362 Poster

DUSP6/MKP3 - a phosphatase between the MAP ERK and mTOR pathways. Regulation of its expression in tumoral cell lines

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Introduction: We had previously shown that the dual specificity phosphatase DUSP6/MKP-3 was phosphorylated and degraded upon growth factor stimulation, in a MEK-dependent manner (Marchetti et al, 2005). Here we investigated the role of the PI3K/mTOR pathway in the phosphorylation and degradation of DUSP6, induced by serum growth factor.

Materials and methods: CCL39, LS174 and A375 tetracyclin-inducible cell lines were used for the study of the phosphorylation and degradation of DUSP6 upon stimulation with serum and specific agonists for the mTOR pathway such as amino-acids or insulin/IGF. In addition, we used specific pharmacological inhibitors for the PI3 Kinase and mTOR pathway. With different mutant constructions of DUSP6, we identify the residues phosphorylated by the mTOR pathway.

Results: upon serum stimulation, DUSP6 is degradated and undergoes a phosphorylation that is evidenced by an electrophoretic shift. This shift is partially blocked by the PI3K and mTOR inhibitors. Moreover, specific agonists for the PI3K/mTOR pathways were also able to induce phosphorylation and degradation of DUSP6. However, a basal activity of MEK was required for the mTOR pathway-mediated phosphorylation to occur. Mutagenesis studies identified serine 159 within DUSP6 as the target of the mTOR pathway. In order to understand the differences of expression of DUSP6 in tumours, we study now the role of the MAP kinase ERK and PI3K/mTOR pathways in the regulation of the expression of DUSP6 at the RNA and protein level.

Conclusion: the ERK phosphatase DUSP6 constitute a novel branch-point of the cross-talk between two major signalling pathways implicated in cell growth in physiological and tumoral processes, the MEK/ERK pathway and the PI3K/mTOR pathway.

363 Poster

Modulation of insulin receptor substrate-1 expression is related to differentiation in human colorectal epithelium and colorectal cancer

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The goal of our study is to test whether insulin receptor substrate 1 (IRS1) plays a role in the differentiation of human colorectal epithelia and whether IRS1 expression and functions are involved in colorectal cancer development and progression. Caco2 and HT29 colorectal cancer cell lines were used as models of colorectal epithelial differentiation. IRS1 gene and protein expression was assessed by Real Time-PCR and western blotting analysis and correlated with proliferating or differentiated status of epithelial cells. IRS1 distribution in colorectal crypts was studied by immuno-histochemistry in 24 non-neoplastic human colorectal mucosa specimens.

The IRS1 gene and protein expression was characterized in the CaCo2 differentiation model, which recapitulates colorectal enterocytic differentiation. Data show that IRS1 gene and protein expression increases during the 14 days differentiation time course. Insulin receptor β (Insulin Rβ) subunit protein is weakly and highly expressed in proliferating and differentiated enterocytes respectively. Insulin-like growth factor I receptor (IGF-IR β) subunit protein is highly and weakly expressed in proliferating and differentiated enterocytes respectively. These results suggest that the IRS1 protein could mediate the IGF-IR pathway in proliferating enterocytes and the IR pathway in differentiated enterocytes. IRS1 immunohisto-chemistry of non-neoplastic human colorectal mucosa shows that the protein is moderately expressed in crypt epithelia, with maximum expression in enterocytes at the upper third of the crypt and luminal surface. IRS1 gene and protein expression was also characterized in pre-confluent, confluent and post-confluent HT29 cell lines. Transmission electron microscopy of HT29 cells demonstrates gradual differentiation. Data show that IRS1 gene and protein levels decreased during the 14 days time course and were highly expressed in proliferating HT29 cells and weakly expressed in more differentiated HT29 cells. Insulin Rβ and IGF-IR β subunit protein were modulated during the HT29 cells time course showing increasing and decreasing expression respectively. A primary CRC tissue microarray containing 190 CRC cases was analyzed for IRS1 protein expression. This showed heterogeneous IRS1 expression in colorectal cancer. Association studies with clinicopathologic features are in progress. These results show that IRS1 gene and protein expression is modulated during in vitro and in vivo normal colorectal epithelial and colorectal cancer differentiation, together with the Insulin R and IGF-IR β subunits.

