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PO-128 IDENTIFICATION OF THE E3 UBIQUITIN LIGASE ITCH AS A NOVEL REGULATOR OF CONNEXIN 43 DEGRADATION AND GAP JUNCTION INTERCELLULAR COMMUNICATION

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Introduction The connexins constitute a family of integral membrane proteins that form intercellular channels, enabling adjacent cells to directly exchange ions and small molecules. The connexin channels assemble into distinct plasma membrane domains known as gap junctions. Intercellular communication via gap junctions has an important role in regulating cell growth, differentiation and in maintaining tissue homeostasis. The most ubiquitously expressed connexin isoform in human tissues, connexin 43 (Cx43), acts as a tumour suppressor in multiple tissue types and is often dysregulated at the post-translational level during cancer development, resulting in loss of gap junctions. However, the molecular basis underlying the regulation of Cx43 degradation remains poorly understood.

Material and methods The cervical cancer cell lines HeLa and C33a were used as model systems. Silencing and ectopic over-expression of proteins were performed by siRNA and plasmid transfection, respectively. The Cx43 ubiquitination status was determined using immunoprecipitation and western blotting. Gap junction intercellular communication (GJIC) was measured using the scrape loading dye transfer assay. Cells were imaged using confocal microscopy.

Results and discussions Here, we identify a member of the NEDD4 (neural precursor cell-expressed developmentally downregulated gene 4) family of E3 ubiquitin ligases, termed ITCH, as a novel regulator of Cx43 degradation and GJIC. Depletion of ITCH resulted in increased Cx43 protein levels, gap junction size and GJIC. Ectopic overexpression of ITCH, but not a catalytically inactive ITCH mutant, led to decreased Cx43 protein levels and loss of gap junctions. The data further indicate that ITCH acts in concert with two other members of the NEDD4 family that previously have been shown to regulate Cx43 degradation, termed NEDD4 and SMURF2. Simultaneous depletion of NEDD4, SMURF2, and ITCH by siRNA was found to result in a significantly lower Cx43 ubiquitination level and reduced Cx43 degradation under basal conditions. The triple knock-down of these three E3 ubiquitin ligases was also found to strongly counteract the TPA (12-O-tetradecanoylphorbol 13-acetate)-induced degradation of Cx43.

Conclusion These data identify ITCH as a novel regulator of Cx43 degradation and GJIC. The data also indicate that ITCH acts together with NEDD4 and SMURF2 to mediate the basal and TPA-induced turnover of Cx43.

PO-129 TRANSFECTION WITH LIVER-TYPE GLUTAMINASE (GAB) SENSITISESHUMAN GLIOBLASTOMA CELL LINES TO HYDROGEN PEROXIDE BY DOWNREGULATION OF THE PI3K/AKT PATHWAY

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Introduction Glutamine (Gln) plays a crucial role in the metabolism of neoplastic cells and its elevated catabolism is observed in tumours of different origin. Glutaminase (GA, EC 3.5.1.2) is an enzyme metabolising Gln to glutamate (Glu) and ammonia. The following GA isoforms are known at present: i) kidney-type isoforms, KGA and GAC, encoded by *GLS* and ii) liver-type isoforms, GAB and LGA, encoded by *GLS2*. GA isoforms play opposite roles in tumorigenesis. In glioblastoma (GBM), an incurable brain tumour, *GLS* is highly expressed, while *GLS2* is hardly detectable. Previous studies showed that transfection of human GBM cells (T98G, U87MG and LN229) with a GAB sequence decreased their survival, proliferation index and sensitised them to hydrogen peroxide (H_2O_2). H_2O_2 treatment has been shown to activate the PI3K/AKT signalling pathway which is upregulated in GBM. Here we tested the hypothesis that GAB transfection sensitises GBM cells to H_2O_2 treatment *via* downregulation of the PI3K/AKT cascade.

