664 KLF4 suppresses estrogen-dependent breast cancer growth by inhibiting the transcriptional activity of ER alpha

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Background: Breast cancer is typically a hormone-dependent tumour, in which exposure to estrogen increases breast cancer incidence and proliferation via estrogen receptor a (ERα). In this study, we screened negative regulator of breast cancer growth and focused on Kruppel-like factor 4 (KLF4/GKLF/EZF). KLF4 encodes a transcription factor that is associated with tumour suppression. Several lines of evidence indicate that KLF4 is an important regulator of cell proliferation. It was also shown that KLF4 is transcriptionally activated following DNA damage in a p53-dependent manner. Here, we show a novel molecular network between p53, KLF4 and ERα in breast cancer.
Material and Methods: To determine the association of KLF4 with tumour progression, we compared the gene expression profiles in breast cancer using Oncomine database. Subsequently, we performed functional analysis of KLF4 using MTT assay, QRTPCR, reporter assay, Co-immunoprecipitation assay and ChIP assay in MCF-7 cells.
Results: We performed an extensive analysis of the Oncomine database and showed that *KLF4* expression was associated with breast cancer tumourigenesis. Analysis of the database also showed a correlation between *KLF4* expression and ERα-positive breast cancers. *In vitro* experiments using the ERα-positive breast cancer cell line MCF-7 revealed that estrogen-dependent cell growth was significantly enhanced by knockdown of *KLF4*. Co-immunoprecipitation experiments revealed that KLF4 binds to the DNA-binding region of ERα and inhibits the binding of ERα to estrogen response elements in promoter regions to reduce the transcription of ERα target genes. We also showed that activation of p53 decreased ERa transcriptional activity by elevating *KLF4* expression.
Conclusion: Our study uncovered a novel molecular network that involves p53, KLF4 and ERα. Our data indicated that p53 up regulate KLF4, and KLF4 suppresses ERα signaling and estrogen dependent breast cancer proliferation. In future, if we could stimulate KLF4, it will be effective therapies for breast cancer.

665 Estrogen receptor ligands regulate prostate tumourigenesis via ER beta/KLF5 pathway

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Background: Prostate cancer is one of the most prevalent malignancies worldwide, resulting in significant mortality among males. Initially, many patients with prostate cancer are treated using androgen ablation therapy. Most neoplasms, however, will eventually become hormone insensitive. Recent clinical studies suggest that antiestrogens inhibit the development of androgen-insensitive prostate cancer, although the underlying mechanism has not been elucidated.
Material and Methods: Anchorage-dependent and -independent proliferation of prostate cancer cells was analyzed by MTT assay and soft agar colony formation assay. DU145 or PC-3 xenograft tumours were grown in BALB/cA-nu mice. Subcutaneously implanted time-release pellets or injection of agents were used for drug delivery. Western blot analysis was used to measure protein expression and quantitative real-time PCR was used to measure mRNA levels. The apoptosis of prostate cancer cells was determined by TUNEL assay.
Results: *In vitro* experiments showed that ER ligands did not affect on anchorage-dependent proliferation of androgen-insensitive prostate cancer cells (DU145 and PC-3). In contrast, anchorage-independent growth was promoted by 17β-estradiol (E2) treatment and suppressed by ER antagonist ICI 182,780 (ICI). Moreover, *in vivo* experiments showed that ICI inhibits prostate tumourigenesis, whereas E2 enhances prostate tumour formation through ERβ. To reveal this mechanism, we screened ERβ-interacting protein and identified Kruppel-like factor 5 (KLF5). Knockdown of KLF5 expression in prostate cancer cells increased tumourigenesis and abolished the effects of ER ligands. In the presence of ICI, ERβ bound to the promoter of FOXO1 through KLF5, enhancing transcription in a manner dependent on CBP recruitment. Elevated FOXO1 levels increased apoptosis in prostate cancer cells and suppressed tumourigenesis. In contrast, estrogen induced the formation of complexes containing ERβ, KLF5, and the ubiquitin ligase WWP1, resulting in the ubiquitination and degradation of KLF5.
Conclusions: Our results demonstrate that prostate tumourigenesis is suppressed by ICI and enhanced by E2. These effects result from ERβ-mediated regulation of the transcriptional activity of KLF5. In addition, we identified FOXO1 as a critical KLF5 target gene that induces cell apoptosis and suppresses prostate tumourigenesis.

