of ILEI in EpH4 and EpRas cells caused EMT, tumor growth and metastasis. RNAi-mediated knock-down of ILEI in EpRas cells before and after EMT (EpRasXT) prevented and reverted TGFbeta-dependent EMT, also abrogating metastasis formation.

ILEI (FAM3C) belongs to the FAM3 family of secreted cytokines. Thus, the simplest explanation for the effects of ILEI overexpression in epithelial cells might be an autocrine action of the secreted protein. However, it was difficult so far to show this with purified recombinant ILEI. Our aim is to understand the way of ILEI action and find possibilities for potential therapeutic interference with the pathway.

Initially, Western blot analysis showed that the secreted form of the ILEI protein is smaller in size than intracellular ILEI. Mass Spec data confirmed the lack of 17 amino acids at the N-terminus in addition to the signal peptide sequence, giving a strong indication for additional proteolytic processing of ILEI. The cleaved form was not detectable in whole cell extracts. In ILEI cleavage assays using purified full length protein we found, that ILEI was cleaved extracellularly, mostly by serum proteases.

To investigate the role of ILEI processing, we generated a series of mutant ILEI forms with the hope being defective in proteolytic cleavage. Using these mutants in overexpression studies we could identify essential amino acids for proteolytic cleavage and secretion. Some mutants were defective in proteolytic processing but not in secretion and we found one mutant which was neither cleaved nor secreted. Surprisingly, all overexpressed non-cleavable ILEI forms were able to induce EMT, including the non-secretable form.

These findings show first, that proteolytic cleavage is not essential for ILEI secretion, providing additional support for extracellular processing of the protein. Secondly, these data indicate that proteolytic processing is not required for ILEI action, raising the question, if full length ILEI might have higher biological activity than the cleaved form. Finally and most unexpectedly, these data show that a sole intracellular action of ILEI can induce EMT in vitro. Currently, we are investigating the capacity of these mutant ILEI forms for metastasis induction, to reveal if autocrine or paracrine functions of this cytokine are required for tumor progression.

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Overactivation of STAT3 by interferon-alpha may negatively influence disease outcome in melanoma patients

L. Adamkova Humpolikova[1], A. Kovarik[2], L. Dusek[3], L. Lauerova[1], V. Boudny[1], J. Kovarik[1]
[1]Masaryk Memorial Cancer Institute, Experimental Oncology, Brno, Czech Republic; [2] Institute of Biophysics, Molecular Epigenetics, Brno, Czech Republic; [3] Institute of Biostatistics and Analyses, Evidence-based Medicine, Brno, Czech Republic

Background: Malignant melanoma is one of the most chemo- and radiotherapy resistant tumours. Few years ago, interferon-α (IFN) has been introduced as an adjuvant treatment of this disease. It stimulates immune defence mechanisms and possesses antiproliferative and proapoptotic activity. However, clinical experiences showed that the response rate was in some patients less than expected to be. Impaired function of some signalling proteins may negatively affect treatment response. Of those, the frequent cancer-associated perturbations were described in two members of STAT family (Signal Transducer and Activators of Transcription) i.e. STAT1 and STAT3. While the former protein behaves as a tumour suppressor, the latter acts as an oncogene. IFN-α activates these proteins by phosphorylation of tyrosine and serine residues. Since STAT 3 transactivates growth-promoting and anti-apoptotic genes we have hypothesized that hyperactivation and/or overexpression of STAT3 induced by IFN-α may negatively affect disease outcome and interfere with the therapeutical effect of this cytokine. No valid data about the association of STAT3 abnormal expression and activation with the clinical parameters of malignant melanoma are available. In this study we investigated the activation response of STAT3 to IFN-α in melanoma cells derived from node metastases and evaluated the possible connection of phosphorylation responses with the course of disease within 5 years follow up.

Material and methods: Melanoma short-term cultures were established from lymph node metastases of 24 patients. Malignant cells as well as normal melanocytes were treated with IFN-α and phosphorylation profiles of STAT3 were determined by Western blot using specific antibodies. STAT 3 phosphorylation responses of individual patients were correlated with disease evolution and statistically analysed.

Results: Our results demonstrated that patients disclosed as activation responders to IFN-α, i.e. whose ex vivo metastatic melanoma cells showed IFN-α-induced STAT3 phosphorylation at Tyr705, exhibited significantly shorter disease-free survival (5 vs. 34,9 months; p=0,049), shorter progression-free interval (26,1 vs. 62,3 months; p=0,041) and shorter overall survival (26,5 vs. 78,4 months; p=0,039) as compared to the non-responder group.

Conclusions: Our data provide evidence that activation of STAT3 at Tyr705 by IFN-α negatively correlates with disease outcome.

