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p53 Action in Suppressing Acute Myeloid Leukemia

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Abstract of the Dissertation

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Acute myeloid leukemia (AML) is the major form of adult acute leukemia. It is characterized by colonial expansion of malignant myeloid progenitors in bone marrow, resulting in insufficient generation of normal blood components to cause sever infection, fatigue, and hemorrhage in patients. p53 is a tumor suppressor that commonly mutates in human cancers. p53 mutations have profound effects on AML biology and are associated with the clinical aggressiveness and drug resistance. However, how p53 loss influences AML development remains to be determined.

Mouse models recapitulating molecular alterations of human cancer are useful tools to dissect the *in vivo* roles of tumor suppressor genes and understand pathophysiology process of tumorogenesis. In my Ph.D. dissertation study, I established a mouse model of AML with p53 deficiency and oncogenic Kras activation (Kras-shp53). By exploiting well-established assays to assess self-renewal capabilities of normal and leukemic cells, I found that p53 deficiency provides one mechanism whereby committed myeloid progenitor cells acquire the capacity for indefinite self-renewal, which contributes to leukemogenesis.

I also established that Kras-shp53 leukemic cells exhibit constitutive activation of Ras signaling and found that suppression of Spry4 gene (one of the negative regulators of Ras signaling) cooperates with Kras activation to induce leukemia/lymphoma. Finally, I established that p53 status determines the level of Ras signaling flux and predisposes the leukemias to differential responsiveness

to MEK inhibition.

All in all, my work has provided new insights into p53 tumor suppressive actions and AML biology, and has lead to design of better therapeutic strategies.

Table of Contents

List of Abbreviations

AML	Acute myeloid leukemia
Ara-C	Deoxycytidine analog cytarabine
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
DOX	Doxycyclin
eIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorting
GEM	Genetically engineered mouse models
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte-macrophage progenitor
H&E	Hematoxylin and eosin stain
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IRES	Internal Ribosome Entry Site
LGmCreER	MSCV LTR Mir30 $CreER_{T2}$
LSL	Loxp-STOP-Loxp cassette
LTR	Long Terminal Repeat
MAPK	Mitogen activated protein kinase
MEF	Mouse mbryonic fibroblasts
MEK	Extracellular regulated kinase kinase
MEP	Megakaryocyte-erythrocyte progenitor
MLP	MSCV LTR Mir30 Puro-IRES-GFP vector
MLS	MSCV LTR Mir30 SV40-GFP vector
MP	Myeloid progenitor cell
MPD	Myeloproliferative disease
MPP	Multi-potential progenitor cell
PBS	Phosphate buffered salin
PCR	Polymerase Chain Reaction
PIG	MSCV LTR Puro-IRES-GFP vector
RNAi	RNA interference
ROMA	Representational Oligonucleotide Microarray Analysis
SCF	Stem cell factor
shRNA	Short hairpin RNA
Spry4	Sprouty 4
tTA	Tetracycline transactivator protein
4-OHT	4-hydroxytamoxife

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Chapter 1

Introduction

1.1 Acute myeloid leukemia (AML)

Hematopoiesis is the process in which cellular components of blood are formed. Disruptions of normal hematopoiesis result in disorders including malignancies such as leukemia. Acute myeloid leukemia (AML) is the major form of acute leukemia in adults and is characterized by colonial expansion of heterogeneous malignant myeloid progenitors ("blasts") resulting from unregulated proliferation and differentiation (McCulloch 1983) (Fig 1.1). The nonfunctional blasts rapidly replace normal bone marrow and cause severe neutropenia, anemia, thrombocytopenia and peripheral hyperleukocytosis due to the insufficient generation of normal blood components. Patients therefore suffer and die from symptoms such as infection, fatigue, and hemorrhage, reviewed by (McCulloch 1983). Disease incidence of AML is 3.5 per 100,000 men and women per year based on cases diagnosed in 2002-2006 (http://www.seer.cancer.gov/statfacts/html/amyl.html). In 2009 it was estimated that 12,810 men and women (6,920 men and 5,890 women) would be diagnosed with, and 9,000 men and women would die of, AML (http://seer.cancer.gov/csr/1975_2006/results_single/ sect_01_table.01.pdf).

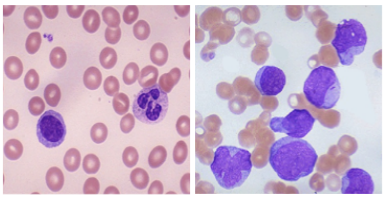

Fig. 1.1 Accumulation of AML blasts in peripheral blood. Left panel is the normal peripheral blood smear showing red blood cells, mature lymphocyte and granular cell. Right panel showing AML blasts lack of differentiation and insufficient erythrocytes.

Current AML treatment protocols consist of several courses of intensive chemotherapy, which mainly include the deoxycytidine analog cytarabine (Ara-C) and an anthracycline antibiotic such as daunorubicin or idarubicin, or the anthracenedione mitoxantrone (Tallman et al. 2005), followed by allogenic bone marrow transplantation (Estey and Dohner 2006). The first therapy phase, so-called “remission induction”, aims at clearing the majority of leukemic blasts and reconstituting normal hematopoiesis, which defines a complete remission (Stone et al. 2004). To maintain this status, intensive “post-remission treatment” (1-2 subsequent cycles of high-dose chemotherapy and allogenic stem cell transplantation) is applied for a long-term cure. Approximately 50-75% of adults with AML achieve a complete remission after induction therapy (Tallman et al. 2005). However, more than half of these patients ultimately relapse and then exhibit refractory disease and very poor prognosis. The overall 5-year relative survival rate for 1999-2005 from 17 SEER geographic areas was 22.8% (http://www.seer.cancer.gov/statfacts/html/amyl.html).

Pretreatment cytogenetic and molecular genetic findings in AML blasts are associated with chemotherapy responsiveness (Focosi 2007). It is established that the patients’ risk could be categorized into three groups by cytogenetic features, favourable, intermediate, or adverse. Each possesses very distinct survival rates. Simply speaking, t(8;21); inv(16)/t(16;16); t(15;17) are confirmed favorable karyotype features, while complex karyotype; inv(3)/t(3;3); del(7) are confirmed adverse features. Beyond cytogenetic risk classification, molecular

genetic markers are clinically significant indexes for chemotherapy response and survival, and are useful to further unravel the heterogeneity within cytogenetic risk groups (Schlenk et al. 2008). For example, mutations that activate receptor tyrosine kinase FLT3 or KIT are associated with inferior prognosis of AMLs within the favorable and intermediate groups respectively, while in normal karyotype AML patients, better survival is found in patients positive for mutation of nucleophosmin (NPM1) but negative for FLT3 (Focosi 2007). In addition, mutations of the tumor suppressor gene p53, together with the complex karyotypes that they are highly associated with, are significant poor prognostic factors (Wattel et al. 1994; Haferlach et al. 2008; Nahi et al. 2008). Other than these observed associations, the genetic basis underlying the heterogeneity in treatment responsiveness and therapy outcome is poorly defined.

1.2 Normal hematopoiesis and Self-renewal

1.2.1 Hematopoietic Hierarchy

The hematopoietic system is complex, in which hematopoiesis is maintained by a developmental cellular hierarchy. Normal hematopoiesis exhibits a successive restriction of self-renewal capacity. At the top of the hierarchy sit hematopoietic stem cells (HSCs) that possess lifelong self-renewal capacity (Harrison and Astle 1982; Morrison et al. 1997). Existence of HSCs was first proposed by Till and McCulloch in 1961 when they found that rare cells from mouse bone marrow could self-renew and form myeloerythroid colonies in the

spleens of irradiated recipient mice (Till and Mc 1961; Becker et al. 1963; Siminovitch et al. 1963). In 1988 Weissman and colleagues first isolated mouse HSCs prospectively using a combination of cell surface markers and fluorescence-activated cell sorting (FACS) technology (Spangrude et al. 1988). Since then, the functional mouse HSCs have been extensively defined and with increasing purities (Ikuta and Weissman 1992; Uchida and Weissman 1992; Christensen and Weissman 2001; Kiel et al. 2005). The first known progenies of HSCs are multi-potential progenitors (MPPs) that lose long-term self-renewal capacity yet maintain full lineage potential (Morrison and Weissman 1994). MPPs in turn give rise to lineage-restricted common lymphoid progenitors (CLP) (Kondo et al. 1997) and common myeloid progenitors (CMP) (Akashi et al. 2000). CMPs then give rise to megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-macrophage progenitors (GMP). Under normal conditions, lineage-restricted progenitors do not detectably self-renew but are continually derived from MPPs and thus HSCs (Chao et al. 2008). The proliferation outcome of these downstream oligopotent progenitors is then to generate all of the lineage-committed mature cells (Chao et al. 2008) (Fig 1.2). Using well-defined combinations of cell surface markers, almost all above-mentioned components of hematopoiesis have been identified and isolated from the heterogeneous bone marrow cell population (Fig 1.2).

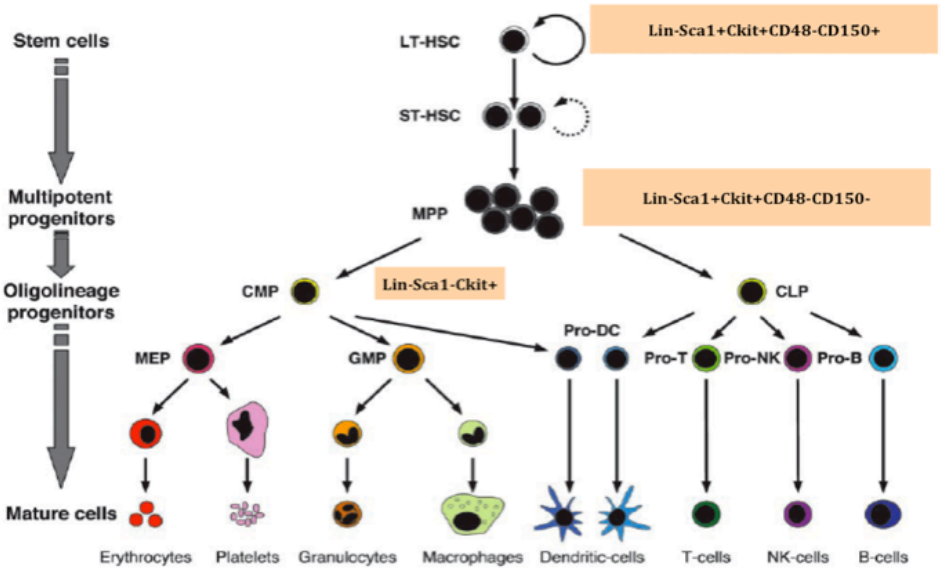

Fig 1.2 Hematopoietic hierarchy in the mouse. On the top of the hierarchy are HSCs that self-renew infinitely. HSCs maintain the pool of stem cells in the hematopoietic system and give rise to all hematopoietic cell types. During differentiation, HSCs first lose self-renewal capacity to become MPPs, then lose lineage potential to become lineage-committed progenitors (CMP, CLP) that further different and eventually commit to matured functional cells of certain lineages (Passegue et al. 2003).

The assays to assess self-renewal of hematopoietic stem and progenitor cells have been well established. HSC self-renew can be measured by long-term multilineage reconstitution of lethally irradiated recipient mice using one single such cell and serial transplantation (Spangrude et al. 1988). In contrast, progenitors such as MPP and MP (myeloid progenitors including CMP, GMP, MEP) do not show persistent *in vivo* proliferation and do not contribute to reconstitution upon transplantation unless they are transformed (Chao et al. 2008). The lifespan and proliferation potential of progenitors are usually analyzed using *in vitro* methylcellulose replating assay. In this assay, MPPs and MPs are propagated in medium containing specific cytokines that promote their proliferation, and they form morphologically distinct colonies composed of lineage

mature cells. After three to four rounds of replating, however, few colonies remain detectable due to the exhaustion of progenitor cells (Lavau et al. 1997). However, if these cells are transformed, they gain self-renewing potential and will continue to form colonies (Cozzio et al. 2003). These simple assays have been very useful in studying the self-renewal potential of hematopoietic cells.

1.2.2 Regulation of normal HSC self-renewal

Self-renewal is defined as a process by which a stem cell divides to generate one (asymmetric division) or two (symmetric division) daughter stem cells with developmental potentials that are indistinguishable from those of the mother cell (Molofsky et al. 2004). Although the molecular and cellular mechanisms of self-renewal have yet to be clearly defined, self-renewal capacity seems to encompass two key features - the limitless replication potential and the identical developmental potential (being able to stay as stem cell or differentiate) (Molofsky et al. 2004). Consequently, stem cells reproduce themselves with high fidelity while preserving their massive replicative and broad developmental potential (Molofsky et al. 2004). Normally only stem cells, including pluripotent embryonic stem cells and multipotent somatic tissue specific stem cells including HSCs, possess self-renewal capability.

I. Known molecular pathways regulating self-renewal of HSC

As might be expected, HSC self-renewal is under strict regulation. In adults and in the absence of injury, the pool size of HSCs remains consistent

(Cheshier et al. 1999; Kondo et al. 2003). At every point of cell division, however, an HSC has to decide its fate – to undergo a process of self-replication or differentiation down toward the committed progenitor (Molofsky et al. 2004). Multiple pathways coordination and intrinsic and extrinsic signals combination are required to balance the decision of cell-cycle progression and cell-fate choices: some pathways regulate only proliferation while others regulate developmental potential and/or differentiation, and some pathways regulate both (reviewed by Molofsky et al. 2004) (Fig. 1.3).

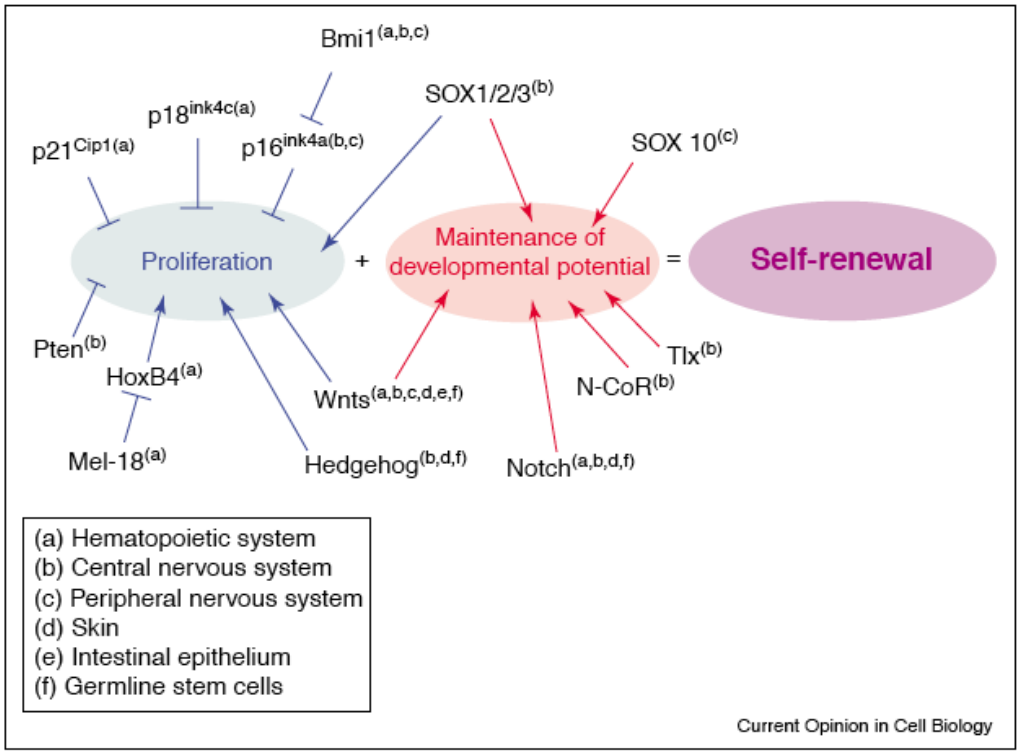

Fig. 1.3 Regulators of somatic stem cell self-renewal. The depicted genes regulate proliferative and/or developmental potential of stem cells from various tissue types (Molofsky et al. 2004).

Pathways that are implicated in regulating self-renewal of embryonic stem cells and other tissue specific stem cells are also required for the self-renewal of HSCs (Kondo et al. 2003; Molofsky et al. 2004). For example, Notch activation promotes the self-renewal of HSCs (Karanu et al. 2000; Varnum-Finney et al. 2000; Calvi et al. 2003) probably through its ability to inhibit differentiation under some settings (Henrique et al. 1997). In contrast, the Sonic hedgehog pathway regulates self-renewal of HSCs (Bhardwaj et al. 2001) possibly by promoting proliferation and survival (Kai and Spradling 2003). Wnt signaling is critical for normal HSC homeostasis (Reya et al. 2003) - soluble Wnt protein acts as a growth factor to induces self-renewal of HSCs (Willert et al. 2003); Wnt pathway activation by overexpressing activated beta-catenin expands the pool of HSCs in long-term culture whereas ectopic expression of inhibitors of the Wnt pathway, such as axin or a frizzled ligand-binding domain, leads to inhibition of HSC growth *in vitro* and reduced reconstitution *in vivo* (Reya et al. 2003). Homeobox transcription factor HOXB4 is a limiting factor in HSC regeneration followed by transplantation, and its overexpression causes the selective expansion of HSC *in vitro* and *in vivo* (Sauvageau et al. 1995; Thorsteinsdottir et al. 1999). Bmi-1, one of the polycomb transcription repressors, is necessary for the postnatal maintenance of hematopoietic stem cells and neural stem cells (Lessard and Sauvageau 2003; Park et al. 2003). Bmi-1 promotes the self-renewal of HSCs through epigenetic repression of the cell cycle regulators $p16^{Ink4a}$ and $p19^{Arf}$ (Park et al. 2003) and p53(Akala et al. 2008). Consistent with these studies, $p16^{Ink4a}$ and p53 were found to partially rescue the self-renewal defects of Bmi-1-

deficient neurual stem cells and HSCs, respectively (Molofsky et al. 2005; Akala et al. 2008). Finally, a number of transcriptional regulators, including Rae28 (Ohta et al. 2002; Kim et al. 2004b), Meis1 (Hisa et al. 2004; Kirito et al. 2004; Azcoitia et al. 2005), c-Myb (Mucenski et al. 1991; Sandberg et al. 2005), Cbp (Rebel et al. 2002), Gata-2 (Tsai et al. 1994; Rodrigues et al. 2005), PU.1 (Kim et al. 2004a; Iwasaki et al. 2005), and Mll (McMahon et al. 2007) are all required for the self-renewal of both fetal and adult HSCs (Levi and Morrison 2008). Clearly, regulation of HSC self-renewal engages complicated networking and cooperation among these pathways to meet requirements during development or in response to (patho)physiological challenges. The mechanisms underlying the control of HSC self-renewal need to be refined and additional programs involved in self-renewal regulation will continually to be identified.

II. Self-renewal regulation by cell cycle status of HSC

Throughout adulthood, HSCs are quiescent (a non-proliferative state of the cell cycle at G_0 stage that can be induced to enter the cell cycle at G_1 stage) most of the time even though all HSCs enter the cycle on a regular basis to contribute to hematopoiesis (Morrison and Kimble 2006; Kiel et al. 2007). It has been proposed that adult self-renewal programs may feature mechanisms that promote quiescence (Levi and Morrison 2008; Orford and Scadden 2008). One potential mechanism by which the stem cell niche maintains stem cell function is through the induction of quiescence (Orford and Scadden 2008). The relationship between adult stem cell quiescence and the maintenance of stem cell function

has been illustrated in the Mef (an important negative regulator of HSC quiescence) conditional knockout mouse model (Lacorazza et al. 2006); deletion of Mef causes increased quiescence and paradoxical HSC expansion that results from reduced HSC exhaustion.

The self-renewal process requires a balance between cell-cycle progression and cell-fate decision (differentiation v.s. self-renewal) (Orford and Scadden 2008). Early G_1 phase seems to be a sensitive period when cell-fate decisions are made (Orford and Scadden 2008). Genes preventing either HSCs from entering G_1 from G_0 or facilitating the transition through the early G_1 phase help minimizing the exposure to exhaustion-inducing stimuli in early G_1 and may favor self-renewal and preserve long-term function of HSCs (Orford and Scadden 2008). For instance, $p18^{Ink4c}$ normally impedes the transition from early to late G_1 by blocking cyclin d–Cdk4 activity and its absence increases the self-renewal of stem cells (Yuan et al. 2004; Yu et al. 2006). In contrast, genes that promote HSC cell cycle entry from quiescent state and that provide the prolonged signaling to G_1 progression favor differentiation (Orford and Scadden 2008). This view is supported by genetically modified mouse models in which increased HSC proliferation compromises long-term and competitive function of HSCs and increases susceptibility to stress-induced depletion of HSC. The first evidence of this came from studies with $p21^{WAF1/CIP1}$-/- mice where HSCs exhibited more active cycling rather than staying quiescent (Cheng et al. 2000). Similarly, HSCs deficient for growth factor independent 1 (Gfi1), phosphatase and tensin homologue (Pten) and forkhead box (Foxo) proteins 1, 3 and 4 all actively cycle

and eventually exhaust (Orford and Scadden 2008). Constitutively activated mitogen activated protein kinase (MAPK) signaling, one effector pathway downstream of activated Ras, provides prolonged signal through G_1 to S phase transition (Jirmanova et al. 2002; Meloche and Pouyssegur 2007) and is a potent differentiation inducer (Marshall 1995; Meloche and Pouyssegur 2007). Activated oncogenic Kras increases cycle entry of quiescent HSCs and possibly endows extended signaling during G1, resulting in a twofold reduction of HSC number by immunophenotyping and a tenfold reduction in reconstitution ability of HSCs (Sabnis et al. 2009).

More recently, p53 was found to be essential for maintaining HSC quiescence during steady-state hematopoiesis, and this function was independent of $p21^{WAF1/CIP1}$ but dependent on Gfi-1 and Necdin (a Rb-alike protein that binds to E2F1 and represses E2F-dependent transcription). It is still puzzling how p53-/- HSCs show concurrent enhanced cycling and self-renewal ability. Probably loss of p53 shortens the early G1 phase, thus outweighs the effects on increased cell cycle progression. As a result, a self-renewal decision is favored. All of the studies discussed above suggest that the balance between cell cycle progression and cell fate decision is delicate and genes that regulate cell cycle progression and proliferation can affect self-renewal without influencing the developmental potential of HSC. However, how these genes cross talk with the pathways that have already been implicated in the self-renewal process is not known.

1.3 Cellular origin of AML and leukemic self-renewal

For decades, cancer research has been focused on identifying the nature of oncogenic mutations and understanding their functions in cellular transformation. Utilizing *in vitro* systems such as primary cells (human and mouse fibroblasts) and transformed cell lines and *in vivo* xenograft based or transgenic mouse models to understand gene function and interactions has lead to the identification of a myriad cancer causing genes and mutations. Although such studies have provided important insights into cancer biology, they have limitations in revealing the full picture of tumorigenesis that oftentimes occurs in a complicated and dynamic cellular context and is strongly influenced by environmental factors. Some of these issues are currently being addressed with the goal to define the specific cellular origin of cancer among a hierarchy of cells that constitute a specific tissue and the mechanisms by which the heterogeneous architecture of a cancer tissue is maintained.

Currently, two models have been proposed to explain the establishment (cellular origin of cancer) and maintenance of cellular heterogeneity within a tumor, the “cancer stem cell” model (Reya et al. 2001) and the “clonal evolution” model (Campbell and Polyak 2007). The “cancer stem cell” model postulates that only a distinct subset of cells with stem-like properties are responsible for generating new tumor cell and for multilineage differentiation, thus accounting for the cellular heterogeneity seen in cancers, whereas most cancer cells have limited proliferative potential and are derived from the cancer stem cells by

aberrant differentiation (Reya et al. 2001). This model suggests that a subset of cells is enriched for the ability to form new tumor cells and should therefore be the therapeutic targets.

In contrast, the “stochastic” (Wang and Dick 2005) or “clonal evolution model” (Campbell and Polyak 2007) argues that most of the tumor cells contribute to tumor maintenance, albeit to varying degrees. This model states that cancer cells acquire various combinations of mutations over time, with the most aggressive cells generated by genetic drift and stepwise natural selection driving tumor progression (Campbell and Polyak 2007). This model also envisions that a tumor is composed of subclones with variable growth and survival advantages that emerge at different stages of neoplastic progression (Adams et al. 2008). Both “cancer stem cell” and “clonal evolution” models possess supporting evidence from human cancer and mouse models (Campbell and Polyak 2007), and it is still under debate that which model more accurately depicts tumor evolution and organization (Adams et al. 2008).

The “cancer stem cell” model and “clonal evolution” model are different in depicting several features of a tumor, such as the frequency of cells with tumorigenic potential (rare to moderate or high), tumor organization (hierarchical or tot necessarily hierarchical), intrinsic differences between tumorigenic and nontumorigenic cells (stable or unstable), and therapy rational (targeting only tumorigenic cells or targeting most cells) (Shackleton et al. 2009). Despite all these disparities, an intrinsic difference between the two models is that they seem to address different states of tumorigenesis. Contrary to the “cancer stem

cell" model that describes the organization of a steady state/chronic stage of a tumor, the concept of "clonal evolution" is more "dynamic"- focusing on the events occurring during the process of tumor progression.

One confusion often arises from the "cancer stem cell" concept. Due to the shared attributes (self-renewal and differentiation) between cancer stem cells (CSCs) and tissue stem cell, CSCs were believed to originate from the stem cells of the tissue where the cancer develops. The notion that tumors arise from stem cells was initially proposed by Cohnheim in 1875 and was first supported by the observation of Dick and colleagues that AML LSCs (leukemic stem cells) are highly enriched in a cell population possessing stem cells markers (Lapidot et al. 1994; Bonnet and Dick 1997). However, subsequent experiments demonstrated that AML LSCs do not express exactly the same markers as normal HSCs (Blair et al. 1997; Miyamoto et al. 2000), which led to the hypotheses that LSC transformation may occur in a downstream committed progenitor cell. In order to test this hypothesis and to determine the leukemia initiating properties of various cell populations, Weissman and colleagues examined CML patients from chronic, accelerated, and myeloid blast crisis phases. They found that (other than the accumulated blasts) granulocyte-macrophage progenitors (GMP) are the only cell population that showed increased frequency in the blast crisis phase (Jamieson et al. 2004). The increased β-catenin level found in GMPs was associated with aberrant methylcellulose replating capability *in vitro* and with the ability to transduce disease into secondary recipient mice (Jamieson et al. 2004).

This study was the first to demonstrate that in human AML, non-self-renewing progenitor cells could be the targets of transformation and to acquire self-renewal capabilities (Jamieson et al. 2004), and has led to the hypothesis of multi-stages leukemogenesis of AML (Passegue and Weisman 2005). The concept is that during the preleukemic phase of disease, such as chronic myeloid leukemia (and corresponding myeloproliferative disease in mice), the HSCs is the only population that is able to self-renew, which in turn allows the cells to accumulate rare oncogenic events. Eventually a leukemic HSC subset attains the transformation state, or a progenitor population derived from the leukemic HSC obtains self-renewal potential and emerges as a novel leukemic stem cells.

Identifying the cell of origin in AML has helped to discriminate between molecular mechanisms that impart premalignant proliferative and survival advantages from those that complete the final transformation process by conferring self-renewal (Passegue and Weisman 2005). Several signaling pathways that have been implicated in normal self-renewal are also implicated in leukemic self-renewal (Chao et al. 2008). Importantly, these pathways endow progenitor cells with the capability to self-renewal. In addition to the activated Wnt/β-catenin pathway mentioned above, Bmi-1 was also found essential for maintenance of leukemic stem cells (Raaphorst 2003). Bmi-1–/– fetal liver cells are resistant to a well-characterized myeloid leukemia induced by Hox9 and Meis1a oncogenes, indicating that Bmi-1 is necessary for AML initiation and maintenance (Lessard and Sauvageau 2003). Enforced expression of homeobox genes Hoxa9, Hoxa10, Hoxb3, Hoxb6, or Hoxb8 confers growth

advantages *in vitro* and *in vivo* and leads to leukemia (Argiropoulos and Humphries 2007). Mixed-lineage leukemia (MLL) transcription factor is required for normal hematopoiesis (McMahon et al. 2007). Retroviral insertion of a leukemogenic MLL-ENL and MLL-GAS7 fusion gene endows the normally non-self-renewing progenitor populations with aberrant self-renewal properties and induces AML (Cozzio et al. 2003; So et al. 2003). Unraveling the molecular mechanisms of leukemic self-renewal, in the context of the specific target cells involved, will be critical for developing therapies that target cancer stem cells while salvaging the function of normal tissue stem cells.

1.4 Genetic alterations in AML

The genetic alterations in AML have been studied more intensively and for longer time comparing to the cellular origin of AML.

1.4.1 Two complementary groups of mutations

AML is a genetically heterogeneous disease. More than 100 mutations, including chromosomal rearrangements, gene mutations, amplifications and expression alterations, are implicated in AML pathogenesis and are thought to cooperate in specific functional patterns to promote leukemogenesis (Gilliland et al. 2004a; Mrozek et al. 2004). Functionally, the molecular lesions that are known to be involved in AML leukemogenesis can be divided into two broad complementation groups (Gilliland et al. 2004b) (Fig. 1.5). Class I mutations are

composed of mutations that activate signal-transduction pathways and thereby enhance the proliferation and/or survival of hematopoietic progenitor cells. Included in this group are mutations that activate receptor tyrosine kinase FLT3 or KIT and components of RAS signaling pathways including NRAS and KRAS, and loss-of-function of the Ras GAP NF1, etc. Class I mutations are viewed as the first complementation group because epidemiologic analyses showed that these mutations rarely co-exist in any given patient (Gilliland et al. 2004a). Class II mutations comprise of mutations and chromosomal rearrangements of transcriptional factors or co-activators implicated in hematopoietic development (Fig 1.4). Dysfunctional fusion proteins such as AML1-ETO, PML-RARα, TEL-AML1, CBFβ-SMMHC, and fusion proteins involving HOX family of transcription factors or MLL methyltransferase are included in this group. Loss-of-function point mutations in AML1, *CEBPA*, and PU.1 are also included. These lesions impair myeloid differentiation by repressing genes involved in lineage commitment and differentiation control (Alcalay et al. 2003).