666 Analysis of microRNAs associated with aggressive subtype of chronic lymphocytic leukaemia harbouring inactivated p53

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Background: In chronic lymphocytic leukaemia (CLL), 15% of patients harbour deletion and/or mutation of tumour suppressor gene *TP53*. The p53 inactivation is strongly associated with aggressive course of the disease and poor response to therapy. We have described a group of three cancer-associated microRNAs (miR-34a, miR-29c and miR-17−5p) differently expressed between patients with wild-type (wt) and inactivated p53 (Mraz et al., Leukemia 2009). The basal level of miR-34a, the direct transcriptional target of p53, is high in CLL cells harbouring wt p53 but low in p53-mutated CLL cells and also in normal B-cells. The contribution of miR-34a, miR-29c and miR-17−5p to CLL aggressiveness and chemoresistance and their role in neoplastic transformation of B-cells remains unclear.
Materials and Methods: B-lymphocytes of CLL patients and healthy donors were separated by negative selection (MACS or RossetteSep; B-cell purity >95%). The expression of miRNAs and p53 target genes was analyzed using Real-time PCR (TaqMan MicroRNA Assay, Applied Biosystems). p53 status was routinely assessed by I-FISH and FASAY in all samples.
Results: (1) We have reported that CLL patients may develop novel TP53 mutations during therapy (Malcikova et al., Blood 2010). In such patient, we have analyzed the expression changes of the three miRNAs previously shown to be influenced by p53 and detected ~6.6-fold decrease in miR-34a expression. This result confirms previously published data that miR34a expression is dependent on p53. The levels of miR-17−5p and miR-29c remained unchanged.
(2) To assess the direct changes of these microRNA expression during cytotoxic response, we performed *in vitro* treatment of malignant and non-malignant B-lymphocytes *in vitro* by fludarabine or doxorubicine. In non-malignant B-cells, we observed 4−6 fold increase in miR-34a levels. However, the miR-34a level was only slightly increased (up to 1.5 fold) in primary cultures from previously untreated CLL patients. The p53 functionality was confirmed by monitoring an induction of PUMA and p21 genes.
(3) To confirm the *in vitro* data, the miRNA-34a, 29c and 17−5p changes during chemotherapy *in vivo* are currently analysed and the results will be were correlated to p53 target genes induction.
Conclusions: Our data support the hypothesis that miR-34a participates in p53-dependent cytotoxic response. However, we suppose that the role of miR34a differs in normal and malignant B-lymphocytes.
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667 APC/C plays a role in the acute response to protein-damaging stress

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The heat shock response promotes cell survival in response to protein-damaging stress. Heat shock transcription factors (HSFs) are a family of transcription factors that are activated by stress and induce increased expression of heat shock proteins, that function as molecular chaperones maintaining protein homeostasis. The regulation of HSF activity is crucial, and HSF1 is mainly regulated through posttranslational modifications, whereas different stresses affect the levels of HSF2 by a so far unknown mechanism. The ubiquitin E3-ligase anaphase-promoting complex/cyclosome (APC/C) drives degradation of mitotic regulators in cycling cells by associating with the coactivators Cdc20 and Cdh1. Although a plethora of APC/C substrates have been identified, only a few transcriptional regulators are described as direct targets of APC/C-dependent ubiquitination.
In vivo and in vitro ubiquitination assays were used to show that HSF2 is ubiquitinated in a APC/C-dependent manner. Interactions between HSF2 and the APC/C complex were studied using co-immunoprecipitation studies. Chromatin immunoprecipitation assays were used to study recruitment of HSF2, Cdc20 and proteasome to the *Hsp70* promoter.
Here we demonstrate that APC/C^{Cdc20} mediates ubiquitination and degradation of HSF2, a transcription factor that binds to the *Hsp70* promoter. The interaction between HSF2 and the APC/C subunits Cdc27 and Cdc20 are enhanced by moderate heat stress and the degradation of HSF2 is induced during the acute phase of the heat shock response, leading to clearance of HSF2 from the *Hsp70* promoter. Remarkably, Cdc20 and the proteasome 20S core α2 subunit are recruited to the *Hsp70* promoter in a heat shock-inducible manner, indicating destruction of the promoter-bound HSF2.
Our results provide the first evidence for a participation of APC/C^{Cdc20} in the acute response to protein-damaging stress, expanding the role of

