61 **Heterozygous deficiency of the oxygen sensor PHD2 prevents metastasis by inducing vessel normalization** Poster

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OBJECTIVES: To study the function of the PHD oxygen sensors in the tumor stroma on tumor growth, angiogenesis and metastasis through in vivo mouse models. **MATERIALS:** B16 F10 melanoma tumor cells, Panc02 pancreatic tumor cells, LLC Lewis lung carcinoma cells were grown in different media and injected subcutaneously or orthotopically in PHD1^{-/-}, PHD2^{-/-}, PHD3^{-/-} and wild type mice, congenic on a C57Bl6/J background. Mice were generated and bred in our institution. **RESULTS:** Mice lacking one allele encoding the HIF prolyl hydroxylase PHD2 (PHD2^{+/-}) but not PHD1 and PHD3 deficient mice were resistant to develop metastasis when challenged with a tumor despite a significant increase of the tumor growth. This effect was mediated by the upregulation of the soluble form of VEGFR-1, sFlt1, in endothelial cells that occurred via stabilization of HIF-2, but not HIF-1. sFlt1 acted as an endogenous trap for VEGF-A. This prevented tumor vessel activation and leakage, therefore improving the perfusion of the tumor and reducing the extent of tumor hypoxia. Although favorable for the growth of the primary tumor, this condition dramatically inhibited cancer cell spreading, intravasation and metastatization. **CONCLUSIONS:** These findings delineate a new role for PHD2 in tumor vessel normalization, and offer a novel mechanistic insight of the anti-angiogenic therapy as a strategy to prevent tumor metastasis.

62 **Tumor stem cells in gliomas - clinical impact of the stem cell marker CD133 and therapeutical strategies** Poster

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A considerable amount of evidence has been gathered supporting the existence of tumor stem cells in a variety of cancers. Glioma-derived tumor stem cells (GTSC) can be enriched by the stem cell surface antigen CD133. Conversely, a controlled, drug-induced depletion of the CD133-positive GTSC pool could have profound therapeutic implications. Retinoids like all-trans retinoic acid (ATRA) have been shown to induce differentiation of GTSCs in vitro. However, it remains unknown whether tumor growth-relevant features of these cells are affected.

We analyzed expression of CD133 in 95 gliomas of various grade and histology by immunohistochemistry. Staining data were correlated with patient outcome. Furthermore, several GTSC lines with high CD133 content were established to investigate ATRA-induced differentiation and potential effects on tumor growth-relevant properties. Proliferation was monitored by BrDU-incorporation assay and CD133 content by FACS-analysis. Impact of differentiation on angiogenic capacity of GTSCs was measured by quantification of angiogenic cytokines and assessed in a HUVEC-based tube formation assay. Potential effects on GTSC invasiveness were studied in a 3D-collagen invasion model. Finally, we studied whether in vitro effects could be confirmed in vivo using a NOD/SCID-mouse xenograft model.

By multivariate survival analysis, both the proportion of CD133-positive cells and their topological organization in clusters were significant ($P < 0.001$) prognostic factors for adverse progression-free (PFS) and overall survival (OS). Also, proportion of CD133-positive cells was an independent risk factor for tumor regrowth and time to malignant progression in WHO II and III tumors. Supporting these clinical data, we present functional evidence that GTSCs exposed to ATRA lower the expression of CD133 in favor of incremented expression of lineage markers. This is accompanied by a significantly reduced VEGF and bFGF secretion, as well as a significantly lowered angiogenic activity following differentiation. Additionally, we show that differentiation elicits strong anti-invasive effects reducing invasion of GTSCs accompanied by a downregulation of invasion-related MMP2 protein. Finally, we report that xenografted tumors of differentiated GTSCs are significantly smaller and less invasive than undifferentiated GTSC tumor xenografts. Correspondingly, animals bearing differentiated cells show both significantly better PFS and OS than mice with GTSC xenografts.

These findings constitute the first conclusive evidence that CD133 expression correlates with patient survival in gliomas, lending support to the current cancer stem cell hypothesis. Additionally, we present functional evidence that differentiation treatment targets the tumor-driving compartment in glioblastoma and constitutes a potential therapeutic approach in the eradication of GTSCs.