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Modulation of cell cycle by extracellular p27

A. Mikecin[1], M. Grdisa[1]
[1]Rudjer Boskovic Institute, Division of Molecular Medicine, Zagreb, Croatia

p27Kip is a cell cycle regulator that, when abundant, binds and inhibits kinase activity of cyclin/cdk complexes necessary for G1/S transition. It has also been proven that p27 is able to induce apoptosis. Through the cell cycle, p27 expression level is maintained by transcriptional, translational and posttranslational mechanisms. Apparently, the most important mechanism of reducing p27 level is ubiquitin-mediated proteolisis. Deregulation in signaling pathway for ubiquitination of p27 is believed to be important for development of cancer in numerous tissues.

In this study the influence of extracellular p27 on proliferation and apoptosis of different cell lines was examined. For that purpose TAT fusion proteins: TAT-p27 (wt), TAT-ptp27 (point mutation) and TAT-N' p27 (truncated form) were transduced in RKO and Raji cell lines as well as in MCF7, which is caspasis 3 negative. The influence of examined proteins on proliferation was monitored by MTT or WST test. Additionally, effect of extracellular p27 on cell cycle and apoptosis was measured using flow cytometry. The expression of different cell cycle and apoptosis regulatory proteins was determined by Western blot.

After the transduction of p27 variants in the investigated cell lines, halt in the proliferation was detected with MTT and WST test. Flow cytometry has shown the elevation of the amount of both, the cells in G1/G0 phase of the cell cycle, as well as dead cells, after the treatment with wt and mutated p27. In RKO and Raji cells treated with p27 wt and p27 mut caspasis 3 activity was found to be raised. On the other hand, in caspasis 3 negative MCF7 cells, treated with p27 wt and p27 mut the expression of proteins in caspasis 3 independent pathway was found to be changed compared to non treated cells. These results show that the influence of extracellular p27 depends on the type of cells and transduced protein.

The extracellular p27 lead to apoptosis in examined cell lines. It seems that in different cell lines, apoptosis was induced by different pathways. According to these results, modulation of p27 expression could be a good candidate for targeted tumor therapy.

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Implication of MAPK signalling pathways on cold stress-induced apoptosis in a multidrug resistant leukaemic cell line

E. Martin-Orozco[1], A.J. Ruiz-Alcaraz[1], V. Gomez-Abellan[1], J.A. Ferragut[2], M. Saceda[2], M. Sanchez[3], P. Garcia-Peñarrubia[1]
[1]University of Murcia, Biochemistry and Molecular Biology B and Immunology, Murcia, Spain; [2] University Miguel Hernandez, Institute of Molecular and Cellular Biology, Elche (Alicante), Spain; [3] University Miguel Hernandez, Vegetal Production and Microbiology, Elche (Alicante), Spain

We have shown that acquisition of Multidrug-resistant (MDR) phenotype by leukaemic cells is accompanied by pleiotropic changes that result on reduced tumour capacity to survive under stress conditions such as hypothermia. Thus, the study of the signalling pathways implicated on it, are fundamental on the design of new approaches to eliminate drug-resistant tumours. For this purpose, we have studied expression and activation of signalling molecules involved on the fate of the cells (survival or cell-death) like Akt/PKB and p38, ERK1/2 and JNK1/2 MAP kinases. We have found that leukaemic cells with MDR phenotype show a different activation profile for Akt/PKB and MAPK signalling molecules versus their sensitive counterparts when exposed to low temperatures. Furthermore, the use of different inhibitors show that Akt or p38 are not involved in cold-stress induced cell death. However, the use of the ERK inhibitor PD98059 and JNK Inhibitor II, partially counteract hypothermia-induced cell-death on resistant cells. Together, these findings indicate the existence of a collateral sensitivity of MDR leukaemic cells to extreme low temperatures due to alterations of the signal transduction pathways involved on regulation of cell death and survival after treatment with anti-neoplasic drugs.

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The proteolysis of ERalpha induced by thiazolidinediones in breast cancer cell lines is a PPAR-independent event

J. Lecomte[1], S. Flament[1], S. Salamone[2], M. Boisbrun[2], S. Mazerbourg[3], Y. Chapleur[2], I. Grillier-Vuissoz[4]
[1]Université Henri Poincaré, EA3442 Aspects cellulaires et Moléculaires de la Reproduction et du Développement, Vandoeuvre les Nancy, France; [2] Université Henri Poincaré, S.U.C.R.E.S UMR 7565 CNRS, Vandoeuvre les Nancy, France; [3] Université Henri Poincaré, EA3442 Aspects Cellulaires et Moléculaires de la Reproduction et du Développement, Vandoeuvre les Nancy, France; [4] Université Henri Poincaré, EA 3442 Aspects Cellulaires et Moléculaires de la Reproduction et du Développement, Vandoeuvre les Nancy, France

The aim of this study was to identify the mechanism leading to ERalpha degradation in breast cancer cell lines exposed to ligands of Peroxisome

proliferator-activated receptor gamma (PPARg). This nuclear receptor can be activated by natural ligands such as 15-deoxy-delta(12,14)-prostaglandin J2 (15d-PGJ(2)) as well as synthetic drugs such as thiazolidinediones. The treatment of human breast cancer cell lines with PPARg agonists is known to have antiproliferative effects but the role of PPARg activation in the process remains unclear.

