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# CURRENT STATUS AND PERSPECTIVE OF CANCER STEM CELL RESEARCH

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## CLOSING REMARKS

# LGR5 STEM CELL-DERIVED ORGANOIDS AND THEIR APPLICATIONS

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The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. *Lgr5*/*Gpr49* was selected from a panel of intestinal Wnt target genes for its restricted crypt expression. Two knock-in alleles revealed exclusive expression of *Lgr5* in cycling, columnar cells at the crypt base. In addition, *Lgr5* was expressed in rare cells in several other tissues including the hair follicle, mammary gland and stomach. Using an inducible Cre knock-in allele and the Rosa26-*LacZ* reporter strain, lineage tracing experiments were performed in adult mice. The *Lgr5*$^{+ve}$ crypt base columnar cell (CBC) generated all epithelial lineages over a 14 month period, implying that it represents the stem cell of the small intestine and colon. Similar obserations were made in hair follicles and stomach epithelium. The expression pattern of *Lgr5* suggests that it marks stem cells in multiple adult tissues and cancers. These predictions have held up and we now know that most if not all adult epithelia utilize Lgr5-marked, tissue-specfic stem cells for their maintenance and repair.

Fate mapping of individual crypt stem cells using a multicolor Cre-reporter revealed that, as a population, Lgr5 stem cells persist life-long, yet crypts drift toward clonality within a period of 1-6 months. Lgr5 cell divisions occur symmetrically. The cellular dynamics are consistent with a model in which the resident stem cells double their numbers each day and stochastically adopt stem or TA fates after cell division.

Intestinal crypts display robust regeneration upon injury. The relatively rare secretory precursors can replace lost stem cells, but it is unknown if the abundant enterocyte progenitors that express the Alkaline phosphate intestinal (Alpi) gene also have this capacity. We created an Alpi-IRES-CreERT2 (Alpi(CreER)) knockin allele for lineage

tracing. Marked clones consist entirely of enterocytes and are all lost from villus tips within days. Genetic fate-mapping of Alpi(+) cells before or during targeted ablation of Lgr5-expressing stem cells generated numerous long-lived crypt-villus "ribbons," indicative of dedifferentiation of enterocyte precursors into Lgr5(+) stems. By single-cell analysis of dedifferentiating enterocytes, we observed the generation of Paneth-like cells and proliferative stem cells. We conclude that the highly proliferative, short-lived enterocyte precursors serve as a large reservoir of potential stem cells during crypt regeneration.

Intestinal cancer is initiated by Wnt pathway-activating mutations in genes such as APC. As in most cancers, the cell of origin has remained elusive. Deletion of APC in in *Lgr5*$^{+ve}$ stem cells leads to their transformation within days. Transformed stem cells remain located at crypt bottoms, while fueling a growing microadenoma in stomach, small intestine and colon. These microadenomas display unimpeded growth and develop into macroscopic adenomas within 4-6 weeks. When APC is deleted in short-lived Transit Amplifying (TA) cells using a different *Cre* mouse, the growth of the induced microadenomas rapidly stalls. Even after 30 weeks, large adenomas are very rare in these mice. We conclude that stem cell-specific loss of APC results in progressively growing neoplasia. Moreover, a stem cell/progenitor cell hierarchy is maintained in early stem cell-derived adenomas, lending support to the “cancer stem cell”-concept.

Lgr5 stem cells are interspersed between terminally differentiated Paneth cells that are known to produce bactericidal products. We find that Paneth cells are CD24+ and express EGF, TGF-a, Wnt3 and the Notch ligand Dll4, all essential signals for stem-cell maintenance in culture. Co-culturing of sorted stem cells with Paneth cells dramatically improves organoid formation (see below for organoid culture conditions). This Paneth cell requirement can be substituted by a pulse of exogenous Wnt. Genetic removal of Paneth cells in vivo results in the concomitantloss of Lgr5 stem cells. In colon crypts, CD24+ cells residing between Lgr5 stem cells may represent the Paneth cell equivalents. We conclude that Lgr5 stem cells compete for essential niche signals provided by a specialized daughter cell, the Paneth cell. In addition, crypt-associated mesenchymal fibroblast-like cells also provide crucial niche factors, such as Gremlins, R-spondins, EGF and Wnts.

In 2009, we reported the establishment of long-term culture conditions under which single crypts undergo multiple crypt fission events, whilst simultaneously generating villus-like epithelial domains in which all differentiated cell types are present. This involvement culturing of stem calls in 3D matrigel and the addition of EGF, the Wnt agonist/Lgr5 ligand R-spondin and the BMP inhibitor Noggin. Single sorted *Lgr5*$^{+ve}$ stem cells can also initiate these crypt-villus organoids. Tracing experiments indicate that the *Lgr5*$^{+ve}$ stem cell hierarchy is maintained in organoids. We concluded that intestinal crypt-villus units are self-organizing structures, which can be built from a single stem cell in the

absence of a non-epithelial cellular niche. The same technology has now been developed for human and mouse *Lgr5*$^{+ve}$ stem cells from stomach, liver, lung, breast, pancreas, prostate, ovary, taste bud, esophagus and others. We have since provided evidence that such human organoids can be used to study hereditary disease and cancer of the pertinent tissues taken from patients. Examples have included Cystic Fibrosis, alpha-1 Anti-trypsin disease, and cancer of the colon, prostate, pancreas and breast.

Organoids also hold promise for regenerative medicine- and gene therapy strategies. In a collaboration with the Watanabe lab, we tested the transplantability of cultured mouse colon organoids into superficially damaged mouse colon. The transplanted donor cells readily integrated into the mouse colon, covering the area that lacked epithelium as a result of the introduced damage in recipient mice. At 4 weeks after transplantation, the donor-derived cells constituted a single-layered epithelium, which formed self-renewing crypts that were functionally and histologically normal. Moreover, we observed long-term (>6 months) engraftment with transplantation of organoids derived from a single Lgr5(+) colon stem cell after extensive in vitro expansion. These data have shown the feasibility of colon stem-cell therapy based on the in vitro expansion of a single adult colonic stem cell.

Increased cAMP levels induce rapid swelling of mouse and human intestinal/rectal organoids by opening the cystic fibrosis transmembrane conductor receptor (CFTR). This response is lost in organoids derived from cystic fibrosis (CF) patients. We have used the CRISPR/Cas9 genome editing system to correct the CFTR locus by homologous recombination in cultured intestinal stem cells of CF patients. The corrected allele is expressed and fully functional as measured in clonally expanded organoids. This study provides proof of concept for gene correction by homologous recombination in organoid-cultured primary adult stem cells derived from patients with a single-gene hereditary defect.

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# ORGANOID-BASED CANCER FUNCTIONAL GENOMICS

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A key challenge in cancer biology resides in the need for robust in vitro systems that accurately capture the diverse homo- and heterotypic cellular interactions between tumor epithelium and stroma within the context of a 3-dimensional tissue microenvironment. We have generated "organoid" models of wild-type gastrointestinal tract tissues combining the 3D architecture of in vivo tissues with the experimental facility of 2D cell lines, using an air-liquid interface (ALI)[1, 2] (Figure 1, 2).

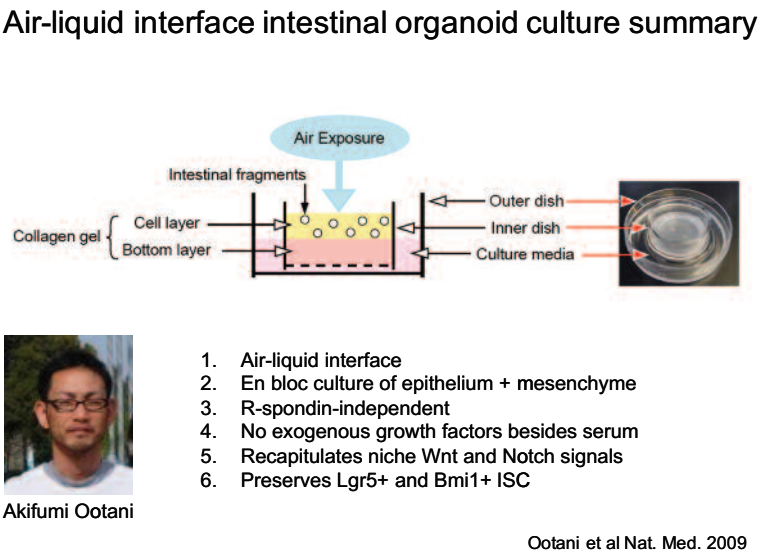


**Figure 1** Summary of ALI method

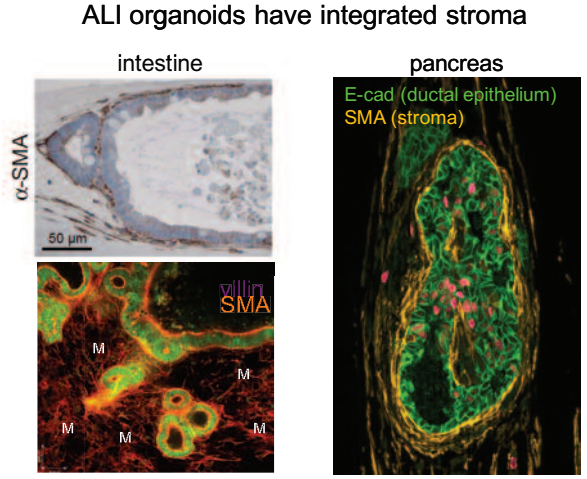


**Figure 2** Demonstration of stromal elements in ALI cultures

In recent years, we have used the ALI method to generate of organoid models that combine tumor epithelium and associated stroma [1, 2]. For the top-down approach this has allowed the in vitro oncogenic transformation of diverse primary wild-type mouse tissues by first creating organoid cultures and then activating oncogenes or deleting tumor suppressors. In the colon, we have used this strategy to demonstrate progressive and stepwise oncogenic transformation with permutations of Apc knockout, $Kras^{G12D}$ overexpression, p53 and/or Smad4 knockdown (Figure 3), recapitulating the Vogelstein model [1].

It has been further possible to extend this paradigm to additional tissues besides colon. We made similar ALI organoids from wild-type mouse stomach and pancreas which also retain epithelial and stromal components. Primary wild-type stomach and pancreas organoids could be similarly transformed with p53 deletion and/or $Kras^{G12D}$ knockin. Further, oncogene-transformed colon, stomach or pancreatic organoids uniformly exhibited histologic dysplasia in vitro and notably could be transplanted in to recipient mice to generate tumors. These organoid methods thus allowed the first in vitro oncogenic transformation of wild-type colon, gastric and pancreatic tissue to adenocarcinoma "in the dish" [1] (Figure 4). These methods importantly allow the in vitro initiation of cancer within wild-type tissues in a "bottom-up" approach.

We have been very interested in the application of organoid methods to the discovery of new driver oncogenic events in cancer. The advent of genome-scale analysis of tumor DNA, RNA and epigenomes through efforts such as TCGA has created a tremendous amount of candidate oncogenic events that now require functional validation. Conventionally, oncogene candidates are evaluated in vitro by manipulation in established cancer cell lines that may have been extensively passaged and/or possess numerous

epi/genetic alterations, which may in turn confound the ability to functionally assess the incremental transforming ability of a given test locus in question. In contrast, the ability to propagate wild-type gastrointestinal tissues as organoids generates a cleaner genetic and epigenetic "tabula rasa" against which the oncogenic functions of a given test locus may be more easily observed. We term such organoid modeling as "bottom-up" cancer gene discovery, as opposed to "top-down" functional evaluation in cancer cell lines [3] (Figure 5).

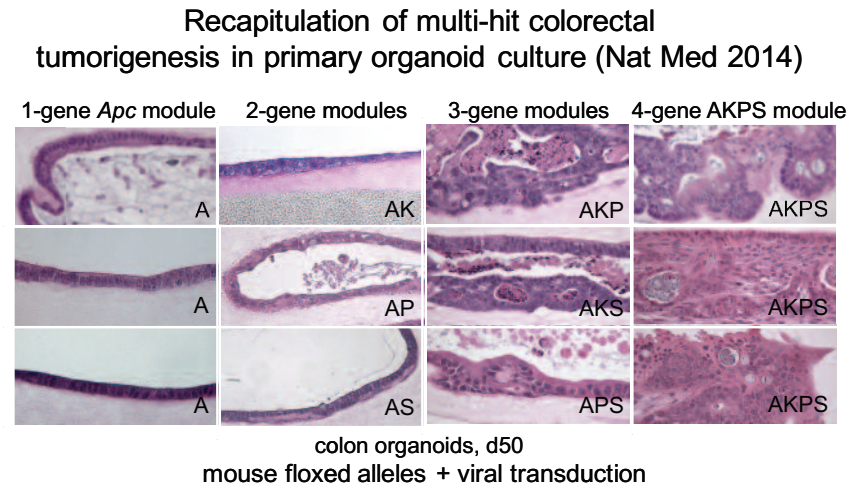


**Figure 3** Recapitulation of mult-hit tumorigenesis in primary colon organoid culture

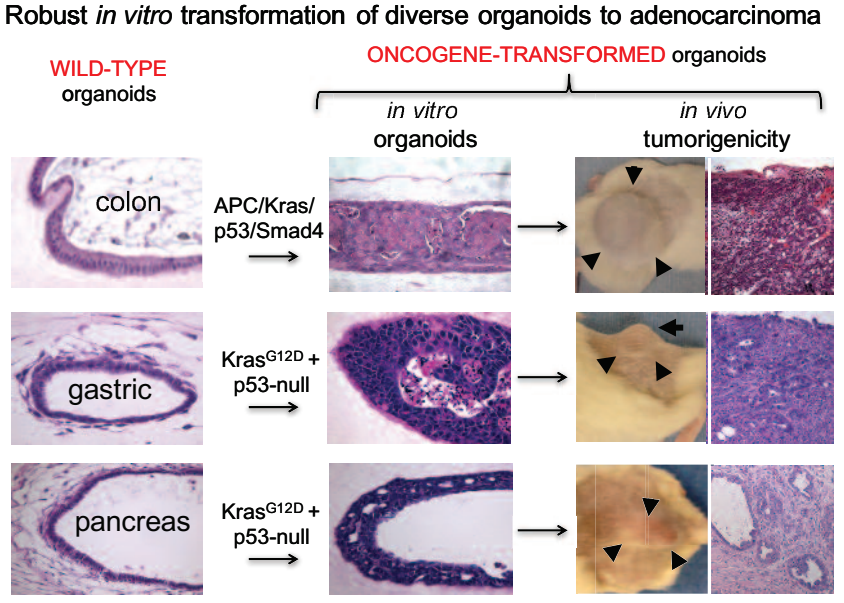


**Figure 4** In vitro transformation of gastrointestinal organoids to adenocarcinoma

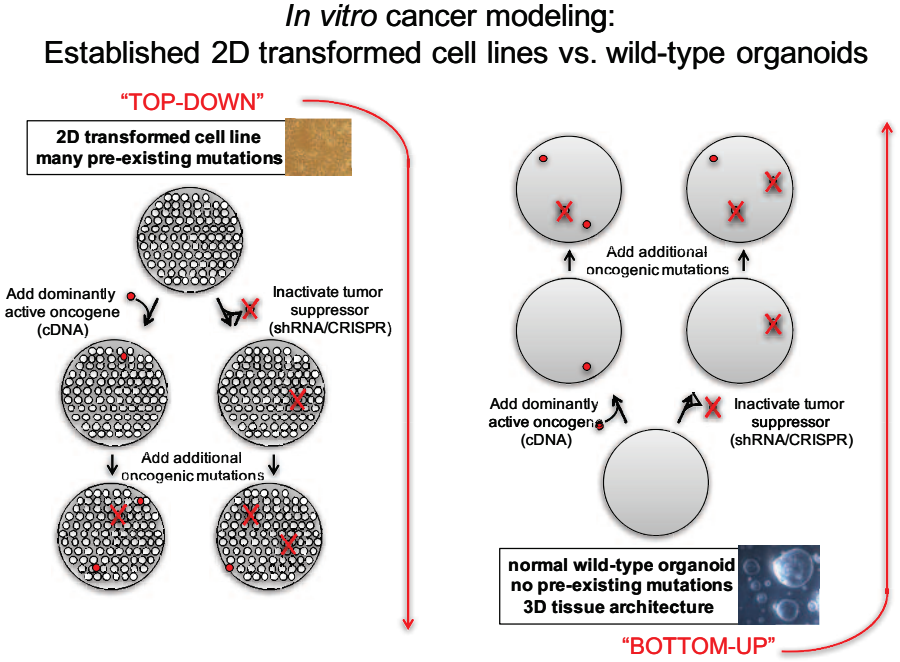


**Figure 5** Top-down versus bottom-up cancer gene validation strategies

Accordingly, we have used organoids to validate miR-483 as a driver oncogene from the 11p15.5 amplicon in human colorectal cancer [1]. Separately, we demonstrated that TGFBR2 is a metastasis suppressor gene in gastric cancer. In both cases, we used contextual modeling to introduce these candidate genes in a minimally-engineered genetic background representing the most relevant clinical context [4].

Overall, organoids represent a highly promising experimental approach to cancer modeling with direct application to functional genomics and novel oncogene discovery. Further, it is increasingly feasible to apply these methods to primary culture of tumor biopsy specimens, with and without stromal components, towards a more holistic in vitro modeling of cancer biology and personalized treatment response [5].

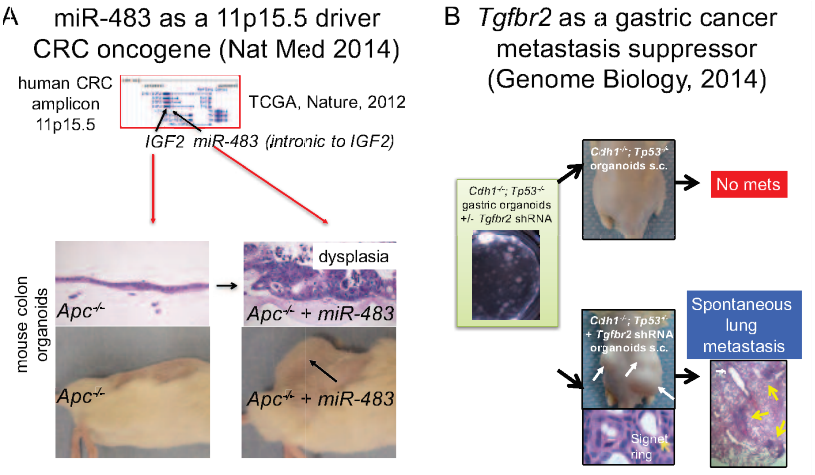


**Figure 6** Novel oncogene validation in organoids

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# VISUALIZATION OF HUMAN COLON CANCER STEM CELLS USING ORGANOID TECHNOLOGY

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Recent advance in next generation sequencing has been providing better understanding of human colorectal cancers, yet the biological interpretation of genetic mutations in the context of malignant progression remains elusive. The difficulty mainly stems from a lack of functional assay system for intestinal epithelium. We identified specific niche environments supporting mouse small intestinal stem cells, which enabled to grow single stem cells into ever-growing organoids [1]. The niche factor based organoid culture system has been broadly applied to various epithelial tissue stem cells from both mouse and human with some modifications optimized to the tissue [2,3]. We found that niche signalling coincides with recurrently mutated oncogenic signalling in human colorectal cancer, suggesting functional connection between niche signalling and colorectal carcinogenesis [4]. By the use of CRISPR-Cas9 genome-editing system, we introduced multiple driver gene mutations into human normal colon organoids [5]. Driver gene (APC, KRAS, SMAD4, TP53 and PIK3CA) mutant organoids were efficiently enriched by selective niche factor culture condition, demonstrating niche factor independent growth in the engineered organoids. Upon xenografting, the engineered organoids exhibited robust tumorigenic capacity in renal subcapsule, but failed to form metastatic colonies in the liver after injection into spleen [6]. In contrast, when introducing equivalent driver gene mutations into chromosome-instable human adenoma organoids, they exhibited aberrant histologic structure and were capable of forming metastatic colonies. These results indicate that aberration of niche factor signalling confers niche independent growth capacity, and metastatic transformation further require genetic lesions other than driver gene mutations.

The organoid culture system was applied to human colorectal cancers with 50-60% establishment efficiency. We optimized isolation and culture protocol for human colorectal tumours and established human colorectal tumour organoid library (CTOL) encompassing a range of histological subtypes and clinical stages [7]. Each line was analysed by gene expression signatures, copy number analysis and exome sequencing. In vitro and in xenografts, the organoids reproduced the histopathological grade and differentiation capacity of their parental tumours. Matched pairs of primary and metastatic organoids had similar genetic profiles and niche factor requirements, but xenograft experiments demonstrated higher metastatic capacity in the metastasis-derived organoids. These observations underscore the importance of genotype-phenotype analyses at a single-patient level.

The cancer stem cell (CSC) theory highlights a rare self-renewing subpopulation of cells that can rebuild tumours after chemotherapy or metastasis. The existence of human CSCs is mainly supported by the results of a xeno-transplantation assay using prospectively isolated cells. However, a direct demonstration of human CSCs in intact cancer tissues has remained elusive owing to a lack of tractable culture system for human primary cancers. We generated knock-in human colorectal cancer organoids in which LGR5 or KRT20 gene expression was visualized by CRISPR-Cas9 mediated genetic knock-in reporter. After xenografting, patient-derived colon cancer organoids reconstituted stem cell hierarchies resembling those in the original patient cancer tissues. We also generated LGR5-CreER knock-in colorectal cancer organoids and their lineage-tracing by fluorescent reporter revealed the self-renewal and differentiation capacity of $LGR5^{+}$ tumour cells. In contrast, $KRT20^{+}$ cancer cells were mostly post-mitotic and rarely formed tumour colonies. The selective ablation of $LGR5^{+}$ cells in LGR5-iCaspase9 knock-in organoids led to tumour regression. Although residual $LGR5^{-}$ cancer cells exhibited some compensatory proliferation, the removal of $LGR5^{+}$ CSCs constrained efficient tumour regrowth. These data provide insights into the functional dynamics of CSCs and their potential as therapeutic targets in human colon cancer.

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# DECIPHERING THE ROLE OF MALIGNANT DEAMINASE ACTIVATION IN CANCER STEM CELL GENERATION

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Cancer is the leading cause of death for individuals in the US under 85 years of age. Mortality is most frequently related to cancer progression in the setting of therapeutic resistance fueled by self-renewing cancer stem cells (CSCs). Thus, there is a pressing unmet medical need for developing novel strategies for early CSC detection coupled with innovative treatment modalities that prevent therapeutic resistance. Comprehensive DNA and whole transcriptome RNA sequencing (RNA-seq) analyses suggest that pre-malignant stem cell populations acquire age-related deficiencies in DNA damage repair followed by RNA processing alterations that promote progenitor self-renewal and malignant transformation.

Recently, RNA-seq analysis revealed that splice isoform signatures could distinguish benign from malignant hematopoietic stem and progenitor cell (HSPC) aging (*Crews et al, Cell Stem Cell 2016*). Splicing deregulation in secondary acute myeloid leukemia (sAML) leukemia stem cells (LSC) was typified by intron retention and distinctive patterns pro-survival and adhesion splice isoform expression patterns. Spliceosome modulation with 17S-FD-895 reverted splicing to a normal aged HSPC pattern in humanized mouse models of sAML thereby providing the impetus for translational research efforts to develop this novel modulator for clinical trials in relapsed or refractory AML.

Further analysis uncovered inflammatory cytokine networks that drive RNA processing deregulation by activating adenosine deaminase associated with RNA (ADAR1) during cancer relapse or progression (*Jiang et al, PNAS 2013*). Notably, the majority of the ADAR1 mediated RNA editing events occur in the context of double stranded RNA loops formed by primate specific Alu repeat sequences, 5′ and 3′ untranslated regions and microRNAs.

Through RNA sequencing, lentiviral overexpression and site-directed mutagenesis, we discovered that ADAR1 contributes to oncogenic transformation of pre-malignant progenitors in chronic myeloid leukemia (CML) by impairing let-7 pri-miRNA biogenesis in response to enhanced JAK2 signaling in malignant niches (*Zipeto et al, Cell Stem Cell 2016*). Treatment with a selective JAK2 inhibitor, fedratinib, and a BCR-ABL1 inhibitor reversed malignant RNA editing in a humanized mouse model of blast crisis CML thereby providing the impetus for development of a combination tyrosine kinase inhibitor (TKI) strategy. Moreover, we have embarked on intensive research efforts to identify selective ADAR1 inhibitors that can be used as monotherapy to target CSCs.

In addition to promoting CSC self-renewal, ADAR1 prevented G1 cell cycle transit (Pineda et al, Scientific Reports 2016) typical of CML by editing primary miRNA 26a at the Drosha cleavage site and thereby impairing biogenesis of precursor and mature miRNA 26a. Lentiviral overexpression of wild-type mir-26a impaired BC CML engraftment in immunocompromised mice and reversed the G1 transit block thereby providing a RNA editing driven mechanism of therapeutic resistance in CML (*Jiang et al, manuscript in preparation*).

To determine if ADAR1 contributed to CSC driven therapeutic resistance in other refractory hematologic malignancies, we analyzed expression and activity of ADAR1 in multiple myeloma (MM). Notably, copy number amplification of chromosome 1q21 that encompasses the ADAR1 and IL-6 receptor locus defined a higher risk subset of patients with MM. These samples harbored a coding region edit in GLI1 that prevented degradation by SUFU and enhanced in vivo serial transplantation potential of high risk MM in immunocompromised mouse models suggesting that ADAR1 activation represents a novel prognostic biomarker and potential therapeutic target in MM (*Lazzari et al, submitted*).

Finally, RNA-seq and RNA editing specific PCR revealed that cytidine deaminase apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC3) transcripts were key ADAR1 editing targets. As essential components of antiviral immune responses elicited by inflammatory cytokines, APOBEC deaminases induce cytidine to uridine base changes that are subsequently read as thymidine (C-to-T). Seminal, reports show recurrent APOBEC mutagenesis patterns in cancer-associated genes. Moreover, the process of kataegis, which involves clustering of DNA mutations, is associated with increased expression of APOBECs capable of inducing DNA base substitutions in tumors. Notably, BC CML CSCs were typified by increased expression of specific APOBEC family members and increased editing of APOBEC3D and 3G. Thus, early detection and inhibition of malignant RNA and DNA deaminase activity may be vital for eradicating therapy resistant CSCs in inflammatory microenvironments.

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# AN AUTOCRINE LOOP INVOLVING β-CATENIN PATHWAY IS CRITICAL FOR DEVELOPMENT OF HUMAN MYELOID LEUKEMIA STEM CELLS

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Acute myeloid leukemia (AML) originates from self-renewing malignant leukemic stem cells (LSCs). It has been shown that purified human LSCs can repopulate human AML in immunodeficient mice after xenogeneic transplantation. Human AML LSCs mainly reside in the $CD34^+CD38^-$ fraction whose phenotype is analogous to normal hematopoietic stem cells (HSCs). We have reported that the T-cell immunoglobulin mucin-3 (TIM-3) is expressed on the surface of LSCs in any FAB type of AML with only exception of M3 (acute promyelocytic leukemia: APL), but it is not expressed in normal human HSCs [1]. Only $TIM\text{-}3^+$ but not $TIM\text{-}3^-$ fraction of human AML cells can reconstitute human AML in immunodeficient mice. We also showed that administration of anti-human TIM-3 killing antibodies cure immunodeficient mice reconstituted with human AML LSCs. Accordingly, we have proposed that TIM-3 should be a strong candidate for therapeutic target to eradicate AML LSCs [1].

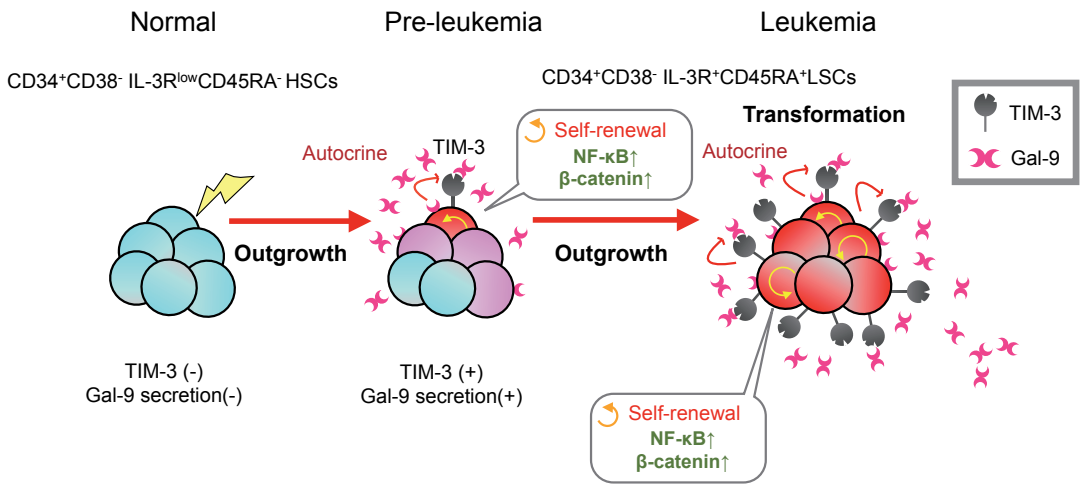
TIM-3 is a type 1 cell-surface glycoprotein, and was identified originally as a surface molecule expressed in $CD4^+$ Th1 cells in mice. In human, it is expressed also in a fraction of T cells, NK cells, monocytes and dendritic cells (DCs). Galectin-9, a ligand of TIM-3, is a β-galactoside binding lectin that contains 2 carbohydrate recognition domains (CRDs), and binds to N-terminal immunoglobulin variable (IgV) domain of TIM-3 through its two CRDs. Ligation of TIM-3 by Galectin-9 has been shown to phosphorylate tyrosine residues of the cytoplasmic tail of TIM-3, and activate Src family kinases through its Src homology 2 (SH2) binding motif in T cells and monocytes. We thus hypothesized that TIM-3 signaling plays an important role in maintenance or self-renewal of human AML LSCs.

We found that serum galectin-9 concentration was significantly elevated in AML

patients (325±54.7pg/ml, n=20) but not in the healthy individuals (25.2±6.8pg/ml, n=7) or patients with B cell malignancies (38.6±32.9pg/ml, n=5). Primitive CD34+ AML cells had abundant galectin-9 protein in their cytoplasms. We then transplanted human $CD34^+$ primitive AML cells into irradiated immunodeficient mice. Strikingly, only in mice reconstituted with human AML, but not in those with normal cord blood or human ALL cells had elevated serum levels of human galectin-9: Serum galectin-9 levels were 234.7±69.0pg/ml (n=8) in mice reconstituted with primary human AML cells, whereas 4.64±4.64pg/ml (n=12) in mice with normal human hematopoiesis. These results collectively suggested that AML cells secreted galectin-9 in vivo in an autocrine manner. We then performed transcriptome analyses of primary $CD34^+TIM\text{-}3^+$ AML cells after galectin-9 ligation. A pathway enrichment analysis showed that both NF-κB and β-catenin pathways were activated. In fact, galectin-9 ligation of $TIM\text{-}3^+$ human AML cells induced activation of the NF-κB pathway via ERK and AKT phosphorylation. Activation of ERK and AKT pathways are known to inhibit the GSK3β activity, and to promote the nucleus translocation of β-catenin in several cancers. To demonstrate significant nucleus translocation of β-catenin of primary AML cells, we established a quantitation system by utilizing the Array Scan VTI system (ThermoFisher Scientific, USA). By this system, we could formally prove that TIM-3 ligation by galectin-9 significantly promoted the nucleus translocation of β-catenin in primary AML cells [2].

We also have extensively analyzed TIM-3 and galectin-9 interaction in cells from patients with myeloid malignancies including myelodysplastic syndromes (n=30), myeloproliferative neoplasms (n=12) and chronic myelogeneous leukemia (n=18). Strikingly, in all cases, frequencies of $CD34^+CD38^-TIM\text{-}3^+$ cells dramatically increased along with disease progression from early/chronic phase to overt leukemias. Furthermore, serum levels of galectin-9 were also dramatically elevated after leukemic transformation. Significant nucleus translocation of β-catenin by galectin-9 ligation was also found in these diseases after leukemic transformation. A recent study has shown that NF-κB and β-catenin pathways could play a cooperative role in conferring the cancer-stem-cell properties to non stem cells in intestinal cancer model.

These data collectively suggest that TIM-3 and galectin-9 constitutes a pan-myeloid autocrine loop to develop malignant stem cells in the vast majority of human myeloid malignancies [2]. Signaling molecules downstream of TIM-3 and galectin-9 ligation, as well as surface TIM-3 itself might be good candidates for cancer stem cell-target therapy common to most myeloid malignancies.

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# INNATE LYMPHOID CELLS IN THE CONTROL OF ORGAN HOMEOSTASIS

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Innate lymphoid cells (ILC) are a recently discovered branch of the lymphoid lineage that belongs to the innate immune system. ILC are tissue-resident cells that are particulary numerous in the intestinal lamina propria. While ILC perform traditional functions associated with immune cells (such as defense against microbial infections), it is an emerging new paradigm, that ILC are involved in processes not normally linked to the immune system. For example, ILC play important roles in maintaining tissue homeostasis and are important regulators of metabolic processes. We have been interrogating two interconnected signaling networks that are involved in these unconventional functions of ILC. ILC express the aryl hydrocarbon receptor (AhR), a transcription factor, activity of which is controlled by environmental (e.g., nutrient-derived) metabolites and toxins that directly initiate transcription of a battery of genes some of which metabolize toxins to non-toxic intermediates. In recent years, the importance of AhR signaling in immune cells has been receiving attention. For example, mice genetically lacking the AhR in group 3 innate lymphoid cells (ILC3) showed a profound defect in the maintenance of ILC3 [1,2]. In addition, AhR signaling in ILC3 is required for the expression of interleukin (IL)-22, an unusual cytokine that exclusively acts on non-hematopoietic cells (e.g., epithelial cells, stroma cells) and has been linked to host resistance to certain types of bacterial [3] and viral infections [4]. Curiously, ILC3 are positioned in proximity to the crypts of *Lieberkuhn* in the small intestine and they produce high levels of IL-22 during steady-state (i.e., in the absence of infections or inflammation). The effects of IL-22 on epithelial cells have not been thoroughly explored. ILC3-derived IL-22 has been shown to induce the production of anti-microbial proteins by the intestinal epithelium, protecting the mucosal layer against

pathogenic infections [3]. In addition, IL-22 has been shown to protect crypt-resident stem cells against damage in a mouse model of graft-versus-host disease, though the IL-22-regulated targets in stem cells remain unidentified [5,6]. Considering the high conservation of the AhR and its importance for IL-22 production, we investigated the function of the AhR-IL-22 axis in response to genotoxic stress in intestinal stem cells caused by carcinogens such as azoxymethane (AOM). Indeed, mice deficient in IL-22 or lacking AhR in ILC3 showed an increased rate of AOM-induced colorectal cancer. Interestingly, IL-22 controlled molecular circuitry that protects the intestinal stem cell niche from genotoxic stress. The implications of these finding for the development of human cancer will be discussed.