63 **The HIF-induced carbonic anhydrase IX and XII regulate intracellular pH promoting tumor survival in a hypoxic and acidic microenvironment** Poster

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Acidosis of the tumor microenvironment is typical of a malignant phenotype. Under hypoxic conditions increased lactic acid secretion together with carbonic acid production contribute to a high acid load in the tumor. All cells express multiple isoforms of carbonic anhydrase (CA), zinc-dependent enzymes capable of catalyzing the reversible hydration of carbon dioxide into bicarbonate and protons. The expression of membrane-bound CAs such as CAIX and CAXII is tightly controlled by oxygen levels via the Hypoxia-Inducible Factor (HIF) in many cancers. In particular tumor expression of HIF-1 and CAIX correlates with poor patient survival. What is the contribution of CAIX and CAXII to the maintenance of the intracellular pH (pHi) in an acidic environment? What advantage do tumor cells derive from their overexpression?

To answer these questions: i) we forced the expression of human CAIX or CAXII in fibroblasts that do not express these isoforms and ii) we silenced CAIX expression combined with or without CAXII silencing in two human tumor cell lines (colon adenocarcinoma LS174, melanoma A375). We demonstrate that cells expressing CAIX strongly acidify the extracellular milieu in response to a 'CO₂-load' under both hypoxic and normoxic conditions. In hypoxic tumor cells that express both CA isoforms, double silencing is necessary to abolish extracellular acidification in response to a 'CO₂-load'. Interestingly, in spite of their capacity to acidify the extracellular milieu, CAIX- or CAXII-expressing cells survive better than control cells. We showed that at low pHe (6 to 7), these cells are able to maintain a resting pHi of 0.2 to 0.3 pH units more alkaline than control cells. Consequently tumor cells expressing high levels of CAIX would survive in an acidic microenvironment much better than normal cells. Preliminary in vivo experiments indicate that CAIX silencing alone leads to a 38% to 45% reduction in the tumor volume. Co-invalidation of both isoforms is in progress. Thus, CAIX and CAXII are major pHi-regulating systems in a tumor hypoxic microenvironment and as pointed out by others, they represent potential targets for anticancer drug development.

64 **Hypoxia-induced autophagy is mediated through the HIF-induction of BNIP3 and BNIP3L via their BH3-domains** Poster

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While HIF is a major actor in the cell survival response to hypoxia, HIF is also associated with cell death. Several studies have pointed the implication of HIF-induced putative BH3 only pro-apoptotic genes bnip3 and bnip3L in hypoxia-mediated cell death. We, like others, do not support this assertion. Here we demonstrate that the rapid induction of BNIP3 and BNIP3L in a hypoxic microenvironment contributes to survival rather than cell death by inducing autophagy. First, whereas siRNA mediated ablation of either BNIP3 or BNIP3L had little effect, combined silencing of the two HIF targets suppressed hypoxia-mediated autophagy. Second, ectopic expression of both BNIP3 and BNIP3L in normoxia activates autophagy. Third, 20-mer BH3-peptides of BNIP3 or BNIP3L, modified with a TAT-like membrane transducing sequence were found to be sufficient to activate the autophagy process in normoxia. We propose a model in which the atypical BH3-domains of hypoxia-induced BNIP3/BNIP3L have been 'designed' for inducing autophagy. They disrupt the Beclin1-Bcl2 complex without inducing cell death.

The identification of BNIP3 and BNIP3L as central mediators of autophagy definitively provides new aspects on their functions as pro-survival proteins in opposition to pro-cell death proteins. These results give us good reasons to think that manipulation of HIF-induced autophagy via BNIP3 and BNIP3L may be a good therapeutic option to investigate in cancer treatment.

65 **HIF-1 links bacterial infection, inflammation and cancer** Poster

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Background: Infection of human intestinal cells with Afa/Dr DAEC C1845 pro inflammatory bacteria induces expression of the VEGF gene, encoding Vascular Endothelial Growth Factor.