We investigated the effects of four PPARg agonists, Rosiglitazone (RGZ), Ciglitazone (CGZ), Troglitazone (TGZ) and the natural agonist 15d-PGJ(2), on two hormone-dependent breast cancer cell lines, MCF-7 and ZR-75-1. ERalpha signalling pathway was studied using transfection and ERalpha was detected by western blotting and immunocytochemistry. In the two cell lines, TGZ, CGZ and 15d-PGJ(2) induced an inhibition of ERalpha signalling associated with the proteasomal degradation of ERalpha. ZR-75-1 cells were more sensitive than MCF-7 cells to these compounds. Treatments that induced ERalpha degradation inhibited cell proliferation after 24 h. In contrast, 24 h exposure to RGZ, the most potent activator of PPARg disrupted neither ERalpha signalling nor cell proliferation. 9-cis retinoic acid never potentiated the proteasomal degradation of ERalpha. PPARg antagonists (T0070907, BADGE and GW 9662) did not block the proteolysis of ERalpha in MCF-7 and ZR-75-1 cells treated with TGZ. ERalpha proteolysis still occurred in case of PPARg silencing as well as in case of treatment with the PPARg -inactive compound Delta2-TGZ.

Taken together, these results demonstrate a PPARg -independent mechanism. Studies are in progress to increase our understanding of the molecular mechanisms leading to ERalpha proteolysis. Thiazolidinedione derivatives able to trigger ERalpha degradation by a PPARg -independent pathway could be an interesting tool for breast cancer therapy.

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Estrogen receptor beta target genes in estrogen receptor alpha negative cells

A. Arumugam[1], L. Murphy[1]
[1]Manitoba Institute of Cell Biology, Department of Biochemistry and Medical Genetics, Winnipeg, Canada

Estrogens exert their action through their receptors (ER) alpha and beta and regulate the expression of genes involved in cell proliferation and differentiation. In recent years, ER beta has been an important focus of researchers because of both its proliferative and antiproliferative properties in breast cancer. To understand the role of ER beta in breast cancer, it is necessary to identify its downstream target genes. To achieve this, a previously developed MDA MB 231-tet-on-ER beta1 cell model system was used. Cells were depleted of estrogens and treated with 2μg/ml doxycycline (DOX) for 48 hrs and then with either 10nM 17-β-estradiol (E2), 500nM of ICI182780 (ICI) or 4 hydroxy tamoxifen (4HT) for 24 hrs. Total RNA was isolated and candidate target gene expression was quantified by real time RT-PCR. Initially five candidate target genes, Trefoil Factor 1 (TFF1), Epidermal growth factor receptor (EGFR), Transforming growth factor (TGF) alpha, lipophilin B and erythroblastic leukemia viral oncogene homolog2 (erbB2) were measured in one clone. ER beta induction (+DOX) resulted in 56 ± 9 (mean ± SD, n= 3) fold increase in TFF1 mRNA and surprisingly, E2 treatment resulted in only a further 2 ± 0.17 fold increase over DOX alone treated cells. EGFR, lipophilin B, TGF alpha and erbB2 mRNA showed 1.7 ± 0.6, 4.6 ± 1.09, 3.2 ± 1.49 and 1.5 ± 0.45 fold increase, respectively, in response to DOX. E2 further increased the expression of EGFR by 1.5 ± 0.05 fold, lipophilin B by 1.8 ± 0.2 fold, TGF alpha by 1.5 ± 0.11 fold and erbB2 by 1.4 ± 0.36 fold. In medium containing 5% FBS, ICI and 4HT resulted in reduction of candidate gene mRNA levels to 16 ± 14.9 % and 13 ± 3 % for TFF1, 70 ± 31.9 % and 40 ± 23.6 % for EGFR, 20 ± 17 % and 20 ± 9 % for lipophilin B, 22 ± 4.1 % and 12 ± 4.2 % for TGF alpha, 20 ± 28 % and 50 ± 42 % for erbB2, respectively compared to untreated cells. After treatment with the transcription inhibitor actinomycin D, induction of all mRNA by DOX +/- E2 was inhibited. The data suggest that ER beta with and without E2 regulated target gene expression primarily at a transcriptional level.

