Curr Stem Cell Rep (2016) 2:183–196
DOI 10.1007/s40778-016-0057-1

STEM CELL SWITCHES AND REGULATORS (KK HIRSCHI, SECTION EDITOR)

MicroRNAs in Control of Stem Cells in Normal and Malignant Hematopoiesis

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Published online: 1 July 2016

Abstract Studies on hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs) have helped to establish the paradigms of normal and cancer stem cell concepts. For both HSCs and LSCs, specific gene expression programs endowed by their epigenome functionally distinguish them from their differentiated progenies. MicroRNAs (miRNAs), as a class of small non-coding RNAs, act to control post-transcriptional gene expression. Research in the past decade has yielded exciting findings elucidating the roles of miRNAs in control of multiple facets of HSC and LSC biology. Here, we review recent progresses on the functions of miRNAs in HSC emergence during development, HSC switch from a fetal/neonatal program to an adult program, HSC self-renewal and quiescence, HSC aging, HSC niche, and malignant stem cells. While multiple different miRNAs regulate a diverse array of targets, two common themes emerge in HSC and LSC biology: miRNA-mediated regulation of epigenetic machinery and cell signaling pathways. In addition, we propose that miRNAs themselves behave like epigenetic regulators, as they possess key biochemical and biological properties that can provide both stability and alterability to the epigenetic program. Overall, the studies of miRNAs in stem cells in the hematologic contexts not only provide key understandings to post-transcriptional gene regulation mechanisms in HSCs and LSCs but also will lend key insights for other stem cell fields.

Keywords miRNAs · Hematopoietic stem cells · Leukemia stem cells · Epigenetic machinery

This article is part of the Topical Collection on *Stem Cell Switches and Regulators*

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Introduction

The field of hematology has initiated the concepts of both tissue stem cells and cancer stem cells [1]. Hematopoietic stem cells (HSCs) have been historically defined as cells that can provide long-term repopulation capacity for the hematopoietic system when transplanted into a new host. Although recent studies in native hematopoiesis have modified views on the role of stem cell activity in an unperturbed healthy mouse [2, 3], the traditional HSC definition is intrinsically related to the success of the life-saving medical procedure, bone marrow transplantation, and will be used in this review.

HSCs are capable of maintaining the hematopoietic system for the lifetime duration of an organism. HSCs are rare hematopoietic cells sitting at the apex of the hematopoietic hierarchy and possess extensive ability to self-renew as well as the capacity to gradually differentiate into downstream hematopoietic lineages. During fetal development, HSCs emerge as part of the definitive hematopoiesis program, best characterized through budding from special populations of endothelial cells [1, 4, 5]. Newly emerged HSCs migrate to and colonize the fetal liver, a stage that is accompanied by HSCs undergoing active cell cycle [4]. In adults, however, HSCs are predominantly localized within bone marrow and are maintained

by a complex set of microenvironment cues provided by the HSC niche [6]. Furthermore, under normal conditions, most HSCs exist in a quiescent state without active cycling [7, 8]. Why must HSCs remain largely quiescent in adults? Although the answer is not fully clear, it has been proposed that stem cell cycling is associated with cancer incidence [9], and quiescence is thus one possible mechanism to avoid the accumulation of DNA replication errors and consequent leukemic transformation. Similar to normal hematopoiesis, leukemia cells are hierarchically organized [10, 11], with relatively rare leukemia stem cells (LSCs) being the only cell population that can re-initiate leukemia in a new host. The bulk of leukemia cells does not have strong self-renewal capacity and is derived from LSCs.

The stem cell concepts of both HSCs and LSCs underscore the importance of the epigenome in defining the functional cellular heterogeneity in normal and malignant hematopoiesis. HSCs and most of their differentiated progenies share the same genetic content, yet they differ strongly on cellular function. Likewise, the bulk population of leukemia inherits genetic contents from LSCs, and the pivotal difference between LSCs and the rest of the leukemia cells on leukemia initiation has to be attributed to their different epigenome.

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides in length. miRNAs are well known to post-transcriptionally regulate gene expression, by degrading target messenger RNA and/or inhibiting protein translation. miRNAs are transcribed as long primary miRNAs, mostly by RNA polymerase II. Primary miRNAs undergo step-wise processing by Drosha-and-DGCR8-containing microprocessor complex in the nucleus to generate precursor miRNAs, which are then transported into the cytoplasm by exportin 5 and further processed by Dicer and its cofactors to generate mature miRNA duplexes. Loading of mature miRNAs into Argonaute (AGO) proteins, with the assistance of HSP90, allows miRNA-guided targeting of AGO proteins to target mRNA and results in downregulation of their binding targets [12–15]. The roles of miRNA in the hematopoietic system, including stem cells, have been documented by a number of excellent reviews [16–27]. In this review, we summarize recent progresses of miRNAs that functionally control HSC and LSC biology. Two emerging themes from these studies are that epigenetic machineries and cell signaling pathways are frequently regulated by miRNAs. We particularly emphasize miRNAs' direct roles on regulating epigenetic enzymes, many of which have been found as somatically mutated in healthy individuals and malignant hematopoiesis. We propose that miRNAs themselves should be considered as an important component of the epigenetic program given their properties. As the focus of this review is on stem cells, we will not be able to cover every key and seminal study on miRNAs in other hematologic contexts, many of which have been reviewed elsewhere.

miRNAs Intrinsically Possess Epigenetic Properties and Serve as Guardians of Cell States

