost in tumor vessels (i.e. pancreas and beast). Using an $\alpha V\beta 3$ targeting nanoparticle to deliver miR-132 to the tumor endothelium, David Cheresh showed that tumor angiogenesis and growth was suppressed in melanoma and breast cancer models. Taken together, he showed that miR-132 and p120RasGAP function as a unique angiogenic switch to maintain endothelial quiescence.

"Identification of tumor dormancy specific microRNAs"

Nava Almog (Boston) studied the involvement of microRNAs in tumor dormancy. For this purpose, she used experimental *in vivo* models of human tumor dormancy. Human tumor cells (glioblastoma, osteosarcoma, liposarcoma and breast carcinoma) generate in these models small tumors when injected into immunodeficient mice. Following long latency periods, they spontaneously emerge from dormancy and initiate rapid growth. Using genome wide transcriptome analysis and advanced microRNA profiling, she compared dormant tumors with tumors that had switched to rapid growth and revealed critical players of tumor dormancy. Among the genes upregulated in the dormant state, she found elevated levels of thrombospondin, angiomotin, EphA5 and Insulin-like growth factor binding protein 5 (IGFBP5). High levels of EGFR, IGFR and Endothelial Specific Marker (ESM) were observed in fast growing tumors. Furthermore, she showed that the plasma levels of EphA5 in mice, similar to human patients, correlated with disease stage. Interestingly, overexpression of miRNAs associated with tumor dormancy in rapid growing tumors resulted in a significant inhibition of tumor growth. In conclusion, this study may lead to the detection and treatment of tumors long before they are symptomatic or their anatomical location is known.

"Using novel mouse genetic approaches to study signaling pathways in tumor angiogenesis"

Gavin Thurston (Tarrytown) presented data regarding the role of Angiopoietin-2 (Ang-2) during tumor angiogenesis. He used embryonic stem (ES) cell-derived teratomas as a new approach to study specific gene knockouts within the tumor entity. Teratomas with the LacZ allele under the control of

muscle (nebulin), epithelial (keratin5), or endothelial (VEGFR-2) specific genes showed distinct patterns and structures. The VEGFR-2-LacZ ES derived cells form networks, which co-express the vascular specific marker CD31 and are connected to the host vasculature. Expression profiling studies comparing ES-derived endothelial cells with host derived endothelial cells showed a similar enrichment of numerous vascular specific genes, both, in teratomas and in host-derived endothelial cells. In addition, Gavin Thurston introduced VE-PTP null ES tumors exhibiting enlarged blood vessels. Treatment of these tumors with systemic Ang-1 resulted in further vessel enlargement. VE-PTP$^{-/-}$ teratomas showed increased Tie2 phosphorylation with or without systemic Ang-1 treatment, whereas inhibition of Ang-1 and Ang-2 reduced Tie2 phosphorylation accompanied by a reduction of tumor vessel diameter. Blockade of Ang-2 in Colo205 a colon carcinoma cell line resulted in decreased tumor vessel diameters. However, no significant difference of tumor vessel numbers was observed. Blockade of Ang-2 was accompanied by increased expression of FOXO target genes that are usually suppressed by the Ang-1-Tie2 axis. Based on these findings, Thurston concluded that Ang-2 acts as a Tie2 agonist in tumor angiogenesis and restores Tie2-AKT signaling.

"Compromised tumor angiogenesis and vessel maturation in mice with broad Junb ablation"

Tobias Nübel (Heidelberg) discussed the function of Junb during angiogenesis. Junb is a subunit of the Ap-1 transcription factor complex and mediates gene regulation. Complete as well as endothelial-cell specific ablation of Junb results in similar angiogenic defects with subsequent embryonic lethality. He showed that Junb acts as a critical regulator of several pro-angiogenic molecules including the major angiogenic growth factor VEGF-A, the CBF transcription factor subunit Cbfbeta and its target MMP13. He injected intradermally B16 mouse melanoma cells into JunbΔ/ΔColl(I)α2-Cre mice, in which Junb was ablated in a variety of cell types, including endothelial cells and fibroblasts. Junb-ablated mice exhibited reduced tumor growth, reduced relative blood volume and decreased blood supply to the tumor. However, the density of the microvasculature and the total vessel number was increased. Furthermore, he observed an altered pattern of pericyte recruitment and maturation of the tumor vasculature in Junb-deficient hosts. Junb-deficient tumor vessels exhibited higher pericyte coverage indices whereas coverage with vascular smooth muscle cells extensively found in tumor vasculature of control animals was almost completely missing in Junb-deficient tumor vessels. In summary, Junb is a critical factor for vessel size regulation and vessel maturation within tumors.

