536 Modeling the human breast stem cell and the breast cancer niche

S. Duss[1], H. Brinkhaus[1], M. Bentires-Alj[1]. [1]*Friedrich Miescher Institute, Growth Control, Basel, Switzerland*

Stem cell/niche interactions are essential for tissue homeostasis by controlling stem cell quiescence and stem cell activation. Recent evidence implies that aberrant stem cell/niche interactions are involved in tumourigenesis and tumour progression. Experiments that functionally describe the cellular compartments responsible for self renewal and quiescence of breast stem- and progenitor cells have not been reported. The nature and role of the breast cancer niche in tumourigenesis, tumour maintenance and metastasis remain speculative.

We established methods that allow for the isolation and propagation of different cellular compartments from normal primary breast and tumour tissue. Co-culture of epithelial and mesenchymal precursors from the human breast enables the assembly of structures displaying functional properties of human breast stem cell niches. We successfully defined novel *ex vivo* three dimensional cell culture conditions that allow for long term maintenance of differentiation and proliferation potential of mammary epithelial stem/progenitor cells.

We are currently validating our culture system *in vivo.* We are also performing gain and loss of function experiments with mammary oncogenes and tumour suppressor genes to assess how oncogenic transformation alters stem cell/niche interactions.

Our experiments should identify molecular pathways involved in aberrant stem cell/niche interactions and breast tumourigenesis and ultimately provide novel therapeutic targets.

537 The role of microRNA molecules in the regulation of aggressive features in melanoma

E. Greenberg[1], O. Itzhaki[2], L. Hershkovitz[2], Y. Nemlich[2], R. Ortenberg[2], M.J. Besser[2], Y. Keisari[1], J. Schachter[2], N. Shomron[3], G. Markel[2]. [1]*Tel Aviv University, Clinical Microbiology and Immunology Sackler Faculty of Medicine, Tel Aviv, Israel,* [2]*Sheba Cancer Research Center, Ella Institute of Melanoma, Ramat Gan, Israel,* [3]*Tel Aviv University, Cell and Developmental Biology Sackler Faculty of Medicine, Tel Aviv, Israel*

Melanoma is a high-grade, poorly differentiated malignant tumour of pigment-producing cells (melanocytes), accounting for more than 70% of the skin cancer related deaths. Unfortunately, the currently available treatments are still mostly ineffective. MicroRNAs (miRs) are short non-coding RNA molecules that function as specific epigenetic regulators of the transcriptome. MiRs regulate various cancer-related functions with clinical importance and significance. Our main objective is to identify a miR pattern involved in the regulation of aggressive features in melanoma and delineate the underlying molecular mechanisms.

We employed qPCR-based miR microarray for analysis of two sublines of a single melanoma cell line, which differ in tumourigenicity and related characteristics. Downregulation of 66 mature miRs was identified in the highly aggressive (HAG) cells. Candidate miRs were analyzed informatically using computational algorithm databases that enabled miR prioritization according to predicted target genes (aggressive phenotype). Selected miRs were cloned into pQCXIP mammalian expression vector and introduced into HAG cells. The transfectants were analyzed functionally in proliferation, invasion and vasculogenic mimicry (VM) formation assays.

Here we describe the roles of two exemplar miRs in melanoma, miR-184 and miR-34a. Over expression of miR-184, which underwent shutdown in the HAG subline, caused a 33% decrement in net cell proliferation and 22% decrement in invasion ability, as compared to mock transfecion. Moreover, overexpression of miR-184 impaired the ability of HAG cells to form VM structures. Over expression of miR-34a, which underwent 11-fold downregulation in the HAG subline, caused a 46% decrement in net cell proliferation as compared to mock transfecion and severely impaired the ability of HAG cells to form VM structures. MiR-34a transfected HAG cells displayed substantially reduced tumourigenicity when injected S.C. to SCID mice. Indeed, 60% of the mice did not develop any tumour, while the rest of the mice developed 10-fold smaller tumour masses.

MiRs provide a strong platform for delineation of cancer mechanisms. Studying miR-mediated regulation of aggressive and tumour related features is expected to provide novel mechanistic insights that may pave the way for new diagnostic tools as well as new molecular targets for future therapy.

538 Autocrine vascular endothelial growth factor signaling has no influence on the apoptosis induction by cisplatin in non-small cell lung cancer cell lines

C. Wohlkoenig[1], K. Leithner[1], A. Hrzenjak[1], A. Olschewski[2], H. Olschewski[1]. [1]*Medical University of Graz, Department of Internal Medicine Division of Pulmonology, Graz, Austria,* [2]*Medical University of Graz, Experimental Anesthesiology University Clinic for Anesthesiology and Intensive Care Medicine, Graz, Austria*

Background: Therapeutic agents blocking cellular pathways are a current promise. Vascular endothelial growth factor (VEGF) is a major protein upregulated in hypoxia and known to promote angiogenesis and tumour cell survival. VEGF blocking antibodies (bevacizumab) are approved for use in non-small cell lung cancer (NSCLC) patients. Recently, it has been postulated that blocking the VEGF pathway could be more proapoptotic than antiangiogenic, by acting on the auto- or paracrine loop of VEGF. The aim of this study was to analyse, whether such a loop exists in NSCLC cell lines and wether its blocking has an effect on chemotherapy and the apoptotic cell death in normoxia and hypoxia.