364 Poster

Signal-dependent control of autophagy and cell death in colorectal cancer cell: the role of the p38 pathway

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Autophagy is a vacuolar process leading to the degradation of long-lived proteins and cytoplasmic organelles in eukaryotes. This process has an important role in normal and cancer cells during adaptation to changing environmental conditions, cellular and tissue remodeling, and cell death. To date, several signaling cascades have been described to regulate autophagy in a cell type-specific and signal-dependent manner.

In an effort to evaluate the impact of p38 signaling on colorectal cancer cell fate, we inhibited p38alfa kinase function by pharmacological blockade or genetic inactivation in several colorectal cancer cell lines and in animal cancer models. Deficiency of p38alfa activity induces a perturbation in the expression profile of a subset of genes that ultimately lead to cell cycle arrest, autophagy and cell death in a cell type-specific fashion. In these cells, a complex network of intracellular kinase cascades controls autophagy and survival since the effect of p38alfa blockade is differentially affected by the pharmacological inhibition of MEK1, PI3K class I and III, and JNK.

We propose that p38alfa can be involved in the management of cellular stress and/or in the regulation of essential metabolic cascades in colorectal cancer cells. Initially, the autophagic response to p38alfa blockade represents a survival pathway, but prolonged inactivation of the kinase leads to cell death. Inhibition of p38alfa-dependent autophagy breaks this process and triggers an apoptotic response. In accordance with this hypothesis, early re-activation of p38alfa induces a significant time-dependent reduction in the autophagic process with a slow re-entry into the cell cycle. Worthy of note, the relationship between metabolic stress, autophagy and cell death in tumorigenesis has been described in epithelial cancer cells both in vitro and in vivo.

Collectively, our results suggest an opportunity for exploiting the pharmacological manipulation of the p38alfa pathway in the treatment of colorectal cancer. Given the number of drugs, currently available or under development, that target the p38 pathway, it stands to reason that elucidating the molecular mechanisms that link p38 and autophagy might have an impact on the clinical translation of these drugs.

365 Poster

MUC1 targeting tissue-specific expression in breast cancer cultures

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Background: Promoter region of carcinoma associated mucine-like glycoprotein (MUC1) known as DF3 promoter was cloned from human breast carcinoma T47D with high expression of MUC1 confirmed previously. MUC1 promoter was cloned in non-viral vector pDsRed2-C1 instead of CMV standard eucariotic promoter. Transfection efficiency of pDsRed2-C1-MUC1 and pDsRed2-C1 vectors was compared in specific and non-specific cell cultures.

Methods: MUC1 promoter region -686 +31 was amplified from a DNA isolate of T47D using two-round nested PCR method with the following primers: "-686 AseI" 5' - AAATTAATGGACCCTAGG GTTCATCGGAG - 3' and "+94" 5' - TGTGAGGAGCAGCAGCAGG - 3'; "-686 AseI" and "+31 NheI" 5' - AAGCTAGCGATTCAGGCAGGCGCTGGCT - 3'. The PCR product was cloned into pTZ57R/T (PCR products cloning kit, Fermentas). Homology of promoter region was confirmed by sequence data using standard M13 primers. The obtained region was cloned into pDsRed2-C1. Homology was confirmed by sequence data using the following primers: "-565-582" 5' - GGAGGAGGAAGAGGTAGG - 3', "-122-139" 5' - ACCCTGAAACCCACAGTC - 3'. MUC1 antigen expression in cell cultures was studied with flow cytometry (FACSCalibour, BD), transfection was performed using GeneJammer (Stratagen). Transfection efficiency and specifity of expression were evaluated with fluorescent and confocal microscopy (Leica DMIRE SP2).

Results: MUC1-inducible FP expression is breast cancer-specific and detectable after 36-48 hours in contrast to 24 hours for CMV promoter.

