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Leukemia Stem Cells in Hematologic Malignancies

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Leukemia Stem Cells in Hematologic Malignancies

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Preface

In many types of cancer, a curative therapy requires eradication of cancer-initiating (stem) cells, and the success relies on a full understanding of survival mechanisms for these stem cells. In this approach, it is equally important to minimize an unwanted effect on normal stem cell counterparts. To realize these goals, a key initial step is to identify and functionally characterize critical target genes and study their associated molecular pathways in cancer stem cells. Relative to solid tumors, a significant number of blood cancers are believed to be derived from leukemic stem cells (LSCs) and responsible for disease progression, relapse, and drug resistance. Therefore, new therapeutic strategies need to be developed by focusing on a complete eradication of LSCs. In this book, we review our current understanding of the biology of LSCs and emphasize the necessity and importance of targeting LSCs in the treatment of hematopoietic malignancies.

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Chapter 1
Signaling Pathways in Leukemic Stem Cells

Lindsay M. Gurska, Kristina Ames, and Kira Gritsman

Contents

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 TGFβ 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 · TGFβ

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-3β	Glycogen synthase kinase-3β
Hes-1	Hairy and enhancer of split-1
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IKK	IκB 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
TGFβ	Transforming growth factor-beta
TKI	Tyrosine kinase inhibitor
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
TβRI	TGFβ-type I receptor
TβRII	TGFβ-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 TGFβ 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 TGFβ 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.

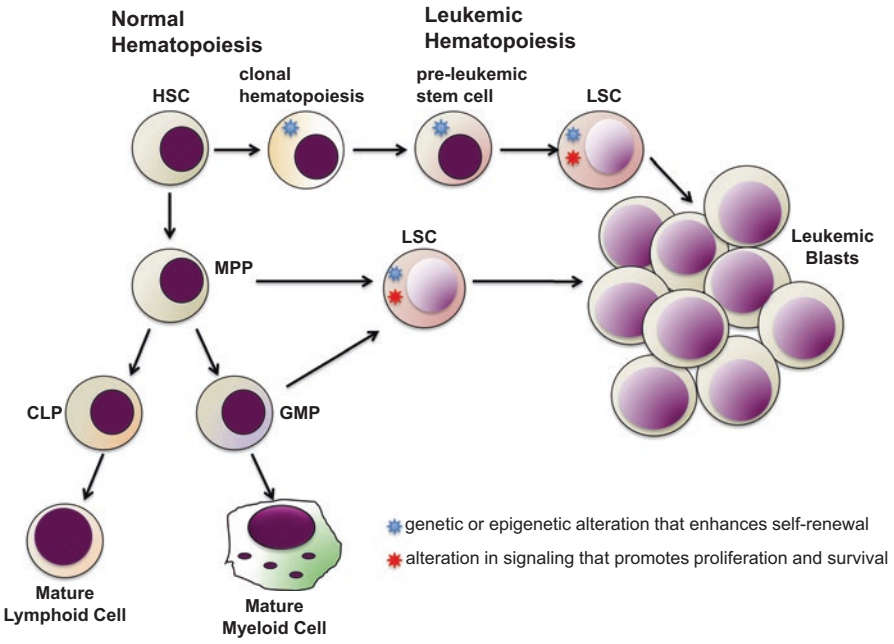

Fig. 1.1 Normal and leukemic hematopoiesis

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 (p110α, p110β, and p110δ), which are encoded by three separate genes (*PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively), and one Class 1B isoform p110ɣ, encoded by the *PIK3CG* gene. The regulatory subunits are encoded by different genes and diversified by alternative promoter usage as follows: *PIK3R1* encodes p85α and the splice variants p55α and p50α, while *PIK3R2* encodes the p85β subunit, *PIK3R3* encodes p55γ, *PIK3R5* gene encodes p101, and *PIK3R6* encodes the p84/p87regulatory subunit. Class 1A p85 regulatory subunits (p85, p55, p50) harbor two SRC homology 2 domains (nSH2, cSH2) and an intervening p110-binding

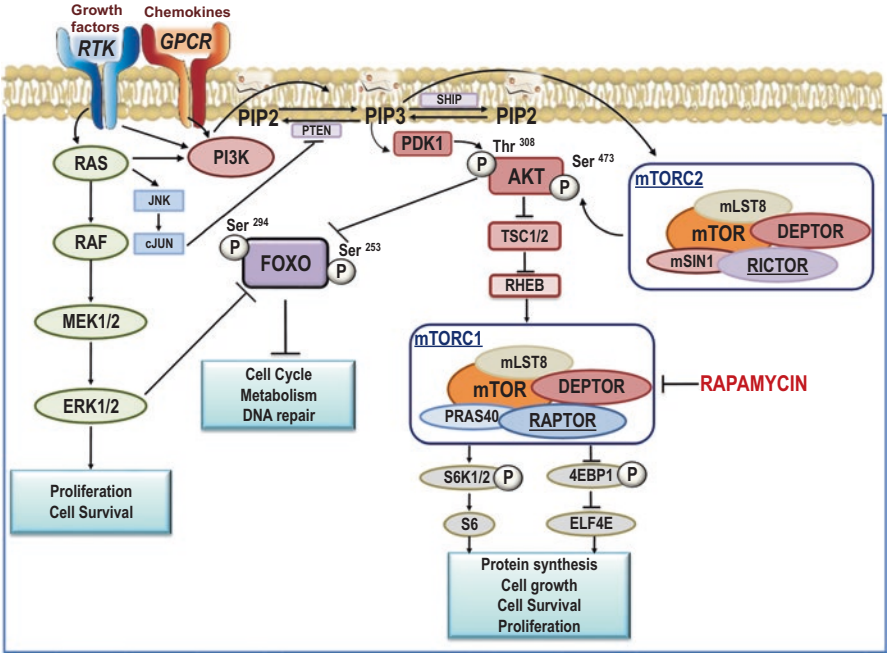

Fig. 1.2 The PI3K/AKT/mTOR and RAS/MEK/ERK signaling cascades and their main downstream effectors

region (iSH2) and constitutively interact with the p110α, p110β, and p110δ 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 Class1B catalytic subunit p110γ does not have a p85-binding domain and is almost exclusively activated by GPCRs. The Class I PI3Ks p110α, p110δ, and p110γ also harbor a RAS-binding domain and all except p110β are thought to be RAS effectors [5]. In contrast, p110β 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 p110α and p110δ isoforms primarily transduce RTK signals, p110β and p110ɣ play a less prominent role in RTK signaling and are functionally redundant in signaling downstream of GPCRs [9]. P110α is essential for growth factor signaling and oncogenic signaling

in fibroblasts [44]. However, functional redundancy between p110α and p110β in transducing RTK signals has also been described in some contexts [45, 46]. Surprisingly, postnatal conditional deletion of p110α in the hematopoietic system results only in mild anemia, without affecting HSC self-renewal, while conditional deletion of p110β in hematopoietic cells does not affect blood counts (Table 1.1) [47, 48]. In fetal liver HSCs, compound deletion of the regulatory subunits p85α and p85β 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 p85α, but not of p85β, 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 p110α 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 p110α 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, p110β was the most important in promoting myeloid leukemogenesis, though a *Rac*-dependent mechanism [48]. Importantly, deletion of p110β 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 p110β [54]. Furthermore, this study showed that a p110β-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
Pik3r1 and Pik3r2	KO	Decreased repopulating ability of fetal liver HSCs	Haneline et al. [49]
Akt	myr-AKT BMT model	Constitutively active AKT depletes HSCs and induces myeloproliferation, AML, and T-ALL	Kharas et al. [58]
Akt1/ Akt2	KO	Compound deletion of Akt1 and Akt2 impairs fetal HSC repopulation	Juntilla et al. [59]
Pik3ca	cKO (Mx1-Cre)	No effect on HSC self-renewal	Gritsman et al. [47]
		Increased survival in $KRAS^{G12D}$JMML model	
Pten	cKO	Pten deletion using Mx1-Cre perturbed HSC function, myeloproliferation, AML, and T-ALL when Pten 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]
Pik3cb	cKO (Mx1-Cre)	p110β deletion prevents myeloproliferative neoplasia in context of Pten deletion and improves HSC function	Yuzugullu et al. [48]
Ship	KO	Myeloproliferative phenotype, hypersensitivity to growth factors	Helgason et al. [70]
Pdk1	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]
TSC1	cKO (Mx1-Cre)	Increased HSC cycling and impaired self-renewal	Chen et al. [79]
mTOR	cKO(Mx1-Cre)	Loss of HSC quiescence	Guo et al. [78]
Rictor	cKO (Mx1-Cre/ Vav1-iCre)	No effect on HSC function	Kalaitzidis et al. [74] and Magee et al. [77]
		Improved survival in Pten−/− model of leukemia	
Raptor	cKO (Mx1-Cre)	Impaired hematopoietic reconstitution	Kalaitzidis et al. [74]
		Improved survival in Pten−/− model of leukemia	
Foxo	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]
		Foxo3a is important for maintenance of CML LICs	Naka et al. [96]
	cKO (Mx1-Cre)	*Foxo1/3/4* deletion induces differentiation of AML cells	Sykes et al. [97]
S6k1	KO	*S6k1* 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.

Kalaitizidis 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 Rimmele 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 IκB 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 "crosstalk 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-3β, 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

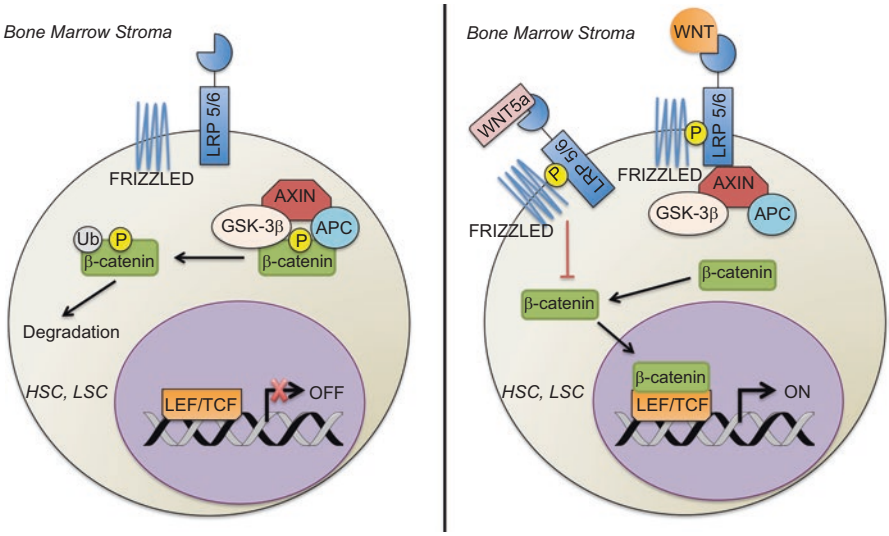

Fig. 1.3 Summary of WNT/β-catenin pathway activation in HSCs and LSCs

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
Ctnnb1	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]
Apc	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]
Lef/Tcf	*Lef* cKO (Vav1-iCre)	Impaired LSC self-renewal in CML model	Yu et al. [118]
	Tcf germline KO	Normal HSC self-renewal	
Wnt5a	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 *Wnt5a* allele leads to spontaneous CML or B cell lymphoma development	Liang et al. [125]
	Haploinsufficiency in the bone marrow niche	Impaired HSC engraftment	Schreck et al. [127]
		Impaired leukemia engraftment in CML model	

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 *Ctnnb1*, 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

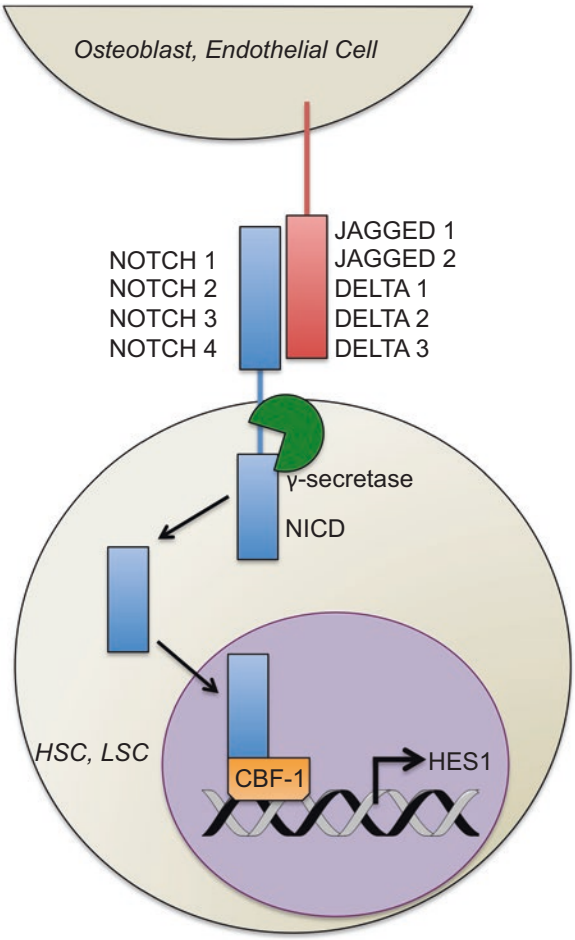

Fig. 1.4 NOTCH signaling in HSCs and LSCs

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

Gene	Alteration	Phenotype	References
Jagged 1/ Delta 1	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	Bowers et al. [149]
		Accelerated leukemia in CML murine model	
Notch 1	Deletion	HSC reconstitution intact, although blockage of T-cell development	Radtke et al. [135]
Notch 2	Deletion	HSC reconstitution impaired only under 5-FU stress conditions	Varnum-Finney et al. [136]
Notch 1/2	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]
Maml	Dominant negative MAML expression	HSC expansion and increased self-renewal at the expense of differentiation	Kannan et al. [143]
Nicastrin	cKO (Mx1-Cre; Vav-iCre)	CMML-like disease	Klinakis et al. [141]
Cbf-1	Dominant negative	Retroviral expression accelerated HSC myeloid differentiation	Duncan et al. [134]
Hes1	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 TGFβ Pathway in HSCs and LSCs

The TGFβ 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 TGFβ family consists of three ligands – TGFβ1, TGFβ2, and TGFβ3 – all of which signal through type I and type II serine/threonine kinase membrane receptors [150]. The primary type I receptor for TGFβ is ALK5/TβRI and the sole type II receptor for TGFβ is TβRII. In short, TGFβ is found in its dormant form within the extracellular matrix, but upon cleavage of its pro-domain, it is able to bind to a TβRII homodimer. TβRII is then able to multimerize with a TβRI homodimer, resulting in subsequent phosphorylation of TβRI. After the required receptor transphosphorylation, the

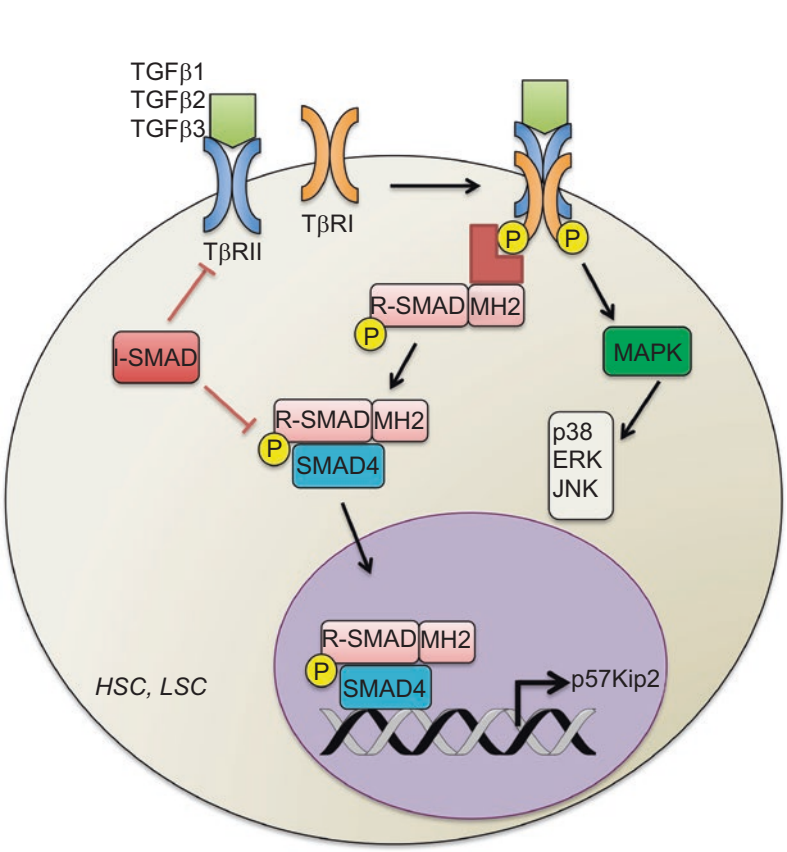

Fig. 1.5 Summary of TGFβ pathway activation in HSCs and LSCs

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

Gene	Alteration	Phenotype	References
TBRI	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 TGFβ 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 TGFβ-neutralizing antibody 1D11 and CXCR4 inhibitor in FLT3-mutated AML model decreases leukemic burden and prolongs survival	Tabe et al. [176]
Tgfβ1	KO	Homozygous mice are embryonic lethal with increased inflammatory response	Kulkarni et al. [160]
Tgfβ1	cKO in megakaryocytes (Pf4-Cre)	HSC activation and proliferation	Zhao et al. [152]
TBRII	KO	Homozygous mice are embryonic lethal with increased inflammatory response	Oshima et al. [161]
TBRII	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 *HOXA9*- and *NUP98-HOXA9*-induced AML model increases LSC frequency	Quere et al. [174]
SMAD 7	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 TGFβ 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 TβRI 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 TGFβ target genes. The inhibitory SMADs turn off TGFβ signaling by either dephosphorylating or recruiting E3 ligases to TβRI or by competitively binding to SMAD 4. TGFβ can also signal through a SMAD-independent pathway, specifically by activating the MAPK pathway and subsequent ERK, JNK, and/or

p38 activity [150]. TGFβ 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 TβRI and TβRII are expressed on hematopoietic cells, and they can induce SMAD activity [153]. Thus, HSCs can both express TGFβ and respond to TGFβ signaling.

It has been well documented that TGFβ is an inhibitor of HSC proliferation and an inducer of HSC quiescence [154–158]. In addition, it was reported that *Tgfβ1*-null mice have enhanced myelopoiesis [159]. Due to this profound regulatory role of the TGFβ pathway, many groups have examined the effect of deleting its components in HSCs. It is evident that TGFβ plays a crucial role in embryonic development, as both *Tgfβ1* and *Tgfβ2* knockout mice have an embryonic lethal phenotype [160, 161]. While conditional deletion of *TβRI* in vivo does not impact HSC self-renewal or long-term repopulating capacity [162], deletion of *TβRII* 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 TGFβ 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 TGFβ signaling in HSCs. This loss of HSC quiescence phenotype could be rescued by injecting TGFβ1 into megakaryocyte-depleted mice. Furthermore, conditional deletion of *TGFβ1* in megakaryocytes induced the same HSC proliferation phenotype as megakaryocyte ablation [152].

Several groups have investigated the mechanisms by which TGFβ 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 TGFβ-SMAD-p57Kip2 axis has been shown to be essential for inducing HSC quiescence. Expression of p57Kip2 increases upon TGFβ 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 TGFβ 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 TGFβ-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 TGFβ-SMAD 2/3-p57Kip2 signaling [167]. The TGFβ-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 TGFβ 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 TGFβ-SMAD-p57Kip2 signaling axis is required for HSC quiescence.

Furthermore, recent evidence suggests that the tyrosine phosphatase SHP-1 is essential for activating the TGFβ pathway in HSCs to induce quiescence, raising the possibility that the TGFβ-SMAD-p57Kip2 axis requires upstream SHP-1 activity. Specifically, it was shown that SHP-1 physically interacts with TβRI 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 TGFβ stimulation [169]. While more work is required to delineate all the possible mechanisms by which TGFβ controls HSC function, it is evident that a TGFβ-induced SMAD-dependent mechanism mediates HSC quiescence and cycling.

Despite the apparent role that TGFβ plays in regulating HSC function, very little information is known about how the TGFβ pathway regulates LSCs. As expected by its accepted role in limiting HSC proliferation, there is evidence that TGFβ 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 TGFβ [170]. It was shown that BCR-ABL-expressing cells have decreased expression of p27 and are incapable of responding to TGFβ stimulation, suggesting that the BCR-ABL fusion protein functions to turn off TGFβ 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 TGFβ stimulation due to dysregulation of the JunB downstream target gene *Hes1*, suggesting a role for the antiproliferative effects induced by the TGFβ 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 TGFβ plays a protective role in myeloid LSCs by regulating downstream parallel pathways that are essential for regulating proliferation.

However, other studies have suggested that TGFβ plays an oncogenic role in LSCs. In one study, TGFβ 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 TGFβ 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 TGFβ can regulate the activity of LYN kinase to protect CML cells from imatinib, and inhibiting the TGFβ pathway can suppress LYN kinase activity and subsequently sensitize CML cells to imatinib treatment [175]. In AML, examining the role of TGFβ in the microenvironment has shown that the hypoxic bone marrow microenvironment reinforced by leukemic cells stimulates the TGFβ 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 TGFβ 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 TβRII play a role in AML. The TβRII splice variant TβRII-B is expressed in normal hematopoietic cells and functions to promote apoptosis and differentiation of AML cells [177]. However, the canonical TβRII receptor, which is the isoform primarily expressed in AML cells, inhibits the ability of TβRII-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 TGFβ pathway is playing in LSC function, displaying the necessity for further exploring the TGFβ pathway in the leukemic context.

1.13 Concluding Remarks

It is evident that proper signaling through the PI3K, WNT/β-catenin, NOTCH, and TGFβ 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 CBFB-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 TGFβ. Exploring this crosstalk in the context of LSCs may help to elucidate the contradictory role of TGFβ 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 TGFβ 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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Chapter 2
Cellular and Molecular State of Myeloid Leukemia Stem Cells

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Contents

Abstract Leukemia stem cells (LSCs) are leukemia-initiating population with the capacity to self-renew, differentiate, and stay quiescent. Human hematopoietic malignancies such as chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) are derived from this cell population. LSCs are also responsible for disease relapse due to its resistance to drug treatment. This rare cell population is phenotypically and functionally heterogeneous. Increasing evidence indicates that this heterogeneous cellular state of LSCs might determine the different drug sensitivity and is the major reason for disease relapse. In here, focusing on myeloid leukemia stem cells, we describe the biological features including cellular and

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molecular state, heterogeneity of LSCs, and the dynamic cross talk between LSCs and bone marrow microenvironment. These specific features of LSCs highlight the dynamic cellular state of LSCs, and further exploring on it might provide potential therapeutic targets that are important for eliminating LSCs.

Keywords Leukemia stem cell · Cellular state · Molecular state · Heterogeneity · Bone marrow microenvironment

2.1 Introduction

Identification and characterization of the cells within a tumor that sustain the growth of the neoplastic clone is one of fundamental problems in the field of cancer research. The original stochastic model postulates that tumorigenesis occurs randomly and is governed by probabilities, and every single cell within tumor has a low but equal probability of proliferating extensively and being able to regrow the tumor. Base on this model, purification of a fraction enriched for tumor-initiating activity is not possible [1, 2]. By contrast, the cancer stem cell (CSC) model postulates that distinct classes of cells with different proliferation capacities exist in tumor, and only a rare definable population functions as CSCs that possess self-renewal and extensively proliferative potential, which are necessary to initiate a new tumor growth and regenerate the cellular hierarchy of tumors [3–5]. According to this model, it is possible to identify a cell population within a tumor that can predictably and reliably contain the capacity to regenerate the entire tumor.

Although the hypothesis of CSCs was first proposed over 50 years ago [4], the progress of CSC study has lagged until the past two decades, dependent on the development of the NOD/SCID xenotransplant assay, quantitative stem cell assays, and fluorescence-activated cell sorting technique (FACS). The first direct evidence for the existence of CSCs was derived by John Dick's laboratory in 1997 through the study of human acute myeloid leukemia (AML). Bone marrow-derived $CD34^+CD38^-$ cells were transplanted into NOD/SCID mice, and these cells could not only initiate AML in NOD/SCID mice but also differentiate in vivo into leukemic blasts [6]. More importantly, serial transplantation demonstrated that these cells have a capacity to self-renew and transfer AML disease into secondary recipients [6]. Therefore, this study showed for the first time that leukemia stem cells (LSCs) are exclusively $CD34^+CD38^-$ cells, which have the same cell-surface phenotype as normal human primitive cells. Subsequently, similar approaches were applied to solid tumors, leading to the identification of CSCs in multiple solid tumors, including the breast [7], brain [8], pancreas [9], colon [10], lung [11], and prostate [12]. Now, it is widely accepted that the existence of CSCs in cancer plays an essential role in cancer initiation, progression, and resistance to traditional chemotherapy or other treatments. Hematologic malignancies, in particular chronic myeloid leukemia (CML) and AML, have been served as important model diseases for CSC study [13, 14]. CML is a myeloproliferative disease that derives from an

abnormal hematopoietic stem cell (HSC) harboring the Philadelphia (Ph^+) chromosome [13, 15, 16]. This chromosomal abnormality is generated by a reciprocal translocation between chromosome 9 and 22 [t (9; 22)(q34; q11)], forming chimeric *BCR-ABL* fused oncogene. BCR-ABL oncoprotein encoding by this fused gene has a constitutive tyrosine kinase activity, consequently resulting in leukemogenesis [15, 17]. AML is a heterogeneous clonal disorder characterized by clonal expansion of myeloid blasts with blocked differentiation into mature cells [18]. It is widely accepted that additional genetic alterations successively occur during the development of AML. These genetic changes aberrantly regulate signaling pathways and influence the function of transcriptional factors, subsequently determining the pathological features of AML and affecting the responsiveness of patients to chemotherapy. An increasing number of recurring genetic lesions have been identified [18].

LSCs are leukemia-initiating population with the capacity to self-renew, differentiate, and stay a quiescent state [14, 19] and have been extensively investigated in both CML and AML fields (Fig. 2.1). Similar to normal hematopoietic stem cells (HSCs), upon dividing, LSCs can decide to self-renew, differentiate, or enter dormancy. Over the last few years, emphasis in the LSC field has shifted more toward

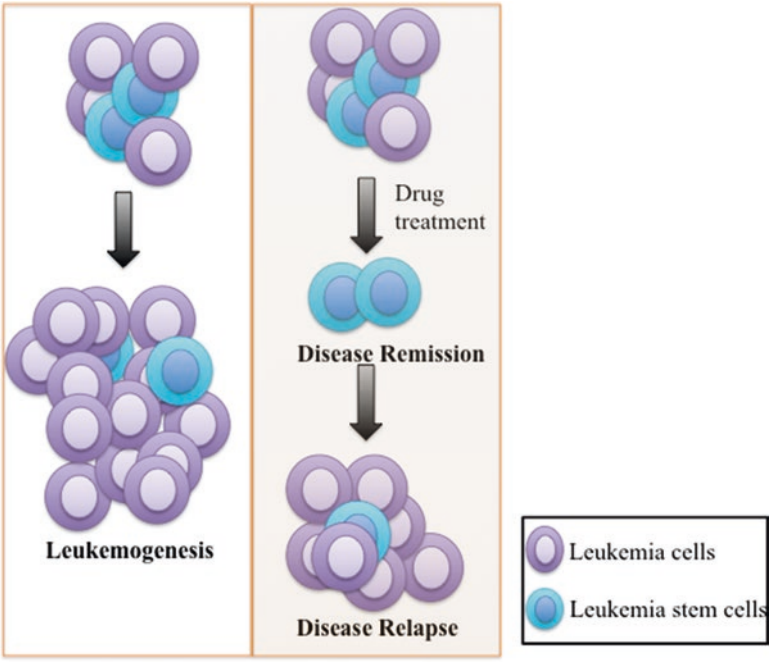

Fig. 2.1 LSCs are responsible for disease relapse. Normally, without treatment, genetic alterations such as BCR-ABL transform normal HSC and subsequently drive the expansion of leukemia cells. Drug treatment including imatinib or other TKIs blocks BCR-ABL activity and eliminates the proliferating leukemia cells. However, LSCs are resistant to TKI and survive and persistent under treatment. These LSCs lead to disease recurrence

understanding the cellular state and molecular mechanism of LSCs, including heterogeneity and drug sensitivity. Furthermore, there is increasing awareness about the critical role of microenvironment in regulating LSC survival. Here, through focusing on LSCs, we will review recent advancements in these topics, including the dynamic phenotypes of LSCs, the heterogeneity of LSCs, and the relationship between bone marrow microenvironment and LSCs.

2.2 Biological Features of LSCs

In the past decade, with self-renewal and multipotency at the hub of what defines a LSC, much research has been invested into understanding the underlying molecular and cellular processes.

2.2.1 Immunophenotype of LSCs

LSCs are generally characterized with surface immunological markers and validated by functional assays. In CML, functional LSCs in mice reside in a cell population that does not express cell lineage markers but express both c-Kit and Sca-1 ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$, LSK) [20], recapitulating cell-surface markers expressed on normal HSCs. LSCs in human CML also reside in the HSC population [21], displaying phenotypically $Lin^-CD34^+CD38^-CD90^+$ with some specific surface markers such as IL-1RAP and CD26 [22, 23]. LSCs of human AML are also considered as $CD34^+CD38^-$ cells, which phenotypically resemble normal human primitive hematopoietic cells [6]. Recent studies identified T cell immunoglobulin mucin-3 (TIM-3) as an AML LSC marker, as TIM-3 is more highly expressed on multiple specimens of AML LSCs than on normal bone marrow HSCs [24–26]. Additional markers have been proposed, including CD123, CD47 [27], CD33 [28], CD96, CD99, CD32 [29], IL1RAP, and CD45RA [22]. It is worth to be noted that identification of patient-derived LSCs is mainly based on the establishment of xenotransplantation assay that facilitates engraftment of patient-derived cells. Thus identification and frequency of LSCs are highly dependent on the model used, which might dictate the observed biological properties of LSCs [30]. Recent study demonstrated the coexistence of at least two distinct $CD34^+$ LSC populations in most AML patients: a $CD34^+CD38^-$ fraction resembling normal lymphoid-primed multipotent progenitors and a $CD34^+CD38^+$ fraction resembling normal granulocyte-macrophage progenitors. Both populations have leukemic stem cell activity and are hierarchically ordered [31]. Therefore, it is reasonable to think that, with improved xenotransplant models, more immunophenotypic and functional LSCs will be identified.

2.2.2 *Cellular State of LSCs*

While transform normal hematopoietic stem/progenitor cells into LSCs, genetic and epigenetic alterations also change the steady cellular state of normal HSCs (Fig. 2.2) [32, 33]. It is commonly accepted that normal HSCs are largely in a state of quiescence with glycolytic, autophagy-dependent, and tightly controlled levels of protein synthesis [34–37]. While LSCs inherit common stem cell characteristics of normal HSCs, they also have some unique functional changes.

LSCs undergo unique reprogrammed cellular metabolism, a hallmark of cancers [38]. Cancer cells rely on glycolysis for energy production instead of oxidative phosphorylation (OXPHOS), a more efficient ATP-producing process. Similarly, normal HSCs also prefer to utilize glycolysis to meet their energy demands [36, 39], LSCs are uniquely reliant on OXPHOS for their maintenance and survival [40–42]. By interrogating the metabolome of human AML stem cells, recent studies found that the majority of functionally defined LSCs isolated from de novo AML patients are characterized by relatively low levels of reactive oxygen species (ROS) [41]. These ROS^{low} LSCs have higher levels of amino acids, which are metabolized in the tricarboxylic acid (TCA) cycle [40]. LSCs of AML are selectively dependent on amino acid metabolism for OXPHOS. In addition, metabolism of branched-chain

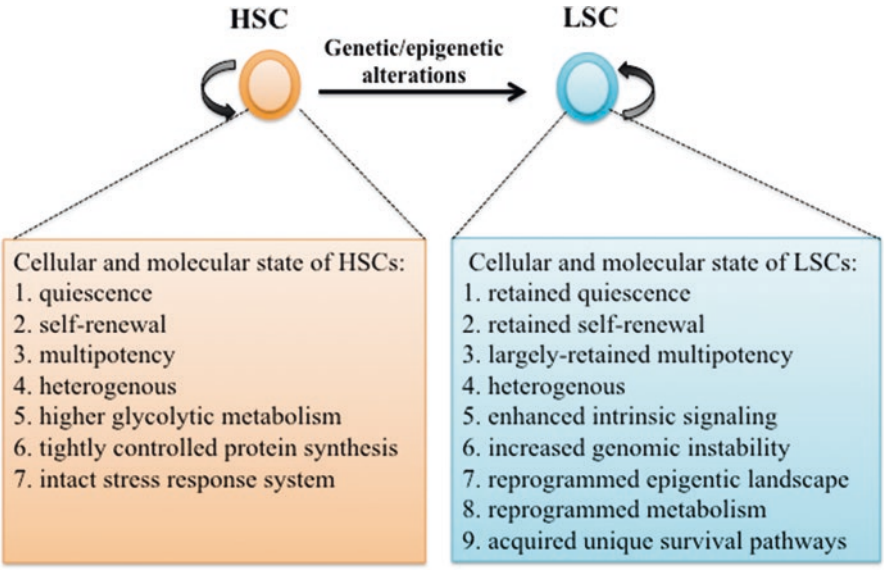

Fig. 2.2 Biological properties of normal HSCs and LSCs. Normal HSCs have three major biological features or stem cell properties: self-renewal, multipotency and quiescence. After acquiring genetic lesions, HSCs undergo cellular transformation to become LSCs that retain the major stem cell properties of HSCs with enhanced signaling activities and also acquire some unique biological features. These biological features define the cellular states of HSCs and LSCs and provide opportunities to develop strategies for specifically targeting LSCs while sparing normal HSCs

amino acids (BCAAs) is also important for LSC survival. BCAA transaminase 1 (BCAT1), a cytosolic aminotransferase for BCAAs, is activated in AML LSCs and regulates intracellular α-ketoglutarate (αKG) homeostasis by transferring α-amino groups from BCAAs to αKG. αKG is an essential cofactor for αKG-dependent dioxygenase such as Egl-9 family hypoxia-inducible factor 1 (EGLN1) and the ten-eleven translocation (TET) family of DNA demethylases [43]. Elevated BCAT1 restricts intracellular αKG and stabilizes HIF1α, which are required for LSC maintenance [44]. Similarly LSCs of CML also display higher oxidative metabolism. A metabolic analysis on both stem cell-enriched ($CD34^+$ and $CD34^+CD38^-$) and differentiated cells ($CD34^-$) derived from CML patients reveals that CML LSCs rely on upregulated oxidative metabolism for their survival [42]. LSCs show an increase in glycerol-3-phosphate, carnitine, and acylcarnitine derivatives, as well as a decrease in free fatty acid such as oleic and stearic acids. Recently, our finding also demonstrated that fatty acid metabolism is essential for the survival of CML LSCs. We found that fatty acid metabolism enzyme stearoyl-CoA desaturase-1 (Scd1) is downregulated in LSCs and plays a tumor-suppressive role in LSCs with no notable inhibitory effect on normal HSCs [45]. In addition, BCAT1 is aberrantly activated and functionally required for chronic myeloid leukemia (CML) in humans and in mouse models of CML. Blocking BCAT1 gene expression or enzymatic activity induces cellular differentiation and impairs the propagation of blast crisis CML both in vitro and in vivo. Direct supplementation with BCAAs ameliorates the defects caused by BCAT1 knockdown, indicating that BCAT1 exerts its oncogenic function through BCAA production in blast crisis CML cells. The oncogenic RNA-binding protein Musashi2 (MSI2) is physically associated with the BCAT1 transcript and positively regulates its protein expression in leukemia [46]. Together, myeloid leukemia stem cells displayed unique cellular metabolic state.

LSCs also display unique epigenetic state. Cancer cells are characterized by aberrant epigenetic landscapes and often exploit chromatin machinery to regulate gene expression programs [47, 48]. For instance, CML LSCs show higher expression of EZH2, the catalytic subunit of polycomb repressive complex 2 [49, 50], which is associated with extensive reprogramming of H3K27me3 targets in LSCs. Inactivation of Ezh2 in conventional conditional mice and through CRISPR-/Cas9-mediated gene editing prevents initiation and maintenance of disease and survival of leukemia-initiating cells (LICs), irrespective of BCR-ABL1 mutational status, and extends survival [49, 51]. Thus, LSCs utilize unique epigenetic state for their survival.

These unique features could provide a promising cellular state-based therapeutic strategy for specifically targeting LSCs. For instance, an EZH2-specific inhibitor promotes apoptosis of LSCs from CML patients without impairing normal HSCs, and combined treatment with tyrosine kinase inhibitors further potentiates these effects and results in significant loss of LSCs [50]. Additionally, targeting the unique metabolic machinery of LSCs also showed exciting effects. As ROS^{low} LSCs aberrantly overexpress BCL-2 [41], inhibition of BCL-2 with venetoclax in combination with azacitidine disrupts the tricarboxylic acid (TCA) cycle manifested by decreased α-ketoglutarate and increased succinate levels. These metabolic perturbations

suppress oxidative phosphorylation (OXPHOS), which efficiently and selectively targets LSCs [40, 52]. Therefore, exploring LSC unique cellular features may result in promising therapeutic intervention that can eradicate LSCs.

2.2.3 Molecular State of LSCs

LSCs display unique state at the molecular level. Using CML as an example, the molecular evolution of CML LSCs initiates from the formation of BCR-ABL oncogene in a HSC [16]. As a result, constitutively active BCR-ABL causes uncontrolled activation of some development-related signaling pathways such as Wnt/β-catenin [53], hedgehog [54], TGFβ-FOXO [55], etc. These intrinsic genetic and signaling alterations increase the abilities of CML LSCs in self-renewal, resistance to apoptosis, and genomic instability [56]. The intrinsic molecular alterations are critical in determining the sensitivity of LSCs to drug treatment in clinic. Currently, it is accepted that the tyrosine kinase inhibitor (TKI)-refractory malignant cells in CML are LSCs [57]. Interestingly, recent studies indicate that survival of CML LSCs is independent of BCR-ABL kinase activity [58, 59], suggesting that CML stem cells utilize signaling pathways that are independent of BCR-ABL kinase activity for their maintenance and survival. Our recent studies have uncovered these signaling pathways. We show that Alox5 is upregulated by BCR-ABL, but not reduced by TKI treatment [60]. Another example is that B lymphocyte kinase (Blk) is significantly downregulated by BCR-ABL in a kinase activity-independent manner, and this pathway plays a tumor-suppressive role in regulating the survival of CML LSCs [61]. Thus, the identification of these BCR-ABL kinase activity-independent pathways may provide novel therapeutic targets for specifically eradicating LSCs.

2.2.4 Heterogeneity of LSCs

Given that clonal heterogeneity of differentiation and self-renewal properties in normal HSCs has been recognized, cellular heterogeneity of LSCs is gradually recognized. The differentiation behaviors of different HSC subtypes including myeloid-biased, lymphoid-biased, or balanced have been distinguished by measuring the contributions of individual HSCs to the circulating cell lineages in serial transplantation experiments [62, 63]. Also, clonal analysis of HSC expansion post-transplant showed that both subtypes display an extensive but variable self-renewal activity with occasional interconversion [63]. Given that HSCs are derived early from embryonic precursors, the heterogeneity of HSCs might inherit from pre-HSCs. In a recent study, the nascent pre-HSCs in the E11 mouse aorta-gonad-mesonephros (AGM) region were captured at high purity using potent surface markers and rigorously validated by single-cell-initiated serial transplantation [64]. Single-cell RNA sequencing (scRNA-seq) analysis reveals remarkable

heterogeneity of pre-HSCs. Pre-HSCs show unique features in transcriptional machinery, arterial signature, metabolism state, signaling pathway, and transcription factor network [64]. Therefore, it is reasonable to consider that heterogeneity could be attributed to the inherent characterization of HSCs that is determined by the intrinsically molecular mechanisms.

LSCs may inherit this feature of normal HSCs, and display functional heterogeneity, which contributes to the different behaviors of LSCs in leukemia initiation, progression, and relapse. Residual BCR-ABL$^+$ stem cells persist but not result in recurrence in some CML patients who have maintained long-term remission even after discontinuing TKI treatment [65–67]. The lack of leukemia recurrence in these patients indicates limited leukemia-initiating potential of the residual LSCs. Normally LSCs survived post treatment have higher leukemia-initiating potential and quickly lead to CML recurrence [20, 21, 68]. The discrepancies in leukemogenic potential between these two types of LSCs can be explained by LSC heterogeneity, which might reflect unique program of LSCs determined by the intrinsically molecular machinery or extrinsically microenvironment. Consistently, using SCL-tTA/BCR-ABL mouse model of CML, a recent study demonstrates that long-term repopulation and leukemia-initiating capacity after transplantation is restricted to cells with the long-term (LT)-LSC. The leukemia-initiating potential of this CML LT-LSC is remarkably heterogeneous [69]. While LT-LSCs from leukemia mice maintain leukemia-initiating ability in serial transplantation, LT-LSCs from non-leukemic mice cannot induce leukemia [69]. However, the mechanisms underlying the heterogeneity of CML LT-LSCs are complex and still poorly understood.

Recent advances in single-cell transcriptomics are ideally placed to unravel LSC heterogeneity. Through comparing the global gene expression in LT-LSCs from leukemia and non-leukemic mice by RNA-seq, this study reveals higher expression of MPL in CML LT-LSCs, and knockdown of MPL expression reduces THPO-induced JAK/STAT signaling and diminishes growth and leukemogenic capacity of CML LT-LSCs [69]. In addition, by combining high-sensitivity detection of BCR-ABL with whole-transcriptome analysis of the same single cell, a recent study analyzed more than 2000 stem cells from CML patients throughout the disease course [70]. While revealed the heterogeneity of CML LSCs, they identified a subgroup of CML LSCs with a distinct molecular signature that selectively persisted during prolonged therapy. These drug-resistant subgroups of LSCs are more close to normal HSCs at the molecular state, showing higher expression of quiescence-related genes. However, compared to normal HSCs, drug-resistant subgroup of LSCs displays hyperactive TGFβ and TNFα signaling [70]. However, the key determinants of heterogeneous leukemia-initiating capacity and drug sensitivity of LSCs need to be further investigated.

Along with the development of in-depth genome sequencing, the true complexity of AMLs is now being understood. The heterogeneity of AMLs is multilayered and involves the genetic and epigenetic dynamics [71]. Each case of AML is a complex mosaic of cells that contain distinct genetic lesions and epigenetic aberrations [72]. Somatic mutations in the epigenetic modifiers *DNMT3A, IDH1/IDH2,* and *TET2* are initiating genetic events in AML, normally occurring in preleukemic

HSCs [73–76]. However, it seems epigenetic mutations alone are not sufficient to transform HSCs, indicating sequential acquisition of mutations is required. Therefore, the multiple levels of genetic and epigenetic heterogeneity not only endow the inherent complexity of LSCs but also challenge us understanding the biology of LSCs.

Fortunately, current technological breakthroughs significantly contribute to understanding heterogeneity of stem cells. In recent years, multiple molecular labeling tools for efficient cell tracking in vivo have been developed. For instance, the cellular barcoding offers a new clonal tracking system [77, 78]. These approaches use either lentiviruses or transposon to introduce unique barcode sequences into the genome of individual HSCs, which enables to label and track HSCs and their progeny in single mice. In the virus-mediated barcode system, cells are labeled by retroviral/lentiviral vectors with random sequence tags, which allow tracing each cell and its progeny over time. In the transposon system, a doxycycline-inducible hyperactive "Sleeping Beauty" (HSB) transposase and a single-copy non-mutagenic transposon are incorporated in the mouse genome through gene targeting [78]. Therefore, upon doxycycline administration, the transposon can randomly mobilize to a different genomic location, which creates an inheritable genomic DNA insertion and serves as a unique tag for tracking individual HSC and its progeny [78]. Therefore, these cellular tracking systems provide feasible and sensitive strategies for examining the clonality and heterogeneity of LSCs. Moreover, the technological improvements at the single-cell level have also greatly contributed to the dissection of the molecular machinery of stem cell heterogeneity [79]. Molecular profiling of the genome and transcriptome at single-cell resolution becomes feasible. For instance, in a recent study, through the single-cell transcriptome profiling of pre-granulocyte-macrophage progenitor, distinct myeloid differentiation pathways were identified, which reveal an early hematopoietic-lineage bifurcation that separates the myeloid lineages before their segregation from other hematopoietic-lineage potential [80]. Thus single-cell molecular profiling would be powerful in dissecting the heterogeneity of stem cells.

2.3 Bone Marrow Microenvironment of LSCs

It is well known that bone marrow niche is a complex ecological system, composed of multiple cell types [81–83], such as osteoblast [84], endothelial cell [85, 86], perivascular cells [64, 87], mesenchymal stem cell (MSC) [88], nonmyelinating Schwann cell [89], and megakaryocyte [90]. Osteoblasts line the surface of the endosteum on the inside of the bone cavity. Nestin$^+$ MSCs are spatially associated with HSCs and adrenergic nerve fibers and highly express HSC maintenance genes, and in vivo nestin$^+$ cell depletion rapidly reduces HSC content in the bone marrow [88]. Recent study identifies that only the Hoxb5$^+$ HSCs exhibit long-term reconstitution capacity after transplantation in primary transplant recipients and, notably, in secondary recipients and in situ imaging of mouse bone marrow, finding that the

perivascular space as a near-homogenous location of LT-HSC, as majority of LT-HSCs are directly attached to VE-cadherin$^+$ cells [64]. Consistently, using a different strategy, Acar et al. found that α-catulin$^+$c-kit$^+$ cells representing HSCs are more common in the perisinusoidal niches in central marrow, and nearly all HSCs contacted leptin receptor positive (Lepr$^+$) and Cxcl12^{high} niche cells, and approximately 85% of HSCs were within 10 μm of a sinusoidal blood vessel [87]. Furthermore, nonmyelinating Schwann cells, a type of glial cells ensheathed autonomic nerves are identified in contact with a substantial proportion of HSCs and responsible for maintaining HSC hibernation [89]. Also, megakaryocytes are critical for maintaining HSC quiescence during homeostasis and promoting HSC regeneration after chemotherapeutic stress via physically associated with HSCs in the bone marrow [90]. These supporting cells secrete factors, including SCF, CXCL12, angiopoietin 1, and thrombopoietin, which promote stem cell maintenance [84, 91]. Mesenchymal stromal cell secrete HSC homing and maintenance factors. Both nonmyelinating Schwann cells and megakaryocytes are major sources of transforming growth factor β1 (TGFβ1), and activated TGFβ1/SMAD signaling is essential in maintaining HSC quiescence [89, 90]. However, in response to stress, fibroblast growth factor 1 (FGF1) signaling from megakaryocytes transiently dominates over TGF-β inhibitory signaling to stimulate HSC expansion [90]. Overall, these observations demonstrate the heterogeneous components that serve as HSC-derived niche cells to dynamically regulate HSC function (Fig. 2.3); and this dynamic nature of HSC niche might also determine the heterogeneity of stem cells.

Alterations in the bone marrow microenvironment or niche that are commonly observed in hematologic malignancies contribute to the abnormal function of LSCs [92, 93]. LSCs are presumed to reside in specific niches in the bone marrow (Fig. 2.3). Through a dynamic in vivo confocal imaging, a recent study found that the adhesion molecule E-selectin and the chemoattractant stromal-cell-derived factor 1 (SDF-1) expressed in the vasculature attract leukemia cells in the specialized vascular area of bone marrow niche [94]. Disruption of the interactions between SDF-1 and its receptor CXCR4 inhibits the homing of leukemia cells to these vessels [94], suggesting that specialized vascular structures might delineate a microenvironment with unique physiology that can be exploited by circulating malignant cells. In addition, compared to normal HSCs, LSCs use different niche signals for their engraftment. CML LSCs rely to a greater extent on selectins and their ligands for homing and engraftment than do normal stem cells. Deficiency of E-/L-selectin in the recipient bone marrow endothelium significantly reduces engraftment by LSCs of CML [95]. More interestingly, signaling from the bone marrow niche might have differential impact on different myeloid leukemias. For instance, osteoblastic cell-specific activation of the parathyroid hormone (PTH) receptor inhibits CML development but promotes MLL-AF9 oncogene-induced AML in mouse transplantation models, which is mediated by the opposing effects of increased TGF-β1 signaling on the respective LSCs, suggesting that bone marrow microenvironment of CML and AML are distinct [96]. These observations clearly indicate the abnormal bone marrow niches of leukemia.