The epigenome has two key properties, its maintenance and its amenability to change, both of which are essential for HSCs and LSCs. During symmetric cell division, the two daughter cells perform similar functions as the mother cells and retain a similar gene expression program, requiring the epigenome to be faithfully copied. The achievement of this feat is by no means simple, the mechanism of which is still far from completely understood. For example, during DNA replication in the S phase of the cell cycle, DNA needs to be unwound from core histones. After replication, histones, including their modifications, need to be re-established at the right place with a high level of precision on both copies of DNA. The best-known mechanism that can potentially maintain the epigenetic state during cell division is DNA methylation at 5 position of cytosine (5mC). DNMT1 is known as a maintenance DNA methyltransferase that is capable of recognizing hemi-methylated DNA and copy the methylation mark onto the newly synthesized strand [28, 29]. Other than active demethylation (to be discussed in the next section), the most effective way to remove DNA methylation marks is by cell division, in which DNA methylation maintenance mechanisms are suppressed to allow passive dilution of DNA methylation in daughter cells. However, DNA methylation cannot be the only mechanism in maintaining the epigenome stability. In model eukaryotes, such as yeast, worms, and fruit flies, DNA methylation is not an actively utilized mechanism, yet their epigenome can be faithfully maintained. In mouse embryonic stem cells, the pluripotency epigenome can be maintained in the absence of all DNMTs (DNMT1, DNMT3a, and DNMT3b) [30, 31]. These facts argue for the existence of additional epigenome maintenance programs that share similar properties as DNA methylation.

miRNAs possess some key properties that are reminiscent to DNA methylation and can be considered an underappreciated component of the epigenetic program. One of the striking features of miRNAs is their stability. Studies from multiple laboratories, including ours, have found that the vast majority of measurable mature miRNAs have half-lives much longer than those of messenger RNAs [32•, 33, 34], possibly due to the protection of mature miRNAs from cytosolic RNAses by AGO proteins. The half-lives of many miRNAs are too long to be determinable in cell culture experiments [32•], but a study on miR-208 in mouse heart tissue shows that the half-life of this miRNA is >12 days [35]. The stability of miRNAs suggests that one of the most effective ways to decrease miRNA expression

in cells is by passive dilution, a property that is similar to DNA methylation marks. Consistent with this notion, we have previously observed that the $Lin^{-}Kit^{+}Sca^{-}$ myeloid progenitor cells, some of which cycle at <8 h per cell cycle [36], have decreased global miRNA expression in comparison to the slower cycling HSCs or more mature cells [37]. In addition to stability, miRNA expression is also amenable to changes during cell-state transitions. Differential miRNA expression, both upregulation and downregulation, has been observed between HSCs, LSCs, and their differentiated progenies [37–41]. Transcriptional upregulation of miRNAs can lead to increased mature miRNA expression. The mechanisms underlying downregulation of miRNA during differentiation are much less understood, which may involve both cell division-based passive dilution in the presence of transcriptional silencing or active destabilization of miRNA. For the aforementioned downregulation of miRNAs in fast cycling myeloid progenitors in comparison to HSCs or $Lin^{-}Kit^{+}Sca^{+}$ hematopoietic stem and progenitor cell (HSPC) population [36, 37], it is currently unknown how many cell divisions are required to go from HSCs or HSPCs to myeloid progenitors, the elucidation of which may reveal the relative contribution of passive dilution to miRNA downregulation in this process. Active miRNA degradation, either through specific enzymes, downregulation of the protective AGO proteins, or secretion through exosomes have been reported in other cellular systems to contribute to active mature miRNA destabilization [33, 34]. However, mechanisms of miRNA downregulation have not been extensively studied within the hematopoietic system, the elucidation of which will add key insights into miRNAs' function in stem cells.

Qualifying miRNAs as an epigenetic component requires additional evidence that they confer functional stability of cell states, in addition to their biochemical properties of being both stable and alterable. Indeed, evidence accumulated in the past decade has identified not only specific miRNAs in control of cell fates but also the overall miRNA pathway as a guardian of cell states. One of the best examples for the latter is the overall miRNA regulation pathway serving as a tumor suppressor for safe-guarding normal cell states from cancer transformation, which we have recently reviewed in detail [42]. Briefly, global decrease of miRNA regulation, by global downregulation of miRNA expression, mutations in miRNA biogenesis pathway, inhibiting miRNA biogenesis through oncogene signaling, or evasion of miRNA regulation by shortening three prime untranslated region (3′UTR), can positively impact tumorigenesis in both mouse and human.

In view of miRNAs as an epigenetic component that helps to define cellular states, we review below both studies that link miRNA directly with the regulation of epigenetic machinery in the context of HSCs and LSCs and miRNA-mediated regulation of HSCs and LSCs through other direct mechanisms, including regulating cell signaling. Table 1 summarizes the function of specific miRNAs in HSC and LSC regulation.