Session 3: CANCER MALIGNANCY

"Inhibition of c-Met and VEGFR suppresses tumor invasion and metastasis and prolongs survival of the RIP-TAG2 mice"

Donald McDonald (San Francisco) investigated the phenomenon that inhibition of vascular endothelial growth factor (VEGF) signaling can promote invasiveness and metastasis in preclinical tumor models. The mechanism underlying this consequence of VEGF inhibition is not fully understood but activation of the hepatocyte growth factor (HGF)/c-Met (HGFR) pathway may be a factor. He reported that selective inhibition of VEGF by a function-blocking antibody increased c-Met expression and exaggerated tumor invasiveness and metastasis. Treatment of tumor-bearing RIP-Tag2 transgenic mice with anti-VEGF antibody reduced tumor growth but increased the expression of c-Met in tumor blood vessels and tumor cells and also increased invasiveness and the number and size of liver metastases. Also, inhibition of HGF/c-Met signaling and VEGF/VEGFR signaling together by the multi-targeted receptor tyrosine kinase inhibitor XL 184 (developed by Exelixis, South San Francisco, CA/Bristol-Myers Squibb, NY) significantly reduced growth and vascularity of RIP-Tag2 tumors and strikingly reduced tumor invasiveness and metastasis accompanied by increased survival rates of RIP-Tag2 mice. Taken together, inhibition of c-Met and VEGFR signaling not only has potent antiangiogenic activity but also reduces tumor growth, invasiveness, and metastasis and improves host survival.

"Phenotypic switching and the anatomy of tumor progression"

George Vande Woude (Grand Rapids) focused his presentation on the phenotypic switching during tumorigenesis. During malignant progression, tumor cells switch their phenotype from proliferative to invasive and then back again to a proliferative phenotype during metastatic growth in their new niche. The acquisition of an invasive phenotype through epithelial mesenchymal transition (EMT) is a critical step in tumor progression, as is mesenchymal epithelial transition (MET) for producing proliferating

metastatic lesions. George Vande Woude evaluated the genes responsible for both, EMT and MET. He showed that a significant proportion of either prostate or ovarian carcinoma cells were epithelial (E) and were non-responsive to hepatocyte growth factor (HGF). E cells were non-invasive *in vitro*, but grew abundantly in soft agar and were highly tumorigenic in athymic nude mice. Beyond the E cells, there was also a significant subpopulation of cells undergoing extensive branching morphogenesis (EMT) in 3D Matrigel in response to HGF. These cells were mesenchymal (M) and, when treated with HGF, were highly invasive *in vitro*, but poorly tumorigenic *in vivo*. With prostate carcinoma cells, he showed data demonstrating that M cells spontaneously emerged from the non-invasive E subclones *in vitro*. Furthermore, he showed that EMT occured with loss of E-cadherin expression and gain of c-Met expression. Interestingly, the β-catenin expression levels did not change with phenotypic switching. However, β-catenin localization shifted from the cell membrane to nuclear/cytoplasm localization suggesting a significant role for β-catenin in modulating c-Met expression and in cell type switching. In summary, George Vande Woude proposed that the regulation of β-catenin location and E-Cadherin expression influences both EMT and MET.

"Molecular characterization of minimal residual cancer"

The detection of disseminated tumor cells (DTC) in bone marrow and lymph node has been associated with reduced disease-free or overall survival and consequently these cells are thought to compromise the metastasis founder cells. **Christoph Klein** (Regensburg) and his group addressed the question whether primary tumors may serve as surrogate for the genetics of DTC and metastasis. However, using comparative genomic hybridization studies and loss of heterozygosity analyses, he found evidence for the parallel tumor progression model in which DTC were genetically less advanced than the primary tumor cells and that these cells disseminated very early during tumor progression. Therefore, the molecular characterization of DTC is essential for developing systemic therapies for cancer. Furthermore, genome-wide characterization of DTCs may uncover the identity and characteristics of metastasis founder cells.

"Searching for origins of breast cancer malignancy in mice"

Katrina Podsypanina (New York) examined the role of the initiating oncogenes *MYC* and $Kras^{D12}$ in the metastatic process using a modified experimental metastasis model and a three-dimensional (3D) culture system. Un-induced mammary cells derived from transgenic mice expressing *MYC* and $Kras^{D12}$ in a mammary-specific doxycycline-dependent manner were intravenously injected into immuno-compromised mice exposed or not exposed to doxycycline. She showed that in the absence of an active oncogene mammary cells homed to and persisted in the ectopic microenvironment of the lung and could give rise to tumors upon activation of *MYC* and $Kras^{D12}$. These metastatic lesions remained dependent on the continuous expression of the initiating oncogenes. However, regulated regression of tumors at ectopic sites left a small number of residual mammary cells that responded to re-induction of the transgenes and may be responsible for tumor recurrency. In a 3D culture system un-induced mammary cells developed into polarized acini. Due to the expression of *MYC* and $Kras^{D12}$, the acini enlarged and generated solid, depolarized spheres. The deactivation of *MYC* and $Kras^{D12}$ resulted in regression of the depolarized structures and the mammary cells showed a mammary epithelium progenitor like phenotype. Moreover, residual mammary cells in the de-induced acini retained the ability to respond to transgene activation and thus may represent the type of cells that give rise to recurrent tumors.

Session 4: INVASION & METASTASIS MEETS IMAGING

"Real-time imaging of tumor cell extravasation at the vascular interface reveals a highly dynamic process regulated by metastatic programming"

Richard Klemke (San Diego) introduced zebrafish embryos as a model to study metastasis of tumor cells. He showed that cancer cell extravasation was a highly dynamic and complex process regulated by pro-metastatic genes that target the cytoskeleton and remodel the endothelium. High resolution time-lapse imaging of arrested tumor cells revealed that these cells were not passively immobilized in the lumen as previously believed, but instead displayed dynamic amoeboid-like movement along the endothelial surface. Surprisingly, cell locomotion could be against or with the blood flow and required integrin-mediated tumor cell adhesion to the blood vessel wall. Extravasating cells did not damage the vessel wall causing leakage as previously suggested, but rather induced local vessel remodeling

characterized by altered endothelial cell-cell junctions. Induction of the pro-metastatic gene, twist, caused a switch from integrin-dependent to an integrin-independent mode of extravasation that required ROCK-mediated formation of dynamic membrane blebs and protrusions that penetrated the vascular wall. In summary, Richard Klemke reported that cancer cell extravasation is a highly dynamic and complex process regulated by pro-metastatic genes that target the cytoskeleton and remodel the endothelium.