Transfection with pDsRed2-C1-DF3 and pDsRed2-C1 vectors was evaluated in breast cancer hormone-dependent adenocarcinoma MCF-7 and carcinoma T47D, hormone-independent adenocarcinoma HBL-100 and carcinoma MDA-MB-435S, ovarian carcinoma Sk-Ov-3, mouse fibroblasts OMEGA-E, green monkey renal epithelium Cos-1 cell cultures. Specific expression of reporting FP was observed in transfected MCF-7, T47D, Sk-Ov-3 and HBL-100 cell lines 36-48 hours after transfection. There was no detectable FP expression in non-specific cells OMEGA-E, Cos-1 and MDA-MB-435S. Transfection efficiency of pDsRed2-C1-DF3 was 20-40% depending on cell culture without great difference with control vector but level of DsRed2 expression from DF3 is only 40-50% of that CMV promoter delivers. Flow cytometry and confocal microscopy analysis showed high presentation of MUC1 receptor in hormone-dependent MCF-7 and T47D, lower in HBL-100, Sk-Ov-3 and MDA-MB-435S and absence of expression in negative control cell lines.

Conclusions: Clinic evidences of MUC1 hyperexpression in 95-98% of breast cancer cases, especially in 30% of ER- and 65% of HER2-neo-negative primary tumors, made this antigen one of the most important diagnostic markers in genotyping and proteomics assays. The enhancement of MUC1 promoter's expression activity is a prospective target for development of selective metastatic breast cancer therapy.

366 Poster

Orchestrating role of bisindolylmaleimide IX in integration of extrinsic and intrinsic apoptosis in COLO 205 cells

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Introduction: Resistance to apoptosis is the strategy used by cancer cells to avoid elimination. We have previously shown that in COLO 205 cells the blockage of TNF-alpha-dependent extrinsic apoptosis results from cFLIP overexpression. Thus, our efforts are focused on the restoration of cell harmony by the use of metabolic inhibitors, such as bisindolylmaleimide-IX (Bis-IX), which is believed to return the balance in apoptosis. Methods: The experimental model was human colon adenocarcinoma COLO 205 cell line. Cell survival was evaluated by MTT assay. The apoptosis induction was visualized by Hoechst/propidium iodide staining. Immunoprecipitation and Western-blot techniques were used to show the expression of proteins and their respective cellular interactions. Additionally, Scan^R Screening System allowed monitoring of the expression of proteins, engaged in apoptosis machinery. Results: The application of Bis-IX sensitized COLO 205 cells to TNF-alpha-mediated apoptosis. The susceptibility of human COLO 205 cells to apoptogenic stimuli resulted from time-dependent reduction in cFLIPL and TRADD protein levels. At the same time, the level of FADD protein was up-regulated. Additionally, the presence of Bis-IX caused caspase-8-independent cytochrome c release and caspase-9 cleavage. In turn, the treatment with bisindolylmaleimide III (Bis-III) did not evoke neither TNF-alpha-dependent nor intrinsic apoptosis. Conclusions: The results of this study indicate that Bis-IX facilitates the death receptor signal mediated by TNF-R1. Moreover, Bis-IX is able to activate mitochondria in caspase-8-independent intrinsic apoptosis. Targeting antiapoptotic protein(s) with TNF-alpha and Bis-IX is a promising tool to activate apoptosis in order to improve efficacy of cancer treatment.

367 Poster

Molecular markers of human brain tumors and their participation in cellular signaling pathways

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The aim of this investigation is identification characterization of genes with significant changed expression in brain tumors and their possible interaction with signaling pathways. Such knowledge is necessary not only for understanding the tumorigenesis, but also the normal brain functioning.

SAGE, Northern, RT-PCR, Western blot analysis, histochemistry were used to identify 129 genes with 5-fold changes of expression in glioblastomas, the most aggressive form of human brain tumors. This altered pattern of gene expression in tumor cells can be viewed as a molecular marker in the analysis of malignant progression of astrocytic tumors, and as possible clues for the mechanism of disease. Moreover, several of genes overexpressed in glioblastomas produce extracellular and membrane proteins or proteins involved in signaling pathways, thereby providing possible therapeutic targets. Next step includes functional analysis of encoded proteins, their potential partners and participation in cellular signaling pathways. High levels of HC gp-39 gene expression, the product of which reveals a mitogenic effect, similar to the effect of insulin-like growth factor I (IGF-I), correlates with unfavorable course of disease.