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# CLINICAL RELEVANCE OF LEUKEMIA STEM CELLS IN AML

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## Acute myeloid leukemia

AML is organized as an aberrant developmental hierarchy maintained by functionally distinct leukemia stem cells (LSC). The capacity for self-renewal is the defining hallmark of all tissue stem cells, responsible for the persistence throughout life of both stem cells and their progeny. LSC are the only leukemic cells with sustained self-renewal capacity. Programs including epigenetic states and the transcription factors and epigenetic modifiers (e.g. Polycomb complexes and miRNA) that govern them are responsible for stem cell properties. The term "**stemness**" is increasingly being used in the literature to refer collectively to the integrated functioning of molecular programs that govern and maintain the stem cell state. LSC exhibit a stemness gene expression program responsible for self-renewal, quiescence, and resistance to apoptosis. These same properties also drive the central role that LSCs play in AML progression, recurrence, and chemotherapy resistance. A common theme from our studies is the strong similarity between the stemness properties of HSC and LSC, a finding that is supported by the recent findings that human AML originates from normal HSC through an intermediate pre-leukemic HSC that possesses a clonal advantage due to mutations in key epigenetic regulation genes such as *DNMT3A*, *TET1*/*TET2* and others. However careful comparison of the transcriptional programs enriched in purified LSC and HSC fractions indicate that there are also differences; LSC fractions more closely resemble primed or activated HSC/MPP. Indeed, comparison of LSC and HSC self-renewal, as measured in clonal serial xenograft transplantation assays, suggest that the capacity for LSC self-renewal is greatly enhanced indicating that regulatory programs must be perturbed during leukemogenesis.

**Clinical relevance of stemness properties in AML**

Despite implementation of combination chemotherapy and hematopoietic cell transplantation (HCT), AML still remains lethal. Most patients respond to initial induction achieving complete remission (CR), but most (>50%) will go on to relapse. The initial success of chemotherapy to seemingly eradicate leukemia, only to have it return in a form resistant to the original chemotherapy points to the survival of leukemia cells that are resistant and regenerative; properties that rest with LSC. Despite decades of research, the cells that cause relapse have not been precisely identified and characterized making it difficult to gain mechanistic understanding of why some cells relapse and to develop better drugs to target them.

Therapeutic strategies in AML rely on the presence or absence of cytogenetic abnormalities and a small set of gene mutations that broadly define favorable, intermediate, and adverse risk categories. The more adverse subtypes require more aggressive therapy such as HCT. However, in the favorable and intermediate risk groups, a subset of patients relapse despite the lack of adverse risk factors. Moreover, patients with cytogenetically- or mutationally-defined adverse risk features and those requiring more than one cycle of therapy to achieve remission have a worse outcome, with or without allogeneic transplantation. Thus there is great need for better biomarkers to identify high-risk patients prior to starting induction chemotherapy. We have now achieved development of the first such biomarker based on LSC stemness that enables rapid and more accurate risk stratification, not only in the post-remission setting but also prior to beginning treatment.

We used the AML xenotransplantation model to identify 138 LSC+ and 89 LSC– cell fractions sorted from 78 AML patients and then through gene expression analysis we derived a 104-gene LSC-specific gene signature. We then applied a statistical-learning algorithm guided by a clinical dataset from 500 patients to extract the core transcriptional components of stemness that related to patient outcomes. This approach resulted in a scoring system that robustly predicted outcome and therapy resistance across multiple AML datasets. Our study identified a 17-gene signature score (LSC17) that is highly prognostic in 5 independent datasets (n=908) across the broad spectrum of AML patients, and improves prediction of initial therapy resistance. Patients with high LSC17 scores had poor outcomes with current treatment strategies including allogeneic transplantation. The LSC17 scoring approach was adapted to the clinically-applicable NanoString platform providing a first-in-class tool that we intend to bring into clinical use to identify AML patients who do not benefit from standard therapy and should be enrolled in trials evaluating novel upfront or post-remission therapies.

**Subclonal genetic diversity and the source of relapse in AML**

In AML, there is good evidence that resistant cells arise prior to chemotherapy and chemotherapy selects these pre-existing cells. Genetic analysis of bulk AML cell populations from paired diagnosis and relapse samples from the same patient have established that often the major clone at relapse shares some but not all mutations with the major clone at diagnosis. With deeper sequencing and specific informatic tools, these *in silico* studies show that the bulk AML population at diagnosis is not uniform; rather it is composed of genetically distinct subclones that are related to each other through branching evolution. In some cases, genetic variants specific to the relapse sample are already present at low frequencies in the bulk diagnosis sample, suggesting that a subclone already present at diagnosis generates relapse. However, such *in silico* depictions of clonal diversity and relapse origins do not provide insight into the actual cells that bear the relapse mutations nor do they provide any biological insight into why they have both therapy resistance and regenerative properties.

To more directly identify relapse originating cells, we undertook whole genome sequencing of 11 paired diagnosis/relapse samples and identified the mutations that were shared, present only in the bulk diagnosis AML population, or only in the bulk relapse population. Then these variants were monitored with high sensitivity by digital PCR on 10 populations purified on the basis of HSPC-like or myeloid blast phenotypes and in multiple xenografts established from the paired samples; we use the xenografts to monitor the genetic identity of the LSC that initiate AML engraftment. Phylogenetic analysis showed that, at diagnosis, the LSC compartment is genetically heterogeneous and composed of LSC that drive the dominant AML population as well as other multiple small LSC subclones. We identified relapse-specific variants in these sorted populations or xenografts derived from the diagnosis sample; thus, in most cases, chemotherapy selects for preexisting subclones present at diagnosis. Our second major finding was that the cellular origin of relapse differed between samples: one group was termed as having primitive-relapse origins and the other group as having committed-relapse origins. Thus, this data is clear evidence that functionally defined LSC are linked to disease recurrence in human patients.

Our findings have considerable implications for the design of better ways to monitor and treat AML patients. The ability to track the cellular origin of relapse will enable biomarker development to better stratify patients to receive the most appropriate therapies. Moreover the isolation of diagnosis subclones fated to cause relapse through xenografting will enable detailed characterization of their vulnerabilities leading to improved therapies.

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# ESSENTIAL ROLES OF EPIGENETIC REGULATOR IN ACUTE MYELOID LEUKEMIA STEM CELLS

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Despite great progress in curing acute leukemia and malignant lymphoma, survival after relapse remains poor. The essential cause of the relapse following conventional chemotherapy is a remaining population of dormant cancer stem cells (CSCs), which is resistant to chemotherapy. Thus, selective targeting of LSCs is a promising strategy for preventing relapse. Polycomb repressive complexes 1 (PRC1) and 2 (PRC2) are important epigenetic regulators that maintain the stemness of embryonic and hematopoietic stem cells. Enhancer of zeste homolog 1 and 2 (EZH1/2) are catalytic components of PRC2, which trimethylates histone H3 at lysine 27 (H3K27) to repress transcription of target genes. Mutation and overexpression of EZH1/2 are associated with cancers, including hematopoietic malignancies.

## 1. Acute Myeloid Leukemia

We found that EZH1/2 are highly expressed in dormant CSCs and are essential for maintenance of dormant CSCs. Double deletion of Ezh1/2 induced differentiation of AML cells and complete remission of AML In AML mice models, which was not achieved by single deletion of Ezh1 or Ezh2. Ezh1/2 suppress expression of Cyclin D1/D2 to maintain dormant CSCs. Dormant CSCs dramatically reduced after deletion of both Ezh1 and Ezh2. Deletion of *Ring1A* and *Ring1B* (*Ring1A/B*) from AML cells caused the loss of self-renewal ability and induced the expression of numerous genes including *Glis2*. Overexpression of *Glis2* caused *MOZ-TIF2* AML cells to differentiate into mature cells, whereas deletion of *Glis2* expression in *Ring1A/B*-deficient *MOZ-TIF2* cells inhibited differentiation. Therefore, Ring1A/B regulate and maintain AML stem cells in part by repressing *Glis2* expression,

which promotes their differentiation. We have developed potent and specific inhibitors against both EZH1 and EZH2.

Oral administration of the EZH1/2 dual inhibitor selectively reduced the number of CSCs and prolonged the survival of the AML mice. Moreover, combination of the EZH1/2 dual inhibitor and Ara-C prolonged survival more dramatically. Taken together, these results strongly suggest that dual inhibition of EZH1 and EZH2 is a promising therapeutic strategy to eradicate CSCs in a wide range of AMLs. The EZH1/2 dual inhibitor was also effective on xenograft models of mantle cell lymphoma and multiple myeloma. Based on these results we have initiated Phase I clinical trial of the dual inhibitor in malignant lymphoma and will also start in AML presently.

### 2. Multiple Myeloma

Multiple myeloma (MM) is largely incurable as the disease eventually relapses despite the recent development of novel therapies. Previous reports show that side population (SP) cells comprise myeloma stem cells. Therefore, targeting SP cells may be a promising strategy for preventing and treating MM relapse. We found that SP cells expressed significantly higher levels of EZH1/2 than non-SP (main population) cells. These results suggest that overexpression of EZH1/2 is important for maintaining the stemness of MM cells and that EZH1/2 could be a potential therapeutic target. the EZH1/2 dual inhibitor suppressed the proliferation of almost all MM cell lines tested, with an IC50 significantly lower than that of the specific EZH2 inhibitor GSK126. Furthermore, flow cytometry analysis revealed that the EZH1/2 dual inhibitor significantly depleted the SP cell population. RNA-seq analysis revealed that the transcriptional profiles of MM cell lines treated with the EZH1/2 dual inhibitor were characterized by up-regulation of genes related to the Wnt pathway. qRT-PCR confirmed that expression of Wnt, Frizzled, and Protein kinase C family members increased markedly after exposure to the EZH1/2 dual inhibitor. Previous studies show that increased activation of the canonical Wnt signaling pathway down-regulates HSC self-renewal and differentiation. Therefore, we generated β-catenin-overexpressing MM cells to examine the effects of increased Wnt signaling on MM cells. Surprisingly, proliferation of these cells was significantly lower than that of control cells. These results suggested that PRC2 directly targets Wnt signaling, and that over-activation induced by EZH1/2 dual inhibition was responsible for the reduced proliferation of MM cells.

Oral administration of the EZH1/2 dual inhibitor to mice bearing MM xenografts led to significant impairment of subcutaneous tumors. Interestingly, long-term administration of the drug at lower doses to mice bearing orthotopic xenografts resulted in complete eradication of minimal residual disease from the bone marrow and complete cure of MM

without any serious side effects. Furthermore, THE EZH1/2 DUAL INHIBITOR treatment of an orthotopic PDX model derived from a relapsed and heavily pretreated MM patient led to a reduction in the levels of human immunoglobulins in the serum.

Taken together, these results strongly suggest that dual inhibition of EZH1 and EZH2 is a promising therapeutic approach to eradicating myeloma stem cells and could lead to important advances in the treatment of MM.

### 3. Mantle cell lymphoma

Mantle cell lymphoma (MCL) is a well-defined and aggressive type of B cell non-Hodgkin's lymphoma that is genetically characterized by the t(11;14)(q13;q32) chromosomal translocation, which results in constitutive overexpression of CYCLIN D1. Although newly developed drugs such as ibrutinib show promising clinical outcomes, relapsed MCL often acquires drug resistance, which is a critical obstacle to treatment. Alternative approaches to overcoming the drug resistance of relapsed MCL are urgently needed. We used a novel dual inhibitor of EZH1/2 to show that inhibiting EZH1/2 is a promising therapeutic strategy for MCL.

First, we developed a xenograft (PDX) mouse model using cells from a heavily pretreated and relapsed MCL patient, and then orally administered an inhibitor of EZH1/2, called THE EZH1/2 DUAL INHIBITOR. THE EZH1/2 DUAL INHIBITOR strongly impaired proliferation of the patient-derived tumors and did not cause any serious side effects. Additionally, an in vitro assay using MCL cell lines (Mino, JeKo-1, Z-138, and REC-1) showed that THE EZH1/2 DUAL INHIBITOR inhibited the growth of MCL cells, and that the effect was much more significant than that using the single EZH2 inhibitor (GSK126). The IC50 of THE EZH1/2 DUAL INHIBITOR was about one tenth that of GSK126. These results strongly suggest that dual inhibition of EZH1/2 could be a promising therapeutic strategy for relapsed MCL.

Next, to investigate the effect induced by dual inhibition of EZH1/2, we conducted further analyses of the MCL cell lines. Cells exposed to THE EZH1/2 DUAL INHIBITOR showed cell cycle arrest (G1 arrest) along with a dose-dependent reduction in phospho-Rb and cell differentiation, coupled with increased cell surface expression of hCD138. We then used RNA-seq analysis of MCL cell lines to compare THE EZH1/2 DUAL INHIBITOR-treated cells with vehicle-treated cells and found that cell cycle-related signaling was significantly affected and that a cyclin-dependent kinase inhibitor, CDKN1C (TP57), was one of the genes most markedly upregulated by THE EZH1/2 DUAL INHIBITOR. ChIP qPCR of MCL cell lines showed that the CDKN1C locus was strongly marked by H3K27 trimethylation, and that THE EZH1/2 DUAL INHIBITOR induced a significant reduction in the level of this histone marker. Furthermore, administration of THE EZH1/2 DUAL

INHIBITOR alone to PDX mice induced increased expression of CDKN1C (as in the in vitro assay). Thus, dual inhibition of EZH1/2 in MCL induces expression of CDKN1C, which in turn causes cell cycle arrest and reduced growth of MCL.

Taken together, these results strongly suggested that dual inhibition of EZH1 and EZH2 is a promising therapeutic strategy for MCL, illustrating the potential of novel epigenetic approaches to overcoming drug resistance of relapsed MCL.

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# THE NOTCH:MYC SIGNALING AXIS IN T CELL DEVELOPMENT AND TRANSFORMATION

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Notch signaling regulates many cell fate decisions in multicellular organisms. Within the lymphoid compartment, Notch induces both T cell and marginal zone B cell fates. Activating Notch mutations increase Notch signaling and within the hematolyhmphoid lineages, occur in T cell acute lymphoblastic leukemia (T-ALL) and muiltiple B cell neoplasms that include mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), splenic marginal zone lymphoma and follicular lymphoma [1-4]. Notch is a transmembrane receptor that normally interacts with a Notch ligand (Jagged, Delta-like) on an adjacent cell [5]. This interaction initiates a series of proteolytic cleavages in the extracellular domain of the Notch receptor, leading to release of the Notch intracellular domain by a gamma-secretase dependent mechanism. The cleaved intracellular domain of Notch (ICN) then translocates to the nucleus where it associates with the DNA-binding factor RBPj (also known as CSL). The Notch:RBPj complex then recruits a member of the Mastermind family (Maml), forming the Notch transcriptional complex (NTC), which recruits transcriptional co-activators leading to gene transcription.

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of immature T cells that affects both children and adults. Though current treatments cure ~70% of pediatric patients, relapsed disease is refractory to treatment and the harsh current treatments have long-term consequences. T cell transformation results from multiple genetic events that lead to dysregulated control of cellular differentiation, growth, survival and metabolism. The most frequently mutated gene in T-ALL is *NOTCH1*, which undergoes gain-of-function mutation in ~60% of tumors [6]. In T-ALL, activating Notch mutations result in either ligand-independent Notch activation and/or decreased Notch

degradation, both of which contribute to dysregulated Notch-induced transcription (Figure. 1).

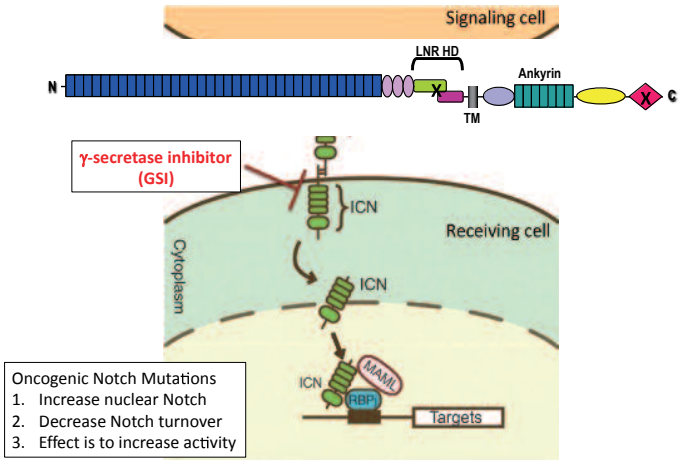


**Figure 1** Oncogenic Notch mutations in T-ALL frequently target the LNR HD domain, leading to ligand-independent signaling and/or the C-terminal PEST domain, leading to decreased nuclear Notch1 turnover. The consequence of either mutation is to increase Notrch signaling.

*MYC*, a transcription factor (TF) intimately tied with cell growth, is one of the most frequently amplified genes in multiple human cancers [7]. MYC is induced by many different growth signals, and is thought to be the master transcriptional regulator of cell growth [8]. The transcriptional program of the *MYC* oncogene comprises more than 15% of all cellular genes including regulators of cell proliferation, DNA replication, protein biosynthesis and metabolism and energy [9-11]. A key function of MYC is to recruit p-Tefb and elements of the RNA Polymerase machinery, which releases transcriptional pausing [12-14]. MYC rapidly drives transcriptional up-regulation of gene programs critical for cell growth through these processes. Despite the central role of MYC, drugs directly targeting this protein have failed for a variety of reasons, thus indirect inhibition via targeting of *MYC* activators might be a more viable strategy. [9-11].

Among the well-characterized direct Notch targets in normal and malignant pre-T cells, *Myc* is particularly important (Figure. 2). *Myc* upregulation by Notch is required for pre-T cells to traverse early developmental checkpoints [15, 16] and for the growth and survival of T-ALL cells [17-19]. Indeed, ectopic Myc expression is sufficient to rescue multiple Notch-dependent T-ALL cell lines from Notch inhibition [20]. Although initial studies showed that NTCs could bind sites within the murine *Myc* proximal promoter [17, 19, 21], both my group and the Ferrando lab recently identified a T cell-specific "Notch-dependent *Myc* enhancer" ("NDME") located ~1.3 Mb 3′ of the *Myc* promoter in mice and humans

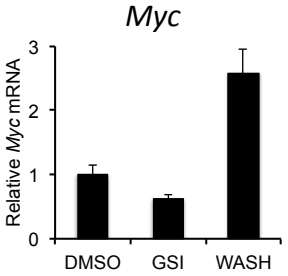


**Figure 2** Myc is a direct Notch1 transcriptional target. Results of GSI "washout" assay (see Ref Z). In the presence of GSI, Notch transcription is reduced ~2X (which is the magnitude of the oncogenic effect. Replacing the GSI medium with vehicle alone (" washout" ) causes increased Myc transcription, even in the presence of cycloheximide. See Ref. 17 for details.

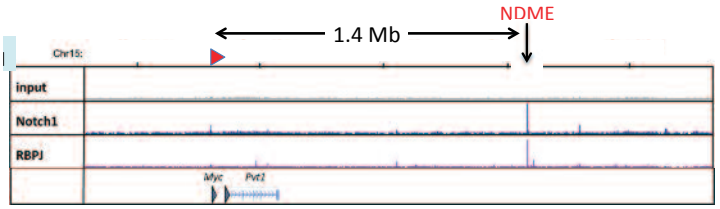


**Figure 3** The Notch Dependent Myc Enhancer (NDME) is located ~1.4 Mb 3′ from the Myc TSS. Notch1 and RBPj ChIP-Seq tracks are shown from a primary Notch1-induced T-ALL. Adapted from Ref. 23.

that is duplicated in ~5% of T-ALLs [22, 23] (Figure. 3). The NDME is located within a regulatory region spanning over 500 kb that includes multiple discrete elements marked by a high content of acetylated H3K27 (H3K27Ac), Brd4 (a bromodomain-extraterminal, or BET protein) and mediator components such as Med1, features that characterize so-called "super-enhancers", One of the far 3′ elements in this region is an enhancer that regulates *Myc* in acute myeloid leukemia cells that is sensitive to small molecule BET inhibitors such as JQ1 [24]; as a consequence, this element is termed the "BET-dependent *Myc* enhancer", or "BDME". Significantly, binding of Notch1/RBPJ complexes to the NDME upregulates H3K27Ac over a ~2 Mb region that includes the *Myc* promoter and the entire Myc 3′ regulatory region comprising the NDME and BDME [23]. Furthermore, T-ALL cell lines selected for growth in the presence of GSI maintain *Myc* expression [25] and looping between the BDME and the *Myc* promoter, suggesting that under the selective pressure of chemotherapy, the BDME can substitute for the NDME to mediate GSI resistance [23] (Figure. 4).

In summary, these data show that an evolutionarily conserved T-cell specific Myc enhancer regulates Notch-dependent Myc expression in both developing T cells and T-ALL. Although, this Myc enhancer is T-cell specific, the T-cell specificity is unlikely to

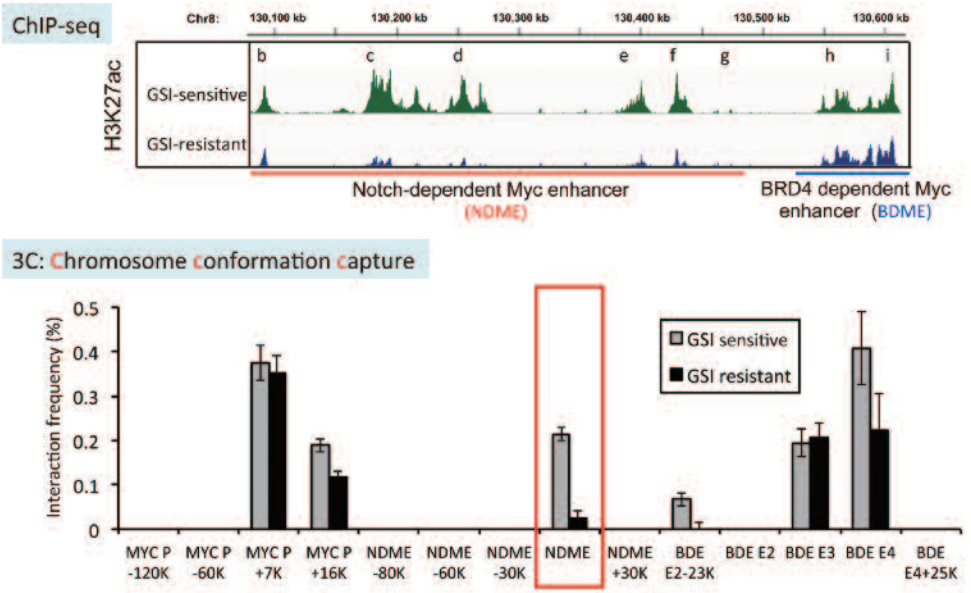


**Figure 4** GSI resistant T-ALL cells (DND41) are devoid of H3K27Ac at the NDME but not the BDME (top) and show decreased looping at the NDME but not the BDME (bottom). Adapted from Ref. 23.

occur via Notch signaling as Myc is a direct Notch target in T-ALL, mantle cell lymphoma and breast cancer [26]. This raises the question of what particular regulatory elements provide the T-cell specificity. We recently identified the Notch dependent enhancer in mantle cell lymphomas and it is distinct from the T cell NDME (data not shown). Another remaining question is how Notch regulates H3K27Ac over such a large region and whether it also regulates additional epigenetic functions. Finally, although we established that enhancer use can "switch" under the selective pressure of chemotherapy in cultured cells, whether this mechanism occurs in vivo remains to be established. Nevertheless, the studies from my group and others of the Notch:Myc axis reveal how a Notch-dependent super enhancer contributes to both normal T cell development and malignant transformation.

**Acknowledgement**: These studies were supported by grants from the NIH (P01CA119070, R01AI047833)

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# MOLECULAR MECHANISM REGULATING STEM CELL PROPERTIES MEDIATED BY NUTRIENT SIGNALS

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It has been demonstrated that shared mechanisms regulate stem cell properties in both hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs). Despite the differing origins of LSCs among different types of leukemia, there appears to be a common regulatory mechanism governing "stemness". On the other hand, understanding critical difference in regulatory manners between HSCs and LSCs is also important for development of LSC-specific therapeutic strategy. For example, there are critical differences in metabolic regulation between these cell types, because normal HSCs utilize glycolysis, whereas LSCs appear to have low glycolytic flow. Therefore, knowledge of the molecular mechanisms of metabolic regulation may provide novel therapeutic approaches for eradiation of LSCs.

Mammalian/mechanical target of rapamycin (mTOR) is a highly conserved serine/threonine kinase in response to environmental determinants such as nutrient availability, energy sufficiency, stress, and growth factor concentration (Figure 1). mTOR forms two different complexes, called mTOR complex 1 (mTORC1) and mTORC2, and these complexes have distinct substrate molecules that function in the many biological processes. mTORC1 consists of several proteins, including an essential component, Raptor, contributes to promotion of anabolic pathways, *i.e.*, enhancement of protein synthesis associated with increased energy production [1]. Several clinical studies reported that high mTORC1 activity is co-related with poor prognosis of solid tumor patients, indicating that the anabolic processes promote malignant phenotypes of cancer cells. To investigate physiological roles of mTORC1 in leukemia cells *in vivo*, we developed several mouse leukemia models based on drug-inducible Raptor deficient mice. As a result, we revealed

an indispensable role of mTORC1 in the development of T-cell leukemia (T-ALL) model driven by oncogenic Kras and Notch [2]. In contrast, in the acute myeloid leukemia (AML) model, although Raptor deletion mainly induced apoptosis in majority of leukemia cells, LSCs survived and maintained in bone marrow long-term [3]. The re-introduction of the Raptor gene into these Raptor-deficient AML cells induced them to propagate and differentiate. Thus, LSCs can survive under the mTORC1 inactivated condition, suggesting that they do not require the anabolic process for their self-renewal.

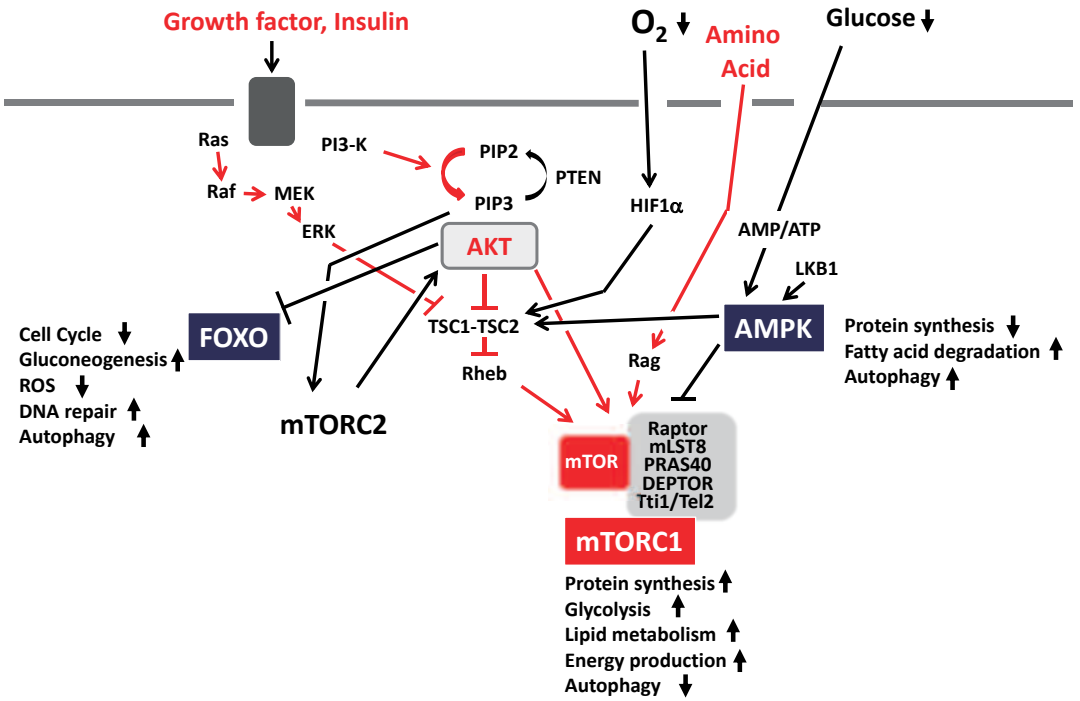


**Figure1** Nutrient-sensing signals for maintenance of LSCs.
mTORC1 is activated by growth factors and amino acids, contributing to catabolic process. In contrast, depletion of nutrients, including growth factors and glucose, induces activation of FOXO and AMPK, respectively. These nutrient sensing signals control behavior of LSCs mediated by metabolic modification.

Depletion of nutrients activates several molecules or pathways that contribute to catabolic process, including AMPK, FOXO and autophagy. Recent studies have revealed that such catabolic pathways are critical for maintenance of LSCs. FOXO family, forkhead family of transcriptional regulators, contribute to maintenance of tissue stem cells including HSCs [4]. In *C. elegans*, exposure to a harsh environmental condition such as nutrient depletion induces entry into the dauer stage, a reversible condition in which the metabolic rate is reduced and life span is markedly enhanced, mediated by activation of Daf-16, a ortholog of the FOXO family, suggesting that FOXO family members have conserved roles in stress resistance in a variety of organisms. We and other groups found that FOXO proteins play critical roles in the resistance of chronic myeloid leukemia (CML) stem cells to tyrosine kinase inhibitor (TKI) therapy [5]. Based on these findings, we have attempted to find chemical compounds that inhibit FOXO activity and investigated how pharmacological inhibition of FOXO pathways affect LSCs. We found that inhibition of FOXO pathway promotes differentiation of LSCs *in vitro* and *in vivo*, through modification of metabolic status (Figure 2). Taking together with recent knowledge about an essential role of AMPK in LSC maintenance, catabolic pathways are critical for maintenance of LSCs *in vivo*. Therefore, we believe that investigation of molecular functions in these pathways will lead to the development of successful therapeutics for eradiation of LSCs.

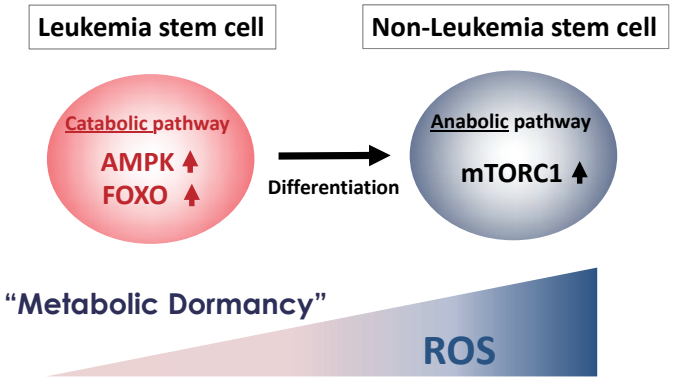


**Figure2** Critical roles of catabolic pathways in maintenance of LSCs.
LSCs are maintained with metabolic dormancy, which is characterized by cell cycle arrest, low oxidative stress and low glycolytic activity. Catabolic molecules, such as AMPK and FOXO, may contribute to the metabolic dormancy.

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# NEW STRATEGY FOR OVERCOMING MULTIFACETED THERAPY-RESISTANT CANCER STEM CELLS

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Although numerous advancements in medical science, such as the emergence of molecular biology techniques in the 1980s and the completion of the Human Genome Project in the early 21st century, have led to substantial progress in the treatment of cancer; however, cancer-related deaths are still increasing. Despite much effort and attention directed toward cancer treatment, cancer survivors still suffer from poor quality of life, even in highly developed countries such as Japan, where this is exacerbated by an increase in population aging. International pharmaceutical companies have developed numerous anticancer agents using molecular targeting strategies and immunotherapies, but resistance develops even for these novel medicines. One of the most important factors that make the present therapeutic strategies ineffective is tumor heterogeneity. It is now widely accepted that a small population of cells within tumors not only contribute to therapy resistance but also to invasion and metastasis, and represents a main determinant of disease recurrence. Several recent studies have revealed that these small populations of cells possess multifaceted characteristics, including self-renewal capacity and multi-functional potential, aspects that are not present in well-differentiated cancer cells; for at least two decades, such critical cells have been referred to as cancer stem cells (CSCs). In order to visualize and collect CSC fractions, we transfected cancer cells with a green fluorescent protein-fused ornithine decarboxylase (ODC) monitoring cassette. This monitoring system allowed visualization of cancer cell populations containing therapy-resistant CSCs. The study revealed that polyamine flux plays a critical role in CSC functions, and polyamine metabolism is linked to epigenetic regulation of downstream gene expression. Global profiling studies have revealed that transcription factors are involved in the maintenance

and function of CSCs. Epigenetic studies, on the other hand, revealed uncharacterized transcriptional mechanisms and underscored the significance of molecular profiling in the discovery of novel therapeutic targets for therapy-resistant cancer cells [1-3]. The novel approaches may open new avenues, especially for treating gastrointestinal cancers that pose challenges for treatment.

