

Chapter 1

Signaling Pathways in Leukemic Stem Cells



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Contents

1.1	Introduction.....	3
1.2	The PI3K/AKT/mTOR Signaling Pathway.....	5
1.3	The PI3K Isoforms in HSCs and in Leukemia.....	7
1.4	AKT in HSCs and Leukemia.....	8
1.5	The Phosphatases PTEN and SHIP in HSCs and LSCs.....	10
1.6	PDK1 in HSCs and Leukemia.....	12
1.7	mTOR in HSCs and Leukemia.....	13
1.8	The FOXO Transcription Factors in HSCs and Leukemia.....	14
1.9	Integration of PI3K/AKT with the MAPK Pathway in HSCs and LSCs.....	15
1.10	The WNT/ β -Catenin Signaling Pathway in HSCs and LSCs.....	16
1.11	The NOTCH Pathway in HSCs and LSCs.....	20
1.12	The TGF β Pathway in HSCs and LSCs.....	24
1.13	Concluding Remarks.....	28
	References.....	30

Abstract Hematopoietic stem cells (HSCs) and leukemic stem cells (LSCs) utilize many of the same signaling pathways for their maintenance and survival. In this review, we will focus on several signaling pathways whose roles have been extensively studied in both HSCs and LSCs. Our main focus will be on the PI3K/AKT/mTOR pathway and several of its regulators and downstream effectors. We will also discuss several other signaling pathways of particular importance in LSCs, including the WNT/ β -catenin pathway, the NOTCH pathway, and the TGF β pathway. For

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each of these pathways, we will emphasize differences in how these pathways operate in LSCs, compared to their function in HSCs, to highlight opportunities for the specific therapeutic targeting of LSCs. We will also highlight areas of crosstalk between multiple signaling pathways that may affect LSC function.

Keywords Hematopoietic stem cell · Leukemic stem cell · Signaling · PI3K · Akt · mTOR · FOXO · WNT · β -Catenin · NOTCH · TGF β

Abbreviations

AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
BCR-ABL	Fusion of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (ABL)
Cbf-1	Centromere-binding protein 1
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
Co-Smad	Common-partner Smad
DEPTOR	DEP domain-containing mTOR-interacting protein
GPCRs	G-protein-coupled receptors
GSK-3 β	Glycogen synthase kinase-3 β
Hes-1	Hairy and enhancer of split-1
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IKK	I κ B kinase
I-Smad	Inhibitory Smad
JNK	Jun N-terminal kinase
LEF	Lymphoid enhancer factor
Lrp5/6	Lipoprotein receptor-related protein 5 or 6
LSC	Leukemic stem cell
LSK	Lineage negative, Sca1+, cKit-
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
MLL	Mixed lineage leukemia
mLST8	Mammalian lethal with sec-13 protein 8
mSin1	Mammalian stress-activated map kinase-interacting protein 1
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NICD	Notch intracellular domain
PDK1	Pyruvate dehydrogenase kinase 1
PH	Pleckstrin homology

PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (3,4)-diphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PORCN	Porcupine
PRAS40	Proline-rich Akt substrate 40 kDa
Protor1/2	Protein observed with Rictor 1 and 2
PTEN	Phosphatase and tensin homolog
Raptor	Regulatory-associated protein of mammalian target of rapamycin
R-Smad	Receptor-mediated Smad
RTK	Receptor tyrosine kinase
SH2	Src-homology 2
SHIP	SH2-containing inositol 5'-phosphatase
SIRT1	NAD-dependent deacetylase sirtuin-1
T-ALL	T-cell acute lymphoblastic leukemia
TCF	T-cell factor
TGF β	Transforming growth factor-beta
TKI	Tyrosine kinase inhibitor
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
T β RI	TGF β -type I receptor
T β RII	TGF β -type II receptor

1.1 Introduction

Hematopoiesis is a hierarchical process, with hematopoietic stem cells (HSCs) residing at the top of the hierarchy. These cells have the ability to both self-renew and differentiate to generate all mature blood cells. To retain homeostasis under normal conditions, as well as to initiate the required responses under stress conditions, hematopoiesis needs to be a tightly regulated process. To maintain homeostasis, HSCs need to respond to extracellular cues, such as growth factors and chemokines within the bone marrow niche, in order to make the decision whether to maintain self-renewal or to enter the cell cycle and differentiate. These growth factors and chemokines are the ligands for key signaling networks that either instruct or facilitate these critical decisions that orchestrate HSC function. Examples of signaling pathways required for HSC regulation include the PI3K, WNT/ β -catenin, NOTCH, and TGF β pathways.

Without proper regulation by such signaling pathways, HSCs and downstream progenitors can acquire unlimited self-renewal potential at the expense of differentiation, as well as increased proliferation and survival. The acquisition of the first genetic or epigenetic alteration in an HSC can give that cell a clonal advantage over its counterparts and may lead to the generation of preleukemic stem cells (pre-LSCs) (reviewed in Corces-Zimmerman et al. [1]) (Fig. 1.1). Pre-LSCs are still capable of multilineage differentiation but have increased self-renewal capacity. They can then acquire additional genetic alterations that will promote full

transformation to leukemic stem cells (LSCs) (Fig. 1.1). Unlike pre-LSCs, LSCs cannot contribute to normal adult blood cells. Instead, they differentiate only into leukemic blasts, which form the bulk of the leukemia. Some studies have shown that LSCs can also arise directly from more committed myeloid progenitors, through genetic alterations that reprogram these progenitors to express a self-renewal program usually associated with HSCs [2–4] (Fig. 1.1). Phenotypically, LSCs may share some of the same cell surface markers as HSCs, but they are defined functionally by their ability to generate leukemia in the bone marrow transplantation setting. As mutations in components of signaling pathways are prominent in leukemias, it is important to understand how these signaling pathways regulate LSC function.

In this review, we will focus on several signaling pathways whose roles have been extensively studied in both HSCs and LSCs and their effects on myeloid LSC self-renewal, cycling, quiescence, and differentiation capacity. Our main focus will be on the PI3K/AKT/mTOR pathway and some of its regulators and downstream effectors, whose roles in HSC and LSC function have been explored. We will also discuss other signaling pathways of particular importance in LSCs, including the WNT/ β -catenin pathway, the NOTCH pathway, and the TGF β pathway. For each of these pathways, we will emphasize differences in how these pathways operate in LSCs, compared to their function in HSCs, to highlight potential opportunities for the specific therapeutic targeting of LSCs. We will also highlight areas of crosstalk between multiple signaling pathways that may affect LSC function.

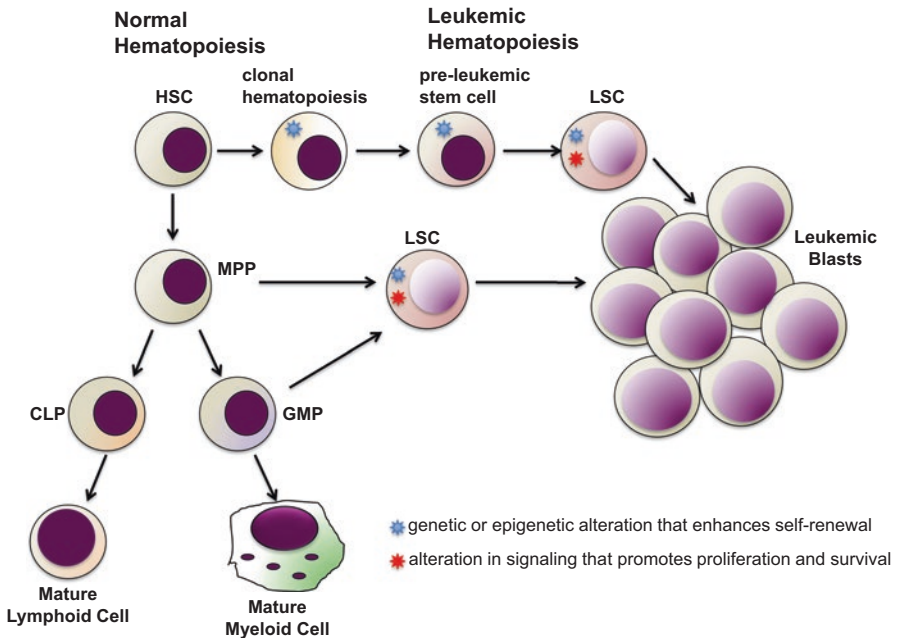


Fig. 1.1 Normal and leukemic hematopoiesis

1.2 The PI3K/AKT/mTOR Signaling Pathway

Many hematopoietic growth factors and cytokines signal through the PI3K/AKT pathway. Phosphatidylinositol 3-kinase (PI3K) can be activated by growth factors through receptor tyrosine kinase (RTK) receptors, by chemokines through G-protein-coupled receptors (GPCRs) or, by RAS family proteins (Fig. 1.2). The PI3K family consists of three distinct classes, and the Class I isoforms have been the most extensively studied in hematopoiesis, so we will focus on Class I PI3K for this discussion. Class I PI3Ks are heterodimeric lipid kinases, which are composed of a p110-kDa catalytic subunit and a regulatory subunit (reviewed in Vanhaesebroeck et al. [5]). Depending on the type of regulatory subunit, Class I PI3Ks are classified as either Class 1A or Class1B enzymes. Mammals have three Class 1A p110 isoforms (p110 α , p110 β , and p110 δ), which are encoded by three separate genes (*PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively), and one Class 1B isoform p110 γ , encoded by the *PIK3CG* gene. The regulatory subunits are encoded by different genes and diversified by alternative promoter usage as follows: *PIK3R1* encodes p85 α and the splice variants p55 α and p50 α , while *PIK3R2* encodes the p85 β subunit, *PIK3R3* encodes p55 γ , *PIK3R5* gene encodes p101, and *PIK3R6* encodes the p84/p87 regulatory subunit. Class 1A p85 regulatory subunits (p85, p55, p50) harbor two SRC homology 2 domains (nSH2, cSH2) and an intervening p110-binding

