THE ROAD TO PATHOGENESIS: CHARTING THE DEVELOPMENT OF LSCs AND PRE-LSCs

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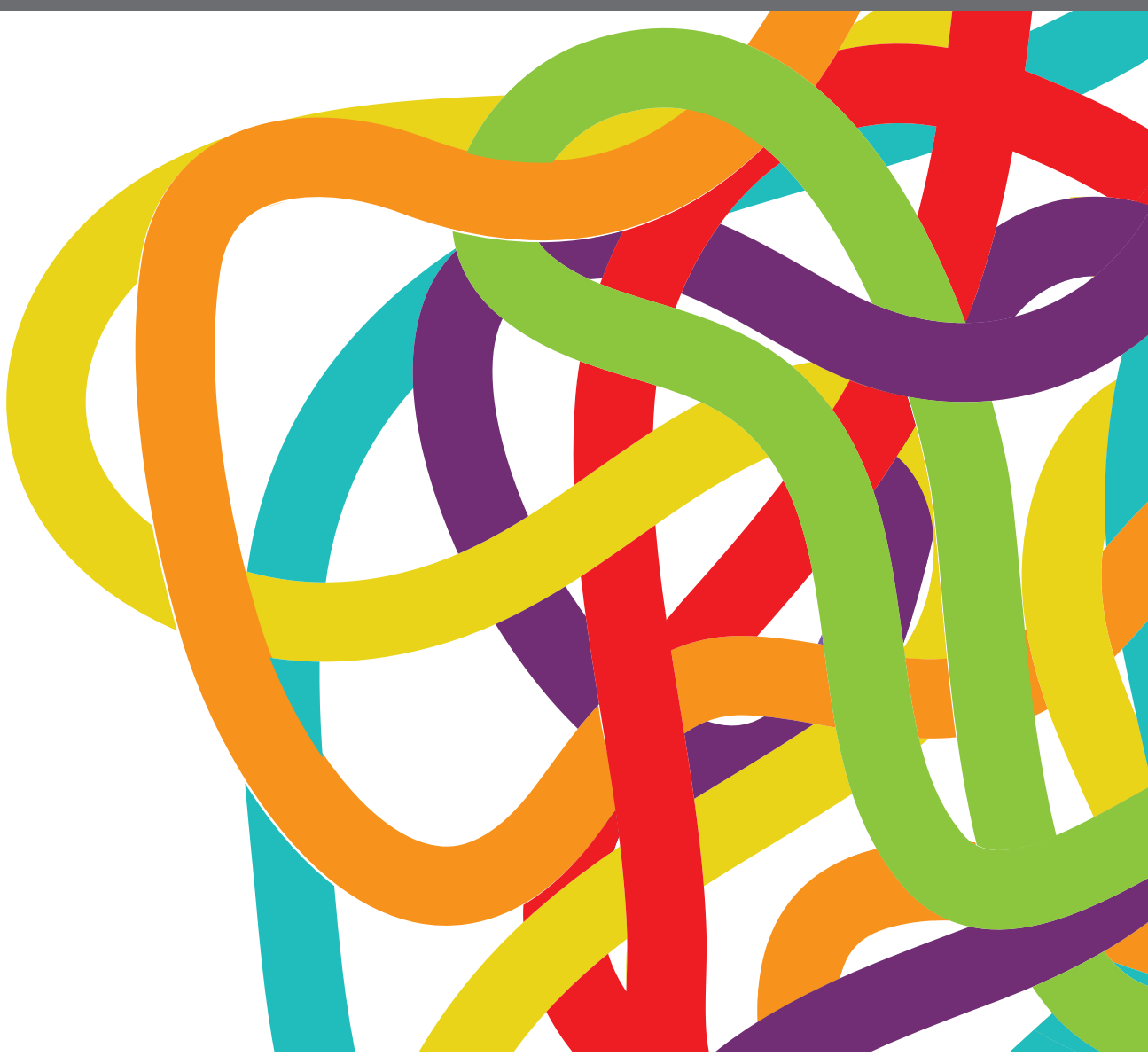

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THE ROAD TO PATHOGENESIS: CHARTING THE DEVELOPMENT OF LSCs AND PRE-LSCs

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MINI REVIEW
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Preleukemia and Leukemia-Initiating Cell Activity in inv(16) Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is a collection of hematologic malignancies with specific driver mutations that direct the pathology of the disease. The understanding of the origin and function of these mutations at early stages of transformation is critical to understand the etiology of the disease and for the design of effective therapies. The chromosome inversion inv(16) is thought to arise as a founding mutation in a hematopoietic stem cell (HSC) to produce preleukemic HSCs (preL-HSCs) with myeloid bias and differentiation block, and predisposed to AML. Studies in mice and human AML cells have established that inv(16) AML follows a clonal evolution model, in which preL-HSCs expressing the fusion protein CBFβ–SMMHC persist asymptomatic in the bone marrow. The emerging leukemia-initiating cells (LICs) are composed by the inv(16) and a heterogeneous set of mutations. In this review, we will discuss the current understanding of inv(16) preleukemia development, and the function of CBFβ–SMMHC related to preleukemia progression and LIC activity. We also discuss important open mechanistic questions in the etiology of inv(16) AML.

Keywords: myeloid, leukemia, CBFB-MYH11, CBFβ-SMMHC, preleukemia, clonal evolution, leukemia-initiating cell, stem cells

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INTRODUCTION

The core-binding factor (CBF) transcription factor has critical roles in hematopoietic stem cell (HSC) maintenance and differentiation by regulating expression of genes associated with cell fate decisions and proliferation in lymphoid and myeloid compartments (1). The CBF has two core subunits and is frequently associated with cofactors that modulate their activity or provide target specificity. The subunit CBFβ increases RUNX affinity to DNA approximately 40-fold and stabilizes RUNX protein from proteasome degradation (2–4). The subunit RUNX (encoded by either *RUNX1*, *RUNX2*, and *RUNX3* genes) binds to DNA at promoters and enhancers (consensus sequence TGYGGT). RUNX is the docking subunit that interacts with CBFβ and cofactors and has the nuclear localization signal (5, 6).

From the clinical and mechanistic points of view, AML is a collection of hematologic malignancies marked by specific driver mutations. *RUNX1* and *CBFB* genes are recurrently mutated in AML. Although a variety of mutations in *RUNX1* have been described in hematologic malignancies, the only rearrangement associated with *CBFB* is the pericentric inversion inv(16)(p13q22), henceforth inv(16), in leukemia (7–9). The inv(16) generates the fusion gene *CBFB-MYH11*, encoding the leukemia fusion protein CBFβ–SMMHC (10). Most of inv(16) AML cases have a myelomonocytic morphology with abnormal eosinophils and are classified as AML subtype M4-Eo,

and in rare occasions as AML subtypes M0, M1, M2, and M5 [French–American–British system (11)]. In spite of the morphology, the inv(16) AML transcriptome clusters as a single entity, suggesting a common underlying molecular alteration (12). The World Health Organization grouped "inv(16) AML" within the "AML with recurrent genetic abnormalities" based on genetic, molecular, and clinical features (13).

The name preleukemia has been used in different contexts in hematologic malignancies and has evolved in the past years (14). The preleukemic HSCs (preL-HSCs) can be considered as HSCs with inv(16) as a founding mutation that generate a clonal expansion of myeloid progenitor cells primed for leukemia (15). In this review, we summarize the current understanding in preleukemia progression of inv(16) AML.

CBFβ–SMMHC DOMAINS THAT REGULATE LEUKEMIA DEVELOPMENT

Two domains in CBFβ–SMMHC that are critical for its leukemogenic function: the *RUNX binding domain* (RBD) and the *assembly competence domain* (ACD) (**Figure 1**). The RBD, corresponding to the 135 N-terminal amino acids of CBFβ region at the N-terminus of the fusion protein, binds to the RUNX factors (16, 17). Genetic evidence, using *Cbfb*$^{+/MYH11}$ knock-in mice, revealed that RUNX activity is essential for CBFβ–SMMHC-associated leukemia function. Accordingly, reduction of *Runx1* or *Runx2* expression inhibited CBFβ–SMMHC-mediated differentiation block in embryos and leukemia onset in mice (18, 19). Furthermore, the increase in Runx2 levels reduced leukemia median latency (20). RUNX1 also interacts with the *high-affinity binding domain* (HABD), at the N-terminus of SMMHC. Surprisingly, RUNX1 binds to CBFβ–SMMHC with approximately 10-fold higher affinity to than to CBFβ. Its dual interaction with the RBD and HABD provides a rationale for the observed dominant negative function of the fusion protein outcompeting CBFβ for RUNX1 binding (21). A later study using *Cbfb*$^{+/MYH11d179\text{-}221}$ knock-in mice expressing CBFβ–SMMHC lacking the HABD established that HABD regulates myeloid differentiation induced by CBFβ–SMMHC but it may actually inhibit leukemia by altering the LIC pool (22). These findings have direct clinical significance because although the majority of inv(16) AML cases include HABD sequence in the *CBFB-MYH11* transcripts, fraction of cases lack HABD sequence due to a different breakpoint on the *MYH11* part of inv(16). The 28 amino acid ACD near the C-terminus is responsible for the oligomerization of CBFβ–SMMHC molecules and formation of filament structures (23–25). The ACD activity is needed for CBFβ–SMMHC's ability to inhibit myeloid differentiation, regulate the expression of CBF targets, and to reduce cell cycle and its nuclear localization *in vitro* (26, 27). Two recent studies using different inv(16) leukemia models have established that the ACD is essential for the expansion of preleukemic cells and for leukemia development (28, 29). Furthermore, the analysis of preleukemic progenitor cells revealed that ACD activity is critical for block in early B-cell differentiation but that sequences outside the ACD in the fusion protein impair T-cell differentiation. Finally, the C-terminal 95 amino acid region of CBFβ–SMMHC, which includes the ACD, binds to the histone deacetylase HDAC8 (30, 31). This interaction is essential for the inv(16) LIC activity because HDAC8 deacetylates p53, rendering it inactive, and modulates the transcription repression function of the fusion protein (31). Finally, inhibition of CBFβ–SMMHC binding to these factors may efficiently reduce preL-HSC and LIC activities, resulting in promising candidates for targeted therapies (32).

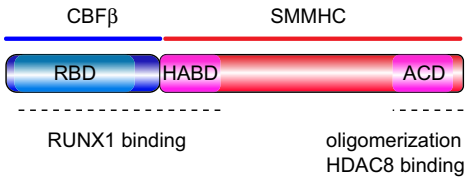

FIGURE 1 | Protein organization of CBFβ–SMMHC. Schematic representation of the CBFβ–SMMHC fusion protein, including the RUNX1-binding domain (RBD) at the N-terminus of CBFβ, the *high-affinity binding domain* (HABD) at the proximal end of SMMHC, and the *assembly competence domain* (ACD) near the C-terminus in the SMMHC region. Functional regions are marked with dash line at the bottom.

THE ORIGIN OF inv(16) PRELEUKEMIA

Our understanding on the origin of AML is still evolving, and in general terms it seems to follow a clonal evolution model (33–35). In inv(16) AML, a small number of studies have tested the origin of inv(16) preL-HSCs in the hematopoietic system. Studies using a *breakpoint backtracking* approach evaluated whether the inv(16) breakpoint identified in the DNA of a patient's inv(16) AML sample is present in the patient's neonatal bloodspot (also called Guthrie card or neonatal heel prick). Two studies identified the inv(16) breakpoint in the bloodspots, demonstrating that preL-HSCs can originate during fetal development and persist quiescent for years (4 to 10 in these studies) before AML diagnosis (36, 37). In a third case with inv(16) AML, the bloodspot analysis was negative suggesting that either the preL-HSCs were infrequent (below the sensitivity of the assay) or that inv(16) occurred postnatally. Of note, since backtracking studies have only been done in pediatric inv(16) AML cases, it is unknown if inv(16) preL-HSCs are prenatal in adult AML. Breakpoint backtracking studies for other leukemia fusion genes, such as RUNX1-RUNX1T1 and TEL-RUNX1, have also confirmed the prenatal origin of preL-HSCs (38–40).

The screening of leukemia fusion transcripts using RT-PCR analysis in healthy individuals revealed that 1 of 10 cord blood and 1 of 58 peripheral blood samples from adult individuals were *CBFB-MYH11* positive (41). These results lack statistical value due to the reduced sample size but suggest that preL-HSCs may persist in the hematopoietic system for years. However, the use of RT-PCR has been disputed because of the challenge in identifying the chromosome breakpoints in fusion transcript positive samples of healthy individuals (42, 43), result that could be explained by transplicing (44, 45).

THE inv(16) PRELEUKEMIC PROGRESSION

The identification of inv(16) preL-HSCs and progenitor cells has important therapeutic value because it is considered the source of leukemia development, drug resistance, and relapse. From a conceptual point, it would shed light on the etiology of disease progression. Studies in mice where allelic *CBFB-MYH11* expression is activated in hematopoietic cells have established that leukemia is preceded by a preleukemic period of 4 to 6 months, and the median leukemia latency can be delayed or render incomplete penetrance by reducing the number of HSCs expressing *CBFB-MYH11* (28, 46). Furthermore, chimeric mice (composed by $Cbfb^{MYH11/+}$ embryonic stem cells and wild-type blastocyst cells) expressing *CBFB-MYH11* in a fraction of their HSCs remained healthy and only developed AML when treated with chemical or retroviral mutagenesis (20, 47). These studies determined that *CBFB-MYH11* expression is necessary but not sufficient for leukemogenesis.

During the preleukemic period, $Cbfb^{MYH11/+}$ HSCs produce abnormal hematopoiesis, with cell compartment-specific defects, myeloid bias, and multilineage differentiation block (**Figure 2**). In the early progenitor compartment, CBFβ–SMMHC expression induces expansion of the short-term HSCs and multipotential

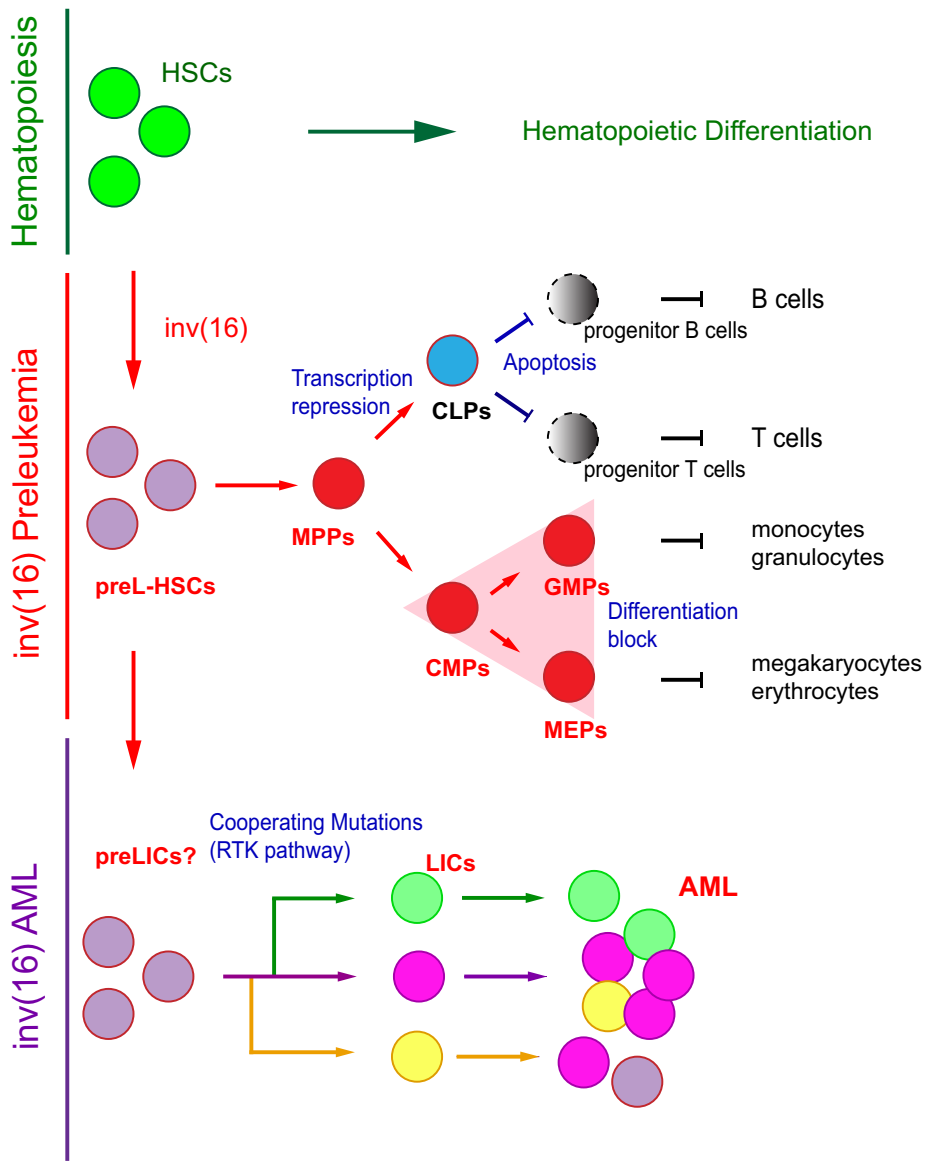

FIGURE 2 | Model of inv(16) associated preleukemia. Normal hematopoiesis is summarized on top. Preleukemia hematopoietic stem cell (preL-HSCs) with inv(16) and myeloid preleukemic progenitors (triangle compartment) are shown in red; preL-HSC-derived lymphoid progenitors and lineages with differentiation block are depicted in gray. Clonal expansion of leukemic cells from a putative preleukemia initiating cell with inv(16) and "cooperating" mutations are shown in green, violet, and orange.

progenitor (MPP) cells, although the frequency of long-term HSCs (putative preLICs) is unchanged, indicating that CBFβ–SMMHC may modulate factors associated with cell-fate decisions (46, 48).

These HSCs undergo normal early lymphoid differentiation, with normal numbers of common lymphoid progenitors (CLPs) but with reduced expression of transcription factors (*Ebf*, *E2a*, and *Pax5*) responsible for the commitment to B and T cell differentiation (49). During B cell commitment, CBFβ–SMMHC induces a marked reduction in pre-pro B cells and in pre-B cells due to apoptosis. These blocks are probably due to repression of RUNX1 activity because similar deficiencies were reported in *Cbfb*- and *Runx1*-knockout mice (50–52). Similarly, differentiation of CBFβ–SMMHC-expressing CLPs to T cell progenitors showed reduced cell number and viability of the double-negative compartments (53). Its repressive function in the production of lymphoid cells in humans was confirmed by fluorescent *in situ* hybridization analysis of lineage sorted inv(16) AML cells (54). Interestingly, the inability of inv(16) preL-HSCs to differentiate to B and T cells provides a mechanism for the myeloid leukemia bias observed in inv(16) AML.

CBFβ–SMMHC-expressing preL-HSCs undergo partial myeloid differentiation, displaying a mixed myeloid-erythroid progenitors (MEPs) and common myeloid progenitors (CMPs) immunophenotype [**Figure 2**, red triangle (46)] and a predominant blast/myeloblast and promyelocyte morphology. Contrary to its strong apoptotic activity on the lymphoid compartment, CBFβ–SMMHC increases the viability of preleukemic myeloid cells and enhances their resistance to genotoxic stress (46, 48, 55). The mechanism by which CBFβ–SMMHC blocks myeloid differentiation is not fully understood. Expression studies suggest that levels of a number of myeloid factors are affected by the fusion protein, including the repression of transcription factors that regulate myeloid lineage commitment (e.g., Cebpa, PU.1, Sox4, Hoxa9, and Irf8), some of which are known Runx1 targets. On the other hand, upregulated factors in preleukemic myeloid cells are implicated in survival and proliferation pathways [e.g., Csf2rb, il1rl1, Fosb, c-Jun, Erg1, and WT1 (28, 55, 56)]. Despite significant progress in this area, it is not clear which of these targets directs differentiation block in inv(16) AML. For example, the myeloid transcription factors C/EBPα and PU.1, both CBF targets, act as tumor suppressors in AML (57–59). In addition, Sox4 has been shown to function as an oncogene in *Cebpa*-mutated AML (60). On the other hand, expression of the colony stimulating factor 2 receptor beta (Csf2rb), is expressed in myeloid progenitor cells of *Cbfb*$^{56M/+}$*;Mx1Cre* mice and has a negative correlation with preL-HSC activity (56).

Transplantation studies of inv(16) preleukemic myeloid cells in mice, revealed that preleukemic cells could not induce leukemia in irradiated recipients (28, 46), indicating that preL-HSCs are not LICs, and that "cooperating" mutations are needed for leukemia transformation. Alternatively, the LIC activity is possibly present at a frequency below 1 in 20,000 preleukemic cells. Therefore, as rare preL-HSCs differentiate to myelomonocytic preleukemic cells and accumulate in the MEP/GMP compartment, additional events seem to be required for leukemia transformation.

LIC ACTIVITY IN inv(16) AML

Our understanding of LIC activity is evolving rapidly with the application of new technologies. Using targeted sequencing techniques in diagnostic inv(16) AML samples, studies have identified an average of 3 (range = 0–6) secondary mutations per sample (61, 62). The majority of inv(16) AML "cooperating" mutations are in genes encoding components of the RTK pathway, with predominance *KIT*, *FLT3*, and *NRAS* (63–65). In contrast, mutations in genes associated with components of cohesin or chromatin complexes are rare (62, 66). Evidence for inv(16) and PU.1 associated leukemia in mice suggests that transformation of preleukemic progenitors could be enhanced by mutations that "weaken" its oncogenic repression activity, thereby moving the differentiation block to a more mature myeloid progenitor that is permissive for transformation (22, 67). This model has been previously illustrated using mouse models for *CEBPA*-mutated AML. *Cebpa*-null mice show differentiation block at the CMPs and remain leukemia free. However, in mice carrying a leukemia-associated *Cebpa* point mutation, differentiation continues to stall at the committed myeloid progenitors and mice succumb with myeloid leukemia (68, 69). The molecular mechanism underlying this perplexing function, however, remains unknown.

inv(16) AML follows the clonal evolution model, whereby *de novo* inv(16) AML samples at diagnosis are composed of multiple leukemia subclones, which have emerged from the same preL-HSCs (**Figure 2**). The subclones share the founding mutation but have a different combination of "cooperating" mutations (70). Each subclone originates from an independent LIC with a different mutation combination and sensitivity to therapies. In addition to the leukemia subclones, the *de novo* AML sample includes preL-HSCs with reduced chemosensitivity, and that may serve as precursors for the expansion of resistant clones at relapse (15, 71). Longitudinal (diagnosis/relapse-matched) studies of AML mutational landscape using whole-genome sequencing have confirmed the clonal evolution model in inv(16) AML (72, 73). In these studies, the AML samples contained 1 to 18 "cooperating" mutations (mean = 6), corresponding to 1 to 3 mutations per subclone. In addition, inv(16) was found in all subclones at both stages of disease progression while a heterogeneity in the "cooperating" mutations indicated clonal evolution and differential sensitivity to therapy. Studies in mice have validated the basic premise of this model in inv(16) AML (48, 74, 75), and the weak LIC activity reported in human and mouse studies was validated in titration dilution transplantation experiments (48).

inv(16) AS A "COOPERATING" MUTATION IN LEUKEMIA

The inv(16) is predominantly a founding mutation that predisposes to *de novo* AML. Accumulating case reports have identified inv(16) in other hematologic malignancies clearly showing that this inversion, at a low frequency, can also originate as a "cooperating" mutation in the progression of other cancers. The inv(16) can emerge in BCR-ABL-positive chronic myelogenous leukemia

(CML) cases transitioning to blast crisis (76–80). The appearance of a inv(16)-positive predominant clone is accompanied by a switch to an immature monocytic morphology and dysplastic eosinophils. In CML cells, the occurrence of inv(16) predicts rapid evolution and poor outcome (77, 80). In addition, inv(16) has been reported in 1–2% of tAML cases that progressed from MDS or solid tumors (81). Probably due to the paucity of these cases, the mechanism of CBFβ–SMMHC function in the LICs from CML-PB or tAML cases has not been studied. However, the understanding of its function when acting as a "cooperating" mutation could open new insights on leukemia progression. It should be noted that CBFβ–SMMHC function in the LIC of CML-chronic phase (i.e., with active proliferative signals) or post-therapy HSCs/MDS (i.e., with higher mutation content) may involve different targets.

CONCLUSION AND PERSPECTIVES

The inv(16) is a somatic mutation that activates CBFβ–SMMHC expression in an HSC, either *in utero* or after birth. Indirect evidence suggests that these preL-HSCs can perdure for years to produce a clonal population with myeloid bias and impaired differentiation. Over time, the preL-HSCs are primed for leukemogenesis after acquiring a relatively small number of "cooperating" mutations, predominantly in components of the RTK pathway. The finding that mutations in genes associated with epigenetic complexes, frequently mutated in other AMLs, are practically absent in inv(16) AML suggests that CBFβ–SMMHC function may deregulate chromatin dynamics.

Future studies are endowed to demonstrate whether preL-HSCs can produce preleukemia initiating cells in inv(16) AML (**Figure 2**). The application of new technologies, such as single cell analysis, next-generation sequencing, CRISPR/Cas9 editing in primary hematopoietic stem and progenitor cells, pharmacology, and sophisticated animal models will greatly enhance our understanding of inv(16) preleukemia biology and minimal residual disease. Considering that each LIC in diagnostic inv(16) AML has a small number of mutations and a heterogeneity of mutations between diagnosis and relapse cases, targeted therapies inhibiting CBFβ–SMMHC binding to RUNX1 and HDAC8, and combination with RTK inhibitors may result in effective treatment. Pharmacologic approaches directly inhibiting specific signals could be valuable to define which components drive preleukemia to leukemia progression. In addition, little is known on the preL-HSC activity in relation with the microenvironment and how changes in the immune system affect LIC activity. The role of RUNX1 in inv(16) AML seems perplexing, as reduction in Runx1 levels decreases leukemia development in mice but loss of RUNX1 levels induce cell death in inv(16) AML cells. It is, therefore, possible that reduction in RUNX1 levels may be required for preleukemia formation and transition to LICs. New strategies designed to force increase in RUNX1 expression may help define new RUNX targets with potential antileukemia functions. The dependence of the RBD and ACD domains in CBFβ–SMMHC in preleukemia and LIC activity clearly indicate that SMMHC-multimerization and RUNX1 binding are critical leukemogenic functions. Interestingly, mutations in both domains interfere with the nuclear localization of the fusion protein. Hence, the development of strategies to directly interfere with the nuclear import of CBFβ–SMMHC may abrogate its leukemic activity. Finally, the study of the inv(16) LIC activity in *de novo* AML versus tAML and CML-blast crisis may shed mechanistic insights on the function of the fusion protein in cells with different mutation composition and proliferation capacity.

AUTHOR CONTRIBUTIONS

All authors listed have contributed to the preparation and editing of the work and approved it for publication.

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MINI REVIEW
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Regulation of Malignant Hematopoiesis by Bone Marrow Microenvironment

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Hematopoietic stem cells (HSCs) that give rise to all kinds of hematopoietic lineage cells on various demands throughout life are maintained in a specialized microenvironment called "niche" in the bone marrow (BM). Defining niche cells and unveiling its function have been the subject of intense study, and it is becoming increasingly clear how niche cells regulate HSCs in normal hematopoiesis. Leukemia stem cells (LSCs), which are able to produce leukemic cells and maintain leukemic clones, are assumed to share common features with healthy HSCs. Accumulating evidence suggests that LSCs reside in a specialized BM microenvironment; moreover, LSCs could control and rebuild the microenvironment to enhance their progression and survival. This article discusses the recent advances in our knowledge of the microenvironment supporting malignant hematopoiesis, including LSC niche.

Keywords: bone marrow microenvironment, niche, leukemia stem cells, hematopoietic stem cells, myelodysplastic syndrome, MPD

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INTRODUCTION

Hematopoiesis needs to be maintained throughout life to supply blood cells on various demands, such as infection, inflammation, blood loss, or hypoxia. Hematopoietic stem cells (HSCs) that reside at the top of hierarchy differentiate into multiple lineage hematopoietic cells through a fine-tuned differentiation process. Each step of differentiation is guided by various extrinsic factors as well as cell-autonomous intrinsic master gene regulations. In adult mammals, HSCs are known to locate in a specific microenvironment termed "niche" that orchestrates HSC function, including self-renewal and differentiation in both physiological and pathological conditions (1). Accumulating evidence reveals that various types of cells in and around the bone marrow (BM) participate in HSC function and its niche regulation (**Figure 1**) (2, 3).

Cell-intrinsic genetic alterations, such as gene mutations, deletions, amplifications, or translocations and epigenetic changes have been postulated mainly as the pathogenesis of hematologic malignancies, including leukemia, myelodysplastic syndrome (MDS), and myeloproliferative neoplasms (MPNs). It is rare, however, that donor cell-derived leukemia (DCL) is a well recognized and vital entity in understanding the process of malignant transformation of hematopoietic cells (5, 6). The possible pathological mechanism of DCL is diverse, such as preleukemic changes in donor cells, oncogene transformation from residual leukemic cells, and impaired immune surveillance. Defects in the BM microenvironment (BMM) in recipient BM have also been assumed as one of the mechanisms, suggesting vital roles of cell-extrinsic factors for malignant clone emergence (1, 7, 8). Recent studies using genetically modified animals indicates that alterations in the BMM could also support the survival of malignant clones or can even be the cause of the evolution of malignant clones (9, 10). In this review, we will summarize the recent achievements uncovering the roles of

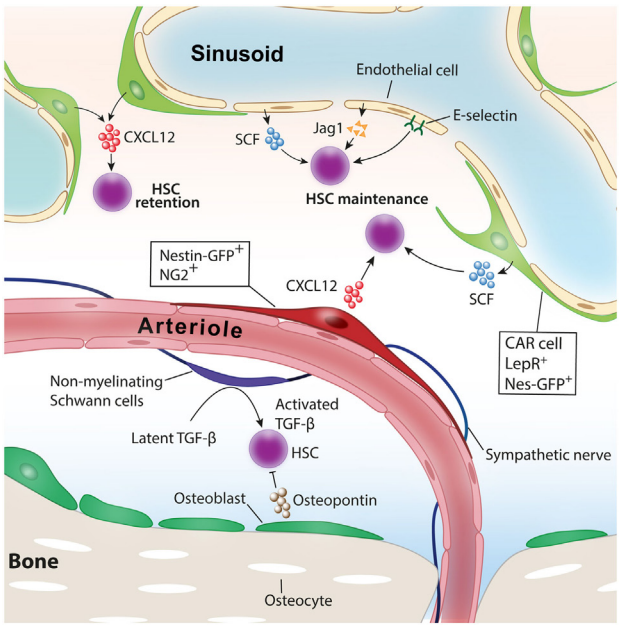

FIGURE 1 | Niche cells for healthy hematopoietic stem cells (HSCs). Various cell types have been identified as niche cells for HSCs in steady-state bone marrow. Perivascular stromal cells such as NG2+ periarteriolar cells and LepR+ perisinusoidal stromal cells differentially regulate HSCs. Nonmyelinating Schwann cells maintain HSC quiescence by activating transforming growth factor-β (TGF-β). Adopted and modified from Ref. (4).

the BMM for the emergence of hematological malignancies and discuss the possibility of therapeutic options targeting the BMM.

KEY PLAYERS IN HSC NICHE IN STEADY-STATE BM

Osteolineage Cells

Since Schofield proposed the concept of the existence of a specific environment for HSCs in the BM, various cell types in the BM have been identified as niche comprising cells. Osteolineage cells, a friendly neighbor of the BM, have been assumed as niche comprising cells for healthy HSCs. Initial *in vitro* studies indicated that bone-forming osteoblasts have the ability to support hematopoietic stem/progenitor cell (HSPC) function (11, 12). In 2003, two reports from different groups showed that osteoblast activation *in vivo* increased the number of HSCs in the BM. One group pharmacologically activated osteoblasts and the other increased the number of osteoblasts by genetic manipulations, and both led to the expansion of HSCs in the BM (13, 14). Conversely, it is reported that osteopontin, a matrix glycoprotein mainly produced by osteoblasts, negatively regulates HSC number in the BM (15). Recent studies using transgenic mouse models in which the major niche factor, such as C-X-C motif chemokine ligand 12 (CXCL12) and stem cell factor (SCF), was deleted specifically in osteoblasts indicated that osteoblasts did not contribute to the maintenance of HSCs at least by producing these niche factors (16–18). The role of bone-embedded osteocytes for hematopoiesis had remained unknown for a long time. The extrinsic administration of granulocyte-colony-stimulating factor (G-CSF), a key cytokine promoting granulopoiesis, facilitates the translocation of HSPC from the BM to peripheral blood. This process is called the "mobilization" of HSPC and mobilized HSPC is collected by apheresis and used for HSC transplantation for the treatment of hematological disorders. A recent study revealed that osteocytes have critical roles in regulating HSPC mobilization by G-CSF. The depletion of osteocytes using transgenic mice in which diphtheria toxin receptor was expressed under the control of dentin matrix protein-1 (*Dmp-1*) promoter led to a suppression of osteoblasts, resulting in a defect of HSPC mobilization by G-CSF.

Endothelial Cells

In mammals, definitive HSCs emerge from the hemogenic endothelium within the aorta-gonado-mesonephros region during embryonic development (19, 20). Like the intimate relationship between endothelium and HSCs during development, endothelial cells lining the BM vasculature support HSC maintenance and regeneration in the BM. *In vitro* coculture experiments indicate that BM endothelial cells expand HSPCs by producing a variety of angiocrine factors, such as insulin growth factor binding protein 2, bone morphogenic protein (BMP) 2 and BMP4, Notch ligands, SCF, CXCL12, and wingless-type MMTV integration site (Wnt) 5a (19–22). *In vivo* evidence in which the functional deletion of niche factors was achieved specifically in endothelial cells revealed that SCF or CXCL12 derived from endothelial cells play an indispensable role for HSC maintenance in the BM (16, 18). Endothelial cells have also been shown to integrate HSC quiescence through surface E-selectin expression (23).

Recent studies in mice identified a distinct subset of BM endothelial cells crucial for HSC function. Endothelial cells with high expression of CD31 and endomucin, referred to as type H endothelium, which distributes in end-terminal arterioles, expressed a higher level of SCF than sinusoid endothelial cells (24). A study done by another group found that endoglin-expressing endothelial cells, referred to as human regeneration-associated endothelial cells (hRECs), are associated with BM regeneration after myelosuppression and support a subset of hematopoietic progenitors through interleukin (IL)-33. Interestingly, gene expression analysis revealed similarities between hRECs and murine type H endothelium (25). A difference of vascular permeability observed between arterioles and sinusoids provides different effects to HSC activities. Arterial vessels are less permeable and maintain HSCs in a low reactive oxygen species (ROS), keeping HSCs quiescent. On the contrary, blood plasma permeabilized from leaky sinusoids promotes a high level of ROS in HSCs, augmenting the ability of differentiation and migration (26).

Stromal Cell-Associated Vasculature

A study defining the location of HSCs in the BM by staining phenotypic endogenous HSCs revealed that HSCs are closely associated with BM vasculature (27). These findings shed light on the vasculature area as HSC niche. Stromal cells that have a potency to differentiate into trilineage mesenchymal cells have been shown to function as HSC niche and are mainly associated

with sinusoids in the BM. Several studies identified different stromal cell types around sinusoids characterized by distinct surface markers or gene expression as niche comprising cells. These cells include CXCL12-abundant reticular (CAR) cells (28–30), which are cells marked by green fluorescent protein (GFP) under the elements of the nestin promoter (Nes-GFP$^+$) (31), leptin receptor (LepR)-expressing cells (16, 17), CD144$^-$CD146$^-$Sca-1$^+$ mesenchymal stromal progenitors (32), and the stromal cells targeted by Cre recombinase promoted by transcription factor osterix (Osx) (18), neural/glial antigen 2 (NG2) (33), or paired related homeobox-1 (17, 18). It has been shown that these cells expressed a high amount of niche factors supporting HSC functions, such as CXCL12, SCF, and VCAM-1, and they exhibit a significant overlap among each other (17, 18, 33, 34). Because the BM is a highly vascularized organ, as a matter of course, they have plenty of arteries and arterioles. A recent study in which the spatial distribution of endogenous HSCs in the BM was analyzed revealed that HSCs are closely and significantly associated with BM arterioles (35). The depletion of NG2-expressing pericytes *in vivo* led to a loss of quiescence and a reduction of HSCs and suggested the roles of periarteriolar stromal cells for HSC maintenance and quiescence. Other studies have argued that HSCs marked by α-catulin GFP and c-kit expression are randomly distributed in the BM and closely associated with sinusoids rather than arterioles (36). Another study has argued the differential contributions of sinusoids and arterioles to HSPC functions (26). Therefore, the contributions of each perivascular stromal cells to HSC niche had been controversial. To delineate the roles of perisinusoidal and periarteriolar stromal cells in HSC niche, we analyzed transgenic mice in which major niche factors, CXCL12 or SCF, were deleted specifically in either perisinusoidal or periarteriolar stromal cells. Whereas CXCL12 deletion in periarteriolar stromal cells led to a reduction of HSC number and alteration of distribution from arterioles, the deletion of CXCL12 in perisinusoidal stromal cells mobilized HSC to peripheral blood and spleen but had no impact on the HSC number or location in the BM. On the contrary, SCF deletion in perisinusoidal but not periarteriolar stromal cells impaired HSC maintenance in the BM (33). These results showed an intriguing mechanism of how different cytokines from distinct perivascular stromal cells contribute to HSC functions.

Nervous System

Bone and BM are extensively innervated by the nervous system. Catecholamine signals released from sympathetic nerve endings finely tune HSC niche functions, integrating HSC mobilization induced by cytokine G-CSF or release of HSCs under the circadian rhythm (37–39). Nonmyelinating Schwann cells wrapping the sympathetic nerves and closely associated with arterioles in the BM have been reported to maintain HSC quiescence by converting transforming growth factor-β (TGF)-β into the active form (40).

Regulatory T (T_{reg}) Cells

It has been well known that HSCs in the BM are resistant to cytotoxic stress and recent studies revealed that T_{reg} cells that suppress the function of effector T cells provide immunoprivileged sites to HSCs in the niche (41, 42). Intravenously transplanted HSCs in the allogeneic mouse transplantation model persisted for 1 month without immunosuppression and most of the HSCs colocalized with T_{reg} cells in the BM. The depletion of T_{reg} cells led to the reduction in the number of surviving donor HSCs after allogeneic transplantation, suggesting a protective function of T_{reg} cells from immune attack to allogeneic HSCs (41). A subsequent study from the same group reported that a distinct fraction of T_{reg} cells that highly expressed CD150 play vital roles for the maintenance of HSC quiescence and engraftment through adenosine (42).

ROLES OF THE BMM FOR MPN

The clinical entity of MPNs is heterogeneous and includes four classic MPNs: polycythemia vera, essential thrombocytopenia, primary myelofibrosis, and chronic myeloid leukemia. As recent studies showed that most cases of MPNs have somatic mutations in the tyrosine kinase Janus kinase 2 (JAK2) (43–46), calreticulin gene (CALR) (47, 48), or thrombopoietin receptor (49), the pathogenesis of these neoplasms appears mostly cell intrinsic. Although the BMM originally regulates differentiation and proliferation of HSCs or immature progenitor cells without aberrant proliferation, recent evidence from mice work suggests that the defect of the BMM can be the cause of abnormal myeloproliferation. The lost of one of the major receptors for vitamin A, RARγ, in the BMM results in increased mature myeloid cells resembling MPNs, which partially depend on tumor necrosis factor-α (TNF-α) production from the BMM (50). Another report showed that the perturbation of interaction between myeloid-derived cells and the BMM by the defect of retinoblastoma protein (Rb), a vital regulator of the cell cycle, led to myeloid cell proliferation (51). The deficiency of Mindbomb-1, an essential component for Notch ligand endocytosis, in the BMM is also shown to cause enhanced myelopoiesis corresponding to MPNs through Notch signaling defects in the BMM (52). All these evidences clearly indicate that nonhematopoietic BMM cells play significant roles in promoting aberrant myelopoiesis; however, the specific cell types contributing to the enhanced myelopoiesis remain largely unknown.

Osteolineage Cells

A recent study by Fulzele et al. reported that osteocytes, which are terminally differentiated osteolineage cells embedded in the calcified bone, participate in myelopoiesis. They found that the specific deletion of Gsα in osteocytes enhanced G-CSF production, leading to the expansion of myeloid-committed cells in the BM (53). As osteocytes are also shown to regulate the BMM and control HSPC activities (54), it might be possible that osteolineage cells participate in the pathogenesis of MPNs.

Stromal Cell-Associated Vasculature and Sympathetic Nerve

A recent study done by Arranz et al. reported that, in both human MPN patients and mice expressing human *JAK2* (*V617F*) mutation in HSCs, the number of sympathetic nerves and Schwann cells ensheathing sympathetic nerves was decreased. In the mice

MPN model, the depletion of Nes-GFP$^+$ perivascular stromal cells accelerated MPN progression. They found that abnormal HSC-derived proinflammatory cytokine IL-1β caused local neuropathy and damaged Nes-GFP$^+$ perivascular stromal cells, leading to the progression of MPN (55). These results suggest that aberrant HSCs in the MPNs rebuild the BMM beneficial for their survival.

Cytokine Milieu

In addition to the cellular players of the BMM, non-cellular components of the BMM have significant contributions to the development or sustainment of MPNs. The increased level of various inflammatory cytokines, including IL-6, IL-8, basic fibroblast growth factor, platelet-derived growth factor, TNF-α, TGF-β, and oncostatin M, has been reported in MPNs (56–58), and these cytokines play a role in the establishment of the disease manifestations. In particular, TGF-β1 mostly produced by megakaryocytes has been implicated in the development of BM fibrosis, a major unfavorable alteration of the BMM in patients with MPNs (59, 60). JAK kinase inhibitors, including ruxolitinib, ameliorate systemic symptoms and splenomegaly in MPN patients (61–63). The reduction of proinflammatory cytokines by the inhibition of JAK-STAT signaling has been identified as one of the mechanisms of ruxolitinib (64). Moreover, a recent study identified a constitutive activation of nuclear factor-κB (NF-κB) signaling in addition to JAK-STAT pathways as a key signaling pathway leading to chronic inflammation in MPNs. Intriguingly, the combined blockade of JAK-STAT and NF-κB pathways with ruxolitinib and JQ1, the bromodomain and extra-terminal motif (BET) bromodomain inhibitor, reduced aberrant cytokine production and improved BM fibrosis in the mice MF model (65).