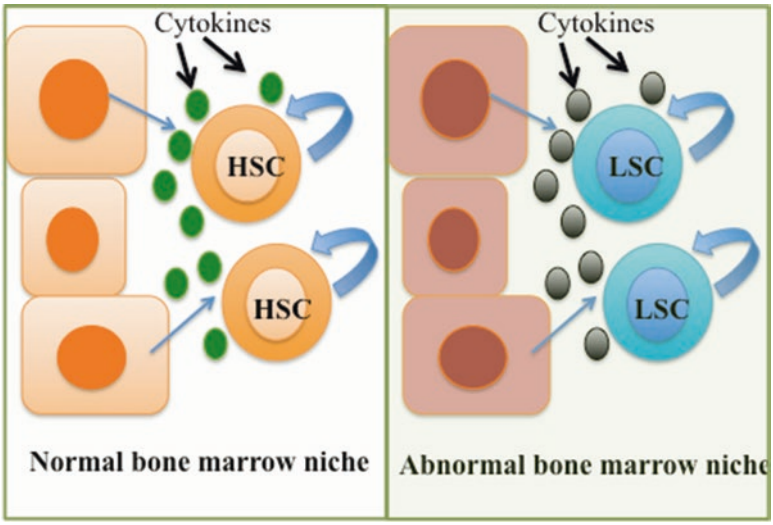

Fig. 2.3 Bone marrow microenvironment is required for LSC maintenance and survival. In both normal and abnormal condition, bone marrow niche is essential for maintaining both normal HSCs and LSCs by direct physical contact and secreting cytokines. In hematologic malignancies, genetic lesion in hematopoietic cells can remodel bone marrow niche to adopt LSC survival and maintenance. Genetic alterations occurred in the niche cells can also induce myelodysplasia and leukemia. Also compared to normal HSCs, LSCs use different niche signals for their engraftment

However, the relationship between leukemogenesis and alteration of bone marrow niche remains complex. In the one hand, bone marrow dysfunction can directly induce myelodysplasia and secondary leukemia by affecting the differentiation, proliferation, and apoptosis of heterologous cells and disrupting hematopoiesis [97]. Deletion of Dicer1 specifically in osteoprogenitors leads to myelodysplasia by reducing expression of Sbds, the gene mutated in Shwachman-Bodian-Diamond syndrome – a human bone marrow failure and leukemia predisposition condition [97]. In addition, an activating mutation of β-catenin in mouse osteoblasts alters the differentiation potential of myeloid and lymphoid progenitors and leads to development of AML by stimulating expression of the Notch ligand Jagged-1 in osteoblasts [98]. On the other hand, genetic lesions of leukemogenesis remodel the bone marrow niche. Using a xenograftment model, a recent study found that patient-derived hematopoietic cells reprogram healthy MSCs to acquire myelodysplasia syndrome (MDS) MSC-like features, which subsequently secrete cytokines to support MDS stem cells and the progeny [99]. Similarly, LSCs of CML display reduced homing and retention in the bone marrow by inhibiting CXCL12 expression of the bone marrow. This alteration also results in suppressive growth of normal HSCs but enhanced growth of LSCs [100]. Interestingly, the treatment with tyrosine kinase inhibitor, imatinib, partially corrects this abnormality of cytokine secretion, suggesting that targeting the niche might represent a new strategy for leukemia stem cells. Together, the cross talk of leukemia and bone marrow niche affects each other

rather than mutually exclusive (Fig. 2.3). Thus further understanding of the differences between normal and malignant bone marrow niches might provide new therapeutic strategies for leukemia stem cells.

2.4 Conclusion

It is increasing recognized that the cellular and molecular state of LSCs is more important than their phenotypic state. In here, we discussed in detail about the biological features including cellular and molecular state, heterogeneity of LSCs, following with bone marrow microenvironment of leukemia. These specific features of LSCs provide a view about the dynamic state of LSCs, which is important in current cancer research. From the developmental perspective, understanding the molecular mechanisms of CSC maintenance and survival could uncover the process of tumorigenesis. And exploring the heterogeneity of LSCs could reveal the dynamic origin of LSCs and the mechanism of drug sensitivity of LSCs. Further investigating the relationship of LSCs with bone marrow microenvironment might enable us fully understand the ecological system of LSC and its niche. From a clinical standpoint, it is important to decipher the mechanisms of drug resistance of LSCs. Therefore, studying these different aspects of LSCs will provide novel therapeutic targets. For instance, the pathways that were identified to be essential for maintaining LSCs provide potential targets in eliminating CML LSCs [54, 101–103]. Also the recent findings of PPARγ as a novel key regulator for CML stem cell survival offer new hope for targeting CML stem cells [45, 104], although some issues such as side effects of PPARγ agonists have to be considered in clinics. Together, based on the recent scientific advances made in the LSC field, it is hopeful that we begin to understand how LSCs utilize unique cellular and molecular features to maintain their abilities of survival and self-renewal, which will lead to future clinical trials for testing new anti-LSC strategies.

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Chapter 3
Metabolic Regulations in Hematopoietic Stem Cells

Dan Huang, Chiqi Chen, Xiaoxin Hao, Hao Gu, Li Xie, Zhuo Yu, and Junke Zheng

Contents

Abstract One of the bottlenecks of the treatments for malignant hematopoietic disorders is the unavailability of sufficient amount of hematopoietic stem cells (HSCs). HSCs are considered to be originated from the aorta-gonad-mesonephros and gradually migrates into fetal liver and resides in a unique microenvironment/niche of bone marrow. Although many intrinsic and extrinsic factors (niche components) are reported to be involved in the origination, maturation, migration, and localization of HSCs at different developmental stages, the detailed molecular mechanisms still remain largely unknown. Previous studies have shown that intrinsic metabolic networks may be critical for the cell fate determinations of HSCs. For example, HSCs mainly utilize glycolysis as the main energy sources; oxidative phosphorylation is required for the homeostasis of HSCs; lipid or amino acid metabolisms may also sustain HSC stemness. Mechanistically, lots of regulatory pathways, such as MEIS1/HIF1A and PI3K/AKT/mTOR signaling, are found to fine-tune the different nutrient metabolisms and cell fate commitments of HSCs. However, more efforts are required for the optimization and establishment of precise

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metabolic techniques specific for the HSCs with relatively rare cell frequency and understanding of the basic metabolic properties and their underlying regulatory mechanisms of different nutrients (such as glucose) during the different developmental stages of HSCs.

Keywords Hematopoietic stem cells · Metabolic regulations · Glycolysis · Oxidative phosphorylation · Stemness maintenance · Bone marrow niche

3.1 Introduction

Hematopoietic stem cells (HSCs), one of the important types of adult stem cells, mainly reside in a unique hypoxic bone marrow microenvironment (niche) [1–3] and self-renew and differentiate to all blood cells. With the development of bone marrow transplantation techniques, HSCs have been widely used in the treatments of various hematopoietic diseases, such as leukemia, lymphoma, aplastic anemia, and immune disorders. Currently, the rare HSC frequency is one of the major bottlenecks that impedes the application of HSCs for different hematopoietic disorders. Moreover, increasing evidence shows that many blood diseases are originally caused by the malfunction or malignant transformation of HSCs. Hence, understanding the intrinsic and extrinsic molecular mechanisms related to HSC self-renewal and differentiation will lead to the development of novel strategies for in vitro or in vivo HSC expansion to provide enough amounts of HSCs for the bone marrow transplantation in clinic and for the treatments of hematopoietic disorders with specific molecular targets.

HSCs originate from a precursor in the endothelial layer lining the ventral side of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region, migrate into fetal liver for a dramatic expansion, and eventually move to and reside in the bone marrow. These cell fate commitments of HSCs are tightly regulated by a large number of intrinsic and extrinsic/microenvironmental factors, which control the occurrence, expansion, differentiation, homeostasis, and aging of HSCs [4–7]. Although many molecules have been identified to regulate HSC activities, it remains largely unknown how cell fate determinations are fine-tuned at different developmental stages. Intrinsic metabolic regulators are considered to play essential roles in maintaining HSC activities under both physiological and pathological situations; how different nutrient metabolisms affect HSC functions awaits for further investigations. In recent years, several lines of evidence have shown that HSCs mainly utilize glycolysis as the energy source, which is well controlled by different intrinsic or extrinsic factors [8, 9]. However, there still exist a large amount of issues to be addressed, for example, which molecules are involved in maintaining HSC glycolysis or other metabolisms, how other metabolic pathways affect HSC stemness, and whether there exist common metabolic properties of HSCs during developmental stages.

3.2 Hypoxic Bone Marrow Niches and HSC Activities

Hematopoietic cells localize in the specific bone marrow niches post birth, which has been well known to play a critical role in normal hematopoiesis. The concept of "niche" was initially proposed by Schofield R in 1978 [10]. The niche to HSC is as the earth to seed. The components/qualities of the "earth" directly influence the life cycle of the "seed" in the bone marrow. Recently, Khan JA et al. report that there also exists a HSC niche in fetal liver, which is mainly composed by $Nestin^+NG2^+$ pericyte cells near portal vessels [11]. The components of HSC niches may contain vascular cells, stromal cells, nerve cells, certain types of hematopoietic cells (such as megakaryocyte), extracellular matrix, and other connective tissues. Regarding that HSCs can produce all the blood cells, the hematopoietic niche is usually referred as HSC niche. Two major types of bone marrow niches have been defined including the endosteal niche and perivascular niche (Fig. 3.1). Quiescent HSCs previously have been reported to reside in the endosteal niche formed by osteoblasts, while cycling HSCs localize close to the sinusoid or arteriole orchestrated by endothelial cells, termed as a perivascular niche. Interestingly, several studies also suggest that these two types of niches are tightly connected to each other to comprise the functional niche networks. Recently, Ding et al. provide intriguing evidence showing that HSCs mainly reside in a perivascular niche, while early lymphoid progenitors localize in an endosteal niche based on the extensive analysis with a *Cxcl12* conditional knockout mouse line [12]. These results indicate HSCs and their derived progenitors may have the distinct distributions in bone marrow niches. Recent studies also imply that both dormant HSCs and proliferating HSCs can simultaneously exist in the perivascular niche. Moreover, other niches

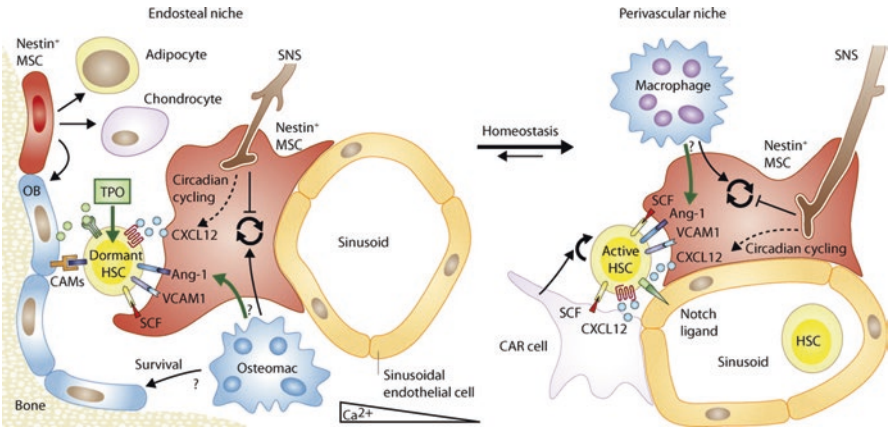

Fig. 3.1 Components of bone marrow niches (cited from Armin E et al. JEM, 2011, 421–428). Dormant HSCs mainly localize in the endosteal niche comprised by the osteoblasts (OB), while active HSC prefers residing close to the sinusoid (perivascular niche) constituted by endothelial cells. Other bone marrow niches formed by distinct stromal cells, including CAR cells, $nestin^+$ MSC, or sympathetic nerve system (SNS), are also indicated

comprised by stromal cells or hematopoietic cells, such as mesenchymal stem cells (MSC), adipocyte and megakaryocyte, are also identified to be able to functionally support HSC activities [13–17].

Bone marrow niches are considered as hypoxic niches and have relatively low oxygen tension due to the unique anatomic structure [18]. Meanwhile, the bone marrow is filled with different categories of hematopoietic cells, which leads to a much high level of total oxygen consumption. There is approximately tenfold decrease of oxygen tension in the region that is several cellular diameters away from capillaries as indicated by the oxygen diffusion model [3]. The average pO_2 in the bone marrow is around 55 mmHg, and the average oxygen saturation is around 87.5% [19]. These findings indicate that HSCs mainly localize in a relatively hypoxic niche. Previous studies showed most of dormant and primitive HSCs exist in the bone marrow niche region with a relative slow blood flow. For example, pimonidazole (a hypoxia indicator) or Hoechst can mark HSCs in the hypoxic region [20, 21]. Hypoxic condition (1–3% oxygen tension) can efficiently promote the differentiation of HSCs to megakaryocytes, erythrocytes, granulocytes, or monocyte progenitors and enhance the in vitro expansion and in vivo engraftment of HSCs [22–24]. Therefore, hypoxic conditions may contribute to the maintenance of HSC stemness.

Bone marrow niche components (such as osteoblasts, macrophages, endothelial cells, Nestin$^+$ MSCs) produce chemokines, growth factors, and adhesion molecules to support HSC activities in the bone marrow. Miharada et al. show that LT-HSCs express high level of the surface receptor, GPR78, to sustain HSCs in the endosteal niche and a relatively high level of glycolysis but low mitochondrial potential. Inhibition of GPR78 in HSCs results in a notable migration out of the endosteal niche [25]. However, it is still not clear where the physical position hypoxic area in the bone marrow is. Recent studies also indicate that the perivascular niche may be more hypoxic compared to that of the endosteal niche [26]. It is conceivable that hypoxic niche may afford a very narrow window of oxygen tension to maintain HSC activities as evidenced that certain hypoxia inducible factor 1 alpha (Hif-1α) level is essential for HSC functions [27]. In summary, these studies suggest that HSCs can tolerate the hypoxic microenvironment with the low oxygen tension in the bone marrow to remodel their metabolic properties. Although the underlying mechanisms are not fully understood, it implies that HSCs have unique metabolic characteristics.

3.3 Metabolisms of HSCs Under Hypoxic Niches

In differentiated cells, oxidative phosphorylation is the major source of ATP under aerobic conditions, which generates ~18-fold more ATP amount than that derived from the glycolysis under anaerobic situations. Oxygen is the final carrier of the electron transport/transfer chain. The proton gradient will be collapsed, followed by the dramatic decrease of ATP production if oxygen supplementation is decreased or

inhibited. Under hypoxic conditions, ATP level is sustained through the cytosolic glycolysis, although only 2 mol ATP is generated from 1 mol glucose and the intermediate metabolite of pyruvate is further converted into lactate to supply NAD^+ for the driving of glycolysis. In 1861, Louis Pasteur first reported oxygen tension could regulate the rate of glucose fermentation. Thereafter, glycolysis under the anaerobic condition is as termed as "Pasteur effect". Glycolytic rate is well regulated by the expression levels and activities of various rate-limiting enzymes. Although hypoxic condition may enhance the energy production derived from glycolysis, it is less important than the fact that most types of cells can downregulate their energy demands under such stress conditions. This phenomenon indicates that it is essential to shut down unnecessary biological activities and functional processes to reserve certain levels of energy, which is also termed as "turning off the pilot light" as suggested by the comparative physiologist Kjell [28, 29]. Studies show that HSCs are metabolically quiescent to maintain their stemness under such "pilot light" condition and satisfied with the limited energy generated from glycolysis. This phenomenon is also consistent with the fact that HSCs are usually dominantly quiescent in a homeostatic microenvironment [29].

Mitochondrial respiration is considered as the major source of reactive oxygen species (ROS). ROS is mainly generated from the electron leaking, which further converts the oxygen to superoxide anion. Although superoxide anion is not a strong oxidant, it serves as the precursor of most other oxygen radicals, such as hydroxyl radical and nitrogen peroxide. ROS may lead to a marked consumption of natural antioxidants and multiple aspects of cellular damage. ROS has been reported to be tightly connected with aging and many degenerative disorders, including HSC dysfunction and aging. In fact, functional HSCs contain relatively low level of ROS, and elevated ROS level impairs HSC self-renewal capacities and quiescent status [30]. For example, loss of function of ATM and FOXOs results in the marked impairment in HSC activities [31, 32]. Therefore, we speculate that the enhanced glycolytic level of HSCs not only protects them from hypoxic stress but also avoids the ROS-mediated oxidative stress from mitochondrial respiration.

In the past decades, due to the limitation of the availabilities of HSC amount, HSC metabolic properties have not been well characterized by using conventional techniques. However, several studies have shown that HSCs may utilize glycolysis as the main energy sources. For example, HSCs with low rhodamine-123 staining (a mitochondria dye) have much higher colony-forming capacities than that of counterparts [33]; mathematics model suggests that HSCs localize in a hypoxic region of bone marrow [3]; more pimonidazole (a hypoxic marker) tends to be incorporated in HSCs [1, 8, 27]; HSCs stably express high level of Hif-1α [8, 27]; injection with the hypoxic toxic regent of tirapazamine into recipient results in the depletion of HSCs [1]. By using proteomics analysis and subsequent comparison of 145 identified differentially expressed proteins, Unwin et al. show that LSK^+ cells (enriched for HSC population) prefer to adopt glycolysis, rather than oxidative phosphorylation, for their energy source, which is opposite to that in LSK^-differentiated hematopoietic cells. Consistently, proteomics analyses from other groups further indicate that more glycolysis related pathways are involved in the HSC population than the

differentiated cells [34]. We also demonstrate that Meis1 (myeloid ecotropic viral integration site 1) transactivates Hif-1α to fine-tune the glycolytic level in both murine and human HSCs, which suggests that metabolic pattern may not only be the consequence regulated by the extrinsic components of bone marrow niches but also be controlled by many intrinsic factors [8].

3.4 Mechanisms Involved in HSC Glycolysis

Hif-1α has been known as the one of the main regulators to enhance the glycolysis under hypoxic conditions, which is quite unstable and promptly undergoes degradation under normoxic situations. Hif-1α is a master regulator that controls the expressions of many downstream targets involved in oxidative stress, glycolysis, and oxidative phosphorylation. Although Hif-1α collaborates with Hif-1β to form the heterodimer and highly exists in differentiated cells under hypoxic conditions, Hif-1α seems to be stably expressed in HSCs even under normoxic conditions. The stability of Hif-1α is tightly controlled by several well-defined signaling pathways. For example, VHL can maintain Hif-1α stability through its mediated deubiquitination pathway [35]; energy sensor AMPK-mediated and redox sensor SIRT1-mediated signaling pathways are important for the certain Hif-1α level [36]; aryl hydrocarbon receptor AhR can form a nuclear localization complex of AhR/aryl to inhibit the ARNT's binding with Hif-1α to reduce its protein level, which further leads to the notion that AhR antagonist may be capable to enhance the ex vivo expansion of HSCs [37]; our previous studies also reveal that cytoskeleton protein PFN1 sustains Hif-1α level through EGR1/Gα13 pathways to regulate the glycolytic status and homeostasis of HSCs in bone marrow niches [38]. All these regulatory networks are critical for the maintenance of HSC functions under hypoxic conditions and prevent the impairment resulting from the oxidative phosphorylation.

Meis1 is one of the HOX family members and evolutionarily conserved DNA binding transcriptional factor. Meis1 is highly expressed in HSCs at different developmental stages and significantly reduced upon differentiation [39]. Meis1deletion in mice is embryonic lethal due to the defects in hematopoiesis and vasculogenesis. So far, the functions of Meis1 are still not fully understood. Our previous studies suggest that Meis1 can transcriptionally activate Hif-1α expression [8]. Moreover, Meis1 regulates HSC glycolysis and self-renewal through Hif-2α/ROS/p16 pathway (Fig. 3.2) [9]. Deletion of Meis1 results in the notable downregulation of Hif-1α and Hif-2α, enhanced ROS level, and loss of HSC quiescence. ROS scavenger NAC can rescue the loss function of Meis1-null HSCs. Interestingly, mobilized human peripheral blood HSCs also adopt glycolysis to obtain energy through Meis1/Hif-1α/Pbx1/HoxA9 pathways [40].

Many Hif-1α targeted genes have been found to play important roles in HSC function, including glycolysis related genes (GLUT1, LDHA, PKM2, PFKL, PDK2, and Cripto). Hif-1α can directly enhance the activity of pyruvate dehydrogenase kinase (PDK) to suppress the entry of pyruvate into tricarboxylic acid cycle

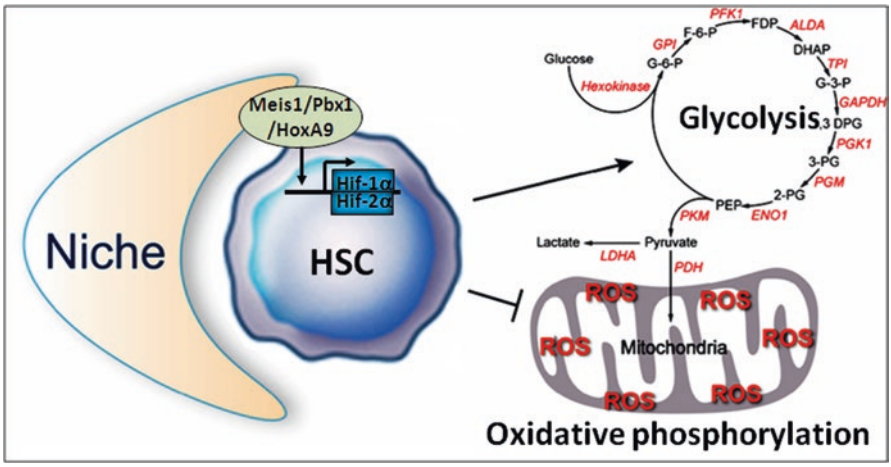

Fig. 3.2 HSCs mainly utilize glycolysis as the energy source. HSCs localize in a unique bone marrow niche and tend to utilize glycolysis, but not oxidative phosphorylation, as main energy sources, which is fine-tuned by Meis1/Pbx1/HoxA9/Hif-1α/Hif-2α at transcriptional level

(TCA cycle) [41]. Wang et al. demonstrate that the deletion of LDHA or PKM2 may lead to an impaired HSC reconstitution ability [42]. Long-term (LT) HSCs highly express Hif-1α, which stably sustains in the migrating HSCs from hypoxic bone marrow to peripheral blood [40, 43]. Takubo et al. show that Hif-1α-null adult HSCs have reduced repopulation capacities and lose quiescent cell cycle status, indicating that appropriate Hif-1α level is critical for sustaining HSC pool [27]. Mutation in the promoter binding sites of Hif-1α of vessel endothelial growth factor impairs HSC reconstitution abilities [44]. Conditional deletion of Hif-1α causes the swift from glycolysis to oxidative phosphorylation in HSCs and the upregulation of Hif-2α that may result from the compensation effect [9]. However, a recent study shows some controversial findings that loss of Hif-1α does not affect HSC stemness [45], although the authors cannot completely exclude the effect of Hif-1α from bone marrow niches.

Other downstream targets, such as VEGF, ADM, SDF1, SCF, ANG2, ANGPTLs, IGF-2, P21, and FOXOs, are also required for sustaining the quiescent status of HSCs. Interestingly, several Hif-1α targets (SDF1, SCF, ANG2, ANGPTLs) are important niche components in regulation of HSC stemness, indicating Hif-1α may regulate HSC functions through both autonomous and non-autonomous manners. Meanwhile, Hif-1α can crosstalk with Notch and Wnt signaling, two crucial pathways for HSC activities, to determine the cell fates of HSCs. Hif-1α is interacted with the intracellular domain of Notch (NICD) to sustain its stability to promote the transactivation of downstream target genes [46, 47]. Interplaying between Hif-1α and β-catenin can reduce the expressions of certain Wnt-mediated target genes while it enhances Hif-1α-targeted gene expressions [47]. This crosstalk suggests the complicated relationships between HSC metabolisms and stemness under hypoxic conditions.

In addition, Hif-1α is reported to be involved in normal hematopoiesis at different developmental stages. Hif-1α deletion in mice is embryonic lethal due to many defects in the neurogenesis, vasculogenesis, or hematopoiesis. The mRNA expression profiles of HSCs share many common features of Hif-1α distribution with human embryonic stem cells and mesenchymal stem cells [48]. Hif-1α deletion in mice causes a significant reduction in size of yolk sac, where it contains much less hematopoietic cells than the control ones [49]. Although current studies have provided intriguing evidence showing how glycolytic level is regulated in HSCs, more efforts are required for the delineation of all the detailed underlying regulatory networks.

3.5 The Roles of Mitochondrial Respiration in HSC Stemness

Although glycolysis is currently considered as the major metabolic manner of HSCs, increasing evidence shows that HSCs contain certain amount of mitochondria and oxidative phosphorylation may also contribute to HSC activities, especially in HSCs at different developmental stages or differentiated states. Bing L and his colleagues recently demonstrate that pre-HSCs originating from AGM or fetal liver HSCs seemingly have markedly higher oxidative phosphorylation level, which is different from that of adult HSCs [39]. It is possible that the metabolic status may dramatically change when dormant HSCs enter into cell cycle for the subsequent expansion, since cycling HSCs need increased demands of energy supply and macromolecular sources to generate amino acids, lipids, and nucleotides. Many of the macromolecular components are the intermediate metabolites derived from TCA cycle, indicating that mitochondrial oxidative phosphorylation lays the foundation for cell fate commitments of HSCs. Although several studies show most of mitochondria in HSCs may be inactive and mainly serve as an energy reserve under certain stress states [50], recent studies suggest that adult HSCs have high mitochondrial mass and enhanced dye-efflux abilities yet possess limited respiratory and turnover capacities [51]. Mitochondrial respiration may also be important for HSC activities [52, 53]. Although glycolysis can quickly generate ATP and to supply the energy for the biomacromolecules synthesis, the fast cell proliferation also heavily relies on many intermediate metabolites derived from TCA cycle. Therefore, slowdown of TCA cycle via the decrease of substance supply and oxygen uptake may be another efficient way to determinate HSC fates.

Mitochondrial respiration is tightly regulated by several key factors, such as LKB1 and PGC-1 [54]. Loss of LKB1 severely impairs HSC function and leads to the phenotype similar to that of Hif-1α-null HSCs, including the loss of quiescence, increase of proliferation of HSCs/progenitors, and decrease in mitochondrial mass and functions. PGC-1, including PGC-1α and PGC-1β, is the agonist of PPARγ.

PGC-1α is transcriptionally regulated by c-Myc and can significantly enhance the expression levels of several antioxidants critical for the ROS detoxication. c-Myc-deficient HSCs still can survive and expand, but not undergo subsequent differentiation, indicating that c-Myc is required for cycling progenitors instead of quiescent HSCs [55]. The E3 ligase Fbxw7 level is upregulated under hypoxic condition, which can further inhibit the expression of c-Myc to regulate cell cycle of HSCs [56]. Deletion of guanine nucleotide exchange factor binding protein 5 reduces mitochondrial potential and causes the loss of quiescence of HSCs [57]. Mitochondrial malfunction may enhance the ROS production, which further impairs HSC stemness. However, it remains unknown whether HSCs with mitochondrial malfunction have abnormal glycolysis and whether there exists crosstalk between mitochondrial regulatory pathways and Hif-1α. More efforts are required for the understanding whether or how oxidative phosphorylation plays a role in the cell fate determinations of HSCs.

Occurrence of mutations in key catalytic enzymes in TCA cycle may result in the malignant transformation of HSCs. One of the famous examples is that the mutations of IDH1 and IDH2 lead to the generation of onco-metabolite, 2-hydroxyglutarate, and initiation of the acute myeloid leukemia (AML) [58]. IDH1 and IDH2 localize in cytoplasm and mitochondria, respectively, and convert isocitric acid into α-ketoglutaric acid. Instead, isocitric acid is catalyzed into 2-hydroxyglutarate in IDH1-/IDH2-mutant cells [59]. α-Ketoglutaric acid is critical for the activities of many oxygenases. For example, TET2 catalyzes 5mC into 5hmC to initiate the DNA demethylation in α-ketoglutaric acid-dependent manner [60]. Loss of TET2 promotes the abnormal self-renewal of HSCs and myeloid-biased proliferation, which eventually leads to the development of myelodysplastic syndrome (MDS) or AML. These results indicate that IDH mutation phenocopies the loss function of TET2 and cytosine methylation acts as a common epigenetic regulation to affect HSC activities.

3.6 Reactive Oxygen Species and HSC Functions

Reactive oxygen species (ROS) is mainly derived from mitochondrial phosphorylation and has been reported to play important roles in cell fate determinations under both physiological and pathological activities. Certain level of ROS is required for sustaining physiological functions of various types of cells. Nevertheless, abnormally accumulated ROS results in the unbalance of redox and oxidative stress, which leads to the oxidation of nuclear acid, lipid, protein, and carbohydrate, aging, cell apoptosis, and malignant transformation of different cell types. Mitochondrion is the major organelle to generate ROS. ROS mainly contains superoxide, hydrogen peroxide, and oxygen radial. Superoxide is produced through the interaction between uncoupled electron released from the electron transfer chain (such as

complex I and III, also termed as electron leaking) and free oxygen during mitochondrial respiration, which is subsequently catalyzed into hydrogen peroxide by superoxide dismutase. Hydrogen peroxide can be further converted into toxic hydroxyl radical or be eliminated by glutathione, peroxidase, and catalase.

As mentioned above, HSCs mainly utilize glycolysis to obtain energy and prevent the impairment derived from the overloading of ROS since HSCs are more sensitive in response to oxidative stress than the progenitors. Long-term exposure to high level of ROS causes the stemness loss or cell death of HSCs. By using DCFDA, a ROS indictor for live cells, HSCs can be divided into $DCFDA^{high}$ and $DCFDA^{low}$ cell populations. Subsequent in vivo transplantation analysis further shows that $DCFDA^{low}$ cell population contributes to much higher donor repopulation than the $DCFDA^{high}$ one, which prefers to a myeloid differentiation [30]. Because the skewness of myeloid differentiation is considered as one of the properties of aging, high level of ROS may serve as a driver in HSC aging. Interestingly, the treatments with either antioxidant or p38 MAPK inhibitor can rescue the in vitro colony-forming ability of $DCFDA^{high}$ cells affected by the overload of ROS. These data suggest that both ROS level and exposure duration may affect the self-renewal and differentiation abilities of HSCs. However, due to the technique limitations, ROS level is usually evaluated by chemiluminescence analysis, which may not fully reflect the redox state of HSCs. It is urgent to develop novel technologies or strategies to precisely and sensitively monitor the subtle and dynamic changes of ROS level in HSCs.

Many studies have shown that ROS level is fine-tuned in different cell types through several pathways. For example, (1) polycomb complex plays an important role in histone modification to maintain ROS level. Bmi1 has been reported to be highly enriched in HSCs and maintains self-renewal abilities of HSCs by downregulating the levels of ROS, as well as $p16^{Ink4a}$/$p19^{Arf}$ [61]. Bmi1 deletion results in mitochondrial dysfunction with decreased ATP production and elevated ROS level and increased DNA damage in HSCs. (2) DNA damage response is another important pathway to sustain appropriate ROS level and genome stability. ATM/p53 signaling pathways have been reported to be critical for the DNA damage repair [62], and loss of ATM impedes normal hematopoiesis, which may result from the enhanced ROS level and deficiency in ATM-mediated DNA damage response and repair. (3) FOXOs signaling pathway is also involved in the regulation of redox state of HSCs [63]. FOXO3a interplays with ATM to suppress ROS production and activates DDR. FOXO3a-deficient HSCs have markedly higher ROS level and decreased self-renewal capacities. (4) HO-1 serves as a stress-dependent antioxidase to sustain the homeostasis of redox state in HSCs. Nrf2 and Keap1 control HO-1 expression at transcriptional level to reduce the ROS production to maintain HSC activities, and HO-1 deletion leads to a dramatic decrease in repopulation abilities of HSCs [64].

3.7 Other Metabolic Signalings in Cell Fate Commitments of HSCs

Many types of stem cells can sense distinct growth signalings or different nutrient levels to activate PI3K/AKT/mTOR pathways to enhance their survival or proliferation [65]. However, constitutive activation of AKT signaling leads to the overexpansion and exhaustion of HSCs. Although AKT1 or AKT2 deletion does not affect abnormal hematopoiesis, AKT1/AKT2 double knockout HSCs cannot exit from G0 and enter cell cycle for further differentiation [66]. In contrast, abnormal activation of PI3K/AKT resulting from PTEN deletion promotes HSCs to enter into cell cycle and eventually causes the HSC exhaustion, which can be rescued by the depletion of p53 or p16 [67]. Conditional knockout of mTOR upstream suppressor TSC1 results in the overactivation of mTOR, which further leads to the notable increased mitochondrial mass and ROS level, and loss of HSC dormancy [68]. These studies indicate PTEN/TSC1/mTOR signaling pathways are crucial for the maintenance of HSC stemness.

Recently, studies also show that there exists AKT/mTOR-independent signaling in the regulation of HSC metabolisms. Tumor suppressor LKB1 can sense the changes of AMP/ATP ratio to regulate AMPK activity, and phosphorylation of AMPK can inhibit mTOR signaling to suppress cell proliferation, indicating LKB1/AMPK regulates cell metabolisms through the subtle changes of ATP level. LKB1-null mice have increased numbers of hematopoietic progenitor cells but compromised HSC self-renewal capacities. However, loss of function of LKB1 in HSCs cannot be rescued by the activation of AMPK signaling, indicating LKB1 maintains HSC pool in an AMPK-independent manner. In fact, LKB1 is required for the normal mitochondrial function to maintain HSC stemness through PGC-1α pathway [54]. Moreover, by using HPLC-MS analysis, Gurumurthy et al. also reveal that LKB1 may restrain the mitochondrial activities to strengthen the lipid metabolism of Lin^- stem/progenitor cells, indicating LKB1 can serve as a sensitive rheostat between anabolisms and catabolisms [69].

3.8 Metabolic Regulations in HSCs at Different Developmental Stages

Increasing evidence indicates that the HSC metabolisms are dynamically changed at different developmental stages. HSCs may have unique metabolic features and molecular signatures during the process of initiation, proliferation, differentiation, homeostasis, and aging [2, 70]. The metabolic dynamics may be tightly controlled by a large number of intrinsic regulators and niche components [39]. For example, the metabolic manner may be different between fetal liver HSCs and adult HSCs

[39]; Wip1 coordinates with p53 and mTORC1 signaling pathways to slowdown HSC aging [71, 72]; cytosolic glycolysis is switched to oxidative phosphorylation, while quiescent HSCs enter cell cycle and differentiate to downstream progenitors or mature hematopoietic cells. Actually, HSC differentiation heavily relies on the level of the mitochondrial respiration as evidenced by the fact that PTPMT1 deletion hinders the differentiation of HSCs and leads to the rapid failure of hematopoiesis [73, 74].

Current studies in HSC metabolisms mainly focus on the glycolysis and oxidative phosphorylation of the glucose metabolism in adult HSCs. Very rare findings related to other metabolic pathways (such as pentose phosphate pathway, lipid metabolism, and amino acid metabolism) and metabolic properties in different developmental stages of HSCs (including HSCs from AGM, fetal liver, and aging stages) have been precisely or systemically evaluated. However, different nutrient metabolisms may also be important for the cell fate commitments of HSCs. For example, Ito et al. find that PML-PPARδ-FAO pathways mediate lipid acid oxidation to preserve the capabilities of self-renewal and asymmetric divisions of HSCs [75]; Signer et al. demonstrate that the protein synthesis rate also influences HSC functions [76]; Yuki et al. and our group show that branched-chain amino acids are critical for the ex vivo expansion and homeostasis of HSCs [77, 78]; Nina et al. report vitamin A-retinoic acid signaling regulates HSC dormancy [79]; many other regulators, such as LKB1, Wnt, FOXOs, c-Myc, OCT1, AKT, and PTEN, may also participate in the metabolisms of various nutrients [54, 80–85]. To precisely dissect these complicated metabolic regulatory networks in HSCs, it is extremely urgent to develop novel and specific tools or techniques for differential analyses of each metabolic pathway.

Owing to the limitations of HSC numbers, routine assays or tools cannot be applied for the analyses of metabolic features of HSCs. In recent years, many improved techniques are being developed to facilitate the detections of different metabolic status of HSCs. For example, glycolysis and oxidative phosphorylation in HSCs can be measured and indicated as extracellular acidification rate (ECAR) and oxygen consumption rate (OCAR), respectively, by a Seahorse XF analyzer [75, 86]. Seahorse XF analyzer has also been used for the evaluation of lipid metabolisms, which may benefit for further unraveling other metabolic characteristics of HSCs with rare frequencies. LC-MS has been developed and improved to detect various intermediate metabolites with reduced cell amounts, which makes it possible to conduct metabonomic analysis in HSCs. Recent studies provide interesting evidence showing that it is feasible to perform a metabolomic analysis with less than 10^4 HSCs to explore the metabolic networks of different types of nutrients [87]. Meanwhile, anabolism or catabolism is a bioreaction of redox in nature in cells, accompanying with electron transfer between different substrates mediated by different coenzymes, such as nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+), and energy formation and release. Therefore, it may be able to accurately monitor the subtle metabolic changes by the sensors that can indicate the ratio changes of NAD+/NADH or NADP+/NADPH as reported by recent studies [88].

3.9 Summary

Due to the rare frequencies and the complicated connections among various nutrients (such as glucose, lipid, and amino acid), it remains largely unknown in the metabolic properties and their underlying regulatory mechanisms of HSCs at different developmental stages. Comprehensive and specific tools/techniques are required for depicturing all the detailed metabolic profiles of HSCs in the near future, including HPLC-MS, single-cell sequencing, metabonomics, genetic murine models, and definitive functional assays. Stem cell metabolisms have become one of the leading scientific fields theses years due to many newly developed techniques and insights related to how intrinsic metabolisms regulate their cell fate determinations. The studies in metabolisms of HSCs or other types of stem cells will open a new avenue to understand the stem cell biology and its related pathogenesis, which may provide another angle to the development of novel strategies for the treatments of hematopoietic disorders or other diseases.

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Chapter 4
RNA N^6-Methyladenosine Modification in Normal and Malignant Hematopoiesis

Hengyou Weng, Huilin Huang, and Jianjun Chen

Contents

Abstract As the most abundant internal modification in eukaryotic messenger RNAs (mRNAs), N^6-methyladenosine (m^{6}A) modification has been shown recently to posttranscriptionally regulate expression of thousands of messenger RNA (mRNA) transcripts in each mammalian cell type in a dynamic and reversible manner. This epigenetic mark is deposited by the m^{6}A methyltransferase complex (i.e., the METTL3/METTL14/WTAP complex and other cofactor proteins) and erased by m^{6}A demethylases such as FTO and ALKBH5. Specific recognition of these m^{6}A-modified mRNAs by m^{6}A-binding proteins (i.e., m^{6}A readers) determines the fate of target mRNAs through affecting splicing, nuclear export, RNA stability, and/or translation. During the past few years, m^{6}A modification has been demonstrated to play a critical role in many major normal bioprocesses including self-renewal and differentiation of embryonic stem cells and hematopoietic stem cells, tissue development, circadian rhythm, heat shock or DNA damage response, and sex determination. Thus, it is not surprising that dysregulation of the m^{6}A machinery is also

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closely associated with pathogenesis and drug response of both solid tumors and hematologic malignancies. In this chapter, we summarize and discuss recent findings regarding the biological functions and underlying mechanisms of m^{6}A modification and the associated machinery in normal hematopoiesis and the initiation, progression, and drug response of acute myeloid leukemia (AML), a major subtype of leukemia usually associated with unfavorable prognosis.

Keywords m^{6}A demethylases · m^{6}A modification · Normal hematopoiesis · AML

Abbreviations

AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
BMT	Bone marrow transplantation
CLIP	Cross-linking immunoprecipitation
CMP	Common myeloid progenitor
EHT	Endothelial-to-hematopoietic transition
GMP	Granulocyte/macrophage progenitor
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
lncRNA	Long noncoding RNA
LSCs	Leukemia stem cells
LSCs/LICs	Leukemia stem/initiating cells
m^{6}A	*N*^{6}-methyladenosine
miCLIP	m^{6}A individual-nucleotide-resolution cross-linking and immunoprecipitation
mRNA	Messenger RNA
MTC	Methyltransferase complex
pri-miRNA	Primary microRNA
rRNA	Ribosomal RNA
snoRNA	Small nucleolar RNA
SNPs	Single-nucleotide polymorphisms
snRNA	Small nuclear RNA
TF	Transcription factor
tRNA	Transfer RNA
TSS	Transcriptional start site

4.1 Introduction

Since the 1960s, over 150 modified RNA nucleotide variants have been identified in both protein-coding and noncoding RNAs, such as N^6-methyladenosine (m^6A) in messenger RNA (mRNA) and primary microRNA (pri-miRNA) [1–4], N^1-methyladenosine (m^1A) in mRNA and transfer RNA (tRNA) [5–7], 5-methylcytosine (m^5C) and 5-hydroxymethylcytosine (hm^5C) in mRNA and long noncoding RNA (lncRNA) [8–11], and pseudouridine (ψ) in tRNA, ribosomal RNA (rRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) [12–16] (Figs. 4.1 and 4.2). Of them, m^6A is the most prevalent and abundant internal

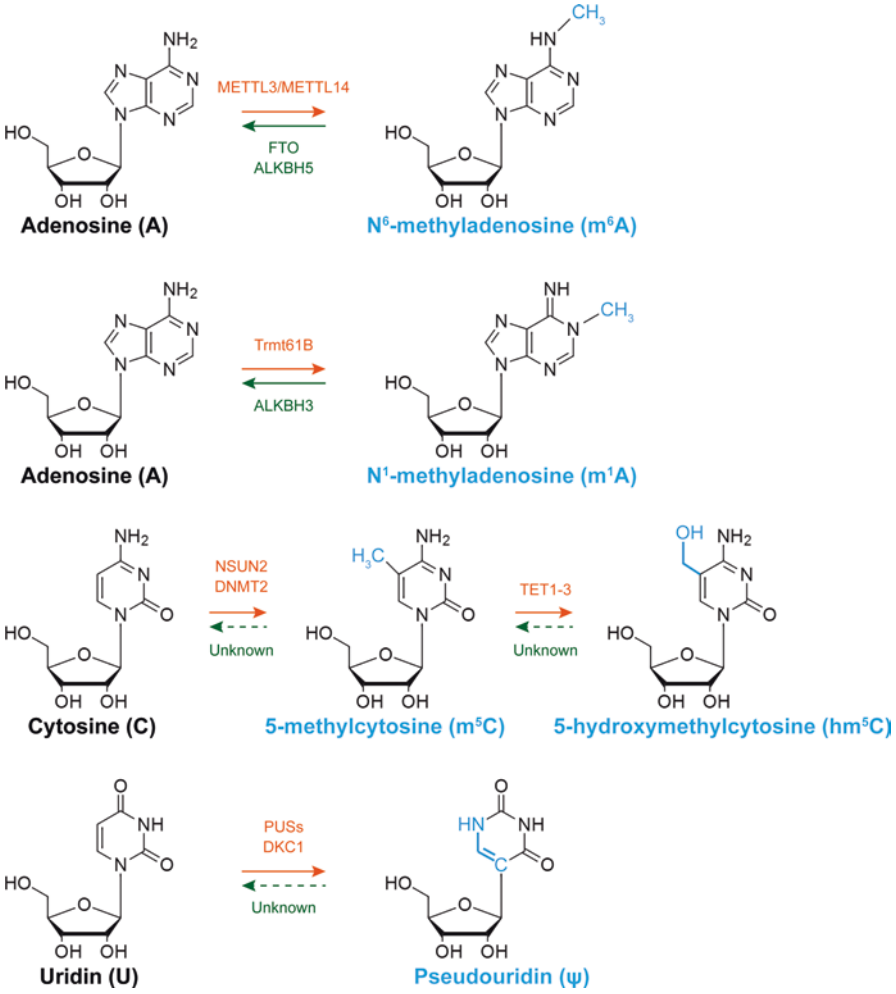

Fig. 4.1 Chemical structures of representative modified RNA nucleotide variants. Modifications are shown in blue. The known writer (in orange) and eraser (in green) proteins are also indicated

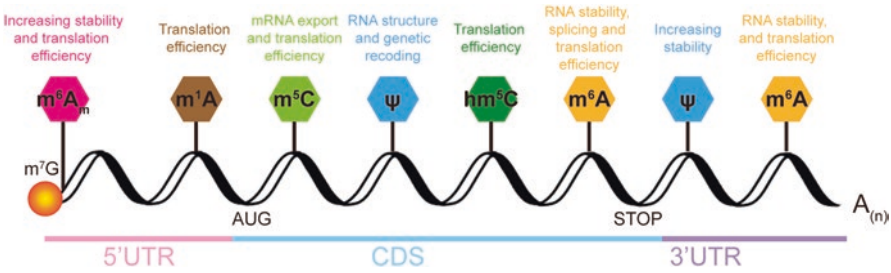

Fig. 4.2 Chemical modifications in eukaryotic mRNA. A schematic representation of common chemical modifications across eukaryotic mRNA transcript including 5′ untranslated region (5′UTR), coding region (CDS), and 3′UTR. Reported roles of these modifications are summarized on top of the corresponding modifications. Note that the same modification in different mRNA regions may have different functions in regulating mRNA fate

modification on eukaryotic mRNAs. m^6A RNA modification was first identified in the 1970s [1–3]. However, due to the lack of knowledge on its dynamic regulation and no high-throughput technology available to map m^6A modification to the RNA transcriptome, little attention had been paid to this RNA mark until 2011, when the fat mass and obesity-associated protein (FTO) was identified as a genuine demethylase of m^6A modification [17], which implies that m^6A modification is a reversible and dynamic process analogous to the well-studied modifications on DNA and histone [18]. Subsequent development of high-throughput m^6A sequencing technologies further facilitates the understanding of m^6A modification in a transcriptome-wide view, revealing that m^6A modification may affect more than 7000 mRNAs in individual transcriptomes of mammalian cells, with a special enrichment in the 3′ untranslated regions (UTRs) near the stop codons of mRNAs and with a consensus sequence of RRACH (R = G or A; H = A, C, or U) [19, 20]. Such findings strongly suggest that m^6A modification may have important biological functions. Indeed, emerging data demonstrate that m^6A modifications in mRNAs or noncoding RNAs influence RNA fate and functions and are critical for many normal and pathological bioprocesses including self-renewal and differentiation of embryonic stem cells, tissue development, circadian rhythm, heat shock or DNA damage response, sex determination, and tumorigenesis, as reviewed elsewhere [4, 16, 21–37]. Evidence is emerging that m^6A modification and the associated machinery also play essential roles in tumorigenesis and drug response (see reviews [16, 38, 39]). Here we summarize recent advances on our understanding of the functions and underlying molecular mechanisms of the m^6A machinery in normal hematopoiesis and AML pathogenesis and drug response.

4.2 Regulators of m^{6}A Modifications

Similar to other epigenetic modifications, m^{6}A modification is regulated by its formation and removal catalyzed by the methyltransferases and demethylases, known as "writers" and "erasers," respectively [16, 40, 41]. m^{6}A marks are installed by a multicomponent methyltransferase complex (MTC) consisting of the core methyltransferase-like 3 and 14 (METTL3 and METTL14) heterodimer and their cofactors including Wilms' tumor 1-associating protein (WTAP), vir like m^{6}A methyltransferase associated (VIRMA, also known as KIAA1429), RNA-binding motif protein 15 (RBM15), and zinc finger CCCH domain-containing protein 13 (ZC3H13) [22, 42–49] (Fig. 4.3). Structural studies demonstrated that METTL3 is the sole catalytic subunit, while METTL14 offers an RNA-binding scaffold to allosterically activate and enhance the catalytic activity of METTL3 [50–52]. WTAP, VIRMA, RBM15, and ZC3H13 are the regulatory subunits of the MTC to facilitate m^{6}A installation *in cellulo*. In addition, the METTL3 homolog METTL16 (methyltransferase-like 16) was recently shown to control cellular S-adenosyl methionine (SAM) level and install m^{6}A marks onto the U6 small nuclear RNA [53, 54]. FTO, previously known to function as a demethylase for N^3-methylthymidine in single-stranded DNA and N^3-methyluridine in single-stranded RNA in vitro [55, 56], was identified as the first m^{6}A demethylase that could demethylate m^{6}A in both DNA and RNA in vivo [17]. A recent study reported that FTO also demethylates m^{6}A$_m$, a modification exclusively found at the first encoded nucleotide after the 7-methylguanosine cap structure of mRNAs [57]. AlkB homolog 5 (ALKBH5) is the second identified m^{6}A demethylase that was found to be highly expressed in the testes [23]. Both FTO and ALKBH5 belong to the AlkB subfamily of the Fe(II)/2-oxoglutarate (2OG) dioxygenase superfamily that requires 2OG and molecular oxygen as co-substrates and ferrous iron Fe(II) as a cofactor to catalyze the oxidation of a substrate [56, 58].