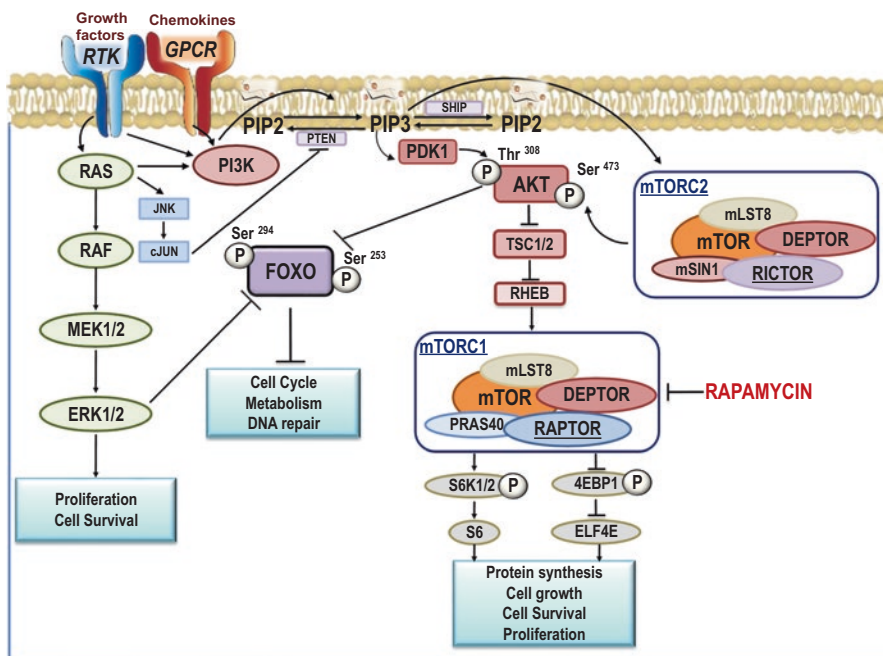


Fig. 1.2 The PI3K/AKT/mTOR and RAS/MEK/ERK signaling cascades and their main downstream effectors

region (iSH2) and constitutively interact with the p110 α , p110 β , and p110 δ catalytic subunits [6]. The Class 1A catalytic isoforms can all bind to the same p85 regulatory subunits, so they can functionally compensate for one another (reviewed in [5]). In contrast, the Class 1B catalytic subunit p110 γ does not have a p85-binding domain and is almost exclusively activated by GPCRs. The Class I PI3Ks p110 α , p110 δ , and p110 γ also harbor a RAS-binding domain and all except p110 β are thought to be RAS effectors [5]. In contrast, p110 β uses its RBD to bind to RAC and RHO GTPase family members and also interacts with Rab5 GTPase [7–9]. Upon stimulation, Class I PI3Ks produce the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP3) from phosphatidylinositol (3,4)-diphosphate (PIP2), and this process can be antagonized by phosphatase and tensin homolog (PTEN) or Src-homology 2 (SH2)-containing inositol 5'-phosphatase (SHIP), both of which dephosphorylate PIP3 to PIP2. PIP3 recruits the inactive serine/threonine-protein kinase AKT and pyruvate dehydrogenase kinase 1 (PDK1) from the cytosol through their pleckstrin homology (PH) domains, where PDK1 then phosphorylates AKT at Thr308. For complete activation, AKT must also be phosphorylated by the mTOR complex 2 (mTORC2) at Ser473 [10]. Intriguingly, activation of PI3K/AKT in tumors can be frequently accompanied by JNK activation, and this activation seems to be PI3K-dependent, since it is promoted by loss of *PTEN*, with constitutive activation of JNK and the transcription factor c-JUN [11]. Furthermore, c-JUN promotes cellular survival by negatively regulating the expression of *PTEN* through direct binding to a variant AP-1 site on the *PTEN* promoter, thus activating the AKT pathway [12]. AKT has multiple downstream effectors, which regulate diverse cell processes, including cellular metabolism, glucose homeostasis, inflammation, apoptosis, cell cycle regulation, protein synthesis, and autophagy [5]. Here we will focus on those AKT effectors that have been shown to play a role in HSCs and LSCs: mechanistic target of rapamycin (mTOR) and FOXO.

mTOR is the major intracellular component that senses and reacts to dynamic environmental changes in response to nutrient and growth factor fluctuation to coordinate cell metabolism and growth. mTOR is a serine/threonine kinase that forms two distinct functional complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (reviewed in Zoncu et al. [13]). mTORC1 has six known protein components, while mTORC2 has seven components [14]. These two complexes share five proteins: the mTOR catalytic subunit, mammalian lethal with sec-13 protein 8 (mLST8, also known as GbL) [15, 16], DEP domain-containing mTOR-interacting protein (DEPTOR) [17], and the TTI1/TEL2 complex counts as 2 proteins [18]. In addition, mTORC1 contains regulatory-associated protein of mammalian target of rapamycin (RAPTOR) [19, 20] and proline-rich AKT substrate 40 kDa (PRAS40) [21–23], while mTORC2 contains the following unique components: rapamycin-insensitive companion of mTOR (RICTOR) [24, 25], mammalian stress-activated map kinase-interacting protein 1 (mSIN1) [24, 26], and protein observed with Rictor 1 and 2 (PROTOR1/2) [27, 28]. mTORC1 integrates signals from nutrients, growth factors, energy, and stress, while mTORC2 is primarily regulated by growth factors [13]. The small GTPase RAS homolog enriched in brain

(RHEB) stimulates mTORC1 activity when it is GTP-bound. AKT activates mTORC1 indirectly, through phosphorylation and subsequent inactivation of tuberous sclerosis 2 (TSC2) in the tuberous sclerosis 1 (TSC1)/TSC2 complex. TSC1-TSC2 acts as a GTPase-activating protein (GAP) for RHEB, thereby inactivating RHEB. Therefore, AKT activation ultimately leads to the stimulation of mTORC1. AKT also increases mTORC1 activity through phosphorylation of proline-rich AKT substrate PRAS40 [13].

The FOXO transcription factors are evolutionarily conserved, and their roles have been implicated in a multitude of fundamental biological processes, including metabolism, stem cell maintenance, lifespan regulation, and tumorigenesis [29, 30]. The mammalian FOXOs include FOXO1, FOXO3, FOXO4, and FOXO6. The FOXOs are potent transcriptional activators that bind to the consensus motif TTGTTTAC (termed DBE for DAF-16a family member binding element) to regulate the transcription of target genes [31–33]. Multiple studies have shown complex regulation of FOXOs by phosphorylation, which determines their subcellular localization and governs their transcriptional activity (reviewed in Tothova et al. [34]). In HSC and progenitor cells, upon growth factor stimulation, AKT phosphorylates FOXO 1, FOXO 3, and FOXO 4 at three conserved residues (in FOXO3 – Thr32, Ser253, Ser315), which inactivates FOXOs by creating a binding motif for the 14-3-3 chaperone proteins. This then facilitates translocation of the FOXOs from the nucleus and to the cytoplasm while preventing nuclear reentry by masking the nuclear localization sequence [31, 35, 36]. It is interesting to note that FOXO phosphorylation by AKT can be overridden by stress stimuli that activate Jun N-terminal kinase (JNK), which phosphorylates FOXOs at distinct sites from that of AKT to facilitate their nuclear import [37–40]. Interestingly, Yang et al. showed that ERK is also able to downregulate FOXO3a activity by phosphorylation of FOXO3a at Ser 294, Ser 344, and Ser 425, consequently promoting cell proliferation and tumorigenesis in a breast cancer model [41]. In addition to phosphorylation, FOXOs are posttranslationally regulated by acetylation/deacetylation, methylation, ubiquitination, and redox modulation. This fine-tuned regulation allows FOXOs to induce specific gene-expression programs in response to many environmental stimuli, such as insulin, growth factors, nutrients, and oxidative stress (reviewed in [42, 43]).

1.3 The PI3K Isoforms in HSCs and in Leukemia

Of the Class I catalytic isoforms, *Pik3ca* and *Pik3cb* are ubiquitously expressed, and their germline deletion in mice results in embryonic lethality (reviewed in Vanhaesebroeck et al. [5]). In contrast, *Pik3cd* and *Pik3cg* are enriched in leukocytes and are not required for development. In fact, mice with germline deletion of *Pik3cd* or *Pik3cg* are viable and have normal blood counts. While p110 α and p110 δ isoforms primarily transduce RTK signals, p110 β and p110 γ play a less prominent role in RTK signaling and are functionally redundant in signaling downstream of GPCRs [9]. P110 α is essential for growth factor signaling and oncogenic signaling

in fibroblasts [44]. However, functional redundancy between p110 α and p110 β in transducing RTK signals has also been described in some contexts [45, 46]. Surprisingly, postnatal conditional deletion of p110 α in the hematopoietic system results only in mild anemia, without affecting HSC self-renewal, while conditional deletion of p110 β in hematopoietic cells does not affect blood counts (Table 1.1) [47, 48]. In fetal liver HSCs, compound deletion of the regulatory subunits p85 α and p85 β decreases HSC frequency and multilineage repopulating capacity [49]. This suggests that there is increased redundancy between the Class IA isoforms in transducing growth factor signals in HSCs.

However, there is evidence that the same level of redundancy may not be present in the context of hematologic malignancies. For example, a requirement for p85 α , but not of p85 β , regulatory subunit was shown to be necessary for myeloproliferative disease driven by an activation loop mutant of proto-oncogene receptor tyrosine kinase (KIT), which encodes the receptor for stem cell factor [50] and has been described in AML [51–53]. An isoform-selective role for the catalytic isoform p110 α was also demonstrated in RAS-mutated myeloid leukemia, in a mouse model of juvenile myelomonocytic leukemia (JMML) driven by oncogenic Kras, and in a xenograft model of human RAS-mutated AML [47]. Furthermore, this study showed that combined pharmacologic inhibition of p110 α and mitogen-activated protein kinase (MEK) could be an effective therapeutic strategy for RAS-mutated myeloid malignancies [47]. In contrast, in a mouse leukemia model driven by loss of *Pten* in HSCs, of all the Class I PI3K isoforms, p110 β was the most important in promoting myeloid leukemogenesis, though a *Rac*-dependent mechanism [48]. Importantly, deletion of p110 β also was able to restore normal HSC function, while depleting leukemia-initiating cells, as was demonstrated in transplantation assays. This is consistent with studies in other tumor models driven by *Pten* loss, such as prostate cancer, which also showed preferential dependency upon p110 β [54]. Furthermore, this study showed that a p110 β -selective inhibitor could prolong survival in leukemia driven by *Pten* loss [48]. All of these studies demonstrate the concept of isoform-selective PI3K dependencies in myeloid leukemia, suggesting that it should be possible to achieve an antitumor effect with a good therapeutic window using selective PI3K isoform inhibition. In fact, several isoform-selective PI3K inhibitors, such as idelalisib, or dual-isoform inhibitors, such as duvelisib, are already in clinical use for indolent lymphoid malignancies, with reasonable toxicity profiles. However, these studies also illustrate that the specific PI3K isoform dependencies may vary depending on the oncogenic driver, as in other tumor types.