Material and methods Human GBM cell lines T98G, U87MG and LN229 transfected with GAB sequence (herein named -GAB) or an empty vector (herein named -pcDNA) were used in this study. The protein levels were measured by Western blot. Mitochondrial activity was assessed by MTT test. Apoptosis was determined using the Caspase-Glo 3/7 Assay Systems (Promega).

Results and discussions Upon H_2O_2 treatment, TGAB, UGAB and LNGAB cells presented diminished phosphorylation level of proteins belonging to the PI3K/AKT cascade: PI3K, PDK1, AKT, as compared to their –pcDNA counterparts. TGAB and UGAB cells showed also lowered phosphorylation level of NFκβ and higher apoptotic rates compared to pcDNA cells. Pretreatment with PDGF, an activator of AKT phosphorylation, reduced H_2O_2-evoked GAB cell death compared to the vehicle-treated counterparts.

Conclusion In conclusion, downregulation of the PI3K/AKT signalling pathway by GAB transfection, an effect likewise observed in T98G, U87MG and LN229 cells, contributes to increased cell sensitivity to H_2O_2 treatment. Combination of GAB upregulation and treatment with oxidising agents is a potential therapeutic strategy against GBM.

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PO-130 SER235 RESIDUE DRIVES EIF6 ONCOGENIC ACTIVITY IN NPM-ALK INDUCED T CELL LYMPHOMAGENESIS

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Introduction Dysregulation of mRNA translational control in cancer leads to cell transformation, metabolic reprogramming and angiogenesis. eIF6 is an oncogenic translation factor, which regulates the initiation phase of translation acting on 60S availability in the cytoplasm and controlling active 80S complex formation. eIF6 activation is mTORC1-independent and driven by PKCβ mediated phosphorylation on Ser235. An increment of eIF6 expression is reported in several cancer cell lines and human tumours, due to amplification or overexpression. In mice, eIF6 haploinsufficiency blocks Myc-driven

lymphomagenesis. Intriguingly, high levels of PKC and eIF6 are found in T-cell lymphomas. In particular, in Anaplastic Large Cell Lymphoma (ALCL) eIF6 is overexpressed and hyperactivated.

Material and methods Here, we aimed to define the role of eIF6 phosphorylation in NPM-ALK mediated T-cell lymphomagenesis, combining multidisciplinary studies on murine and cellular models. We used a conditional eIF6^{SA}KI mouse model in which Ser235 is replaced by an Ala.

Results and discussions First, we addressed the effect of eIF6 mutated protein expression in all tissues: homozygosity is lethal after gastrulation while heterozygous mice are viable but resistant to NPM-ALK driven lymphomagenesis. Then, we investigated the role of Ser235 phosphorylation specifically in T-cell lineage, crossing eIF6^{SA}KI mice with CD4-Cre mice. Physiological T-cell development and subsets composition are not affected by the eIF6 mutated protein. In cancer, eIF6$^{SA/SA}$ CD4-Cre NPM-ALK mice have a significant increase in survival time, compared to wt with a delay in the appearance of lymphoma up to 6 months. Histological analysis and *ex vivo* cultures confirm the delay in disease development. eIF6$^{SA/SA}$ CD4-Cre NPM-ALK thymocytes are smaller respect to wt counterparts and show a striking senescence-like phenotype *in vitro*. Similarly, *in vitro* generated eIF6$^{SA/SA}$ MEFs show a markedly reduced proliferation and increased SA β-gal positivity. This phenotype is completely rescued by transducing eIF6 wild-type, but not by eIF6^{SA}. Currently, we are investigating the molecular mechanisms by which eIF6 phosphorylation affects ALK-induced malignancy and whether it may modulate premature cell senescence, thus establishing an effective barrier to T-cell lymphomagenesis.

Conclusion Our work demonstrates for the first time that eIF6 phosphorylation plays an essential role in mammals development, cell homeostasis and is rate-limiting for T-cell lymphomagenesis *in vivo*.