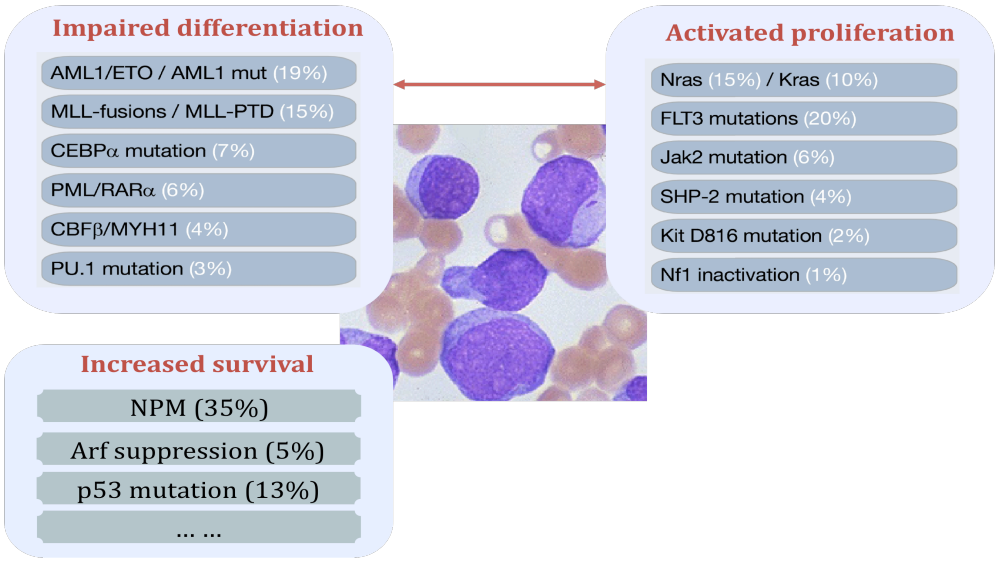

Fig. 1.4 Frequent AML lesions are grouped into functional classes. Arrow indicates cooperation between two complementation groups.

It has been suggested that cooperation between the above two classes of mutations is a major theme of AML pathogenesis (Gilliland et al. 2004a). Evidence supporting this “two-hit” model comes from clinical demonstration that class 1 and class 2 lesions occur together more commonly than two class 1 or two class 2 lesions do (Estey and Dohner 2006), and several mouse models of AML illustrated the cooperation between lesions from these two groups in driving the oncogenic process (Schessl et al. 2005; Zuber et al. 2009).

This “two-hit” model, however, oversimplifies the situation. Indeed, disruptions of almost every cellular process, including proliferation, differentiation, self-renewal, survival, cell cycle checkpoint control, DNA repair and chromatin stability, and cell dissemination have been found to occur during AML pathogenesis (Licht and Sternberg 2005). Therefore, these mutations may

provide additional advantages than the above two functional groups do, and lesions outside of these two major complementation groups could also play significant roles in leukemogenesis.

An example of the first scenario involves the self-renewal capability of leukemic cells. Increasing lines of evidence suggest that Class II mutations, in addition to differentiation blockage, also endow hematopoietic progenitors with the ability to self-renew by activating self-renewal regulating pathways. For instance, expression of AML1-ETO or PML-RARa fusion protein results in activation of Notch signaling through the Jagged1 ligand (Alcalay et al. 2003) and Wnt/β-catenin signaling through expression of β-catenin and γ-catenin proteins (Muller-Tidow et al. 2004b). MLL rearrangements promote self-renewal by inducing HOX genes that are highly expressed in hematopoietic stem cells (Dorrance et al. 2006). Activation of these known self-renewal pathways provides a cell-autonomous mechanism of self-renewal for leukemic cells.

Examples for the second scenario include p53 and NPM1 (nucleophosmin 1) mutations, etc. Deficiency of p53 is traditionally considered to contribute to AML due to failure to induce apoptosis and the G1 checkpoint, or to maintain genomic stability (Renneville et al. 2008). Recently, p53 was found to suppress self-renewal of stem and progenitors in several tissues (Armesilla-Diaz et al. 2008; Cicalese et al. 2009; Krizhanovsky and Lowe 2009; Liu et al. 2009). This “newly” discovered function for p53 might provide additional insights into how p53 deficiency promotes leukemogenesis.

1.4.2 The p53 tumor suppressor and p53 mutations in AML

Mutations of p53 tumor suppressor gene are common in human cancers and frequently associated with clinical aggressiveness and drug resistance (Wattel et al. 1994; Buttitta et al. 1997; Levine and Oren 2009). Germline p53 mutations predispose mice and humans to diverse tumors (Malkin et al. 1990; Donehower 1996) and p53 loss cooperates with a variety of other oncogenic lesions, including oncogenic Ras, deregulated Myc, and PTEN loss, to drive cells toward the malignant state (Hermeking and Eick 1994; Eischen et al. 1999; Johnson et al. 2001; Chen et al. 2005). Other mutations that attenuate p53 function, including those that act upstream and downstream of p53, are also common in human cancers (Bueso-Ramos et al. 1993; Esteller et al. 2001; Lowe and Sherr 2003; Brown et al. 2009). Thus, mutational disruption of the p53 network may occur in virtually all aggressive end-stage cancers (Brown et al. 2009).

p53 possesses a range of biological activities that may contribute to its tumor suppressive function, including its ability to promote various cell cycle checkpoints, apoptosis, autophagy, differentiation and cellular senescence (Meek 2009). Moreover, loss of p53 fuels genomic instability to further facilitate tumor evolution. p53 is best understood for its role in response to cellular stress, whereby it elicits one or more of the above biological responses.

Recent studies have indicated that p53 is involved in controlling the self-renewal and quiescence of embryonic and adult stem cells (van Os et al. 2009). In the hematopoietic system, p53 promotes HSC quiescence and its loss

enhances self-renewal of HSCs and myeloid progenitor cells (Liu et al. 2009). Although the underlying mechanism is not known, the increased number of HSCs in p53 deficient mice seems be due to abnormal self-renewal but not decreased apoptosis or senescence (Dumble et al. 2007; Liu et al. 2009). In neural stem cells, p53 negatively regulates proliferation and survival, and therefore the self-renewal of these cells (Meletis et al. 2006; Medrano et al. 2007; Armesilla-Diaz et al. 2008). Transcriptome analyses identified the dysregulation of several cell cycle regulators in p53 deficient neural stem cells, including $p21^{WAF1/CIP1}$ (Meletis et al. 2006; Medrano et al. 2007; Armesilla-Diaz et al. 2008). However, the molecular mechanism that underlies normal self-renewal regulation by p53, whether this is a novel p53 tumor suppressing function, and whether p53 regulates oncogenic self-renewal, have not been evaluated.

p53 mutations have profound effects on the biology of AML. Although mutations in the p53 gene itself occur in only approximately 4-15% of cases at diagnosis (Melo et al. 2002), they are associated with the most aggressive forms of AML and drug resistance (Wattel et al. 1994; Haferlach et al. 2008; Nahi et al. 2008). p53 mutations are positively correlated with transformation from myelodysplastic syndromes (MDS) to AML (Castro et al. 2000), and are commonly found in blast crisis of chronic myeloid leukemia (CML) (Calabretta and Perrotti 2004). It was shown that p53 mutations present in a very small proportion of cells at diagnosis but in a larger group upon relapse, suggesting that the p53 defective population is resistant to chemotherapy and/or facilitates the relapse (Wada et al. 1994). On the other hand, using paired AML samples

obtained from patients with AML at the stages of diagnosis and first relapse, it has been suggested that relapse was not merely a re-growth of the initial leukemic blast (Nakamura et al. 2004). In addition, acquisition of p53 mutations affected the leukemic development from the initiation stage of AML all the way to relapse (Nakamura et al. 2004). Therefore, it is unclear how mutations of p53 contribute to AML leukemogenesis, relapse and therapy resistance.

In addition to direct mutations, p53 function can be impaired in AML through other mechanisms, such as dysfunction of ARF (Kojima et al. 2005), which binds and inactivates negative regulator of p53 MDM2. Alterations of ARF appear in about 5% of AMLs and have been correlated with poor survival (Muller-Tidow et al. 2004a). Arf haploinsufficiency also cooperates with inv (16) to induce AML in a mouse bone marrow transplantation model (Moreno-Miralles et al. 2005). Mutations of NPM1, which recently have been identified as the most common genetic lesions in AML (Falini et al. 2005), also perturb p53-dependent and -independent functions of ARF by causing it to re-localize to the cytoplasm (Colombo et al. 2005; den Besten et al. 2005). Given that p53 mutations are associated with disease agressiveness and poor prognosis, understanding how p53 loss influences AML development may provide insights into the most aggressive features of this disease.

1.4.3 Ras signaling pathway and mutations in AML

RAS oncogenes encode a family of small GTPase proteins that relay signal transduction from a variety of membrane receptors to various downstream

effectors, and play important roles in proliferation, differentiation and apoptosis (Malumbres and Barbacid 2003). Activation of Ras signaling cascade begins with ligands binding and activation of receptor tyrosine kinases, which in turn associate with Shc that is responsible for recruiting the Ras-GEF Grb2/Sos (guanine nucleotide exchanging factor) complex. The Ras-GEF complex activates Ras by loading membrane-bound Ras with GTP. Ras-GTP binds to and activates different classes of effector molecules, including Raf, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS). There are three Raf serine/threonine kinases (ARAF, BRAF and RAF1), which phosphorylate mitogen associated extracellular regulated kinase-1 (MEK1) (Xu et al. 1995). MEK1 in turn phosphorylates extracellular regulated kinases 1 and 2 (ERKs 1 and 2) on specific threonine and tyrosine residues (Xu et al. 1995) (Fig 1.5).

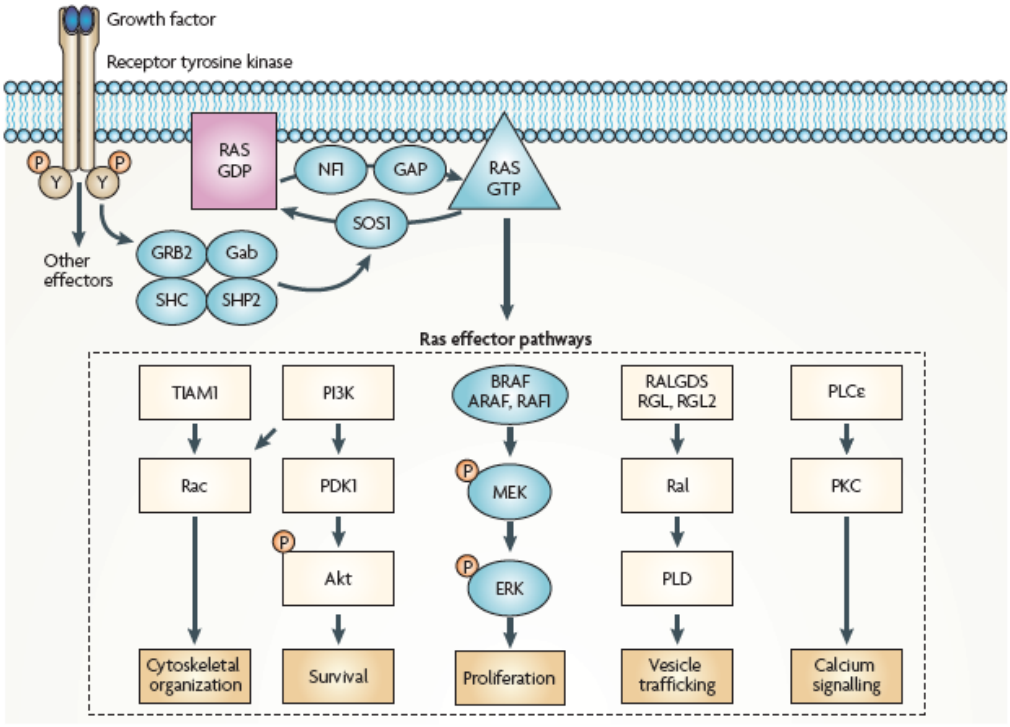

Fig 1.5 The Ras signalling pathway. Growth factor binding to cell-surface receptors results in activated receptor complexes containing adaptors SHC, GRB2 and Gab proteins to recruit SHP2 and SOS1, which is Ras-GEF and increases Ras–GTP levels by catalysing nucleotide exchange on Ras. The Ras-GAP neurofibromin (NF1) binds to Ras–GTP and accelerates the conversion of Ras–GTP to Ras–GDP. Ras effector pathways: BRAF– MEK– ERK pathway; PI3K– 3-phosphoinositide-dependent protein kinase 1 (PDK1)–AKT pathway; RALGDS, RALGDS-like gene (RGL), RGL2- Ral- phospholipase D (PLD) and TIAM1-Rac pathway. P, phosphate; Y, receptor tyrosine. (Suzanne Schubbert et al, Nature Reviews Cancer 2007)

Activated ERK1/2 phosphorylates and activates a variety of cytosolic and nuclear substrates that control cell cycle progress and cell proliferation. After translocating to the nucleus, ERK1/2 phosphorylates numerous transcription factors regulating genes encoding proteins that control cell-cycle progression, such as cyclin D (Treinies et al. 1999) and growth factor and cytokines important for the growth and survival of hematopoietic cells (Steelman et al. 2008). ERK1/2

also phsophorylates cytosolic 90 kDa ribosomal S6 kinase 1 (p90Rsk1), which stimulate cells enter the cell cycle from G_0 by activating multiple transcription factors participating in the immediate-early response (transient and rapid response to a wide variety of cellular stimuli) (Roux and Blenis 2004). Activated ERK also promotes cellular growth (increased cell mass or size) by phosphorylating cytosolic substrates such as MNK1 and MNK2 kinases (Waskiewicz et al. 1997). MNK1/2 kinases in turn phosphorylate eukaryotic initiation translation factor 4E (eIF-4E), an event thought to stimulate binding of eIF4E to mRNA 5' end cap structures, thereby enhancing polysome assembly and facilitating protein synthesis to meet increased biosynthetic requirements (Ueda et al. 2004).

Activation of Ras signaling can also increase cellular survival by modulating the activity of many proteins involved in apoptosis (Steelman et al. 2008). Several pro-apoptotic proteins are known to be inactivated by phosphorylation; ERK phosphorylates the BH3-only domain protein Bim, Akt phosphorylates Caspase 9 (Harada et al. 2004) (Cardone et al. 1998), and p90Rsk1 phosphorylates another BH3-only domain protein Bad (Bonni et al. 1999). The antiapoptotic gene Mcl-1 and Bcl-2 are up-regulated by PI3K signaling pathway through a transcription factor complex containing CREB (Wang et al. 1999; Pugazhenthi et al. 2000). Mcl-1 is also phosphorylated and stabilized by ERK (Domina et al. 2004). In summary, Ras signaling cascades are important signals for cell proliferation, survival and differentiation (discussed in section 1.2), therefore unchecked activation of Ras results in uncontrolled

proliferation and enhanced survival that facilitates malignant transformation.

RAS mutations occur almost exclusively as a single nucleotide change at codon 12, 13 or 61; these changes abrogate intrinsic GTPase activity and confer resistance to GAPs (GTPas activating protein) leading to constitutive activation of RAS proteins and downstream effectors, reviewed (Malumbres and Barbacid 2003). In AML, oncogenic RAS mutations can be detected in 30-40% patients (Reuter et al. 2000), with NRAS and KRAS mutations found in 10%-20% and 5%-15% of patients, respectively (Valk et al. 2004). RAS mutations are relatively overrepresented in French-American-British (FAB) type M4eo/inv(16) AML (Bowen et al. 2005; Bacher et al. 2006) and M4 AML (Radich et al. 1990; Goemans et al. 2005), which are both characterized by myelomonocytic differentiation. In contrast, Ras mutations are less common in AML with t(15;17) (2%) or with complex aberrant karyotypes (1.5%) (Bowen et al. 2005). Most large studies documented no significant prognostic implications of RAS mutations in overall survival, complete remission rate, or duration of AML disease (Ritter et al. 2004; Bowen et al. 2005; Goemans et al. 2005; Bacher et al. 2006; Boissel et al. 2006).

In addition to point mutations in RAS, RAS effector pathways are also activated by various mechanisms including 1) constitutive activation of receptor tyrosine kinases such as FLT3 and KIT; 2) loss of function of the Ras GAP protein NF1; 3) the presence of point mutations in the SHP-2 gene (an adaptor protein that enhances the phosphatase activity PTPN11 and thereafter activation

of MAPK); 4) generation of fusion proteins that acquire constitutive kinase activity (e.g. t(5;12)-TEL/PDGFRβ, t(9;12)-TEL-ABL, t(12;15)-TEL/TRKC)(Gilliland et al. 2004a; Renneville et al. 2008). Taken together, the RAS pathway is activated in more than half of all AML cases.

1.5 Targeting Ras signaling in AML

Similar to the self-renewal ability that is essential for AML oncogenesis and maintenance, deregulation of RAS signaling cascades is conceivably as important in AML pathogenesis. The dependence on RAS oncogenic signaling of leukemic cells for their sustained proliferation and/or survival (so called "oncogene addition") may serve as a "Achilles' heel" of AML and has been used to exploit therapeutic approaches (Sharma and Settleman 2007). Because normal cells do not have these aberrant signaling events they are less reliant on them (discussed later), and thus small molecule inhibitors that specifically switch off these crucial signaling pathways could selectively affect tumor cells while sparing normal cells (Sharma et al. 2006; Sharma and Settleman 2006). Remarkable success was examplified by imatinib, a drug that is now routinely used for CML and gastrointestinal stromal carcinoma patients (Druker et al. 2001a; Druker et al. 2001b; Joensuu et al. 2001).

1.5.1 Elevated Ras signaling in AML

If we focus on MAPK signaling downstream of RAS in AML, constitutive

activation of ERK has been found in 50 -80% of patients (Towatari et al. 1997; Kim et al. 1999; Lunghi et al. 2001; Milella et al. 2001). Flow cytometry analyses with increased sensitivity have revealed even a higher frequencies of constitutive ERK activation- with approximately 90% of primary AML samples from both *de novo* and relapsed/refractory patients showing elevated phospho—ERK levels (Ricciardi et al. 2005). Conversely and interestingly, activation of ERK is absent in highly purified normal CD34+ progenitors (Ricciardi et al. 2005), indicating that activation of MAPK signaling is dispensable for normal function of hematopoietic progenitors. These studies together highlight the lineage committing effects of MAPK signaling over its role in self-renewal of stem cells (Fichelson et al. 1999; Bonati et al. 2002). Therefore, constitutive ERK activation is crucial for AML blasts but not normal hematopoietic stem/progenitor cells, and targeting MEK/ERK can specifically eliminate leukemic cells.

1.5.2 Targeting RAS signaling in AML with MEK inhibitor

Given the high frequency of RAS pathway alterations in human AMLs, selective small molecule inhibitors targeting RAS signaling could be of considerable value. MEK-ERK is an important node to target for several reasons. Multiple mitogenic signaling transductions converge at the level of MEK and the only know substrate of MEK is ERK. The level of ERK phosphorylation can be measured directly *in vivo* as biomarker for targeting effects (Milella et al. 2005). Activation of MEK requires phosphorylation; relative few phosphorylation sites on MEK make it easy for an inhibitor to achieve high potency.

First-generation MEK inhibitors include PD98059 and U0126, which are non-competitive with ATP and act on the MAPK cascade by binding and preventing the activation of MEK1/2 (Morgan et al. 2007). PD98059 and U0126 show greater than 70% growth inhibition in liquid culture and methylcellulose colony assays for AML cell lines but are less potent *in vivo* (Morgan et al. 2001). CI-1040 (PD184352) is another first-generation non-competitive MEK1/2 inhibitor and inhibits tumor growth *in vivo*. CI-1040 was the first MEK inhibitor in the clinical trial (Messersmith et al. 2006). In a phase II, multicenter, parallel arm study carried out in patients with advanced breast cancer, colon cancer, non-small cell lung cancer CI-1040 was found to be well tolerated although it demonstrated insufficient antitumor activity- with only a mild positive association ($P<0.055$) between baseline pERK expression and stabilized disease (Rinehart et al. 2004).

A second-generation oral MEK inhibitor, PD 0325901, was improved from CI-1040 and has displayed superior potency and pharmacologic properties (Lorusso et al. 2005). The first human trial of PD 0325901 enrolled 35 breast, colon, non-small cell lung cancer, and melanoma patients. Two partial responses were observed in the melanoma patients, while 8 patients achieved stabilized disease for 3–7 months (Wang et al. 2007).

Interestingly, CD34+ cells that are enriched for hematopoietic stem/progenitor cells and total normal BM cells do not show constitutive ERK phosphorylation, and their viability and clonogenic growth are not affected by treatment with either PD98059 or CI-1040 (Milella et al. 2001) or U0126 (James

et al. 2003). These studies collectively imply that leukemic but not normal cells would show selective susceptibility to MEK inhibition.

MEK inhibitors are potential anti-cancer agents in that they lower the apoptotic threshold of leukemic cells thereby sensitizing them to the pro-apoptotic action of classical cytotoxic drugs, ionizing radiation, and other biological agents that modulate apoptosis (Milella et al. 2005). Notably, in most instances, MAPK inhibition has cytostatic rather than cytotoxic effects (Milella et al. 2005). MEK inhibition in sensitive AML cells resulted in inhibition of cell cycle progression and accumulation of cells at the G0/G1 phase, which was accompanied by $p27^{KIP1}$ protein dephosphorylation and accumulation as well as down regulation of $p21^{WAF1/CIP1}$ and cyclins D2 and E (Milella et al. 2001). Accordingly, precise timing is critical when combining MEK inhibition with classical chemotherapy, as chemotoxic drugs often need cell cycling to take effect. Therefore, rather than co-administration with cytotoxic drugs, MEK inhibitors should be delivered after chemotherapy to obtain a synergistic response (Milella et al. 2005).

Despite the dramatic progression in pre-clinical and clinical trials, small molecule inhibitors including the MEK inhibitors have not achieved great clinical success. Identification of “sensitivity prediction biomarkers” that reflect the dependency on the signaling pathway and that predict drug sensitivity would allow the pre-selection of patient populations that are most likely to respond to therapy. Therefore, deeper insights into the mechanisms of molecular action and regulation are needed from preclinical models in order to maximize success in

the clinics. Theoretically, this could be accomplished through the use of genetically defined mouse models of cancers and in a well-controlled manner. Furthermore, mouse models could be useful for evaluating “clinical response” biomarkers that monitor anti-tumor effects of inhibitors, and to design/optimize an effective pharmacodynamic schedule for single inhibitor application or in combination with the chemotherapeutic or another targeting regimens.

1.6 Mouse models of AML

Cancer is a disease arising as a consequence of multiple genetic mutations (Hanahan and Weinberg 2000) and has been modeled in mice. The mouse models used for cancer research have significantly evolved over the last few decades (Frese and Tuveson 2007). Xenograft models are in an intermediate step between cell culture and true mouse cancer models and are now regarded as “animal culture”. They enable rapid *in vivo* assessment of tumor tissue and cell lines in immunocompromised mice, but diminish the representation of tumor architecture and genetic heterogeneity (Frese and Tuveson 2007). Carcinogen driven spontaneous tumor models are implicated in identification of cancer genes and compounds assessment, but they suffer from drawbacks such as restricted tumor types, incomplete penetrance, and variable latency (Frese and Tuveson 2007). Genetically engineered mouse models (GEM), in contrast, are the most sophisticated animal models of human cancer so far, and have been adapted to dissect the *in vivo* genetic complexity of human tumors, identify cancer related genes and evaluate new compounds and

combinatorial therapies pre-clinically. In the last few years, more than 35 GEM harboring individual human cancer gene have been generated and used to obtain invaluable insight into their cellular function and genetic interactions [for review see (Zender et al. 2007)].

A complex cellular hierarchy maintains the hematopoietic system and AML studies require creation of models that accurately mimic the biological and genetic process of the equivalent human diseases. Correspondingly, various technologies have been employed to generate GEMs of AML, including embryonic stem cell-mediated or retroviral-mediated gene transfer strategies. Consequently a large number of genetically engineered AML models have been described (McCormack et al. 2008).

Transgenic mice are generated through pro-nuclear injection of exogenous DNA into fertilized zygotes that are subsequently implantated into a pseudo-pregnant female, or via injection of genetically modified mouse embryonic stem (ES) cells into a blastocyst. In the later case, by targeting a mutant version of the exogenous DNA fragment to the endogenous locus through homologous recombination “knock-in” mice harboring oncogenic lesions at their native genomic locus are created (Smithies et al. 1985). These are the most precise endogenous GEMs (Frese and Tuveson 2007) and often utilize the Cre-lox system to control the onset of mutations in a spatio-temporal manner. However, a major limitation of using these models is that generating diseased mice based on compound mutations requires time- and resource- consuming genetic crosses.

Retroviral transduction and transplantation models, on the other hand, take advantage of the availability of bone marrow or fetal liver cells that contain hematopoietic stem and progenitor cells (HSPCs), as well as the well established *ex vivo* genetic manipulation and *in vivo* reconstitution protocols. Because genetic combinations could be achieved easily and rapidly by varying the genotype of the HSPCs and the transgenes, mosaic models developed through this strategy could serve as invaluable tools to study cancer gene cooperativity. In addition, the environmental interactions between donor cells and host marrow niche could be studied by altering the genotype of the recipients. Several murine mosaic models mimicking Burkitt lymphoma, hepatocarcinoma and myeloid leukemia have been developed in our laboratory (Schmitt et al. 1999; Zender et al. 2006; Zuber et al. 2009), and used with success to understand the mechanisms underlying cooperation between genetic lesions during tumorigenesis and to valid oncogenic and tumor suppressive genes identified using oncogenomic information derived from patient materials.

Retroviral co-delivery methods have also evolved to incorporate more sophisticated components, such as RNA interfering that functions in a constitutive or regulatable manner to control gene expression and incorporation of single or dual fluorescent markers or bioluminescent proteins to readily trace the gene of interest and disease progression/regression. Incorporating Cre recombinase that is commonly used to direct loxP-mediated recombination can further enhance flexibility. As described later in Chapter 2 of this dissertation, the combined delivery of self-deleting Cre, shRNA and GFP into hematopoietic

stem/progenitor cells has provided a novel and fast approach to simultaneously: (1) model endogenous oncogene activation independent of oncogene overexpression or complex crosses; (2) minimize Cre genotoxicity; (3) GFP-tag the resulting disease; and (4) directly test gene cooperativity using RNAi.

1.7 Retrospective of my graduate research

When I first started my graduate dissertation research, I was interesting in studying chemotherapy responsiveness and the genetic determinants underlying differential responsiveness using mouse models of AML. We initially utilized AML samples provided by our collaborators, Dr. Kevin Shannon (University of California, San Francisco) and Dr. David Largaespada (University of Minnesota) to generate protocols for AML cell isolation and transplantation. These AMLs were generated by viral insertional mutagenesis in Nf1-deficient- or AML1-ETO knock-in mice. Thereafter, the AML1-ETO viral mutagenesis model was chosen for pilot experiments to establish *in vivo* treatment regimens using conventional drugs of clinical AML therapy such as cytarabine (Ara-C) and doxorubicin. After an extensive effort to optimize drug doses, I was able to establish a protocol involving treatment with 3.3 mg/kg Doxorubicin daily for first three days and 160mg/kg Ara-C daily for 10 days administered by intraperitoneal injection. Mice have responded to this regimen by partial remission (i.e. the majority of leukemic cells disappears from the peripheral blood) and a prolongation of survival by approximately two weeks.

A major problem we encountered with these pre-existing models, however,

was acquisition of treatment resistance after serial passaging, in part due to the ongoing selection of cells harboring selective advantages. In addition, although these models involved important genetic alterations in AML including Ras pathway mutations and AML1-ETO, viral insertional mutagenesis complicated the genetic background of the diseases. Finally, material was often limited for these studies. Based on these limitations, we planed to generate genetically defined AML models that were independent of viral insertional mutagenesis and whose generation was based on common human AML lesions.

In generating genetically defined AML models, our primary focus was on testing the cooperation of oncogenic Kras and lesions in the p53 network. As previously shown, a Cre-mediated activation of the mutant Loxp-STOP-Loxp $Kras^{G12D}$ allele in the hematopoietic system induced a fatal myeloproliferative syndrome (Braun et al. 2004). As mentioned above, rapid retroviral gene transfer protocol has been established in our lab by infecting hematopoietic stem/progenitor cells (HSPCs) with genes of interest and transplantation into lethally irradiated syngenic recipient mice. To make this strategy more flexible, methods for mimicking loss of function of tumor suppressor genes through stable RNA interference was also included (Hemann 2003; 2004; Xue 2007; Zender 2008).

We developed a transplantation-based “mosaic” mouse model in which hematopoietic stem/progenitor cells derived from LoxP-STOP-LoxP $Kras^{G12D}$ mice could be transduced with a retrovirus harboring a short hairpin RNA targeting p53 (Dickins et al. 2005; Zuber et al. 2009) and an inducible CreER

(shp53Cre). Within an observation period of 120 days, more than 70% of recipients of Kras-shp53Cre HSPCs developed an aggressive fatal leukemia with a median survival of 79 days. The acute leukemia was characterized by increased mature myeloid cells in the bone marrow and peripheral blood, severe anemia, and dissemination of neoplastic cells in the spleen, liver and lung. Importantly, the leukemic cells were transplantable to sublethally irradiated recipient mice. These leukemias displayed virtually undetectable levels of p53 and increased activity of Ras effectors. In addition to the constitutively activated p53 short hairpin, inducible RNAi technology was used to regulate p53 suppression in this system. It is worth mentioning that using the similar methods we found that Pten deletion also cooperated with p53 knock down in AML leukemogenesis since $Pten^{floxed}$-shp53Cre recipients developed aggressive fatal leukemia. In conclusion, we have used the mosaic transplantation strategy to rapidly generate a mouse model of AML with deficiency in the p53 gene and endogenous levels of oncogenic Kras.