1.4 AKT in HSCs and Leukemia

Constitutive activation of PI3K/AKT signaling is observed in many cancers, including AML. Interestingly, unlike in solid tumors, mutations in members of the PI3K pathway are rarely detected in AML. However, increased AKT phosphorylation has been observed in up to 72% of AML cases [55]. In many cases, AKT activation in

Table 1.1 HSC and LSC phenotypes resulting from genetic alterations in the PI3K pathway

Gene	Alteration	Phenotype	References
<i>Pik3r1</i> and <i>Pik3r2</i>	KO	Decreased repopulating ability of fetal liver HSCs	Haneline et al. [49]
<i>Akt</i>	myr-AKT BMT model	Constitutively active AKT depletes HSCs and induces myeloproliferation, AML, and T-ALL	Kharas et al. [58]
<i>Akt1/</i> <i>Akt2</i>	KO	Compound deletion of <i>Akt1</i> and <i>Akt2</i> impairs fetal HSC repopulation	Juntilla et al. [59]
<i>Pik3ca</i>	cKO (Mx1-Cre)	No effect on HSC self-renewal Increased survival in KRAS ^{G12D} JMML model	Gritsman et al. [47]
<i>Pten</i>	cKO	<i>Pten</i> deletion using Mx1-Cre perturbed HSC function, myeloproliferation, AML, and T-ALL when <i>Pten</i> is deleted in adult HSCs	Yilmaz et al. [63], Zhang et al. [64], and Magee et al. [73]
		Accelerated disease progression in CML model (using BCR-ABL-iCre retrovirus)	Peng et al. [68]
<i>Pik3cb</i>	cKO (Mx1-Cre)	p110 β deletion prevents myeloproliferative neoplasia in context of <i>Pten</i> deletion and improves HSC function	Yuzugullu et al. [48]
<i>Ship</i>	KO	Myeloproliferative phenotype, hypersensitivity to growth factors	Helgason et al. [70]
<i>Pdk1</i>	cKO (Vav1-iCre)	Impaired repopulating potential and differentiation of fetal liver HSCs	Wang et al. [75]
	cKO (Mx1-Cre)	Prolonged survival in MLL-AF9 AML model	Hu et al. [76]
<i>TSC1</i>	cKO (Mx1-Cre)	Increased HSC cycling and impaired self-renewal	Chen et al. [79]
<i>mTOR</i>	cKO(Mx1-Cre)	Loss of HSC quiescence	Guo et al. [78]
<i>Rictor</i>	cKO (Mx1-Cre/ Vav1-iCre)	No effect on HSC function	Kalaitzidis et al. [74] and Magee et al. [77]
		Improved survival in <i>Pten</i> ^{-/-} model of leukemia	
<i>Raptor</i>	cKO (Mx1-Cre)	Impaired hematopoietic reconstitution	Kalaitzidis et al. [74]
		Improved survival in <i>Pten</i> ^{-/-} model of leukemia	
<i>Foxo</i>	KO or cKO (Mx1-Cre)	Increased HSC cycling, reduced repopulation activity, myeloproliferative phenotype, increased ROS levels	Tothova et al. [88], Yalcin et al. [89], Rimmele et al. [90], and Liang et al. [91]
		<i>Foxo3a</i> is important for maintenance of CML LICs	Naka et al. [96]
		<i>Foxo1/3/4</i> deletion induces differentiation of AML cells	Sykes et al. [97]
<i>S6k1</i>	KO	<i>S6k1</i> KO impairs HSC self-renewal and improves survival in MLL-AF9 AML model	Ghosh et al. [86]

AML is thought to be secondary to mutations or chromosomal rearrangements causing constitutive activation of upstream RTKs, such as FLT3 or c-KIT. Inactivation of PTEN, either through genetic or epigenetic mechanisms, may also play a role in AKT activation. However, the prognostic significance of AKT phosphorylation is controversial. Phosphorylation of the AKT at Thr308 was shown to confer a poor prognosis in AML [56], whereas phosphorylation of AKT at Ser473 correlates with a favorable response to chemotherapy [57]. To determine whether AKT activation is a true mechanism of transformation, or simply a marker of the transformed state, Kharas et al. generated a murine retroviral bone marrow transplantation model in which they expressed myristoylated AKT in mouse bone marrow cells [58]. The transplant recipients developed a myeloproliferative phenotype, as well as transplantable AML or T-ALL, demonstrating that AKT activation is an important driver of transformation in leukemia. Interestingly, this study also showed that PI3K/AKT pathway activation is deleterious to normal HSC function, due to increased HSC cycling and depletion of the stem cell pool. Both the T-ALL disease phenotype and the HSC maintenance phenotype could be rescued with the mTORC1 inhibitor rapamycin, highlighting the important role of mTORC1 in HSCs and in T-ALL, but not in AML. Interestingly, this study did not show any change in ROS levels in HSCs expressing myristoylated AKT [58].

In a different study, Juntilla et al. have shown that AKT1 and AKT2 play essential redundant roles in fetal HSC maintenance. *Akt1/2* double knockout fetal HSCs are unable to enter cell cycle and persist in the G0 phase, causing long-term functional defects due to increased quiescence. This increased quiescence of AKT1/2-deficient HSCs was associated with decreased ROS levels, suggesting that AKT1 and AKT2 are important for regulating ROS levels in hematopoiesis [59]. This work confirmed the important roles of AKT in the regulation of HSC homeostasis.

1.5 The Phosphatases PTEN and SHIP in HSCs and LSCs

PTEN is commonly deleted or otherwise inactivated in diverse cancers (reviewed in [60]), including hematologic malignancies [61, 62]. The phenotype observed with conditional deletion of *Pten* in the hematopoietic system using Mx1-Cre resembles the phenotype observed with activation of AKT in hematopoietic cells [63, 64], also reviewed in [65]). *Pten* deletion in hematopoietic cells led to the development of MPN, AML, and T-ALL and resulted in enhanced LSC function while compromising normal HSC function [63]. Zhang and colleagues also demonstrated that *Pten* inactivation in murine bone marrow causes short-term expansion, followed by the subsequent long-term decline of HSCs, primarily due to enhanced HSC activation, followed by exhaustion of the HSC pool [64]. Although *Pten*-deficient HSCs engraft normally in recipient mice, their ability to sustain hematopoietic reconstitution is impaired due to cell cycle dysregulation and decreased bone marrow retention [64]. Both phenotypes, the HSC exhaustion and leukemia initiation, are mediated by mTORC1, because they can be rescued by rapamycin [63]. However, it was later

shown by Guo et al. that in established T-ALL, the effects of *Pten* deletion in LSCs couldn't be completely reversed by rapamycin [66]. This likely reflects the different roles of mTORC1 in leukemia initiation, as opposed to leukemia maintenance. Interestingly, Lee et al. showed that the effects of rapamycin on *Pten*-deficient HSCs and LSCs can be partially rescued by increased expression of cyclin-dependent kinase inhibitor 2A, p16(Ink4a) and p53 in HSCs. However, while both p53 and p16(Ink4a) promoted the depletion in *Pten*^{-/-} HSCs, only p53 could suppress leukemogenesis [67]. In a CML model driven by BCR-ABL expression, Peng et al. demonstrated that *Pten* deletion accelerates disease development, while *Pten* overexpression delayed disease progression by suppressing LSC function [68]. This study further supports the role of PTEN as a tumor suppressor in myeloid LSCs. All of these studies highlight the distinct effects of PTEN deletion in HSCs and in LSCs, again suggesting that there is a therapeutic window for targeting elevated AKT signaling in LSCs.

In another study, Li et al. showed that PTEN protein levels are similar among different HSC populations and multipotent progenitors (MPPs), but that there is increased PTEN phosphorylation (p-PTEN) during the transition from long-term to short-term HSCs, and further to MPPs, where p-PTEN levels are inversely correlated with the self-renewal capacity and long-term repopulation capacity of HSPCs [69]. Serial transplantation experiments demonstrated that unphosphorylated Pten augments the self-renewal of wild-type long-term repopulating cells and promotes myeloid over lymphoid differentiation. Moreover, unphosphorylated Pten restricts HSCs to a quiescent state in the bone marrow niche by dual inhibition of PI3K/AKT and SRC signaling. In contrast, p-PTEN represses PI3K/AKT, but promotes the niche contact-stimulated signaling that regulates the proliferation and lodging of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow niche, whereas unphosphorylated PTEN represses both types of signaling [69]. Thus, this study highlights the dual cell-autonomous and non-cell-autonomous regulation of HSCs by PTEN.

Conditional deletion of another PIP3 phosphatase, *Ship*, also led to the development of a myeloproliferative disease, and bone marrow progenitor cells from *Ship*^{-/-} mice were hypersensitive to hematopoietic growth factors [70, 71]. Luo et al. evaluated the potential tumor suppressor role of *SHIP* in myeloid leukemogenesis, using AML patient samples and myeloid leukemia cell lines [72]. Strikingly, they discovered in one AML patient a dominant negative mutation in the *SHIP* gene, *SHIP*^{V684E}, which replaces Val with Glu at the phosphatase active site, reducing its catalytic activity.

Leukemia cells bearing this mutation have enhanced AKT phosphorylation following IL-3 stimulation. These cells also have a growth advantage, and are resistant to apoptosis upon serum deprivation, and in response to the chemotherapy drug etoposide [72]. These results suggest that mutated SHIP is a potential mechanism for the development of chemotherapy resistance through the PI3K/AKT signaling pathway.

Magee et al. addressed the effects of patient age on activation of the PI3K/AKT pathway in HSCs [73]. They asked why *PTEN* is sometimes mutated in adult, but

rarely in early childhood leukemias, and showed that there is a different effect of *Pten* deletion on the neonatal vs. adult HSCs. They revealed that PTEN is required by adult HSCs to dampen AKT signaling and prevents leukemia development in adults. However, neonatal HSCs do not activate AKT signaling as a result of *Pten* deletion. Moreover, they showed that in adult mice, *Rictor* deletion in *Pten*^{-/-} HSCs prevents leukemogenesis and HSC depletion, which for the first time implicates mTORC2 in HSC maintenance in the context of *Pten* inactivation. On the contrary, in normal HSCs, deletion of *Rictor* has little effect. Together with the fact that in neonatal HSCs, *Pten* deletion does not activate PI3K/AKT pathway and does not promote HSC proliferation, depletion, or leukemogenesis, this demonstrates the difference in critical tumor suppressor mechanisms and in the roles of PI3K/AKT signaling between adult and neonatal HSCs [73]. This study highlights the importance of the developmental changes in key signaling pathways that confer temporal alterations upon stem cell self-renewal and tumor suppressor mechanisms.