"Brain tumor imaging using a novel peptide targeting malignant brain tumors"

Pirjo Laakkonen (Helsinki) used phage displayed peptide libraries to map disease-specific differences in the vasculature. Using this technology, she isolated several peptides homing specifically to the tumor blood vessels, lymphatic vessels and/or to tumor cells. She compared low grade astrocytoma with malignant brain tumors, where the tumor cells propagated and spred efficiently by cooperating with existing vessels. Thereby, she identified a 9 amino acid peptide, CooP, which specifically targeted a subset of malignant brain vasculature. The receptor molecule was expressed in a grade-dependent manner in human brain tumor specimens. The CooP-peptide, covalently linked with a drug, was used for tumor therapy in a mouse model of brain tumors. The targeted treatment induced a prolonged survival and decreased tumor burden in the mice. In addition, a radio labeled CooP peptide could be used to image brain tumors using the SPECT-CT technology.

"The role of HIF hydoxylases in tumor progression and metastasis"

Ben Wielockx (Dresden) - content embargoed.

"The endogenous TLR4 ligands regulate pre-metastatic soil"

Yoshiru Maru (Tokyo) reported on the identification of ligands involved in the bi-directional signaling between primary tumor cells and pre-metastatic tissues. Using DNA-array analysis, subcutaneously injected tumor cells representing an experimental system for the pre-metastatic phase were compared with tumor cells injected into the tail vein representing the metastatic phase. S100A8 was found to be induced in the metastatic phase. He showed that VEGF and TNFα from primary tumors stimulated pulmonary resident cells in lungs to secrete the endogenous TLR4 agonists S100A8 and SAA3. SAA3 caused production of TNFα in the lungs, which made TNF from the primary tumors dispensable for the stimulation of SAA3 production in the lungs. Both, S100A8 and SAA3 activated the two transcription factors NF-κB and ATF3. ATF3 induced expression of the angiogenic molecules PAI-1, Eph/ephrin family of proteins, but not VEGF. Abrogation of the S100A8-SAA3-TLR4 cascade inhibited lung metastasis. Given the embryonic lethality of S100A8.deficient mice, the endogenous TLR4 agonists could play essential roles even in physiological settings.

Session 5: EMT

"Molecular dissection of epithelial-mesenchymal transition (EMT)"

Gerhard Christofori (Basel) presented data regarding the role of E-cadherin signaling during EMT. Loss of E-cadherin function appears to play a central role in triggering full EMT in a number of epithelial cell types *in vitro* and in transgenic mouse models of carcinogenesis *in vivo*. He showed that the transcription factor distal-less homeobox 2 (Dlx2) was upregulated during EMT by TGFβ signaling. Dlx2 knockdown cells still underwent EMT. However, these cells died especially after TGFβ induction indicating that Dlx2 knockdown cells were resistant to TGFβ which induced cell survival. Furthermore, knockdown of Dlx2 in tumors resulted in reduced tumor growth. Dlx2 induced the transcriptional repression of TGFβRII, leading to attenuated TGFβ signaling and thus reduced expression of TGFβ target genes. Simultaneously, Dlx2 promoted cell survival and proliferation through EGFR-mediated activation of the MAPK and the PI3K signaling pathways and, thus, was critical for primary tumor growth and metastasis. In conclusion, these results establish a mechanistic link between Dlx2 gene expression, resistance against TGFβ mediated growth inhibition, and promotion of cell survival and invasion.

"Tumor invasion and metastasis: EMT and cancer stem cells"

Thomas Brabletz (Freiburg) highlighted in his talk the role of ZEB1 in epithelial mesenchymal transition (EMT). His concept of malignant progression and metastasis is based on migrating cancer stem cells (MSCs). Tumor cells at the invasive front of colorectal adenocarcinomas (CRC) accumulated nuclear β-catenin, underwent EMT and aberrantly expressed EMT-associated transcriptional repressors, like ZEB1. ZEB1 is known to be involved in EMT during development. In addition, ZEB1 repressed target genes involved in basement membrane formation and epithelial cell polarity. Knockdown of ZEB1 induced MET in tumor cells and thereby inhibited invasion and metastasis in tumor xenografts. ZEB1 directly suppressed transcription of members of the miRNA-200 family. He showed that miR200c, a MET promoting factor, reduced migration and invasion of tumor cells. ZEB1 directly bound and inhibited the miR200c promoter. Both, miR200c and miR141 negatively regulated ZEB1 and TGFβ2. Furthermore, he demonstrated *in vitro* that knockdown of ZEB1 reduced the cancer stem cell (CSC) population, outgrowth of potential CSC spheres, and the expression of stem cell factors, like Bmi1 and Sox2. Furthermore, expression of ZEB1 correlated with Bmi1 expression in human pancreatic cancer. Taken together, Thomas Brabletz proposed that EMT-associated tumor cells at the invasive front act as "migrating cancer stem cells" which can re-differentiate and depending on the range of dissemination, give raise to primary carcinoma metastases.