Next, I set out to dissect the mechanism(s) that underlie the cooperation between Kras activation and p53 knockdown. As overexpression of mutant Ras causes senescence as part of a failsafe mechanism that ultimately prevents malignant transformation, we wanted to test whether the endogenous levels of oncogenic Kras were sufficient to induce senescence in our system. I found that Kras activation induced $p16^{Ink4a}$ expression in cultured HSPCs and senescence associated *beta-gal* (SA-b-gal) positive staining in spleen sections of recipients.

Unfortunately, due to the lack of defined markers of senescence in the hematopoietic system to confirm the senescence phenotype, I was not able to formally implicate the senescence phenotype in a defined population of bone marrow and thereby the direct relevance of senescence in preventing AML development.

Next, I started to evaluate self-renewal capability of the premalignant and leukemic cells. Using *in vitro* methylcellulose based colony forming and replating assays I found that loss of p53 dramatically increased replating efficiency and thus enhanced the self-renewal capability of Kras-expressing cells. By transplanting Kras-shp53 meyloid progenitor cells to recipient mice, I confirmed that these cells were able to initiate AML *in vivo*. Thus, I showed that p53 deficiency – which is associated with a particularly adverse prognosis in AML patients – provide one route to self-renewal during the course of AML leukemogenesis. The results of these studies are being submitted for publication.

During the course of characterizing AML induced by Kras-shp53, I found that (unlike Kras activation alone that increases the level of Ras-GTP but not the activities of downstream effectors such as pERK and pS6) Kras-shp53 leukemic cells exhibited constitutive activation of Ras signaling. I proceeded to identify Sprouty4 (Spry4, a negative regulator of Ras signaling pathway), among several negative regulator of Ras signaling, was induced by Kras activation. Using similar strategies as has applied to p53 knock down, I found that Spry4 knockdown cooperated with $Kras^{G12D}$ to induce T cell leukemia/lymphoma in mice. In parallel, using other genetically defined AML models involving AML1-ETO and Nras

mutations, I found that p53, as in the Kras-shp53 AML model, might control the level of signaling output, and the level of signaling flux in different AML models predisposed them to differential responsiveness to MEK inhibition. We are currently in the process of completing experiments for the eventual publication of these results.

In summary, throughout my dissertation research, I generated several genetically defined mouse models of AML. With these models, I discovered that p53 plays role in suppressing oncogenic self-renewal and Ras signaling output, and established Spry4 as a potential tumor suppressor. I also established myeloid progenitors that gain self-renewal capabilities are the AML initiating cell population in our model. Finally, using AML mouse models harboring different activating levels of Ras signaling, I found that p53 dependent signaling output predetermines sensitivity to targeted therapy. Together, I believe my dissertation research has provided new insights into the previously underappreciated role of p53 in AML.

Chapter 2

p53 deficiency provides a route to AML oncogenic self-renewal

Introduction

Studies examining the consequences of Ras deregulation in the myeloid compartment have utilized bi-transgenic mice harboring a Kras "lox-stop-lox" (LSL) allele (LSL-Kras^{G12D}) (Tuveson et al. 2004) and an interferon-responsive Mx1-Cre transgene (Kuhn et al. 1995). In this system, the activated Kras allele is knocked into the endogenous locus but kept silent by the LSL cassette, which can be excised by Cre-mediated recombination following injection of pIpC. Following Kras activation, mice develop a fatal myeloproliferative disease (MPD) between two and three months (Braun et al. 2004; Chan et al. 2004). However, unlike leukemia associated fusion proteins but similar to other signaling activating lesions such as BCR-ABL, Kras activation does not enhance or confer self-renewal capability to hematopoietic stem/progenitor cells, and cannot initiate hematopoietic malignancies without additional genetic alterations (Huntly et al. 2004; Zhang et al. 2008; Sabnis et al. 2009).

Hyperactivity of pro-proliferative signaling, such as mutations in Ras, Braf, loss of Pten or Nf1, triggers p53-dependent failsafe responses in various tissues, and loss of p53 cooperates with them to achieve the malignant transformation (Hermeking and Eick 1994; Eischen et al. 1999; Johnson et al. 2001; Chen et al. 2005). It remains unclear if such interplay between p53 and pro-proliferative mutations (Class I AML mutations) exists and contributes to AML leukemogenesis.

In this study, we explored the genetic and biological interactions between

activation of Kras and disruption of p53 in AML oncogenesis. By exploiting the well-characterized LSL-Kras^{G12D} system and established assays to assess self-renewal capabilities of normal and leukemic cells, we found that p53 mutations provide one mechanism whereby differentiated myeloid progenitor cells acquire the capacity for indefinite self-renewal during the course of leukemogenesis. Our results therefore provide new insights into AML biology and p53 action in tumor suppression.

Results

A self-excising Cre vector that simultaneously delivers an shRNA and GFP reporter

To test the leukemogenic effect of p53 loss in this model, we tried to generate compound Mx1-Cre;LSL-Kras^{G12D};p53-/- mice by intercrossing individual mutant strain. Already time consuming and expensive, the 4-allele cross did not yield sufficient offspring for studies examining leukemia onset, since mice of the desired genotype were born at sub-Mendelian ratios and frequently died shortly after birth of uncharacterized causes (data not shown). We also attempted to control p53 deletion by production of Mx1-Cre;LSL-Kras^{G12D};p53loxp/loxp mice, where both activation of Kras and inactivation of p53 are triggered by Cre-mediated recombination. Shortly following pIpC administration, these mice succumbed to complicated phenotypes including myeloid hyperplasia, atypical lymphoid hyperplasia in thymus and spleen and histiocytic sarcoma and were sacrificed (data not shown). Similar phenotypes were observed in untreated mice (data now shown), perhaps owing to leaky cre expression in both hematopoietic and non-hematopoietic cell types (Sabnis et al. 2009).

The difficulties we encountered producing mice harboring conditional germline Cre, Kras, and p53 alleles prompted us to explore a more time and cost effective strategy to examine the effects of p53 inactivation on leukemogenesis. Our laboratory has developed methods for mimicking loss of tumor suppressor

gene in mice through stable RNA interference (Hemann 2003; 2004; Xue 2007; Zender 2008). We therefore set out to develop a transplantation-based "mosaic" mouse model in which hematopoietic stem/progenitor cells derived from LSL-$Kras^{G12D}$ mice could be transduced with a retrovirus harboring a short hairpin RNA targeting p53 (Dickins et al. 2005; Zuber et al. 2009). We constructed a Murine Stem Cell Virus (MSCV)-based retroviral vector (LGmCreER, see Figures 2.1a) harboring a well characterized p53 shRNA based on the mir30 design, which efficiently triggers the RNA machinery and can be expressed in the same transcript as green fluorescent protein (GFP) (Dickins et al. 2005; Stegmeier et al. 2005). To enable co-expression of Cre-recombinase from the same vector, we introduced a tamoxifen inducible $CreER_{T2}$ expressed from a constitutive PGK promoter. Importantly, to avoid toxicity associated with long term Cre exposure (Loonstra et al. 2001; Forni et al. 2006) but to retain the capacity for GFP-shp53 in the same construct, we implemented a novel self-excising design by flanking the PGK-$CreER_{T2}$ cassette with two lox511 sequences, which also prevent the recombination with the loxP sites in targeted cells (Hoess, 1986). Southern blotting of DNA derived from mouse embryo fibroblasts (MEFs) infected with virus collected within 24-36 hours after packaging cells transfection revealed that more than 70% of the integrated proviruses were full length (Figure 2.1b); as predicted, the addition of 4-hydoxytamoxifen (4-OHT) led to the efficient excision of the PGK-CreER cassette. Moreover, immunoblotting revealed that cells transduced with the LGshp53CreER vector showed potent suppression of p53 expression (Figure 2.1c).

We next compared the effectiveness of LGmCreER vector at excising a lox-stop-stop (LSL) cassette. NIH3T3 cells containing a LSL-LacZ reporter were transduced with LGmCreER and a non-self excising MSCV-CreER$_{T2}$-IRES-GFP vector, and cell populations were examined in the absence and presence of 4-OHT (Fig 2.1d). Each vector was efficiently delivered into target cells as assessed by GFP fluorescence; however, in the absence of 4-OHT, a substantial percentage of cells harboring the non-self excising vector were β-Gal positive, suggesting a leakiness of LTR-driven CreER$_{T2}$. Moreover, 4-OHT addition induced substantial cell death, presumably owing to Cre toxicity as previously reported in fibroblasts (Silver and Livingston 2001). By contrast, cells transduced with the LGmCreER construct showed low basal β-Gal activity that was sinduced by 4-OHT (Fig 2.1d). Thus, the LGmCreER vector could deliver a reporter (GFP) and shRNA to target cells and at the same time induce Cre-mediated recombination with minimal toxicity.

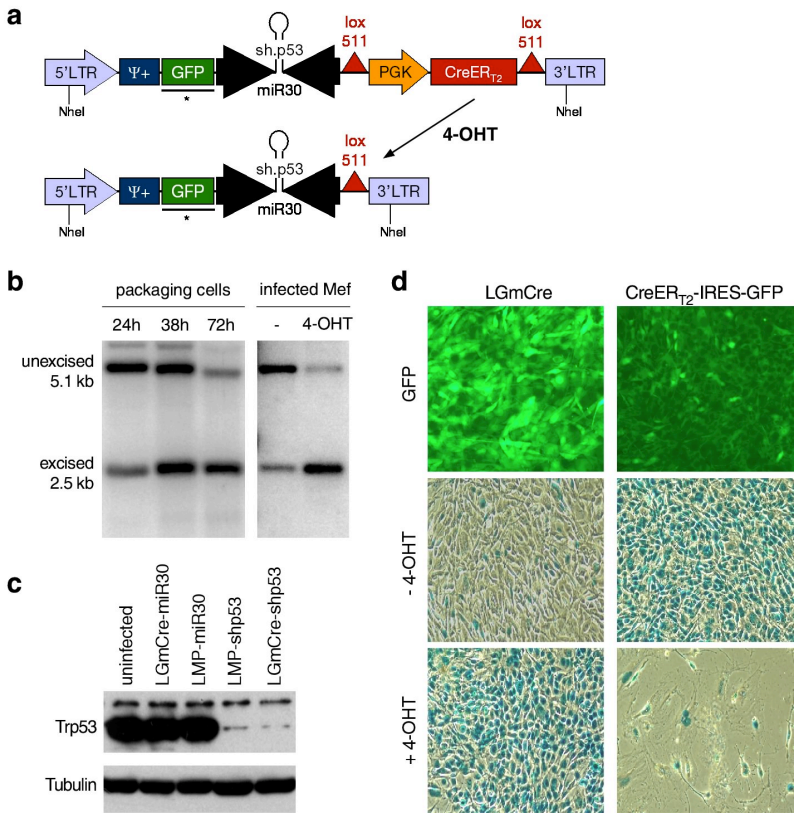

Fig. 2.1 Functional characterization of the retroviral LGmCreER for co-transduction of GFP, miR30-based shRNAs and 4-OHT inducible self-excising CreER$_{T2}$

(a) Schematic of the MSCV-based retroviral vector LGmCreER for co-transduction of GFP, miR30-based shRNAs and 4-OHT-inducible self-deleting CreER$_{T2}$. Treatment with 4-OHT activates CreER$_{T2}$ resulting in deletion of the lox511-flanked PGK-CreER$_{T2}$ cassette. *Probe used in Southern blot analysis.

(b) Southern blot analysis of the excision of PGK-CreER$_{T2}$ in LGmCreER in Phoenix packaging cells at various time points after transfection and infected MEFs with and without 4-OHT- induction of CreER$_{T2}$. A GFP-specific probe (*) detects a 5.1 kb fragment before, and a 2.5 kb fragment after excision of PGK-CreER$_{T2}$. In phoenix packaging cells PGK-CreER$_{T2}$ is slowly excised over time due to leaky activity of CreER$_{T2}$. MEFs infected with viral supernatant harvested not later than 36h after packaging cell transfection show mostly full-length integrations of LGmCreER, while 4-OHT treatment induces efficient excision of the PGK-CreER$_{T2}$ cassette.

(c) Immunoblotting of p53 in adriamycin-treated MEFs infected with LGmCreER or the established miR30-shRNA expression vector LMP with or without p53 shRNA. LGshp53CreER induces potent knockdown of p53 comparable to conventional LMP-shp53.

(d) Evaluation of Cre recombination efficiency using LGmCreER and conventional MSCV-CreER$_{T2}$-IRES-GFP in LSL-LacZ-NIH3T3 reporter cells. GFP fluorescence indicating efficient transduction of both constructs. Beta-Gal staining of untreated and 4-OHT treated reporter cells demonstrates that LGmCreER efficiently induces Cre-mediated recombination after 4-OHT induction with minimal leakiness and no toxicity, while conventional MSCV-CreER$_{T2}$-IRES-GFP-transduced reporter cells show leaky target recombination in the absence of 4-OHT and severe Cre-induced cytotoxicity after 4-OHT treatment.

p53 shRNAs cooperate with Kras^{G12D} to promote AML

To test whether p53 suppression cooperates with activated Kras^{G12D} in promoting AML, we isolated hematopoietic stem and progenitor cells (HSPC) from fetal livers of LSL-Kras^{G12D} embryos and infected these cells with LGmCreER containing either a p53 shRNA (LGshp53CreER) or the control empty miR30 cassette (LGmCreER). Cells were then transplanted into lethally irradiated syngeneic recipient mice (Fig 2.2 a). To induce Cre activity, 4-OHT was either directly added to the infected HSPCs *in vitro* or administrated to mice by intraperitoneal injection 4 weeks after transplantation *in vivo*. Recipient mice were monitored for signs of neoplasia at different times using sonographic spleen imaging, whole body fluorescence imaging, blood examination (WBC, blood smear cytology), and examined for overall morbidity at which time they were sacrificed.

As early as 6 weeks post transplantation and well before the median onset of blood pathology, only mice reconstituted with Kras-shp53 HSPCs showed advanced splenomegaly, with spleen sizes more than 3-fold larger than any of the other recipient mice (Fig 2.2 b). At the same time, Kras^{G12D}-shp53 mice had an accumulation of GFP positive cells in bone marrow, spleen and liver (Fig 2.2 c). Eventually, mice receiving Kras^{G12D}-shp53 HSPCs developed lethal neoplasias with a median survival of 79 days post-transplantation (n=45, Fig 2.2

d). Bone marrow isolates from animals with advanced disease were capable of transplanting lethal malignancies to recipient mice, indicating that the neoplasias produced by cells harboring $Kras^{G12D}$ and p53 shRNAs were bona fide leukemias rather than an aggressive myeloproliferative disease (Kogan et al. 2002) (Fig. 2.2 d). Of note, similar phenotypes were observed regardless of whether 4-OHT was administered before or after transplantation with *in vivo* Kras activation resulting in about two weeks delay of disease onset and progression. By contrast, mice carrying wt-shp53 or $Kras^{G12D}$ alone mainly developed T-cell lymphomas with much lower penetrance and longer latency comparing to $Kras^{G12D}$-shp53 leukemias (Fig 2.2 d and data not shown; $p<0.0001$for $Kras^{G12D}$ alone and $p=0.0692$ for wt-whp53).

To further characterize leukemias arising from $Kras^{G12D}$-shp53 HSPCs, we analyzed leukemic cells and tissues from moribund mice using flow cytometry and histopathology. GFP positive leukemic cells were present in the bone marrow, spleen and peripheral blood, ranging between 60-90% of the total cell population. Immunophenotyping revealed that most leukemias were of myeloid origin, with the majority (13/14) consisting of cells expressing Mac-1 and/or Gr-1 (Fig 2.2 e and data not shown). In about 40% of cases (9/22), leukemic mice also had enlarged thymi infiltrated with $GFP^+/Thy1^+$ lymphoblasts, suggesting they also suffered from thymic lymphoma. Leukemic mice displayed elevated white blood cell counts and signs of severe anemia (Fig 2.2 f, A). Approximately two thirds of the animals presented with an MPD-like myeloid leukemia composed of

mature neutrophilic and monocytic cells and less than 20% blasts (Fig. 2.2 f, A-D), while the other third of the mice presented acute myelomonocytic leukemia or AML without maturation (Fig. 2.2 f, E-F) (Kogan et al. 2002). In all cases spleens from leukemic mice were massively enlarged (800-2500 mg at sacrifice) and harbored virtually no white pulp but extensive extramedullary hematopoiesis and infiltration of myelomonocytes as well as maturing myeloid elements (Fig 2.2 f, C). In summary, using this transplantation-based "mosaic" approach, we quickly developed AML mouse models recapitulating various types of human AMLs.

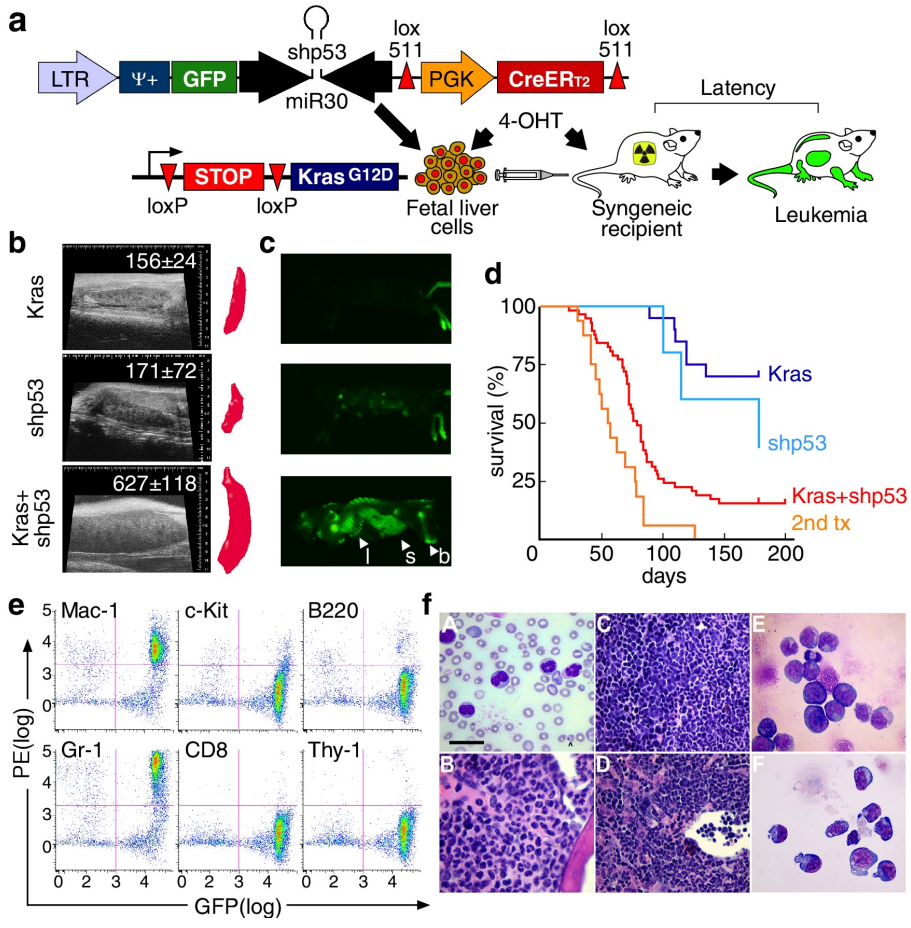

Fig. 2.2 Activation of endogenous Kras^{G12D} and shRNA mediated suppression of p53 cooperate to induce AML in mice
(a) Schematic of the experimental strategy. Retroviral LGmCreER is used to co-transduce GFP, miR30-based shRNAs and 4-OHT-inducible self-excising CreERT2 into HSPCs isolated from ED13.5-15.5 fetal livers of LSL-KrasG12D embryos. Cre-mediated recombination is induced by treatment with 4-OHT in vitro or in vivo.
(b) Representative spleen sonogram and 3D reconstruction volumetry in recipient mice of HSPCs of indicated genotypes six weeks after transplantation. Numbers indicate average spleen volume and standard deviation in 6 mice for each genotype. Only KrasG12D-shp53 recipient mice show severe splenomegaly at this preleukemic stage.
(c) Whole body imaging of representative mice reconstituted with KrasG12D, shp53 and KrasG12D-shp53 HSPCs. Only KrasG12D-shp53 mice show strong accumulation of GFP positive cells in bones (b), spleen (s) and liver (l).
(d) Kaplan-Meier curve showing the survival of mice reconstituted with KrasG12D-shp53 HSPCs (n=57) and controls. **p=0.0022 and ***p<0.0001 (log-rank test) indicate highly significant survival reduction compared to recipients of shp53 (n=12) and KrasG12D (n=20) control HSPCs, respectively. Secondary transplantation (2nd tx) curve indicating survival of sublethally irradiated recipient mice transplanted with KrasG12D-shp53 leukemias (n=16).
(e) Representative immunophenotyping of KrasG12D-shp53 leukemic bone marrow showing strong infiltration of GFP positive cells that are positive for Mac1 and Gr1.
(f) Representative histopathology of KrasG12D-shp53-induced AMLs, including MPD-like leukemias (A-D) and acute myelomonocytic leukemia (E) and AML without maturation (F). A, peripheral blood containing elevated numbers of neutrophilic and monocytic cells. Arrowhead denotes Howell-Jolly body. B, sternal bone marrow filled with maturing myeloid cells. C, spleen with vastly expanded red pulp filled with erythroid cells (upper left) and maturing myeloid elements (lower right). D, liver with extensive perivascular and sinusoidal hematopoiesis. E, bone marrow cytospin showing blasts and monocytic cells. F, bone marrow cytospin showing immature blast cells. Blood smear and bone marrow cytospin: Wright-Giemsa, scale bar 20 μm. Liver and spleen: hematoxylin & eosin, scale bar 50 μm.

We also characterized the resulting AMLs at the molecular level. To confirm activation of oncogenic Kras^{G12D}, we used allelic specific PCR to verify excision of loxp-STOP-loxp (LSL) cassette in the infected LSL-Kras^{G12D} HSPCs and AML tissue samples. Using an optimized PCR strategy that includes an optional secondary nested PCR step allowing for enhanced detection of rare LSL-recombination events in small subpopulations, we documented excision of the LSL cassette in peripheral blood of Kras^{G12D}-shp53 recipient mice 7 days after 4-OHT treatment (Fig 2.3 a, top panel). We also confirmed efficient excision of the LSL cassette in leukemic cells from multiple recipient mice (Fig 2.3 a,

bottom panel).

In $Kras^{G12D}$/Mx1-Cre induced MPDs, activated Kras causes elevated Ras-GTP levels that do not result in constitutive activation of the downstream signaling cascades (Braun et al. 2004), suggesting that Kras activation may induce a feedback loop to control the signaling output negatively. Ras transmits proliferative signals through the mitogen activated protein kinase (MAPK) cascade as well as the phosphoinositide 3-kinase (PI3K) pathway, which can be monitored by increased phosphorylation of the Erk1/2 and S6 proteins, respectively. To determine the output of Ras signaling cascades in the Kras-shp53 AML cells, we used flow cytometry (Van Meter et al. 2007) to quantify the intracellular phosphorylation of different Ras effectors in the absence and presence of GM-CSF, a growth factor important in normal myeloid cell proliferation. All analyzed $Kras^{G12D}$-shp53 leukemias displayed a significant elevation of basal p-Erk1/2 and p-S6 levels in comparison with wild type bone marrow in both bulk and stem/progenitor ($lineage^-/ckit^+$) populations (Fig 2.3 c, d and data not shown). In many cases GM-CSF stimulation could not further enhance p-Erk1/2 levels, indicating constitutive and saturating activation of MAPK signaling. These results suggest that the constitutively activated Ras effector signaling features the malignant state of leukemias. It'll be interesting to understand it is causal or consequential effect, and whether p53 involves in the negative regulation.

p53 knockdown was confirmed by immunoblotting of total bone marrow lysates extracted from Kras^{G12D}-shp53 leukemic mice. Following ex-vivo treatment with the DNA damaging agent adriamycin we observed a strong induction of p53 in wild type bone marrow and p53 wild type control AMLs, while p53 expression was undetectable in Kras^{G12D}-shp53 leukemias (Fig 2.3 b, compare to MLL/ENL leukemia from p53 null mice). This result indicates a strong selective advantage of leukemic cells with effective shRNA-mediated p53 suppression and underlines the potential of miR30-based RNAi for probing tumor suppressor genes in AML. Taken together, these results demonstrate that RNAi mediated knockdown of p53 cooperates with endogenous Kras^{G12D} to promote aggressive myeloid leukemia.

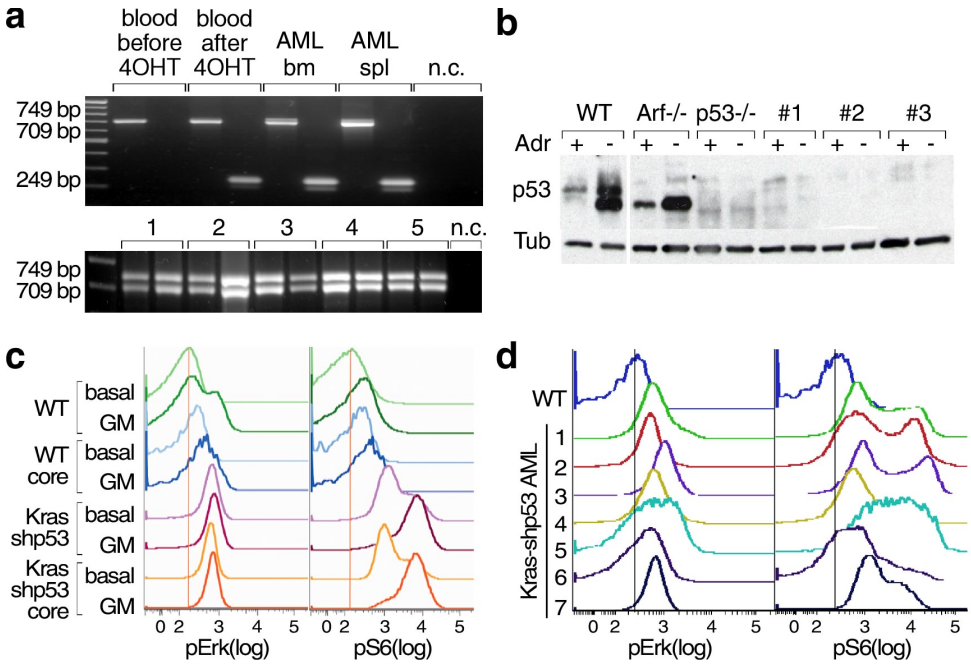

Fig. 2.3 Molecular analysis of leukemias of Kras^{G12D}-shp53 mice
(a) PCR analysis to verify Cre-mediated recombination of the LSL-cassette. Top panel: two-step PCR in peripheral blood of a representative KrasG12D-shp53 HSPC recipient mouse before and 5 days after 4-OHT treatment in vivo and after leukemia onset in bone marrow (bm) and spleen (spl). Recombination is indicated by the appearance of a 749 bp band after the first (I), and a 249 bp band after the second PCR (II). Bottom panel: LSL-KrasG12D recombination in leukemic mice (1-5) analyzed in bone marrow and spleen (left and right lane in each sample, respectively). nc, negative control.
(b) Western blotting of p53 in wildtype bone marrow, Arf-/- and p53-/- MLL/ENL-induced control leukemias and 3 independent KrasG12D-shp53 AMLs (#1, #2, #3) without (-) or with (+) Adriamycin (Adr) treatment in vitro.
(c) Flow cytometry analysis of intracellular phosphorylated Mapk1/Mapk3 (pErk) and Rps6 (pS6) in full and core (Lin-cKit+) populations of wildtype bone marrow and KrasG12D-shp53 leukemia.
(d) Elevated basal levels of intracellular phosphorylated Mapk1/Mapk3 (pErk) and Rps6 (pS6) in multiple independent KrasG12D-shp53 AMLs.

p53 suppression enables self-renewal of Kras^{G12D}-expressing hematopoietic progenitor cells

As a first step toward examining the cell of origin of AML and the basis for the p53 effects, we examined how p53 loss impacted the proliferation and survival of various cells of the hematopoietic lineage, either alone or in combination with endogenous Kras^{G12D}. The hematopoietic develops through a cellular hierarchy that can be readily distinguished using well-established cell surface markers (Chao et al. 2008). Self-renewing hematopoietic stem cells [(HSCs) are enriched in cells expressing KSL markers (cKit$^+$Sca1$^+$Lin$^-$) and purity can be further increased with SLAM markers (CD150$^+$CD48$^-$KSL). HSCs give rise to multipotential progenitors [MPP (CD150$^-$CD48$^-$KSL)] that eventually differentiate into the lineage-restricted common lymphoid progenitor (CLP) or common myeloid progenitor (CMP) cells. Notably, CLPs and CMPs – and all of their decedents – are committed to differentiate and are unable to self renew

(Chao et al. 2008). CMPs give rise to megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) (Chao et al. 2008). Myeloid progenitors (MP), including CMP, GMP, and MEP, can be purified as Lin^- $Ckit^+Sca\text{-}1^-IL7R\alpha^-$ cells (Akashi et al. 2000; Miyamoto et al. 2002; Akala et al. 2008; Zhang et al. 2008), and used in subsequent experiments to represent a non-self renewing progenitor cell population with myeloid identity. In the following experiments, wild type and $Kras^{G12D}$ HSPCs were transduced with LGshp53CreER or a control vector (LGshlucCreER, expressing a ontrol shRNA targeting firefly luciferase) and, following 4-OHT mediated Cre activation, wt-shluc, wt-shp53, $Kras^{G12D}$-shluc or $Kras^{G12D}$-shp53 cell populations were generated.