Kalaitzidis et al. confirmed the observation of the importance of RICTOR in the context of *Pten* loss in adult mice in their study of *Raptor* and *Rictor* deletion in the hematopoietic system (74). They found that deletion of *Raptor* in *Pten*^{-lox-lox};Mx1-Cre; mice significantly improved survival, but without much improvement in HSC function in transplantation assays. This is consistent with prior studies showing that rapamycin can prevent leukemia initiation in the context of hematopoietic *Pten* deletion [63]. However, in contrast to *Raptor* deletion, rapamycin was also able to rescue HSC function in the context of *Pten* loss, suggesting that RAPTOR may have some rapamycin-insensitive functions that are required for HSC maintenance [74]. Interestingly, conditional deletion of only *Rictor*, but not *Raptor*, after disease onset could significantly improve HSC function in the context of *Pten* loss [74]. Overall, these studies underscore the importance of both mTORC1 and mTORC2 in leukemic transformation induced by *Pten* loss, while only mTORC1 appears to play an important role in the maintenance of normal HSCs. Therefore, components of mTORC2 should be explored further as potential therapeutic targets for myeloid malignancies.

1.6 PDK1 in HSCs and Leukemia

It has been reported that pyruvate dehydrogenase kinase 1 (PDK1), which contributes to AKT activation by phosphorylating AKT on Thr308, also plays important roles in the hematopoietic system. Wang et al. revealed an indispensable role for PDK1 in fetal liver HSCs. Using the Vav-iCre system, they showed that *Pdk1* is crucial for HSC expansion, and *Pdk1* deletion severely impairs the repopulating potential and differentiation capacity of fetal liver HSCs [75]. Interestingly, another study showed that conditional deletion of *Pdk1* in the MLL-AF9 murine AML model prolonged survival due to increased apoptosis of LSCs, which was accompanied by reduced expression of *Stat5*, which is often activated in leukemia [76]. These data highlight the essential role of PDK1 during fetal hematopoiesis and in

LSC maintenance. It would be interesting to further delineate the differences between the function of PDK1 in adult HSCs and in LSCs, given the differences in PI3K/AKT signaling previously reported between neonatal and adult HSCs [77].

1.7 mTOR in HSCs and Leukemia

mTOR has been shown to play a central role in both embryonic and adult hematopoiesis. Guo et al. showed that conditional mTOR deletion using Mx1-Cre results in loss of HSC quiescence, with the consequent exhaustion of HSCs, as well as impaired HSC engraftment and repopulation [78]. Interestingly, conditional deletion of *Tsc1*, and consequent mTOR activation, also causes HSC cycling and premature depletion, due to increased mitochondrial biogenesis followed by elevated levels of reactive oxygen species (ROS) [79]. This suggests that TSC1 is an essential regulator of HSC quiescence. In a follow-up study, Chen et al. also showed that mTOR activity in HSCs increases with age, and that *Tsc1* deletion mimics the characteristics of aged HSCs in young mice [80]. Furthermore, they showed that decreasing mTOR signaling with rapamycin restores HSC function in old mice, thus highlighting a fundamental function of mTOR signaling in HSC aging [80]. However, activation of mTOR signaling by conditional deletion of *Tsc1* is insufficient to cause hematologic malignancies in mice [79]. Interestingly, Kalaitzidis et al. also showed that Raptor deficiency leads to HSC expansion in *Pten*-wild-type HSCs and promotes the transition of short-term HSCs from the G0 to G1 phase [74]. Furthermore, Raptor-deficient BM cells cannot reconstitute hematopoiesis in lethally irradiated recipient mice [74]. Although under homeostatic conditions mTORC1 is not required for HSC maintenance, its loss leads to HSC and progenitor mobilization. A similar phenotype was reported by Hoshii et al. for *Raptor* deletion in the hematopoietic system; they observed myeloid expansion, but no effects on progenitor proliferation or survival [81]. Both groups also showed that deletion of Raptor, but not of Rictor, caused an increase in AKT activation in hematopoietic stem and progenitor cells (HSPCs). This is consistent with the previously described effects of prolonged rapamycin treatment on AKT activation, which can occur through the unopposed activity of mTORC2, leading to phosphorylation of AKT on Ser473 [82]. Given the known effects of AKT activation on HSC function, this could potentially explain the HSC cycling phenotype in *Raptor*^{-/-} mice [58, 74].

Surprisingly, the GTP-binding protein RHEB, which is known to activate mTORC1 activity in other cell types, was found to only moderately activate mTORC1 in HSCs and progenitors [83]. Peng et al. showed that *Rheb* deletion did not significantly affect HSC maintenance in mice, in contrast to *Raptor* deletion, signifying that under steady-state conditions, RHEB activity is expendable for mTORC1 activity and HSC self-renewal [83].

Dysregulation of the mTORC1-S6K1 signaling pathway occurs frequently in AML patients. Primary AML cells derived from patients show increased activation of S6K1 [84]. The role of mTORC1-S6K1 signaling and its role in regulation of

HSCs and LSCs were recently reviewed in detail by Ghosh and Kapur, where they highlight recent progress in identifying the roles of different components of the pathway and pharmacological targeting of mTORC1 and S6K1 [85]. Ghosh et al. showed that S6K1 deficiency in hematopoietic cells results in the reduced quiescence of L-HSCs, and their impaired self-renewal, concomitant with the reduced expression of cyclin-dependent kinase inhibitor 1A (Cdkn1a) [86]. They showed that loss of S6K1 increases the susceptibility of mice to repeated stress. Moreover, the authors reported that the leukemic potential of cells driven by the mixed lineage leukemia (MLL) fusion oncogene MLL-AF9 is similarly dependent on S6K1. Inhibition of S6K1 activity reduced the proliferation of MLL-rearranged human AML cells, and loss of S6K1 impaired *in vivo* LSC function in MLL-AF9 AML model, by modulating AKT and 4E-BP1 phosphorylation [86]. This data demonstrated that S6K1 loss decreases mTOR activation, consequently leading to reduced phosphorylation of AKT and 4E-BP1 in HSCs. Thus, S6K1 is a potential therapeutic target in AML LSCs. Hoshii reported similar findings with *Raptor* deletion in MLL-AF9 LSCs and also showed that loss of Raptor led to differentiation of LSCs [81]. Also, consistent with the results of Kalaitzidis et al. in the *Pten*^{-/-} leukemia model, they reported that loss of *Raptor* failed to rescue normal hematopoiesis in the MLL-AF9 AML model. However, Hoshii et al. also reported the intriguing finding that *Raptor* deletion compromised the leukemia-initiating cells in MLL-AF9 AML mice, without compromising the self-renewal of LSCs, arguing that these are two different cell populations with differential dependence on RAPTOR [81]. This finding brings into question the usefulness of targeting mTORC1 alone in AML.

1.8 The FOXO Transcription Factors in HSCs and Leukemia

In the hematopoietic system, FOXO1 and FOXO3 are thought to be the main players, with FOXO3 being more active than FOXO1, and they have been shown to be key regulators of both HSC and LSC maintenance. It was shown that FOXOs regulate the intracellular levels of reactive oxygen species (ROS), where loss of FOXO activity elevates ROS levels, leading to increased HSC cycling and apoptosis [87, 88]. Yalcin et al. also showed that loss of FOXO3 leads to an increased number of hematopoietic progenitors with hypersensitivity to cytokines and led to a myeloproliferative phenotype. These progenitors had increased ROS levels, increased AKT/mTOR signaling, and a relative deficiency of LNK, a negative regulator of cytokine receptor signaling [89]. More recently, it was shown by Rimmel et al. that in *Foxo3*^(-/-) HSCs and HSPCs, mitochondrial metabolism is suppressed, affecting HSC long-term repopulation activity [90]. This repression was associated with altered expression of mitochondrial and metabolic genes, which was independent of ROS levels or mTOR signaling [90].

Remarkably, accumulating evidence in the field raises the possibility that in stem cells, including in HSCs, AKT is not the only regulator of FOXO. It has been demonstrated that nuclear FOXO localization and activity in HSPCs are dependent on other upstream regulators, such as P38 mitogen-activated protein kinases (p38MAPK) or NAD-dependent deacetylase sirtuin-1 (SIRT1) [87, 91]. In fact, at steady state, FOXO seems to be primarily regulated independently from PI3K/AKT signaling. As Miyamoto et al. have shown using their *Foxo3a* conditional knockout mouse model, *Foxo3a*^{-/-} HSCs have normal proliferation and differentiation in the bone marrow, but lose their self-renewal capacity due to loss of HSC quiescence, associated with increased p38-MAPK phosphorylation [87]. However, during myelosuppressive stress and aging, FOXO3a in HSCs is regulated via AKT and ERK signaling [92].

It has been reported that high levels of FOXO3a mRNA and phosphorylation of FOXO3a are adverse prognostic factors in AML [93, 94]. Interestingly, Chapuis et al. showed that in AML samples, the localization of FOXO3a is able to escape AKT control and is also independent of the ERK/MAPK signaling pathway, but it is dependent on FOXO3a Ser644 phosphorylation by I κ B kinase (IKK) [95]. They proposed an interesting possibility that IKK activity retains FOXO3a in the cytoplasm, thereby inactivating FOXO3a and affecting the proliferation and survival of AML cells. Although FOXOs are primarily known as tumor suppressors, the roles of FOXOs in myeloid malignancies are controversial. High level of FOXO3 expression is associated with an adverse prognosis in AML [93], and genetic ablation of *Foxo3* has been shown to reduce disease burden in a murine model of CML [96]. Strikingly, Sykes et al. demonstrated the opposite roles for AKT/FOXOs in AML [97]. There is a generally accepted concept that activated AKT signaling, which should lead to FOXO inhibition, is associated with hematopoietic transformation, but they observed that in 40% of AML patient samples, regardless of genetic subtype, FOXOs are active. In these patients, instead of decreased FOXO activity, FOXO is active and inversely correlated with c-Jun N-terminal kinase of (c-JUN)/JNK signaling. Moreover, leukemic cells resistant to FOXO inhibition responded to JNK inhibition, which revealed a molecular role for AKT/FOXO and JNK/c-JUN in maintaining the differentiation blockade, opening an exciting avenue for the inhibition of leukemias with a range of genetic lesions [97].