Material and Methods: A549 and H358 cells were preincubated for 3 days at 1% O2 or ambient O2 in serum starved medium (0.5% FCS) supplied with predefined concentrations of bevacizumab. After preincubation fresh bevacizumab was added and the cells were treated with cisplatin [32 μM] for another 3 days. Apoptosis rate was analysed by measuring active caspase-3 with flow cytometry (PhiPhiLux®). Regulation of VEGF under hypoxia was assessed via qRT-PCR. Expression of VEGF-Receptor 1 and 2 was investigated with qRT-PCR, western blot and immunocytochemistry.

Results: VEGF was expressed in both cell lines but only in hypoxic A549 cells it was significantly upregulated by a factor of 2 ($p = 0.037$) compared to normoxic cells. VEGF-Receptor 1 was found on mRNA- and protein level in both cell lines. In contrast VEGF-Receptor 2 was found only in H358 cells. Cisplatin led to 44.9% (±5.6%) apoptosis rate in A549 cells and 27.9% (±2.4%) in H358 cells in normoxia. In hypoxia, cisplatin induced an apoptosis rate of 7.6% (±3.1%) in A549 cells and 13.56% (±8.8%) in H358 cells. Co-incubating the cells with up to 250 μg/ml bevacizumab led to an apoptosis rate of 48.0% (±4.1%; $p = 0.48$ to cisplatin alone) in normoxic and 8.8% (±3.3%; $p = 0.67$) in hypoxic A549 cells and 24.69% (±2.6%; $p = 0.34$) in normoxic and 12.51% (±9.9%; $p = 0.92$) in hypoxic H358 cells, respectively.

Incubating the cells with up to 250 μg/ml bevacizumab without cisplatin induced no apoptosis in normoxia and hypoxia (data not shown).

Summary and Conclusion: Although the components for an auto- or paracrine loop of VEGF are present in A549 and H358 cells the apoptosis rate of the cells could not be influenced by blocking soluble VEGF with bevacizumab. A combination of cisplatin and bevacizumab had no beneficial effect on apoptosis induction, neither in normoxia nor in hypoxia. Either the auto- or paracrine VEGF loop is not functional in A549 and H358 cells or its blocking has no proapoptotic effect on these cell lines.

539 Elevated IGF-1R signaling induces anti-estrogen resistance and provokes a switch from antagonistic to agonistic effect of tamoxifen in breast cancer cells

J.H.N. Meerman[1], Y. Zhang[2], M. Moerkens[2], B. van de Water[2]. [1]*Leiden/Amsterdam Center for Drug Research, Pharmacogenomics, Leiden, The Netherlands,* [2]*Leiden/Amsterdam Center for Drug Research, Toxicology, Leiden, The Netherlands*

Background: Acquired resistance to anti-estrogens is a consequent setback leading to relapse and poor prognosis of estrogen receptor (ER) positive breast cancers. Emerging data indicate the relevance of altered expression and modification of growth factor receptors and downstream signaling to the anti-estrogen resistance. Here, we have investigated the role of elevated insulin-like growth factor receptor type 1 (IGF1R) in tamoxifen resistance.

Material and Methods: We used human breast cancer MCF7 cells (MCF7/IGF1R) overexpressing human IGF1R by retroviral transduction generated in our laboratory. The cells were exposed to estradiol and various antiestrogens in 96 well plates. Proliferation was measured colometrically with the sulphrhodamine B assay.

Results: Overexpression and phosphorylation of IGF-1R in ER positive human MCF7 breast cancer cells induces acquired resistance to the anti-estrogens tamoxifen and fulvestrant (ICI 182780).

Particularly, increased IGF-1R signaling can convert tamoxifen, but not fulvestrant, from an antagonist into agonist, thereby further enhancing proliferation of MCF7/IGF1R cells. Auto-phosphorylation of tyrosine kinase domains in IGF-1R was fully activated upon exposure to IGF-1, concurrently initiating downstream ERK signaling significantly stronger in MCF/IGF1R cells than that in parental MCF7 cells. By use of IGF-1R inhibitor BMS-536924, MEK/ERK inhibitor U0126 and PI3K inhibitor BEZ235 respectively, it was shown that IGF-1R signaling involves both downstream ERK and PI3K/Akt pathways activation.