ROLES OF THE BMM FOR THE PATHOGENESIS OF MDS

By definition, MDS are a heterogeneous group of clonal HSC diseases characterized by cytopenia, dysplasia in one or more of the major myeloid lineages, ineffective hematopoiesis, recurrent genetic abnormalities, and increased risk of developing acute myeloid leukemia (AML) (66, 67). As various types of recurrent cytogenetic abnormalities in hematopoietic aberrant clone have been identified, it is broadly accepted that the pathogenesis of MDS is mainly cell intrinsic. Some studies indicated that cultured BM stromal cells isolated from MDS patients harbor cytogenetic abnormalities distinct from hematopoietic cells (68–70). Because stromal cells analyzed in these studies were cultured *in vitro* and most of them were analyzed after several passages, observed abnormalities could be acquired *in vitro* rather than originating from primary stromal cells. Emerging evidence from sophisticated mice studies strongly suggests that defects in the BMM could promote at least a partial initiation of malignant clone or advance the disease progression.

Stromal Cell-Associated Vasculature

Genetically engineered mice in which *Dicer 1*, the RNase III endonuclease essential for microRNA biogenesis and RNA processing, was deleted explicitly in osteoprogenitor cells were marked by Osx-Cre-developed MDS accompanied by osteoblastic dysfunction (9). In addition to *Dicer 1*, the deletion of Shwachman–Diamond–Bodian syndrome (*Sbds*) gene in osteoprogenitor cells resulted in cytopenia and dysplastic changes in neutrophils and megakaryocytes. A subsequent study using the same mouse model done by the same group demonstrated that S100A8/9 protein, proinflammatory molecules referred to as damage-associated molecular pattern or alarmins, secreted by osteoprogenitor cells in *Sbds*-deficient mice induces genotoxic stress mediated by mitochondrial dysfunction, oxidative stress, and DNA damage response activation in HSPCs (71). Although *Osx* is one of the master regulator genes that lead mesenchymal progenitors to osteoblast lineage differentiation (72), stromal cells marked by Cre promoted by *Osx* showed a significant overlapping with other stromal cells that are closely associated with sinusoids, such as CAR cells (18), Nes-GFP$^+$, or LepR-expressing stromal cells (34). Collectively, these results indicate that the dysfunction of perisinusoidal stromal cells that have osteoblastic differentiation potential might induce dysplasia in hematopoiesis through undefined mechanisms.

Cytokines and Immune Cells

It has been well recognized that both cellular and non-cellular immune systems are perturbed in MDS patients (73). The increased levels of various proinflammatory cytokines, such as IL-6, IL-8, TNF-α, TGF-β, and interferon-γ in MDS patients have been reported and implicated in the pathogenesis of MDS (74).

With regard to the roles of immune cells, immunoregulatory T_{reg} cells might be involved in the pathogenesis of MDS. The increased number of T_{reg} cells has been reported to correlate with unfavorable factors, such as high percentage of BM blasts, high International Prognostic Scoring System score, and disease progression (75). A recent study identified that high numbers of effector memory T_{reg} cells that have more potent immunosuppressive function are associated with higher risk disease, increased blast percentage, and reduced overall survival (76). Although these evidences indicate crucial roles of T_{reg} cells in the pathogenesis or the mechanism of disease progression in MDS, further studies will be necessary to determine whether T_{reg} cells participate in the pathogenesis of MDS or a merely reactive consequence of hematological dysregulation.

ROLES OF THE BMM FOR THE PATHOGENESIS OF LEUKEMIA

Similar to the normal hematopoietic system, stem or progenitor cells reside at the top of the hierarchy and produce descendant leukemic cells and self-renew to propagate leukemia and sustain clonal tumor burden (77, 78). Although leukemia stem cell (LSC) seems to be less dependent on their niche than normal HSCs, the leukemogenic process does not completely abrogate niche dependence for LSCs. Cumulative evidence suggests that BMM influences LSC behavior in many ways similar to normal hematopoiesis (73).

Non-Cellular Component

It is shown that human AML stem cells (LSCs) expressed CXCR4, a counter-receptor for CXCL12 that is a potent chemoattractant for HSCs secreted from BM stromal cells, and the blockade of CXCR4–CXCL12 axis abrogated the homing of LSCs and propagation of leukemic cells in a xenotransplantation murine model (79). Another study reported that the level of CXCL12 in the BM with chronic myelogenous leukemia (CML) was decreased, which impaired the homing efficacy of both exogenous transplanted LSCs and healthy HSCs. Plasma isolated from the BM of CML mice BM impeded the growth of healthy HSCs but not LSCs *in vitro* culture, leading to a growth advantage for the leukemic clone (80). AMD3100 (plerixafor), a small-molecule inhibitor of CXCR4, have been tested in a phase 1/2 study combined with chemotherapy for relapsed/refractory AML with encouraging response rates (81). However, a subsequent trial testing the additive effect of G-CSF on AMD3100 combined with chemotherapy in AML patients failed to improve the response rate (82). More potent CXCR4 inhibitors have been developed and *in vitro* studies revealed that they could induce the apoptosis of AML, which is favorable to eradicate LSCs (83, 84).

In addition to CXCR4–CXCL12 interaction, the adhesion molecule CD44 on LSCs also has been documented to be involved in the crosstalk between LSCs and BMM. The ligation of CD44 by the monoclonal antibody specifically prevented LSCs to home and engraft to the BM without disturbing normal HSC function (85). The phase I study of an anti-CD44 antibody that blocks the interaction between LSCs and BMM revealed that the drug was safe and well tolerated but had limited activity to leukemia (86). These series of evidences highlighted the significant roles of the BMM for leukemia pathogenesis and LSC biology. Defining the exact cell types of LSC niche and the mechanism how niche cells regulate LSCs have been under intense study (**Figure 2**).

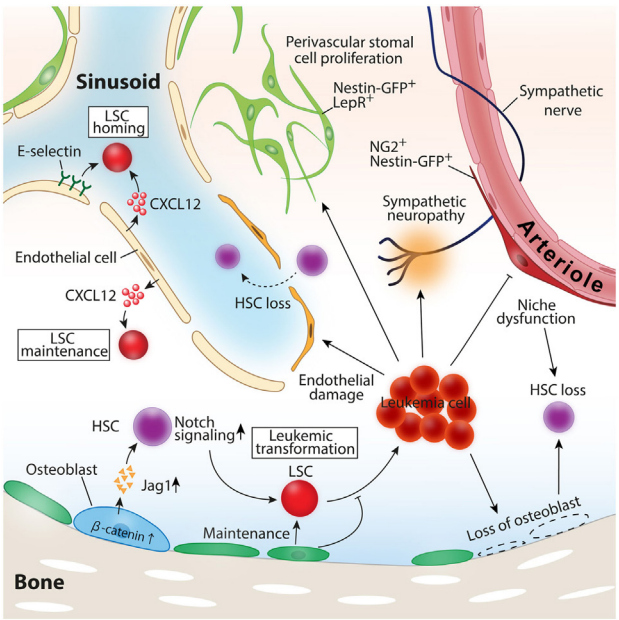

FIGURE 2 | Roles of the BM microenvironment in leukemia pathogenesis. Constitutive activation of β-catenin in osteoblast-induced leukemia transformation in mice model. Leukemic cells induce loss of osteoblasts, vascular endothelial cells, and periarteriolar NG2+ stromal cells, leading to healthy hematopoietic stem cell (HSC) loss. CXCL12 and E-selectin expressed by vascular endothelial cells function as inducers of leukemia stem cell (LSC) homing to the bone marrow, and CXCL12 secreted by endothelial cells also contributes to LSC maintenance and the propagation of leukemia.

Endothelial Cells

Ample evidence suggests the indispensable roles for vascular endothelial cells in supporting LSCs and leukemia cell progression. Although most of the leukemia are disseminated diseases when they cause clinical symptoms, the initial clonal evolution should occur at a certain site in the BM. After the initial proliferation of aberrant clones, leukemic cells extravasate from the original BM to the bloodstream and spread to other BMs throughout the body. Similar to healthy HSCs, LSCs are required to have the ability to home and engraft to the BM for their expansion. Sipkins et al. analyzed the spatial distribution pattern of externally transplanted mice leukemic cells and revealed that leukemic cells homed and colonized around E-selectin and CXCL12 expressing BM endothelial cells, suggesting the importance of distinct vascular endothelial cells as a supporter of leukemic cell expansion (87). The deletion of CXCL12 specific from vascular endothelial cells impeded T-cell acute lymphoblastic leukemia (T-ALL) growth in both mice leukemia model and human T-ALL xenografts (88). A recent study showed that LSCs expressed a high level of CD98, an integrin binding glycoprotein, mediated adhesion of LSCs to vascular endothelial cells where LSCs were maintained. Moreover, the blockade of CD98 by monoclonal antibodies abolished leukemia engraftment and proliferation in the mice AML model, suggesting a therapeutic potential of the agents targeting CD98 (89). The antileukemic effect of anti-CD98 antibody in relapsed or refractory AML patients has also currently been under investigation.

In terms of the number of the vasculature in leukemia, the increased density of BM vasculature has been observed both in murine aggressive AML model and in leukemia patients (90, 91). However, it seems that we should take into consideration the location of vasculature rather than the magnitude of the increase of endothelial cells. Duarte et al. demonstrated that endosteal vascular endothelial cells were depleted in MLL-AF9-driven mouse AML model, which was associated with healthy HSC loss through the increase of transendothelial migration of HSCs. The prevention of endosteal endothelium impairment with a small-molecule deferoxamine or a genetic approach rescued HSC loss and prolonged the survival of the mice treated with chemotherapy (92).

Osteolineage Cells

In the human acute leukemia xenograft model, residual leukemic cells were located in the vicinity of the endosteal area after chemotherapy, implying the existence of a distinct microenvironment for chemotherapy-resistant dormant leukemic stem cells around osteolineage cells (93). Reduced numbers of mature osteoblasts and osteocalcin in the blood, one of the surrogate markers of osteoblast function, were reported in both AML patients and the MLL-AF9 mouse aggressive AML model, resulting in

reduced healthy hematopoiesis (94, 95). Targeted ablation of mature osteoblasts in the mouse transgenic leukemia model representing human chronic phase CML accelerated leukemia progression possibly due to the loss of quiescence of LSCs and led to a deterioration of LSC ability to generate leukemia in the recipient mice (96). These results suggest that osteoblasts have indispensable roles to inhibit leukemia expansion and to sustain stemness of LSCs in mouse CML (96). Consistent with this idea, osteoblast activation by the treatment of parathyroid hormone decreased LSC proliferation in a transduction-transplantation model of CML (97).

A recent study by Kode et al. showed that osteoblasts are involved in not only the regulation of established leukemic cells but also the evolution of leukemia. In this study, the authors showed that the constitutive activation of β-catenin in mature osteoblasts stimulated the expression of Notch ligand jagged 1 in osteoblasts, which in turn led to the activation of Notch signaling in HSPCs, and induced malignant transformation of HSPCs to leukemic cells (10).

Perivascular Stromal Cells

As discussed in the niche cells for healthy HSCs, perivascular stromal cells in the BM have attracted much attention as a vital niche player. However, it remains elusive whether these cell types contribute to the evolution or growth of leukemia. One study showed in a transduced mouse T-ALL model that perivascular stromal cells did not contribute to leukemia propagation at least through CXCL12–CXCR4 signals between BMM and leukemia cells (88). A more recent study analyzing the dynamic interaction of T-ALL leukemic cells with the niche component across the leukemia progression demonstrated that leukemic cells had any spatial preference with any niche component including perivascular stromal cells represented by Nes-GFP$^+$ stromal cells (98). In the BM with advanced T-ALL, the number of Nes-GFP$^+$ cells was maintained, whereas mature osteoblasts and osteoprogenitor were completely lost (98). In contrast to the T-ALL model, the robust expansion of Nes-GFP$^+$ cells with impaired niche factor expression for healthy HSCs has been observed in mice with transduced MLL-AF9 aggressive AML cells (91). NG2$^+$ perivascular stromal cells closely associated with arterioles that have been shown to maintain healthy HSCs were reduced, which was consistent with the diminished number of healthy HSCs. Intriguingly, these dramatic alterations of niche components induced by AML were mediated by the disruption of sympathetic nerves in the BM induced by leukemic cells, and treatment of β2-adrenergic receptor agonist led to the reduction of LSCs in the BM and prolonged the survival of leukemic mice (91). Altogether, these evidences suggest that the roles of perivascular stromal cells in leukemia pathogenesis may vary among the subtypes of leukemia, and further studies are necessary.

In the context of leukemia evolution, although transgenic mice in which *Dicer 1* or *Sbds* was abrogated in perivascular stromal cells presented myelodysplastic changes and subsequent evolution to leukemia (9, 71), there is, so far, no evidence clearly demonstrating that dysfunction in perivascular stromal cells causes *de novo* leukemia *in vivo*.

Immune Cells

As is the case in normal hematopoiesis, immune cells modulate BMM in leukemia. In the mice AML model, immunosuppressive T_{reg} cells presented at the AML site and impaired the function of adoptively transferred cytotoxic T cells (CTLs). The depletion of T_{reg} cells in turn restored CTL function and reduced leukemia progression in the mice model (99).

CONCLUDING REMARKS

Over the past decade, a significant advancement in understanding the roles of the BMM in the pathogenesis of hematologic malignancies has been achieved. Because even the mechanisms by which niche cells orchestrate healthy HSCs or hematopoiesis are not completely understood, the involvement of the BMM to malignant hematopoiesis must be diverse and complicated. For instance, the results gained thus far from murine studies indicated that a different type of leukemia interacts with a distinct BMM differently. Further studies clarifying the detailed mechanisms that underlie each type of hematopoietic malignancy will lead us to our final goal to improve therapeutic strategies and conquer hematopoietic malignancies.

AUTHOR CONTRIBUTIONS

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REVIEW
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Epigenetic Regulators in the Development, Maintenance, and Therapeutic Targeting of Acute Myeloid Leukemia

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The importance of epigenetic dysregulation to acute myeloid leukemia (AML) pathophysiology has become increasingly apparent in recent years. Epigenetic regulators, including readers, writers, and erasers, are recurrently dysregulated by way of chromosomal translocations, somatic mutations, or genomic amplification in AML and many of these alterations are directly implicated in AML pathogenesis. Mutations in epigenetic regulators are often discovered in founder clones and persist after therapy, indicating that they may contribute to a premalignant state poised for the acquisition of cooperating mutations and frank malignancy. Apart from the proto-oncogenic impact of these mutations, the AML epigenome is also shaped by other epigenetic factors that are not mutated but co-opted by AML oncogenes, presenting with actionable vulnerabilities in this disease. Targeting the AML epigenome might also be important for eradicating AML leukemia stem cells, which can be critical for disease maintenance and resistance to therapy. In this review, we describe the importance of epigenetic regulators in AML. We also summarize evidence implicating specific epigenetic regulators in AML pathobiology and discuss emerging epigenome-based therapies for the treatment of AML in the clinic.

Keywords: acute myeloid leukemia, epigenetic therapy, leukemia stem cell, epigenome, chromatin modification

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INTRODUCTION

Acute myeloid leukemia (AML) is a clonal malignancy resulting from the transformation of hematopoietic stem and progenitor cells. AML is marked by enhanced proliferation and impaired differentiation of immature myeloid progenitors. Over the past few decades, strategies for treating AML have remained largely unchanged, although survival outcomes have improved, especially in younger patients (1). Despite these improvements, approximately 60% of young patients with AML eventually succumb to disease even after treatment with intensive therapies (2). In patients over 60 years of age, a population that has an increased frequency of AML, survival outcomes are much more dismal; less than 5% of patients are alive 5 years after diagnosis (3). There are several reasons why AML cure rates have plateaued. First, therapeutic approaches that have shown success in younger patients are often extremely aggressive and are, therefore, not tolerated well by elderly patients with frailty and other comorbidities. Treatment-related toxicity also results from the fact that standard therapies do not discriminate between normal and leukemic cells, resulting in severe

toxicities. Second, although patient selection based on morphologic and cytogenetic features is routinely used for guiding treatment strategies and risk stratification, current therapeutic approaches do not adequately address the inherent molecular heterogeneity of AML. Last, current treatments that target the leukemic bulk may spare leukemia stem cells (LSCs) that provide a reservoir of premalignant or malignant clones that can regenerate the tumor. This is of great significance for AML therapy. Most patients who go into remission after treatment will relapse within the first few years, which diminishes their rate of survival substantially. Therefore, safer and more effective therapies are urgently required for the majority of AML patients with severely limited effective treatment options. A better understanding of the molecular landscape of AML and the biology of LSCs may, therefore, aid the design of much more targeted therapies for AML. We will discuss advances in our understanding of these processes in more detail in the following section with a focus the contribution of epigenetic regulators to AML heterogeneity and for the emergence and sustenance of LSCs.

Epigenetic Regulators and the AML Mutational Landscape

Acute myeloid leukemia is highly heterogeneous in terms of its underlying genetics, pathobiology, and clinical manifestation. Even though the morphological and cytogenetic heterogeneity of AML has been recognized for several years, the marked molecular heterogeneity has only come to the fore recently. Emerging evidence from genome-scale studies propelled by advances in next-generation sequencing (NGS) has substantially broadened our knowledge of the spectrum and frequency of mutations in AML. Characterization of the genomic AML landscape has led to the identification of recurrent mutations in a number of previously uncharacterized genes in AML. The classes of genes mutated in AML include transcription factors, kinases, cell cycle regulators, spliceosomal genes, and epigenetic regulators. The observation that genes encoding epigenetic regulators are among the most commonly occurring mutated factors in AML, strongly points to a role of epigenome dysregulation in AML pathogenesis. These mutations in epigenetic regulators encompass a broad spectrum of epigenetic writer, eraser, and reader proteins which will be the focus of this review. The epigenome is dynamically regulated through chemical modification of DNA and RNA as well as the histone proteins around which DNA is packaged. Our genomes harbor a number of enzymes that deposit these chemical marks (writers), or remove them (erasers), dedicated to specific modifications of DNA or chromatin. Proteins with specialized domains that can selectively bind to specific DNA, RNA, or histone modifications (readers) also abound, indicating a well-orchestrated mechanism for relaying epigenetic marks to downstream effectors. The coordinated action of epigenetic reader, writer, and eraser proteins is important for regulation of various cellular processes, including transcription, DNA replication, cell cycle control, and the DNA damage response. Recurrent genomic alterations in epigenetic writer, reader, and eraser proteins, such as DNA methyltransferase 3A (DNMT3A), TET1/2, IDH1/IDH2, EZH2, mixed-lineage leukemia (MLL), NSD1/3, AF10, ENL, and other epigenetic regulators have been cataloged in AML, inspiring a wave of preclinical studies aimed at uncovering causal links between epigenome dysregulation and leukemogenesis (see **Figure 1** and **Table 1**). These studies are yielding important actionable information that can be rationally applied to the development of epigenome-based therapies for AML patients.

LSCs and the Epigenome

The failure of "debulking" strategies in AML can now at least partly be attributed to AML–LSCs. Several lines of evidence demonstrate that AML emerges from a subset of cells with stem-cell-like properties [reviewed in Ref. (25, 26)]. It is now well documented that long-lived normal hematopoietic stem cells (HSCs) can accumulate mutations bearing the potential to trigger myeloid transformation in later life (27–30). These mutant HSC clones can eventually transform into LSCs, a population of cells with stem-cell properties that have the ability to sustain and propagate the tumor. Alternatively, certain AML-specific mutations in downstream hematopoietic progenitors can also initiate a transcriptional program reminiscent of HSCs, converting them to self-renewing LSCs (31, 32). It is now clear that stemness attributes in cancer are much more fluid than previously imagined, especially in constantly evolving neoplastic cells that display an enormous amount of genetic and epigenetic instability. Therefore,

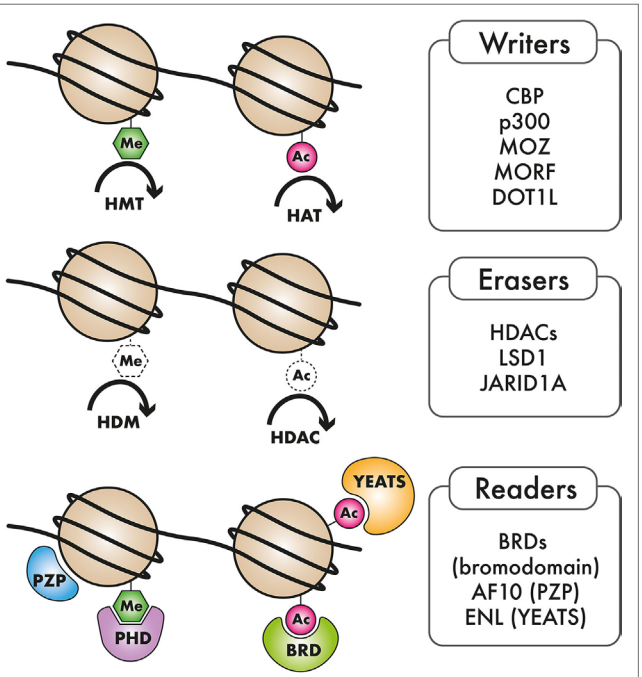

FIGURE 1 | Types of epigenetic regulators mutated in AML: Epigenetic "writers," such as DNA methyltransferases, histone methyltransferase (HMT), and histone acetyltransferases (HAT), deposit methylation and/or acetylation on DNA or on histones. These epigenetic marks may be removed by epigenetic "erasers," including histone demethylase (HDM) and histone deacetylase complexes (HDACs). Epigenetic "readers" are highly specialized proteins that specifically bind to distinct epigenetic marks to convey this information to downstream effectors.

TABLE 1 | Frequency and role of recurrently mutated epigenetic regulators in acute myeloid leukemia (AML).

Genes (reference)	Frequency in AML	Mutation type	Description
DNMT3A (4–6)	~12–22%	Point mutation/indel (~60% R882H) Loss-of-function	DNMT3A mutations cause genome-wide DNA hypomethylation *in vitro* and may have dominant negative effects
IDH1/IDH2 (7–10)	~10–20%	Missense point mutation (R132-IDH1, R140/172-IDH2) Gain-of-function	Mutants of cytoplasmic (IDH1) and mitochondrial (IDH2) decarboxylase convert isocitrate to 2-HG, which inhibits TET2, result in genome-wide DNA hypermethylation
TET2 (11–13)	~14%	Point mutation/indel Loss-of-function	A 5-mC-dioxygenase that converts 5-mC to 5-hmC, an intermediary process for demethylation. *TET2* mutations phenocopy *IDH* mutations
EZH2 (14–16)	<1%	Point mutation/indel Loss-of-function	An enzymatic component of PRC2 and H3K27 methyltransferase. Biological mechanism unclear
MLL-fusion proteins (17–21)	~3–5% PTD/~5–10%	Partial tandem duplication (PTD)/translocation Gain-of-function	Duplication of an internal N-terminal region of MLL, retains SET domain/fusions of MLL N-terminal region to several different partner proteins, create dominant transcriptional activators
CBP/p300-MOZ/MORF fusion (22–24)	<1%	Translocation Gain-of-function	Acetyltransferases involved in rare but recurrent chromosomal translocations with elevated *HOX* gene expression and adverse prognosis

DNMT3A, DNA methyltransferase 3A; Indel, insertion and/or deletion; IDH, isocitrate dehydrogenase; 2-HG, 2 hydroxyglutarate; TET2, tet methylcytosine dioxygenase; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressive complex 2; H3K27, lysine 27 of histone H3; MLL, mixed-lineage leukemia; CBP, CREB-binding protein; MOZ, monocytic leukemia zinc-finger protein; MORF, MOZ homolog; HOX, homeobox.

instead of the presence of a fixed, immutable population of cancer stem cells, there is evidence suggesting that cancer cells can switch between stem-like and non-stem-like states within the tumor, making the cancer stem cell a "moving target." Such extraordinary plasticity of tumor cells requires rapid adaptations to changing micro-environmental cues as well as to the selective pressures mounted by aggressive therapeutic interventions typically used in cancer patients. This exceptional plasticity is likely to be provided by rapid and reversible epigenetic, rather than genetic changes in cancer cells. This is especially likely since epigenetic changes govern key steps in the transition of stem cells to their differentiated progeny in the process of normal hematopoiesis (33). Consistent with this notion, it is no surprise that almost all of the epigenetic regulators with recurrent AML-associated mutations have important roles in HSC self-renewal, survival, or differentiation. Importantly, studies have shown that mutations in epigenetic modifiers, including *DNMT3A* and *IDH1/IDH2*, occur in early pre-leukemic HSCs (29, 34, 35), while signaling pathway mutations in genes that confer proliferative advantage, such as *NPM1* (nucleophosmin 1), *FLT3-ITD* (internal tandem duplication of the *FLT3* gene), and *KRAS/NRAS*, are acquired later during the development of AML (36). Strikingly, there is increasing evidence that mutations that lead to clonal expansion of HSCs are acquired during normal aging, a process that is termed "clonal hematopoiesis." Individuals with clonal hematopoiesis have an increased risk of progression to myeloid neoplasia and lower overall survival. Interestingly, a large proportion of the mutations observed in normal elderly individuals with clonal hematopoiesis are in epigenetic regulators (27–30). These striking observations indicate that mutations in epigenetic regulators may establish a leukemia-predisposing epigenetic state in premalignant HSC clones. These HSC clones may then be poised to transform into fully leukemic LSCs upon acquisition of secondary mutations with complementary oncogenic activities. Taken together, therapeutic targeting of the epigenome may turn out to be an attractive strategy for targeting AML–LSCs and may provide lasting curative benefit, especially in combination with traditional "debulking" strategies.

EPIGENETIC REGULATORS IN AML PATHOGENESIS

Ever since chromosomal translocations and fusion oncogenes were discovered in AML several years ago, it was apparent that chromatin modulators such as the "writers" MLL1/KMT2A, CBP/p300, and NSD1/KMT3B might have causative roles in AML pathogenesis. MLL1, CBP, and NSD1/3 are involved in recurrent chromosomal translocations in a fraction of AML patients. These translocations were discovered early because they could be observed using methods, such as karyotyping and fluorescence *in situ* hybridization, that enabled identification of gross genetic aberrations in AML cells. However, these chromatin modifier mutations only accounted for a minor fraction of AML patients. There was little evidence for the direct genomic alteration of epigenetic regulators in the vast majority of AML. This scenario changed dramatically with the recent explosion in NGS, whereby mutations in several novel genes not previously implicated in AML pathogenesis were identified. Recent NGS-based discovery efforts in AML have demonstrated that epigenetic regulators comprise one of the most frequently mutated classes of genes in AML, accentuating the role of the epigenome in AML pathogenesis. Recurrent mutations in DNA methyltransferases (*DNMTs*), isocitrate dehydrogenases (*IDH1/IDH2*), methylcytosine dioxygenases of the ten-eleven-translocated (TET) family, and human homologs of the Drosophila polycomb complex such as Enhancer of Zeste 2 (*EZH2*) and additional sex-combs like genes (*ASXL1/2*) have been discovered in AML and myelodysplastic syndromes and myeloproliferative neoplasms (MDS/MPN), and many of these mutations have been causally linked

to myeloid transformation in murine models. The role of these epigenetic modifiers in AML pathobiology and studies exploring these proteins as druggable targets will be described in detail below. Apart from genes mentioned above, there are a number of examples of epigenome modulators that are not directly mutated but nevertheless implicated in AML pathogenesis. Several chromatin modifiers have been discovered as selective dependencies in specific AML subtypes as discussed in the Section "*DNMT* Mutations."

DNMT Mutations

DNA methylation is an important process in development that involves the addition of a methyl group to the carbon-5 position of cytosine in CpG dinucleotides, leading to the formation of 5-methylcytosine (5-mC). The DNMT family, including *DNMT1*, *DNMT3A*, and *DNMT3B* encode methyltransferases that catalyze this reaction. DNMT3A and DNMT3B are largely *de novo* DNMTs, whereas DNMT1 predominantly plays a role in the maintenance of DNA methylation (37). CpG clusters are enriched in regions upstream of genes (CpG islands) and increased methylation of CpG islands leads to transcriptional silencing of the downstream gene. Recurrent mutations in *DNMT3A* are observed in 12–22% of AML and always present as heterozygous mutations. *DNMT3A* mutations are associated with poor prognosis and decreased overall survival (4). A majority of these mutations lead to premature truncation of DNMT3A protein through nonsense or frame-shift mutations in the protein-coding region. Approximately 60% of *DNMT3A*-mutated AML patients harbor a missense mutation in the arginine 822 residue that diminishes its methyltransferase activity while reducing its binding affinity to DNA, which has been proposed to have a dominant negative function over the wild-type DNMT3A protein (5). *DNMT3A* mutations have been observed in non-leukemic T-cells from AML patients as well as in normal elderly individuals with no signs of leukemia, suggesting their provenance from an early, premalignant multipotent cell (27, 35). The mechanisms of leukemogenesis by DNMT3A are not entirely clear; however, studies have shown that heterozygous *Dnmt3a* ablation in mice leads to an expansion of the HSC pool (38), myeloid skewing and a predisposition to myeloid malignancies that may require additional genetic alterations. These studies reinforce the notion that the *DNMT3A* mutation, perhaps like mutations in other epigenetic regulators, do not lead to frank leukemic transformation on their own, but rather create a premalignant state that lays the ground for malignancy. Recently, it was also reported that mutant DNMT3A (R882H) interacts with the Polycomb repressive complex 1 (PRC1) to silence genes, suggesting that PRC1 activity could be an attractive target in DNMT3A-mutant tumors (39).

Isocitrate Dehydrogenase (*IDH*) Mutations

Isocitrate dehydrogenases are key components of the tricarboxylic acid cycle responsible for oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG). The IDH1 and IDH2 proteins are nicotinamide adenine dinucleotide phosphate (NADP+)-dependent enzymes that mediate a number of important cellular processes including lipid metabolism, glucose sensing, and oxidative phosphorylation (7). *IDH1* and *IDH2* mutations are found at a frequency of 10–20%, and these mutations are more common in the cytogenetically normal sub-group of AML. *IDH1* and *IDH2* mutations are mutually exclusive and result in a gain of neomorphic activity (8). Specifically, gain-of-function *IDH* mutations convert the metabolite α-KG to the structurally similar I-2-hydroxyglutarate (2-HG). 2-HG acts as an "oncometabolite" since its accumulation in leukemic cells interferes with the enzymatic functions of several chromatin modifiers that use α-KG as a cofactor. Mechanistic investigations into the model of action of *IDH* mutations have shown that hematopoietic specific IDH1 (R132H) mutation using a conditional knock-in strategy expands HSC and myeloid progenitor compartments but fail to show signs of overt AML (40). Similar results were demonstrated by Heuser and colleagues using a retroviral bone marrow transplantation model which showed that mutant *IDH* overexpression was not sufficient to cause AML, but could do so in the presence of the Hoxa9 oncogene (9). These results suggest that similar to DNMT3A, *IDH* mutations may also need secondary mutations for initiation of frank malignancy in AML. Strikingly, the same group also demonstrated that *in vivo* injection of the oncometabolite 2-HG, could recapitulate most, but not all of the oncogenic effects of the *IDH1* mutation (10). These interesting observations reinforced the role of 2-HG as an oncometabolite but also suggested that IDH1 may have additional oncogenic functions beyond its role in 2-HG accumulation. The exact role of chromatin modifying enzymes and epigenomic modifications in relaying the consequences of *IDH* mutation to oncogenic transcription remains to be determined.

TET Family Mutations

One of the most important classes of enzymes affected by *IDH* mutations is the TET family of methylcytosine dioxygenases. Normally, TET2, with the cofactor molecule α-ketoglutarate (α-KG), converts 5-mC to 5-hydroxymethylcytosine (5-hmC), which can then be demethylated back to cytosine *via* a series of intermediate steps (11) This TET-enzyme catalyzed CpG demethylation is an important step in the dynamic regulation of DNA methylation associated regulation of cellular processes. Inactivating mutations in TET enzymes lead to decreased hydroxylation of methyl-CpG sites (12, 41) resulting in aberrant CpG hypermethylation, decreased expression of key differentiating enzymes, and inhibition of normal cellular differentiation (42). Several studies have examined the function of TET2 inactivation in mice, *Tet2* deletion leads to hematopoietic defects including enhanced HSC self-renewal and myeloid expansion, correlating with global loss of 5-hmC in primitive hematopoietic populations (43–45). It was recently described that restoration of TET function using an inducible shRNA model of TET-induced AML or through the administration of Vitamin C, which is a cofactor for α-KG dependent dioxygenases reverses leukemogenicity induced by the mutant TET protein (13). These exciting results imply that metabolic control of TET activity could be harnessed for therapeutic benefit in patients with TET mutations. Notably, cytosine methylation signatures of *TET2*-mutated AML show significant overlaps with those found in *IDH1/IDH2* mutated patients and *IDH1/IDH2* and *TET2* mutations are mutually exclusive in AML

(8), signaling a common mechanism of leukemogenesis based on aberrant DNA methylation. Recently another addition to this sub-group was made due to the discovery that mutations in the Wilms tumor gene *WT1*, which are found in approximately 10% of AML, are also mutually exclusive with *TET* and *IDH* mutations and display global cytosine hydroxymethylation profiles reminiscent of *IDH* and *TET* mutated AML. Levine and colleagues, who reported these observations, went on to demonstrate that WT1 physically interacts with TET proteins, TET2 and TET3, and compromises TET functions. It was concluded in this study that *IDH1*/*IDH2*, *TET2*, and *WT1* mutations define a common AML subtype with overlapping disordered DNA 5-hmC profiles (46). Taken together, these results imply that dysregulated DNA methylation, achieved either through mutations in *DNMT3A*, *IDH1*/*IDH2*, *TET2*, or *WT1*, play an important role in the pathogenesis of a large proportion of AML patients. This information may help identify common targeted therapies for patients with mutations in these functionally related genes.

MLL/KMT2A Tandem Duplications

The *MLL/KMT2A* gene was one of the first epigenetic regulators known to be involved in leukemia pathogenesis. MLL is a chromatin writer, a SET-domain containing lysine methyltransferase belonging to the *Drosophila* Trithorax family of proteins. Approximately 3–5% of *de novo* AML present with in-frame partial tandem duplications of MLL exons 3–9 or 3–11 (17). This mutation is associated with a poor prognosis (18, 19). The MLL partial tandem duplication (MLL-PTD) duplicated the N-terminal AT-hook region of MLL, in addition to a domain that preferentially binds to unmethylated CpG sites and a transcriptional repression domain (20, 21). Mice carrying the *MLL-PTD* mutation show developmental abnormalities and dysregulated *Hox* gene expression similar to AML patients with the *MLL-PTD* mutation (47), but require additional leukemogenic driver mutations such as the Flt3-internal tandem duplication (48) for overt leukemogenesis. Intriguingly, a recent study from Koeffler and colleagues aimed at capturing the mutational landscape of *MLL-PTD* AML demonstrated that *MLL-PTD* mutations co-occur with several other mutations, including *FLT3-ITD*, *DNMT3A*, *IDH1*, *TET2*, cohesion genes, and splicing factors, but not *NPM1* which is the most commonly mutated gene in AML (49). These studies suggest that *MLL-PTD* and *NPM1* mutations may act through overlapping mechanisms. Furthermore, ordering of mutations in this study suggested that the *MLL-PTD* mutation was a secondary mutation that was undetected in remission in contrast to persistent mutations in epigenetic regulators, such as IDH2/DNMT3A and TET2.

MLL/KMT2A Translocations

In addition to tandem duplications of MLL that are observed in AML, the chromosomal band 11q23 is also involved in chromosomal translocations that fuse MLL to a partner gene on another chromosome. MLL fuses to several different partner genes; more than 80 different MLL-fusion partners have been discovered to date (50, 51). MLL-fusions are observed in 5–10% of adult AML and approximately 15–20% of AML in infants (50). In infant ALL, the frequency of MLL-rearrangements is as high as 70% (50), highlighting the role of these fusions in leukemogenesis. The binding of MLL-fusions to their target promoters is contingent upon the interaction of the N-terminal part of MLL with the LEDGF protein, an interaction that is bridged by the protein Menin (MEN1). The MLL–Menin interaction, therefore, is an attractive target for therapy and small-molecule compounds targeting this interaction have been developed (52–55). MLL-fusion protein expression activates a cascade of downstream transcriptional programs, one of the most important of which is the clustered homeobox (*HOX*) genes and their cofactor MEIS1. These *HOX/MEIS* genes are crucial for perpetuating the highly self-renewing state that is triggered by MLL-fusion protein expression in transformed hematopoietic progenitors. Indeed, several recent studies have shown that oncogenesis by MLL-fusion proteins requires the coordinate action of a number of chromatin factors that are essential and rate limiting for the transcriptional activation of *HOX/MEIS* genes. A prime example of this is the histone methyltransferase (HMT) DOT1L. The DOT1L protein biochemically interacts with several of the most common MLL-fusion partners, including AF4, AF9, ENL, AF10, and AF17 (56–59). All of these fusion partners retain the DOT1L interacting motif in their respective MLL-fusion events, and this interaction has been shown to be necessary and sufficient for oncogenic transcriptional activation functions by MLL-fusion proteins. Based on structure–function assays, genetic studies, and small-molecule inhibitor investigations, DOT1L has emerged as a clear therapeutic target in MLL-rearranged AML and clinical trials are currently ongoing (60) as described later in the review. Interestingly, DOT1L seems to be generally involved in *HOX/MEIS* regulation and other models of AML where *HOX/MEIS* activation is observed are sensitive to genetic and/or pharmacological DOT1L inhibition. These include AML driven by MLL-fusion proteins that do not recruit DOT1L, MLL-tandem duplications, nucleoporin 98 (NUP98)–NSD1 fusions, *NPM1* mutations or mutations in the *DNMT3A* gene (59, 61–63). Strikingly, MLL–Menin inhibitors also seem to show broad activity against diverse HOX-activating AML oncogenes, suggesting that both these proteins are involved in an epigenetic network that is broadly essential for sustaining HOX gene expression (61). MLL-fusion transformed cells have also been shown to be sensitive to the depletion of several other chromatin factors, including PRC1 and polycomb repressive complex 2 (PRC2) complex proteins (64–69), the histone acetyltransferases (HATs) MOF, the arginine methyltransferase PRMT1 (70), and the MLL methyltransferase paralog MLL2 (71). Another interesting aspect of MLL-rearrangements is the involvement of chromatin readers. Many of the common fusion partners of MLL have chromatin-reading domains that recognize specific histone modifications and these reader–histone interactions and their transcriptional consequences are only recently being uncovered. AF9 and its paralog ENL harbor YEATS domains in their N-terminal region that bind to specific acetylated or crotonylated histone residues (72, 73). AF10 and AF17 on the other hand, have N-terminal PHD-zinc finger-PHD (PZP) domains that specifically recognize unmethylated H3K27 (74). Even though the chromatin reader modules of these MLL-fusion partners are excluded from MLL-fusion proteins themselves, chromatin reading by some

of the wild-type, non-rearranged MLL-fusion partners, such as AF10 and ENL, have been shown to be important for MLL-leukemogenesis (73–75). Intriguingly, MLL-rearranged AML cells, which were dependent on AF10 or ENL for their proliferation, were found to be insensitive to the inactivation of their closely related paralogs AF17 or AF9, respectively. Even though this mystery of differential sensitivity is still unresolved, the fact that chromatin reading by specific PZP and YEATS domains are critical for MLL-leukemogenesis opens up the exciting possibility of targeting MLL-rearranged leukemias using selective small-molecule inhibitors of these chromatin-reading modules that are likely to be developed in the near future.

PRC Dysregulation in AML

Polycomb group (PcG) proteins are transcriptional repressors that regulate key fundamental processes, including cellular identity, differentiation, and stem cell plasticity (76). PcG proteins have highly conserved roles throughout evolution in the silencing of transcription through specific histone modifications. PcG proteins are constituents of two major multi-subunit complexes, PRC1 and 2, which have distinct effects on chromatin, gene expression, and developmental regulation. The PRC2 complex consists of four core constituents: The *Drosophila* enhancer of zeste homolog (EZH2), embryonic ectoderm development, suppressor of zeste homolog, and RbAp46/48, also known as RBBP4. PRC1 composition is more variable with only two core components RING1A and RING1B which complex together with the proteins BMI1, MEL18, or NSPC1 (76). The PRC2 complex is involved in histone 3 lysine 27 mono, di, and trimethylation, a function that shows high evolutionary conservation as a major facilitator of gene silencing. EZH2, the enzymatic component of PRC2 is mutated in myeloid malignancies, most commonly in MDS, chronic myelomonocytic leukemia (CMML), and primary myelofibrosis and rarely in AML (14–16). These mutations are missense or frame-shift mutations, which are predicted to lead to EZH2 loss of function. Interestingly, in diffuse large B-cell lymphoma (DLBCL), approximately 20% of patients bear activating *EZH2* mutations (77), suggesting that PRC2 may have contrasting context-dependent roles in oncogenesis. Wild-type *Ezh2* depletion in murine hematopoietic progenitors leads to myeloproliferative effects (78), whereas depletion of non-enzymatic PRC2 components such as *Eed* leads to severe lethal myelo- and lympho-proliferative disorders (79). These results indicate that further investigations are required to clarify the roles of EZH2 and PRC2 activity in leukemogenesis.

Of the PRC1 components, the *BMI1* oncogene is implicated in the self-renewal of normal as well as leukemic stem cells in AML (80). Despite the apparent importance of BMI1 in normal and leukemic stem cells, mutations in this PRC1 component or any other members of the PRC1 complex have not been identified in AML.

Demethylase Mutations

Mutations in the histone 3 lysine 27 demethylase *UTX* are found in a variety of human cancers, including multiple myeloma, esophageal squamous cell carcinomas, and renal cell carcinoma (81). In myeloid malignancies, *UTX* mutations are found in 8% of patients with CMML and approximately 10% of patients with CMML-derived secondary AML. Most of these mutations were adjacent to the Jumonji C domain of UTX, which is required for the demethylase activity of UTX, suggesting that UTX loss of function may contribute to leukemogenesis. The JARID1A (KDM5A) H3K4 demethylase is fused to NUP98 in approximately 10% of pediatric acute megakaryoblastic leukemia resulting in the cytogenetically cryptic NUP98–JARID1A translocation. These fusions are believed to compromise normal functions both of NUP98 as well as JARID1A, leading to leukemogenesis (82). Exact consequences of demethylase mutations in these rare AML subtypes and their role in leukemogenesis remain to be discovered.