1.9 Integration of PI3K/AKT with the MAPK Pathway in HSCs and LSCs

Baumgartner et al. recently showed that during emergency hematopoiesis, the MEK/ERK and PI3K pathways are synchronously activated in HSCs [98]. Moreover, MEK1 phosphorylation by activated ERK provides a feedback mechanism to counterbalance AKT/mTORC1 activation, presenting HSCs with the rate-limiting feedback mechanism that controls their fitness and reentry into quiescence (Fig. 1.2)

[98]. In myeloid leukemogenesis, oncogenic NRAS^{G12D} activates both MAPK and PI3K signaling and can promote the expansion of preleukemic clones in some contexts [99–102]. It is interesting to consider that disruption of this feedback loop that controls the duration of PI3K signaling and the production of PIP3 could exhaust the pool of fast-proliferating HSCs induced by chemotherapy, while at the same time this could benefit the expansion of a pool of self-renewing preleukemic clones.

Many of the growth factors and cytokines that orchestrate hematopoiesis and HSC maintenance signal through the PI3K/AKT canonical signaling pathway, and dysregulation of this pathway can lead to hematopoietic transformation. It is becoming clear that tight regulation of PI3K/AKT pathway activation is critical for the maintenance of HSC homeostasis, at least in adults. There is also sufficient evidence that activation of AKT in HSCs, either directly or indirectly through inactivation of PTEN or SHIP, can lead to hematopoietic transformation. However, modulation of the downstream components of AKT generally is not sufficient to promote full transformation, though it can cause significant alterations in HSC homeostasis. This highlights the importance of understanding the roles of the individual components of the PI3K/AKT pathway in hematopoietic transformation, as well as their crosstalk with parallel pathways, such as with the MAPK pathway. Exciting studies have already provided evidence that the FOXOs can act as a “cross-talk hub” between several signaling pathways, including the PI3K/AKT, MEK/ERK, and JNK/c-JUN pathways, suggesting that any mutation that alters FOXO3 function in HSCs might contribute to malignant transformation. Further research in this area should focus not only on further delineating the roles of PI3K/AKT pathway members in LSCs but also on the interplay between PI3K and other signaling pathways.

1.10 The WNT/ β -Catenin Signaling Pathway in HSCs and LSCs

The WNT signaling pathway has been well characterized for its stimulatory role in adult HSC maintenance and self-renewal [103, 104] (Fig. 1.3, Table 1.2). The canonical WNT signaling pathway is activated when WNT ligands within the bone marrow stroma interact with the FRIZZLED-LRP5/6 receptor complex, which is expressed on the HSC surface (reviewed in Staal et al. [105]). This leads to inhibition of the multi-protein destruction complex composed of GSK-3 β , AXIN1 or AXIN2, and APC, which normally phosphorylates β -catenin, leading to its proteasomal degradation. β -Catenin is then able to accumulate in cytoplasm, leading to its translocation into the nucleus, where it activates the TCF/LEF transcription factor family to activate the expression of genes essential for expanding the HSC pool [105].

Despite the conserved role of WNT signaling in regulating HSC self-renewal, there has been conflicting evidence regarding the effects of β -catenin signaling on

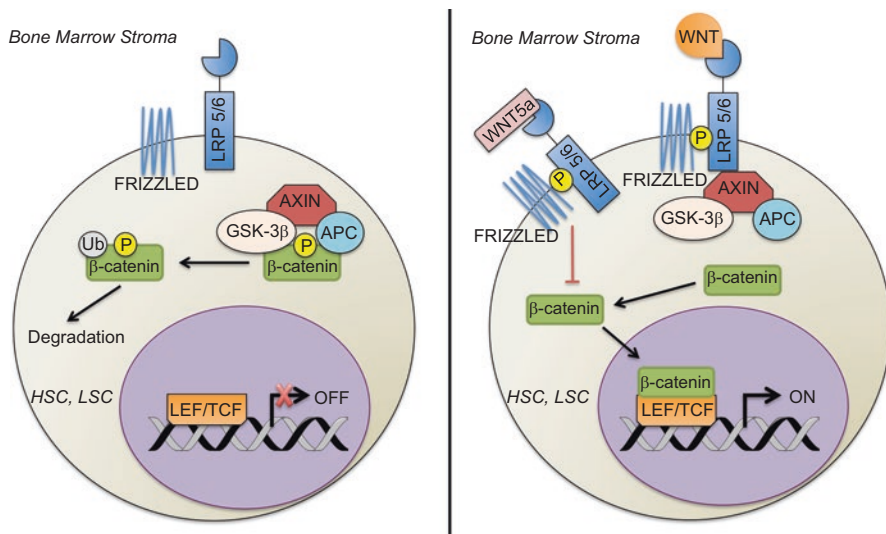


Fig. 1.3 Summary of WNT/ β -catenin pathway activation in HSCs and LSCs

the HSC population. Knockout studies have demonstrated that WNT signaling is dispensable for HSC function. For example, deletion of β -catenin in bone marrow progenitors did not impair self-renewal or differentiation [106]. In support of this, deletion of *Porcupine* (*Porcn*), an O-acyltransferase essential for all WNT ligands, in HSCs did not impair their ability to self-renew, proliferate, or differentiate [107]. A recent review has covered the concept that the proper dosage of WNT signaling must be achieved to moderate HSC self-renewal versus differentiation decisions [108]. Using transgenic mouse lines expressing different doses of the negative regulator APC, they elegantly demonstrated that slightly enhanced levels of WNT signaling can enhance HSC reconstitution, while much higher levels of WNT signaling can result in the failure to engraft and reconstitute lethally irradiated mice [109]. This supports earlier work, which showed through conditional expression of a stable constitutively active form of β -catenin that HSCs lose their ability to repopulate and also lose the capacity for myeloid lineage commitment [110]. Scheller et al. also observed a similar HSC exhaustion phenotype when they expressed a different constitutively active mutant of β -catenin in hematopoietic cells [111]. Notably, using the SCL-Cre-ER mouse model, β -catenin activation more specifically in HSCs resulted in HSC apoptosis [112]. However, when β -catenin activation was coupled with *Pten* deletion, there was an expansion of LT-HSCs at the expense of differentiation, suggesting that the WNT and PI3K/AKT pathways cooperate in regulating LT-HSC proliferation, apoptosis, and differentiation [112]. Altogether, it is clear that the HSCs require some level of activation of the WNT signaling pathway for self-renewal, survival, and proliferation and that this level must be tightly regulated.

Table 1.2 HSC and LSC phenotypes resulting from genetic alterations in the WNT/ β -catenin pathway

Gene	Alteration	Phenotype	References
<i>Ctnnb1</i>	cKO (Mx1-Cre)	No effects on HSC function	Cobas et al. [106]
		Delays initiation of disease in mouse model of CML, compromised LSC self-renewal	Zhao et al. [114] and Hu et al. [115]
		Impaired LSC function in MLL-AF9 AML	Wang et al. [119]
		Pre-LSCs have impaired ability to generate LSCs in MLL-ENL AML model	Yeung et al. [120]
	Conditional expression of activated mutant (Mx1-Cre)	Impaired HSC repopulation, loss of myeloid lineage commitment	Kirstetter et al. [110] and Scheller et al. [111]
	Conditional expression of activated mutant (Scl-Cre-ER)	HSC apoptosis, which can be rescued by activation of the PI3K pathway	Perry et al. [112]
<i>Apc</i>	cKO (hypomorphic allele)	Mildly increased WNT signaling enhances HSC reconstitution in vivo, but higher levels of WNT signaling impair engraftment and HSC reconstitution	Luis et al. [109]
<i>Lef/Tcf</i>	<i>Lef</i> cKO (Vav1-iCre)	Impaired LSC self-renewal in CML model	Yu et al. [118]
	<i>Tcf</i> germline KO	Normal HSC self-renewal	
<i>Wnt5a</i>	Overexpression	Inhibits HSC expansion by inducing a quiescent state at the expense of canonical WNT signaling	Nemeth et al. [124]
	Heterozygous deletion	Loss of one <i>Wnt5a</i> allele leads to spontaneous CML or B cell lymphoma development	Liang et al. [125]
	Haploinsufficiency in the bone marrow niche	Impaired HSC engraftment Impaired leukemia engraftment in CML model	Schreck et al. [127]

The WNT signaling pathway has also been shown to play important roles in LSCs, as this population also requires the WNT signaling pathway for self-renewal and survival. In human CML LSCs, it has been shown that β -catenin is localized in the nucleus and that in vitro LSC activity could be reduced by expression of AXIN [113]. Transplantation of BCR-ABL LSCs depleted of β -catenin into mice results in a decreased disease burden and impaired serial transplantation [114, 115]. Importantly, it has been shown that progression of CML from the chronic phase to blast crisis occurs through the stimulation of β -catenin activity through activation of the PI3K pathway by BCR-ABL [116]. Hu et al. also showed that inhibiting BCR-ABL or the PI3K pathway results in decreased levels of β -catenin.

Subsequently, *in vivo* inhibition of this pathway delays CML development and impairs the tumor-initiating ability of CML LSCs in secondary transplantation, implying that the crosstalk between the PI3K pathway and WNT pathway is important for LSC maintenance in CML [116].

Importantly, β -catenin also plays a role in BCR-ABL tyrosine kinase inhibitor (TKI) resistance, as it has been reported that β -catenin is stabilized in CML cells to promote TKI resistance through a BCR-ABL kinase-independent resistance mechanism [117]. It has been reported that CML cells treated with imatinib maintain their β -catenin expression and have subsequent activation of downstream targets, promoting their survival and colony formation ability [117]. In addition, it has been shown that CML LSCs require the transcription factors TCF1 and LEF1 to propagate CML in secondary recipients, and combining imatinib with induced deletion of *Tcf1* and *Lef1* *in vivo* diminishes the LSC population [118]. Importantly, HSCs are less sensitive to loss of *Tcf1* and *Lef1*, suggesting that targeting TCF1 and LEF1 in combination with a TKI could be a novel therapeutic strategy to eliminate CML LSCs, without much toxicity to HSCs [118].