Taking advantage of the GFP marker in the retroviral construct and lineage specific markers, we were able to track $Kras^{G12D}$ and/or p53 deficient cells in different cell types by flow cytometry. We analyzed BrdU incorporation and overall representation of GFP positive stem cell (HSCs) and myeloid progenitors (MPs) in culture four days after 4-OHT-mediated $Kras^{G12D}$ activation (Fig 2.4 a, b). Consistent with previous reports that in Mx1-Cre/$Kras^{G12D}$ MPD bone marrow HSCs show increased proliferation but are reduced in frequency (Van Meter et al. 2007), we found that $Kras^{G12D}$ activation resulted in a mild increase in BrdU incorporation in KSL ($cKit^+Sca1^+Lin^-$) cells that are enriched for HSCs (designated as HSC^{KSL})(Fig 2.4 a), while the overall frequency of GFP+ $Kras^{G12D}$ HSC (CD150+CD48-KSL) among all GFP+ cells was reduced

compared to wild type (Fig 2.4 b, top panel). Similar results were observed in cells cultured in STIFA medium (StemSpan serum-free media containing the growth factors SCF, TPO, IGF-2, FGF-1 and Angptl3), which supports the expansion of normal HSCs *in vitro* (Zhang et al. 2006; Akala et al. 2008) (data not shown). These results are consistent with previous reports that enforced Ras signaling favors differentiation and not self-renewal of HSCs (Marshall 1995; Meloche and Pouyssegur 2007)

p53 loss showed opposite effects of Kras on HSC and MP population. Increased the percentage of BrdU positive cells was found in p53 knock down HSC (Fig 2.4 a). As a consequence, p53 suppression alone had mild increase in HSC, consistent with previous reports (Akala et al. 2008; Liu et al. 2009) (Fig 2.4 b). Moreover, p53 knock down partially canceled the effects of Kras on HSCs depletion (Fig 2.4 b, top panel), although not to the level of p53 suppression alone (Fig 2.4 b, top panel), indicating loss of p53 did not override the differentiation effect of Kras. As for MPs, p53 loss enhanced their proliferation and overall representation in both wt and Kras context (Fig 2.4 b, bottom panel). These results indicate p53 loss promotes proliferation of HSCs and MPs, while show different effects on HSCs and MPs in combination with Kras activation.

Studies in mice and humans imply that leukemia initiating cells can derived from cell types that normally do not possess self renewal capabilities, suggesting these cells must acquire such capabilities during leukemogenesis (Passegue et al. 2003; Braun et al. 2004; Jamieson et al. 2004). We wanted to

determine whether p53 inactivation aberrantly provides such a self-renewal capability that underlies its contribution to the cooperation with Kras activation. One classical well-established assay to tackle this question is the methylcellulose serial replating assay, with which the lifespan and replication potential of progenitors could be measured *in vitro*. Using this assay, previous work showed $Kras^{G12D}$ expressing bone marrows do not exhibit enhanced self-renewal ability and exhaust their proliferation potential after three rounds of replating (Braun et al. 2004; Chan et al. 2004).

We plated 5,000 GFP+ wt-shluc, wt-shp53, $Kras^{G12D}$-shluc and $Kras^{G12D}$-shp53 HSPCs in methylcellulose culture and scored the number of GFP+ colonies 7 days later. Cells were then harvested and the same procedure was repeated. As expected, both wild type and $Kras^{G12D}$ HSPCs lost their capacity in reproducing colonies after the 4^{th} round of plating, indicating that the progenitor cell compartment within these populations was exhausted (Fig 2.4 c,d). Consistent with previous reports (Liu et al. 2009), wt-shp53 cells showed a modest enhancement in their colony forming and replating ability. In sharp contrast, the effects of p53 loss on replating capacity in the presence of $Kras^{G12D}$ were profound: $Kras^{G12D}$-shp53 cells continued forming numerous robust CFU-M like GFP^{+} colonies through 6 rounds of replanting when the experiments were terminated (Fig 2.4 c,d). Interestingly, over the period of replating, only wt-shp53 and $Kras^{G12D}$-shp53 cells were enriched as indicated by gradually increasing GFP%, and GFP+ $Kras^{G12D}$-shp53 cells dominate the culture eventually (Fig 2.4 e and data not shown). The $Kras^{G12D}$-shp53 colonies could be easily adapted

into liquid culture thereafter and two million $Kras^{G12D}$-shp53 cells collected from the final replating were capable of initiating AML and thymic lymphomas following transplantation into sublethally irradiated recipient mice (data not shown), indicating they were enriched for leukemia initiating cells.

Since the progenitor compartments are known to be responsible for the colony formation in this assay (Lavau et al. 1997), we harvested colonies from the fourth round and stained them with combination of cell surface markers for MPs and MPPs to investigate their presence in the continuously formed colonies. Both MP and MPPs in outgrowing $Kras^{G12D}$-shp53 colonies were detected (Fig 2.4 e). Importantly, all cells with progenitor immunophenotype were positive for GFP (Fig 2.4 e), demonstrating that only cells with both Kras activation and p53 knockdown had contributed to colony formation. Taken together, these *in vitro* results indicate p53 dysfunction enhances proliferation of myeloid progenitor cells and, in combination with oncogenic Kras, confers self-renewal capability *in vitro*.

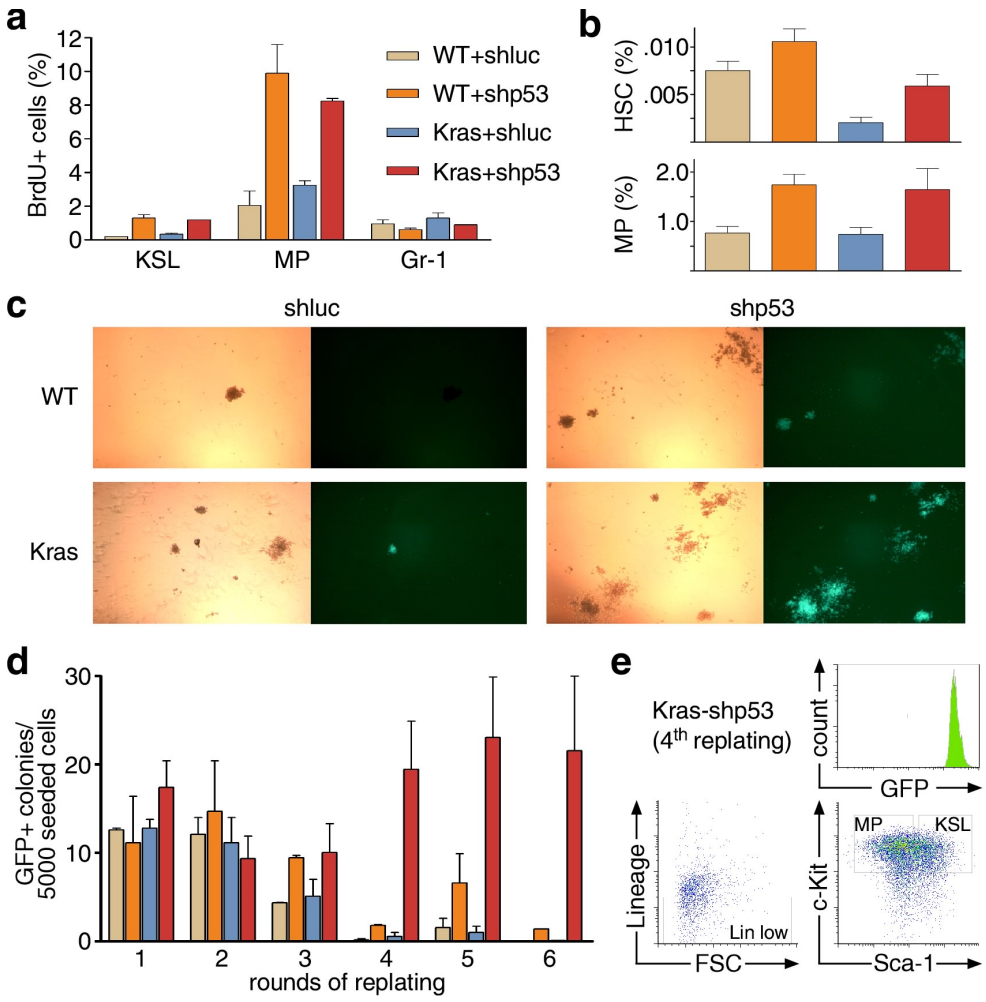

Fig 2.4. p53 suppression and endogenous Kras^{G12D} induce self-renewal of myeloid progenitor cells *in vitro*

(a) BrdU incorporation in transduced mature myeloid cells (Gr1+), MPs (Lin$^-$cKit$^+$Sca1$^-$), and KSL (Lin$^-$cKit$^+$Sca1$^+$) in indicated genotypes 4 days following 4-OHT treatment.

(b) Percentage of transduced (GFP+) myeloid progenitors (MP; Lin$^-$cKit$^+$Sca1$^-$) and hematopoietic stem cells (HSC; Lin$^-$cKit$^+$Sca1$^+$CD150$^+$CD48$^-$) in all transduced (GFP+) HSPCs 4 days following 4-OHT treatment-mediated activation of Kras^{G12D}.

(c) Methylcellulose colony formation and serial replating assay of transduced and 4-OHT treated HSPCs of indicated genotypes. Bright field and GFP fluorescent images showing colonies formed after the fourth round of replating.

(d) Quantification of colonies of 6 rounds of replating. Only combined activation of endogenous Kras^{G12D} and suppression of p53 results in robust colony formation beyond 4 rounds of replating.

(e) Progenitors analysis of representative Kras^{G12D}-shp53 cells after the fourth round of replating. Kras^{G12D}-shp53 colonies are strongly enriched for MPs and multipotential progenitors (MPP; Lin$^-$cKit$^+$Sca1$^+$CD150$^-$CD48-) that are contained in KSL population, and MPs are positive for GFP.

p53 suppression enhances reconstitution and in concert with endogenous Kras^{G12D} expands myeloid progenitor cells that promote leukemogenesis *in vivo*

Since Kras-shp53 MPs showed increased proliferating rate and enhanced proliferation potential reminiscent self-renewal capability *in vitro*, we speculate this population may serve as the leukeumia initiating cells for the AML. We then asked how loss of p53 and Kras activation affects myeloid progenitors *in vivo* and whether they ultimately contribute to leukemia formation. To this end, we first analyzed how the two lesions individually and in combination influence hematopoietic reconstitution *in vivo* and the afterwards frequency of MPs as well as HSCs. We transduced wild type and LSL-Kras HSPCs with LGlucCreER and LGshp53CreER, and transplanted these cells into lethally irradiated recipients. Transduction efficiencies were comparable across all genotypes and a similar fraction of non-transduced (GFP-) cells was preserved as internal control for the following *in vivo* competition studies. Kras^{G12D} activation was triggered following host reconstitution in order to prevent the potential deleterious effects of Ras activation on engraftment (Sabnis et al. 2009). Three weeks after 4-OHT treatment, bone marrow were harvested and analyzed for: 1) GFP+ percentage in bone marrow, and 2) GFP+ percent in MPs and HSCs. These three parameters reflect the relative competition between infected GFP+ cells that contain Kras and/or p53 knockdown and those uninfected internal control cells, within the total bone marrow and the two specific populations. We also analyzed the frequency of GFP+ MPs and HSCs amongst totoal GFP+ cells, which reflects the overall growth of Kras and/or shp53 expressing myeloid progenitors or HSCs

among the total donor derived infected GFP+ cells.

We first noticed in comparison with wt-shluc HSPC recipients that showed approximately 50% GFP+ cells in total bone marrow, MPs and HSCs (Fig 2.5 a), recipients of $Kras^{G12D}$-shluc HSPCs showed a consistent although not statistically significant reduction of GFP+ cells in all these compartments (Fig 2.5 a for quantification and 2.5 b for representative FACS profile), indicating that Kras activation did not confer competitive advantages to these cells *in vivo*. In addition, the frequency of Kras MPs (Fig 2.5 c) and HSCs (Fig 2.5 d) among total GFP+ cells reduced, in accordance with the *in vitro* culture results described earlier and the previous results on reduction of hematopoietic stem and progenitor cells in bone marrow of Kras MPD mice (Van Meter et al. 2007). By contrast, shp53-exprssing cells were strongly enriched in overall bone marrow and both HSCs and MPs (Fig 2.5 a,b) to levels consistently >75%, which was independent of the $Kras^{G12D}$ status and indicates that suppression of p53 confers selective advantage in these compartments during the process of transplantation and *in vivo* proliferation. Of great interest, in the context of $Kras^{G12D}$ activation loss of p53 had opposing effects on HSCs and MPs: while GFP+ HSCs became even more underrepresented in the absence of p53 (Fig. 2.5 d), the Kras-induced reduction of GFP+ MPs was fully rescued (Fig. 2.5 c). Importantly, these MPs persist in established AMLs, since immunophenotyping of bone marrow from leukemic mice revealed the presence of MPs that are purely positive for GFP, while GFP+ HSCs were hardly detectable (data not shown).

In the normal hematopoietic system, only the hematopoietic stem cells

have the capacity to self-renew and contribute to long-term reconstitution in recipient hematopoietic ablated mice, reviewed (Chao et al. 2008). This holds true for $Kras^{G12D}$ mice as injection of HSCs but not myeloid progenitor including common myeloid progenitor cells provides durable multi-lineage reconstitution and leukemia initiation (Zhang et al. 2008; Sabnis et al. 2009). To confirm that the 'rescued' MPs seen in Kras-shp53 mice acquire aberrant self-renewal ability and induce AML, we examined their self-renewal and leukemia initiating capacity *in vivo*. GFP+ MPs were isolated from pre-leukemic (3 weeks post 4-OHT treatment) and mice succumbed to leukemia based on the combination of $GFP^{+}Lin^{-}Sca1^{-}CKit^{+}$ $IL7R\alpha^{-}$. 2,000-5,000 such cells together with 200,000 wild type syngeneic helper cells were transplanted into lethally irradiated recipients. Whereas Kras-shluc MP recipients remained healthy, those received Kras-shp53 MPs from both early stage (3 weeks post Kras activation) and advanced leukemia succumbed to disease (Fig 2.5 e) with phenotypes resembling primary AMLs except no accompanied thymic lymphoma (data not shown). About 60% GFP positive leukemic cells were positive for Mac1 and Gr1 (data not shown), indicating the Kras-shp53 MPs proliferated *in vivo* and produced downstream progenies that account the majority of leukemic burden. These results collectively indicate myeloid progenitors with Kras activation and p53 deficiency have gained *in vivo* self-renewal capability, and served as leukemia initiating cells in $Kras^{G12D}$-shp53 mediated AML leukemogenesis.

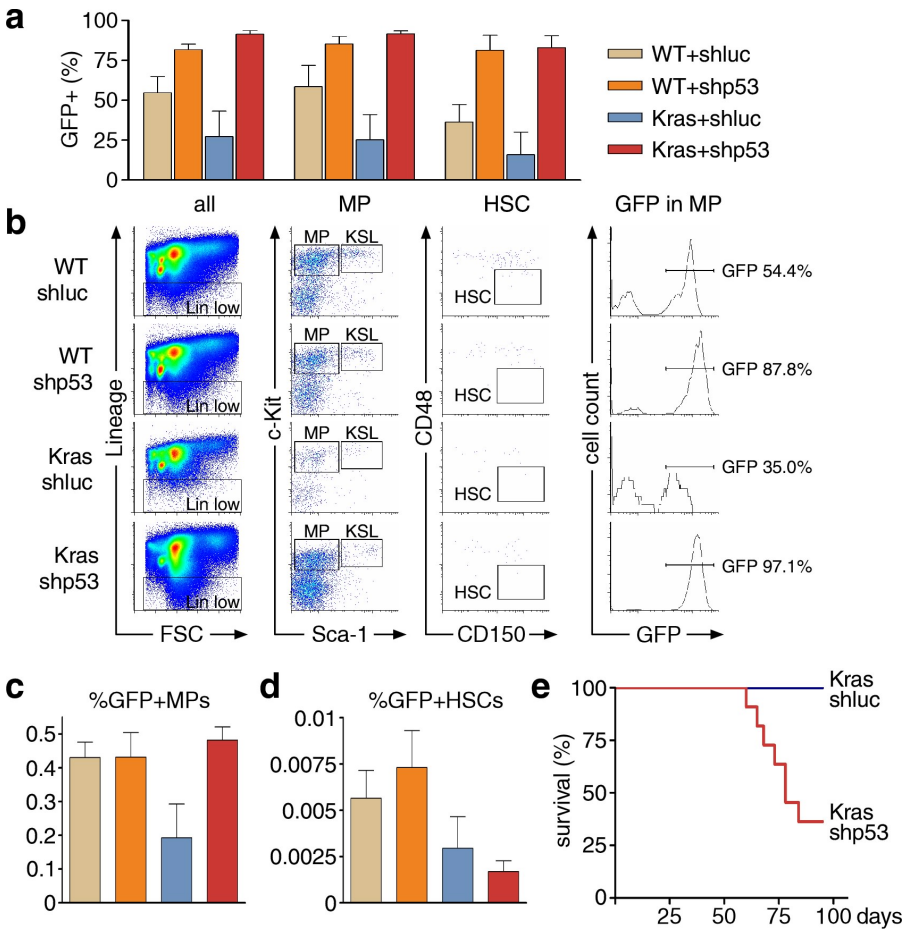

Fig. 2.5 p53 suppression enhances reconstitution ability and expands myeloid progenitors *in vivo*, which promote leukemogenesis in concert with endogenous Kras^{G12D}

(a) Quantification of GFP^{+} WBCs, MPs and HSCs in bone marrow of mice reconstituted with HSPCs of indicated genotype 3 weeks after *in vivo* 4-OHT treatment to induce Kras^{G12D}.

(b) Representative flow-cytometry profiles of bone marrow HSCs and MPs of mice reconstituted with HSPCs of indicated genotypes, including conventional Lin/cKit/Sca1 staining (left two panels), SLAM markers (third panel) and histogram indicating the percentage of GFP^{+} in MPs (right panel).

(c) Quantification of GFP+ MPs within GFP^{+} bone marrow cells of mice reconstituted with HSPCs of indicated genotypes. Harvests were performed at the same time as in A.

(d) Quantification of GFP+ HSCs within GFP^{+} bone marrow cells of mice reconstituted with HSPCs of indicated genotypes. Harvests were performed at the same time as in A.

(e) Kaplan-Meier curve of lethally irradiated recipient mice transplanted with 2,000-5,000 doubly sorted myeloid progenitor cells from primary Kras^{G12D}-shp53 and Kras^{G12D} mice.

p53 gene deletion and p53 knockdown have similar effects on the self-renewal and leukemia initiating potential of Kras^{G12D} expressing myeloid progenitors

The above results illustrate how combining ectopic Cre delivery and RNAi provide a rapid method to study the impact of deregulated Kras signaling and p53 dysfunction on leukemogenesis and self-renewal. To rule out the possibility that technical aspects of the experimental systems involved may affect our observations (isolation of fetal liver HSPCs, Cre delivery, transplantation/reconstitution or RNAi off target effects), we repeated key experiments in an entirely transgenic setting. To produce the precise genetic configurations approximated above, we intercrossed Mx1-Cre (C), LSL-Kras^{G12D} (K) and p53$^{loxp/loxp}$ (P) mouse strains. Mice were treated at around 6 weeks of age with pIpC to activate Kras^{G12D}, 10 days later bone marrow were harvested from each genotype for colony forming and serial replating assay as well as FACS analysis for the frequency of mature myeloid cells (Mac1^{+}Gr1^{+}) and stem/progenitor populations in bone marrow. In addition, myeloid progenitors were isolated and assessed for their leukemia initiation capacity *in vivo* (for see overview Fig 2.6).

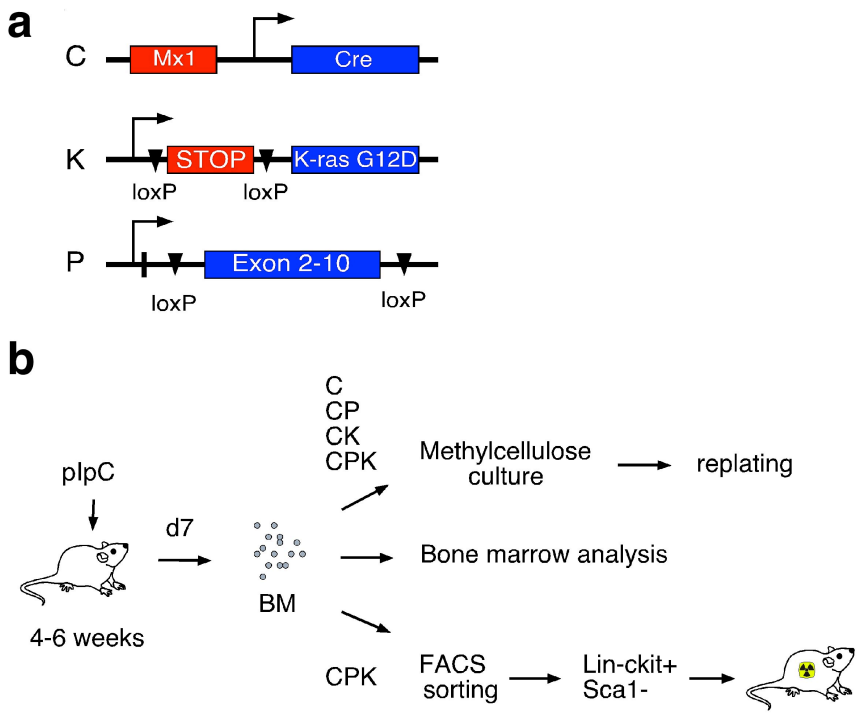

Fig. 2.6 Schematic overview of mice used for fully transgenic validation experiments. Existing transgenic mice harboring Mx1-Cre (M), LSL-Kras^{G12D} (K) and p53$^{F2\text{-}10/F2\text{-}10}$ (P) mice were crossbred to generate Kras^{G12D} or/and p53^{floxed} transgenic bone marrow cells for indicated subsequent *in vitro* and *in vivo* assays. Transgenic mice were treated with pIpC at 4-6 weeks of age and bone marrow was harvested 7-10 days following treatment.

Precisely paralleling observations from the retroviral HSPC transduction approach, p53 deficient bone marrow cells (homozygous deletion p53$^{\Delta 2\text{-}10}$) displayed mildly enhanced colony forming and replating capacity, while combined Kras^{G12D} and p53 deficiency induced a dramatic increase in colony formation and long-term replating capabilities (Fig 2.7 a, b). Similar to published results (Braun et al. 2004; Chan et al. 2004; Van Meter et al. 2007) and observations in the our retroviral system, we found that all Kras^{G12D} activated mice showed an expansion

of mature myeloid cells (Fig 2.7 c, top), as well as an about twofold reduction in HSC frequency that was not rescued but rather aggravated by loss of p53 (Fig 2.7 c, bottom). Although MPs frequency in $Kras^{G12D}$ was not reduced at this time point (10 days post pIpC) as in fully developed MPD (around 50 days post pIpC) (Van Meter et al. 2007), like in the retroviral system, combined $Kras^{G12D}/p53^{\Delta 2\text{-}10}$ mice showed the most dramatic expansion of MPs (Fig 2.7 c, middle) *in vivo*.

As seen in the retroviral system that combined $Kras^{G12D}$ activation and p53 loss render MPs self-renewal potential and competence to induce and transplant AML, most recipient mice of myeloid progenitors harboring combined lesions succumbed to leukemias with a median survival of 62 days (Fig 2.7 e), while all recipients of MPs harboring $Kras^{G12D}$ as a single lesion remained disease-free. Histopathology analysis showed a massive mononuclear infiltration into bone marrow, spleen and liver, indicating presence of myeloid leukemia (Fig 2.7 e and data not shown).

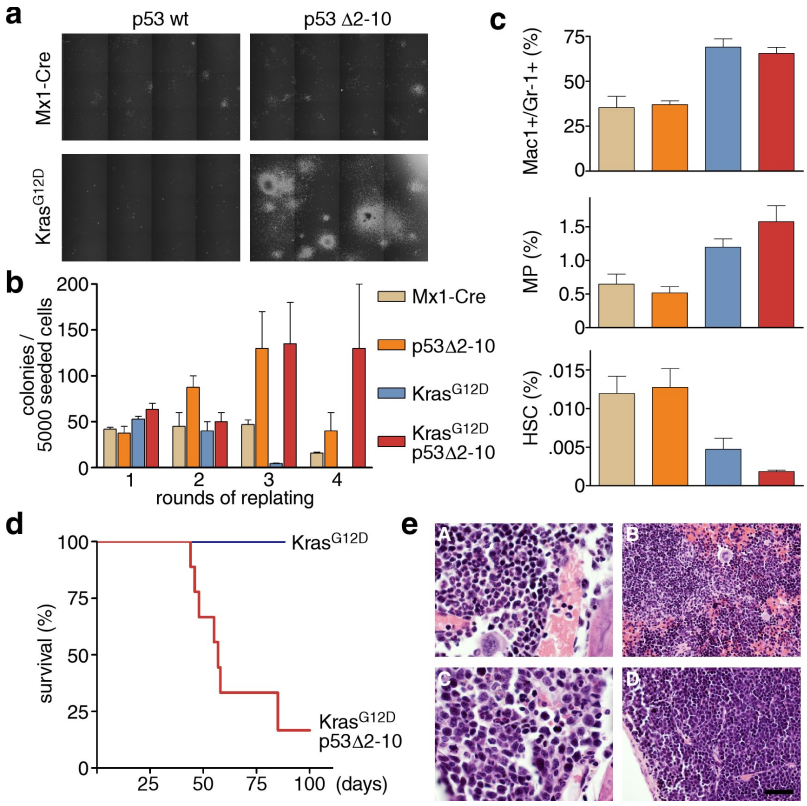

Fig. 2.7 Conditional knockout of p53 confers self-renewal ability on LSL-Kras^{G12D} myeloid progenitors *in vitro* and induce AML *in vivo*

(a) Bright field images showing colony forming ability by bone marrow cells of each genotype at the third round of replating and the robust CFU-M, CFU-G and CFU-GM like colonies formed by Kras^{G12D}-p53^{floxed} marrow.

(b) Quantification of colony replating assay.

(c) Percentages of mature myeloid cells (Mac1^{+}Gr1^{+}), MPs (Lin^{-} cKit^{+}Sca1^{-}) and HSCs (Lin^{-} cKit^{+}Sca1^{+}CD150^{+}CD48^{-}) in bone marrow of mice with indicated genotype 7-10 days after pIpC treatment.

(d) Kaplan-Meier curve of lethally irradiated recipient mice transplanted with 2,000-5,000 myeloid progenitor cells doubly sorted from primary Mx1Cre-LSL-Kras^{G12D} and Mx1Cre-LSL-Kras^{G12D}-p53^{floxed} mice.

(e) Histopathology analysis illustrating the progression from a relatively normal marrow and spleen in the donor to acute leukemia in the recipient. A: marrow section (100X) of donor shows mixed hematopoeisis with large megakaryocyte at lower middle, and mixed erythroid (dark nuclei) and myeloid (irregular nuclear contours) elements. Marrow sinusioids filled with RBCs are also seen. B: splenic section (40X) of donor shows normal lymphoid white pulp at upper 2/3's on left, and normal red pulp with mixed hematopoiesis in remainder of image. C: marrow section (100X) of recipient shows blastic cells and includes some mitotic figures and apoptotic bodies. D: splenic section (40X) of recipient shows loss of normal architecture and mixture of blasts and darker staining interspersed erythroid precursors. (Scale bar represents 20 microns for 100X images and 50 microns for 40X images.)

Our studies have demonstrated that physiological levels of oncogenic

Kras cooperated with p53 suppression to promote AML. Various *in vitro* and *in vivo* assays using both retroviral transplantation model and transgenic model indicated p53 suppression in combination with Kras endowed myeloid progenitors with self-renewal capabilities, which were responsible for the initiation, maintenance and propagation of AML phenotype. These results suggest that p53 is a negative regulator for self-renewal of HSCs and myeloid progenitors in normal condition, and serves as a barrier to prevents the MPD progressing toward AML in response to the oncogenic stress such as Kras activation.

Discussion

In this study, we combined the use of a mosaic mouse model with *in vivo* RNAi and Cre mediated recombination to examine the effects of oncogenic Kras activation and p53 deficiency in the hematopoietic system, and showed that these two lesions cooperate to induce AML in mice. Using this novel model of AML, I demonstrated that p53 deficiency and Kras activation collectively transformed myeloid progenitor cells by enabling these cells to reproduce themselves while seem to maintain their differentiation potential. This work has illustrated an improved Cre technology with a novel design of Cre retroviral vector, and has provided insights into several aspects of p53 and AML biology.

From a technical perspective, the mosaic mouse model we developed provided a rapid platform to study interactions between ras and p53 during leukemogenesis, using a novel LGmiRCreER construct with improved Cre performance. For example, concerned about genotoxicity of Cre reported *in-vitro* (Loonstra 2001) and *in-vivo* (Forni&Scuoppo, 2006), we applied self-deleting method to limit intensity and duration of Cre expression. Most existing self-deleting Cre vectors harbour a single loxP or lox511 site in the 3'LTR, which is very potent in limiting Cre toxicity but prevents continued co-expression of other transgenes (e.g. fluorescent markers, shRNA). We improved the design by: (1) expressing CreER under a weak PGK promotor instead; (2) flanking the Cre and

its promotor with two lox511 sites to achieve self-excising, and (3) using tamoxifen-inducible $CreER_{T2}$ to keep Cre activity dormant in packaging cells (Feil et al. 1997). Moreover, to avoid recombination between Cre-flanking lox sites and the ones in the target cell genome, we flanked Cre by lox511 sites, which do not recombine with commonly used loxP sites (Hoess et al. 1986). All these designs helped to improve the Cre activity while reduce its toxicity while, at the same time, enabling the analysis of only endogenous lesions on leukaemia development.