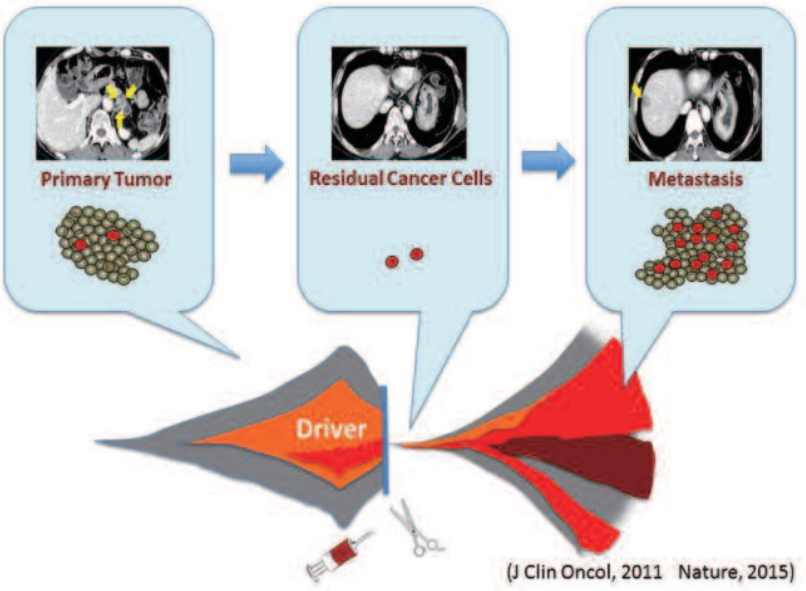


**Figure 1** Therapy-resistant Cancer Cells
Primary tumor heterogeneity is characterized by the presence of actively growing cancer cells and a small population of dormant CSCs in addition to supportive stromal cells. After administration of anticancer agents and/or surgery, complete tumor remission may be achieved, but therapy-resistant CSCs still remain. In this process, tumor-driving mutations accumulate, and after some period of time, the tumor relapses and metastasizes to other organs.

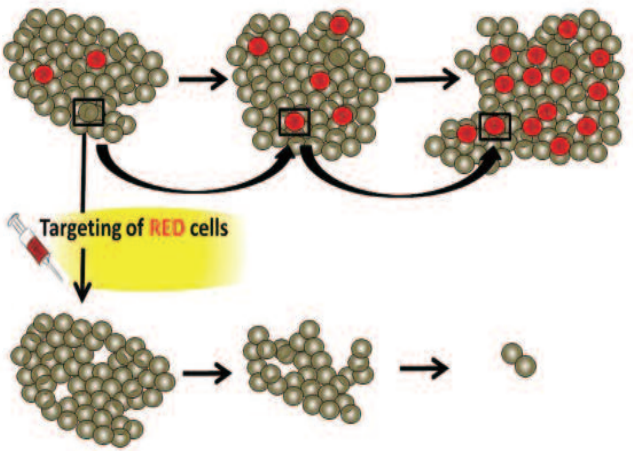


**Figure 2** Therapeutic Strategy against Dormant Slow Cycling Cancer Cells
In tumors, a small fraction of cancer cells (marked red in the figure) proliferate at a slow rate. The elimination of such cells would result in the suppression and eradication of tumors. Thus, the development of novel anticancer agents against slow cycling CSCs is highly beneficial to achieve better prognosis through effective cancer treatment.

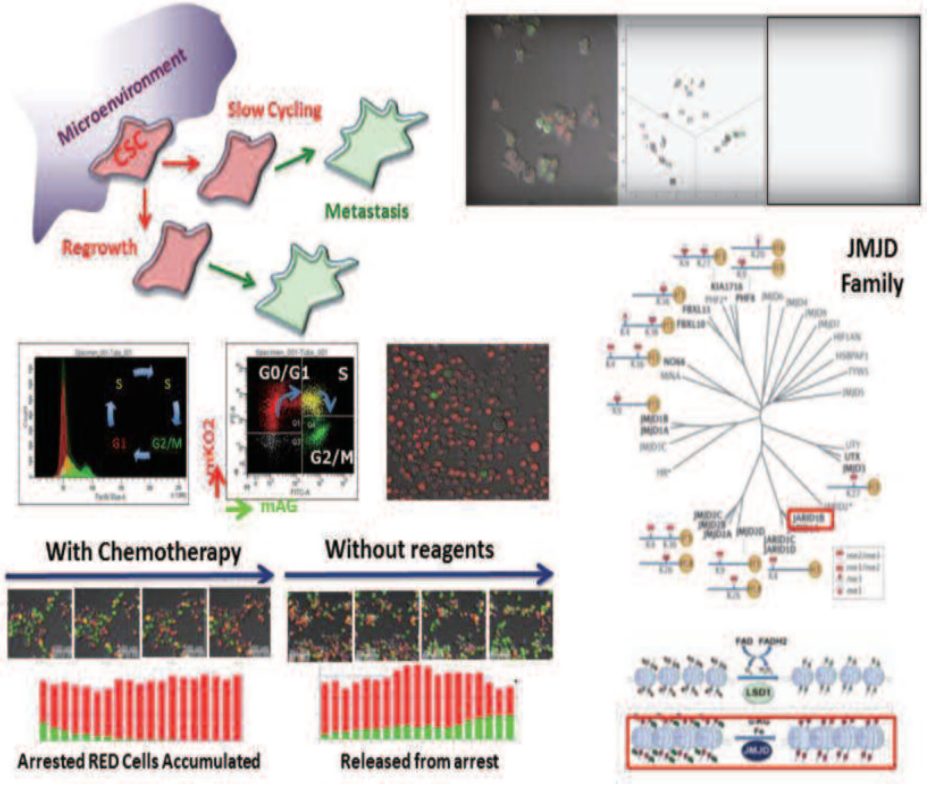


**Figure 3** Single Cell Tracking of Therapy-resistant Cancer Cells
The single cell tracking study indicated that exposure to chemotherapeutic agents results in the accumulation of dormant or slow cycling cancer cells (shown in red). This approach allowed the identification of the Jumonji domain-containing histone demethylase family KDM5/Jarid1, which removes tri- and dimethyl groups from lysine 4 on histone H3.

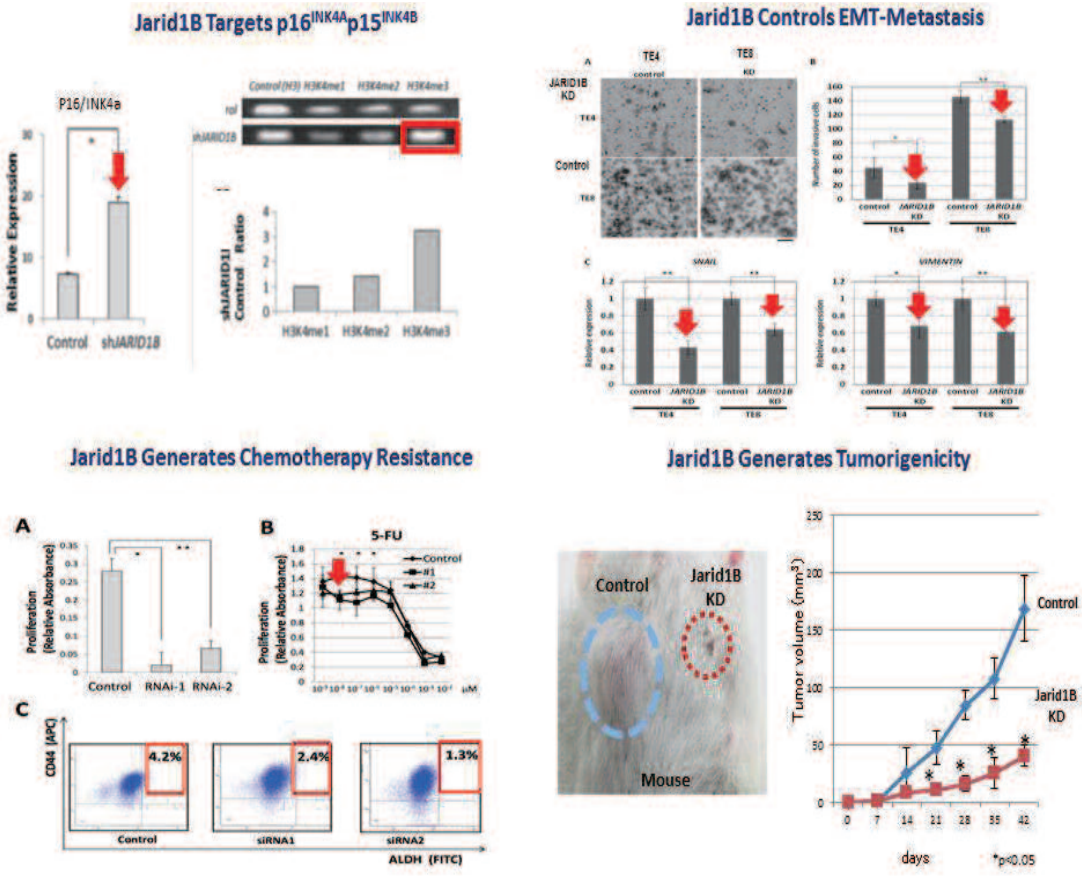


**Figure 4** Jarid1B, a Therapeutic Target for Slow cycling Cancer Cells
Knockdown of Jarid1B using shRNA resulted in the induction of H3K4 tri-methylation and stimulation of p16/INK4A gene expression (upper left). Inhibition of Jarid1B translation also led to a decrease in the CD44+ cancer stem cell fraction, sensitization of cells to the anti-cancer agent 5-fluorouracil (FU) (lower left), and suppression of the epithelial mesenchymal transition (EMT) markers SNAIL (lower right), and VIMENTIN (upper right). The inoculation of Jarid1B-deficient cancer cells in immunocompetent mice resulted in the suppression of tumors in vivo. These data indicate that suppression of Jarid1B expression might be efficient for the eradication of therapy-resistant cancer stem cells.

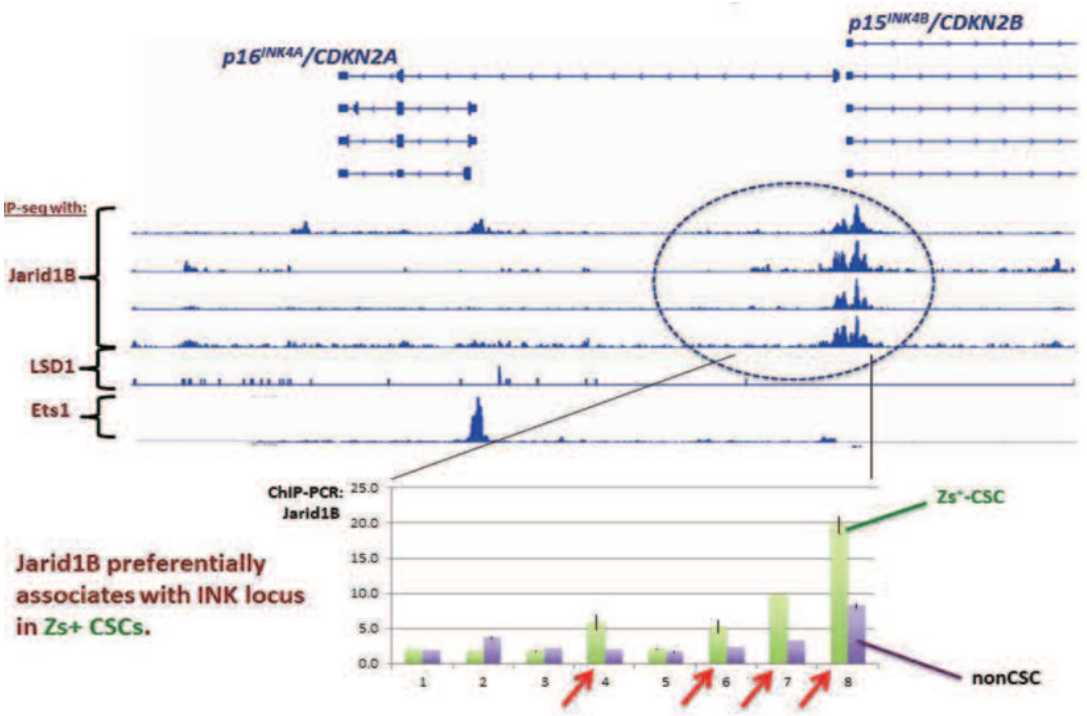


**Figure 5**

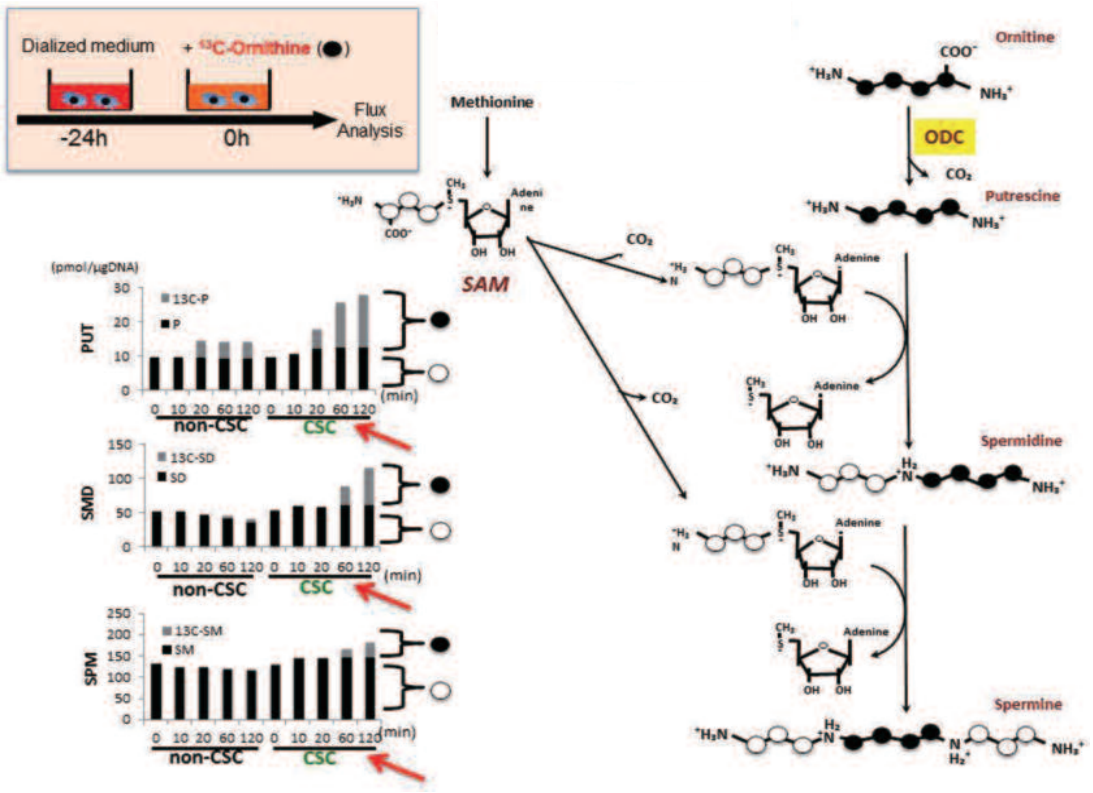


**Figure 6**

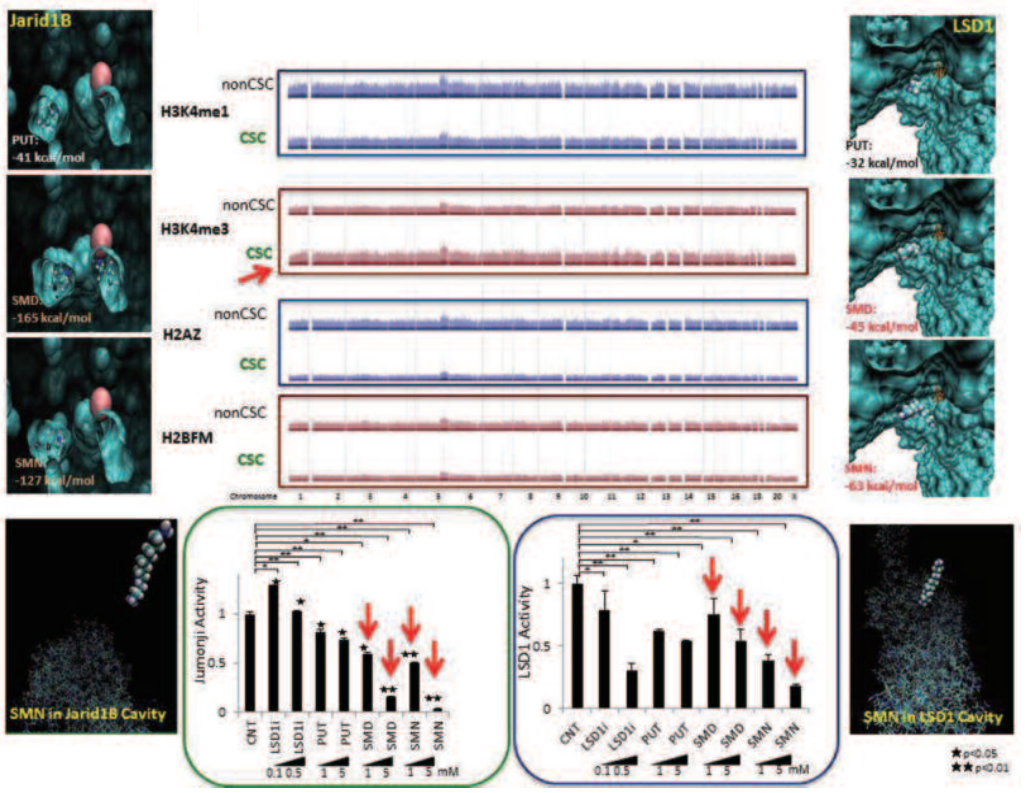


**Figure 7**

**Figure 5** *INK* Locus in H3K4 Demethylases
The chromatin immunoprecipitation study indicated that Jarid1B interacts with the promoter region of the p16/INK4A gene, whereas LSD1 and Ets proteins do not. The association with the promoter was particularly high in $Zs^{+}$-CSCs (Green).

**Figure 6** Preferential Activation of the ODC Pathway in CSCs
The study of metabolic labeling with non-radioactive isotopes indicated an increased polyamine flux in ODC-expressing CSCs compared with non-stem cancer cells (control), suggesting the importance of polyamine flux in the regulation of one-carbon metabolism in CSCs.

**Figure 7** H3K4 methylation is Increased in $Zs^{+}$-CSCs
Free energy calculations by computer simulation of Jarid1B and LSD1 binding interactions indicated that both these proteins have the ability to bind to polyamines, suggesting that polyamines could interfere with the demethylation reactions of these enzymes.

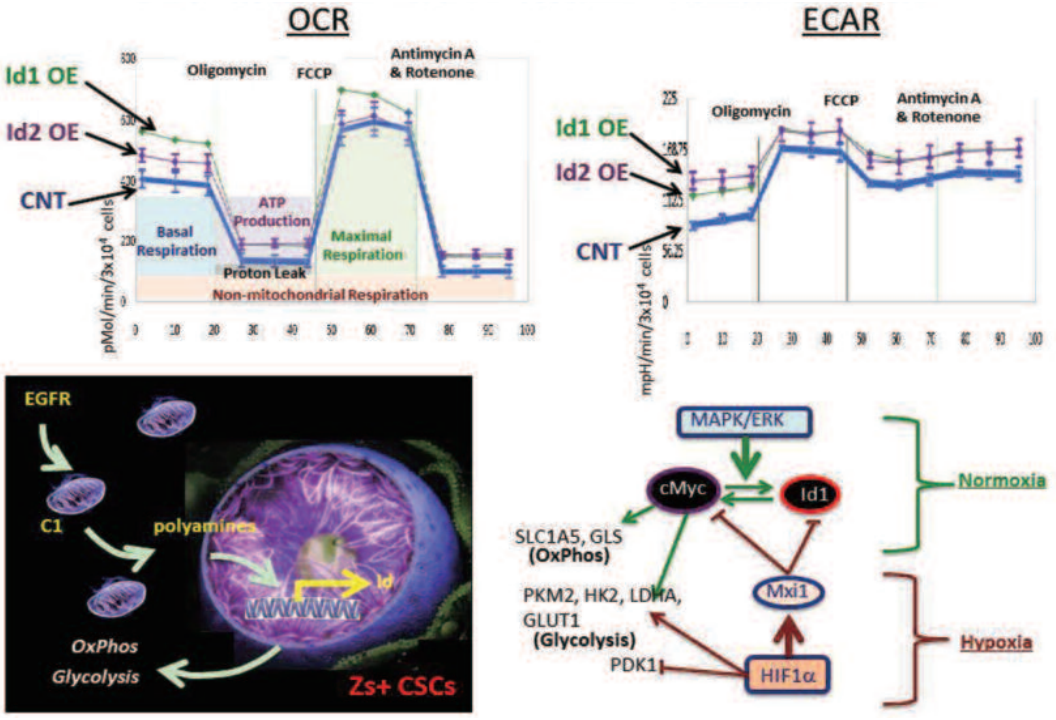


**Figure 8**

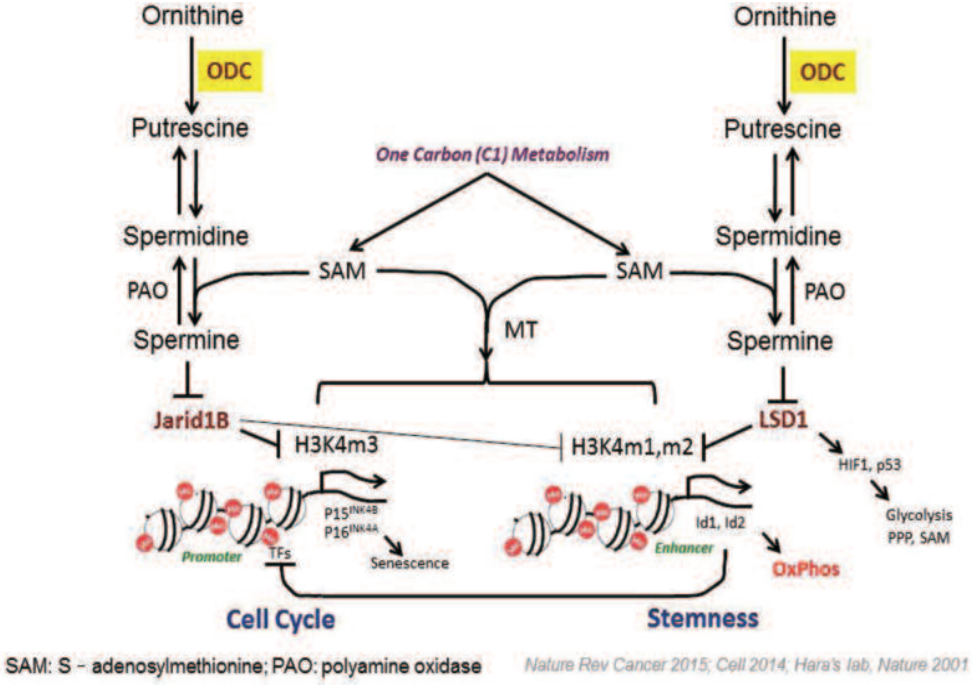


**Figure 9**

**Figure 8** Id Stimulates Cancer Metabolism in $Zs^{+}$-CSCs
The present study allowed the identification of Id genes that play a critical role in the maintenance and progression of cancer stem cells by modulating mitochondrial respiration and glycolysis. Extracellular stimuli such as epidermal growth factor (EGF) stimulate the flux of polyamines into the one-carbon metabolic pathway, contributing to the specific metabolism of cancer stem cells. The association between Id and c-Myc may be important for this process.

**Figure 9** *Double-Faced*, Metabolite-dependent Epigenetic Response in Therapy-resistant Cancer Stem Cells
The epigenetic marking of the fourth lysine of histone 3 (H3K4) is important for the maintenance and progression of cancer stem cells. Jarid1B contributes to the control of demethylation at the promoter of the p16/INK4A gene, a cell cycle regulator. LSD1 in turn binds to multiple enhancer sites and controls the expression of many genes, including the Id family, which alters tumor metabolism by modifying oxidative phosphorylation (OxPhos) in CSCs. The present study identifies novel druggable targets for cancer stem cell therapy and suggests that the discovery of small new compounds acting on the KDM family provides a breakthrough in the treatment of CSC-related refractory cancers thereby significantly improves the patients' clinical outcome.

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(See http://www001.upp.so-net.ne.jp/CancerProfiling/index.htm#, for the previous publications)

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# THE CANCER STEM CELL NICHE OF SQUAMOUS CELL CARCINOMAS: BIOLOGY AND IMPACT FOR THERAPEUTICS

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Adult tissue stem cells have the ability to self-renew long term and differentiate into one or more tissues. Many stem cells are used sparingly to replenish cells during normal homeostasis. However, even stem cells that are quiescent must be able to respond quickly to injury in order to fuel rapid tissue regeneration. How stem cells balance self-renewal and differentiation is of fundamental importance to our understanding of normal tissue maintenance and wound repair. Increasing evidence suggests that the regulatory circuitry governing this balancing act is at the root of some types of cancers.

The hair follicle is an excellent model system to understand how stem cells remain quiescent during times of minimal wear and tear, how these cells become mobilized during the cyclical bouts of hair growth and wound-repair, and how the normal process of stem cell activation goes awry in cancer. Hair follicles have the remarkable capacity to undergo cycles of growth (anagen), degeneration (catagen) and rest (telogen). In mice, the first several cycles are synchronized, making hair follicles an ideal system for understanding how stem cells interact with progeny and heterologous cell types in their niche to transition between quiescence and active tissue (hair) regeneration.

The quiescent hair follicle stem cells reside in a single layer of the bulge niche, located at the bottom of the non-cycling portion of the hair follicle. Within the bulge niche is a layer of BMP-expressing differentiated cells that anchor the hair and transmit inhibitory signals to maintain stem cell quiescence. The stem cells adhere to a basement membrane flanked by a dermal sheath. Beneath the bulge is a mesenchymal stimulus called the dermal papilla. Stem cells at the base of the bulge, closest to this stimulus are the first to be activated at the start of the hair cycle. These cells communicate with the dermal papilla.

As Valentina Greco, a former postdoc in my lab, now at Yale, showed, the 'primed' stem cells make increasing levels of WNTs; the dermal papilla makes increasing levels of BMP inhibitory signals, and together these two signals are needed to overcome the inhibitory BMP signal that otherwise keeps the stem cells in quiescence.

When the activating threshold is reached, the stem cells at the base of the bulge begin to proliferate and generate short-term progeny called matrix cells. The first group of these cells produces Sonic Hedgehog (SHH). As shown by Ya-Chieh Hsu, a former postdoc in my lab, now at Harvard, showed, SHH then acts in two ways: first it acts on the dermal papilla, which elevates BMP inhibitors and pro-activating FGFs; second, it acts on the quiescent upper bulge stem cells, which self-renew and produce a cellular shaft (the outer root sheath) which pushes the SHH-DP signaling center downward, resulting in a return of the bulge to quiescence until the next hair cycle. Meanwhile the signaling center fuels the proliferation of the short lived progeny to produce the hair and its channel, the inner root sheath. After a period of hair growth, the lower two thirds of the hair follicle differentiates and degenerates, bringing the DP upward to make contact again with the bulge. In this way, the hair follicle becomes self-propelling to undergo these cyclical bouts of activity.

Over the years, we developed methods to purify the bulge stem cells and their progeny and we carried out RNA-seq on these populations straight from fluorescence activating cell sorting (FACS). The stem cells expressed high levels of SOX9, LHX2, NFATc1, FOXC1, IDs, TCF3 and TCF4 transcription factors, which distinguished them from their committed counterparts and from the epidermal stem cells residing in the basal layer of the epidermis. We used conditional knockout technology to ablate each of these factors in the skin. Our findings showed that some factors, e.g. NFATc1, IDs and FOXC1, acted downstream from BMP signaling, and promoted stem cell quiescence. We found that TCF3/4 antagonize WNT-beta catenin to repress HF fate differentiation, while LHX2 suppresses sebaceous gland differentiation and SOX represses epidermal differentiation. In carrying out a combination of in vivo chromatin immunoprecipitation and high throughput DNA sequencing (ChIP-seq) with antibodies against not only the transcription factors but also epigenetic marks including H3K27ac, Med1, H3K27me3, H3K4me1, H3K4me3 and H3K36me2, we identified ~350 HFSC genes which are controlled by large open chromatin domains, or 'super-enhancers,' a word coined by Rick Young at MIT. Within these super-enhancers are small regulatory elements, which we call 'epicenters,' that contain clustered binding sites for the cohort of HFSC transcription factors. In work carried out by one of my graduate students, Rene Adam, we've cloned these regulatory elements and tested them in mice and found that they act to target reporter gene expression to the bulge stem cells.

In looking at the endogenous group of HFSC genes regulated by super-enhancers, we found that they encode the majority of the stemness genes, including the genes regulating

SOX9, TCF3/4 and LHX2 as well as Integrins alpha 6 and beta4, LGR5, BMP6 and the receptors for BMPs, WNTs and FGFs. There are many other human disease genes that are found on this short-list comprising ~5% of the total number of genes expressed by HFSCs.

Intriguingly, when HFSCs were cultured, their chromatin landscape changed dramatically as a new cohort of super-enhancers were gained while others were lost. In seeking mechanisms, we discovered that many HFSC transcription factor genes were silenced in vitro while new wound-induced transcription factors appeared. Moreover, the new super-enhancers were regulated by these wound-induced factors, corresponding to the new proliferative migratory state of the cultured hair follicle stem cells.

This work, led by Rene Adam and Hanseul Yang in my lab, gave us new insights into the sensitivity of stem cells to their microenvironment and the importance of niche-stem cell interactions in dictating stem cell behavior. Hanseul along with Daniel Schramek, a former postdoc in my lab now at the Lunefeld Cancer Center in Toronto, then asked the question of how the chromatin landscape changes as hair follicle stem cells acquires the mutations that lead to malignant progression. When they carried out ChIP seq analyses on the tumor-initiating (stem) cells of squamous cell carcinoma chromatin, they unearthed new super-enhancers regulating oncogenes such as Src and Myc. These super-enhancers were regulated by a new cohort of transcription factors whose genes were also regulated by super-enhancers, thereby generating a feed forward loop. Notably were ETS2 and ELK3, because these super-enhancer regulating transcription factors are known to be phosphorylated and activated by RAS-MAPK, the pathway upregulated in the SCCs. Indeed, mice engineered to express a phosphomimic ETS2, the epidermis became hyperproliferative, invasive and generated a chromatin landscape that was similar to the SCC state. Again, these findings underscored the importance of a now new microenvironment characteristic of the SCC state.

Finally, we looked at how the microenvironment changes during malignant progression. We discovered that wherever blood vessels invade the stroma and approach the tumor, the tumor-initiating cancer stem cells at the tumor-stroma interface respond to TGF-beta brought in by the perivasculature, rich in immune cells such as monocytes. The outcome is striking—these stem cells become slow-cycling and break down the basement membrane and invade the stroma. By contrast, stem cells more distant from blood vessels proliferate rapidly, generating the bulk of the tumor. Does this matter? To test this possibility, we treated mice with cisplatin, the drug of choice for many human squamous cell carcinomas. Most of the tumor cells died, except for those cancer stem cells responding to TGF-beta. Moreover, these stem cells evaded the chemotherapy and regrew the cancer when cisplatin was washed out. This was the work of Naoki Oshimori, who has now moved on to start his own lab at Oregon Health Science Cancer Center. The studies

beautifully underscore the importance of heterogeneity in the tumor microenvironment.

I don't want to leave you with the notion that genetic variation in tumor progression doesn't matter. Clearly, it does, as there are hundreds of mutations in these solid tumors. To this end, we've used our powerful in utero lentiviral technology to carry out in some cases, genome-wide screens for oncogenic drivers, tumor suppressors and oncogenic microRNAs involved in SCC progression. Together, these multipronged approaches should be useful in our ever growing endeavors to understand the underlying basis for squamous cell carcinomas, which are not only one of the most common but also one of the most life-threatening cancers world-wide.

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# EVIDENCE AND MECHANISM FOR THE TRANSDIFFERENTIATION FROM LUNG ADENOCARCINOMA TO SQUAMOUS CELL CARCINOMA

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Lineage transition between lung adenocarcinoma (ADC) and lung squamous cell carcinoma (SCC) as implicated by clinical observation of mixed adenomatous and squamous pathologies in lung adenosquamous cell carcinoma (Ad-SCC), remains as a fundamental and yet unsolved question. We provide the first in vivo evidence showing the ADC to SCC transdifferentiation (AST) in three mouse models based on Lkb1 homozygous deletion: the Kras/Lkb1 (KL) adeno-Cre nasal inhalation mouse model (Figure 1), the Kras/Lkb1/Spc-CreERT2 mouse model as well as Kras/Lkb1 lung ADC serial transplantation in nude mice [1]. In KL mouse model, we find that Lkb1-deficient lung ADC can progressively transdifferentiate into SCC, via a pathologically mixed Ad-SCC intermediate. Our data show that lysyl oxidase (Lox) plays an important role in regulating the AST process. LOX is originally up-regulated by Lkb1 inactivation in mouse lung ADC, which is known to promote collagen deposition, tumor malignant progression, and even lung cancer metastasis [2]. However, LOX expression is significantly down-regulated during the AST process, which results in the reduction of collagen deposition and extracellular matrix (ECM) remodeling. Pharmacological LOX inhibition promotes the AST process, whereas ectopic Lox expression significantly inhibits this process [1].

