by the ability of cells to cross porous inserts covered in matrigel. Membrane localization was determined by sucrose gradients and immunocytochemistry.
Results: Herein, we report the surprising observation that the TF protein increased by progesterone localizes to the heavy portion of the plasma membrane and does not contribute to coagulation. Instead progesterone-increased coagulative activity is localized to lipid rafts. This activity of progesterone is dependent on transcription, the progesterone receptor and is independent of caveolin-1 presence. In the presence or absence of progesterone, TF cannot be detected in lipid rafts by western blotting, but blocking antibodies against this protein eliminate coagulation. These results suggest that progesterone increases the capacity of basal levels of TF located in the lipid raft region to cleave coagulation factor X in the presence of its ligand Factor VIIa. In confirmation of this theory, the use of either 2-methoxyestradiol or inhibitors of the c-src pathway, which we have to shown to eliminate the increase in TF by progesterone, do not inhibit the capacity of progesterone to increase coagulation. Interestingly both these inhibitors eliminate the ability of progesterone to increase breast cancer cell invasion, shown previously by us to be dependent on TF.
Conclusion: We demonstrate that TF levels do not correlate to coagulative ability in breast cancer cells and show that progesterone can modulate coagulation without increasing TF levels.
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452 A novel method to enrich for glioma stem cells from glioma cell lines

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Background: Glioma stem cells (GSC) are inherently similar to stem cells except they can transform into tumours reminiscent of the pathological features of the originated tumour mass. GSCs serve as an excellent pre-clinical model to comprehend tumour re-growth and treatment resistance. Several approaches were previously described to purify GSCs, but seemingly appeared to be laborious, costly and sometimes with poor yield. Our objective was to investigate alternative strategies to cost-effectively and efficiently enrich for GSCs.
Methods-Results: We grew 3 glioma cell lines in a modified serum free media that promotes the growth of stem cells over a 10 day period and with ease of harvesting from the supernatant. The tumour spheres had cell line specific morphologies. For instance, those from U87 and DB54MG were significantly larger with tightly associated spheres, in comparison to those from U251. The tumour spheres expressed stem cell markers and in fact were 80−96% rich in CD133+ve cells. Upon growth in DMEM/10% FCS tumoursphere diffentiation occured. In addition, the tumour spheres can transform in in-vitro and with the ability to grow into tumours having similar pathological hallmarks but faster growth in comparison to xenograft tumours derived from the growth of glioma cell lines. These findings were overall similar with passages 1, 10 and 30 GSCs examined.
Conclusions: We have discovered an alternative strategy to enrich for glioma stem cells from glioma cell lines in a cost-effective, easy and efficient manner. Current efforts are undertaken to utilize our protocol to enrich for glioma stem cells from surgical tissues.

453 The effectiveness of Fas apoptosis signalling pathway determined by the combined action of functional polymorphisms at Fas, FasL and Fadd

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Background: In previous reports we described germ-line functional polymorphisms that differentiate *Fas* and *FasL* genes in two mouse strains (SEG/Pas and C57BL/6J) exhibiting extreme differences in susceptibility to gamma-radiation induced T-cell lymphomas. In this study we provide new data about the functional significance of the intra-cellular and extra-cellular polymorphisms of *Fas* and *FasL*, and report new polymorphisms in the coding sequence of *Fadd*, another key element of this pathway.
Material and Methods: Chimerical Fas and FasL proteins were constructed, combining the intra- and extra-cellular regions derived from C57BL/6J and SEG/Pas. TUNEL apoptosis assay was used to evaluate the induction of apoptosis in cells bearing wild-type/chimerical Fas and FasL molecules. Caspases cleavage assessment through Western Blot served to confirm the TUNEL assay results. C57BL/6J and SEG/Pas-*Fadd* cDNAs were genotyped and sequenced. Fadd-FLAG and Fas-HA constructs were used in co-immunoprecipitation assays.
Results: When assaying TUNEL in the chimerical systems, we found significant reductions in the levels of apoptosis they induce, as compared with those of the SEG/Pas system. This suggests evidence that the polymorphic residues we identified at the intra- and extra-cellular regions of both the Fas receptor and its ligand exhibit a different functionality. As expected, the accumulation of polymorphisms, represented in the double-chimerical systems, produces the highest differences of apoptosis. Strikingly significant seems as well the functionality of those polymorphic amino acids located on the intracellular region of Fas, through which it interacts with Fadd. These might determine different affinities of interaction between Fas and Fadd, given that none of the polymorphic residues found at *Fadd* cDNA between C57BL/6J and SEG/Pas lie in its death domain. Co-immunoprecipitation experiments show that the interaction Fas-Fadd is stronger when Fas derives from SEG/Pas, confirming the different functionality of the polymorphic residues at the intracellular region of Fas.
Conclusions: Our results support the functionality of polymorphisms located at the intra- or extra-cellular regions of Fas and FasL, but prompt us to consider that the functional consequences of any of those changes should be assessed within the general context of the system.