This work also has provided insights into several aspects of p53 action in AML biology. Firstly, p53 is involved in preventing leukemogenic self-renewal and its deficiency provides a route to AML leukemogenesis. Oncogenic Kras induces proliferation of hematopoietic stem and progenitor cells (Van Meter et al. 2007; Sabnis et al. 2009). Consequently, differentiation is activated as a default ground state (Braun et al. 2004; Chan et al. 2004), probably to limit the tumorigenic effects of Kras activation. We found the myeloid differentiation induction seems to be, at least at the level of myeloid progenitors, dependent on the presence of functional p53. Loss of p53 prevents the reduction in the number of myeloid progenitors caused by Ras-mediated differentiation at both early and late stage of AML development (Fig 2.5 c and data not shown), and $Kras^{G12D}$-shp53 collectively transforms myeloid progenitor cells by endowing them with *in vivo* self-renewal and leukemogenic capability.

In addition to the known proteins that have been implicated in leukemic

self-renewal including β-catenin, Hox family members, MLL fusion proteins and Bmi1, our work indicates that p53 loss provides a new route to leukemic self-renewal. It has been accepted that stem and progenitor cells have a greater opportunity to cause cancers than differentiated cells because they undergo extensive cell divisions that might cause multiple different mutations to accumulate (Puzio-Kuter and Levine 2009). Our results support the hypothesis that one of the functions of the p53 pathway is to enforce a "one-way street sign" upon development (differentiation), especially in the face of oncogene activation, and this might be another tumor suppressor function of p53 (Puzio-Kuter and Levine 2009).

Secondly, our studies have highlighted similarities between the immortalization process – which p53 has long been associated with – and the more physiological process of self-renewal. Self-renewal reflects a physiological process whereby cells can reproduce themselves without differentiation, or retain the choice to differentiate into a more specialized cell type. In leukemia, it has been established that the aberrant property of self-renewal must be acquired by more differentiated progenitors that do not normally harbor this capacity (Chao et al. 2008). Here, using well-established assays, we associate p53 with the acquired capability of myeloid progenitor cells to self-renew.

p53 loss has been associated with immortalization, which is an aberrant process in which senescence, a permanent program of cell cycle arrest, is bypassed and cells can grow indefinitely. This acquired capability may

conceptually overlap with the physiological process of self-renewal and, indeed, p53 is proposed to modulate the self-renewal capacity of some normal cell types. One potential difference, based primarily on the study of fibroblasts in culture, is that immortalization leads to the indefinite propagation of the same cell type without the retention of differentiation capabilities. However, if some immortalized cells do indeed retain the capability to differentiate, p53 mutations may be a key factor in the formation of the so-called “cancer stem cells” that, in some situations, represent a sub-population of tumor-initiating cells within cancers.

Thirdly, our results may help to explain the high mutation frequency of p53 in CML blast crisis. CML begins with an indolent chronic phase characterized by the presence of BCR-ABL but inevitably progresses to a fatal blast crisis (CML-BC). The most common genetic alterations in CML-BC involve the p53 gene, which is mutated in 25%-30% of myeloid CML-BC, or suppressed by the activation of MDM2 negative regulator in CML-BC. Given the similarity between $Kras^{G12D}$ activation and BCR-ABL in driving proliferation of HSCs and myeloid progenitors (Schemionek et al. ; Van Meter et al. 2007), the cooperating effect of p53 loss in promoting self-renewal of myeloid progenitors might explain the mechanism for p53 mutations in CML.

Finally, the ability of p53 deficiency to enable self-renewal of myeloid progenitor cells might explain the aggressive and chemo-resistant features of

these AML. p53 mutation is correlated with poor prognosis of AML patients because of resistance to chemotherapy and high relapse rate (Wattel et al. 1994). However, most p53 deficient cells still die in response to treatment with cytotoxic drugs (Schmitt et al. 2002b). Our work indicates that p53 loss contributes to the generation of leukemic stem cells. It has been suggested that the leukemic stem cells are relatively quiescent and are not targeted by cytotoxcic drugs (Guan and Hogge 2000; Jordan and Guzman 2004; Holtz et al. 2005), therefore it is conceivable that the poor prognosis of AML with deficient p53 is correlated with the presence of the LSCs that are responsible for the resistant clone and the minimum residual disease.

Another interesting observation arising from our work is that p53 deficiency has opposing effects on Kras expressing HSCs and myeloid progenitor cells: loss of p53 and Kras activation seem to have synergetic detrimental effects on the self-renewal ability of HSCs (Fig 2.5 d, 2.7 c), while enable myeloid progenitor cells to self-renewal.

The signals such as Kras activation that promote quiescent HSCs to enter cell cycle increase the exposure of these cells to early G1 phase- a sensitive period during which cell-fate decisions are made, and usually result in HSCs depletion (Orford and Scadden 2008). In wild type HSCs, p53 promotes quiescent state of HSCs through Gfi-1 and Nectin, and HSCs deficient in p53 are induced to enter cell cycle (Liu et al. 2009). Paradoxically, p53 deficient HSCs show concurrent enhanced cycling and increased self-renewal ability, probably in

the same manner as loss of $p18^{Ink4c}$ that promotes the transition from early to late G1 and increases the self-renewal of stem cells (Yuan et al. 2004; Yu et al. 2006). However, with the additional presence of $Kras^{G12D}$, HSCs are further prompted to enter cell cycle and then exposed to the prolonged activated MAPK signaling (differentiation signal), which could not be counteracted by p53 loss. Therefore, HSCs with both $Kras^{12D}$ and p53 knockdown are more prone to differentiation. Whether this hypothesis could explain further depletion of HSCs has not been examined, and warrants further investigation.

In contrast, for myeloid progenitors the default fate of cell proliferation is differentiation; p53 in these cells may contribute to self-renewal suppression (Akala 2008), but probably also be "set" to respond to abnormally increased and sustained hyperproliferative signals. Similar regulation of p16/p19 in progenitors has been observed (Williams and Sherr 2008). Kras activation provides persistent signaling for proliferation, of which the outcome is the accumulation of more mature cells. However, when p53 is further deleted, these cells obtain the self-renewal capability. The mechanisms underlying the opposing effects of p53 loss on HSCs and MPs harboring Kras could help to understand how p53 and Kras controls normal self-renewal and in combination promote leukemic self-renewal.

In conclusion, we demonstrated that RNAi mediated gene inhibition can be combined with Cre in GEMs to quickly generate mice harboring combinations of genetic lesions, which has enable us to generate a novel mouse model of AML

involving activated $Kras^{G12D}$ and p53 deficiency. Using this model, we demonstrated myeloid progenitor showed competitive advantages *in vitro* and *in vivo*, and exhibited self-renewal ability by continuously reforming colonies upon serial replating *in vitro* and inducing leukemogenesis in recipient mice. These results suggest that myeloid progenitor cells are the cellular origin of AML oncogenesis in this model and indicated for the first time that p53 loss contributes to leukemic self-renewal.

Chapter 3

p53 deficiency enhances Ras signaling and sensitivity to MEK inhibition in acute myeloid leukemia

Introduction

In human or mouse diploid fibroblasts overexpression of RAS (Hras and Kras) oncogenes induces constitutive activation of Ras signaling through several effector pathways including RAF-MEK-ERK, PI3K-AKT and RALGDS pathways (Van Aelst et al. 1994; White et al. 1995). Unlike ectopic Ras expression, however, $Kras^{G12D}$ expressed at a physiological level was associated with attenuated steady-state levels of phosphorylated Erk1/2 in MEFs (Guerra et al. 2003; Tuveson et al. 2004). Similarly, in bone marrow cells of myeloproliferative disease (MPD) induced by the same endogenous $Kras^{G12D}$ activation, increased the levels of RAS GTP but not phosphorylated Mek and Akt were observed (Braun et al. 2004). The initial hypotheses to explain these results proposed that endogenous level of Ras activation is not sufficient to activate downstream effectors, or a negative feedback regulation restricts signaling intensity (Guerra et al. 2003; Braun et al. 2004; Tuveson et al. 2004).

Later, work by Cichowski and colleagues identified a multifaceted feedback signaling network that negatively controlled the levels of Ras signaling output (Courtois-Cox et al. 2006). In this particular work, the authors found activation of Ras effector pathways were rapidly suppressed following Nf1 knockdown (another physiological level of Ras activation) or Braf activation, through engaging the expression of negative regulators of Ras pathways, including Sprouty 2/4, dual specific phosphotase (DUSP) 1/2/3/6 and several Ras GAPs (Courtois-Cox et al. 2006). Collectively, these studies suggest that

signaling cascade downstream of endogenous level of Ras signaling is under a restriction of a negative feedback control, which could be breached by overexpression of oncogenic Ras.

In Chapter 2 of this dissertation, I described that p53 deficiency and $Kras^{G12D}$ activation jointly promote self-renewal of myeloid progenitor cells, which justifies their cooperation in promoting MPD progression to AML. Consistent with results from previous studies, we found p53 deficiency alone only mildly enhanced methycellulose replating ability *in vitro,* suggesting co-occurrence of p53 loss and Kras activation is necessary for the self-renewal property and the eventual malignant transformation. In this chapter, we investigate how unregulated Ras signaling contributes to this process.

In human primitive cells like stem and progenitor cells (HSPC), high level of Ras-MAPK signaling appears to promote differentiation. MEK-MAPK pathway is not activated in self-renewing hematopoietic stem and progenitor cells in culture (Bonati et al. 2002; Ricciardi et al. 2005); high level of activation of HRAS enhances differentiation of these cells towards monocytes/macrophages lineage (Majeti et al. 2009); compared to normal HSCs, leukemic stem cells (LSC: Lin-CD34+CD38-) up-regulate MAPK signaling (Majeti et al. 2009). These studies suggested that HSPCs do not tolerate high level of Ras signaling activation unless they are transformed. As described in Chapter 2, we found leukemic cells with both $Kras^{G12D}$ and p53 knock down exhibit high-level activation of Ras

effector signaling pathways (Fig 2.3 c, d). Therefore, we hypothesized that p53 deficiency allows the elevated signaling flux of Ras, which thereby contributes to their cooperation.

Theoretically, MAPK signaling could be activated by mutations from any upstream level (receptor tyrosine kinase-RTK, RAS and BRAF). However, Rosen and colleagues found that mutant BRAF evaded the feedback inhibition on RAF-MEK that down-regulated MAPK output in cells with mutation of RAS or RTK (Solit et al. 2006; Pratilas et al. 2009). They also implicated that the level of RAS signaling out put was correlated with the sensitivity of MEK inhibition, as cells with mutations of BRAF but not RAS or RTK showed enhanced and selective sensitivity to MEK inhibition (Solit et al. 2006; Pratilas et al. 2009). Shannon and colleagues recently found that enhanced MAPK signaling plays a role in AML progression; and MEK inhibitors are ineffective in Nf1-deficient MPD but induce objective regression of many AMLs induced from MPD by retroviral insertional mutagenesis (Lauchle et al. 2009). These results indicate that the cooperating lesions emerged during the course of progression from MPD to AML render the aggressive leukemia cells to an increased dependency to activation of Ras pathways (MEK activity particularly) for proliferation and survival (Lauchle et al. 2009).

As described in chapter 2, using a sensitive flow cytometry based method, I detected constitutively elevated levels of phosphorylated Erk and S6 in

$Kras^{G12D}$-shp53 leukemic cells, which were in sharp contrast to the cells harboring only $Kras^{G12D}$. In this chapter I will describe the results in detail. Inspired by the possibility that p53 might be involved in control of Ras signaling, I took advantage of two existing AML mouse models to further investigate this role of p53 in modulating ras effector pathways and, in turn, the sensitivity of AML cells to MEK inhibition. Our results provide insights between the cooperative interactions between ras and p53 in transformation and identify factors that influence cellular sensitivity to MEK inhibition.

Results

Ras signaling output is increased in Kras-shp53 AML

Initial characterization of Ras effector signaling in Kras driven MPD was performed by Braun et al using bone marrow mononuclear cell lysates and immunoblotting (Braun et al. 2004). Kras mutant cells showed elevated levels of Ras-GFP as expected, which was surprisingly not associated with constitutively activated MEK and AKT (Braun et al. 2004). Later on applying the multi-parameter flow cytometric (FACS) analysis based intracellular phosphorylation analysis (phospho-FACS), the same group confirmed this result and established the protocol to examine the activation of Ras effector signaling with much improved sensitivity and in various populations in bone marrow (Van Meter et al. 2007).

After generating the AML model with $Kras^{G12D}$ and p53 knockdown, we employed the same method to assess the level of pErk and pS6 of leukemic cells and evaluate Ras signaling activation. For this purpose, I shortly starved leuekmic cells from bone marrow after collection to reveal the intrinsic basal pErk and pS6 levels, and then used cytokines GM-CSF and SCF to disclose the maximum levels of activation.

Consistent with published results (Braun et al. 2004; Van Meter et al. 2007), $Kras^{G12D}$ cells did not display any increase in basal or induced pErk or

pS6 level, whereas $Kras^{G12D}$-shp53 cells showed enhanced level of pErk and pS6 that could not be further enhanced by cytokine stimulation in most of the cases analyzed (Fig 3.1a and Fig 2.2). This result was nicely justified using the GFP negative population that served as internal uninfected wild type control and showed normal basal level and response to cytokines stimulation (Fig 3.1b). In addition, we further analyzed the population enriched for a stem/progenitor (Lin-Kit+) or mature myeloid cell (Mac1+) populations. As shown in Fig 3.1c, the levels of pErk and pS6 of stem/progenitor cells (core) under basal and stimulated conditions were both higher than those of the wild type control cells. Of note, Mac1+ cells that are enriched for mature myeloid cells also showed substantially enhanced pS6 and slight elevated pErk compared to wt Mac1+ cells (data not shown). These results indicate that $Kras^{G12D}$-shp53 leukemic cells have managed to break the feedback suppression on flux of Ras effector pathways leading to the their constitutive activation and saturation, and I would hypothesize that unlike normal progenitor cells, progenitors bearing deficient p53 tolerate persistent MAPK activation, which may contribute to their self-renewal capability and transformation.

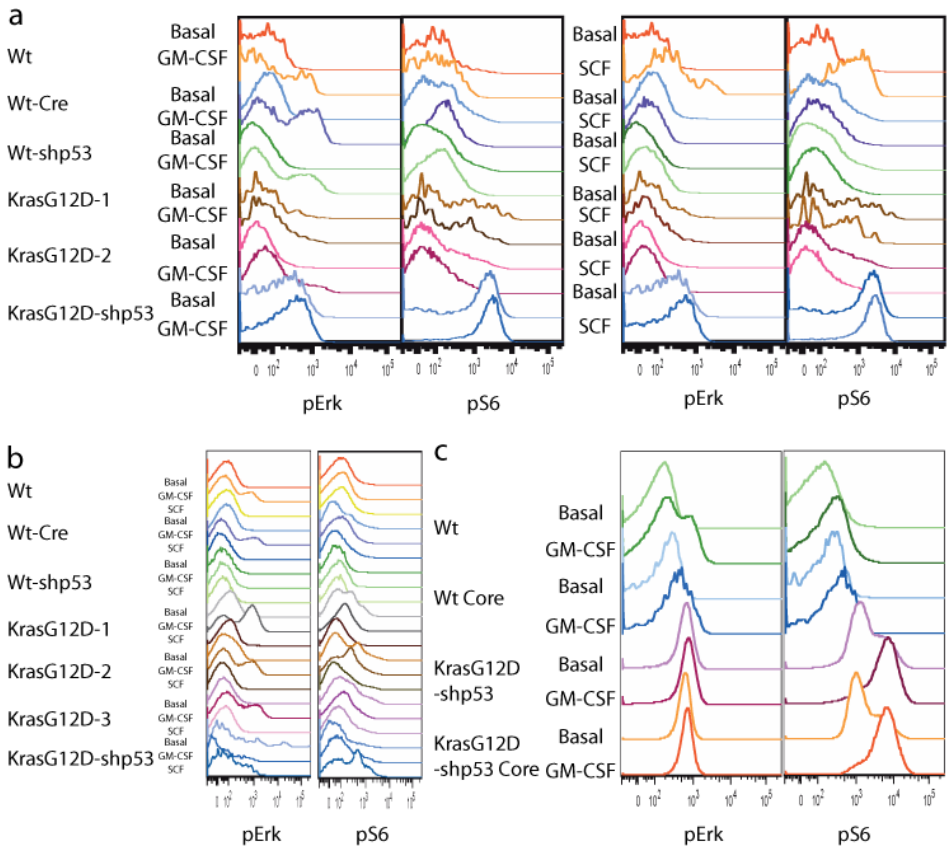

Fig 3.1 Activation of Ras effector signaling pathways in Kras^{G12D}-shp53 leukemia cells.

(a) The level of pErk and pS6 in the GFP positive population of Wt-Cre, Wt-shp53, Kras^{G12D}, and Kras^{G12D}-shp53 cells. Cells were starved briefly after collection from bone marrow and then stimulated with GM-CSF (left panel) and SCF (right panel). Only Kras^{G12D}-shp53 cells showed elevation in basal level of pErk and pS6, which could not be further induced by cytokines GM-CSF and SCF.

(b) The level of pErk and pS6 in the GFP negative population of Wt-Cre, Wt-shp53, Kras^{G12D}, and Kras^{G12D}-shp53 cells. Similarly low basal level of pErk and pS6 and the responsiveness to cytokines stilmulations were observed in all samples.

(c) Lin-Kit+ stem/progenitor cells (core) showing similar elevation as the bulk population in Kras^{G12D}-shp53 leukemic cells.

Ras signaling in reactivated shp53 model

Next, we attempted to ask whether the sustained signaling output in Kras^{G12D}-shp53 leukemias was a direct consequence of p53 deficiency or a

secondary consequence of transformation. To this end, a pilot experiment was designed to determine the requirement of p53 loss in maintaining elevated Ras signaling. I took advantage of a regulatable short hairpin targeting p53 that is under the control of the tetracycline transactivator protein tTA ('tet-off') promoter. Previous work in our lab has shown that p53 expression can be efficiently suppressed in the absence of doxycycline (Dox) and rapidly restored following Dox addition (Dickins et al. 2005). To modify our system to enable Dox control of a p53 shRNA, I replaced the short hairpin from the LGshp53CreER vector with a tTA sequence. In another vector shp53 was placed downstream of a DsRed fluorescent marker and under the control of the tet-responsive promoter TRE (Fig 3.2a).

LSL-Kras^{G12D} HSPCs co-transduced with these two vectors were transplanted into lethally irradiated recipients to induce AML development. The leukemias generated using the regulatable shp53 showed disease onset, progression and phenotype indistinguishable from the ones induced by constitutive shp53 (Fig 3.2b). Due to the presence of dsRed expression in the leukemia cells, AML mice could be imaged *in vivo* readily (data not shown). Leukemic cells were then harvested from bone marrow and cultured in the absence or presence of Dox. As shown in Fig 3.2c, untreated leukemias were positive for dsRed, indicating the presence of shp53. Following Dox addition cells lost dsRed expression, an index of turning off of the shp53 expression (Fig 3.2c). Cell cycle analysis uncovered the reduction of G2M population and increased sub-G1 population, suggesting the leukemic cells stopped proliferating

and underwent apoptosis (Fig 3.2d). A control leukemic sample induced with constitutive shp53 did not show the same cell cycle alterations, indicating these were not due to Dox treatment (data not shown). The mechanism resulting in the cell cycle changes is not clear. It has been shown that transduce of wild type p53 into BCR-ABL-positive p53-negative cells causes a decreased ability to arrest in G2 and an increase in apoptosis in response to etoposide treatment (Cummings et al. 2002). Probably Kras expressing cells are unable to undergo proper mitosis when p53 exerts G2M checkpoint and subsequently execute apoptosis.

Using western blotting analysis, I observed that the levels of pErk were decreased upon Dox treatment (p53 restoration) (Fig 3.2 e), consistent with the earlier results that p53 loss enables persist activation of MAPK in leukemias. However, the impact of p53 restoration on pAkt level was ambiguous. Although these results are supportive to the possibility that p53 acts to repress ras signaling, these experiments have two caveats. First, the level of DsRed decreased following Dox treatment, indicating the expression of shp53 was turned off as shp53 is expressed from the same transcript as DsRed. However, the level of p53 was not sufficiently restored and this may mask the real effects of p53 on signaling output. Second, because leukemic cells seem to stop/slow down proliferation (loss of G2M) upon Dox addition, down-regulation pErk might be correlated with the non-proliferating state but not the presence of p53 activity. Therefore, whether p53 directly controls the feedback suppression of Ras signaling needs further evaluation.

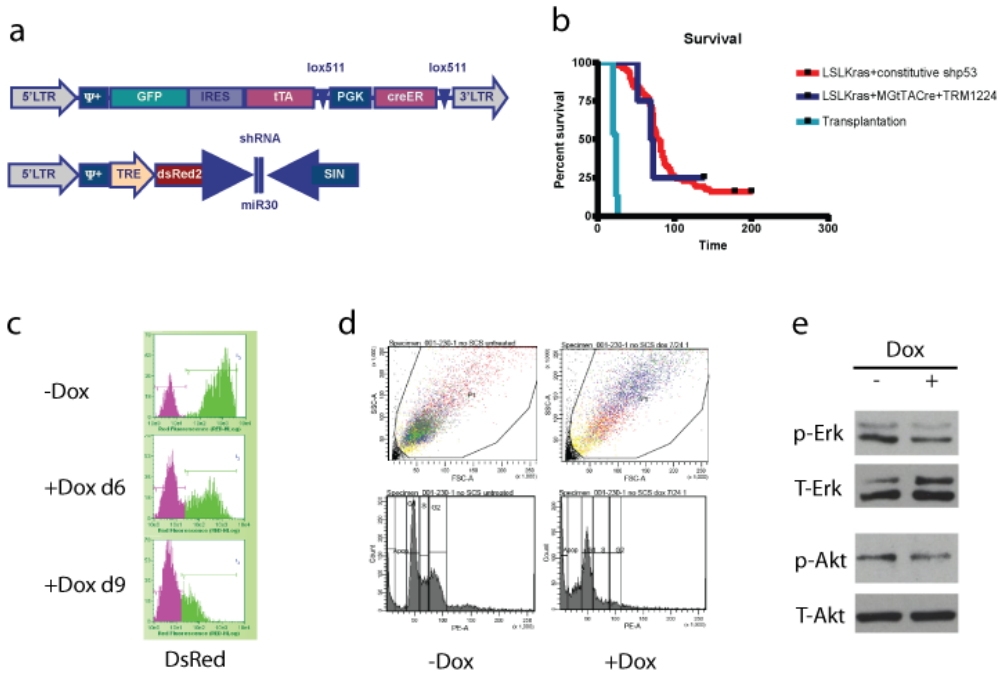

Fig 3.2 Characterization of cell proliferation and signaling output with p53-regulatable leukemias

(a) The two retroviral constructs used to introduce Tet regulatable shp53 and CreER into LSL-Kras HSPCs.

(b) Similar kinetics of disease development and transplantability of AMLs generated with constitutive and regulatable shp53.

(c) Upon Dox addition, DsRed positive leukemic cells losing the Red signal (expression of the hairpin).

(d) Dot plots showing the increased granularity (SSC) and size (FSC) of G1 population (blue in color) upon Dox treatment (top panel); cell Cycle analysis showing reduction of G2M phase and appearance of subG1 population when cells were treated with Dox (bottom panel).

(e) Western blotting analyses showing down-regulation of pErk upon Dox treatment (p53 reactivation), while the change of pAkt was minimum.

p53 loss is associated with elevated Ras signaling in response to cytokine stimulation in two other leukemia genotypes

To confirm whether p53 plays roles in restraining the Ras signaling output, I took advantage of an isogenic pair of AMLs generated with AML1-ETO9α fusion

protein and $Nras^{G12D}$ overexpression on the p53 wild type (p53+/+) or p53 deficient (p53-/-) background (Zuber et al. 2009). Of note, $Nras^{G12D}$ was co-expressed with a luciferase marker under the LTR promoter, but was translated from an internal ribosomal entry site (IRES), which reduced expression of the ectopic Nras gene to near physiological levels (Parikh et al. 2006; Zuber et al. 2009). Loss of p53 accelerated AML1/ ETO9α+ Nras-induced leukemogenesis (median survival 44 and 84 days for p53-/- and p53+/+ recipients, respectively), but did not affect the typical disease morphology and immunophenotype (Zuber et al. 2009). Unlike their p53 wild-type counterparts, p53-/- leukemias expressing AML1/ETO9α + Nras responded poorly to chemotherapy regimens (Zuber et al. 2009).

To determine whether p53 loss affected ras signaling in this genetic setting, I investigated the levels of phophorylated Erk, S6 and eIF4E in these two leukemias. Leukemia cells were deprived from the cytokines briefly by starvation and stimulated with GM-CSF to test their sensitivity to Ras activation. Under this condition, two independent AML1/ ETO9α; Nras; p53-/- primary leukemias both exhibited elevated levels of phophorylated Erk, S6 and eIF4E comparing to AML1/ ETO9α; Nras; p53+/+ (Fig 3.3). This result supports the notion that loss of p53 allows the sustained elevated Ras signaling flux.

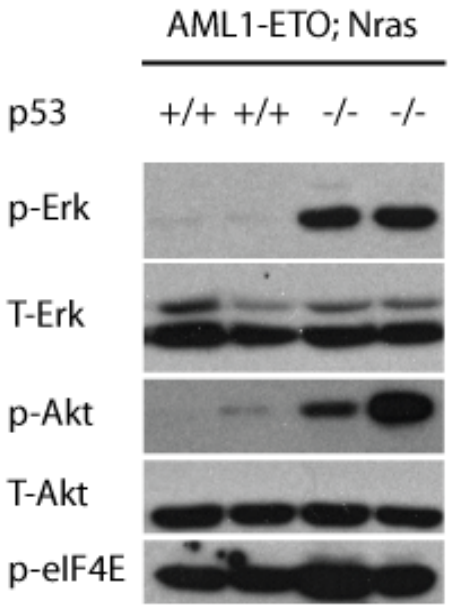

Fig 3.3 Enhanced activation of Ras effector signaling pathways in AML1/ ETO9α; Nras; p53-/- leukemias. Two independently harvested primary cells from AML1/ ETO9α; Nras; p53+/+ or AML1/ ETO9α; Nras; p53-/- leukemia were analyzed for level of phosphorylation of Erk, Akt and eIF-4E in response to GM-CSF stimulation following cytokine starvation, as well as total amount of Erk and Akt. Both lines of AML1/ ETO9α; Nras; p53-/- leukemia showing elevated level of pErk, pAkt and p-eIF4E in comparison with AML1/ ETO9α; Nras; p53+/+ leukemias, suggesting p53 loss allows enhanced Ras signaling output downstream of Nras over expression.

AML1/ ETO9α; Nras; p53-/- leukemias show sensitivity to treatment with the MEK inhibitor PD 0325901

Given that Shannon group has shown that Nf-1 deficient MPD is not sensitive to MEK inhibitor but AML derived from the Nf-1 deficient MPD initially is, and the results from my study that p53 loss enhances Ras signaling in the above isogenic pair of AMLs, We hypothesize that loss of p53 might sensitize leukemic cells to MEK inhibitor. Therefore, we obtained MEK inhibitor PD 0325901 and the dose/schedule that has been optimized in mice from our collaborators Drs. Lauchle and Shannon in UCSF (personal communication). In two independent

trials, I transplanted four independent primary leukemias (two lines of AML1/ ETO9α; Nras; p53+/+ and two lines of AML1/ ETO9α; Nras; p53-/-) into cohorts of sublethally irradiated recipient mice. Since the oncogenic $Nras^{G12D}$ was co-expressed with a luciferase marker, the mice were imaged by bioluminescence to monitor the onset of the leukemia (Zuber et al. 2009). Once the clear signal was detected (approximately 10-14 days for p53-/- AML and 3 weeks for p53+/+), we started daily administration of PD 0325901 at 12.5 mg/kg body weight or vehicle control orally for two weeks. For the chemosensitive AML1/ ETO9α; Nras; p53+/+ leukemia I also included standard chemotherapy regimen (3.3 mg/kg Doxorubicin daily for first three days and 100mg/kg Ara-C daily for 7 days) to validate the experimental procedures such as transplantation; for the chemoresistant AML1/ ETO9α; Nras; p53-/- leukemia I conducted chemo therapy followed by PD 0325901 to examine the potential synergistic effects.

As expected, AML1/ ETO9α; Nras; p53 +/+ leukemias were sensitive to chemotherapy as indicated by the reduced bioluminescent signaling (Fig 3.4a), the survival of these mice was prolonged for approximately 20 days by chemo regimen (34 days v.s. 52 days, $p<0.05$) (Fig 3.4c). When these leukemias were treated with PD 0325901, however, they did not gain any survival benefit (median survival 34 days for vehicle control and 32 days for PD 0325901).

By contrast, AML1/ ETO9α; Nras; p53-/- leukemias were progressive-mice succumbed to disease shortly with vehicle treatment (Fig 3.4d). Zuber et al showed that these leukemias responded poorly to chemotherapy and gained only a minor survival benefit for about 10 days (Zuber et al. 2009). Interestingly and

surprisingly, however, they responded to PD 0325901 by a stabled/delayed disease progression (the bioluminescent imaging signaling persisted following treatment) (Fig 3.4b), which eventually led to an approximately 20 days of overall survival benefit (median survival 26.5 days v.s. 42 days, p=0.0109) (Fig 3.4d). This result is in sharp contrast to AML1/ ETO9α; Nras; p53 +/+ leukemias that did not show response and survival advantage to PD 0325901. The combination of PD 0325901 and chemotherapy did not provide further survival benefit (Fig 3.4d), indicating no synergistic anti-leukemic effects between them in these chemo resistant leukemias. Taken together, these studies confirmed that p53 status is correlated with different levels of Ras signaling activation, which predisposes the leukemias to differential responsiveness to MEK inhibition.