Session 6: STEM CELLS

"Wnt/β-catenin in stem and cancer stem cells"

Walter Birchmeier (Berlin) gave a talk highlighting the essential functions of Wnt/β-catenin in stem cells and cancer stem cells. He used genetic mouse models to study the necessity of Wnt/β-catenin and Bmp signaling in stem cells of the salivary gland. β-catenin loss-of-function prevented stem cell differentiation towards the hair lineage and led to less precursor cells and more differentiated cells in the skin. In contrast to this, β-catenin gain-of-function produced dozens of teeth from one tooth bud. Wnt/β-catenin controlled the balance between self-renewal and differentiation of precursor cells and the specification of dorsal neurons in the central nervous system. In addition, β-catenin was required for DMBA/TPA-induced tumor formation in the skin. He showed that β-catenin signaling was essential to maintain cancer stem cells in the skin. Combined activation of Wnt/β-catenin with blockage of Bmp signals produced highly aggressive squamous cell carcinomas. These tumors contained a high number of CD24+CD29+ stem cells and as few as 500 of these cells induced tumors following transplantation into NOD/SCID mice. If the Wnt/β-catenin and Bmp pathways were individually mutated, stem cells showed an increased potential for tissue regeneration but no tumors occurred. Walter Birchmeier concluded that his work defines a switch in stem cell potency from single to double mutations.

"A hypoxic niche regulates glioma stem cells"

Till Acker (Giessen) presented data regarding CD133+ tumor stem cells (TSCs). Glioma growth and recurrence have been shown to depend on CD133+ tumor stem cells. He demonstrated that side population (SP) cells display TSC characteristics. SP cells were able to self-renew, regenerated SP as well as non-SP cells within a cellular hierarchy and were highly tumorigenic *in vivo*. SP cells fulfilled the criteria of tumor stem cells proven by FACS analysis and they formed tumors with a fivefold increased probability compared to non-SP cells. The SP is characterized by a distinct gene signature and he identified 73 genes using a comprehensive transcriptional profiling analysis of SP and non-SP cells. These signature genes were overexpressed by TSCs in vascular and perinecrotic/hypoxic niches. Specifically, the hypoxic microenvironment played a key role in the regulation of the TSC phenotype, through the hypoxia-inducible factor (HIF)-2α and subsequent induction of specific TSC genes. In conclusion, Till Acker proposed that TSCs are maintained within a hypoxic niche, providing a functional link between the well-established role of hypoxia in stem cell and tumor biology.

"The vascular wall as a source of stem cells in adult organs"

Bruno Péault (Los Angeles) focused on mesenchymal stem cells (MSC) whose identity, frequency and location have remained obscure. He suggested that these multi-lineage progenitors originated in

blood vessel walls. He documented anatomic, molecular and developmental relationships between endothelial cells and myogenic cells within human skeletal muscle. Myoendothelial cells coexpressing myogenic and endothelial cell markers (CD56, CD34 and CD144) were identified by immunohistochemistry and flow cytometry. Cultured myoendothelial cells proliferated, retained a normal karyotype, were not tumorigenic and survived better under oxidative stress than regular myogenic cells. Clonally derived myoendothelial cells differentiated into myogenic, osteogenic and chondrogenic cells in culture. He identified in multiple human organs perivascular cells expressing CD146, NG2 and PDGF-Rβ which did not express hematopoietic, endothelial, and myogenic cell markers. Pericytes purified from skeletal muscle or non-muscle tissues were myogenic *in vivo*. Long-term cultured pericytes retained myogenicity, expressed MSC markers and migrated by chemotaxis. Furthermore, pericytes reduced myocardial fibrosis after acute myocardial infarction. In conclusion, Bruno Péault showed that blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of MSCs and other related adult stem cells.

Session 7: BONE MARROW-DERIVED CELLS

"Bone marrow derived progenitor cells in angiogenesis and tumor growth"

Petri Salven (Helsinki) investigated the mobilization of BM-derived cell populations to the circulation. Therefore, he inoculated VEGF polypeptides, adenoviral vectors expressing VEGF or syngeneic tumors in wild type mice. The current concept indicates that a significant part of neovascular endothelial cells (ECs) originates from circulating "precursor" or "progenitor" cells. These cells are first mobilized from the BM and subsequently differentiate to mature ECs and incorporate into the growing vasculature. These cells were originally defined as VEGF receptor 2-expressing (VEGFR-2) cells that are mobilized from the BM by VEGF or by tumors. Using a genetically tagged syngeneic BM stem cell transplantation model Petri Salven showed that systemic VEGF or tumors did not promote the mobilization of VEGFR-2+ BM cells to the circulation. GFP+ BM derived cells infiltrated the tumor and were recruited close to blood vessel wall ECs. However, they did not form parts of the endothelium. He showed that all BM-derived cells in angiogenic tissue were perivascular. Using endothelial cell specific genetic reporter systems Petri Salven identified no BM-derived VEGFR-2+ or Tie1+ ECs in angiogenic neovasculature. He concluded that endothelial differentiation is not a typical *in vivo* function of normal BM-derived stem cells and it has to be an extremely rare event if it occurs at all. In addition, he showed that angiogenesis during tumor growth did not involve or require contribution from BM-derived circulating progenitors for vascular ECs. Instead, large numbers of perivascular cells derived from CD117(c-kit)+/Sca-1+/lin-/Thy1.1low hematopoietic stem cells of the BM were recruited to angiogenic sites during neovascularization or tumor growth. Taken together, while BM-derived cells may play a significant role in the formation of neovasculature and in cancer growth, their contribution does not involve differentiation of BM stem cells to vascular endothelial cells.

