of clonal evolution tumour heterogeneity is caused by genetic instability and phenotypic drifting. Thus, tumours arise from a single "mutated" cell which upon subsequent additional alterations gives rise to more aggressive subpopulations within the original neoplastic clone. These cells may leave a large number of offspring by chance, or new mutations may provide a growth advantage over the other tumour cells. Waves of such clonal expansion and selection drive the process. Therefore, any cancer cell can potentially become invasive and cause metastasis. This stochastic model predicts that the evolution of cancer cells is influenced by intrinsic (e.g. signaling pathways) or extrinsic (e.g. microenvironment) factors. These influences are unpredictable or random and result in heterogeneity in the cell phenotype or in the tumour initiating capacity. A key tenet of this model is that all cells of the tumour are equally sensitive to such stochastic influences. Moreover, tumour initiating cells cannot be identified prospectively or enriched for by for sorting cells based on intrinsic characteristics.

Recently, our understanding of tumour heterogeneity has been expanded through "the hierarchy model" which predicts that cancers contain a minority population of tumour initiating cells or cancer stem cells (CSC) that resist treatment and give rise to the bulk of the more differentiated tumour cells. Thus, a tumour can be considered a hierarchy defined by a maturation process analogous to normal tissue homeostasis. Therefore heterogeneity arises as a consequence of the presence of biological distinct classes of cells with differing functional abilities and behavior within the hierarchy. As opposed to the stochastic model the hierarchy model predicts that tumour-initiating cells can be identified prospectively and purified from the bulk of non-tumourigenic population based on intrinsic characteristics. The fact that most epithelial cancers are composed of cells that retain at least some level of differentiation suggests that the cancer stem cell generates a linage restricted progeny with a finite life span which nevertheless constitute the majority of the tumour. It follows that the bulk of the tumour would die out without being replenished from the cancer stem cells. Other than that little is known about the function of differentiated cancer cells.

Evidence will be presented here for the existence of a stem cell hierarchy in the normal breast and in breast cancer.

631 The effect of TGF-beta on glioma initiating cells

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The oncogenic effect of the TGF-beta pathway has prompted the design of several compounds to be used as anti-TGF-beta therapies in cancer. However, it is crucial to understand the molecular pathways implicated in the malignant role of TGF-beta in oncogenesis in order to select the patient population that may benefit from an anti-TGF-beta therapy. We have focused our studies on the role of TGF-beta in glioma. We have demonstrated that high TGF-beta-Smad activity is present in aggressive, highly proliferative gliomas and confers poor prognosis in patients with glioma. Moreover, we have observed that TGF-beta induces the self-renewal capacity of glioma-initiating cells (GICs). GICs are considered to be responsible for glioma initiation, maintenance and recurrence, and hence are optimal therapeutic targets against this deadly disease. We have discerned the mechanisms and molecular determinants of the regulation of GICs by TGF-beta using a transcriptomic approach and analyzing human glioma biopsies, primary cultured patient-derived tumour cells, and patient-derived GICs.

632 Regulation of self-renewal in cancer stem cells

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Recent findings support the concept that cells with the properties of stem cells (SC) are integral to the development and perpetuation of several forms of human cancer, and that eradication of cancer stem cells (CSC) may be essential to achieve cancer cure. However, direct proof of these concepts is still lacking, mainly due the scarcity of appropriate model systems.

We have recently defined a number of CSC-specific biological properties and underlying molecular mechanisms. These findings were generated using mouse models of two types of human cancer: (i) leukaemia, obtained by transgenic expression of the PML-RAR or AML1-ETO leukemia-associated oncogene; and (ii) mammary tumour, obtained by transgenic expression of the ErbB2 oncogene, and represent the rationale for the research activities proposed in this grant application.