Tamoxifen resistance and tamoxifen agonistic effect were induced mainly via ERK pathway activation, while the PI3K pathway is linked to cell proliferation.
Conclusions: Our data provided the first evidence for the causal role of IGF-1R signaling in acquired anti-estrogen resistance and agonistic action of tamoxifen in human breast cancer cells.

540 Post-translational mechanisms involved in downregulation of the gap junction protein Connexin43 in colorectal cancer

E. Leithe[1], S. Sirnes[1], J. Bruun[1], A. Kjenseth[1], G.E. Lind[1], M. Kolberg[1], H. Stenmark[2], A. Brech[2], R.A. Lothe[1], E. Rivedal[1]. *[1]Institute for Cancer Research Radiumhospitalet Oslo University Hospital, Department of Cancer Prevention, Oslo, Norway, [2]Institute for Cancer Research Radiumhospitalet Oslo University Hospital, Department of Biochemistry, Oslo, Norway*

Background: The purpose of this study was to elucidate the molecular mechanisms involved in down-regulation of the gap junction protein Connexin43 in colorectal carcinogenesis.
Materials and Methods: The expression level, localization and phosphorylation status of Connexin43 was analyzed in normal colon tissue and colorectal tumours, as well as in colorectal cancer cell lines. As a model system for studying the post-translational mechanisms involved in regulation of Connexin43, the IAR20 rat liver cell line was used. These cells express Cx43 endogenously and form functional gap junctions. The cells were exposed to the tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which induces endocytosis and degradation of Connexin43. Candidate proteins involved in Connexin43 endocytosis and degradation were depleted by small interfering RNA (siRNA). Connexin43 localization was analyzed by confocal microscopy. The Connexin43 protein level and ubiquitination status were analyzed by western blotting and immunoprecipitation.
Results: Connexin43 was expressed in the plasma membrane in normal colon tissue, while in colorectal tumours Connexin43 was found to localize in intracellular compartments. Among 19 colorectal cell lines examined, 7 were found to express Connexin43 protein. None of the cell lines were able to form functional gap junctions, and Connexin43 was localized intracellularly, indicative of enhanced gap junction endocytosis and degradation. To elucidate the molecular mechanism involved in aberrant trafficking of Connexin43, IAR20 cells were used as a model system. Connexin43 organized in gap junction plaques was found to undergo ubiquitination in response to TPA treatment. Depletion of the ubiquitin-binding proteins Hrs or Tsg101 by siRNA counteracted trafficking of Connexin43 from early endosomes to lysosomes. Under these conditions, Connexin43 was able to undergo dephosphorylation and deubiquitination, locate to the plasma membrane, and form functional gap junctions.
Conclusions: Colorectal cancer cells are unable to form functional gap junctions, and express Connexin43 in intracellular compartments, indicative of aberrant endocytic trafficking. Using the IAR20 cell line as a model system, the TPA-induced endocytosis and degradation of Connexin43 organized in gap junctions was found to involve ubiquitination and to be mediated by the ubiquitin-binding proteins Hrs and Tsg101.

541 Huntingtin interacting protein 1 (HIP1) induces Epithelial-to-Mesenchymal Transition (EMT) in prostate cancer cells

R. Bantval Rao[1]. *[1]Cancer Research UK Cambridge Research Institute, Oncology, Cambridge, United Kingdom*

Background: HIP1, an adaptor protein classically involved in clathrin mediated endocytosis affecting cell signalling, is overexpressed in prostate cancer and drives fibroblast and prostate epithelial cell transformation by perturbing growth factor receptor signalling. It has also been shown to translocate to the nucleus and have a role as an androgen receptor coactivator.
Methods: To explore its role in prostate cancer we used HIP1 overexpressing ($HIP1^+$) prostate cell lines in soft agar colony formation, invasion, migration, and adhesion assays, gene expression arrays, and real-time PCR.
Results: $HIP1^+$ epithelial PNT1a cell lines showed cell transformation. $HIP1^+$ PNT1a and $HIP1^+$ LNCaP showed significantly increased anchorage independent cell growth. $HIP1^+$ LNCaP also showed significantly increased cell adhesion to ECM protein fibronectin, implicated in cancer growth/survival and drug resistance. Epithelial-to-mesenchymal transition (EMT) is associated with increased propensity for cell migration, invasion, and metastasis. Here, we show a >2 fold upregulation of Wnt7b, Snail, and vimentin in $HIP1^+$ cell lines, which have been implicated in EMT. Gene expression arrays showed enrichment of pathways involved in cell-cell signalling, cell movement and metabolic pathways in these $HIP1^+$ cell lines. Furthermore, $HIP1^+$ PNT1a cell lines showed a resistance to paclitaxel treatment in soft agar colony formation assays.
Conclusion: HIP1 may contribute to prostate cancer progression by altering cell-cell interaction, migration, and invasion through the induction of an EMT-like phenotype.
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542 Tumour stem cells in oncogenic RAS-dependent rhabdomyosarcoma