PO-131 OVER EXPRESSION OF EXTRACELLULAR MATRIX PROTEIN 1 (ECM1) IN MUSCLE-INVASIVE BLADDER CANCER

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Introduction Bladder cancer (BC) is the ninth most common cancer worldwide. A quarter of all cases present as muscle invasive BC (MIBC). Treatment for this group has remained largely unchanged for the past 20 years limited mainly to cisplatin based therapy and radical cystectomy. Prognosis for these patients is poor, with 5 year survival <50% and identification of potential new therapeutic approaches is urgently needed. We have explored the possible role of extracellular matrix protein 1 (ECM1) overexpression in MIBC. ECM1 overexpression has been linked to carcinogenesis and progression to a more aggressive phenotype in a number of carcinomas, but its role in BC has not yet been examined. In a previous study, ECM1 was proposed to interact with and stabilise EGFR in a breast cancer cell line leading to increased resistance to ERBB targeting drugs. Despite repeated implication of EGFR in the progression of BC, EGFR targeting agents have so far had little success in the treatment of MIBC in part due to resistance. Improved understanding of the mechanisms of resistance to such agents is vital. The current study examined ECM1 overexpression and the relationship between ECM1 and EGFR in BC.

Material and methods ECM1 expression was assessed at the mRNA and protein levels in 47 bladder tumour derived cell lines using qRT-PCR and western blot analysis. ECM1 knockdown cell lines were established using shRNA lentiviral transduction in a panel of cell lines which overexpress the protein. ECM1 knockdown cell lines were analysed for phenotypic effects. Phosphorylation of EGFR and downstream effectors was examined following recombinant ECM1 treatment. Publicly available microarray data was mined to assess the clinical significance of ECM1 expression in patients, relating high expression to tumour stage and overall survival.

Results and discussions ECM1 knockdown had an inhibitory effect on wound-healing ability. Evidence suggests ECM1 initiates phosphorylation and thus activation of EGFR and downstream effectors in the pathway, however we have been unable to detect a direct interaction with EGFR as described in breast. Mining of publicly available data revealed a significant association between high ECM1 expression and reduced overall survival.

Conclusion ECM1 overexpression in MIBC appears to be associated with poorer prognosis in patients. While ECM1 has been shown to influence EGFR signalling, the mechanism of EGFR activation by ECM1 in BC may be different from that previously described in breast cancer.

PO-132 ALPHA5 BETA1 INTEGRIN PROVIDES GLIOMA CELL RESISTANCE TO EGFR TYROSINE KINASE INHIBITORS. ROLE OF MEMBRANE TRAFFICKING

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Introduction Overexpression of epidermal growth factor (EGFR) drives glioblastoma (GBM) cell invasion and tumour progression. EGFR has been a major therapeutic target in GBM. However, clinical trials were disappointing, and we are still missing molecular basis to explain these poor results. In other solid tumours, extracellular matrix proteins found in the tumour microenvironment and their heterodimeric integrin receptors trigger resistance to EGFR targeted therapy. We recently established that the fibronectin receptor, α5β1 integrin is a pertinent therapeutic target in GBM. Therefore, we seek to evaluate whether α5β1 integrin is involved in therapy targeting EGFR in GBM.

Material and methods We used GBM cell line (U87) overexpressing or down expressing α5 integrin subunit. EGFR was inhibited by clinically approved tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib, lapatinib). For cell motility assays, we performed spheroid dissemination assays, in 2D and 3D environment and chemotaxis assays through Boyden chambers. We also evaluated the impact of integrin expression on cell clonogenicity and cell grow th (2D, spheroids) in presence of EGFR inhibitors. Confocal microscopy confirmed integrin, EGFR and endosomes markers localization. Protein colocalization in early-endosomes was confirmed by confocal image analysis and dSTORM super-resolution.

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