We further find that decreased ECM potentially causes the nuclear exportation of YAP, the major effector downstream of Hippo pathway, which in turn results in down-regulation of ZEB2 expression [3]. Interestingly, ZEB2 is intrinsically activated to repress

DNp63 transcription in lung ADC in a default manner. During the AST process, YAP is inactivated, which in turn relieves ZEB2-mediated default repression of DNp63 and triggers squamous transdifferentiation. Our data show that overexpression of DNp63

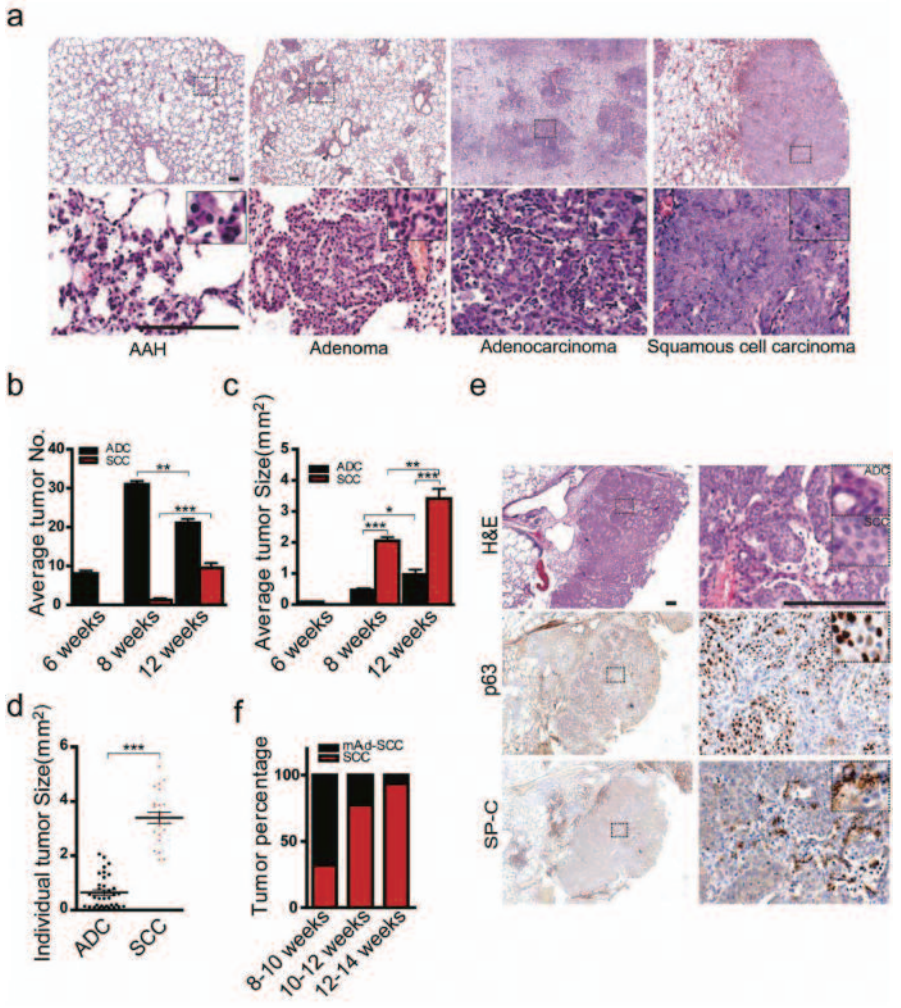


**Figure 1** *Lkb1*-deficient Lung ADC progressively Transdifferentiates into SCC.
(**a**) Representative histology for AAH, adenoma, ADC and SCC lesions in *Kras*/*Lkb1* mice at a serial of time points post Ad-Cre treatment. Scale bar, 150 μm. (**b** and **c**) Quantification of average tumor number (**b**) and tumor size (**c**) per mouse for ADC and SCC in *Kras*/*Lkb1* mice at 6 weeks (n=6), 8 weeks (n=12) and 12 weeks (n=8) post Ad-Cre treatment. Data were shown as mean ± s.e.m. *P <0.05, **P < 0.01, ***P < 0.001. (**d**) Quantification of individual tumor size for ADC and SCC in *Kras*/*Lkb1* mice at 10-12 weeks post Ad-Cre treatment. Data were shown as mean ± s.e.m. *P <0.05, **P < 0.01, ***P < 0.001. (**e**) Representative mAd-SCC shown with H&E staining, p63 and SP-C immunohistochemical staining on serial sections. Scale bar: 150 μm. (**f**) Quantification of tumor percentage for mAd-SCC and SCC in *Kras*/*Lkb1* mice at indicated time points post Ad-Cre treatment. n=8 for each time point. Scale bar: 150 μm. Data were shown as mean ± s.e.m. *P <0.05, **P < 0.01, ***P < 0.001.

alone is able to partially drive the squamous transdifferentiation. YAP knockdown significantly accelerates squamous transdifferentiation, whereas constitutive YAP activation conversely inhibits this transition (Figure 2). More importantly, ectopic DNp63 expression rescues the inhibitory effect of YAP on squamous transdifferentiation. These

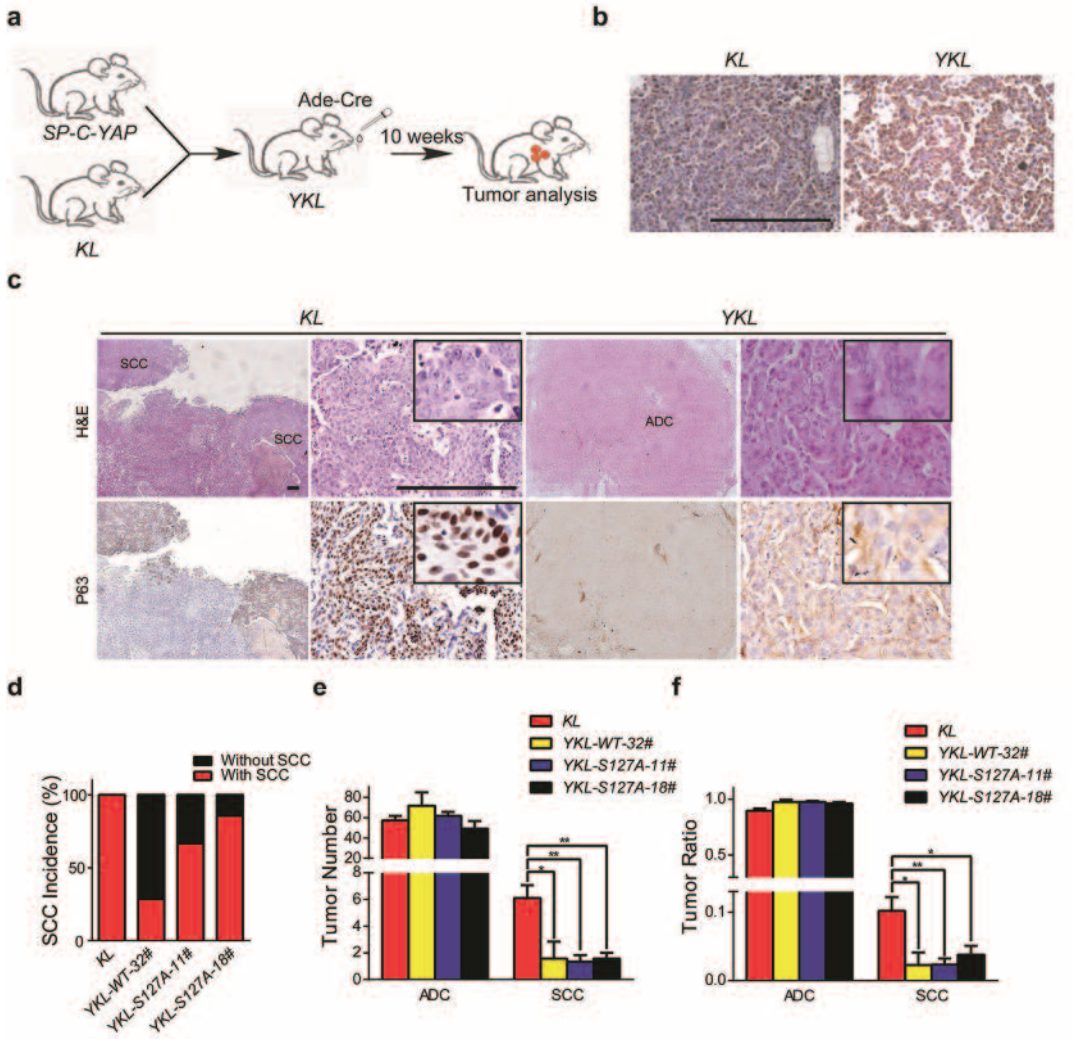


**Figure 2** Lineage-specific YAP expression inhibits ADC to SCC transdifferentiation.
**(a)** Scheme of Ad-Cre treatment in established *YKL* mouse model. **(b)** Yap immunohistochemical staining in lung tumors from *KL* or *YKL* mice at 10wks post Ad-Cre treatment. Scale bar, 150 μ m. **(c)** Representative images for H&E and p63 immunohistochemical staining in *KL* or *YKL* mice at 10wks post Ad-Cre treatment. Scale bar, 150 μ m. **(d)** Quantification of SCC incidence of *KL* (n=9) or *YKL* mice from three *SP-C-YAP* transgenic lines including *YKL-WT-32#* (n=7) , *YKL-S127A-11#* (n=6) and *YKL-S127A-18#* (n=7). **(e-f)** Tumor number **(e)** and tumor ratio **(f)** quantification for ADC and SCC from *KL* (n=9) or *YKL* mice from three *SP-C-YAP* transgenic lines including *YKL-WT-32#* (n=7), *YKL-S127A-11#* (n=6) and *YKL-S127A-18#* (n=7). Data are shown as mean ± SEM.*, $P$ <0.05; **, $P$ <0.01.

findings establish YAP as an essential barrier for lung cancer cell fate conversion.

It intrigues us that the downstream signaling triggered by LKB1 inactivation such as LOX up-regulation and collagen deposition is conversely shut down during the AST process. We find that the oxidative stress triggered by LKB1 inactivation significantly contributes to the AST process and regulates cancer plasticity [4]. A differential reactive oxygen species (ROS) level is observed in lung ADC and SCC from KL model. We find that down-regulation of ROS level through N-acetyl cysteine (NAC) treatment or NRF2 expression inhibits the AST process, highlighting the functional importance of ROS in regulating cancer plasticity. Our data further show that pentose phosphate pathway deregulation and impaired fatty acid oxidation collectively contribute to the redox imbalance and functionally affect the AST process. Similar tumor and redox heterogeneity are also found in human LKB1-inactivated lung cancer. These data thus uncover a critical redox control of tumor plasticity which contributes to the AST process through the signaling reversal.

Interestingly, we find that phenformin treatment, known to effectively inhibit lung ADC progression in KL model, promotes the AST process and results in the development of drug resistance [4]. This indicates that the AST process might represent a novel cellular mechanism for drug resistance. Our work in animal models is further supported by recent clinical observation about the potential link between AST and drug resistance [5]. Up to now, a total of 11 cases of EGFR-mutant lung ADC patients have been reported: all of them are shown to have ADC from first biopsy and initially respond to tyrosine kinase inhibitor (TKI) or chemotherapy very well; however, after the relapse, the second biopsy shows the squamous pathology frequently maintaining the same TKI-sensitive EGFR mutations [5].

With the efforts to overcome drug resistance conferred by AST, we have established over 20 patient derived xenograft (PDX) models in past years and found one with LKB1 inactivating mutation. This LKB1-mutant PDX model develops drug resistance towards phenformin treatment, potentially through up-regulation of Wnt pathway. This leads us to develop the combinational treatment of JW-55, the Wnt pathway inhibitor, together with phenformin. We find that this combinational treatment efficiently inhibits the LKB1-mutant tumor growth in PDX model.

In summary, we have provided convincing evidence in supporting the AST in animal models. Our data have further provided important mechanistic insights into the process of AST and indicated the AST might represent a novel mechanism for drug resistance. Moreover, we have provided a potential therapeutic strategy for overcoming drug resistance in linking to the AST process.

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# DYNAMIC COMPLEXITY OF GLIOMA STEM CELLS

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Recent studies have redefined the designation of gliomas, based on molecular classification using mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2), chromosome 1p and 19 co-deletion, and mutation of ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked). Collectively, these mutations appear to resolve adult gliomas into three different disease states: glioblastoma (wild-type for IDH1/2, with the worst prognosis), astrocytomas (harboring mutations in IDH1 or IDH2 and ATRX, with an intermediate prognosis), and oligodendrogliomas (harboring mutations in IDH1 or IDH2 and co-deletion of chromosomes 1p and 19, with a relatively better prognosis). In pediatric gliomas, there have been additional driver mutations in histone variants 3.1 and 3.3, as well as fusion events in BRAF. Collectively, there are now a series of distinct diseases within the overall rubric of gliomas, which display differences in underlying biology, response to therapy, and prognosis. Within the most common tumor in adults, the glioblastoma, there have been numerous attempts to better separate patients based on transcriptional profiling and other molecular events (e.g. MGMT promoter methylation), based these efforts have had limited impact on clinical practice.

Against this background, it is useful to consider the role of the tumor cell hierarchy in tumor biology and patient outcome. Gliomas and other brain cancers were among the early cancer types for which cancer stem cells were defined. There have been both substantial advances and challenges in the field. One area of (unnecessary) conflict has been in the definition of the cell-of-origin in gliomas. Numerous genetically engineered mouse models have shown that different cells may yield gliomas, based on molecular drivers. While the eventual biology of tumors may reflect the cell-of-origin, it remains

unclear how these findings will be reflected in the tumor hierarchy. Glioblastomas commonly contain a subset of tumor cells that are self-renewing, express stem cell markers, and are tumorigenic when transplanted into recipient hosts. Many investigators have used these criteria as sufficient to define cancer stem cells, but this fails to address the presence of a hierarchy of tumor initiation. Prospective enrichment and depletion using markers has been used, but is controversial due to the lack of uniform markers across all tumors, but CD133 (Prominin1) has been the most widely used. Although not defining characteristics, we and others have shown the brain tumor stem cells also exhibit a number of other phenotypes that are clinically relevant, including invasion into normal tissues, promotion of tumor angiogenesis, and modulation of the immune system [1, 2]. Previously, we also showed that glioma stem cells display a relative resistance to radiotherapy, the most effective non-surgical treatment for glioblastoma [3]. Our original report described a mechanism linked to activation of the DNA damage checkpoint, but subsequent studies from our lab and others has enriched these observations to include Notch activation, PARP activation, and recruitment of meiotic repair mechanisms [3-7]. Therefore, glioma stem cells appear to a potential important population for designing therapeutic paradigms to improve the outcome of patients.

Glioblastomas, like most cancers, contain regions that are stressful to the inhabiting cellular populations, due to the presence of hypoxic, acid, and limited nutrient availability. We have previously shown that each of these aspects can both maintain glioma stem cells and induce the acquisition of stem-like features in more differentiated progeny [8-11]. The hypoxic response in glioma stem cells is accompanied by the specific induction of HIF2 at both the transcriptional and post-translational regulatory levels, but tumor propagation depends on the expression of the expression of both HIF1 and HIF2 [9]. Acidic stress is an even stronger effector of cancer stem cell maintenance than hypoxia and also induces HIF2 [10]. Glucose levels in standard cell culture conditions is designed to promote cellular proliferation, but when we used physiologic levels of glucose (approximately 10% of standard levels), we found that there was both a relative survival of the glioma stem cells and an acquisition of stem-like features in differentiated tumor cells [11]. We hypothesized that glioma stem cells may have a competitive advantage in extracting glucose from the microenvironment. We therefore differentially labeled glioma stem cells and differentiated gliomas cells with fluorescent reporters, implanted them into brain slices and administered fluorescent glucose. Glioma stem cells display a strong preferential uptake, which we subsequently linked to the expression of GLUT3, a high affinity glucose transporter normally expressed by neurons. Targeting GLUT3 expression depletes glioma stem cells and GLUT3 expression is a negative prognostic factor for glioblastoma patients [11]. In unpublished studies, we have performed glucose tracing experiments that have revealed a

strong enrichment of de novo purine biosynthetic intermediates (data not shown). We found that the entire pathway is up-regulated and expression of the components in this pathway portend a poor prognosis (data not shown). Selective uptake in glioma stem cells extends beyond glucose, as we have found that transferrin, transferrin receptor and the ferritins are preferentially expressed in glioma stem cells with strongly increased iron uptake and dependence on the uptake and storage of iron [12]. In additional studies of glioma stem cell metabolism, we find that glioblastomas display strong transcriptional regulation of enzymes that act to deplete the available methyl donors, with preferential expression in the glioma stem cell population (data not shown). Targeting these pathways attenuates tumor growth, whereas depleting the upstream source of methyl donors (methionine) accelerates tumor growth (data not shown). Finally, the overall structure of the mitochondria differs between the stem and differentiated tumor cell populations. Mitochondria in glioma stem cells are fragmented, whereas differentiated progeny contain tubular mitochrondria [13]. This process is driven by differential post-translational regulation of the essential mediator of mitochondrial fission, DRP1 (dynamin-related protein 1). In glioma stem cells, DRP1 is activated by CDK5 (cyclin-dependent kinase 5), whereas DRP1 is inhibited in differentiated tumor cells by CAMKII (Ca2+/calmodulin-dependent protein kinase II) family members [13]. Genetic or pharmacologic targeting of DRP1 disrupts glioma stem cell maintenance, whereas normal brain cells may be better able to survival stress with DRP1 inhibition, suggesting that DRP1 may be a therapeutic target with a high therapeutic index [13]. Collectively, these results and additional ongoing studies suggest that glioma stem cells display differential metabolic behaviors that offer potential nodes of fragility to be exploited by novel therapeutic paradigms.

Normal tissue-specific stem cells may reside in multiple niches, either because there are multiple pools of stem cells or progeny with plasticity of differentiation state. In parallel, in several studies we and others have found that brain tumor stem cells reside in at least two distinct niches: a perivascular niche and a hypoxic niche in necrotic regions. In fact, we find that necrotic regions offer the most effective areas for glioma stem cell derivation (unpublished observations). Vascular proliferation represents a histologic criterion for glioblastoma, and the initial discovery of VEGF (vascular endothelial growth factor) was in part in glioblastoma. We previously demonstrated that glioma stem cells selectively promote tumor angiogenesis due to increased VEGF secretion, which was validated by others and extended to include CXCRL12 (C-X-C motif chemokine 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF1)) secretion [1]. Subsequently, other groups have shown that glioma stem cells may contribute structurally to the tumor vasculature through transdifferentiation towards endothelial cells. However, in collaboration with Shideng Bao, we found in lineage tracing and targeting studies that glioma stem cells rarely

transdifferentiate towards endothelial cells, but rather become pericytes through attraction by CXCL12 and then fate transition due to TGF-beta (transforming growth factor-beta) [14].

Based on the recognition that the environment is critical to define glioma stem cell biology, we have generated glioma organoids [15]. These organoids display structural complexity with an outer region with proliferating cells and frequent SOX2-positive cells and an inner region with little proliferation and fewer SOX2-positive cells. Radiation treatment of organoids caused preferential cell death in the outer region with only SOX2-negative cells undergoing cell death. Implantation of organoid-derived tumor cells into immunocompromised mice recapitulates the histology of the original tumor from which they were derived much better than matched tumorspheres grown in serum-free conditions. Glioblastomas display regional variance in histology, radiographic appearance, and therapeutic response. Therefore, we have derived tissues from different regions in patient tumors, revealing distinct organoids with histology maintained upon implantation.

Based on the importance of the in vivo environment, we performed an inducible shRNA screen for chromatin modifiers (manuscript under re-review) in matched orthotopic brain tumors and identical cells grown under stem cell (sphere) conditions. Surprisingly, there were fivefold more hits in vivo than in vitro, and the identity of the hits was almost completely non-overlapping. The in vivo hits converged onto the enhancer-mediated pause-release/elongation initiation complex. Matched RNA sequencing (RNA-seq) revealed in vitro transcriptional profiles aligned with proliferation and metabolism in vitro, whereas in vivo profiles show evidence of cell-cell and microenvironmental interactions. Super enhancer analysis reveals distinct regulation of critical loci based on growth conditions and reveal selective dependencies of tumor cells in vivo. These studies raise the specter that discovery efforts in vitro may not only miss critical tumor maintenance factors, but may also identify targets that are not essential to in vivo tumor growth.

As noted above, glioblastomas are defined by angiogenesis and pseudopalisading necrosis. In studies under revision, we demonstrate that these features are associated with distinct transcriptional programs, with vascular regions showing a Proneural profile and hypoxic regions a Mesenchymal pattern. As these regions harbor glioma stem cells, we investigated the epigenetic regulation of these two niches. Proneural, perivascular glioma stem cells activated EZH2 (Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit), whereas Mesenchymal glioma stem cells in hypoxic regions expressed BMI1 (BMI1 Proto-Oncogene, Polycomb Ring Finger) protein, which promoted cellular survival under stress. Using both genetic and pharmacologic inhibition, we found that Proneural glioma stem cells are selectively sensitive to EZH2 disruption, whereas Mesenchymal glioma stem cells are sensitive to BMI1 inhibition. Given that glioblastomas contain both Proneural and Mesenchymal glioma stem cells, combined EZH2 and BMI1 targeting proved more

effective than either agent alone both in culture and in vivo, suggesting that strategies that simultaneously target multiple epigenetic regulators within glioblastomas may be necessary to overcome resistance to therapies caused by intratumoral heterogeneity.

In conclusion, brain tumor stem cells are important to critical tumor behaviors, including therapeutic resistance, invasion, and immune modulation. They are regulated by complex interactions between the tumor hierarchy and the environment with integration of signal transduction, metabolism, genetics, and the epigenetic cell state.

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# IDENTIFICATION OF CRITICAL DRIVERS OF TUMOR MAINTENANCE THROUGH IN VIVO FUNCTIONAL GENOMICS

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Despite truly significant advancements in recent years towards our understanding of cancer pathogenesis and its underlying genomic complexity, our field is still plagued by an inability to improve around the development of truly effective therapeutic agents. Notable exceptions are represented by the discovery of certain immune checkpoints-targeting therapies and molecular targeted therapies that have provided long lasting responses in some disease types, but this certainly is not sufficient.

Several critically important factors have emerged, including the realization that genomic, and therefore, functional heterogeneity is a common feature of essentially any cancer and that conquering the disease requires a better understanding of which cells within a tumor may be sensitive or resistant to treatment. Rapid adaptation to therapy because of cell populations within the tumor being resistant to treatment seems to represent the rule more than an exception in many cases.

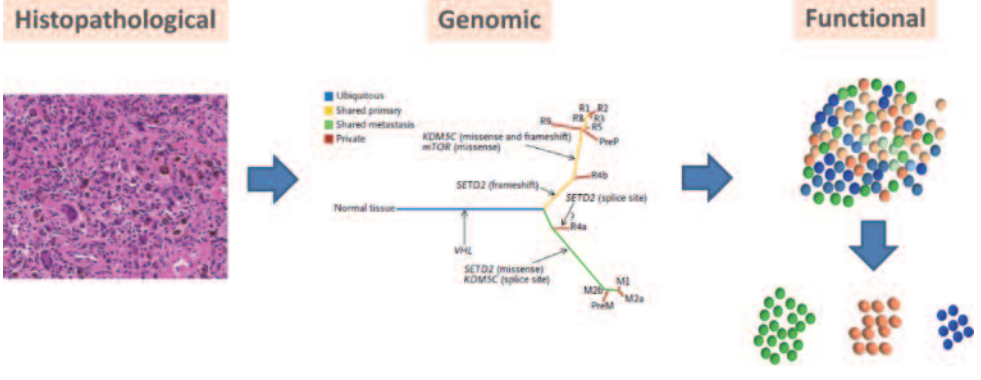


**Figure 1** Towards functional elucidation of tumor heterogeneity

Our laboratory at MD Anderson focuses on the identification of gene products essential for tumor growth in vivo and on the elucidation of critical dependencies in selected tumor cell subpopulations. One critical finding has been the discovery that in pancreatic cancer, a subpopulation of tumor epithelial cells can survive KRAS inactivation, remain dormant in the tumor and ultimately give rise to recurrence. These cells seem to carry characteristics of cancer stem cells, being able to aggressively form tumors upon serial transplantation and carrying surface markers that are characterizing cancer stem cells. Though several different approaches we could determine that these cells are critically dependent on mitochondrial function for their survival and that combination inhibition of KRAS signaling, through either genetic or pharmacological intervention, and of mitochondrial function will results in dramatic inhibition of tumor growth [1]. This work has inspired the initiation of a drug discovery project aimed at identifying potent and selective inhibitors of mitochondrial oxidative phosphorylation. This compound, IACS-10759, has recently entered the clinic.

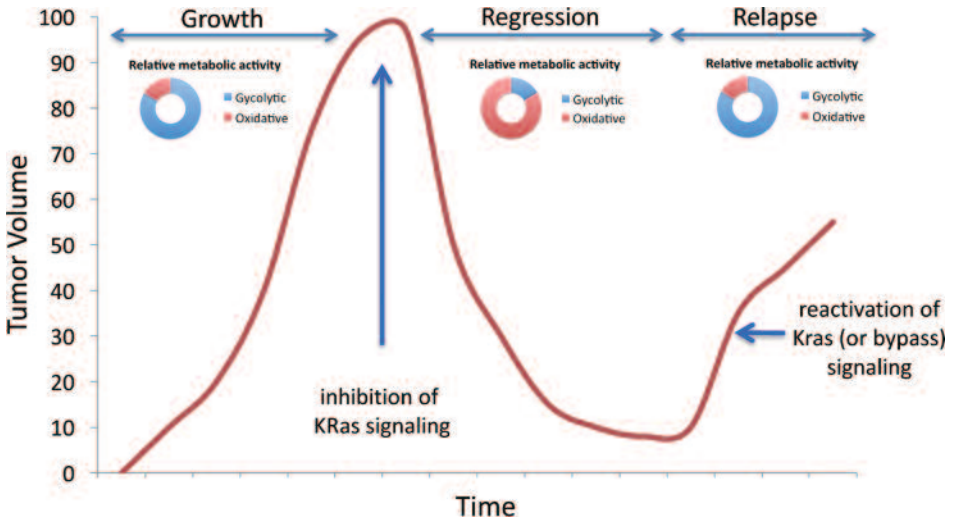


**Figure 2** Tumor relapse depends on failure to eliminate cells surviving inhibition of KRAS dependent signaling

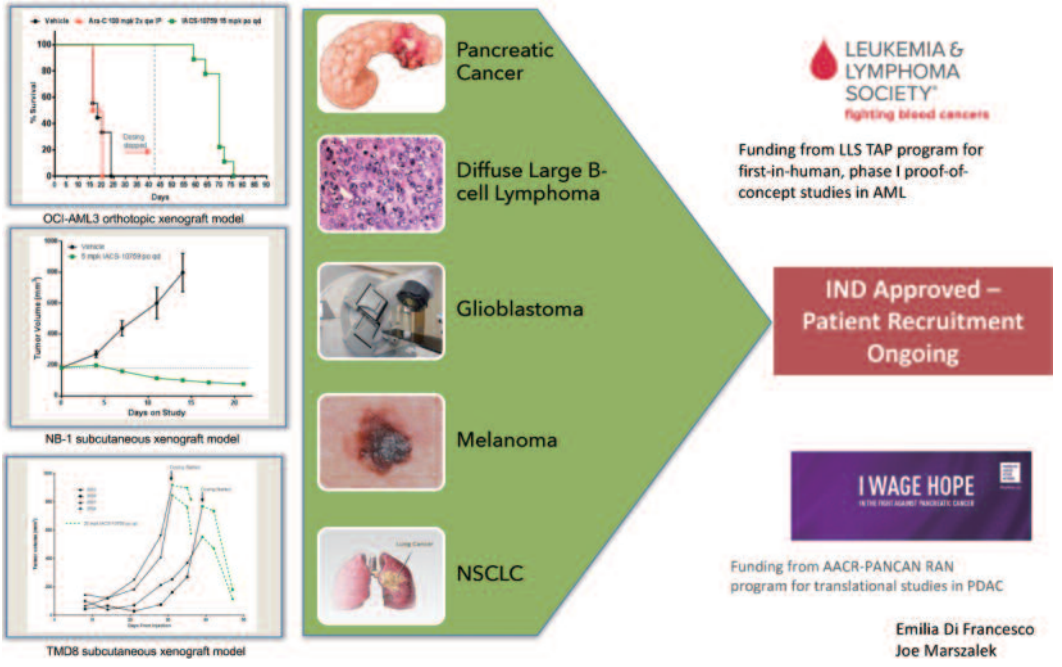


**Figure 3** The mitochondrial OXPHOS inhibitor IACS-10759 is entering the clinic in multiple indications

Recently we have also discovered another critical vulnerability of pancreatic tumors. Through in vivo mouse genetics studies and the clinical pathological assessment of human tissues, we have found that certain most aggressive pancreatic adenocarcinomas lose expression of the chromatin-modifying protein SMARCB1, activate C-Myc and carry alterations of proteostasis and consequently, while being resistant to treatment with KRAS signaling inhibitors, are sensitive to HSP90 and proteasome inhibitors, as well as to JNK inhibitor treatment [2]. We plan to conduct clinical trials in this population.

We have also developed an unbiased strategy to identify potential drug discovery targets in pancreatic adenocarcinoma, as well as in other tumors. Through functional in vivo screens performed using both human tumors-derived tissue in xenografts as well as mouse tumors in syngeneic hosts we have identified several candidate disease drivers and are pursuing the elucidation of their mechanism of action and their suitability as drug discovery targets [3, 4]. In summary, using complementary genetics approaches, we hope to continue to contribute to the identification and validation of mechanisms that keep tumor cells alive and of novel agents that might conquer these devastating diseases.

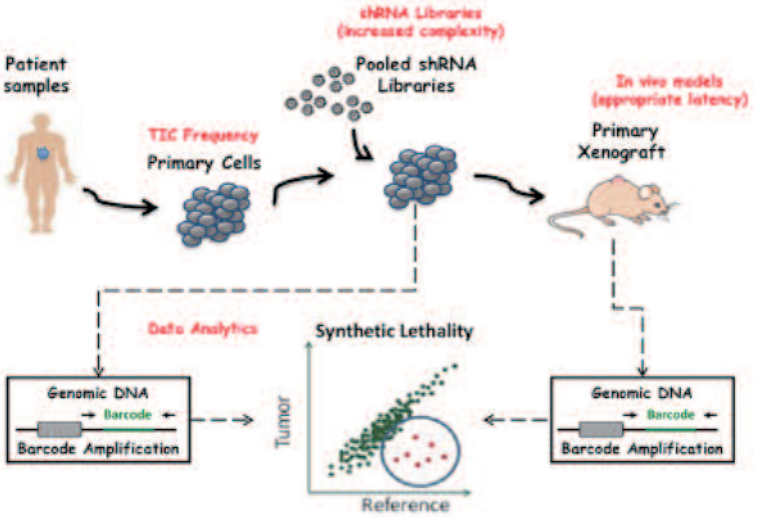


**Figure 4** Development of an in vivo genetic screening platform to identify critical vulnerabilities in primary human tumor derived tissue

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# CYP3A5 MEDIATES BASAL AND ACQUIRED THERAPY RESISTANCE IN DIFFERENT SUBTYPES OF PANCREATIC DUCTAL ADENOCARCINOMA

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Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive disease with dismal prognosis[1]. In both Europe and the USA pancreatic cancer is the fourth leading cause of cancer death[2,3]. Treatment with gemcitabine[4], FOLFIRINOX scheme[5] or the albumin-paclitaxel conjugate nab-paclitaxel[6] only offer a modest increase in overall survival. Despite extensive testing of targeted therapies in clinical trials, thus far all of the examined compounds confer little or no survival benefit in unselected cohorts of PDAC patients[1,7,8]. Although patient stratification according to molecular characteristics has not yet been performed in clinical trials for PDAC, molecular subtypes for PDAC were recently described[9-12]. We established patient-derived models representing the full spectrum of previously identified quasi-mesenchymal (QM-PDA), classical and exocrine-like PDAC subtypes, and identified two markers—HNF1A and KRT81—that enable stratification of tumors into different subtypes by immunohistochemistry (Figure 1a, b)[13]. Patients bearing tumors of these subtypes show substantial differences in overall survival (Figure 1c)[13].