miRNA-Mediated Control of Epigenetic Program in HSCs and LSCs

A complex epigenetic program safeguards HSCs by maintaining the self-renewal potential and limiting overproliferation, the disruption of which leads to HSC exhaustion. Studies in the past several years have revealed striking levels of somatic mutations in the form of clonal hematopoiesis, particularly in healthy elderly individuals [96–100]. Among the most frequently mutated genes in clonal hematopoiesis are DNMT3A and TET2 [101–103], both of which are recognized as the founding genetic mutations in hematopoietic malignancies such as acute myeloid leukemia [100, 104, 105]. In healthy populations aged 65 or older, ~3 % contains somatic DNMT3A mutations and ~0.5–1 % with TET2 mutations, with mutant alleles detectable in ~20 % or more of mononuclear hematopoietic cells in peripheral blood. Both DNMT3A and TET2 are involved in the DNA methylation pathway. DNMT3A is a "de novo" methyltransferase that is not dependent on existing methylation marks. In contrast, TET2 is a member of the ten-eleven translocation (TET) family that catalyzes the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC). TET2 proteins can further oxidize 5hmC into 5 formylcytosine (5fC) and then 5-carboxyl cytosine (5caC) that can be subjected to excision repair-mediated active demethylation [106–108]. In addition, due to DNMT1 not recognizing 5hmC, oxidation of 5mC into 5hmC effectively leads to site-specific suppression of maintenance methylation activity and hence proliferation-based passive demethylation [109]. Functionally, despite opposite biochemical activities, genetic ablation of DNMT3A and TET2 can both lead to HSC amplification that outcompete normal HSCs [110–115]. In particular, TET2 demonstrates haplo-insufficiency both in human malignancy samples and in mouse models, which is interesting given that miRNAs often only partially suppress target gene expression. In this section, we will review the roles of miRNAs that target epigenetic machinery, with a focus on discussing their regulation of DNA methylation/demethylation enzymes.

miRNAs Regulating TET2 and Other DNA Demethylation Genes

TET2 has been demonstrated to be under extensive regulation by miRNAs. The miR-125 family miRNAs are among these TET2-targeting miRNAs. miR-125 family consists of miR-125a and miR-125b, with the latter encoded by two separate genomic loci on human chromosomes 11 and 21. Members of the miR-125 family appear to play redundant roles in mouse hematopoiesis [116]. Both miR-125a and miR-125b show

Table 1 The roles of specific miRNAs in HSC and LSC biology

miRNA	Species	Category	HSC/ LSC	OE phenotype	KD/KO phenotype	Target	Reference(s)
let-7	Mouse	Self-renewal	HSC		Lin28 overexpression in adult HSCs leads to increased self-renewal	HMGA2	[43•]
miR-101	Human/mouse		HSC	OE bone marrow transplant, myeloid expansion hematopoietic expansion		TET2	[44••]
miR-105	Human	Differentiation	HSC	OE CD34+ cells enhanced megakaryocyte production		c-Myb	[45]
miR-106a	Human	Differentiation	HSC	OE progenitor cells proliferation increases, monocytic differentiation block	KD repressed proliferation and increased differentiation/maturation of monocytes	AML1	[46]
miR-99a/ 100~125b	Human/mouse	Expansion	HSC	OE in HSC expansion		TGFbeta pathway	[47]
miR-125a miR-125b-2 miR-125b-2	Human/mouse	Expansion Proliferation, differentiation, oncogenic transformation, self-renewal	HSC	OE in HSC increases colony formation, increase in HSCs in vivo, leukemia, myeloid-biased proliferation, increased proliferation and self-renewal	Full-body miR-125a heterozygous KO leads to myeloproliferative phenotypes	Bak1, WT1, Lin28A, TET2, Bak1, Stat3, c-Jun, Dicer1, ST18, PTPN7, PTPN18, PPP1R16A	[24, 37, 48–53]
miR-126	Human/mouse	Proliferation	LSC/ HSC	OE in HSC impaired cell cycle entry, reduced hematopoietic contribution self-renewal OE in LSC quiescence, self-renewal	KD HSC Enhanced self-renewal LSC Proliferation, differentiation	PI3K, AKT, GSK3beta pathway, HOXA9	[54••, 55••, 56, 57]
miR-139-3p	Mouse/human	Proliferation	HSC	OE in CD34+ cells strongly inhibited proliferation/cell cycle arrest and apoptosis	KD restored defective proliferation of Ercc1-deficient progenitors	HuR, EIF4G2	[58, 59]
miR-142-3p	Zebrafish/mouse/ xenopus	Development	HSC		KD regulates HSC formation and differentiation	Irf7, TGFbeta signaling	[60•, 61, 62]
miR-145	Human/mouse					TIRAP	[63]
miR-146a	Zebrafish/mouse/ human	Differentiation	HSC	OE bone marrow transplant transient myeloid expansion, decreased erythropoiesis, impaired lymphopoiesis	KO decreased HSCs number, excessive myeloproliferation, HSC exhaustion, and hematopoietic neoplasms	TRAF6, NFkappaB, IL-6	[63–66]
miR-150	Mouse	Engraftment reconstitution	HSC	OE bone marrow transplant slowed recovery rates across major blood lineages after 5-fluorouracil treatment.	KO marrow recovers faster after 5-fluorouracil treatment.	c-Myb	[67]
miR-152	Human	Oncogenic transformation	LSC			MLL and DNMT1	[68]
miR-155	Human/mouse	Differentiation	HSC/ LSC	OE bone marrow transplant blocked both erythroid and myeloid colony formation in vitro.	KO resistance to experimental autoimmune encephalomyelitis	Pu.1, SHIP1	[38, 69–73]
miR-15a	Human	Differentiation	LSC	OE CD34+ cells blocked both erythroid and myeloid colony formation in vitro.		c-Myb	[74]
miR-15a/16-1 cluster	Human/mouse	Proliferation	LSC		KO accelerates proliferation		[75]
miR-17-92 cluster	Human/mouse	Oncogenic transformation	LSC	OE oncogenic transformation		c-Myc, Bim, p21	[76–78]
miR-181 family	Human/mouse	Differentiation/oncogenic transformation	LSC	OE CD34+ granulocytic and macrophage-like differentiation block/increased B-lineage	KD bone marrow transplant CD34+ improves myeloid differentiation, inhibited engraftment/infiltration	PRKCD, CTDSPL, CAMKK1	[79, 80]
miR-196b	Human	Oncogenic transformation	LSC	OE increased proliferation, survival, differentiation block	Antagomir reduced self-renewal in vitro		[81]
miR-199a-3p	Mouse	Proliferation	HSC	OE increased proliferation	KD reduced proliferation	Prdx6, Runx1, Suz12	[58]
miR-204	Human					HOXA10, MEIS1	[82]
miR-21	Mouse	Oncogenic transformation	LSC	Knock in OE leads to leukemia		STAT3	[76, 83]
miR-212/132	Mouse	Proliferation	HSC	OE rapid HSC cycling and depletion	KO reduced proliferation and survival	FOXO3	[84]
miR-22	Mouse/human	Self-renewal/oncogenic transformation	HSC/ LSC	Knock in OE increased self-renewal, differentiation block, MDS, and hematological malignancies	KD reduced proliferation	TET2	[85••]
miR-223	Human	Differentiation/oncogenic transformation	LSC	OE CD34+ enhanced differentiation	KD CD34+ cell differentiation block/aberrant differentiation	NFI-A, Mef2c	[86–88]