While the prevalence and distribution of m^{6}A are determined by writers and erasers, the m^{6}A-dependent functions are mediated by m^{6}A-binding proteins, the so-called readers, which through specific recognition and binding to m^{6}A-modified mRNAs determine the fate of these transcripts [16, 21, 59] (Fig. 4.3). The YT521-B homology (YTH) domain family of proteins, including YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2, are among the first identified m^{6}A readers that possess a conserved m^{6}A-binding pocket [19, 60–66]. Of them, YTHDC1 was found to be located in the nucleus, playing a role in splicing regulation, XIST-mediated X-chromosome silencing, and nuclear export of m^{6}A-modified mRNAs [46, 63, 67]. The other YTH family proteins are all cytoplasmic m^{6}A readers regulating mRNA fate through different mechanisms: YTHDF2 promotes degradation of target mRNAs [60, 68], and YTHDF1 promotes translation of target mRNAs [61], while YTHDF3 and YTHDC2 can both mediate mRNA decay and enhance translation [66, 69–73]. We recently identified insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; including IGF2BP1/2/3) as a new family of m^{6}A readers that could promote stability and translation of their target mRNAs [32], distinct

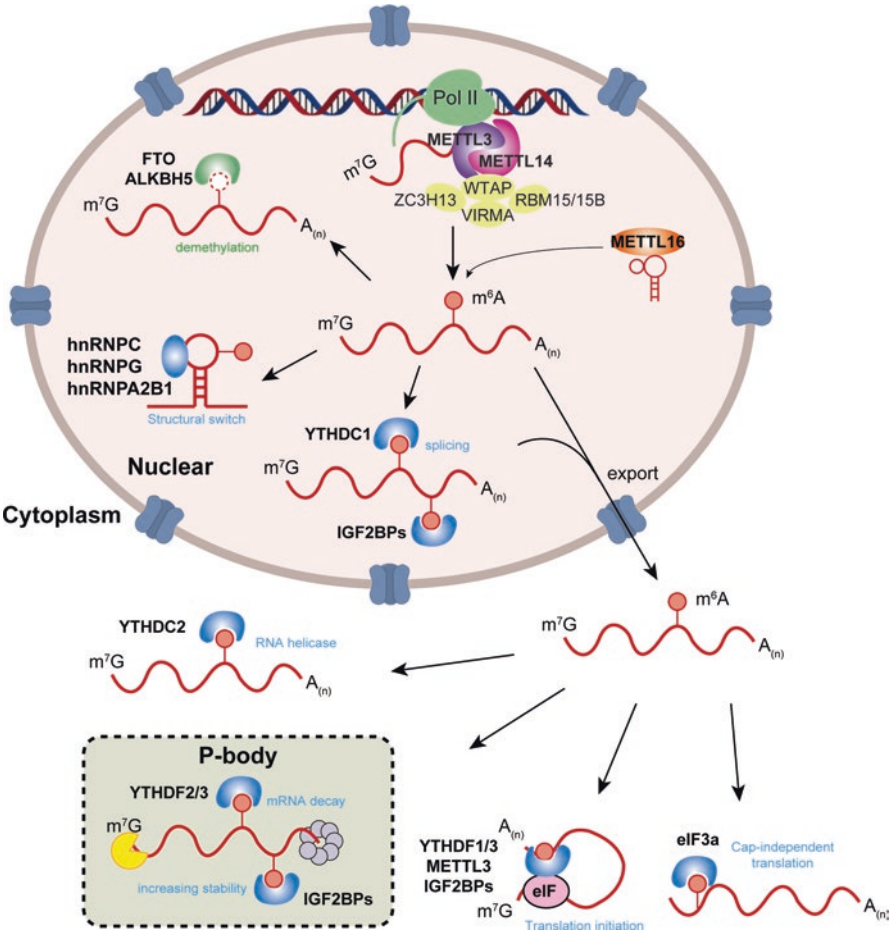

Fig. 4.3 Roles of m^{6}A RNA modification in determining mRNA fate. In nuclear, the methyltransferase complex composed of METTL3, METTL14, WTAP, VIRMA, RBM15, and ZC3H13 deposits m^{6}A marks co-transcriptionally onto newly transcribed RNAs, while METTL16 is responsible for m^{6}A deposition on the U6 snRNA. FTO and ALKBH5 function as m^{6}A demethylases to remove m^{6}A marks in selected sites of RNA. M^{6}A-mediated structural switch of mRNA recruits hnRNP family proteins including hnRNPC, hnRNPG, and hnRNPA2B1. Nuclear reader protein YTHDC1 recognizes m^{6}A to mediate alternative splicing. IGF2BP reader proteins bind to m^{6}A mRNAs to stabilize these nascent transcripts. After exporting to cytoplasm, m^{6}A-modified mRNAs could be subjected to degradation by YTHDF2 or YTHDC2 or protected by IGF2BP proteins and loaded to translation machinery. YTHDF1, YTHDF3, as well as METTL3 and eIF3a could also promote translation of m^{6}A mRNAs

from the functional manners of the YTH family proteins. The K homology (KH) domains of IGF2BPs are required for their recognition of m^{6}A and are critical for their oncogenic functions. Interestingly, ELAV-like RNA-binding protein 1 (ELAVL1, also known as HuR), an mRNA stabilizer that was previously reported

as an indirect m^{6}A-binding protein [22], was found to be a cofactor of IGF2BPs and may mediate the mRNA stabilizing effect of IGF2BPs [32]. The heterogeneous nuclear ribonucleoprotein (HNRNP) HNRNPA2B1 was previously reported to regulate alternative splicing and primary microRNA processing as an m^{6}A reader [74]; however, recent structural study suggested an "m^{6}A switch" mechanism rather than direct m^{6}A binding for the protein [75]. Two other members of the HNRNP family proteins, namely HNRNPC and HNRNPG, were shown to recognize m^{6}A-induced changes in mRNA secondary structures [76]. Other proteins were also recently reported to be m^{6}A interactors, including FMR1 and LRPPRC [77, 78], although the exact mode of binding still needs to be clarified.

4.3 Roles of m^{6}A in Normal Hematopoiesis and HSC Self-Renewal

Hematopoiesis is a tightly regulated dynamic process where mature blood cells are generated from a small pool of multipotent hematopoietic stem cells (HSCs) [79, 80]. During the past few decades, it has been well acknowledged that transcriptional regulation by a variety of hematopoietic transcription factors (TFs) plays a big role in regulating the multistep normal hematopoiesis [81–83]. In particular, during myelopoiesis where HSCs are differentiated into myeloid progenitors and eventually mature myeloid cells, the sequential actions of master TFs are required to specify and re-enforce each cell fate decision. For instance, PU.1 (also known as SPI1, the product of the oncogene SPI1) is a transcriptional master regulator of myeloid cells which plays an essential role in generating early myeloid progenitors (i.e., common myeloid progenitors, CMPs), while the basic region leucine zipper transcription factor C/EBPα is required for the production of granulocyte/macrophage progenitors (GMPs) from CMPs [80, 84].

In recent years, emerging studies reveal m^{6}A modification at the RNA level as an additional layer of the posttranscriptional regulation in governing HSC activity and normal hematopoiesis (see Fig. 4.4). During the endothelial-to-hematopoietic transition (EHT) of zebrafish embryogenesis, a key developmental event leading to the formation of the earliest hematopoietic stem and progenitor cells (HSPCs), m^{6}A modification was reported to play a role [30]. Deficiency of mettl3 in zebrafish embryos leads to decreased levels of m^{6}A and blockage of HSPC emergence, likely due to the reduced m^{6}A modification on the arterial endothelial genes *notch1a* and *rhoca* that delayed YTHDF2-mediated mRNA decay of these transcripts [30]. Vu and colleagues used short hairpin RNAs (shRNAs) to knock down *METTL3* expression in human HSPCs and observed cell growth inhibition and increase of myeloid differentiation [34]. Conversely, overexpression of wild-type, but not catalytically dead mutant of METTL3, promotes proliferation and colony formation and inhibits myeloid differentiation. We recently showed that METTL14 is highly expressed in murine HSCs and Lin$^-$ Sca-1$^+$c-kit$^+$ (LSK) cells and was downregulated during

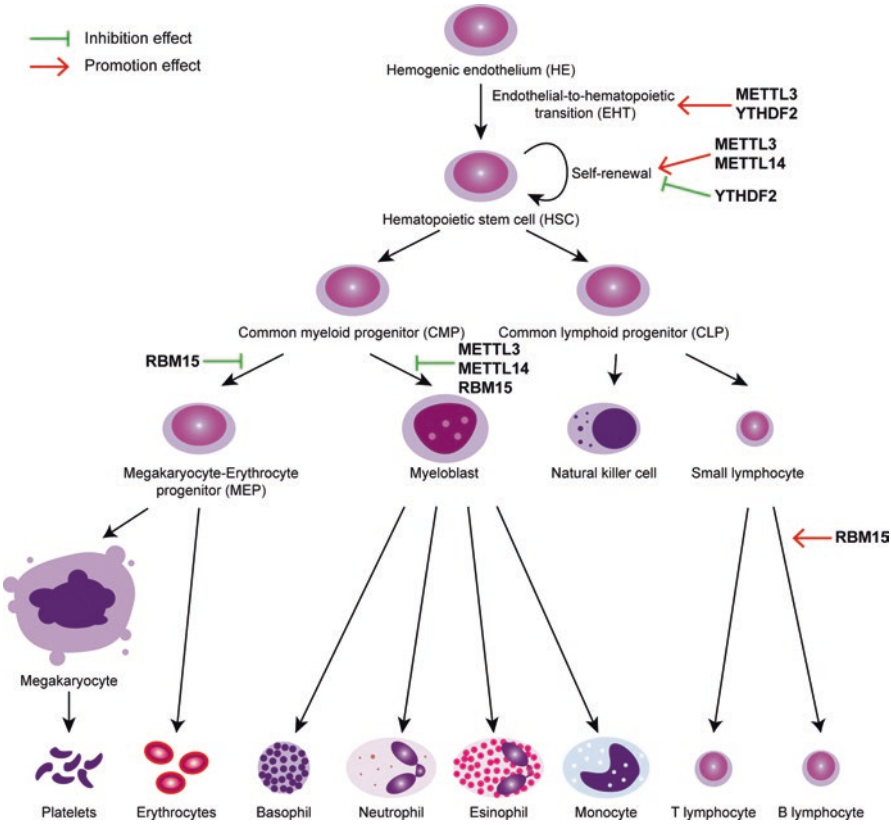

Fig. 4.4 Modifiers of m^{6}A RNA modification in normal hematopoiesis. METTL3 and METTL14 promote self-renewal of HSC and inhibit myeloid differentiation. In contrast, YTHDF2 inhibits HSC self-renewal. RBM15 inhibits myeloid and megakaryocytic differentiation while promotes B cell expansion, although it is unclear whether these effects are m^{6}A-related

myelopoiesis, showing reduced expression in CMP and GMP progenitors and especially in mature myeloid cells [33]. Consistent with the expression pattern, depletion of *METTL14* expression in human HSPCs by shRNAs promotes myeloid differentiation in vitro. Moreover, by utilizing a *Mettl14* conditional knockout mouse model, we demonstrated that induced deletion of *Mettl14* impairs HSC self-renewal ability in vivo. Such effects were mediated by decreased *MYB* and *MYC* expression owing to the reduction of m^{6}A modification on these transcripts upon *METTL14* knockdown/knockout. Considering the role of MYB [85, 86] and MYC [87, 88] transcription factors in regulating HSC self-renewal and differentiation, METTL14-mediated m^{6}A regulation on the mRNA transcripts of these TFs adds a new layer of complexity to the regulatory networks in normal hematopoiesis.

A very recent study took advantage of *Mettl3* and *Mettl14* conditional knockout mice to investigate the roles of these m^{6}A writer proteins on regulation of HSC self-renewal in adult mouse bone marrow [89]. They found that deletion of *Mettl3*

alone or together with *Mettl14* in the hematopoietic system substantially increases HSC frequency in the bone marrow; in contrast, deletion of *Mettl14* alone has little effect [89]. Conditional deletion of *Mettl14* and especially that of *Mettl3* suppresses HSC self-renewal activity in recipient mice. Notably, although deletion of either *Mettl3* or *Mettl14* deletion leads to significant reduction of donor-derived myeloid cells in the peripheral blood, only deletion of *Mettl3* results in a significant reduction of B- and T-cell lineage [89].

RBM15, recently identified as a component of the m^{6}A methyltransferase complex, was also reported to play a role in normal hematopoiesis [90–92]. Conditional knockout of *Rbm15* in adult mice blocks B cell differentiation and results in an increase of the Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) HSPCs and an expansion of myeloid and megakaryocytic cells in spleen and bone marrow, demonstrating a role of RBM15 in hematopoietic development [90]. Rbm15 is expressed at highest levels in HSCs and inhibits myeloid differentiation and megakaryocytic expansion through stimulation of the Notch signaling and regulation of *MYC* expression, respectively [91, 92].

YTHDF2 is the first well-characterized m^{6}A reader that promotes mRNA decay of target transcript with m^{6}A modification [60]. Li and colleagues studied the role of YTHDF2 in adult stem cell maintenance and reported an increase of HSCs in *Ythdf2* conditional knockout mice as well as in human umbilical cord blood upon *YTHDF2* knockdown [93]. Such effects are partially mediated by the stabilization of mRNA transcripts encoding TFs critical for stem cell self-renewal. Overall, the studies of m^{6}A in normal hematopoiesis are just in the beginning. It is of great interest to explore the functions and underlying mechanisms of other m^{6}A modulators, including other writers, erasers, and readers, in stem cell biology and normal hematopoiesis.

4.4 Dysregulation of m^{6}A Regulators in Malignant Hematopoiesis

Dysregulation of the regulatory networks of normal hematopoiesis, such as incorrect activity of the hematopoietic TFs attributed to either aberrant expression or mutation, breaks the balance between HSC self-renewal and differentiation and places the progenitor cells at a higher risk of developing leukemia [80, 94]. Acute myeloid leukemia (AML) is a clonal hematopoietic disorder where a stem cell-like self-renewal capacity is gained and the differentiation capacity is blocked [95, 96]. According to the Cancer Statistics 2017, the 5-year survival of AML between 2007 and 2013 is 26.9%, much lower than other types of leukemia and many other common cancer types [97]. For instance, during the same period, the 5-year survival rate is 89.7% for breast cancer and 66.9% for chronic myeloid leukemia (CML). Therefore, there is an urgent need to better understand the mechanisms underlying AML leukemogenesis and, based on the gained knowledge, to develop effective targeted therapies.

The first evidence of a role of m^{6}A modification on leukemia, specifically on AML, came from the study of FTO, a major m^{6}A demethylase [31]. *FTO* was previously known as a gene associated with fat mass, adipogenesis, and body weight [98–100], and single-nucleotide polymorphisms (SNPs) in *FTO* were linked to higher risk of developing cancers including leukemia and lymphoma by large-scale epidemiology studies [101–103]. We found that *FTO* is highly expressed in certain subtypes of AML, including those carrying t(11q23)/*MLL* rearrangements, t(15;17)/*PML-RARA*, *FLT3*-ITD, and/or *NPM1* mutations. Modulation of *FTO* expression by depletion of *FTO* or forced expression of wild-type FTO (but not catalytically inactive mutant) could significantly influence AML cell survival and leukemogenesis and affect the response of AML cells to all-trans retinoic acid (ATRA) [31]. Importantly, the oncogenic function of FTO in AML relies on its m^{6}A demethylase activity. By reducing m^{6}A abundance on the transcripts of *ASB2* and *RARA*, two genes with reported roles in cell proliferation and drug response of leukemia cells, FTO posttranscriptionally regulates expression of *ASB2* and *RARA* through reducing m^{6}A abundance, thereby decreasing mRNA stability of these transcripts. These data provide compelling evidence on the role of FTO in leukemogenesis and establish a first link between m^{6}A modification and leukemia pathogenesis (Fig. 4.5) [31].

More recently, we showed that, by targeting FTO directly, R-2-hydroxyglutarate (R-2HG), a previously reported oncometabolite produced by mutant isocitrate dehydrogenase 1/2 (IDH1/2) enzymes [104–106], exhibits a broad and intrinsic antitumor activity in leukemia. By inhibiting the m^{6}A demethylase activity of FTO, R-2HG results in an increase of m^{6}A abundance on FTO target genes, such as *MYC* and *CEBPA*, leading to decay of these transcripts in R-2HG sensitive cells (see Fig. 4.5). Our data indicate that FTO/MYC homeostasis controls the sensitivity of leukemic cells to 2HG: high abundance of FTO confers R-2HG sensitivity, whereas hyper-activation of MYC signaling renders leukemic cells resistant to R-2HG [37]. Consistent with this notion, pharmaceutical or genetic inhibition of MYC signaling by JQ1 or *MYC* shRNAs can resensitize R-2HG-resistant leukemic cells to R-2HG. In addition, we showed that R-2HG treatment or FTO inhibition also sensitized AML cells to first-line chemotherapy drugs such as ATRA, Azacitidine (AZA), Decitabine, and Daunorubicin ([37] and unpublished data). Collectively, our studies demonstrate the critical role of FTO in leukemia pathogenesis and drug response and also highlight the therapeutic potential of targeting FTO signaling for AML treatment.

Subsequently, the dysregulation of the m^{6}A installing machinery was also reported to be involved in AML pathogenesis [107] (see Fig. 4.6). METTL3 and METTL14 were both expressed at a higher level in AML than in the vast majority of other cancer types according to The Cancer Genome Atlas (TCGA) genome-wide gene expression datasets [33–35]. We found that METTL14 is aberrantly overexpressed in certain subtypes of AMLs, such as those carrying t(11q23)/*MLL* rearrangements, t(15;17)/*PML-RARA*, and t(8;21)/*AML1-ETO* [33]. We next conducted in vitro and in vivo gain- and loss-of-function studies and demonstrated that METTL14 plays a critical oncogenic role in AML pathogenesis [33]. Depletion of

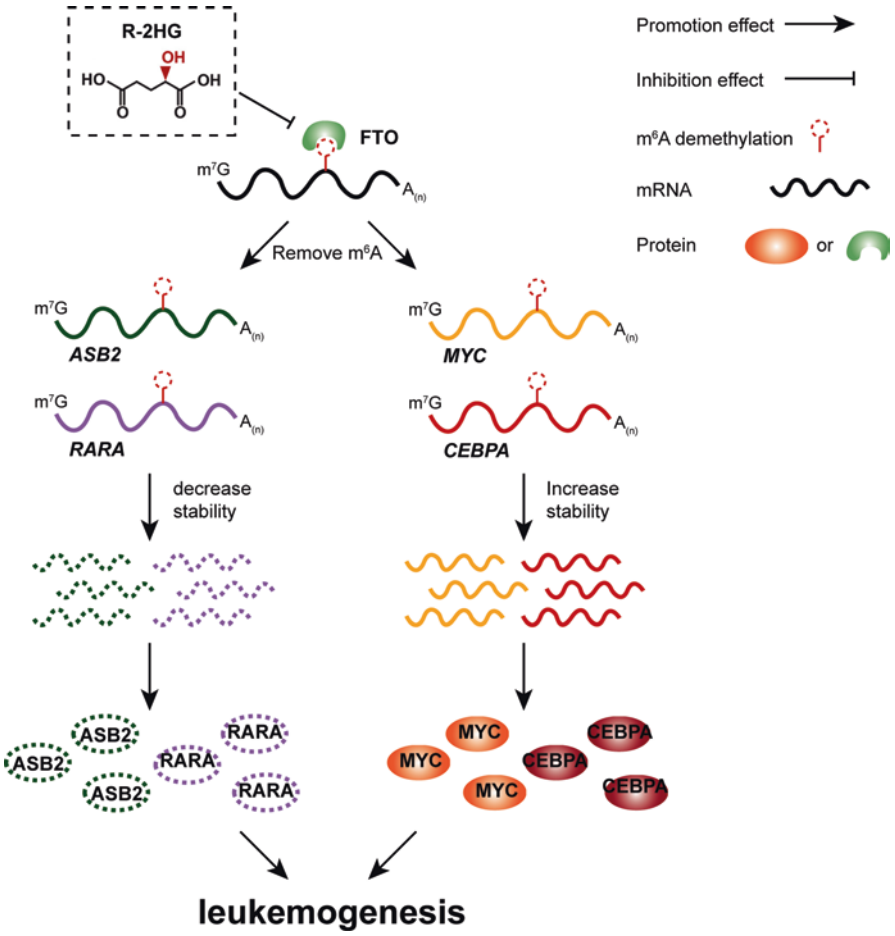

Fig. 4.5 Role of FTO in leukemogenesis. FTO removes m^6A modification on its target mRNAs (e.g., *ASB2*, *RARA*, *MYC*, and *CEBPA*), resulting in the decreased or increased stability of these transcripts, and promotes leukemogenesis. R-2HG inhibits FTO and exhibits antileukemia effects

METTL14 inhibited survival and proliferation of AML cells, promoted myeloid differentiation, and suppressed leukemic oncofusion protein (e.g., MLL-AF9, MLL-AF10, and AML1-ETO9a)-mediated immortalization of normal HSPCs. The opposite is true when wild type, but not mutant METTL14 (i.e., R298P), was forced expressed. Moreover, knockdown of *METTL14* significantly inhibited progression of human AML cells in xenotransplantation recipient mice, while inducible knockout of *Mettl14* greatly inhibited AML development and maintenance in bone marrow transplantation (BMT) recipient mice [33]. High-throughput RNA-seq and transcriptome-wide m^6A-seq, coupled with gene-specific m^6A-qPCR assays, cross-linking and immunoprecipitation (CLIP) assays, luciferase reporter and mutagenesis assays, mRNA stability assays, and polysome profiling assays, demonstrated

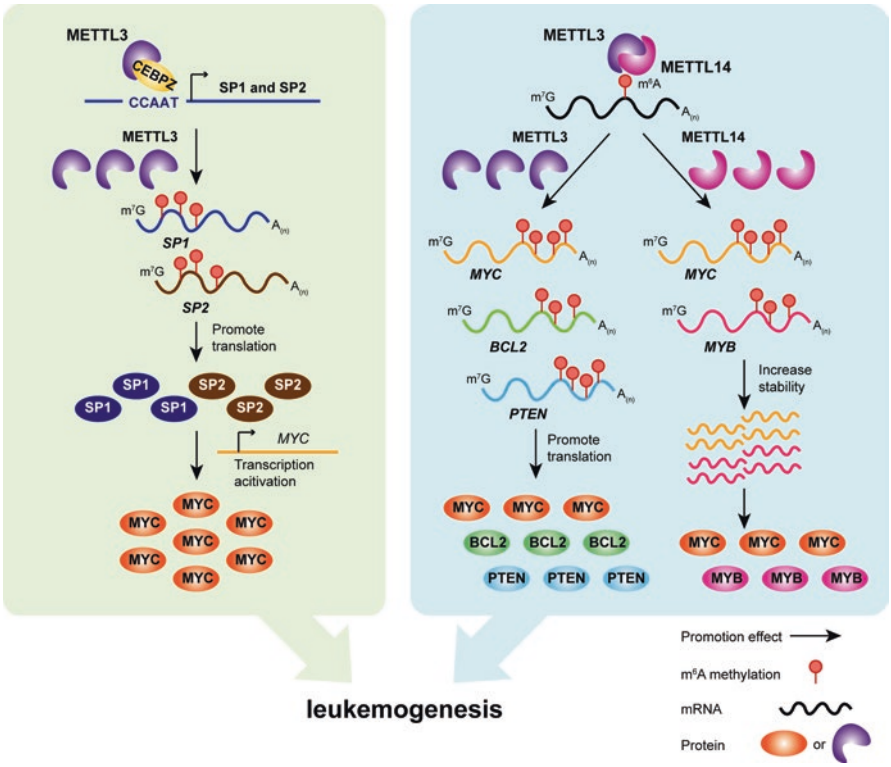

Fig. 4.6 Roles of m^{6}A writer genes in leukemogenesis. METTL3 and METTL14 function as oncogenes in AML through depositing m^{6}A modification on their target transcripts (e.g., *MYC*, *BCL2*, and *PTEN* for METTL3 and *MYB* and *MYC* for METTL14) to enhance translation and/or increase mRNA stability of these transcripts. METTL3 can also be recruited to promoter of its target gene by CEBPZ and deposit m^{6}A modification on the coding region of the target transcripts (e.g., *SP1* and *SP2*) to promote their translation and eventually leads to *MYC* activation

that METTL14 posttranscriptionally regulates the expression of its critical target mRNA transcripts, such as *MYB* and *MYC*, two well-known TF genes involved in leukemogenesis, in an m^{6}A-dependent manner [33]. Silencing of *METTL14* reduces m^{6}A abundance of *MYB* and *MYC* transcripts, especially near the 3′ end of the mRNAs, resulting in decreased mRNA stability and translation of these transcripts. Such effects were not mediated by the YTH family proteins, and therefore other readers (such as IGF2BPs [32]) may mediate the effect of METTL14 on *MYB* and *MYC*. Furthermore, we also identified SPI1 (also called PU.1), a transcriptional master regulator of myelopoiesis, as a negative regulator of *METTL14* expression in AML [33]. Taken together, our work reveals a previously unappreciated SPI1-METTL14-MYB/MYC signaling axis in leukemogenesis and highlights the critical roles of METTL14 and m^{6}A modification in malignant hematopoiesis [33].

Meanwhile, two other groups have demonstrated that METTL3 also plays an essential oncogenic role in AML, by showing that depletion of *METTL3* expression

results in cell growth inhibition, cell cycle arrest, and induction of differentiation and apoptosis, whereas overexpression of wild-type *METTL3*, but not a catalytically inactive mutant, promotes proliferation in AML cells and primary blasts [34, 35]. Similar to METTL14, METTL3 is also required for AML development as demonstrated by the xenotransplantation assay data that shRNA-mediated knockdown or CRISPR/Cas9-mediated knockout of *METTL3* substantially inhibited AML progression and prolonged survival in recipient mice [34, 35]. Such findings support the oncogenic role of METTL3 as an m^{6}A-catalyzing enzyme. Despite of the similar functions of METTL3 reported by the two groups, distinct underlying mechanisms of METTL3 in AML were reported [34, 35]. Vu et al. performed m^{6}A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP), RNA-seq, and Ribo-Seq, and identified *MYC*, *BCL2* and *PTEN* as direct RNA targets of METTL3, whose m^{6}A abundances were substantially reduced when *METTL3* was knocked down [34]. However, upon *METTL3* depletion, while expression of *MYC*, *BCL2*, and *PTEN* transcripts showed a great increase, the corresponding protein levels decreased on day 3 after shRNA transduction and recovered 1 day later [34]. Although the authors proposed that an alternative internal ribosome entry site (IRES)-mediated translational mechanism may be involved to reactivate translation of *MYC* and *BCL2* under METTL3 depletion-induced cell apoptosis, it remains unclear why the expression changes of these target genes at the mRNA level are inconsistent with that at the protein levels. A systematic understanding of the changes and functions of m^{6}A readers under this circumstance may help to address this question. Different from Vu's study [34], Barbieri et al. found that METTL3 and METTL14 could bind chromatin, mainly localizing to the transcriptional start sites (TSSs) of coding genes characterized by bimodal H3K4me3 peaks [35]. However, METTL3 and METTL14 did not bind the same TSSs, suggesting independent regulation on target genes. CEBPZ, a transcription factor critical for hematopoietic differentiation, recruits METTL3, but not METTL14, to chromatin [35]. Promoter-bound METTL3 induces m^{6}A modification within the coding region of the associated mRNA transcript, such as *SP1* and *SP2*, and enhances their translation by relieving ribosome stalling [35].

In addition to METTL3 and METTL14, other m^{6}A methyltransferases or cofactors of the m^{6}A methyltransferase complex have also been implicated to function in AML. A genome-wide CRISPR-Cas9 screening identified METTL16, as well as METTL3 and METTL14, as critical genes for AML survival [35], while detailed study is yet to be done. Before it was identified as a component of the m^{6}A methyltransferase complex [43], WTAP was shown to be upregulated in AML and is a novel client protein of HSP90 [108]. Knockdown of WTAP in AML cells led to reduced proliferation and promotion of phorbol 12-myristate 13-acetate (PMA)-induced myeloid differentiation and inhibited growth of human leukemia cells in xenograft mice [108]. RBM15 was found to be a fusion partner of the MKL1 gene in t(1,22)(p13;q13) acute megakaryoblastic leukemia (AMKL), a subtype of pediatric AML [109]. It is therefore reasonable to speculate that RBM15 may play a role in leukemogenesis, possibly related to m^{6}A modification, for which further studies are warranted.

4.5 m^{6}A and Leukemia Stem Cells

Malignant stem cells are considered as the potential origin of and a key therapeutic target for AML, similar to what is believed for other cancer types [110–115]. Leukemia stem cells (LSCs) are defined as cells with two important properties: (1) capable of engrafting and initiating the disease when transplanted into immunodeficient animals and can self-renew by giving rise to leukemia in serial transplantations and (2) produce non-LSC bulk blasts that resemble the original disease but are unable to self-renew [116]. During the last few decades, investigators have been dedicated to the characterization of LSCs using different combinations of cell surface markers. It was found that LSCs immunophenotypically resemble certain normal hematopoietic progenitor populations and usually reside in the CD34$^+$CD38$^-$ fraction, although approximately 25% of AML cases lack CD34 expression [116]. It is believed that therapeutic targeting and eliminating of LSC is the key to eradicate leukemia and achieve long-term remissions.

Retroviral transduction of the *MLL-AF9* oncofusion gene into mouse HSPCs followed by transplantation into recipient mice represents one of the best mouse models for AML LSC studies [116]. By using this model, we have shown that depletion of either *Fto* or *Mettl14* in mouse HSPCs could inhibit leukemia initiation in primary BMT recipients and maintenance in secondary transplantations [31, 33], suggesting a role of these m^{6}A modifying proteins on AML LSC function. Indeed, we conducted limiting dilution assays using bone marrow cells harvested from MLL-AF9 primary leukemia mice to directly evaluate the effect of *Mettl14* depletion on the frequency of leukemia stem/initiating cells (LSCs/LICs) and found that the estimated LSC/LIC frequency was significantly reduced when *Mettl14* was knocked out [33]. While our data provide the first link between m^{6}A modification and LSC self-renewal [33], further systematic studies are warranted to better understand the role of m^{6}A modification on LSC biology.

4.6 Conclusions and Perspectives

Evidence is emerging that m^{6}A modification and the associated machinery play essential roles in both normal and malignant hematopoiesis, including the self-renewal and differentiation of normal and malignant HSCs. Systematical studies of the functions and underlying molecule mechanisms of individual m^{6}A writer, eraser, and reader genes will further advance our understanding of the complex networks and molecular mechanisms underlying normal and malignant hematopoiesis. In addition, it is also important to understand how expression of individual m^{6}A regulators is regulated during normal and malignant hematopoiesis.

The impact of m^{6}A modification on the fate of its embedded RNA mediates the functions of such modification. Therefore, it is of great importance to identify key functionally important RNA targets (including mRNA, lncRNA, etc.) that when

manipulated could phenocopy or reverse the effects of knockdown or overexpression of individual m^{6}A regulators. For instance, recent studies show the oncogenic *MYC* transcript as an important target of many m^{6}A regulators in cancer including leukemia [32–34, 37], highlighting the importance of precise regulation of *MYC* expression at the posttranscription level in normal development and the big impact of its dysregulation in tumorigenesis. Further systematic identification and functional studies of all critical target genes of m^{6}A modification by combined use of high-throughput sequencing such as CLIP-seq, m^{6}A-seq, and RNA-seq, followed by the validation assays and in vitro and in vivo functional studies, are warranted. Such knowledge will be not only important for the understanding of the effects of manipulation of individual m^{6}A regulator but also may lead to the discovery of novel therapeutic targets for AML and other types of leukemia.

As demonstrated by the published data thus far, m^{6}A regulators represent promising targets for treatment of cancer, including leukemia. Development of effective and selective inhibitors targeting oncogenic m^{6}A regulators (e.g., FTO and METTL14) that play more essential roles in leukemogenesis, especially in self-renewal of LSCs/LICs, than in normal hematopoiesis may hold great therapeutic potential in treating leukemia, especially when in combination with other therapeutic agents.

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Chapter 5
Leukemia Stem Cells in the Pathogenesis, Progression, and Treatment of Acute Myeloid Leukemia

Kanak Joshi, Lei Zhang, Peter Breslin S.J., and Jiwang Zhang

Contents

Abstract Despite the significant progress that has been made in understanding the biology of leukemia stem cells (LSCs), some key questions regarding the concept of LSCs have not as yet been satisfactorily addressed experimentally. As a result, the clinical relevance of LSCs remains less than clear due to controversies caused largely by technical limitations in efficiently identifying LSCs. This has impeded

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our ability to fully address the features of genetic heterogeneity and metabolic/epigenetic plasticity of pre-LSCs and LSCs. With the development and use of humanized immunocompromised mice, we are able to more precisely analyze LSCs for their functions and interaction with the bone marrow niche. In addition, some promising targets in LSCs have recently been identified, including Sonic Hedgehog (SHH) and BCL-2, which are highly expressed in AML cells. It is hopeful that new anti-LSC compounds will be tested fully in clinical trials for their efficacy in treating human leukemias.

Keywords Leukemia stem cell · Humanized immunocompromised mice · AML

5.1 Introduction

Acute myeloid leukemia (AML) is a common type of hematopoietic malignancy which primarily develops in the elderly population. The average age at diagnosis for AML is 67 years. The 7 + 3 regimen of intensive chemotherapy consists of 3 days of an anthracycline antibiotic or an anthracenedione + 7 days of standard-dose cytarabine (ARA-C). This has been considered the first line of treatment for AML for over 45 years. 7 + 3 treatment can induce an overall response (OR) including complete remission (CR) or CR with incomplete count recovery (CRi) in 60–80% of patients. However, most elderly patients cannot tolerate such an intense regimen of antineoplastic agents. Currently, the 5-year overall rate of survival (OS) for AML is 35–40% for patients under 60 years of age and <10% for patients over the age of 60 years at the time of diagnosis [1–6].

For patients who fail to achieve CR/CRi and may die of either treatment-related complications or primary drug resistance-related disease progression, novel medications and medication combinations are critically needed, especially for elderly patients. Over the past several years, many new regimens such as CPX-351 (a liposomal formulation of cytarabine and daunorubicin encapsulated at a synergistic 5:1 molar ratio) and hypomethylating agents (HMAs), including azacitidine (AZA) and decitabine (DEC), have been approved by the FDA for the treatment of elderly patients who cannot tolerate 7+3 chemotherapy [7]. CPX-351 treatment significantly increases the CR/CRi rate (47.7% for CPX-351 vs. 33.3% for 7+3) and OS (median 9.56 months for CPX-351 vs. 5.95 for standard 7+3) in elderly AML patients [7]. Although HMAs can only induce CR/CRi in 15–25% of elderly AML patients when used as a monotherapy, such treatment can significantly extend OS (median 10.4 months for HMAs vs. 6.5 months for 7+3) [8–12]. Most recently, the successful use of the BCL-2 inhibitor Venetoclax (VEN) and AZA in combination to treat elderly patients represents a potentially revolutionary change in the treatment of AML [13–16].

For those AML patients who have achieved CR/CRi, disease relapse is one of the major causes of death, owing to the inability to achieve complete elimination of leukemia-regenerating cells (LRCs), which form the basis of minimal residual

disease (MRD) and are responsible for the relapse of leukemia [17–22]. The prevention of disease relapse in such patients by maximally eliminating LRCs in order to reduce the potential for MRD is the most urgent issue that needs to be addressed clinically in order to arrive at the goal of a cure for this disease. Most previous studies suggested that LRCs are derived from leukemia stem cells (LSCs), which are resistant to chemotherapy and are responsible for disease relapse. Thus, a combination strategy involving targeting LSCs plus chemo-debulking therapy has been proposed as an effective treatment for AML [23–31]. However, such a concept is challenged by two recent research reports which demonstrate that these chemoresistant LRCs in MRD are not derived from naïve LSCs [32, 33]. Thus a more complete understanding of the nature of the LRCs in MRD and an elucidation of the mechanism by which MRD occurs will help us to develop therapies to better target LRCs in MRD by (1) preventing the possibility of MRD occurring, (2) eliminating MRD when it does develop, and (3) inhibiting the propagation of LRCs to significantly decrease the likelihood of disease relapse.

Although the classical LSC model provides a plausible explanation for clinical observations in AML and also includes relevant implications for therapy, yet due to technical limitations as well as the dynamic and heterogeneous nature of LSCs, this model still lacks direct evidence to fully support the notion that LRCs in MRD are derived from LSCs. As a consequence, inconsistencies and even controversies have been associated with previous studies [34–36]. In this review, we will update the most recent advances in LSC research and discuss the significance and limitations of the LSC concept, as well as those related to the potential targeting of LSCs to treat AML. Thus, we hope to be able to provide reasonable explanations for the controversial aspects of LSC research, as well as cancer stem cells (CSCs) in solid tumors, and propose useful concepts that might guide future LSC and CSC studies.

5.2 The Concept of LSCs

It is now well-established that almost any cancer, including AML, can be conceptualized as a heterogeneous mixture of malignant types of cells which share some properties but which also allow them to be distinguished morphologically, genetically, and functionally [37]. The LSC concept was introduced in the 1990s, first for AML and subsequently for other hematopoietic malignancies, to explain the heterogeneity of malignant cells by reference to the hierarchical model of cellular organization of tissues described for normal hematopoiesis [23, 38, 39]. According to this model, there is a subpopulation of leukemic cells (LCs) in AML tissues which is located at the apex of the differentiation hierarchy which gives rise to highly proliferative leukemic progenitors (LPs) and subsequently to partially differentiated leukemic blasts (LBs) which maintain disease progression and allow for metastasis [40]. It is still generally accepted that most current chemotherapeutic agents target rapidly dividing LPs and LBs, which constitute the bulk of malignant cells in leukemia. But these medications cannot kill LSCs because the latter are relatively slow

cycling (i.e., quiescent) cells [40]. The LSC concept is primarily intended to focus on and explain several critical questions in leukemia research and attempts to identify factors which lie at the root of attempts to cure leukemia. Such questions include the following: (1) From which cell(s) do(es) the malignancy originate? (2) During the progression of leukemia, which cells propagate the leukemic tissue in both primary tumors and distant metastases? (3) During treatment, which cells are resistant to therapy? (4) After therapy-induced remission of leukemia, which cells remain and form the basis of MRD and ultimately lead to disease relapse? [23, 41]

Using fluorescence-conjugated antibody staining and fluorescence-activated cell sorting (FACS) techniques, molecular oncologists are able to separate hematopoietic cells into multiple subpopulations based on their unique combinations of cell surface antigens. Then, using transplantation assays, they can identify a small subset of hematopoietic cells with a unique phenotype, the hematopoietic stem cells (HSCs), which are capable of reconstituting multiple lineages of hematopoiesis in recipient animals over the long term [42, 43]. Using exactly this technical strategy, John Dick and colleagues demonstrated in 1994 and again in 1997 that there is a small subset of malignant cells in the bone marrow (BM) or peripheral blood (PB) of most AML patients which can regenerate the same type of AML in recipient animals upon transplantation [38, 39]. Because severe combined immune deficiency mice (SCID mice) were used as recipients in their first study, Dick and colleagues named such cells SCID leukemia-initiating cells or SL-ICs [38]. These cells are $Lin^{-}CD34^{++}CD38^{-}$ and share a similar phenotype with normal HSCs [39]. Due to their unique ability to regenerate AML in recipient mice, it was speculated that SL-SCs are the LSCs which lie at the root of AML development. In addition, many follow-up studies suggested that LSCs are relatively resistant to most types of chemotherapeutic agents [44–47]; thus it was conjectured that LSCs are the cause of relapse of leukemia following treatment-initiated remission. Based on these early studies, the combination of FACS purification and in vivo transplantation has been used as the standard method to define the phenotype of LSCs, the limited dilution and competitive transplantation assay has been used as a standard method to evaluate the frequency of LSCs in a given population of AML cells, while the serial transplantation assay has been used to detect the self-renewal capacity of LSCs [48]. Exactly the same series of assays has been extended to studies of CSCs in solid tumors [41]. This LSC concept has been most clearly documented in chronic myeloid leukemia (CML) in the chronic stage and provides the best explanation for the clinical observations seen in malignancies such as CML, which shows a relatively simple genetic abnormality and a linear clonal evolution program [49–55]. LSCs in CML are genetically homogeneous with only one driver mutation, *BCR-ABL*, in the chronic stage [56].

It was known that CML is initiated from an HSC that harbors a chromosomal translocation (t9:21) that creates the BCR-ABL fusion protein [57]. The LSCs in CML have a phenotype similar to normal HSCs as demonstrated by similar cell surface markers, slow cell cycling kinetics, and low levels of reactive oxygen species (ROS). Tyrosine kinase inhibitors (TKIs) such as imatinib [58], the first type of drug to directly target BCR-ABL, effectively induce a major or complete molecular remission (CMR) and prolong survival in most patients with CML. However, only

10–20% of patients achieve successful treatment-free remission (TFR). Approximately one-fourth of patients fail TKI therapy due to unique *BCR-ABL* kinase mutations, alternative oncogene activation, or because of progression to an accelerated phase or blast crisis [49], whereas the remaining 50–60% of patients experience disease relapse after discontinuation of TKI therapy due to the insensitivity of their LSCs to TKIs [53, 59, 60]. LSCs in CML are also phenotypically and epigenetically heterogeneous, just as normal HSCs are. The most TKI-resistant LSCs are $Lin^{-}CD34^{+}CD38^{-/low}CD45RA^{-}cKIT^{-}CD26^{+}$ [61]. In most patients, TKI treatment fails to completely eliminate LSCs, thus rendering TKI therapy a lifelong requirement for them [62–65]. Studies have suggested that in contradistinction to LPs and LBs, LSCs in CML are not reliant upon BCR-ABL activity for their survival [53, 59]. CML LSCs express high levels of the thrombopoietin (THPO) receptor MPL and high levels of BCR-ABL protein, and also show high levels of autophagic activity [66–71]. It was proposed that LSCs in CML might depend upon THPO-MPL-stimulated activation of JAK-STAT-BCL-xL and MAPK signaling, BCR-ABL kinase activity-independent activation of the JAK2/PP2A/β-catenin network, or digested products of autophagy for their survival [66–71]. Thus to achieve a sustained, deep CMR and TFR, a combination of a TKI together with inhibitors of these signaling pathways is necessary in order to completely eliminate LSCs in CML [66–71].

However, in AML, the LSC concept is complicated by the existence of a founder clone and the subsequent development of subclones which can be found in patient samples and is explainable by the complicated clonal evolutionary history and complex genetic abnormalities that occur during the pathogenesis of AML. In addition, to complicate matters further, compared to LSCs in CML, LSCs in AML possess relatively greater plasticity [23–25, 27, 29, 31]. The controversies which arose from previous studies might be traceable to limitations imposed by the techniques used, which resulted in a failure to integrate these phenotypic and genetic heterogeneities with the functions of LSCs.

5.3 Genetic Heterogeneity of LSCs in AML

AML can be classified into several distinct subtypes based on cellular morphology and stage of differentiation. The phenotypes and xenograft capacities of LSCs from different subtypes of AML are not the same [72–74]. More importantly, genetic mutations observable in different patients' blood and BM are also not identical even when they are found within the same classification subtype.

Recurrent somatic myeloid malignancy-associated mutations (including chromosomal translocations, cytogenetic abnormalities, and point mutations) are detected in BM and peripheral blood (PB) samples from almost all AML patients. Studies have suggested that AML develops from an original mutant HSC by clonal evolution and dynamic disease developmental processes [75–78]. Mutations of DNA methylation regulator proteins, such as DNMT3A, TET2, and IDH1/IDH2,

histone modifiers such as ASXL1, splicing factors (including SF3B1, SRSF2, and U2AF1), and p53 are detected in ∼10% of all individuals by the age of 70 and increase further as aging proceeds further [79–81]. This is referred to as age-related clonal hematopoiesis (ARCH) or clonal hematopoiesis of indeterminate potential (CHIP), a pre-myelodysplastic syndrome (MDS) condition without a clear diagnosis of any specific hematologic abnormality. HSCs with such founder mutations are predisposed to lead eventually to MDS, a preleukemic condition which can subsequently transform to actual AML by accumulating additional mutations. The average number of mutations observed in MDS patients' cells is 3, while AML patients' cells harbor 5–12 mutations. In cases of ARCH, if mutations of *SF3B1*, *SRSF2*, *U2AF1,* and *TP53* are observed, it is predictive of progression to MDS and subsequent transformation to AML. However, if mutations of *EZH2*, *IDH2*, *ASXL1*, *DNMT3A*, *TP53*, and *SRSF2* are detected in MDS patients, the likely predictable outcome will be AML transformation and shorter survival time [82–88]. The most common additional mutations during the MDS-to-AML transformation process are inactivating mutations in core hematopoietic transcription factor genes (such as *RUNX1*, *CEBPA*, *EVI1*, *GATA2*, and *ETV6*) and activating mutations in proliferative signaling pathways (such as *FLT3*, *N-RAS/K-RAS*, *PTPN11*, *KIT*, *JAK2*, *MPL*, *CBL*, and *NF1* family members) in a subclone of diseased HSCs or hematopoietic progenitor cells (HPCs) [89–91]. Studies suggested that it might take >10 years for HSCs with founder mutations to accumulate sufficient additional mutations to lead to a full transformation to AML [92]. This suggests that these hematologic malignancies proceed by a dynamic developmental process of clonal evolution from mutant HSCs in ARCH to MDS and finally to AML (Fig. 5.1). Importantly, such clonal evolution is not a strictly linear process. Multiple subclones are generated along the way. Thus, in addition to the founder clone (with 1–2 mutations) which is generated in ARCH and the intermediate preleukemic clone(s) that is (are) generated during the MDS stage, more than one leukemic subclone likely exists in any AML patient. Each of these leukemic subclones shares the same founder mutation(s) but likely has distinct additional mutations. In most cases, one of the subclones might become dominant due to the growth advantage it has acquired by virtue of its unique set of mutant genes, while in other cases there exists more than one detectable codominant clone. Theoretically, each subclone of LCs might have its own distinct LSCs. The phenotype and leukemogenic activity of LSCs from different subclones might differ. In addition, there are also stem cells for the founder clone (pre-pre-LSCs) and pre-LSCs for the preleukemic clones [93–95]. The phenotype and leukemogenic capacity of pre-LSCs in low-risk MDS clones are closer to pre-pre-LSCs, whereas the phenotype and leukemogenic capacity of pre-LSCs in high-risk MDS clones are closer to LSCs [96, 97]. Thus LSCs in AML patients are heterogeneous both phenotypically and genetically. In the same patient's specimen, multiple phenotypic LSCs can be detected. LSCs from most cases have an lymphoid-primed multipotent progenitor (LMPP)-like, GMP-like, or even committed myeloid precursor-like phenotype, while pre-pre-LSCs and pre-LSCs normally present with an HSC phenotype [27, 98, 99]. To further complicate these issues, distinct genomic abnormalities can be detected in LSCs of the same phenotype. Thus, in some cases,

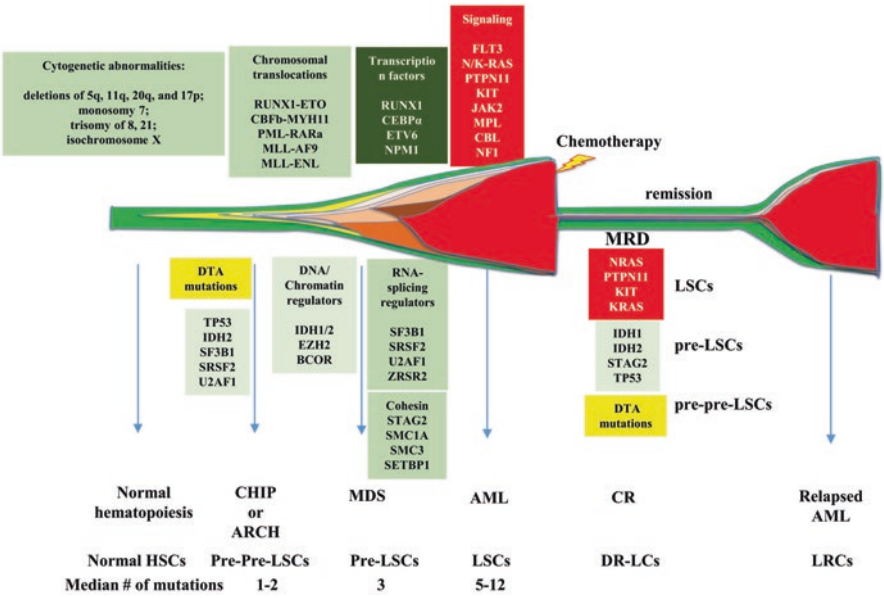


Fig. 5.1 The dynamic pathogenesis and genetic heterogeneity of AML. From the founder clone, harboring the first genetic alteration, to AML transformation, multiple genetic abnormalities must accumulate sequentially, which leads to the generation of multiple subclones of pre-LCs and LCs. Pre-LCs and LCs from different subclones share the founder genetic alteration(s) and have distinct additional genetic abnormalities. Chemotherapy kills most of the LCs and induces CR in patients. However, MRD can be detected in most CR patients. If the MRD contains LSCs derived from either the major or minor leukemic clones, it will predict early disease relapse, while if the MRD contains pre-LSCs, it will predict a later disease relapse. If the MRD contains pre-pre-LSCs, it will predict no disease relapse

only the LSCs for the dominant clone(s) might able to survive and regenerate AML in xenograft models, while in some other cases, LSCs from both the dominant and minor clone(s) can grow. In addition, in most cases, pre-pre-LSCs and pre-LSCs are not able to grow in xenograft models [100]. Thus the AML seen in xenografts based on current models (which will be discussed below) might not fully represent the true genetic heterogeneity that is a feature of all AML in human patients.