1. Extended self-renewal of leukemic SCs, due to p21 up-regulation (Viale et al., Nature 2009). The self-renewal potential of normal SCs (measured as the number of times a single SC replicates during a life span without losing its regenerative potential) is intrinsically limited. This limit becomes experimentally evident as exhaustion of their regenerative potential when SCs are synchronously induced to hyper-proliferate, such as in serial transplantation. Self-renewal of CSCs, instead, is virtually unlimited, as inferred by their ability to support continuous expansion of the cancer clone and to be propagated inexhaustibly during serial transplantation. We have demonstrated that up-regulation of the cell-cycle inhibitor p21 is indispensable for maintaining self-renewal of leukaemia SCs (LSCs). Expression of leukaemia-associated oncogenes in normal hematopoietic SCs (HSCs) induces DNA damage and activates a p21-dependent cellular response that, in turn, imposes cell-cycle restriction and triggers repair of the damaged DNA. This effect of p21 prevents the physiological exhaustion of HSC self-renewal, which occurs in time owing to accumulation of DNA damage, and confers an advantage to HSCs when they hyper-proliferate, as it occurs during stress or after full transformation (for example, in the LSCs), thus explaining the role of p21 in the maintenance of the self-renewal potential of LSCs. These findings imply that cell-cycle-restricted LSCs are critical for the initiation and/or maintenance of the leukaemic clone, suggesting that targeting this compartment might be critical to disease eradication, and suggest that inhibition of DNA repair might be synthetic lethal with oncogene expression. However, it is not clear: (i) whether targeting of p21 in growing leukemias can lead to tumour regression, e.g. whether p21 or the p21-pathways are molecular targets for therapeutic intervention; (ii) which are the molecular mechanisms underlying the effect of p21 on LSCs; (iii) whether p21-upregulation in CSCs is a general mechanism of transformation and is also critical in the more common epithelial tumours.

2. Increased frequency of symmetric self-renewing divisions in ErbB2-mammary CSCs, due to attenuated p53-signaling (Cicalese et al., Cell 2009). Normal SCs accomplish their functions of self-renewal and differentiation through a single mitotic division ('asymmetric division'), in which one progeny retains SC identity, while the other (progenitor) undergoes multiple rounds of divisions before entering a post-mitotic fully differentiated state. We found that self-renewing divisions of CSCs are more frequent than normal counterparts, unlimited and symmetric, thus contributing to increasing numbers of SCs in tumoural tissues. SCs with targeted mutation of the tumour suppressor p53 possess the same self-renewal properties of cancer SCs, and their number increases progressively in the p53-null pre-malignant mammary gland. We showed that p53 signaling is attenuated in ErbB2-driven tumours, and that pharmacological re-activation of p53 induced restoration of asymmetric divisions in cancer SCs and tumour growth reduction, without affecting rates of apoptosis or proliferation on additional cancer cells. These data demonstrate that p53 regulates polarity of cell division in mammary SCs and suggest that loss-of-p53 in epithelial cancers favors symmetric divisions of CSCs, contributing to tumour growth. Molecular mechanisms underlying the effects of p53 on mammary SC polarity remain unknown. 3. Biological heterogeneity of breast cancers correlates with their cancer stem cell content (Pece et al., Cell 2010). Emerging evidence suggests that the number of CSCs within a given tumour can significantly vary from tumour to tumour. This is true not only in human tumours when using xenotransplantation assays, as discussed above, but also in syngeneic mouse models. In this context, CSCs can denote a small subpopulation of tumour cells (0.0001−0.1%, as for ErbB2 mammary tumours o leukemias associated with loss of Pten) or rather constitute a substantial proportion of the tumour mass (>10% in Ras- or myc-induced lymphomas). It is not clear though if the variation in the relative frequencies of CSCs is due to tumour type, specific genetic aberrancies in a given tumour, stage of disease progression or the specific experimental system used. We have recently showed that the heterogeneous phenotypical and molecular traits of human breast cancers are a function of their CSC content. Using an expression signature derived from purified normal mammary SCs (MSCs), we analysed breast cancer expression data sets, and found that we can stratify breast cancers on the basis of their biological (poorly differentiated G3 vs well differentiated G1) and molecular (basal-type tumours from other molecular types of breast cancers: ErbB2-type, luminal-A or -B) characteristics. Xenotransplantation experiments and immunohistochemistry using markers from the SC-signature directly demonstrated that G3s are enriched in CSCs. Notably, genes annotated as putative p53 targets were significantly enriched ($p < 0.002$) in the MSC-signature, suggesting that p53 is one component of the genetic program that accounts for the diversity of abundance of CSCs in tumours.