Table 1 (continued)

miRNA	Species	Category	HSC/LSC	OE phenotype	KD/KO phenotype	Target	Reference(s)
miR-27a	Human/zebrafish/mouse	Differentiation	HSC			GATA-2	[89]
miR-29a miR-29b-1 miR-29b-2	Mouse	Self-renewal oncogenic transformation	LSC	OE increased self-renewal, myeloid-biased differentiation, development of MDS/leukemia	KO reduced HSC numbers transcriptional deregulation	DNMT3a, DNMT3b, TET2	[44••, 90, 91•],
miR-33	Mouse	Self-renewal	HSC		OE of p53 in HSCs leads to downregulation	p53	[92]
miR-451	Zebrafish	Differentiation	HSC		KD red blood cell differentiation block		[93]
miR-495	Human	Oncogenic transformation	LSC	OE decreased cell viability and increased apoptosis		PBX3 and MEIS1	[94]
miR-7	Human/mouse					TET2	[44••]
miR-9	Human/mouse	Oncogenic transformation	LSC	OE promotes MLL fusion-mediated leukemia formation in vivo	KD (sponge) inhibits MLL fusion-induced leukemia formation		[95]

enriched expression in HSCs, and their expression decreases in more differentiated cell populations [37, 117, 118]. Interestingly, miR-125b is involved in rare cases of genomic rearrangements in both myeloid and lymphoid leukemia, which leads to increased miR-125b expression [48, 119]. Furthermore, increased expression of miR-125b from the miR-125b-2 locus has been observed in trisomy 21 cases, which demonstrate increased susceptibility toward acute megakaryocytic leukemia [49]. Even in the absence of direct genomic rearrangements involving miR-125 loci, overexpression of miR-125 family miRNAs has been observed frequently in acute myeloid leukemia and other hematopoietic malignancies and occasionally under the control of other oncogenes such as ETV6-RUNX1 fusion [120]. The functions of miR-125 family miRNA in the hematopoietic system have been reviewed previously [24]. In short, constitutive overexpression of either miR-125a or miR-125b leads to HSC expansion in vivo, and skewed myeloid and monocyte differentiation, with the latter phenotype dependent on continued miR-125 overexpression [50]. Among many targets of miR-125 family miRNAs that have been studied [24, 44••], miR-125-TET2-targeting relationships have been identified through a systematical screen for TET2-targeting miRNAs, and miR-125 is capable of altering the global 5hmC levels in hematopoietic cells [44••]. TET2 knockout displays similar phenotypes as miR-125 overexpression, and enforced expression of TET2 corrects nearly all miR-125 overexpression phenotypes including hematopoietic expansion and lineage differentiation skewing, supporting TET2 as an important target of miR-125 family miRNAs [44••]. Interestingly, other than TET2, other targets involved in attenuation of cytokine signaling have been reported [24, 50], placing this family of miRNAs as regulators of both epigenetic and signaling pathways. Germline knockout of miR-125a has recently been published. Curiously, heterozygous miR-125a knockout mice develop myeloproliferation similar to miR-125a overexpression, but this phenotype cannot be observed in homozygous miR-125a knockout mice [51]. Whether this is due to miR-125a function in the microenvironment, rather than within hematopoietic cells, remains to be determined.

Another miRNA family that target TET2 is the miR-29 family [44••, 121], which consists of miR-29a, miR-29b, and miR-29c. Unlike miR-125, miR-29 targets all three TET family members and regulates DNA methyltransferases and other components within DNA demethylation pathway. While the function of miR-29 family will be discussed in detail in the next section, overexpression of TET2 can also correct miR-29b-induced myeloproliferation [44••] supporting a role of TET2 as a downstream effector of miR-29 family miRNAs.

miR-22 overexpression, which is often seen in myelodysplastic syndrome (MDS), can also lead to direct targeting of TET2. Furthermore, in a genetic mouse model, overexpression of miR-22 leads to increased HSC activity and