In AML patients, morphologic CR can be achieved by eliminating >95% of LCs. The persistence of LRCs from dominant and minor clones in MRD predicts early disease relapse [21, 101]. To achieve deep CR and long-term disease-free survival, all LCs in the dominant and minor subclones, including LSCs, need to be completely eliminated. Fortunately, in most cases, the LCs are relatively sensitive to standard chemotherapies due to their high proliferation status. LSCs can also be eliminated in many cases. However, pre-pre-LSCs and pre-LSCs are quiescent in terms of the cell cycle and are therefore much less susceptible to chemotherapeutic interventions [88, 99, 102]. Thus evidence of pre-pre-leukemic clone and pre-leukemic clones can be detected in MRD in most patients after chemotherapy-induced CR/CRi. The persistence of pre-LSCs in MRD predicts a later disease

relapse due to the additional mutations that must accumulate in order for active AML to be regenerated. Fortunately, the persistence of pre-pre-LSCs in MRD does not predict disease relapse [103, 104]. Thus, more detailed analyses of patient samples using more sensitive single-cell techniques prior to and after treatment in order to separate founder clone, pre-leukemic clones, and leukemic clones will provide useful information to more accurately predict disease relapse and better instruct clinical treatment choices and decisions. From the founder clone, harboring the first genetic alteration, to AML transformation, multiple genetic abnormalities need to be accumulated sequentially, which leads to the generation of multiple subclones of pre-LCs and LCs. Pre-LCs and LCs from different subclones share the founder genetic alteration(s) and have distinct additional genetic abnormalities. Chemotherapy kills most of the LCs and induces CR in patients. However, MRD can be detected in most CR patients. If the MRD contains LSCs derived from either the major or minor leukemic clones, it will predict early disease relapse, while if the MRD contains pre-LSCs, it will predict a later disease relapse. In the most favorable cases, the MRD contains pre-pre-LSCs and no disease relapse will be predicted.

It should be emphasized that, although approximately 80% of AML are de novo and develop without an MDS stage (lack of hematopoietic symptoms before AML development), the dynamic and multistep clonal evolution process is also confirmed in the pathogenesis of de novo AML by demonstrating the hierarchies and patterns by which the mutant clones evolve [92, 93, 95, 102, 105–108]. The reason why only ~20% of AML patients have an MDS stage might have to do with the types, the emergence time sequence, and the variant allele frequency of the genetic abnormalities. For example, mutations of epigenetic regulators such as *DNMT3A*, *ASXL1*, and *TET2* are most commonly detected in ARCH; these are founder mutations for most hematopoietic malignancies including MDS and AML [80]. Such mutations promote HSC self-renewal and clonal expansion by resetting the epigenetic landscape. In addition, HSCs with *DNMT3A*, *ASXL1*, and *TET2* mutations are also predisposed to MDS and AML due to increased genomic instability. However, mutations in genes for splicing factors (including *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*) and chromatin regulators (such as *EZH2*, *IDH1/IDH2*, and *BCOR*), as well as cytogenetic abnormalities (including deletions of 5q, 11q, 20q, and 17p, monosomy 7, trisomies of 8 and 21, and isochromosome X), are the most commonly detected gene alterations in MDS. Such genetic abnormalities lead to inefficient hematopoiesis and cytopenia by inducing programmed death in hematopoietic progenitor cells such as MEPs and GMPs. In support of this notion, significantly more cell death was detected in BM hematopoietic cells in low- and intermediate-risk MDS patients. It was found that extrinsic pathway apoptosis was increased in *SF3B1*- and *SFSR2*-mutant HPCs due to the abnormal splicing of *MAP3K7* and *Caspase-8* [109, 110]. As a consequence, despite the expansion of the primitive HSC compartment observed in most MDS patients, both the long-term and short-term hematopoietic reconstitutive capacities of MDS HSCs are comprised [111, 112]. This explains why it has been so challenging to generate MDS xenografts using current animal models. Additional mutations are required for disease progression and AML trans-

formation by preventing cell death and promoting proliferation of HPCs. In addition, when AML presents with such MDS-related genetic abnormalities, it usually suggests secondary AML which developed from MDS [85, 88, 97, 113, 114]. However, in de novo AML, chromosomal translocation-associated leukemic fusion genes and/or mutations in *NPM1*, transcription factors such as *RUNX1* and *CEBPα*, and/or proliferative signaling pathway genes (including *N-Ras*, *K-Ras*, *FLT3* or *c-Kit*) are typically observed. Such genetic abnormalities promote self-renewal in HSCs and proliferation in HPCs without inducing cell death. This explains why AML patients having such mutations normally do not go through an MDS stage [106, 107].

5.4 Plasticity of LSCs

Compared to HPCs and mature blood cells, HSCs are relatively more plastic, which explains why they are able to give rise all lineages of blood cells. During normal hematopoiesis, HSCs differentiate to produce MPPs and, subsequently, to committed progenitors, which then produce mature blood cells, a process in which they lose both their self-renewal capacity and multi-lineage plasticity. Such processes are regulated by BM microenvironmental factors which induce dynamic gene expression by initiating specific transcriptional and epigenetic programs [115].

Mutations of epigenetic modifiers are commonly detected in AML patients (described above). In addition, mutations of signaling molecules and transcription factors also induce changes in epigenetic patterns. Thus during AML development, genetic lesions induce a dynamic epigenetic reprogramming (including histone modifications and DNA methylation), unleashing cellular plasticity and leading to oncogenic cellular reprogramming. Therefore, compared to normal hematopoietic counterparts, LCs show increased epigenetic plasticity. Although distinct epigenetic alterations and gene expression profiles were identified in patient LCs with distinct genetic mutations, suggesting a genetic mutation-driven, unique epigenetic reprogramming [106, 116, 117], LSCs from almost all patients share a similar "stemness" epigenetic signature which is largely independent of genetic mutations [118, 119]. Such an epigenetic signature is correlated to a unique expression profile of "stemness" genes and xenograft ability which predicts adverse patient outcome [119, 120]. These studies suggested that LSC properties are regulated by a unique epigenetic signature which is independent of genetic mutations. Such an epigenetic signature and stemness gene expression pattern can be induced by the BM niche microenvironment in non-LSCs which may convert them to LSCs, suggesting a relatively high degree of plasticity for LCs [36]. Interestingly, such LSC signatures are associated with relative hypomethylation compared to LBs, which might explain why HMA therapy fails to induce a durable CR in AML and MDS patients since HMAs are unable to eradicate LSCs [17].

5.5 Technical Limitations of Research on LSCs

Although the immune rejection of transplanted human AML cells is significantly reduced in NOD/SCID mice due to the lack of T- and B-cell-mediated adaptive immune responses, the fact that natural killer (NK) and Mφ-/dendritic cell-mediated innate immune reactions also restrict the growth of human AML cells explains the low efficiency of engraftment and low frequency of LSCs detected when utilizing NOD/SCID mice as transplantation recipients. In addition, due to the short lifespan of NOD/SCID mice (~8 months), such mice are not useful for the study of LSCs when long-term time courses are required in order to regenerate leukemia. To solve such a problem, several additional mouse strains were generated. NSG and NRG mice are the result of crossing NOD.CB17-Prkdc^{scid}/J mice with B6.129S4-IL-2Rγ^{null} or NOD-Rag1^{null} and NOD-SCID IL-2Rγ^{null} mice. These mouse strains are deficient not only in mature lymphocytes but also in NK cell cytotoxic activity and Mφ/dendritic cell activity due to the targeted mutation of the *interleukin 2 (IL-2) receptor common gamma chain* gene (IL-2Rγ^{null}); thus these newer strains are much more efficient in supporting the growth of patients' LSCs compared to NOD/SCID mice. In addition, the lifespans of both NSG and NRG mice are significantly extended compared to NOD/SCID mice, which makes them suitable hosts for studying the long-term effects of transplanted xenoengraftments [121–123]. As a consequence, higher frequencies of LSCs in AML samples can be detected using NSG and NRG mice compared to what had been predicted by earlier studies using NOD/SCID mice [73]. Moreover, multiple phenotypes of LSCs were identified and coexisted within the same patient samples. It was documented that LSCs exist not only among LCs within the HSC phenotype but also in LCs with phenotypes of multipotent progenitors (MPPs), granulocyte-monocyte progenitors (GMP), and even more committed granulocyte or monocyte precursors. For example, Goardon et al. demonstrated that in most cases LSCs are enriched in the CD34$^+$ population. At least two types of LSCs, Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^+$ LMPP-like and Lin$^-$CD34$^+$CD38$^+$CD123$^{+/lo}$CD110$^-$CD45RA$^+$ GMP-like, were found to coexist within the same patient sample [98]. In some cases, especially in *NPM1*-mutant and *TET2*-mutant AML, LSCs are enriched in the CD34$^-$ population [124, 125]. By comparing gene expression profiles among LSCs, non-LSCs, and HSCs, several cell surface markers for LSCs have been identified, including CD123 (IL-3R), CLL-1, TIM3, CD44, CD96, CD47, CD133, IL-1RAP, CD117, CD32, CD93, GPR25, and CD25. Some of these have been used to further separate true functional LSCs from non-LSCs [108, 126–142]. Some of these markers, such as CD123, are associated with *FLT3-ITD* mutations [96, 143, 144] and together predict disease progression and relapse in both AML [45, 145] and MDS [92, 93]. These studies all suggest that there is significantly greater phenotypic diversity among LSCs from AML patients compared to those from CML patients.

Technically, NSG and NRG mice are comparable in terms of xenograft efficiency (Table 5.1). However, due to the lack of the DNA damage repair gene *Prkdc*, NSG mice are highly sensitive to treatment with DNA-damaging agents such as radiation

Table 5.1 Animal models used in LSC research

Mouse strains	Mutant or transgenic genes	T	B	NK	Mϕ	DNA repair	Lifespan
Nod/Scid	NOD/Prkdc^{scid}	–	–	+	+/–	Defective	~8.5 mos
NSG/NOG	NOD/Cg-Prkdc^{scid}/Il2Rγ^{null}/SzJ	–	–	–	+/–	Defective	~20 mos
NRG	NOD/Cg-Rag1^{null}/IL2Rγ^{null}	–	–	–	+/–		~20 mos
NSGS	NSG-Tg hIL3, SCF, GM-CSF	–	–	–	+/–	Defective	~18 mos
NRGS	NRG-Tg hIL3, SCF, GM-CSF	–	–	–	+/–		~18 mos
MISTRG	Rag2^{null}IL2Rγ^{null} -Tg hM-CSF,IL-3,GM-CSF, SIRPα,TPO	–	–	–	+/–		~7 mos
SCID-Hu BLT mouse	NSG or NRG mice with humanized BM, liver, and thymus	–	–	+	+/–		~7 mos
NSG-human BM ossicle	NSG mice with ectopic human BM ossicles	–	–	–	+/–		

and chemotherapeutic medications, more so than NRG mice are [146]. Thus NRG mice might be more appropriate experimental subjects when radiation and/or chemotherapy are used in follow-up treatment studies. However, due to the relatively low homology of many key cytokines such as IL-3, GM-SCF, and SCF, between humans and mice, murine cytokines cannot effectively stimulate the growth of human AML cells. *To wit.*, low ratios of AML chimeras were detected in the PB of NSG and NRG mice. In many cases, although successful engraftment of AML cells was detected in the BM, low numbers of human AML cells were detected in spleens and PB [147]. To solve such problems, scientists developed NSGS (NSG-SGM3) and NRGS (NRG-SGM3) mice by crossing NSG and NRG mice with mice that transgenically express human IL-3, GM-SCF, and SCF (~2000–4000 pg/ml). Both NSGS and NRGS mice express the human IL-3, GM-SCF, and SCF cytokines, which significantly enhance the growth of human AML cells (~1.5 to fivefold relative to NSG mice). The success of engraftment can be detected within 4 months, which is a shorter latency period than exists for NSG and NRG mice. In addition, a higher ratio of AML chimeras can be detected in the BM, spleens, and PB of successfully engrafted mice, therefore better recapitulating the patients' AML [147]. However, there is also a concern that higher physiological levels of these cytokines in such mice might be altering the behaviors of LCs and LSCs. Moreover, the addition of more human cytokines might further promote the growth of human LCs such as in MISTRG mice [148]. However, more human cytokines seem not to be good for the normal development of mice, which consequently reduces their lifespan. Thus, better models, in which normal physiological levels of key human cytokines are expressed, such as in NOG-EXL mice (which express ~35 pg/ml hGM-CSF and ~80 pg/ml hIL-3), would appear to be indicated for future studies.

It should be noted that only 50–75% of patient samples were able to successfully generate xenografts in the models described above [98, 147, 149–152]. More recently, in order to better recapitulate AML development as it occurs in human patients, several humanized animal models have been developed. These

models were developed by prior implantation of human hematopoietic cells, such as in SCID-Hu BLT mice, or by ectopic BM environmental tissues such as the humanized BM ossicle xenotransplantation model [153–155]. These can significantly enhance the successful engraftment of human AML cells in localized regions. With such models, some AML samples which failed to grow in NSG or NSGS mice can still be successfully engrafted. It has also been speculated that a higher frequency of LSCs might be detectable in such models compared to the NSG and NSGS models. It is also expected that even pre-LSCs, which normally cannot grow in most other model animals, might be capable of growth in such humanized models. Thus humanized mouse models likely represent a better system with which to study LSCs as they not only increase the possibility of successful engraftment but may also maintain the heterogeneous features of AML cells [154, 155]. The limitation of these models is that they are technically very sophisticated and therefore require experienced personnel to conduct experiments; they are also difficult to be standardized.

Despite the significant efforts that have been made during the past two decades, LSC research has been significantly hampered by a lack of adequate models. Theoretically, all LSCs should be capable of regenerating leukemia if an optimized model is used. However, up until now, even when humanized models were used, in order to obtain successful engraftment and regeneration of leukemia, almost all studies involved the transplantation of large numbers of cells ($>10^3$), suggesting that the precise identification of LSCs remains a challenge. In addition, due to the genetic heterogeneity of leukemic clones and also due to the high degree of plasticity of LSCs, LSCs are also genetically heterogeneous even within the same phenotype, which blurs precise determination of distinct leukemic clones. Such a complicated situation might explain some of the controversies that hamper current LSC research because the leukemogenic capacities of LSCs with different genetic mutations are not the same [34]. Recently, Craig Jordan's laboratory demonstrated that low levels of intracellular ROS might be a better bioindicator of LSCs than cell surface markers by showing that functional LSCs are significantly enriched in the ROS-low subpopulation. The phenotypes of LSCs are not always correlated to their ROS levels and leukemogenic activities. In order to more precisely understand the molecular pathways and genetic/epigenetic diversities of LSCs, an optimized model system that can detect LSCs and pre-LSCs at the resolution of single cells is urgently needed. This will require the use of more advanced techniques such as mass cytometry [156] and single-cell transcriptomics. In addition, an improved system is required in order to best integrate both the phenotypic and genetic heterogeneities of LSCs with their biologic properties and leukemogenic capabilities.

5.6 LSCs in Drug Resistance, MRD, and Disease Relapse

Many early studies suggested that LSCs from AML patients are relatively quiescent with respect to the cell cycle and are also resistant to chemotherapy when compared to the bulk of leukemic blasts [157, 158]. A high frequency of LSCs at the time of

diagnosis of AML (naïve LSCs, as defined by either phenotype or function, as well as high stem cell gene expression programs) predicts a high probability for MRD, disease relapse, and a poor prognosis [150, 159–165]. These studies might provide useful biomarkers to predict outcomes for AML patients. For example, Metzeler et al. identified a panel of 86 stem cell-like genes the expression signature of which predicts survival in cytogenetically normal AML [166, 167]. Ng et al. further limited such a panel to 17 stemness genes and found that the expression pattern of such genes can reliably determine risk in AML [168, 169]. Paired studies of AML samples at diagnosis and relapse using deep sequencing analysis of genetic abnormalities suggested that the LRCs in MRD could be derived from LSCs of the dominant genetic subclone, LSCs of minor genetic subclones, or pre-LSCs [102, 170–172]. These studies suggested that the origin of LRCs in AML patients is potentially heterogeneous [37, 51, 52]. Using a primary human AML NSG xenotransplantation model, Ishikawa et al. demonstrated that LSCs home back to the endosteal region of the BM niche shortly after transplantation to propagate for leukemia regeneration. After leukemia developed, the endosteal BM niche maintained self-renewal and a quiescent state in these LSCs and protected them from the cytotoxic, cell cycle-dependent effects of ARA-C treatment [3]. They also demonstrated that when G-CSF stimulation was used to induce LSCs to enter the cell cycle, it enhanced their sensitivity to ARA-C treatment in such models [138, 149]. These studies suggested that therapy-resistant LSCs are already present in the BM at diagnosis and that these cells are protected by the endosteal stem cell niche. In addition, LSCs might also have an intrinsic partial resistance to chemotherapy owing to their distinct biological properties (Fig. 5.2). LSCs are responsible for disease relapse. However, the

	LSCs	bulk of LCs	healthy HSCs
OXPHOS	High	Low	Low
Glycolysis	Low	High	High
BCL-2	High	Low	Low
MCL1	patient-dependent	patient-dependent	High
Mito mass	Reduced	Increased	Reduced
Mitophagy	High	Low	High
ROS	Low	High	Low
AA	High	Low	Low
Glutathione	High	Low	High
Proliferation	Low	High	Quiescent
CFU ability	High	Low/absent	High
Leukemogenesis	High	Low/absent	N/A
Chemo-resistance	Relative	None	High

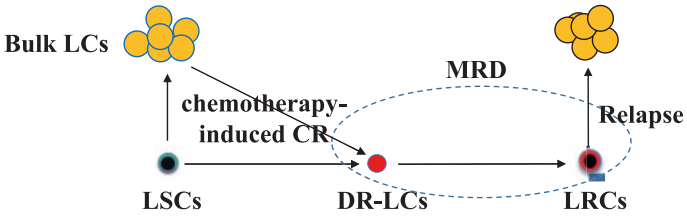
Fig. 5.2 The biological properties of LSCs, bulk LCs, and normal HSCs

prevailing explanation for the role of LSCs in leukemia relapse was challenged by two very recent studies.

Using patient-derived xenograft models, two recent studies showed that by 2–3 days post-chemotherapy, drug-resistant leukemic cells (CR-LCs) in MRD are neither quiescent nor LSCs [32, 33]. Both studies demonstrated that at the time of maximal chemotherapy-induced AML repression, which occurred 2–3 days after the last ARA-C administration, neither quiescent cells nor LSCs were enriched in MRD in the BM as determined by phenotypic analyses, colony-forming assays, functional transplantation studies, and gene expression signatures. In fact, the frequency of LSCs is lower in BM early during chemotherapy-induced AML inhibition and rebound to pretreatment levels at the onset leukemia relapse (15–18 days after the last administration of ARA-C) [33]. The LRCs derived from CR-LCs also reacquire almost all of the phenotypes and stemness gene expression profiles found in naïve LSCs. Thus the authors claimed that CR-LCs are not derived from naïve LSCs. However, despite their significantly reduced colony-forming ability in vitro and leukemia-regenerating capacity in vivo, CR-LCs primarily use oxidative phosphorylation (OXPHOS) to meet their energy needs, in which behavior they are comparable to both naïve LSCs and LRCs but are distinct from the bulk of LCs which primarily use glycolysis for their energy needs. In addition, CR-LCs share most of the features of ROS-low LRCs described by Jordan's group, including high expression of the fatty acid (FAA) transporter CD36 and increased intracellular FAA metabolism (Fig. 5.3). Studies suggested that the stem cell properties of LSCs are better correlated to low ROS levels rather than to any specific phenotype. Thus there is a possibility that the CR-LCs identified in these two studies might be still selected by chemotherapy from among the naïve ROS-low LSCs. Chemotherapeutic drugs convert LSCs to CR-LCs by inducing DNA damage and transient oxidative stress which temporarily repress stem cell properties, including cell surface CD34 expression, stem cell gene expression, and clonogenic/leukemogenic ability. It was suggested that the increased ROS levels might mediate drug resistance in CR-LCs. During the interphase between treatments, CR-LCs reacquire stemness properties through metabolic and epigenetic reprogramming to reset to a ROS-low status. In the future, a label-tracing assay or single-cell technique will be essential to determine the exact origins of CR-LCs and LRCs. In addition, it will be necessary to carefully account for both the phenotypic and genetic heterogeneity features of LSCs and pre-LSCs.

5.7 Mitochondrial Metabolic Regulation of LSCs

Most recent studies have suggested that the activities of LSCs are most likely regulated by mitochondrial dynamics, through metabolic and/or epigenetic reprogramming mechanisms. Functionally transplantable LSCs are enriched in the ROS-low state. The maintenance of low ROS levels is required for preserving the undifferentiated/self-renewal-competent status of LSCs. ROS-low LSCs maintain a low rate



OXPHOS	High	High	High
Glycolysis	Low	Low	Low
BCL-2	High	High	High
Mitochond. mass	Reduced	Increased	Reduced
Mitophagy	High	Low	High
ROS	Low	High	Low
AA con.	High	Low	Low
CD36	Low	High	High
FAO	Low	High	High
Proliferation	Low	High	Low
CFU capacity	High	Low/absent	High
Leukemogenesis	High	Low/absent	High
Chemo-resistance	relative	High	High

Fig. 5.3 The biological properties of CR-LCs compared to LSCs and LRCs

of energy metabolism which renders them relatively quiescent. In addition, compared to ROS-high non-LSCs, ROS-low LSCs express high levels of BCL-2 and are resistant to chemotherapy. Interestingly, although more $CD34^{+}CD38^{-}$ cells can be detected in the ROS-low population than in the ROS-high population, yet there is no clear association of other LSC surface markers such as including CD123, CD33, CD117, CD90, and CD44 with the lower oxidative state. This suggested that, compared to phenotypic biomarkers, biological properties of LSCs such as mitochondrial dynamics, oxidative status, and energy production are more consistent among all patients irrespective of disease stage and genetic heterogeneity. Studies suggested that the ROS-low state is most likely a common feature for both normal tissue stem cells and CSCs [173, 174]. Thus, it might be more reliable to detect LSCs by examining their unique biological properties such as ROS level or relative quiescence with respect to the cell cycle rather than by indexing the molecular markers on their surfaces.

In normal hematopoietic tissue, HPCs are primarily reliant upon mitochondrial OXPHOS of glucose, an economical way to produce energy, while HSCs efficiently utilize anaerobic glycolysis for energy homeostasis, thereby producing low ROS levels [175, 176]. ROS are a by-product of OXPHOS, which is required for the proliferation and differentiation of HPCs [177]. However, maintaining a low reliance on OXPHOS, lower mitochondrial biogenesis, and a ROS-low status is very impor-

tant for maintaining the quiescent state and self-renewal ability of HSCs [178–180]. Inducing OXPHOS in HSCs such as by cytokine stimulation or oxygen stress will increase ROS production and comprise the self-renewal ability of HSCs. It is believed that HSCs are localized in a hypoxic BM niche which protects HSCs from oxygen stress [175]. In addition, HSCs maintain a ROS-low status by using intrinsic mechanisms such as HIF-1α-PDK pathway-mediated OXPHOS repression [176, 181] and clearing of damaged mitochondria by mitophagy [182, 183]. Such mechanisms are regulated by the nutrient-sensitive PI3K-AKT-mTOR and LKB1-AMPK pathways, as well as by glutamine and PML-PPARγ- fatty acid metabolism [184].

Alterations in cellular metabolism and energetics are among the hallmarks of cancer [185, 186]. It was generally accepted that, in contrast to their normal counterparts, most types of tumor cells, including AML cells, are reliant upon aerobic glycolysis and not OXPHOS for energy production even in oxygen-rich conditions, a phenomenon known as the Warburg effect [187]. Studies suggested that glycolytic metabolism is required for proliferation and survival in most cancer cells because in the presence of sufficient nutrients, in addition to providing sufficient cellular ATP, anaerobic glycolysis can also provide important metabolites necessary for the biosynthetic demands of continuous cellular proliferation. However, recent studies demonstrated that CSCs from multiple tumor types including AML, glioblastoma, melanoma, and pancreatic cancer display distinct metabolic features compared to cancer cells without stem cell features (cancer blasts) [174, 188]. CSCs from most cancer types including LSCs are dependent on OXPHOS for energy production and have lower glycolytic reserves than cancer blasts [189–191]. Thus the use of OXPHOS is greater in CSCs than in cancer blasts. Intriguingly, despite the high OXPHOS feature, CSCs maintain a ROS-low status, suggesting a very efficient ROS-scavenging system is used in such cells for maintaining redox balance. For example, increased antioxidant responses have been detected in some CSCs owing to the upregulation of HIF-1α expression. Studies suggested that LSCs might maintain ROS-low status and mitochondrial dynamics by inducing AMPK-FIS-mitophagy pathway-mediated clearance of damaged mitochondria [192], which might be induced by the hypoxic BM niche [193, 194].

In most tissue cells, OXPHOS is fueled by glucose; however naïve LSCs are primarily dependent on amino acids, primarily glutamine, glutamate, and proline, to fuel OXPHOS and ATP synthesis. More intriguingly, when exposed to chemotherapy, LSCs reprogram their metabolic machinery and utilize FAA to fuel OXPHOS and energy production. It was known that most cancer cells consume FAA for survival when glucose metabolism becomes limiting [195–198]. Through β-oxidation, FAA not only sustain ATP levels but also produce cytosolic NADPH, a key to counteracting oxidative stress [199, 200]. This might explain why more LSCs can be detected in either BM or extramedullary adipose tissues after chemotherapy [44]. It might also help to explain the poorer prognosis for AML in obese patients [201]. Furthermore, because LSCs appear to be deficient in their ability to employ glycolysis and are highly reliant on OXPHOS, it is suggested that inhibition of mitochondrial OXPHOS might be an effective therapeutic strategy to treat human AML by targeting LSCs [202].

5.8 Targeting LSCs as an Important Component of Therapy for AML

Therapeutic targeting of LSCs is still difficult to accomplish [25]. To develop drugs that can eliminate LSCs, significant efforts have been made to identify their unique vulnerabilities. By comparing gene expression profiles among LSCs, bulk LBs, and normal HSCs, several LSC-specific surface molecules (including CD123, IL-1RAP, CD47, TIM3, and CD44) and self-renewal/proliferation pathways (such as NF-κB, Wnt-β-catenin, Sonic Hedgehog, Bmi1, and PI3K-AKT/mTOR) [203–205] have been identified. Antibody-drug conjugates and CAR-T cells that are specific for some of these cell surface molecules, as well as small molecule inhibitors that are specific for components of these pathways, have been developed and tested in preclinical studies for anti-LSC efficacy. There have been some promising preliminary results, especially when these types of molecules were combined with various classes of chemotherapeutic agents [128, 131, 134, 139, 206–208]. In addition, targeting the microenvironment in order to enhance anti-LSC therapy by inhibiting BM niche-emergent signals (e.g., CXCL12-CXCR4 and HIF-1α) has also been tested preclinically [209, 210]. Most of these new treatments are currently in the early stages of clinical trials. Detailed information regarding these studies has been summarized well and is discussed in several review articles [29, 206, 208, 211–214]. Here we will discuss only the inhibition of Hedgehog signaling, one of the most promising therapies to target LSCs.

5.9 Targeting the Hedgehog Pathway to Treat AML

Sonic Hedgehog (SHH) signaling is activated in LSCs isolated from most AML samples. This signaling entity is essential for the maintenance of LSCs [215, 216]. Overexpression of GLI1, the key transcriptional mediator of SHH signaling, is associated with disease relapse, drug resistance, poor prognosis, and reduced OS in AML patients [217]. Preclinical studies have suggested that the inhibition of SHH sensitizes LSCs to ARA-C or AZA and represses AML development [218]. Based on these studies, clinical trials were conducted to test the anti-AML effects of glasdegib, an SHH signaling inhibitor, in combination with low-dose cytarabine (LDAC) or standard 7 + 3 treatment. In a Phase Ib study, it was shown that glasdegib + LDAC induced CR/CRi in 31% (16/54) of patients, with an average OS of 8.3-months in patients with either AML or high-risk MDS; this CR/CRi rate is significantly better than the 11%–28% rate previously reported in studies using either an HMA or LDAC alone [219]. Based on this study, in November, 2018, the USFDA granted accelerated approval of glasdegib + LDAC for the treatment of newly-diagnosed AML in adults 75 years of age or older. However, in a Phase II randomized multicenter study, glasdegib + LDAC induced CR/CRi in only 15% of patients (17/132). Nevertheless, the 8.8-month median OS reached in the glasdegib + LDAC

arm is significantly better than the 4.9-month OS in the LDAC-alone arm [220]. Although another study suggested that glasdegib plus standard 7 + 3 therapy induced CR/CRi in 46.4% of patients with a 14.9-month median OS, such treatment is limited only to those patients who can sustain the standard 7 + 3 regimen of chemotherapy [221]. Interestingly, a preclinical study suggested that glasdegib + AZA might synergistically inhibit AML and MDS [222]. Future studies will need to determine whether glasdegib can facilitate the elimination of LSCs and prevent disease relapse when combined with VEN + AZA (described below).

5.10 Inhibition of BCL-2 to Target LSCs in AML

BCL-2 is highly expressed in most types of cancer cells [223, 224]. Unlike normal HSCs which depend on MCL1 for survival [225], most cancer cells, including AML cells, are reliant on BCL-2 for their survival [223, 224, 226–228]. Thus, BCL-2 has been approved as a safe and reliable target for the treatment of BCL-2-expressing cancer [223, 224, 229]. Several BCL-2 inhibitors have been developed and tested in clinical trials as antineoplastic therapy. ABT-737 [230] and its derivative Navitoclax (NAV, ABT-263) [231] are small molecule mimetics of the BH3 domain of the BH3-only sensitizer protein BAD, which efficiently binds to BCL-2, BCL-xL, and BCL-W, thus permitting the release of mitochondrial-bound pro-apoptotic proteins to induce apoptosis in BCL-2-dependent cancer cells [231]. Venetoclax (VEN, ABT-199), another modified derivative of ABT-737 developed by AbbVie in 2010, maintains specificity for BCL-2 but lacks affinity for BCL-xL [232]. Although preclinical studies suggested that ABT-737 and NAV might have greater anticancer activity in BCL-2-expressing cancers compared to VEN, clinical use of ABT-737 and NAV was hindered by dose-limiting thrombocytopenia induced by these drugs, as platelets are dependent upon BCL-xL for their survival [233]. VEN treatment spared platelets with tolerable side effects at its effective anticancer dosage, and in 2016 it was granted accelerated approval by the FDA for the treatment of chronic lymphocytic leukemia (CLL) patients whose cells show a 17p deletion; full approval was granted in June 2018 for patients with CLL or small lymphocytic leukemia regardless of 17p deletion [232, 234–236]. Recent studies demonstrated that VEN treatment is also effective in myeloid malignancies including AML, as demonstrated in cell lines, primary patient samples, and clinical patient treatment.

In AML, LSCs express high levels of BCL-2 compared to non-LSCs [51]. In addition to its anti-apoptotic activity, BCL-2 also regulates energy production in LSCs by maintaining their relatively high reliance on OXPHOS without increasing ROS production. BCL-2 inhibition impedes OXPHOS and thus hinders ATP production [190]. Thus BCL-2 inhibition selectively kills LSCs by inhibiting the production of energy-storing molecules and also by inducing mitochondrial apoptosis, all while having minimal effects on normal HSCs [13, 51]. Preclinical studies suggested that targeting BCL-2 leads to the preferential elimination of LSCs in xenografts from most AML patient samples. However, as a single-agent treatment, VEN

induced CR/CRi in only 19% of AML patients, and its treatment effects were not durable [237]. It was suggested that elevated MCL1 is the major reason for VEN resistance in AML cells and patient samples [115, 116, 238–241]. Supporting this notion, it was demonstrated that MCL1 inhibition sensitizes VEN-resistant AML cells to treatment with this drug [238, 239, 241–243]. The anti-AML effect of VEN in combination with an MCL1 inhibitor needs to be evaluated clinically, however. Recent studies suggest that HMA, PI3K/AKT inhibitors, MEK inhibitors, cytarabine, or idarubicin might indirectly repress MCL1 expression and synergize with VEN to increase the latter's overall anti-AML efficacy [242, 244–247].

As single agents, the HMAs azacitidine and decitabine only induce a response in 15–25% of elderly AML patients [248]. Interestingly, the combination of VEN + AZA or DEC induced CR/CRi in 67% (97/145) and 85% (28/33) of elderly patients with newly-diagnosed AML, respectively [13–15], and in 64% (21/33) of patients with chemotherapy-relapsed or refractory AML [16, 226]. This is significantly superior to results obtainable with traditional chemotherapy. Of importance, ~20% of patients achieved an MRD-negative deep CR, suggesting that LSCs may indeed have been maximally ablated. These studies suggested that 400 mg is an effective and tolerable/safe dose of VEN and the VEN + AZA combination is better than the VEN + DEC combination as indicated by earlier responsiveness and longer survival. Although the long-term effects of such treatment need to be further evaluated by randomized clinical trials and long-term observations, we believe that VEN + AZA ought to and will likely become the first-line of treatment for elderly AML patients and may alter the treatment strategy for younger AML patients as well. Mechanistically, AZA and DEC both sensitize AML cells to VEN by upregulating NOX1 and repressing MCL1 [246, 249–251]. Thus VEN + either AZA or DEC synergistically eliminates the bulk of LCs by inducing apoptosis, which leads to disease remission. AZA + VEN also synergistically removes LSCs in many patients by inhibiting OXPHOS and disrupting glutathione-dependent ETC complex II-related energy metabolism [13, 14]. Patients with *IDH1*/*IDH2* mutations were more likely to respond better due to the inhibition of cytochrome C oxidase by 2-hydroxyglutarate, the abnormal product of mutant IDH1/IDH2 [237, 252], whereas *K-RAS/N-RAS* or *PTPN11* mutations may predict a poorer response or disease progression because of the upregulation of MCL1 in such patients. It is notable that, in contrast to chemotherapy which induces p53-dependent apoptosis, the VEN + AZA treatment program kills AML cells in a p53-independent fashion [236]. Thus most patients with *TP53* mutations might still respond to VEN + AZA therapy. In addition, VEN + LDAC induces CR/CRi in 54% (44/82) of patients with a median OS of 10-months, which is superior to LDAC alone (CR/CRi 11% and OS 5 months) [253]. Whether VEN + LDAC can also eliminate LSCs and induce a deep and long-term CR in patients needs to be evaluated. In response to these studies, which were reported in November of 2018, the USFDA has granted accelerated approval to VEN + AZA and VEN + LDAC for the treatment of newly-diagnosed AML in adults age 75 years and older.

5.11 Prospective

Despite the significant progress that has been achieved in research into LSCs, several key questions regarding the concept of an LSC have not been satisfactorily addressed experimentally. Thus the clinical relevance of LSCs remains in question due to controversies extant from many previous studies. These controversies are primarily attributable to the technical limitations inherent in trying to unequivocally identify LSCs, which results in the inability to fully address the features of the genetic heterogeneity and metabolic/epigenetic plasticity of pre-LSCs and LSCs.

It is well-accepted that the founder mutations leading to AML normally occur in primitive HSCs, suggesting the HSC origin of AML. However, the immunophenotypes of LSCs are {LMPP-like} or GMP-like or even resemble more differentiated precursors in almost all AML cases, which implies that LSCs likely arise from a mutant progenitor that has acquired self-renewal capabilities rather than originating directly from a single HSC. It is also well-accepted that the pre-LSCs in most AML cases share a phenotype similar to normal HSCs and are quiescent with regard to the cell cycle, which explains the relatively chemoresistant characteristic of pre-LSCs. However, direct evidence is still lacking to demonstrate that the tendency toward cell cycle quiescence is also a consistent feature of LSCs because earlier studies failed to consider in their analyses the possibility that pre-LSCs are separate entities from LSCs [99, 138, 149, 254].

Studies have suggested that leukemia could relapse from either a preleukemic clone or leukemic subclones [102]. Future studies need to address whether the CR-LCs and LRCs detectable during MRD are derived from LSCs, pre-LSCs, or certain types of drug-resistant LBs. Fully addressing this question will allow us to design and develop still better strategies to treat AML by killing the bulk of LCs to induce disease remission and, importantly, eliminating LRCs from MRD to prevent disease relapse (Fig. 5.4).

Furthermore, due to the dynamic metabolic reprogramming of LSCs in response to exposure to chemotherapeutic pharmaceuticals, and also at the onset of disease relapse, different treatment strategies will almost certainly be necessary in order to target LSCs at different treatment stages. For example, naïve LSCs, CR-LCs, and LRCs all use OXPHOS to generate ATP; therefore the inhibition of mitochondrial OXPHOS might be a useful strategy to kill all of these cells. However, naïve LSCs rely on amino acids to fuel OXPHOS reactions for the generation of ATP, while after chemotherapy, CR-LCs and LRCs convert to the use of FAA to fuel OXPHOS for energy production. Therefore, to target LSCs, CR-LCs, and LRCs, distinct metabolic pathways such as amino acid-OXPHOS or CD36-FAO-OXPHOS must be targeted [255]. In addition, compared to LSCs and LRCs, the CR-LC subpopulation lacks leukemogenic capacity and shows some chemotherapy stress-induced transient metabolic features such as high ROS levels. Thus targeting CR-LCs might represent a very critical therapeutic component to prevent disease relapse either by

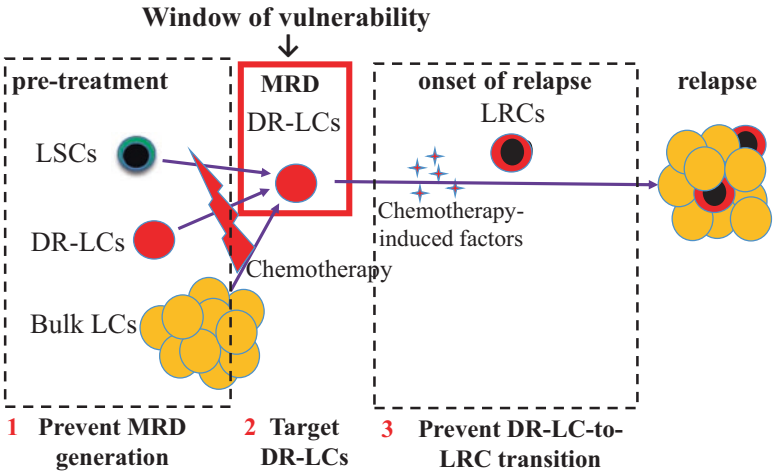


Fig. 5.4 Strategies to prevent disease relapse by targeting drug-resistant cells. (1) There are three potential origins for drug-resistant leukemic cells (DR-LCs): LSCs, pre-existing DR-LCs, or cells which may be selected from the existing bulk of LCs. Thus, determining the actual origin of DR-LCs will help in the design of specific strategies to prevent the development of MRD because LSCs, DR-LCs, and bulk LCs might have distinct biological properties (see Figs. 5.2 and 5.3). We may able to add a specific targeted therapy to standard chemotherapy to prevent the production of MRD without affecting the activity of normal HSCs. (2) Immediately after chemotherapy, the DR-LCs in MRD have distinct metabolic properties (see Fig. 5.3) which might provide a window to target DR-LCs by inhibiting these unique metabolic pathways. (3) Since DR-LCs need to regain leukemogenic potential in order to become LRCs for disease relapse, we might be able to prevent such relapse by inhibiting the DR-LC-to-LRC transition

eliminating CR-LCs or by inhibiting the CR-LC-to-LRC transition. One study suggested that patients' serum factors such as dopamine and its receptor DRD2 stimulate CR-LCs to reacquire the properties of LSCs. Thus targeted inhibition of DRD2 signaling was shown to lead to the suppression of AML growth, providing direct evidence for the potential benefit of anti-CR-LC targeted therapy.

Lastly, although BCL-2 inhibition targets LSCs by inhibiting mitochondrial OXPHOS, AZA might not target LSCs due to their hypomethylated epigenetic feature. Of interest, a combination of VEN + AZA seems to synergistically kill both bulk LCs and LSCs. In the future, more detailed molecular mechanisms need to be elucidated in order to fully explain how AZA enhances the anti-AML feature of VEN. Such information will help us to design more effective strategies to improve treatment of therapy-resistant AML and also for patients who fail to achieve a deep CMR. Taken together, it is hoped that these newer therapeutic strategies, based as they must be on a clearer, sounder technical appreciation of the nature of bulk LCs as well as the component cells of MRD, will significantly improve the outlook for AML patients and will do so sooner rather than later.

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Chapter 6
Tissue "Hypoxia" and the Maintenance of Leukemia Stem Cells

Persio Dello Sbarba and Giulia Cheloni

Contents

Abstract The relationship of the homing of normal hematopoietic stem cells (HSC) in the bone marrow to specific environmental conditions, referred to as the *stem cell niche* (SCN), has been intensively studied over the last three decades. These conditions include the action of a number of molecular and cellular players, as well as critical levels of nutrients, oxygen and glucose in particular, involved in energy production. These factors are likely to act also in leukemias, due to the strict analogy between the hierarchical structure of normal hematopoietic cell populations and that of leukemia cell populations. This led to propose that leukemic growth is fostered by cells endowed with stem cell properties, the leukemia stem cells (LSC), a concept readily extended to comprise the cancer stem cells (CSC) of solid tumors. Two alternative routes have been proposed for CSC generation, that is, the

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oncogenic staminalization (acquisition of self-renewal) of a normal progenitor cell (the "CSC in normal progenitor cell" model) and the oncogenic transformation of a normal (self-renewing) stem cell (the "CSC in normal stem cell" model). The latter mechanism, in the hematological context, makes LSC derive from HSC, suggesting that LSC share SCN homing with HSC. This chapter is focused on the availability of oxygen and glucose in the regulation of LSC maintenance within the SCN. In this respect, the most critical aspect in view of the outcome of therapy is the long-term maintenance of the LSC subset capable to sustain minimal residual disease and the related risk of relapse of disease.

Keywords Leukemia stem cell maintenance · Hypoxia · Oxygen · Stem cell niche

6.1 Introduction

The relationship of the homing of normal hematopoietic stem cells (HSC) in the bone marrow (BM) to specific environmental conditions, referred to as the "stem cell niche" (SCN), has been the object of intensive study over the last three decades. These conditions include the action of a number of molecular and cellular players as well as critical levels of nutrients, oxygen and glucose in particular, involved in energy production [1, 2]. These factors are likely to act also in leukemias. A strict analogy indeed emerged between the hierarchical structure of normal hematopoietic cell populations and that of leukemia cell populations [3]. This led to propose [4] that leukemic growth is fostered by cells endowed with stem cell properties, the leukemia stem cells (LSC), a concept readily extended to comprise the cancer stem cells (CSC) of solid tumors [5, 6]. More than one model exists to explain the relationship of CSC to their normal counterpart. Two alternative routes have been proposed for CSC generation [6], that is, the oncogenic staminalization (acquisition of self-renewal) of a normal progenitor cell (the "CSC in normal progenitor cell" model) and the oncogenic transformation of a normal (self-renewing) stem cell (the "CSC in normal stem cell" model). The latter mechanism, in the hematological context, makes LSC derive from HSC, an assumption which suggests that LSC share with HSC the homing behavior within SCN of BM [7, 8]. This chapter is focused on the role of the availability of oxygen and glucose in the regulation of LSC maintenance. In this respect, the most critical aspect in view of the outcome of therapy is the long-term maintenance of the LSC subset capable to sustain minimal residual disease (MRD) and the related risk of relapse of disease following successful induction of remission. The description of aspects of the regulation of LSC maintenance related to the symbiosis with stromal cells, to the action of soluble or cell surface/matrix-bound cytokines or growth factors, and to the modulation of intracellular signaling is left to other chapters of this book and/or to independent reviews.

6.2 Why Hypoxia Is Written "Hypoxia"

Tissue zones where stem cell potential is maintained are commonly referred to as "hypoxic" SCN [9], being "hypoxia" an inappropriate term in this context, as it is used to define in vitro or in vivo situations where O_2 concentration is lower than that of sea-level atmosphere (21% O_2). Indeed, O_2 concentration in normal tissues, even in the lung, is always significantly lower than 21% [10–13]. The papers we are referring to here provided convincing arguments for the use of the term "relatively (to 21%) low O_2 concentration" (to be shortened to "low O_2"), instead of "hypoxia," to define conditions corresponding to physiological tissue O_2 concentrations. Accordingly, any O_2 concentration typical of any tissue under physiological conditions should be considered as "*in situ* normoxia" [13]. On the other hand, the term hypoxia can be correctly used to refer to pathological situations where O_2 concentration is lower than that of the corresponding normal tissue. Therefore, it is important to point out that the concept of in situ normoxia applies to significantly different O_2 concentrations, between different tissues and different sites within the same tissue [13]. In BM, for instance, the normal overall O_2 concentration is lower than in most other tissues. BM cells are indeed physiologically distributed along a gradient of partial O_2 pressure (ppO_2) that ranges from around 5% O_2 close to blood vessels to about 0 ("anoxia") in the most distant regions. Thus, within BM, "normoxic" conditions correspond to O_2 concentrations as different as 5% from 0.1%, being even anoxia to be considered as in situ normoxia in some zones of BM [13].

6.3 The Low-Oxygen Stem Cell Niche in the Bone Marrow

The key feature of SCN is to enable HSC to proliferate without losing (or losing as slowly as possible) stem cell potential, i.e., to undergo the so-called self-renewal, which is a defining property of stem cells. Such a pattern of HSC proliferation is what sustains long-term maintenance of HSC, yet allowing their contribution to active hematopoiesis. The SCN concept emerged as a theoretical model [14], yet based on experimental ex vivo data indicating an uneven compartmentalization in BM of HSC and less immature hematopoietic progenitor cells (HPC), being HSC located preferentially close to the bone surface and HPC instead in proximity of the central sinus. This concept evolved later from the HSC/HPC contraposition to a model where different HSC subsets are distributed between "endosteal SCN," where the stem cell potential of HSC is maintained, and "vascular SCN," where HSC commitment to clonal expansion and differentiation is driven [15–17]. The relationship of SCN function to proximity to blood vessels is in keeping with a regulation based on nutrient supply, O_2 and glucose in particular [18, 19]. It emerged that SCN where HSC are physiologically long-term maintained are placed in BM zones where O_2 concentration is lower than in the surrounding tissue and that in situ normoxic conditions for HSC maintenance are ensured in sites at the lower extreme of ppO_2

gradient in BM [20]. It is this HSC-maintaining SCN that will be referred to hereafter with the acronym SCN, according to the "restrictive" original Schofield's definition [14].