APC/C beyond the cell cycle regulation. We propose that the acute response to proteotoxic stress is delicately modulated by adjusting the abundance of promoter-bound HSF2. This spatiotemporal regulation is facilitated by recruitment of Cdc20 to the *Hsp70* promoter and subsequent degradation of HSF2, suggesting that APC/C^{Cdc20} actively participates in the heat shock response.

668 Vitamin D receptor and colon cancer: effect of the Snail family of transcription factors

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Background: 1alpha,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and a number of less calcemic analogs are in clinical trials as anticancer agents against colon cancer and other neoplasias based on their antiproliferative, prodifferentiation, pro-apoptotic and antimetastatic activity in cultured cells and experimental animal models. Most, if not all, $1,25(OH)_2D_3$ actions are mediated by vitamin D receptor (VDR). Thus, VDR expression is the major determinant of cell responsiveness to $1,25(OH)_2D_3$. VDR is expressed in normal colon epithelial cells and in some colon cancer cells. However, VDR expression is lost during colon cancer progression, possibly causing unresponsiveness to $1,25(OH)_2D_3$.
Material and Methods: We ectopically expressed Snail1 or Snail2 in human colon cancer cells to analyze the effect of these transcription factors on VDR RNA and protein expression and $1,25(OH)_2D_3$ action. In addition, we study VDR, Snail1 and Snail2 RNA expression using quantitative-RT-PCR in one hundred human colon cancer samples and their normal counterparts.
Results: The transcription factors Snail1 and Snail2 repress VDR expression and block $1,25(OH)_2D_3$ action in human colon cancer cells. By contrast, other inducers of epithelial-to-mesenchymal transition such as Twist1, Zeb1, Zeb2 and E47 did not affect VDR levels. Snail1 and Snail2 have a strong additive effect and cooperate to repress VDR expression. In addition, we found that Snail1 and/or Snail2 overexpression in human colon tumours correlates with VDR downregulation. Accordingly with data from cultured cells, the strongest VDR repression was found in those colon tumours that overexpress both transcription factors.
Conclusions: Our results suggest that Snail1 and Snail2 are probably responsible for VDR downregulation and $1,25(OH)_2D_3$ unresponsiveness in advanced colon cancer. Our data indicate that patients with high levels of these transcription factors will be poor responders to therapy with $1,25(OH)_2D_3$ or its analogs, and may contribute to generate more rational protocols for the clinical use of these compounds.
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669 Quantitative expression analysis of nine ETS transcription factors and of the MYC and PTEN genes in a consecutive series of 200 prostate carcinomas

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Background: Genomic rearrangements involving the androgen regulated gene TMPRSS2 and several members of the ETS family of transcription factors are early events in prostate carcinogenesis and gain of MYC and loss of PTEN have been associated with disease progression. We aimed to evaluate whether ETS genomic changes and expression correlate with MYC and PTEN expression using a consecutive series of 200 prostatectomy specimens.
Material and Methods: We used TaqMan Low Density Arrays (TLDAs) to simultaneously assess the expression levels of nine ETS transcription factors, MYC, PTEN and the common fusion between TMPRSS2 exon 1 and ERG exon 4. The panel of ETS transcription factors was chosen according to either the chromosomal localization or the involvement in genomic rearrangements in different cancer models and included ERG, ETV1, ETV4, ETV5, ELK4, FLI1, FEV, ETV6 and ETS2. Whenever necessary, the presence of a genomic rearrangement was assessed by FISH analysis on the correspondent paraffin-embedded sections using dual color or tricolor probe combinations.
Results: The TMPRSS2-ERG transcript was found in 104 cases ($Ct \leqslant 30$). Four samples that were negative for the fusion between TMPRSS2 exon 1 and ERG exon 4 showed high expression of ERG. FISH analysis using a tricolour probe flanking ERG and the 5′ region of TMPRSS2 revealed that two of these cases are also TMPRSS2-ERG rearranged (expressing a different TMPRSS2-ERG transcript), whereas in the other two cases ERG is rearranged with a different 5′ partner. Outlier expression was found for ETV1 in 16 cases (8%), for ETV4 in two (1%) and for ETV5 in one case. FISH analysis with BAC probes is being used to identify the 5′ fusion partners. No outlier expression was found for FLI1, FEV, ETV6 or ETS2. Correlation analysis between TMPRSS2-ERG and MYC expression shows a week positive association ($r_s = 0.197$, $p < 0.01$), while correlation of TMPRSS2-ERG with PTEN expression shows a week negative association ($r_s = -0.167$, $p < 0.02$).
Conclusions: Assessment of gene expression proved to be an efficient approach to identify prostate cancers with ETS rearrangements. We confirm that the pattern of ETS fusion genes in prostate carcinomas is heterogeneous and show that the TMPRSS2-ERG rearrangement is associated with MYC overexpression and PTEN downregulation.