MDS-like phenotypes, whereas overexpression of TET2 suppressed miR-22 phenotypes in vitro and in vivo, including a partial rescue of mouse survival [122]. In contrast to these findings, miR-22 has recently been described as a tumor suppressor in acute myeloid leukemia (AML), where it negatively regulates the expression of a number of oncogenes including CRTC1, FLT3, and MYCBP. In this study, loss of miR-22 could occur either via genetic lesion or TET1-mediated silencing [123] suggesting that TET family of proteins may be negatively regulating miR-22. In addition to the abovementioned miRNAs, miR-26 family has been shown to downregulate multiple TET family genes, including TET2, in both hematological and pancreatic contexts [124], and overexpression of miR-26a in wild-type bone marrow cells leads to a transient myeloid expansion in vivo [44••]. Similarly, overexpression of miR-101, which can target both TET2 and EZH2, leads to myeloid skewing in differentiation [44••, 125]. Other TET2-targeting miRNAs have been identified through the 3′ UTR screen of ~460 mouse and human miRNAs, some of which selectively target human TET2 over mouse TET2 (for example, miR-7 had a much weaker effect on mouse TET2), indicating a species-specific mode of regulation. In a cohort of cytogenetically normal AML, overexpression of miR-125b, miR-29b, miR-29c, miR-101, and miR-7 is significantly associated with TET2 wild-type cases than TET2 mutant cases, suggesting that these miRNAs may also underlie human leukemia formation [44••]. Why is TET2 under the regulation of so many miRNAs? Although the answer is unclear, it is possible that there might be a pressure to attenuate TET2 expression in multiple tissue types during development so that overdemethylation of the epigenome does not occur. In addition to TET family-mediated demethylation, activation-induced cytidine deaminase (AID) and APOBEC can also lead to active DNA demethylation, although their roles may be more context-specific. Interestingly, AID can be regulated by miR-155 [126] and miR-181b [127] in the context of B cells, and both of these miRNAs are elevated in MDS and leukemia CD34+ cells [69, 70, 79]. Whether these targeting relationships regulate HSCs needs further investigation.

miRNAs Regulating DNA Methylation

In addition to targeting TET family genes, the miR-29 family miRNAs have been demonstrated to regulate multiple DNA methyltransferase genes. miR-29b was initially identified as a potential tumor suppressor in established AML cells. When ectopic miR-29b was introduced into established human AML cell lines or patient blasts, induction of apoptosis and partial differentiation were observed [128, 129]. One of the studies found that miR-29b can target DNMT3A and DNMT3B directly and target DNMT1 indirectly through suppressing Sp1 [128] and miR-29b overexpression altered DNA methylation landscape in AML cells with re-expression of tumor suppressor genes. In contrast to these reports of miR-29b as a tumor suppressor in AML, miR-29a overexpression in wild-type hematopoietic cells leads to myeloproliferation and occasional AML progression, accompanied by aberrant self-renewal activity [90]. Recently, the effect of ablating miR-29a/miR-29b-1 cluster has been examined in the hematopoietic context. Their deletion leads reduced HSCs with reduced self-renewal activity. Reintroduction with miR-29a, but less so with miR-29b, leads to rescue effects, suggesting that miR-29a deficiency underlies this phenotype. Crossing miR-29a/miR-29b-1 heterozygous knockout mice with DNMT3A heterozygous knockout mice convincingly establishes that DNMT3A is an important target of miR-29 in this context [91•]. Notably, in miR-29a/miR-29b-1 knockout HSCs, DNMT3A is only upregulated mildly, at 1.55-fold the level seen in wild-type HSCs, suggesting a quantitative sensitivity of HSCs to small changes in DNMT3A levels. Furthermore, DNMT3A knockout cannot fully rescue the HSC deficiencies in miR-29 knockout, suggesting that other targets, such as TET2, may be involved. In addition to miR-29a, overexpression of miR-29b in wild-type bone marrow cells leads to myeloid expansion [44••]. Why do some studies found miR-29 family miRNA to be potential tumor suppressors and others assign oncogenic functions? One possibility is the cellular context differences, in which oncogenic functions are seen when miR-29 is examined in wild-type HSCs, whereas tumor suppressor functions are seen in established AML cells. The second possibility is that other targets of miR-29, such as TET2, MCL-1, or other targets [44••, 129], may alter biological output. It is thus interesting to note that some subtypes of AML are dependent on the oncogenic function of TET1 [130], which can also be targeted by miR-29 family miRNAs.

In addition to miR-29 family miRNAs, miR-199a has been demonstrated to target DNMT3A in other tissue contexts [131]. When overexpressed in the hematopoietic system, miR-199a leads to an expansion of myeloid progenitors [58]. Whether DNMT3A is functionally involved in the miR-199a effect is unknown. DNMT1 is also regulated by other miRNAs. miR-152, which potentially regulates DNMT1, is often heavily methylated in leukemia [68]. miR-21 [83] and miR-126 [54••] both of which play roles in leukemia have been shown to regulate DNMT1 in lymphoid cells [132, 133].

miRNAs Regulating Cell Signaling and Other Targets in HSC and LSC Biology

miRNA Regulation of Hematopoietic Development

In mammals, hematopoiesis is initially observed in the developing yolk sac, termed “primitive hematopoiesis.” This early wave can produce red blood cells and populations of

macrophages. HSCs emerge from the subsequent waves of definitive hematopoiesis, with HSC activity detected in the aorta-gonad mesonephros (AGM) region by budding off the hemogenic endothelium. These cells then migrate to the fetal liver and finally the bone marrow where they predominantly reside in the adult organism. A proliferation switch occurs in mouse roughly 2–3 weeks post-birth, with HSCs changing from actively cycling in the fetal and neonatal stage to largely quiescent in the adult stage [134].

miR-126 has been reported to regulate the primitive wave of erythroid differentiation through modeling embryoid body differentiation from murine embryonic stem cells [135•]. Gain of miR-126 expression increases the number of erythroid progenitors, whereas knocking out miR-126 reduces these progenitors. The mechanism is through non-cell-autonomous regulation of Vcam-1 in mesenchymal cell that, in turn, impacted erythroid progenitor development.