In AML LSCs, inhibiting β -catenin in either the HOXA9/MEIS1 or MLL-AF9 mouse models prevents AML initiation from either the HSC or GMP populations, suggesting that WNT signaling is essential for LSC function [119]. In addition, conditional deletion of *Ctmb1*, the gene that encodes β -catenin, in MLL-rearranged AML mouse models reduces the number of LSCs and overall incidence of leukemia development [120]. In both CML and AML LSC populations, β -catenin- and WNT pathway-associated genes are increased in comparison to normal HSCs [113, 120], further supporting the important roles of the WNT signaling pathway in LSCs. More recently, it has been shown that GPR84, a positive regulator of β -catenin, is also upregulated in LSCs in comparison with normal HSCs, and suppression of GPR84 reduced MLL-rearranged LSC functionality *in vitro* and *in vivo* [121]. WNT signaling has also been studied in FLT3-mutant AML models, as FLT3 signaling can increase nuclear β -catenin levels [122]. High levels of β -catenin were found in bone marrow resident leukemic cells in patients with FLT3-mutated AML. Notably, the combination of a β -catenin antagonist and a FLT3 TKI impaired the engraftment of FLT3-mutated leukemic cells into immunodeficient mice, suggesting that β -catenin inhibitors can synergize with FLT3 inhibitors in FLT3-mutated AML, due to the effects of this drug combination on LSC function [122].

Noncanonical WNT signaling has also been shown to play a role in myeloid LSCs. The noncanonical WNT ligand WNT5a can signal through both the planar cell polarity and WNT/calcium noncanonical pathways, both of which are extensively reviewed in Komiya and Habas [123]. In short, WNT5a signaling through the WNT/calcium pathway results in increased intracellular calcium levels and subsequent inhibition of β -catenin [123]. This has been shown in HSCs, as WNT5a can inhibit the activation of canonical WNT signaling, and this inhibition results in an increase of quiescent HSCs [124]. WNT5a has implications in AML, as WNT5a levels are low or absent in primary AML samples, and WNT5a hemizygous mice develop myeloid leukemias [125]. In addition, high levels of methylation of the WNT5a gene locus correlate with lower expression of WNT5a, and this high meth-

ylation status correlates with poor prognosis of a subset of AML patients [126]. More recently, the role of WNT5a in the niche has been explored. It was shown that both HSCs and BCR-ABL LSCs are impaired in engrafting into a WNT5a-deficient niche [127]. Furthermore, transplanting LSCs exposed to a WNT5a-deficient niche reduces the incidence of leukemia in mice, as well as the ability to propagate leukemia to secondary recipient mice [127]. A more recent study has confirmed a role for both canonical and noncanonical WNT signaling in leukemia, showing in vivo that the canonical WNT signaling pathway is upregulated in the leukemic HSPC compartment at the expense of a downregulated WNT5a signaling axis [128]. Altogether, it is evident that myeloid LSCs, like HSCs, rely on WNT signaling for functionality, but that different levels of WNT/ β -catenin signaling may be required to promote HSC and LSC function.

1.11 The NOTCH Pathway in HSCs and LSCs

The NOTCH pathway is best characterized for its crucial role in embryonic development and in HSC emergence during embryogenesis. However, the efforts to elucidate the roles of NOTCH signaling in adult hematopoiesis and HSC maintenance have expanded in an effort to understand how dysregulated NOTCH signaling affects the leukemic state (Fig. 1.4, Table 1.3). Within this signaling network is a family of four NOTCH receptors expressed on the cell surface that can interact with one of five ligands (Jagged 1, Jagged 2, Delta 1, Delta 2, and Delta 3), which are presented on the surface of a neighboring cell (reviewed in Pajcini et al. [129]). Ligand binding results in cleavage of the intracellular domain of the NOTCH receptor by a γ -secretase complex to allow this portion of the receptor to translocate into the nucleus. In the nucleus, the intracellular domain of NOTCH (NICD) can interact with co-activator proteins such as CBF-1 and MAM1, resulting in transcriptional activation of NOTCH target genes, such as the HES family [129].

NOTCH signaling has been shown to play a stimulatory role in adult HSCs, and promotes HSC expansion. Notably, constitutive activation of NOTCH1 signaling via expression of NICD in hematopoietic cells results in immortalized, cytokine-dependent HSCs capable of differentiating into both myeloid and lymphoid progeny [130]. This result was confirmed by additional work showing that expression of NICD in vivo resulted in HSC expansion and self-renewal at the expense of differentiation [131]. Several groups have shown that NOTCH pathway activation also leads to expansion of the hematopoietic progenitor compartment. For example, Karanu et al. showed that adding soluble forms of the human ligands Delta 1 and Delta 4 results in increased proliferation of hematopoietic progenitors in vivo, and transplantation of Jagged 1 or Delta 1 into immunodeficient mice expands HSPCs that are capable of pluripotent hematopoietic reconstitution [132, 133]. Supporting this work, it was shown in transgenic *Notch* reporter mice that NOTCH signaling is upregulated in HSCs, but that this signaling is lost upon lineage commitment [134]. Moreover, inhibiting NOTCH signaling was able to accelerate the differentiation of

HSCs *in vitro*, and this inhibition resulted in the depletion of HSCs *in vivo* [134]. Thus, activation of the NOTCH pathway, either through overexpression of NICD or by exogenous ligands, promotes HSC expansion and self-renewal.

Surprisingly, examination of the role of NOTCH through genetic deletion has suggested that NOTCH signaling is not required for adult HSC function. It has been shown that genetic deletion of *Notch1* does not affect the reconstitution capacity of HSCs, though it does lead to a block in T-cell development [135]. Studying the effects of *Notch2* deletion in the hematopoietic context has also shown that HSC function is NOTCH-independent [136]. However, *Notch2*-deficient mice have impaired hematopoietic recovery after 5-fluorouracil (5-FU), suggesting a requirement for NOTCH signaling for HSC function during stress hematopoiesis [136]. In addition, NOTCH may regulate HSC function through the niche. For example, osteoblasts within the niche express high levels of JAGGED 1, correlating with an increase in HSC number *in vivo* [137]. In addition, endothelial cells within the niche promote the proliferation and survival of LT-HSCs via expression of NOTCH

Fig. 1.4 NOTCH signaling in HSCs and LSCs

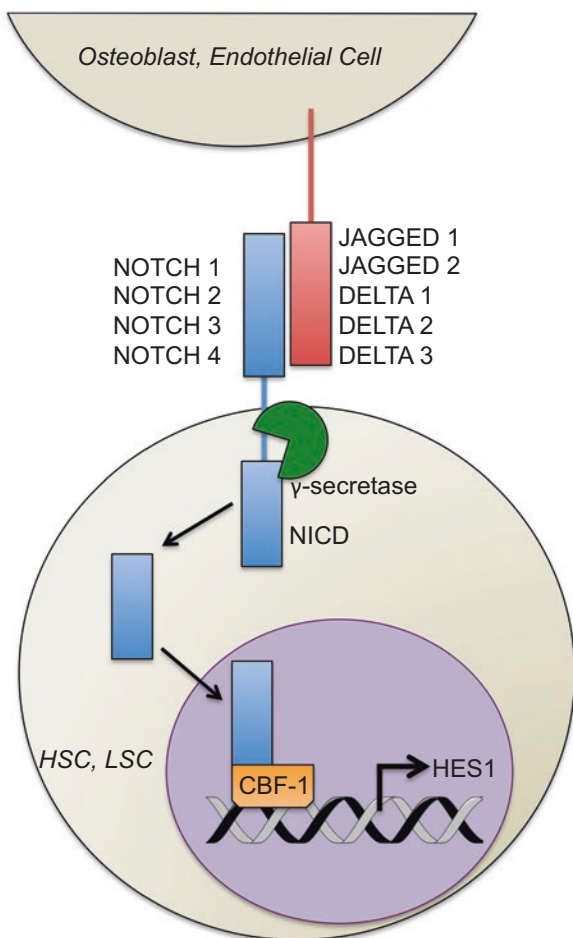


Table 1.3 HSC and LSC phenotypes resulting from genetic alterations in the NOTCH pathway

Gene	Alteration	Phenotype	References
<i>Jagged 1/ Delta 1</i>	Soluble ligand administration	HSC expansion and enhanced hematopoietic reconstitution	Karanu et al. [132, 133]
	Osteoblast ablation	Expands LT-HSC population with impaired self-renewal capacity Accelerated leukemia in CML murine model	Bowers et al. [149]
<i>Notch 1</i>	Deletion	HSC reconstitution intact, although blockage of T-cell development	Radtke et al. [135]
<i>Notch 2</i>	Deletion	HSC reconstitution impaired only under 5-FU stress conditions	Varnum-Finney et al. [136]
<i>Notch 1/2</i>	Overexpression or cKI of constitutive active ICD	Immortalization of HSCs capable of both myeloid and lymphoid differentiation	Varnum-Finney et al. [130]
		HSC expansion and increased self-renewal at the expense of differentiation	Stier et al. [131]
		Growth arrest of AML cell lines, decreased AML engraftment	Kannan et al. [143]
		Decreased disease burden and LSC function in MLL-AF9 murine model of AML	Lobry et al. [139]
		Inhibits proliferation of CML K562 cell line	Yin et al. [148]
<i>Maml</i>	Dominant negative MAML expression	HSC expansion and increased self-renewal at the expense of differentiation	Kannan et al. [143]
<i>Nicastrin</i>	cKO (Mx1-Cre; Vav-iCre)	CMML-like disease	Klinakis et al. [141]
<i>Cbf-1</i>	Dominant negative	Retroviral expression accelerated HSC myeloid differentiation	Duncan et al. [134]
<i>Hes1</i>	Deletion	Accelerates development of leukemia in MLL-AF9 model	Kato et al. [144]
	Overexpression	Immortalizes CMPs and GMPs	Nakahara et al. [146]
		Promotes progression of CML to blast crisis in BCR-ABL murine model Decreased AML engraftment	Kannan et al. [143]

ligands *in vivo*, further suggesting a role for NOTCH signaling dependence for HSC function [138].