454 c-Met endosomal signalling and breast cancer cell migration

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Background: c-Met, the receptor of HGF (Hepatocyte Growth Factor), is a tyrosine kinase receptor overexpressed or mutated in various cancers. In breast cancer, c-Met has been associated with cancer progression and metastasis and is considered to be a marker for poor prognosis. Therefore specific targeted therapy against c-Met may provide a valuable therapy for patients.
The mechanisms of c-Met signalling that promote breast cancer progression are poorly understood. Recently, it has been shown that tyrosine kinase receptors, including c-Met, continue to signal from the endosome after internalisation. This endosomal signalling may have unique consequences on cellular outcome due to the spatial and temporal activation of downstream signalling pathways.
The objectives of this study were to investigate and compare the role of c-Met endosomal signalling in the migration of a range of human breast cancer cell lines, ranging from a pre-invasive to a highly invasive phenotype.
Material and Methods: c-Met internalisation, intracellular trafficking and downregulation were compared using FACS analysis and confocal microscopy. The relationship of c-Met trafficking to signalling, cell migration and invasion was determined using western blot analysis, transwell migration assays and 3D organotypic invasion assays.
Results: We find that the requirement of c-Met for endocytosis in the stimulation of several signalling pathways and in cell migration varies significantly between the cell lines. The more aggressive cell lines seem more reliant on c-Met endocytosis for the full activation of Gab1, ERK and AKT downstream of c-Met. Consequently, these cells require c-Met trafficking for their full migration and invasion.
Conclusions: Our results suggest that c-Met endosomal signalling might play a role in breast cancer progression.

455 Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90

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Background: Hypoxia-Inducible Factor 1 (HIF-1) is a transcription factor that is a critical mediator of the cellular response to hypoxia. Enhanced levels of HIF-1α, the oxygen-regulated subunit of HIF-1, is often associated with increased tumour metastasis, therapeutic resistance and poor prognosis. In this context that we previously demonstrated that the antiapoptotic protein bcl-2 cooperates with hypoxia topromote HIF-1/Vascular Endothelial Growth Factor (VEGF)-mediated tumour angiogenesis.
Material and Methods: Expression vectors encoding human bcl-2, *wild type* or hydroxylation resistant HIF-1α were used for stable and transient transfections of M14 human melanoma line. The effect of bcl-2 stable transfection will be evaluated in cells under hypoxic conditions in terms of bcl-2 and HIF-1α protein expression and localization (*western blot and confocal microscopy analyses*) HIF-1α protein stability and ubiquitination (*Western blot and immunoprecipitation analyses*) and HIF-1 transcriptional activity (*reporter assay*). The role of Heat Shock Proteins (HSPs) in the bcl-2-mediated regulation of HIF-1α expression and transcriptional activity (*Western blot analysis and reporter assay*) was evaluated by using chemical inhibitors. Immunoprecipitation experiments were also performed to investigate the possible effect of bcl-2 protein on the interaction of HIF-1α and HSPs.
Results: By using M14 human melanoma cell line and its derivative bcl-2 overexpressing clones, we demonstrated that bcl-2-induced accumulation of HIF-1α protein during hypoxia was not due to an increased gene transcription. In fact, it was related to a modulation of HIF-1α protein expression at a post-translational level, indeed its degradation rate was faster in the control lines than in bcl-2 transfectants. The bcl-2-induced HIF-1α stabilization in response