miRNAs also play important roles in the development of HSCs during definitive hematopoiesis. One important miRNA in this process is miR-142-3p. miR-142 is abundantly expressed and enriched in adult hematopoietic cells [80]. The precursor miR-142 gives rise to two mature miRNAs, miR-142-5p and miR-142-3p, from the 5′ and 3′ arm of the miRNA hairpin, respectively. Unlike many other miRNAs, both miR-142-5p and miR-142-3p are abundantly expressed. In xenopus, miR-142-3p is strongly upregulated in newly emerged HSPCs from hemogenic endothelium and is functionally required for the development of the hemangioblast through targeting TGF-beta receptor and its signaling [60•]. In zebrafish, miR-142-3p is abundantly expressed in RUNX1 and MYB-positive HSCs. Inhibition of miR-142-3p in zebrafish by morpholino leads to decreased HSCs in AGM, a defect that can be corrected by inhibiting Irf7 [61], suggesting that interferon signaling might be involved. Similarly in mouse, inhibition of miR-142-3p in AGM cells leads to reduced colony formation in vitro and reduced spleen colonies in vivo [61].

In fetal liver HSCs and adult HSCs, despite their cell cycle differences, a shared expression program regulates the expression of the maternally expressed imprinted Dlk1-Gtl2 locus [136]. This non-coding region harbors a large number of miRNAs as well as other non-coding transcripts. Inactivation of the expression of this region leads to defective fetal HSC maintenance, resulting in reduced long-term HSC function. Mechanistically, the miRNAs expressed within this region collectively target the phosphoinositide 3-kinase (PI3K)-mechanistic target of rapamycin (mTOR) signaling pathway. In the absence of this non-coding mechanism, overactivation of mitochondria metabolism occurs with excessive reactive oxygen species.

The post-natal switch between the fetal/neonatal hematopoiesis and the adult hematopoiesis program is controlled by a Lin28b-let-7-HMGA2 axis. let-7 is a family of miRNAs initially identified in *Caenorhabditis elegans* that controls the timing of larval development [137]. let-7 is highly conserved during evolution, and its processing from primary miRNA to precursor miRNA, and from precursor miRNA to mature miRNA, is inhibited by the RNA-binding protein Lin28A and Lin28B [138–140]. In most tissues, let-7 is a tumor suppressor miRNA that downregulates a plethora of oncogene targets, including HMGA2 [141], Ras [142], and Myc [143]. Both Lin28B and HMGA2 are expressed higher in fetal HSCs versus adult HSCs, whereas a few members of let-7 family miRNAs show opposite expression. Overexpression of Lin28B and HMGA2 leads to increased self-renewal of adult HSCs, whereas HMGA2 KO inhibits the heightened self-renewal of wild-type fetal HSCs [43•]. Consistent with this role, ectopic expression of Lin28 in adult HSPCs causes the adaptation of a fetal lymphopoiesis-like program [144], whereas overexpressing let-7 can drive fetal pro-B cells to become more adult-like, through repression of Arid3a [145].

miRNA Regulation of Adult HSC Self-Renewal and Quiescence

Balance of HSC self-renewal, quiescence, and differentiation is tightly controlled in order to maintain a relatively constant HSC pool size over the lifespan of an organism [146]. HSCs are largely quiescent, and the cell cycle status of the HSC reflects their functional output [7, 147, 148]. A number of miRNAs function to positively or negatively regulate HSC self-renewal and/or quiescence. In addition to the aforementioned miR-125 and miR-29 family miRNAs, miR-196b has enriched expression in HSPCs, although expression of this miRNA is higher in short-term HSCs than long-term HSCs. miR-196b is located within the HoxA cluster, and its expression is controlled by mixed-lineage leukemia (MLL). Overexpression of miR-196b in vitro leads to increased c-Kit$^+$ myeloid progenitor cell expansion with hypersensitivity to cytokine stimulation, suggesting modulation of cell signaling [81]. However, the impact of miR-196b on stem cell activity and its targets was not characterized. In contrast to the above miRNAs, overexpression of miR-126 inhibited the self-renewal of HSCs in both human and mouse models, whereas lentiviral sequestering of miR-126 leads to increased HSC cycling and expansion without evidence of exhaustion. This miRNA suppresses multiple members of the PI3K-AKT signaling pathway, and a PI3K inhibitor can reduce cell proliferation upon miR-126 inhibition in vitro [55••]. miR-155 is also capable of expanding HSCs when overexpressed. However, sustained miR-155 overexpression leads to MDS [69] through regulation of PU.1 [71] and SHIP1 [72].

Role of miRNAs in HSC Aging

In mammals, aging leads to an increase in the number of immunophenotypic HSCs but an overall decrease in HSC competitiveness and a skewing toward myeloid differentiation [149]. The miRNA-212/132 cluster shows enriched expression in HSCs and Lin^- cells compared to more differentiated progenies. The expression of this cluster further increases during aging [84]. Knocking out this cluster does not strongly impact HSCs under normal conditions, but under conditions of inflammatory stress by LPS, knockout HSCs show reduced cycling [84]. LPS is a gram-negative bacteria endotoxin that can lead to both HSC cycling and a premature aging-like phenotype [150]. Furthermore, miRNA-212/132 knockout modestly improved competitive repopulation of aged HSCs but not young HSCs [84]. Related phenotypes have been observed in miR-146a knockout mice. miR-146a was initially discovered as an LPS-inducible miRNA in macrophages [64] and is thus involved in immune responses [151]. It is also located on chromosome 5q and, together with miR-145, has been demonstrated to induce MDS-like conditions in mice when both miRNAs are inhibited [63]. miR-145 has been reported to cooperate with another 5q gene, RPS14, in regulating erythroid and megakaryocyte differentiation [152]. Knockout of miR-146a does not affect HSCs at 6 weeks, but with increased age, miR-146a knockout mice develop myeloproliferation with a gradual decrease in HSC number and function, leading to HSC exhaustion and neoplasm [153••]. The function of miR-146a can be attributed to miRNA-mediated regulation of inflammatory signaling by targeting TRAF6 and resulting in IL-6 deregulation [153••].