The SCN model started to be enriched with mechanistic details in the early 1990s, on the basis of results of in vitro studies [21]. In murine BM cell cultures incubated in atmosphere at 1% O_2, HSC maintenance was found enhanced, while HPC were suppressed and the overall hematopoietic output markedly reduced. Thus, low ppO_2 emerged as a regulatory aspect of SCN function, and the concept of "hypoxic" SCN was introduced. An interesting aspect of this study was that pooled data obtained using a number of different progenitor/stem cell assays revealed a precise hierarchical "gradient" of resistance to low O_2, being the progenitor the more resistant, the higher its hierarchical level [21]. This means that an environment which is "hypoxic" for the bulk of hematopoietic cells and HPC is actually "normoxic" for HSC. Thus, the first mechanistic feature of SCN to emerge pointed to metabolic issues, and to the role of ppO_2 in particular, in the regulation of HSC maintenance. Similar data were then collected for human hematopoiesis and long-term repopulating HSC [22–24], and the existence of "hypoxic" SCN in vivo was later confirmed experimentally [9], leading to a complete systematization of the issue [13]. That said, it is necessary here to underscore a point relative to the abovementioned "gradient" of resistance to low ppO_2. To home selectively within SCN placed in tissue zones at the lowest ppO_2, HSC need to exhibit a metabolic profile that is not shared by HPC. This profile cannot simply consist of the compatibility with a generic tissue "hypoxia," which refers to a wide range of tissue O_2 concentrations. Either HSC or HPC exhibit indeed a "hypoxic" metabolic profile [25]. Thus, what qualifies HSC from HPC is that HSC (but not HPC) are capable to stand the lowest physiological ppO_2 and for extended times. The question invariably slides toward the upregulation of HIFα signaling, the best known driver of "cell adaptation to hypoxia." However, HIFα stabilization threshold is around 3% O_2 [26], and HIFα signaling is active in both HSC and HPC, so that it cannot sustain alone the critical metabolic differences between HSC and HPC or confer upon HSC all the features enabling their selective homing in low ppO_2 SCN. Only a small minority of cells where HIFα is stabilized is indeed capable to stand the low ppO_2 typical of SCN [9]. Thus, HIFα stabilization is a necessary, although not sufficient, condition for HSC maintenance, in keeping with what demonstrated for the maintenance of LSC [27] of chronic myeloid leukemia (CML).

One of the mechanisms operating within the SCN to sustain hematopoiesis while making HSC lose stem cell potential as slowly as possible consists in the regulation of balance between HSC quiescence and cycling. It was therefore crucial to establish whether low O_2 modulates this balance. The issue was addressed using murine BM cell cultures incubated at 1% O_2 and two different strategies, i.e., determining the suppression of cycling HSC following treatment with 5-fluoro-uracil (5FU) [28] and the coupling between mitotic history and maintenance of stem cell potential [29]. It was found that, after 5 days in low O_2, 1/3 of HSC are induced to quiescence (5FU-resistant), while 2/3 are 5FU-sensitive, suggesting that low ppO_2 of SCN in vivo is compatible with the cycling of a substantial proportion of HSC [28]. It also emerged that one replication cycle at 1% O_2 boosts stem cell potential and that this

effect is lost when cycling is sustained for more than one cycle at 1% O_2 or cells are incubated in "normoxia" or in the presence of interleukin-3. This indicates that HSC self-renewal occurs immediately upon HSC recruitment from quiescence to cycling, provided this happens in low O_2, suggesting that low ppO_2 of SCN in vivo is a crucial factor for the maintenance of stem cell potential of HSC, helping HSC to proliferate as stem cells (self-renewal), and preventing their commitment to clonal expansion. On the contrary, stem cell potential is typically lost when proliferation is extensively stimulated, such as in the presence of IL-3 or relatively high ppO_2 [29].

While all the earlier studies of the effects of low O_2 on HSC maintenance were carried out incubating cells at 1% O_2 (or even higher O_2%), the question emerged of whether O_2 concentrations lower than 1%, detectable in vivo [10, 11, 13, 20], should be considered to play a regulatory role in SCN. An affirmative answer was obtained in vitro, showing that at 0.1% O_2 not only HSC survival but also HSC cycling, a crucial aspect of SCN function, is maintained, although induction of quiescence is favored [30, 31]. Actually, O_2 concentrations ranging between 0.1% and 1.0% were classified even as "moderate hypoxia" [26]. A direct measurement of O_2 concentration in BM showed that ppO_2 is around 10 mmHg, equivalent to 1.3% O_2, within the vascular SCN [32], the SCN type where ppO_2 is believed to be relatively high. This suggests that ppO_2 of endosteal SCN should be well below the 1% O_2 concentration used for the earlier studies in vitro of the effects of low O_2 on HSC maintenance. The compatibility of O_2 concentrations as low as 0.1–0.2% with SCN function led to the use these concentrations for the studies of leukemias summarized below.

6.4 Low Oxygen and "Oncogene Suppression" in Leukemias

Working with CML stabilized cell lines or primary explants, it was shown that the culture in atmosphere at 0.1% O_2 for relatively long incubation times (7–10 days) markedly reduced cell bulk with respect to time zero, while in the residual population stem cell potential was integrally maintained on a per-cell basis. This parallels strictly what observed for normal hematopoiesis. A crucial outcome of these experiments was that incubation in low O_2 also completely suppressed $BCR/Abl_{protein}$, the product of the fusion oncogene responsible for CML pathogenesis, but not BCR/Abl_{mRNA} [33]. Consequently, the CML cell subset capable to stand incubation in low O_2 is independent of BCR/Abl for persistence in culture but remains genetically leukemic (Figs. 6.1 and 6.2). Thus, when BCR/Abl-independent cells were transferred to growth-permissive incubation conditions ("normoxia"), their stem cell potential was exploited, as expected, via $BCR/Abl_{protein}$ re-expression, which is capable to ensure a maximal expansion of cell population. It is extremely relevant that the suppression of driver oncogenic proteins ("oncogene suppression") following cell incubation in low O_2 is not restricted to CML (Fig. 6.1) but is extended to other types of leukemias [34, 35], such as murine Friend's erythroleukemia (MEL; erythropoietin receptor and glycoprotein 55), human acute myeloblastic leukemia (AML; AML1/ETO), and human acute promyelocytic leukemia (APL; PML/

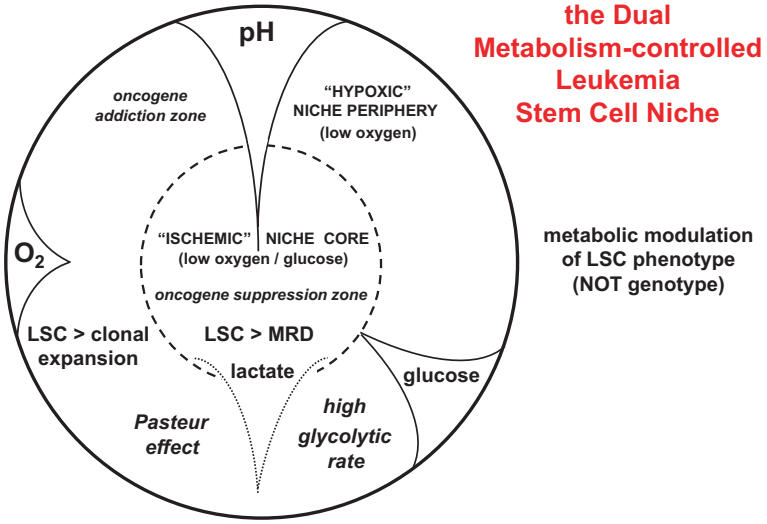

Fig. 6.1 Schematic representation of the "Dual Metabolism-controlled CML Stem Cell Niche," as hypothesized on the basis of published data and hypotheses [41, 49–51, 66, 86]. The "hypoxic" stem cell niche (SCN) periphery, where ppO_2 is low but glucose concentration is relatively high, allows the expression of the oncogenic protein (maintenance of oncogene addiction), steering leukemia stem cells (LSC) to clonal expansion. In the "ischemic" SCN core, characterized by both O_2 and glucose shortage, the oncogenic protein is suppressed, boosting LSC self-renewal and maintanance of minimal residual disease (MRD)

RARα). In all leukemias tested, oncogene suppression is relatively slow (3–7 days after the beginning of incubation in low O_2), indicating that the phenomenon cannot be considered as a direct consequence of HIFα stabilization, which is triggered within few minutes of cell transfer to low O_2 [9]. Nevertheless, HIFα activity has been shown necessary for the maintenance of LSC of CML [27]. As for the downstream, effector mechanisms of oncogene suppression in low O_2, they belong to a complex panel which includes transcriptional, translational, and posttranslational events [36–39]. It is important to point out that the downregulation of these processes occurring in low O_2 is not a generalized phenomenon and that the block of protein synthesis does not apply to a number of factors, many of which are apparently important for cell maintenance under metabolic restriction [39, 40].

A key point of the scenario summarized above is that oncogene suppression occurring in low O_2 does not correlate with a loss of stem cell potential, at least in the case of CML [33, 41] and MEL [34] cells. This points to the existence of a leukemia cell subset endowed with LSC properties and capable to survive and cycle (34 and Tanturli M, Cheloni G, Bono S and Poteti M, unpublished data) in the absence of oncogenic signaling (Fig. 6.1). The selection of such an LSC subset under metabolic pressure suggests that maintained oncogenic stimulation makes LSC unfit to home the SCN, due to the detrimental effects of growth-promoting

stimuli in a growth-limiting environment. On the contrary, oncogene suppression would enable LSC to persist as such within the SCN, without risking to be pushed by oncogenic signaling to commitment to clonal expansion and differentiation. Thus, the loss of “oncogene addiction” [42–44] would be a necessary step of LSC adaptation to SCN (Fig. 6.1). In other words, in order to earn their long-term maintenance within the SCN, LSC are actually required to revert to a normal, HSC-like phenotype. On the other hand, when the homeostatic balance within the LSC compartment shifts from the maintenance of stem cell potential to commitment to clonal expansion and then differentiation, LSC would shift from the core to the periphery SCN environment, which is permissive for the expression of the oncogenic protein and the boosting of clonal expansion by oncogenic signaling. Under this perspective, two points are worth being underscored: (a) oncogene suppression is not a genetically blocked event but a fully reversible phenotypical adaptation, like the one proposed to describe the relationship between HSC and HPC [45]; (b) the withdrawal of oncogenic signaling in LSC appears functionally equivalent to that of cytokine signaling in HSC [3]. With respect to (b), it is to note that BCR/Abl-induced growth factor independence of hematopoietic cells is mediated at least in part by IL3 signaling [46], which indeed impairs HSC maintenance in low O_2 [29].

6.5 The Dual “Hypoxic/Ischemic” LSC Niche in Chronic Myeloid Leukemia

When the metabolic consequences of incubation of CML cells in low O_2 were addressed, it was glucose consumption from culture medium to emerge as the crucial condition for the reduction of total cell number with respect to time zero, as well as for $BCR/Abl_{protein}$ suppression. By varying time zero cell density and glucose concentration in cultures incubated in low O_2, it was established that the kinetics of this suppression is strictly related to that of glucose exhaustion [41]. Deepening the issue, it was shown that the lack of glucose is capable to drive $BCR/Abl_{protein}$ suppression independently of O_2 shortage [40]. Thus, glucose availability emerged as a converging trait of the metabolic control of $BCR/Abl_{protein}$ expression, suggesting that it is severe energy restriction in general, rather than low O_2 in particular, to trigger the $BCR/Abl_{protein}$ suppression machinery that qualifies the SCN environment (Figs. 6.1 and 6.2). If this is the case, why do we focus on low O_2 environments? For two reasons: (a) because the lower solubility of O_2 when compared to glucose, affecting their diffusion in tissues, makes O_2 shortage precede glucose shortage physiologically while getting away from blood vessel [47]; (b) because the enhanced glucose consumption rate (the Pasteur effect) typical of cell metabolism at low ppO_2 makes of low O_2 a powerful modulator of glucose availability. The time lapse between the onset of glucose shortage and that of O_2 shortage (a) well explains the markedly delayed kinetics of $BCR/Abl_{protein}$ suppression with respect to that of HIFα stabilization. Point (b) is affected by HIFα stabilization itself, glucose exhaustion being more

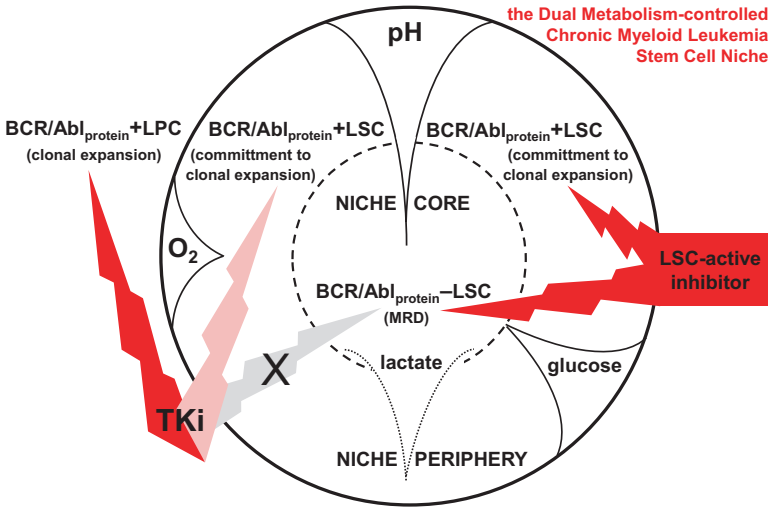

Fig. 6.2 Schematic representation of the "Dual Metabolism-controlled CML Stem Cell Niche," as hypothesized on the basis of published data and hypotheses [41, 49–51, 66, 86]. The "hypoxic" stem cell niche (SCN) periphery, where ppO_2 is low but glucose concentration is relatively high, allows $BCR/Abl_{protein}$ expression, making BCR/Abl-dependent self-renewal of leukemia stem cells (LSC) sensitive to tyrosine kinase inhibitors (TKi) active on BCR/Abl signaling (pink bolt). In the "ischemic" SCN core, characterized by both O_2 and glucose shortage, $BCR/Abl_{protein}$ suppression and BCR/Abl-independent self-renewal of LSC maintain minimal residual disease (MRD) protected from TKi action (grey bolt/X). On the other hand, TKi reach maximal activity outside SCN (red bolt on the left) against leukemia progenitor cells (LPC) engaged in CML clonal expansion (100% in replication). When $BCR/Abl_{protein}$ is suppressed, the question of the presence or the absence of primary or secondary *BCR/abl* mutations determining BCR/Abl resistance to TKI is irrelevant. Many non-TKi, LSC-active inhibitors act irrespective of $BCR/Abl_{protein}$ expression or suppression, thus succeeding in targeting both MRD and clonal expansion (red bolts on the right)

efficiently driven in low ppO_2 zones than in zones at higher ppO_2 where HIFα is not stabilized. The role of HIFα in the control of leukemic growth is reviewed in [48].

What summarized above led to define two different nutrient-restricted SCN environments, the low O_2 and the low O_2/glucose, playing distinct roles in the biology of leukemias (Fig. 6.1). These two SCN environments can be referred to, respectively, as the "hypoxic" and the "ischemic" SCN zones [41, 49–51]. The term "ischemic" sounds as inappropriate as "hypoxic" in the context of SCN. Indeed, "ischemia" refers to in vivo (but not in vitro) pathological occurrences where the tissue is damaged as a consequence of nutrient shortage, whereas in the SCN this shortage has a precise regulative role. A permanently insufficient glucose diffusion, affecting cell viability, has been shown in solid neoplasias [47] and is likely to characterize also the hyperplastic BM of leukemias, CML in particular. A simple model drawing the relationship between the two SCN zones predicts the onset of ischemia-like conditions within the core of hypoxic SCN [49–51]. In the SCN core, glucose would get close to exhaustion due to the combined effects of the high rate of consumption (in the SCN periphery) and the scarce diffusion (higher distance from

blood vessels of SCN core than SCN periphery). While HIFα is surely stabilized in both the SCN core and SCN periphery, it is most probably the HIFα-dependent metabolic reprogramming of the latter which conditions the asset and function of SCN core. It is worth pointing out here that the existence of ischemia-like conditions emerged in normal BM from studies of the role of blood perfusion, rather than O_2 diffusion, in the control of hematopoiesis and in the hierarchical distribution of HSC in BM. These studies showed that the most primitive HSC reside in BM zones where blood perfusion is lowest, implying that HSC maintenance within the SCN is supported by a strongly reduced supply of nutrients as well as of blood-borne soluble factors [52, 53]. The capacity of LSC to nest in HSC niches has been also demonstrated [54, 55].

The dual "hypoxic/ischemic" SCN delineated above impacts LSC modeling in CML. As mentioned in the *Introduction*, Reya et al. summarized two alternative models for CSC generation, the cancerization of a normal stem cell or the staminalization of a normal progenitor cell, meaning that cells exhibiting either the stem or the progenitor cell phenotype may long-term sustain cancer growth [6]. The identification of two possible "metabolism-driven" phenotypes for LSC of CML led us to propose that, short of being alternative to each other, *both* models fit with CML biology, i.e., that two LSC subsets with different functional roles coexist in CML (Fig. 6.2). The "stem cell LSC model" seems indeed adequate to describe a BCR/$Abl_{protein}$-negative LSC subset capable of BCR/Abl-independent self-renewal, while the "progenitor cell LSC model" suits an LSC subset where BCR/$Abl_{protein}$ is expressed and self-renewal is sustained by BCR/Abl-dependent signaling [49–51]. It is worth reminding here that CML and MEL include an LSC subset capable to cycle in the absence of oncogenic signaling (Tanturli et al. unpublished data; [34]). It is reasonable to think that this subset cycles more slowly than that where oncogenic signaling is active, as it is deprived of the growth advantage oncogenic signaling confers. Such an advantage, however, while being useful to maximize clonal expansion whenever possible, would be highly detrimental to the long-term maintenance of LSC. The dual CML stem cell compartment described above is well in keeping with the view of CML as a stem cell-derived but progenitor cell-driven disease [56], if one transposes the concepts of "stem" and "progenitor" cells to the two different LSC subsets described above.

6.6 The LSC Niche and the Refractoriness of MRD to Treatment in CML

The studies where the response of CML to low O_2 was initially characterized also addressed the effects of treatment with imatinib mesylate (IM; Gleevec®), the inhibitor of the constitutive enzymatic activity of BCR/Abl which is the prototype of tyrosine kinase inhibitors (TKi) used for CML therapy. As expected on the basis of what explained above, cells where BCR/$Abl_{protein}$ had been reversibly suppressed

following incubation at 0.1% O_2 were resistant to treatment, obviously because devoid of the molecular target of IM [33, 41]. The crucial aspect of such an outcome is that the maintenance of CML stem cell potential was completely insensitive to IM (Fig. 6.2). This insensitivity is appropriately referred to as "refractoriness," while the term "primary resistance" should be reserved to genetically determined (and therefore irreversible) insensitivity to IM already present before the treatment starts, and the term "secondary resistance" to the irreversible insensitivity acquired by a cell subset via mutations under treatment pressure. It is worth discussing resistance to IM under the light of the SCN model delineated in the previous section.

Metabolic pressure within the SCN controls LSC sensitivity or refractoriness to IM as a phenotypical adaptation, rather than a genetically blocked event, depending on whether LSC reside within the "hypoxic" or the "ischemic" SCN zones where $BCR/Abl_{protein}$ is expressed or suppressed, respectively (Fig. 6.2). The concept of resistance to IM as a phenotypical change is in keeping with the marked phenotypical heterogeneity of CML cells as for BCR/Abl expression and sensitivity to IM [57, 58] and with the fact that relapse of disease upon discontinuation of IM most often occurs without signs of development of mutation-driven secondary resistance to IM, so that the relapsed patient responds well to the reintroduction of IM [59]. Accordingly, the TKi-resistant CML progenitor cells shown to be BCR/Abl-positive by FISH or PCR [60–62] are likely to be BCR/Abl_{mRNA}-positive/$BCR/Abl_{protein}$-negative. This is a conceptually simple way to resolve the controversy of whether TKi inhibit or not BCR/Abl kinase in LSC [60–63], by concluding that TKi are always effective but LSC do not respond due to the lack of the TKi molecular target. The presence of $BCR/Abl_{protein}$-negative LSC also explains easily the lack of LSC-suppressive effects of not only IM but also "second-generation" BCR/Abl-active TKi, despite their enhanced action on CML cell bulk [64, 65]. The ineffectiveness of IM as well as dasatinib on LSC was confirmed using CML cells, from stabilized lines or explanted from patients, kept at 0.1% O_2 for incubation times sufficient to drive glucose exhaustion from culture medium [66]. In this scenario, it was predicted that even next generations of BCR/Abl-active TKi will be useless to suppress LSC [35, 50, 51].

SCN zones where LSC are capable to cycle slowly and self-renew independently of BCR/Abl signaling and are therefore protected from TKi action represent ideal sites for the long-term maintenance of treatment-resistant MRD of CML (Fig. 6.2). Such an outline facilitates MRD modeling, because it prevents us from restricting MRD to quiescent LSC, although there is no doubt that quiescence boosts resistance to treatment when drugs generically active against cycling cells are used. What we call a "dynamic maintenance" of MRD [49–51] appears indeed better suited than LSC quiescence to explain the combination of refractoriness to TKi with liability to neoplastic progression. First, the finding that cycling of HSC in low O_2 fosters self-renewal rather than clonal expansion [29] suggests that the same may apply to LSC. A relatively high stem cell potential of the mutated CML subclone stored in the SCN before relapse of disease reaches the clinical level may be behind the often very rapid and aggressive course of relapse once it is triggered. Second, neoplastic progression while disease remains at a subclinical level is best explained if LSC

cycle, because cell cycling is necessary to transmit mutations to progeny, which are undoubtedly frequent in the genetically unstable CML cells.

The process leading from MRD to relapse of disease in CML can be summarized hypothetically as follows: (a) BCR/Abl-independent, IM-insensitive self-renewal within the “ischemic” SCN core would maintain (MRD) non-mutated or mutated LSC; (b) migration out of the SCN core or (transient?) increase of glucose supply therein would make LSC switch to conditions typical of the “hypoxic” SCN periphery, where ppO_2 is still very low but glucose concentration is higher, which allow the rescue of $BCR/Abl_{protein}$ expression; (c) BCR/Abl-dependent signaling in the SCN periphery would enhance LSC self-renewal and enable commitment to clonal expansion; and (d) a move of LSC to better oxygenated BM zones (the “vascular” niches) would drive clonal expansion and differentiation (if any), making relapse of disease emerge at the clinical level (Fig. 6.2). Of course, from phase (b) on, CML growth returns to be TKi-sensitive in the majority of cases, so that there is risk of relapse only in the absence of therapy [59]. The majority of relapsed patients indeed does not present mutations, or, if it does, the mutations are incapable of conferring secondary TKi resistance. It is likely that the shift from chronic to accelerated to blast clinical phase of CML is founded on the above biological frame.

6.7 To Exploit the Metabolic Control of LSC Compartment for Therapeutic Purposes

The long-standing use for cancer therapy of drugs most of which are cell cycle-specific antiblastic agents obviously leads to trace back treatment-resistant MRD to quiescence, to quiescent stem cells in particular. Furthermore, especially in the hematopoietic field, quiescence is a substantial component within the SCN of the mechanisms protecting stem cells from premature commitment to clonal expansion and differentiation [14]. Therefore, the combination of treatments capable of inducing (stem) cell exit from quiescence with cycle-specific antiblastic agents has been proposed to try to suppress MRD [67]. A more advanced approach to the therapy of neoplasias is based on the use, instead of drugs acting generically against cycling cells, of selective inhibitors of disease-specific targets, such as BCR/Abl in CML, with obvious advantages in terms of reduced toxicity on normal cells (therapeutic index). In theory, such an approach should be also active on quiescent cells sustaining MRD, as neoplastic cells may depend on oncogenic signaling not only for proliferation but also survival (oncogene-addicted cells). An opposite, less optimistic perspective springs from the SCN model we proposed for CML, which also applies to MEL. In these leukemias, the loss of oncogene addiction driven under metabolic pressure makes not only survival but also self-renewal independent of oncogenic signaling (see above). Thus, the pool of cells resistant to drugs targeting the oncogenic protein may be larger than the quiescent LSC subset, to include LSC capable to cycle in the absence of oncogenic signaling. Therefore, to push LSC to exit from

quiescence would not be equivalent to make them sensitive to treatment. In this case, a possible alternative strategy would be to combine the targeting of the oncogenic protein with treatments capable to prevent or revert its suppression. In CML, either the treatment with specific drugs [68] or the interference with the metabolic adaptation of LSC to the SCN (Poteti M et al., manuscript in preparation) was shown to prevent $BCR/Abl_{protein}$ suppression following incubation at 0.1% O_2.

A perhaps more straightforward approach to suppress MRD maintained under conditions where oncogenic signaling is suppressed is represented by the targeting of mechanisms driving the overall LSC adaptation to these conditions (rather than those specifically related to oncogene suppression, discussed in the previous paragraph). As those conditions are likely to consist of a low O_2/glucose environment, HIFα appears an obvious potential therapeutic target (see text above and, as examples, [69–71]), and HIFα targeting was tested. Echinomycin was the first HIFα inhibitor to be used to treat leukemias. The drug, capable to block the DNA-binding activity of HIFα, was found to target human AML and LSC in particular in a xenogeneic model in vivo, reducing leukemia burden and extending mouse survival, as well as, more importantly, suppressing disease development following transplantation into secondary recipients. Equally important is the finding that echinomycin does not interfere with HSC self-renewal, thus exhibiting a high therapeutic index [72, 73]. In murine APL models driven by both PML-RARα and PLZF-RARα, the HIF-1α transcription antagonist EZN-2968 or the HIF-1α inhibitor EZN-2208 are capable alone of debulking leukemia and prolonging mouse survival and, when administered in combination with all-trans retinoic acid, suppressing LSC and eradicating leukemia [71, 74]. Furthermore, L-ascorbic acid inhibits HIF-1α transcription and reduces growth of APL and CML cell lines [75]. Finally, acriflavine, which decreases HIF transcriptional activity by inhibiting α/β dimerization, reduces growth and LSC maintenance in cultures incubated at low O_2 of CML cell lines and cells explanted from a number of patients as well as in *BCR/abl*-induced mice used as a stem cell-driven disease model [66].

An alternative strategy to the block of HIF-dependent LSC adaptation to SCN consists of taking advantage of low O_2 to activate prodrugs which then target LSC already adapted to SCN. TH-302 is a "hypoxia"-activated prodrug that is specifically cytotoxic in low O_2 via multiple mechanisms and is capable to reduce leukemia burden and to prolong recipient survival in murine models of human AML [76]. Similar results were obtained by treating acute lymphoblastic leukemia (ALL) cells with the "hypoxia"-activated DNA cross-linking agent PR-104; in a phase I/II trial study, PR-104 was shown effective in the treatment of AML or ALL patients, even refractory to standard therapy or in relapse [77, 78].

As explained above, HIFα is a necessary, but not sufficient, mediator of LSC maintenance. Indeed, other critical regulators of stem cell compartment unrelated to HIFα signaling, or more in general to the metabolic control of LSC homing within the SCN, have been identified as potential therapeutic targets. The product of the arachidonate-5-lipoxygenase (5-LO) gene (alox5) is one of these regulators, and Zileuton, a specific 5-LO inhibitor, induces depletion of short-term LSC and multipotent progenitor cells of CML, likely by blocking the commitment of long-term

LSC, in the murine CML model mentioned above [79, 80]. Zileuton also impairs stem cell function in polycythemia vera [81]. Likewise, alox15 inhibition by PD146176 causes LSC depletion and prevents CML initiation [82]. Omacetaxine (formerly homoharringtonine) markedly reduces both LSC and leukemia bulk in vitro and in vivo, also when *BCR/abl* is mutated [83], exemplifying a general advantage of non-BCR/Abl-targeting drugs. The hedgehog signaling inhibitors cyclopamine or GDC-0449 target LSC in CML [84]. BMS-214662 induces apoptosis of both proliferating and quiescent LSC of CML via mechanism not associated with the farnesyltransferase-inhibitory activity of the drug [85]. Finally, the inhibition of MEK5/ERK5 pathway reduces growth and LSC maintenance in cultures incubated at low O_2 of CML cell lines and cells explanted from a number of patients and in the murine CML model mentioned above [86]. Very often, normal HSC were found much less sensitive than LSC to these treatments. The vast array of new options for leukemia therapy summarized above, once its translatability to clinical practice is verified, induces a reasonable hope to succeed in suppressing LSC and MRD in patients affected by a number of different leukemias, thus aiming at cure rather than care.

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Chapter 7
DNA Damage Response in Quiescent Hematopoietic Stem Cells and Leukemia Stem Cells

Wenjun Zhang, Guangming Wang, and Aibin Liang

Contents

Abstract In humans, hematopoietic stem cells (HSCs) adopt unique responsive pathways counteracting with the DNA-damaging assaults to weigh the balance between the maintenance of normal stem cell poor for whole-life blood regeneration and the transformation to leukemia stem cells (LSCs) for leukemia initiation. LSCs also take actions of combating with the attack launched by externally therapeutic drugs that can kill most leukemic cells, to avoid extermination and promote

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disease relapse. Therefore, the collection of knowledge about all these underlined mechanisms would present a preponderance for later studies. In this chapter, the universal DNA damage response (DDR) mechanisms were firstly introduced, and then DDR of HSCs were presented focusing on the DNA double-strand breaks in the quiescent state of HSCs, which poses a big advantage in promoting its transformation into preleukemic HSCs. Lastly, the DDR of LSCs were summarized based on the major outcomes triggered by different pathways in specific leukemia, upon which some aspects for future investigations were envisioned under our currently limited scope of knowledge.

Keywords DNA damage · Hematopoietic stem cell · Leukemia stem cell

7.1 Introduction

As the most important material in a cell of the whole organism, DNA is very fragile and constantly under attack of various, endogenous, and exogenous assaults which cause different kinds of DNA lesions [11, 66]. To counteract the deleterious action of DNA damage, organisms have developed multiple and robust DNA damage response (DDR) mechanisms through which the DNA defects are either removed or maintained to induce differential cellular outcomes [10, 68].

Hematopoietic stem cells (HSCs), which are crucial for the maintenance of homeostasis and regeneration of the blood system over the lifetime of an individual, inevitably suffer damaging factors leading to genome instability. Accelerating evidence highlights the occurrence of DDR and the presence of DNA lesions being the underlined factors contributing to the functional decline and the leukemogenic accumulation of HSCs [9, 31, 75]. More strikingly, an emerging model proposes mutations in HSCs giving rise to the emergence of aberrant preleukemic HSCs that, with the additional oncogenic/environmental events, are finally transformed into leukemic stem cells (LSCs); in this way, the mutations in HSCs seem to serve as the time bomb for the leukemia [38]. LSCs, being the rare populations in leukemia with the capacity of self-renewal to drive the expansion of leukemia and taking the responsibility for the relapse of leukemia, also employ various means to cope with DNA damage, which now is garnering widespread interest of investigators [34].

7.2 The DNA Damage Response

All living cells suffer the DNA lesions constantly caused by endogenous and exogenous resources and combat with them through a well-organized process called DDR. Different DNA-damaging insults generate specific type of DNA lesions that are processed by distinct DDR leading to quite different outcomes for a cell, which depends on, at the cellular level, the developmental stage, e.g., stem or mutual cell;

cell cycle state, e.g., dividing or nondividing cell; and tissue origin, e.g., blood or neural cell. Here, we firstly give a brief description of DNA damage induction, and then we make a summary of the universally reactive DDR based on current understanding.

7.2.1 Various Insults Generate Different Types of DNA Lesions

DNA damage is normally classified based on the lesions that occurred at the base or strand level of the DNA resulting from various endogenous or exogenous factors [11, 66] (Fig. 7.1). At the DNA base level, the bases within the normal sequences of the DNA can be base-base mismatched, dimerized (e.g., pyrimidine dimers), deleted (e.g., abasic sites), and abnormally modified (e.g., Uracil, 8-oxoguanine, O-6-methylguanine) [3]. At the strand level, the damage may take place within one single strand, such as single-strand nick (SSN) or single-strand break (SSB), or between two individual strands, such as interstrand cross-link (ICL) and double-strand break (DSB).

DNA base-base mismatches are endogenously generated during the leading- and lagging-strand replication of DNA by DNA polymerase picking up the incorrect dNTPs while posing a very low frequency due to the high selectivity of substrates by the DNA polymerase and the proofreading mechanism in vivo [53, 60]. The pyrimidine dimers are produced between adjacent pyrimidine residues in one strand of the DNA upon ultraviolet (UV) radiation [74, 92]. Abasic sites, also known as AP sites (apurinic/apyrimidinic site), form spontaneously when the glycosidic linkage between the deoxyribose and its nitrogenous base is broken due to the enzymatic reaction induced by chemical compounds [67]. The abnormally modified bases, e.g., oxidized/reduced bases, alkylated bases, and deaminated bases, are majorly introduced directly by agents of external origin such as chemotherapeutic drugs, and reactive oxygen species (ROS) are generated internally by metabolic processes or externally by ionizing radiation and UV [19, 36].

DNA SSNs and SSBs which predominantly resulted from the oxidative attack by ROS are produced endogenously by metabolisms or exogenously upon radiation, also generated by cellular enzymes such as DNA topoisomerase 1 (TOP1) that poses a DNA nick to relax DNA for transcription and replication, or produced as intermediates containing a 1–30 nucleotide break during base and nucleotide excision repair [20]. The DNA ICLs are mainly created by the involvement of agents covalently binding to two base sites in the DNA duplex arising from endogenous metabolites such as nitrous acid and bifunctional aldehydes or exogenous chemicals such as cisplatin, nitrogen mustard, and mitomycin D and also oxidatively generated by free oxygen radicals from internal and external sources [30, 77]. As the most severe lesions to DNA, DSBs are broadly induced by various factors under physiologic condition such as V(D)J recombination and class switch during early lymphocytic development and pathologic situation such as ionizing radiation, oxidative

stress, replication fork, inadvertent enzymatic action, topoisomerase failures, and mechanical stress, which was well summarized by Michael R. Lieber [65].

In a cell, DDR is a multicomponent, well-organized biological process employed by cells when encountering with DNA lesions. Different DNA lesions will be primarily repaired by different means (Fig. 7.1) and trigger other mechanistically distinct DDR, composed of signaling pathways in a hierarchical manner, which similarly involves in the following steps: sensors recognize the damage; mediators and transducers process the signal; and effectors regulate the cellular outcome [10, 89]. As the exact pathway of DDR between prokaryotes and eukaryotes, on the other hand, lower and higher eukaryotes, represents some difference in details, we mainly take higher eukaryotes for the following illustration under current understanding.

7.2.2 DNA Base Damage Is Primarily Repaired

DNA base-base mismatches are predominantly processed by DNA mismatch repair (MMR), a highly conserved biological process between prokaryotes and eukaryotes, which involves in the collaboration of MMR-specific proteins and replication and/or recombination components [60, 63]. In eukaryotic cells, MutSα recognizes a mismatch, followed by recruitment of MutLα in an ATP-dependent manner, which is then activated by PCNA to incise at both sides of the mismatched base giving rise

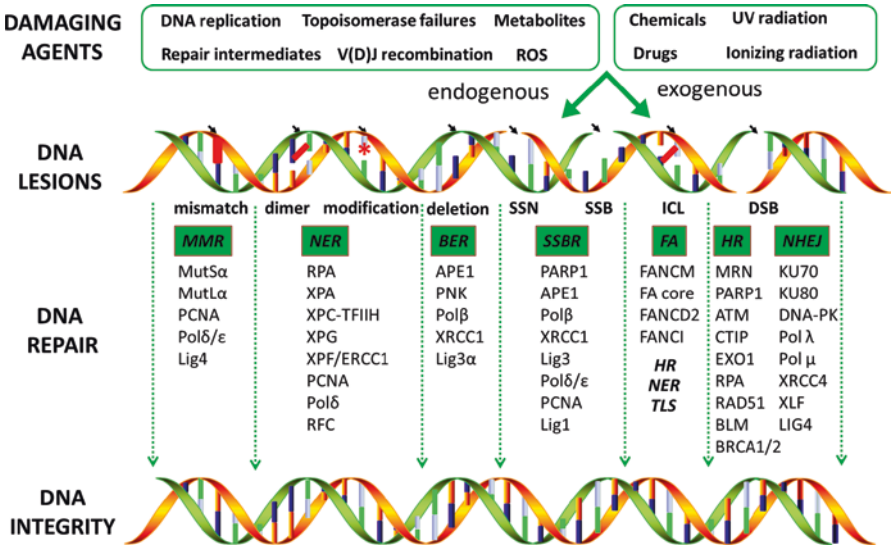

Fig. 7.1 The direct induction and major repair pathway of DNA damage. Various factors from endogenous and exogenous stimulus can cause different types of DNA lesions that are primarily repaired by distinct pathways to sustain the DNA integrity

to the error-containing strand, and then the repair is completed following error removal, DNA resynthesis, and ligation [60, 63]. The pyrimidine dimers are directly recognized and repaired by photolyase in *E. coli*, which is consisted of a photoantenna cofactor in the form of 5,10-methenyltetrahydrofolate (5,10-MTHF) and a catalytic cofactor in the form of $FADH^-$. Photolyase interacts with the backbone of DNA duplex and forms specific contacts with pyrimidine dimers, initiated by light; 5,10-MTHF absorbs and transfers energy to $FADH^-$, which then transfers the electron to dimer to split it into individual pyrimidines [78, 88].

In higher eukaryotes, the AP sites are majorly repaired by base excision repair (BER), during which AP-endonucleases (APE1) firstly recognize and incise the AP sites producing SSBs containing a 5′-deoxyribosephosphate (5′-dRP) end, which are then removed by DNA polymerase β or 5′-flap endonuclease resulting in gaps of one or few nucleotides that are finally processed by DNA polymerase and ligase [55]. Other responses to AP sites may involve nucleotide excision repair (NER), recombination, DNA translesion synthesis (TLS), or cell death, which is revealed by studies based on BER deficiency [12]. The abnormally modified bases are primarily repaired by specific process depending on the type of modification. For example, uracil bases and 8-oxoguanine are efficiently repaired by BER and O-6-methylguanine by methylguanine DNA methyltransferase in a direct way [59, 89]. NER is the major system to deal with bulky DNA adducts in which for human cells, RPA, XPA, and XPC-TFIIH forming a complex recognizes the DNA lesion and makes successively preincision complex 1, 2 and 3 through ATP hydrolysis of damaged duplex, replacement of XPC with XPG and recruitment of XPF/ERCC1, respectively. Then the damaged strand is incised by XPG and XPF/ERCC1 leaving a gap that is taken cared by replication machinery including Polδ, PCNA, and RFC [95].

7.2.3 DNA Strand Damage Impacts Cell Fate

Most SSBs in a cell are rapidly repaired by global SSB repair process [20, 21]. In the SSB repair procedure, PARP1 recognizing SSBs from oxidative reaction or abortive TOP1 activity or APE1 remaining bound to the cleaved SSB intermediate during BER stimulates the recruitment of XRCC1 protein complexes that are composed of constitutive Polβ and Lig3 and in addition either PNKP, APTX, or APLF for DNA end processing. Then Polβ replaces the single missing nucleotide, and XRCC1/Lig3α complex ligates the patch in a more commonly short-patch repair model. While in a less frequent long-patch model, the gap usually 2–12 nt is filled by Polβ and/or Polδ/ε leaving a single-strand flap that is removed by flap endonuclease-1 (FEN1), and this patch is then ligated by PCNA/Lig1 complexes. Aside from direct repair, SSBs may lead to paused replication forks or DSBs during S phase that are processed by PARP1 augmented homologous recombination (HR) in cycling cells [15, 61, 98] or stalled transcription resulted in altered transcription profiles and/or cell death in nonproliferating cells [54, 73].

The DNA ICLs have very bad impacts on cells, in the worst case, leading to cell death. The unrepaired ICLs can block replication by preventing the progression of the replisome or stall transcription through impeding the interaction between proteins and DNA resulting in p53-dependent apoptosis; moreover, accumulated ICLs directly cause cell death due to the loss of vital DNA integrity [30]. Nevertheless, cells employ sophisticated repair mechanisms, which involves the coordination of several repair processes including homologous recombination (HR), NER, and TLS, to protect themselves from the death fate [30, 56, 77]. The repair of ICLs is majorly a concomitant Fanconi anemia (FA) pathway in which FANCM and associated proteins recognize ICL followed by the recruitment of the FA core complex, the monoubiquitylation of FANCD2-FANCI, and the subsequently coordinative action of repairing components including nucleases and polymerases, such as BRCA2 and RAD51C of HR and FAN1 and Polν of TLS. This procedure is recently found to be aided by a non-erythroid alpha spectrin (αIISp) serving as a scaffold for the recruitment of repair proteins to sites of damage [62].

The responses to DSBs that are particularly well understood due to its great importance and accordingly deep investigation of researchers in the past few decades are still the hot area drawing attention of the followers for further exploration [41, 106]. DSBs processed by distinct pathways can lead to various cellular outcomes including error-prone repair, error-free repair, cell cycle arrest, apoptosis, senescence, and specifically differentiation for stem cells, depending majorly upon the cell cycle status and the activity of p53 [24, 35, 68, 103, 106] (Fig. 7.2). DSBs recognized by the KU dimer (KU70–KU80) are repaired by nonhomologous end joining (NHEJ) which is an error-prone repair system employed by proliferating and nonproliferating cells [64]. In its classically simplest way, the DNA-dependent protein kinase (DNA-PK) catalytic subunits (DNA-PKcs) are recruited by KU70–KU80 to activate DNA-PK that conducts end processing and resection with the participation of the nuclease Artemis, which is followed by gap filling with Pol λ and Pol μ and subsequent ends ligation performed by a ligase complex XRCC4/XLF/LIG4 (Fig. 7.3).

The detection of DSBs by PARP and MRE11-RAD50-NBS1 (MRN) complex leads to a higher-fidelity repair named HR or other consequences including cell cycle arrest, apoptosis, and senescence in cycling cells [10, 68]. In this complicated process, briefly the protein kinase ataxia-telangiectasia mutated (ATM), recruited by MRN complex and activated by autophosphorylation, phosphorylates and activates downstream targets that can operate repair or define the cell fate [10, 68]. The cell fate determinants include checkpoint kinase 2 (CHK2) and p53 leading to cell cycle arrest through p21 transactivation, bcl2 gene family members resulting in apoptosis, and p16/Ink4a and p19/ARF causing senescence. In the procedure of HR, ATM phosphorylates histone H2AX and other members of the DDR leading to the following repair [7] (Fig. 7.3), in which the two short stretches of single-stranded DNA (ssDNA) at DSBs are processed by nucleases including CTIP, EXO1, and

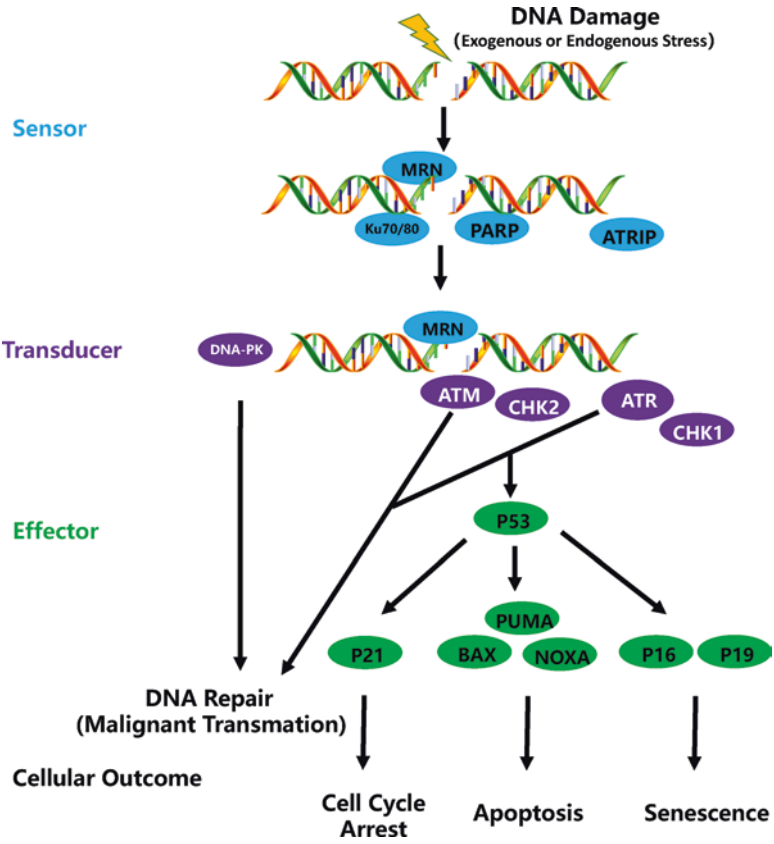

Fig. 7.2 DNA damage response pathways. DNA damage is caused by a variety of sources. There are distinct sensors, transducers, and effectors in the DNA damage response pathways. This DNA damage response converges upon p53 which, depending on the target genes activated, regulates different cellular outcomes, which may involve the cell cycle arrest, senescence, execution of DNA repair, or, when the damage is severe, initiation of apoptosis

BLM leaving the long ssDNA overhangs bound by RPA. RPA is then replaced by RAD51 recombinase, with the assistance of BRCA1, BRCA2, and PALB2, forming multimers to produce the nucleoprotein filament that invades the sister chromatid and promotes the formation of Holliday junctions through the generation of a D-loop intermediate and primed DNA synthesis of the invading strand when met with the homology. The Holliday junctions are lastly processed by some enzyme complex such as BLM, MUS81, EME1, and GEN1 resulting in the final crossover or noncrossover products.

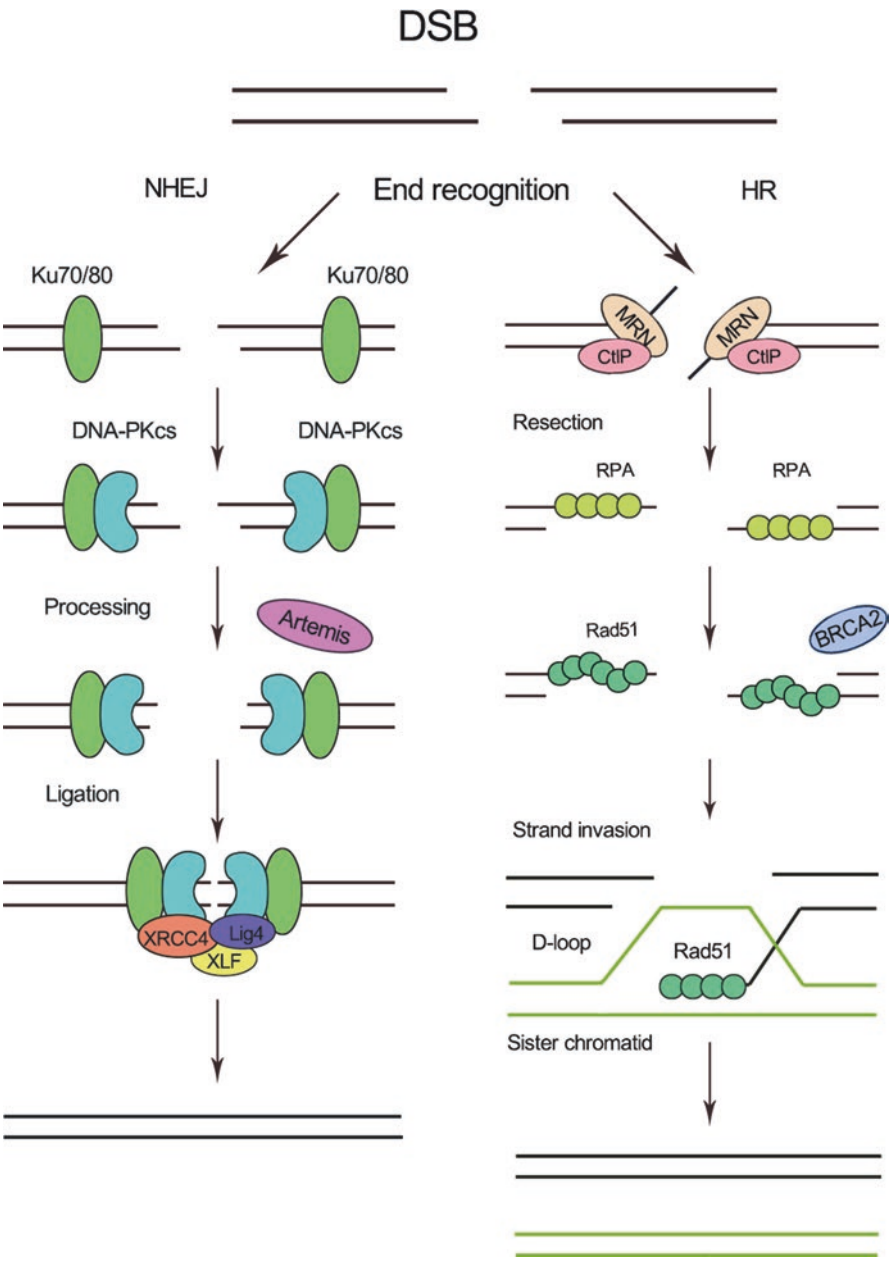

Fig. 7.3 NHEJ and HR repair pathway. In NHEJ pathway, the KU70-KU80 heterodimer firstly recognizes the DNA ends and subsequently recruits DNA-PKcs. If the ends are incompatible, nucleases such as Artemis can trim the ends. The XRCC4-DNA Ligase IV-XLF ligation complex seals the break. In HR pathway, the MRN-CtIP complex resects the DNA breaks to generate single-stranded DNA (ssDNA). After resection the break can no longer be repaired by NHEJ. RPA first coats the ssDNA and subsequently replaces Rad51 with the help of BRCA2. These Rad51 nucleoprotein filaments mediate strand invasion on the homologous template. Extension of the D-loop and capture of the second end lead to repair

7.3 DNA Damage Response in Hematopoietic Stem Cells

Lifelong blood production depends on HSCs-a subset of primitive hematopoietic cells endowed with high self-renewal potential. HSCs give rise to committed progenitors (CPs) with limited or no self-renewal, which, in turn, differentiate into various mature blood cells. Mounted evidence have revealed that, aside from DSBs, DDR of various DNA lesion is involved in leukemia [32] and quiescence serves as the most prominent property of adult stem cells [23]; here we take DSBs in quiescent HSCs for detail illustration.