NSD Gene Fusions

Nuclear receptor-binding SET domain protein 1 is a HMT that is involved in recurrent chromosomal translocations with the NUP98 gene that are usually cryptic. NUP98–NSD1 fusions are found at a significantly increased frequency in pediatric as compared to adult patients (approximately 5 vs 1.4% of AML, respectively) (83, 84). In both adult and pediatric AML, NUP98–NSD1 translocations confer a poor prognosis and are enriched in the cytogenetically normal AML cohort. Mechanistically, NUP98–NSD1 fusions drive abnormal expression of *HOX/MEIS* oncogenes and this activation is dependent on the H3K36 methyltransferase activity of NSD1. NSD1-driven H3K36 methylation repels PRC2 complex proteins from the HOX/MEIS and other NUP98–NSD1 target genes, leading to sustained transcriptional activation and oncogenesis. NUP98-fusions with NSD3, a close homolog of NSD1 have also been reported in AML (85), further highlighting the role of this family of proteins in AML pathogenesis.

CBP/p300 and MOZ–MORF Fusions

The *mo*nocytic leukemia *z*inc-finger MOZ (MYST3) protein and its paralog MORF (MYST4) are HATs involved in recurring chromosomal rearrangements in AML. The balanced chromosomal translocation t(8;16)(p11;p13), which is found in <1% of AML patients, leads to in-frame fusions of MOZ with the HAT CBP (22). Another common partner of MOZ is TIF2, a member of the p160 family of nuclear receptor co-activators (86). MOZ-TIF2 expression in murine hematopoietic progenitor cells leads to aberrant *Hoxa* gene activation, increased self-renewal, and transformation in *in vitro* and *in vivo* assays. Notably, TIF2 interacts with CBP, indicating a common thread that links MOZ-fusions is the enlisting of CBP/p300 HAT activity. Consistent with this notion, MORF–CBP fusions, as well as fusions of either MOZ or MORF to the CBP homolog p300 have also been observed in AML, signifying common mechanisms linking these paralogous pairs of HATs to leukemogenesis. *HOX* gene activation is also observed in AML cells bearing MOZ–CBP fusions, similar to MLL and NUP98-fusion proteins (23). Even though patterns of *HOX* gene activation vary depending on which *HOX*-activating fusion protein is present in AML cells, *HOX* gene activation seems to be causally linked to transformation in all these AML subtypes based on preclinical studies.

Hijacking of Chromatin Modulators by AML Oncogenes

Apart from the epigenetic regulator mutations mentioned above, there are a number of examples of epigenome modulators that are not directly mutated but nevertheless implicated in AML pathogenesis. In recent years, several chromatin modifiers have been discovered as selective dependencies in specific AML subtypes as discussed briefly in the Section "The Advantage of Epigenetic Therapies." Some of the most striking examples of epigenetic regulator hijacking for AML pathogenesis are observed in studies with oncogenic fusion proteins. Co-option of histone methyl and acetyltransferases, such as DOT1L by MLL-fusion proteins has been discussed in detail in the Section "CBP/p300 and MOZ–MORF Fusions." In addition, a number of AML fusion proteins interfere with functioning of the PRC1 and PRC2 complexes. The promyelocytic leukemia–retinoic acid receptor (PML–RAR) fusion, which is seen in approximately 95% of the cases of acute promyelocytic leukemia (APL) (87, 88) can participate in biochemical interactions with several PRC2 complex proteins, recruiting repressive epigenetic modifications on target loci, while the other PML fusion oncoprotein PLZF–RARA binds to PRC1 complex members (89, 90). In separate studies, the PML–RARA fusion protein has also been shown to enlist the gene silencing activity of DNMT3A and HDAC3 complexes through biochemical interactions with the fusion protein (91–93). Similarly, the AML1/ETO fusion protein, a product of the recurrent t(8;21)(q22;q22) translocation, one of the most common cytogenetic abnormalities in AML, participates in biochemical interactions with chromatin modulatory proteins. AML1–ETO interacts with the protein arginine methyltransferase PRMT1. PRMT1 knockdown reduces the transcription of AML1–ETO target genes, implicating PRMT1 activity in AML1–ETO pathogenesis (94). AML1–ETO also acts as a transcriptional repressor and the repressive mechanisms of AML1–ETO have been shown to be facilitated by biochemical interactions with repressive complexes, such as N-CoR, mSin3A, SMRT, and HDAC1 (95–101).

THE ADVANTAGE OF EPIGENETIC THERAPIES

The last few years have seen a wave of unprecedented activity in the development of novel therapeutic agents and treatment strategies for AML. These include novel monoclonal antibody-based therapies, potent small-molecule inhibitors of signaling pathway mutations, tyrosine kinases, nuclear export, and immunotherapy. Most of these approaches are guided by specific mutations found recurrently in AML patients, which may herald a new era of precision medicine in AML. This strategy has been used with great success for more than a decade in the treatment of chronic myeloid leukemia and APL, but has largely failed in AML due to the absence of a single defining mutation event or hitherto intractable molecular targets. The recurrent prevalence of epigenetic regulator mutations in subsets of AML as well as broad epigenomic reprogramming across AML subtypes has ignited vigorous efforts to therapeutically target the AML epigenome. One of the biggest advantages of exploiting the epigenome as a therapeutic target is that, in contrast to the genomic alterations observed in AML cells that are difficult to reverse, epigenetic abnormalities can be reverted using pharmacological agents. Many epigenetic regulators such as DNA and histone modifying proteins have enzymatic activity, which is considered more amenable to therapeutic targeting using small-molecule inhibitors than other classes of proteins such as transcription factors. Another consideration is that since mutations in chromatin modulators are often observed in founding AML clones, targeting mutated epigenetic regulators may also eliminate LSCs, thereby striking at the root of AML and prevent relapse. For all of these reasons, the AML epigenome has emerged as one of the most exciting frontiers for drug discovery in recent years. Recent advances in preclinical and clinical development of epigenome-based therapies in AML will be discussed in the Section "Emerging Epigenome-Based Therapies in AML."

Emerging Epigenome-Based Therapies in AML

Some of the early epigenome-based strategies have focused on broad-based epigenomic reprogramming aimed at restoring the altered epigenomic configurations in AML cells. This kind of broad epigenomic reprogrammig—for example, with the use of DNMT or histone deacetylase complex (HDAC) inhibitors—has been shown to reverse the commonly observed silencing of tumor suppressor genes (TSG) and restore normal differentiation. Since these epigenetic processes are involved in both silencing as well as activation of transcription dependent on the epigenetic mark and the chromatin context, it may be very difficult to identify which subset of AML may benefit most from broad-based epigenomic reprogramming therapies. More targeted therapies require the identification of specific silenced TSG or activated oncogenes for targeted therapeutics (**Figure 2**). Nevertheless, broad-based inhibition of DNA methylation and histone deacetylation using DNMT and HDAC inhibitors has been explored extensively as a therapeutic strategy in AML. The DNMT inhibitors azacitidine (AZA) and decitabine (DAC) are extensively used in MDS and also in patients with AML, where they show benefit, especially in elderly AML patients (102). Drugs, such as valproic acid (VPA), panobinostat and vorinostat, are some of the HDAC inhibitors approved for clinical use. More recently, after the identification of epigenetic regulator mutations, efforts have intensified to precisely target the oncogenic activity of those mutant proteins. This approach is particularly promising, as it may finally lead to precisely targeted therapies in patients with non-APL AML. Finally, as mentioned previously, there is compelling evidence that some AML-activated oncogenic transcriptional programs are specifically dependent on chromatin regulatory proteins, marking these chromatin regulators as attractive candidates for therapy. Prominent examples are the HMT DOT1L that regulates *HOX* gene expression and the bromodomain-containing protein BRD4, which regulates the expression of super-enhancer linked genes in AML and other cancers. These newly discovered dependencies present hitherto unexplored epigenetic vulnerabilities for therapeutic

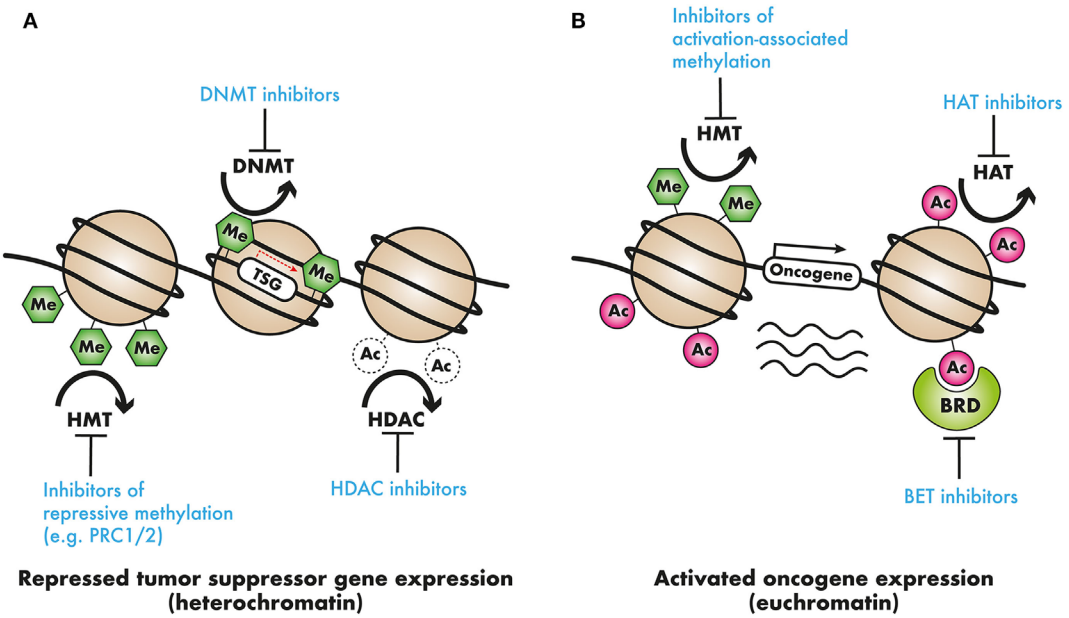

FIGURE 2 | Epigenetic modifiers in cancer as clinical targets: **(A)** tumor suppressor genes (TSG) may be silenced by chromatin compaction resulting from DNA methylation or histone deacetylation, or repressive histone methylation. DNA methyltransferase (DNMT) inhibitors, demethylating agents, histone deacetylase (HDAC) inhibitors, or inhibitors of repressive histone modifying complexes such as PRC2 may restore the expression of these TSGs. **(B)** In contrast, oncogene activation by means of activation-associated histone hypermethylation, or histone hyperacetylation could be countered with the use of selective histone methyltransferase (HMT) or acetyltransferase histone acetyltransferase (HAT) inhibitors. Additionally, readers that recruit these activation-associated marks, such as the AF10 PZP domain, the AF9 or ENL YEATS domain, and the BRD4 bromodomains, and recruit transcriptional complexes present targets for pharmacological intervention.

TABLE 2 | Current status of select pharmacological agents targeting epigenetic regulators.

Epigenetic target	Agent (reference)	Clinical trials	Mechanism
DNMT3A	Azacitidine (103, 104) Decitabine (105) Guadecitabine (106)	Phase 3 Phase 2/3 Phase 3	Nucleoside analogs that incorporate into DNA to inhibit DNMTs and prevent hypermethylation of tumor suppressor genes (TSG)
HDAC	Panobinostat (107) Vorinostat (108) Entinostat (109) Mocetinostat (110)	FDA FDA Phase 3 Phase 2	Reduction of oncogene transcription and signaling to promote cell cycle arrest and apoptosis
BET	OTX015 (111) INCB054329 (112) FT-1101 (113) GSK525762 (114)	Phase 1/2 Phase 1/2 Phase 1 Phase 1	Reversibly bind to BRDs of BET proteins to prevent acetylated histone binding and inhibit enhancer-mediated oncogene expression
IDH1/IDH2	AG-120 (115, 116) Enasidenib (117, 118)	Phase 3 FDA	Inhibition of mutant IDHs to restore TET2 activity and reduce DNA hypermethylation
EZH2	CPI-1205 (119) Tazemetostat (120)	Phase 1 Phase 1/2	Inhibition of H3K27 methylation to induce apoptosis or differentiation
DOT1L	EPZ-5676 (121, 122)	Phase 1	Inhibition of H3K79 methylation and induces synthetic lethality to cells with MLL rearrangement
LSD1	GSK2879552 (123, 124)	Phase 1/2	Inhibition of H3K4 and H3K9 demethylation to facilitate TSG expression and cell differentiation
MLL–Menin	KO-539 (125)	Preclinical	Selective inhibition of MLL-rearranged cell growth

DNMT3A, DNA methyltransferase 3A; HDAC, histone deacetylases; BET, bromodomain and extra-terminal motif; BRD, bromodomain; IDH, isocitrate dehydrogenase; TET2, tet methylcytosine dioxygenase; EZH2, enhancer of zeste homolog 2; H3K27, lysine 27 of histone H3; DOT1L, disruptor of telomere silencing 1-like; H3K79, lysine 79 of histone H3; MLL, mixed-lineage leukemia; LSD1, lysine-specific demethylase 1A; H3K4, lysine 4 of histone H3; H3K9, lysine 9 of histone H3.

intervention (see **Table 2**). In the Section "Broad Epigenomic Reprogramming As a Therapeutic Strategy in AML," pharmacological strategies that employ broad epigenetic reprogramming, specific targeting of mutated epigenetic regulators, or selective inhibition of cancer-specific epigenetic vulnerabilities will be discussed.

Broad Epigenomic Reprogramming As a Therapeutic Strategy in AML

DNMT Inhibitors

DNA methylation is dysregulated in most cancers including leukemia and has been the preferred target for cancer therapy since the development of hypomethylating agents (HMA). The HMAs AZA and DAC are nucleoside analogs and inhibitors of the DNMT enzymes DMNT1 and DMNT3. Investigational treatment with AZA and DAC in AML started more than 40 years ago [reviewed in Ref (102).]. AZA and DAC are now established as standard options for the treatment of older patients who do not tolerate standard intensive therapy. HMAs are thought to reactivate epigenetically silenced TSG through hypomethylation. Interestingly, HMAs seem to act indirectly through epigenetic reprogramming, rather than through direct cytotoxicity, as indicated by the delayed and prolonged responses (126, 127). Yet, a few caveats exist to the first-generation HMAs. Primary and secondary resistance to HMAs has been commonly reported (128, 129) and both AZA and DAC are degraded in plasma by the enzyme cytidine deaminase. This has promoted the development of second-generation HMAs with enhanced pharmacology and pharmacodynamic properties like guadecitabine, which has shown encouraging results in early clinical trials (130). Even though HMAs have provided much-needed options for older patients, their efficacy as single agent is limited. A number of studies have reported successful early findings from combination trials with HMAs with other agents used in AML such as tyrosine kinase inhibitors (102).

HDAC Inhibitors

Histone deacetylase complex inhibitors were initially identified in screens aimed at identifying factors that induce differentiation in leukemia cells (131). Histone acetylation is a major epigenetic mechanism that is carefully maintained by the interplay of HDACs and HATs (132). HDACs enzymatically remove the acetyl group from histones to serve as critical regulators of gene expression. Besides histones, many non-histone proteins that can be reversibly acetylated have been identified and are reported to be involved in a wide range of cellular processes, including gene expression, translation, DNA repair, metabolism, and cell structure (133). Many of these acetylated proteins are known to play roles in tumorigenesis, tumor progression, and metastasis (134). Along with HMAs, histone deacetylase inhibitors (HDACi) were the first epigenetically targeted inhibitors to be FDA approved for the treatment of cancer in the United States. HDAC inhibitors were historically identified based on their ability to induce tumor cell differentiation (135). Inhibition of class I HDACs targets expression of genes involved in cell cycle protein expression, cell cycle arrest in the G2/M phase, and apoptosis. HDAC inhibitors may help reactivate epigenetically silenced TSG including p21 and TP53. VPA was investigated in AML as the inhibitor of class I histone deacetylases. Unfortunately, the response rates of VPA for monotherapy in AML have been relatively low. Several other HDACi have been also tested as monotherapy in myeloid cancers, including romidepsin/depsipeptide (136, 137), entinostat (138), and mocetinostat (139). Likewise, these were found to be insufficient to further develop as a single agent in AML with the overall response rate ranging from 0 to 16%, with transient blast clearance and hematological improvement. Instead, when used in combination with agents with known antileukemia activity, including DNMTi (e.g., AZA, DAC) and chemotherapies, HDACi have shown a decreased time to response and an increase of overall response (107, 108, 140–150). Combination therapy based on the second-generation HDACi vorinostat or entinostat yielded an increased complete remission rate as compared to historical controls (151). The second-generation pan-HDACi panobinostat modulates gene expression by inducing hyperacetylation of core histone proteins, H3 and H4, and was shown to exhibit antitumor activity against several hematologic tumors, both *in vitro* and *in vivo* (107, 152). Even though potent and orally bioavailable, panobinostat yields modest result as a single agent in elderly patients with AML. Adding non-selective HDACi to combination schedules often results in increased toxicities which can lead to dose reduction and early treatment discontinuation (144, 153–157). Therefore, isozyme-selective HDACi with improved safety profiles may overcome this hurdle and provide additional clinical benefit to patients.

Bromodomain and Extra-Terminal Motif Protein (BET) Inhibitors

The bromodomain and extra-terminal (BET) protein family serve as transcriptional adapter molecules that facilitate transcription (158–160). They comprise bromodomain-containing protein (*BRD*) *2*, *BRD3*, and *BRD4*, which are universally expressed, while *BRDT* expression is limited to the testes (160, 161). Diverse functions of BET proteins include histone modification to chromatin remodeling and ultimately lead to transcriptional activation (162) and are essential for cellular homeostasis (160, 163–166). The most well-characterized function of BET proteins is their binding to acetylated lysine restudies through tandem N-terminal bromodomains, These bromodomains are specialized epigenetic reader modules that are essential for high-level expression of oncogenes such as Myc by promoting enhancer activity (167, 168). Recently, they have also been implicated in transcriptional dysregulation in many cancer types, with BRD4 identified as a key player in AML (167, 169–172). BET inhibitors (BETi) reversibly bind the bromodomains of BET proteins. In a variety of human AML cell lines, suppression of BRD4 was shown to suppress MYC effectively suggesting a potential target for cancer treatment (111, 172). OTX015, a thienotriazolodiazepine, is a small-molecule oral inhibitor of BRD 2/3/4 demonstrated to induce apoptosis in a variety of leukemia cell lines and human AML samples (173). BETi have raised great interest as a novel treatment approach, and ongoing phase 1 trials are investigating their single-agent activities along with combination therapies with other novel agents.

Lysine-Specific Demethylase 1 (LSD1) Inhibitors

Lysine-specific demethylase 1 has emerged as a promising therapeutic target in multiple cancers, notably in AML (174–179). Its main role is demethylation of H3K4me1/2 and H3K9me1/2 and LSD1 has been shown to dynamically affect a wide range of transcriptional programs in a context-specific manner, acting either as a transcriptional repressor or as an activator (180–183).

Pharmacologic inhibition or genetic knockdown of LSD1 in human leukemia cells induces differentiation (123). GSK2879552, an oral LSD1 inhibitor, is currently being investigated as a monotherapy in a phase 1 study for patients with relapsed/refractory AML (NCT02177812). In leukemia cell lines, there appears to be synergism between HDAC and LSD1 inhibitors which supports a clinical trial for further exploration (124). To date, the only HDACi to be evaluated preclinically in combination with an LSD1 inhibitor (SP2509) in AML is the pan-HDACi panobinostat. Treatment with SP2509 and panobinostat resulted in synergistic *in vitro* cytotoxic effects and significantly improved the survival of mice engrafted with AML cells without overt toxicity (178).

EZH2 Inhibitors

As mentioned previously, the exact role of EZH2 in AML is not entirely clear. Studies using an MLL-AF9 leukemia model have shown that PRC2 activity is required for MLL-rearranged AML. Inactivation of *Eed*, the critical component of PRC2 prolonged survival and reduced tumor burden in leukemic mice (22). These results were recapitulated with the use of UNC1999, a small-molecule inhibitor of both EZH1 and 2 which upregulated PRC2 target genes such as p16 and p19 in MLL-rearranged leukemia cells and strongly suppressed transformation (64, 184). A number of potent and selective EZH2 and PRC2 inhibitors are being tested in clinical trials in other malignancies where PRC2 activity has demonstrated proto-oncogenic roles such as DLBCL and synovial sarcoma, and it remains to be studied which subsets of AML may benefit from PRC2 antagonist therapies.

Targeting of Mutated Epigenetic Regulators

IDH Inhibitors

Given the high prevalence of *IDH* mutations in AML as well as in low-grade glioma, intensive efforts are on to develop clinical-grade IDH inhibitors. AGI-6780, a potent and selective allosteric inhibitor of the IDH2-R140Q mutations was recently reported to significantly induce differentiation in primary AML cells bearing IDH2-R140Q in *ex vivo* cultures. More recently another potent small-molecule inhibitor AG-221 (enasidenib) was developed that was shown to confer significant survival benefits in a mouse model of IDH mutant leukemia and also in a xenografts model of primary human AML (185). These exciting studies catapulted IDH inhibitors into clinical trials with very encouraging results, leading to the FDA approval of AG-221 for the treatment of patients with relapsed or refractory AML with *IDH* mutations. Considering that the IDH mutations were only first discovered less than 10 years ago (186), the fact that IDH inhibitors have already been approved for use is an astonishing success story for precision medicine in AML, although the long-term benefits of IDH inhibitors for AML patients remain to be seen.

Targeting Epigenetic Dependencies

DOT1L Inhibitors

An S-adenosyl-methionine competitive inhibitor of DOT1L (EPZ-4777) was developed by Epizyme Inc. as a potent and selective inhibitor of the methyltransferase activity of DOT1L (187). Using this compound as a tool, several studies preclinical studies were performed to show that MLL-rearranged AML was highly sensitive to pharmacological DOT1L inhibition (59, 187–190). Subsequently, using structure-guided design and optimization of a series of aminonucleoside compounds, the small-molecule EPZ-5676 was developed as a more potent DOT1L with better pharmacological properties than EPZ-4777 (92). Preliminary studies demonstrated potent single-agent antitumor effects of EPZ-5676 in preclinical models of *MLL*-rearranged AML, and synergistic effects with other standard chemotherapeutic drugs (63, 191). EPZ-5676 is being evaluated in clinical trials for adult and pediatric patients with relapsed or refractory AML with *MLL*-rearrangements (122, 187). EPZ-5676 was well-tolerated in initial studies and showed efficacy in a few patients, but several other patients showed moderate to no response, possibly due to pharmacokinetic limitations of the drug. Continued investigation of EPZ-5676 in patients with *MLL* gene rearrangements is warranted and results from the Phase I/II trials are awaited. Next-generation DOT1L inhibitors with improved pharmacological properties are being developed and are likely to show more pronounced efficacy in the clinic.

MLL–Menin Inhibitors

The MLL–Menin interaction is retained in all MLL-fusion proteins (192–195). Preclinical studies have demonstrated a critical role for Menin in leukemic transformations mediated by numerous MLL-fusion proteins. Genetic disruption of the MLL–Menin fusion protein interaction abrogates oncogenic properties of MLL-fusion proteins and blocks the development of acute leukemia *in vivo* (195). Recently, small-molecule inhibitors of the MLL–Menin interaction MI-463 and MI-503 were developed, and they were used to demonstrate that pharmacologic inhibition of the MLL–Menin interaction blocks progression of MLL leukemia *in vivo* without impairing normal hematopoiesis (54). These studies have prompted the development of more potent clinical-grade MLL–Menin inhibitors.

CONCLUDING REMARKS

These are still early days for targeted epigenetic therapies, but the prospects are very exciting. There are several challenges ahead that warrant consideration before epigenetic therapies become the mainstay of AML treatment strategies. First, a lot more needs to be done in terms of preclinical and basic research in order to define exact consequences of epigenetic regulator mutations that have been discovered in AML. This will require the development of faithful genetically engineered mouse models that recapitulate AML mutations, combined with detailed studies on normal and leukemic hematopoiesis. Characterization of the impact of these mutations on normal physiological processes in general and hematopoiesis, in particular will be helpful in predicting potential toxicities. Second, barring few exceptions, it is not entirely clear which subsets of AML may benefit from a particular epigenome-based therapy. Matching patients appropriately to epigenetic therapies will require detailed characterization and sensitivity studies including *in vitro* and *in vivo* inhibitor or genetic screens or epigenomic studies aimed at identifying specific "epigenetic lesions" and their respective drivers. Finally, there is an urgent need for the

development of more potent and more selective small-molecules targeting epigenetic regulators. This is a rapidly developing field and selectively small-molecule inhibitors of class-specific HDACs, HATs, as well as DNA and HMT are being developed by several academic investigators and pharmaceutical companies. The next decade will see unprecedented activity in preclinical and clinical investigation of epigenome-based therapies.

AUTHOR CONTRIBUTIONS

AD, YS, and BC conceived and drafted the manuscript. YS prepared the illustrations.

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MicroRNA, an Antisense RNA, in Sensing Myeloid Malignancies

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Myeloid malignancies, including myelodysplastic syndromes and acute myeloid leukemia, are clonal diseases arising in hematopoietic stem or progenitor cells. In recent years, microRNA (miRNA) expression profiling studies have revealed close associations of miRNAs with cytogenetic and molecular subtypes of myeloid malignancies, as well as outcome and prognosis of patients. However, the roles of miRNA deregulation in the pathogenesis of myeloid malignancies and how they cooperate with protein-coding gene variants in pathological mechanisms leading to the diseases have not yet been fully understood. In this review, we focus on recent insights into the role of miRNAs in the development and progression of myeloid malignant diseases and discuss the prospect that miRNAs may serve as a potential therapeutic target for leukemia.

Keywords: acute myeloid leukemia, microRNAs, microRNA-based therapeutics, myelodysplastic syndrome, myeloid malignancies

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INTRODUCTION

MicroRNAs (miRNAs) in Cancer

MicroRNAs are small non-coding RNAs with 19–22 nucleotides to control gene expression through binding to mRNA of their cognate target genes and thereby participate in numerous biological processes such as cell proliferation, differentiation, development, metabolism, apoptosis, survival, and hematopoiesis (1). *miRNA* is transcribed by RNA pol II/III to generate a primary miRNA followed by nuclear cleavage by the RNase III endonuclease Drosha and its binding to the double-stranded RNA-binding protein DGCR8 to form a precursor miRNA (pre-miRNA) (2, 3). Subsequently, the pre-miRNA is transported by Exportin-5/RanGTP to the cytoplasm to be further cleaved by the RNase III endonuclease Dicer, leaving an unstable miRNA duplex that unwinds. The 5′ guide strand containing the mature miRNA sequence is incorporated into a ribonucleotide silencing complex, while the 3′ passenger strand undergoes rapid degradation (4–6).

While miRNAs located within chromosomes deleted in cancer play roles as tumor suppressors, miRNAs located in genomic regions amplified in cancer function as oncogenes. Deregulated miRNAs found in both solid tumors and hematopoietic malignancies target the transcripts of essential protein-coding genes involved in tumorigenesis (7, 8). Fingerprints of miRNAs' expression are linked to clinical and biological characteristics of tumors including tissue type, aggressiveness, and therapy response. Abnormal expression of pre-miRNA is also found in various types of human cancer. Because sequence abnormalities of *miRNAs*' genes and transcripts are also observed in the germline (8), the inherited subtle variations in miRNAs may have a great effect on the expression profiles of protein-coding genes in cancer.

miRNAs in Hematological Malignancies

Hematological malignancies comprise a collection of heterogeneous diseases, all originating from cells of the bone marrow or lymphatic system. Hematological malignancies include leukemias,

lymphomas, myelodysplastic syndromes (MDS), and myeloproliferative neoplasms (9). Myeloid malignancies are clonal disorders that are characterized by excessive proliferation, abnormal self-renewal, and/or differentiation blocks of hematopoietic stem cells (HSCs) and myeloid progenitor cells (10, 11). miRNA expression profiling in myeloid malignancies has revealed distinct signatures associated with diagnosis, stage classification, progression, prognosis, and response to treatment of leukemias (**Table 1**). miRNAs can be regulated by epigenetic modifiers including DNA methylation and histone modification in leukemias, suggesting that aberrant expression of miRNAs by epigenetic mechanisms may trigger hematopoietic cell transformation.

In this review, we focus on recent advances in understanding the roles of miRNA deregulation in the pathogenesis of myeloid malignancies and discuss the prospect that miRNAs may serve as potential therapeutic targets for leukemias.

miRNA DEREGULATION IN MDS

Myelodysplastic syndromes are HSC disorders characterized by ineffective hematopoiesis and a high risk of progression to acute myeloid leukemia (AML) (50). More than 70% of all human miRNAs are located within regions of recurrent copy-number alterations in MDS and AML cell lines (51). The targeted ablation of *Dicer1* in murine hematopoietic system leads to abnormal hematopoiesis and MDS, supporting the relevance of miRNA deregulation to the pathogenesis of MDS (52).

TABLE 1 | MicroRNAs (miRNAs) in myeloid malignancies.

miRNA	Expression profiles in leukemias	Reference
miR-22	High in AML/high in MDS	(12, 13)
miR-99	High in AML	(14)
miR-128a	High in AML	(15)
miR-155	High in AML	(16)
miR-182	High in AML	(17)
miR-221/miR-222	High in AML	(18)
miR-4262	High in AML	(19)
miR-29	Low in AML	(20)
miR-34a	Low in AML	(21)
miR-34b	Low in AML	(22)
miR-137	Low in AML	(23)
miR-142-3p	Low in AML	(24)
miR-194-5p	Low in AML	(25)
miR-204	Low in AML	(18)
miR-217	Low in AML	(26)
miR-223	Low in AML with t(8;21)	(18, 27–29)
miR-302a	Low in AML	(30)
miR-451	Low in AML	(31)
miR-650	Low in AML	(32)
miR-125b	High in AML	(33, 34)
miR-192	Low in AML	(35, 36)
miR-193	Low in AML	(37)
miR-124	Low in AML	(38, 39)
miR-181a	Low in AML	(18, 40)
miR-196b	High in AML	(34, 41)
miR-21	High in acute lymphoblastic leukemia, high in MDS	(42, 43)
miR-17–92/miR-20	High in CML/high in MDS	(44, 45)
miR-10a	Low in CML, high in MDS	(18, 46)
miR-126	High in AML	(47, 48)
miR-155	High in MDS	(46)
miR-130	High in MDS	(46)
MiR-144/451	Low in MDS	(12, 49)
miR-146a	Low in MDS	(46)
miR-150	Low in MDS	(46)
let-7a	Low in MDS	(46)

AML, acute myeloid leukemia; MDS, myelodysplastic syndromes.

Several recent studies have addressed the role of miRNAs in MDS pathogenesis. Vasilatou et al. have shown that miR-17-5p and miR-20a, as members of the miR-17–92 cluster, repress the transcription factor E2F1, which is highly expressed in 67% of patients with MDS (44). Similarly, let-7a downregulates KRAS, which is aberrantly expressed in high-risk MDS (53, 54). A subset of miRNAs involved in stage-specific regulation of erythropoiesis are also deregulated in MDS (55). Overexpression of miR-181, miR-221, miR-376b, miR-125b, miR-155, or miR-130a inhibits erythroid cell growth (56), and this event might be responsible for disease-associated ineffective erythropoiesis. miR-155 targeting CEBPB and CSF1R is significantly upregulated in high-risk MDS (57). High expressions of miR-155, miR-126, and miR-130 in MDS restrain megakaryopoiesis and may account for higher frequency of thrombocytopenia observed during disease progression (46). However, recent evidence reveals that reduction of Rho family members by miR-155 contributes to impaired neutrophil migration in MDS (58). miR-21 expression has been found to be increased in MDS, and its interaction with SMAD7 mRNA leads to ineffective, MDS-like hematopoiesis *via* overactivating TGFβ signaling (42). In addition, serum miR-21 level appears to act as a potential non-invasive biomarker that predicts a response following treatment with hypo-methylating agents, such as azacytidine or decitabine in MDS patients (59). In contrast, decreased expression of the miR-144/451 members targeting the erythroid transcription factor GATA-1 is closely associated with high-risk MDS (12, 49). Overall, both the aberrant expression and the function of miRNAs are the important factors contributing to MDS pathogenesis and prognosis.

Despite significant amount of evidence demonstrating miRNA expression and role in tumorigenesis is available, a very few studies illustrate mechanisms of miRNA deregulation and related mechanisms underlying MDS. miR-22 has been found to be overexpressed in MDS patients with poorer survival outcome (60). Furthermore, transgenic mice expressing hematopoietic miR-22 exhibit decreased global 5-hydroxymethylcytosine levels and increased HSC self-renewal along with defective differentiation and develop MDS and myeloid leukemia over time. miR-22 directly targets the DNA demethylating enzyme ten-eleven-translocation 2 (TET2) and affects the epigenetic landscape in the hematopoietic compartment, while forced expression of TET2 suppresses the miR-22-induced malignant phenotypes. A significant inverse correlation between miR-22 and TET2 observed in MDS patients suggest the miR-22-TET2 regulatory network as a reliable factor for MDS pathogenesis (60, 61). A better understanding of miR-22 deregulation in MDS disease progression and AML transformation will provide insight into the mechanisms of MDS pathogenesis and provide new therapeutic strategies against leukemia transformation.

EMERGING ROLES OF miRNA DEREGULATION IN THE PATHOGENESIS OF AML

Acute myeloid leukemia is characterized by the accumulation of immature myeloid cells in the bone marrow and shows genetic abnormalities including mutations and chromosomal translocations (10). Distinctive miRNA expression profiles have been demonstrated for cytogenetic subtypes and mutations in *CEBPA*, *FLT3*, and *NPM1* of AML (62–64). miRNA profiles are also associated with AML prognosis, underscoring the importance of miRNAs in AML (65). As such, miRNAs impact AML development and progression through targeting known oncogenes or tumor suppressors or collaborating with them to promote or suppress myeloid malignancy.

miR-9 has shown to be overexpressed in MLL-rearranged AML and play a critical oncogenic role in MLL fusion-mediated leukemogenesis (66). Ectopic expression of miR-9 blocks neutrophil development in myeloid cell lines and in murine primary lineage-negative bone marrow cells by inhibiting ETS-related gene (67). Also, miR-9 exerts its tumor-suppressive effects through the cooperation with let-7 to repress the oncogenic Lin28b/HMGA2 axis in AML (68).

miR-125b is upregulated in AML patients and blocks the differentiation of AML blast cells by directly targeting the cytoplasmic tyrosine-protein kinase FES that is expressed exclusively in myeloid cells (33). Overexpression of miR-125b also leads to a reduction in expression of the RNA binding protein Lin28A (69), which is known to play an important role in stem cell biology.

miR-181 and miR-128 target Lin28, leading to the progression of myeloid leukemia and differentiation blockage of hematopoietic cells to their lineage (15, 70, 71). The inhibition of miR-181 expression partially reverses the lack of myeloid differentiation in AML patients and in the mice implanted with CD34+ hematopoietic stem/progenitor cells (HSPCs) from AML patients (72).

The targeted miR-126 reduction in cell lines and primary AML samples results in decreased AML growth through inhibiting multiple components of the PI3K/AKT/mTOR pathway (47, 73, 74). The attenuated expression of miR-126 also leads to expand normal HSC (75), suggesting that miR-126 dictates opposing self-renewal outcomes in normal and leukemic HSC. Furthermore, both gain- and loss-of-function *in vivo* studies of miR-126 in murine models demonstrate that either overexpression or knockout of miR-126 promotes development of AML in mice (76). This result suggests that miR-126 plays a dual role in leukemogenesis and supports a new layer of miRNA regulation in AML.

Overexpressed miR-155 is associated with poor outcome in AML patients. miR-155 promotes FLT3-ITD-induced myeloproliferative disorder through inhibition of the interferon (IFN) response, inositol 5-phosphatase 1 (SHIP1), CEBPB, and PU.1, while it is upregulated in FLT3-ITD+ and MLL-rearranged AML (57, 77–80). These results suggest that miR-155 can collaborate with FLT3-ITD to promote myeloid cell expansion, and this involves a multi-target mechanism that includes repression of IFN signaling.

miR-22 is overexpressed in AML, and its aberrant expression correlates with silencing of TET2 in AML patients (60). Approximately 70% of miR-22 transgenic mice develop AML by 2 years of age. Also, miR-22 impairs the MLL-AF9-induced leukemogenesis through repressing CREB and Myc pathways and relieves the monocyte/macrophage differentiation and the growth of AML by targeting MECOM (81, 82). Therefore, in AML, miR-22 can be both oncogenic and tumor-suppressive, depending on the specific individual backgrounds (e.g., early HSCs versus the committed myeloid progenitors).

The accumulation of peroxiredoxin III caused by decreased miR-26a leads to a marked reduction in reactive oxygen species (ROS) in primary AML granulocyte samples (83). Growing evidence demonstrates that ROS plays a key role in regulating the balance between self-renewal and differentiation of HSCs (84). Thus, the reduced ROS levels might drive HSCs toward differentiation into myeloid lineage fates, providing a potential mechanism for miR-26a's role as a tumor suppressor.

miR-29a appears to be significantly increased in peripheral blood mononuclear cells and bone marrow white blood cells from AML patients. Increased miR-29a promotes differentiation into granulocytes and monocytes, while reduction of miR-29a suppresses myeloid differentiation in leukemic cells (85). In myeloid leukemogenesis, c-Myc inhibits miR-29a expression, resulting in increased AKT2 and Cyclin D2 expressions in AML (86). Conversely, ectopic expression of miR-29a in murine HSPCs leads to acquisition of self-renewal capacity by myeloid progenitors, biased myeloid differentiation, and the development of a myeloproliferative disorder that progresses to AML (87).

miR-34a is downregulated in AML and induces apoptosis *via* inhibition of autophagy by targeting HMGB1 in leukemic cells (21). miR-34b plays a critical role in AML pathogenesis by targeting CREB, and its expression is repressed due to its promoter hypermethylation in AML patients (22). The methylation of the miR-124a family, including miR-124a-1 and miR-124a-3, is also observed in AML patients independently of their cytogenetic subtypes (88). It is also noted that epigenetic silencing of miR-124a is associated with the expression of EVI1 in AML (89, 90).

While studies of miR-125b suggested that it has oncogenic role in AML, miR-125a is considered as a tumor-suppressive miRNA. miR-125a expression in cytogenetically normal AML appear to be most decreased in favorable and intermediate prognostic populations and associated with decreased survival (91).

In the context of AML caused by toxic DNA interstrand crosslinks (ICLs), miR-139 and miR-199a have opposite roles in hematopoietic cell expansion and leukemogenesis (92). The levels of miR-139 and miR-199a are elevated with age in myeloid progenitors from the nucleotide excision repair gene (*Ercc1*)-deficient mice. Ectopic expression of miR-139 inhibits proliferation of myeloid progenitors, whereas increased miR-199a enhances proliferation of progenitors and augments the AML phenotype. This study supports the oncogenic role of miR-199a and also indicates that the elevated miR-139 as a tumor suppressor is involved in the defective hematopoietic function in ICL-induced AML.

miR-223 decreases cell proliferation and enhances cell apoptosis in AML *via* targeting FBW7 (93, 94). miR-223 was originally identified as a critical regulator in granulopoiesis and

transactivated by NFI-A and C/EBPα in acute promyelocytic leukemia (27). AML1/ETO oncoprotein induces epigenetic silencing of miR-223 through directly binding to the *pre-miR-223* gene in AML (28). miR-223 targets E2F1 to inhibit cell cycle progression, thereby resulting in myeloid differentiation, and in turn, E2F1 represses miR-223 transcription, forming a negative feedback loop in AML (95–97). In summary, scientific evidence supporting the role of miRNAs in the pathogenesis of AML with proven tumor suppressors or oncogenic activities is becoming increasingly clear.

miRNA-BASED THERAPEUTICS IN MYELOID MALIGNANCIES

As the understanding of miRNA expression and action in myeloid malignancies continues to evolve, miRNAs have a great potential to serve as both the non-invasive biomarkers and a potential therapeutic target for leukemia. For example, miRNA expression signatures classify leukemias of uncertain lineage as either AML or acute lymphoblastic leukemia (98). miRNA expression profiles can also predict progression of MDS to AML (99) and survival outcome of AML patients (100, 101). Furthermore, circulating miRNAs have been recently demonstrated as an economical, non-invasive, and sensitive tool to monitor for minimal residual disease, which refers to the persistence of a small number of leukemic blasts in the bone marrow after chemotherapy and can ultimately cause disease relapse. Indeed, AML patients have a distinctive circulating miRNA expression profiles compared to healthy controls (102, 103), and an altered expression signature of serum miRNAs is observed after standard chemotherapy (104).

Yet, cancer therapy is based on a therapy targeting a single gene or pathway: "one target, one drug" model. A treatment effectively targeting multiple genes and pathways of cancer concomitantly may be an important innovation. Such an approach would not only more effectively suppress cancer cell growth but also would inhibit the common emergence of resistance in a single gene or pathway. miRNAs form a complex network where each miRNA can regulate multiple genes and pathways and each gene or pathway can be regulated by multiple miRNAs. Thus, miRNAs hold promising potential for "multi-targeted therapy" in cancer patients. To date, miRNA replacement therapy has largely made use of synthetic miRNA mimics to restore lost tumor suppressor expression (105). Restoration of lost tumor suppressor miRNAs using synthetic double-stranded RNAs (with a delivery agent) has been successful in preclinical models of leukemia. For example, the targeted delivery of miR-29b mimics by transferrin-conjugated lipid nanoparticles in mice engrafted with human AML cells shows a significantly longer survival compared to control nanoparticles or free miR-29b (106).