Role of miRNAs in HSC Niche

A complex niche environment regulates HSC and HSPC behavior in vivo. Can miRNAs in niche cells regulate hematopoiesis? The first supporting evidence was revealed in a Dicer knockout model. Mice lacking Dicer1 in their osteoprogenitors, via Sbds expression reduction, develop an MDS-like condition, which progresses to leukemia [154••]. Transplanting wild-type bone marrow cells into mutant host can induce the MDS phenotypes, whereas transplanting MDS bone marrow cells from mutant mice into wild-type recipients corrects the phenotype, supporting a non-hematopoietic role of Dicer1 in modulating HSPC differentiation activity. Although Dicer1 processes additional RNA species other than miRNAs, a HITS-CLIP study performed in murine bone marrow endothelial cell and mesenchymal cells revealed extensive endogenous binding and regulation by miRNAs on potential niche-contributing signaling factors. These include miR-193a regulating JAG1, miR-200a regulating WNT5A, miR-9 regulating MMP2, and miR-185 regulating VEGFA [155]. Other candidate miRNAs that may regulate HSPCs within niche has been reported by a study comparing stromal cell lines that support HSC/HSPC activity in vitro versus those that do not. miRNAs that positively correlate with supportive activity include miR-143, miR-214*, miR-9*, miR-222, miR-342 3p, miR-193*, miR199b*, miR-214*, miR674*, mIR-351, miR-7a-1, miR-148b, miR-455, and miR-199a-3-p, whereas miR-155 and miR-411 are anti-correlated with HSC/HSPC supportive activity [156]. The function of miR-155 in the bone marrow microenvironment has been studied comprehensively in a myeloproliferative neoplasm (MPN) model induced by knocking out Notch-signaling component recombination signal binding protein for immunoglobulin kappa J region (RBPJ). RBPJ knockout leads to an elevation of miR-155 in bone marrow endothelial cells, which, in turn, upregulates pro-inflammatory signaling through repressing kB-Ras1 and hence NF-kB activation, resulting in myeloproliferation in a non-hematopoiesis-autonomous fashion [157•]. It is currently unclear whether the anti-correlation between miR-155 and non-supportive stromal lines is related to this functional activity of miR-155 regulating inflammatory signaling in the bone marrow microenvironment.

Another area with active miRNA research that relates to HSC niche is inter-cellular signaling/communication by secretory exosomes, which contain miRNAs. For example, exosomes from mesenchymal stromal cells contain a number of miRNAs, including miR-451, miR-1202, miR-630, miR1207-5p, miR-33b, miR-1268, miR-638, miR-575, and miR-1225-5p [158]. Whether exosomal miRNAs from bone marrow stromal cells regulate hematopoiesis in vivo is currently unknown. However, a recent study shows trans-cell delivery of cre in vivo [159], suggesting that this mode of epigenetic material exchange between cells may functionally impact hematopoiesis.

Role of miRNAs in LSCs

Comparisons between LSC-enriched populations versus their normal counterparts have revealed a long list of potential miRNAs that are differentially expressed in LSCs versus HSCs and have been discussed elsewhere [20]. Given the inability to isolate either LSCs or HSCs to perfect purity, whether these miRNAs are indeed differentially expressed between functional LSCs and HSCs is unknown. Additional studies sought to stratify phenotypic AML LSCs based on their ability to engraft in an immune compromised mouse. A comparison of miRNA expression and the engraftment ability identified miRNAs both positively (miR-99a, miR-125b, miR-155, miR-409, miR-100, miR-320, miR-126, miR-1, miR-542, and miR-15b) and negatively (miR-451, miR-103,

miR-200c, miR-423-3p, let7g, miR-30e, miR-26b, miR-140, miR-22, and miR-21) correlate with AML engraftment and LSC activity [54••]. These candidate miRNAs provide a perfect list for functional validation as only engraftment and leukemia reconstitution is a reliable way of defining LSCs.

Some LSC-enriched miRNAs have been studied and positively influence leukemia development. Remarkably, most of these miRNAs can target enzymes that control DNA methylation. Overexpression of miR-125b-1 [24], miR-125b-2 [49], miR-155 [69], and miR-126 [54••] results in leukemia phenotype, and all of these miRNAs are capable of regulating enzymes which control DNA methylation as discussed previously. The best-studied miRNA in human AML LSCs is miR-126, whose high expression in leukemia specimens correlates with AML survival [54••]. However, opposite to its role in human HSCs discussed earlier, this same miRNA enhances LSC self-renewal and prevents differentiation in vivo. Intriguingly, miR-126 also seems to exert its effect in LSCs through the same PI3K-AKT-mTOR pathway as in HSCs, even though the biological outcome in HSCs and LSCs is different [54••]. These data suggest that the human LSCs behave similarly as fetal liver HSCs [136], in which unchecked PI3K-mTOR pathway damages stem cells, whereas adult HSCs may be opposite.