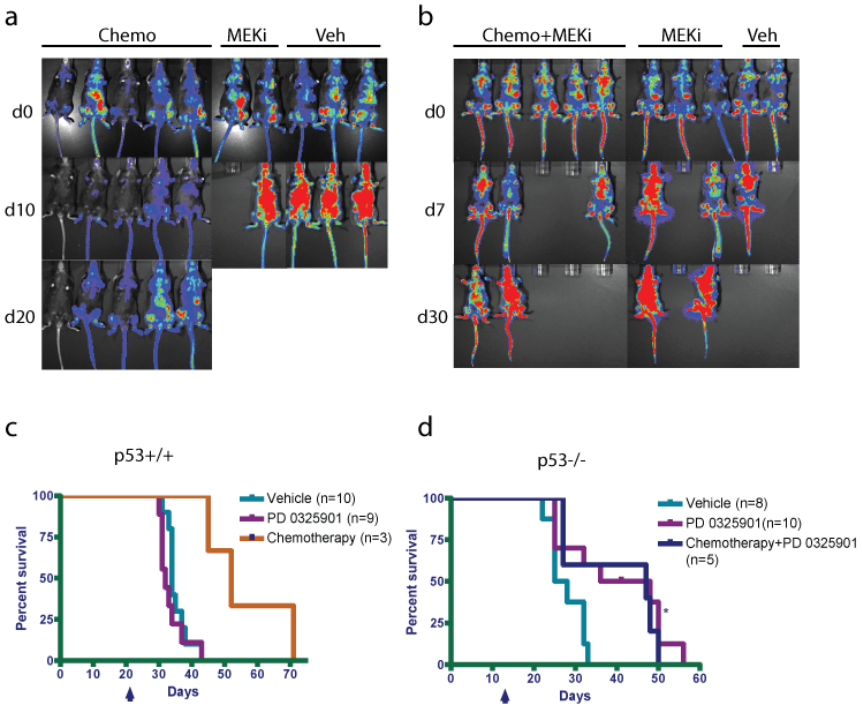

Fig 3.4 AML1/ ETO9α; Nras; p53-/- leukemias (p53-/- for short) but not AML1/ ETO9α; Nras; p53+/+ (p53+/+ for short) leukemias respond to MEK inhibitor PD0325901

(a) Representative bioluminescent imaging of AML1/ ETO9α; Nras; p53+/+ leukemias treated with chemotherapy, PD 0325901 or vehicle. These leukemias showing reduced luciferase signaling (leukemia burden) following chemotherapy but not PD 0325901 treatment, in comparison with vehicle treated controls.

(b) AML1/ ETO9α; Nras; p53-/- leukemias treated with PD 0325901 showing prolonged survival with the presence of leukemia burden.

(c) Kaplan-meier survival curve showing no survival advantage of PD 0325901 treatment for AML1/ ETO9α; Nras; p53+/+ leukemias comparing to vehicle control (median survival 32 day and 34 days respectively, p=0.2677).

(d) AML1/ ETO9α; Nras; p53-/- leukemias showing response to PD 0325901 by overall survival benefit (median survival 26.5 days v.s. 42 days for vehicle and PD 0325901 respectively, p=0.0109). No synergistic effects found when combining MEK inhibition and chemotherapy (median survival 47 days for combination, p=0.6452 comparing to PD 0325901). The survival curves were generated from two independent experiments. The arrow indicates the start of treatment.

Discussion

Endogenous $Kras^{G12D}$ activation in MEFs causes proliferation and loss of contact inhibition, but these MEFs are unable to form colonies when cultured at low density or form tumors in nude mice (Tuveson et al. 2004). Similarly, $Kras^{G12D}$ expressing bone marrow cells show enhanced proliferation but cannot transmit MPD into secondary sub-lethally irradiated recipient mice in the absence of other genetic mutations (Guerra et al. 2003; Braun et al. 2004; Tuveson et al. 2004). These studies suggest the transforming effect of endogenous $Kras^{G12D}$ is partial. This level of $Kras^{G12D}$ expression is insufficient to drive constitutive phosphorylation of effctors in both MEFs and total bone marrow (Guerra et al. 2003; Braun et al. 2004; Tuveson et al. 2004). Therefore, it is conceivable that the feedback restriction on signaling flux limits the transforming effects of Ras activation at physiological level. As discussed below, this can be the case, and has clinical implications.

Using the hematopoietic stem/progenitor cells (HSPC) gene transduction and mosaic transplantation approach, I showed that the $Kras^{G12D}$-shp53 expressing leukemic cells exhibited elevated level of Ras signaling output (pErk and pS6). The elevation seemed to be dependent on p53 deficiency, as the level of pErk and pS6 in $Kras^{G12D}$ expressing cells with functional p53 were indistinguishable from wild type controls. These observations could raise two

mutually exclusive hypotheses to explain the link between Ras signaling and cellular transformation. First, elevated signaling output is necessary and causal for the malignant transformation, and second, genetic alterations occur during the course of malignant transformation resulting in generation of and selection for cells that tolerate increased Ras signaling (or resistant to the negative feedback mechanisms that prevent tumor cells outgrowth). Whether p53 is related is an interesting question in both hypotheses. One hypothesis is that in response to aberrant Ras activation p53 is induced (by DNA replication stress or through activated Arf) (Serrano et al. 1997; Bartkova et al. 2006; Gonzalez et al. 2006), and thus directly or indirectly up-regulates the members of negative feedback network to tune down the signaling flux. Alternately, loss of p53 provides transformed cells with other growth advantages, such as loss of cell cycle checkpoint (expedited proliferation), which are associated with enhanced signaling flux.

Recently our lab established that mouse models of AML capable of recapitulating the genetic and pathological alterations of common forms of AML are useful tools to study standard induction chemotherapy and to dissect determinants for the differential therapy responsiveness (Zuber et al. 2009). We speculated these models could be powerful pre-clinical means to exploit targeted therapeutic strategies. Using the isogenic AML mouse models of AML1/ ETO9α; Nras with only difference in p53 status while showing different levels of Ras signaling activation, I performed a pilot study to test whether MEK inhibitor would have differential anti-leukemic effects in these two models. The results for these

experiments showed p53 seemed to be dispensable for the action of MEK inhibitor PD 0325901, and surprisingly, the chemotherapy-refractory AML1/ ETO9α; Nras; p53-/- leukemias but not the chemotherapy-sensitive AML1/ETO9α; Nras; p53+/+ leukemias showed responsiveness to PD 0325901. These results have important implications. 1) elevated steady state level of signaling output is positively correlated with effect of MEK inhibition- higher level of pErk correlates with better response to therapy; 2) patients resistant to chemotherapy through loss of p53 might be sensitive to targeted therapy such as MEK inhibitor, and p53 status could be a biomarker to decide should chemotherapy or targeted therapy be the primary therapy choice; 3) genetically defined mouse models are powerful tools to pinpoint biomarkers that could be used to guide therapy in patients.

One other interesting observation from this part of work is that although the overall difference was not statistically significant, when AML1/ ETO9α; Nras; p53+/+ leukemias were treated with PD 0325901 some mice showed accelerated disease progression and succumb to disease earlier than untreated control. I suspect that this adverse effect of MEK inhibition might be more obvious if mice are treated earlier. This result is interesting, in that members of our lab have recently showed that Mek1 scored as a context-dependent tumor suppressor whose knock down accelerated Myc driven lymphomagenesis (Bric et al. 2009). How Mek1 suppression accelerates tumorigenesis has not been defined yet. The results I showed here suggest that the AML1/ ETO9α; Nras; p53+/+

leukemia model could be another genetic setting to study the impact of Mek1 inhibition on tumor suppression and thus provide insight in why these leukemias are resistant to MEK inhibition. More importantly, these results collectively indicate that a thorough understanding of the biological consequence of MEK inhibition is important, as in some instance the result might be unfavorable.

Chapter 4

A Negative Regulator of Ras Signaling Sprouty 4 is a Tumor Suppressor

Introduction

The Ras effector signaling pathways integrate extracellular stimuli and respond by engaging diverse cellular functions critical for organism development and tissue homeostasis, and thereby require precise regulation to ensure appropriate biological outcomes. Chichowski and colleagues found that in response to a persistent oncogenic stimulus 14 genes among a negative feedback loop of Ras signaling, including several RasGAPs, Sproutys, Spreds (Sprouty related proteins) and DUSPs, were transcriptionally induced and correlated with the attenuation of ras signaling output (Courtois-Cox et al. 2006).

Sprouty was originally identified as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development in *Drosophila* (Hacohen et al. 1998), and later was deemed as a general inhibitor of MAPK signaling pathways involved in *Drosophila* development and organogenesis (Casci et al. 1999; Kramer et al. 1999; Reich et al. 1999). In mammals, four Sprouty orthologs (Spry 1,2,3 and 4) (Minowada et al. 1999) and three Spreds (Kato et al. 2003) have been identified to inhibit RTK-dependent MAPK signaling pathway (Gross et al., 2001; Impagnatiello et al., 2001; Lee et al., 2001; Sasaki et al., 2001, 2003; Yigzaw et al., 2001; Hanafusa et al., 2002; Yusoff et al., 2002). The interaction between Spry proteins and several components of RTK-Ras-MAPK cascade have been reported, and several mechanisms for the Sprouty-mediated inhibition have been proposed, including interfering with Grb2–Sos recruitment (Gross et al., 2001; Hanafusa et al., 2002) and inhibition of Raf

(Yusoff et al., 2002; Sasaki et al., 2003). However, the view of how Spry proteins suppress MAPK activity is not fully understood.

Mammalian Sprys and Spreds have recently been found to be tumor suppressors. Reduced expression of Sproutys was found in several cancers (Fong et al., 2006; Lo et al., 2006). Down-regulation of Spry2 in non–small cell lung cancer contributed to tumor malignancy (Berger, et.al, 2007), and in a mouse model of lung tumorigenesis mediated by $Kras^{G12D}$ Spry2 was found to function as a tumor suppressor to limit tumor number and overall tumor burden (Shaw et al. 2007).

SPRY4 is located at 5q31.3, making it a gene of great interest to us because deletion of whole chromosome 5 or the long arm of it [del (5q)] are recurring abnormalities in myeloid malignancies such as myelodysplastic syndrome (MDS) or AML (Le Beau et al. 1986; Thirman and Larson 1996). Cytogenetic and FISH analyses of 177 patients with AML/MDS or t-AML/t-MDS defined a 4-Mb CDS on 5q where SPRY4 is located (Le Beau et al. 1993). As described earlier in Chapter 3, constitutively elevated MAPK signaling was found in $Kras^{G12D}$-shp53 AMLs and may contribute to AML development. Given that SPRY4 is a negative regulator of signaling output and resides in a chromosomal location relevant to myeloid malignancies, I hypothesized that suppression of Spry4 might also promote enhanced MAPK signaling and leukemogenesis in the $Kras^{G12D}$ mouse model.

Results

Spry4 expression is induced by Kras activation

When analyzing the Ras signaling output in $Kras^{G12D}$-shp53 leukemias, I noticed that cells bearing $Kras^{G12D}$ alone did not show elevated level of pErk or pS6, consistent with the findings from Van Meter, et al that endogenous level of mutant Kras does not induce constitutive effector pathways activation (Van Meter et al. 2007). In searching for the factors that are potentially involved in the negative suppression of Ras signaling output in hematopoietic system, I infected the LSL-$Kras^{G12D}$ fetal liver cells that contain hematopoietic stem progenitor cells (HSPC) with the control shluc-CreER or the shp53-CreER, and activated $Kras^{G12D}$ by treating the cells with 4-OHT. 4 days later cells were collected to exact RNA for cDNA syntheses. Using a candidate approach and quantitative PCR (qPCR), the expression of genes that were found to be transcriptionally induced in response to oncogenic Raf activation- Spry1, 2, 4, Spred 1, 2, Rasa1, and DUSP 5, 6, 14(Courtois-Cox et al. 2006) were analyzed. Among these genes, Spry4 was found to be induced at transcription level by $Kras^{G12D}$ to approximately twofold in comparison with wt cells (Fig 4.1a). Later on, the experimental procedure was refined by sorting the GFP positive cells (the transduced cells of four genotypes) for RNA extraction and the induction of Spry4 by mutant Kras was confirmed (Fig 4.1b)

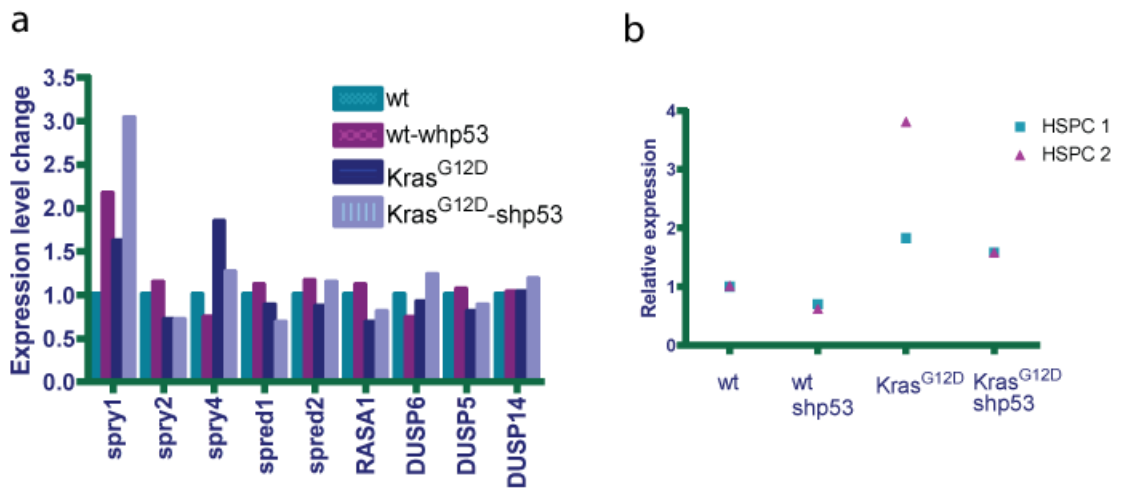

Fig 4.1 Spry4 expression is induced by Kras^{G12D} in hematopoietic cells.

(a) LSL-Kras^{G12D} fetal liver cells infected with control CreER vector or shp53-CreER were analyzed for expression of indicated negative regulators. The expression of each gene was calculated by normalizing to the level of actin using the ΔCt approach, and each sample was normalized to wt sample that was set to 1. Among the genes tested, Spry4 showing a close to twofold increase in mRNA level following Kras^{G12D} activation.

(b) Infected cells positive for GFP were sorted with FACS before RNA extraction and then the level of Spry4 was analyzed. Two independent primary lines of Kras^{G12D} cells (HSPC1 and HSPC2) were used in the same infection for duplication, and induction of Sry4 was confirmed in the both lines.

For different experimental purposes, Dr. Wen Xue and Dr. Agustin Chicas in our laboratory have performed expression microarray analyses to identify genes stimulated by overexpression of Ras. Dr. Xue transformed mouse hepatocytes with Hras and a regulatable p53 short hairpin and then restored the level of p53 by shutting off the hairpin expression, while Dr. Chicas infected human IMR90 fibroblasts with Hras to induce senescence. In order to check whether the negative regulators of Ras signaling pathways are induced by Ras, Dr. Xue and Chicas kindly re-analyzed their list of Ras-responsive genes and found the presence of several these negative regulators. Among them, Spry4 was identified in both experiments (Table 4.1). Collectively, our RNA expression

results (Table 4.1 and Fig 4.1) support that Spry4 is induced in response to oncogenic stimuli, and thereby may play roles in restricting the oncogenic potential of $Kras^{G12D}$.

Table 4.1 Summary of two expression arrays analyses and AML ROMA

Gene	Microarray analysis		ROMA analysis of AML patients
Spry1	WX (6.23)		Deletion in 2 samples
spry2		AC (7.32)	
Spry4	WX (3.27)	AC (4.10)	Deletion in 3 samples (MS023 AML-M2, MS109 t-AML, DE533 t-MDS)
Spred1	WX (2.03)	AC (1.59)	chr15:34,922,348-36,061,777 deletion in one sample upstream to this gene
Spred2	WX (2.83)	AC (3.86)	
Dusp10	WX (2.07)		
Dusp22	WX (2.14)		
DUSP1			
DUSP3		AC (2.47)	
DUSP4		AC (16.54)	
DUSP5		AC (3.38)	
DUSP6		AC (7.06)	
RASA1		AC	55 Mb deletion in KN0904
Dusp14	WX (0.23)		Focal deletion at 17q12 (chr17:31,488,966-31,492,922) in KN0904

Table 4.1 Re-analysis for known negative regulators of Ras signaling pathway with data derived from two expression microarray experiments performed by Drs. Wen Xue and Agustin Chicas (WX and AC for abbreviation). WX showing induction (green in font color) or suppression (red in font color) of indicated genes in response to p53 reactivation in Ras expressing mouse hepatocytes, and AC showing expression induction of indicated genes in response to Ras in human IMR90 fibroblasts. Number indicates the change in expression level. In WX the expression level was normalized to when p53 was suppressed by short hairpin. In AC the expression level of genes following Ras expression was normalized to that in control vector expressing cells. ROMA was performed and analyzed by Dr. Mona Spector.

Spry4 deficiency cooperates with Kras activation to cause malignant T cell lymphoma/leukemia

To test this hypothesis by asking whether Spry4 deficiency potentiates $Kras^{G12D}$ transforming ability, I performed a pilot study by infecting LSL-$Kras^{G12D}$ HSPCs with three Spry4 short hairpins as a pool and found mice developed disease reminiscent lymphoma/leukemia (Fig 5.1a). Based on these results, several more short hairpins targeting Spry4 were cloned and tested for inhibitory efficiency. To this end, we cloned a Flag-tagged Spry4 and expressed it in NIH 3T3 cells to generate a stable cell line, which was then infected with MLP retroviral vectors containing shSpry4 at low MOI or high MOI (multiplicity of infection) by controlling the infection frequency. Hairpin expressing cells were then selected and analyzed for Spry4 level using Flag antibody. At high MOI that is normally used when HSPC are transduced for mosaic transplantation, hairpin #3, #4 and #5 showed substantial knockdown (Fig 4.2). We then cloned these three hairpins into the LGmCreER construct described in Chapter 2 of this dissertation and used them to infect LSLKrs^{G12D} HSPC to characterize the cooperation between $Kras^{G12D}$ activation and Spry4 deficiency.

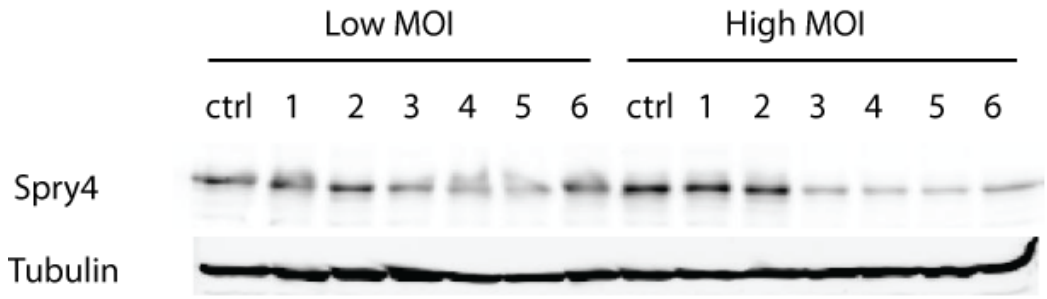

Fig 4.2. Western blotting showing hairpin 3, 4 and 5 efficiently knock down Spry4 at high MOI. Spry4 (with a N terminal Flag-tag) expressing NIH 3T3 cells were infected with MLP construct containing Spry4 short hairpin 1 through 6 for once (low MOI) or three times (high MOI). Cells were then selected with puromycine for hairpin expression and lysed for protein extraction. Spry 4 level was revealed with Flag antibody and β-tubulin was used as the loading control. Hairpin #3, #4 and #5 showing substantial knock down of Spry4. (Dr. Johannes Zuber performed the Hairpin design and cloning, and Mrs Silvia He and Dr. Mona Spector cloned Flag-Spry4)

The mice received $Kras^{G12D}$-shSpry4 cells developed and succumbed to T cell lymphoma/leukemia. The disease was manifested as massive enlargement of thymus composed of GFP+ blasts that also accumulated in liver, spleen, bone marrow and peripheral blood. Of note, all three Spry4 hairpins accelerated the disease onset comparing to the control hairpin, suggesting the cooperating outcome was not due to off-target effects. Hairpin #5, which exhibited the best Spry4 knockdown (see fig 4.2), was also the most potent at producing malignancy, as mice developed fatal disease more rapidly and at higher penetrance than mice harboring the other two spry4 shRNAs (Fig 4.3b). Histopathology and immunophenotyping analyses of the lymphoma/leukemia are still in progress. Transplantation of 1 million blasts derived from the thymus of sp-Spry4 reconstituted mice, however, could transplant a phenotypically similar disease to sublethally irradiated secondary recipient mice, indicating the

malignant feature of the disease.

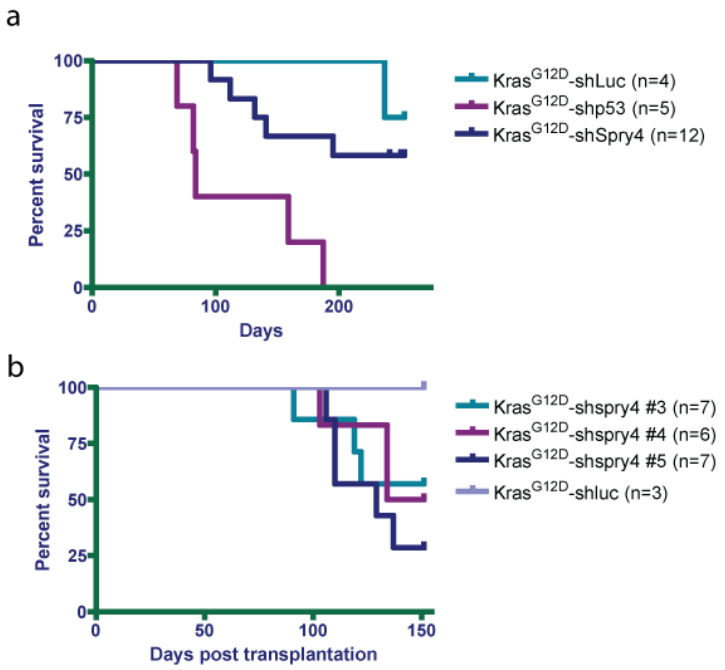

Fig 4.3 Spry4 deficiency accelerating Kras^{G12D} driven T cell lymphoma/leukemia.

(a) Kaplan-Meier survival curves generated from early experiments when LSL-Kras^{G12D} HSPCs were infected with pooled Shspry4 showing shortened survival of Kras^{G12D} -shSpry 4 mice in comparison with Kras^{G12D} -shluc, although the disease penetrance was low. Positive controls (Kras^{G12D}-shp53) and negative (Kras^{G12D}-shluc) were included in these experiments.

(b) Survival curve of mice induced with new designed individual shSpry4 #3, #4, and #5 that showed substantial knockdown by western blotting analysis, among them #5 inducing the highest penetrance in approximately 75% mice.

Enhanced signaling in Kras-shspry4 lymphoma cells

As discussed in Chapter 3, the incomplete transforming effect of endogenous Kras^{G12D} may be in part due to the feedback restriction on signaling output by the negative regulators. Therefore, I next asked whether shSpry4 accelerates the onset and potentiates the penetrance of Kras^{G12D} T cell

lymphoma/leukemia through releasing the signaling constraint. Using the phospho-FACS technology mentioned in Chapter 3, I analyzed the pErk and pS6 level of bone marrow and thymic blasts from $Kras^{G12D}$-shSpry4 lymphoma/leukemia mice, and compared them to the level of their wild type counterparts. As expected, the GFP positive Kras-^{G12D}-shSpry4 thymic blasts exhibited elevated level of pErk (Fig 4.4a) and pS6 (Fig 4.4b). pErk was mainly enhanced at basal level (following cytokine deprivation) relative to wild type thymocytes, whereas the pS6 level was elevated at both basal and steady state (untreated condition). In bone marrow, an increase in pS6 at basal level was also observed, although change in pErk was minimum. These results are still preliminary, and further experiments are needed using $Kras^{G12D}$ expressing cells instead of wild type cells as controls to confirm the impact of Spry4 deficiency on Ras signaling output. If confirmed, it will also be interesting to determine the impact of Spry4 knockdown on leukemia cells sensitivity to MEK inhibition.

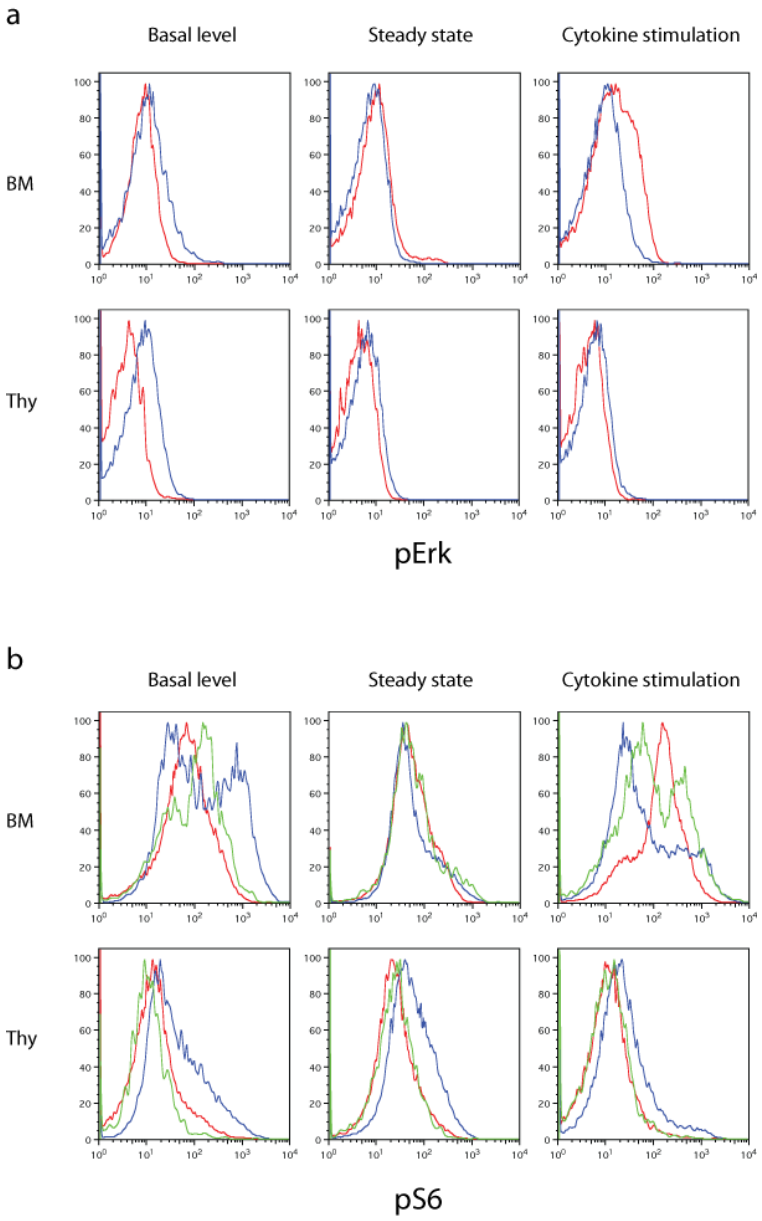

Fig 4.4 Phospho signaling analyses of bone marrow and thymic blasts from Kras^{G12D}-shSpry4 T cell lymphoma/leukemia mice

Bone marrow and thymocytes were harvested from wild type and diseased Kras^{G12D}-shSpry4 mice, and were analyzed for levels of pErk and pS6 using flow cytometry based intra cellular phosphorylation analysis (phospho-FACS, see material and method for details). Level of pErk and pS6 are showing in histogram (x-axis represents the intensity of phospho signaling and y-axis represents percentage of cell).

(a). Level of pErk in bone marrow and thymocytes at basal level, steady state and in response to cytokine stimulation. Kras^{G12D}-shSpry4 thymocytes (GFP+ blasts, blue in line color) showing enhanced basal level of pErk in comparison with wild type thymocytes.

(b). Levels of pS6 in Kras^{G12D}-shspry4 GFP positive cells (blue in line color), GFP negative cells (green in line color) and in wt cells (red in line color) under the same three conditions.

Enhanced level of pS6 in $Kras^{G12D}$-shSpry4 thymocytes (GFP+ blasts) was found in all three conditions.

Human genomic information suggests Spry4 as a tumor suppressor

Spry4 is an intriguing candidate tumor suppressor for the hematopoietic system, in that it is located in a region commonly deleted in AML and MDS patients(Le Beau et al. 1993; Thirman and Larson 1996). In order to identify potential tumor suppressor in 5q and other common deletions in AML patients, Dr. Mona Spector from our lab analyzed the genomic DNA samples derived from these patients using oncogenomic strategies. These strategies include ROMA (representational oligonucleotide microarray analysis, which detects chromosomal deletions and amplifications), and high throughput deep sequencing that uncovers the DNA nucleotide alterations. With ROMA, Dr. Spector found that three AML patients harbored the deletion of 5q region covering Spry4 (Table 4.1 and Fig 4.5a). By deep sequencing the remaining 5q arm, she found one of these three patients patient #32 contained a novel single nucleotide polymorphism (SNP), which was subsequently confirmed by capillary sequencing (Fig 4.5b). This novel SNP is a non-reference allele “A” resulting in a non-synonymous amino acid change S217T (Fig 4.5b), which is close to the Raf1 binding domain (RBD) of SPRY4. According the Polyphen (Polymorphism Phenotyping), a tool that predicts possible impacts of an amino acid substitution on the structure and function of a protein, this change was predicted to be “possibly damaging” (ranking top second among all four possibilities). With the available DNA material it could not be determined whether this SNP was disease

related or an unrelated germ line SNP, and the genome of this patient contained many other alterations. However, given that we have showed that Spry4 deficiency cooperated with oncogenic $Kras^{G12D}$ in mouse model (Fig 4.3), these results collectively provide a rationale to further validate the tumor suppressing function of Spry4 in myeloid malignancies and to investigate the functional consequence of this particular mutation of Spry4 in mouse models and myeloid cell lines to evaluate its impacts on signaling control.