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Symposium

Gene expression and regulation

633 The contribution of dysregulated ribosomal gene transcription to malignant transformation

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Background: c-MYC plays a prominent role in cancer. Intriguingly, many of the genes regulated by this oncoprotein are associated with ribosome biogenesis and we have previously demonstrated that Myc regulates a major rate limiting step in this pathway, transcription of the 45S rRNA genes by RNA Polymerase I

(Pol I). These observations lead us to hypothesise that cMyc's regulation of rRNA synthesis may contribute to its oncogenic properties. We have tested this hypothesis in a mouse model of Myc-driven lymphoma, the EμMyc transgenic mouse.

Methods and Results: B-cells purified from EμMyc mice display an increased growth rate in comparison to their wild-type littermates, with increased cell volume, total RNA and protein per cell. This phenotype is characterised by higher rates of 45S rRNA transcription and increased expression of factors specific for Pol I transcription. Knockdown of one of these factors, UBF, by RNAi in EμMyc lymphoma cell lines results in a selective proliferative disadvantage of cells *in vitro*, in a competition assay, and *in vivo,* in a transplant model. This phenotype is driven by an increased rate of apoptosis associated with a reduction in 45S rRNA transcription.

Based on these findings we explored the potential therapeutic effectiveness in this model of a novel specific small molecule inhibitor of Pol I, CX-5461, currently in preclinical development. Transplanted EμMyc tumours showed marked sensitivity to CX-5461 *in vivo*, with a dramatic reduction in tumour burden in the peripheral blood (97.54%±0.56) and lymph nodes (94.96%±0.90) due to induction of apoptosis 24hrs following a a single oral dose at 75 mg/kg. Importantly a normal B-cell population was preferentially maintained in treated mice (13%±1.39 wt B220+ cells versus 1.04%±0.24 tumour B220+ cells, as a percentage of total WBC) indicating specificity of the compound for tumour cells. Four doses of CX-5461, 75 mg/kg orally every third day, significantly delayed time to endpoint by 9.5 days ($P < 0.0001$) compared to untreated animals This delay was accompanied by a period of complete remission with normal white blood cell counts ($6.76 \pm 0.48 \times 10^9$cells/L) and no identifiable tumour cells in the peripheral blood. Interestingly, *in vitro* dose curves indicate a dependence of CX-5461 sensitivity on wild-type p53 function (p53 wt and ARF−/− cell line $IC_{50} = 9.28$ nM±1.53 in comparison to p53 mutant and p53−/− $IC_{50} = 1.70$ uM±0.03), which can be reduced with over expression of Bcl2 (Bcl2 $IC_{50} = 2.33$ uM±1.3). Notably even in the more resistant p53 mutant and p53−/− cell lines, cell death also occurred via apoptosis, suggesting p53-dependant and independent mechanisms to be involved in CX-5461 mediated cell death.

Conclusions: In summary, this work with UBF RNAi and CX-5461 identifies inhibition of RNA Pol I transcription as a novel and effective target in the treatment of cMyc-driven malignancies and for the first time establishes that dysregulation of rDNA transcription can directly contribute to malignant transformation.

634 Regulation of alternative splicing by the Ewing Sarcoma Protein (EWS) and DNA damage

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Background: The Ewing sarcoma protein EWS belongs to the FET family (FUS, EWS, TAF15) of polypeptides, which can bind RNA as well as DNA and are implicated in transcription, splicing, RNA transport, signalling and maintenance of genomic integrity. Translocations of the EWS gene are a landmark of Ewing sarcomas and are common in other tumours. EWS interacts with several core splicing factors like U1C and SF1 as well as with several splicing regulators of the SR and hnRNP families. Our goal was to identify alternative splicing events regulated by EWS, whose alteration could contribute to the biology of cancers in which expression of EWS is altered.

Materials and Methods: To identify alternative splicing events regulated by EWS, RNA from HeLa cells depleted of EWS or the corresponding controls were hybridized to a splicing sensitive microarray developed in our laboratory, which analyzes 1804 events in 482 genes relevant for cancer progression and RNA processing. RNA immunoprecipitation (RIP), Crosslinking-Immunoprecipitation (CLIP) and Chromatin-immunoprecipitation (ChIP) assays were used to assess the association of EWS with DNA and RNA of regulated genes.

