Immunoprecipitation and western analysis were conducted in order to detect the formation of each growth factor-integrin complex. Confocal microscopy was used in order to perform immunofluorescence, detecting the above mentioned complex formations.
Results: The application of both agents either alone or in combination showed significant reduction in proliferation and chemotactism in both cell lines. There was also an induction of apoptosis in both cell lines. MMP levels were down regulated in M059K cells while there was no change of MMP levels in U87 cells. Lapatinib intercepted the formation of EGFR-integrinb1 complex, in both cell lines while sunitinib intercepted VEGFR-integrinb3 complex formation in U87 cells. Immunofluorescence revealed colocalisation of molecules in the above mentioned complexes and their disengagement after application of agents in a time course manner.
Conclusions: Lapatinib and Sunitinib have a strong inhibitory effect. Combinational dosing of these agents has a better and stronger effect in the above mentioned parameters than each one of them on its own. The current data showed an implication of the tested agents in the integrin – growth faxtor's pathway.

511 Caveolin-1, TGFβ/Smad2 and Alpha5 Beta1 integrins connection in human glioblastoma

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Background: Caveolin-1 (cav1) plays a crucial role in cancer development and progression. Although caveolin-1 expression is increased in glioma, cav1 negative ($cav1_{low}$) and positive ($cav1_{high}$) cells coexist in glioblastoma (GBM). We reported that $cav1_{low}$ GBM cells exerted a more aggressive phenotype than $cav1_{high}$ GBM cells, suggesting that cav1 is a tumour suppressor in brain tumours. Transcriptomic analysis showed that cav1 represses integrins especially $\alpha_5\beta_1$ integrin so that cav1 and $\alpha_5\beta_1$ integrin expressions were inversely correlated. We identified $\alpha_5\beta_1$ integrin as the mediator of cav1's effect in GBM. This study focused on the mechanisms by which cav1 regulate $\alpha_5\beta_1$ integrin expression.
Material and Methods: 6 different GBM cell lines were used. Some were silenced (using $si/shRNA_{cav1}$) or forced to express ($pEGFP_{cav1}$) cav1. TGFβ was quantified using a commercially available kit. Protein expression and activity was determined by western blot. Drugs used were SB431542, LY294002 and U0126 (inhibiting the TGFβ receptor, Pi3K and MEK1 respectively), K34c (a $\alpha_5\beta_1$ integrin antagonist), TGFβ and activin. Surviving fraction after drug treatment was determined by clonogenic assays. Gene expression was studied by qPCR.
Results: Cav1 affects the TGFβ/Smad2 pathway, previously identified as a regulator of integrin expression. Silencing cav1 increased the secretion of TGFβ, the expression of TGFβ receptor and the activity of its downstream effector Smad2. Conversely, forced expression of cav1 repressed the TGFβ/Smad2 pathway so that cav1 expression and TGFβ/Smad2 activity are inversely correlated. Using selective inhibitors, we showed that the TGFβ/Smad2 pathway was involved in the regulation of $\alpha_5\beta_1$ integrin expression by cav1. Two Smad2-dependent signaling pathways were involved; one independent on the TGFβRI (cav1 $\rightarrow$ ERK $\rightarrow$ Smad2 $\rightarrow$ $\alpha_5\beta_1$ integrin) and one dependent on the TGFβRI (cav1 $\rightarrow$ TGFβRI $\rightarrow$ Pi3K/Akt $\rightarrow$ Smad2 $\rightarrow$ $\alpha_5\beta_1$ integrin). Therefore, $cav1_{low}$ cells exert high level of TGFβRI/Smad and $\alpha_5\beta_1$ integrin and vice and versa. The reverse correlation between cav1 and $\alpha_5\beta_1$/TGFβ/Smad2 was confirmed in different GBM cell lines. Finally, we showed that $cav1_{low}/\alpha_5\beta_1$/TGFβ/$Smad_{high}$ cells (identified as being the most aggressive) are highly sensitive to SB431542 and K34c.
Conclusions: Cav1 controls $\alpha_5\beta_1$ integrin expression through the TGFβ/Smad2 pathway. The status of cav1/$\alpha_5\beta_1$/TGFβ/Smad2 might be a useful marker of the tumour behavior and a predictor of anti-TGFβ or anti-$\alpha_5\beta_1$ integrin therapies.