670 Characterisation of LSAMP, a novel candidate tumour suppressor gene in osteosarcomas

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Background: Osteosarcomas are the most common primary malignant tumours of bone. The tumours are highly aggressive and show complex genomic aberrations. We have recently identified a small frequently deleted region in 3q13.31in osteosarcoma tumours and cell lines. This region contains the limbic system-associated membrane protein (*LSAMP),* which has previously been reported to be a candidate tumour suppressor gene in other cancer types. Interestingly, our data shows that low expression of *LSAMP* is statistically correlated with shorter patient survival. We are further investigating the potential use of *LSAMP* as a biomarker for osteosarcomas, as well as its role in osteosarcoma development.
Material and Methods: The gene copy number and expression level of *LSAMP* are being investigated in a larger panel of osteosarcomas using qRT-PCR. The promoter methylation status will be further investigated using bisulfite sequencing, and the protein level will be analysed using immunohistochemistry on tissue microarrays and Western blotting. The expression of LSAMP protein will be restored in cell lines showing deletion and no expression in order to identify transcriptional and phenotypic changes, using microarray expression profiling and cell assays.
Results: We are currently analysing the gene copy number and expression level of *LSAMP* in a larger panel of osteosarcoma tumours. The results will be correlated with different clinical variables, including patient survival, in order to elucidate the potential use of *LSAMP* as a biomarker for osteosarcomas.
In addition, we have examined the expression level of other genes and non-coding RNAs located in the small deleted region, identifying two other genes and one non-coding RNA that may be additional candidate targets for this deletion. The expression level of these genes will be examined in a larger panel of osteosarcomas as well.
We have identified a number of osteosarcoma cell lines showing deletion and no expression of *LSAMP*, which will be used to identify transcriptional and phenotypic changes when expression of LSAMP protein is restored. Currently, we are making constructs in order to over-express LSAMP and the three other candidate targets in these cell lines.
Conclusion: We have previously identified *LSAMP* as a novel candidate tumour suppressor gene in osteosarcomas. Further studies are being done in order to elucidate the potential use of *LSAMP* as a biomarker for osteosarcomas, as well as its role in osteosarcoma development.

671 The role of retrovirally-tagged microRNAs in glioma development

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Background: The importance of non-coding RNAs in cancer has become evident in recent years. A previous screen for brain tumour genes in a PDGF-driven mouse model identified retroviral integrations close to microRNAs, which suggests that they have a role in glioma development.
Materials and Methods: The expression of three of the identified microRNAs was evaluated with a stem-loop real-time TaqMan PCR and Northern blotting. Potential target genes of the microRNAs were estimated using bioinformatic tools.
Results: The expression of mature mir-21 was increased in mouse glioma cell lines, compared to normal adult brain. The expression of mature mir-29a and mir-29b was decreased in the same set of samples indicating a tumour-suppressive role of the mir-29 family. One of the potential targets of mir-21 according to bioinformatic prediction was Sox2. This transcription factor is known to be essential in maintenance of self-renewal of embryonic stem cells and has been implicated to have a role in cancer initiating cells. Intriguingly, our results indicate that levels of Sox2 are decreased upon siRNA treatment of glioma cells as determined by Western blot. This finding suggests that Sox2 is positively regulated by mir-21 and that the direct target of mir-21 is upstream of