Among other miRNAs present on the LSC-enriched list, miR-99a and miR-100 are located in close proximity to the miR-125 family members [47] and often co-expressed with miR-125 miRNAs. Interestingly, elevated miR-1 expression is strongly correlated with IDH1/2 mutation status [160], and IDH1/2 mutation negatively regulates TET2 activity. miR-320 [161] and miR-15 [75] have been demonstrated to inhibit cell proliferation, which might be relevant to stem cell quiescence. Further work will be needed to assign functional roles for LSC-enriched miRNAs.

Most miRNAs whose expression is anti-correlated with engraftment have not been studied carefully in the context of HSCs of LSCs. Nevertheless, miR-22 has been discussed above which causes an MDS-like phenotype when overexpressed in wild-type cells and regulates TET2 gene expression.

Summary, Outlook, and Challenges

As evidenced above, miRNAs are functionally involved in most, if not all, aspects of stem cell behaviors in both normal and malignant hematopoiesis. Mechanistically, two common themes emerge from the diverse array of miRNAs and their targets. The downstream pathways of miRNAs that regulate HSC and LSC biology are overall enriched for factors that regulate other epigenetic machineries and/or cell signaling. Some miRNAs regulate both. For example, miR-125 family miRNAs target both TET2, an epigenetic enzyme [44••], and control the strength of cytokine signaling with inhibition of multiple protein phosphatases [50]. miRNA regulation of these two categories of targets makes much sense. miRNAs are known as quantitative regulators of target gene expression, and thus, the functional output of miRNAs must be mediated through factors or combinations of factors that are sensitive to quantitative alterations, rather than black and white binary distinctions. Many of the epigenetic machinery enzymes are intricately controlled in expression. A subtle change in their expression may cause widespread genome-level alterations in epigenetic landscape. For example, TET2 is a well-known haplo-insufficient tumor suppressor [162]. Loss of function of Ezh2, which occurs predominantly on a single allele, is observed in myeloid malignancies [163]. Similarly, signaling pathways are naturally balanced with both activation signaling and deactivation proteins, and quantitative alteration in signaling factors may lead to much amplified functional output than the level of miRNA-mediated target regulation.

In addition to their direct regulation of epigenetic enzymes, we propose that miRNAs should be considered as epigenetic regulators themselves, as they possess key biochemical and biological properties that may provide both stability and alterability to the epigenetic program. This is consistent with a proposed role of miRNAs as buffers for noise in biological systems overall [164].

While exciting progresses have been made, we also highlight three challenging areas that require more intensive research to further elucidate and capitalize on the roles of miRNAs in normal and malignant stem cells. First is on the regulation of miRNAs themselves. Overall, there lacks a clear understanding on what attributes to the specific expression patterns of miRNAs in HSCs and LSCs. The expression of miRNAs can be regulated on the level of primary miRNA transcription, miRNA processing, miRNA loading into AGO proteins, and miRNA stability. A few studies have examined the transcriptional control of miRNAs. For example, miR-146a can be regulated by NF-kB signaling [165], miR-125b can be regulated by HoxA10 [47], and MLL regulates the expression of multiple miRNAs, including miR-196b and miR-150 [81, 94]. However, what genetic elements and transcriptional programs that endow HSC-enriched expression patterns for most of the functional miRNAs are largely unknown. Even less is known about the post-transcriptional control of miRNAs in hematopoiesis. We have shown that human miR-125b-1 behaves differently from other miRNAs in that it has a much-decreased reliance on HSP90 activity when loading into AGO proteins [32•]. As another example, it is interesting to note that a human SNP in the XRN1

gene, which actively regulates miRNA let-7 degradation in *C. elegans* [166], is associated with mean corpuscular volume of red blood cells in human [167]. These data argue for the importance of examining the post-transcriptional regulation of miRNAs in hematologic systems, which can help reveal mechanisms that underlie stem cell-enriched expression of miRNAs and their downregulation during cell fate changes. Second, for the mechanisms of miRNAs, there is an overreliance on a single miRNA single target scheme, which unfortunately is a common problem across most disciplines of miRNA studies. As each miRNA is known to quantitatively regulate multiple targets, it is conceivable that the function of miRNAs may be contributed by small changes of multiple target genes. Of course, functional integration of quantitative regulation is challenging, and new methodologies are required to push this area forward. The fast development in the CRISPR-based gene editing and gene expression controls may provide a viable approach to control multiple downstream targets of a miRNA of interest. Third, while the functional analysis of miRNAs in HSCs and LSCs have provided mechanistic insight, multiple miRNAs could be interesting candidates for therapeutic modulation. For example, loss of miR-126 and gain of miR-125 family miRNAs both can enhance HSC self-renewal and could potentially be used in clinical amplification of stem cells if they or their inhibitors can be delivered into HSCs. While multiple methods of miRNA or miRNA inhibitor delivery in vitro has been possible [168], small RNA delivery in vivo is overall challenging. Recent studies, however, have showcased a few examples in which nanoparticles have been used to successfully deliver miRNAs or miRNA inhibitors in vivo (e.g., [169]), which may become a route toward successful translation of the basic biological insights on miRNAs.

In summary, exciting progresses in the past decade have provided ample evidence supporting the functions of miRNAs in multiple facets of HSC and LSC biology, including HSC emergence during embryogenesis, HSC switch from a fetal/neonatal program to an adult program, HSC self-renewal and quiescence, HSC aging, HSC niche, and malignant stem cells. The studies of miRNAs in stem cells in the hematologic contexts not only provides key insights into the mechanisms of HSC and LSC controls but also will actively influence the studies of other stem cell areas by establishing key principles.

Compliance with Ethical Standards

Conflict of Interest Christine Roden and Jun Lu declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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