to low oxygen tension conditions was achieved through the impairment of ubiquitin-dependent HIF-1α degradation involving the molecular chaperone HSP90 but it was not dependent on the prolyl hydroxylation of HIF-1α protein. Notably, we also showed that bcl-2, HIF-1α and HSP90 proteins form a tri-complex that may contribute to enhancing the stability of the HIF-1α protein in bcl-2 overexpressing clones under hypoxic conditions.
Conclusions: We identified the stabilization of HIF-1α protein as a mechanism through which bcl-2 induces the activation of HIF-1 in hypoxic tumour cells, involving the molecular chaperone HSP90.

456 Identification of oncoantigens associated to breast cancer stem cells for preventive antitumour vaccination

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Background: Characterization of genes differentially expressed during the stages of tumour progression may lead to the identification of "oncoantigens", tumour-associated molecules that play important roles in driving tumour progression and constitute potential targets for preventive antitumour vaccination. Until now, we have identified putative oncoantigens (POAs) as molecules expressed by mammary cells in pre-neoplastic lesions and over-expressed in evident neoplastic lesions. However, many human malignancies, including breast cancer, are organized in a hierarchical network of rare slowly dividing cancer stem cells (CSCs), rapidly dividing amplifying cells and differentiated tumour cells. CSCs constitute the source of the tumour and could be responsible for tumour progression, metastasis, resistance to therapy and recurrence, so preventive vaccination should target them. Thus, analysis of CSCs transcriptional profiling may identify new POAs, more suitable for effective vaccination.
Material and Methods: Mammary tumour specimens were obtained from a cell line derived from BALB-neuT breast carcinomas (Ag12). Cells were plated in differentiative conditions to obtain tumour epithelial cells (e0) or under specific conditions to generate mammospheres (p1), which were then disaggregated and plated to obtain second (p2) and third (p3) passage mammospheres. Expression of CSCs markers on mammospheres was checked by cytofluorimetry. Transcription profiling was performed on e0 and p1−3 using Illumina microarray platform MouseWG-6 v2.0.
Results: Mammospheres generated from Ag12 displayed clonogenicity, self renewal, CSCs markers and ability to differentiate in mammary epithelial cells and maintained the tumourigenic potential. 452 deregulated transcripts were detected in mammospheres using rank product statistics, comparing e0 with p1−3. To detect CSC vaccination targets, a subset of 183 transcripts (POAs) which expression increased from p1 to p3 were selected by K-mean clustering. Vaccination targets for breast cancer prevention were selected ranking the 183 transcripts on the basis of the relation between their expression and survival in 7 public human breast cancer transcription profiles. The actual protein increase of some of these POAs in p1−3 was confirmed in ELISA and cytofluorimetric experiments.
Conclusions: Mammospheres transcription profiling led to the identification of new POAs. Future experiments will validate these POAs in preventive vaccination in BALB-neuT mice.

457 Lysophosphatidic acid induces cell-cell adhesion disassembly and actin cytoskeleton disorganization through an event that requires RhoA-Rock and Src signaling in colon cancer cells