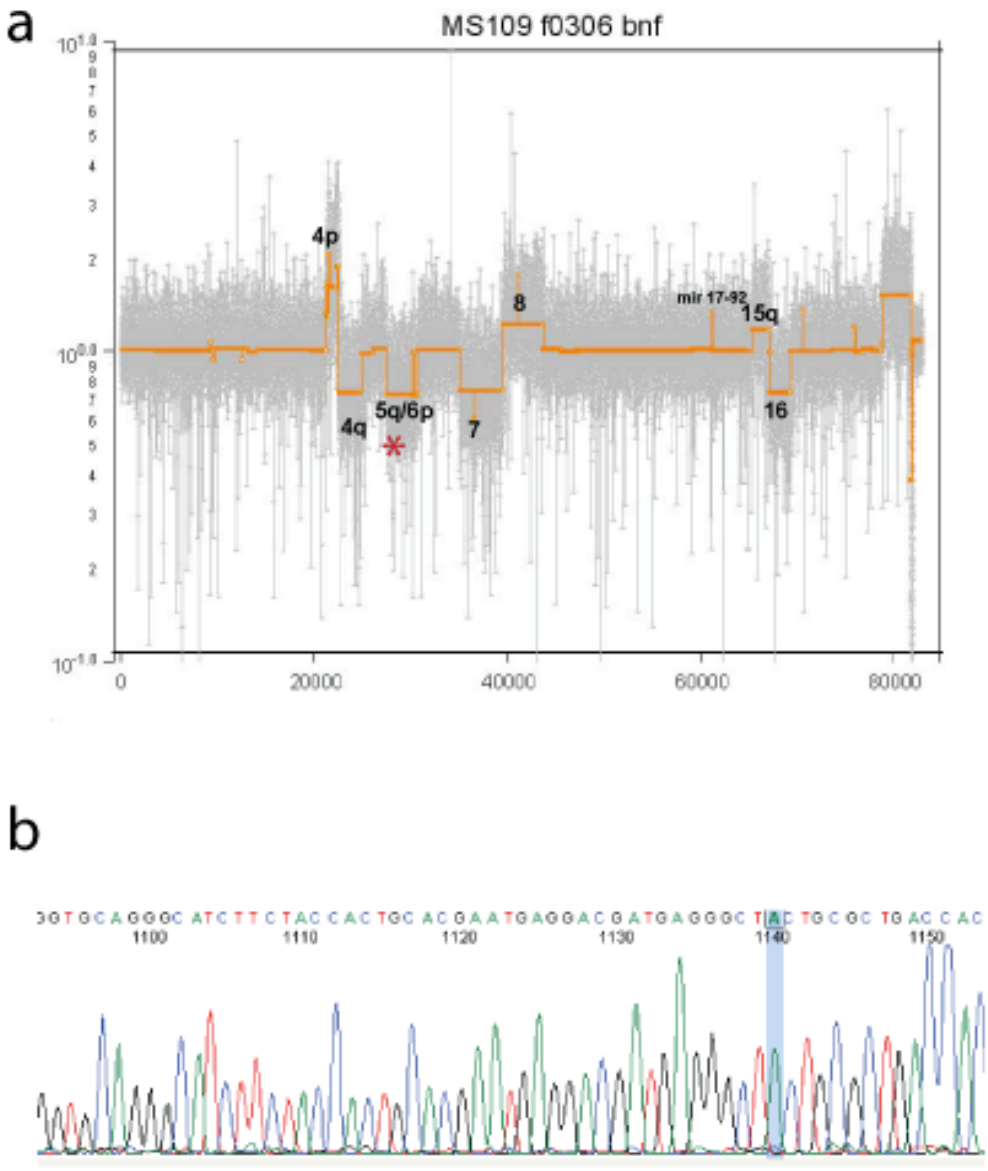

Fig 4.5 Oncogenomic analyses identifying a novel SNP of Spry4 on the remaining 5q chromosome arm of patient #32

(a) ROMA plot showing several chromosomal alterations including 5q deletion (*) of this patient using DNA probes

(b) Capillary sequencing plot confirming the novel non-reference nucleotide change ("A" highlighted with the blue bar) that was identified by deep sequencing of the remaining 5q arm of the same patient. (Both ROMA and sequencing experiments were performed by Dr. Mona Spector)

Discussion

(Sasaki et al. 2001)Receptor tyrosine kinase signaling, especially through MPAK cascade, induces the expression of Spry proteins (Ozaki et al. 2001; Sasaki et al. 2001). In cell culture, Spry1 is down regulated by FGF and PDGF, whereas Spry2 and Spry4 are up regulated by EGF, FGF and PDGF (Gross et al. 2001; Impagnatiello et al. 2001). During vertebrate development, Sprys are often expressed at sites of FGF activity associated with the mesenchymal-to-epithelial transition (Minowada, G. et al. 1999; Chambers, D. and Mason, I. 2000; Chambers, D. et al. 2000; Zhang, S. et al. 2001). Tissue-specific transcription factors also control the expression of Sprys. For example, Spry1 is induced by Wilms tumor-suppressor gene 1 (WT1) transcription factor by direct binding (Gross, I. et al. 2003), and promoters of Spry1, Spry2 and Spry4 contain GC-rich regions that include putative binding sites for numerous transcription factors (Gross, I. et al. 2003; Ding, W. et al. 2003; Ding, W. et al. 2004). Other than these, however, the factors that control the tissue- specific expression of these Spry genes have yet been defined and activation of the Spry promoters following MAPK signaling activation is not well understood.

To identify components of the negative feedback loops that control Ras signaling output in the hematopoietic cells, I took a candidate approach and found Spry4, among all other negative regulator genes analyzed, was induced by $Kras^{G12D}$ activation. This result suggested that Spry4 might be a key modulator of

ras signaling flux in hematopoietic cells, especially in response to aberrant signaling activation. A 69-bp Ras-responsive element has been mapped in Spry4 promoter (Ding, W. et al. 2004); whether this underlies the induction of Spry4 in our system is unknown. However, I found in wt-shp53 cells the level of Spry4 was slightly reduced comparing to wt cell (Fig 4.1 a and b), and in Kras-^{G12D}-shp53 cells, the Spry4 mRNA was down regulated close to the wild type. These results suggest that p53 might be involved in controlling/modifying expression of Spry4 and thus signaling output, and the effect might be more prominent and critical under the "signaling-responsive" condition. Using bioinformatics prediction, two putative p53 binding sites have been mapped to intron 1 of Spry4 by Dr. Zhenyu Xuan, therefore ChIP (chromatin immunoprecipitation) analysis could be performed to investigate whether p53 regulates Spry4 expression by direct binding, especially in response to oncogenic stimuli. While these results raise the exciting possibility that p53 might directly attenuate ras signaling by inducing spry4, patients with heterozygous deletions in spry4 often also harbor p53 mutations (Schoch et al. 2005; Haferlach et al. 2008), thus not supportive a simple linear pathway.

Recently, Bergo and colleagues found that Nf1 deficiency cooperated with Kras^{G12D} to promote a myeloid malignancy that could not be achieved by either lesion as a single mutation (Cutts et al. 2009). This result is surprising in that Nf1 deficiency is regarded equivalent to RAS mutation in activating Ras signaling pathways, and should have limited role in potentiating oncogenesis driven by

Ras mutation. The synergistic oncogenic effects between Ras activation and Nf1 deficiency suggested Nf1 might have Ras-independent function. Alternately, in a manner similar to overexpression of Ras, concurrent Nf1 deficiency and Kras activation may enable cells to more efficiently evade the negative feedback mechanisms that normally constraint Ras signaling, and eventually promote leukemia progression. The same mechanism might apply for Kras-shSpry4 cooperation as well, as loss of Spry4 disables the negative regulation on the signaling pathways allowing persistent oncogenic signal flux that facilitates tumorigenesis.

Using the same HSPC gene transduction and transplantation described in Chapter 2, I confirmed that Spry4 deficiency cooperated with $Kras^{G12D}$ to induce T cell lymphoma/leukemia. Endogenous level of $Kras^{G12D}$ expression in hematopoietic system was first found to cause MPD with complete penetrance and around 30% T cell lymphoblastic lymphomas (Braun et al. 2004; Chan et al. 2004). Later work from the Braun laboratory showed that the phenotypes in primary $Kras^{G12D}$ mice were different from those in recipients of transplanted $Kras^{G12D}$ expressing HSCs, where much higher frequency of T cell leukemia/lymphoma was observed (Zhang et al. 2008; Sabnis et al. 2009). Two possible explanations for these observation have been proposed: (1) hematopoietic microenvironment in young mice may be more permissive for MPD than the irradiated bone marrow of an adult; (2) pIpC administration in Mx1-Cre mice quickly creates a large number of $Kras^{G12D}$ myeloid progenitors that

contribute to the rapid evolution of MPD in primary mice (Sabnis et al. 2009). Indeed, $Kras^{G12D}$ induced T cell lymphoma/leukemia has been associated with secondary Notch1 mutations in thymocytes (Sabnis et al. 2009). These hypotheses and results help to explain in our mosaic transplantation model $Kras^{G12D}$ mostly induced T cell lymphoma/leukemia (Chapter 2), which was then accelerated by Spry4 loss. The relationship between Spry4 deficiency and Notch signaling and the mechanism by which they promote T cell lymphoma/leukemia are interesting questions that warrant further investigation.

The combined application of genetically defined cancer mouse models and oncogenomics represents a powerful approach for pinpointing and validating new cancer genes (Zender and Lowe 2008). In this particular part of work of my dissertation study, this approach indeed guided the process of validating Spry4 as a tumor suppressor. Initially, we noticed Spry4 was induced by $Kras^{G12D}$ (Fig 4.1), and it further drew our further attention because of its genomic location and thus the frequent deletion in AML patients (Table 4.1). Using a mouse model, Spry4 deficiency was found to potentiate the oncogenic effects of mutant Kras (Fig 4.4). Then advanced genomic sequencing revealed a potentially functional SNP S217T in AML patient with deletion of the other allele (Fig 4.5), which is worthy of functional characterization.

I plan to perform several experiments to further characterize the potential mutant spry4 found in human AML. (1) Wt and the S217T Spry4 will be evaluated

for their abilities in suppressing Ras signaling output and tumor growth in $Kras^{G12D}$-shp53 leukemic cells, as well as AML cell lines derived from patients. (2) Spry4 knock out cells (MEFs or HSPCs) will be used to compare wt and mutant Spry4 for their ability in rescuing defects of these cells if there is any, or in signaling regulation if no obvious defects would be observed. (Spry4 null mice showed reduced viability due to mandible defects (Taniguchi, K et al. 2007]). (3) Wild type and S217T Spry4 could be tested for their potential anti-proliferative ability in cells expressing other oncogenes (mutations of Kit or Braf), and in other tumor type (lung cancer, pancreatic cancer) as well. Regardless of the outcome of these effects, our work validates spry4 as a tumor suppressor in hematopoietic malignancies, raising the possibility that it acts as a one of several genes on chromosome 5 important for suppressing the development of AML.

Chapter 5

Discussion and Perspective

Before going to graduate school, I was trained to be a physician. One important reason that led me to a graduate program was that I did not feel the "power" to help the patients then, especially those young patients with cancers. In medical school, I learned that AML can be divided into 8 subtypes according to the French-American-British (FAB) classification, based on appearance of the leukemic cells under microscope. This classification was proposed in 1976 and was an important progress at that time to generate a uniform system of AML classification and nomenclature (Bennett et al. 1976). Indeed, for almost every disease taught to me (including cancer), such pathological- or pathophysiological- based classifications were applied, and many of them are still being used in clinical practice. How useful, and to what extent, these information are in determining the treatment and care of patients, especially cancer patients were uncertain to me.

In searching for a "power" to improve the treatment outcome of cancer patients by understanding cancer better, I chose to pursue cancer genetics, with the belief that most cancers are the consequences of somatic genetic disorders and that genetic studies of cancers will lead to better cancer classifications, risk analyses and therapeutic options. With this purpose in mind, I joined Dr. Scott Lowe's Laboratory for my dissertation research. After about five years of study and research, I have gained knowledge on cancer biology, grasped certain capabilities on basic research and extended my visions about cancer, especially AML. In this chapter, I would like to update my view about cancer research based

on my dissertation work.

I will discuss the following three main points, which are intimately linked to my work. (I) It is importance to study cancer in the context of tissue or organ (composed of a heterogeneous populations of cells) to gain insight into cancer biology that could not be achieved purely by tissue culture or subcutaneous “animal culture”. (II) The comparison between self-renewal and immortalization, normal self-renewal and oncogenic self-renewal, and how p53 is implicated in self-renewal; (III) Bio-markers for targeted therapy approaches are required to guide “mechanism-based-personalized-medicine”. Since all my studies were performed and substantially facilitated by genetically defined mouse models, I will begin with discussing these.

5.1 Generation of novel AML models using genetically engineered mouse models

Genetically defined mouse models of cancers have been powerful tools to dissect the *in vivo* genetic complexity of human tumors, identify cancer related genes and evaluate new compounds and combinatorial therapies pre-clinically. In Chapter 2 of this dissertation, I described a recombinant CreER construct that simultaneously activates Kras and inactivates p53 *in vivo* to generate a novel mouse model of AML. In addition to the biological insight it provided, a number of technological advances were accomplished in this model.

First this novel AML model is independent of oncogene overexpression and does not require complex genetic crosses. The LSL-$Kras^{G12D}$ mice used in this study are conditional endogenous GEMs. These mice mimic the pathophysiological and molecular features of human malignancies to the best level currently, and provide tractable experimental tools to study cancer genetics, pathogenesis and therapy in a physiological environment. To control the activation of Kras spatially and temporally, these mice are usually crossed to a strain that expresses Cre in a tissue specific manner. If additional genetic lesions are desired, very complex genetic crosses are often needed. Instead, in my study, I applied a combined retroviral Cre and RNAi mediated gene-silencing technology to simultaneously achieve endogenous Kras activation and p53 knockdown.

RNAi mediated gene-silencing increases the speed and flexibility with

which compound mouse models can be generated. In Chapter 2, I exploited shRNA-mediated knockdown of p53 to demonstrate that p53 deficiency cooperates with endogenous level of Kras activation and conditional loss of Pten to transform hematopoietic cells. The same strategy was used in Chapter 4 to confirm the cooperation of Spry4 deficiency and $Kras^{G12D}$ in inducing T cell lymphoma/leukemia. In addition, using a regulatable shRNA to control the expression of p53, effects of p53 on Ras signaling output were evaluated in Chapter 3. Importantly, RNAi mediated p53 knock down faithfully recapitulated p53 loss in cooperating with Kras activation and impact on *in vivo* behavior of hematopoietic stem and progenitor cells - highlighting the implications of RNAi mediated gene silencing in probing gene function *in vivo*.

Taken together, the strategies we used demonstrated that RNAi mediated gene inhibition can be combined with Cre in GEMs to quickly generate mice harboring various combinations of genetic lesions. In addition to their applications in studying hematopoietic malignancies, these strategies can be employed to model any other type of cancer where the mosaic transplantation method can be applied, such as breast, brain and liver cancers. When combined with cell separation technology such as flow cytometry- or magnetic beads-based cell separation, the LGmiRCreER construct can deliver gene knockdown and Cre activity to a specific cell type without requiring the tissue-specific Cre expressing strains. This is important since it has been appreciated that human cancers arise from a limited cell population and new efforts are needed to restrict the mutagenic events to fewer, but more relevant cells, as opposed to the entire

organ (Frese and Tuveson 2007). Finally, it is imaginable that these easily made endogenous GEMs will be useful for studies to: (1) identify the cellular origin of leukemic stem cells and define the cellular and molecular mechanisms that influence their generation and function; (2) assess how interactions among genes and environmental effectors contribute to cancer susceptibility, disease progression, response to interventions; (3) integrate and validate oncogenomic information such as somatic copy number alterations, genomic rearrangements and sequence mutations; and (4) identify biological markers that can distinguish among previously unrecognized patient groupings and provide better patient stratification.

5.2 Cellular origin of AML

Cancer is heterogeneous at many levels. At the clinical level, patients with the same type of cancer present with different tumor burden, histopathological type and grade, degree of invasiveness and metastasis, and show difference in responsiveness to treatment. At the genomic/genetic/epigenetic level, cancer heterogeneity is manifested as numerous alterations such as gene mutations, deletions, and amplifications in a given tumor. Heterogeneity from this perspective underlies the clinical heterogeneity to some extent. At the tissue/cellular level, cells within a tumor vary in their type, size, morphology and biology function (Campbell and Polyak 2007), and cellular heterogeneity is a common and prominent feature of most human solid tumors (Weiss 2000). In liquid cancers such as AML, based on cell surface marker expression, the apparent homogenous “blasts” can be divided into subpopulations that belong to different developmental stages and exhibit different proliferation and tumorigenic ability (Lapidot et al. 1994; Jamieson et al. 2004).

Currently, there are two models that have been proposed to explain the cellular origin of cancer and maintenance of cellular heterogeneity within a tumor: the “cancer stem cell” model (Reya et al. 2001) and the “clonal evolution model” (Campbell and Polyak 2007). Both models possess supporting evidence from human cancer and mouse models (Campbell and Polyak 2007), and which

model more accurately depicts tumor evolution and organization is still under debate (Adams et al. 2008). One idea to reconcile these two models is that they may represent different stages of tumor progression (Adams et al. 2008). At an early/pre-malignant stage of cancer development, a tumor might be driven primarily by rare cells of one (immuno)phenotype, highly possibly the stem cells in that they self-renew inherently. However, later on a mutation enhancing self-renewal occurs in a differentiated derivative and creates a dominant subclone that exhibit different (immuno)phenotype (Adams et al. 2008).

Using the $Kras^{G12D}$-shp53 AML model, I showed that $Kras^{G12D}$ and p53 deficiency cooperate to transform a subpopulation of hematopoietic cells-myeloid progenitor cells, by enabling these otherwise non-self renewing cells to do so. Ras activity is a proliferation/differentiation signal for HSCs (Dorrell et al. 2004; Shen et al. 2007). $Kras^{G12D}$ activation causes cell cycle entry and increases proliferation of HSCs, even though long-term expression is deleterious-reduction in HSCs number and function (Sabnis et al. 2009). Because HSCs are the only self-renewing population in hematopoietic system, $Kras^{G12D}$ expressing HSCs, but not downstream myeloid progenitor cells, are able to induce secondary malignancies in mice (Zhang et al. 2008; Sabnis et al. 2009), therefore when only Kras is activated, HSCs are the leukemic stem cells (LSC). However, when p53 is also knocked down, the defects on HSCs caused by $Kras^{G12D}$ are not rescued and HSCs carrying these two lesions show further exhaustion and a competitive disadvantage. In contrast, the myeloid progenitor

population with both $Kras^{G12D}$ and p53 deficiency takes charge: they show competitive advantages *in vitro* and *in vivo*, and consequently increase representation in bone marrow. They exhibit self-renewal ability by continuously reforming colonies upon serial replating, and induce *in vivo* proliferation and leukemogenesis in recipient mice. Therefore, for AML progression, myeloid progenitor cells are the LSCs. These results suggest that within a hematopoietic hierarchy, myeloid progenitors serve as the cellular origin of AML progression even though the initial target of $Kras^{G12D}$ are HSCs.

Our results support the multi-stages model of AML oncogenesis (Passegue and Weisman 2005), and seem to reconcile the "cancer stem cell model" and "clonal evolution model". At the early stage of leukemognesis, such as CML and MPD in our study, the disease is primarily driven by rare cells of one (immuno)phenotype (highly possibly the stem cells in that they self-renew inherently). The "cancer stem cell" model seems to fit in this stage of leukemogenesis. At the later stage such as CML blast crisis or AML, additional transforming events enhancing self-renewal occur in a differentiated downstream derivative and create a dominant novel clone of LSC, which exhibits a different (immuno)phenotype than the original founding clone (Passegue and Weisman 2005). This multi-stage leukemognesis model suggests the evolution of LSC during leukemia progression and reflects the "clonal evolution" model. LSC identity, therefore, seems to be largely dictated by the nature of the oncogenic events and by which of these events perturb essential processes such as self-renewal, proliferation, differentiation, and survival (Passegue and Weisman

2005).

An interesting question to follow up in our study is, even though the self-renewing myeloid progenitor cells persist in the advanced stage of leukemia, once their final transformation has been completed, are they the exclusive population to maintain AML phenotype? Alternately, can downstream mature myeloid cells obtain/inherit the self-renewal capability and propagate the leukemia? Another related issue is whether mature myeloid cells can also be transformed directly (as opposed to derived from upstream progenitors) to cause AML progression. These are questions that were not addressed in this dissertation. Indeed, these questions attempt to uncouple self-renewal ability from the cellular origin of cancer where the transformation process initiates. Clear answers to these questions will help in understanding the process of AML leukemogenesis and reveal the “cancer stem cells” required for the maintenance of an existing leukemia. Ultimately, such answers will facilitate the rational design of therapeutic strategies that eradicate all leukemic cells. Several mouse models of AML have shown that the numbers of leukemic cells needed to propagate AML in secondary mice vary based on the initiating oncogene and can be as low as about 50 cells - suggesting that downstream progenies of leukemia initiating cells might be able to self-renew and be responsible for AML maintenance/propagation (Kroon et al. 1998; Somervaille et al. 2009).

5.3 p53 and self-renewal

5.3.1 p53 loss provides a route to AML oncogenic self-renewal

Regardless of the initiating cellular origin of AML mentioned above, a unifying feature of AML is its capacity to self-renewal, an attribute of LSC (Chao et al. 2008). Enabling myeloid progenitor cells to self-renew explains the transforming ability of MLL oncogenes and MOZ-TIF2 (Cozzio et al. 2003; Huntly et al. 2004), whereas in contrast BCR-ABL and $Kras^{G12D}$ are insufficient for transforming cells that lack intrinsic self-renewal capacity (Braun et al. 2004; Chan et al. 2004; Passegue 2005). In chapter 2 of this dissertation, I demonstrated that p53 suppression increased the replating ability of hematopoietic progenitor cells derived from fetal (fetal liver) and adult tissue (bone marrow), and in combination of $Kras^{G12D}$ p53 suppression dramatically increased the proliferation (size of the colonies) and replicative potential (round of replating) of myeloid progenitor cells - features reminiscent of cells with self-renewal qualities. By sorting and transplanting myeloid progenitor cells that normally do not self-renew or repopulate recipients, I demonstrated that myeloid progenitors with $Kras^{G12D}$ activation and p53 deficiency proliferated in recipient mice and eventually induced AML development.

As discussed earlier in this chapter, whether mature myeloid cells downstream of myeloid progenitors inherit/obtain the self-renewal potential to induce AML still needs to be investigated. Nonetheless, our results confirmed

that p53 is a negative regulator of normal self-renewal of HSCs and myeloid progenitors, and demonstrated for the first time that p53 serves as a barrier to prevent AML progression in response to oncogenic stresses, such as Kras activation.

5.3.2 Immortalization and self-renewal

Recent studies have linked p53 to the process of stem cell self-renewal (TeKippe et al. 2003; Meletis et al. 2006; Medrano et al. 2007; Armesilla-Diaz et al. 2008; Liu et al. 2009). Using cells derived from p53 knockout mice, p53 loss was shown to increase the self-renewal capacity of hematopoietic stem cells and mammary stem cells in culture and in mice, and in the later instance, doing so by enhancing symmetric cell division (Rambhatla et al. 2001; Dumble et al. 2007; Cicalese et al. 2009; Liu et al. 2009). Moreover, suppression of p53 or the p53 pathway can enhance the production of induced pluripotent stem (iPS) cells from a variety of differentiated cell types (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). It remains to be determined whether these effects reflect a physiologic role for p53 in repressing reprogramming or is merely a consequence of stress inherent in the experimental design (Krizhanovsky and Lowe 2009). Nevertheless, the ability of p53 loss to "immortalize" cells in culture has long been linked to its role in tumorigenesis. Whether or not p53 action in stem cell biology is important for its tumor suppressive role has not been directly tested.

In the six hallmarks of cancer proposed by Hanahan and Weinberg

(Hanahan and Weinberg 2000), three of them - growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis- uncouple the growth of a cell from its environmental influence and theoretically enable expansive tumor growth (Hanahan and Weinberg 2000). However, without limitless replicative potential, which is absent in most types of cells, generation of the vast cell populations that constitute macroscopic tumors cannot be achieved (Hanahan and Weinberg 2000). For AML cells and iPS cells, this limitless replication potential may occur following acquisition of an aberrant self-renewal capability normally restricted to stem cells.

Literally self-renewal and immortalization both contain the concept of limitless replicative capability. These two concepts have been used in two biology research fields, stem cells research and cancer research, respectively. Recent studies on iPS cells connect the two fields by showing that the process of producing iPS cells and tumor cells are similar- both can be induced by oncogenes and accelerated by disabling the p53 network (Takahashi and Yamanaka 2006; Hanna et al. 2009; Hong et al. 2009; Krizhanovsky and Lowe 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). Therefore, whether these two concepts are essentially the same and depicting the similar biological properties in different scientific territory, or whether they reveal distinct biological process is a question of academic and biomedical importance.

Except for embryonic and some adult stem cells that self-renewal limitlessly, most types of mammalian cells possess an intrinsic, cell-autonomous program that constrains their multiplication (Hanahan and Weinberg 2000).

Senescence (a permanent state of cell cycle arrest at G0) induction is a key restriction of replication potential. Senescence was initially observed when primary cells propagate extensively *in vitro* (Hayflick, 1997), while premature type of senescence can be induced in response to high level expression of ras oncogene (Serrano et al., 1997). Occasionally cell that has acquired the ability to multiply without limitation emerges that is termed immortalization (Wright et al., 1989).

Immortalization, e.g., limitless replicative potential, is essential for the malignant growing of tumor cells, evidenced by most types of tumor cells appear to be immortalized when propagated in culture (Hayflick, 1997). This phenomenon suggests that at certain point during the course of multistep tumorigenesis, premalignant cells exhaust their ability of replication and can only complete tumorigenic transformation if they escape the replicative limitation (Hanahan and Weinberg 2000). Because immortalization was initially observed in cultured fibroblasts that circumvent replicative senescence, it has been conceptually seen as circumvent of senescence.

In addition to senescence, there are other types of postmitotic states, including various forms of terminal differentiation. Terminal differentiation is fundamental to the development of a multicellular organism as many cells commit to permanently exit the cell cycle and enter a postmitotic state early in life to perform their specialized functions (Buttitta, L., and B. Edgar. 2007). Indeed, self-renewal is the ability to replicate and maintain the developmental potential for differentiation, whereas differentiation reflects a cellular “choice” to give up the

capability to self-renewal.

Our studies highlight the similarities and difference between the immortalization process – which p53 has long been associated with – and the more physiological process of self-renewal. Immortalization can be achieved through bypassing senescence, where the differentiation state of the cell remains the same. As such, it can be viewed as a one-dimensional change. In contrast, self-renewal is a two-dimensional concept because it encompasses both limitless replication and developmental identity, and at least two kinds of cells are involved (parental and daughter cells). So immortalization describes the limitless replication of homogenous cell population in cell culture (immortalized primary cells or tumor cell lines), while self-renewal describes the maintenance of an *in vivo* heterogeneous hierarchy. Therefore, the concept of self-renewal builds in both the aspect of the immortalization phenotype and the differentiation potential. I am not certain whether the immortalized cells have been tested for their differentiation ability without induction, if they posses the differential potential, then they should be regarded as cancer stem cells and then the “so-called” immortalization is the same as self-renewal.

Based on the concepts defined above, I conclude that the ability obtained by myeloid progenitor cells from bearing Kras activation and p53 knock down is self-renewal but not simply immortalization. In this study, transplantation of Kras-shp53 HSPC induces AML with a relatively differentiated phenotype in most of

the cases we analyzed (leukemic cells express mature myeloid markers Mac1 and/or Gr1). These differentiated cells seem to be derived from their upstream ancestors as purified myeloid progenitor cells also give rise to leukemias composed of various levels mature myeloid cells. These results suggest that, rather than "blocking" myeloid progenitor cells at an undifferentiated stage, Kras activation and p53 deficiency enable myeloid progenitor cells to reproduce themselves, which is the ability normally devoid in these cells, while do not impair the mechanisms through which differentiation is achieved.

5.3.3 Normal self-renewal and leukemic self-renewal

To better understand how loss of p53 might contribute to leukemic self-renewal in AML, a precise definition of leukemic self-renewal is required. Comparing to normal self-renewal that features two important components: the limitless replicative potential and the identical developmental potential of the stem cell (Molofsky et al. 2004), the concept of leukemic self-renewal is less clearly defined and seems to have different emphasis from normal self-renewal or been "over-used" in the leukemic field. First, leukemic self-renewal has been used mainly to describe the limitless replication rather than together with developmental potential. Second, leukemic self-renewal seems to be a comprehensive "readout" of the leukemic state (enhanced proliferation, block of differentiation and resistant to apoptosis) rather than the replicative potential and differentiation potetntial mentioned above.

As leukemic stem cells are found to be more numerous and mature than

originally proposed and the AML developmental hierarchy is less clear than that of normal hematopoiesis (Kelly et al. 2007; Somervaille et al. 2009), it has been difficult to understand whether self-renewing leukemic cells give rise to normal downstream progenies. MLL-ENL transduced HSCs only yielded leukemic cells with a FcRIIhigh/CD34+ phenotype (one population of progenitors) instead of all myeloid progenitor populations; the “blasts” generated from MLL-ENL transformed myeloid progenitors (CMP, GMP) were downstream to GMP and above terminal different myeloid cells (Cozzio et al. 2003). MOZ- TIF2 conferred properties of self-renewal to myeloid progenitors (CMP, GMP) generating “blasts” expressing Mac-1 to varying degrees of Gr-1 and c-kit positivity, while the myeloid progenitor groups within this leukemia population were reduced (Huntly et al. 2004).