7.3.1 DNA Damage Response in Quiescent Hematopoietic Stem Cells

Given that HSCs maintain the homeostasis and regenerate the blood system over the lifetime of an individual, any exogenous or endogenous factor leading to genome instability would lead to shortening of organism survival. In order to preserve HSCs' genomic integrity to ensure lifelong function, some strategies were proposed to focus on minimizing endogenous DNA damage. Firstly, endogenous DNA damages were mostly from ROS of oxidative stress and DNA replication stress. In order to be protected from these DNA damages, a quiescent phase of the cell cycle mostly kept in the human and mouse contributes to their overall maintenance and protection of their genomic integrity by minimizing DNA damage associated with ROS production, cellular respiration, and cell division [107]. Compared to quiescent HSCs, activated HSCs showed a higher levels of ROS and ROS-induced DNA lesions [104]. Secondly, the distinct niches where adult HSCs reside helped to reduce the level of ROS, as exampled by hypoxic niches in the bone marrow [45] where active ROS transportation occurs in adjacent mesenchymal stromal cells [101]. Despite reducing the endogenous damages to capacity, accumulation of DNA damage and formation of γH2AX foci and comet tail lengths as the markers for SSBs and DSBs are increased in old HSCs from both mice and humans [6]. In contrast with HSCs, the number of γH2AX foci is similar between progenitors in old animals and those in young animals [86]. The follow-up researches indicate that DNA damage accumulation in HSCs was associated with broad attenuation of DNA repair and response pathways that were dependent upon HSC quiescence. Accordingly, cycling fetal HSCs and adult HSCs driven into cycle upregulated these pathways leading to repair of strand breaks [6, 8]. Therefore, HSC quiescence and concomitant attenuation of DNA repair and response pathways underlie DNA damage accumulation in HSCs (Fig. 7.4).

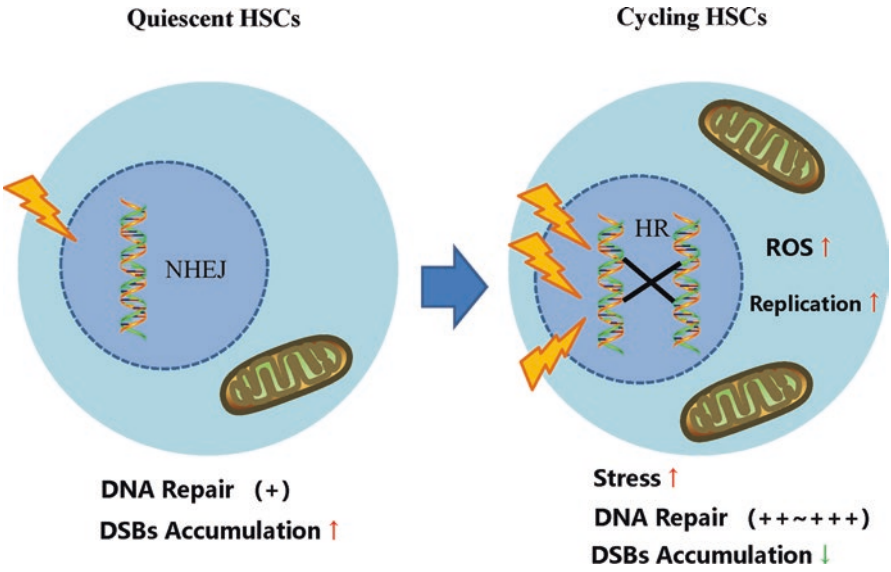

Fig. 7.4 Differences between quiescent and cycling HSCs in DNA repair. Although quiescent HSCs are more likely to utilize NHEJ as a repair mechanism, the attenuation of DNA repair and response pathways underlies DNA damage accumulation in these cells. Compared to quiescent HSCs, elevated ROS and ROS-induced DNA lesions were observed in those cycling HSCs; the enhanced HR could still reduce the DNA damage accumulation in these activated cells

The continuous DNA damage accumulations in HSCs are bound to activate the cellular DNA damage machinery and lead to the corresponding DDR outcome. Two studies uncover different outcomes of irradiation-induced DDR between mouse HSCs and human HSCs (Fig. 7.5). Murine hematopoietic stem and progenitor cells (HSPCs) were more resistant to apoptosis than differentiated progenitor cells [71]. The unique DNA damage response of mouse HSPCs involves the tumor suppressor protein p53 and is lost when stem cells are forced out of quiescence and into the cell cycle by treatment with chemotherapy or cytokines. In human umbilical cord blood cells, an enhanced sensitivity to apoptosis was observed in contrast with differentiated cells. Survival and the clonogenic and reconstitutive capacity of the irradiated human HSPCs were rescued by blocking p53 expression or by overexpression of the antiapoptotic factor Bcl-2. However, irradiated human HSPCs lacking p53 were unable to sustain hematopoiesis and showed evidence of persistent DNA double-strand breaks when serially transplanted into recipient mice. Therefore, a short-term gain in survival could be achieved by human hematopoietic stem cells as found in the mouse, but the default setting for irradiated human HSPCs is an increase in p53 expression resulting in apoptosis [70].

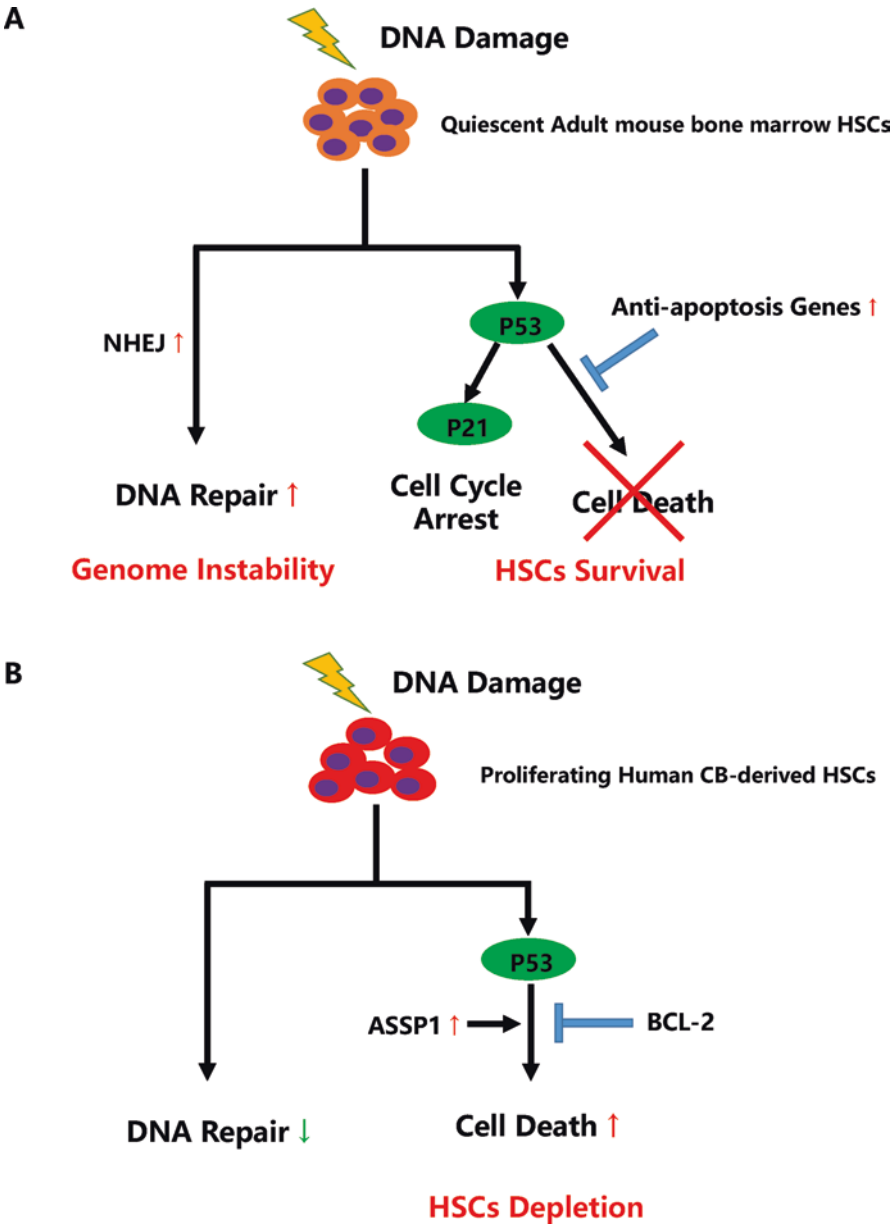

Fig. 7.5 DNA damage response in murine and human HSCs. Quiescent murine HSCs (**a**) and human CB-derived HSCs (**b**) exhibit opposite outcomes following DNA damage induced by low-level ionizing radiation, with different consequences for their overall maintenance and genomic integrity

7.3.2 NHEJ Pathway Was Responsible for the Mutagenesis of Quiescent HSCs

DSBs, which are considered the most lethal form of DNA damage, can be repaired by two major pathways, homology-directed repair (HR) and nonhomologous end joining (NHEJ) [42]. HR is active during the late S and G2 phases of the cell cycle and uses the intact sister chromatid as the template for repair. And NHEJ, which joins DNA ends of DSBs directly and occurs throughout the cell cycle (G0, G1, and early S phase), is the major DSB repair pathway in mammalian cells. However, this error-prone repair pathway could lead to accumulation of mutation in cells. On the one hand, in order to maintain their genome integrity and survival, HSCs must employ the repair pathway to reduce the accumulated DNA damages caused by exogenous or endogenous factors. Due to the cell cycle status of quiescence, repair of DNA damage through homologous recombination (which has a lower error rate than NHEJ) requires that cells enter the cell cycle; thus, quiescent HSCs must rely on NHEJ as an alternative to repair the DNA. In one study, it was revealed that irradiated quiescent HSCs display high levels of chromosomal abnormalities after the rapid DNA repair through the error-prone NHEJ pathway and their progeny show persistent genomic instability associated with some of the same abnormal cytogenetic findings and engraftment defects in secondary mouse recipients. These events would pose an obvious risk to a long-lived organism as serial exposure of stem cells to genotoxic agents could readily result in leukemia or aplasia. As mentioned above, NHEJ appears to be the most commonly used DNA repair pathway in quiescent HSCs, and this result is in line with the fact that the deficiency of functional components of DSB recognition and repair pathways affected the function of HSCs upon genotoxic stress in mouse models. Recurrent chromosome translocations occur frequently in both de novo and therapy-related in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Cloning of the genomic breakpoints in the common chromosome translocations in leukemia reveals that most of the genomic breakpoints tend to cluster in a restricted intronic region. In addition, sequencing of the translocation junctions identified regions of microhomology, strongly indicative of the involvement of ALT-NHEJ in the repair of DSBs and the generation of these chromosomal abnormalities [111]. It may explain why cancer patients treated with radiotherapy or chemotherapy may develop blood cancer or myelodysplasias bone marrow failure because of the use of error-prone DNA repair in quiescent HSCs.

On the other hand, in order to reduce the risk of mutagenesis, quiescent HSPCs exhibit reduced NHEJ activities in comparison with cycling progenitors. In contrast with the two- to sixfold difference in total NHEJ capacity, Alt-EJ activity was more moderately reduced by 30% in HSPCs compared with cycling progenitors, thus supporting the notion that canonical NHEJ capacity rises with the degree of differentiation in hematopoietic cells [8]. In line with these results, the global transcriptional analysis showed decreased expression of multiple DNA damage checkpoint and DNA repair pathways which are involved in DNA DSB repair and can be detected in highly purified murine and human quiescent HSCs with much less in

their cycling progeny [6, 8, 18].Secondly, there might be a link between quiescence status and DNA repair gene expression. For example, NF-kB and PI3K/AKT/mTOR signaling pathways have recently been implicated in the transcriptional regulation of a group of DNA repair genes that mediate HR and NHEJ in human primary and cancer cells [22, 57]. Collectively, for one thing, in order to reduce the accumulated DNA damage and survive, quiescent HSCs rely on NHEJ repair pathway that has the potential to cause mutations for malignant hematology; on the other hand, high rigidity of DSB repair in HSCs reduces the risk of mutagenesis.

7.3.3 Preleukemic HSC Emergence: The Cost of Long-Term Genomic Integrity

The mutagenesis caused by the error-prone NHEJ pathway is a short-term survival strategy at an expense of their long-term genomic integrity in quiescent HSCs. Misrepair or unrepair of DNA damage in these cells can disrupt normal hematopoiesis and promote leukemogenesis. In some studies, attempts were made to conduct next-generation sequencing on leukemia cells and highly purified HSCs from the same AML patients with a goal of identifying the initiating lesions and determining the order of their acquisition of the lesions and their impact on the function of HSCs [27, 47, 94]. In human HSCs, a set of leukemia-related recurrent mutations could be detected prior to the onset of leukemia for years in the same patient. These HSCs with mutations are called preleukemic HSCs and retained normal stem cell activity, showing a multilineage repopulation advantage over non-mutated HSCs in xenografts [94]. Preleukemic HSCs preferentially display mutations in "landscaping" genes involved in DNA methylation, chromatin modification, and cohesin complex (such as IDH1, IDH2, IKZF1, DNMT3A, and ASXL1), indicating that these functional mutations represent initiating events in leukemogenesis [37, 46, 69, 109]. The existence of preL-HSCs with certain frequencies that meet the definition of clonal hematopoiesis poses a high risk for developing hematologic cancers (hazard ratio, 11.1–12.9) [37, 46]. In sum, preleukemic mutations that are essential for the initial development of hematologic malignancies, such as AML, are critical for the early stages of leukemogenesis.

7.4 DNA Damage Response in Leukemia Stem Cells

It was proposed that LSCs are some rare cells possessing the ability to self-renew to drive the expansion of leukemia from the entire population of leukemia cells [34]. Both as stem cells, LSCs are different from HSCs in that they can only differentiate into specific lineage. Being the two most outstanding characterizations of LSCs, including other CSCs, self-renewal is unlimited [25, 79], and quiescent state is a

major barrier for regular therapeutic approaches such as chemotherapy and radiotherapy [17, 58, 99]. Although DNA damage can be various for one cell and is processed by very different responding, most studies focused on DSBs as which are the most severe lesions for DNA, and the cell employs the most sophisticated means to deal with them [10, 89]. On the other hand, the general response of leukemia cells to DNA damage was detailedly documented in a recent nice review [76], so here we summarized the major DDR to particular DSBs in stem cell compartment of leukemia.

7.4.1 Leukemia Stem Cells Adopt a Quiescent State

LSCs are now thought to be in a quiescent state that, on the one hand, protect them from exhaustion and responsible for continuous activation to maintain leukemic progression under normal leukemogenesis and, on the other hand, render them resistance to cell cycle-dependent chemotherapeutic drugs and relapse [33, 39, 44]. Many studies have suggested that cell cycle restriction could be one of the distinctly protective mechanisms that LSCs utilize to their advantage.

In an early study, through the isolation of a highly quiescent subpopulation of primitive leukemic cells in CML, Holyoake et al. proved that LSCs, which continuously overproduces myeloid progenitors and mature cells, are maintained as a quiescent, self-renewing, nonexpanding population [43]. Since then, accelerating evidence support the quiescence of LSCs being a prominent mechanism underlying resistance to cell cycle-dependent cytotoxic therapy [51]. Recently, using the humanized mouse models, the researchers have revealed that the transplanted human LSCs in immunodeficient mice pose a quiescent state rendering them anti-chemotherapy, and based on which they developed the valuable research tools for LSC specific therapeutic schedule [44, 87]. In these studies, Ishikawa and colleagues firstly developed a human AML xenotransplantation model with NOD/SCID/IL2r-gamma (null) mice and then demonstrated that it was the transplanted LSCs which remained the self-renewal capacity and homed to and engrafted within the osteoblast-rich area of the bone marrow, contributed to the escape of chemotherapy-induced apoptosis [44]. Next, they proved that these LSCs harboring in the endosteal region are predominantly quiescent and can be stimulated to proliferation following in vivo cytokine treatment such as granulocyte colony-stimulating factor (G-CSF), upon which they can be effectively eliminated by treatment with chemotherapeutic agents such as cytarabine (AraC) [87]. Here, particularly worth mentioning is that the NOD/SCID/IL2r-gamma (null) mice have become the most commonly used research models for leukemia studies nowadays.

7.4.2 *Cancer Stem Cells Are More Resistant to DNA Damage Induction*

In a cell, DDR is a multicomponent joined and fine-regulated processing, if damage not being repaired, leading to distinctly cellular outcomes such as transient cell cycle arrest, apoptosis, and senescence depending upon DNA damage extent, cell type, cell cycle stage, and p53 activity [10, 89]. On the other hand, DNA damage being the hallmark of cancer is less well processed in cancer cells compared to normal ones [40, 48]. Consequently, the components of DDR has long been proposed as a relatively ideal therapeutic target for almost all kinds of cancers with the combination of DDR inhibitors and cytotoxic agents, as well as the more promising drugs based on synthetic lethality [13, 29, 80]. Even with these promising therapeutic strategies, cancer is still one of the most refractory diseases with which we are combating, probably due to the existing CSCs that are responsible for relapse which are thought to give rise to therapy resistance through various mechanisms including drug efflux, DDR enhancement, quiescence status, and so on [26, 96, 97, 110]. The enhanced DDR in CSCs was majorly investigated in solid tumors under radiotherapy, such as carcinoma from glioma, prostate, and breast [1, 2], which could be achieved by inhibition of cell cycle progressing, promotion of DNA repair, and scavenging of reactive oxygen species [26, 81]. Based on these studies, it was suggested that CSCs majorly being a quiescent state are less sensitive to radiation-induced DNA damage reasoning probably from that the fast dividing cells were effectively eradicated, while the slowly growing ones remained undamaged upon irradiation [72, 97]. As quiescence is not limited to CSCs, which also resides in other normal tissues [23], the cellular outcome and related molecular mechanism of DDR in different tissue compartments are distinctly various, so as to that of normal stem cells [10, 103]; it will be of great value to identify the specific DDR of any kinds of stem cells, including LSCs.

7.4.3 *Cell Cycle Arrests Upon DNA Damage in Leukemia Stem Cells*

When DNA damage occurs in a cell, it can be sensed, transduced, and mediated to the ultimate effectors to induce the cell cycle arrest for later repair, not only in cycling stem cells but also in quiescent ones. Although LSCs are primarily in quiescent state, the induction of cell cycle arrest is their powerful weapons to defend the attack of anticancer drugs as discussed below.

Many studies have revealed that DNA damage-induced cell cycle arrest is p53 mediated and p21 dependent. For example, two decades ago, Wilson et al. had proved that induction of DNA damage with irradiation upregulating p53 and p21

expression suppressed cell proliferation in cycling stem cells of intestinal tissue through the detection of p53-/p21-positive cells along with [^{3}H] thymidine incorporation [108]. The work form Takubo et al. manifested that the undifferentiated spermatogonia, as the stem cell compartment in spermatogenesis, lost self-renewal and gained arrested cell cycle due to accumulated DNA damage under ATM deficiency, which could be restored by suppression of p21 [100]. Also in HSCs, DNA damage induced by iron radiation caused transient growth arrest through p53-mediated induction of p21 expression for the following DNA repair, which protected them from apoptosis compared to the progenitors [71]. So far, in these stem cells, the p21-dependent cell cycle arrest upon DNA damaging may serve as a protective mechanism employed by them to cope with DNA damage. However, this kind of protection could be utilized by LSCs in AML to get themselves more comfortable to survive, which was well illustrated by the study from Viale et al. [102]. The abovementioned response emphasized the fact that p21-dependent cell cycle arrest was induced by DNA damage induction exogenously; on the contrary, as revealed by Viale and colleagues, p21 loss caused increased DNA damage in HSCs. In preleukemic HSCs, high levels of DNA damage were introduced by expression of PML-RAR or AML1-ETO fusion oncoproteins endogenously, which resulted in upregulation of p21-induced cell cycle restriction, which is also applied to LSCs. These findings highlighted that LSCs can respond to DNA damage endogenously through the induction of p21-dependent cell cycle arrest limiting DNA damage accumulation and preventing exhaustion of LSCs, which indicates some novel antileukemic strategies based on DNA repair; however, studies about the cell cycle progressing response of LSCs upon direct DNA-damaging agents are lacking.

7.4.4 Differentiation Is Induced by DNA Damage in Leukemia Stem Cells

Differentiating into descendent cells being one of the hallmarks of stem cells can also be induced by DNA damage, which serves as a DDR specific to stem cells [93]. DNA damage-induced differentiation of stem cells can have a bad effect on the normal stem cell compartment by accelerating the exhaustion of stem cell pool or reducing the product of proper cell types for homeostasis; on the other hand, it may serve a positive role in anticancer treatment to exhaust CSCs. In neural stem cell (NSC) compartment, Schneider and colleagues found that ionizing radiation-induced DNA damage promotes NSCs into astrocytic differentiation in normal brain tissue while through which the tumorigenic potential of CSCs in glioblastoma is affected [91]. An interesting phenomenon was observed in muscle stem cells in which a differentiation checkpoint exists whose target, MyoD, is controlled by DNA-damaging induction that causes a reversible inhibition of myogenic differentiation [83]. In HSCs, previous studies seemed to indicate that DNA damage may lead to myeloid differentiation of HSCs, since DNA damage, including DSBs,

accumulates with age [84] and aging HSCs prefer to differentiate into myeloid lineage rather than lymphoid descendants [85]. Now the direct connection between DNA damage and differentiation in HSCs was clearly revealed by the study from Wang et al., who proved that DNA damage induces lymphoid differentiation of HSCs through a basic leucine zipper transcription factor, BATF, in a G-CSF-/STAT3-dependent manner [105].

More recently, the link between differentiation and DNA damage in LSCs was also well characterized by Santos and colleagues [90]. In this outstanding work, upon the elucidation of histone methyltransferase MLL4 playing the suppressing role in AML by regulating antioxidant response, Santos et al. demonstrated that expression of MLL-AF9 oncogene in LSCs induces DNA damage directly leading to cell cycle exit and terminal differentiation through the activation of p21 in AML. Besides, a balance between the differentiation and self-renewal may exist in LSCs to account for the final decision of cellular outcomes (Fig. 7.6).

7.4.5 The Damages Are Repaired in Leukemia Stem Cells

DNA repair is a major molecular process responding to DNA damage for all cell types, including LSCs, which were deeply studied particularly in CML. The progress of CML can be divided into two stages, the chronic phase (CML-CP) and the blastic phase (CML-BP). The generally accepted fact is that BCR-ABL1 emerging in one single HSC initiates the genesis of CML-CP, in which LSCs or LPCs (leukemia progenitor cells) progress to CML-BP upon the acquisition of mutations and chromosomal aberrations [14, 28, 82]. LSCs in CML-CP, defined as $Lin^{-}CD34^{+}CD38^{-}$ population [52], can survive from the treatment with tyrosine kinase inhibitor (TKI)-based drugs, such as imatinib, by adopting a quiescence, high expression of BCR-ABL1 and dysregulated DNA repair, which is rooted in the progression of CML-BP [4, 50, 82].

BCR-ABL1 can cause enhanced DNA damage but, on the other hand, unfaithful and inefficient DNA repair universally in leukemia cells [16], which is also applied to LSCs in CML-CP to promote CML progression. The treasonable DNA repair in LSCs can be well illustrated by the prominent genomic instability of LSCs or LPCs in CML. BCR-ABL1 of LSCs itself may gain mutation in CML-CP, as described by Jiang et al., who revealed that LSCs from patients with CML-CP displayed massive mutation corresponding to frameshift or premature stop codon at the kinase domain [49]. Also, LPCs from patients in CML-CP displayed ABL kinase domain mutations preceding the CML-BP [5], so it was suggested that genomic instability in CML may occur at LSC/LPC level which can be passed on to the successive generation promoting the CML progression, furthermore contributing to the drug resistance [82].

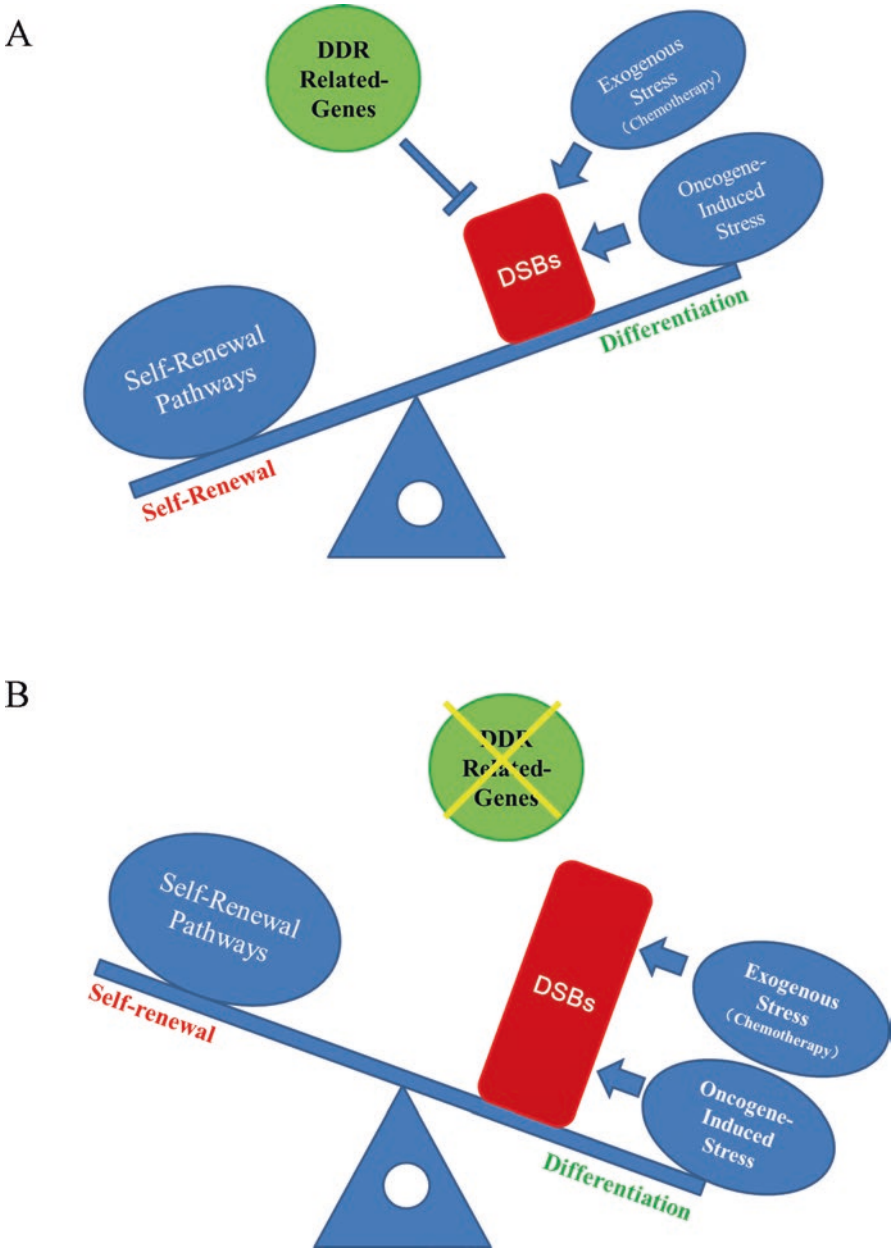

Fig. 7.6 Model showing the balance between the self-renewal and differentiation in leukemia stem cells. (**a**) Exogenous (chemotherapy et al.) or endogenous (oncogene-induced replication et al) stresses underlie the accumulation of DSBs in LSCs. In order to maintain the capabilities of self-renewal, DNA damage response machinery is used to reduce the DSBs. (**b**) Disruptions of DNA damage response of LSCs impair the above balance and lead to the genome instabilities, which are sufficient to induce the differentiation of LSCs

7.5 Perspective

In the past few decades, the studies about DDR of somatic cells, tissue stem cells, cancer cells, and cancer stem cells have made great progress, with which many novel therapeutic drugs aiming to cancers have been developed. However, the systemic understanding of DDR in LSCs hurdled by various factors is still ill defined to us. The first is the sophistication of DDR system in which one specific DNA lesion could be processed by more than one pathway and different repair processes may share enzymes and reaction intermediates. The second difficulty is the complexity of leukemia that poses so many types; the exact DDR of one specific LSC of leukemia may not be applied to another. The third and perhaps most challenging obstacle is the real modeling of leukemia in humans; so far, so many studies are based on humanized mouse model with expression of oncogenes, which can hardly capture the exact process in human leukemia. So even with the big achievement on DDR in organisms, the direct DDR of specific LSCs upon DNA damage has less been elucidated, which needs the endeavor from current and following researchers, with which we can better understand the regulation of DDR in LSCs and efficiently create novel weapons to exterminate leukemia.

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Chapter 8
Epigenetic Abnormalities in Acute Myeloid Leukemia and Leukemia Stem Cells

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Contents

Abstract Recently advances in cancer genomics revealed the unexpected high frequencies of epigenetic abnormalities in human acute myeloid leukemia (AML). Accumulating data suggest that these leukemia-associated epigenetic factors play critical roles in both normal hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs). In turn, these abnormalities result in susceptibilities of LSC and related diseases to epigenetic inhibitors. In this chapter, we will focus on the mutations of epigenetic factors in AML, their functional roles and mechanisms in normal hematopoiesis and leukemia genesis, especially in LSC, and potential treatment opportunities specifically for AML with epigenetic dysregulations.

Keywords Epigenetic changes · Leukemia stem cell · AML

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8.1 Introduction

Epigenetics is heritable phenotype changes beyond the DNA sequence. Waddington coined the term "epigenetics" in 1942 and proposed the famous Waddington's epigenetic landscape for embryonic development [1]. These changes include DNA modifications and histone modifications, called epigenetic marks. These epigenetic markers are added to specific DNA and histones by a variety of enzymes, called epigenetic writers. Another group of proteins, called epigenetic readers, can recognize and bind these modified DNA and histones to regulate the expressions of involved genes. The third group of proteins, called erasers, is able to remove these epigenetic modifications [2].

Histones help DNA to fold into structural units called nucleosomes. Posttranslational modifications on histones can change chromosomal folding and regulate gene expressions. Each nucleosome contains two copies of core histones H2A, H2B, H3 and H4, and histone H1 is the linker histone between nucleosomes. Frequent histone modifications include methylation, acetylation, phosphorylation, and ubiquitination. Histone methylation are catalyzed by histone methyltransferases (HMTs), including two major types, lysine-specific and arginine-specific HMTs. There are many lysine-specific HMTs, such as SET domain-containing ASH1L, EZH1/2 and MLL family members (MLL1–5), SET family and NSD1, and non-SET domain containing DOT1L [3, 4]. Arginine-specific HMTs can be divided into three subtypes, dependent on methylation form on arginine. H3 and H4 lysine methylations can either activate or repress the expressions of related genes, dependent on the lysine sites and also gene regions. Lysine methylations can be mono-, di-, or trimethylations, which can have different effects on gene expressions. H3K4 methylations are generally active marks. Histone methylations are removed by histone demethylases, which can have different catalytic domains with various functions. There are two major types of histone demethylases. One is lysine demethylases (KDMs), and the other one is the Jumonji C domain-containing family. The first discovered KDM is LSD1. LSD1 can remove methyl groups from H3K4 or H3K9 [5]. The level of histone acetylation is mainly determined by histone acetyltransferase (HATs) and histone deacetylase (HDAC). Both HATs and HDACs can form protein complexes with transcription factors to coordinate the fate of DNA transcripts.

DNA methylation is the most studied epigenetic modification on DNA. DNA methyltransferase enzymes (DNMTs) are the writers of DNA methylations. There are three DNMTs, DNMT1, DNMT3A, and DNMT3B, in mammals. They catalyze the additions of methyl group to the 5-cytosine to form 5-methylcytosine (5mC) in CpG. DNMT3A and DNMT3B are the major de novo methyltransferases, while DNMT1 is mainly responsible for methylation maintenance during DNA replication. In contrast, TET genes (TET1, TET2, and TET3) can successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). TDG and BER can remove carboxyl group from 5caC and then complete the "erasion" of cytosine methylation. 5mC at CpG sites on a

gene promoter generally leads to the expression silencing, while the functions of other methylation modifications or 5mC on other regions are less clear.

It has been shown that there is critical crosstalk between DNA methylation and histone modifications, especially histone methylations, to regulate the expressions of associated genes. First discovered in plant, there is a self-reinforcing loop between DNA and histone methylation mediated by DNA methyltransferase CMT3 and histone methyltransferase KYP [6]. DNA methylation can inhibit H3K4 methylation [7], and, in turn, H3K9 methyltransferase can also reduce DNA methylation [8].

Numerous studies have showed that these epigenetic regulators are essential for normal development and healthy hemostasis. Mutations and dysregulations of these regulators are involved in multiple human diseases, including metabolic disorders, autoimmune diseases, and various cancers. In recent years, with the rapid advances and applications in high-throughput sequencing, amounting data have described a more and more clear picture of human cancer genomics. Genomic studies have identified novel numerous recurrent mutations in hematopoietic malignancies. Among them, the most interesting ones are a group of genes which can affect the epigenetic landscapes or the readouts of the abnormal epigenetic modifications in leukemia and lymphoma cells. The landmark studies by The Cancer Genome Atlas (TCGA) research group revealed unexpected high frequent mutations of DNA methylation-related genes and chromatin-modifying genes in adult de novo AML [9]. Forty percent AML contained mutations in DNA methylation-related genes, including DNMT3A, DNMT3B, DNMT1, TET1, TET2, IDH1, and IDH2. And more than 30% AML had mutations on chromatin modifiers, such as MLL fusions, ASXL1, EZH2, and KDM6A. To add up, more than 70% AML, at least in this cohort, had epigenetic-related mutations. Similarly, epigenetic genes are also involved in lymphoid neoplasms. About 50% of early T-cell precursor acute lymphoblastic leukemia (ALL) had mutations on histone-modifying genes, such as EZH2, EED, SUZ12, SETD2, and EP300 [10]. Thus, understanding the functional roles and underlying molecular mechanisms of these epigenetic factors in both normal hematopoiesis and hematopoietic malignancies would have great impact on understanding the pathology of these diseases and exploring novel treatment options.

8.2 Epigenetic Regulations of Normal Hematopoietic Stem Cells

Hematopoietic stem cells can self-renew to maintain the hemostasis of the HSC pool and also differentiate into all blood and immune cell lineages. Dysregulations of HSC have been linked to numerous human diseases, including multiple hematopoietic malignancies. Epigenetic factors play critical roles in the tightly regulated self-renewal and gradual differentiation of HSC.

It has been shown that there are dramatic DNA methylation patterns in HSC, lineage-specific progenitors, and differentiated cells [11]. DNA methylation maintenance is critical in these processes. DNMT1 mutant HSCs have severe defects in their functions, including self-renewal, niche retention, and differentiation [12]. DNA de novo methylation is important for gradually silencing of self-renewal genes during HSC differentiation. DNMT3A deficiency leads to impaired HSC differentiation and enhanced self-renewal [13]. Loss of both DNMT3A and DNMT3B had more dramatic phenotype, possibly through regulating the DNA methylations on multiple self-renewal genes, including b-catenin [14]. Interestingly, erasers of DNA methylation also play significant roles in HSC self-renewal. TET2 loss mice have progressive enlargement of their HSC pool, associated with both enhanced HSC self-renewal and block of differentiation. Differentiation block by TET2 deficiency can also happen at multiple stages besides HSC, including myeloid progenitors [15]. Beyond gene expression regulations, DNA methylation seems to play roles in maintaining the genome integrity. It has been shown that TET2-mutant HSC and progenitors display hypermutagenicity [16].

The functions of HSC and the hematopoietic system decline during aging, which might contribute to the hematopoietic pathophysiology in old people. It seems that the global DNA methylation levels don't significantly change in aged HSC. However, site-specific DNA methylation can vary a lot, especially on genes involving HSC self-renewal, which are largely affected by the proliferation history of HSC over time [17]. Consistent with the association of DNA methylation patterns with HSC aging, importantly, recurrent TET2 and DNMT3A mutations have been observed in normal elder with clonal hematopoiesis, a syndrome associated with abnormal self-renewal of HSC [18–20].

Among histone modifiers, HMTs are the best studied ones in hematopoietic system, given their frequent involvement in hematopoietic malignancies. One example is the polycomb repressive complex 1 and 2 (PRC1 and PRC2). The catalytic components in PRC1 and PRC2 are EZH1 and EZH2, respectively. PRC1 can ubiquitylates H2AK119 in mammalian cells and thus promotes chromatin folding. While PRC2 is responsible for H3K27 methylation, BMI1 is a unique component in PRC1. It has been shown that BMI1 is critical for the self-renewal of HSCs in adult largely through repressing the expression of p16INK4A and p19ARF [21] and balancing the symmetric and asymmetric divisions of HSCs [22]. BMI1 also represses the production of reactive oxidative species to prevent the premature of HSCs and plays roles [23]. Interestingly, EZH1 can repress primitive and EMP-biased hematopoiesis in early embryos [24]. The roles of PRC2 in HSCs are more complicated. EZH2 loss in adult HSCs doesn't significantly affect HSC self-renewal or hematopoiesis, except lymphoid differentiation. However, deletion of EZH2 in embryonic HSCs dramatically reduces their self-renewal and functions, associated with H3K27 methylation reduction [25]. However, knockout of Eed, a component of PRC2, has minimal effect on fetal liver HSCs but depletes both neonatal and adult HSCs [26]. MLL genes are H3K4 mono- and di-methyltransferases. MLL1, the founding member of

this gene family, is frequently involved in chromosome translocations in both lymphoid and myeloid leukemia. MLL1 is essential for the hemostasis of adult HSCs. MLL1 knockout drives quiescent HSCs into cell cycle, and thus these mutant HSCs lose their long-term self-renewal and repopulation capacity. Interestingly, MLL1-deficient progenitor cells show reduced proliferation [27].

Histone methylations in HSCs are maintained by the balance between histone methyltransferases and demethylases. Dysregulations of histone demethylases may also lead to HSC defects. One isoform of LSD1, a H3K9 and H3K4 demethylase, can maintain HSC quiescence [28]. It also controls the differentiation of erythroid progenitors by regulating the expressions of GATA2 and HOX genes. Interestingly, LSD1 can also interact with MLL1 complex. JARID1B, a Jumonji C domain-containing histone H3K4 demethylase, is not required for steady-state hematopoiesis but essential for long-term HSC self-renewal and maintenance [29]. More significantly, KDM6A and KDM6B, which show similar biochemical activity to remove methyl groups from H3K27, reverse to EZH2 activity, have contrasting functions in hematopoietic system. KDM6B knockout mice are largely normal with minimal phenotype, but it is essential for long-term repopulating ability of HSCs revealed by competitive transplant assay. However, KDM6A is critical for regulating the self-renewal of HSCs, and loss of KDM6A can promote the transformation into both myeloid and lymphoid malignancies through both demethylation activity-dependent and demethylation activity-independent mechanisms [30]. Interestingly, KDM6A is also a component of the MLL3/4-containing COMPASS-like complex. Thus, fine-tuning the histone modifications is essential for normal HSC self-renewal and functions.

8.3 DNA Modifications in Leukemia and LSCs

Abnormal DNA methylation pattern is a common feature of blood malignancies. Profiling the genome-wide methylation of 344 AML sample showed that these patients could be divided into separate subgroups with distinct DNA methylation patterns, which were associated with different driver mutations [31]. Methylations at promoter CpG islands and CpG shores and enhancers had numerous changes during leukemogenesis, though the functional significance of individual sites remains largely unknown. Strikingly, genomic profiling revealed unexpected high frequent mutations in the DNA methylation factors, such as DNMT3A, TET2, and IDH1/2 [9]. Accumulating data suggest that the DNA modifiers play significant roles in leukemia pathology and also opportunities for novel therapy options.

8.3.1 DNMT3A in AML

DNMT3A alters gene expression by de novo methylation of 5′-position of cytosine at CpG dinucleotides, which are among the most frequently mutated epigenetic regulators in hematological malignancies [32]. It was shown that approximately 22% of cytogenetically normal AML cases carry DNMT3A mutations, and the hotspot mutation lies on arginine 882 in the DNMT3A catalytic domain, with around 60% mut cases affecting this codon [33]. This somatic missense mutation is usually associated with poor prognosis in AML patients and, particularly when accompanied by FLT3-ITD positivity, is an extremely poor prognostic factor even after allogeneic hematopoietic stem cell transplantation [34].

Relative studies have identified DNMT3A as a tumor suppressor gene in many hematological malignancies. In mice transplanted with Dnmt3a-KO HSCs, both lymphoid and myeloid malignancies emerged, and the latency was 3–14 months [35, 36]. Mice transplanted with Dnmt3a-R882H represented a disease phenotype mimicking chronic myelomonocytic leukemia (CMML) in humans 1 year after bone marrow transplantation [37]. Loss of Dnmt3a synergizes with the Flt3 internal tandem duplication (ITD) in mice to initiate both AML and T-ALL, with Dnmt3a dosage influencing leukemia type [38]. In another genetic background, Dnmt3a mutation cooperates with NRAS mutation to transform hematopoietic stem/progenitor cells and induce acute leukemia development [39].

DNMT3A functions primarily at the stem cell level. Dnmt3a is highly expressed in mice HSC, where its loss promotes HSC self-renewal and inhibits differentiation [40], the mechanism of which was proposed as the interruption of downstream regulators such as RUNX1 [41]. HSC acquires a DNMT3A mutation prime a preleukemic clone to promote the impact of secondary hits, such as FLT3-ITD, to initiate myeloid or lymphoid leukemia. The underlying mechanism of DNMT3A in leukemogenesis is intricate. Despite the global 5mC level in HSC is unaffected, DNMT3A mutation induces focal epigenetic alteration. CpG hypomethylations are significantly enriched at gene regulatory sites, notably, putative enhancers marked by H3K4me1 as well as binding sites of master hematopoietic transcription factors [39]. DNMT3A R882H directly binds to and potentiates transactivation of stemness genes for leukemogenicity, including a Meis1-Mn1-Hoxa regulatory node, which encodes a set of transcription factors and cofactors crucial for sustaining self-renewal of LSCs [39, 42, 43]. Demethylation increases the representation of transcription factors, such as FLI1, GFI1B, and PU.1, which determine cell fate during stem, myeloid, and lymphoid cell differentiation [38]. In DNMT3A-mutated CMML, disturbed DNA methylation – either hypomethylation or hypermethylation – was detected. Most hematopoiesis-related genes, such as Hoxa3 and Hoxa6, were hypomethylated in the gene body, while genes that regulate lymphocyte development, such as Notch1 and Notch3, have shown more hypermethylated status in gene body regions. Thus, the relative balance of transcriptional controls between proliferation and differentiation of hematopoietic cells may be broken [37]. In addition, DNA methylation-independent manner of mutated DNMT3A is explored as

well. Aberrant increase of CDK1 in the presence of the DNMT3A mutant can promote cell-cycle activity [37]. Junji Koya revealed aberrant recruitment of polycomb repressive complex 1 (PRC1) to specific differentiation-associated gene loci, such as PU.1 and Cebpa, resulting in the differentiation block of LSCs [44].

8.3.2 TET2 (Ten-Eleven Translocation 2)

While DNA methylation is carried out by DNMT enzymes, the removal of methyl group and conversion to unmodified state are catalyzed by TET gene family. TET2 is the only mutated gene among TET family members reported in a variety of human hematological malignancies such as leukemia, myelodysplastic syndrome (MDS), and malignant lymphoma, with nearly 8–23% of adult AML patients carrying such mutation.

TET2 mutation alone is not sufficient for disease formation but rather leads to a generation of premalignant clones, setting a critical basis for malignant transformation. Additional mutations are required for developing full-blown diseases. Most Tet2-mutant mice develop CMML-like myeloid disease or T-cell lymphoma after a long latency, usually more than several months [45, 46]. The combination of Tet2 disruption and AML1-ETO expression can induce AML [47].

TET2 is critical for the function of hematopoietic stem cells. Disruption of TET2 results in the expansion of multipotent as well as myeloid progenitors, leading to the accumulation of pre-LSCs [48]. However, the detailed mechanism of how TET2 mutations propagate preleukemic and leukemic states remains poorly understood. Study showed that the catalytic activity of TET2 was essential and critical for its myeloid malignancy-suppressive function in HSC/HPCs [49]. Thus, DNA demethylation processes mediated by the disruption of TET2 suggest a causative role of TET2 in disease initiation. Hypermethylation at non-CpG islands is reported in TET2 mutation in multiple studies [50, 51]. TET2 disruption predominantly affects the epigenetic state of enhancers. Hypermethylation at enhancers can be directly linked to deregulation of specific genes that in turn results in oncogenic transformation, including downregulated putative tumor suppressor genes such as Mtss1, Las2, Lxn, Ctdspl, and Grap2 and upregulated putative oncogenes such as Aff3, Pim2, Nepn, Notch3, and Igf1r [47]. Disruption of TET2 activity confers enhanced self-renewal in HSC resulting in the expansion advantage in the mutated clone to form pre-LSCs population. Moreover, sustained Tet2 deficiency is required to maintain self-renewal of pre-LSCs, since its effects are reversed upon Tet2 restoration [52].

8.3.3 IDH1/2

Isocitrate dehydrogenases (IDHs) are metabolic enzymes that catalyze the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate, a critical step in the citric acid cycle. However, because alpha-ketoglutarate is the critical cofactor of multiple epigenetic regulatory factors, including TET and Jumonji C domain-containing histone demethylases, IDHs can also affect the epigenetic regulations beyond metabolism. Surprisingly, IDH1 and IDH2 have been found to be mutated in about 20% AML and many other human cancers [9]. IDH mutations are associated with intermediated prognosis and more frequent in normal karyotype AML. Majority of these mutations are on IDH1 R132, IDH2 R140, and R172. These mutations not only disrupt the normal catalytic function of IDHs to generate alpha-ketoglutarate from isocitrate but consume alpha-ketoglutarate to produce a new metabolite 2-hydroxyglutarate [53, 54]. It turns out that this new metabolite greatly impairs the functions of the epigenetic factors TET and histone demethylases which require alpha-ketoglutarate as cofactor. 2-Hydroxyglutarate leads to DNA hypermethylation, abnormal histone methylations, and, subsequently, aberrant gene expressions and HSC dysregulated self-renewal and differentiation block [55, 56]. Thus 2-hydroxyglutarate is an onco-metabolite, and IDH mutations provide a direct unique link between cancer metabolism and epigenetics.