While resistance of PDAC to therapy is well described[1], little is known about the molecular mechanisms mediating it. Members of the cytochrome P450 (CYP) enzyme family have been previously only investigated with regard to a role in systemic drug metabolism[14,15] or their up- or down-regulation in solid tumors compared to normal tissues[16]. Thus, the functional role and impact of CYPs on tumor-cell autonomous drug resistance remains largely unknown[16,17]. We could show that the three PDAC subtypes significantly differ in drug sensitivity, with the exocrine-like subtype being resistant to tyrosine kinase inhibitors and paclitaxel[13]. The xenobiotic biotransformation enzyme,

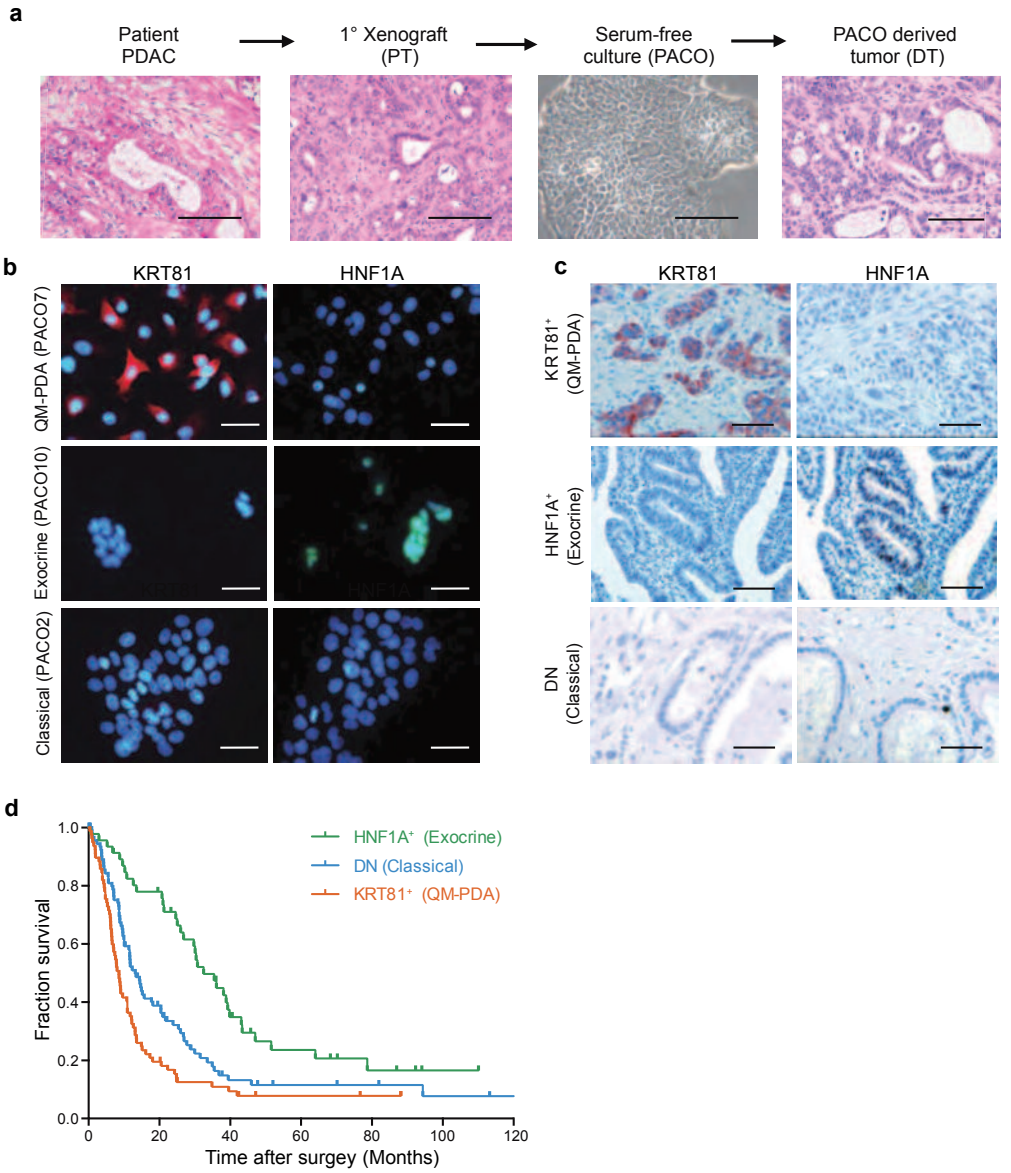


**Figure 1** Subtype stratification of PDAC models and patients by two markers[13].
(a) Schematic overview of the experimental workflow used to generate orthotopic xenografts and PACO cells. H&E staining of a human PDAC tumor, the corresponding first passage xenograft (PT), phase contrast image of the derived cell line (PACO10) and the respective derived xenograft (DT). Scale bar, 100 μM. (b) KRT81 and HNF1A immunofluorescence staining on PACO lines from the three different subtypes (n = 3). Scale bar, 50 μM. (c) KRT81 and HNF1A immunostaining on sections from a TMA of individuals with PDAC (n = 241). Scale bar, 100 μM. (d) Kaplan-Meier analysis of overall survival of subjects with PDAC (n = 217). Tumor sections on the TMA were retrospectively subtyped into three groups based on KRT81 and HNF1A expression as determined by immunostaining (HNF1A$^+$: n = 46; DN: n = 92; KRT81$^+$: n = 79). *P* value was determined by log-rank test.

cytochrome P450 3A5 (CYP3A5), metabolizes these compounds in tumor cells of the exocrine-like subtype, and pharmacological or small hairpin RNA (shRNA)-mediated CYP3A5 inhibition sensitizes tumor cells to these drugs (Figure 2)[13]. Additionally, retrospective analysis of a large patient cohort confirmed that CYP3A5 is predominantly found in those patient tumors classified as exocrine-like[13]. Whereas the hepatocyte nuclear factor 4, alpha (HNF4A) controls basal expression of CYP3A5, drug-induced CYP3A5 upregulation is mediated by the nuclear receptor NR1I2[13]. Interfering with these regulatory mechanisms may provide an alternative approach to suppress this CYP3A5 mediated resistance pathway. CYP3A5 also contributes to acquired drug

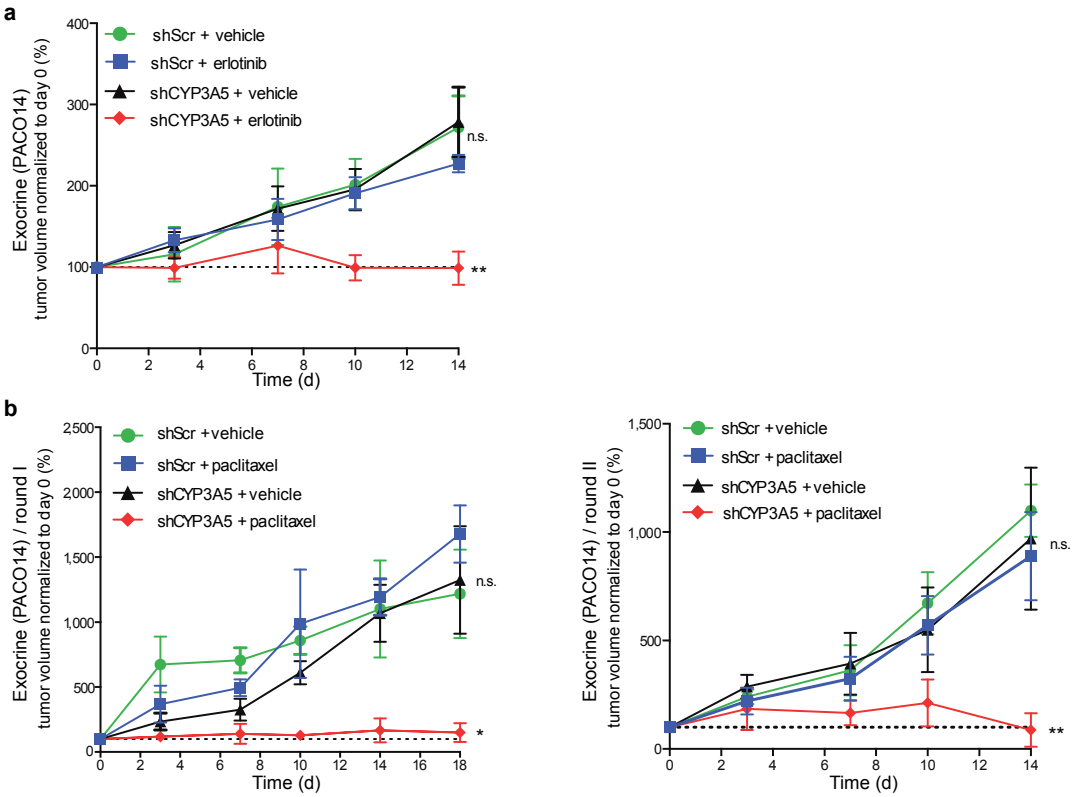


**Figure 2** CYP3A5 mediates drug resistance in exocrine-like PDAC cells in vivo[13].
(a) Growth curves of PDAC xenografts from exocrine-like shScr and shCYP3A5 cells, treated for two cycles of 5 days with erlotinib (100 mg/kg) and 2 days recovery. (b) Growth curves of PDAC xenografts from exocrine-like shScr and shCYP3A5 cells treated with two cycles of 5 days paclitaxel (2 mg/kg) and 2 days recovery followed by 4 days of paclitaxel (left panel, round I). Cells from one xenograft per treatment group were re-injected and treated for two cycles of 5 days paclitaxel and 2 days recovery (right panel, round II). Tumor volume was measured with a digital caliper. Shown are tumor volumes normalized to baseline (day 0) and depict mean ± SEM. P values were determined at the end point using one-sided Mann-Whitney U test. (n = 6 mice per treatment group; *P < 0.05; **P < 0.01; n.s. = not significant).

resistance in some QM-PDA and classical PDAC cases. In other cases CYP3A5-independent pathways mediate acquired drug resistance in QM-PDA and classical cells. However, the mechanistic details of the pathways mediating the observed resistance are yet to be determined. To note, CYP3A5 is also highly expressed in several additional malignancies[13]. Taken together, these findings designate CYP3A5 as predictor of therapy response and as a tumor cell-autonomous detoxification mechanism that must be overcome to prevent drug resistance (Figure 3).

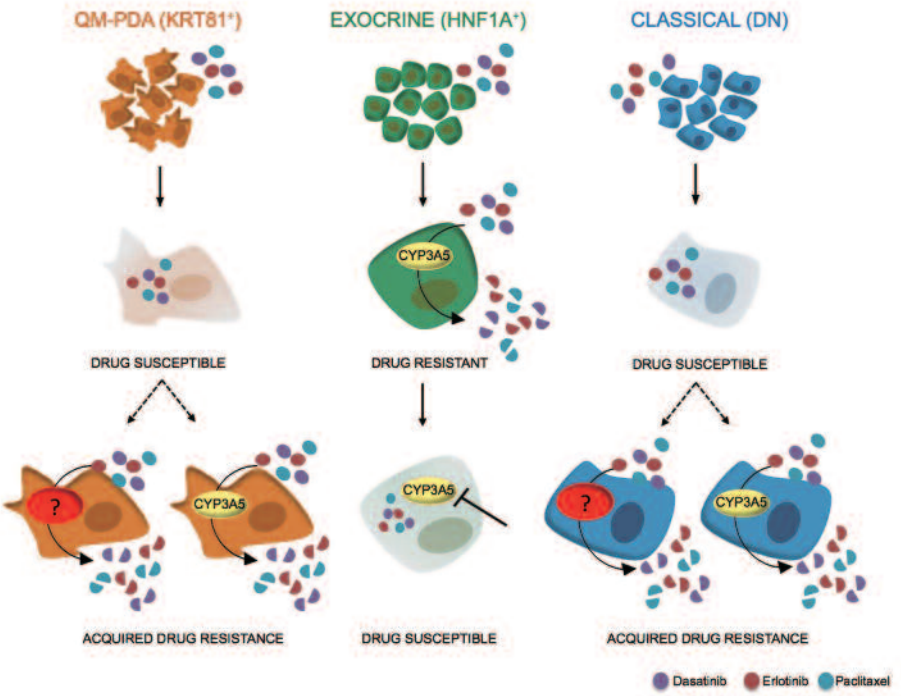


**Figure 3** CYP3A5 mediates basal and acquired drug resistance in different subtypes of PDAC. Patient-derived models of pancreatic ductal adenocarcinoma (PDAC) represent the full spectrum of previously identified quasi-mesenchymal (QM-PDA), exocrine-like and classical subtypes. According to the expression of the surrogate markers HNF1A and KRT81, PDAC models and tumors can be stratified into three distinct biological groups, which are enriched in the respective PDAssigner genes{Collisson, 2011 #5}. These subtypes significantly differ in drug sensitivity; with the exocrine-like subtype being resistant to the small molecule drugs erlotinib, dasatinib and paclitaxel. The observed resistance can be attributed to the cytochrome P450 3A5 (CYP3A5) enzyme, which actively metabolizes and detoxifies these small molecule drugs. Pharmacological or short hairpin RNA (shRNA)-mediated CYP3A5 inhibition sensitizes tumor cells to erlotinib, dasatinib and paclitaxel *in vitro* and *in vivo*. Hence, CYP3A5, previously mainly implicated in systemic drug metabolism in hepatocytes, plays a critical role in mediating resistance to various small molecule drugs in exocrine-like tumor cells. Additionally, CYP3A5 contributes to acquired drug resistance in some cases of the QM-PDA and classical PDAC subtype after long-term treatment with small molecule drugs. Also CYP3A5-independent pathways mediate acquired drug resistance in QM-PDA and classical cells. However, the mechanistic details of the pathways mediating the observed resistance are yet to be determined. Taken together, CYP3A5 not only mediates drug resistance in the exocrine-like subtype, but also contributes to acquired drug resistance in the some cases of the QM-PDA and classical PDAC subtypes.

**Acknowledgements**

This work was supported by the German Bundesministerium für Bildung und Forschung (BMBF) funded e:Med consortium "PANC-STRAT" (grants 01ZX1305B, 01ZX1305C), the Helmholtz pre-clinical comprehensive cancer center (PCCC) and the Dietmar Hopp Foundation.

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# TRANSDIFFERENTIATION APPROACH FOR TARGETING CANCER STEM CELLS

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Cancer stem cells (CSCs) are a subset of tumor cells that are responsible for initiating and maintaining the disease. In the clinical point of view, the most important characteristics of CSCs include their resistance to various therapeutic interventions. However, the underlying mechanisms of the resistance remain unclear. To address this question, we established a mouse osteosarcoma (OS) model by overexpressing c-MYC in bone marrow stromal cells (BMSCs) derived from *Ink4a/Arf (-/-)* mice (Figure 1) [1]. Single-cell cloning revealed that c-MYC-expressing BMSCs are composed of two distinctly

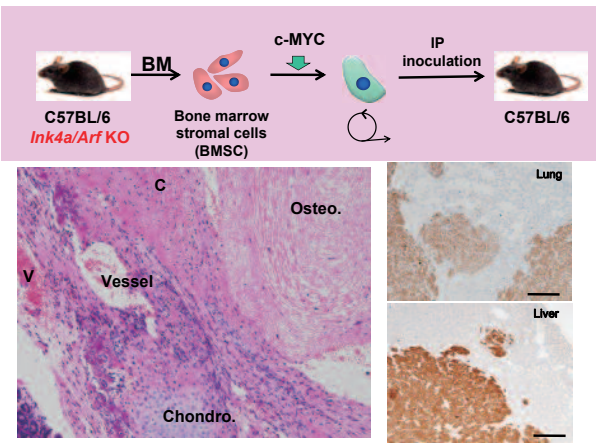


**Figure 1** Development of mouse osteosarcoma cells. *c-MYC* gene was introduced into the mouse bone marrow stromal cells (BMSCs) derived from *Ink4a/Arf* knock-out C57BL/6 mice by retroviral transfer, and we enriched infected cells positive for green fluorescent protein (GFP). GFP positive cells were inoculated in syngeneic C57BL/6 mice. The pathological analysis of the tumor developed after the inoculation shows heterogenous tumor cell composition. All mice inoculated the tumor cells had metastasis of osteosarcoma cells in lung and liver.

different clones: highly tumorigenic cells (termed AX cells), similar to bipotent-committed osteochondral progenitor cells, and low-tumorigenic tripotent cells (termed AO cells), similar to mesenchymal stem cells. We found that the loss of adipogenic potential is an essential event for AO cells to become AX-like tumorigenic cells. Although AO cells are not tumorigenic as far as they maintain the adipogenic differentiation potential, AO cells were found to be highly resistant to chemotherapeutic agents such as Adriamycin. Then, AO cells survived after the chemotherapy eventually change to AX-like cells by losing their adipogenic differentiation potential to become tumorigenic. Therefore, our understanding of regulatory mechanisms of adipocyte differentiation would greatly contribute to control OS tumorigenesis.

We have recently found a novel regulatory mechanism of adipocyte differentiation. Regulation of the transcriptional coactivator MKL1 (megakaryoblastic leukemia 1) by actin cytoskeleton dynamics drives adipocyte differentiation mediated by peroxisome proliferator–activated receptor γ (PPARγ), a master transcriptional regulator of adipogenesis [2]. We found that disruption of actin stress fibers through the inactivation of RhoA-ROCK signaling induces the rapid increase in monomeric G-actin, leading to the interaction of G-actin with MKL1, which prevents nuclear translocation of MKL1 and allows expression of PPARγ followed by adipogenic differentiation (Figure 2). Our findings thus provide new mechanistic insight into the relation between the actin dynamics and transcriptional regulation during cellular differentiation.

Based on this discovery, we attempted to induce adipocyte differentiation in OS stem cells by treatment with Rho kinase inhibitors. Rho kinase inhibitors induced adipocyte differentiation of OS stem cells and significantly suppressed their *in vitro* growth and *in vivo* tumorigenesis. Our findings suggest that induction of trans-differentiation of cancer

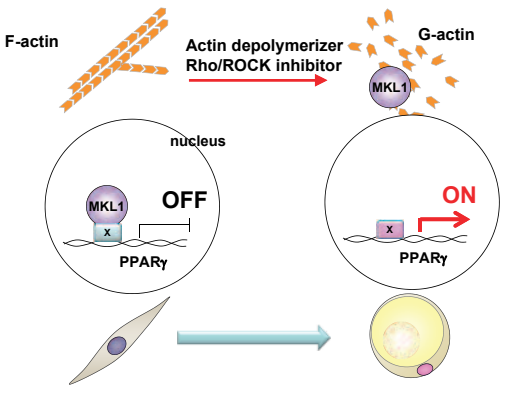


**Figure 2** Mechanism of adipocyte differentiation driven by actin cytoskeleton dynamics.

stem cells by regulating actin cytoskeleton dynamics is a potential approach for some tumor types.

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# MECHANISMS CONTROLLING TUMOR HETEROGENEITY

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For the vast majority of cancers, the cell at the origin of tumour initiation is still unknown [1]. Here, we used mouse genetics to identify cells at the origin of basal cell carcinoma (BCC), which is one of the most frequent types of cancer in human and results from the activation of hedgehog signalling pathway [2]. Using mice conditionally expressing constitutively active Smoothened mutant (SmoM2), we activated hedgehog signalling in different cellular compartments of the skin epidermis, and determined in which epidermal compartments hedgehog activation induces BCC formation. Activation of SmoM2 in hair follicle bulge stem cells (SCs) and their transient amplifying progenies did not induce cancer formation, demonstrating that BCC do not originate from bulge SC as previously thought. Using clonal analysis, we found that BCC arise from long-term resident cells of the interfollicular epidermis and the upper infundibulum. Our studies uncover the cells at the origin of BCC in mice and demonstrate that expression of differentiation markers in tumour cells is not necessary predictive of the cancer initiating cells [3, 4].

The skin interfollicular epidermis (IFE) is the first barrier against the external environment and its maintenance is critical for survival [5]. Quantitative analysis of clonal fate data and proliferation dynamics demonstrate the existence of two distinct proliferative cell compartments composed of slow-cycling SC and committed progenitors (CPs), both of which undergo population asymmetric self-renewal. However, following wounding, only SCs contribute substantially to the repair and long-term regeneration of the tissue, while CP cells make a minimal and transient contribution [6].

The changes that occur in cell dynamics following oncogenic mutation leading to the development of invasive tumors are currently unknown. Here, using skin epidermis as a model, we assessed the impact of oncogenic Hedgehog (HH) signalling in distinct cell populations and their capacity to induce basal cell carcinoma (BCC), the most frequent cancer in humans. We found that only K14-CreER targeted Stem Cells (SCs), and not Inv-CreER targeted committed progenitors (CPs), were competent to initiate tumor formation upon oncogenic HH signalling in the different skin regions. Interestingly, this difference was due to the hierarchical organization of the tumor growth in oncogene targeted SCs, characterized by increased symmetric self-renewing division, but also to a higher resistance of oncogene-targeted SCs to apoptosis and growth arrest compared to oncogene-targeted CP. Combined, these properties induced a much more rapid cell expansion in oncogene-targeted SCs, allowing them to progress to BCCs. Our work reveals that the capacity of oncogene-targeted cells to induce tumor formation is not only dependent on the long term survival and increased fitness of these cells, but also depends on the specific clonal dynamics of the cancer cell of origin [7] (Figure 1).

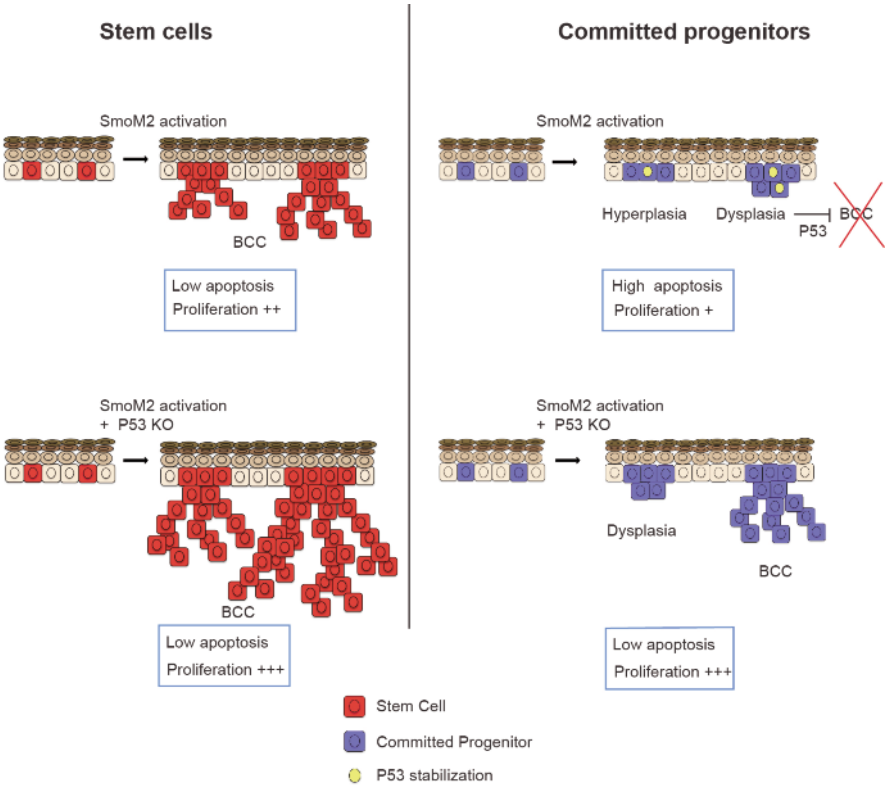


**Figure 1** Stem cells but not progenitors are competent to initiate BCC formation
Activation of oncogene in SCs leads to the generation of BCC due to an increase in symmetric renewal and resistance to apoptosis. However, activation of p53 in SmoM2-expressing CPs restricts the progression of dysplastic clones to BCC by promoting apoptosis and cell cycle arrest. Deletion of p53 in CPs allows them to progress into BCC.

Squamous cell carcinoma (SCC) represents the second most frequent skin cancer. The cellular origin of SCC remains controversial [1]. Here, we used mouse genetics to determine the epidermal cell lineages at the origin of SCC. Using mice conditionally expressing a constitutively active KRas mutant (G12D) and an inducible CRE in different epidermal lineages, we activated Ras signaling in different cellular compartments of the skin epidermis, and determined from which epidermal compartments Ras activation induces squamous tumor formation. Expression of mutant KRas in hair follicle bulge stem cells (SCs) and their immediate progeny (hair germ and outer root sheath), but not in their transient amplifying matrix cells, led to benign squamous skin tumor (papilloma). Expression of $KRas^{G12D}$ in interfollicular epidermis also led to papilloma formation, demonstrating that squamous tumor initiation is not restricted to the hair follicle lineages. Whereas no malignant tumor was observed following $KRas^{G12D}$ expression alone, expression of $KRas^{G12D}$ combined with the loss of p53 induced invasive SCC. Our studies demonstrate that different epidermal lineages including bulge SC are competent to initiate papilloma formation and that multiple genetic hits in the context of oncogenic KRas are required for the development of invasive SCC [8].

Epithelial to mesenchymal transition (EMT) in cancer cells has been associated with metastasis, tumor stemness and resistance to therapy. Here, we developed genetically engineered mouse models allowing lineage tracing and activation of the same oncogenic hits in different epidermal lineages, and assessed whether the cancer cell of origin controls EMT in mouse skin squamous cell carcinoma (SCC). Surprisingly, while SCCs arising from the interfollicular epidermis consisted essentially of well-differentiated tumors, SCCs that arose from hair follicle lineages exhibited very frequently EMT. Transplantation assays showed the intrinsic priming of HF derived tumor epithelial cells to undergo EMT. Transcriptional and chromatin profiling of the different tumor cell populations and their cell of origin unravelled the changes in the chromatin landscape and gene regulatory network associated with tumorigenesis and EMT and demonstrated the importance of epigenetic and transcriptional priming of the cancer cell of origin to undergo EMT upon oncogenic transformation [9] (Figure 2).

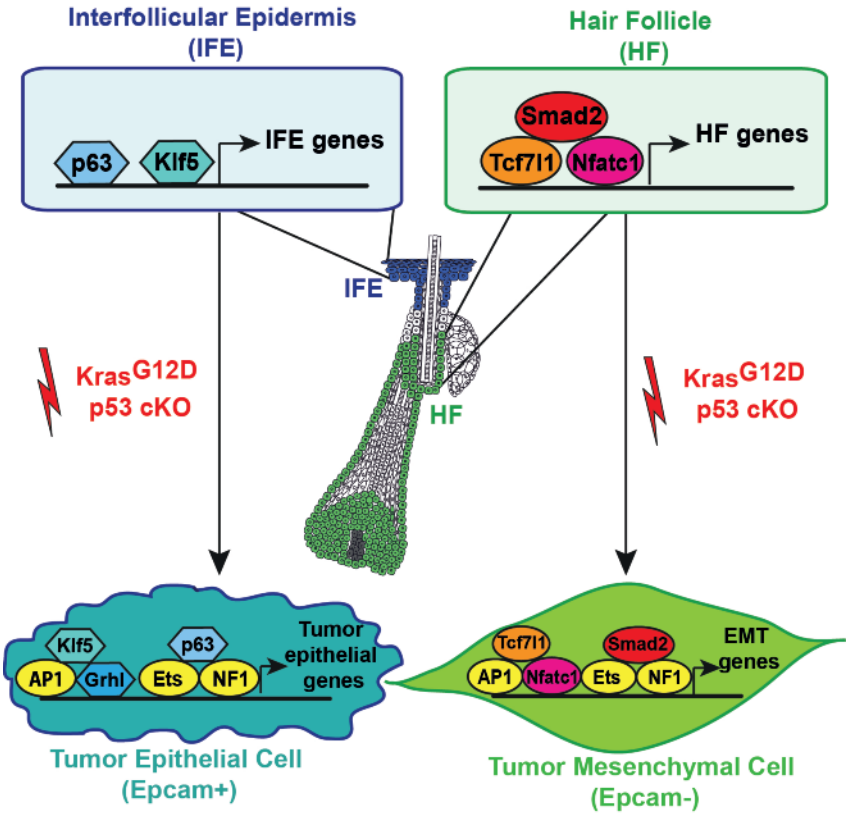


**Figure 2** The cancer cell of origin primes tumour-initiating cells to undergo EMT
Squamous cell carcinomas (SCCs) derived from interfollicular epidermis (IFE) are generally well-differentiated, while hair follicle (HF) stem cell-derived SCCs frequently exhibit EMT. Transcriptional and chromatin profiling revealed IFE and HF tumor-initiating cells possess distinct epigenetic landscapes and gene regulatory networks that prime the cancer cell of origin to undergo EMT during tumorigenesis.

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# NEW INSIGHTS INTO CELLULAR PLASTICITY: TARGETING METAPLASTIC CANCERS

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## Cellular Plasticity

Cellular plasticity is a universal and fundamental biological process that generates non-genetic variation and allows cells to explore multiple phenotypic states. Embryonic stem cells (ESCs) exhibit cellular plasticity during differentiation. Ensuing epigenetic changes then dictate cell fate and generate differentiated progeny. Cellular plasticity can also be activated by cellular stress responses that are initiated upon wound healing. Current research is now indicating that one population of regenerative cells responds to homeostatic cues and keeps tissue in balance while an independent population of cells responds to wounding cues and regenerates functional tissue after damage [1-3]. In areas of chronic wounding, caused by repeated irritation, injury or inflammation, cells respond by generating metaplastic tissues. Metaplastic tissues are healthy "normal" tissues that are formed in inappropriate sites. For example, exposure of esophageal tissue to repeated acid reflux injures the squamous esophageal cells which are then replaced by intestinal tissue [4]. The intestinal tissue is properly differentiated to secrete protective mucins from goblet cells thereby protecting the underlying cells from acid damage. The origin of the cells that give rise to metaplastic tissues is unknown. The extent of cellular plasticity is underappreciated as a mechanism to generate tumor heterogeneity independently of genetic mutations. Cellular plasticity allows the adoption of altered cellular states that can fuel metastasis (epithelial to mesenchymal transition; EMT) as well as drug resistance (persister or drug-tolerant cells) [5].

Interestingly, cellular plasticity is activated by conditions known to promote malignancy and in places where malignancy often arises, such as areas of chronic

wounding. The cells that exhibit plasticity must be able to bypass proliferative arrest that is induced by negative growth signals as well as exhibit multi-lineage potential. Here we describe our studies to isolate such cells and characterize a rare population of human cells that exhibit cellular plasticity under stress conditions.

**Rare Cells Can Bypass Negative Growth or Injury Signals and Exhibit Cellular Plasticity**

The majority of cells within an injured tissue activate cell cycle checkpoint controls and proliferative arrest until resolution of cellular damage releases this inhibition or apoptosis removes these cells from the injured field [6]. Only cells with compromised cell cycle checkpoints can continue to proliferate in the presence of damage signals. We reasoned that cells exhibiting plasticity would reveal or unveil themselves under stress conditions and make their evaluation possible. This proved to be correct.

Evaluation of primary isolates of disease-free human breast tissue unmasked a cell population which, when placed in culture conditions that activate stress responses, exhibits cellular plasticity. The major population, human mammary epithelial cells (HMEC), is composed of luminal and myoepithelial cells. These cells undergo cell cycle arrest when they encounter stress conditions and remain in a static state for an extended period of time. Strikingly, a rare subpopulation of cells could be identified by a reversible repression of p16 transcription [7]. Realizing that repression of p16 activity was a defining characteristic of pluripotent and adult stem cell populations [8], we evaluated this population of cells for their ability to exhibit stem cell traits or plasticity. Pluripotent cells (human embryonic stem cells (hESCs), induced Pluripotent Stem Cells (iPSCs)) and adult tissue-specific stem cells have the potential to self-renew and generate multiple lineage derivatives. Surprisingly, we found that the rare population of p16-repressed cells we had identified had the ability to self-renew and generate mature cells of all three embryonic lineages (Figure 1), far exceeding the plasticity of tissue-specific cell types that one might have expected [7]. Further study of these **endogenous Plastic Somatic** (ePS) cells revealed that, when activated, they expressed canonical pluripotent factors including co-incident expression of Sox2, Oct3/4 and Nanog (SON) and existed in multiple tissues of the adult human body.

We identified cell surface markers and growth conditions that allowed prospective enrichment and isolation of these rare ePS cells. Rare cells (0.15% of breast parenchyma), expressing NT5E/CD73 and EpCAM but devoid of expression of THY1/CD90, were extensively characterized. We showed that ePS cells coincidently express SON within the nucleus when in their activated state at levels similar to those measured for hESCs (Figure 2 and [7]) and, when placed in differentiation conditions, can generate glial and neuronal cells, bone, cartilage, adipocytes, cardiomyocytes, endothelial, pancreatic and intestinal cells (Figure 1). When SON is expressed, ePS cells demonstrate (a) pluripotency without

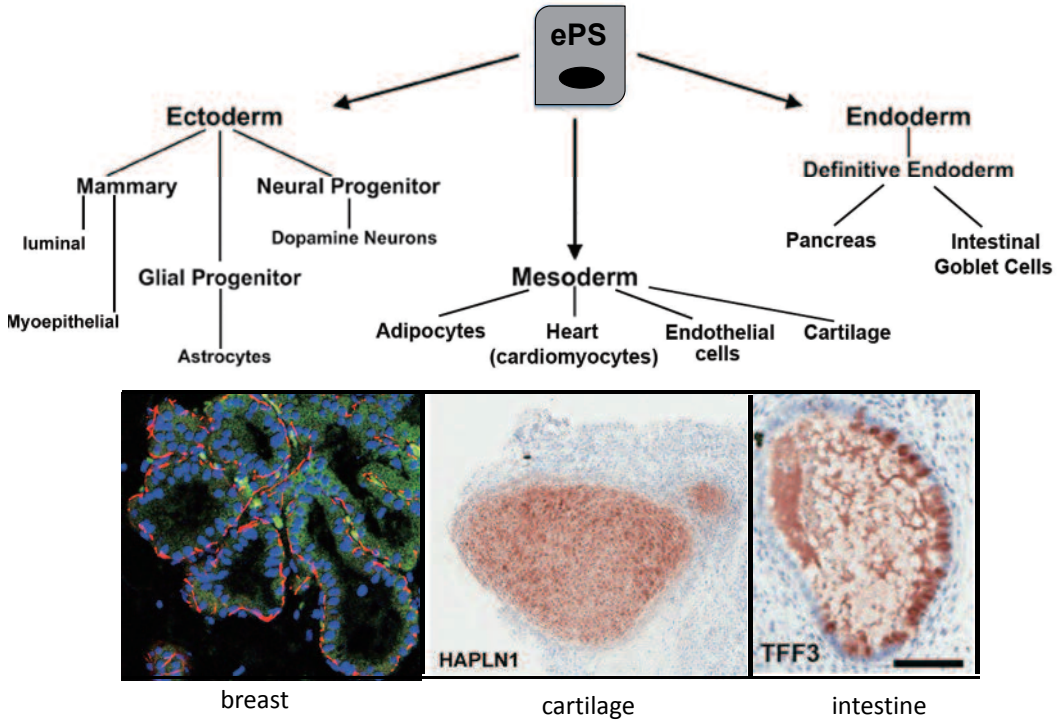


**Figure 1** Characterization of endogenous Plastic Somatic Cells

culture *in vitro*, (b) clonal evidence of pluripotency *in vitro* and *in vivo*, (c) cell type-specific gene expression and (d) functionality of all three lineage derivatives (ectodermal: secretion of human milk in transplanted mice, mesodermal: lipid-accumulating adipocytes, tubule-forming endothelial cells and beating cardiomyocytes, and endodermal: intestinal goblet cells). Finally, exclusion of cell-cell fusion or contamination events (through Short Tandem Repeat (STR) analysis and karyotyping of multiple cell populations before and after differentiation) confirms the origin of the cells. Together, these studies provide morphological, molecular and functional evidence of lineage plasticity of these cells. The ePS cells are distinct from hESCs, iPS cells and adult stem cells as assayed by expression profiling, functional tests, growth conditions and expression of cell surface markers [7]. For example, when we isolated tissue-specific breast stem cells using previously published markers [9], the isolated cells easily generated breast derivatives but failed to make mesodermal or endodermal derivatives.

Notably, we also demonstrated the mortal and non-malignant state of the ePS cells isolated from breast tissue obtained from multiple disease-free women of various ages and ethnicities. The ePS cells exhibit normal diploid 46,XX karyotypes, low telomerase

expression and activity and ultimately enter replicative senescence, distinguishing them from immortal, genomically unstable tumor cells and from hESCs (Figure 2). While ePS cells exhibit enough proliferation potential to generate fully expanded and differentiated tissues in the context of (non-malignant) teratomas, their mortality suggests a short-term contribution to tissue function under yet-to-be-determined conditions such as tissue repair during wound healing. We have found evidence of these cells in multiple tissues of the human body (Pan and Caruso, unpublished results). Finally, colleagues have provided independent validation that these cells can be isolated and exhibit the characteristics reported in Roy et al. [7].