"Tumor-mediated education of bone marrow-derived cells in tumor angiogenesis and tumor progression"

Curzio Rüegg (Lausanne) presented the role of bone marrow derived (BMD) cells during tumor angiogenesis. It is unclear whether angiogenic activity of BMD cells is acquired only upon local recruitment to the tumor or it may occur before these cells reach the tumor site. Using *in vitro* and *in vivo* studies and correlative analyzes of patient material he characterized the effect of tumor-derived factors on the generation of angiogenic myelomonocytic cells from hematopoietic progenitor cells. He observed that breast cancer cells could educate monocytes to develop angiogenic capacity during differentiation from hematopoietic progenitor cells. This angiogenic education was associated with the expression of $CCR5^+$ on $CD11b^+$ monocytes in cell culture and with the increased frequency of circulating $CCR5^+/CD11b^+$ monocytes in breast cancer-bearing mice and breast cancer patients. $CD11b^+$ cells educated by breast cancer cells induced endothelial cell (EC) sprouting in co-culture experiments. Furthermore, circulating $CD11b^+$ cells that were also Tie2 positive promoted EC sprouting in breast cancer patients. Curzio Rüegg demonstrated that MMP-9 was highly upregulated during BMD cell education. In addition, MMP inhibitors blocked the angiogenic activity of educated BMD cells. He concluded that breast cancer can influence hematopoiesis towards the generation of angiogenic monocytes and that this provides a new mechanism on how tumor cells hijack normal host events to their advantage.

Session 8: LYMPHANGIOGENESIS VERSUS ANGIOGENESIS

"Interfering with growth factor crosstalk for angiogenesis vs. lymphangiogenesis"

Kari Alitalo (Helsinki) investigated the crosstalk between angiogenesis and lymphangiogenesis via growth factors. Vascular endothelial growth factor (VEGF) stimulates angiogenesis via its two receptors VEGFR-1 and VEGFR-2, but it has only little lymphangiogenic activity. The VEGFR-3 receptor does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. Transgenic mice expressing the VEGFR-3 ligand VEGF-C or VEGF-D showed evidence of lymphangiogenesis. In addition, VEGF-C knockout mice exhibited defective lymphatic vessels. The proteolytically processed form of VEGF-C also bound to VEGFR-2 and was angiogenic. He introduced the molecule Claudin like protein 24 (CLP24) which interacted with VEGFR-2 activating it and leading to its internalization. CLP24 and VEGFR-2 had the same expression pattern except in the retinas. In addition, CLP24 expression was increased in lymphatic vessels and had been shown to be important for lymphatic and vascular development in *Xenopus*. Furthermore, soluble VEGFR-3 inhibited embryonic and tumor lymphangiogenesis and lymphatic metastasis. He showed the role of VEGFR-3 signaling in the settings of physiological and pathological angiogenesis. VEGFR-3 blocking antibodies provided significant inhibition of tumor angiogenesis and growth in several xenograft models. He found that angiopoietin-1 could stimulate lymphatic sprouting while secretion of angiopoietin-2 by endothelial cells led to hyperplasia in developing lymph vessels. Kari Alitalo concluded that receptor trafficking and compartmentalization determine the angiopoietin-specific signals via cell-cell and –pericellular matrix interactions.

"Vascular Endothelial Growth Factor-C protects prostate cancer cells from oxidative stress by activation of mTORC-2 and AKT-1"

Michael Muders (Dresden) presented data regarding the protective role of vascular endothelial growth factor-C (VEGF-C) on reactive oxygen stress induced cell death by activation of mTOR complex 2 (mTORC-2). Recurrence and subsequent metastatic transformation of cancer developed from a subset of malignant cells which showed the ability to resist stress and to adopt to a changing microenvironment. These tumor cells had distinctly different growth factor pathways and anti-apoptotic responses compared to the vast majority of cancer cells. Both microarray and immunohistochemical analysis of human prostate cancer tissue samples have shown an increased expression of vascular endothelial growth factor-C (VEGF-C) in metastatic prostate cancer. He discovered that VEGF-C acted directly on prostate cancer cells to protect them against oxidative stress. VEGF-C increased the survival of prostate cancer cells during hydrogen peroxide stress by activation of AKT-1/PKBα. This activation was mediated by mTORC-2 and was not observed in the absence of oxidative stress. Finally, the transmembrane non-tyrosine kinase receptor Neuropilin-2 was found to be essential for the VEGF-C-mediated AKT-1 activation. His findings suggest a novel and distinct function of VEGF-C in protecting cancer cells from stress-induced cell death, thereby facilitating cancer recurrence and metastasis. This is distinctly different from the known function of VEGF-C in inducing lymph-angiogenesis.