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Transcription factor PAX3 isoforms differentially regulate expression of the proto-oncogene c-Met in vitro

S. Kumar[1], M. Slevin[2], P. Kumar[2], Q. Wang[2]
[1]Manchester University, Pathological Department School of Medicine, Manchester, United Kingdom; [2] Manchester Metropolitan University, School of Biology Chemistry and Health Sciences, Manchester, United Kingdom

The paired box gene, PAX3 encodes a transcription factor involved in myogenesis, melanogenesis and neurogenesis and has significant roles in cell migration, proliferation and survival in early embryonic development. PAX3 has seven differently spliced isoforms. Studies by our group and others have shown these isoforms exhibit different transcriptional specificities, activities and functions.

The proto-oncogene c-Met is a well established target gene regulated by PAX3. HGF/Met signalling is involved in many aspects of embryogenesis and abnormal Met signalling has been implicated in both tumour development and progression. In particular, this signalling is important in promoting tumour cell invasion and metastasis. We investigated the relationship between PAX3 isoforms and the regulation of c-Met expression using RT-PCR and Western blotting in PAX3 isoforms transfectants of mouse melanocytes. It was found that the expression of c-Met was up-regulated in PAX3c and PAX3d transfectants, but was unaffected in PAX3e, PAX3g and PAX3h isoform transfectants. A luciferase assay revealed that only PAX3c and PAX3d isoforms were capable of activating the c-Met promoter in vitro; PAX3e, PAX3g, and PAX3h had no effect on the c-Met promoter. Thus, some PAX3 isoforms differentially affect c-Met expression through varied transactivations of c-Met promoter. Our results indicate the different roles of PAX3 isoforms in tumourigenesis.

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Cyclophilin A is located at the centrosome and functions in the maintenance of genome stability

J.H. Bannon[1], F.T. Bane[1], M.M. Mc Gee[1]
[1]Conway Research Institute, School of Biomolecular and Biomedical Science, Dublin, Ireland

Cyclophilin A (cypA) belongs to the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family which includes the structurally distinct FK-506 binding proteins, the parvulins, and the recently identified PTPA. The PPIase family catalyse the cis-trans isomerisation of peptide bonds located to the N-terminus of proline residues in polypeptide chains thereby altering protein conformation. As such, PPIases are believed to play a role in protein folding and transport, however their true cellular function remains unclear.

In recent years the parvulin, pin1, has been shown to regulate the activity of important cell cycle proteins and thus exhibit both tumour promoting and tumour suppressor activity. These contradictory observations raise the possibility that pin1 can function as a tumour promoter or as a conditional tumour suppressor in a cell-type-selective manner [1].

Recently cypA was found to be overexpressed in pancreatic and lung cancer cells suggesting a possible role during tumourigenisis [2], however its function during tumour development and progression is unknown. In this study we have found that cypA is overexpressed in cells derived from a number of solid tumours including breast, prostate and cervix, and haematopoietic malignancies such as chronic myeloid leukaemia, Jurkat T lymphoma and HL-60 pro-myelotic leukaemia.

Investigation into a possible function of cypA has shown that it is localised at the centrosome in interphase haematopoietic cells. During mitosis, cypA concentrates at the spindle poles and migrates to the midbody during cytokinesis. Centrosomal localisation of cypA was confirmed by double staining of cells with anti-cypA and anti-γ-tubulin, an important component of the conserved γ-tubulin ring complex (γ-TuRC) that regulates microtubule function during mitosis. Merged images illustrate co-localisation of cypA and γ-tubulin suggesting a potential role for cypA during cell division. In support of this, it was found that leukaemia and lymphoma cells that do not express cypA undergo defective cell division and become multinucleated. Furthermore cypA-/- cells display a weakened mitotic spindle checkpoint in response to the microtubule-disrupting agents, taxol and nocodazole. Measurement of mitotic index using phosphorylated histone H3 has revealed that cells that lack cypA exit mitosis prematurely without completing cytokinesis and subsequently re-enter mitosis leading to the accumulation of cells with >4N. Collectively, this study strongly suggests a novel role of cypA in the maintenance of genome stability and provides valuable information that will help elucidate the precise role of PPIases during tumourigenesis.

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563 Poster

Hypothesis of mechanism of microRNA-directed DNA methylation and its evolutionary descent

V. Halytskiy[1]
[1]Palladin Institute of Biochemistry of NAS Ukraine, Molecular Immunology Department, Kiev, Ukraine

We examined microRNA sequences in order to identify possible mechanism of sequence-specific targeting of genes during the transcriptional silencing. Human, mouse and rat sequences of mature microRNAs - in all 1083 sequences - were extracted from database miRBase, http://microrna.sanger.ac.uk/. We detected that only 20.96% microRNA molecules did not contain any 5’-CG-3’ or 5’-CNG-3’ site. The