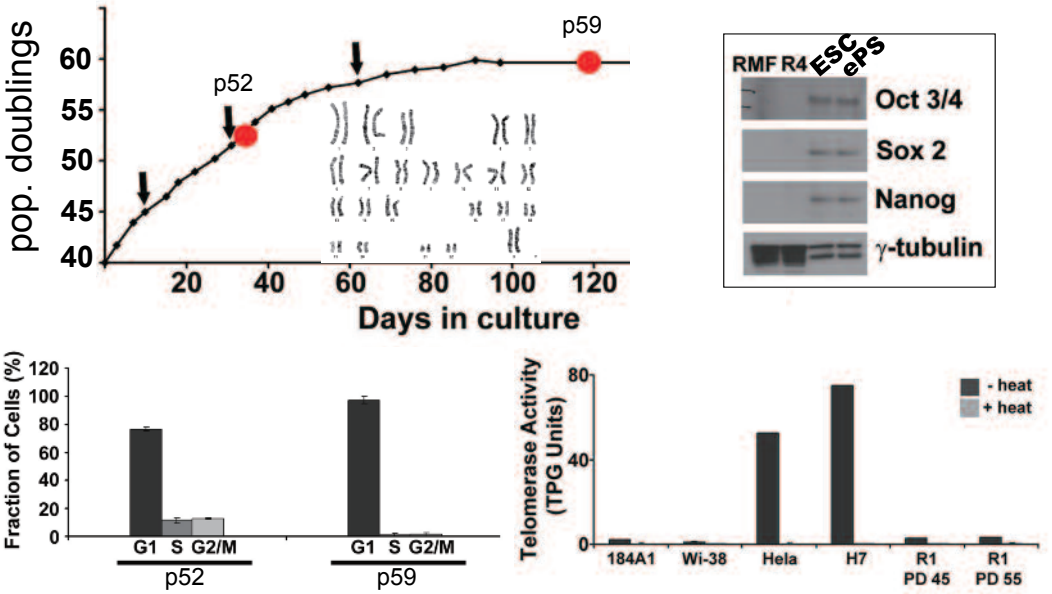


**Figure 2** ePS cells express SON, yet, are mortal, expand ~59 population doublings, maintain low telomerase activity, exhibit a normal diploid karyotype and arrest in G1

### Studying The Regulation and Biological Relevance of ePS Cells *In Vitro* and *In Vivo*

We have pursued these unexpected observations in several directions. First, we found that ePS cells need the activated expression of SON to exhibit pluripotency; knockdown of any of these three genes ablates the plastic potential of ePS cells and triggers a caspase-dependent apoptotic response [10]. Second, we determined that the well-known signaling pathways that regulate and maintain pluripotency in hESCs and iPSCs (the activin A and FGF2 pathways) are necessary but not sufficient to activate or maintain SON expression and plasticity in ePS cells and that additional unique signaling pathways are required to

activate the SON program. One of these pathways relies on CD73 enzymatic activity. We have now defined the signaling necessary and sufficient to activate SON and plasticity in ePS cells [10] (Figure 3). Thus, ePS cells can enter into a pluripotent state using pathways that are novel and not utilized by hESC or iPSC.

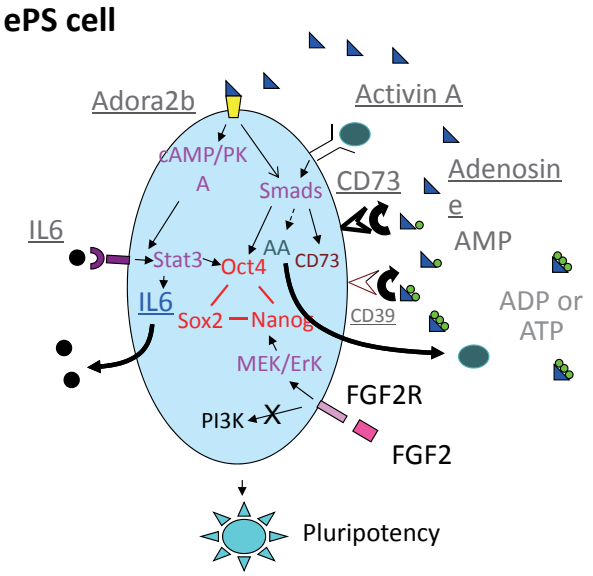


**Figure 3** Novel Circuitry Controls Pluripotency in ePS Cells

## Potential in Disease?

The most lethal of tumors are the rare metaPLASTIC tumors that are highly undifferentiated and exhibit embryonic properties of cell movement (as seen in metastasis) and heterogeneity (known to drive drug resistance). Strikingly, these tumors exhibit the most aggressive phenotypes. A metaplastic breast cancer can contain, not only malignant breast tissue, but also additional malignant tissues such as bone, pancreas and cartilage tissue derivatives – all in one localized area (Figure 4). Since previous studies demonstrated that these multiple tissue types all contain the same mutations and chromosomal abnormalities (for example identical mutations in p53), investigators have long postulated that they arose from a single pluripotent cell that became malignant [11] (Figure 4). Until now, that cell had been hypothetical.

The characterization of ePS cells described above suggests the hypothesis that ePS cells may provide a source of cells and/or factors that contribute to the unusual phenotypic and behavioral features that typify metaplastic tissues in areas of chronic wounding and their

associated carcinosarcomas. If correct, we hypothesize that ePS cells may ultimately be used for modeling these disease states and that understanding their biology may provide critical leads in screening for useful drugs to prevent or intervene in the generation of lethal malignancies that contain metaplastic tissue derivatives. Since metaplastic tumors are resistant to all known therapeutics, an unmet need in this field is to find a therapeutic approach that can be used for patients suffering from metaplastic cancers.

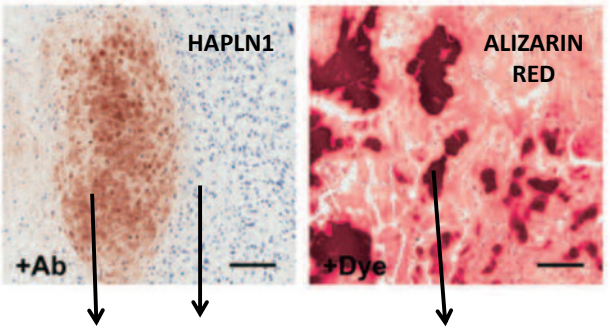


**Figure 4** Lineage Plasticity in Metaplastic Breast Carcinoma

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# METABOLIC EXCHANGE BETWEEN STEM CELLS AND NICHE CELLS

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Hematopoietic stem and progenitor cells (HSPCs) maintain the hematopoietic system for an organism's entire lifetime under homeostatic or in stress settings. At steady state, long term hematopoietic stem cells (LT-HSCs), the most undifferentiated cells in the hematological system, are kept quiescent or in G0 of the cell cycle. Hematological stresses deplete the body of blood cells, and if stresses remain below the lethal dose, bone marrow (BM) LT-HSCs enter the cell cycle and robustly repopulate the entire hematopoietic system via multi-lineage differentiation and self-renewal. Such hematological repopulation activity is termed "stress hematopoiesis," an activity essential for homeostatic maintenance of blood production. Understanding stress hematopoiesis is also critical to establish efficient methods enabling expansion in vitro of HSPCs, since forced activation of the cell cycle by cytokine supplementation induces a stress-like cellular state. p38MAPK inhibition is thought to suppress aberrant HSPC proliferation and protect HSPCs from exhaustion or replicative senescence in stress settings or in the context of damage associated with aging. However, genetic evidence supporting this hypothesis is still limited.

To identify the predominant p38MAPK isozyme in BM compartments, we assessed expression of mRNAs encoding the four p38MAPK isozymes by qPCR of cDNAs from LT-HSCs, short-term (ST)-HSCs, multipotent progenitors (MPPs), lineage markers$^-$ c-Kit$^+$ Sca-1$^-$ (LKS$^-$) myeloid progenitors, and lineage markers$^+$ (Lin$^+$) fractions of wild-type BM. Among them, p38$\alpha$ was highly expressed in BM mononuclear cells (BMMNCs), and p38$\alpha$ expression levels were significantly higher in HSPC fractions than in fractions of differentiated cells. To test p38$\alpha$ function, we took an inducible conditional knockout approach using the CAG-CreERT2:p38$\alpha^{flox/flox}$ (p38$\alpha^{fl/fl}$) mouse, in which p38$\alpha$ deletion in

vivo (p38 $\alpha^{\Delta/\Delta}$) is induced globally by intraperitoneal tamoxifen administration. Frequencies of various primitive hematopoietic cells (LT-HSCs, ST-HSCs, MPPs, and progenitors) were also indistinguishable in p38 $\alpha^{+/+}$ and p38 $\alpha^{\Delta/\Delta}$ BM. Thus p38 $\alpha$ is dispensable for steady state hematopoiesis in young mice, despite the fact that it is expressed in wild-type hematopoietic cells. Given the function of p38 $\alpha$ in the context of stress, we monitored how tolerant p38 $\alpha^{\Delta/\Delta}$ HSCs were of hematological stress by performing serial BMT. We observed lower relative PB and BM chimerism in the p38 $\alpha^{\Delta/\Delta}$ compared to the p38 $\alpha^{+/+}$ cell-transplanted group in the primary and secondary transplantation. We next evaluated survival rate after injection of mice of both genotypes with 5-fluorouracil (5-FU), which eliminates differentiated hematopoietic cells and induces proliferation of quiescent HSCs. After a single administration of 5-FU, p38 $\alpha^{\Delta/\Delta}$ mice showed higher mortality than did p38 $\alpha^{+/+}$ mice. Taken together, p38 $\alpha$ loss in mice results in defects in stem cell capacity during stress hematopoiesis in vivo.

Next, we focused on cell cycle progression after BMT using the fluorescent label carboxyfluorescein succinimidyl ester (CFSE). CFSE-labeled cells lose fluorescence at each cell division; thus CFSE intensity reflects cell division history of transplanted cells after BMT. For this analysis we stained freshly isolated p38 $\alpha^{+/+}$ or p38 $\alpha^{\Delta/\Delta}$ BMMNCs with CFSE and transplanted them into lethally-irradiated mice. We then analyzed donor-derived LT-HSCs after BMT. CFSE fluorescence levels were significantly higher in mice transplanted with p38 $\alpha^{\Delta/\Delta}$ compared to p38 $\alpha^{+/+}$ cells from days 1 to 3 after BMT, whereas CFSE fluorescence levels prior to BMT were identical between genotypes. These defects were seen not only in LT-HSCs but also in ST-HSCs and MPPs. Taken together, loss of p38 $\alpha$ results in defective control of LT-HSC cell cycle progression in stressed settings. Given that altered metabolic activities can change cell cycle status [1,2], we asked whether p38 $\alpha$ regulation of a particular metabolic pathway could initiate HSPC cycling under stress conditions. To do so, we collected p38 $\alpha^{+/+}$ or p38 $\alpha^{\Delta/\Delta}$ LSK cells either at steady state or after BMT and analyzed metabolites using mass spectrometry. Among metabolites surveyed, we focused on glycine and aspartic acid as they were the only two of four metabolites assessed whose levels increased in p38 $\alpha^{\Delta/\Delta}$ cells. Levels of both increased in p38 $\alpha^{\Delta/\Delta}$ as compared with p38 $\alpha^{+/+}$ LSK cells after BMT. Also, mice transplanted with p38 $\alpha^{\Delta/\Delta}$ compared with p38 $\alpha^{+/+}$ LSK cells showed lower levels of allantoin, a product of purine catabolism. These findings suggest that p38 $\alpha$ loss suppresses purine metabolism during stress hematopoiesis. Expression of both inosine-5′-monophosphate dehydrogenase (Impdh) 2, the rate-limiting enzyme of guanosine monophosphate (GMP) synthesis, and guanosine monophosphate synthetase (Gmps) was significantly decreased in p38 $\alpha^{\Delta/\Delta}$ relative to p38 $\alpha^{+/+}$ LT-HSCs on day 1 after BMT.

To identify how p38 α regulates purine enzymes, we searched open-access databases for transcription factors capable of binding to promotor regions of genes that encode purine-related enzymes and are highly expressed in HSCs. Among candidates, we found that the microphthalmia-associated transcription factor (Mitf) commonly binds promoters of purine-related genes and is highly expressed in HSCs. To evaluate Mitf function in vivo and in vitro, we used a mouse line homozygous for the vitiligo-associated spontaneous Mitf mutation ($Mitf^{vit/vit}$), a point mutant encoding a protein with reduced transcriptional activity. In vitro EdU incorporation into LT-HSCs decreased in $Mitf^{vit/vit}$ relative to $Mitf^{+/+}$ LT-HSCs and also transplanted $Mitf^{vit/vit}$ LT-HSCs showed higher CFSE-fluorescence, suggesting delayed proliferation immediately after BMT. We also evaluated expression of Impdh and Gmps by qPCR. Expression levels of Impdh2 and Gmps significantly decreased in $Mitf^{vit/vit}$ relative to $Mitf^{+/+}$ LT-HSCs, an effect similar to that seen following transplantation of p38 $\alpha^{\Delta/\Delta}$ LT-HSCs.

To verify that Impdh2 functions as a p38 α effector in HSC proliferation in vitro and in vivo, we transduced LSK cells with retrovirus expressing Impdh2. Impdh2 overexpression restored short-term EdU incorporation and CFSE fluorescence levels in p38 $\alpha^{\Delta/\Delta}$ LSK cells. Next, Impdh2-overexpressing LT-HSCs were sorted and transplanted into lethally-irradiated recipient mice along with competitor MNCs. Impdh2 overexpression rescued short-term repopulation defects in p38 $\alpha^{\Delta/\Delta}$ LT-HSCs to levels seen in non-mutant cells. However, Impdh2-overexpression did not rescue long-term repopulation defects. Next, we asked whether Impdh2 functions as a Mitf effector in HSC proliferation. Impdh2 overexpression restored short-term EdU incorporation in $Mitf^{vit/vit}$ $Lin^-$ cells. Unlike the case in p38 $\alpha^{\Delta/\Delta}$ LT-HSCs, Impdh2 overexpression restored both short- and long-term repopulation defects seen following $Mitf^{vit/vit}$ transplantation. We conclude that after transplantation, p38 α promotes HSC cycling through Mitf-dependent Impdh2 expression.

In summary, we have identified p38 α as a cell cycle and metabolic regulator of HSPCs in hematological stress. p38 α is required for HSPC cell cycle initiation, especially during stress settings including transplantation, bone marrow recovery, and ex vivo proliferation [3]. In addition, hematological stresses activate p38 α-dependent purine metabolism to initiate cycling of quiescent HSCs. Artificial regulation of the p38 α/Mitf/purine metabolism axis could be a novel modulator of HSPCs during hematological stress or ex vivo expansion (Figure 1) [3]. This study reinforces the importance of cellular metabolism in stem cell regulation and is the first study of metabolic requirements of HSPCs during stress.

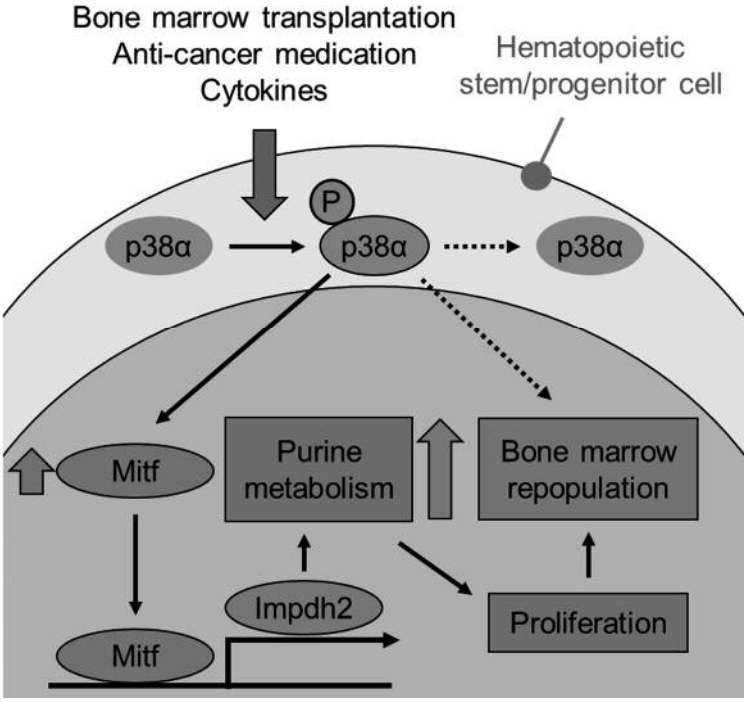


**Figure 1** Regulation of stress hematopoiesis by p38α-activated purine metabolism

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# HEDGEHOG SIGNALING AND THE STEM CELL NICHE IN TISSUE RENEWAL AND MALIGNANCY

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The Hedgehog signaling pathway plays a critical role in the postnatal control of tissue-specific stem cell activity for the repair of injured tissues and maintenance of tissue integrity. In organs such as the urinary bladder, epithelial injury triggers augmented expression of the Hedgehog signal, which then elicits signals from underlying stromal cells that in turn trigger epithelial cell proliferation and differentiation. This feedback between tissue layers enables cells of the undamaged stromal layer to serve as a stem cell niche that templates the maintenance and repair of damaged epithelia. Surprisingly, in the context of malignancy, this feedback signaling from stromal to epithelium serves to restrain tumor growth and progression. This protective effect is due to Hedgehog induction of stromal signals, often BMP family proteins, that induce epithelial differentiation and thus prevent further tumor growth and spread. Similar roles for Hedgehog signaling in restraining cancer growth have been found in other endodermal malignancies, such as those arising from pancreas and colon.

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# INDUCTION OF CANCER STEMNESS AND DRUG RESISTANCE BY EGFR INHIBITORS AND CELLULAR STRESS

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Previous studies in our lab revealed that integrin avb3 become upregulated on blood vessels during tumor angiogenesis. We found that targeting this integrin with antagonists could block angiogenesis. Our current findings reveal that avb3 expression leads to endothelial cell reprogramming to an immature state and following its down regulation endothelial cells mature into vascular structures. However, avb3 also plays a role in tumor growth and metastasis. Here we show that avb3 expression on tumor cells is upregulated in response to drug treatment or microenvironemental stress. Tumor cells encounter stresses including therapeutic intervention and overcome these stresses to achieve disease progression and metastasis. Previous work has established that cancer therapy can select for the expansion of drug resistant cancer stem cells or induce an epithelial-to-mesenchymal transition leading to tumor progression. We recently reported that epithelial cancers exposed to receptor tyrosine kinase inhibitors such as Erlotinib showed an upregulation of integrin αυβ3 on the cell surface. We found that αυβ3 was both necessary and sufficient to account for cancer stemness and drug resistance by initiation a KRAS/Ralb/TBK1/NFkB pathway (Seguin et al, Nature Cell Biology, 2014). In fact, αυβ3 formed a membrane complex with the multivalent lectin Galectin 3 allowing it to drive KRAS addiction and a dependency on micropinocytosis for nutrient uptake and survival. A clinically active Galectin 3 inhibitor was able to selectively destroy αυβ3 positive drug resistant lung tumors by suppressing micropinocytosis in these cells.

While αυβ3 is necessary and sufficient to account for erlontib resistance and tumor stemness it is not clear whether αυβ3 expression was due to a selection of preexisting drug resistant cells or the induction of αυβ3 gene expression due to drug treatment or cellular

stress. Here we show the surprising finding that EGFR inhibitor treatment represents one form of cellular stress such as hypoxia, nutrient deprivation, oxidative stress that directly induces the reprogramming of epithelial carcinoma cells, converting them to a stress-tolerant, stem-like, drug-resistant state. Each of these forms of stress induces integrin β 3 (ITGB3) transcription that is necessary and sufficient to drive expression of pluripotency maintaining factors OCT4 and NANOG, representing a plastic state that endows cells with stress tolerance and drug resistance. These reprogrammed cells can be detected in regions of tumor hypoxia and are highly enriched in the circulation of tumor bearing mice after acquired resistance to systemic therapy. Mechanistically, stress induces a series of histone modifications that promote an open chromatin state on the β 3 promoter, allowing binding of the transcription factor HNF4A that is itself induced by stress. Histone acetyltransferase or demethylase inhibitors not only block stress-mediated histone modifications on the β 3 promoter to prevent the induction of β 3, but block the induction of OCT4/NANOG and sensitize cells to the effects of stress. Our findings reveal β 3 both as a marker and mediator of stress tolerance and cellular reprogramming that drives tumor progression, likely accounting for its enrichment on metastatic tumor lesions. The induction of β 3 and HNF4A by stress highlights the ability of epithelial tumor cells to rapidly adapt to therapeutic intervention, thus challenging the notion that drug resistance primarily results from a small population of pre-existing stress-tolerant cells. Consequently, this work reveals how certain anti-cancer therapies may accelerate cancer progression by upregulating integrin β 3 as an adaptive response to stress.

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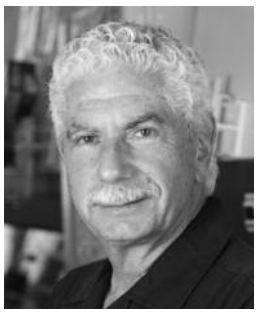

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# GROWTH FACTOR SIGNALING IN CANCER STEM-LIKE CELLS AND THEIR NICHE

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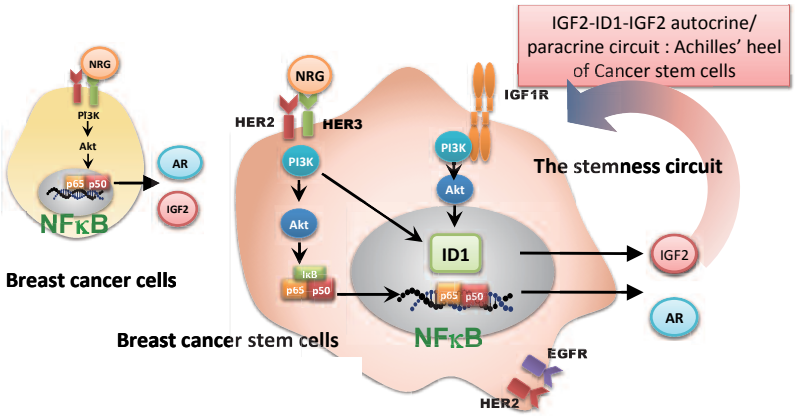
Cancer stem cells (CSCs) have characteristics in common with normal stem cells from tumor-prone tissue. For instance, CSCs can self-renew and simultaneously produce differentiated daughter cells that proliferate strongly until they reach their final differentiated state. Apparent differences also exist between CSCs and normal stem cells. The latter are maintained under tight homeostatic regulation and are passively protected in the surrounding microenvironment or stem cell niche in adult tissues. However, the former may actively contribute to tumor formation and may use the CSC niche for their own survival and proliferation. It is now believed that many solid tumors have cancer stem-like cells.

Growth factor signaling plays important roles for a variety of biological aspects in physiological and pathological conditions, including tumorigenesis. It is thought that inflammatory microenvironment creates a pro-tumorigenic state through production of growth factors or cytokines. However, it is obscure how growth factor- or cytokine-signaling contributes to cancer stem-like cells and the CSC niche.

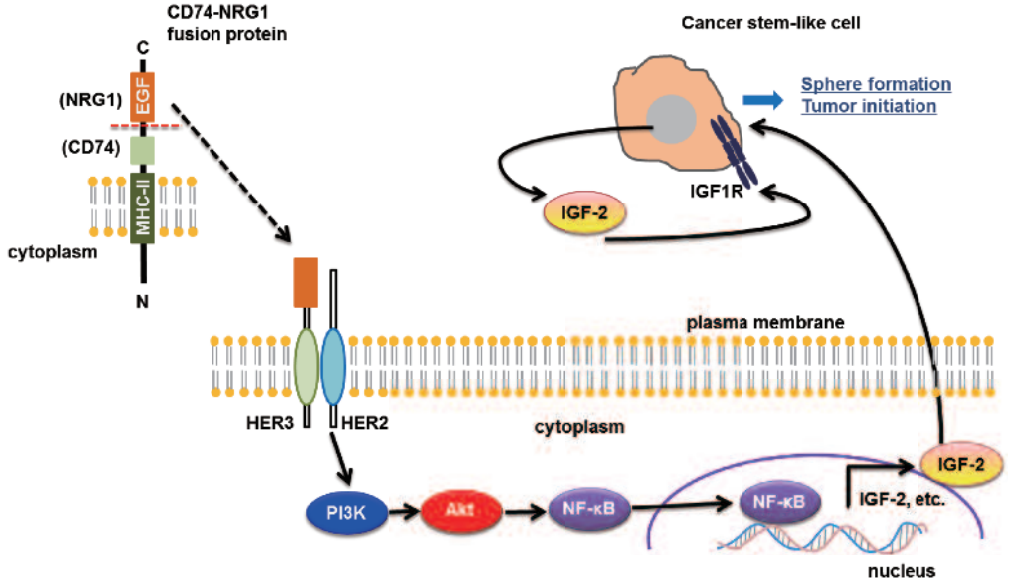
We found that growth factor signaling plays critical roles for the communication between cancer stem-like cells and their niche in order to maintain the cancer stem-like cells in the CSC niche. We have established conditions of culturing patient-derived tumor spheres and generated patient-derived xenograft (PDX) models derived from the breast cancer tissues. By using these patient-derived cells and PDXs, we uncovered the various key mechanisms mediated by signaling through NFκB, IGF1 receptor, beta-catenin, semaphorin, FRS2beta, growth differentiation factor (GDF) 15, MTHFD2, and so on. There are various mechanisms: autocrine-paracrine mechanisms, regulation of stemness,

regulation of symmetric-asymmetric division and regulation of metabolism. Cancer stem-like cells appear to survive and divide by using these mechanisms in order to adapt in the different conditions of the CSC niche. Among them, we have been able to identify proper molecular targets for cancer stem-like cells to eradicate tumors. In this symposium I would like to talk about several key mechanisms we have recently identified for maintenance of cancer stem-like cells in the CSC niche.

1. Many breast cancer patients suffer from relapse that is potentially due to cancer stem cells that are not eliminated by treatment. Not only cancer stem cells do not easily disappear, but also cancer cells appear to have plasticity by which they undergo dedifferentiation and even acquire cancer stem cell properties! However, it is largely unknown how cancer stemness is maintained. Here we have shown the fundamental mechanisms to stabilize cancer stemness. Since the HER2/3-PI3K-NFκB pathway is important for cancer stemness, we examined expression of downstream molecules in this pathway by comprehensive analysis of gene expression profiles over time after addition of the HER3 ligand heregulin (HRG) that is also called as neuregulin (NRG)1. Insulin-like growth factor 2 (IGF2) was identified as a key downstream molecule, since anti-IGF2 antibody treatment blocked tumor sphere formation, a characteristic of stemness, even under conditions where other growth factors/cytokines were present. IGF2-PI3K signaling induced tumor sphere formation and enhanced the expression of genes favoring stemness, including the transcription regulator *ID1* and *IGF2* itself. Consistent with these data, ID1 and the IGF2 receptor IGF-1R were expressed at high levels in cancer stem cell population of the breast cancer PDXs. Moreover, *ID1* knockdown suppressed IGF2-induced expression of *IGF2*. Finally, treatment with anti-IGF1/2 antibodies blocked tumorigenesis derived from the IGF1R$^{high}$ CSC-enriched population in a PDX model. Thus, NF κ B may trigger IGF2-ID1-IGF2 positive feedback circuits that allow cancer stem-like cells to appear. Then, they may become addicted to the circuits. Since the circuits are the Achilles' heels of CSCs, it will be critical to break them for eradication of CSCs.



2. Cancer stem cells (CSCs) are thought to be responsible for the initiation and recurrence of tumors. Therefore, targeting molecules that have a critical role in maintenance of CSCs would be a useful strategy. CD74-NRG1 fusion gene was identified in 5-15% of in invasive mucinous adenocarcinoma of the lung, a malignant type of lung adenocarcinoma. CD74-NRG1 protein contains the CD74 transmembrane domain and the EGF-like domain of the NRG1/HRG protein, suggested to mediate juxtacrine signals through HER2:HER3 receptors. In this study, we expressed cDNA of CD74-NRG1 fusion gene in H322 human lung cancer cell line and BT20 human breast cancer cell line by using a lentivirus system, and investigated whether this fusion gene is involved in the promotion of CSC phenotype using a CSC assay. First, we examined the self-renewal ability of CD74-NRG1 expressing breast cancer cells by performing the sphere forming assay. CD74-NRG1 expressing cancer cells were able to form tumor spheres without adding any growth factors, while cells infected with the lentivirus carrying control vector were not. Then, we analyzed the population of BCSCs by flow cytometry using CD44 and CD24 antibody. The percentages of $CD44^{high}/CD24^{-/low}$ CSC-enriched population increased from 1.94% to 9.47% (variant 1) or 8.21% (variant 2). These results suggest that expression of CD74-NRG1 fusion gene promotes cancer stem cell properties and is involved in stem cell function of several types of cancers including lung and breast cancer.



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# IMAGING STEM CELL SIGNALS IN CANCER HETEROGENEITY AND THERAPY RESISTANCE

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Malignant progression of pancreatic adenocarcinoma is critically dependent on the RNA binding prorein Musahi [1]. Pancreatic cancer is a disease for which treatment is rarely curative and in developed countries, it is the fourth leading cause of cancer-related deaths [2]. Because patients are asymptomatic at early stages, by the time a diagnosis is made, standard treatments have limited impact [3-5]. Four genes are commonly altered in pancreatic cancer: activating mutations of KRAS2 are found in greater than 90% of tumors, while the tumor suppressors p16/INK4A, p53, and SMAD4 [6-10] are frequently inactivated by mutation, deletion or epigenetic silencing. These mutations have proved difficult to target and currently no targeted therapies are available for use in pancreatic cancer. In the absence of targetable driver mutations, defining downstream epigenetic programs that are critical signaling nodes for disease progression could identify new therapeutic targets.

Pancreatic cancer progresses from a more differentiated non-invasive precursor stage called pancreatic intraepithelial neoplasia (PanIN) to a more undifferentiated and malignant invasive adenocarcinoma [11]. Although the aberrant proliferation and survival in pancreatic cancer cells have been targeted by several approaches[12], the molecular programs that disable differentiation events during pancreatic cancer progression are not as well understood. This undifferentiated state is, however, a hallmark of adenocarcinoma and as integral to its maintenance as proliferation and survival; thus, identifying the pathways that mediate this differentiation arrest can be an important avenue for understanding the basis of pancreatic cancer progression and may reveal new vulnerabilities. To address this, we have focused on signals that can antagonize

differentiation in stem and progenitor cells during normal development. One particularly powerful but little studied signal is the RNA binding protein Musashi (Msi), a molecule that governs stem cell fate. Msi is an evolutionarily conserved RNA binding protein originally identified in drosophila [13]. A gene duplication event in teleosts led to the creation of two Msi family members, Msi1 and Msi2. Mouse Msi1 and mouse Msi2 share a 75% similarity in amino acid sequence with the highest sequence similarity (85%) in the critical RNA Recognition Motifs [14,15]. Each family member displays 95-99% sequence similarity between their mouse and human homologs [16]. Msi is highly expressed in mammalian stem and progenitor cells across many tissues, such as the brain, mammary glands and the gut, and has been long used as a marker of immaturity in many contexts [17-19]. However, its functional impact is only beginning to emerge: genetic loss of function models of Msi1 or Msi2 have shown that Msi signaling is important for maintaining stem cells in the mammalian nervous system [20], and, more recently, in normal and malignant hematopoiesis [21-23]. Thus, whether and how Msi contributes to adenocarcinoma development in primary pancreatic and other solid cancers and whether it may be a tractable therapeutic target remains unknown.

To assess the contribution of Msi in pancreatic carcinogenesis, we created knock-in fluorescent reporter mouse strains that allow visualization and tracking of Msi+ cells *in vivo* and crossed them to the Ras/p53 driven mouse models of pancreatic cancer. This served as a unique new tool for image based and functional tracking and showed with remarkable precision that Msi reporter activity marks cancer stem cells that propagate adenocarcinoma (Figure 1), circulating tumor cells with preferential capacity to metastasize, and cells that are gemcitabine resistant. The fact that Msi+ cells are such a "high risk" population highlighted the need for targeting them: deletion of either Msi1 or Msi2 in genetically engineered mouse models delayed tumor initiation (Figures 2 and 3) and dramatically impaired transition from PanIN to adenocarcinoma, and markedly improved survival, suggesting that disease progression is deeply dependent on Msi signaling (Figures 4 and 5).

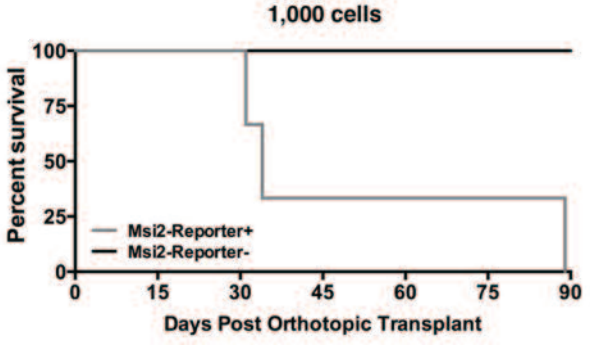


**Figure 1** Survival curves of mice orthotopically transplanted with Msi-Reporter+ or Msi-Reporter- $KP^{ff}C$ pancreatic tumor cells reflect the tumor-propagating ability of Msi-expressing cells.

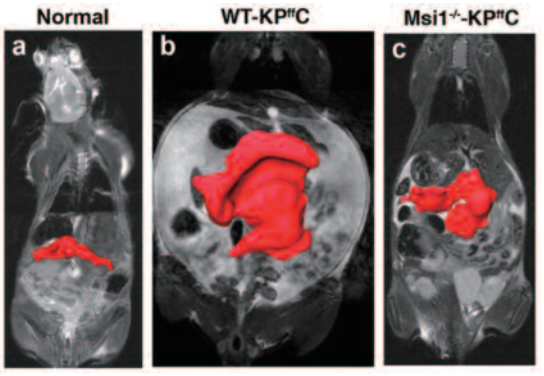


**Figure 2** MRI images of normal, WT-KP$^{ff}$C and Msi1-/-KP$^{ff}$C mice, with 3-dimensional volume rendering of the tumor mass shown in red. Tumors are significantly smaller in the absence of Msi1.