512 Tetraoxanes induced ROS production and activation of caspase 3 in HeLa cells

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Background: It was demonstrated that mixed steroidal tetraoxanes inhibit cancer cell proliferation at micromolar level through an apoptotic mechanism. It will be interesting to see if these compounds may possibly induce oxidative stress, which could lead to induction of apoptosis in tumour cells. As tumour cells contain more iron than other normal tissues it is reasonable to suggest that tetraoxanes could react with iron, generating alkoxy radicals or even highly reactive hydroxyl radicals in a Fenton-like reaction. To gain further insight into the mechanism of cell death induced by tetraoxane endoperoxides, we tested production of reactive oxygen species (ROS) and level of activation of caspase 3 in tumour cells treated with several newly synthesized tetraoxanes.
Material and Methods: Stock solutions of investigated tetraoxanes, were prepared in DMSO at concentrations of 10 mM and afterwards they were diluted with complete nutrient medium to various final concentrations. Target cells used were malignant cervix carcinoma HeLa cells.
Production of intracellular ROS was measured using the fluorescent dye 2′,7′-dichlorofluorescein diacetate, a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to 2′,7′-dichlorofluorescein (H_2DCFDA), a nonfluorescent polar compound. In the presence of an oxidizing compound, H_2DCFDA is converted into highly fluorescent 2′,7′-dichlorofluorescein.
Level of active caspase 3 is measured using the caspase 3 fluorimetric assay kit (Sigma Chemicals), based on the hydrolysis of the peptide substrate by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin moiety.
Results: After treatment with investigated tetraoxanes ROS level in HeLa cells significantly increased indicating possible oxidative stress, maybe as a result of the production of reactive alkoxy or hydroxyl radicals. Significant increase in activity of caspase 3 was observeded after incubation of HeLa cells with all investigated tetraoxanes.
Conclusion: Taken together, these results demonstrate that tetraoxanes potently generates ROS, and strongly inhibits the growth of HeLa cells throughout apoptosis. Although the mechanisms by which mixed tetraoxanes activates caspase 3 in HeLa cells remains unclear, those results provide correlation between ROS production, caspase 3 activity and tetraoxanes-induced apoptosis.

513 DNA copy number changes in radiation-induced mammary carcinoma of (SD x COP) F1 hybrid rats

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Background: Epidemiological studies indicate that breast is one of the most susceptible organs to radiation-induced carcinogenesis. Most studies, however, have failed in identifying clear genetic alterations in radiation-induced breast/mammary cancers. The Copenhagen (COP) rats are completely resistant, whereas Sprague-Dawley (SD) rats are highly susceptible, to chemically-induced mammary carcinogenesis, and this resistance of the COP background is regarded as a dominant trait.
Material and Methods: Mammary cancer-prone SD, -resistant COP, and their hybrid (SD $\times$ COP) F1 rats were irradiated with gamma-rays at 4 Gy and underwent autopsy at the time of spontaneous death or at 1.5 years post-irradiation. Genomic DNA was extracted from mammary cancers and ear skins of corresponding individuals. Genome-wide DNA copy number was analyzed by array comparative genomic hybridization (aCGH).
Results: COP rats were resistant to radiation-induced mammary carcinogenesis. Interestingly, F1 rats showed a relatively susceptible trait, suggesting recessive inheritance of the resistance to radiation-induced mammary carcinogenesis. The preliminary results of aCGH analysis indicated that partial deletions of the proximal region of chromosome 2, where Mcs-1 (mammary cancer susceptibility gene-1 for chemical carcinogenesis) is mapped, were occasionally observed, suggesting a tumour suppressive role of Mcs-1 in radiation carcinogenesis. Other aberrations including small deletions and aneuploidy were also frequent but scattered throughout the genome.
Conclusions: These findings implicate that radiation-induced rat mammary cancers are rather heterogeneous with regard to copy number changes. The frequent deletion of COP alleles suggests that these tumour suppressive alleles may be readily targeted by radiation but not carcinogenic chemicals.