"The tumor-lymphatic interface: CCL21 as an important player in invasion and immune escape"

Melody A. Swartz (Lausanne) proposed a synergistically crosstalk between the chemokine (C-C motif) ligand 21 (CCL21) and the vascular endothelial growth factor (VEGF)-C which enables tumor cells to escape the immune surveillance as well as to obtain an increased invasiveness and thereby promoting metastasis via the lymphatic route. The secretion of VEGF-C by tumor cells, tumor-associated macrophages and stromal cells resulted in an increased expression of the CCL21 on lymphatics, which in turn drove CCR7-dependent tumor chemoinvasion towards lymphatics. Furthermore, CCL21 was also expressed by invasive tumor cells. Several *in vivo* experiments indicated the recruitment of lymphoid tissue inducer cells to invasive $CCL21^+$ tumors and the upregulation of HEV marker expression on intratumoral vessels, lymph node stromal cell markers on surrounding fibroblasts and M2 macrophage switching. These lymphoid-like changes together with the tolerogenic cytokine profile of the tumor microenvironment led to the recruitment of naïve $CCR7^+$ T-cells and their priming to regulatory T-cells. Thus, the tumor escaped the immune response and spread via lymphatics.

Session 9: TUMOR MICROENVIRONMENT

"Exploring new strategies to target the pro-angiogenic tumor stroma"

Kristian Pietras (Stockholm) talked about bone morphogenetic protein (BMP) 9 as a new player in activin receptor-like kinase (ALK) 1 signaling. He used the RIP1-TAG2 insulinoma mouse model. He found progressive upregulated expression of TGFβ and BMP9 during tumorigenesis, which correlated with highest ALK1 expression during the angiogenic state. Furthermore, impaired ALK1 signaling retarded tumor progression and reduced tumor vascular density in RIP1-TAG2; ALK1+/- mice. He generated RAP-041, an ALK1-Fc fusion protein that neutralized specifically BMP9. RAP-041 inhibited physiological vessel formation *in vitro* and *in vivo*. RAP-041 treatment of RIP1-TAG2 mice resulted in a therapeutic benefit. Genetic as well as pharmacological targeting of ALK1 led to reduced down-stream signaling *in vivo*. To analyze the mechanistic basis of these findings he analyzed the endothelial cell behavior in proliferation and apoptosis assays *in vitro.* He demonstrated that TGFβ and BMP9 acted only in concert to enhance VEGF-A function resulting in increased proliferation rates and reduced apoptosis. The same TGFβ and BMP9 interaction was found in an *ex vivo* angiogenic islet sprouting assay and in an *in vivo* assay using Matrigel plugs. In both cases the cytokines stimulated endothelial cell sprouting. Inhibition of ALK1 signaling by phosphorylated SMAD2 affected prototypical ALK5 downstream target genes. Kristian Pietras further introduced endoglin as a co-receptor of ALK1, which was expressed by endothelial cells in the RIP1-TAG2 tumors. Endoglin deficient mice showed improved pericyte coverage of blood vessels and increased formation of liver micrometastases. In summary, Kristian Pietras concluded that the TGFβ-BMP9-ALK1-endoglin pathway is important for the angiogenic switch.

"Tenascin-C in the tumor microenvironment triggers oncogenic signaling"

Gertraud Orend (Strassburg) introduced the extracellular matrix molecule tenascin-C (TNC) that is highly expressed in most cancers and which correlates with bad survival prognosis and tamoxifen resistance. TNC played a role in promoting tumor cell proliferation, angiogenesis, invasion and metastasis but the molecular mechanisms are poorly understood. She crossed Rip-Tag mice that express the SV40 antigen under the control of the insulin promoter with TNC transgenic animals and analyzed the tumor progression in the pancreas of these mice. The animals showed enhanced lung micrometastases with insulin positive areas and increased β-catenin expression and nuclear β-catenin localization. TNC stabilized β-catenin and induced tumor vasculature with an aberrant structure. Tumors overexpressing TNC exhibited tubular structures in the matrix resembling structures described in human tumors (e.g. human insulioma, certain colon carcinoma). In addition, TNC stimulated endothelin receptor type A (EDNRA) expression which maintained cell rounding. FAK, paxilin, RhoA and Tropomyosin-1 were critical targets of TNC downstream of syndecan-4 and EDNRA. Taken together, TNC in the tumor microenvironment triggers oncogenic signaling.

"Diverse role of mesenchymal cells in cancer progression and metastasis"

Raghu Kalluri (Boston) talk presented the previously unappreciated presence of fibroblast diversity and its influence on primary tumor growth and metastasis formation. He showed an exclusive anatomical distribution patterns in fibroblast lineage cells in mice in which fluorescent reporter genes were expressed under the control of alpha-SMA, NG2 or FSP1 promoter respectively. The functional relevance of the above finding was shown using a 4T1 breast cancer model in which 90% of cancer associated fibroblast were FSP1+ and were alpha-SMA-. Additionally, he demonstrated that in contrast to bone morrow derived cells, the local fibroblast population formed the premetastatic niche. To confirm the functional relevance of the diverse fibroblast cells in tumor growth, transgenic mice expressing thymidine kinase under the control of alpha-SMA, NG2 and FSP promoter were generated and the cells were selectively ablated by ganglocyclovir administration. In the 4T1 orthotropic breast cancer model, the depletion of alpha-SMA+ cells resulted in reduced tumor growth but had no effect on metastasis. Absence of NG2+ cells caused leaky vessels, reduced primary tumor and increased metastasis due to increased levels of HIF1alpha, p38 and TWIST. Mice deprived of FSP1 cells exhibited no changes in primary tumor growth but altered metastasis. Finally, he addressed the contribution of mutations occurring in fibroblast cells in pathophysiologcal conditions. Transgenic mice harboring mutant KrasG12D gene under the control of alpha-SMA promoter predominantly died shortly after birth and surviving pups developed transformed breast epithelial cells during the first

month. Taken together, he demonstrated that the diversity of fibroblast cells plays an important role in primary tumor growth and metastasis development.