Conversely, targeting overexpressed oncomiRs can be conducted mainly by three approaches: anti-miRNA oligonucleotides (AMOs; antagomiRs), miRNA masking, and miRNA sponges (**Figure 1**). AMOs that are chemically modified with the locked nucleic acid (LNA) can be systemically delivered to affect cancer-related pathway *via* the binding and inhibiting oncomiRs in leukemia. For instance, targeting nanoparticles containing miR-126 antagonists (antagomiR-126) results in an

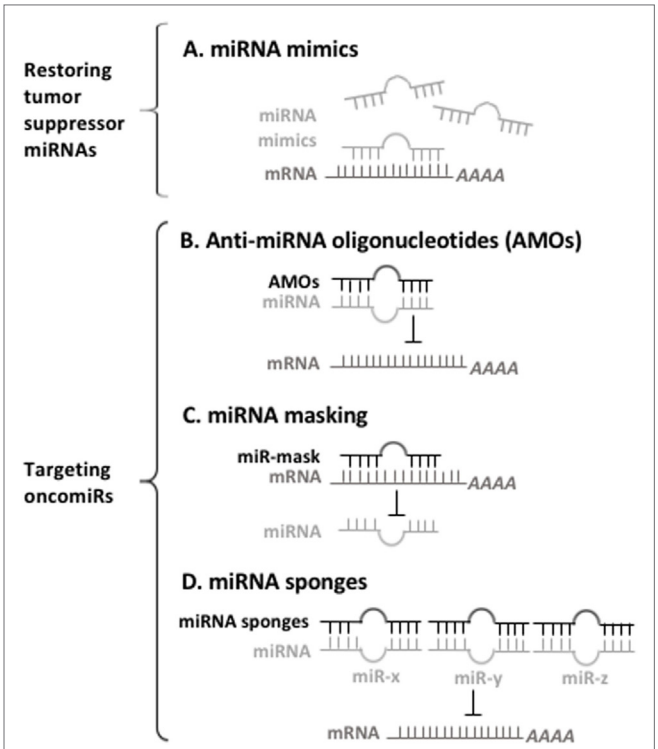

FIGURE 1 | A prospect of miRNA-based therapy for myeloid malignancies. **(A)** Synthetic oligonucleotides used for restoring the depleted microRNAs (miRNAs) bind to their target mRNAs for inhibiting mRNAs of oncogenes. **(B)** Anti-miRNA oligonucleotides (AMOs) interact with oncomiRs, thus preventing them from interacting with their target mRNA. **(C)** miRNA-mask is designed to bind to 3′UTR of mRNAs, thus preventing oncomiRs recognition their target mRNAs. **(D)** miRNA sponges have multiple complementary sites against targeted miRNA, thereby inhibit the functions of oncomiRs.

in vivo reduction of leukemic stem cells by depletion of the quiescent cell sub-population (74). miR-21- and miR-196b-specific antagomiRs inhibit *in vitro* leukemic colony-forming activity and *in vivo* leukemia-initiating cell activity of HOX-based leukemias, which have led to improved survival and delayed disease onset in murine AML models (107). miRNA-masking antisense oligonucleotides (miR-mask) can be used to achieve a gene-specific anti-miRNA therapy that masks the specific target mRNA from endogenous miRNA, and thus prevent the inhibitory action of miRNA. miRNA sponges are another approach to silence miRNAs with potentially important clinical utility, and have complementary binding sites to seed sequences of target miRNAs. This advantage gives them the ability to inhibit multiple miRNAs that have the same sequence in their seed region. It has been shown that using miR-22 sponges, both the leukemic cell proliferation and the activity of miR-22 are markedly impaired (60).

In addition to these encouraging outcomes with the use of miRNA-based therapy in preclinical, animal models, the first therapies targeting miRNAs have now entered clinical trials (108). Treatment of an LNA inhibitor of miR-122 (known as Miravirsen) in patients with hepatitis C virus infection holds great promise of miRNA-based therapeutics (https://ClinicalTrials.gov, NCT01200420) (109). Furthermore, the first cancer-targeted

miRNA drug—MRX34, a liposome-based miR-34 mimic—has entered clinical trials in patients with advanced or metastatic liver cancer (https://ClinicalTrials.gov, NCT01829971) (110). These studies provide a proof of principle that should encourage future endeavors of miRNA-directed therapy for leukemia.

CONCLUSION AND PERSPECTIVES

There are many more miRNAs, shown in publication, that are involved in the pathogenesis of myeloid malignancies, suggesting intense enthusiasm for research in this area in recent years. However, the regulatory changes in miRNA levels are often small and might get lost in the biological noise when using a small number of samples. Using *in vitro* systems to study the miRNA phenotypes might be different from what happens *in vivo*. Also, the efficacy of overexpression or antagomiR tools should be validated using downstream target readout to convince the endogenous interaction between the miRNA and the targets.

MicroRNAs have emerged as the potential targets for therapeutic applications. Circulating miRNAs in exosomes/extracellular vesicles from serum or plasma represent a new source of promising biomarkers that may be applied to clinical settings. A specific MDS/AML-associated serum miRNA profiles could not only provide an exciting screening tool for early detection of leukemia in the clinic but could also be used to track leukemic blasts relapsed after chemotherapy. However, exosomal miRNAs loaded from leukemic cells can be transferred to stromal or normal HSC recipient cells and alter their functions, thereby promoting leukemic phenotypes. A further investigation of the relevance of exosomal miRNAs to the pathogenesis of myeloid malignancies is clearly warranted.

Although the case of miRNA-based therapeutics entering clinical trials continues to grow, no miRNA-based therapy has yet made its way to clinical trials particularly for the treatment of AML. A main obstacle of applying miRNA-based therapeutics for clinical use is the limitation of more efficient and specific delivery methods. Thus, many new approaches are currently being explored for improved delivery of miRNA-based therapies, including liposomes, nanoparticles, LNAs with increased stability, and peptide-based inhibitors. Further, how to precisely deliver miRNA mimics or antagomiRs into the targeted cells *in vivo* has also become another major barrier preventing the establishment of miRNA-directed strategies. But nevertheless, miRNA-based therapies may be available soon for the treatment of myeloid malignancies, and miRNA-based therapeutics may be efficacious when used in a combination with current chemotherapy regimen for leukemia.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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RUNX1 Mutations in Inherited and Sporadic Leukemia

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RUNX1 is a recurrently mutated gene in sporadic myelodysplastic syndrome and leukemia. Inherited mutations in *RUNX1* cause familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML). In sporadic AML, mutations in *RUNX1* are usually secondary events, whereas in FPD/AML they are initiating events. Here we will describe mutations in *RUNX1* in sporadic AML and in FPD/AML, discuss the mechanisms by which inherited mutations in *RUNX1* could elevate the risk of AML in FPD/AML individuals, and speculate on why mutations in *RUNX1* are rarely, if ever, the first event in sporadic AML.

Keywords: *RUNX1*, leukemia, myeloid neoplasms, leukemia predisposition, familial platelet disorder with predisposition for acute myeloid leukemia, pre-leukemia

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INTRODUCTION

Acute leukemia is caused by the acquisition of mutations in hematopoietic stem and/or progenitor cells (HSPCs) that promote their clonal expansion and impair downstream differentiation. The earliest initiating step in leukemia is a mutation that generates a hematopoietic stem cell (HSC) that is, in some poorly understood way, primed for leukemic transformation by secondary mutations. The mutations in HSCs that initiate sporadic leukemia in adults generally occur in genes encoding epigenetic regulatory proteins such as *DNMT3A, ASXL1, IDH2*, and *TET2* (Papaemmanuil et al., 2016). Disease progression is caused by the acquisition of secondary mutations in HSCs containing initiating mutations. These secondary, driver mutations involve genes encoding several functional categories of proteins including transcription factors (e.g., *CEBPA, RUNX1, GATA2*, and *ETV6*), signaling molecules (*FLT3, NRAS, PTPN11, KRAS, KIT, CBL*, and *NF1*), splicing factors (*SRSF2, SF3B1*, and *U2AF1*), and proteins with other functions (*NPM1, SMC1A*) (Cancer Genome Atlas Research Network et al., 2013; Papaemmanuil et al., 2016).

The order of mutations in sporadic acute leukemia in adults, where mutations in epigenetic regulators generally precede those in genes encoding other categories of proteins, is upended in individuals who have inherited a leukemia predisposition gene. Leukemia predisposition genes are constitutional mutations that greatly elevate the lifetime risk of leukemia, which in the general population is 1.5% (Howlader et al., 1975–2014), but in individuals with inherited leukemia disposition genes, the risk of myeloid malignancy is much higher depending on the mutation and other genetic, epigenetic, and environmental factors (Owen C. et al., 2008; Liew and Owen, 2011; West et al., 2014). For instance, the lifetime risk of myeloid malignancy is about 44% with germ line *RUNX1* mutations; however, the lifetime risk approaches nearly 100% for germ line *CEBPA* mutations (Godley, 2014). The importance of these inherited mutations in leukemia, their distinct clinical features, and the implications for treatment were recently recognized by the World Health Organization in their 2016 revision to the classification scheme for myeloid neoplasms and acute leukemia, which includes a new category of "myeloid neoplasms with germ

line predisposition" (Arber et al., 2016; **Table 1**). Of note, mutations in several of the genes that confer leukemia predisposition (e.g., *CEBPA, ETV6, GATA2, NF1, RUNX1, PTPN11, CBL*, and *RAS*) are also frequently found in sporadic acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) (Cancer Genome Atlas Research Network et al., 2013). However, an important distinction, for which the mechanistic basis is not well understood, is that these inherited mutations are rarely initiating events in sporadic AML in adults but are, by definition, the initiating events in myeloid neoplasms with germ line predisposition.

Here we will focus on *RUNX1,* a leukemia predisposition gene that is also frequently mutated in sporadic leukemia. We will describe the inherited and acquired mutations in *RUNX1* and speculate on why mutations in *RUNX1* can be initiating events when inherited but are rarely initiating events in sporadic leukemia.

SOMATIC MUTATIONS IN *RUNX1*

RUNX1 encodes a sequence-specific transcription factor that is essential for HSC formation in the conceptus and is important for the differentiation of cells of the lymphoid, myeloid, and megakaryocytic lineages (Cai et al., 2000; Ichikawa et al., 2004; Growney et al., 2005; Tober et al., 2016). *RUNX1* is a recurrent target of somatic mutations in *de novo* AML, myelodysplastic syndrome (MDS), acute lymphocytic leukemia (ALL), atypical chronic myeloid leukemia (aCML), and secondary AML (Mangan and Speck, 2011). There are two broad categories of mutations in *RUNX1*: monoallelic chromosomal translocations and mono- or biallelic somatic mutations. The most common chromosomal translocations are t(8;21)(q22;q22) in *de novo* AML and t(12;21)(p13;q22) in acute lymphocytic leukemia (B-ALL). Both of these translocations are initiating events in sporadic leukemia and can be acquired *in utero* as evidenced by their presence in Guthrie card samples from newborns diagnosed in childhood with AML or B-ALL (Wiemels et al., 1999, 2002). The t(8;21) and t(12;21) translocations generate fusion proteins (RUNX1-RUNX1T1 and ETV6-RUNX1, respectively) with neomorphic activity (Miyoshi et al., 1991, 1993; Chang et al., 1993; Nucifora et al., 1993; Golub et al., 1995; Romana et al., 1995; Shurtleff et al., 1995; Yergeau et al., 1997; Okuda et al., 1998; Wildonger and Mann, 2005; Schindler et al., 2009). Both translocations confer a favorable prognosis in their respective diseases (Grimwade et al., 1998; Byrd et al., 2002; Schlenk et al., 2003). AML with t(8;21)(q22;q22) or with inv(16)(p13;q22) or t(16)(p13;q22), which disrupt *CBFB* the non-DNA-binding partner of RUNX1, are included under the category of "AML with recurrent genetic abnormalities" in the 2016 WHO classification scheme (Arber et al., 2016) and together are often referred to as "core-binding factor acute myeloid leukemia" (CBF-AML).

Mono- and biallelic mutations in *RUNX1* include deletions, missense, splicing, frameshift, and nonsense mutations. These mutations are mechanistically distinct from the chromosomal translocations and confer a worse prognosis (Osato et al., 1999; Imai et al., 2000; Harada et al., 2003; Steensma et al., 2005; Gelsi-Boyer et al., 2008; Kuo et al., 2009; Bejar et al., 2011; Mangan and Speck, 2011; Gaidzik et al., 2016). Some mutations truncate the RUNX1 protein N-terminal to or within the DNA-binding domain and consequently inactivate the protein. Other mutations confer weak dominant negative activity to RUNX1. For example, mutations in DNA-contacting residues that disrupt DNA binding without perturbing the structure of the DNA-binding domain behave as weakly dominant negative mutations (Michaud et al., 2002; Matheny et al., 2007; Owen C. J. et al., 2008; Preudhomme et al., 2009; Bluteau et al., 2011). The mechanism by which this occurs is not known but probably involves RUNX1 binding to and sequestering a limiting protein through an interaction that requires RUNX1 to be properly folded. A possible candidate for this limiting protein is the non-DNA-binding subunit partner of the RUNX proteins, CBFβ, which associates with the RUNX1 DNA-binding domain. Another type of dominant negative mutation removes the C-terminal transactivation domain while leaving the DNA-binding domain intact, which allows mutant RUNX1 to occupy its target sites and block occupancy and transactivation by full length RUNX proteins (RUNX1, RUNX2, and RUNX3; Harada et al., 2003; Satoh et al., 2008). For simplicity's sake, we will refer to both the weakly dominant negative and inactivating *RUNX1* mutations as "mutations" to distinguish them from the chromosomal translocations. AML with mono- and biallelic *RUNX1* mutations has been provisionally classified by the WHO as "AML with mutated *RUNX1*" (distinct from CBF-AML) to reflect the possible worse prognosis as compared to other AML types (Tang et al., 2009; Gaidzik et al., 2011, 2016; Schnittger et al., 2011; Mendler et al., 2012; Arber et al., 2016).

TABLE 1 | World Health Organization classification of myeloid neoplasms with germ line predisposition.

Myeloid neoplasm classification	Genes involved	Recurrently mutated in sporadic AML, largely initiating or secondary event
Myeloid neoplasms without a preexisting disorder or organ dysfunction	*CEBPA* *DDX41*	Yes, secondary No
Myeloid neoplasms and preexisting platelet disorders	*RUNX1* *ANKRD26* *ETV6*	Yes, secondary No Yes, secondary
Myeloid neoplasms and other organ dysfunction		
Germ line *GATA2* mutation	*GATA2*	Yes, secondary
BM failure syndromes*	*Multiple*	No
Telomere biology disorders	*TERT, TERK*	No
JMML associated with neurofibromatosis, Noonan syndrome or Noonan syndrome- like disorders, Down syndrome	*NF1, PTPN11, CBL, KRAS*	Yes, secondary

*Includes Fanconi anemia (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANKF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN), dyskeratosis congenita (DKC1, TERC, TERT, TIN2, NOP10, NHP2), Schwachman-Diamond syndrome (SBDS), Diamond Blackfan anemia (RPS19, RPS24, RPS17, RPL5, RPL11, RPL35A, RPS7, RPS10, RPS26, GATA1), congenital amegakaryocytic thrombocytopenia (MPL), and severe congenital neutropenia (ELA2, GFI1, HAX1).

The order of mutation acquisition in sporadic AML can be discerned by whole-genome sequencing, which reveals not only which mutations are present but also the allelic fraction of each mutation. Clonal mutations, present in all leukemia cells, represent initiating events, while subclonal mutations are secondary events. Whereas mutations in epigenetic modifiers such as *DNMT3A*, *ASXL1*, *IDH2*, and *TET2* have been consistently identified as early mutations in studies examining the temporal order of mutation acquisition in sporadic AML (Jan et al., 2012; Krönke et al., 2013; Corces-Zimmerman et al., 2014; Shlush et al., 2014; Hirsch et al., 2016; Papaemmanuil et al., 2016), *RUNX1* mutations have rarely been identified as "early" or "initiating" mutations in these studies (Jan et al., 2012; Corces-Zimmerman et al., 2014; Shlush et al., 2014; Hirsch et al., 2016; Papaemmanuil et al., 2016). The largest and most recent analysis of the order of mutation acquisition was conducted using 1540 *de novo* AML samples and validated the previous identification of *DNMT3A*, *ASXL1*, *IDH2*, and *TET2* as the earliest mutations in sporadic AML (Papaemmanuil et al., 2016). In this large analysis, as in prior smaller analyses, acquired *RUNX1* mutations were not identified as the earliest mutations and were rarely clonal. In a calculation to determine the relative order of mutation acquisition based on pairwise precedences, sporadic *RUNX1* mutations occurred 11th out of 28 genes analyzed. Thus, *RUNX1* mutations in sporadic AML are usually intermediate secondary events that drive disease progression (Jan et al., 2012; Corces-Zimmerman et al., 2014; Shlush et al., 2014; Hirsch et al., 2016; Papaemmanuil et al., 2016).

Myelodysplastic syndrome (MDS) is a clonal hematopoietic disorder characterized by ineffective hematopoiesis and is a harbinger of AML in approximately 25–30% of patients (Tefferi and Vardiman, 2009; Steensma, 2015). MDS is characterized by cytopenias of one or more peripheral blood lineages, bone marrow cells with dysplastic features, and $\leq$19% blast cells (Arber et al., 2016). Mutations in *RUNX1* are usually secondary events in MDS, although in a small number of patients they were identified as initiating events in clonal analyses (Papaemmanuil et al., 2013; Thota et al., 2014; da Silva-Coelho et al., 2017).

The paucity of initiating *RUNX1* mutations is particularly striking in the age-related phenomenon of clonal hematopoiesis of indeterminant potential (CHIP) (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014). CHIP is caused by a mutation in an HSC that causes it to selectively expand in the bone marrow relative to normal HSCs. The presence of CHIP is detected by an increase in the fraction of a mutated allele in peripheral blood (Busque et al., 2012; Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014; McKerrell et al., 2015). Despite having an expanded HSC clone, individuals with CHIP have normal hematopoiesis. The prevalence of CHIP increases with age—it is rare in individuals less than 40 years of age, increases to approximately 5–10% incidence in people over the age of 70, and reaches an incidence of 20% in individuals $\geq$90 years of age (Genovese et al., 2014; Jaiswal et al., 2014; McKerrell et al., 2015). CHIP confers a modestly elevated risk of MDS and AML (0.5–1% per year). The most common mutations in CHIP are the same as those identified as initiating mutations in sporadic adult AML: *DNMT3A*, *TET2*, and *ASXL1* (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014). CHIP is thought to represent a first step in leukemogenesis in adults that at a low, but discernably elevated, rate progresses to leukemia. Strikingly, in three separate studies not a single mutation was found in *RUNX1* in individuals with CHIP (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014). Mutations in several other germ line leukemia predisposition genes (*CEBPA, GATA2, ETV6*) were also rare or undetected.

RUNX1 mutations are more common in clonal cytopenia of undetermined significance (CCUS) (Kwok et al., 2015; Malcovati et al., 2017). CCUS is a subcategory of idiopathic cytopenias of undetermined significance (ICUS) (Valent et al., 2012; Cargo et al., 2015; Kwok et al., 2015), which include unexplained cytopenias that fail to meet the diagnostic criteria for MDS. CCUS is distinct from CHIP by virtue of its abnormal hematopoiesis. Approximately 2–4% of CCUS patients had *RUNX1* mutations (Kwok et al., 2015; Malcovati et al., 2017).

In summary, mutations in *RUNX1* are rarely initiating events in AML, have not been observed in CHIP, and have been identified at a low frequency in MDS and CCUS.

INHERITED MUTATIONS IN *RUNX1*

Inherited mono-allelic *RUNX1* mutations are associated with "familial platelet disorder with predisposition to AML" (FPD/AML) (Song et al., 1999). FPD/AML individuals usually present with mild to moderate thrombocytopenia and bleeding disorders (epistaxis, easy bruising, excessive bleeding during minor surgery, menorrhagia) (Luddy et al., 1978). The bleeding disorder is caused by impaired proplatelet formation, functional aspirin-like platelet activation defects, and abnormal megakaryocyte differentiation and polyploidization. FPD/AML individuals also have a strikingly elevated (∼44% lifetime risk) of MDS or acute leukemia (AML and to a lesser extent T-ALL) (Godley, 2014). Since the bleeding disorder is usually manageable and in most individuals does not greatly compromise the quality of life, bone marrow transplantation from a non-affected family member or an unrelated matched donor is generally not recommended unless the patient has leukemia (University of Chicago Hematopoietic Malignancies Cancer Risk Team, 2016).

The ability to monitor HSC expansion by changes in variant allele frequencies in FPD/AML individuals by next generation sequencing may offer the opportunity to intervene at the pre-leukemic stage, prior to the appearance of overt MDS or leukemia. Knowing when to intervene and treat a healthy individual at an early stage of disease, however, requires knowing which secondary mutations are likely to lead to disease progression. In sporadic leukemia, it is assumed that the most potent cooperating mutations are those that most frequently co-occur due to selection. For *RUNX1* in sporadic leukemia, these include mutations in *ASXL1, BCOR, KMD2A, PHF6,* and *SRFS2,* and less significantly in *IDH2, STAG2, SF3B1,* and trisomy 13 (Gaidzik et al., 2016; Papaemmanuil et al., 2016). On the other hand, *RUNX1* mutations are inversely correlated with mutations in *CEBPA*, *NPM1*, *TP53*, t(8;21)(q22;q22), inv(16)(p13;q22)/t(16)(p13;q22), and t(15;17)(q24;q21), presumably because the mutations are

either functionally redundant or antagonistic. Other common MDS or AML mutations (e.g., in *DNMT3A)* were neither enriched nor inversely correlated with *RUNX1* mutations.

Unfortunately, the rarity of FPD/AML makes definitive large-scale sequencing studies impossible, and the handful of smaller studies that have been conducted reported somewhat different results. One group sequenced AML samples from 9 Japanese FPD/AML patients and reported somatic mutations in *CDC25C* in 4 patients, concluding that somatic mutations in *CDC25C* (which are rarely observed in sporadic AML) were the most common genetic event in AML arising from FPD/AML (Yoshimi et al., 2014; Sakurai et al., 2016). A second group analyzed somatic mutations in 8 leukemia patients from four French FPD/AML families, identified loss of function mutations in the second *RUNX1* allele in 6 patients, and concluded that mutation of the remaining wild type *RUNX1* allele was the most common secondary event (Antony-Debré et al., 2016). Strikingly, the French group found no mutations in *CDC25C*. A third group (Churpek et al.) from the United States analyzed somatic mutations in 7 FPD/AML patients with MDS or AML and found no mutations in either *CDC25C* or in the second *RUNX1* allele, but instead, identified mutations in a collection of other genes including *BCOR*, *PHF6*, *DNMT3A, TET2, CREBBP, U2AF1, NUP214, SMC3,* and *PDS5B* (Churpek et al., 2015).

In the Churpek et al. study, the peripheral blood of 9 FPD/AML individuals with no evidence of leukemia was also analyzed (Churpek et al., 2015). Six of these 9 individuals had clonal hematopoiesis, and a missense mutation in *DNMT3A* was found in one person (Churpek et al., 2015). A *DNMT3A* mutation was also identified in an asymptomatic FPD/AML individual in a separate study (Antony-Debré et al., 2016). The asymptomatic FPD/AML individuals with evidence of clonal hematopoiesis ranged in age from 8 to 54 (Churpek et al., 2015), whereas CHIP in the general population is extremely rare in this age group (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014). It appears that the acquisition of secondary mutations is greatly accelerated in FPD/AML individuals and presumably contributes to the elevated risk of leukemia in these patients. An important question raised by these studies is why clonal hematopoiesis is so greatly accelerated in individuals with FPD/AML. In the next several sections we will describe experimental data that begin to address this question.

DEFECTS IN DNA REPAIR PATHWAYS IN *RUNX1* MUTANT CELLS

Inefficient DNA repair could contribute to the rapid accumulation of mutations seen in non-leukemic FPD/AML patients. This could result from the dysregulated expression or activity of proteins in DNA repair pathways, which include mismatch repair (MMR), base excision (BER), nucleotide excision (NER), double-strand break repair [homologous recombination (HR), non-homologous end joining (NHEJ), alternative non-homologous end-joining (Alt-NHEJ)], and interstrand DNA crosslink repair by the Fanconi Anemia (FA) / BReast CAncer susceptibility (BRCA) pathway (Cheung and Taniguchi, 2017). Thus far, RUNX1 has been implicated in regulating the BER, HR, and FA/BRCA pathways.

Most evidence for RUNX1's role in DNA repair has been generated using cells expressing the neomorphic RUNX1-RUNX1T1 (AML1-ETO) protein. Several studies showed that expression of RUNX1-RUNX1T1 in primary HSPCs or cell lines resulted in down regulation of a number of genes involved in BER, including *OGG1* which encodes the primary enzyme responsible for excising oxidized bases (Alcalay et al., 2003; Krejci et al., 2008; Liddiard et al., 2010; Forster et al., 2016). RUNX1-RUNX1T1 was shown to occupy the *OGG1* gene and presumably directly regulates *OGG1* expression (Krejci et al., 2008; Forster et al., 2016). Functional defects in BER were observed in RUNX1-RUNX1T1 expressing cells, such as slow repair of DNA damage by alkylating agents (Alcalay et al., 2003) and accumulation of somatic mutations more rapidly over time (Forster et al., 2016).

Inefficient DNA repair by the HR pathway has also been documented in RUNX1-RUNX1T1 expressing cells. RUNX1-RUNX1T1 expressing cells are very sensitive to poly (ADP-ribose) polymerase (PARP) inhibitors (Esposito et al., 2015), which have potent activity specifically on cells with defective HR repair (Helleday, 2011). Treatment with PARP inhibitors suppressed the colony forming ability of RUNX1-RUNX1T1 expressing cells, consistent with a defect in HR repair (Esposito et al., 2015). A very early event in the repair of DNA double-strand breaks and a surrogate marker for breaks is phosphorylation of the histone variant H2AX (the phosphorylated form is γ-H2AX) by the ATM serine-threonine kinase (Mah et al., 2010). DNA double-strand breaks trigger a cascade of events that recruit DNA repair proteins, including the recombinase RAD51 through BRCA1 and BRCA2. RUNX1-RUNX1T1 expressing cells cultured *in vitro* gradually acquired more γ-H2AX foci than normal cells over time, indicating that they more rapidly accumulated unrepaired double-strand breaks (Krejci et al., 2008; Wichmann et al., 2015). Following acute induction of DNA damage, the γ-H2AX foci that formed disappeared more slowly, indicative of slower resolution or repair of γ-H2AX+ double strand breaks (Esposito et al., 2015). The slow repair of DNA correlated with decreased recruitment of the recombinase RAD51 to the DNA damage sites (Esposito et al., 2015). This deficiency in HR in RUNX1-RUNX1T1 expressing cells was associated with lower expression of genes responsible for HR, including *BRCA1, BRCA2, ATM,* and *RAD51* (Esposito et al., 2015). Whether RUNX1-RUNX1T1 regulates these DNA repair genes directly or indirectly is unclear.

Defects in DNA repair have also been observed with the types of *RUNX1* mutations found in FPD/AML. Retroviral transduction of a dominant negative truncated RUNX1 protein lacking its transactivation domain into HSPCs resulted in a greater proportion of cells with evidence of DNA damage, as detected by the presence of an increased number of cells containing γ-H2AX$^+$ foci and slower decay of γ-H2AX$^+$ foci following DNA damage induction (Satoh et al., 2012). The defect in DNA repair was accompanied by the downregulation of several DNA repair genes, including *RAD51* and the growth arrest and DNA damage gene *GADD45*. The RUNX proteins may also directly regulate the FA/BRCA pathway (Krejci et al.,

2008; Wang et al., 2014). The FA/BRCA pathway is essential for repairing interstrand crosslinks in DNA. Inherited mutations in multiple FA/BRCA pathway genes cause bone marrow failure and predispose to myeloid leukemia and other cancers (Cheung and Taniguchi, 2017; **Table 1**). The FA/BRCA pathway is strongly activated by the crosslinking agent mitomycin C. When RUNX1 and its homolog RUNX3 were together deleted or knocked down, the sensitivity of cells to mitomycin C increased, indicating inefficient repair of the damage (Wang et al., 2014). RUNX1 was shown to co-immunoprecipitate with FANCD2, a protein in the FA/BRCA pathway that localizes to sites on interstrand crosslinks (Wang et al., 2014). Recruitment of FANCD2 to DNA decreased upon combined knockdown of RUNX1 and RUNX3, and repair was depressed (Wang et al., 2014).

In addition to direct effects on DNA repair pathways, *RUNX1* mutations may promote the survival of HSCs with somatic mutations. For example, lower p53 levels have been documented in RUNX1 deficient HSPCs (Cai et al., 2015). The p53 transcription factor protects cells from DNA damage by arresting the cell cycle to allow DNA repair to take place and by inducing apoptosis and senescence of unrepaired cells. The reduction in p53 protein levels in RUNX1 deficient HSCs was not accompanied by reduced *Tp53* mRNA levels; therefore, the mechanism of the reduction is post-transcriptional (Cai et al., 2015). Other studies showed that RUNX1 forms a complex with p300-p53 to acetylate p53 and thereby activate p53 target genes (Wu et al., 2013). Hence loss of RUNX1 could affect both p53 levels and activity. RUNX1 deficient HSCs contained a decreased percentage of apoptotic cells, both in the absence and presence of DNA damaging agents (Motoda et al., 2007; Cai et al., 2011, 2015). Thus, inefficient elimination of cells that have acquired DNA damage through impaired p53 activity (Cai et al., 2015) could contribute to the accumulation of HSCs with unrepaired DNA damage and acquired secondary mutations in FPD/AML. Other survival pathways and apoptotic pathways are also likely to be dysregulated in RUNX1 deficient HSCs, as increasing p53 levels by inhibiting its interaction with MDM2 using the MDM2:p53 inhibitor nutlin-3 did not completely correct the low apoptotic phenotype (Cai et al., 2015). All of the above-mentioned studies were performed with cells that have more severe perturbations in functional RUNX1 than would be expected with the mono-allelic mutations in FPD/AML, and the DNA repair and apoptotic defects are likely to be more profound in experimental models. Nevertheless, the median age for leukemia development in FPD/AML individuals is 33 years (Owen C. J. et al., 2008). Thus, even minor defects in DNA repair could contribute to the increased accumulation of mutations over a period of several decades.

FORCES THAT MAY DRIVE CLONAL SELECTION OF HSCs WITH SECONDARY MUTATIONS IN FPD/AML

For an HSC with a somatic mutation to clonally expand, there must be selective pressures favoring that HSC that provide it with a competitive advantage. The nature of selective pressures can change with age; for example, the selective pressure that drives the expansion of somatically mutated cells is thought to increase with age and contribute to the age-associated increased incidence of cancer (Rozhok and DeGregori, 2016). An aged inflammatory niche, for instance, was a critical determinant in selecting for HSPCs with oncogenic driver mutations in mice (Henry et al., 2015). The ability of HSPCs transduced with oncogenes (*$NRAS^{V12}$*, *BCR-ABL,* and *MYC)* to promote leukemogenesis was greatly enhanced by transplantation into old recipient mice, whereas when transplanted into young hosts, leukemogenesis was largely suppressed (Henry et al., 2015). The expansion of the transduced HSPCs in the aged bone marrow niche could be prevented by transgene expression of the anti-inflammatory mediators α-1-antitrypsin (AAT), or interleukin-37 (IL-37) (Henry et al., 2015). These findings suggest that not only do mutations need to be acquired in HSCs but also that the leukemic clone must be in the inflammatory environment of an aged bone marrow for the mutations to provide a fitness advantage that can be selected for. Consistent with this, recent studies showed that inflammatory cytokines can promote the selective *in vitro* growth of human AML cells. In a large functional screen of primary human AML samples, several inflammatory cytokines, including IL-1, promoted the growth of AML progenitors while suppressing the growth of normal HSPCs (Carey et al., 2017). Strikingly, despite genetic and clinical heterogeneity, the growth of 40 out of 60 primary AML samples was stimulated by IL-1, suggesting that selective growth driven by inflammatory cytokines is a common feature of many AML cells (Carey et al., 2017). Differentiated myeloid cells were identified as the primary source of IL-1, highlighting the critical role that an inflammatory niche may play in leukemogenesis (Carey et al., 2017).

Increased production of inflammatory cytokines by myeloid cells has been observed in individuals with CHIP and in mouse models of the mutations found in CHIP. Evidence of inflammation and increased serum levels of IL-8 were identified in CHIP associated with mutations in *TET2* (Jaiswal et al., 2017). Myeloid cells in mice were shown to be a source of inflammatory cytokines, as a myeloid cell specific disruption of *Tet2* with Lyz2-Cre elevated mRNAs encoding multiple chemokines and inflammatory cytokines; furthermore, endotoxin stimulation of *Tet2* deficient macrophages resulted in more robust secretion of IL-1β and IL-6 (Cull et al., 2017; Fuster et al., 2017; Jaiswal et al., 2017). Hence, mutations in HSCs may increase the production of inflammatory cytokines by downstream myeloid lineage cells, which could potentially feedback on and selectively promote the growth of the mutated HSCs.

Clinical evidence hints that elevated inflammation may contribute to disease severity in FPD/AML individuals. In one FPD/AML pedigree, affected family members were reported to have eczema, the severity of which correlated directly with the severity of thrombocytopenia (Sorrell et al., 2012). Strikingly, eczema was most severe in those family members who went on to develop frank leukemia (Sorrell et al., 2012). In another study, hypersensitivity to G-CSF was observed in peripheral blood mononuclear cells from an FPD/AML patient (Chin et al., 2016). This correlates with findings in mouse models, where increased

sensitivity of HSPCs to G-CSF upon mono- or biallelic deletion of *Runx1* was proposed to result from an increase in STAT3 signaling (Chin et al., 2016; Lam et al., 2016).

Further suggestive evidence that mutations in RUNX1 may elevate inflammation can be seen in studies of its homolog RUNX3, which binds the same DNA sequence as RUNX1 and is a well-documented negative regulator of inflammation. As is well reviewed elsewhere, genome wide association studies (GWAS) linked *RUNX3* with diseases of inflammation in humans including ulcerative colitis, celiac disease, ankylosing spondylitis, psoriasis, and asthma (Lotem et al., 2015). *Runx3* knockout mice exhibit spontaneous colitis and lung inflammation, in part due to dysregulated TGF-β signaling (Brenner et al., 2004; Fainaru et al., 2004). RUNX1 also negatively regulates inflammatory signaling in the lung (Tang et al., 2017). Deletion of *Runx1* in lung epithelium resulted in constitutive activation of NF-κB pro-inflammatory signaling and increased susceptibility to LPS-induced acute lung injury *in vivo* (Tang et al., 2017). Given these findings in lung epithelium, germ line mutations in *RUNX1* could have more pleiotropic effects in patients with FPD/AML than previously appreciated.

The mechanisms by which RUNX1 regulates inflammation are unclear. RUNX1 is reported to have opposing effects on NF-κB signaling output due to interactions with different members of the NF-κB signaling pathway. Nakagawa *et al.* showed that RUNX1 interacted with the IκB kinase (IKK) complex in the cytoplasm, and deletion of *Runx1* activated NF-κB signaling (Nakagawa et al., 2011). The ability of RUNX1 to inhibit IKK activity was lost in three RUNX1 mutants identified in MDS patients (Nakagawa et al., 2011). However, a different group reported a contrasting activity of RUNX1 in regulating NF-κB, in that knock down of RUNX1 in myeloid cell lines and activated primary mouse peritoneal macrophages attenuated NF-κB signaling (Luo et al., 2016). The latter study also showed that RUNX1 binds to p50 (a NF-κB family member) in the nucleus and may lead to enhanced NF-κB signaling via recruitment of RNA polymerase II at some p50-occupied sites (Luo et al., 2016). Further, use of a RUNX1 inhibitor provided some protection against LPS-induced septic shock in an *in vivo* murine model (Luo et al., 2016). Therefore, RUNX1 appears to interface with the NF-κB pathway at multiple points and to be capable of both interfering with and augmenting NF-κB signaling.

RUNX proteins can also modulate inflammatory signaling through their regulation of T cell development and function (Djuretic et al., 2009; Wong et al., 2011). RUNX1 and RUNX3 influence the development and function of T_H1, T_H2, T_{REG}, and T_H17 cells, which regulate autoimmunity, asthma, allergic responses, and tumor immunity (Djuretic et al., 2007, 2009; Naoe et al., 2007; Ono et al., 2007; Zhang et al., 2008; Bruno et al., 2009; Wong et al., 2011). T cell specific deletion of *Runx1* caused spontaneous hyperactivation of CD4+ T cells resulting in severe lung inflammation that evolved into a lethal systemic inflammatory disease (Wong et al., 2012).

Putting all this together, we speculate that systemic inflammation may be elevated in FPD/AML and that an inflammatory bone marrow microenvironment may provide a positive selective pressure for HSCs that have acquired secondary mutations.

EFFECTS OF *RUNX1* MUTATIONS ON HSCs

The mutations in epigenetic regulator genes that initiate AML and predominate in CHIP have the capability of selectively expanding HSCs. This phenomenon of clonal HSC expansion has been reproduced in several mouse models. Deletion of *Dnmt3a* in mice skewed the balance of self-renewal versus differentiation of HSCs, causing an accumulation of HSCs in the bone marrow, inefficient output of HSCs to differentiated hematopoietic progeny in the periphery, and a repopulation advantage of *Dnmt3a* deficient HSCs over normal HSCs in transplant experiments (Challen et al., 2012; Shlush et al., 2014). Mutations in *Tet2* similarly conferred a competitive advantage to murine HSCs, allowing them to outcompete normal HSCs in transplantation experiments (Ko et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011).

RUNX1 mutant HSCs do not appear to enjoy the same competitive advantage over normal HSCs as do HSCs with *Dnmt3a* or *Tet2* mutations. *RUNX1* mutations may require the context of additional mutations in order to provide a fitness advantage for the leukemic clone. Initial reports showed that deletion of *Runx1* in hematopoietic cells caused an expansion of HSPCs (lineage negative $Sca1^+$ Kit^+ cells) and committed myeloid progenitors in the bone marrow (Ichikawa et al., 2004; Growney et al., 2005). However, the majority of studies in which careful analyses were carried out on functional long-term repopulating HSCs (LT-HSCs) reported modest, 2-4-fold decreases in their frequency upon *Runx1* mutation (Sun and Downing, 2004; Jacob et al., 2009; Cai et al., 2011). For example, a pan-hematopoietic homozygous deletion of *Runx1* resulted in a 3-4-fold decrease in functional LT-HSCs (measured in limiting dilution transplantation experiments) in the bone marrow of both young and aged mice (Jacob et al., 2009; Cai et al., 2011). Similarly, monoallelic germ line mutations in *Runx1* reduced the frequency of functional LT-HSCs in the bone marrow by about 50% (Sun and Downing, 2004). *Runx1* deficient ($Runx1^{\Delta/\Delta}$), $Runx1^{+/-}$, and wild type HSCs performed equivalently well in serial transplantation experiments, in that by the fourth round of transplantation, HSCs of all three genotypes were essentially exhausted, and hence had similar self-renewal potential (Sun and Downing, 2004; Cai et al., 2011). By comparison, *Dnmt3a* deficient HSCs expanded with each subsequent round of transplantation (Challen et al., 2012). Therefore, the inability of *RUNX1* mutations to induce clonal HSC expansion like that seen with *DNMT3A* and *TET2* mutations may account for the rarity of *RUNX1* mutations as initiating events in AML and in CHIP.

An interesting and potentially clinically consequential feature of RUNX1 mutant HSPCs is that they exhibit a greater selective advantage over normal HSPCs following exposure to radiation. Irradiation of a mixture of *Runx1* deficient ($Runx1^{\Delta/\Delta}$) and normal bone marrow cells followed by transplantation into mice resulted in a greater selective expansion of *Runx1* deficient

HSPCs (lineage negative Sca1$^+$ Kit$^+$ cells) and myeloid lineage cells in the bone marrow compared to irradiated wild type or non-irradiated *Runx1* deficient cells (Cai et al., 2015). This was likely due to the increased resistance of *Runx1* deficient HSPCs to endogenous and genotoxic stress (Cai et al., 2015). A very similar phenomenon of radiation-dependent HSC expansion was also observed in HSPCs with a *Tp53* mutation, consistent with this interpretation (Marusyk et al., 2010). *Runx1* deficient HSPCs were less apoptotic, grew more slowly than normal HSCs, had a lower rate of protein translation, and had markedly decreased ribosome biogenesis. Consistent with decreased ribosome biogenesis, *Runx1* deficient HSPCs were smaller than normal HSPCs, and preliminary data suggested that this small cell phenotype was shared with HSPCs from FPD/AML individuals (Cai et al., 2015). This stress resistant, slow growth, low apoptotic phenotype could presumably confer resistance to treatment and may explain why *RUNX1* mutations confer a relatively poor prognosis in AML. It may also explain the observation that *RUNX1* mutations occurred at a higher frequency in MDS patients previously exposed to low-level radiation as compared to all MDS patients (Harada et al., 2003; Zharlyganova et al., 2008).

CONCLUSIONS

It was originally thought that inherited *RUNX1* mutations cause leukemia predisposition because every HSC in an FPD/AML individual is already one mutation along the path to AML. However, it is likely that inherited *RUNX1* mutations play a more active role in promoting leukemia progression. The dysregulation of DNA repair and decreased apoptosis of RUNX1 mutant HSCs along with an increased inflammatory microenvironment may contribute to the markedly increased incidence of MDS, AML, and T-ALL in FPD/AML individuals. We envision a model whereby *RUNX1* mutations increase the rate at which secondary mutations are acquired; moreover, increased inflammatory signals delivered by RUNX1 mutant myeloid lineage cells or other cells in the RUNX1 mutant bone marrow microenvironment provide a selective pressure that confers a competitive advantage to FPD/AML HSCs that have acquired secondary mutations (**Figure 1**). On the other hand, in a normal individual a single HSC with a *RUNX1* mutation does not have a competitive advantage over normal HSCs and hence does not expand and initiate leukemia.