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Background: Lysophosphatidic acid (LPA), an extracellular lipid mediator of multiple cellular responses, acts as a potent stimulator of tumour progression triggering different cell signaling pathways that stimulate cell proliferation, migration and survival in colorectal cancer (CRC). Adherens junctions (AJ) disassembly and actin cytoskeleton alterations are initial events of cancer development; however, the cellular mechanisms underlying these phenomenon remain to be defined. The aim of this study was to examine the cell signaling pathways triggered by LPA to mediate alterations of cell-cell adhesion and actin cytoskeleton reorganization during CRC progression.
Material and Methods: Cell monolayers of Caco-2, a colon adenocarcinoma cell line, were used as CRC model. Cells were serum starved for 24 h and then treated with 10 μM of LPA for 15 to 60 min or pretreated for 1 h with inhibitors of Rho GTPases, Rho-kinase (Rock), PI3K, PKA, EGFR and Src, before LPA treatment. Changes in the location of AJ proteins E-cadherin, b-catenin and p120-catenin were examined by immunofluorescence, and actin cytoskeleton organization by confocal microscopy using rhodamine-phalloidin. RhoA and Rac1 activation was assessed by the pull-down assay, and Src and FAK activation through immunoblotting of the phosphorylated protein forms. Cell migration was analyzed through the wound-healing technique, and cell viability through the crystal violet assay.
Results: LPA treatment induced cell-cell adhesion disassembly, alteration of the actin cytoskeleton organization with stress fibers formation. Pharmacological inhibition of Rho with toxin A from *Clostridium difficile* and Rock with Y-27632 prevented AJ disassembly and actin reorganization caused by LPA treatment. Additionally, Src inhibition with PP2 abrogated p120-catenin redistribution from cell-cell contacts to cytosol induced by LPA. We observed that LPA treatment caused RhoA, Src and FAK activation as evidenced by immunoblotting, however RhoA activation was not prevented by Src inhibition with PP2. Furthermore, by the wound-healing technique we demonstrate that Rho, Rock and Src chemical inhibition also prevented the increase in cell migration LPA-mediated.
Conclusions: Our finding indicates that LPA modulates AJ disassembly, actin disorganization and cell migration through a regulatory cascade that integrates RhoA-Rock and Src-FAK signaling pathways in colon tumour cells.

458 Inhibition of TGFβ2 production in mouse dedifferentiated hepatoma cells leads to decrease of their tumourigenic and metastatic potential

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Background: Cytokines of Transforming Growth Factor (TGF) β family are involved in regulation of cell proliferation, apoptosis, motility and differentiation. Also TGFβ plays dual role in carcinogenesis acting as tumour suppressor or promoter depending on stage of tumour progression and tissue context. Increased levels of TGFβ1 were detected in serum and urine of patients with advanced stages of hepatocellular carcinoma (HCC). While the role of TGFβ1 in hepatocarcinogenesis is actively investigated, the impact of other isoforms in this process is underestimated. Our aim was to investigate the role of TGFβ2 in HCC progression. We have shown that in experimental model of mouse HCC progression highly invasive fast-growing HCC (fgHCC) was characterized with overexpression of TGFβ2 and downregulation of HNF4α, liver enriched transcriptional factor playing a central role in maintenance of hepatocyte morphology and differentiation.
Material and Methods: To inhibit production of TGFβ2 in H33 cells culture obtained from fgHCC we used shRNA technique. The effects of TGFβ2 inactivation in H33 cells in vitro were studied by RT-PCR gene expression analysis, proliferation and cell motility tests. To analyze TGFβ2 effects on tumourigenic and metastatic potential of HCC tumour cells were injected subcutaneously into syngenic recipient mice.
Results: TGFβ2 inactivation in H33 cells induced re-expression of HNF4α and C/EBPα, transcription factor also essential for the maintenance of hepatic differentiation, and alterations in several TGFβ2 responsive genes expression. Inhibition of TGFβ2 in H33 cells led to growth retardation and decrease of cell motility in vitro. After subcutaneous injection into mice H33-siTGFβ2 cells showed delay in tumour formation and decrease of metastatic potential.
Conclusions: TGFβ2-induced activation of TGFβ signaling in HCC cells can contribute to tumour progression increasing tumourigenic and metastatic potential of tumour cells. It can be explained by involvement of TGFβ signaling in regulation of such key properties as proliferation, cell motility and differentiation, probably due to repression of HNF4α and C/EBPα.
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459 The FGF-2 binding domain of thrombospondin-1: functional characterization and exploitation to design antiangiogenic compounds

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New blood vessels formed by angiogenesis supply oxygen and nutrients to solid tumours and provide a gateway for metastatic cells to enter the bloodstream and disseminate to distant organs. Thrombospondin-1 (TSP-1), an endogenous inhibitor of angiogenesis, restrains angiogenesis through different mechanisms, including the direct binding to and sequestration of angiogenic factors, in particular fibroblast growth factor-2 (FGF-2). TSP-1 binds FGF-2 through a site located in its type III repeats domain.
We hypothesized that this domain might serve as a template for the development of inhibitors of angiogenesis. Using a peptide array approach, we identified a FGF-2 binding sequence in the type III repeats of TSP-1.