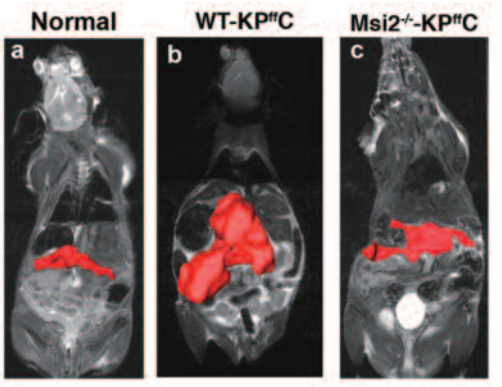


**Figure 3** MRI images of normal, WT-KP$^{ff}$C and Msi2-/- KP$^{ff}$C mice, with 3-dimensional volume rendering of the tumor mass shown in red. Tumors are significantly smaller in the absence of Msi2.

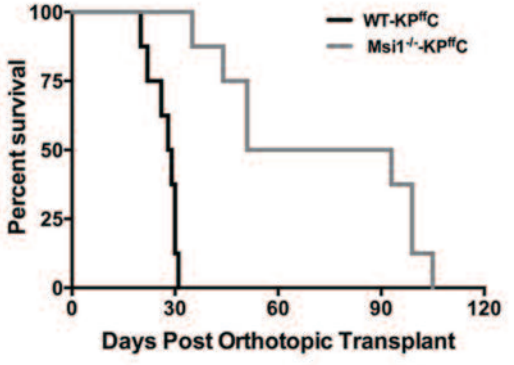


**Figure 4** Survival curves of mice orthotopically transplanted with WT or Msi1-/- KP$^{ff}$C pancreatic tumor cells demonstrate improved survival in the absence of Msi1.

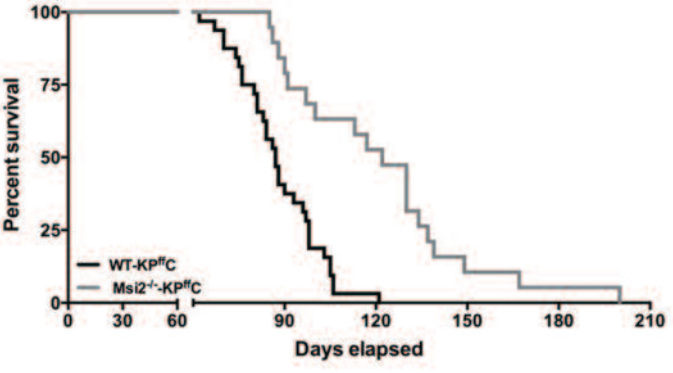


**Figure 5** Survival curves of WT and Msi2-/- KP$^{ff}$C mice demonstrate improved survival in the absence of Msi2.

Mechanistically, MSI1 and MSI2 controlled the expression of shared and distinct genetic programs; among these, Msi could bind the 3′UTR and regulate expression of the proto-oncogene c-MET, which could rescue defects triggered by Msi loss. In human lines, Msi expression generally rose with aggressiveness, and drove enhanced growth. Msi inhibition not only blocked growth of these lines, but also adenocarcinoma growth in patient-derived pancreatic cancer xenografts, demonstrating that Msi is important in the context of multiple and varied somatic mutations that occur in human disease. Finally, to explore the translational potential of this work, and define if Msi can be targeted with a deliverable small molecule, we used a newly emerging paradigm to block its expression. Thus we designed, screened, and developed therapeutic antisense oligonucleotides against Msi that effectively downregulated Msi expression and blocked growth of established primary pancreatic tumors *in vivo* (Figure 6). Our data suggests that MSI-directed ASOs should be considered for further development, not only for potential use in pancreatic cancer, but also for broad use in other aggressive cancers with highly upregulated Msi expression, such as glioblastoma and breast cancer. Finally, we also found that Msi rises in both human pancreatitis and in caerulein induced mouse models of the disease, raising the intriguing possibility that blocking Msi via ASO delivery could prevent or reduce risk of progression from pancreatitis to pancreatic cancer and thus could contribute to prevention efforts as well. In the long term, defining Msi as a new target in pancreatic cancer together with methods to block this pathway could provide a new approach to control cancer growth and progression.

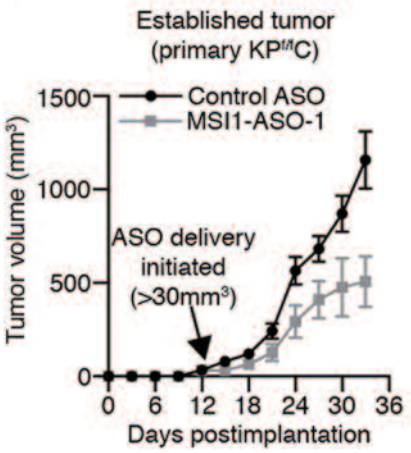


**Figure 6** Msi1-ASO delivery reduces growth of established pancreatic tumor in vivo.

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# REGULATION OF MYELOPOIESIS AND LEUKEMIA BY NONCODING RNA

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Tight regulation of myeloid transcription factors PU.1 and C/EBPa is necessary for proper hematopoietic stem cell function and granulopoiesis, and dysregulation of these genes contributes to leukemia. We have focused on regulation of these two genes by noncoding RNAs (ncRNAs). In the case of PU.1, we previously described a long antisense RNA that is initiated from a discrete promoter in intron 3 and extends past the transcription start site. This transcript is expressed at highest levels in T cells, in which PU.1 is not expressed, and can inhibit PU.1 protein. siRNAs which target this antisense transcript can increase PU. 1 mRNA and protein, and induce differentiation of leukemic cells. A second nuclear long noncoding RNA locus is initiated in the upstream regulatory element (URE), extending greater than 10 kb toward the transcription start site. We are testing the function of the URE enhancer RNA using human BAC transgenics in which transcription terminators have been used to abrogate expression of the noncoding RNA.

In the case of C/EBP alpha, we identified a ncRNA extending beyond the polyadenylation signal. In contrast to PU.1, this extracoding transcript correlates positively with C/EBP alpha mRNA, and siRNA knockdown of the ncRNA leads to a decrease in C/EBP alpha mRNA and increase in methylation of the locus. Overexpression of this ncRNA leads to an increase in expression of C/EBP alpha in a cell line (K562) in which C/EBP alpha is methylated and not expressed.

In summary, we have initiated studies of long noncoding RNAs in both the PU.1 and C/EBP alpha genes. The function of these RNAs appears to be completely different. In the case of PU.1, an antisense noncoding RNA downregulates PU.1 expression. In the case of C/EBP alpha, an extracoding RNA inhibits methylation of the locus and increases mRNA

levels. Targeting the PU.1 antisense RNAs with shRNAs or inducing gene selective demethylation of tumor suppressors using RNAs represent novel approaches to inducing differentiation of leukemic cells.

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# NORMAL AND NEOPLASTIC STEM CELLS

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Blood-forming stem cells are responsible for the development and lifelong maintenance of normal blood and for blood diseases such as leukemia (Figure 1) [1]. Thus, to understand leukemia, one should examine the changes occurring in the blood-forming stem cell.

The cell surface molecules that define HSC have been well described in the scientific literature. In mice, these cells are positive for Thy1-lo, CD150, c-kit, Sca-1; and negative for B220, Mac-1, Gr-1, CD3, 4, 8, Ter119, Flk2, CD34 [2]. In humans, HSC are positive for CD34, Thy1-lo, CD150, c-kit; and negative for CD3, CD4, CD8, CD10, CD14, CD15, CD16, CD19, CD20, CD 38, glycophorin A [3]. However, at least in mice these markers were not sufficient to define and completely purify long term HSC [4]. We recently found that a

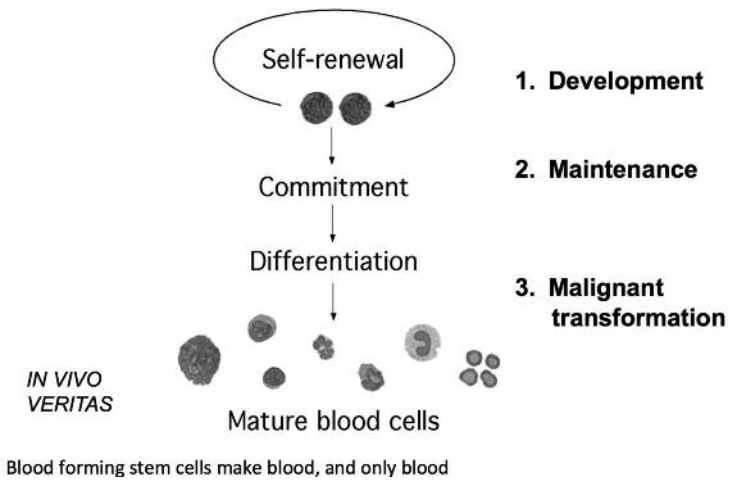


**Figure 1** Blood formation by Hematopoietic Stem Cells [HSCs].

gene called HoxB5 is expressed on only a subset of the cells we have previously defined as HSC. To test the utility of this marker, we generated a reporter mouse in which cells express mCherry along with the HoxB5 gene. Among the cells previously defined as HSC, only a small fraction were positive (high or low) for HoxB5. We assessed the developmental outcome of the different cell subsets by transplantation into myeloablated recipient mice and found that the HoxB5-positive (high and low) cells, but not HoxB5–negative, could reconstitute hematopoiesis for the life of the animal. Therefore, HoxB5 is not only a marker for blood-forming stem cells, but defines the true long-term [LT] HSC [4].

These reporter mice allowed us to visualize LT-HSC in their bone marrow niche for the first time and ask: where are stem cells in the blood-forming tissue? We published that within the bone marrow, 94% of the HoxB5 + blood-forming stem cells are attached to the outside of one kind of blood vessel, the bone marrow sinusoids (Figure 2). Hence, this area around the HoxB5+ cell has to be the bone marrow niche. Given this finding, the first published claims that the hematopoietic niche was located next to the bone or endosteium, cannot be explained, because no HoxB5+ cells were found close to the bone. Other claims that the niche was next to the arterioles also cannot be the explanation, because the HoxB5+ cells were only detected next to venous sinusoids or venules. A quantitative analysis showed that there were only about 100 LT-HSC in each mouse femur. In sum, we have established the identity and location of the long-term stem cells [4].

Many years ago we became interested in the clinical importance of blood-forming stem cells. We analyzed blood-forming tissue—bone marrow or mobilized peripheral blood (circulating blood into which HSC have been mobilized from the bone marrow). Bone marrow and mobilized blood were and still are used in autologous transplantations in cancer patients. In this scenario, a sample of mobilized blood is removed from the patient; very high dose combination chemotherapy is administered, which removes most of the

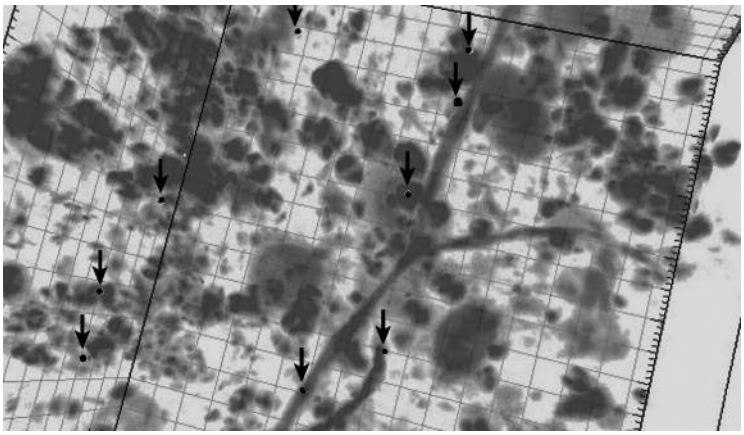

**Figure 2** HoxB5+ blood forming stem cells (arrows) found next to bone marrow sinusoidal vessels [4].

patient's blood-forming tissue and most of the cancer cells throughout the body; and then the mobilized blood is injected back into the same patient to restore blood-forming function. We thought that since we found cancer cells contaminating the mobilized blood this approach could not be effective at curing cancer. When we purified blood-forming stem cells from the mobilized blood and transplanted those into patients, we had decreased the numbers of contaminating cancer cells by a factor of 250,000 (Figure 3) and had removed T cells [5]. We then conducted a clinical trial (initiated 20 years ago): we purified stem cells from mobilized blood in women with breast cancer that had metastasized to the lymph nodes and distant tissues, such as liver, lung, and bone. No surgery or radiotherapy could eliminate the disease, so a cure required high-dose chemotherapy. Figure 4 shows the long-term results of that trial, comparing transplants of HSC purified from mobilized blood vs. transplants of unpurified mobilized blood [6]. Amazingly, in 2011 and 2017, one third of the pure HSC-transplanted patients are still alive and free of disease. Therefore,

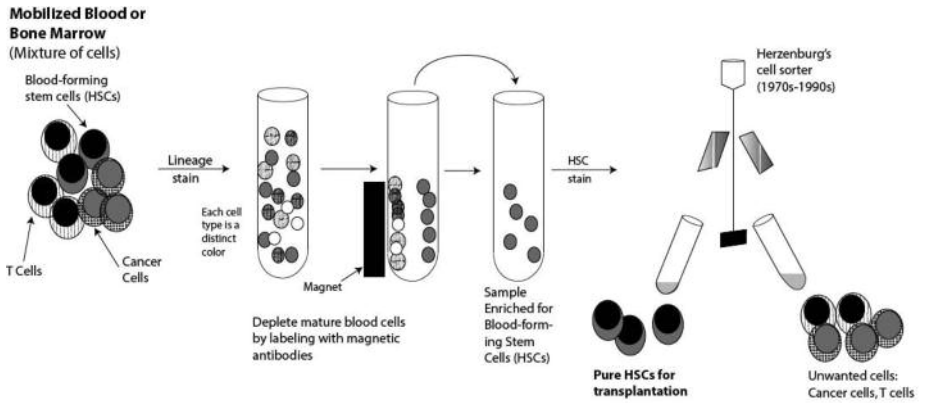


**Figure 3** Removal of contaminating cancer cells from blood-forming tissue grafts. [5]

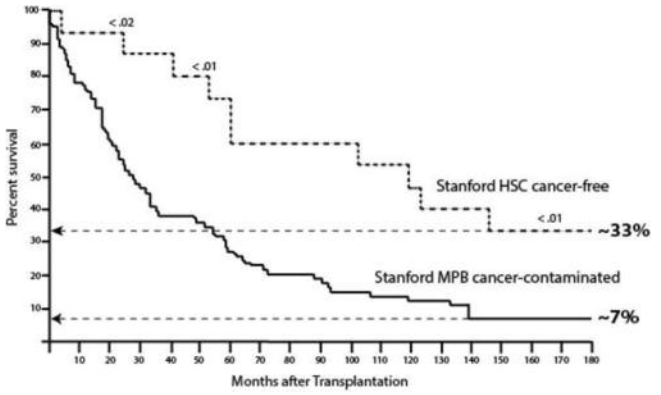


**Figure 4** Survival of patients with metastatic breast cancer treated with high dose combination chemotherapy followed by autologous transplantation of purified blood-forming stem cells from mobilized blood VS. unpurified mobilized blood [6].

when high-dose chemotherapy followed by autologous transplantation of blood-forming tissue is used to treat diseases (such as leukemia, lymphoma, metastatic breast cancer, and melanoma), one should use cancer free purified HSC. Stanford University is opening a pure stem-cell transplantation laboratory and clinic and we intend to extend those initial clinical trials.

We also examined the role of purified blood-forming stem cells in transplantations from one person to another (donor to recipient). In Figure 5, on the left, when T cells from the donor contaminate the transplant, they attack the tissues in the recipient causing multi-organ system graft versus host disease (GVHD). These patients have to be contained in a nearly germ-free environment, while immunosuppressive drugs that increase the risk of infections are used to decrease the severity of GVHD. Amongst the T cells that are causing GVHD, some are usefully combating the cancer. This was the forerunner of current T-cell therapy for cancer. If we wish to cure diseases of the blood-forming system with donor-to-recipient (allogeneic) transplantation, using purified HSC will not cause GVHD, and thus not necessitate immunosuppression. Moreover, allogeneic HSC transplantation induces transplantation tolerance. Therefore any organ or cells from the HSC donor could be subsequently transplanted to the same recipient and tolerated without immunosuppression [7,8]. In sum, purified HSC transplantation eliminates GVHD and enables other tissue transplantation without immunosuppressive drugs. This would expand the use of HSC transplantation to treat blood diseases such as severe combined immunodeficiency (SCID), thalassemia and sickle cell anemia. It could also cure, as we showed in animal models, genetic autoimmune diseases such as type I diabetes [9] and systemic lupus erythematosus [10] by replacing a blood-forming system prone to autoimmunity with a healthy one (Figure 5, right) [7,8].

Typically blood-forming tissue transplantation requires prior conditioning of the patient with high-dose radiation and chemotherapy to eliminate the existing blood-forming cells.

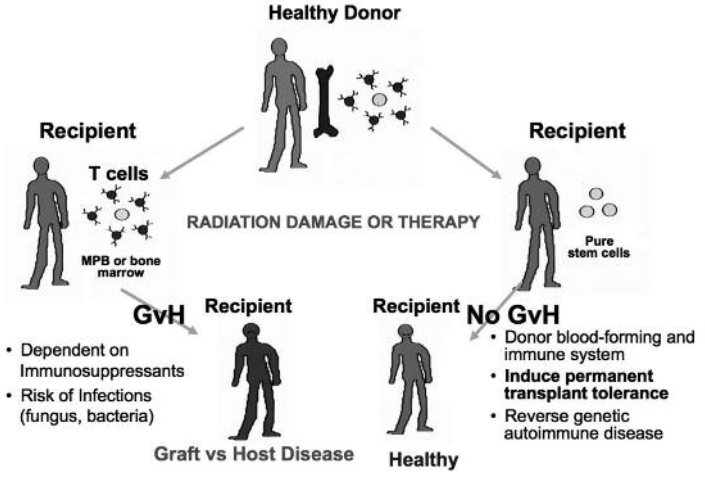


**Figure 5** HSC transplantation from a healthy donor to a recipient. [7-10]

Because this radiation is toxic to other systems, we have sought to develop, through the use of antibodies, a less toxic conditioning method to enable transplantation. As Figure 6 shows, anti c-kit antibody (ACK2; which targets the recipient's blood-forming stem cells), and anti-CD4 and anti-CD8 antibodies (which target recipient T cells), are not on their own sufficient to eliminate those cells in mice and allow a transplant to engraft [11]. However, combining these antibodies with anti-CD47 antibody allows the purified donor's blood-forming stem cells to engraft in an unrelated recipient, leading to full reconstitution of all blood cell types without GVHD (Figure 6). In sum, we want to limit the use of cytotoxic conditioning and radiation conditioning in the future, and use far less toxic antibody-only conditioning. Because of the fact that HSC from a donor induce tolerance in the recipient to all other tissues from that same donor, in the far future, we want to use cells rather than whole tissues or organs from a donor to generate HSC and other needed tissue-forming stem cells to replace diseased or damaged tissue for life.

To understand the role of HSC in the development of leukemia we first analyzed the genetic makeup of leukemia cells from patients in Japan after the atom bomb [12]. In AML1-ETO leukemia, the of leukemia stem cells had the distinctive AML1-ETO chromosomal translocation plus other mutations. Furthermore, the AML1-ETO translocation had to occur in a stem cell, because, the initial genetic changes that we found in leukemias do not change a non–self-renewing cell into a self-renewing cell. We

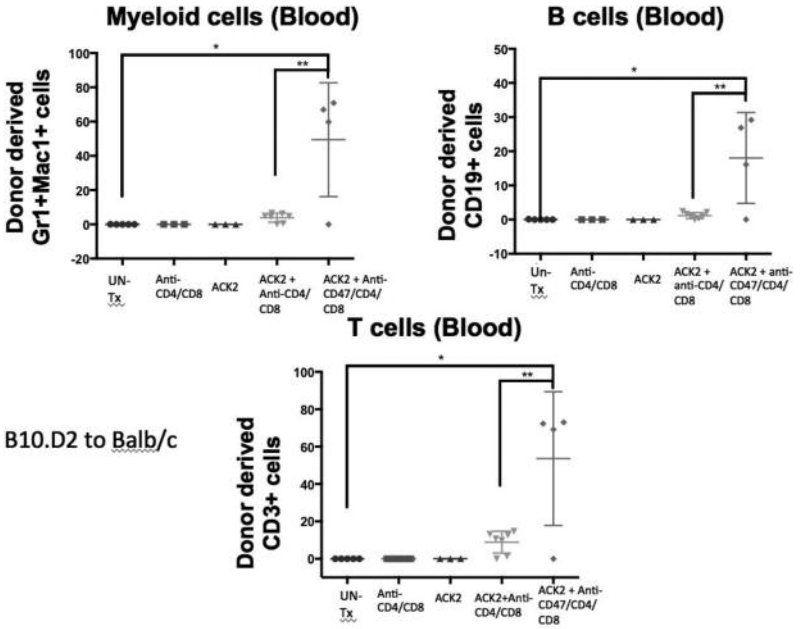


**Figure 6** Successful engraftment of a mouse transplant from a donor to recipient (with minor mismatch) using antibody conditioning rather than radiation conditioning. (Antibodies: anti-c-kit [ACK2], anti-CD4, anti-CD8, anti-CD47) [11].

hypothesized that therefore the only cells that must accumulate the changes and pass them on to daughter cells, are the self-renewing stem cells [13]. If this were true, one could trace back from the leukemia stem cell (Figure 7, bottom row, cells marked "1-7"), and find a clone that has n-1 (in this case, 6) mutations, another with n-2, another with n-3, etc. Therefore, knowing all the mutations in the leukemia from a particular patient could allow one to determine the order of the mutations. Another important point: the number of stem cells and the number of niches are constant. Thus, in order for a stem cell clone with a given mutation or set of mutations to advance to a point where it is dangerous, it must outcompete the normal stem cells for limited niche sites.

We tested this hypothesis by sequencing the DNA to find mutations that were in the leukemia but not in normal tissue (Figure 8, top half). We prepared DNA primers that would identify the normal or mutant stem cells when we analyzed one stem cell at a time [14,15]. The bottom row of Figure 8 shows some of the mutations in the first patient whose bone marrow we analyzed. The first mutation was TET2 Y1649stop, which stopped the protein from being expressed, leaving haploexpression of TET2. The second mutation (T1884A) was in the second allele of that TET2 gene, leading to no expression of this gene. We could find mutant cells in bone marrow from anywhere in that patient, because not only did the clone expand, but, like normal blood-forming stem cells, it migrated from one bone marrow cavity to another. Moreover, the mutation that was associated with the initial

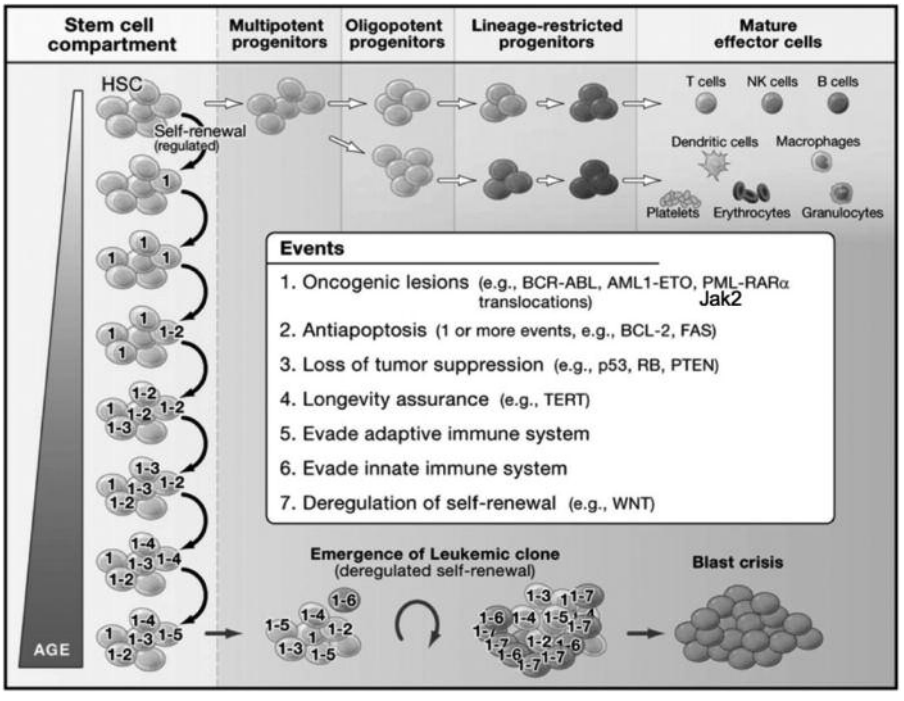


**Figure 7** Schematic diagram of the development of leukemia. The numerals superimposed over the blood-forming stem cells (1, 1-2, 1-3, etc.) indicate the number of mutations present [13].

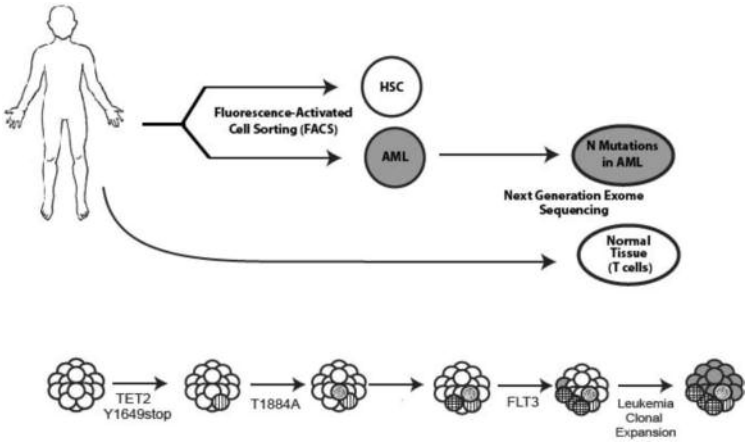


**Figure 8** (Top) Strategy for analyzing leukemia stem cells compared to normal blood forming stem cells (HSCs) and other normal stem cells to identify sequence of mutations leading to leukemia. (Bottom) Example of the results of this analysis applied to a patient. (Striped and hatched stem cells acquired mutations that did not lead to proliferation; final mutation in grey cells [leukemia stem cells] led to proliferation) [14].

diagnosis—FLT3-ITD (internal tandem repeat)—was not found in the stem cell clone but only in the leukemia stem cell derived from that clone. In looking at 21 other patients, we could generalize: the initiating mutations are in those genes that are required for normal differentiation from the stem cell toward a blood cell. A knockout of TET2 lowers the frequency of differentiation but does not limit proliferation, so the cells can compete for the niche. The last mutated gene in different patients (e.g., K-ras, N-ras, FLT3) appears as the last step and allows massive increase in proliferation, with perhaps self-renewal. Thus, the initial mutations have prepared the cells to finally become leukemic once the last mutation occurred [14]. This important principle also probably applies to other cancer in any tissues with stem cells and progenitor cells.

Elimination of all cells in a cancer except the cancer stem cell will allow the disease to grow. Therefore, successful therapy must eliminate all the cancer stem cells. Thus, we wanted to know whether there are targets on leukemia stem cells but not normal stem cells. The very first we discovered was CD47, a signal that protects a cell that expresses it from phagocytosis by macrophages. Tht is, CD47 is a "don't eat me" signal to macrophages. As shown in Figure 9, we proposed that in order for an aging normal or cancer cell to survive, its "don't eat me" signal must be overcoming a contrary "eat me" signal that the cell expresses. To study this, we transplanted human leukemias from patients into the blood-forming system of immune deficient mice and allowed them to engraft and grow (Figure 10). [16] When we then treated for 2 weeks with a blocking antibody to CD47, 90% of the leukemias were cured. In 10% of cases the leukemia cells managed to escape elimination by macrophages.

CD47 was also in breast cancer. As shown in Figure 11, when breast cancer stem cells isolated by Michael Clarke were transplanted into an immune deficient mouse and allowed

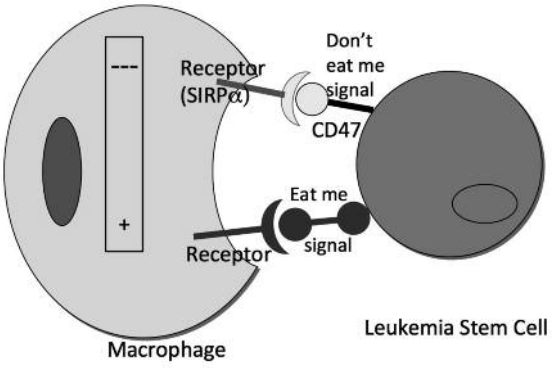


**Figure 9** Expression of CD47 on myeloid leukemia cells contributes to pathogenesis by providing a "don't eat me" signal to macrophages that counteracts other "eat me" signals on the leukemia cells [16].

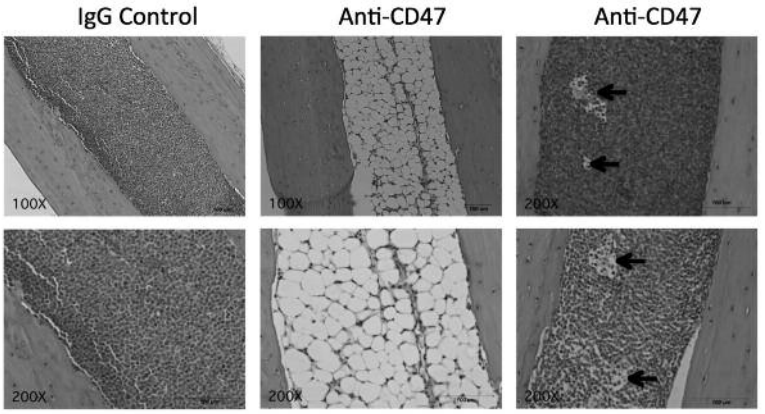


**Figure 10** Anti-CD47 antibody treatment depletes human acute myeloid leukemia transplanted and allowed to take over the bone marrow of mice. (Right panel: arrows point to macrophages containing leukemia cells) [16].

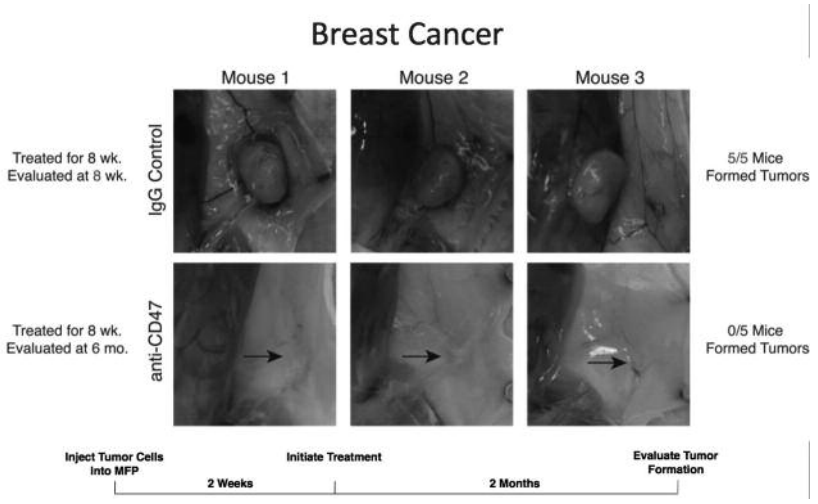


**Figure 11** Anti-CD47 antibodies inhibit the growth of breast cancer tumors transplanted from patients into mice. [17]

to grow into a tumor, treatment with a blocking antibody to CD47 cleared the tumor. In fact for every type of cancer we transplanted into a mouse and then treated with anti-CD47 antibody, if the tumor was small enough, it was cleared; if it was not small enough, at least the metastases were eliminated [17].

There are two parts to the strategy to increase phagocytic removal of tumor cells: block their "don't eat me" signal and provide or strengthen their "eat me" signal. Figure 12 shows that antibodies like rituximab (anti-CD 20) have a binding site on the back of the antibody for the phagocytic receptor—the high affinitity Fc receptor—on macrophages. When an aggressive human lymphoma was transplanted into an immune deficient mouse and allowed to grow, treatment with rituximab plus anti-CD47 antibody (but neither agent alone) cured the lymphoma [18].

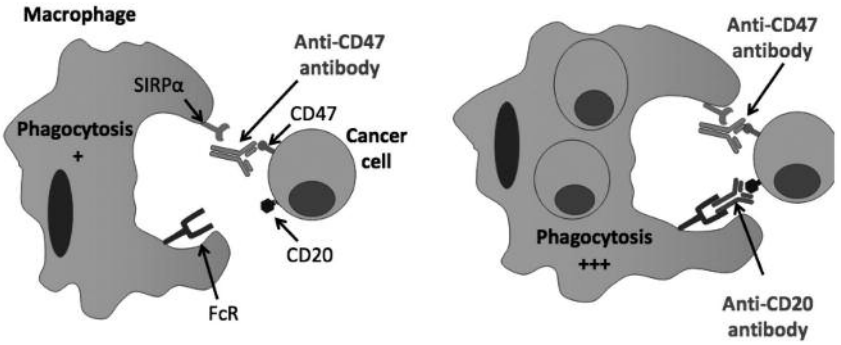


**Figure 12** Combination therapy with anti-CD47 antibody + cancer-targeting monoclonal antibodies (e.g., rituximab [anti-CD 20]). (SIRPa=receptor for CD47; FcR = receptor for Fc fragment of anti-CD20 antibody) [18,19].

In summary, by taking stem cells as a way of looking at the biology of the human blood-forming system (and later brain-forming, breast-forming, etc.), we discovered that by purifying the cells you can begin to understand the events that would: allow a normal stem cell to regenerate the system; allow physicians to switch from chemotherapy and radiation conditioning to less toxic antibody conditioning prior to performing transplant; and allow us to compare cancer stem cells to normal tissue stem cells to discover therapeutic targets like CD47.