Since deregulation of the IGF system/HC-gp39 is a frequent pattern in tumours, IGFs/ IGFBPs/HC-gp39 should be included in the panel of tumour markers used for the diagnosis and serological surveillance in various malignancies.

As a functional antagonist to the potential oncogene HC gp-39, gene TSC-22 has significantly lower expression in astrocytic gliomas. Differential expression of TSC-22 was confirmed by histochemical analysis. TSC-22 may serve as a mediator of TGF-β signals. A substantial decrease of TSC-22 expression on the RNA and protein levels revealed in glial tumors together with known negative role of TSC-22 in the cell proliferation regulation have evidenced about its tumor-suppressed function, it allows to offer TSC-22 as a prognostic factor for gliomas.

Further characterization of these genes will thus allow them to be exploited in molecular classification of glial tumors, diagnosis, prognosis, and anticancer therapy. Novel antisense and iRNA strategies targeting components of cellular signaling pathways may offer additional options for treatment of malignant gliomas.

368 Poster

Full-length tissue transglutaminase is a resistance factor for cell differentiation in neuroblastoma

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Transcriptional activation of tissue transglutaminase (TG2) is essential for neuroblastoma cell differentiation induced by retinoic acid (RA). We have previously shown that the MYCN oncogene suppresses neuroblastoma cell differentiation through repressing TG2 gene transcription. Due to the alternative splicing of pre-mRNA, there exists at least 4 isoforms of TG2.

In this study, we aim to determine the effects of the full-length isoform of TG2 (TG2-L) on neuritic differentiation and cell viability in neuroblastoma cells. MYCN amplified neuroblastoma BE(2)-C and LAN-1 cells were transiently transfected with scrambled control siRNA, total-TG2 siRNA targeting all isoforms of TG2 or TG2-L siRNA specific for TG2-L, followed by treatment with 1μM of all-trans RA (atRA) or control for 5 days, to determine the effect of TG2-L on cell differentiation and the combinational effect of repression of TG2-L and retinoid. siRNA transfection efficiency was determined by competitive RT-PCR. Cell viability was assessed by trypan blue assay.

Treatment with atRA induced transcriptional activation of TG2-L in both BE(2)-C cells and LAN-1 cells. Repression of TG2-L with siRNA alone induced neuritic differentiation with morphological transition to neuronal type in the cells within 48 hours, and more dramatically 5 days post-transfection. The induction of differentiation was further amplified when cells were transfected with TG2-L siRNA and treated with 1μM atRA, compared with atRA alone or TG2-L siRNA alone ($p < 0.05$). Moreover, combination of TG2-L siRNA and atRA resulted in a dramatic decrease in cell viability 5 days post-treatment in both BE(2)-C and LAN-1 cells.

Taken together, our data suggests that TG2-L is a resistance factor to neuritic differentiation in MYCN-amplified neuroblastoma cells, and that decrease in cell viability after TG2-L repression is secondary to terminal differentiation.

369 Poster

Jab1 and estrogen receptor alpha (ERa) in human breast cancer

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Jab1 (Jun activation domain-binding protein 1) may have a role in the development and progression of breast cancer. Interestingly in a recent study conducted in our laboratory with 283 ERα-+ve breast tissues, examined by immunohistochemistry, we found a significant positive correlation between Jab1 and ERα expression. This result was unexpected given previous reports in the literature. To investigate the potential mechanisms underlying this relationship, we determined the expression of Jab1 in breast cancer cell lines after estrogen (E2) and anti-E2 treatment by western blot. Exposure of cells to 4-Hydroxy-tamoxifen (4-HT) resulted in a little up-regulation of Jab1 after 24h. As expected we observed an increased expression of ERα protein after 4-HT treatment at > 24 hours, and a strong down-regulation of ERα due to treatment with ICI 182,780 (ICI). However, no significant change in Jab1 expression was found due to