The initiation and evolution of leukemia in FPD/AML individuals can be carefully followed by next generation sequencing. Individuals diagnosed with FPD/AML could be monitored on a regular basis for evidence of clonal HSC expansion driven by acquired secondary mutations and treated before they progress to frank AML. However, we still do not know which secondary mutations are the most deleterious and whether they are the same as or different than the co-occurring mutations in sporadic AML. For example, *DNMT3A* mutations have been reported in two asymptomatic FPD/AML individuals (Churpek et al., 2015; Antony-Debré et al., 2016). Does the presence of a *DNMT3A* mutation, which in a normal individual only modestly increases the risk of leukemia, confer a much greater risk in the context of an inherited *RUNX1* mutation? More data and longitudinal studies correlating the stepwise accumulation of mutations in individuals with FPD/AML with clinical outcome are needed to predict which mutations most likely portend a rapid progression to MDS or AML. Mechanistic studies in animal and cell models will also be necessary to understand what is driving leukemia progression in FPD/AML

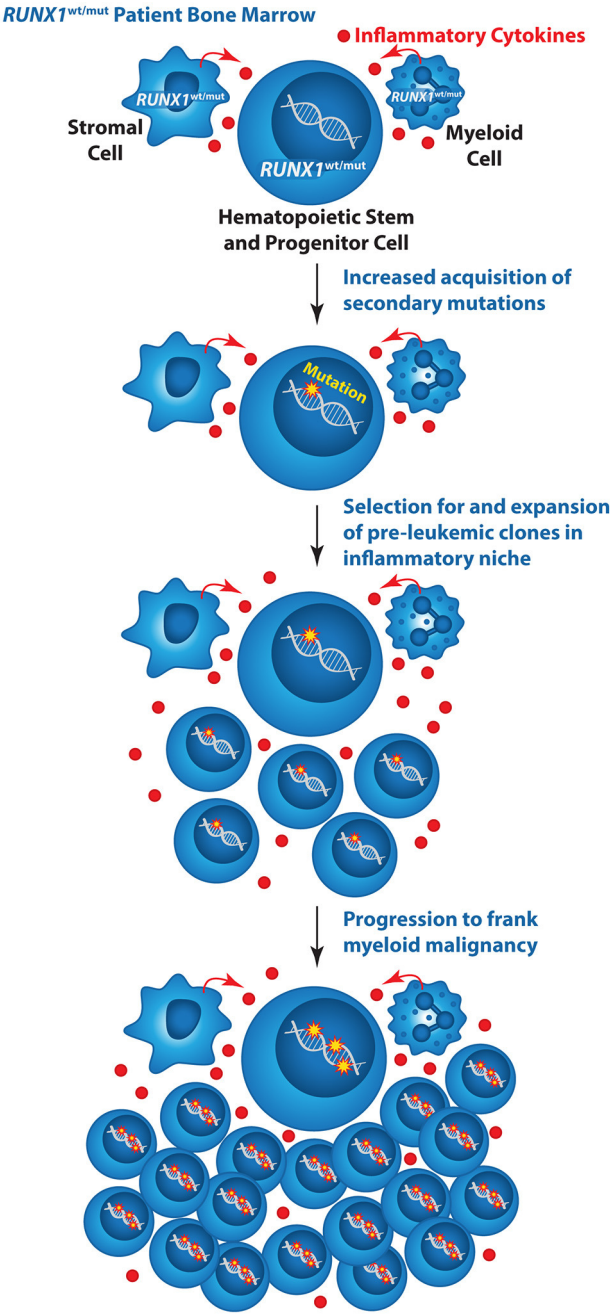

FIGURE 1 | Model illustrating the mechanism by which inherited mutations in *RUNX1* could elevate the risk of AML in FPD/AML individuals. Wildtype (wt), mutant (mut).

and to identify strategies that can avert, delay, or reverse this progression.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Modeling Myeloid Malignancies Using Zebrafish

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Human myeloid malignancies represent a substantial disease burden to individuals, with significant morbidity and death. The genetic underpinnings of disease formation and progression remain incompletely understood. Large-scale human population studies have identified a high frequency of potential driver mutations in spliceosomal and epigenetic regulators that contribute to malignancies, such as myelodysplastic syndromes (MDS) and leukemias. The high conservation of cell types and genes between humans and model organisms permits the investigation of the underlying mechanisms of leukemic development and potential therapeutic testing in genetically pliable pre-clinical systems. Due to the many technical advantages, such as large-scale screening, lineage-tracing studies, tumor transplantation, and high-throughput drug screening approaches, zebrafish is emerging as a model system for myeloid malignancies. In this review, we discuss recent advances in MDS and leukemia using the zebrafish model.

Keywords: splicing, myelodysplastic syndrome, acute myeloid leukemia, zebrafish, hematopoiesis, malignancies

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INTRODUCTION

Myeloid malignancies are clonal disorders of hematopoietic stem and progenitor cells in which there is bone marrow failure, an overgrowth of blasts, differentiation arrest, and lineage skewing. These malignancies include chronic disorders such as myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), and chronic myeloid leukemia (CML) and acute conditions such as acute myeloid leukemia (AML). These disorders are distinguished by the prevailing cell type, pathogenic severity, prognosis, and molecular underpinning. The etiology of most myeloid malignancies is poorly characterized; however, recent large-scale sequencing of patient samples has uncovered key recurrent classes of mutated factors (1–4). Through these studies, researchers identified genetic alterations in factors involved with gene expression regulation including hematopoietic transcription factors, spliceosomal components, and epigenetic regulators. Although genotype–phenotype correlations between a mutated gene and disease state are highly suggestive of causation, model organisms provide a controlled approach to demonstrate the connections between genetic alteration and blood defects as well as to determine the underlying mechanism in more uniform genetic backgrounds. Since the establishment of the first model of transplantable *c-myc*-driven T-cell acute lymphoblastic leukemia (T-ALL) (5), the zebrafish *Danio rerio* has emerged as a useful animal model to explore the control of both normal and malignant hematopoiesis.

ZEBRAFISH HEMATOPOIESIS

Most of the core regulators of hematopoiesis are evolutionarily conserved between teleosts, such as zebrafish, and mammals, such that findings in zebrafish can be directly translated into mouse and human systems. According to the recently completed and updated annotation of the zebrafish

genome, approximately 70% of protein-coding genes in humans have at least one ortholog in the zebrafish, and 84% of disease-associated genes have a zebrafish equivalent (6). This extent of homology further demonstrates the potential utility of zebrafish to define critical regulators of malignancies and the underlying genetic causes. Having an in-depth understanding of the normal processes and signaling requirements underpinning hematopoietic lineage emergence and development provides a solid framework to understand how genetic perturbations exert their influence in disease states. Studying hematopoiesis during embryonic development can be advantageous to minimize the accumulation of environmental influences acquired through the life of an organism. Utilizing the zebrafish model to study embryonic hematopoiesis has a myriad of advantages including high fecundity, rapid external embryonic development, organismal transparency, numerous hematovascular fluorescent reporter lines, genetic tractability, and similar chronological and spatial lineage emergence kinetics and regulation to mammals.

Like other vertebrates, zebrafish hematopoiesis develops in three discrete waves: primitive, erythro-myeloid progenitor (EMP)-derived, and definitive (7). All three waves of hematopoiesis arise from lateral mesoderm-derived cells that possess different hematopoietic differentiation capacity. The primitive hematopoietic wave arises during the first 24 h post fertilization (hpf) from two locations: the anterior lateral mesoderm generates myeloid lineages, and the intermediate cell mass generates primitive myeloid and erythroid cells. Emergent primitive myeloid cells then migrate around the yolk sac and differentiate into distinct lineages, up-regulating expression of *spi/pu.1*, *colony stimulating factor 1 receptor* (*csf1r/fms)*, *csf3r*, *l-plastin*, and *myeloperoxidase* (*mpo/mpx*), while the primitive *gata1*-expressing erythroid cells upregulate the levels of *erythropoietin receptor* (*epor)* and *globin* genes then enter circulation (8–14). The transient EMP wave derives from the posterior blood island and differentiates to form definitive erythroid and myeloid cells that lack self-renewal or multilineage differentiation capacity (15, 16). Despite the mostly transient nature of these waves, new findings from the past several years indicate that some myeloid progenitors from the primitive and EMP wave could persist in adulthood and provide the pool for microglia (macrophages in the brain) and other tissue-resident macrophages (17–19). It will be interesting to see if these embryonically derived cells play a role in human disease.

The final wave of hematopoietic specification gives rise to definitive adult-like hematopoietic stem cells (HSCs), which possess both self-renewal capacity and erythroid, myeloid, and lymphoid potential. Starting from approximately 30 hpf, HSCs emerge from *kinase insert domain receptor-like (kdrl)*-positive endothelium lining the ventral wall of the dorsal aorta, equivalent to the mammalian aorta-gonad-mesonephros region (16, 20, 21). The newly emergent HSCs transiently co-express endothelial markers, such as *kdrl*, and HSC markers, such as *cd41* and the transcription factors *cmyb* and *runt-related transcription factor 1* (*runx1*) (15, 16). From approximately 48–72 hpf, HSCs then migrate *via* the circulation to the caudal hematopoietic tissue (CHT) (22), the functional equivalent of the mammalian fetal liver. Between 48–96 hpf, cells from the CHTs will then seed the thymus for T-cell production or the kidney marrow, which functions as the adult hematopoietic niche similar to the mammalian bone marrow (15).

The zebrafish model affords many advantages for investigating mechanisms underlying normal and malignant myelopoiesis [reviewed in Ref. (23)]. The fate determination, differentiation, and maturation of myeloid cells are highly similar from embryonic development to adulthood. As such, studies of myeloid development in zebrafish have been informative not only for understanding embryonic development but also for adult myeloid malignancies [reviewed in Ref. (23)]. Myeloid precursors express conserved transcription factors such as *spi1/pu.1*, *runx1*, and *ccaat-enhancer binding protein alpha* (*cebpα*) that are critical in myeloid lineage commitment (11, 24, 25). Mature myeloid cell types with similarities or equivalence to well-defined mammalian lineages have been identified in zebrafish development based on their expression of signature genes, histochemical staining properties, and morphology: macrophages that express genes such as *l-plastin*, *lysozyme (lyz)*, *and csfr1/fms*; neutrophils that express *mpx and matrix metalloproteinase 9 (mmp9)*; and basophils/eosinophils that have high levels of *gata2* expression (9, 24, 26, 27).

Zebrafish also possess many technical advantages. Targeted genetic manipulations allow for rapid alteration of gene function, including anti-sense morpholino knockdown (MO), zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technologies for precision genome editing [reviewed in Ref. (28)]. Exogenous expression of proteins of interest is also possible in zebrafish, either through transient introduction of *in vitro* transcribed mRNA or through stable integration of DNA, most commonly *via* the Tol2 transposon-based transgenesis system (29, 30). Phenotype-driven genetic or chemical screening approaches are commonly employed due to the large clutch size, rapid generation time, and ease of drug treatment by infusion in the water. External and transparent embryonic development in combination with the multitude of fluorescent reporter lines enables sophisticated *in vivo* live imaging of lineage emergence and cell dissemination. Transparent mutants, such as the *casper* line (31), improve imaging capacity in adult zebrafish. Transplantation of zebrafish-derived hematopoietic tumor cells has been utilized to quantify and define subsets of leukemic propagating cells, as well as to image tumor microenvironmental interactions (32–34).

We can therefore take advantage of the technical and genetic advantages of the zebrafish model to study the genetic basis of malignancy and translate the findings to inform a better understanding of human cancer biology for therapeutic application. Current pathways to develop therapeutics for disease treatment are costly, labor and animal intensive, and take 10–30 years from discovery of the molecule or pathway to having a drug in the clinic. It is therefore essential to utilize streamlined processes for *in vivo* testing of drug targets. The zebrafish model, with high fecundity, conservation of many key genes, and an extensive experimental toolbox, provides a high-throughput model for such *in vivo* analysis.

MYELOID MALIGNANCIES

Myelodysplastic syndrome and AML are among the most common myeloid malignancy of the elderly each affecting 3–5 out of 100,000 people in the USA with approximately 10,000–20,000 newly diagnosed cases per annum (35–39). Both malignancies stem from clonal HSC disorders and are characterized by bone marrow failure and peripheral blood cytopenias. A major distinguishing characteristic of AML is the presence of excessive (>20%) undifferentiated myeloid blast in the bone marrow or peripheral blood, which are generally low in MDS patients. MDS is thought to be a precursor syndrome to AML with up to 30% of MDS cases progressing to secondary AML.

ZEBRAFISH MODELS OF AML

The classic model of AML development states that cells accumulate molecular alterations (large chromosomal rearrangements or genetic point mutations) in two classes: those that promote proliferation (class I) and those that impair differentiation (class II) (40). Prognostic risk is stratified based on the cytogenetic and molecular mutation profile present in the leukemia. For example, cytologically normal *FMS-like tyrosine kinase 3* (*FLT3*)-*internal tandem duplication* (*FLT3-ITD*) correlates with an adverse prognosis, while *nucleophosmin 1* (*NPM1*) mutations are linked with favorable outcomes. Investigating the molecular mechanisms driving AML is difficult in human samples, thus disease models are examined in model organisms including mouse and zebrafish. The first cancer model established in zebrafish in the early 2000s was *c-myc*-driven T-ALL (5), but since then robust myeloid leukemia models have finally been established. Most of these models are based on exogenous expression of prominent human AML fusion oncogenes derived from chromosomal translocations. These oncogenes are generally considered to be potent drivers of AML, with expression of such mutations in zebrafish often resulting in severe, early-arising, embryonic lethal hematologic anomalies, which preclude the study of adult leukemia. Despite this limitation, by employing the pliable genetic and chemical advantages of studies in embryonic zebrafish, much has been discovered regarding underlying mechanisms of these AML-like phenotypes.

CHROMOSOMAL REARRANGEMENTS IN ZEBRAFISH AML MODELS

The chromosomal translocation t(8;21)(q22;q22) was one of the first molecular alterations identified in AML, with a frequency of 5–15% of all human AML cases. It results in the fusion of two nuclear proteins: acute myeloid leukemia 1 protein (AML1, also called RUNX1/CBFα2/PEBPαB) and eight twenty one [ETO, or myeloid translocation gene on chromosome 8 (MTG8/RUNX1T1)]. AML1 is a master transcriptional regulator of definitive hematopoiesis that binds enhancers and activates hematopoietic gene expression (41). Chromosomal translocations and mutations involving *AML1* are associated with several forms of adult leukemia and childhood MDS (42, 43). ETO is broadly expressed in hematopoietic cells, including $CD34^+$ progenitors, and acts as a nuclear localized zinc finger containing protein that normally recruits the nuclear receptor co-repressor/SIN3/histone deacetylase (HDAC) complex to induce transcriptional repression, including of *AML1* (44–47). The AML1-ETO molecular subtype of leukemia is characterized by granulocyte precursor accumulation (48, 49). In zebrafish, transient induction of the human *AML1-ETO* oncogene during development could recapitulate the granulocytic lineage skewing observed in human patients (**Figures 1A,B**) (50). *AML1-ETO* expressing embryos displayed a biased expression of *spi1/pu.1* in myelo-erythroid progenitors at the expense of *gata1*, resulting in an expansion of granulocytes (**Figure 1C**). This largely recapitulated both the differentiation changes observed in human patients and the phenotype in the mouse model (51), demonstrating the utility of the zebrafish system. Mechanistic studies revealed that the observed lineage skewing was mediated *via* modulation of the early fate choice transcription factor *stem cell leukemia (scl)*. Yeh and colleagues then took advantage of the screening capability of the zebrafish and performed an unbiased chemical suppressor screen to find small molecules that could reverse the myeloid expansion in *AML1-ETO*-expressing zebrafish. They identified that cyclooxygenase 2 (COX-2) and β-catenin pathways were downstream of AML1-ETO and that HDAC inhibition by trichostatin A could therapeutically target the AML-like effects in the zebrafish model (**Figure 1C**).

A zebrafish model of the chromosomal translocation t(9;12)(p24;p13) fusion oncogene ETS leukemia virus 6 (ETV6) and Janus kinase 2 (JAK2) has also been generated. ETV6 (also known as TEL) is an E26 transformation-specific (ETS) family transcription factor involved in early embryonic yolk sac angiogenesis and multilineage adult hematopoiesis including HSC survival (46, 53, 54). JAK2 is a non-receptor tyrosine kinase commonly involved in hematopoietic cytokine signaling cascades and crucial in erythromyeloid differentiation and HSC maintenance and function (55). The TEL-JAK2 fusion product leads to constitutive activation of JAK2 kinase activity (56). TEL-JAK2 has been identified in lymphoid and myeloid malignancies, with fusion between TEL exon 5 and JAK2 exon 9 occurring in T-ALL, while TEL exon 5 is found to fuse with JAK2 exon 12 in CML (57). To generate a myeloid-restricted mutant zebrafish line, Onnebo and colleagues expressed the *tel-jak2a* fusion oncogene under the control of the *spi1/pu.1* promoter (58). These transgenic animals have disrupted embryonic hematopoiesis, including anemia and expansion of the myeloid compartment. Of note, a subsequent study found that expression of the TEL exon 5-JAK2 exon 9 variant led to lymphoid-restricted defects, while expression of the TEL exon 5-JAK2 exon 12 variant produced myeloid-restricted phenotypes, consistent with prior clinical observations (59). These results indicate that the lineage selection for the specific TEL-JAK2 variant occurs *via* regulation of the downstream signaling rather than at the level of the chromosomal aberration.

NON-FUSION ONCOGENES IN ZEBRAFISH AML MODELS

Gain-of-function mutations in *FLT3* occur in ~30% of AML cases and correlate with poor prognosis (60). Mutations include the internal tandem duplication (FLT3-ITD) and point mutations in the tyrosine kinase domain (FLT3-TKD), both of which result in

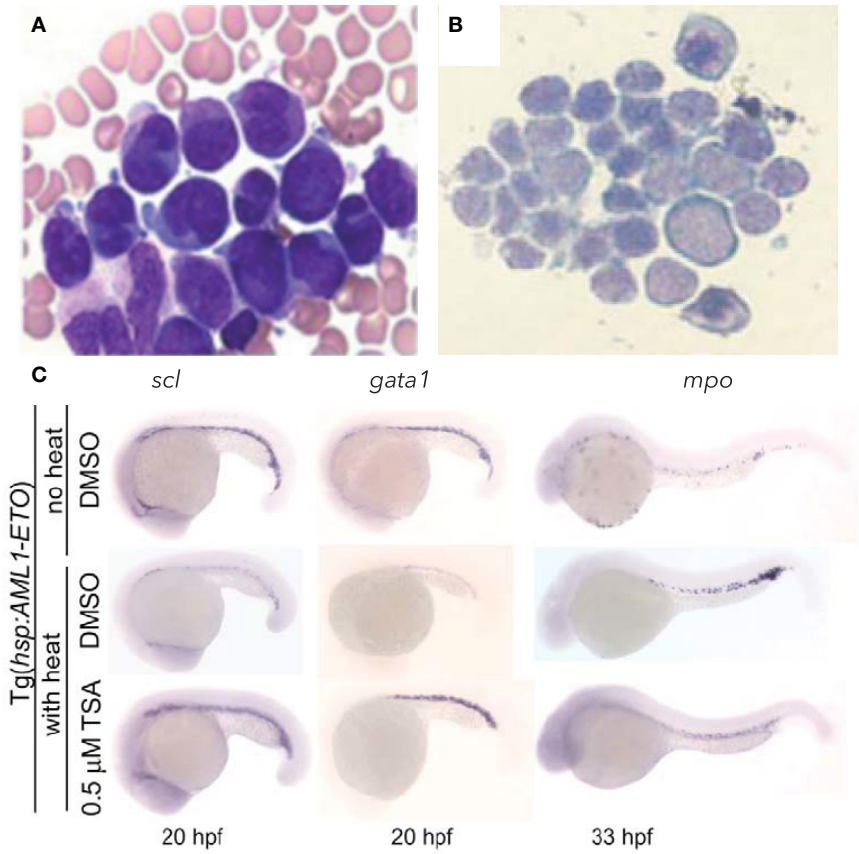

FIGURE 1 | Hematopoietic phenotype conserved in zebrafish model of AML-ETO driven AML. **(A,B)** Wright–Giemsa stained blood cells from **(A)** human acute myeloid leukemia (AML) patient bone marrow smear demonstrating accumulation of promyelocytes [modified and published with permission from Ref. (52)] and **(B)** zebrafish peripheral blood smear showing accumulation of myeloid blasts from AML-ETO overexpressing embryos at 40 hpf. **(C)** Rescue of hematopoietic phenotype with trichostatin A (TSA) treatment. Inducible Tg[*hps:AML1-ETO*] line utilized, such that heat-shocked Tg embryos treated with DMSO develop AML-like phenotype, which can be reversed with TSA treatment. *scl* marks hematopoietic stem and progenitor cells; *gata1* marks erythroid lineage; *mpo* marks myeloid lineage. Panels **(B,C)** modified and published with permission from Yeh et al. (50).

elevated tyrosine kinase activity (61, 62). FLT3 (also known as FLK2 and STK1) is expressed in human HSCs and is essential for adult HSC and immune hemostasis (63). He et al. established the function of zebrafish *flt3* in hematopoietic development, demonstrating that MO knockdown of endogenous *flt3* in zebrafish significantly impaired progenitor and myeloid differentiation (52). Transient expression of human *FLT3-ITD via* mRNA injections into embryos resulted in expansion of myeloid progenitors (*pu.1*$^+$) and mature cells (*mpx*$^+$ and *cebpα*$^+$). Elevation of downstream signaling such as phosphorylation of Stat5, Erk1/2, and Akt was also observed indicating human FLT3-ITD can trigger established endogenous signals of Flt3 in the zebrafish. Transient expression of human FLT3-TKD (D835Y) also resulted in myeloid cell expansion, but to a lesser extent than the FLT3-ITD. To demonstrate the ability of the zebrafish to test relevant human drugs, He et al. treated FLT3-ITD and FLT3-TKD expressing zebrafish embryos with AC220, a tyrosine kinase inhibitor shown to have potent selectivity for FLT3 (52, 64). Consistent with inhibiting the kinase domain of FLT3, they found that AC220 partially rescued the myeloid effects of FLT3-ITD; however, this did not abrogate the FLT3-TKD phenotype. Subsequently, Lu and colleagues generated a stable transgenic line with myeloid-restricted (*spi1/pu.1* promoter-driven) FLT3-ITD and found that these animals develop adult AML symptoms, further illustrating the conservation of function of this oncogene from zebrafish to humans (65).

Modeling of non-fusion oncogenes is also underway in zebrafish. NPM1 is a ubiquitously expressed nucleolar phosphoprotein that regulates multiple cellular processes and is the most frequently mutated gene in adult AML, occurring in ~30% of cases (4, 66). Mutations in *NPM1* result in altered protein localization from the nucleus to the cytoplasm (termed *NPMc*$^+$). Zebrafish have two *NPM1* orthologs, *npm1a* and *npm1b* (67). Double MO knockdown of both paralogs results in the production of fewer myeloid cells. Global transient expression of human *NPMc*$^+$, the human mutant cytoplasmic protein, but not wild-type *NPM1*, resulted in increased *spi1/pu.1*$^+$ myeloid precursors, *mpx*$^+$ granulocytes and *csf1r*$^+$ macrophages (67). Of note, the myeloid expansion from *NPMc*$^+$ expression was enhanced in a *p53*-deficient background, suggesting that too much *NPMc*$^+$ could trigger apoptosis. In line with this finding, a recent study showed that NPM1 acts as

a scaffold for the apoptotic apparatus termed the PIDDosome [p53-induced death domain-containing protein 1—receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a death domain (PIDD-RAIDD)-caspase-2 complex] (68). *NPMc*$^+$ expression also increased HSC levels within the dorsal aorta, indicating a possible role for mutated NPM1 in leukemic stem cell development. Additionally, *NPMc*$^+$ was shown to activate canonical Wnt signaling in early zebrafish development, which contributed to hematopoietic cell expansion (69). The elevation of WNT signaling was confirmed in human *NPMc*$^+$ AML blasts, which was reversed by knockdown of the mutant *NPMc*$^+$ transcript. Together these finding illustrate how studies in zebrafish embryogenesis can inform mechanism in human AML.

Mutations in *isocitrate dehydrogenase 1 and 2* (*IDH1/2*) are found in ~8% of AML cases (70). IDH1/2 are enzymes that catalyze the oxidative decarboxylation of isocitrate producing α-ketoglutarate. AML-associated mutations in *IDH1/2* perturb this function, altering the citric acid cycle, and leading to production of the oncometabolite 2-hydroxyglutarate, which alters DNA methylation *via* inhibition of ten-eleven translocation 2 (TET2) (70, 71). When *idh1* levels were diminished in zebrafish using morpholino or TALEN approaches, Shi et al. observed expansion of *pu.1*$^+$ precursors, impaired myeloid differentiation and reduced HSC formation (72). In contrast, when *idh2* was diminished, the zebrafish displayed similar myeloid cell defects to *idh1* mutants, but normal formation of HSCs, indicating a functional redundancy between the two *idh* factors during early embryonic HSC formation. Expression of human oncogenic IDH1-R132H in wild-type zebrafish induced myeloid compartment expansion that was suppressed by treatment with the potent and selective IDH inhibitor AGI-5198. These studies demonstrate that leukemogenic pathways are conserved between humans and zebrafish and illustrate how zebrafish can be used for therapeutically relevant drug studies.

ADULT AML MODELS IN ZEBRAFISH

These embryonic models demonstrate that partial AML phenotypes can be recapitulated in embryonic zebrafish, which can be useful for mechanistic studies and drug discovery, but do not represent a full adult-arising leukemia. The first adult model of AML in zebrafish was based on the inv(8)(p11;q13) chromosomal translocation resulting in the oncogenic fusion of MYST3 (also known as MOZ, YBFR2, SAS2, TIP60 family histone acetyltransferase monocytic leukemia 3) and nuclear co-activator 2 [NCOA2, also called transcriptional mediator/intermediary factor 2 (TIF2)]. MYST3 is in the MYST family of histone acetyltransferases, while NCOA2 is a member of the p160 HAT family [reviewed in Ref. (73)]. To promote AML formation in zebrafish, Zhuravleva and colleagues expressed the human MYST3-NCOA2 (referred to as MOZ-TIF2) oncogene under the zebrafish *spi1/pu.1* promoter (74). This resulted in development of AML after 14–26 months with immature myeloid blast accumulation in the kidney marrow, but decreased progenitors and lymphocytes in the spleen. However, such an AML phenotype was a rare event (2/180), indicating inefficient transformation, insufficient expression levels driven from the *pu.1* promoter, or perhaps the requirement for a secondary mutation for disease development. Due to the long latency and low penetrance, there have not been further studies with this model.

The t(7;11)(p15;15) chromosomal translocation leading to *nuclear pore complex protein 98* (*NUP98*)-*homeobox protein A9* (*HOXA9*) oncogenic fusion is widely observed in hematological pathologies including MDS, CML, and AML, and correlates with poor prognosis. NUP98 is involved in nuclear trafficking (75), and HOXA9 is a vertebrate transcription factor essential in hematopoiesis with >80% of human AML showing overexpression of *HOXA9* (76). Utilizing a novel Cre-LoxP system that allowed for both myeloid-restricted and heat-shock inducible expression [T*g*(*spi1:loxP-EGFP-loxP:NUP98-HOXA9*); *Tg(hsp70:cre)*], Forrester and colleagues were able to explore the effects of conditional expression of the human *NUP98-HOXA9* both in the embryo as in the studies described above, but also in adulthood. The later inducible expression is key to circumvent any deleterious events from early embryonic expression that could preclude analysis of disease in older animals. Similar to the mouse model (77), induction of human *NUP98-HOXA9* expression resulted in ~23% of transgenic fish developing preleukemic MPN by 2 years (78). Examination of embryos with *NUP98-HOXA9* expression revealed early lineage skewing where *pu.1*$^+$ myeloid progenitors were enhanced at the expense of *gata1*$^+$ erythroid progenitors, with perturbed myeloid lineage differentiation. A follow-up study that employed chemical approaches to determine the driving mechanism as well as potential new therapeutics for AML revealed that *NUP98-HOXA9* upregulates *prostaglandin synthase 2* (*ptgs2*) to expand HSC numbers (79), a pathway identified in prior studies to be important for normal HSC formation (80, 81). Blocking prostaglandin production with COX inhibitors could reverse the increase in HSCs, suggesting a role for this pathway in leukemic stem cell expansion. Additionally, gene expression analyses showed *NUP98-HOXA9* elevated the expression of the epigenetic modifier *dna methyltransferase 1 (dnmt1)*, which lead to hypermethylation. Treatment with an HDAC inhibitor reversed this phenotype. Both of these pathways were also identified as suppressors of myeloid expansion in the AML-ETO model, suggesting that they could play a more general role in AML induction.

MODELING MDS IN ZEBRAFISH

Myelodysplastic syndromes are a diverse group of chronic myeloid pathologies defined by perturbed clonal hematopoiesis, impaired differentiation and peripheral blood cytopenias with the potential to transform into AML. Substantial research efforts have been invested in understanding the drivers of MDS toward improving diagnosis and stratification of subtypes, which will improve treatment of patients. Until recently the etiology of the heterogeneous clinical outcomes of MDS was unclear. Extensive genomic analyses in recent years have revealed that some subtypes of MDS correlate strongly with mutations in spliceosomal or epigenetic factors (1–3, 82). Mutations in spliceosomal machinery are common and thought to be critical drivers in MDS pathogenesis. They have been identified in approximately 60% of all MDS cases (2), with mutations in splicing factor 3B, subunit 1 (SF3B1) observed in

80–90% of cases with the refractory anemia with ringed sideroblast (RARS) subtype (1, 3, 83, 84). Epigenetic factors are mutated in approximately 45% of MDS cases (2) with mutations in the methylcytosine dioxygenase TET2 being the most prevalently observed in 30% of MDS (2, 82). The function of splicing and epigenetic factors in MDS is still elusive as their role in normal hematopoietic development is unclear. The best way to clarify their function is to generate and study *in vivo* animal models to gain an organism-wide context of normal and perturbed gene function throughout lineage emergence, differentiation, and niche interactions.

SPLICEOSOMAL COMPONENTS IN MDS

The spliceosome is a large complex within eukaryotic nuclei encompassing five small nuclear ribonucleo-proteins (snRNPs) comprising RNAs and the associated protein molecules [reviewed in Ref. (85)]. The components, structure, and the function of the spliceosome are highly conserved throughout evolution in yeast, teleosts, and mammals. The high conservation of the spliceosome permits experiments from across eukarya to inform human spliceosome function and regulation. The function of the spliceosome is to remove introns from newly transcribed pre-mRNAs, resulting in mature mRNAs that are then translated by ribosomes to generate proteins. Splicing is a dynamic, highly coordinated process, thus its correct action is essential for normal functioning of cells. The major U2-type spliceosome comprises U1, U2, U4, U5, and U6 snRNPs (**Figure 2A**) and catalyzes the majority of splicing events, while the U12-type minor spliceosome has a specific target subset. Alternative splicing to generate multiple transcript variants for each gene occurs normally throughout

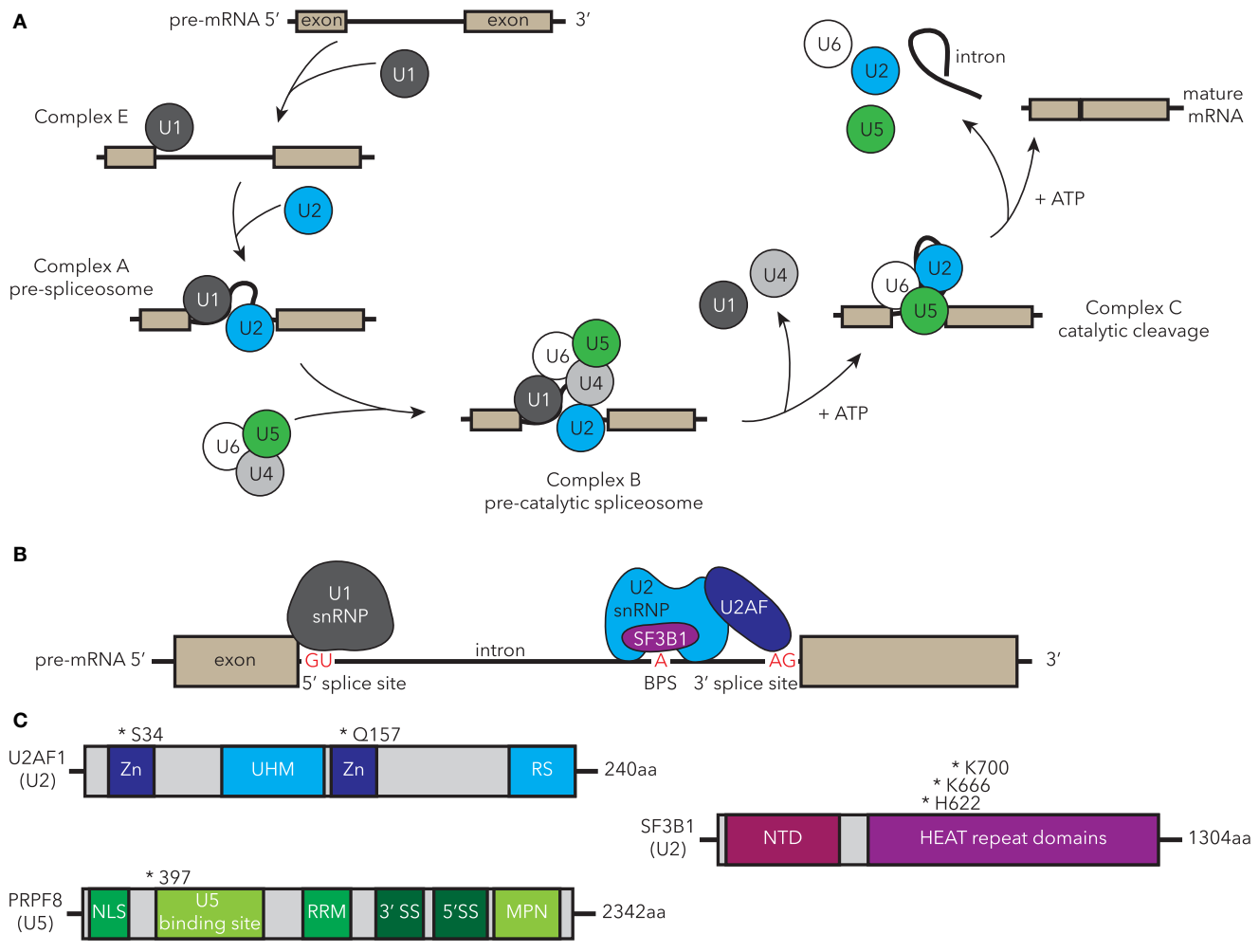

FIGURE 2 | Human MDS-associated mutations in essential components of the spliceosome are conserved in zebrafish disease models. **(A)** Spliceosomal processing of pre-mRNA to mature transcript, indicating recruitment of snRNP complexes. Complexes containing MDS-mutated factors that have been studied in zebrafish are highlighted in blue (U2) and green (U5). **(B)** Essential components of complex A, including binding of the U1 snRNP to the 5′ splice site (SS), and U2 snRNP U2AF recognition and binding of AG in the 3′ SS, while SF3B1 recognizes the branch point site (BPS). **(C)** Structure and common MDS-associated mutations in U2-associated components U2AF1 and SF3B1 and U5 PRPF8. Zn, zinc finger domain; UHM, U2AF homology motif; RS, arginine-serine domain; NTD, N-terminal domain; NLS, nuclear localization sequence; RRM, RNA recognition motif; MDS, myelodysplastic syndrome; snRNP, small nuclear ribonucleo-protein; U2AF1, U2 small auxiliary factor 1; SF3B1, splicing factor 3B, subunit 1; PRPF8, pre-mRNA processing factor 8.

development and is regulated in a tissue-specific manner. However, it can also occur as a result of spliceosomal dysfunction. MDS-associated mutations in spliceosomal components can lead to specific alternative splicing events, which correlate with their function in splicing. For example, SF3B1-containing complexes bind the branch point site within introns, and cells with MDS-associated SF3B1 mutations show defects in branch site selection, which can result in alternative proteins or unstable mRNA (**Figure 2B**) (86–90).

Splicing factor 3B, subunit 1 is a core component of the U2 snRNP and is one of the most highly mutated spliceosomal factors in MDS (2, 82). In addition to MDS, mutations in *SF3B1* have been identified in other types of leukemia such as CLL (91, 92), and several solid organ malignancies, including pancreatic cancer (93), breast cancer (94, 95), and uveal melanoma (96, 97). Mutations in *SF3B1* are strongly correlated with the ring sideroblast phenotype in MDS and are associated with better prognostic outcomes including a decreased risk of AML evolution (82, 83, 98). In SF3B1, most mutations cluster within the HUNTINGTON-ELONGATION FACTOR 3-PR65/A-TOR (HEAT) repeats in the C-terminus of the protein particularly in residues K700, K666, and H662 (**Figure 2C**) (83, 99). Recent data suggest that the HEAT repeat domains mediate protein–protein interactions (90). How these point mutations alter SF3B1 function and why this leads to hematologic dysfunction is unclear in part due to the limited understanding of the general signaling mechanism through which SF3B1 usually regulates hematopoiesis. To address this latter question, an *sf3b1* loss-of-function zebrafish mutant was studied to understand the normal function of Sf3b1 in hematopoiesis and development (100). The homozygous *sf3b-1*^{hi3394a} loss-of-function mutants displayed an arrest of primitive hematopoiesis in both myeloid and erythroid lineages, which occurred after specification presenting as a block in differentiation and proliferation. In contrast, specification of definitive HSCs was hindered, despite the normal specification and differentiation of the non-hemogenic endothelial cells within the dorsal aorta. The lower production of mature blood cells coupled with poor HSC output was reminiscent of an MDS phenotype. HSC emergence from hemogenic endothelium is a NOTCH-dependent process (101). NOTCH signaling was normal in *sf3b1* mutant zebrafish, indicating that the HSC induction defect is downstream or NOTCH-independent. These studies establish the importance of Sf3b1 somewhat selectively in hematopoiesis as other tissues such as the vasculature develop normally. How MDS-associated point mutants behave in this context requires further study. Recently, murine models of the most common MDS-associated point mutation (*Sf3b1*$^{+/K700E}$) were generated and can be used to follow-up potential mechanisms identified in unbiased screening systems such as the zebrafish or human cell culture (102, 103).

U2 small auxiliary factor 1 (U2AF1) is mutated in 8–20% of MDS patients with the most common mutations occurring at residues S34 and Q157 (**Figures 2B,C**) (2, 99, 104, 105). Mutated U2AF1 in MDS causes aberrant splicing and is associated with increased risk of AML evolution. During splicing, SF3B1 interacts with the U2AF complex to help establish the 3′ splice site and splicing fidelity (**Figure 2B**) (106). Similar to *sf3b1* mutants, homozygous loss-of-function *u2af1*^{hi199} mutant zebrafish have fewer definitive HSCs, develop anemia, and have elevated *tp53* transcript levels, phenotypes which are all observed in MDS (107). Knockdown of *tp53 via* MO injections suppressed these hematologic defects suggesting it as a downstream mediator of *u2af1* phenotypes. This model can therefore be used to further dissect the mechanism underlying U2AF1 and p53 activation in MDS.

Recurrent point mutations in the pre-mRNA processing factor 8 (PRPF8) have been reported in MDS and AML, correlating with increased myeloid progenitors, ring sideroblasts, and overall poor prognosis (108, 109). PRPF8 is a highly conserved component of the U5 snRNP that plays a role in both U2- and U12-spliceosomal processing (**Figures 2A,C**) (110). A zebrafish loss-of-function *prpf8* mutant (called *cephalophonus/cph*^{gl1}) was identified through a forward genetic screen for factors that regulate embryonic myelopoiesis (111). The *cph/prpf8* homozygous mutants have defective myeloid and erythroid development, but unlike *sf3b1* and *u2af1* mutants, they show normal formation of definitive HSCs.

These spliceosomal mutants have some overlapping, but also distinct phenotypes. These findings are consistent with what is observed clinically; patients harboring mutations in different splicing factors share some disease features, but also have distinguishing characteristics. This suggests that although all factors are part of the spliceosome, their individual functions either within or outside of the spliceosome contribute to specific facets of disease. Using these zebrafish models will permit unbiased mechanistic explorations into these functions.

TET2 IN MDS

Epigenetics is the study of changes in gene expression patterns regulated by non-genomic modifications without altering the DNA sequence. Epigenetic marks are transmitted through DNA methylation, histone modifications including acetylation and methylation of histone tails, RNA interference, and nuclear organization, thereby modulating transcriptional activation and silencing [reviewed in Ref. (112)]. Such epigenetic marks are heritable, allowing for transgenerational inheritance of non-genetic traits. Epigenetics has established critical roles in embryonic development, maternal/paternal gene imprinting, X inactivation, and disease. In cancer, there is a high prevalence of DNA hypermethylation and histone modification [reviewed in Ref. (113)]. Specifically in MDS and AML, many of the top class of mutated factors are epigenetic modifiers, which have opened the hematology field to delve into the role of epigenetics in normal and diseased hematopoiesis. The factors controlling epigenetic patterns and inheritance are highly conserved between teleosts and mammals, making zebrafish an excellent model to explore how mutations in this process lead to hematologic dysfunction.

Ten-eleven translocation proteins (TET1/2/3), a family of methylcytosine oxidases, function as epigenetic regulators of the genome methylation state. They catalyze the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine (114), which are key intermediates in DNA demethylation. Controlled methylation and demethylation are crucial for embryonic development and control of gene expression (115). Somatic deletions and loss-of-function

mutations in TET2 frequently occur in myeloid malignancies: ~30% of MDS and ~10% of *de novo* AML cases. *Tet2*-deficient mouse models have shown the function of TET2 in HSC self-renewal and differentiation, with myeloid defects reminiscent of MDS and AML (116, 117). Zinc finger nuclease technology was utilized to generate a homozygous *tet2* loss-of-function zebrafish (118). Consistent with *Tet2* null mice, *tet2*-deficient zebrafish are viable and have intact embryonic hematopoiesis. Similar to murine models and humans, the *tet2*-mutant zebrafish develop progressive clonal myelodysplasia, anemia, and myeloid progenitor expansion as they age. By 24 months, they develop a more severe MDS phenotype including peripheral blood erythrocyte dysplasia. A study in compound mutants for *tet* family members uncovered a redundancy of *tet2* and *tet3* in HSC formation (119). The underlying mechanism for the diminished levels in *tet2;tet3* double mutants was *via* regulation of NOTCH signaling in aortic endothelial cells (119). In mammalian blood cells, TET2 and TET3 are the predominantly expressed TET family members and might act redundantly (120). These data suggest a high degree of similarity in zebrafish and mammalian TET usage in hematopoiesis. Thus, the zebrafish *tet2* single and *tet2;tet3* double mutants will be useful for screening for new treatment targets of this epigenetic driver of MDS.

5q− SYNDROME AND RIBOSOMOPATHIES

5q− syndrome is an MDS subtype with macrocytic anemia arising due to large deletions within chromosome 5 [reviewed in Ref. (121)]. The deleted chromosomal segment includes two common deleted regions (CDRs) encompassing many genes expressed by HSCs including hematopoietic cytokines, protein phosphatase 2, ribosomal protein S14 (RPS14), heat shock protein family A member 9B (HSPA9B), and more distally NPM1, but which factors are involved in disease phenotypes was unknown for quite some time (122).

The zebrafish mutant *crimsonless (crs)* presents with MDS-like hematological defects from 33 hpf, including anemia with a block in maturation, increased apoptosis and multilineage (erythroid and myeloid) cytopenia (123). The mutation in *crs* was determined to be a point mutation in the *hspa9b* (*hsp70*) gene likely generating a null allele. Hspa9b is a mitochondrial matrix chaperone whose loss leads to blood-restricted oxidative stress and apoptosis. In humans, the *HSPA9B* gene is located within the 5q31 CDR in human MDS. A recent study in human hematopoietic progenitors showed that similar to the zebrafish mutant depletion of HSPA9B in human hematopoietic progenitors also leads to apoptosis (124). Combined these studies suggest that loss of *HSPA9B* could contribute to 5q− syndrome MDS.