Session 10: TUMOR SIGNALING

"EphrinB reverse signaling contributes to endothelial and mural cell assembly into vascular structures"

Giovanna Tosato (Bethesda) presented her data about ephrinB and EphB receptor and their influence on vascular development through bidirectional cell-to-cell signaling. She showed that ephrinB was a critical mediator of post-natal pericyte-to-endothelial cell assembly into vascular structures. This function was dependent upon extracellular matrix-supported cell-to-cell contact, engagement of ephrinB by EphB receptors on cells and Src-dependent phosphorylation of the intracytoplasmic domain of ephrinB. Phosphorylated ephrinB marked angiogenic blood vessels in the developing and hypoxic retina, in tumor tissue and at contact points between endothelial cells and pericytes. Inhibition of ephrinB activity prevented proper assembly of pericytes and endothelial cells into vascular structures. Thus, ephrinB signaling orchestrates pericyte/endothelial cell assembly, suggesting that therapeutic targeting of ephrinB may be useful to inhibit angiogenesis when it contributes to disease.

"EphrinB2 regulates VEGFR trafficking to mediate tip cell filopodial extension during angiogenesis"

Amparo Acker-Palmer (Frankfurt) demonstrated in her talk that ephrinB2 signaling involving PDZ interactions regulated tip cell guidance to control angiogenic sprouting and branching in mouse retina and tumor angiogenesis. *In vivo*, ephrinB2 PDZ signaling deficient mice exhibited reduced number of tip cells with less filopodial extensions. In pathological settings, blockade of the ephrinB2 PDZ signaling pathway decreased tumor vascularization and led to reduced angiogenic sprouting and branching of tumor vasculature. At cellular level, time-laps microscopy revealed that ephrinB2 signaling induced the extension of filopodia by regulating the internalization of VEGFR-2 in endothelial cells. Interestingly, activation of ephrinB2 rescued the blockade of VEGFR-2 by soluble Flt1 regarding tip cell filopodia dynamics both in retinal explants and in cultured endothelial cells. In summary, Amparo Acker-Palmer showed that ephrinB2 has an essential role in the guidance and function of tip cell filopodia during development and also during sprouting angiogenesis.

"The bone morphogenic protein antagonist DRM/Gremlin as a novel pro-angiogenic factor"

Marco Presta (Brescia) introduced the protein Drm/gremlin, a member of the Dan family of bone morphogenetic protein (BMP) antagonists. Drm/Gremlin binds to BMP 2, 4, and 7 on the endothelial surface. He purificated Drm/gremlin from conditioned medium of FGF2-overexpressing endothelial cells. Recombinant Drm/gremlin stimulated endothelial cell migration and invasion on fibrin and collagen gels. Moreover, Drm/gremlin exerted a potent angiogenic activity in the chick embryo chorio-allantoic membrane and in Zebrafish embryos. Sequence alignment analysis showed sequence similarities to VEGF-A. Drm/gremlin was also able to bind to VEGFR-2 and induces dimerization *in vitro*. This interaction mediated the angiogenic activity of Drm/gremlin *in vitro* and *in vivo* by phosphorylation of intracellular proteins including Paxillin, focal adhesion kinase and ERK1/2. Drm/gremlin also induced Ang-1 upregulation in endothelial cells which was mediated by the activation of the transcription factor NF-kB. Marco Presta demonstrated that Drm/gremlin was highly overexpressed in human tumors (e.g. lung, pancreas, prostate) where it stimulated tumor growth. He concluded that Drm/gremlin has a novel, previously unrecognized capacity to modulate angiogenesis *in vitro* and *in vivo* by interacting directly with VEGFR-2 on endothelial cells.

"EGFR/Ras/ERK-signaling-dependent production of the chemokine CCL20 in tumor cells critically contributes to angiogenesis and tumor progression"

Andreas Hippe (Düsseldorf) presented his data about the EGFR/Ras/ERK-signaling regulated chemokine CCL20. Tumors enhance angiogenesis by upregulating the production of CCL20. *In vivo*, the chemokine CCL20 was overexpressed in areas of melanoma, breast cancer, colon cancer and head and neck squamous cell carcinoma with increased ERK phosphorylation. Using large scale tissue microarrays he identified increased CCL20 expression in most advanced or aggressive tumors

and the expression of CCL20 also correlated with tumor progression and lymph node metastases. Its specific corresponding receptor, CCR6, was abundantly expressed on endothelial cells *in vitro* and *in vivo*. Activation of CCR6 signaling in endothelial cells induced migration, *in vitro*-repair and led to enhanced vessel formation. *In vivo*, CCL20 specifically induced increased vascularization of Matrigel plugs in wildtype mice, which was abrogated in CCR6-deficient mice. In addition, CCL20 expressing B16F10 melanomas showed significantly decreased tumor growth and vessel density in CCR6-deficient compared to wildtype mice. Andreas Hippe propagates CCL20 as a novel chemokine-driven mechanism of tumors to promote angiogenesis and tumor progression.