IDH1 R132H knockin mice have increased numbers of HSC and progenitor cells, suggesting this mutation impairs the differentiation of HSC and early progenitor cells. Probably due to the defects of these mutant HSCs and progenitors, these mice also have anemia and extramedullary hematopoiesis [57]. However, IDH mutations themselves can't lead to full-blown AML. IDH mutations frequently occur with NPM1, DNMT3A, and FLT3 mutations. Our work, together with others' work, has shown that IDH2 R140 or R172 mutant, cooperating with FLT3ITD or NRASG12D, can promote leukemogenesis by blocking the differentiation of HSC and progenitors [58]. IDH1 R132 or IDH2 R140 mutations can also cooperate with JAK2 mutations to promote myeloproliferative neoplasm progression [59]. Further, we showed that pharmacologic or genetic inhibition of IDH2 triggers the differentiation and death of AML cells, albeit only with prolonged IDH2 inhibition. Inhibition of the bromodomain-containing protein Brd4 triggers rapid differentiation and death of IDH2 mutant AML. Combined inhibition of JAK2 and IDH2 in JAK2 and IDH2 double mutated NPM can normalize the hematopoiesis and impede MPN progression. Thus, IDH mutations are putative AML drivers and also therapeutic target in diseases with these mutations. Indeed, the first IDH mutant inhibitor has been approved for AML recently.

8.4 Histone Modifications

Despite the great heterogeneity among leukemia, abnormal histone modifications and mutations in histone modifiers are common in these diseases. Recently cancer genomics studies show that more than 30% AML had mutations on chromatin modifiers, such as MLL fusions, ASXL1, EZH2, and KDM6A. MLL fusions are among the most studied histone modifiers in AML [9].

8.4.1 MLL Fusions

MLL1, located at chromosome 11q23, is among the most frequently rearranged genes in both pediatric and adult leukemias, associated with intermediate prognosis. MLL fusions drive leukemia by regulating HOX gene expressions through histone modifications and also direct promoter binding as transcriptional factor. Hox genes, such as HOX B4 and A9, are important regulators of hematopoiesis and act, in part, by promoting stem cell renewal. MEIS1, the common transcriptional factor for HOX genes, is also a direct target of MLL-fusion proteins and involved in AML pathology. The serine-threonine-rich sequence of MLL in the fusion proteins is required for HSC transformation by DNA binding. Most MLL-fusion proteins have gain of functions that the truncated MLL as a transcriptional activator directed by its partners. MLL fusions can have numerous partners with distinct functions. ENL and AF9 can recruit SWI-SNF chromatin-remodeling complexes. And MLL-fusion transcriptional activity to regulate the expressions of HOX genes is SWI-SNF dependent [60].

DOT1L, a histone H3K79 methyltransferase, forms a complex with multiple MLL fusions and is required for the aberrant transcriptional activity of these MLL-fusion proteins. Inhibition of DOT1L can reduce the expressions of HOX genes and further inhibit the transformation capacity of MLL fusions [61, 62]. Thus, DOT1L can be a potential therapeutic target in AML with MLL fusions.

Interestingly, 10% of adult AML with normal cytogenetics have internal tandem duplications of MLL. This duplication can alter the DNA binding capacity of MLL, which is required for its transformation activity. However, MLL PTD doesn't lead to upregulate HOX genes, and the functional targets of MLL PTD are still largely unknown [63]. MLL copy number variations are also observed in some MDS and AML. AML with MLL amplifications shares some dysregulated gene expression signature, including HOX genes, with those with MLL fusions, suggesting similar molecular mechanisms of transformation [64]. But how wild-type MLL in MLL-amplified AML is recruited to its targets is still unclear.

8.4.2 EZH2 (Enhancer of Zeste Homolog 2)

EZH2, the catalytic subunit of polycomb repressive complex (PRC2), trimethylates H3K27 to promote transcriptional repression of target genes, which plays a major role in normal hematopoiesis. Aberrant expression of EZH2 differentially contributes to the tumor initiation in different types of leukemia. EZH2 acts as a double facet factor either as a tumor suppressor or oncogene in acute and chronic myeloid and/or lymphoid leukemias. Overexpressing of EZH2 in many different malignancies and mutations that elevate its trimethyltransferase activity is found in subtypes of lymphoma, suggesting a pro-tumorigenic role. However, EZH2 loss-of-function mutations have also been observed in MDS, suggesting a tumor suppressor function in certain cellular contexts.

Comparing the gene expression profiles of human CML LSC to normal HSC, EZH2 is found to be upregulated in LSC at all three phases of disease. It was reported that the survival of CML LSC relies on the expression of EZH2, and more specifically its enzymatic activity, since inactivation of EZH2 affects the survival of LSC and prevents disease from initiation and maintenance. Expression of the EZH2 homolog EZH1 is reduced in EZH2-deficient CML LSC, creating a context resembling complete loss of PRC2 [65]. The mechanism by which EZH2 regulates stemness of LSC is proposed as EZH2 binds to and trimethylates H3K27 at the promoter of PTEN, thus repressing its gene transcription and, meanwhile, activating its major downstream AKT/mTOR signaling [66].

Leukemia generated from fetal liver (FL) and adult bone marrow (BM) differed dramatically in their LSC activity with FL leukemia characterized by relatively poorer transplantability and more rapid cycling. EZH2 plays a critical role in this phenomenon. FL leukemia possesses robust NOTCH1-driven autocrine IGF1 signaling, which drives cells into cycling and thereby depletes the LSC compartment. However, the IGF1 pathway is restricted in BM leukemia by EZH2-dependent H3K27 trimethylation. Therefore, EZH2-mediated epigenetic downregulation of IGF1 signaling promotes LSC activity [67].

Other mechanisms concerning the requirement of EZH2 in LSC are proposed as well. The following listed pathways involving in the LSC initiation and maintenance are all regulated by the histone-modifying function of EZH2. Koki Ueda et al. and J Shi et al. found that inhibition of EZH2 depletes LSC of MLL-fusion leukemia through upregulation of Cdkn2a and its product p16 [68, 69]. The oncogenic function of Ezh2 in AML indicated that besides the aforementioned pathway, Ezh2 plays a critical role in the maintenance of LSC by reinforcing their differentiation blockage through the regulation of its direct targets including EGR1, which is a positive regulator of differentiation of myeloid cells and a tumor suppressor that transactivates tumor suppressor genes, including TGFβ1, PTEN, and p53 [70].

On the other hand, the tumor suppressor role of EZH2 is analyzed as well. A study performed in a murine model demonstrated that PRC2, along with MLL-AF9, is responsible for blockade of myeloid differentiation in AML [68]. In the model of ETP-ALL generated by deleting EZH2 in p53-null hematopoietic cells, EZH2 loss

propagates hypermethylation at T-cell differentiation-regulating genes to promote leukemic transformation [71].

8.5 COMPASS Family (Complex of Proteins Associated with Set1)

In human, COMPASS family consists of six macromolecular complexes which are capable of methylating H3K4. The core components – Set1a/b, MLL1/MLL2, and MLL3/MLL4 – acting as scaffolds of their respective complexes, are critical for the enzymatic activity. MLL1/MLL2 are necessary for gene-specific H3K4 trimethylation, while MLL3/MLL4 have been accredited as key H3K4 monomethyltransferases at enhancers. MLL3/MLL4 associates with WRAD (WDR5, RbBP5, ASH2L, and DPY30), NCOA6, PTIP, PA1, and UTX in its own separate protein complex. MLL1/MLL2/MLL3/MLL4 together with UTX have been documented as the most frequently mutated histone modifiers in human cancers [72].

A model of AML induced by the MLL-AF10 oncogene was established by Wong et al. to interrogate the LSC epigenome. They reported that c-kit+ cells enriched for LSCs were maintained in a global epigenetic state characterized by relative H3K4 hypermethylation and H3K79 hypomethylation. H3K4 hypermethylation is the major epigenetic alteration that sustains the LSC maintenance transcriptional program and the expression of Hoxa and Meis1 genes, which are direct targets of MLL oncoproteins required for AML pathogenesis. Conversely, global erasure of H3K4me3 is associated with LSC terminal differentiation [73].

MLL3 (also known as KMT2C) is validated as a haploinsufficient 7q tumor suppressor in AML in our study. MLL3 is required for efficient differentiation of HSPC from long-term hematopoietic stem cells (LT-HSC) to short-term HSC (ST-HSC), multipotent progenitors (MPP), and more committed myeloid progenitors. Genes differentially expressed in shMll3;p53 −/− HSPC suggest that Mll3 suppression affects a LSC signature that is linked to Myc activity. Mll3 suppression produces a repressive chromatin context on genes linked to differentiation [74].

8.6 Epigenetic Therapy

AML is the most prevalent acute leukemia in adults. With the exception of a few specific AML subtypes, the mainstay of treatment for the majority of AML subtypes has not significantly changed over the last 20 years and continues to be based on standard cytotoxic chemotherapy. As a result, clinical outcome remains poor for the majority of patients, with overall long-term survival in the region of 20–30%, posing the demand for novel therapies [75].

Leukemia stem cells initiate hematopoietic malignancies, and epigenetic alterations are early events during LSC formation. LSCs are resistant to conventional chemotherapy, and persistent LSCs, which are insensitive to upfront regimens, are the root of resistance and relapse. Patients harboring epigenetic mutations are usually associated with poor prognosis. Therefore, therapies aimed at eradicating LSCs are supposed to be imperative for curing leukemia. Nowadays, significant progress has been made in the exploring of LSCs-targeted therapies. Monoclonal antibodies targeting LSC surface markers, agents blocking the interactions between LSCs and their microenvironment, and agents targeting signaling pathways involved in the regulation of survival and self-renewal of LSCs have been either approved or investigated in preclinical and clinical trials [76]. Typically, epigenetic genes have been considered as potentially attractive targets for new therapies, due to the reversible nature of epigenetic signatures.

Two DNMT inhibitors, azacitidine (5-azacytidine) and decitabine (5-aza-2′-deoxycytidine), have already been approved by the Food and Drug Administration for the treatment of patients with MDS and are also being routinely used for AML based on results from several clinical trials. AML patients with DNMT3A mutation are found to be associated with increased response to azanucleoside DNMT inhibitors. One study reported that 75% of patients with DNMT3A mutations achieve complete response (CR), while the CR rate in patients with wild-type DNMT3A was 34% [77]. The mechanisms underlying the inhibition of DNA methylation by Aza and Aza-dC are mainly focused on their apoptotic- and differentiation-inducing effects on LSC [78]. For instance, 5-AZA-dC upregulates the expression of DIF2 through the demethylation of its promoter and thus facilitates TNFα-induced transcriptional pathways leading to differentiation [79]. Similarly, AZA treatment significantly demethylates upstream regulatory element within PU.1 promoter, leading to upregulation of PU.1, followed by derepression of its transcriptional targets and onset of myeloid differentiation [80]. Another study showed that Aza-dC induces expression of OLFM4, of which an overexpression leads to apoptosis and differentiation in HL60 cell line [81].

Apart from the DNA methylome, histone methylome is another major epigenetic modification as the target in epigenetic drugs (Fig. 8.1). HDAC inhibitors were among the first epigenetic drugs developed, and several have been tested in clinical trials for malignancies including AML, such as valproic acid (VPA), panobinostat, and vorinostat. HDAC inhibitors alone have shown limited clinical activity in MDS and AML. Clinical trials evaluating the combination of HDACi with DNMTi are still ongoing. EZH2 was highly overexpressed in primary human CML CD34 + cells. Study showed that targeting EZH2 by either small molecule GSK126 or specific shRNA displayed a remarkable inhibitory effect on LSCs and drastically prolonged the survival of CML mice. EZH2 knockdown resulted in elevation of PTEN and led to impaired recruitment of EZH2 and H3K27me3 on the promoter of PTEN gene [66]. Another study found that EZH2 inhibition not only suppressed MLL-fusion leukemia proliferation but also reduced LSC frequency through upregulation of p16 [69]. Tyrosine kinase inhibitor (TKI) combined with EZH2 inhibitor significantly upregulates H3K27me3 level compared to either TKI or EZH2i alone, resulting in

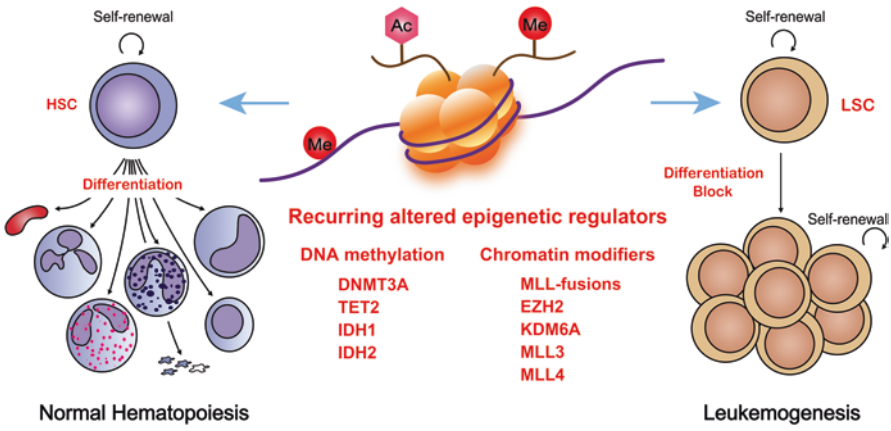

Fig. 8.1 Epigenetic regulation of LSCs. LSCs undergo epigenetic changes during leukemogenesis, providing new therapeutic strategies for leukemias

further loss of LSCs. Combined effects provide an approach to eradicate TKI-persistent LSCs in refractory patients with CML [82].

8.7 Perspective

Since LSCs were firstly found in AML in 1997, extensive studies have been contributed to identification and characterization of such cell populations. During the course of malignant transformation, both genetic and epigenetic abnormalities play an essential role in the pathogenesis. However, despite the genetic programs that maintain LSCs self-renewal have been greatly explored, the comprehensive epigenetic landscape that sustains LSCs' cellular identity and functionality is not clear yet. Epigenetic-targeting therapies raise the possibility of eliminating LSC, but these are still early day for epigenetic therapies.

On the one hand, further studies are needed to shed light on the role of epigenetic modifications in leukemia and leukemia stem cells. Preclinical and basic research are required in order to define exact consequences of epigenetic regulator mutations that have been discovered in AML and their molecular mechanism in leukemia. On the other hand, efficient LSC-based treatments of leukemia remain to be explored which can eventually benefit leukemia patients. The next decade will see unprecedented activity in preclinical and clinical investigation of epigenome-based therapies. More efforts have to be made and the prospects are very exciting.

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Chapter 9
Leukemia Stem Cells in Chronic Myeloid Leukemia

Yi Shan, Ngoc DeSouza, Qiang Qiu, and Shaoguang Li

Contents

Abstract Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by a chromosome translocation that generates the BCR-ABL oncogene encoding a constitutively activated tyrosine kinase. Although BCR-ABL tyrosine kinase inhibitors (TKIs) are highly effective in treating CML at chronic phase, a number of patients develop drug resistance due to the inability of TKIs to kill leukemia stem cells (LSCs). Similar to other types of hematopoietic malignancies, LSCs in CML are believed to be a rare cell population responsible for leukemia initiation, disease progression, and drug resistance. Therefore, a full understanding of the biology of LSCs will help to develop novel therapeutic strategies for effective treatment of CML to possibly reach a cure. In recent years, a significant progress has been made in studying the biology of LSCs in both animal models and human patients at cellular and molecular levels, providing a basis for designing and testing potential molecular targets for eradicating LSCs in CML.

Keywords CML · Leukemia stem cell · TKIs · Therapeutic targets

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9.1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm, characterized by clonal expansion and accumulation of differentiated myeloid cells in the bone marrow and blood [82]. CML usually begins in a chronic phase with leukemia cells in the bone marrow and then, over time, spreads to the blood. If the disease is not controlled, leukemia cells will eventually spread to other areas of the body. Initially, CML cells spreads and grows slowly but can change from a slowly progressing into a rapidly growing form of acute leukemia that can spread and invade almost all major organs in the body. Specifically, CML is a triphasic disease, initiating from a chronic phase (CP) and then progressing to an intermediate accelerated phase (AP) and a terminal blastic phase (BP) (Fig. 9.1a). In CP, the myeloid cell compartment expands, and the cells still retain the capacity to differentiate. In general, CML patients in CP have mild symptoms with many patients remaining asymptomatic. In AP, patients begin to show more immature cells in the blood with frequent constitutional symptoms and a reduced sensitivity to therapy. In BP, immature cells become dominant and survival is poor [83]. The American Cancer Society estimates about 8990 new cases will be diagnosed with CML, and about 1140 people will die of CML in the United States in 2019. Overall, about 15% of all new cases of leukemia are CML, and about 1 person in 526 will get CML in their lifetime in the United States.

A hallmark of CML is the existence of "Philadelphia chromosome" resulting from the reciprocal translocation of the *c-ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (Fig. 9.1b). The molecular basis of Philadelphia chromosome is the formation of BCR-ABL, an oncogenic fusion gene encoding a constitutively activated ABL tyrosine kinase [71, 81]. In general, tyrosine kinases are important mediators of signaling cascades in cells and play key roles in diverse biological processes including growth, differentiation, and apoptosis of cells in response to internal and external stimuli. Deregulation of protein kinase activity has been shown to play a central role in the pathogenesis of human cancers [46]. In normal cells, the activity of c-ABL tyrosine kinase is typically regulated in an autoinhibitory fashion in native c-ABL due to the binding of its myristoylated N-terminal "cap" (N-cap) to a deep hydrophobic pocket in the C-terminal lobe of the kinase domain. After the formation of the chimeric BCR-ABL fusion protein occurs, the myristoylated N-cap of c-ABL is replaced with a truncated portion of the BCR protein rendering the C-terminal kinase domain active as a consequence of the conformational change [62]. BCR-ABL is the driving force of leukemogenesis in CML and is responsible for initiating and maintaining leukemia phenotype of CML as well as the phosphorylation, activation, and dysregulation of intracellular signaling pathways that regulate the survival and growth of leukemia cells [93]. CML is considered as a paradigm of a disease to be caused and marked by a single acquired chromosomal translocation.

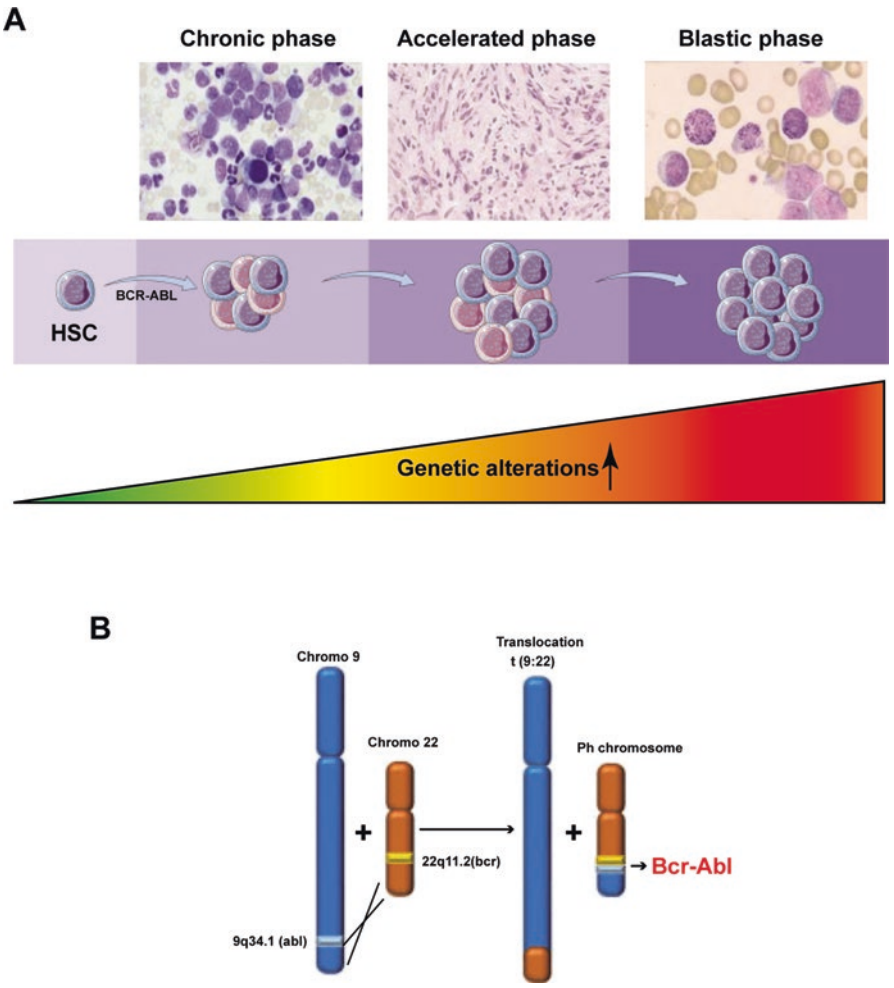

Fig. 9.1 Clinical features of chronic myeloid leukemia. (**a**) Progression of CML from chronic phase (CP) to blastic phase (BP). Biologically, the transition is associated with the accumulation of additional hits in Bcr-Abl itself or in other genes/chromosomes. (**b**) Philadelphia chromosome. A piece of chromosome 9 and a piece of chromosome 22 break off and trade places. The BCR-ABL gene is formed on chromosome 22 where the piece of chromosome 9 attaches. The changed chromosome 22 is called the Philadelphia chromosome

9.2 BCR-ABL Inhibitors: The First-Line Therapy in CML

The standard of care for CML is the BCR-ABL tyrosine kinase inhibitors (TKIs) that are widely used as the first-line agents for CML treatment, because TKIs frequently induce complete hematologic response and dramatically improve patient outcome in chronic phase CML. The advances in biology and therapy of CML have

set a gigantic milestone in the history of anticancer precision medicine and targeted therapy. BCR-ABL is one of the first tyrosine kinases to be implicated in a human malignancy and to be successfully targeted. In the late 1990s, the first-generation tyrosine kinase inhibitor, imatinib (IM; also known as "Gleevec" or "Glivec"), was discovered by biochemist Nicholas Lyndon through random screening of a library of thousands of compounds and then was developed into a clinically usable drug by oncologist Brian Druker to treat CML through targeting BCR-ABL kinase activity. The mechanism of action of IM is based on the fact that the active sites of ABL tyrosine kinase each have a binding site for ATP and the enzymatic activity catalyzed by the kinase triggers the transfer of the terminal phosphate from ATP to tyrosine residues on the ABL kinase, leading to tyrosine phosphorylation that keeps the ABL kinase active in signaling and cellular transformation. IM works by binding closely to the ATP binding site, locking the ABL kinase in a closed or self-inhibited conformation, resulting in inhibition of the enzymatic activity of the kinase [24] (Fig. 9.2a). The ultimate result of IM binding to the ABL tyrosine kinase is "switching-off" the downstream signaling pathways that promote leukemogenesis.

The first IM clinical trial took place in 1998 and the drug received FDA approval in 2001. The advent of IM as an effective BCR-ABL kinase inhibitor rapidly and dramatically modified the treatment of CML patients and also led to important changes in the disease management [28]. The initial landmark studies showed high response rates to IM in patients with advanced CML [26] and in a randomized trial involving 1106 CP-CML patients [72]. IM induced complete hematological

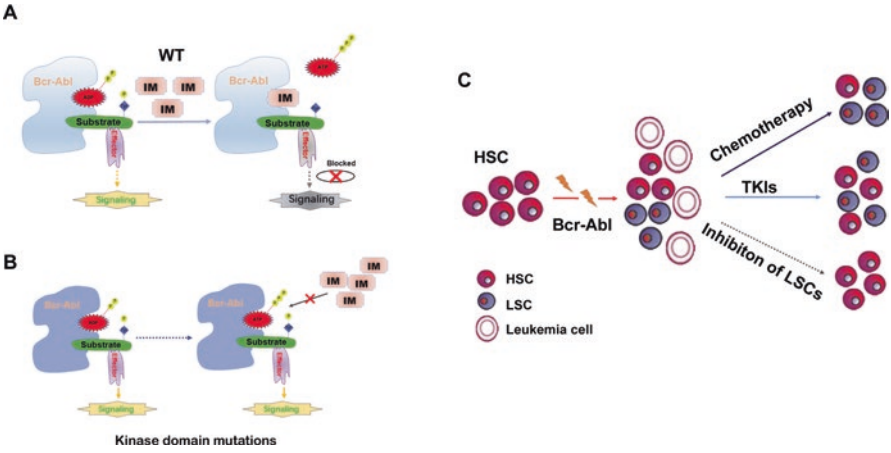

Fig. 9.2 Therapeutic strategies for the treatment of CML. (**a**) Mode of action of the Bcr-Abl kinase inhibitors imatinib (IM). Imatinib blocks the ATP binding to Bcr-Abl, thus inhibiting its phosphorylation and kinase activity. (**b**) When Bcr-Abl kinase domain acquires mutations, imatinib can no longer bind to the kinase domain efficiently to inhibit Bcr-Abl kinase activity, resulting in imatinib resistance. (**c**) Effects of different therapeutic strategies on CML. Chemotherapy and TKIs are effective in killing proliferating Bcr-Abl-positive leukemia cells but fail to eradicate LSCs. Therefore, inhibition of LSCs in CML therapy is the key to curing the disease

response (CHR) in 95.3% of patients and complete cytogenetic response (CCR) in 73.8% of patients. In addition, patients on IM had an improved quality of life [36]. At 6-year follow-up of IRIS trial, IM induced CHR in 98% of patients in chronic phase and CCR in 87% of patients. As a result, IM was described as a "magical bullet" due to its role in converting a fatal cancer into a manageable disease. It is appropriate to say that the use of IM in CML patients revolutionized the treatment of the disease as a consequence of concept breakthrough in targeted therapy of cancer.

Despite a major clinical advance in the treatment of CML through the specific targeting of BCR-ABL kinase activity using TKIs, not all patients experience an optimal response to IM due to a conventional challenging problem associated with the development of drug resistance. In fact, the existence of patients resistant to IM was evident soon after the introduction of the drug into clinical practice. On the other hand, the initial TKI responses were unsatisfactory in patients with advanced-phase (AP) disease, and responses tended to be transient in most responders in AP [84, 92]. Primary resistance is defined as an inability to achieve CHR at 3 months and complete molecular response (CMR) at 6 months. Primary resistance may be caused by differential drug metabolism and/or drug transport, while acquired resistance is defined as progression to advanced disease or loss of response with a five- to tenfold increase in BCR-ABL transcripts. Acquired resistance may be caused by mutations in the BCR-ABL kinase domain, amplification of the BCR-ABL fusion gene, overexpression of drug transporter genes, and overexpression of other tyrosine kinases such as the SRC family kinases [8, 50] (Fig. 9.2b). Consequently, second- and third-generation TKIs, namely, dasatinib, nilotinib, bosutinib, and ponatinib, were rationally designed and approved for clinic use to override the problem of drug resistance. These TKIs are more potent but have been associated with more serious side effects and complications [5]. Patients achieving stable optimal responses to TKI therapy are predicted to have the same life expectancy of the general population. However, it has come to a common recognition that TKIs do not "cure" CML and most CML patients will have to face the perspective of life-long TKI treatment. It is also well established that leukemia stem cells (LSCs) in CML are intrinsically insensitive to TKIs, mainly because their survival does not completely depend on BCR-ABL kinase activity [17] (Fig. 9.2c). LSCs can survive TKI therapy and represent a dangerous reservoir from which TKI resistance and disease relapse may originate. In addition, LSC persistence is thought to be one of the major reasons why treatment-free remission may not be achieved in approximately half of the CML cases [5, 17]. In an attempt to eradicate LSCs, some key molecules/pathways in LSCs have been identified, but the development of new compounds against these molecules/pathways is still ongoing, although we are closer to testing candidate anti-LSC compounds in CML patients.

9.3 LSCs: The Root of CML

The majority of CML patients in CP are highly responsive to TKI therapy that targets BCR-ABL kinase activity. However, a significant number of CML patients treated with TKIs eventually develop drug resistance and relapse. It has been well accepted that LSCs are the root of CML and TKI treatment unlikely leads to a cure of CML due to the ineffectiveness of TKIs in eradicating LSCs. It is necessary to develop new therapies aiming to target LSCs. Typically, stem cells can self-renew to maintain themselves and can also differentiate and proliferate to produce various types of specialized cells [66]. In hematopoietic system, hematopoietic stem cells (HSCs) differentiate and produce multiple lineages of progenitors and mature blood cells [104]. In some cancers, a small population of cells called "cancer stem cells" (CSCs) in bulk of tumor cells is believed to be responsible for maintaining the uncontrolled cell growth. CML is a classic example of a stem cell cancer and arises when the t9;22 translocation occurs in HSCs that function as CSCs or LSCs in CML. This event results in the constitutive expression of the fusion tyrosine kinase BCR-ABL [27, 70, 81], transforming the HSCs into the LSCs, which then gives rise to a clonal myeloproliferative disease (Fig. 9.3). Early evidence regarding the HSC origins of CML came from observations that transfusion of peripheral blood cells from CML patients into severely neutropenic recipients resulted in temporary homologous bone marrow engraftment and Ph1 progeny in the blood. This was later explained by the presence of high numbers of mobilized LSCs in the peripheral blood of chronic phase CML patients. Until very recently, BCR-ABL expression was considered sufficient to cause a CML-like disease in mouse models using retrovirus transduction or transgene insertional mutagenesis to express the oncogene in LSCs [42].

9.4 General Features of the LSCs in CML

9.4.1 Resistance to the Treatment of TKIs

Although it is well accepted that CML is derived from a LSC, biological features of LSCs are largely unclear. For example, LSCs are in general less sensitive or even resistant to inhibition by TKIs, and the underlying mechanisms are poorly understood. CML is caused by the acquisition of the tyrosine kinase BCR-ABL in HSCs, transforming them into LSCs that are able to self-renew, proliferate, and differentiate to give rise to a myeloproliferative disease [75]. Although TKIs that target the kinase activity of BCR-ABL have transformed CML from a once-fatal disease to a manageable one for the vast majority of patients in chronic phase (CP), about only 10% of them can discontinue TKI treatment and maintain a therapy-free remission. Available evidence shows that CML LSCs are resistant to TKIs and persist during long-term therapy, and as a result, LSCs may promote acquired TKI resistance and

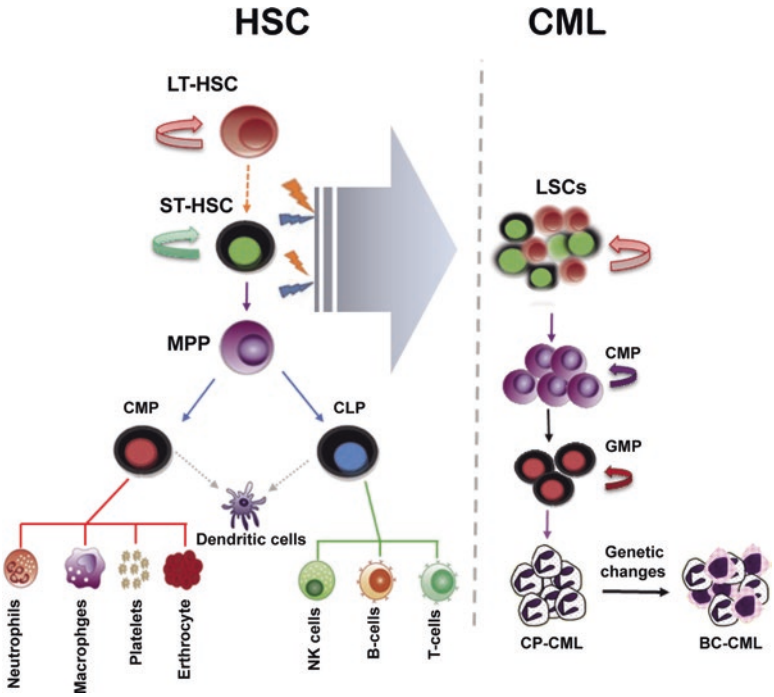

Fig. 9.3 Schematic illustration of the normal and leukemic hematopoietic hierarchies. Hematopoiesis is organized in a hierarchical manner, and rare HSCs give rise to various types of progenitor cells, which proliferate extensively and generate mature blood cells at the bottom of the hierarchy. If HSCs acquire the transforming mutation known as BCR-ABL, the cells gain uncontrolled self-renewal capacity to become LSCs that give rise to CML. Aberrant developmental hierarchy, finally leading high count abnormal white cells in the blood and bone marrow, these cells are determined as LSCs. After the formation of BCR-ABL in CML chronic phase (CP), additional genetic changes can occur, and the disease eventually progresses to fatal CML blastic phase called blastic crisis (BC)

drive relapse or disease progression [5]. The earliest evidence of CML LSCs predated the introduction of TKIs followed by definitive evidence of a deeply, but reversibly, quiescent subpopulation of leukemic cells in patients with CML [41]. In the subsequent years, the consensus view has emerged that virtually all CP patients on TKI therapy and in MMR are not cured of CML and show signs of residual disease burden from the presence of LSCs in the bone marrow (termed "LSC persistence"). In a typical cohort of 100 CP CML patients who undertake TKI therapy over a 5-year period, almost two-thirds will have this "LSC persistence" phenotype. Researchers have consistently detected BCR-ABL primitive cells in the bone marrow of TKI-treated patients in major molecular response (MMR), which are capable of growth in colony-forming cell and stem cell culture assays, even in patients in deep molecular response with no detectable BCR-ABL transcripts by quantitative polymerase chain reaction [17]. The most recent studies have shown that, although

LSCs are not always detectable in cases of deep molecular response, most likely due to technical limitations, some patients with no detectable LSCs can subsequently relapse after TKI discontinuation [16]. Since their discovery in patients almost two decades ago, CML LSCs have become a well-recognized example of CSCs and have been characterized extensively as a significant bottleneck to cure, with the aim of developing new curative therapeutic approaches based on LSC eradication [42].

9.4.2 Quiescence of LSCs

The molecular mechanism of LSC-mediated resistance to current therapies is still largely unknown, but it appears that quiescence of LSCs allows the cells to avoid to be targeted by current therapies [21]. In general, quiescence of CSCs is highly relevant for cancer therapy, because quiescent CSCs are often resistant to both conventional chemotherapy and targeted therapy and contribute to disease relapse following discontinuation of therapies. Therefore, improved understanding of stem cell quiescence helps to develop new strategies for targeting quiescent CSCs [60]. In the case of CML, recent studies have shown that LSCs, particularly those in a quiescent state, are highly resistant to available chemotherapy and targeted therapies, which is closely associated with disease relapse [49, 54]. It is believed that LSC quiescence is controlled by both intrinsic regulatory mechanisms and extrinsic signals from the microenvironment. In addition, signaling molecules involved in cell survival and self-renewal which are the two critical characteristics of quiescent LSCs have been linked to key regulators of cell cycle progression. In CML, BCR-ABL aberrantly phosphorylates and mislocalizes cell cycle proteins which have been implicated in cell cycle deregulation of leukemia cells [21]. Additionally, the interaction of LSCs with bone marrow niche plays a critical role in the long-term maintenance of LSC quiescence. LSCs in the niche are largely dormant and resistant to common chemotherapy. Increasing evidence supports an idea that specific signals from the surrounding stromal cells induce cell cycle arrest of LSCs, allowing them to persist even during TKI treatment. On the other hand, cell-fate decision in HSCs is regulated by several oncogenic transcription factors, including MEIS1 and HOXA9, and is overexpressed in BP of CML [51, 52]. In particular, HOXA9 collaborates with MEIS1 to transform primary bone marrow cells [57], and HOXA9 forms ternary complexes with PBX2 and MEIS1 in myeloid leukemia cells [89]. In addition, *HIF1α*, a master transcriptional regulator of the cellular and systemic hypoxia response, plays a role in cancer progression by activating transcriptional programs for maintaining the ability of self-renewal and multipotency of CSCs in a hypoxic environment [23, 64, 91]. Furthermore, *HIF1α* has been shown to be required for stem cell functions of mouse lymphoma and human acute myeloid leukemia [96]. Some recent studies demonstrate that *HIF1α* is upregulated in BCR-ABL-expressing LSCs and is required for survival maintenance of LSCs in CML. Specifically, deletion of *HIF1α* impairs the cell cycle progression of LSCs with an accumulation of cells in the G0–G1 phases and a concomitant reduction in the S phase of the cell

cycle through the upregulation of cyclin-dependent kinase inhibitors $p16^{Ink4a}$, $p19^{Arf}$, and p57 [100]. LSCs that survive TKI treatment are sensitive to inhibition by a HIF1 inhibitor acriflavine (ACF), and the reduced survival and growth of CML cells by ACF are mediated through downregulation of c-Myc and stemness-related genes and inhibition of cell cycle progression of LSCs [10].

9.4.3 Critical Signaling Pathways Involved in Self-Renewal and Survival of LSCs

Wnt/β-Catenin Pathway Self-renewal is one of key characteristics of LSCs, and recent studies have reported that a set of critical genes control the self-renewal and survival of LSCs through a wide range of signaling pathways [37]. Among these pathways, the Wnt/β-catenin signaling pathway plays a fundamental role in maintaining the HSC population. Wnt genes encode small secreted proteins existing in all animal genomes, and Wnt signaling is involved in virtually every aspect of embryonic development and also controls homeostatic self-renewal of cells in a number of adult tissues. The activation of the Wnt/β-catenin pathway leads to the translocation of β-catenin into the nucleus, where it induces expression of its target genes such as c-Myc, c-Jun, and cyclin D1 [80] (Fig. 9.4a). A number of studies have demonstrated that Wnt/β-catenin signaling pathway acts as a key regulator in controlling proliferation, survival, and differentiation of hematopoietic cells [19]. Aberrant activation of the Wnt/β-catenin signaling pathway has also been found in CML [43, 102]. Further studies have shown that the Wnt/β-catenin signaling pathway is required for efficient self-renewal of LSCs, indicating that targeting of the Wnt/β-catenin signaling is an attractive therapeutic strategy for CML [43]. It has also been shown that Wnt signaling pathway and the polycomb group protein BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) are involved in the expansion of LSCs [59].

Hedgehog Pathway The hedgehog (Hh) pathway is a highly conserved developmental pathway that regulates the proliferation, migration, and differentiation of cells during development [45]. Three distinct ligands, sonic (Shh), Indian (Ihh), and desert (Dhh) hedgehog, exist in humans. Upon ligand binding to the receptor patched (Ptch), inhibition of smoothened (Smo) receptor is relieved. Smo then activates members of the Gli family of zinc-finger transcription factors, which translocate to the nucleus to regulate the transcription of Hh target genes including Gli1, Gli2, Ptch, and regulators of cell proliferation and survival [94] (Fig. 9.4b). The role of Hh signaling in hematopoietic malignancies has been studied in CML animal models. The $Smo^{-/-}$ CML mice showed a significant decrease in the population of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$(LSK) cells, which contains CML LSCs, whereas activated SmoM2 stimulated an increase in LSK cells and acceleration of CML progression [44]. Hh inhibition by cyclopamine, similar to a situation in the $Smo^{-/-}$ cells, caused

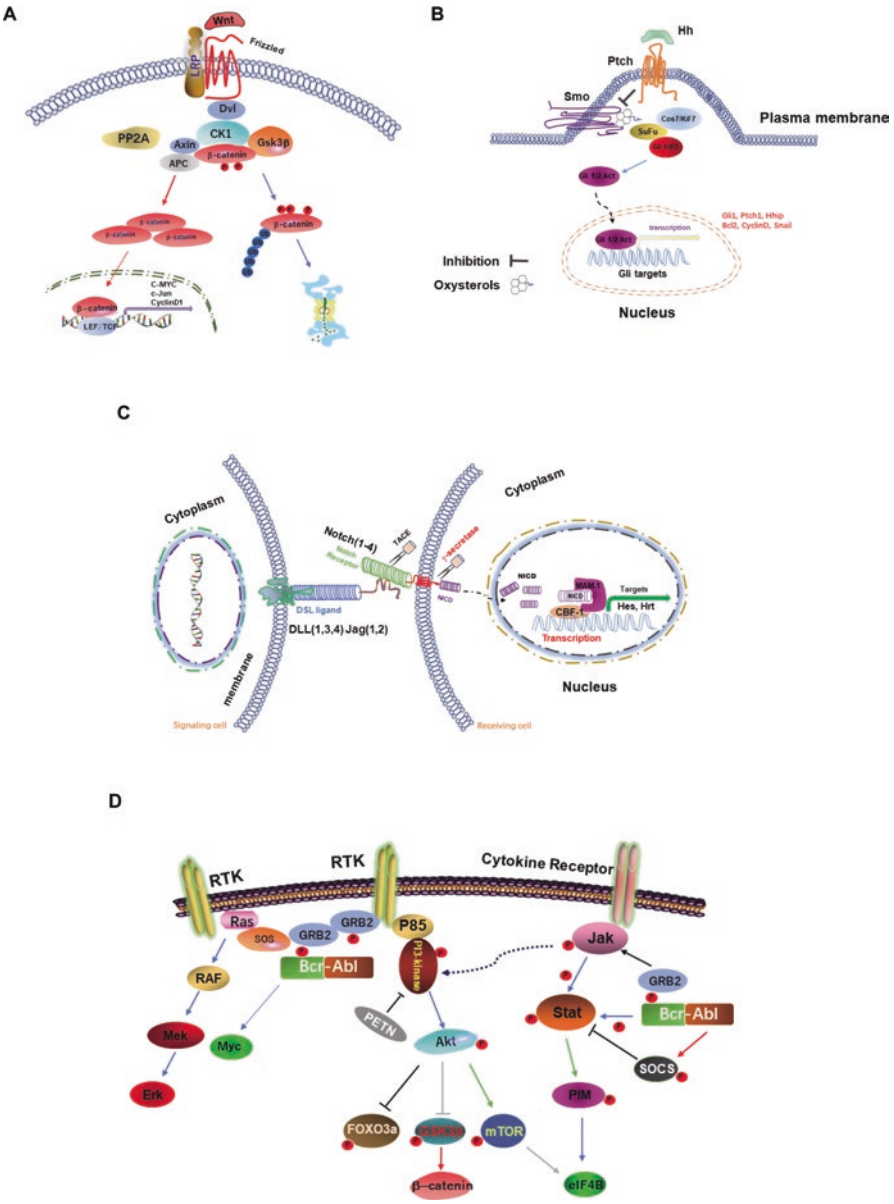

Fig. 9.4 Critical signaling pathways involved in self-renewal and survival of LSCs. (**a**) In the absence of Wnt ligands, APC (adenomatous polyposis coli) and Axin are recruited into the "β-catenin destruction complex," and then Axin is dephosphorylated by the protein phosphatase 2A (PP2A). β-Catenin is phosphorylated by CK1α (casein kinase 1α) and GSK3β (glycogen synthase kinase 3β) and recognized by E3 ubiquitin ligase which subsequently degrades β-catenin via the proteasome. Low cytoplasmic levels of β-catenin ensure activation of TCF/LEF (T-cell factor/lymphoid enhancer factor) transcription factors and transcriptional repression of Wnt target genes. In the accumulation of the extracellular Wnt ligands, the association of Axin with phosphorylated

increased latency of disease and a decrease in the LSK population, even in CML with the imatinib-resistant T315I mutation. In the secondary recipients, no mice receiving $Smo^{-/-}$cells developed disease, even after 9 months of observation [25]. A combination of cyclopamine and the BCR-ABL inhibitor nilotinib had an additive effect in reducing colony-forming units in vitro, and these CML mice took longer to relapse after discontinuation of therapy than those treated with nilotinib alone. A combination of the Smo antagonist NVP-LDE225 and nilotinib reduced replating efficiency of colony formation even further and also improved survival of the treated CML mice after drug discontinuation, similar to the cyclopamine results [47, 98]. These reports support an effect of Hh inhibition on CML stem cells.

Notch Pathway Another pathway that has been shown to have cross talk with other major "stemness pathways" described above in regulation of self-renewal is the Notch signaling pathway. Notch receptors are an evolutionarily conserved family of transmembrane receptors known to be expressed and activated in HSCs [29]. Binding of its physiological ligands of the Delta and Serrata families leads to separation of an intracellular portion of Notch that can enter the nucleus where it binds to the transcriptional factor CBF-1 that interacts with the cofactor MAML-1 (mastermind-like-1) and leads to transcriptional activation of target genes [1] (Fig. 9.4c). There appears to be an interaction between Notch and Wnt signaling-mediated maintenance of HSCs, suggesting a role of Notch in regulation of

Fig. 9.4 (continued) LRP5/6 (lipoprotein receptor-related protein 5/6) and recruitment of phosphorylated DVL (disheveled) to FZD (frizzled) lead to the dissociation of the destruction complex. β-Catenin is stabilized and translocated into the nucleus to form the complex with TCF or LEF and subsequently activates the target genes, including C-MYC, C-Jun, cyclin D1, etc. (**b**) In the absence of Hh, Ptch inhibits the activation of Smo by removing oxysterol from Smo, resulting in Smo inactivation and degradation. In the presence of high level of Hh ligand binding with Ptch, the pump function of Ptch is turned off to allow accumulation of oxysterols around Smo, leading to Smo accumulation at the plasma membrane. In turn, this accumulation of sterols allows SMO to become active or stay on the membrane for a longer period of time. Activated Smo stimulates Hh pathway, ultimately leading to the transcription of Gli target genes, Gli1, Ptch1, Hhip, Bcl2, CyclinD, and Snail. (**c**) There are five Notch ligands in mammals: Delta-like1 (Dll1), Delta-like3 (Dll3), Delta-like4 (Dll4), Jagged1, and Jagged2. All ligands have an extracellular domain called DSL (Delta, Serrate, and Lag-2) that is involved in receptor binding. There are four Notch receptors in mammals: Notch1-4. Binding of the Notch ligand on one cell to the Notch receptor on another cell results in two proteolytic cleavages of the receptor. The ADAM10 or TACE (TNF-α-converting enzyme) metalloprotease catalyzes the S2 cleavage to generate a substrate for S3 cleavage by the γ-secretase complex. This proteolytic processing mediates release of the Notch intracellular domain (NICD), and then NICD translocates into the nucleus and interacts with the transcription factor CBF-1, thereby displacing corepressors (CoR) and recruiting coactivators such as MAM-1. This results in the activation of downstream target genes including Hes and Hrt. (**d**) The cellular effects of BCR-ABL are exerted through interactions with various proteins that transduce the oncogenic signals responsible for the activation or repression of gene transcription. The key pathways implicated so far are those involving RAS, mitogen-activated protein (MAP) kinases, signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K), and MYC. Most of the interactions are mediated through tyrosine phosphorylation and require the binding of BCR-ABL to adapter proteins such as growth factor receptor-bound protein 2 (GRB-2)

self-renewal capacity [29]. Although Notch-associated self-renewal has not been clearly demonstrated in CML, the Notch target Hes1 was found to be highly expressed in CML-blast crisis [67]. Recently, inactivating mutations of Notch signaling have been described in patients with chronic myelomonocytic leukemia (CMML). Notch inhibition in HSCs leads to accumulation of aberrant myeloid progenitors and a CMML-like disease in mice [56]. It is necessary to point out that the role of Notch signaling in myeloid leukemia stem cells has not yet been examined adequately and seems to be context dependent, and its role in self-renewal of LSCs needs future investigations.

JAK/STAT and PI3K/AKT Pathway It is well established that JAK/STAT and PI3K/AKT are two crucial signaling pathways involved in regulating cellular survival and proliferation in LSCs [30, 78] (Fig. 9.4d). In CML, JAK/STAT/PIM and PI3K/AKT/mTOR pathways are constitutively activated by BCR-ABL, resulting in uncontrolled cellular proliferation [63, 78]. BCR-ABL can also activate tyrosine phosphorylation of suppressors of cytokine signaling 1 and 3 (SOCS-1 and SOCS-3), two potent suppressors of JAK/STAT signaling, leading to a reduction of their inhibitory effects on JAK/STAT activation [77]. In addition, BCR-ABL causes constitutive repression of FoxO3a by continued activation of the PI3K/AKT signaling axis, providing another mechanism of BCR-ABL-mediated apoptotic resistance [55, 87]. Furthermore, AKT inhibits GSK3β activity, which is involved in apoptotic regulation of CML cells. Finally, it was observed that other members of the PI3K/AKT/mTOR pathway, including PTEN (phosphatase and tensin homolog) and mTOR, play a role in the maintenance of LSCs [33, 73], and there is a cross talk between the JAK/STAT/PIM and PI3K/AKT/mTOR pathways that converge on eukaryotic translation initiation factor 4B (eIF4B) to regulate the activity of the ABL oncogene [11, 97].