In 2008, Ebert and colleagues identified RPS14 as a major driver of 5q− anemia (125). A zebrafish homozygous *rps14* loss-of-function mutant also develops anemia with a terminal erythroid maturation defect equivalent to that observed in 5q− syndrome (126). The *rps14* mutant displayed elevated p53 activity, which was shown to contribute to the later events of the anemia. Mutations in another ribosomal protein RPS19 are linked with the childhood disease Diamond–Blackfan anemia (DBA) (127, 128). Similar to depletion of *rps14*, zebrafish homozygous

TABLE 1 | Zebrafish models of human AML and MDS.

Human mutated factors	Correlating human disease	Zebrafish manipulation	Reference
AML-ETO t(8;21)(q22;q22)	AML	Transgenic expression of human AML-ETO fusion	(50, 133)
TEL-JAK2 t(9;12)(p24;p13)	AML	Transgenic expression of human TEL2-JAK2 fusions	(58, 59)
FLT3-ITD, FLT3 TKD	AML	Transgenic expression of human FLT3-ITD or FLT3-TKD	(52, 64, 65)
NPM1c	AML	Knockdown of zebrafish *npm1* homolog; transgenic expression of human NPM1c	(67, 69)
IDH1/2	AML	Knockdown of zebrafish *idh1* and idh2 homologs; transgenic expression of human IDH1 point mutant	(72)
MYST3-NCOA2 inv(8)(p11;q13)	AML	Transgenic expression of human MYST3-NCOA2 fusion under the *spi1/pu.1* promoter	(74)
NUP98-HOXA9 t(7;11)(p15;15)	AML	Transgenic expression of human NUP98-HOXA9 fusion under the *spi1/pu.1* promoter	(78, 79)
SF3B1	MDS	*sf3b1*^{hi3394a} mutant	(100)
U2AF1	MDS	*u2af1*^{hi199} mutant	(107)
PRPF8	MDS	*prpf8*^{gl1}*/cephalophonus*^{gl1} mutant	(111)
TET2/3	MDS	*tet2*^{zdf20}*, tet2*^{mk17}*, and tet3*^{k18} mutants	(118, 119)
RPS14	Ribosomopathy (5q− MDS)	*rps14*^{zf624} mutant	(126)
RPS19	Ribosomopathy (DBA)	*rps19*^{zf556} mutant	(129, 134)
HSPA9B	Ribosomopathy (5q− MDS)	*hspa9b*^{unspecified}*/crimsonless*^{unspecified} mutant	(123)

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ETO, eight twenty one; JAK2, Janus kinase 2; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; TKD, tyrosine kinase domain; NPM1, nucleophosmin 1; IDH1/2, isocitrate dehydrogenase 1 and 2; NCOA2, nuclear co-activator 2; NUP98, nuclear pore complex protein 98; HOXA9, homeobox protein A9; SF3B1, splicing factor 3B, subunit 1; U2AF1, U2 small auxiliary factor 1; PRPF8, pre-mRNA processing factor 8; TET2/3, Ten-eleven translocation; HSPA9B, heat shock protein family A member 9B; DBA, Diamond–Blackfan anemia.

rps19 loss-of-function mutants develop a p53-dependent anemia (129). These models will therefore be useful to dissect the signaling pathway intermediates between ribosomal proteins and p53-mediated factors that drive anemia. Indeed, L-leucine, a drug in testing for DBA (130, 131), has already proven effective in treatment of both mutant Rps14- and Rps19-driven anemia in zebrafish (126, 132).

CONCLUSION AND FUTURE DIRECTIONS

The zebrafish model has great utility for investigating driver mutations underlying disease pathogenesis in MDS and AML. In particular, the high frequency of spliceosomal mutations identified in human MDS and AML and the conservation of myelo-erythroid phenotypes in the mutants studied to date indicates that this is a useful system to investigate the role of the spliceosome and epigenetics in myeloid malignancies. The above discusses some of the zebrafish myeloid disease models currently being studied (**Table 1**), and with the ability to rapidly generate mutant lines utilizing technologies such as CRISPR/Cas9, many more genes can be investigated in a relatively high-throughput manner. Furthermore, mechanistic studies are faster, cheaper, and higher throughput with *in vivo* testing of drug pathways feasible. This will facilitate more robust testing of targets *in vivo* in a whole organism setting. From this, we can identify and test rational, targeted pathways and therapeutics rather than the aggressive, non-specific cytotoxic chemotherapies utilized in current MDS and AML treatment regimes.

Unlike murine models, which often faithfully recapitulate human leukemias, zebrafish myeloid malignancy models frequently fail to develop the full adult disease state as observed in human MDS and AML, which is a limitation in the system to date. To address this, xenograft models using human leukemic cell lines or primary leukemic cells are now being used in zebrafish to investigate human disease progression (135). As zebrafish younger than a week have an innate immune system, but do not yet have a functioning adaptive immune system, xenografts in larvae are particularly useful to examine cancer progression *in vivo* without the need for damaging pre-conditioning regimens to permit human cell engraftment (23, 136). Thus, xenografts in zebrafish provide a new dimension for analysis of disease states and causative mechanisms, and will be extremely useful moving forward to screen human cells for drug susceptibility within an *in vivo* environment.

Currently, ubiquitous loss-of-function mutants are used to investigate the normal function of genes of interest in development. In human myeloid malignancies, mutations in genes often arise somatically and are missense rather than null. Genetic approaches in murine models permit tissue-specific expression of point mutants, which more closely resembles the human condition. With the advent of CRISPR/Cas9 technologies, the next step in zebrafish is to generate specific knock-in models of disease-associated point mutants [reviewed in Ref. (137)] and to induce mutations in a tissue-specific manner (138). These advances will expand our understanding of MDS and AML, including how faithfully the loss-of-function mutants recapitulate the phenotype of point mutants, and for screening of potential treatment molecules. The recent development of clonal lineage tracing capabilities for the blood system in zebrafish (139) opens the door to uncover drivers of the initial clonal events prior to hematologic dysfunction. Additionally, using live animal imaging, the initiation of cancer at the single-cell level was recently demonstrated in zebrafish melanoma (140). Combining these clonal and genetic approaches in myeloid malignancies can help examine the earliest events of disease formation not readily accomplished in other animal models.

AUTHOR CONTRIBUTIONS

KP and TB designed and wrote the manuscript. TB gave final approval of the manuscript.

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REVIEW
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Inflammatory Signaling Pathways in Preleukemic and Leukemic Stem Cells

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Hematopoietic stem cells (HSCs) are a rare subset of bone marrow cells that usually exist in a quiescent state, only entering the cell cycle to replenish the blood compartment, thereby limiting the potential for errors in replication. Inflammatory signals that are released in response to environmental stressors, such as infection, trigger active cycling of HSCs. These inflammatory signals can also directly induce HSCs to release cytokines into the bone marrow environment, promoting myeloid differentiation. After stress myelopoiesis is triggered, HSCs require intracellular signaling programs to deactivate this response and return to steady state. Prolonged or excessive exposure to inflammatory cytokines, such as in prolonged infection or in chronic rheumatologic conditions, can lead to continued HSC cycling and eventual HSC loss. This promotes bone marrow failure, and can precipitate preleukemic states or leukemia through the acquisition of genetic and epigenetic changes in HSCs. This can occur through the initiation of clonal hematopoiesis, followed by the emergence preleukemic stem cells (pre-LSCs). In this review, we describe the roles of multiple inflammatory signaling pathways in the generation of pre-LSCs and in progression to myelodysplastic syndrome (MDS), myeloproliferative neoplasms, and acute myeloid leukemia (AML). In AML, activation of some inflammatory signaling pathways can promote the cycling and differentiation of LSCs, and this can be exploited therapeutically. We also discuss the therapeutic potential of modulating inflammatory signaling for the treatment of myeloid malignancies.

Keywords: inflammatory, preleukemic, leukemic stem cell, toll-like receptor, tumor necrosis factor, interferon, interleukin, NF-κB

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INTRODUCTION

Several known preleukemic disorders, including myelodysplastic syndrome (MDS) and the myeloproliferative neoplasms (MPNs), are characterized by acquired cytogenetic abnormalities or molecular alterations in hematopoietic stem cells (HSCs) (1, 2). These alterations result in altered or ineffective hematopoiesis, and varying degrees of bone marrow fibrosis, ultimately leading to morbidity and decreased life expectancy. Preleukemic stem cells (pre-LSCs) have a selective growth advantage over normal HSCs, but are still capable of normal differentiation [reviewed in Ref. (3)]. In acute leukemias, LSCs have the distinct property to undergo self-renewal, but these cells can only differentiate into leukemic blasts [reviewed in Ref. (4)]. Both pre-LSCs and LSCs have been implicated in posttreatment relapse in leukemia patients. Abnormalities in inflammatory signaling have been noted in both preleukemic conditions (MDS/MPN) and in acute myeloid leukemia

(AML), suggesting an important role for inflammatory signaling in these conditions. Inflammatory signaling can occur both in hematopoietic cells and in the hematopoietic niche, significantly altering the crosstalk between hematopoietic cells and their microenvironment [reviewed in Ref. (5)]. Interestingly, some of the same inflammatory pathways may actually promote the differentiation and loss of self-renewal of LSCs in AML.

CLONAL HEMATOPOIESIS, MUTATIONS IN EPIGENETIC MODIFIERS, AND INFLAMMATION

Clonal hematopoeisis of indeterminate potential (CHIP) is a recently characterized entity, which describes the increased rate in the acquisition of somatic mutations in hematopoietic cells with increasing age, in the absence of cytopenias or morphologic bone marrow fibrosis or dysfunction (6). While many of these mutations are commonly seen in MDS or AML, and their presence in hematopoietic cells is associated with an increased risk of developing hematologic malignancies, the majority of these patients (99–99.5%) will never progress to frank MDS or AML (6, 7). Since aging has been associated with a chronic inflammatory state, it is possible that clonal hematopoiesis is also promoted by inflammation (5, 8, 9). This could occur through increased genomic instability, which can lead to the acquisition of mutations, followed by the positive selection of mutant clones. It was recently shown in mouse models that hematopoietic stress, such as that induced by serial polyI-polyC injection, which activates toll-like receptor (TLR) signaling and induces HSCs to exit quiescence, can precipitate DNA damage in HSCs (10). This could be a potential mechanism for the increased acquisition of mutations in HSCs with age.

MDS-related mutations are the most commonly found in clonal hematopoiesis, including the "first hit" mutations that are thought to initiate clonality, such as those seen in genes that affect DNA methylation (TET2, DNMT3A) or histone acetylation (ASXL1) (11). The understanding of how epigenetic modifiers may regulate inflammatory signaling is evolving. Recent data suggest a causal link between some epigenetic mutations seen in clonal hematopoiesis or MDS and inflammation. In a study of over 17,000 blood samples from unselected patients, the patients with clonal hematopoiesis not only had a higher rate of hematologic malignancies but also a higher rate of death from cardiovascular disease and stroke compared to those without clonal hematopoiesis (7). TET2 (ten-eleven translocation-2) and DNMT3A (DNA methyltransferase 3A) are known to cause abnormalities in hematopoiesis, including in the monocyte-macrophage lineage, derangements of which are also seen in atherosclerotic disease and diabetes (7, 12). In fact, Jaiswal et al. recently showed that loss of *Tet2* in hematopoietic cells could promote atherosclerosis in the LDL-receptor knockout mouse model due to activation of macrophages (13). They found that macrophages from *Tet2*−/− bone marrow secreted increased levels of several chemokines, including CXCL1, CXCL2, CXCL3, PF4, and PBBP, some of which are known to promote atherogenesis. In patients with CHIP with TET2 mutations, they also found serum elevations of the inflammatory chemokine interleukin 8 (IL-8) (13). Another recent study also identified increased interleukin 1 beta (IL-1β) and inflammasome activation in mice with *Tet2* deficiency (14). Furthermore, Cull et al. found constitutive activation of the lipopolysaccharide (LPS)-related inflammatory pathway *in vivo* in peritoneal fluid in a *Tet2* knockdown mouse model, and increased IL-1β and interleukin 6 (IL-6) levels from bone marrow-derived macrophages *in vitro*, suggesting that chronic inflammation and dysregulation in the immune microenvironment is a result of Tet-2 loss (15).

Leoni et al. recently reported on the role of DNMT3A, another epigenetic modifier, in regulating mast cell inflammatory responses (16). They found that *Dnmt3a* knockout mast cells were more responsive to stimuli than wild-type mast cells, and secreted higher levels of inflammatory cytokines, such as IL-6, tumor necrosis factor alpha (TNF-α), and IL-13, leading to increased acute and chronic inflammatory responses *in vivo*. Together, these studies directly link inactivation of *Tet2* or *Dnmt3A*, two of the most commonly mutated genes in patients with clonal hematopoiesis or myeloid malignancies, with the initiation of an inflammatory state. Activation of inflammatory signaling can then lead to further expansion of mutant clones, by increasing cell cycling or enabling evasion from apoptosis, thereby promoting progression to MDS, MPN, and/or AML. Here, we discuss several mechanisms by which specific inflammatory signaling pathways can promote the clonal expansion of pre-LSCs and modulate disease progression, including pathways driven by interferon (IFN) I and II, TLRs, TNF-α, and ILs, in particular IL-1β, IL-6, and IL-8. Several of these pathways include potential therapeutic targets for the treatment or prevention of MDS, MPN, or AML.

TYPE I IFNS (IFN-α/IFN-β)

Interferons are known as key regulators of HSCs. They are categorized into type I IFN, including IFN-α and IFN-β, and type II IFN (also known as IFN-γ). Type I IFNs are produced endogenously in response to a program set by TLR3 activation in response to a variety of host challenges, such as viruses, and also to double stranded DNA from bacteria and tumors (17). Type I IFNs signal via the ubiquitously expressed IFN-α/β receptor (IFNAR, see **Figure 1**). IFNAR is composed of an extracellular heterodimer of two receptor tyrosine kinases, IFNAR2 (TYK2) and IFNAR1 (JAK1), whose binding leads to phosphorylation of STAT1 and STAT2, forming a trimer with unphosphorylated IRF9 (17). This trimer then enters the nucleus to direct transcriptional activity of a variety of antiviral cell programs, also leading to the production of the chemokine CXCL10 and induction of apoptosis pathways. IFN-α was initially tested as a therapy for chronic myelogenous leukemia (CML) in the 1970s, following *in vitro* studies showing inhibition of CML growth with IFN treatment. Subsequent clinical trial data showed up to a 60% complete cytogenetic response and improved overall survival compared to traditional chemotherapy. Rare complete long-term remissions post-IFN treatment were reported in a subset of patients who were treated without allogeneic stem cell transplantation (SCT), making this the standard of care for the treatment of CML prior to the era of tyrosine kinase inhibitors (TKIs) (18). IFN has also

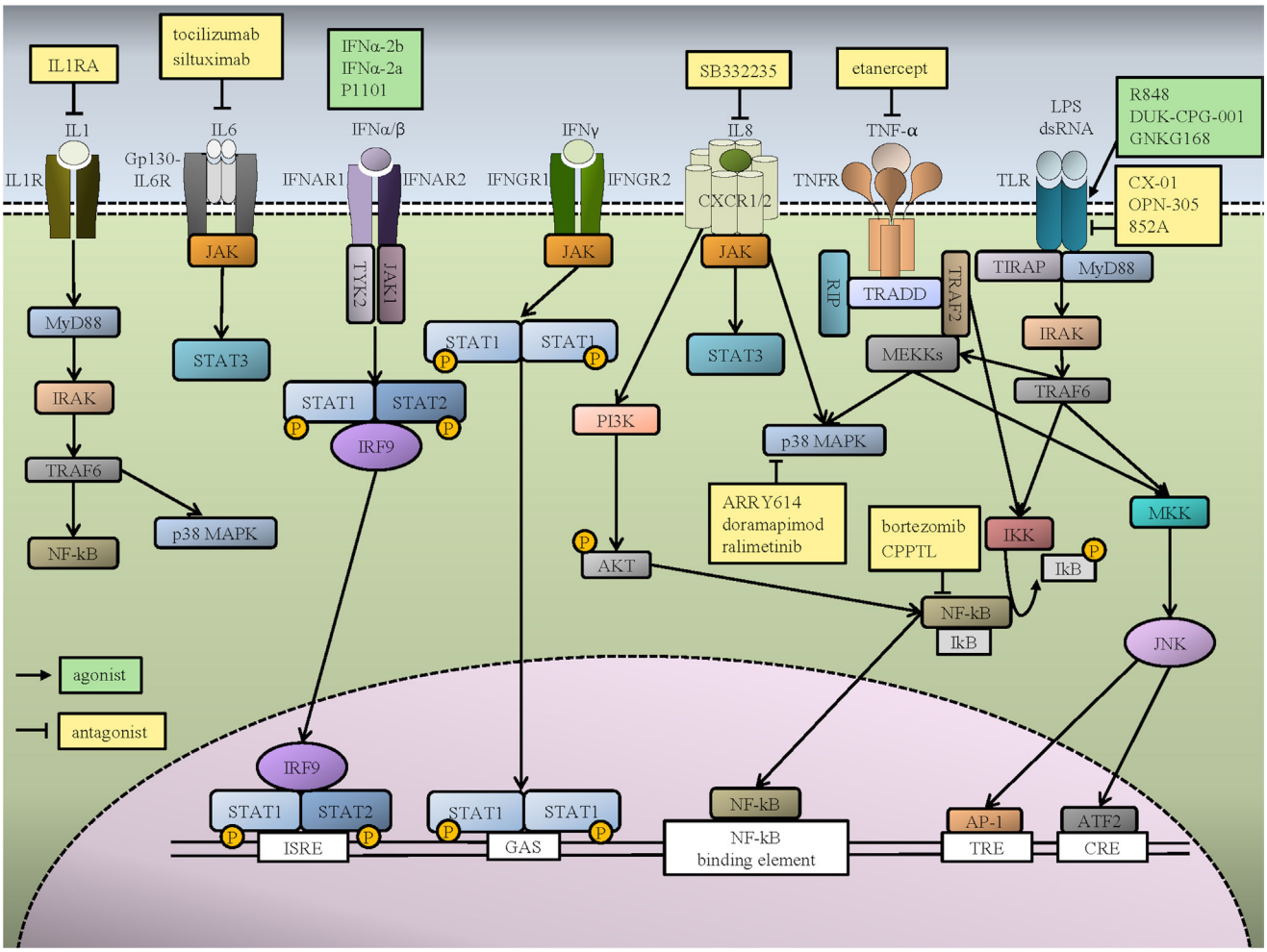

FIGURE 1 | Inflammatory signaling pathways in hematopoietic cells and potential therapeutic targets for myeloid malignancies. Interleukin (IL)-1β activates the IL-1 receptor (IL-1R), which causes dimerization and intracellular downstream signaling *via* MYD88 and IRAK. This activates multiple downstream pathways, including NF-κB and p38 MAPK. Two interleukin 6 (IL-6) molecules form a hexamer with two IL-6 receptors (IL-6R) and two GP-130 molecules, which signal *via* the JAK1–STAT3 pathway. The binding of IFN-α/β to IFNAR receptors activates TYK2 and JAK1, which phosphorylate STAT1 and STAT2. The association of IRF9 and phosphorylated STAT1 and STAT2 activates transcription by binding to IFN-stimulated response elements (ISREs). IFN-γ binding to IFNGR receptors promotes STAT1 phosphorylation by JAK. The STAT1 homodimer translocates to the nucleus and activates IFN-γ-activated site (GAS) sequences. IL-8 binds to its receptor, either CXCR1 or CXCR2, which can activate various downstream signaling pathways, including PI3K/AKT, JAK/STAT, and MAPK. There is extensive crosstalk between tumor necrosis factor alpha (TNF-α) and Toll-like receptor (TLR) signaling pathways. TNF-α binds to its receptor TNFR and activates IKK *via* RIP and TRAF2 recruitment by TRADD. IKK activation promotes IKB phosphorylation and release of NF-κB, which can then translocate to the nucleus. TNF-α binding also activates p38 and MEKK. The activation of MEKK causes JNK to stimulate AP-1, which binds to TPA DNA-response elements (TRE) and ATF2, which binds to cAMP-responsive elements (CRE). Activation of TLR by infectious molecules initiates the signaling pathway through MyD88, which recruits IRAK to bind TRAF6 and activate NF-κB and JNK pathways. Representative pathways agonists (green boxes) and antagonists (yellow boxes) that are either in preclinical or clinical investigation are shown.

been used clinically in the treatment of Philadelphia chromosome (Ph)-negative MPNs, including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF) (19, 20). While successful as the first biologic treatment in cancer, the mechanism of action of IFN-α in the treatment of MPN, or its effects on hematopoiesis in general, remained elusive.

One proposed mechanism of action for IFN-α in hematopoiesis is through activation of the p38MAPK-mediated apoptosis pathway. It was demonstrated that IFN-α treatment of KT-1 cells led to activation of p38 MAPK-mediated growth inhibition, which was reversed with p38 inhibitor treatment (21). Treatment of patient-derived CML cells grown in culture with IFN-α also showed decreased cell growth via upregulation of p38, and this effect was also reversed with a p38 inhibitor. Therefore, activation of p38 MAPK is likely to be one of the therapeutic effects of IFN-α in CML (21, 22).

Additional mechanisms of IFN-α action are likely to be important *in vivo*. Short-term IFN-α treatment induced most hematopoietic stem and progenitor cells (HSPCs) in mice to exit the quiescent state and enter active cell cycling (23).

However, chronic IFN-α administration causes irreversible HSC dysfunction, as demonstrated by the inability to repopulate in a competitive repopulation assay. Furthermore, activation of IFN signaling in mice impairs hematopoietic recovery after 5-fluorouracil chemotherapy (23). Therefore, chronic activation of type I IFN signaling can deplete HSPCs by repeatedly driving them out of the quiescent state and impeding their return to steady state after activation.

Two studies using knock-in JAK2V617F murine models of PV suggest that IFN-α may play a similar role in MPN stem cells (24, 25). Both studies reported that HSPCs in the JAK2V617F model become more proliferative and lose quiescence after IFN-α treatment, leading to the depletion of MPN stem cells. Furthermore, IFN-α treatment can prevent disease initiation in secondary transplantation, suggesting that IFN-α treatment has a direct effect on MPN disease-initiating cells (24, 25).

CLINICAL APPLICATIONS OF MODULATING IFN-α/β SIGNALING

The proliferative effects of IFN-α on HSCs could have therapeutic utility in the treatment of myeloid malignancies, and this strategy is currently being explored in multiple clinical trials (**Table 1**). By inducing dormant LSCs to enter the cell cycle, IFN-α could make them more sensitive to chemotherapy or kinase inhibitors. Data from a few CML patients who were treated with IFN-α, followed by imatinib, shows that these patients achieved prolonged remissions, suggesting the depletion of pathologic LSCs with drug administration (26). Data from clinical studies in PV, ET, and MF patients show that treatment with IFN-α can cause cycling and differentiation of pathologic stem cells, and lead to a decrease of JAK2 allelic burden (9, 27). Similar results have been obtained in patients with calreticulin-mutated ET and MF (28). IFN treatment resulted in transfusion independence, decreased spleen size, and improved symptoms and quality of life in a significant proportion of MPN patients (29). There is also a strong rationale for combining IFN-α with JAK2 inhibitors in patients with JAK2-mutated MPNs (9, 25, 30). IFN-α is clinically approved for several indications, and there are numerous ongoing clinical trials that incorporate IFN into the treatment of MPN and AML, specifically in the post-transplant setting for relapse prevention (see **Table 1**). A recent interim report of one such clinical trial that examined the role of IFN-α in AML patients with minimal residual disease (MRD) after SCT demonstrated that 75% of the patients converted to MRD-negative status after IFN-α treatment (31). These promising early results suggest that IFN-α may be an effective approach in the postremission setting to eliminate LSCs or pre-LSCs in AML and in MPNs.

TYPE II IFN (IFN-γ)

Type II IFN (also known as IFN-γ) activates the receptors IFNGR1 and IFNGR2, which signal through STAT1 (see **Figure 1**). It is produced by immune effector cells, such as NK and T-cells, and is important in the response to several intracellular pathogens, including some viral infections. The role of IFN-γ in HSCs is controversial [reviewed in Ref. (32)]. Originally, IFN-γ was demonstrated to inhibit the growth of human CD34+ cells, and to

TABLE 1 | Clinical trials targeting interferon α/β MDS, MPN, and AML.

Drug	Clinical trial	Status
IFN-α-2b	NCT03121079: IFN-α prevents leukemia relapse of AML patients after SCT	Phase I
IFN-α-2a	NCT02328755: PEG-INFα-2a to enhance antileukemic responses after allogeneic transplantation in AML	Phase I/II
IFN-α	NCT02027064: IFN-α for the intervention of molecular relapse in t (8;21) AML after allo-HSCT	Phase IV
IFN-α-2b	NCT02331706: IFN-DLI for relapsed acute leukemia after Allo-SCT	Phase I
IFN-α-2b	NCT00548847: Immunotherapy for AML, ALL, blast phase CML, and MDS, relapsed after allogeneic SCT	Phase II, completed
IL-12 + IFN-α	NCT00003451: IL-12 followed by IFN-α in treating patients with advanced cancer	Phase I, completed
IL-2 + IFN-α	NCT00002504: IL-2 plus IFN-α in treating adults with metastatic cancer (including leukemias, MDS, and MPN)	Phase II, completed
IL-2 + IFN-α	NCT00003408: Biological therapy (GM-CSF, interleukin 2, and IFN-α) following chemotherapy and SCT in treating patients with cancer (including MDS and MPN)	Phase II, completed
IFN-α-2a	NCT00452023: Pegasys® in patients with MPNs	Phase II
IFN-α-2a	NCT02742324: Ruxolitinib and Peg-IFN-α-2a combination in patients with primary myelofibrosis RUXOPeg (RUXOPeg)	Phase I/II
PEG-proline-IFN-α-2b	NCT02370329: P1101 [polyethyleneglycol (PEG)-proline-IFN-α-2b] in treating patients with myelofibrosis	Phase II
PEG-proline-IFN-α-2b	NCT03003325: The benefit/risk profile of AOP2014 in low-risk patients with PV (low-PV)	Phase II
Nilotinib and PEG-IFN-α-2b	NCT02001818: Peg-IFN-α-2b and nilotinib for augmentation of complete molecular response in CML (PInNACLe)	Phase II
IFN-α-2b	NCT01657604: Tasigna and IFN-α Evaluation Initiated by the German CML Study Group—the TIGER Study (TIGER)	Phase III
IFN-α-2a	NCT02201459: Nilotinib ± Peg-IFN for first-line chronic phase CML patients (PETALs)	Phase III
PEG-proline-IFN-α-2b	NCT01933906: Addition of P1101 to imatinib treatment in patients with chronic phase CML not achieving a complete molecular response	Phase I
PEG-IFN-α-2a	NCT02381379: Malaysia stop tyrosine kinase inhibitor trial (MSIT): IFN-α vs. observation in CML patients off TKI after deep molecular remission × 2 years	Phase III

Representative studies are listed. Source: www.clinicaltrials.gov.

IL, interleukin; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; AML, acute myeloid leukemia; SCT, stem cell transplantation; HSCT, hematopoietic stem cell transplantation; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; PV, polycythemia vera; TKI, tyrosine kinase inhibitor.

induce differentiation and apoptosis (33). However, more recent studies of both human and mouse HSCs have shown that IFN-γ can stimulate HSCs to proliferate, while promoting myeloid differentiation, suggesting that IFN-γ is important to maintain normal myeloid development in the setting of viral infections (34, 35). Subsequently, it was shown that only chronic IFN-γ exposure leads to HSC depletion (36).

Excessive IFN-γ signaling has been associated with hematopoietic dysfunction in humans. For instance, polymorphisms in the *IFNγ* gene have been linked with high production of IFN-γ, and occur more frequently in patients with aplastic anemia (37). Increased IFN-γ signaling has also been reported in MDS patients (38). In addition, previous studies have demonstrated the expansion of abnormal auto-reactive CD8 T cells in the bone marrow of MDS patients, which suggests a mechanism for increased production of myelosuppressive cytokines that affect hematopoietic cells in MDS (39–41). Furthermore, treatment with hypomethylating agents has been associated with elevated IFN-γ secretion in lower risk MDS (42). However, the detailed mechanisms through which IFN-γ promotes HSC incompetence in bone marrow failure syndromes are not well understood. To better understand the response to decitabine in MDS, Zhang et al. examined the levels of PD-L1, PD-1, and STAT1 in T-cells after decitabine therapy in lower risk MDS. They found that, although the level of STAT1 expression did not predict treatment response, an increase in the PD-1/STAT1 ratio was associated with hematopoietic improvement and prolonged survival in MDS patients treated with decitabine (43). Therefore, elevated IFN-γ/STAT1 signaling has also been associated with progression of MDS and treatment response.

Sharma et al. tested the engagement and functional role of protein kinase R (PKR) in the generation of IFN-γ effects on primitive hematopoietic progenitors and MDS cells. PKR is an IFN-inducible double-stranded RNA-activated serine-threonine protein kinase, which is a major mediator of the antiviral and antiproliferative activities of IFNs. Using a specific PKR inhibitor or siRNA-mediated PKR knockdown on bone marrow or peripheral blood mononuclear cells from MDS patients, they observed an increase in myeloid (CFU-GM), erythroid (BFU-E), and hematopoietic progenitor colony formation. Their data suggest that drugs that target PKR might be novel candidates for MDS therapy (44).

The role of IFN-γ in LSCs is still uncertain. To examine the role of IFN-γ in CML cells in the context of TKI treatment, Madapura et al. treated CML cell lines and primary human CML CD34+ cells with IFN-γ with and without imatinib. They showed that IFN-γ upregulates BCL6 *via* STAT1, as well as several antiapoptotic family members of the BCL2 family, including MCL-1L, the long isoform of MCL1. Interestingly, IFN-γ treatment also increased colony formation by CD34+ CML cells. These data support a protumorigenic effect of IFN-γ in CML and suggest that IFN-γ may contribute to TKI resistance. Their data suggest that combining TKIs with inhibitors of BCL6 or MCL1 is a potential approach to eradicate CML stem cells (45).

In contrast to the tumor-promoting effects of IFN-γ reported in MDS, Fatehchand et al. demonstrated that in AML, IFN-γ could induce cytotoxicity. IFN-γ treatment promotes myeloid differentiation of myeloblasts, and thereby potentiates the antibody-mediated cytotoxicity effect of daratumumab in several AML cell line-derived models. They also showed that IFN-γ treatment promotes the myeloid differentiation and phagocytic activity of primary AML patient cells. The combination of IFN-γ and FCγR activation enhanced the production of granzyme B, suggesting that IFN-γ can induce AML cells to differentiate into immune effector cells (46).

SOCS1 is an important negative regulator of IFN-γ signaling. The RIP1/RIP3 kinases, which are activated by TNF-α, inhibit the degradation of SOCS1, which limits the extent of IFN-γ signaling. Induction of RIP1/RIP3-mediated necroptosis has been proposed as an alternative strategy for treating apoptosis-resistant leukemia (47). In a recent study, Xin et al. demonstrated that, despite a high basal level of TNF-α secretion and RIP1/RIP3 signaling in the majority of French-American-British (FAB) subtype M4 and M5 AML samples, most AML cells do not undergo apoptosis. Using genetic and pharmacologic approaches, they showed that AML cells with inactivated RIP1/RIP3 signaling exhibit increased sensitivity to IFN-γ-induced differentiation, which leads to decreased clonogenic activity and apoptosis. Therefore, they suggested that the combination of IFN-γ with other inducers of differentiation could be a novel therapeutic strategy for AML (48).

CLINICAL APPLICATIONS OF MODULATING IFN-γ SIGNALING

While IFN-γ1β is an approved treatment for preventing infections in chronic granulomatous disease, and is currently being tested in multiple autoimmune conditions, solid tumors, and lymphoma, its use in myeloid malignancies has not yet been investigated in clinical trials. Because the roles of IFN-γ in pre-LSC and LSC function in MDS and AML are still unclear, further preclinical investigation with *in vivo* models is needed to better understand the appropriate clinical setting for targeting IFN-γ signaling in these diseases.

TLR SIGNALING

Toll-like receptors are a family of pattern recognition receptors that are important in innate immunity. TLRs 1, 2, 4, and 6 transduce signals via the myeloid differentiation primary response gene 88 (MYD88), which leads to activation of IRAK1, 4, and 2, and TRAF6. This mediates an acute proinflammatory response through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), AP-1, and p38 MAPK pathways [reviewed in Ref. (49), see **Figure 1**]. TLRs are expressed on HSPCs, effector immune cell populations, and stromal cells (50–52). Several classes of TLRs, including TLR4, TLR7, TLR8, and TLR9, are expressed on human CD34+ cells, suggesting that HSCs have an early mechanism to immediately detect infection (32). Although normal TLR signaling plays an important role in the immune response to injury or infection, enhanced or abnormal TLR signaling has been linked with defective hematopoiesis and hematopoietic malignancies (53, 54). Takizawa et al. recently demonstrated that LPS, the best

known ligand for TLR signaling, can directly stimulate HSCs *in vivo* and increase their cycling. They showed that, while LPS induces the proliferation of dormant HSCs, prolonged LPS exposure impairs HSC regenerative capacity (55). They also found that this process is mediated *via* the TLR4-TRIF-ROS-p38 pathway, and not through MyD88 signaling. This suggests that inactivation of the TRIF-ROS-p38 signaling axis could prevent the induction of HSC dysfunction by LPS, while not affecting emergency myelopoiesis (55).

In addition, LPS increases the number of colony forming units in the blood, spleen, and bone marrow (56, 57). Activating mutations, increased expression of TLRs and TLR signaling pathway intermediates, and loss of repressors of TLR signaling have all been reported in MDS (49, 58–64). Interestingly, while increased expression of TLR2 and TLR9 has been reported in MDS, their expression decreases with progression to AML (49). This suggests that TLRs may play different roles in pre-LSCs in MDS and in LSCs in AML.

Recent studies have shed more light onto the mechanisms of activation of TLR signaling in del (5q) MDS. The S100 calcium-binding protein family members S100A8 and S100A9, which are released during the activation of phagocytes, have been described as endogenous activators of TLR4 (65). Schneider et al. have linked S100A8 and S100A9 to HSC dysfunction and impaired erythroid differentiation in del (5q) MDS. They showed that impaired erythropoiesis in del (5q) MDS is associated with heterozygous deletion of RPS14 (ribosomal protein small subunit 14). Using conditional knockout Rps14 mice, they detected a p53-dependent defect in erythroid differentiation. This differentiation defect resulted in age-dependent progressive anemia, megakaryocyte dysplasia, and loss of HSC quiescence. Using proteomic profiling, they observed a higher level of S100A8 and S100A9 expression in Rps14 mutant erythroblasts. By genetically inactivating S100A8 expression they rescued the erythroid differentiation defect in Rps14 haploinsufficient HSCs (66). This suggests that secretion of S100A8 and S100A9 by activated immune effector cells could promote hematopoietic dysplasia *via* TLR signaling.

Starczynowski et al. have shown that, in del (5q) MDS patients, loss of miR-145 and miR-146a can also cause the abnormal activation of TLR signaling (62). These micro-RNAs normally inhibit the TLR signaling intermediates TIR-domain-containing adaptor protein and TRAF6, a TLR effector with ubiquitin (Ub) ligase activity. Moreover, overexpression of TRAF6 or knockdown of miR-145 or miR-146 mimics some of the features of del (5q) MDS in mouse models, suggesting that aberrant activation of TLR pathway signaling in HSCs contributes to disease pathogenesis (62, 67).

It has also been shown previously that TLR-driven pathways are involved in coordinating RNA processing during hematopoietic differentiation (68, 69). Fang et al. demonstrated the role of TRAF6 in RNA processing in hematopoietic cells by examining RNA ubiquitination. Using a global ubiquitination screen, they identified hnRNPA1, an RNA-binding protein and auxiliary splicing factor, as a substrate of TRAF6. TRAF6-mediated ubiquitination of hnRNPA1 regulates the alternative splicing of *Arhgap1*, which activates the GTP-binding Rho family protein Cdc42, and contributes to the HSPC dysfunction observed in the TRAF6 overexpression mouse model (67).

Varney et al. investigated the role of the TRAF-interacting protein with forkhead-associated domain B (TIFAB) in MDS, which is a haploinsufficient gene in del (5q) MDS. Loss of heterozygosity of TIFAB causes bone marrow failure and significant changes in myeloid differentiation. Gene expression analysis in TIFAB knockout HSPCs revealed the upregulation of immune and infection response signatures, which suggests hypersensitivy to TLR4 stimulation. However, TNF and endotoxin signatures were downregulated. Using a global proteomic analysis, this study revealed that TIFAB forms a complex with TRAF6 and decreases the stability of TRAF6 *via* a lysosome-dependent mechanism. Therefore, loss of TIFAB increases TRAF6 protein levels and thereby activates NFKB, which leads to ineffective hematopoiesis. Furthermore, the authors observed that deletion of both TIFAB and miR-146a increases the expression of TRAF6, suggesting that these factors cooperate in promoting dysfunctional hematopoiesis (70).

Recent work has further highlighted the importance of TLR signaling in the bone marrow microenvironment in the initiation of preleukemic disorders. Using the preleukemic Shwachman-Diamond syndrome (SDS) mouse model driven by deletion of the *Sbds* gene in mesenchymal progenitor cells, Zambetti et al. have shown that *Sbds* deletion drives mitochondrial dysfunction, oxidative stress, and activation of the DNA damage response in HSPCs. The authors performed RNA sequencing of purified mesenchymal cells from SDS mice and also of sorted mesenchymal cells from patients with three preleukemic diseases: SDS, low-risk MDS, and Diamond-Blackfan anemia. When comparing the overexpressed genes in each case, they identified the p53-S100A8/9-TLR inflammatory signaling pathway as a common driving mechanism of genotoxic stress in these diseases. Remarkably, they also demonstrated that overexpression of S100A8 and S100A9 in mesenchymal cells is sufficient to induce DNA damage and apoptosis in wild-type HSPCs in a paracrine manner via activation of TLR signaling. Furthermore, S100A8/9 expression in mesenchymal cells, associated with activated p53 and TLR signaling, predicted leukemic evolution and decreased progression-free survival in low-risk MDS patients (71). Since MDS is a heterogeneous disease with a variable prognosis (1), this finding could have significant clinical relevance. This study provides strong evidence that TLR signaling may play an important role in the premalignant microenvironment, which promotes HSPC dysfunction, and leads to the generation of pre-LSCs in MDS and other bone marrow failure disorders. However, none of the mice in this study developed AML, possibly because intrinsic HSPC factors likely also play a role in disease progression.

Dimicoli et al. have recently demonstrated that MYD88, a key mediator of TLR innate immune signaling, is potentially involved in the pathogenesis of MDS. While this study did not find any mutations in MYD88 in MDS, they detected higher expression of MYD88 in 40% of MDS patient cells compared to normal CD34+ cells. MYD88 blockade caused increased erythroid colony formation, and suppressed the secretion of IL-8. They concluded that MYD88 mediates innate immune signaling in MDS, and that

inhibition of MYD88 could potentially improve erythropoiesis in this disease (63).

While TLR signaling has been implicated in the emergence of pre-LSCs in MDS, TLR signaling in AML appears to play a different role. Ignatz-Hoover et al. found that resiquimod (R848), a TLR7/8 agonist, promotes the differentiation of AML blasts in a TLR8/MyD88/p38-dependent manner. They also observed antileukemic activity of R848 in a xenograft mouse model of AML (72). Furthermore, Zhong et al. showed that combining R848, LPS, and TNF-α or the combination of TNF-α, and R848, caused significantly higher cytotoxicity to AML cells than TNF-α or R848 alone (73). These data suggest that stimulating TLR8, particularly in combination with other inflammatory signaling pathways, can offer a potential therapeutic strategy for AML (72).

The S100A8 and S100A9 calcium-binding proteins are also highly expressed in AML, and their expression has been linked to poor prognosis in this disease (74). To investigate the roles of S100A8 and S100A9 in AML, Laouedj et al. examined their protein expression in two mouse models of AML and in AML patient samples. They found that S100A8/A9 are secreted by leukemic blasts, and not by the microenvironment. While S100A proteins were not required for AML initiation in the HoxA9-Meis1 mouse model of AML, treatment with an anti-S100A8 antibody induced AML cell differentiation *in vivo* and impaired AML progression. Interestingly, treatment with recombinant S100A9 protein prolonged survival in the same mouse model of AML, suggesting an antagonistic relationship between S100A8 and S100A9. Investigating the pathways involved in S100A9-induced AML cell differentiation revealed that S100A9 induces differentiation *via* TLR4 and several downstream factors, including p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1/2), Jun N-terminal kinase (JNK), and NF-κB. The authors concluded that S100A9 induces differentiation of AML, while S100A8 prevents S100A9-induced differentiation, and that the ratio of S100A9 to S100A8 determines the degree of differentiation in AML (75).

CLINICAL APPLICATIONS OF MODULATING TLR SIGNALING

While a great deal of preclinical evidence supports that activation of TLR signaling promotes the emergence of pre-LSCs in MDS, the clinical utility of inhibiting TLR signaling in MDS is unclear. Several clinical trials have focused on inhibiting TLRs in hematologic malignancies (**Table 2**). The humanized anti-TLR2 antibody is being studied in a phase I/II study as a second-line treatment for lower risk MDS (NCT02363491). In addition, the TLR2/4 antagonist CX-01 is being tested in a phase I trial for relapsed/refractory MDS and AML in combination with azacitidine (NCT02995655).

In contrast, another emerging approach in clinical trials is to use TLR agonists to induce differentiation in MDS and AML (**Table 2**). Weigel et al. performed a phase II study on the TLR7

TABLE 2 | Clinical trials targeting inflammatory signaling pathways in MDS, MPN, and AML.