G. Schaaf[1], F. Sage[1], A. Chan[1], F. Chen[2], M. Hamdi[3], D. Zwijnenburg[3], S. Boutet[2], M. Kool[3], T. Rando[2]. *[1]Erasmus M.C., Stem Cell Institute for Regenerative Medicine, Rotterdam, The Netherlands, [2]Stanford School of Medicine, Neurology and Neurological Services, Palo Alto, USA, [3]Academic Medical Center, Human Genetics, Amsterdam, The Netherlands*

Tumour heterogeneity reflects the hierarchical organization of normal tissues. Many tumours have now been shown to retain cells with stem cell activity, also referred to as tumour stem cells (TSC). Our aim was to indentify TSC in rhabdomyosarcoma (RMS), childhood tumours displaying skeletal muscle differentiation. Recently, we found activating RAS mutations in nearly half of embryonal RMS cases, indicating that RAS mutations appear early and are relevant for RMS development. We showed that the putative tumour suppressor sprouty1 becomes essential for the maintenance and survival of RMS harboring oncogenic RAS. Following shRNA-mediated inactivation of sprouty1 RMS cells became apoptotic *in vitro*, while fully established RMS-grafts completely regressed upon sprouty1 silencing. Although these data show novel therapeutic promise for RMS, the clinical value of this finding depends largely on the efficacy against different tumour subpopulations.
RMS has been suggested to originate from muscle satellite cells. Therefore, we decided to express oncogenic RAS in normal muscle progenitors and determine if cells with stem cell activity were maintained after transformation. This approach resulted in the generation of transformed myoblasts that upon orthotopic transplantation generate tumours resembling embryonal RMS. Normal and transformed mouse myoblasts were first screened for the expression of (tumour) stem cell markers (including c-kit, CD24, CD44 and CD133) as well as for satellite cell markers (b-integrin and CXCR4). By using a combination of these markers, designated as SIG, we could discriminate between stem cell populations and their differentiated progeny in both normal and malignant muscle.
In normal myoblast cultures the cells with myogenic activity were contained in the SIG-positive population. SIG-positive-sorted myoblasts restored the original culture after replating. And again cells with myogenic activity were predominantly found within the SIG-positive population of these 'restored' cultures, suggesting that these cells selfrenew. In RAS-transformed myoblasts only the SIG-positive population generated colonies in soft agar, generated heterogeneous tumours and self-renewed *in vivo* as demonstrated by serial passaging in NOD/SCID mice. We conclude that RAS-driven RMS contains a population of highly malignant selfrenewing cells with an immunophenotype resembling stem cells in normal muscle. The identification of TSC is essential for development of stem cell-targeted therapies for the treatment of RMS.

543 RNA Polymerase III transcription deregulation in cancer: study of Brf1 expression in prostate cancer

N.A. Nam[1], I. Ahmad[2], J. Edwards[3], H.Y. Leung[2], R.J. White[1]. *[1]Beatson Institute for Cancer Research, Gene Transcription Lab, Glasgow Scotland, United Kingdom, [2]Beatson Institute for Cancer Research, Urology Research Lab, Glasgow Scotland, United Kingdom, [3]Glasgow Royal Infirmary, Department of Surgery, Glasgow Scotland, United Kingdom*

Background: Aberrant RNA Polymerase III (Pol III) transcription has been linked to increased cellular proliferation and progression to cancer. The importance of elevated Pol III transcripts and its associated transcription factor Brf1 in cancer has been demonstrated in cell culture and in mice. However, the significance of this deregulation has yet to be determined in patients. This study examines Brf1 expression in prostate cancer (PCa) tissues in comparison to benign prostate hyperplasia (BPH) to determine if elevated levels of Brf1 protein are detected in tumour samples and if there is any correlation with severity of disease.
Materials and Methods: Immunohistochemical staining was performed on Tissue Micro Arrays (TMAs) containing 149 cases of PCa and 21 BPH samples and the Brf1 expression was evaluated using the weighted Histoscore method. The proliferation status of these samples was assessed using Ki-67 staining.
Results: Brf1 expression was detected as heterogenous staining and localised predominantly in the nucleus. Brf1 expression is elevated in PCa compared to BPH ($p < 0.001$), however there is no association between Brf1 expression with increasing Gleason Grade ($p = 0.545$) and no significant correlation with Ki-67 expression was observed.
Conclusions: Assessment of Brf1 levels in prostate cancer tissues revealed higher expression compared to benign hyperplasia and suggests that it may be a potential biomarker for cancer therapy. However a larger patient cohort that includes both hormone sensitive and hormone resistant prostate cancer patients will be of further value to determine Brf1 significance in the clinical settings.