The roles of the NOTCH pathway in LSCs have been controversial, as NOTCH signaling has been reported to play both a tumor suppressor and oncogenic role in LSCs – depending on the lineage context. The roles of NOTCH signaling in LSCs have been extensively studied in the context of T-ALL and CLL, in which NOTCH signaling is considered oncogenic (reviewed in Lobry et al. [139]). Work is still

being done to fully elucidate the role of the NOTCH pathway in myeloid LSCs. However, several groups have begun to unravel that NOTCH signaling may play a tumor suppressor role in the myeloid context [140–143]. NOTCH signaling has an accepted role in promoting myeloid differentiation by upregulation of the transcription factor PU.1 [140]. It was shown that *Notch 1* expression is downregulated in AML cell lines and in primary patient blasts, and this downregulation correlates with a decrease in PU.1-mediated differentiation, suggesting that NOTCH signaling may be responsible for maintaining the immature myeloid compartment seen in leukemia [140]. Furthermore, data suggests that induced activation of the NOTCH signaling axis in AML in vivo results in cell growth arrest, differentiation, and apoptosis [142]. Consistent with this, activation of NOTCH signaling in AML by NOTCH ICN1 expression in vivo decreases AML cell proliferation, and this phenotype is reversed through the inhibition of NOTCH signaling using a dominant-negative MAML construct [143]. Additionally, inactivation of NOTCH signaling in HSCs in vivo can promote a chronic myelomonocytic leukemia (CMML)-like phenotype, further supporting a tumor-suppressive function [141]. Additionally, recent work has shown that the NOTCH target HES1 binds to the FLT3 promoter to suppress its transcription, and loss of HES1 results in AML development in vivo [144]. Importantly, in MLL-AF9 leukemic cells, FLT3 was significantly upregulated upon HES1 deletion [144]. However, the role of NOTCH signaling as a tumor suppressor pathway in AML LSCs was recently challenged by results revealing that crosstalk between the WNT and NOTCH signaling pathways in osteoblasts of the bone marrow results in activation of NOTCH signaling between osteoblasts and HSPCs, actually promoting leukemic transformation [145]. Thus, more work is still needed to fully elucidate the roles of NOTCH signaling in AML LSCs.

There is also evidence to suggest that the NOTCH signaling pathway plays a role in CML LSCs, although the specific effects of NOTCH in this context also require further exploration. For example, induced expression of *Hes1* was shown to cooperate with BCR-ABL to induce blast crisis CML in vivo [146]. Importantly, HES1 is highly expressed in CML patients in blast crisis, suggesting its potential role in the progression from chronic phase to blast phase in CML [146]. In addition, genetic deletion of the transcription factor *Twist1* in the microenvironment activates the NOTCH signaling pathway via upregulation of *Jagged 2*, and this activation is required for MLL-AF9-induced AML [147]. In contrast, it was shown that overexpression of NICD was sufficient to inhibit the proliferation of the CML blast crisis cell line K562 [148]. A tumor-suppressive role for NOTCH has also been demonstrated within the CML niche. Specifically, ablation of osteoblasts in a CML transgenic mouse model results in accelerated development of CML [149]. Since it was shown that JAGGED 1 levels were increased in CML osteoblasts, this group went on to show that the addition of JAGGED 1 to CML LSCs reduced their cycling, thus supporting the hypothesis that NOTCH signaling promotes an antileukemic environment [149]. However, while it is clear that NOTCH signaling is important in normal HSC and myeloid LSC function, understanding and deciphering the contexts in which it promotes or suppresses the leukemic state still remains an active area of research.

1.12 The TGF β Pathway in HSCs and LSCs

The TGF β pathway is known to play an important role in controlling HSC function, but its potential role in myeloid LSCs has only been explored within the last decade (Fig. 1.5, Table 1.4). Recently reviewed by Vaidya and Kale [150], the TGF β family consists of three ligands – TGF β 1, TGF β 2, and TGF β 3 – all of which signal through type I and type II serine/threonine kinase membrane receptors [150]. The primary type I receptor for TGF β is ALK5/T β RI and the sole type II receptor for TGF β is T β RII. In short, TGF β is found in its dormant form within the extracellular matrix, but upon cleavage of its pro-domain, it is able to bind to a T β RII homodimer. T β RII is then able to multimerize with a T β RI homodimer, resulting in subsequent phosphorylation of T β RI. After the required receptor transphosphorylation, the

Bone Marrow Stroma

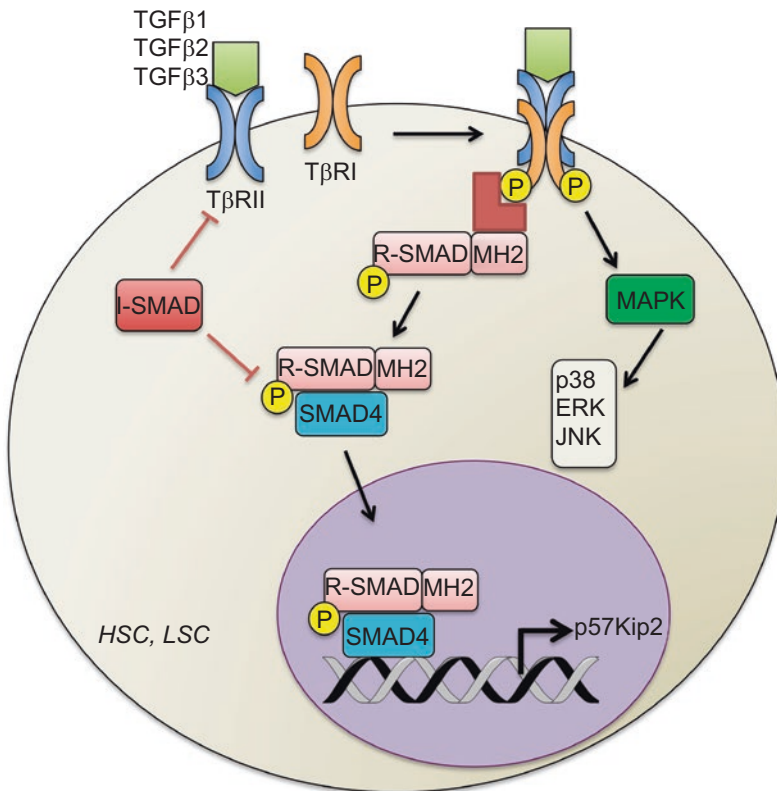


Fig. 1.5 Summary of TGF β pathway activation in HSCs and LSCs

Table 1.4 HSC and LSC phenotypes resulting from genetic alterations in the TGF β pathway

Gene	Alteration	Phenotype	References
<i>TBRI</i>	cKO (Mx1-Cre)	No effect on HSC self-renewal, differentiation, or long-term repopulation capacity	Larsson et al. [162]
	Pharmacological inhibition	Inhibitors Ly364947 and SD208 suppress CML LSC colony formation	Naka et al. [96]
		Combination of TGF β inhibition and imatinib decreases CML LSC function	
		Inhibitor SB431542 sensitizes CML cell lines to imatinib treatment by reducing LYN kinase turnover	Smith et al. [175]
		Combination of TGF β -neutralizing antibody 1D11 and CXCR4 inhibitor in FLT3-mutated AML model decreases leukemic burden and prolongs survival	Tabe et al. [176]
Tgf β 1	KO	Homozygous mice are embryonic lethal with increased inflammatory response	Kulkarni et al. [160]
Tgf β 1	cKO in megakaryocytes (Pf4-Cre)	HSC activation and proliferation	Zhao et al. [152]
<i>TBRII</i>	KO	Homozygous mice are embryonic lethal with increased inflammatory response	Oshima et al. [161]
<i>TBRII</i>	cKO (Mx1-Cre)	Increased HSC cycling and reduces long-term repopulation capacity	Yamazaki et al. [163]
SMAD 4	cKO (Mx1-Cre)	Impaired HSC self-renewal and reconstitution capacity	Karlsson et al. [153]
		Deletion of SMAD 4 in <i>HOXA9</i> - and <i>NUP98</i> - <i>HOXA9</i> -induced AML model increases LSC frequency	Quere et al. [174]
<i>SMAD 7</i>	Retroviral overexpression	Increased HSC self-renewal, but no impact on reconstitution or differentiation capacities	Blank et al. [164]
p57Kip2	cKO (Mx1-Cre)	Impaired HSC self-renewal and reconstitution capacity	Matsumoto et al. [166]

canonical downstream TGF β pathway involving the SMAD transcription factors is activated. The SMAD family consists of five receptor-mediated SMADs (R-SMADs 1, 2, 3, 5, and 8), one common-partner SMAD (Co-SMAD 4), and two inhibitory SMADs (I-SMADs 6 and 7). The R-SMADs contain MH2 domains that can bind via adaptor proteins to activated T β RI receptors. This interaction results in the phosphorylation of the R-SMAD, which is now able to dissociate and bind to SMAD 4. This heterodimeric complex is able to translocate into the nucleus to regulate the transcription of TGF β target genes. The inhibitory SMADs turn off TGF β signaling by either dephosphorylating or recruiting E3 ligases to T β RI or by competitively binding to SMAD 4. TGF β can also signal through a SMAD-independent pathway, specifically by activating the MAPK pathway and subsequent ERK, JNK, and/or

p38 activity [150]. TGF β is expressed by both hematopoietic and bone marrow stromal cells [151], as well as by other niche components, such as megakaryocytes [152]. It has also been shown that both T β RI and T β RII are expressed on hematopoietic cells, and they can induce SMAD activity [153]. Thus, HSCs can both express TGF β and respond to TGF β signaling.

It has been well documented that TGF β is an inhibitor of HSC proliferation and an inducer of HSC quiescence [154–158]. In addition, it was reported that *Tgfb1*-null mice have enhanced myelopoiesis [159]. Due to this profound regulatory role of the TGF β pathway, many groups have examined the effect of deleting its components in HSCs. It is evident that TGF β plays a crucial role in embryonic development, as both *Tgfb1* and *Tgfb2* knockout mice have an embryonic lethal phenotype [160, 161]. While conditional deletion of *T β RI* in vivo does not impact HSC self-renewal or long-term repopulating capacity [162], deletion of *T β RII* results in impaired HSC long-term repopulation [163]. Consistent with this finding, conditional deletion of *Smad 4* also results in impaired HSC self-renewal and reconstitution capacity [153]. In an attempt to remove the major source of TGF β from HSCs, Zhao et al. depleted megakaryocytes, which resulted in the loss of HSC quiescence and subsequent HSC proliferation [152]. Importantly, these HSCs had reduced SMAD 2 and SMAD 3 phosphorylation, supporting the hypothesis that megakaryocyte ablation is sufficient to reduce TGF β signaling in HSCs. This loss of HSC quiescence phenotype could be rescued by injecting TGF β 1 into megakaryocyte-depleted mice. Furthermore, conditional deletion of *TGF β 1* in megakaryocytes induced the same HSC proliferation phenotype as megakaryocyte ablation [152].