514 Co-expression of E- and P-cadherin in breast cancer: role as an invasion suppressor or as an invasion promoter?

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Background: Cadherins are cell-cell adhesion molecules. During tumour progression, their expression and/or function are frequently altered. E-cadherin down-regulation is often associated with tumour initiation and progression in breast cancer, whereas P-cadherin overexpression is associated with a worse patient survival, as well as with invasive breast cancer cells. In this study, we aimed to understand if P-cadherin overexpression could interfere with E-cadherin invasion suppressor role in breast cancer.
Materials and Methods: E- and P-cadherin expression was evaluated in a series of invasive breast carcinomas. The results were correlated with prognosis and clinico-pathological parameters. To study the functional value of E- and P-cadherin co-expression, we silenced the transcription of both cadherins in BT-20 breast cancer cells, and investigated the *in vitro* effects

on cell motility, invasion and apoptosis. Gene expression profiles were studied by cDNA microarrays.
Results: We confirmed that P-cadherin overexpressing tumours often do not loose E-cadherin. Interestingly, tumours co-expressing both cadherins showed a more aggressive behavior and were related with the worst patient survival. *In vitro*, we showed that cadherins co-expressing breast cancer cells demonstrated increased cell invasion and migration capacities, when compared with the ones expressing only one cadherin. In addition, P-cadherin silencing led to increased levels of apoptosis. Microarrays of breast cancer cells, after E- and/or P-cadherin silencing, generated a large amount of data, which is now being analyzed and validated. However, it was already possible to conclude that both these molecules are important in the regulation of different signaling pathways. As an example, the apoptotic pathway was enriched in cells with P-cadherin silencing, confirming the *in vitro* results obtained.
Conclusions: E- and P-cadherin co-expression has an invasion promoter role in breast cancer cells and is a poor patient prognostic biomarker. In addition, P-cadherin overexpression constitutes a cancer cell survival signal. It was still proven that the role of each cadherin alone is completely distinct from when these are co-expressed in the same cell, conferring different transcriptional programs.

515 Number of stem-like cells and the genetic susceptibility to mammary carcinogenesis in rats

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Background: The rat mammary tumour model has been used for the study on the biology of human breast cancer. Genetic predisposition for mammary carcinogenesis plays a significant role in the rats. Female Sprague-Dawley and Wistar-Furth (WF) rats are sensitive to DMBA- and MNU-induced mammary carcinogenesis, but Copenhagen (COP) rats are completely resistant. F1 hybrids (WF x COP) show resistance, suggesting a dominant tumour suppressive trait of the COP background. The underlying genetic components are complex and not completely understood.
Stem cells and their immediate progeny are considered as the targets for malignant transformation. To elucidate the cellular basis for resistance to mammary carcinogenesis in COP rats, we performed transplantation assays to examine the number of stem-like cells, previously referred to as clonogens, and their response to cancer promoting condition (glucocorticoid deficiency and high prolactin) in comparison with susceptible WF rats.
Materials and Methods: Young-adult female WF and COP rats and their F1 hybrids (WF x COP) were used. The number of stem-like cells was determined by a transplantation assay. Two types of donor rats were used: untreated rats and adrenalectomized, pituitary-transplanted rats. Serially diluted monodispersed mammary epithelial cells from donor rats were transplanted into the interscapular fat pad of recipient F1 rats grafted with mammotrophic pituitary tumour cells. Three weeks after transplantation, the fat pads were removed and examined for the presence of alveolar units (AUs) and branching ductal units (DUs) developed at graft sites. The total number of AU- or DU-forming cells per mammary gland was calculated.
Results: The total number of AU-forming cells per normal female mammary gland was much smaller in COP than WF, being coincided with tumour susceptibility. However, the number of AU-forming cells of F1 rats was comparable to those of WF, which failed to account for the differential tumour susceptibility between WF and F1. On the other hand, the number of DU-forming cells of F1 was one third of those of WF, in good agreement with their tumour susceptibility. More importantly, DU-forming cells in COP and F1 were not stimulated to expand by glucocorticoid deficiency and high level of prolactin, in contrast with the marked response by the WF cells.
Conclusion: The DU-forming cells may be the targets for chemically induced mammary carcinomas.