Results: We have identified 39 alternative splicing events in 31 genes affected by EWS knockdown. RIP, CLIP and ChIP data document direct association of EWS with at least some of the target genes. These changes are enriched in alternative acceptor choices and underrepresented in exon skipping events. Interestingly, alternative splicing changes were identified that affect key genes involved in the response to DNA damage. Remarkably, one third of these changes were also induced upon UV irradiation of control cells. We have also observed a remarkable change in subnuclear localization of the EWS protein upon UV irradiation. While ATM and p38 activities are apparently not required for this effect, inhibition of Erk and Jnk kinases partially impairs EWS translocation.

Conclusions: Some key genes important for response to DNA damage are regulated postranscriptionally by EWS. The EWS protein itself is re-localized upon UV irradiation. These results suggest the possibility that changes in EWS activity are part of the mechanisms underlying the changes in alternative splicing induced by genotoxic stress. They also offer a potential explanation for the observation that EWS knockout mice show hypersensitivity to ionizing radiation and premature senescence.

635 Transcription and RNA Processing: links to cancer

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Changes in gene expression patterns are characteristic of cancer cells and play important roles in facilitating cell proliferation. These changes occur at essentially all steps in the gene expression pathway. I will describe studies from my lab that examine molecular mechanisms by which changes in alternative splicing and polyadenylation of mRNA precursors occur in cancer. With respect to splicing, I will describe a pathway that is activated in cancer cells that results in alternative splicing of pyruvate kinase (PK) mRNA. This switch in splicing, which allows production of the embryonic PK isoform that is necessary for cancer cell proliferation, is mediated by the action of several hnRNP proteins that repress inclusion of an adult-specific exon while favoring inclusion of an exon specific to the embryonic form of the enzyme.

Alternative polyadenylation, which occurs in expression of over 50% of human genes, is also known to change in cancer. This results in shortening of the 3′UTRs of many proliferation-associated mRNAs, removing negative miRNA sites and contributing to their enhanced expression. I will describe studies from my lab that address how changes in both transcription efficiency and the make-up of the complex polyadenylation machinery can lead to changes in 3′ processing efficiency, and as a results the use of upstream, promoter-proximal polyadenylation sites.

Together, these studies illustrate how changes in mRNA processing can contribute to alterations in gene expression that contribute to enhanced proliferation of cancer cells.

636 Mechanisms by which the p53 tumour suppressor protein selects its target genes

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The p53 tumour suppressor protein mediates various cellular processes such as cell cycle arrest, senescence, DNA repair, cell death and even survival. Serving as a sequence specific transcriptional regulator, p53 has a large number of well validated transcriptional target genes that facilitate these outcomes. Yet some p53 outcomes are contradictory (eg. survival vs apoptosis) suggesting that not all target genes are equivalently induced by p53 under conditions that produce a specific and exclusive outcome. We have spent several years striving to elucidate the basis for target gene selectivity by p53. The experimental approaches we take include biochemical and cell-based assays. We and other have identified modifications, co-factors and cellular states that play roles in such selectivity. Recent work from our group continues to focus on this area. We have discovered that transcriptional activity of p53 is regulated at a surprising number of levels. These include (1) binding site recognition in the context of naked vs nucleosomal DNA, (2) the extent and sites of modification of the protein by acetylation, (3) selective repression by Mdm2 and MdmX (4) initiation vs. elongation of target gene RNA and (4) post-transcriptional mechanisms. We have also identified novel p53 target genes that play roles in cell cycle progression, energy metabolism, and even pro-oncogenic functions. Our work reveals the complexity of the p53 network and thereby poses challenges for future studies to deconstruct the key processes that are required for p53 tumour suppression.

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637 Integrative high throughput analysis for the identification of novel therapeutic targets in breast cancer

No abstract received.

638 Tyrosine kinase inhibitor resistance mechanisms: molecular & histologic correlates

No abstract received.

639 Optimizing pathological diagnosis with new biological tools: examples in breast cancer

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In current clinical practice, the majority of patients with early breast cancer will receive some form of systemic adjuvant therapy (chemo- and/or endocrine therapy). A variety of clinical and pathological factors are being used as