9.5 Novel Therapeutic Targets in LSCs

The TKIs that inhibit BCR-ABL kinase activity are widely used for treatment of CML and induce a complete hematologic and cytogenetic response in the majority of chronic-phase CML patients. Although these TKIs can readily kill a significant number of leukemia cells, they fail to completely eradicate the primitive LSCs in CML [39]. Also, $CD34^+$ cells from CML patients with complete cytogenetic responses are capable of driving CML to BP [5]. These results demonstrate that LSCs remain in CML patients, even though they do not show evidence of the disease, suggesting that TKIs are unable to eradicate quiescent LSCs that are responsible for disease progression [4, 18, 20, 40, 53]. It is generally accepted that eradication of LSCs is required for curing CML, but the major challenge lies in the identification of differences between LSCs and their normal stem cell counterparts, HSCs. For example, some developmental genes essential for self-renewal and survival of stem cells, including the Wnt/β-catenin and hedgehog signaling pathways

described above, are involved in the regulation of both HSCs and LSCs [102, 103]. Success of an anti-stem cell strategy relies on complete inhibition of genes that are required functionally and specifically for the maintenance of LSCs. Because LSCs in CML express markers similar to those on normal stem cells, the major difference between them should be related to the leukemia-initiating genetic changes such as acquiring an oncogene or accumulating a DNA mutation [7, 58]. It is reasonable to hypothesize that these genetic changes (like acquiring BCR-ABL) cause aberrant regulation of signaling pathways, consequently turning a normal stem cell into a LSC. Some studies have shown that although certain genes play roles in both leukemia and normal stem cells, they are functionally more critical for leukemia than for normal stem cell counterpart [25, 103]. In this situation, the difference in the degree of dependence on the same genes for survival between leukemia and normal stem cells provides a therapeutic window for more specifically targeting LSCs [14].

Some novel candidate targets in CML LSCs have been identified, including Wnt, Notch, Hox, Bcl-2 and Bmi-1, etc. It is exciting to see that small molecular inhibitors for some of these targets are currently under development or being tested in clinical trials, aiming to selectively eradicate LSCs while sparing normal HSCs in patients with CML [6, 34, 35, 65, 68]. Three research groups have shown that activators of the peroxisome proliferator-activated receptor γ (PPARγ) have increased antileukemic activities in combination with TKIs [32, 76, 99]. Activators of PPARγ result in transcriptional downregulation of STAT5, whereas TKIs block phosphorylation of STAT5, with the combined effects of both drugs significantly downregulating this pathway and causing LSCs to exit quiescence where they were eradicated by TKIs [69]. Also, the eukaryotic translation initiation factor 4E (eIF4E) is a potent oncogene elevated in an estimated 30% of human cancers [22, 90]. Recent studies showed that targeting of the MNK-eIF4E axis in blast crisis CML inhibits LSC function [61]. It has been demonstrated that RAD51 (Rec A homolog of *E. coli*) recombinase activity plays a critical role for cancer cell survival, proliferation, and drug resistance. IBR2, a novel small molecule RAD51 inactivator, significantly prolonged survival in a murine imatinib-resistant CML model and effectively inhibited the proliferation of $CD34^+$ progenitor cells from CML patients [105].

Hsp90 is an ubiquitously expressed chaperone protein which accounts for about 1–2% of total cellular protein in mammalian cells [86]. It is a chaperone of several oncoproteins, such as Her2, v-Src, and BCR-ABL, and plays a role in regulating survival, proliferation, and apoptosis of cancer cells [2]. Hsp90 is constitutively expressed at a level that is two- to tenfold higher in tumor cells than in their normal stem cell counterparts, suggesting that Hsp90 is a potential therapeutic target in cancer [3, 48]. It has been shown that IPI-504, an inhibitor of Hsp90, efficiently induces dissociation of BCR-ABL and Hsp90 in BCR-ABL-expressing 32D cells as quickly as 30 min after the treatment. In vivo, treatment with IPI-504 alone significantly prolonged the survival of mice with CML induced by BCR-ABL and had a dramatic inhibitory effect on LSCs in CML mice. These studies demonstrate that inhibition of Hsp90 can effectively inhibit the survival and proliferation of LSCs in CML [74].

The arachidonates 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) genes (*Alox5* and *Alox15*) have been shown to regulate numerous physiological and pathological progresses, including inflammation and cancer [9, 12, 79]. The essential role of *Alox5* and *Alox15* in LSCs was originally discovered from a study using a murine model of BCR-ABL-induced CML. These studies showed that BCR-ABL fails to efficiently induce CML in mice in the absence of *Alox5* or *Alox15* due to the impairments of the functions of LSCs [13, 15] (Figs. 9.5 and 9.6). In support of the role of these two genes in the maintenance of LSCs, specific 5-LO and 15-LO inhibitors cause the impairments of the functions of LSCs in a similar manner. Most importantly, the lack of *Alox5* or *Alox15* does not significantly affect the functions of normal HSCs, indicating that *Alox5* and *Alox15* are required specifically for survival regulation of LSCs with a minimal effect on normal HSCs. These findings provide supporting evidence to the notion that it is possible to develop an effective therapeutic strategy for leukemia therapy through specific targeting of LSCs.

HIF1 belongs to the family of basic helix-loop-helix (bHLH) transcription factors and is a heterodimer that consists of a constitutively expressed HIF1β subunit and a HIF1α subunit [88]. Recent studies demonstrated that *HIF1*α plays a role in cancer progression by activating transcriptional programs for maintaining the ability of self-renewal and multipotency of CSCs in a hypoxic environment and that *HIF1*α was required for stem cell functions of mouse lymphoma and human acute myeloid leukemia [23, 64]. *HIF1α* was identified as a critical regulator for survival maintenance of LSCs in CML. Deletion of HIF1α impairs the propagation of CML through impairing cell cycle progression and inducing apoptosis of LSCs [100] (Fig. 9.7). Deletion of HIF1α also results in elevated expression of $p16I^{nk4a}$ and $p19^{Arf}$ in LSCs; knockdown of $p16^{Ink4a}$ and $p19^{Arf}$ rescues the defective colony-forming ability of $HIF1\alpha^{-/-}$ LSCs. Compared with normal HSCs, LSCs appear to be more dependent on the *HIF1α* pathway. Together, these results demonstrate that *HIF1α* represents a critical pathway in LSCs, and inhibition of the *HIF1α* pathway provides a therapeutic strategy for eradicating LSCs in CML.

On the other hand, it has been reported that some tumor suppressor-like genes play a critical role in survival regulation of LSCs in CML. It has been shown that the stearoyl-CoA desaturase 1 (*Scd1*) and B lymphoid kinase (*Blk*) negatively regulate the functions of LSCs in CML mice [99, 101] (Figs. 9.8 and 9.9). Scd1 catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids, which are the most abundant fatty acids present in mammalian organisms [85]. Scd1 expression is detected in almost all tissues, with a predominant expression in the liver, and is involved in regulating metabolic pathways related to preadipocyte differentiation, insulin sensitivity, metabolism, and tumorigenesis [31, 38]. In the BCR-ABL-induced CML mouse model, *Scd1* expression is downregulated in LSCs, and the deletion of *Scd1* accelerated CML development through affecting the function of LSCs with no effect on normal HSCs [99]. In addition, Pten, p53, and Bcl2 are regulated by *Scd1* in LSCs, and the induction of Scd1 expression by a PPARγ agonist suppresses LSCs and delays CML development, suggesting that *Scd1* plays a tumor-suppressive role in functional regulation of LSCs [99]. Blk is a SRC-family protein tyrosine kinase that is typically involved in cell proliferation and differentiation [95].

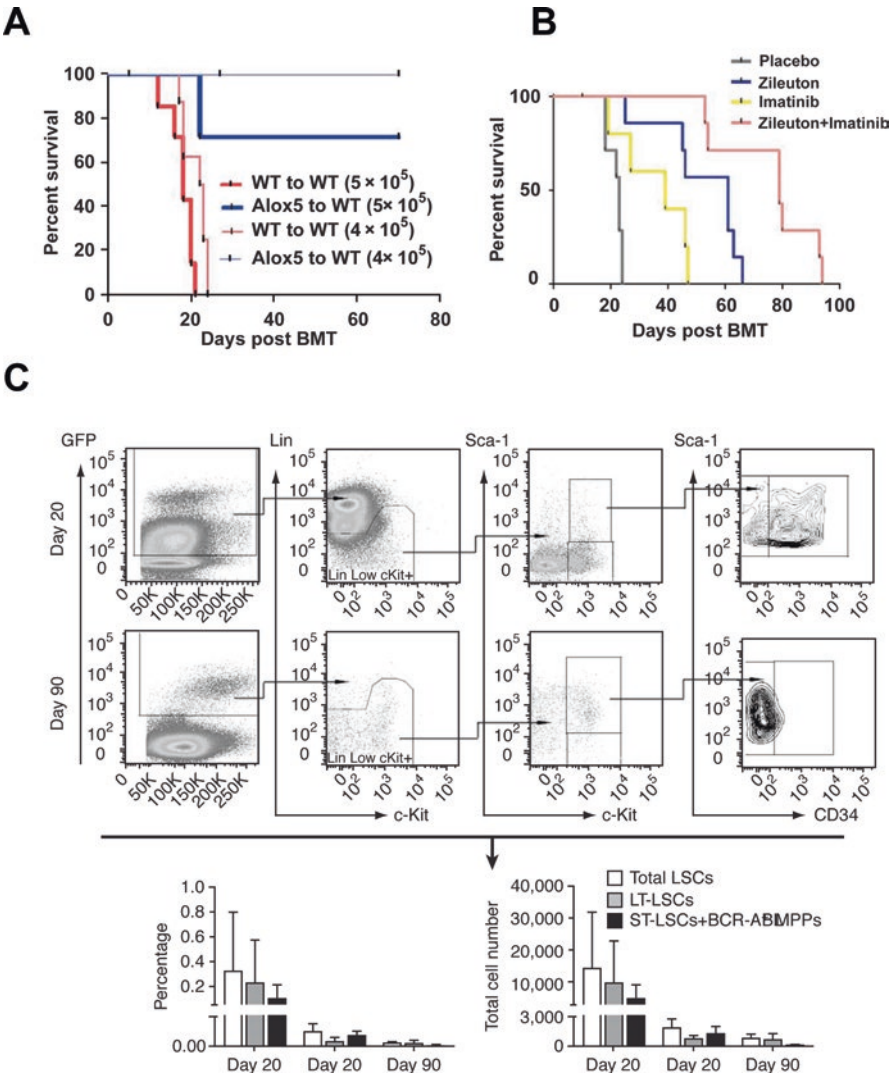

Fig. 9.5 *Alox5* is essential for the functions of LSCs in CML mouse model induced by BCR-ABL. (**a**) Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from wild-type or *Alox5*$^{-/-}$ donor mice (10 mice per group). All recipients of *BCR-ABL*-transduced bone marrow cells from wild-type donor mice developed CML and died within 4 weeks after bone marrow transplantation (days post BMT), whereas recipients of *BCR-ABL*-transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice survived. (**b**) Kaplan-Meier survival curves for CML mice treated with a placebo, zileuton alone, imatinib alone, or both zileuton and imatinib in combination. Zileuton-treated CML mice were much healthier than placebo-treated CML mice, and inhibition of Alox5 by zileuton significantly prolonged survival of CML mice. (**c**) Bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild-type or *Alox5*$^{-/-}$ donor mice, and FACS analysis showed that both percentages and total numbers of LT-LSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$) and ST-LSCs /BCR-ABL expressing MPP cells (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$) in recipients of BCR-ABL-transduced *Alox5*$^{-/-}$ (middle and right panels) donor BM cells were much lower than those in recipients of BCR-ABL-transduced wild-type (left panel) donor BM cells ($n = 4$). (Adapted from Chen et al. [13])

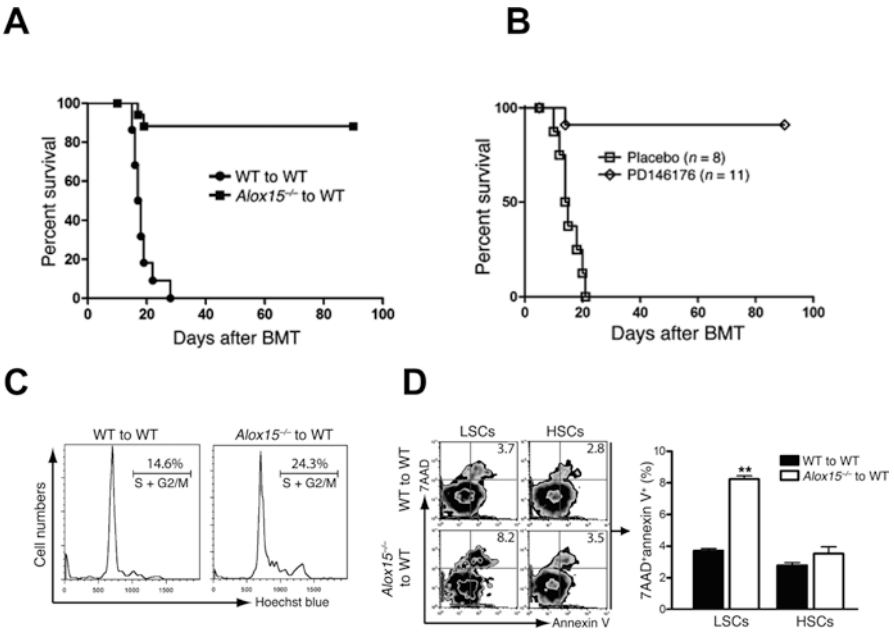

Fig. 9.6 *Alox15* plays a critical role in regulating LSC function and CML development. (**a**) Kaplan-Meier survival curve for recipients of BCR-ABL-transduced bone marrow cells from *Alox15*$^{-/-}$ mice was significantly prolonged compared to recipients of BCR-ABL-transduced bone marrow cells from WT mice (8 mice per group). (**b**) Kaplan-Meier survival curves showed that inhibition of *Alox15* by PD146176 dramatically prolonged survival of the CML mice. (**c**) Cell cycle analysis showed a higher percentage of LSCs in the S + G2/M phase in BM cells of *Alox15*$^{-/-}$ versus WT CML mice. BM cells were stained with Hoechst blue for FACS analysis. The mean percentage for each cell population is shown. (**d**) *Alox15* deficiency caused increased apoptosis of LSCs over normal HSCs by staining the cells with 7-AAD and annexin V. Results represent the mean ± SD. (Adapted from Chen et al. [15])

Inhibition of the Blk pathway accelerates CML development, and overexpression of the Blk delays CML development through suppressing LSC functions via Pax5 and p27, and Blk has no inhibitory effect on normal HSCs [101]. These results indicate that Blk functions as a tumor suppressor specifically in LSCs.

9.6 Perspective

CML is a classic stem cell disease, in which the BCR-ABL oncoprotein is considered essential for abnormal growth and accumulation of neoplastic cells. During the past two decades, despite the BCR-ABL tyrosine kinase inhibitors have successfully been introduced in the treatment of the disease, intrinsic and acquired drug resistance has been recognized as a challenge in clinical practice due to the persistence of CML LSCs that are a small cell population required for the initiation and

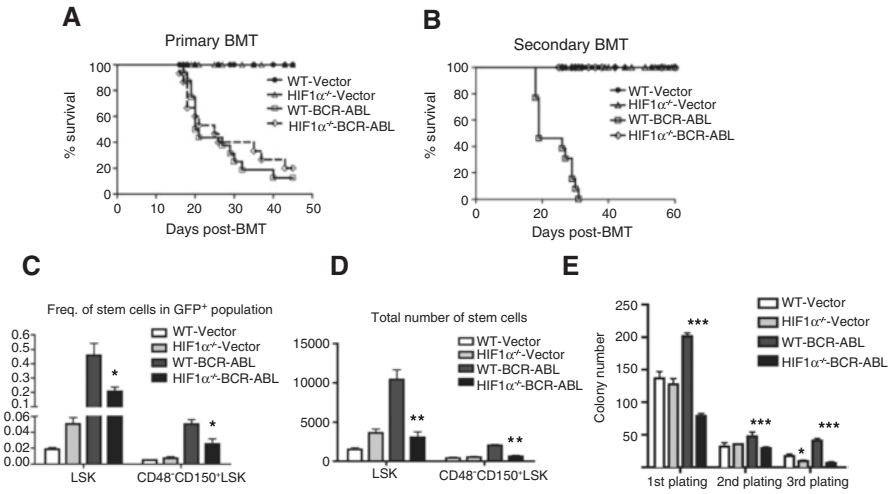

Fig. 9.7 *HIF1α* is required for survival maintenance of CML stem cells. (**a**, **b**) Kaplan-Meier survival curves for primary and secondary recipients of empty vector or *BCR-ABL*-transduced BM cells from WT or *HIF1α*$^{-/-}$ donor mice. For secondary BM transplantation, BM cells from primary control and CML recipient mice which received empty vector or *BCR-ABL*-transduced WT and *HIF1α*$^{-/-}$ BM cells were analyzed by FACS, and BM cells containing equal number of WT or *HIF1α*$^{-/-}$ GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ cells along with 2X10^5 WT BM cells (CD45.1) were transplanted into each lethally irradiated secondary recipient mouse. (**c**, **d**) The percentages and total numbers of stem cells (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ cells and GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ CD150$^+$CD48$^-$ cells) in BM of secondary recipient mice receiving BM cells from WT or *HIF1α*$^{-/-}$ mice were analyzed by FACS; $*P < 0.05$; $**P < 0.01$. (**e**) The loss of *HIF1α* causes a decrease in the colony-forming ability of BCR-ABL-expressing LSK cells. Sorted normal LSK cells and BCR-ABL-expressing LSK cells (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$ cells) were plated into methycellulose medium, colonies were counted, and cells were serially replated. Data show results from representative experiments. Mean values (± SEM) are shown; $*P < 0.05$; $**P < 0.01$. (Adapted from Zhang et al. [100])

maintenance of leukemia. LSCs cannot be eradicated through inhibiting BCR-ABL tyrosine kinase activity by TKIs. Importantly, the insensitivity of LSCs to TKIs is, in some cases, not associated with the development of drug-resistant mutations on BCR-ABL, suggesting that BCR-ABL kinase activity-independent pathways also contribute to the maintenance of survival and self-renewal of LSCs. Therefore, identification of novel genes that play critical role in regulating the function of LSCs will help to develop new therapeutic strategies through targeting LSCs for the treatment of CML, leading to a long-lasting clinical response with the absence of drug resistance and disease relapse. Identification of LSC targets in CML requires a full understanding of genetic changes in BCR-ABL-expressing LSCs. In addition, identification of surface biomarkers and signaling pathways associated with LSCs may provide more LSC targets for eradicating LSCs. On the other hand, in the development of anti-LSC strategies, we should pay much attention to the potential harm of an anti-LSC therapy to normal HSCs.

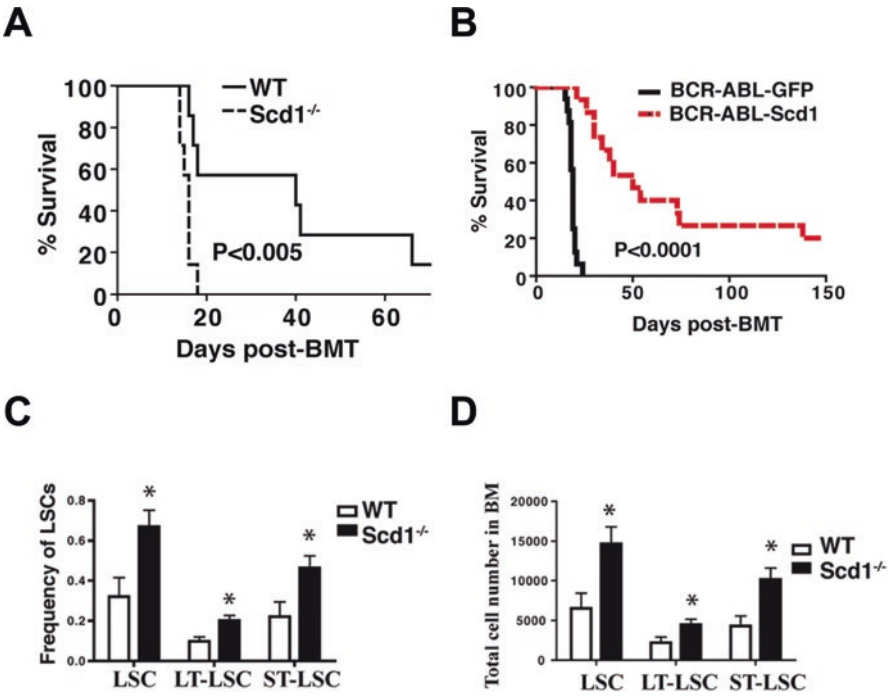

Fig. 9.8 *Scd1* plays a tumor-suppressive role in survival of LSCs in CML. (**a**) Kaplan-Meier survival curves showed that recipients of *BCR-ABL*-transduced *Scd1*$^{-/-}$ bone marrow cells developed and died of CML significantly faster than recipients of *BCR-ABL*-transduced WT mice bone marrow cells (n = 7 to 8 mice per group; $P < 0.005$). (**b**) Kaplan-Meier survival curve for secondary recipients of equal number of bone marrow cells from primary CML mice receiving *BCR-ABL*-transduced or *BCR-ABL-Scd1*-transduced WT bone marrow cells. Scd1 overexpression caused a significant delay of CML development in secondary recipients of bone marrow cells from the primary CML mice. (n = 14–16 mice per group; $P < 0.0001$). (**c**) Bone marrow cells from recipients of BCR-ABL-transduced WT or *Scd1*$^{-/-}$ bone marrow cells were analyzed by FACS at day 14 after BMT. The percentages and numbers of total LSCs, LT-LSCs, and ST-LSCs in the bone marrow of recipients of *BCR-ABL*-transduced *Scd1*$^{-/-}$ donor bone marrow cells were dramatically higher compared to those in the bone marrow of recipients of *BCR-ABL*-transduced WT donor bone marrow cells (n = 4 to 7 mice per group). The data are representative of one of four independent experiments, and mean values (± SEM) are shown (∗, $P < 0.05$). (Adapted from Zhang et al. [99])

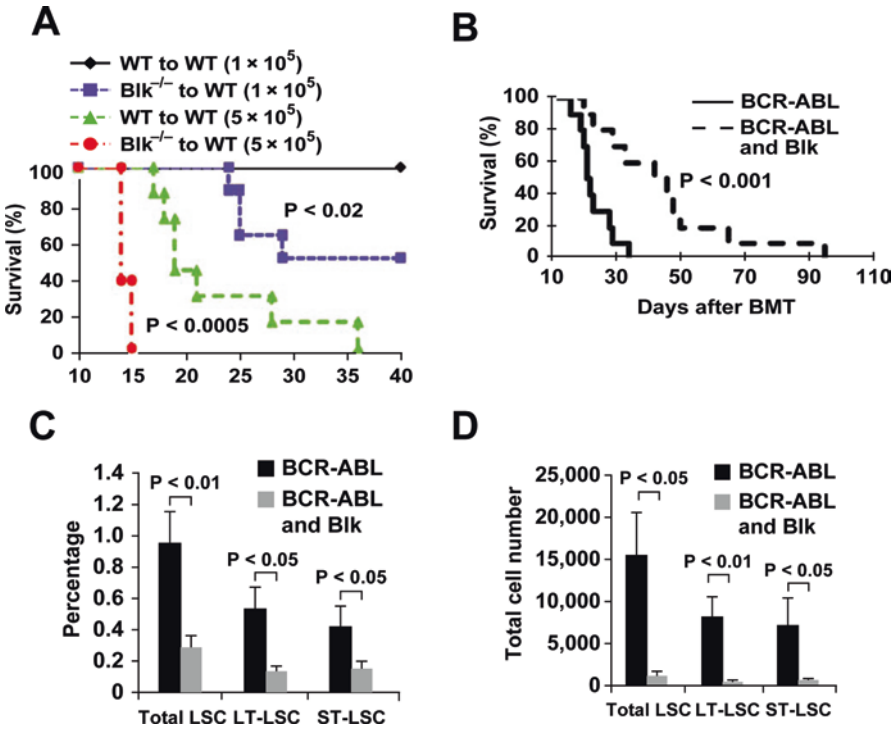

Fig. 9.9 The Blk pathway functions as a tumor suppressor in CML stem cells. (**a**) Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from WT ($n = 7$) or *Blk*$^{-/-}$ ($n = 8$) donor mice. Recipients of *BCR-ABL*-transduced bone marrow cells from *Blk*$^{-/-}$donor mice developed CML significantly faster than did recipients of *BCR-ABL*-transduced WT mice bone marrow cells. (**b**) Kaplan-Meier survival curves for recipients of *BCR-ABL* ($n = 10$) or *BCR-ABL-Blk* ($n = 10$) transduced bone marrow cells. Overexpression of *Blk* in *BCR-ABL*-transduced bone marrow cells delayed the CML development of recipient mice. (**c**, **d**) The percentages and numbers of total LSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$), LT-LSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$), and ST-LSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$) in the bone marrow of recipients of *BCR-ABL*- or *BCR-ABL-Blk*-transduced bone marrow cells were analyzed at day 15 after transplantation. Mean values ± SEM ($n = 5$). (Adapted from Zhang et al. [101])

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Chapter 10
Cellular Immunotherapy in the Treatment of Hematopoietic Malignancies

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Contents

Abstract Cancer immunotherapy has been shown to be an efficacious therapeutic approach in the treatment of cancers including hematopoietic malignancies. Induction of T cell cytotoxicity against tumors by adoptive cell therapies (ACT), cancer vaccines, gene therapies, and monoclonal antibody therapies has been intensively studied. In particular, immune checkpoint blockade and chimeric antigen receptor T (CAR-T) cell therapies are the recent clinical successes in cancer immunotherapy. This article introduces the main concepts and addresses the most relevant clinical modalities of cellular immunotherapies for hematological malignancies: antigen non-specific T cell therapy, genetically modified T cell receptor (TCR) T cell therapy, chimeric antigen receptor (CAR) T cell therapy, and CAR-T cell clinical trials in leukemia, lymphoma, and multiple myeloma. Clinical trials have shown encouraging results, but future studies may need to incorporate novel CAR constructs or targets with enhanced safety and efficacy to ensure long-term benefits.

Keywords Adoptive cell therapies · CAR-T · Leukemia

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10.1 Introduction

Much interest has risen for cancer immunotherapy as a new and efficacious therapeutic approach in the last decade. Especially, adoptive cell therapies (ACT), cancer vaccines, gene therapies, and monoclonal antibody therapies have all been used to induce T cell cytotoxicity against tumors.

Immune checkpoint blockade and chimeric antigen receptor T (CAR-T) cell therapies are the recent clinical successes in cancer immunotherapy. In 2017, CAR-T cell therapy (Kymriah, Novartis) for juvenile acute lymphoblastic leukemia (ALL) was approved by the FDA as the first CAR-T cell therapy for the treatment of B-cell ALL patients that does not respond to treatment or relapsed under 25 years old. Thereafter the FDA approved the use of CAR-T cell (Yescarta, Kite) for large B-cell lymphoma, including diffuse large B-cell lymphoma (DLBCL), primary mediastinal large B-cell lymphoma, high-grade B-cell lymphoma, and transformed follicular lymphoma. In 2018, Kymriah was also approved for the treatment of non-Hodgkin lymphoma. These CAR-Ts are genetically modified autologous T cells made to recognize and lyse cancer cells in a very specific fashion. This article introduces the main concepts and addresses the most relevant clinical modalities of cellular immunotherapies for hematological malignancies (see Table 10.1).

10.2 Antigen Non-specific T Cell Therapy

The immune system, especially T lymphocytes cells (T cells), plays an important role to detect and eliminate "nonself" such as pathogens and transformed cells like cancers. Adoptive T cell therapy (ACT) is based on infusion of tumor-specific T cells. Many different cell types can be used as killer cells. Lymphokine-activated killer cells were utilized for the first trial of ACT in human. Patients with metastatic melanoma were treated with the combination of lymphokine-activated killer cells plus interleukin-2 in that study [1]. The survival and sustained activation of the infused lymphokine-activated killer cells were ensured by IL-2. The approach has resulted in marked tumor regression in up to or approximately 30% of the patients with renal cell, melanoma, colorectal, non-Hodgkin's lymphoma, and adenocarcinoma of the lung [2].

ACT with tumor infiltration lymphocytes (TILs) has provided promising results in clinical studies in metastatic melanoma [3]. However, ACT with TILs faces three problems: (1) It remains limited to particular tumor types, (2) it takes 4–6 weeks for TILs to grow, and (3) it requires specialized cell production facilities and staff [4, 5]. There are approaches being developed to attempt overcoming some of these hurdles. Among the most promising ones are the T cell receptor (TCR) engineered therapy and chimeric antigen receptor (CAR) therapy (Fig. 10.1).

Table 10.1 Selected clinical trials for cellular immunotherapy

Immunotherapy type	Cell type	Antigen	Disease	Trial ID	Compound name
Cell therapy	CMV-specific T cells	CMV	CMV-positive hematological malignancies	NCT03067155	N/A
	Mismatched immune cells	CD3/CD28	Leukemia, lymphoma, multiple myeloma	NCT00558675	AlloStim
	NK -92 cell line	Irradiated NK cells	Leukemia, lymphoma, myeloma, Hodgkin's disease	NCT00990717	N/A
	NK cells	NK	Acute myeloid leukemia	NCT02809092	N/A
TCR-T cell therapy	T cells	NY-ESO-1, LAGE-1	Multiple myeloma	NCT01352286 [1]	N/A
	T cells	NY-ESO-1, LAGE-1	Multiple myeloma	NCT01892293	N/A
	T cells	NY-ESO-1	Solid or hematological malignancies	NCT03391778	GSK3377794
	T cells	NY-ESO-1	Multiple myeloma, other metastatic solid cancers	NCT02457650	N/A
	T cells	WT1	Acute myeloid leukemia	NCT00052520 [2]	N/A
	T cells	WT1	Acute myeloid leukemia, chronic myeloid leukemia	NCT01621724	
	Central memory/naive CD8+ T cells	WT-1	Acute myeloid leukemia	NCT02770820	N/A
	T cells	PRAME TCR	Acute myeloid leukemia	NCT02743611	BPX-701
	T cells	HA-1 T TCR	Acute myeloid leukemia	NCT03326921	N/A
CAR therapy	T cells	CD19 CAR	B-cell acute lymphoblastic leukemia, B-cell non-Hodgkin lymphoma	Approved [3]	KYMRIAH
	T cells	CD19 CAR	Large B-cell lymphomas	Approved [4]	YESCARTA
	T cells	CD19 CAR	B-lineage acute lymphoblastic leukemia	NCT02028455 [5]	N/A
	T cells	Muc1/CLL1/CD33/CD38/ CD56/CD123	Acute myeloid leukemia	NCT03222674	N/A

(continued)

Table 10.1 (continued)

Immunotherapy type	Cell type	Antigen	Disease	Trial ID	Compound name
	T cells	CD123	Acute myelocytic leukemia	NCT03556982	N/A
	T cells	CD19	CD19+ refractory or relapsed B-ALL patients	NCT03173417	IM19
	T cells	CD22	B-cell malignancies	NCT03262298	N/A
	T cells	CD19/CD22	B-acute lymphoblastic leukemia	NCT03241940	N/A
	T cells	CD20	B-cell malignancies	NCT02710149	N/A
	T cells	BCMA	Multiple myeloma	NCT02658929	bb2121
	T cells	BCMA	Multiple myeloma	NCT02215967 [6]	N/A
	APRIL CAR-T cells	BCMA and TACI	Multiple myeloma	NCT03287804	AUTO2
	T cells	CD133	Acute myeloid and lymphoid leukemias and solid tumor	NCT02541370	N/A
	T cells	CD138	Multiple myeloma	NCT01886976 [7]	N/A
	T cells	CD19	Multiple myeloma	NCT02135406 [8, 9]	N/A
	T cells	CD30	Hodgkin lymphoma and anaplastic large cell lymphoma	NCT01316146 [10]	N/A
	T cells	CD20	Diffuse large B-cell lymphoma	NCT01735604 [11]	N/A
	T cells	CD20	Non-Hodgkin's lymphoma	NCT00012207 [12]	N/A
	T cells	CD5	T cell leukemia or lymphoma	NCT03081910	N/A
	NK cells	CD7	CD7-positive leukemia and lymphoma	NCT02742727	N/A
	NK cells	CD19	CD19-positive leukemia and lymphoma	NCT02892695	PCAR-119
	NK cells	CD19	Relapsed/refractory B-lymphoid malignancies	NCT03056339	N/A

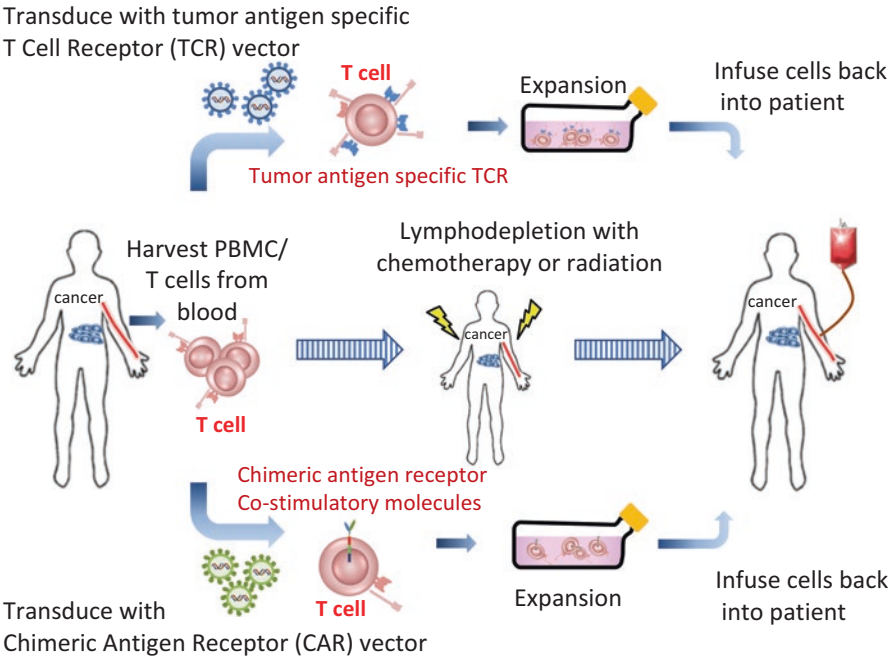

Fig. 10.1 Engineered T cell therapy process. The most promising cellular immunotherapies are the T cell receptor (TCR)-engineered therapy and chimeric antigen receptor (CAR) therapy

10.3 Genetically Modified T Cell Receptor (TCR) T Cell Therapy

In this modality of cellular therapy, T cells are modified to express new T cell receptors that recognize tumor-specific antigens. These receptors can be modified to improve their binding avidity and affinity against their target antigens in an MHC-specific context. PBMC/T cells from patients are harvested and transduced with a viral vector to incorporate the new T cell receptor. TCR therapies have shown tumor regression in some patients with advanced solid tumors, e.g., metastatic melanoma, colorectal cancer, and synovial cell sarcoma [6, 7]. One of the difficulties in TCR therapy is that the TCR must be genetically matched to the patient's MHC type. A possible safety issue is the unpredictable specificities of heterodimer formation of the recombinant α and β TCR chains with the respective chains of the endogenous TCR [8]. Unpredictable and unideal mispairing of TCR chains may not only result in loss of specificity but may also induce potential autoreactivity [9, 10]. Introduction of murine-human hybrid T cell receptor (TCR) [11, 12] and cysteines into the

constant region of the α and β chains [13] enforces preferential pairing and improves both the efficacy and safety of TCR therapy to generate tumor-reactive T cells. The first treatment with genetically engineered T cells against MART1 was administrated in metastatic melanoma patients and reported in 2006 [14]. In this study, 2 of 15 patients demonstrated objective regressions of metastatic melanoma lesions, and no toxicities were observed in any patients [14]. A second-generation TCR with greater affinity for MART1 was conducted in a subsequent clinical trial [15]. Six of the 20 patients (30%) treated with MART-1 TCR and 3 of 16 (19%) treated with gp100 TCR experienced an objective antitumor response; however several patients displayed autoimmune T cell-mediated destruction of normal melanocytes [15].

Cancer-testis (CT) antigens are a very attractive class of proteins to be targeted in cancer. These antigens have restricted tissue expression in germ cells and in a significant number of different malignancies. NY-ESO-1 is a CT antigen expressed by many malignancies, including multiple myeloma, melanoma, sarcoma, non-small cell lung cancer, cholangiocarcinoma, and breast and ovarian cancer [16, 17]. A TCR against NY-ESO-1 with single and dual CDR3α and CDR2β amino acid substitutions dramatically enhanced tumor recognition [18]. The response rates of patients with melanoma and synovial cell sarcoma were 45% and 67%, respectively, and no toxicities were observed in a trial [6]. Another CT antigen, MAGE-A3 had been targeted with a high avidity TCR. Five of nine patients demonstrated cancer regression following infusion of anti-MAGE-A3 TCR gene-engineered T cells [19]. However, three of nine patients developed severe neurologic toxicity, probably due to the recognition of the MAGE-A12 protein which is expressed in human brain. Another MAGE-A3-specific TCR led to cardiac shock in two patients and caused death from heart failure when the TCR cross-reacted with Titin, a protein expressed in myocardial tissue [20]. Regarding the use of TCRs in hematologic malignancies, a phase I/II trial for multiple myeloma (MM) patients accessed the safety and efficacy of NY-ESO-1 and LAGE-1-specific TCR-engineered T cells [21]. In this study, 14 out of 20 MM patients showed a near complete response (nCR) or complete response (CR), 2 patients showed a very good partial response, 2 showed a partial response, 1 showed stable disease, and 1 showed progressive disease at day 100 after transplantation [22]. Interestingly, long-term engraftment/persistence of engineered cells was observed up to 2 years after infusion in this study. Another promising target for hematologic malignancies, Wilms' tumor 1 (WT1), is known to be expressed in most acute myeloid leukemia (AML) patients [22, 23]. Since WT1 expression levels correlate with poor clinical prognosis, it would be an attractive target for ACT [22]. WT1-specific TCR-engineered T cell therapy was conducted in a phase I clinical trial for AML and high-risk myelodysplastic syndromes (MDS) [24]. In the study, five out of eight patients showed persistence of engineered cells, and four of these five patients survived over 12 months. These results indicate that targeting WT1 antigen for AML and MDS has the potential to be of clinical benefit [24].

10.4 Chimeric Antigen Receptor (CAR) T Cell Therapy

The chimeric antigen receptor (CAR) is composed of an extracellular domain with a single-chain variable fragment (scFv) from monoclonal antibody for antigen binding and an intracellular part with a TCR/CD3ζ endodomain for the transmission of ζ signal in response to recognition of antigen and provision of T cell activation [25]. Second- and third-generation CARs have been developed for enhanced cytotoxicity, proliferation, cytokine secretion, resistance, and development of memory by including combinations of costimulatory domains, such as CD28, CD137 (4-1BB), and CD134 (OX40) [26]. A fourth-generation CAR construct called "T cells redirected for universal cytokine-mediated killing" (TRUCKs) is equipped with genetic modifications to induce expression of a transgenic product like IL-12 or another cytokine [27].

10.5 CAR-T Cell Clinical Trials in Leukemia

CD19-specific CAR-T cells have been administered in recurrent/refractory CD19+ ALL in children and young adults, and there was 93% minimal residual disease-negative (MRD−) complete remission (CR) rate in treated patients, resulting in 89% overall efficacy [28]. In this study, 45% of patients who achieved an MRD− CR relapsed, and 39% of those relapses showed a CD19-negative escape variant leukemia [28]. In chronic lymphocytic leukemia (CLL), a report of a patient who received ACT with second-generation anti-CD19 CAR-modified T cells demonstrated a complete remission associated with the tumor lysis syndrome [29]. In addition to tumor lysis syndrome, the patient had B-cell depletion and hypogammaglobinemia as a chronic toxic effect [29]. Severe toxic effects with CAR-T cell therapy have been observed from unexpected presence of the target antigens on normal tissues [30]. To address this issue, a novel construct that incorporated the interleukin-15 (IL15) gene and an inducible caspase-9-based suicide gene was developed. It had improved antitumor effects and efficiently eliminated CAR CD19 T lymphocytes [31]. Suicide gene may be useful for treatment of patients, except when acute toxic effects occurred within hours after administration [32, 33]. The most common adverse events, cytokine release syndrome (CRS), and neurologic toxicities (NT) have been observed in CAR-T cell trials. Effective ways to prevent these toxicities remain poorly understood [34]. Modification of T cell composition and dose reduction have been successful in B-cell ALL patients [35], and blocking IL-6 was found to be effective [36, 37]. Myeloid cells, including macrophages and monocytes, were found to be the major cells mediating CRS and NT by releasing IL-1 and IL-6 among other cytokines [38]. Blocking IL-1 receptor effectively abolished CRS as well as NT in mouse model [38]. CD22 targeting CAR-T might be an option to CD19-relapsed patients [39] and is currently under

clinical investigation. Another attractive target is CD123. Preclinical studies with CD123-specific CAR-T cells demonstrated tumor eradication and long-term disease-free survival in PDX mice model [40]. A pilot safety study of CD123 CAR-T exhibited a decrease of blasts, and the patient achieved partial remission, although cytokine release syndrome developed [41]. Over ten clinical trials are accruing for leukemia patients with CD123 CAR-T, and further exploration is needed.

10.6 CAR-T Cell Clinical Trials in Multiple Myeloma

Candidate antigens of immunotherapies for multiple myeloma include B-cell maturation antigen (BMCA)/CD269, CD138, and CD19. The expression of BCMA/CD269, a member of the TNF receptor superfamily, has been reported mostly in plasma cells and subsets of mature B cells including multiple myeloma [42–45]. Preclinical studies showed that BCMA-CAR-T exhibits cytotoxicity and in vivo tumor eradication [42]. One clinical trial showed that the overall response rate was 81%, with 63% very good partial response or complete response [46]. In this study, two out of ten patients who received 0.3–3 × 10^6 CAR+ T cells/kg showed partial response (PR) and very good partial response (VGPR). The overall response rate for patients who received 9 × 10^6 CAR+ T cells/kg was 81%, 3 PR, 8 VGPR, 2 stringent complete response (SCR) among 16 patients. Eleven patients were found to be minimal residual disease (MRD) negative, 5 were not evaluated among 16 patients who received 9 × 10^6 CAR+ T cells/kg. The toxicity was mild and no cytokine release syndrome (CRS) at lower dose (0.3–3 × 10^6 CAR+ T cells/kg). However, 6 of 16 patients at the dose of 9 × 10^6 CAR+ T cells/kg had CRS grade 3 or 4 [46]. CD138 (syndecan-1) is a member of the transmembrane heparan sulfate proteoglycan family and is expressed on normal and malignant plasma cells [47, 48]. Five patients were infused CD3+ CD138-CAR-T cells (average of 0.756 × 10^7 cells/kg), and there was no intolerable toxicity observed [49]. Four of five patients had stable disease that lasted over 3 months. CD19 is not typically considered a target in multiple myeloma; however, minor subset of the multiple myeloma cells with drug-resistant, disease-propagating properties has a CD19-positive phenotype [50, 51]. Ten patients received CD19-CAR-T cells following salvage high-dose melphalan and autologous stem cell transplantation (ASCT) with one, two and four patients exhibiting SCR, PR and VGPR, respectively, 100 days after ASCT [52]. Two of ten patients who had VGPR exhibited significantly longer PFS after ASCT + CD19-CAR-T compared to prior ASCT alone (479 vs. 181 days; 249 vs. 127 days). Most of grade 3 or higher adverse events were unrelated to CD19 CAR-T cells. CRS was observed in one patient only and it was grade 1 [52]. This study indicated that CD19-CAR-T may improve duration of response and targeting CD19+ myeloma-propagating cells could be of clinical benefit [52].

10.7 CAR-T Cell Clinical Trial Therapy in Lymphoma

CD30 is a member of the tumor necrosis factor receptor (TNFR) family, and it is a cell surface receptor for activated T cells, Hodgkin lymphoma (HL), anaplastic large cell lymphoma (ALCL), and a few other non-Hodgkin's lymphomas (NHL) [53]. Anti-CD30 monoclonal antibodies in HL and ALCL showed clinical success. This encouraged the development of CD30-CAR-T cell therapy. CD30-CAR-T cell were infused without a conditioning regimen into 7 HL and 2 ALCL patients [54]. Two had CR, one had continued CR (CCR), and three had transient stable disease in this study. One CR lasted over 3 years and another CR lasted for 2 years. These CR responses were mainly achieved in patients infused with CD30-CAR-T cells at the dose of 2×10^8. No severe adverse events were observed, and no patients developed CRS [54]. CD20 is a tetra-transmembrane protein that is expressed in >90% of B-cell lymphomas [55]. Anti-CD20 antibody combined with chemotherapy will produce superior clinical effects in most NHL patients compared to chemotherapy alone. CD20-CAR-T 1×10^7 cells/kg were infused into seven patients, one had CR, and four had PR, and in total five of seven showed tumor regression during the trial with four of seven experiencing grade 3 or 4 delayed adverse events [56]. Seven patients received CD20-CAR-T cells at the dose of 1×10^8 for infusion 1, 1×10^9 for infusion 2, and 3.3×10^9 for infusion 3 in another trial [57]. Two of seven patients achieved CR to cytoreductive chemotherapy administered before the CAR-T cell infusions and remained disease-free 3 months and 13 months after T cell infusions. One patient had PR and lasted for 3 months after treatment with CAR-T cells. Four other had SD. There were no grade 3 or 4 toxicities observed and no adverse events attributable to the T cell infusions [57]. In vitro and in vivo mouse characterization experiments indicated that bispecific CAR-T platform with CD19-CD20 enhanced cytotoxicity by producing more IFNγ, TNFα, and IL-2 compared to original CAR. Bispecific CD19-CD20 CAR-T would trigger robust cytotoxicity against tumor expressing CD19 or CD20 and potentially prevent antigen escape [58].

10.8 Future Prospects for Cellular Immunotherapy

Checkpoint-blocking antibodies and small molecule inhibitors have demonstrated promising potential, but these new treatment modalities are still in their early stages, and persistence of response is short-lived, limiting their overall clinical benefits. Therefore, a combinatorial approach with ACT may offer a number of potential synergies; therefore a number of clinical trials are underway.

Although CAR-T cell therapy has shown great success in hematological malignancies, autologous T cell therapies are costly and labor-intensive, and its safety prediction still remains difficult. Some alternative cellular immunotherapies have been developed to address some of those issues. CAR-NK and CAR-iPSC-NK cells

might extend these possibilities since natural killer (NK) cells do not require HLA matching and show cytotoxicity against tumor. The advantages of using CAR-NK are that allogeneic NK cells should not cause significant GVHD; NK cells would have a relatively limited life-span, limiting toxicity. The NK92 cell line was used for a clinical trial as it can easily expand. However, its use requires irradiation prior to infusion to patients due to the potential for tumor engraftment following infusion. And irradiation of CAR-NK can affect its cellular proliferation and persistence in vivo [59, 60]. Cord blood (CB) can be a source of NK cells; however they are phenotypically and functionally immature. Using GMP-grade K562-based artificial antigen-presenting cells (aAPCs) expressing membrane bound IL-21 and 4-1BB ligand, clinically relevant doses of GMP-grade NK cells were generated from a CB [61]. In another approach, NK-CAR-iPSC-NK cells significantly inhibited tumor growth and prolonged survival in an ovarian cancer mouse model [62]. The iPSC-derived NK cells also induced less cytokine release, lymphocyte-related pathological damage, and GVHD [62]. CAR-iPSC-NK cells may be a promising cellular therapy, although further clinical studies will reveal its own safety concerns.

10.9 Conclusions

An ACT, along with the engineering of the TCR or CAR to recognize tumor antigens, and potentially in combination with checkpoint blocking Abs or small molecule inhibitors, has the potential to be very beneficial in assisting in the eradication of various hematological malignancies. However, there are still several gaps in knowledge to be addressed before it becomes a mainstream therapeutic choice. Clinical trials have shown encouraging results, but future studies may need to incorporate novel CAR constructs or targets with enhanced safety and efficacy to ensure long-term benefits.

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