Several groups have investigated the mechanisms by which TGF β signaling controls HSC quiescence and self-renewal. Most evidence suggests a SMAD-dependent mechanism, as seen in one study in which the inhibitory SMAD 7 was overexpressed in murine HSCs, leading to an increase in their self-renewal capacity [164]. Furthermore, the TGF β -SMAD-p57Kip2 axis has been shown to be essential for inducing HSC quiescence. Expression of p57Kip2 increases upon TGF β stimulation in primary human hematopoietic cells, and this upregulation is essential to drive cell cycle arrest [165]. It was later shown that quiescent murine CD34- HSCs exhibit both increased SMAD 2/SMAD 3 and p57Kip2 activity as opposed to their cycling CD34+ counterparts [163], further suggesting that SMAD-dependent TGF β signaling is required to maintain a dormant HSC population. In support of this, conditional deletion of p57 was shown to impair HSC self-renewal and reduce the size of the LSK population [166]. Additionally, Musashi 2 (MSI2), an RNA-binding protein that is important for HSC function and myeloid malignancies, was shown to maintain HSC function and regulate HSC self-renewal fate decisions by regulating TGF β -SMAD 2/3-p57Kip2 [167]. *Msi2*-deficient HSCs have reduced reconstitution capability due to enhanced myeloid differentiation at the expense of HSC maintenance, and this is accompanied by decreased TGF β -SMAD 2/3-p57Kip2 signaling [167]. The TGF β -SMAD-p57 axis has also been implemented in restoring HSC quiescence after stress-induced cycling. After 5-FU treatment, murine HSCs exhibit a transient increase in TGF β expression that correlates with the amount of time required for HSCs to reenter a quiescent state [168]. In summary, there is clear evi-

dence that the TGF β -SMAD-p57Kip2 signaling axis is required for HSC quiescence.

Furthermore, recent evidence suggests that the tyrosine phosphatase SHP-1 is essential for activating the TGF β pathway in HSCs to induce quiescence, raising the possibility that the TGF β -SMAD-p57Kip2 axis requires upstream SHP-1 activity. Specifically, it was shown that SHP-1 physically interacts with T β RI to potentiate downstream signaling, and SHP-1-depleted HSCs lose their quiescence and subsequently enter cycling until exhaustion [169]. As expected, these *Shp-1*-depleted HSCs have impaired long-term self-renewal, and, importantly, they are incapable of becoming quiescent upon TGF β stimulation [169]. While more work is required to delineate all the possible mechanisms by which TGF β controls HSC function, it is evident that a TGF β -induced SMAD-dependent mechanism mediates HSC quiescence and cycling.

Despite the apparent role that TGF β plays in regulating HSC function, very little information is known about how the TGF β pathway regulates LSCs. As expected by its accepted role in limiting HSC proliferation, there is evidence that TGF β acts as a tumor suppressor. The cyclin-dependent kinase p27 is critical for regulating the cell cycle of hematopoietic cells, and one growth factor that regulates p27 expression is TGF β [170]. It was shown that BCR-ABL-expressing cells have decreased expression of p27 and are incapable of responding to TGF β stimulation, suggesting that the BCR-ABL fusion protein functions to turn off TGF β signaling to maintain a proliferative advantage [170]. It was previously reported that loss of the transcription factor JunB in HSCs is adequate to transform HSCs and induce myeloid malignancies in vivo [171, 172]. One possible mechanism for this phenotype is that loss of JunB renders HSCs incapable of responding to TGF β stimulation due to dysregulation of the JunB downstream target gene *Hes1*, suggesting a role for the antiproliferative effects induced by the TGF β pathway in HSCs in protection against myeloid malignancies [173].

The SMADs have also been shown to play tumor suppressor roles. For example, it was shown that HOXA9, a key transcriptional regulator of HSC self-renewal that is commonly dysregulated in AML, can be stabilized in the cytoplasm by SMAD 4, thereby losing its ability to translocate into the nucleus and mediate events required for leukemic transformation [174]. Importantly, transplanting SMAD 4-deficient HSCs transduced with the oncogenic NUP98-HOXA9 fusion into mice resulted in rapid onset of a more pronounced myeloproliferative disease, and secondary transplantation of these cells displayed a competitive advantage compared to wild-type cells, with faster development of leukemia. Notably, reintroducing the SMAD 4 domain required for HOXA9 cytoplasmic stabilization induced apoptosis of the HOXA9-transduced cells in vitro and limited their ability to initiate leukemia in vivo [174]. In summary, it is plausible that TGF β plays a protective role in myeloid LSCs by regulating downstream parallel pathways that are essential for regulating proliferation.

However, other studies have suggested that TGF β plays an oncogenic role in LSCs. In one study, TGF β was shown to be a critical regulator of AKT activity, and

AKT-dependent suppression of active nuclear FOXO3a is required for eliminating CML LSCs [96]. Importantly, the combination of inhibiting TGF β signaling and imatinib was sufficient to impair CML transformation, and this was shown to be due to decreased nuclear FOXO levels [96]. It has also been suggested that TGF β can regulate the activity of LYN kinase to protect CML cells from imatinib, and inhibiting the TGF β pathway can suppress LYN kinase activity and subsequently sensitize CML cells to imatinib treatment [175]. In AML, examining the role of TGF β in the microenvironment has shown that the hypoxic bone marrow microenvironment reinforced by leukemic cells stimulates the TGF β pathway, which in turn induces the expression of CXCR4 – a critical factor involved in promoting the survival of resident chemoresistant LSCs [176]. Furthermore, the inhibition of both TGF β and CXCR4 in a FLT3-mutated AML model was sufficient to decrease the leukemic burden and prolong survival [176]. Lastly, it has recently been suggested that several different isoforms of T β RII play a role in AML. The T β RII splice variant T β RII-B is expressed in normal hematopoietic cells and functions to promote apoptosis and differentiation of AML cells [177]. However, the canonical T β RII receptor, which is the isoform primarily expressed in AML cells, inhibits the ability of T β RII-B to mediate its tumor suppressor role *in vivo*, overall blocking the differentiation of the leukemic cells [177]. This further complicates the potential role that the TGF β pathway is playing in LSC function, displaying the necessity for further exploring the TGF β pathway in the leukemic context.

1.13 Concluding Remarks

It is evident that proper signaling through the PI3K, WNT/ β -catenin, NOTCH, and TGF β pathways is fundamental for regulating the fate decisions and proliferation of HSCs. With the frontline treatment of AML still being chemotherapy and stem cell transplantation for most patients, and given the resistance observed in CML patients to targeted therapies like imatinib, it is critical that new treatment options become available to fully eradicate LSCs. Key advances in considering new therapeutic avenues include the emerging field of studying the characteristics of the LSC, as well as the acceptance of a pre-LSC that has the ability to contribute to normal hematopoiesis, while accumulating key mutations that eventually push it toward the threshold of transforming to a LSC. Mutations in components of each of these signaling pathways are common in myeloid leukemia, further validating studying these signaling pathways in the context of regulating the function of the LSC.

A classic “two-hit” model for the accumulation of mutations in AML to generate a fully functional LSC posits that two distinct types of mutations are required for full transformation (Fig. 1.1) [178]. First, a mutation in a gene critical for regulating HSC differentiation and self-renewal decisions is required. Traditionally, genes within this class were thought to be transcription factors, but it is now understood that alterations in multiple other types of genes, including splicing factors and epigenetic regulators, could also serve a similar function. This type of mutation will

confer properties of self-renewal to the LSC at the expense of differentiation. Second, the LSC also requires an activating mutation in a signaling pathway essential for regulating HSC proliferation. This mutation will confer a proliferative and survival advantage to the LSC. This requirement for second mutations, the so-called “two-hit hypothesis”, has been supported by multiple studies. For example, it was shown that signaling mutations alone, such as BCR-ABL and FLT3-ITD, are not sufficient to impair LSC differentiation and confer self-renewal, suggesting that the proliferative advantage alone is not adequate to generate a fully functional LSC [179]. On the other hand, mutations and/or rearrangements in transcription factors, such as AML1-ETO or CBFβ-MYH11, are sufficient to confer self-renewal properties to LSCs, but insufficient to trigger leukemogenesis without additional mutations [177, 180, 181]. Given this, since the PI3K, WNT/β-catenin, NOTCH, and TGFβ signaling pathways play essential roles in regulating HSC cycling and self-renewal decisions, mutations in components of either of these pathways can directly impact the functionality of the LSC.

Epigenetic mutations also play an essential role in leukemia development at the stem cell level. However, their potential cooperation with signaling pathway mutations is often overlooked. Mutations in enzymes that affect DNA methylation or chromatin modifications, including TET2, DNMT3a, IDH1/2, and ASXL1/2, are considered to be “initiating” mutations, which are acquired at or before the pre-LSC stage (reviewed in Corces-Zimmerman et al. [1]). It is clear how such mutations may cooperate with mutations in transcription factors in leukemogenesis, since these epigenetic modifiers function in establishing the genomic landscape required for the recruitment of transcription factors. However, little has been explored regarding how epigenetic mutations cooperate with signaling mutations in LSCs. The “two-hit” model of leukemogenesis still helps us to understand the requirements for leukemic transformation. However, perhaps it would be helpful to think of these two, or more, classes of mutations not as simply additive, but as potentially synergistic in promoting hematopoietic transformation. The specific nonrandom patterns of co-occurrence of genetic alterations in AML, such as AML1-ETO with C-KIT mutations, or MLL rearrangements with RAS mutations [182], could provide clues about such potential synergistic relationships that should be further explored. For example, in other tumors, evidence for the direct regulation of epigenetic modifying enzymes by PI3K/AKT signaling is emerging (reviewed in Spangle et al. [183]). Thus, it is necessary to explore the interactions between signaling pathways and epigenetics in the context of LSCs, ultimately with the goal of refining the standard of care for myeloid leukemia patients to prevent relapse.

Lastly, given that the PI3K, WNT/β-catenin, NOTCH, and TGFβ pathways all play important roles in regulating HSC self-renewal and proliferation, it is not surprising that there would be crosstalk between these pathways. One example of crosstalk in other cell types includes the NOTCH and PI3K pathways, in which NOTCH signaling requires activation of the PI3K/AKT pathway to induce megakaryocyte differentiation [184]. A second example is between the PI3K and WNT pathways, as it has been shown that β-catenin is a direct substrate of AKT [185]. Lastly, it has been demonstrated extensively that PI3K/AKT signaling is a

SMAD-independent pathway that can be activated by TGF β . Exploring this cross-talk in the context of LSCs may help to elucidate the contradictory role of TGF β in LSCs. Although such crosstalk between signaling pathways has been studied in hematopoietic cells and in other tissues, it may be different in LSCs, and it is crucial to decipher these targetable differences. In summary, the PI3K, WNT/ β -catenin, NOTCH, and TGF β pathways are all important signaling pathways in HSCs, but with distinct roles in LSCs in some contexts. Further characterization of these pathways in the context of LSCs is important for understanding leukemogenesis and for developing new treatment paradigms with a safe therapeutic window.

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