516 Isolation and functional characterization of tumour-initiating cells using a let-7c micro RNA cellular reporter system

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A growing body of evidence suggests that a few stem cell-like cancer cells, also termed tumour-initiating cells (TICs) or cancer stem cells (CSCs), have the ability to self-renew and continuously produce differentiated cancer cells that make up the bulk of a tumour. Expression of multi-drug resistance (MDR) proteins that pump out toxic substances, enhanced activity of enzymes that confer resistance to toxic agents, increased levels of telomerase that prevent telomere-shortening, as well as the quiescent state of TICs may cause resistance to traditional therapy leading to relapses or metastasis. Importantly, such particular properties of TICs can also be exploited to isolate and characterize them to ultimately uncover potential targeting strategies.
Let-7 micro RNA (miRNA) family members are either not expressed or expressed at low levels in TICs, whilst higher expressed in differentiated normal and cancer cells. We take advantage of this feature by using a reporter system consisting of an expression plasmid in which a fluorescent reporter protein (DsRed) is placed under the control of a CMV promoter and a 3′ untranslated region (3′UTR) harboring a perfect complementary let-7c binding site [1]. Hence, the level of DsRed expression is regulated by the endogenous levels of let-7c, allowing us to isolate strongly fluorescent TICs (which have low levels of endogenous let-7) from cancer cell lines, using flow cytometry.
We are currently using the let-7c miRNA reporter system to isolate stem-like cell populations from different human breast cancer cell lines. Colony assays, stemness and differentiation surface marker expression analyses, cell cycle analyses, and examination of stem-like gene levels (e.g. *Nanog, Oct-4, Sox-2*) at mRNA and protein level will reveal whether our approach selects for TICs. Furthermore, we are evaluating the expression of let-7 targets such as the cell cycle regulators CDC25A and CDK6, growth promoters including RAS and c-Myc, as well as known regulators of stemness and differentiation during embryogenesis including HMGA2 and Lin28(B). Lastly, the DsRed reporter assay approach will be employed in mouse models to assess tumour-initiating potential *in vivo.*

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517 3D culture of oesophageal cancer cells in extracellular matrix: morphology correlates with invasiveness

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Background: Oesophageal cancer is known for its intrinsic resistance to current systemic therapies. Thus far, no comprehensive in vitro or animal models exist for this cancer. Therefore, we investigated systematically a panel of nine oesophageal cancer cell lines in a 3D culture system to establish a more comprehensive model system to study this cancer in vitro.
Material and Methods: We used the on-top Matrigel®-assay to analyse five oesophageal squamous cell carcinoma cell lines (KYSE-30, KYSE-270, KYSE-410, KYSE-520 and COLO-680N) and four adenocarcinoma cell lines (OE19, OE33, LN1590 and PT1590) for their behaviour in contact with extracellular matrix (ECM) in a 3D culture system. The phenotype was compared with conventional 2D culture. The invasiveness was assessed with Matrigel®-coated Transwell System (Boyden Chamber) whereas the Fence-Assay was applied to evaluate the migration of the oesophageal cell lines. Expression of genes related to proliferation and cell adhesion were investigated via quantitative RT-PCR.
Results: Upon the on-top Matrigel®-assay KYSE-30, OE33, LN1590 showed a round mass growth pattern, KYSE cell lines -270, -410, -520 and COLO-680N exhibited tumour mass like pattern, OE19 a grape-like growth pattern and PT1590 grew in stellate spheroids. Interestingly, the distinct growth pattern of the oesophageal cancer cell lines correlated significantly with the invasive capacity as analysed with the Matrigel®-Invasion Chambers ($p = 0.048$). In contrast, the migratory capacity analysed with the Fence-Assay did not correlate with the phenotype observed in the 3D culture. We also noted an impact of the ECM-culture condition on the expression profile of some of the analysed genes.
Conclusion: Compared to monolayer cell culture grown on plastic, the on-top Matrigel 3D culture provides a more realistic environment and led to a distinct growth pattern of the investigated cell lines. Their observed behaviour upon 3D culture covered a comprehensive range from low- to high-aggressive phenotypes. Thus, these nine cell lines cultured in the on-top Matrigel®-assay seem to provide a suitable model to perform therapeutic in vitro studies in oesophageal cancer.

518 The relevance of the therapeutic target EpCAM (CD326) for the progression of esophageal carcinoma

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Background: Oesophageal cancers frequently express EpCAM (CD326) and its strong expression was correlated to poor prognosis in squamous cell carcinomas. Here we tested in cell-based experiments whether EpCAM expression is an epiphenomenon or whether it actively contributes to the aggressive phenotype this cancer type.