Session 11: NOTCH SIGNALING

"Notch signaling – new mechanism and role in resistance to anti-VEGF therapy"

Adrian Harris (Oxford) investigated the role of Dll4 and Avastin on tumor growth. MDA231 cells transfected with Dll4 exhibited increased tumor volume and led to reduced survival of the mice. Simultaneous administration of Avastin for one and a half week significantly reduced tumor volume. He also showed that inhibition of Notch signaling sensitized tumors to anti-VEGF therapy. Avastin did not affect the vessel morphology, but instead it decreased the number of vessels. Resistance of tumors to VEGF blockade was due to poor antibody penetration. Upregulation of Dll4 in tumors resulted in resistance to Sorafenib treatment. He demonstrated that Dll4/Notch signaling affected perivascular coverage. Dll4 regulated EphB4 and blockade of ephrinB2 re-sensitized tumor response to anti-VEGF therapy. The second story Adrian Harris presented was the role of exosomes in tumor angiogenesis. He investigated in U87 glioblastoma cells transfected with Dll4 the production of exosomes. The exosomes contained beside Dll4 the exosomal markers Rab5, Tsg101 and Lamp1. Addition of Dll4 containing exosomes to HUVECs inhibited Notch signaling and enhanced branching. The mechanism was related to removal of Notch1 from the cell surface. He showed that the exosomes were incorporated into mouse tumor endothelial cells *in vivo.* This incorporation could be one of the possible mechanisms regulating blood vessels at a distance from tumors and enhancing vessel branching. Therefore, Notch signaling either produced from stromal cells, endothelial cells themselves or cancer cells have a highly significant role in tumor angiogenesis and are likely to modify the response to many different anti-angiogenic therapies.

"Decoding tumor-host interactions in dormancy: Notch3-mediated regulation of MKP-1 promotes tumor cell survival"

Stefano Indraccolo (Padova) presented data regarding the regulation of the phosphatase MKP-1 by Notch3. The Notch ligand Dll4, induced by angiogenic factors in endothelial cells, triggers Notch3 activation in neighboring T-ALL leukaemia cells and promotes tumorigenesis. He showed new data that MKP-1 levels were controlled by Notch3 by protein ubiquitination and stability and not by gene expression. Notch3 and MKP-1 levels were consistently upregulated in aggressive compared to dormant tumors. A good correlation between Notch3 ICD and MKP-1 levels was observed in T-ALL primary samples from patients and in a panel of T-ALL cell lines. Silencing Notch3 by RNA interference or by γ-secretase treatment or stimulation of Notch3 by the Dll4 ligand had marked effects on MKP-1 levels in T-ALL cells *in vitro*. Stefano Indraccolo also showed that MKP-1 was downregulated by anti-DLL4 therapy. On the one hand Notch3 inhibition lowered MKP-1 levels and on the other hand Notch3 activation increased MKP-1 levels in T-ALL cells. Attenuation of MKP-1 levels by shRNA did not affect proliferation, whereas it significantly increased T-ALL cell death and thereby controlled leukemia outgrowth. In summary, Stefano Indraccolo presented a novel mechanism downstream of Notch3 by which the direct interplay between endothelial and tumor cells promote survival of T-ALL cells.

"Two Notch ligands with opposing effects on angiogenesis"

Expression of the ligand Delta-like 4 (Dll4) in tip cells activates Notch receptors in adjacent endothelial cells and downregulates VEGF receptor expression in these cells. **Ralf Adams** (Münster) demonstrated that sprouting was also controlled by a second Notch ligand, Jagged1, which is a potent pro-angiogenic regulator with the opposite role of Dll4. Sprouting and tip cell numbers were reduced in endothelial cell-specific *Jag1* loss-of-function mice, whereas overexpression of Jagged1 produced the reverse effect. Notch target genes were upregulated in the Jagged1-deficient endothelium and blocking of Notch signaling in these mutants restored a wild-type-like response. Ralf Adams also

showed that loss of Jag1 led to downregulation of VEGFR-3. These findings and the spatial expression patterns of Dll4 and Jagged1 suggested that the balance between Notch ligands with opposing roles controlled physiological and perhaps also pathological angiogenesis. In addition, he showed new data regarding the expression of ephrinB2 and EphB4 in the retinal endothelium. Knockdown of ephrinB2 in zebrafish resulted in sprouting defects. He demonstrated that ephrinB2 controlled VEGFR-3 endocytosis and signaling. EphrinB2 is also a regulator of lymphangiogenesis. EphrinB2 expressing lymphatic vessels exhibited altered VEGFR-3 signaling. Taken together, ephrinB2 is a regulator of endothelial cell invasiveness and sprouting.

Sabine Gesierich, Heidelberg

(supported by: Ilse Hofmann, Vijayshankar Sivanandam, Sonja Breuninger, Anja Runge, Soniya Savant, Miriel Teichmann, Anja Weick, Matthias Wieland)