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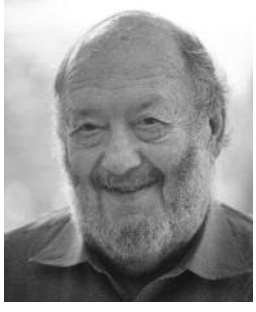

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# CELL OF ORIGIN AND TUMOR STEM CELLS IN MOUSE DIGESTIVE ORGAN TUMORS

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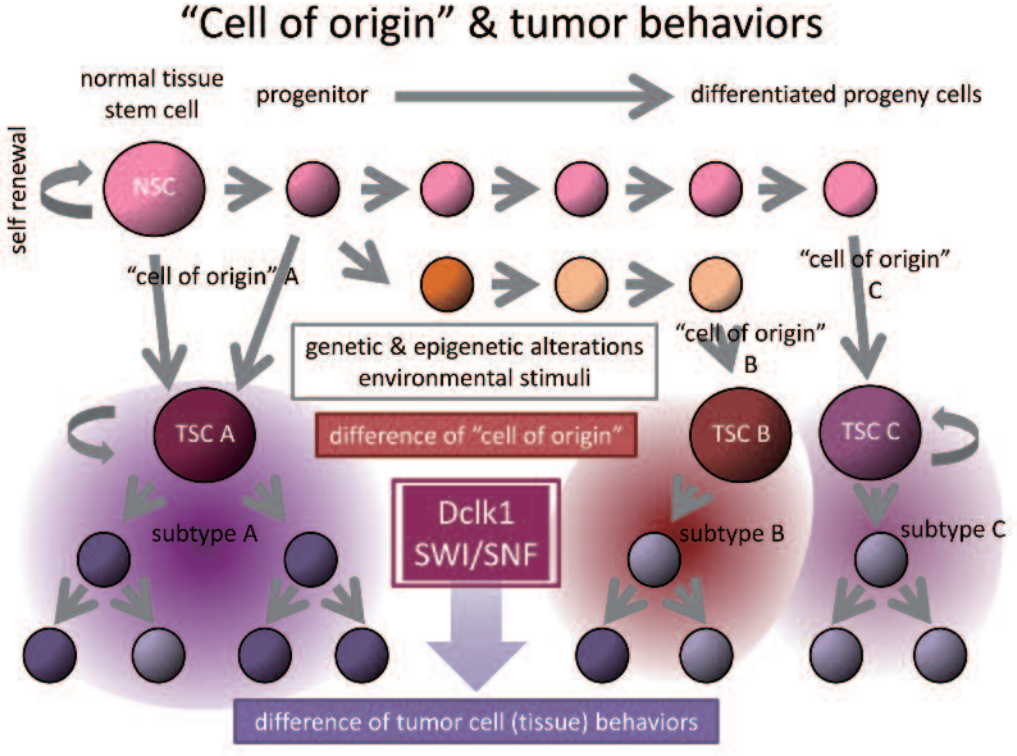
Accumulating evidence suggests the existence of tumor stem cells that resemble normal stem cells. There is great interest in tumor stem cells as potential therapeutic targets; however, cancer therapies targeting tumor stem cells are limited. One of the drawbacks is that tumor stem cell markers are often shared by normal stem cells; thus, therapies that target these markers may cause severe injury to normal tissues. In this respect, we reported that doublecortin-like kinase 1 (Dclk1) is a unique marker that distinguishes tumor stem cells from normal stem cells in the intestine [1]. In *Dclk1*$^{creERT2/+}$*; Rosa26-LacZ; Apc*$^{Min/+}$ mice, intestinal tumors were occupied by LacZ-labeled progeny tumor cells derived from Dclk1-expressing tumor stem cells with scattered LacZ expression in the normal intestinal mucosa after tamoxifen injection. Consistent with this, specific ablation of Dclk1-expressing tumor stem cells resulted in a marked regression of polyps without apparent damage to the normal intestine. Therefore, targeting Dclk1-expressing cells appears to be a potential therapeutic strategy against intestinal tumors.

Recent studies have also shown that digestive organ tumors are complex and can be derived from both normal tissue stem cells and differentiated cells. Another factor complicating the tumor hierarchy is that genetic or epigenetic alterations caused by intrinsic and extrinsic events conceal the identity of the "cell of origin" of tumors including tumor stem cells. This raises the question of whether "cell of origin" affects the biological behaviors of tumors in the presence of the same genetic or epigenetic alterations.

In this symposium, I presented the examples of pancreatic tumors in the association with Dclk1 and SWI/SNF chromatin remodeling complexes. In the pancreas, pancreatic acinar cells with oncogenic Kras formed pancreatic intra-epithelial neoplasia (PanIN) via

acinar-to-ductal metaplasia (ADM). Additional inactivation or loss of p53 led PanIN to progress to pancreatic ductal adenocarcinoma (PDA). During this process, Kras activation in Dclk1-expressing duct or islet cells did not affect their proliferation. However, under caerulein-induced inflammatory conditions, Kras activation in Dclk1-expressing cells resulted in PanIN formation. Furthermore, Brg1, a subunit of SWI/SNF complexes found in both eukaryotes and prokaryote, in acinar cells suppressed the development of ADM and PanIN as in the case in intestinal tumors [2, 3]. In contrast, loss of Brg1 in pancreatic duct cells cooperated with oncogenic Kras to form intraductal papillary mucinous neoplasm (IPMN), a distinct precursor that progresses to PDA as in the case in PanIN. As well, loss of Arid1a, another subunit of SWI/SNF complexes, also showed resembling phenotypes. Importantly, IPMN-derived PDA was less lethal than PanIN-derived PDA in both mouse and human. In addition, Brg1 deletion inhibited Kras-dependent PanIN development from adult acinar cells, but promoted Kras-driven preneoplastic transformation in adult duct cells. Thus, "cell of origin" may skew the initiation, developmental process, and prognosis of PDA even in the presence of the same genetic changes.

Based on these data, we discussed how digestive organ tumors are initiated and develop in a "cell of origin" context dependent manner especially focusing on the pancreas.



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# LGR5+ STEM CELLS IN EPITHELIAL HOMEOSTASIS, REGENERATION & DISEASE OF THE STOMACH

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The gastric epithelium continuously self-renews throughout life, driven by limited reservoirs of resident Lgr5$^+$ adult stem cells [1-5]. In vivo ablation of Lgr5$^+$ cells severely impairs epithelial homeostasis in both the pyloric antrum and the corpus. Transcriptome analysis of the pyloric Lgr5$^+$ stem cells reveals novel gastric stem cell-specific markers that can be used to selectively target cancer-causing mutations to the Lgr5$^+$ stem cell compartment in the stomach as a means of evaluating their contribution to gastric cancer initiation. These new pyloric stem cell markers include membrane-expressed genes that can be used to isolate human gastric stem cells for therapeutic applications. Genes including AQP5 are currently being validated as stem cell markers via in vivo lineage tracing using new AQP5-EGFP-ires-CreERT2 mouse models. Using antibodies against AQP5, we can isolate endogenous pyloric stem cells from wild-type mice that are highly enriched for Lgr5 expression and selectively generate gastric organoids in ex vivo culture. Future efforts will be directed at using anti-AQP5 antibodies to isolate the equivalent human pyloric stem cells for evaluation via organoid assay. We hope that this will provide a source of pure human stomach stem cells for regenerative medicine applications in the clinic.

We additionally document Lgr5 expression on a subset of Chief cells in the corpus. In vivo lineage tracing using a novel Lgr5-2A-CreERT2 model and ex vivo organoid culture assays reveals these to be a damage-inducible stem cell population contributing to epithelial repair following Parietal cell atrophy. In comparison with other published markers such as Troy, Sox2 and Mist1, Lgr5 exhibits the most restricted expression pattern in both mouse and human corpus. Conditional mutation of these damage-inducible Lgr5+

corpus stem cells drives the formation of SPEM, a pre-cancerous lesion considered to be the precursor to invasive gastric cancer in humans, identifying these cells as a source of gastric cancer. We also present a new mouse model of human metastatic gastric cancer, incorporating the first stomach-specific Cre line, Cldn-18-CreERT2. Using this Cre to selectively introduce conditional mutations into the stomach epithelium efficiently drives invasive, metastatic gastric cancer throughout the glandular region. These new mouse models of human gastric cancer will be employed to derive invaluable mechanistic insight into gastric cancer initiation and progression, for evaluating potential Lgr5+ cancer stem cells and as an accurate pre-clinical model for evaluating novel gastric cancer drugs. Finally, we present preliminary data on the identification of new Lgr5+ stem cell populations and their evaluation as cancer cell origins in various tissues using a new Lgr5-2A-CreERT2 mouse model.

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# CELL CYCLE REGULATION IN CANCER STEM CELL

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Most cancer stem cells (CSCs) are quiescent, and this characteristic provides these cells with resistance to conventional anticancer therapies that preferentially target dividing cells. CSCs that persist in spite of therapies may result in relapses and metastases. Therefore, elucidation of the mechanism by which CSCs maintain quiescence is critical for the development of new therapeutic approaches toward the elimination of cancer.

Fbxw7 is the F-box protein component of an SCF-type ubiquitin ligase, in which it functions as a receptor responsible for the recognition of many targets including c-Myc and Notch. We found that Fbxw7 plays a pivotal role in maintenance of quiescence in leukemia-initiating cells (LICs) of chronic myeloid leukemia (CML) (Figure 1). Our findings reveal that ablation of Fbxw7 in a mouse model of CML results in accumulation of c-Myc and disruption of quiescence in LICs. Furthermore, we demonstrate that Fbxw7-deficient LICs are sensitive to currently available anticancer drugs and combination therapy with Fbxw7 depletion and these drugs is able to eradicate LICs, leading to a decreased relapse rate and a significant survival advantage. Finally, we present data that such combination therapy is also effective for human CML LICs, supporting our conclusion that Fbxw7 is a promising target for the treatment of human leukemia. Therefore, combination of Fbxw7 suppression and anti-cancer drugs may provide the basis for new approaches to eradication of CSCs.

However, we found that Fbxw7 suppression might increase the rate of metastasis. We generated mice that are deficient in Fbxw7 specifically in bone marrow–derived cells (Fbw7 cKO mice). Intravenous injection of melanoma (B16/F10 or B16/F1) cells or of lung cancer (Lewis lung carcinoma) cells into these mutant mice revealed that lung metastasis was

enhanced and that the animals died sooner compared with injected wild-type controls. Orthotopic transplantation of E0771 murine breast cancer cells also showed that metastasis to the lungs was markedly enhanced in Fbw7 cKO mice, whereas the size of the primary tumors did not differ significantly between the mutant and wild-type animals (Figure 2).

Deletion of *Fbxw7* in mouse bone marrow-derived stromal cells induced the accumulation of Notch and consequent transcriptional activation of the CCL2 gene. Increased serum levels of the chemokine CCL2 in Fbxw7-deficient mice resulted in the recruitment of both monocytic myeloid-derived suppressor cells and macrophages and promoted metastatic tumor growth (Figure 3).

Administration of propagermanium, an antagonist of the CCL2 receptor CCR2, blocked the enhancement of metastasis in Fbxw7-deficient mice. Furthermore, *FBXW7* expression in peripheral blood was associated with the serum CCL2 concentration and prognosis in human breast cancer patients. These results suggest that modulation of the Fbxw7-Notch-CCL2 axis may provide the basis for new approaches to suppression of cancer metastasis (Figure 4).

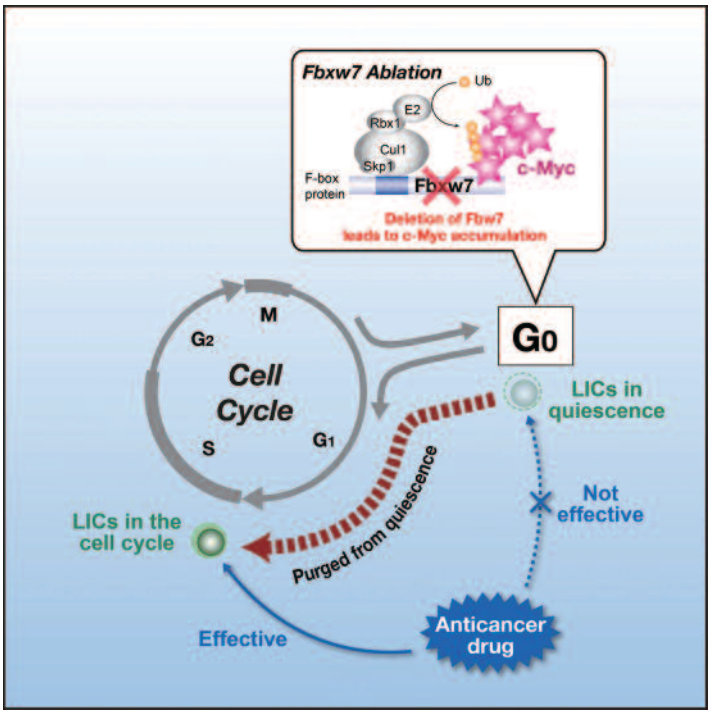


**Figure 1** The Fbxw7 – c-Myc axis is pivotal for maintenance of quiescence ($G_0$ phase) and stemness in LICs. Combination of Fbxw7 ablation and anticancer drugs is effective for LIC eradication by purging LICs from quiescence.

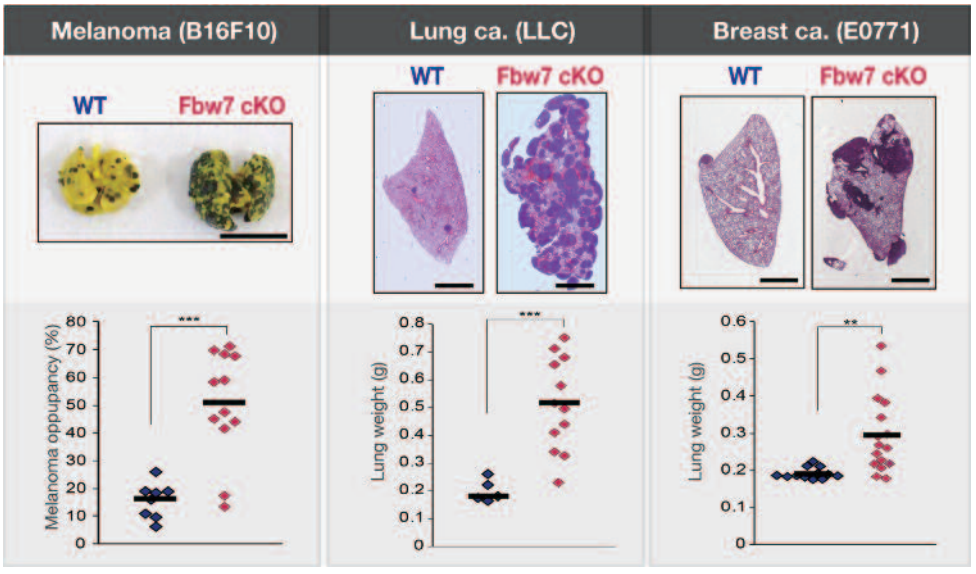


**Figure 2** *Fbxw7* deletion in bone marrow promotes cancer metastasis in an intravenous tumor cell transplantation model (melanoma and lung cancer) and in an orthotopic transplantation model (breast cancer).

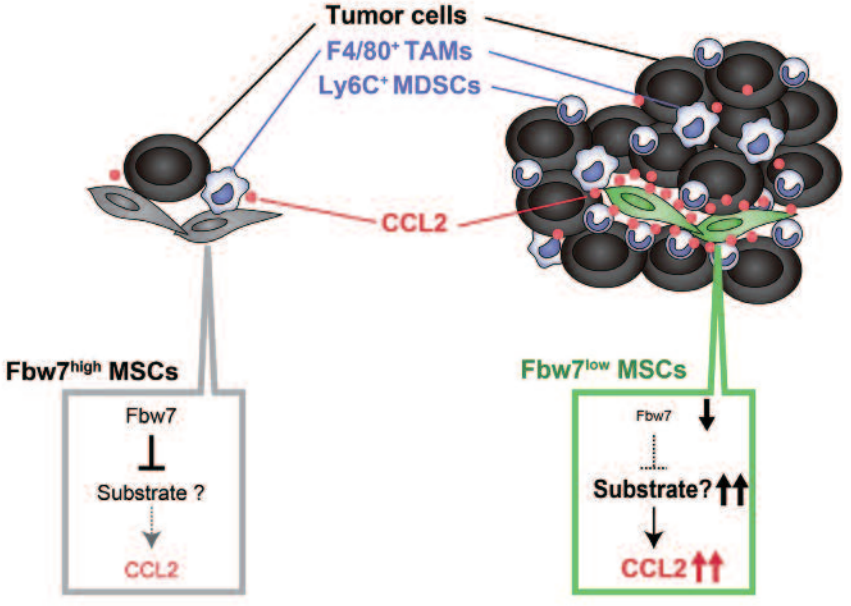


**Figure 3** Model for the promotion of cancer metastasis by loss of Fbxw7 in the host environment. Excessive signaling by Notch1 due to the impairment of its degradation caused by Fbxw7 ablation gives rise to increased production of CCL2 by bone marrow – derived mesenchymal stem cells (MSCs). The consequent recruitment of myeloid-derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) facilitates metastatic tumor growth.

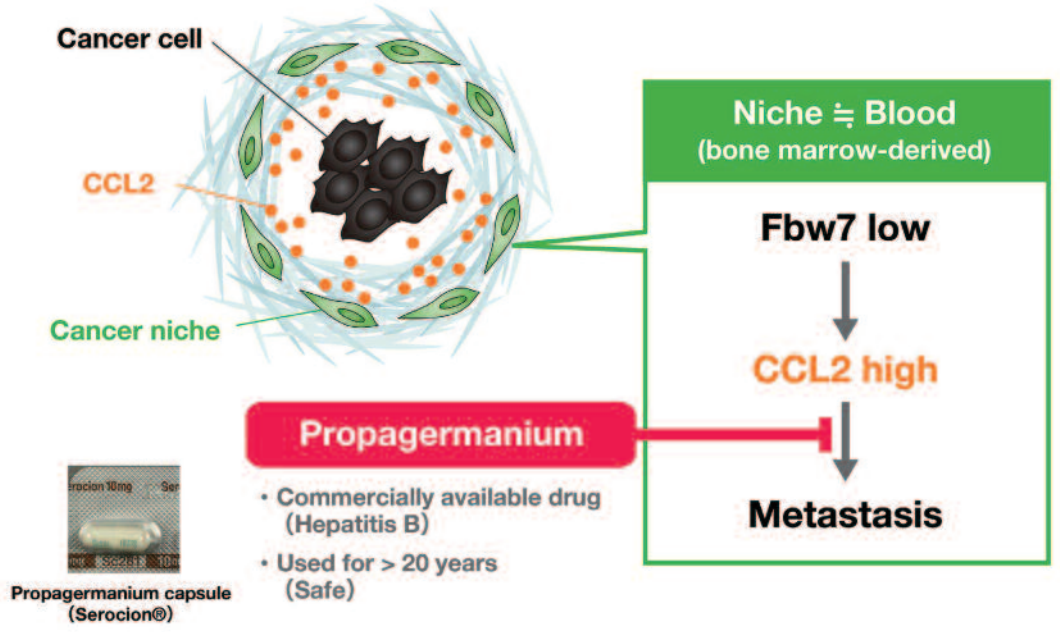


**Figure 4** Propagermanium prevents cancer metastasis induced by CCL2 overproduction. Propagermanium is a commercially available drug approved for hepatitis B treatment, and has been used for more than 20 years.

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# CANCER STEM CELL BIOLOGY ENTERS THE CLINIC

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Most common cancers, such as cancers of the breast and colon, arise in organs that contain a small population of stem cells that constantly replenish the mature cells of the tissue. Stem cells are defined by the ability to divide and give rise to a new stem cell (self-renewal), as well as the ability to give rise to the differentiated cells of an organ, and thus are the only long-lived cell population in many tissues. It is becoming increasingly apparent that cancers utilize stem cell pathways, particularly self-renewal pathways. Indeed, these stem cell pathways contribute to critical features of cancers.

Because cancers arise in tissues with stem cells and stem cell pathways contribute to the morbidity and mortality of cancers, it is important to understand self-renewal pathways. Hematopoiesis is the best understood stem cell system. Although the details of each organs stem cell and differentiation hierarchy functions might differ, understanding the hematopoietic stem cell (HSC) can serve as a foundation for understanding the other tissues. In the blood, transplantation of a single HSC can regenerate the blood system of a mouse for life, and can generate thousands more stem cells which can also regenerate the blood system [1]. No other hematopoietic cell has this capability. The unique ability to self-renew underlies the ability of an HSC to maintain the blood system. *Bmi1* was the first gene that was identified that regulates self-renewal. It is a member of the polycomb family, and it regulates self renewal by repressing senescence, apoptosis, and differentiation pathways specifically in stem cells, but not in more differentiated progeny (Figure 1) [2]. Subsequent work has shown that Bmi1 is required for the self-renewal of brain, mammary gland and mesenchymal stem cells [3, 4].

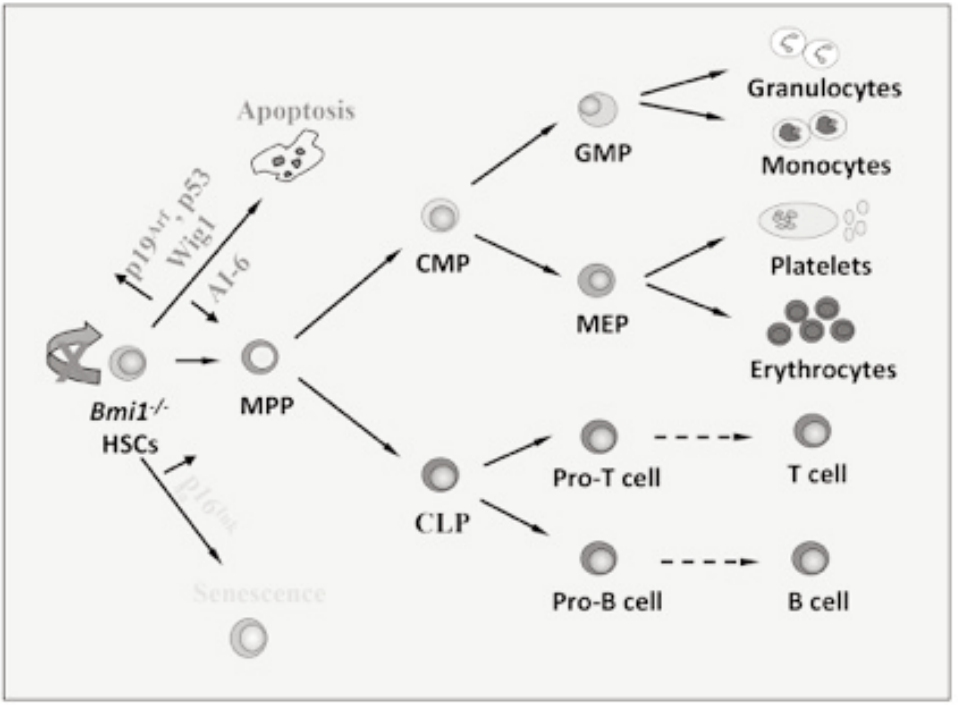


**Figure 1** Bmi1 is necessary for HSC self-renewal. Bmi1 represses the Tp53, Cdkn2a (p16) and Hox differentiation pathways specifically in HSCs. Loss of Bmi1 results in exhaustion of the HSC pool.

Although expression of Bmi1 is necessary for the self-renewal of stem cells from multiple tissues, it is not sufficient. Thus, a major question is why Bmi1 expresssion in cells other than stem cells cannot self-renew. Yohei Shimono et al. looked in the mammary gland, and found that other than stem cells, most epithelial cells in the mammary gland stem express members of the miR-200 microRNA family. He found that miR-200 targets Bmi1 and prevents the self renewal of the stem cells [5]. Similarly, Adorno et al. found that expression of Usp16, which modulates Bmi1 by removing the Bmi1-mediated ubiquitination of proteins, is expressed at higher levels in non stem cells [6]. This shows that stem cells can be defined by two properties. First, by the expression of genes such as Bmi1 which are required for self-renewal. Second, by the lack of expression of genes such as miR-200 and Usp16 that prevent self renewal (Figure 2).

Recent evidence suggests that colon and breast cancers arise from normal stem or early progenitor cells [7]. Our laboratory was the first to isolate breast and colon cancer stem cells, demonstrating that these cells are critical for the growth and metastasis [8, 9]. These findings have implications for the treatment of these cancers. We reasoned that the frequency of cancer stem cells in early tumors could be used to identify high-risk Stage-II

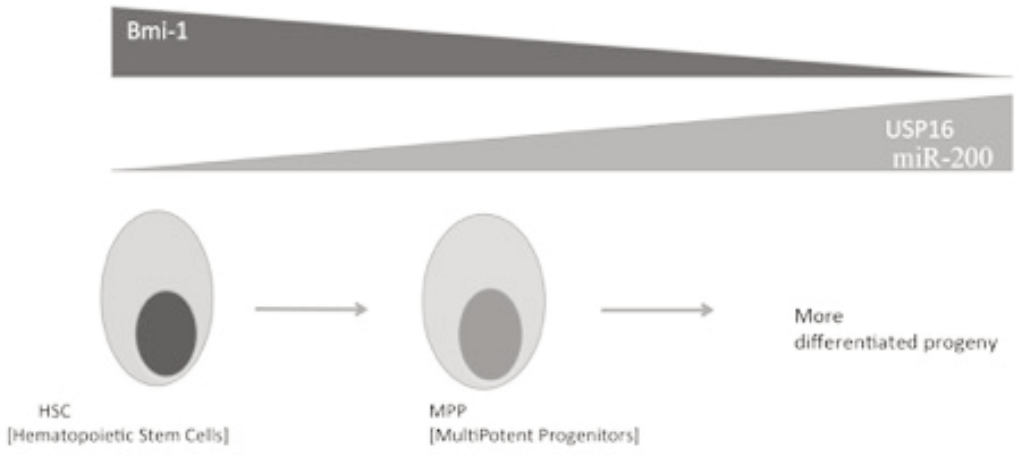


**Figure2** Bmi1 modulators prevent self-renewal in non stem cell populations. Bmi1 is expressed in the HSC and its immediate non-stem cell progenitor, the MPP. The MPP expresses Usp16 and miR-200, which modulate Bmi1 and prevent the self-renewal of the MPP.

colon carcinomas patients who need adjuvant treatment after surgery. Microarray-based multi-gene expression signatures derived from stem/progenitor cells hold promise, but are difficult to implement in clinical practice. We therefore used a novel bioinformatics approach to search for robust, individual biomarkers of colon epithelial differentiation across gene-expression arrays. Based on the availability of a clinical-grade diagnostic assay, the top candidate was tested for association with disease-free survival (DFS) and benefit form adjuvant chemotherapy using subgroup analysis on a collection of independent and retrospective cohorts of colon cancer patients. The transcription factor CDX2 ranked first in our screen. A small population of colon carcinomas lacking CDX2 expression ($CDX2^{neg}$) were associated with reduced 5-year DFS independently of stage, grade, age or gender, in both discovery (n=32/466, 6.9%; HR=3.44, 95%CI=1.60-7.38, p=0.002) and validation datasets (n=38/314, 12.0%; HR=2.42, 95%CI=1.36-4.29, p=0.003). The difference in 5-year DFS between $CDX2^{neg}$ and $CDX2^{pos}$ tumors was statistically significant in Stage-II patients (discovery dataset: 49% vs. 87%, p=0.003; validation dataset: 51% vs. 80%, p=0.004). Stage-II $CDX2^{neg}$ tumors treated with adjuvant chemotherapy were associated with improved 5-year DFS as compared to surgery alone in a pooled database of all patient cohorts (chemotherapy vs. no-chemotherapy, 56% vs. 91%, p=0.006). Lack of CDX2 expression appears to identify a subset of high-risk Stage-II colon cancer patients who appear to benefit from adjuvant chemotherapy. To our knowledge, this is the first time that the investigation of cancer stem cells has led to improvement of patient outcomes and has informed therapeutic decisions in the clinic (Figure 3) [10].

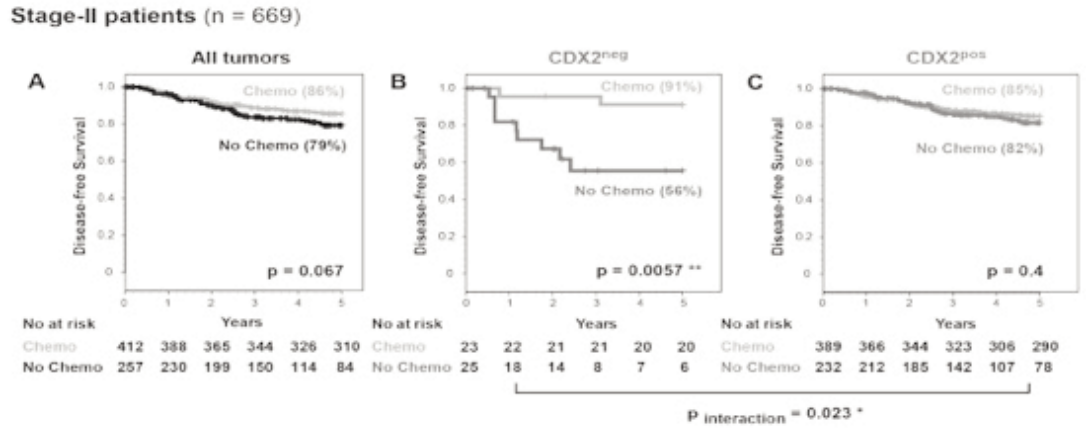


**Figure3** CDX2 identifies patients who benefit from therapy. A) Most patients with stage II colon cancer do not benefit from adjuvant chemotherapy (top line). B) Patients with CDX2 minus tumors do benefit from chemotherapy (top line). C) When patients with CDX2 minus tumors are removed from the dataset, there is virtually no benefit from adjuvant chemotherapy. Note that there still might be an unidentified small group of patients who do benefit. The top line in each panel is patients who received adjuvant therapy.

In summary, stem cell self-renewal pathways are used by cancer cells to maintain tumors and enable them to metastasize. Recent evidence demonstrates that cancer stem cell biology indeed has clinical significance. In can be used to identify patients with poor prognosis tumors. More importantly, in can be used to inform therapeutic decisions and save lives. Finally, identifying drugs that more effectively target cancer stem cells should improve clinical outcomes and save lives.

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# CLOSING REMARKS

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The 47th international symposium of the Princess Takamatsu Cancer Research Fund provided an excellent opportunity for scientists to appreciate where we stand regarding cancer stem cell research, as well as highlighting the real prospect of treating cancer by targeting cancer stem cells. It was most encouraging to see the emerging evidence supporting the original notion that cancer stem cells are responsible for treatment resistance and disease recurrence, and that at least in some cancers, targeting cancer stem cells could cure what would otherwise be a terminal disease. Here are some of the highlights from the symposium.

Large scale clinical studies show that haematological and solid cancers with signatures of stemness are more likely to recur and be associated with worse prognosis, supporting the original hypothesis that cancer stem cells are responsible for treatment resistance and cancer recurrence. Thus stemness can also serve as a useful biomarker, and CDX2 expression was found to be a useful marker in stage-II colon cancer for identifying individuals who would benefit from adjuvant chemotherapy.

The organoid system and the development of novel approaches to studying cancer stem cells in situ through creative tracing methods have allowed us to understand about the origin and fate of cancer stem cells, namely that cancer stem cells appear to arise from transformed stem or pluripotent cells. Investigation of the origin of metaplastic cancers also showed that they originate from normal pluripotent cells.

Recent success with immune check point inhibitors for cancer treatment have refocused our attention to the role of the immune system in carcinogenesis. Cancers are thought to arise through the evasion of immune-surveillance that would normally destroy transformed cells. If we are able to answer how the transformed stem cells (i.e. the cancer stem cells) are able evade the immune system

in the first place, we may be able to effectively treat and prevent cancer. One such mechanism appears to be the expression of the "don't eat me" protein CD47 on the cell surface of cancer cells and cancer stem cells, preventing macrophages from attacking these cells. Blocking CD47 was able to eliminate human cancer stem cells, and this approach is currently undergoing investigation in a clinical trial.

It was very much evident from the lectures that we now know much more about the biology of cancer stem cells, and this has lead to many other promising strategies that could target cancer stem cells. The targeting of quiescence, which is one of the main features of cancer stem cells through the suppression of Fbxw7, the F-box protein component of an SCF-type ubiquitin ligase, was one elegant example of making leukemia-initiating cells in chronic myeloid leukemia become more sensitive to the currently available anticancer drugs. Others have directly targeted the epigenetic mechanisms involved with the maintenance of stemness and/or chemoresistance, thereby inducing differentiation or transdifferentiation of cancer stem cells. Cancer stem cells were found to be located in certain places within tumours, reinforcing the importance of the cancer stem cell niche, and other studies demonstrated the importance of the metabolic crosstalk between the cancer stem cells and their niche.

We now have a marker, DCLK1, that can reliably differentiate cancer stem cells from normal stem cells, which asides from research purposes, could allow for precise treatment of cancer while leaving the healthy tissues intact. That organoids required mutations only in four genes (APC, SMAD4, TP53 and KRAS) to generate intestinal cancer was most intriguing and provides support that cancer stem cells could be targeted through the manipulation of a finite number of signalling pathways.

Much has therefore been achieved in the field of cancer stem cells in the last few years, and we sincerely hope that the cancer stem cell concept will be put through its final test one day soon, that is to target them in cancers to hopefully achieve long-term cure in patients. One concern shared by many is the plasticity of stemness, and most importantly the ability of differentiated cancer cells to revert to cancer stem cells. Therefore, the eradication of cancer stem cells alone may only be transient and insufficient.

We may also need to be wary of the way in which cancer stem cell therapy is evaluated, given it may not necessarily lead to the disappearance of tumours. Instead, the long-lasting growth inhibition of tumours may be a more appropriate and meaningful endpoint. Also, we may need to generate reliable animal models of cancer recurrence in order to evaluate cancer stem cell therapy.

On behalf of the organisers I would like to thank the Princess Takamatsu Cancer Research Fund for hosting this international symposium, and our distinguished speakers and guests for making this symposium a great success.

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