Target	Drug	Mechanism	Clinical trial	Status
TLR	CX-01	Inhibitor of TLR2 and TLR4	NCT02995655: CX-01 combined with azacitidine in the treatment of relapsed refractory MDS/AML	Phase I
TLR	OPN-305	Humanized anti-TLR2 antibody	NCT02363491: A phase I/II study of OPN-305 as second line in lower risk MDS	Phase I/II
TLR	DUK-CPG-001	TLR9 agonist	NCT02452697: Phase II NK cell-enriched DLIs with or without DUK-CPG-001 from donors following allogeneic SCT (NK-DCI)	Phase II
TLR	GNKG168	Oligonucleotide that acts as TLR9 agonist	NCT01743807: Phase I study of GNKG168 in pediatric acute lymphoblastic leukemia (ALL) and AML	Phase I, terminated
TLR	852A	TLR7 agonist	NCT00276159: Phase II study of 852A administered subcutaneously in patients with hematologic malignancies not responding to standard treatment (76)	Phase II, completed
p38-MAPK	ARRY614	Inhibitor of p38 MAPK and Tie2	NCT0149649: Hematological improvement in lower risk MDS patients who previously failed azanucleoside treatment (77)	Phase I, completed
IL-6	Tocilizumab	Anti-IL-6 antibody	NCT02057770: Allogeneic or haploidentical SCT followed by high-dose cyclophosphamide in treating patients with relapsed or refractory AML	Phase I
IL-6	Siltuximab	Anti-IL-6 antibody	NCT02805868: Siltuximab in treating patients with primary, post-PV, or post-ET MF	Phase I, withdrawn
IL-6	Siltuximab	Anti-IL-6 antibody	Phase II study comparing siltuximab plus best supportive care (BSC) with placebo plus BSC in anemic patients with IPSS low- or int-1-risk MDS (78)	Phase II
TNF-α	Etanercept	IgG inhibitory antibody against TNFR	NCT00118287: Azacitidine and etanercept in treating patients with MDS	Phase I/II, completed
NF-κB	Bortezomib	Proteasome inhibitor inhibits NF-κB	Phase I study of bortezomib in combination with idarubicin and cytarabine in patients with AML (79)	Phase I, completed
NF-κB	Bortezomib		Phase I study using bortezomib with weekly idarubicin for treatment of elderly patients with AML (80)	Phase I, completed
NF-κB	Bortezomib		NCT00262873: Bortezomib in treating patients with MDS	Phase II, completed
NF-κB	Bortezomib		Phase II study of bortezomib combined with chemotherapy in children with AML (81)	Phase II, completed

Representative studies are listed. Source: www.clinicaltrials.gov.

IL, interleukin; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; AML, acute myeloid leukemia; TLR, toll-like receptor; DLI, donor lymphocyte infusion.

agonist imidazoquinoline (852A) on patients with recurrent hematologic malignancies, including six AML patients. They assessed the activity of 852A when administered with prolonged dosing and its safety and ability to activate the immune system. They observed that 852A can be safely administered twice weekly with prolonged tolerability. However, only one partial remission was observed in this small cohort of AML patients (76). Another phase I trial (NCT01743807) tested the oligonucleotide GNKG163, which acts as a TLR9 agonist, in acute lymphoblastic leukemia (ALL) and AML with MRD, but the trial was terminated. Because the roles of TLR signaling in MDS and AML are complicated, and largely dependent on disease stage and clinical context, future design of clinical trials incorporating TLR-modulating agents will need to take into account these context-dependent effects.

IL-1β

Interleukin 1 beta is the first of 11 ILs of the IL-1 family, a pro-inflammatory cytokine produced by myeloid cells in response to TLR stimulation by infection and by non-infectious stressors. IL-1β is activated by caspase-1, which activates the IL-1R1 receptor, causing dimerization with IL-1 accessory protein (IL-1RAP). This leads to the dimerization of intracellular TIR complexes, which then engages the MYD88-IRAK4 complex, thereby activating multiple downstream pathways, including NF-κB and p38 MAPK, in multiple organ systems (see **Figure 1**) (82). IL-1β is an important stromal growth factor in the maintenance of multipotent mesenchymal stromal cells and enhances the ability of stromal cells to maintain HSCs, as shown in both *in vitro* mouse studies and long term culture-initiating cell assays on human cells (83, 84). A study by Pietras et al. elucidated the function of IL-1β in normal HSC *in vivo*. They found that short-term or acute IL-1β administration caused rapid myeloid differentiation of HSCs, and also facilitated recovery of the myeloid lineage after 5-FU myeloablation *via* activation of NF-κB, which leads to activation of the myeloid transcriptional program orchestrated by the transcription factor PU.1(85). However, chronic or long-term administration of IL-1 caused decreased competitive repopulation activity, suggesting a replication challenge and impaired self-renewal of HSCs. This effect was reversible upon withdrawal of IL-1 (85). Furthermore, Hérault et al. also showed that IL-1 signaling in HSPCs, induced by IL-1 secreted by the bone marrow niche after 5-FU treatment, is required for myeloid regeneration (86). However, IL-1β cannot induce differentiation of human HSC *in vitro* (87). Therefore, it appears that IL-1β is required but not sufficient for normal myeloid differentiation in the setting of myeloablation, but that chronic administration of IL-1 impairs normal HSPC function.

Several recent studies have elucidated the functional role of IL-1β in myeloid malignancies. IL-1β levels are elevated in the serum of patients with several preleukemic and leukemic conditions, which makes IL-1β a potential therapeutic target [reviewed in Ref. (82)]. In Ph-MPNs such as PV and PMF, high IL-1 levels are associated with a worse prognosis, and may suggest a higher likelihood of progression to fibrosis (88). In CML, levels of IL-1 and IL-1RAP are elevated, seen more often in blast crisis, and predict a poor prognosis (89). In a murine model of MPN driven by JAK2-V617F, it was observed that MPN stem cells secrete IL-1β, which induces mesenchymal stem cell (MSC) death and resultant disease expansion (90). This effect on the bone marrow microenvironment was partially reversible with IL-1β-inhibitor treatment (90). Hérault et al. showed that the preleukemic niche in two different mouse models of MPN secretes elevated levels of IL-1, which drives the differentiation of HSPCs into proliferative granulocyte/macrophage clusters (86). In CML, Zhang et al. demonstrated that LSCs have increased expression of IL-1 receptors and IL-1RAP and that treatment with an IL-1 receptor antagonist (IL-1RA) in a CML mouse model inhibits IL-1 signaling and growth of CML LSCs *in vivo* (91). Furthermore, they showed that IL-1RA cooperates with TKIs in the elimination of CML LSCs. Therefore, IL-1β is a promising therapeutic target in MPNs.

The role of IL-1β in AML is more complicated. Several previous studies have shown that IL-1β is expressed by AML blasts, associated with poor prognosis, and promotes the proliferation of AML blasts [reviewed in Ref. (82)]. A recent study by Katsumura et al. elucidates how IL-1 might become upregulated in AML. They found that p38 MAPK and MEK1 induce hyperphosphorylation of the master HSC transcription activator GATA-2 in human AML cell lines (92). This leads to increased expression of IL-1β, which is a transcriptional target of GATA-2. Because IL-1β activates p38 MAPK, this produces a p38-GATA-2-IL-1β positive feedback loop (92). They also observed a correlation between GATA-2 and IL-1β expression levels in AML patients. Furthermore, they observed that a higher IL-1 level in the bone marrow of AML patients portends a poor prognosis (92). However, serum data from AML patients in other studies showed a 10-fold lower level of IL-1 expression compared with normal controls (93). The different subtypes of AML examined in these two studies could partially account for these contradictory results regarding IL-1 expression levels. Katsumura et al. found higher IL-1 expression in M4-M5 AML, while Su et al. focused on patients with M0-M2 AML (92, 93).

Interestingly, Yang et al. found that expression of IL-1β was downregulated in the CD34+CD38− LSC-enriched population of AML cells compared with mature blasts and normal CD34+ cells, and that lentiviral expression of IL-1β in these cells inhibited their self-renewal and promoted cell cycle progression (94). However, low dose IL-1β exposure in the same study could stimulate colony formation by AML cells (94). In contrast, Carey et al. recently identified IL-1β in a functional screen as one of the factors that promoted the growth of patient AML cells *ex vivo,* while suppressing the role of normal HSPCs. They found that IL-1β is mostly produced by macrophages, and its levels are elevated in the serum of AML patients, most consistently in patients with the FAB M4 or M5 subtypes of AML. Furthermore, siRNA knockdown of IL-1R1 decreased the proliferation of AML blasts, and genetic deletion of *il1r1* prolonged survival in a murine model of AML driven by AML1-ETO9a and $Nras^{G12D}$. They also found that IL-1β-sensitive AML samples have increased phosphorylation of p38-MAPK, and that the p38-MAPK inhibitors doramapimod (BIRB-796) or ralimetinib could inhibit the IL-1β-dependent growth of AML patient mononuclear cells or AML CD34+ cells. Interestingly, treatment of normal CD34+ cells *ex vivo* with doramapimod also

rescued the inhibitory effects of IL-1β, suggesting that treatment with p38-MAPK inhibitors could improve normal hematopoiesis while inhibiting leukemic growth (95).

Carter et al. demonstrated that coculture of several human AML cell lines with MSCs could lead to an increase in IL-1β expression by AML cells, and that *in vitro* inhibition of IL-1β by IL-1βRA in cultured OCI-AML3 cells suppressed leukemic cell migration and sensitized to cytarabine chemotherapy (96). In MLL-rearranged AML, Liang et al. showed that IL-1 signaling promotes degradation of the wild-type MLL protein *via* phosphorylation of the ubiquitin ligase UBE20 (97). Inhibiting the degradation of wild-type MLL using IRAK1/4 and IRAK4 inhibitors increased the stability of wild-type MLL in MLL-AF9 AML, which displaces the MLL fusion protein from some of its chromatin targets and leads to deregulation of the gene regulatory network in MLL-rearranged AML. As a result, IRAK1/4 inhibitor treatment caused increased survival and impaired LSC function in the MLL-AF9 mouse model of AML (97). This novel mechanism could account for some of the activity of IL-1 pathway inhibitors reported in other studies in secondary AML, and specifically in MLL-AF9 AML (98). Together, these results suggest that there may be differences in how leukemic blasts and LSCs regulate IL-1β expression and respond to IL-1β, and that there may be differential dose-dependent effects of IL-1β on both normal HSPCs and LSCs. In MPN and in at least some subtypes of AML, it is clear that IL-1β promotes the proliferation and maintenance of LSCs. However, the role of IL-1 signaling in the emergence of pre-LSCs and in disease progression to AML is less clear.

CLINICAL APPLICATIONS OF MODULATING IL-1β SIGNALING

Overall, inhibition of the IL-1 signaling pathway appears to be a promising approach to the treatment of myeloid malignancies. Multiple potential therapeutic agents that target IL-1 signaling have been tested preclinically in myeloid malignancies, but only a few are currently under clinical investigation. The IL-1R1 receptor antagonist IL-1RA, a competitive inhibitor of IL-1α and IL-1β, has preclinical activity in JAK2-V617F positive MPNs and CML (82, 91). IRAK1/4 inhibitors also have preclinical activity in MLL-rearranged AML (97). There have also been preclinical studies using antibodies to target IL-1RAP in CML and AML, but in many cases the mechanism of action is to eliminate IL-1RAP-expressing leukemic cells, rather than direct inhibition of IL-1 signaling (99–101). In addition, the IL-1β-specific blocking antibody canakinumab and the IL-1 receptor antagonist anakinra are both approved by the FDA for the treatment of some inflammatory disorders, but have not yet been studied clinically in patients with hematologic malignancies. Finally, the p38 MAPK inhibitor ralimetinib, which had preclinical activity in reducing the proliferative effects of IL-1 in AML, is in clinical trials for ovarian cancer, but has not been tested clinically for hematologic malignancies (95). Given the potential dose-dependent effects of IL-1β on both normal HSPCs and LSCs, and the differences in IL-1 expression among AML subtypes, the design of clinical trials to target IL-1 signaling may be challenging.

INTERLEUKIN 6

Interleukin 6 is a proinflammatory cytokine that is released by monocytes and macrophages as part of the acute phase response to viruses and bacteria, in response to signals such as TLR, IL-1, and TNF, and by T cells during chronic inflammation. This leads to the recruitment of neutrophils to sites of injury (102, 103). The effects of IL-6 can be mediated by binding to the IL-6 receptor on the cell membrane (the classical signaling pathway, as in acute inflammation) or by binding to a soluble IL-6 receptor (causing a trans-signaling pathway that is believed to cause chronic inflammation). In both cases, a hexamer is formed with two IL-6 molecules, two IL-6 receptors (either membrane bound or soluble) and two membrane-bound gp130 molecules, which cause identical downstream effects via the JAK1–STAT3 pathway (see **Figure 1**) (102, 103). In embryonic HSC development, IL-6 mediates the increased production of HSCs, working downstream of the HIF1α/PGDFRβ signaling pathway, but may also respond to hypoxia independently as a proinflammatory cytokine (104). IL-6 is also produced by adult HSCs in response to stressors, including sepsis, chemotherapy, or in the post-transplant setting, and promotes myelopoiesis (105). Chronically increased IL-6, as seen in inflammatory conditions, has been shown to decrease hemoglobin production in late stage erythroid precursors and causes direct mitochondrial impairment (102). This is one of the likely etiologies of anemia of chronic inflammation. In addition, aged MSCs secrete IL-6, which disrupts their crosstalk with HSCs and impairs HSC quiescence in the bone marrow (106).

Reynaud et al. have shown that IL-6 also plays an important role in pre-LSCs and LSCs. In a murine model of CML driven by BCR-ABL expression in HSCs, they demonstrated that CML is induced and sustained by high IL-6 levels produced by BCR-ABL-expressing cells (107). This leads to expansion not only of BCR-ABL mutated cells, but also of wild-type hematopoietic cells, which accelerates disease progression in a paracrine fashion. The dysfunction of normal HSCs in response to high IL-6 levels in this CML model can be rescued with an anti-IL-6 antibody (108). Furthermore, studies with CML patient samples also confirmed the importance of IL-6 in promoting the proliferation and differentiation of CML cells (108). These studies highlight the importance of the proinflammatory changes in the microenvironment induced by leukemic cells, and suggest that IL-6 could be an important component of that pathologic microenvironment. In several different mouse models of Ph-MPNs, including MF driven by MPL-W515L or JAK2-V617F, single cell analysis showed high expression of IL-6, which was most highly expressed from mutated stem cells, but also expressed by wild type stem cells, suggesting an important role for IL-6 in the pathogenesis of Ph-MPNs (109).

Elevated IL-6 levels have been observed in many patients with preleukemic and leukemic conditions, though its predictive value as a biomarker is unclear. In patients with JAK2-V617F PV and PMF, the levels of several inflammatory cytokines were elevated, including IL-6 and IL-8 (110). Furthermore, Reikvam et al. showed that co-culture of patient-derived AML cells with healthy donor-derived MSCs led to increased IL-6 secretion into the media (111). In addition, Lopes et al. found that IL-6

levels in MSCs correlate with disease progression from MDS to AML, with only slight IL-6 elevations in the MSCs of MDS patients, and higher levels in MSCs from AML patients (112). However, another study found that MDS patient samples have increased IL-6 levels compared to healthy controls, but this does not correlate with disease stage (113). Elevated serum IL-6 levels have also been reported in AML patients compared to healthy controls (93, 114). These studies suggest that IL-6 may have a role in promoting the progression of MPN and MDS to AML, but more functional preclinical data are needed to better understand its role in preleukemic stem cells and LSCs.

Interestingly, Zhang et al. found that Tet2 represses the transcription of IL-6 in dendritic cells and macrophages during inflammation. They showed that Tet2 mediates this repression by recruiting histone deacetylase 2 (HDAC2) to prevent constant transcriptional activation of IL-6 in response to inflammation (115). This suggests that targeting Tet2/Hdac2-mediated gene-specific repression could be a novel therapeutic approach to decrease IL-6 signaling in patients with hematologic malignancies.

CLINICAL APPLICATIONS OF MODULATING IL-6 SIGNALING

The anti-IL-6 antibody tocilizumab, which is approved by the FDA for use in several rheumatologic diseases, is currently being tested for its possible anti-inflammatory effects in haploidentical SCT (NCT02206035). However, there are currently few clinical trials testing IL-6 inhibitors in hematologic malignancies (see **Table 2**). A phase II double blind randomized controlled study of siltuximab, an anti-IL-6 inhibitor, studied in transfusion-dependent low risk MDS, was terminated early due to futility (78). IL-6 levels may also be affected by several kinase inhibitors that are currently in clinical use for hematologic malignancies, such as ruxolitinib, a JAK inhibitor in the treatment of MF and PV (109). The clinical significance of elevated IL-6 levels in myeloid malignancies, both as a predictive biomarker and as a therapeutic target, needs to be further elucidated.

IL-8

Interleukin-8 is a proinflammatory chemokine that is released in response to IL-1 or TNF-α as a result of environmental stressors such as infection, hypoxia and chemotherapy, and can act as a neutrophil chemoattractant. IL-8 promotes homing of neutrophils to the site of injury, entrapment and killing of bacteria by promoting neutrophil extracellular traps, phagocytosis, and oxidative burst, and also facilitates healing *via* angiogenesis (116, 117). IL-8 binds to one of its two G protein-coupled receptors, CXCR1 or CXCR2. These receptors are often present on endothelial and myeloid lineage cells, but can also be present on tumor cells (see **Figure 1**) (116). Once coupled with its receptor, the IL-8 program signals *via* activation of several downstream pathways, including PI3K/AKT, PLC/PKC, MAPK, FAK, and JAK/STAT (117). The CXCL8 gene that encodes IL-8 is not expressed in rodents, so its role in hematopoiesis cannot be studied through genetic inactivation in murine models. Therefore, the understanding of the roles of IL-8 in HSC function is limited.

However, emerging data suggest that IL-8 plays an important role in hematologic malignancies. Serum IL-8 levels were found to be increased in MDS and also in PV and ET, independent of JAK2V617F mutation status (118). In CML patients, it was reported that high serum IL-8 levels with low serum TGFβ3 could predict treatment outcome better than the traditional Sokal score (119). In addition, Schinke et al. reported that IL-8 and its receptor CXCR2 are expressed at higher levels in pre-LSCs from patients with MDS than in normal human CD34+ cells (120). Furthermore, knockdown or pharmacologic inhibition of CXCR2 with the inhibitor SB332235 in AML cell lines and in MDS and AML patient samples led to G0/G1 cell cycle arrest, and also inhibited leukemia progression in a xenograft mouse model (120).

Corrado et al. showed that exosomes secreted by CML cells stimulate the bone marrow microenvironment to produce IL-8, which in turn promotes survival of a CML cell line *in vitro* and in a xenograft mouse model *in vivo* (121). In a coculture study with AML patient samples, IL-8 was secreted by the bone marrow microenvironment as a result of hypoxia (O_2 1% for 48 h) by AML cells more than by normal cells (122). Among AML subtypes, acute promyelocytic leukemia had the lowest levels of IL-8 secretion, while FLT3-ITD AML had the highest IL-8 levels, which can predict for poor prognosis in FLT3-ITD AML (122). Abdul-Aziz et al. also demonstrated that AML cells cocultured with bone marrow stromal cells secrete macrophage inhibitory factor, which stimulates IL-8 production by the stroma, which in turn promotes the survival of AML cells (123). Furthermore, shRNA knockdown of IL-8 inhibited the prosurvival effects of the stroma on AML cells (123). Cordycepin, an adenosine analog, blocks mesenchymal stromal/stem cells from expressing VCAM-1 or IL-8 via impaired NF-κB signaling. The inhibitory effects of cordycepin in preclinical AML models support the importance of targeting the crosstalk between AML cells and the bone marrow niche in the treatment of AML. Combined with an adenosine deaminase inhibitor, cordycepin prolonged survival in U937 and K562 xenograft mouse models of AML (124). While IL-8 could be useful as a biomarker in multiple hematologic malignancies and could be a promising therapeutic target for MDS and AML, no clinical trials have been initiated targeting IL-8 or its downstream mediators.

TNF-α/NF-κB PATHWAY

TNF-α is a major proinflammatory cytokine produced by macrophages upon stimulation with endotoxin or bacterial antigens. TNF-α signaling is mediated through the p55 receptor (TNFRSF1A), which is expressed on all nucleated cells and the p75 receptor (TNFRSF1B), which is only present on hematopoietic cells (see **Figure 1**) (32). The roles of TNF signaling in HSCs are controversial [reviewed in Ref. (32)]. While baseline TNF signaling is known to be important for normal HSC maintenance, excessive TNF-α signaling is associated with bone marrow failure and MDS (32). TNF-α stimulates NF-κB, which has a well-described role in malignancy (125–128). The important roles of TNF-α and NF-κB in MDS have been extensively reviewed elsewhere (129), so we will

focus on more recent studies describing their roles and regulation in pre-LSCs and LSCs in MDS and AML.

It has been previously reported that TNF-α is upregulated in the bone marrow plasma and peripheral mononuclear cells of MDS patients, and is positively correlated with apoptosis in early stage/low risk MDS (129). Hence, TNF-α upregulation can play a crucial role in the impairment of hematopoiesis during MDS progression. To uncover the mechanism of TNF-α elevation in hematopoietic malignancies, Shikama et al. studied the expression of c-Fos under the regulation of its targeting miRNAs, miR-34a and miR-155. They demonstrated a significant decrease in stability of c-Fos mRNA as a consequence of miR-34 overexpression in AML cells. Higher levels of miR-34a expression in the blood of MDS patients correlated with increased TNF-α overexpression in granulocytes upon LPS stimulation (130).

Several recent studies have implicated TNF signaling in myeloid LSC function. In a study of the TNF superfamily ligand-receptor pair CD70/CD27 in AML, Riether et al. found that AML blasts and AML stem/progenitor cells express both CD70 and CD27. Moreover, soluble CD27 is expressed at significantly higher levels in the sera of newly diagnosed AML patients than in healthy controls, and can be used as a strong prognostic biomarker for survival. They also demonstrated that blocking the CD70/CD27 interaction with an anti-CD70 monoclonal antibody leads to increased differentiation and survival in a patient-derived AML xenograft model, including in secondary transplantation experiments without additional treatment. This suggests that the CD27/CD70 interaction is also important for LSC function. On the other hand, HSPCs from healthy human bone marrow did not express CD70/CD27, and were not affected by antibody treatment. Therefore, blocking the interaction between CD70/CD27 could be a novel therapeutic strategy to inhibit TNF signaling in AML with potential for a good therapeutic window (131).

Zhou et al. demonstrated that the trans-membrane form of TNF-α (tmTNF-α) is expressed specifically on LSCs in AML and ALL, using a monoclonal antibody termed C1, which specifically recognizes tmTNF-α and not its secretory form (132). They also found that leukemia cells are more sensitive to chemotherapy *in vitro* after tm-TNF-α knockdown, and that tm-TNF-α inhibition with the C1 antibody delays the onset of leukemia in patient-derived AML xenografts. Importantly, they demonstrated that treatment with the C1 antibody in primary transplant mice led to reduced engraftment of leukemic cells and a decreased disease burden, supporting the important role of tmTNF-α in LSCs *in vivo*. Additionally, they showed that *in vivo* targeting tmTNF-α with C1 antibody does not affect normal hematopoietic cells, suggesting a favorable therapeutic window for the use of this antibody in patients (132).

NF-κB is a transcription factor that is well known as an important regulator of cell survival, proliferation, and differentiation. It is both activated by TNF-α signaling, and can also regulate the expression of TNF-α (133–135). Although the activity of NF-κB is not detectable in normal unstimulated CD34+ HSCs, and NF-κB levels were reported to be low in low-risk MDS, they are increased in high-risk MDS patients, and correlate with increased blast counts, suggesting a role for NF-κB in the transition from pre-LSCs to LSCs (136). The activity of NF-κB has been observed in several molecular subtypes of AML, and it is likely that this pathway is involved in the progression to AML (137). Furthermore, it has been shown that NF-κB is highly expressed in primitive CD34+CD38− cells in AML, the population that is enriched in LSCs (138–140).

It has been demonstrated that inhibition of NF-κB can effectively eradicate LSCs while sparing normal HSPCs (138, 141). However, *in vivo* inhibition of NF-κB cannot completely eliminate AML cells, indicating that there are parallel survival signals in leukemic cells. To identify compensatory survival signals for NF-κB inhibition, Volk et al. demonstrated that AML stem and progenitor cells can be sensitized to NF-κB inhibition by inhibiting TNF-JNK signaling (142). They also reported that in some subtypes of AML, including M3, M4, and M5, leukemic cells produce endogenous TNF-α, leading to an increase in proliferation and survival of AML blasts through an autocrine mechanism *via* downstream signaling through both NF-κB and JNK-AP1 (142).

Recently, Li et al. reported that CD34− leukemic blasts in M4 and M5 AML also secrete IL-1β, while more immature CD34+ cells primarily secrete TNF-α, and that IL-1β can induce JNK signaling independently of NF-κB signaling. Inhibition of both IL-1 and TNF sensitizes the LSCs and leukemic progenitors to NF-κB inhibition. Furthermore, they showed that combined inhibition of TNF-α, IL-1, and NF-κB *in vivo* significantly impaired LSC function in the MLL-AF9 mouse model of AML, and prolonged survival in the secondary transplantation setting. Therefore, they suggested that inhibiting both TNF and IL-1β signaling could be a promising treatment for the M4/M5 subtypes of AML and for therapy-related AML (98).

However, the mechanism of TNF-α secretion and NF-κB activation in pre-LSCs and LSCs in MDS and progression to AML has been unclear. Gañán-Gómez et al. identified a cluster of microRNAs that regulate the expression of NF-κB in MDS (143). They detected significantly higher expression of miR-125a in MDS patients, and described a correlation between the expression of miR-125a and miR-99b, which is in the same cluster, with prognosis in MDS. They described the activation of NF-κB by miR-125a and miR-99b *in vitro*. However, the expression level of miR-99b and miR-125a showed a negative correlation with TLR2 and TLR7 RNA expression levels, which suggests that the activation of NF-κB by the miRNA clusters is independent of TLR signaling. In addition, they suggested that mir-125a inhibits NF-κB upon TLR stimulation, which could act as a fine-tuning mechanism for regulating NF-κB expression in MDS. Moreover, they demonstrated the inhibition of erythroid differentiation by miR-125a in MDS and in leukemia cell lines, which might make it a potential therapeutic target and prognostic marker for MDS (143).

CLINICAL APPLICATIONS OF MODULATING TNF-α/NF-κB SIGNALING

The potential of therapeutic targeting of NF-κB in AML has been explored in several early phase clinical trials (**Table 2**). One strategy used to inhibit NF-κB signaling is the proteasome inhibitor bortezomib, which is approved by the FDA for the

treatment of multiple myeloma. Bortezomib inhibits NF-κB signaling by blocking the ubiquitin-proteasome pathway, which prevents degradation of phosphorylated IKB, the inhibitory protein of NF-κB. Stabilization of IKB prevents the nuclear translocation of NF-κB, which is necessary for its function as a transcription factor (144). Howard et al. reported the results of a phase I study with weekly bortezomib in combination with idarubicin in elderly patients with AML, most with preceding MDS. They demonstrated the feasibility of the combination of bortezomib and idarubicin and showed that this combination was well tolerated (80). Some clinical activity was observed, with a 20% complete remission rate, and with a decrease in circulating blasts in most patients. Their data suggest that inhibition of NF-κB and activation of p53 were associated with the activity of bortezomib and idarubicin in AML blasts. In another phase I study of bortezomib used in combination with induction chemotherapy in an older AML patient population, Attar et al. reported a CR rate of 61%, and a tolerable toxicity profile (79). However, in a phase II trial for pediatric relapsed refractory AML testing bortezomib in combination with chemotherapy, minimal clinical responses were observed (81). Additional clinical trials are underway testing bortezomib in combination with chemotherapy in pediatric AML. Therefore, the use of bortezomib needs to be further investigated, and may be effective in the correct clinical context. However, bortezomib is likely to have additional biological and clinical effects besides the inhibition of NF-κB signaling (144).

More specific inhibitors targeting NF-κB are currently in preclinical development. For example, the natural compound parthenolide has also been shown to inhibit NF-κB signaling, and was found to have preclinical activity in AML LSCs (139). Furthermore, Dai et al. showed that parthenolide enhances the lethality of pan-histone deacetylase inhibitors in AML, and that this strategy can target leukemic progenitor cells (145). In a recent study, Gao et al. determined that the small molecule CPPTL, a novel analog of parthenolide, causes cytotoxicity and apoptosis of AML cells *in vitro* (146). Furthermore, the CPPTL prodrug DMA-CPPTL prolonged survival in a patient-derived AML xenograft model. Their findings demonstrated that this drug induces the generation of ROS, followed by JNK pathway activation, which then promotes mitochondrial damage. These results suggest that CPPTL may be a promising drug candidate for the treatment of AML. The clinical testing of more specific inhibitors targeting TNF and NF-κB will shed more light on the clinical significance of this pathway in disease progression in myeloid malignancies.

CONCLUDING REMARKS

There is now substantial evidence that a proinflammatory microenvironment, which can be initiated through cytokine and chemokine secretion both by hematopoietic cells and by stromal cells, can promote the emergence of pre-LSCs and progression to AML. While intriguing, further studies are needed to make a clear connection between inflammation and the pathogenesis or minimally known clinical manifestations of clonal hematopoiesis. It is unclear why the majority of patients with clonal hematopoiesis do not progress despite having mutations that are common in MDS and AML. It is possible a "second hit" is needed to generate pre-LSCs or LSCs, and that cells with a single mutation have increased self-renewal properties but limited clonal expansion capacity (147). Furthermore, studying clonal progression and pre-LSCs in aseptic mouse models can be difficult, as these models do not accurately represent the more infection-prone environment in which most humans normally reside. The low-grade chronic inflammatory state of aging may contribute both to the initiation of preleukemic mutations, as well as to the clonal outgrowth of mutated cells, which in some cases can progress to the preleukemic or leukemic state. Current efforts are underway to generate better murine models of clonal and preleukemic hematopoiesis to improve our understanding of the roles of inflammatory signaling pathways in leukemic progression.

Preleukemic or leukemic cells can then propagate the disease by interacting with the microenvironment, leading to the increased secretion of proinflammatory factors, such as S100A8/S100A9, IL-1β, IL-6, and IL-8. Furthermore, while inflammatory signaling is a critical component of the crosstalk between HSPCs and their microenvironment during infection and hematopoietic stress, chronic activation of inflammatory signaling pathways such as IFN-α, IFN-γ, TLR, and TNF-α can also suppress normal HSC function and lead to bone marrow failure. In the case of IFN-α/β, the ability to drive HSPCs into the cell cycle similarly affects LSCs, and can be exploited to impair their self-renewal to aid in their elimination, leading to long-term remissions in MPNs and other myeloid malignancies. Many clinical trials are now testing this strategy for relapse prevention, in some cases in combination with other therapeutic agents, with some early promising results. Currently, combinations strategies that include IFN-α/β appear to be the most promising immunomodulating approaches for the treatment of hematologic malignancies, such as those combining JAK2 inhibitors with IFN-α/β for MPN, with a real possibility of inducing long-term remissions. The experience with the JAK2 inhibitor ruxolitinib as a single agent in MPN suggests that inhibiting a single inflammatory signaling pathway may not be sufficient to achieve long-term results (9). Similar combination strategies including antiproliferative therapies together with immunomodulating agents need to be more widely explored both preclinically and in the clinic.

There is convincing preclinical data that dampening of some inflammatory signaling pathways, such as IL-1β, IL-6, and IL-8, can eliminate LSCs and in some cases induce differentiation of AML blasts. While inhibitory antibodies or pharmacological inhibitors are available to block these pathways (see **Figure 1** and **Table 2**), their clinical translation has been challenging, due to the context-dependent effects of some of these inflammatory signals. For example, ILβ is likely to have dose-dependent effects on the proliferation and differentiation of hematopoietic cells, and appears to have differential effects on normal HSPCs, pre-LSCs in MPN, and LSCs and leukemic blasts in AML. On the other hand, other inflammatory signals, particularly TLRs, can also become downregulated with progression to AML, and re-expression of these factors in leukemic cells can lead to differentiation. In this

case, TLR antagonists may be effective in preventing the progression of early MDS, while TLR agonists may be more useful for promoting the differentiation of AML blasts. Therefore, each potential therapeutic agent modulating one of these inflammatory signaling pathways needs to be tested in the correct disease stage and context, and careful attention must be paid to dosing and timing of treatment, since levels of pathway activation may have distinct effects on the proliferation and differentiation of pre-LSC and LSCs.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception, writing, and editing of the manuscript, and have approved it for publication.

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Transcriptional and Microenvironmental Regulation of Lineage Ambiguity in Leukemia

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Leukemia is characterized by the uncontrolled production of leukemic cells and impaired normal hematopoiesis. Although the combination of chemotherapies and hematopoietic stem cell transplantation has significantly improved the outcome of leukemia patients, a proportion of patients still suffer from relapse after treatment. Upon relapse, a phenomenon termed "lineage switch" is observed in a subset of leukemia patients, in which conversion of lymphoblastic leukemia to myeloid leukemia or *vice versa* is observed. A rare entity of leukemia called mixed-phenotype acute leukemia exhibits co-expression of markers representing two or three lineages. These two phenotypes regarding the lineage ambiguity suggest that the fate of some leukemia retain or acquire a certain degree of plasticity. Studies using animal models provide insight into how lineage specifying transcription factors can enforce or convert a fate in hematopoietic cells. Modeling lineage conversion in normal hematopoietic progenitor cells may improve our current understanding of how lineage switch occurs in leukemia. In this review, we will summarize the role of transcription factors and microenvironmental signals that confer fate plasticity to normal hematopoietic progenitor cells, and their potential to regulate lineage switching in leukemias. Future efforts to uncover the mechanisms contributing to lineage conversion in both normal hematopoiesis and leukemia may pave the way to improve current therapeutic strategies.

Keywords: lineage switch leukemia, mixed-phenotype acute leukemia, hematopoietic stem cells, acute myeloid leukemia, acute lymphoid leukemia, CAR-T cells

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INTRODUCTION

Hematopoietic stem cells (HSCs) establish and maintain the hematopoietic system through differentiation into the multi-lineage progenitors and committed progenitors from which all the mature lineage cell types arise. In the classical model of hematopoiesis, long-term HSCs, short-term HSCs, and multipotent progenitors (MPPs) reside at the apex of the hierarchy (1–5). MPPs are able to differentiate into lineage-committed progenitors, including common lymphoid progenitors (CLPs) (6) and common myeloid progenitors, which further differentiate into granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) (7). A characteristic feature of this model is that, as progenitors differentiate through this pathway, their developmental potential narrows. For example, MEPs lack the granulocyte-monocyte (GM) potential of GMPs and instead have differentiation potential that is restricted to the megakaryocyte and erythroid (Meg/E) lineages.

Importantly, although many studies have provided evidence supporting this classical hierarchy, studies have shown that the committed state can be canceled or reprogramed by the action of lineage specifying cytokines and transcription factors. This raises the question to what extent the committed states are fixed, and whether or not oncogenic mutations exploit the lineage promiscuous state of normal progenitor cells to change their phenotypes upon therapies.

LINEAGE COMMITMENT AND SWITCH IN NORMAL HEMATOPOIESIS

The increasingly narrowed lineage potential results from a precise combination of gene expression signatures and epigenetic modification. Several transcription factors have been found to be involved in the fate decision of hematopoietic progenitors (8, 9). Among these are the lineage-specific master regulator transcription factors, such as Pu.1 (also known as Spi-1; spleen focus forming virus proviral integration oncogene 1), C/ebp-α, Gata1, Pax5, and Ikaros (**Figure 1**). Pu.1 and C/ebp-α are master regulators of the myeloid cell fate, and not only do these transcription factors promote myeloid differentiation of progenitor cells (10) but also ectopic expression of these transcription factors confer a myeloid cell fate to cells of other lineages, such as T-cells, B-cells, or fibroblasts (11–14). Gata1 is a master regulator of erythroid cell fate that is required and sufficient to confer the erythroid fate. Deletion of Gata1 in mice causes defective erythropoiesis (15–21), whereas ectopic expression of Gata1 confers Meg/E fate to cells of other lineages, such as monocytic cells (22, 23). Loss of B-cell master regulators Pax5 and Ikaros disrupts the B-cell transcriptome and reprograms B-cells into myeloid (24, 25) or epithelial-like cells (26), respectively. Lineage conversion by ectopic expression or loss of these master regulator transcription factors is often associated with a change in a network of transcription factors that governs cell fate. Pu.1 promotes multipotent hematopoietic progenitors to differentiate into myeloid cells by activating multiple myeloid-lineage-related genes, including C/ebp-β and suppressing erythroid factors such as Gata1 (10),

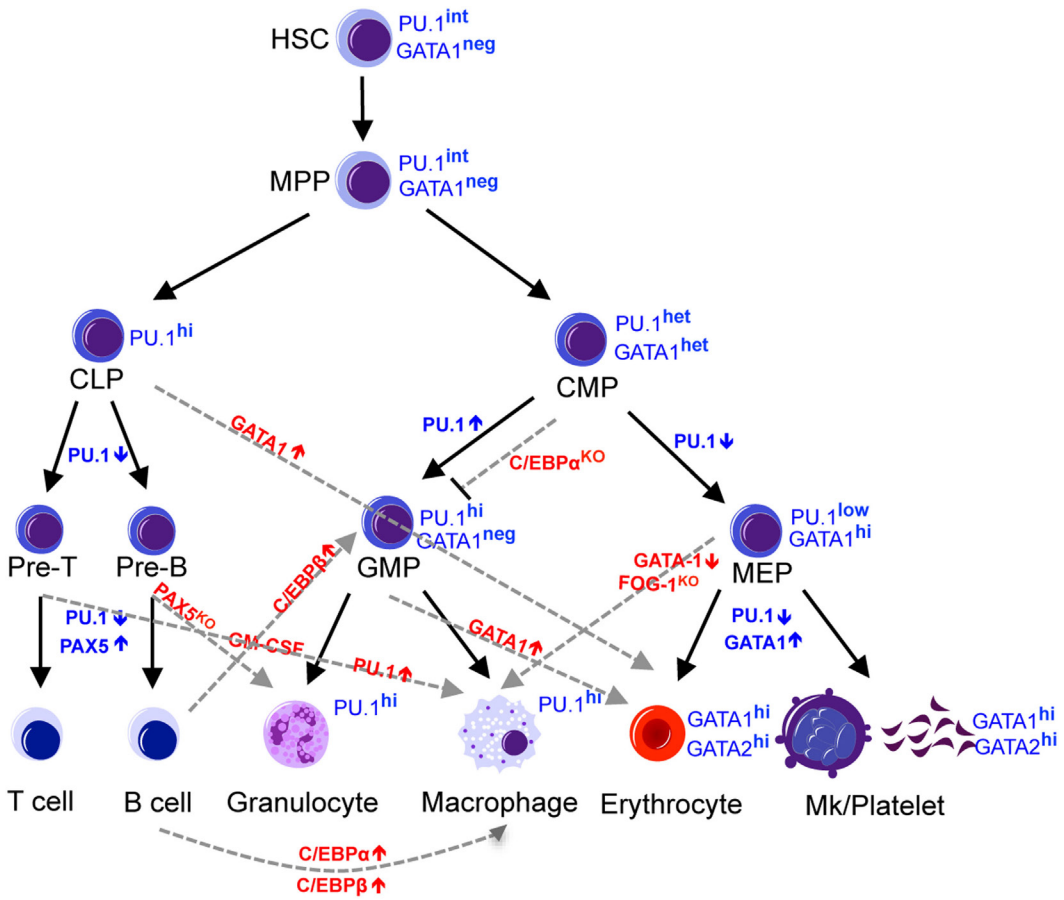

FIGURE 1 | Regulators of lineage commitment in normal hematopoiesis. The hematopoietic system is maintained by HSCs, which gradually lose developmental potential through differentiation into downstream progenitors and mature cells. This narrowed lineage potential is controlled by a precise combination of transcription factors and can be reprogrammed by manipulating the expression level of certain transcription factors. Black solid lines indicate the normal commitment steps, while the gray dashed lines indicate altered lineage potential by manipulating the levels of transcription factors. HSC, hematopoietic stem cells; MPP, multipotent progenitors; CLP, common lymphoid progenitors; CMP, common myeloid progenitors, MEP, megakaryocyte-erythroid progenitors; GMP, granulocyte-monocyte progenitors.

while C/ebp-α and C/ebp-β converts differentiated B-cells into macrophages by inhibiting Pax5 (12). The extent to which these transcription factors over-ride the lineage-committed state of differentiated cells illustrates the extensive potential these master regulators possess.

LINEAGE AMBIGUITY IN MALIGNANT HEMATOPOIESIS

Leukemias develop as a consequence of mutations that cooperatively confer aberrant self-renewal capacity to leukemic cells and allow them to proliferate indefinitely without differentiation. Recent advances in high-throughput sequencing of leukemia genomes have revealed numerous mutations in cytokine signaling, epigenetic regulators, and transcription factors (27, 28). Genetic studies using murine models have established that mutations in epigenetic and transcriptional regulators upregulate self-renewal and block differentiation of hematopoietic stem/progenitor cells (HSPCs) (29). The ability of transcription factor levels and external cytokine milieu to influence hematopoietic progenitor plasticity raises the question of whether lineage conversion plays any role in malignant hematopoiesis that often carry mutations in these regulators. In fact, a phenomenon called lineage switch has been reported, in which patients with acute leukemia that meet the French–American–British classification for being lymphoid or myeloid leukemias relapse with acute leukemia of the other lineage. Most cases of lineage switches are from ALL to AML (30–34), but AML to ALL switches have also been reported (33, 35–37). Additionally, some leukemias show no clear evidence of differentiation into a single lineage. These leukemias, termed mixed-phenotype acute leukemia (MPAL) (38) exhibit cells of at least two lineages; MPALs involving B-cell and myeloid lineages are the most frequent but some rare cases involve B- and T-cells, or B/T/myeloid cells. Patients with lineage switch leukemia or MPAL have poor prognosis, due to the difficulty in diagnosis and the lack of set protocols to guide treatments (38–41). Understanding the molecular mechanism behind lineage switch and ambiguity should pave a way for better treatment. We will discuss several hypotheses that have been proposed to explain the lineage switch and ambiguity in leukemias. These mechanisms are not mutually exclusive and likely occur in parallel. For example, dysregulation of lineage-specific transcription factors may generate an aberrant bi-potential leukemic clone, and therapies may facilitate the selection of bi-potential clones that are better equipped to survive the therapy by changing their phenotype.

Multipotency of Leukemic Clones

One potential mechanism to explain how some leukemias switch their lineages is that these leukemias were derived from bi-potential clones. When leukemia cells from a patient who exhibited T-cell acute lymphoblastic leukemia (T-ALL) to AML switch upon chemotherapy were transplanted into SCID mice, engrafted AML cells exhibited myeloid cell markers (such as CD33) as expected, but the cells exhibited T-cell markers (such as CD2, CD4, and CD7) similar to the T-ALL at diagnosis if the recipient mice were treated with cytokines GM-CSF or interleukin 3 (IL-3) (42). Interestingly, although a common NRAS mutation was identified at every time point during the study (T-ALL at diagnosis, AML upon lineage switch, and T-ALL or AML in SCID mice) suggesting that both T-ALL and AML were derived from a common founding clone, the TCR rearrangement observed in T-ALL at diagnosis was not observed in patient's AML cells upon switch nor the cells in SCID mice. This results indicate that the AML emerged in the patient upon lineage switch were not derived from T-ALL cells with TCR rearrangement, and suggests that a common *NRAS* mutated bi-potent leukemia clone with T-ALL and AML potential existed (42).