Two conclusions can be made from the experiments described above: 1) leukemogenic self-renewal is aberrant, and depending on the driving oncogene gives rise to progenies blocked at different stages of myeloid differentiation; 2) in these two mouse models, myeloid progenitors gained self-renewal ability but an additional transforming event seemed to be required for the accumulation of the “blasts”. Therefore, in order to understand leukemic self-renewal, especially whether self-renewing leukemic stem cells form normal or aberrant downstream progenies, clear definitions of both the leukemic stem cells and the downstream bulk “blasts” population (based on the leading oncogenic alterations) are needed.

In my thesis research, I showed that the majority of AMLs induced by

Kras-shp53 HSPC show a differentiated phenotype - leukemic cells express mature myeloid markers Mac1 and/or Gr1, and purified myeloid progenitor cells also induce leukemias composed of mainly mature myeloid cells. These results suggested that p53 loss enables Kras expressing progenitor cells to self-renew, but does not impair their differentiation capability. Therefore, it seems that, instead of “locking” the myeloid progenitor cells at a non-differentiated stage, p53 loss and Kras activation together transform the myeloid progenitor cells by enabling them to regenerate themselves while maintain their differentiation potential - namely self-renewal capability.

5.3.4 Potential mechanisms underlying self-renewal control by p53

p53 can be activated in response to DNA damage, oncogene activation or hypoxia to induce apoptosis, cell-cycle arrest, senescence, or modulation of autophagy for tumor suppressing (Yee and Vousden 2005; Riley et al. 2008; Green and Kroemer 2009). These tumor suppressor functions of p53 are context-dependent: the critical nodes of the p53 network differ and engage different tumor suppressing programs based on the tumor cell context (Zilfou 2010). However, the molecular mechanism that underlies normal self-renewal regulation by p53, whether it is another novel tumor suppressor function of p53, and whether p53 regulates oncogenic self-renewal are not well understood.

Although the mechanisms by which p53 regulates self-renewal were not systemically investigated in this dissertation research, there are a few preliminary results that may help to provide some hints into the mechanism. Firstly, p53 loss

partially blocked induction of p16 by Kras activation (data not shown). p16 induction has been a hallmark for ras-mediated senescence (Shay and Roninson 2004). As discussed in Chapter 3, senescence and terminal differentiation share similarities in that they are both post mitotic stages of cell division and senescence has been demonstrated to be a tumor suppressing mechanism (Narita and Lowe 2005). Whether senescence is induced by oncogenic Kras in the hematopoietic system has not been confirmed, but rather myeloid differentiation has been observed (Braun et al. 2004; Chan et al. 2004). Myeloid differentiation of human CD34+ hematopoietic stem and progenitor cells caused by oncogenic NRAS has been associated with up-regulation of p16 and $p21^{WAF1/CIP1}$ (Shen et al. 2007). Bmi-1 mediated suppression of INK4-ARF locus is implicated in the self-renewal ability of HSCs (Park et al. 2003) and in progenitor and their progenies, the INK4-ARF locus seems to become poised to respond to various forms of stress, including sustained oncogenic signaling (Williams and Sherr 2008). These studies collectively suggest that p16 might be involved in myeloid differentiation induced by Kras in our system and the presence of p53 seems to be required for induction of p16 and Kras induced myeloid differentiation.

Secondly, in a pilot study aimed to understand the relationship between p53 loss and activation of Wnt pathway, I found levels of axin2, a negative regulator of Wnt signaling, were down-regulated in Kras-shp53 cells (data not shown) - potentially leading to enhanced Wnt signaling activity. Given the importance of Wnt signaling in normal and malignant self-renewal (Reya et al.

2003; Willert et al. 2003; Molofsky et al. 2004), whether self-renewal of $Kras^{G12D}$-shp53 myeloid progenitor cells is dependent on Wnt signaling is worthy of further studying.

Recently, actions of p53 in the regulation of malignant stem/progenitor cell differentiation, self-renewal and tumorigenic potential have been investigated in a few mouse models of solid tumors including glioblastoma (GBM-a highly lethal brain tumor) (Zheng et al. 2008) and breast tumor (Cicalese et al. 2009). Concomitant central nervous system (CNS)-specific deletion of p53 and Pten in the mouse generated a penetrant GBM by blocking the neural stem/progenitor cells at an undifferentiated state, and this effect was dependent on the induction of Myc (Zheng et al. 2008). This study, however, did not show direct evidence of self-renewal of the neural stem/progenitor cells. In an ErbB2 transgenic model of breast cancer, p53 loss increased the self-renewal properties of mammary stem cells, which is similar to its effect on HSCs and myeloid progenitor cells in our system (Cicalese et al. 2009). This study also found p53 regulated polarity of cell division in mammary stem cells and loss of p53 favored symmetric divisions of cancer stem cells that contributed to tumor growth (Cicalese et al. 2009), which seems to be one potential mechanism how p53 control self-renewal.

Other mechanisms underlying how p53 loss promotes self-renewal emerged recently from studies on induced pluripotent stem (iPS) cells. iPS cells harbor the same pluripotent potential as embryonic stem cells, and can be generated from MEFs by the enforced expression of genes encoding four

transcription factors: c-Myc, Klf4, Sox2 and Oct4, although the efficiency of the process is very low (Takahashi and Yamanaka 2006). Inactivation of p53 or p16/p19 remarkably increases the efficiency and kinetics of iPS-cell production (Takahashi and Yamanaka 2006; Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). These studies did not provide consistent mechanisms by which p53 blocks reprogramming (p53 loss promotes self-renewal), but suggested stress response inducing p53-mediated apoptosis and senescence following DNA damage and oncogene activation could be responsible for the low efficiency of iPS production (or for preventing self-renewal) (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). These studies also raised the possibility that p53 might communicate with epigenetic regulators in a cell and promote differentiation, therefore loss of p53 would tilt the epigenetic states towards self-renewal (Puzio-Kuter and Levine 2009). This hypothesis might help to understand how Kras driven differentiation is dependent on p53 in our study.

In contrast, another independent study used quantitative analyses to distinguish cell-division-rate-dependent and -independent modes of iPS cell reprogramming (Hanna et al. 2009). The authors found that the number of cell divisions was a key parameter driving iPS cells generation. Unlike Nanog overexpression that accelerated reprogramming in a predominantly cell-division-rate-independent manner, additional inhibition of the p53/$p21^{WAF1/CIP1}$ pathway increased the cell division rate and resulted in an accelerated kinetics of iPS cell formation that was directly proportional to the increase in cell proliferation (Hanna

et al. 2009). These results suggest that p53 loss might be irrelevant to direct reprogramming and self-renewal control but rather to shorten the cell cycle and bring cells to the required number of cell divisions faster. However, as introduced in Chapter 1 of this dissertation, cell cycle regulation might control self-renewal by balancing the cell fate decision (self-renewal v.s. differentiation). Indeed, I found HSC and myeloid progenitor cells exhibit distinct responses to p53 knockdown and Kras activation, suggesting the role of cell cycle regulation in the process of self-renewal (see Chapter 2).

5.4 Ras signaling and AML

5.4.1 Ras signaling output and Spry4

Feedback loops play important roles in controlling the duration and intensity of signaling output. Positive-feedback loops can induce switch-like or bistable behavior (Ferrell, 2002), whereas negative-feedback loops maintain the signaling stability (Kholodenko, 2000). Disruption of signaling regulation could result in developmental defects and cancer. As an example of the latter case, Ras pathways are often activated to cause abnormal proliferation and survival, therefore the negative feedback should play important roles in limiting oncogenic effects of constitutively activated Ras. Recent recognition of inhibitory feedback loops through phosphatases (DUSPs) and binding proteins with no obvious catalytic activity such as the Sprouty proteins have added a new dimension to our understanding of the MAPK pathway (Tuveson 2009), and have suggested an overlooked tumor-suppressing avenue.

In Chapter 3 of this dissertation, I illustrated that, consistent with several published studies, bone marrow cells expressing $Kras^{G12D}$ from its endogenous locus did not show elevated activity of effector pathways, as measured by the levels of phosphorylated Erk and S6. In contrast, in leukemic cells induced by $Kras^{G12D}$ and p53 deficiency, pErk and pS6 levels are increased and oftentimes saturated. These results are consistent with the hypothesis that Ras signaling

(especially the MAPK pathway) is subject to a negative feedback control. More importantly these results suggested that, in addition to its role in limiting self-renewal, p53 might act in parallel to promote a negative feedback loop in response to oncogene Kras activation; hence, p53 loss dampens the induction of the negative feedback loop and thus enhanced signal flux is allowed. Since several negative regulators (Sprys, Dusps, and RasGaps) have been shown to be induced transcriptionally following oncogene stimulation (Courtois-Cox et al. 2006), it is conceivable that p53 is the transcription (co)activator of these genes and induces their expression when p53 is stabilized upon oncogenic activation. Thus, the consequences of p53 loss during leukemogenesis might be to promote self-renewal and enable the higher flux of ras signaling.

If the failure of feedback signaling induced (directly or indirectly) by p53 loss is important for leukemogeneis, one predicts that disruption of specific feedback components will contribute to disease. Indeed, in Chapter 4 of this dissertation, I identified Spry4 as a candidate negative regulator in suppressing signaling intensity in hematopoietic cells in response to Kras activation. Expression of Spry4 (but not the other Sprouty genes or DUSPs) was induced by $Kras^{G12D}$ and reverted to near wild-type levels when p53 is knocked down. Deficiency of Spry4 was then shown to accelerate the $Kras^{G12D}$ driving T cell lymphoma/leukemia, probably through elevated Ras signaling output. These results demonstrated for the first time in a mouse model that Spry4 could be a tumor suppressor, and that its tumor suppressing function could be to inhibit Ras

signaling output.

Spry1 and Spry2 are found down regulated in breast cancer and prostate cancer samples (Lo et al. 2004; McKie et al. 2005; Lo et al. 2006). As well, Spry2 down-regulation has also been reported in liver cancers (Fong et al. 2006). Surprisingly, a fraction of prostate cancer patients showed a higher expression of Spry1 in cancer tissues compared to the corresponding normal epithelium (Lo et al. 2004). Similarly, in a mouse model of $Kras^{G12D}$-driven lung cancer, Spry2 levels were found to be elevated (Shaw et al. 2007). These results suggest that Spry might be induced at an early stage during tumor evolution but down regulated as a cause or consequence of tumor progression. Consistent with this hypothesis, our results showed that Spry4 is induced in response to Kras activation, probably as the negative feed back mechanism to suppress aberrant Ras signaling flux. Spry4 deficiency strongly potentiates the oncogenic capability of $Kras^{G12D}$ and leukemic cells exhibits elevated Ras signaling output. These results suggest the tumor suppressing function for Spry 4.

5.4.2 p53, Ras signaling output, and personalized medicine

As described in Chapter 3, I found that loss of p53 correlated with increased Ras signaling output. Activated oncogenic Ras induces p53 through activating Arf (Dimri et al. 2000) or DNA damaging response (Ries et al. 2000; Lin and Lowe 2001) and induce senescence (Serrano et al. 1997). However, whether p53 reversely restricts Ras signaling flux is unknown. One connection between Ras signaling and p53 was suggested in oncogene- induced

senescence (OIS): attenuation of Ras signaling output achieved by negative feedback loop contributes to the establishment of senescence, for which p53 activity is also required (Courtois-Cox et al. 2006). Therefore, it is very interesting to understand whether p53 cross talks to the mechanisms by which the negative feedback is exerted.

One hypothesis is, as mentioned earlier, that under stress of aberrant signal activation, p53 transcriptionally activates negative regulators in a direct manner (direct binding) or indirectly (by promoting the activity of transcriptional factor/coactivator of the negative regulator). One candidate to test this hypothesis is Spry4, which is induced by $Kras^{G12D}$ in hematopoietic cells. In scanning for transcription binding sites, we found two putative p53 binding sites in intron 1 of Spry4. Whether p53 binds to these sites and transactivates Spry4 following Ras activation needs to be experimentally examined, and may provide insight into how p53 regulates Ras signaling throughput.

Despite the need to understand the mechanisms underlying how p53 controls Ras signaling output, the correlation between p53 deficiency and enhanced Ras signaling appears a good starting point to evaluate whether p53 status could be employed as a biomarker for activation of Ras effector pathways in AML and thus the sensitivity to MEK inhibition. Indeed, using two isogenic AML mouse models harboring activated Ras and that only differ in p53 status (p53 wt v.s. p53-/-), I found that loss of p53 positively correlated with enhanced steady state/cytokine response signaling of Ras effectors. In agreement with the

above hypothesis, p53-/- AMLs showing higher levels of Ras signaling responded to MEK inhibition, in sharp contrast to their lack of response toward cytarabine (Ara-C) and doxorubicin, whereas AMLs harboring wt p53 exhibiting lower Ras signaling showed no, if not worse response to MEK inhibitors, despite the fact that they were chemosensitive.

These results have two profound implications. First, they support the "oncogene addition" hypothesis (Weinstein 2000, 2002; Weinstein and Joe 2006) and suggest that targeting molecules upon which AML are dependent on (MEK in this study) can have devastating effects on cancer cells while sparing normal cells. Indeed, they suggest that one basis for such addiction is the breakdown of normal feedback signaling that may attenuate signaling imbalances in normal cells.

The second implication is that they indicate the potential of "mechanism-based-personalized" cancer care. Most conventional cancer treatment applies standard therapy protocols (chemotherapy induction and consolidation in AML), and it is well recognized in oncology practice that patients respond differently to anticancer therapy, with only a subset of patients benefit. The ability to understand the variations in responses and to identify the subpopulation of patients who are most likely to respond in advance is critical. One principle underlying variations of therapy response is the heterogeneity of cancer. Theoretically, with advances in large-scale and high-throughput oncogenomic technologies such as ROMA and deep sequencing, individual genomes could be translated into "maps" that indicate integrated changes in critical pathways and

used to match patient tumors to corresponding targeting drugs or drug combinations.

A substantial effort is now underway to identify additional drugable kinases, and to develop and test inhibitors of these (McDermott and Settleman 2009). It has also been recognized that even for targeted therapy, the clinical benefit is typically limited to a subset of patients (McDermott and Settleman 2009). Therefore, the genomic or biochemical biomarkers that could stratify patients before applying specific target inhibitors will not only maximize the chance for success during clinical trial development, but also provide more effective personalized targeting strategies.

In this dissertation, I found that p53-/- AMLs with high levels of Ras signaling responded to MEK inhibition even though they are resistant to conventional chemotherapy, whereas p53 wt AMLs that were chemosensitive and exhibited low Ras signaling, showed no, if not worse response to MEK inhibition. These results may suggest that: (1) p53 deficiency is a “yellow or red” light for chemotherapy, but may become “green” when confronted with a MEK inhibitor, as it allows enhanced signaling output resulting in addition of the cells to MAPK signaling; (2) not all patients, even though those sensitive to chemotherapy, will benefit from MEK inhibition, and levels of pERK can be used as a bio-marker for screening patients who might benefit; (3) in preclinical trials, genetically defined mouse models, but not the normally used xenograft models harboring by cell lines without a clear genetic background, might better tools to

identify and refine bio-markers for a targeting regimen.

The value of pErk as a positive bio-marker for MEK inhibitor needs to be confirmed. Mouse models with other tumor types (e.g.- lung cancer and pancreatic cancers) where Ras mutations are common should be employed to validate whether p53 loss facilitates higher Ras signaling output. Activation of the Ras signaling cascade using mutations involving “upstream” RTK and Nf1, or “downstream” Raf and Mek players could be used to exam whether pErk is a universal bio-marker for MEK inhibition. Nonetheless, our work has suggested the potential “mechanism-based-and-biomarker-guided” personalized cancer medicine in future oncology practice, where the “right” bio-marker could guide more efficient pre-clinical and clinical trials and eventually enable individual therapy design.

In conclusion, throughout my dissertation research, I: (1) established a novel mouse model of AML by cooperation of Kras oncogene and p53 deficiency; (2) used this model to demonstrate that myeloid progenitor cells that acquire aberrant self-renewal capability could serve as the leukemic initiating population; (3) showed that p53 deficiency is correlated with enhanced levels of Ras signaling cascade and responsiveness to MEK inhibition; and (4) identified a negative regulator of Ras signaling, Spry4, as a tumor suppressor in the hematopoietic system. These work provides novel insights into how p53 acts to suppress AML oncogenesis, and suggests therapeutic strategies for aggressive

p53 deficient AMLs.

Chapter 6

Material and methods

Retroviral constructs

The self-deleting Cre vectors were MSCV-based constructs containing either the empty mir30 cassette, amir30 based short hairpin RNA targeting luciferase or Trp53 gene (Dickins et al. 2005). These vectors were applied in generating leukemia models. The non self-deleting Cre vectors were generated by removing the second Lox511 site, and used in experiments where GFP positive cells were traced for *in vitro* proliferation, colony forming, stem/progenitor cells analysis and sorting. LMP vectors were from previous work (Hemann et al. 2003).

Mouse strains

Lox-stop-lox (LSL) Kras ^{G12D} and Mx1Cre mice (kind gifts from Dr. Shannon in UCLA) were backcrossed onto C57BL/6 background for more than six generations. Trp53$^{F2\text{-}10}$ mice (Jonkers et al. 2001) were backcrossed onto C57BL/6 for 4 generations before generating quadruple Mx1CreLSL-Kras^{G12D}p53$^{F2\text{-}10/F2\text{-}10}$ (CPK)mice. All mouse related experiments were performed under the approval of the Cold Spring Harbor Laboratory Animal Care and Use Committee.

HSPC isolation, retroviral transduction, mosaic transplantation and leukemia transplantation

Fetal liver cells (HSPCs) of E13.5-15.5 were harvested and genotyped. HSPCs infection procedures were as described (Schmitt et al. 2002a). Of note, phoenix cells were plated and transfected with retroviral plasmids for 24 hours. Afterwards virus-containing supernatants were collected every 6 hours for three times and added to HSPCs for infection. To induce Cre activity *in vitro*, infected HSPCs were treated with 4-hydroxytamoxifen (4-OHT) (SigmaH6278, dissolved in 95% cold Ethanol) at the concentration of 0.2-0.5μM for 36-48 hrs. Approximately 2-3×10^6 cells were then injected by tail vain to 8- to 10-wk-old lethally irradiated syngeneic recipient mice (split doses of total 9.0 Gy administrated with Gammacell 40 Exactor, MDS Nordion). For *in vivo* Cre activity induction, infected HSPCs were transplanted within 24 hrs after last infection. 3-4 mg 4-OHT (distributed at 40mg/ml in sunflower seeds oil [SigmaS5007] by sonication) were administrated i. p. for 4 serial days after recipient mice had been reconstituted. For secondary leukemia induction, recipients were irradiated with 4.5 Gy and 2 million freshly harvested bone marrow leukemic cells were transplanted.

Cytological and molecular characterization of leukemia

Peripheral blood smears (Wright's Giemsa stained) of recipient mice were monitored starting from 4 weeks after transplantation. Ultrasonic imaging

screening was performed on Vevo 770 Imaging System (Visualsonics) imager 7 weeks after transplantation. Fluorescence-based whole-body GFP imaging (Illumatool Bright Light System LT-9900, Encinitaas, CA) was conducted to illustrate *in vivo* leukemia distribution as described (Schmitt et al. 2002b). Animals were sacrificed by CO_2 euthanasia upon clear leukocytosis and blood blast appearance as well as disheveled appearance, hunching, abnormal gait, et.c. Statistical evaluation of overall survival was based on the log-rank (Mantel-Cox) test for comparison of the Kaplan-Meier event-time format. Organs were fixed in 10% neutral buffered formalin and processed to obtain paraffin sections for histological staining by the Mouse Pathology Shared Resource at CSHL and the University of California, San Francisco Comprehensive Cancer Center. Photographs were taken on a Nikon Eclipse 80i microscope with a Nikon Digital Sight camera using NIS-Elements F2.30 software at a resolution of 2560 ◊ 1920. Using Adobe Photoshop CS2, autocontrast was applied, unsharp mask was used to improve image clarity, and images were re-sized and set at a resolution of 300 pixels/inch. Bone marrow cells were flushed from tibias and femurs with PBS with 2% heat-inactivated bovine serum. Erythrocytes were lysed and nucleated cells were filtered through nylon screen (100μm) to obtain a single-cell suspension. Whole nucleated bone marrow cells were stained with PE conjugated antibodies to Sca-1, Ckit, CD34, Mac1, Gr1, B220, Thy1 and Ter119 (BD Bioscience) for single staining. Data was collected on LSRII flow cytometer (BD Bioscience) and analyzed with Flowjo software (TreeStar, Ashland, OR).

Immunoblotting analysis

Wild-type MEFs were infected with control plasmids MLP, MLPshp53, LGmCreER and LGmCreER-shp53. All infection efficiencies were above 95% indicated by GFP positive percentage analyzed 48 hours post infection. Cells were then incubated with presence or absence of 0.5uM 4-OHT for another 48 hours. Bone marrow cells were harvested as above from wild-type mice, Kras-shp53 leukemias and control leukemias mice (MLL/ENL fusion protein overxpression driven leukemias on background of Arf deficiency and p53 deficiency). To induce functional level of p53, all samples were treated with 1 μg/ml adrymycin for 5 hrs and then harvested for whole cell lysates. p53 immunoblotting analysis was then performed as described (Hemann et al. 2003). For pErk and pS6 analyses, the AML1-ETO;$Nras^{G12D}$;p53+/+ or AML1-ETO;$Nras^{G12D}$;p53-/- leukemias were thawed from cryopreserved samples, washed with PBS, and then starved with IMDM containing 1% bovine serum albumin (BSA; Sigma Aldrich USA) at 37°C for 45mins. The cells were stimulated with murine GM-CSF 10mg/ml for 15 minutes before lysis.

For Spry4 knock down analysis, NIH 3T3 cells were infected with MSCV-5xFlag-$Spry4^{Ser}$-hygro construct and selected with hygromycin to generated a stable line expressing Flag-Spry4. Then the cells were infected with MLP based shSpry4 once for low MOI and three times for high MOI. After selection with puromycin, cells were lysed for protein extraction. F1804 monoclonal anti-flag M2 antibody (Sigma) was used for detecting expression of Spry4.

Southern blotting analysis

Wild-type MEFs were infected with LGmCreER and incubated with absence or presence of 0.5uM 4-OHT for 4 days. Genomice DNAs were harvested from these cells as well as packaging phoenix cells at indicated time points after transfection, and Southern blotting analysis was performed with a probe that recognizes GFP.

PCR analysis

To assess the Cre-mediated recombination of LoxP sites in LSL-Kras^{G12D} cells, 2 steps allelic specific PCR were designed. For PCR I, primers were KLSLfw3 (AAGCAAGGCAGAAGTCACAGAGG) and LoxedRasRv (TCCGAATTCAGTGACTACAGATGTACAGAG). Amplification conditions was 95°C 10min followed by 34 cycles of 95°C 30sec, 64°C 45sec, 72°C 1min, and end up with 72°C 7min. PCRI yielded products of 709 and 749 bps for wild-type and recombined alleles respectively. If the recombined allele couldn't be identify clearly, 1 to 100 dilution of PCR I products were used for PCRII. Primers for PCRII were KLSLfw4 (GTGCAGTTTTGACACCAGCTTCG) and KLSLrev4 (CGCATGAGCTTGTCGACATAACTTCG). Amplification conditions was 95°C 10min followed by 28 cycles of 95°C 30sec, 65°C 30sec, 72°C 1min, and end up with 72°C 7min. A 249 bps PCR product from PCRII indicates recombined allele. To track excision of LSL cassette in one mouse reconstituted with Kras-shp53 HSPCs, genomic DNAs were extracted from its peripheral WBCs prior to and

after *in vivo* 4-OHT treatment, and at sacrifice for PCR analysis. Multiple Kras -shp53leukemic mice were harvested for bone marrow and spleen DNAs (with more than 80% of GFP positive leukemic cells on average) to analyze recombination event.

RT-QPCR analysis

Fetal liver cells were infected and $Kras^{G12D}$ was activated for 48 hours and then kept in culture for 6 days. In the scanning experiment where multiple genes were tested, the whole infected population was collected for RNA extraction; in the validation of Spry4 experiment, GFP positive transduced cells were sorted before RNA extraction. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Gene-specific primer sets were designed using Primer Express 1.5 (sequences are available from the authors uponrequest). Real-time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on the Roche's IQ5 ICycler. GAPDH or β-actin serves as an endogenous normalization control. The quantification was done using Δ Ct method.

pFACS analysis

Bone marrow cells were harvested from femora and tibiae into IMDM

containing 1% bovine serum albumin (BSA; Sigma Aldrich USA). Erythrocytes were lysed and mononuclear cells were incubated in the harvesting medium at 37°C for 45mins. The cells were stimulated with murine GM-CSF 10mg/ml for 15 minutes before fixation. Antibodies used and following procedures performed were exactly as described (Van Meter et al. 2007).

Stem/progenitor cells analysis, sorting and transplantation

Antibodies used were lineage markers (Mac-1, Gr-1, CD3, CD4, CD8, B220 and Ter119), Sca-1, c-kit, IL-7R, CD150 and CD48. Cells were analyzed or sorted using ARIAII (BD Biosciences). $CD150^{+}CD48^{-}Sca\text{-}1^{+}Lin^{-}c\text{-}kit^{+}$ and $CD150^{-}CD48^{-}Sca\text{-}1^{+}Lin^{-}c\text{-}kit^{+}$ were used to identify hematopoietic stem cells (HSCs) or multipotent progenitors (MPPs) respectively as previously described(Kiel et al. 2005; Kim et al. 2006; Akala et al. 2008). For myeloid progenitors (MPs) we used $c\text{-}kit^{+}Sca\text{-}1^{-}Lin^{-}$ $IL\text{-}7R^{-}$ (Akala et al. 2008). Antibodies were directly conjugated or biotinylated and purchased from e-Bioscience, BD Biosciences, or Biolegend. HSCs and MPs were doubly sorted into PBS and transplanted to C57Bl/Ka-CD45.1 (lethally irradiated at 8.2 gy single dose) by retro-orbital venous sinus injection, together with a radioprotective dose of 2×10^{5} bone marrow cells from unirradiated C57Bl/ Ka-CD45.1 mice. For mosaic transplantation recipients, GFP positive HSCs or MPs were sorted.

In vitro proliferation and colony formation analysis

HSPCs of LSL-Kras^{G12D} and Wt control were infected with the non-self deleting version of LGmCre vectors containing shLuc or shp53. For BrdU analysis, cells were then treated with 4-OHT for 48 hours and cultured for 4 days. BrdU (5'-bromodeoxyuridien) was added 2 hrs before analysis at the concentration of 31ul/ml. The following procedure for staining and analysis was performed according to company protocol (BD Pharmingen™Cat#557892). For colony formation, cells were treated with 4-OHT for 48 hours and 5,000 GFP positive cells were plated into Methocult GF M3534 (Stem cell technology). Cultured cells, grown on 35mm dishes, were imaged with the GFP fluoresence and brightfield phase contrast optics of a Zeiss Observer Z.1 inverted microscope. Using a 5x objective and the Zeiss MRm monochrome CCD camera, images of the entire surface of dishes were collected with the Mosaic automation function of the Zeiss Axiovision software(Version 4.7.3) on day 7 of culture and analyzed by ImageJ. Colonies were harvested to repeat the same procedure for serial replating. Cultured cells, grown on 35mm dishes, were imaged with the GFP fluoresence and brightfield phase contrast optics of a Zeiss Observer Z.1 inverted microscope. Using a 5x objective and the Zeiss MRm monochrome CCD camera, images of the entire surface of dishes were collected wtih the Mosaic automation function of the Zeiss Axiovision software (Version 4.7.3).

Leukemic mice imaging and treatment

Approximately 1 million of AML1-ETO;Nras^{G12D};p53+/+ or AML1-ETO;Nras^{G12D};p53-/- leukemic cells were transplanted into sublethally irradiated recipient mice (4.5 Gy, 24 h prior to transplantation) by tail-vein injection. Recipient mice were maintained on Ciprofloxacin- supplemented drinking water (Ciprofloxacin 125 mg/L, sucrose 20 g/L; both Sigma) until 2 wk after the end of chemotherapy. This antibiotic regimen has been shown to efficiently decon- taminate aerobic Gram-negative pathogens without major effects on hematopoiesis (Velders et al. 2004). Mice were mon- itored by bioluminescent imaging starting 10 d after transplantation. Chemotherapy was initiated upon detection of clear signals in pelvis, tail, and both femurs, and initial stage of hepatosplenic infiltration, which correlated with 30%–60% bone marrow infiltration as assessed by flow cytometry. Mice were treated for five consecutive days every 24 h with i.p. injections of 100 mg/kg cytarabine (Bedford Laboratories); during the first 3 d, 3 mg/kg doxorubicin (Bedford Laboratories) was administered in a separate i.p. injection. Immediate response and long-term treatment effects were monitored by weekly luciferase imaging starting the first day after treatment and by histopath- ological analysis of representative mice at various time points. PD 0325901 was administrated by oral gavages daily at the dosing of 12.5mg/kg body weight. As a single reagent, it was used for 2 weeks or until the mice succumbed to disease; in combination with chemotherapy, it was administrated after the last dose of cytarabine and used for 4 doses.

Representational Oligonucleotide Microarray Analysis

Human AML samples were obtained from the University of Michigan. Genomic DNA was isolated using PureGene DNA Isolation Kit (Gentra). Hybridizations were carried out on 85K arrays (NimbleGen) (Lucito et al., 2003; B. Lakshmi, I.M. Hall, C. Egan, J. Alexander, J. Healy, L.Z., W.X., M.S.S., S.W.L., M.W., and R.L., unpublished data). The genome position was determined from the UCSC GoldenPath browser (freezes April 2003 for hu- man and February 2003 for mouse). Focal gains or losses were defined as spanning <5 Mb.

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