On the other hand, other studies have shown the presence of TCR rearrangements in myeloid leukemia cells upon lineage switch from lymphoid leukemias (43, 44). In these cases, it remains unclear whether the lymphoid leukemia clones with TCR rearrangements had bi-potential at diagnosis, or whether the lymphoid clones gained myeloid potential through potential mechanisms discussed below. Similarly, reports on B-cell precursor acute lymphoblastic leukemia (BCP-ALL) to AML switch have suggested that a bi-potential B-myeloid progenitor, which has been detected in fetal and adult mice (45, 46), may have become transformed, but evidence that such bi-potential progenitor cells are the origin of the disease and existed at diagnosis is lacking. It is equally possible that a B-ALL clone changed the phenotype upon treatment due to selective pressure.

Clonal Selection and Therapies

Lineage switch is often associated with therapy relapse, suggesting that clones with altered phenotypes emerge as a consequence of the selective pressure imposed by the therapy. Therapies can eradicate the dominant clone(s) but select for a latent clone that survived the therapy, or the dominant clone may acquire additional mutations to evolve. While this has been elegantly demonstrated in relapsed AML (47), therapy-related selection has also been reported in lineage switched leukemia. For example, Podgornik et al. reported a B-ALL patient who relapsed with AML from a separate clone that survived the B-ALL therapy (48), while Mantadakis et al. reported on a pediatric patient with T-ALL who relapsed with AML after chemotherapy (49). Recent findings with B-ALL immunotherapy further lend insight into this mechanism (**Figure 2**). Chromosomal rearrangements at 11q23 are found in both AML and ALL and result in the fusion of the MLL1 gene with approximately 80 partner genes, among which *AF4* is the most common partner (50). MLL–AF4 fusion is mostly associated with pro-B-ALL expressing some myeloid cell markers such as CD15 (51). Recent studies reported cases of MLL–AF4 rearranged pro-B-ALL patients treated with blinatumomab, a bispecific antibody that targets CD19 on B-cells, who relapsed with AML (52–54). Transplantation of the relapsed AML into NSG mice caused $CD19^+$ B-ALL that was genetically related to the relapsed AML, suggesting that the anti-CD19 treatment selected for clones that downregulated CD19 and acquired phenotypic changes toward the myeloid lineage (52). Moreover, CD19 CAR-T therapy caused relapse accompanied by a phenotypic change from pre-B-ALL to a myeloid phenotype (55, 56). The authors also used a mouse E2a–PBX1 transgenic B-ALL model and demonstrated that mouse CD19 CAR-T treatment relapses by either causing an

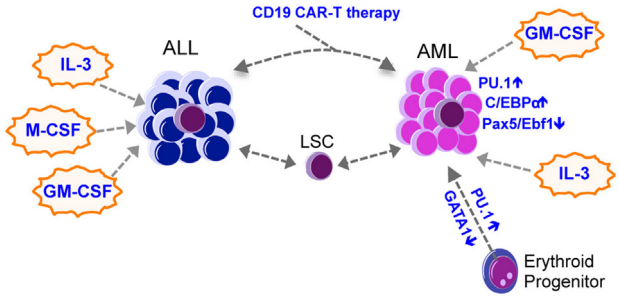

FIGURE 2 | Lineage switch in leukemia. Most lineage switch in leukemia is from ALL to AML, with occasional AML to ALL switches being observed. Lineage switch could be mediated by therapy-mediated (such as CAR-T immunotherapies) selection of heterogeneous leukemic clones that survive the therapy and expand to produce an altered lineage output. Transcription factors such as Pu.1, C/ebp-α, Pax5, and Ebf1, together with cytokines such as IL-3, M-CSF, and GM-CSF, may mediate the lineage switch. AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; LSC, leukemia stem cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IL-3, interleukin 3.

alternative splicing of CD19 in B-ALL cells to escape CAR-T cells, or by causing a switch to myeloid leukemias with low Pax5 and Ebf1 expression and increased expression of CD11b and Gr-1 (56). Relapsed myeloid leukemia cells were not detected in E2a–Pax5 pre-B-ALL cells using single cell approaches, suggesting that CAR-T unlikely selected for rare myeloid leukemia cells but instead reprogrammed the B-ALL cells into a myeloid fate. These findings reinforce the idea that some leukemias retain the plasticity to drastically change their phenotype in the face of a strong selection imposed by therapies, and that lineage switch might represent a novel mechanism of resistance against immune therapies.

Cell Reprogramming by Transcription Factors

Similar to how lineage-specific transcription factors reprogram the fate of normal hematopoietic progenitors, these transcription factors have a large impact upon the fate of leukemia cells. Leukemogenic mutations appear to tip the balance created by the network of transcription factors to block and/or bias the differentiation program toward a particular lineage. Dysregulated expression of Pu.1, Gata1, and C/ebp-α all contribute to leukemogenesis (57), and some of these factors are used by leukemic fusion genes to promote aberrant self-renewal of leukemia. For example, AML caused by MLL-fusion genes or MOZ-TIF2 depends upon Pu.1 for their maintenance (58, 59). Recent studies also indicate that these lineage-specific transcription factors regulate the fate choice of leukemia, and may contribute to the lineage switch of leukemias observed upon therapies.

In zebrafish models, AML1–ETO upregulates Pu.1 and downregulates Gata1 to convert the fate of erythroid cells into granulocytic cells, causing a phenotypic change similar to human AML (60). Overexpression of Pu.1 was reported in a rare case of adult Philadelphia chromosome-positive bilineage leukemia (myeloid and T-cell), in which TCR rearrangement was detected in both the myeloid and T-cell compartments of the disease, suggesting that the AML population emerged from T-ALL cells, potentially due to Pu.1 expression (61). Similar to Pu.1, C/ebp-α is also found to be involved in leukemia lineage switch. Slamova et al. reported cases of BCP-ALL that underwent lineage switch upon therapy to monocytic leukemias, which carried the same Ig/TCR rearrangements as the original BCP-ALL (43). The monocytic leukemias with Ig/TCR rearrangements had C/ebp-α promoter hypomethylation accompanied by increased C/ebp-α expression compared to the original BCP-ALL (43). These results suggest that myeloid transcription factors, such as Pu.1 and C/ebp-α, are involved in promoting lymphoid leukemias to switch their fate to a myeloid fate. Additional evidence suggests that the lineage switch can be promoted by the loss of lymphoid transcription factor expression. In the reported case of CD19 CAR-T-induced lineage switch of B-ALL to AML (56), expression levels of lymphoid transcription factors Pax5 and Ebf1 were reduced. Mouse model of CAR-T-induced lineage switch also revealed epigenetic changes leading to loss of Pax5/Ebf1 and increase of C/ebp-α expression and demonstrated that deletion of Pax5 or Ebf1 promoted the lineage switch from B to myeloid fate without CAR-T therapy (56). However, it is still unclear to what extent myeloid transcription factors contribute to the switch toward the myeloid fate, and whether the lineage-specific transcription factors can be exploited to block switching or target the switched leukemias.

Microenvironment

The local microenvironment of HSPCs regulates the maintenance of HSPCs and is often altered in hematological malignancies (62–64). The microenvironment influences disease initiation (65), progression (63), and the efficacy of the therapies (66) by modulating the cytokine milieu and the metabolic parameters. Moreover, similar to how lineage-instructing cytokines can affect the fate of normal HSPCs, leukemia cells with certain plasticity exhibit different lineage output depending on the cytokine milieu.

MLL-translocated leukemias appear to retain lineage plasticity that can be tapped to direct the differentiation toward either B-cell or myeloid lineages using different cytokines. Expression of MLL-fusion oncogenes in cord blood HSPCs induces B-ALL upon xenotransplantation (67). The types of leukemias these oncogenes caused was affected by the culture conditions, as MLL–ENL expressing cells that are prone to cause B-ALL initiated AML with rearranged IgH when cultured in myeloid-promoting conditions. The ability of MLL–AF9 oncogene to produce B-ALL or AML is also affected by the culture condition, as well as the humanized cytokines expressed in the recipient immunocompromised mice (68). The fusion product of human MLL and murine Af4 (MLL–Af4) initiates pro-B-ALL that recapitulates the human pathology but causes AML when the cells were culture in myeloid-promoting conditions (52). Interestingly, MLL–Af4 transformed myeloid cells cultured in a myelopoietic condition had increased expression of lymphoid regulators, such as Ebf1, compared to AML cells transformed by MLL–AF9, suggesting that the myelopoietic condition cannot fully rewire the lymphoid program imposed by MLL–Af4.

A recent study demonstrated that BCR–ABL1 rearranged B-ALL can be reprogrammed to a myeloid fate by myeloid-instructing and proinflammatory cytokines (69–73). Purified B-ALL blasts exhibited myeloid cell marker expression and phagocytotic phenotypes upon stimulation by IL-3, M-CSF, and GM-CSF. The reprogrammed macrophage-like cells (termed MLCs) had increased expression of myeloid master regulators C/ebp-α and Pu.1, and corresponding overexpression of C/ebp-α and Pu.1 significantly induced myeloid reprogramming, suggesting that the myeloid cytokines and myeloid transcription factors cooperate to confer a myeloid cell fate to B-ALL, consistent with the ability of these two transcription factors to confer a myeloid fate to lymphocytes (11, 12, 14). Interestingly, although the original B-ALL cells that failed to reprogram into MLCs had the ability to cause B-ALL in recipient mice upon xenotransplantation, the reprogrammed MLCs had negligible ability to engraft. Since lineage switch is often associated with relapse and worse clinical outcome, it is unclear whether promoting myeloid reprogramming can be used as a therapeutic strategy. Nonetheless, depending on how deeply leukemias can be directed to differentiate, instructing cells to differentiate into other lineages may provide a novel therapeutic option.

CONCLUSION

Similar to normal hematopoietic cells, leukemia cells also exhibit lineage plasticity and reversibility aided by master transcriptional regulators that control lineage determination of normal hematopoietic progenitor cells, by the instructive cytokine milieu and also by the strong selective pressure imposed by therapies. Although some improvements in treatment outcome have been reported by the use of intensified ALL therapy followed by AML therapy upon lineage switch, treatment of these leukemia remains challenging (74, 75). The ability of B-ALL cells to change their lineage-specific cell surface marker expression in response to immunotherapies underscores the clinical challenges posed by the plasticity of leukemia. Molecular characterization of the fundamental requirements of leukemias may reveal new strategies to target the disease regardless of their lineages.

AUTHOR CONTRIBUTIONS

TH, RM, and DN reviewed the literature and wrote the manuscript. TH designed the figures.

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Preleukemic Hematopoietic Stem Cells in Human Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is an aggressive malignancy of the bone marrow characterized by an uncontrolled proliferation of undifferentiated myeloid lineage cells. Decades of research have demonstrated that AML evolves from the sequential acquisition of genetic alterations within a single lineage of hematopoietic cells. More recently, the advent of high-throughput sequencing has enabled the identification of a premalignant phase of AML termed preleukemia. Multiple studies have demonstrated that AML can arise from the accumulation of mutations within hematopoietic stem cells (HSCs). These HSCs have been termed "preleukemic HSCs" as they represent the evolutionary ancestors of the leukemia. Through examination of the biological and clinical characteristics of these preleukemic HSCs, this review aims to shed light on some of the unexplored questions in the field. We note that some of the material discussed is speculative in nature and is presented in order to motivate future work.

Keywords: leukemia, myeloid, acute, preleukemic hematopoietic stem cell, clonal hematopoiesis, clonal evolution, premalignant lesions

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IDENTIFICATION OF PRELEUKEMIC HEMATOPOIETIC STEM CELL (HSC)

The earliest evidence for a preleukemic phase of acute myeloid leukemia (AML) came from clonality studies in adult and pediatric patients (1–15). Collectively, these experiments demonstrated that leukemogenic mutations arise in multipotent hematopoietic cells and have been thoroughly reviewed previously (16, 17). The current model for preleukemic clonal evolution has resulted from multiple lines of scientific evidence ranging from mouse models to high-throughput sequencing of primary human specimens. This model (18) posits that the first leukemogenic mutation must either occur in a cell that is capable of self-renewal or confer self-renewal upon the cell. If the first mutation fails to meet one of these two criteria, it will be lost over time due to terminal differentiation.

This model has been investigated over the past 5 years, beginning with the first prospective identification of preleukemic HSCs (19). These initial observations were enabled by the identification of cell surface markers, TIM3 and CD99, which allow for prospective separation of normal HSCs from leukemic cells (20, 21). Utilizing these markers, immunophenotypic HSCs isolated from leukemia patients are capable of generating bi-lineage engraftment in immunodeficient mice, demonstrating that they represent bona fide HSCs (19). From targeted deep sequencing, these HSCs were identified to harbor some, but not all, of the leukemia-specific mutations. Moreover, single-cell-derived colonies

generated from patient HSCs allowed for the determination of the order of mutation acquisition (19). Collectively, this work provided the first modern proof of the existence of preleukemic HSCs in AML.

GENETIC, MOLECULAR, AND CELLULAR CHARACTERISTICS OF PRELEUKEMIC HSCs

Follow-up studies provided additional support for these conclusions through investigation of expanded patient cohorts and targeted sequencing experiments (22, 23). In particular, these studies identified patterns of mutation acquisition whereby the earliest mutations in leukemia evolution occur predominantly in genes that regulate the epigenome, while the latest mutations occur predominantly in genes that lead to activated signal transduction and proliferation pathways (22–28). The most common preleukemic mutations occur in the DNA methyltransferase 3A (*DNMT3A*) and ten-eleven translocated 2 (*TET2*) genes (22–24). Additional genes mutated during the preleukemic phase include isocitrate dehydrogenase 1 and 2 (*IDH1/2*) (22, 29) and the members of the cohesin complex (30). The most common late (non-preleukemic) mutations occur in Fms-like tyrosine kinase 3 (*FLT3*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*). Mutations in other common leukemia-related genes such as nucleophosmin 1 (*NPM1*), CCAAT/enhancer-binding protein alpha (*CEBPA*), and Wilms tumor 1 (*WT1*) have been found to occur as both preleukemic and late events (22, 23).

In addition, recent work has demonstrated that the penetrance of preleukemic mutations varies greatly across patients (31). We have previously introduced the concept of "preleukemic burden," which we define as the percent of HSCs in a leukemia patient that harbor at least the first preleukemic mutation. In this way, patients whose preleukemic HSCs have expanded greatly will have a high preleukemic burden (**Figure 1A**). It is now clear that the preleukemic burden across AML patients can vary from 100% to below the limit of detection of standard high-throughput sequencing methodologies (~1%) (**Figure 1B**) (31, 32). To illustrate this point clearly, a preleukemic burden of 100% indicates that a single HSC expanded to outcompete all other HSCs after acquisition of the first preleukemic mutation. This highlights some of the key characteristics of preleukemic HSCs—the ability to survive, outcompete normal HSCs, and undergo clonal evolution through the acquisition of multiple additional mutations, eventually leading to frank leukemia. Mutations in both *TET2* and *DNMT3A* have been shown to be significantly associated with high preleukemic burden in AML (31). Nevertheless, it remains unclear how some patients develop AML with undetectable preleukemic burden while others exhibit full reconstitution of their HSC pool with mutated HSCs. Some of this difference may be mediated by the preleukemic mutations acquired and the time since mutation acquisition, but this is only one piece of a very complicated puzzle (33). Moreover, the same mutations can sometimes lead to highly divergent preleukemic burdens. For example, mutations in DNMT3A have been shown to lead to preleukemic burden ranging from undetectable to 100% (31). One intriguing hypothesis is that this difference is mediated by epigenetic differences in the cell of origin, with certain epigenetic profiles being more primed for clonal competition than others. Future work investigating how and why preleukemic burden is so variable will be crucial to our understanding of this phase of the disease.

The precise mechanisms that mediate this clonal outcompetition remain incompletely understood. From an evolutionary standpoint, an increase in the "fitness" of a stem cell would likely come from an increase in self-renewal. More specifically, a stem cell that produces more daughter cells whose self-renewal potential is at least as great as the parental cell would have an increased fitness. In the context of a preleukemic stem cell, it is not sufficient to merely produce more daughter cells. Rather, those daughter cells must retain the ability to self-renew if they are to persist long enough to acquire additional preleukemic and eventually leukemic mutations. This idea has been functionally tested in preleukemic HSCs isolated from AML patients, demonstrating

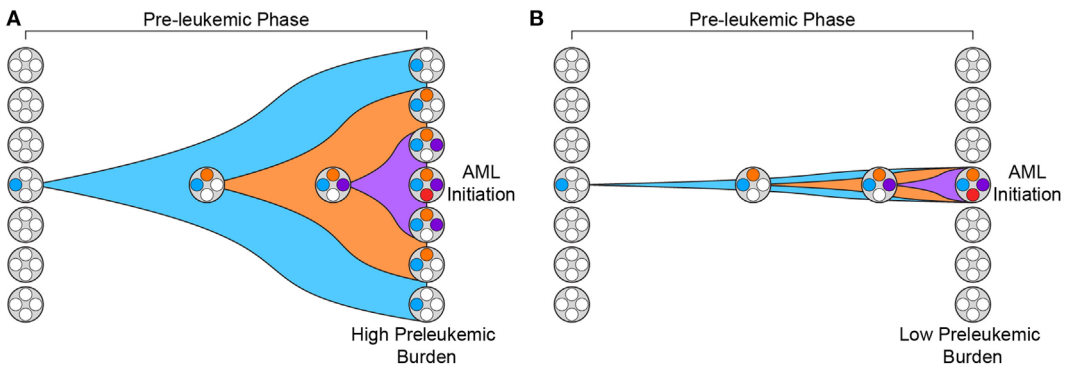

FIGURE 1 | Preleukemic burden is highly variable in acute myeloid leukemia (AML) patients. **(A)** Preleukemic burden is defined as the percentage of hematopoietic stem cells (HSCs) in an AML patient that harbor at least the earliest preleukemic mutation. This diagram depicts the preleukemic phase of evolution with the acquisition of three distinct mutations represented by three distinct colors (blue, orange, and purple). Eventually, the first mutation (blue) is present in every HSC, leading to a preleukemic burden of 100%. **(B)** This diagram depicts the acquisition of the same three mutations shown in panel **(A)** but the resulting HSCs fail to expand. In this scenario, only a minority of the HSCs harbor mutations, and therefore, the preleukemic burden is low.

that preleukemic HSCs resist enforced differentiation *in vitro* in comparison to both cord blood- and adult bone marrow-derived hematopoietic stem and progenitor cells (31). This observation supports the hypothesis that mutations in certain genes (i.e., *DNMT3A*, *TET2*, *IDH1/2*, and the cohesin complex) occur predominantly during the preleukemic phase because they function in part to prevent differentiation. Presumably, these mutations simultaneously enable the HSCs to persist long enough to acquire additional mutations and prevent full differentiation during the leukemic phase of AML. Mechanistically, mutations in these epigenetic regulators could lead to modest but impactful alterations in key lineage defining genes that lead to clonal outcompetition (34, 35). This model of preleukemic evolution is supported by additional studies that demonstrate that certain preleukemic mutations prevent differentiation, both in mouse models and in *in vitro* culture (30, 36–42).

CLONAL HEMATOPOIESIS (CH) AND PRELEUKEMIA

Since the discovery of preleukemic HSCs, multiple groups have identified an age-associated syndrome that has been termed clonal hematopoiesis of indeterminate potential (CHIP) (43–50). CHIP was identified by searching for mutations in genes that occur in hematologic malignancies in blood cells from individuals with no history of hematologic disease that had been sequenced for genomic studies of other conditions. CHIP is characterized by the clonal outgrowth of mutated hematopoietic cells. The most frequently mutated genes in CHIP are *DNMT3A* and *TET2*, echoing their role during the preleukemic phase of AML (43, 44, 50). These studies have shown that the incidence of CHIP is associated with age, with very few individuals under the age of 40 showing detectable CH and more than 10% of individuals over the age of 70 showing detectable CH (43, 44). In fact, a small-scale follow-up study using targeted error-corrected sequencing for more sensitive mutation detection (≥0.0003 VAF) identified CHIP in 95% of individuals between the ages of 50 and 60 years old (51). Most individuals only have one detectable mutation in a gene known to be involved in hematologic malignancy. Importantly, the presence of CHIP with a variant allele fraction of at least 0.10 is associated with a 49-fold higher relative risk of developing a hematologic malignancy. However, the absolute risk of hematologic malignancy remains small, with only 4% of persons with CHIP progressing to malignancy (43, 44). These findings raise the possibility of leukemia prevention if therapeutics are developed that can target these pre-malignant cells (discussed below).

In addition to being associated with an increased risk of hematologic cancer, CHIP is also associated with other adverse health outcomes. Of particular note, after controlling for age, sex, and diabetes, the presence of CHIP is associated with an increased all-cause mortality (hazard ratio, 1.4). Contributing to this increase in all-cause mortality, carriers of CHIP have a 1.9-fold higher risk of coronary heart disease, potentially due to an increased secretion of several cytokines and chemokines from mutant hematopoietic cells that contribute to atherosclerosis (52, 53). Intriguingly, a recent study of more than 8,000 individuals has shown an association of CH with solid tumor malignancies (54). Of all cancer patients, 25% carried CH, with 4.5% harboring a presumptive leukemia driver mutation. In this study, CH was associated with increased age, prior radiation therapy, and tobacco use. This indicates that CH may be caused by environmental factors as well as age-dependent stochasticity. The mechanisms accounting for the increased association of CH with solid tumors are unclear, but we propose the intriguing possibility that CH affects the immune system in such a way as to inhibit immune surveillance of cancer. Similar studies have implicated CH as a risk factor for the development of therapy-related myeloid neoplasms (55, 56). Certainly, this will be an important area for further investigation.

DURATION OF THE PRELEUKEMIC PHASE

An important topic that remains poorly understood is the duration of the preleukemic phase of AML. To date, no studies have provided concrete evidence to suggest an upper and lower bound for the period of time between the acquisition of the first leukemogenic mutation and the onset of disease. However, multiple lines of anecdotal evidence exist to provide an estimate. Recently, multiple studies have tracked the development of leukemia in allogeneic bone marrow transplant donors and recipients (57, 58). In one study, both the donor and the recipient were diagnosed with AML more than 7 years posttransplant. Both patients harbored mutations in *DNMT3A* and this mutation was retrospectively identified in the donor prior to transplant (VAF = 41%) (58). In the second study, both donor and recipient developed *DNMT3A*-mutant AML within 2 years of transplantation and the donor was retrospectively found to have a mutation in *DNMT3A* (VAF = 46%) at the time of transplant (57). These studies indicate that preleukemic evolution takes at least 7 years and, in reality, probably many more as the DNMT3A HSC clone had already expanded substantially in the donor at the time of transplant. Research from our own group has identified a single patient where we believe the preleukemic phase lasted for at least 15 years (31). This particular patient was diagnosed with AML at age 29 and harbored a preleukemic IDH1 mutation. Intriguingly, this mutation was also present at high penetrance (VAF = 25%) in T cells. As the vast majority of an individual's T cell repertoire is established prior to puberty and progressive thymic involution (59), this indicates that this preleukemic clone likely arose prior to adolescence. In this case, the preleukemic phase could have lasted more than 15 years.

These temporal dynamics of the preleukemic phase of AML raise multiple interesting, and as of yet unanswered questions. Even if the preleukemic phase lasts 20–30 years, why are the majority of AML patients over the age of 65? If leukemia is capable of developing in just 20 years, why do we not observe more cases of AML in younger adults? Are aged HSCs more susceptible to mutation? Are aged HSCs more capable of accepting the epigenetic consequences of a mutation in *DNMT3A* or *TET2*? Perhaps, the progressive myeloid bias observed during aging (60, 61) plays a role in this process as well. Answering these questions will lead

to clear advances in our understanding of the preleukemic phase and identify opportunities for therapeutic intervention prior to the onset of AML.

PRELEUKEMIC HSCs IN REMISSION AND RELAPSE

The identification of preleukemic HSCs as the reservoirs for mutation acquisition prior to the onset of AML raises the question of whether these cells have a clinical relevance beyond the preleukemic phase. We hypothesized that preleukemic HSCs could survive standard induction chemotherapy, persist during remission, and contribute to relapsed disease through the acquisition of a small number of additional mutations (17). Several studies demonstrated that preleukemic HSCs did, indeed, survive standard induction chemotherapy (6, 22, 23, 62, 63). However, no formal proof of the ability of preleukemic HSCs to seed relapsed disease in AML has been provided. This is likely due to the inadequacy of our standard treatment regimens which fail to eradicate every AML cell, making it difficult to distinguish rare minimal residual disease (MRD) from residual preleukemia. Currently, relapsed disease most frequently originates from re-emergence of a clone present at diagnosis or further evolution of a clone present at diagnosis (64, 65). Without full eradication of the AML, it remains unlikely that additional mutations would accumulate in preleukemic HSCs more rapidly than the expansion of an existing AML clone that has survived therapy. One intriguing possibility is that preleukemic HSCs may acquire additional mutations with delayed kinetics and perhaps give rise to late relapses (16, 17). Nevertheless, we believe that preleukemic HSCs do represent an important clinical entity and have the ability to generate relapsed disease if our therapies improve to the point of sufficiently eradicating all frankly leukemic cells.

PRELEUKEMIC BURDEN AND PATIENT SURVIVAL

Recently, multiple studies have identified a correlation between high preleukemic burden and a worse overall or relapse-free survival. In a broad characterization of preleukemic HSCs in a cohort of nearly 40 AML patients, high pre-leukemic burden was defined as greater than 20% of HSCs harboring at least the first mutation. Overall and relapse-free survival was significantly shorter in patients with high pre-leukemic burden with hazard ratios of 3.3 and 2.99, respectively (31). Similarly, a second study of patients with lympho-myeloid clonal hematopoiesis (LM-CH) showed that the preleukemic clone was refractory to chemotherapy, leading to a higher incidence of relapse than patients without LM-CH (63). This association is somewhat paradoxical in that, at diagnosis, preleukemic HSCs make up less than 1% of the total cells, and that the relapsed disease of these patients did not necessarily originate directly from preleukemic HSCs. One possible explanation for this observation is that a higher preleukemic burden predisposes for a more aggressive leukemia. This would be consistent with the increased competitive advantage that leads to a higher preleukemic burden. As mentioned previously, it is possible that a higher preleukemic burden could be associated with an epigenetic profile that is primed to mediate out-competition. Additional mechanisms including both cell-intrinsic and cell-extrinsic effects could be involved. Further studies on larger patient cohorts will need to be performed in order to validate these observations and motivate future work into understanding why high preleukemic burden is associated with poor outcomes in AML.

MRD AND PRELEUKEMIC MUTATIONS

In situations where standard induction chemotherapy regimens can be implemented, the majority of AML patients are able to achieve a complete morphologic remission (66). However, many of these patients inevitably relapse and succumb to less responsive relapsed disease. As mentioned previously, this relapsed disease largely originates from leukemic clones present at diagnosis (64, 65). The key clinical decision is to determine which patients should receive transplants during first remission. One avenue that is being explored to inform this decision is the monitoring of MRD, sub-microscopic levels of persistent leukemic cells that can be monitored with flow cytometry, quantitative PCR, or sequencing methods (67). MRD has been most successfully tracked using detection of mutated *NPM1* transcripts (68–70). Recent work has shown that the persistence of *NPM1*-mutated transcripts in peripheral blood during remission is associated with a significantly higher risk of relapse at 3 years than is the absence of such transcripts (82 vs. 30%, univariate hazard ratio = 4.80) and a lower rate of survival (24 vs. 75%, univariate hazard ratio = 4.38) (68). Similar results have been shown for other AML-specific mutations occurring in genes such as *DNMT3A, TET2, IDH1/2, KRAS*, and *FLT3* (71).

The clinical relevance of MRD and the persistence of preleukemic HSCs during remission illustrate the potential for preleukemic mutations to confound MRD detection. For example, if a mutation in *DNMT3A* occurred in a preleukemic HSC, the persistence of this mutation during remission may demonstrate the persistence of preleukemic cells rather than frankly leukemic cells. As relapse from a preleukemic clone is likely rare, one might reason that detection of preleukemic cells during remission may not be relevant to treatment decisions. This would suggest that late occurring mutations will be more effective markers for MRD. However, one recent study has shown a clear difference in event-free survival between patients with any mutation detectable above 5% VAF at 30 days post therapy compared to patients with no mutation detectable above 5% VAF (71). Some of these mutations were clearly being detected in preleukemic cells as the blast count showed a strong response to therapy but no corresponding change in VAF was observed. This study indicates that patients with high preleukemic burden have a poorer prognosis and that detection of preleukemic mutations during remission may also be an indicator of poor survival outcome. This is consistent with the previously mentioned retrospective studies (31, 63) showing that patients with high preleukemic burden have poorer outcomes than patients with low preleukemic burden. While there are many reasons to suggest that preleukemic cells should not be considered "disease" during MRD monitoring, these results should serve to

motivate future work on the impact of persistent preleukemic cells during remission on patient outcome.

TARGETING PRELEUKEMIC MUTATIONS AND PRELEUKEMIC HSCs

The identification and characterization of preleukemic HSCs has raised the question of how this knowledge should influence therapy development and treatment decisions. As discussed above, recent work on CHIP has shown that carriers have an increased risk for developing hematologic malignancies. This indicates that, if these cells could be targeted without adverse side effects, it could be possible to prevent the onset of AML. However, it remains unclear how best to approach this problem. First and foremost, successful targeted therapeutic intervention would require identification of a dependency unique to preleukemic HSCs. Recent work has identified minimal consistent transcriptional and epigenetic differences between healthy and preleukemic HSCs (31), making it unlikely that these cells will be universally sensitive to the same intervention. This means that any targeted preleukemic therapy would likely be based on the genetic mutations present in preleukemic cells and would therefore target DNMT3A or TET2. Though no approved targeted therapeutics exist for either of these genes, studies have demonstrated proof-of-concept targeting of *DNMT3A*-mutant cells with an inhibitor of the DOT1-like histone lysine methyltransferase (72). Effective therapies would preferentially tip the scales in favor of differentiation of preleukemic HSCs, leading the mutations to exhaust as the clone undergoes lineage commitment (**Figure 2A**). Importantly, no therapies have been designed with preleukemic HSCs as the primary target and it is likely that therapies that are effective against AML cells would be ineffective against preleukemic cells (**Figure 2B**). Even if a targeted preleukemic therapy existed, there are situations where therapeutic intervention during the preleukemic phase could be highly detrimental. For example, in patients with very high preleukemic burden, up to 100% of HSCs could harbor preleukemic mutations. If these HSCs were induced to differentiate, the patient could suffer widespread bone marrow failure that would only be treatable by bone marrow transplantation (**Figure 2C**). As most of these individuals would be of advanced age, this type of therapy would likely be poorly tolerated. Some of these problems are exemplified by the treatment

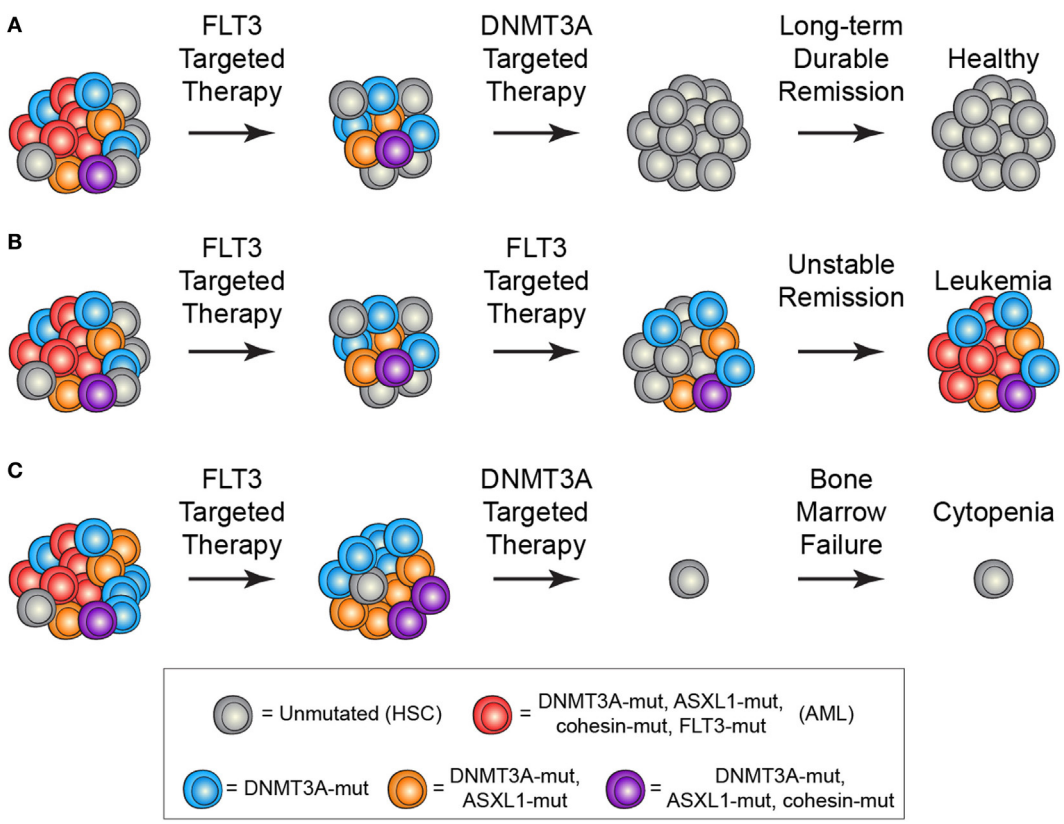

FIGURE 2 | Treatment scenarios in acute myeloid leukemia (AML) and the impact of preleukemic hematopoietic stem cells (HSCs). **(A)** The ideal treatment would combine a therapy targeted against the frankly leukemic cells (such as anti-FLT3 therapy) to eradicate AML cells followed by a targeted therapy against the preleukemic cells (such as anti-DNMT3A therapy). This would lead to long-term durable remission and disease cure. **(B)** Current AML therapies largely target late mutations, such as FLT3-ITD, which are not present in preleukemic HSCs. In the event that all AML cells are eradicated, the preleukemic HSCs could eventually lead to relapsed disease. **(C)** Targeting of preleukemic HSCs in the context of high preleukemic burden could lead to bone marrow failure and cytopenias as the vast majority of HSCs would be targeted.

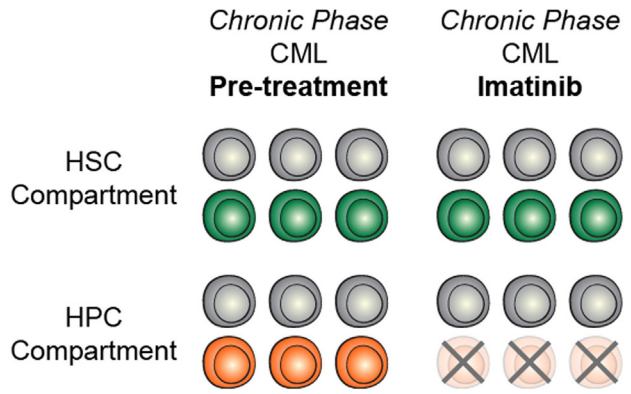

FIGURE 3 | Targeted treatment of preleukemic mutations may be context dependent. In chronic phase CML, treatment with imatinib efficiently targets heamtopoietic progenitor cells (HPCs) but does not target HSCs, likely due to context specificity.

of chronic phase chronic myelogenous leukemia (CML) with the tyrosine kinase inhibitor imatinib. Imatinib targets the BCR-ABL tyrosine kinase fusion protein that is present in every CML cell, including the preleukemic HSCs. However, while progenitor cells are highly sensitive to treatment (73), BCR-ABL-positive HSCs remain resistant (**Figure 3**) (74). CML patients treated with imatinib can achieve complete morphologic remission, durable for many years, but retain HSC clones that harbor the BCR-ABL translocation that is the hallmark of the disease (75). Upon removal of tyrosine kinase inhibitor therapy, these patients can relapse due to the residual HSCs harboring the BCR-ABL fusion (75, 76). Successful eradication of preleukemic HSCs will require consideration of these many caveats to therapy design.

CONCLUSION

Our understanding of preleukemia is still in its infancy. Much of the work that has been performed has aimed at understanding the genetic component of preleukemia, identifying which mutations occur during this protracted evolutionary phase and which mutations occur during the progression to frank leukemia. We have learned that these cells persist during remission, contribute to remission hematopoiesis, and have the potential to generate relapsed disease. We have identified associations between preleukemic burden and patient outcome. However, there is still much to learn about the clinical relevance of these preleukemic HSCs. Future work will serve to demonstrate whether therapeutic intervention during the preleukemic phase is feasible and safe, potentially opening the door to preventative treatments for AML. A more rigorous understanding of these cells could lead to therapeutic interventions that have the potential to stop AML before it starts.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript and contributed to the design and overall outline.

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MINI REVIEW
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Pharmacologic Targeting of Chromatin Modulators As Therapeutics of Acute Myeloid Leukemia

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Acute myeloid leukemia (AML), a common hematological cancer of myeloid lineage cells, generally exhibits poor prognosis in the clinic and demands new treatment options. Recently, direct sequencing of samples from human AMLs and pre-leukemic diseases has unveiled their mutational landscapes and significantly advanced the molecular understanding of AML pathogenesis. The newly identified recurrent mutations frequently "hit" genes encoding epigenetic modulators, a wide range of chromatin-modifying enzymes and regulatory factors involved in gene expression regulation, supporting aberration of chromatin structure and epigenetic modification as a main oncogenic mechanism and cancer-initiating event. Increasing body of evidence demonstrates that chromatin modification aberrations underlying the formation of blood cancer can be reversed by pharmacological targeting of the responsible epigenetic modulators, thus providing new mechanism-based treatment strategies. Here, we summarize recent advances in development of small-molecule inhibitors specific to chromatin factors and their potential applications in the treatment of genetically defined AMLs. These compounds selectively inhibit various subclasses of "epigenetic writers" (such as histone methyltransferases MLL/KMT2A, G9A/KMT1C, EZH2/KMT6A, DOT1L/KMT4, and PRMT1), "epigenetic readers" (such as BRD4 and plant homeodomain finger proteins), and "epigenetic erasers" (such as histone demethylases LSD1/KDM1A and JMJD2C/KDM4C). We also discuss about the molecular mechanisms underpinning therapeutic effect of these epigenetic compounds in AML and favor their potential usage for combinational therapy and treatment of pre-leukemia diseases.

Keywords: epigenetic modulator, small-molecule inhibitors, acute myeloid leukemia, bromodomain, MLL, EZH2, DNMT3A, DOT1L

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INTRODUCTION

Epigenetic modifications, including DNA methylation and a myriad of post-translational modifications of the DNA-packaging histone proteins, represent a fundamental means for regulating gene expression and other DNA-templated processes (1–4). These modifications of DNA or histones are increasingly appreciated to be dynamically regulated by epigenetic modulators, a broad class of proteins that consist of "epigenetic writer" enzymes catalyzing chromatin modification, "epigenetic eraser" enzymes removing the modification, "epigenetic readers" or "effectors" recognizing the

modification to elicit biological consequences, and various other cellular regulators that indirectly influence the level or readout of epigenetic modification (2, 5). While the dynamic regulation of epigenetic modification enables cells to adapt and function differently in response to developmental and environmental cues, their mis-regulation often perturbs gene expression and cellular function leading to pathogenesis of human disease such as cancer. Indeed, recent deep sequencing of human cancer patient samples has identified novel recurrent mutations in genes encoding a wide range of epigenetic modulators and even histones themselves (6–9).

Acute myeloid leukemia (AML), a common malignancy of myeloid-lineage precursor cells in the blood, is characterized by two hallmarks, uncontrolled cell proliferation and impaired differentiation. Previously, progression and characteristics of AML were linked to several key pathways (10, 11), including inactivation of tumor suppressors [such as TP53 and *Wilm's Tumor-1* (WT1)], gain-of-function mutation of oncogenic kinases (such as FLT3, NRAS, and KRAS), and stem cell transcription factors (TFs) [such as rearrangement and/or overexpression of HOX cluster genes and their cofactors such as MEIS1 (12–14)], as well as inactivating mutation of differentiation-promoting TFs (such as PU.1 and CEBP/α). Recently, deep sequencing of samples from human patients with AML and pre-leukemia diseases such as myelodysplastic syndrome (MDS) and clonal hematopoiesis of indeterminate potential (CHIP) additionally revealed frequent somatic mutations of genes involved in epigenetic modulation or RNA splicing (11, 15–26). Among the various affected epigenetic pathway genes include the *DNA (cytosine-5)-methyltransferase 3 A* (DNMT3A, a DNA methylation "writer"), *Tet Methylcytosine dioxygenase 2* (TET2, a DNA methylation "eraser" or demethylase), *Enhancer of zeste homolog 2* [EZH2/KMT6A, a "writer" mediating methylation of histone H3, Lys27 (H3K27)], *Additional Sex Combs Like 1 and 2* (ASXL1 and ASXL2, an EZH2-associated cofactor family), the Cohesin complex (SMC3-SMC1-RAD21-STAG) genes, and *Isocitrate Dehydrogenase 1* and *2* (IDH1 and IDH2). These newly identified somatic mutations of DNA/chromatin modifiers and structural organizers are in agreement with previous karyotyping/FISH-based analyses of AML patients, which already identified recurrent chromosomal translocation or abnormality of genes encoding various members of epigenetic "writers" (MLL/KMT2A, NSD1/KMT3B, NSD3/WHSC1L1/KMT3F) (27–31), "erasers" (JARID1A/KDM5A) (32, 33), and "readers" (PHF23) (32, 34). Importantly, mutations of *DNMT3A*, *TET2*, *IDH1/2*, or *ASXL1* were frequently detected among apparently healthy individuals with clonal hematopoiesis or CHIP (22, 24, 35, 36) and in AML patients who received complete disease remission after chemotherapy (26, 35, 37–39), supporting the pivotal roles of epigenetic deregulation in initiation, clonal evolution and relapse of AMLs.

In contrast to significant advances in molecular appreciation of human AML's mutational landscape and putative "driving" pathways, chemotherapy remains as the frontline treatment for most AML patients, with an exception of all-trans retinoic acid (ATRA) used as targeted therapy of the acute promyelocytic leukemia (APL) subtype. AML patients still suffer from low overall survival and a high rate of recurrence, demanding new treatments to be developed. Recent studies of AML and other tumors have increasingly shown that genetic lesion of epigenetic modulator often induces a subsequent chain reaction leading to aberrations in chromatin modification/remodeling, gene-expression program, and cellular states during tumorigenesis (2, 5, 29, 40–43). Thus, pharmacologic targeting of epigenetic players responsible for the above chromatin/gene mis-regulation shall represent new mechanism-based strategies for therapeutic intervention. This review aims to summarize recent advances in specific inhibition of histone-modifying enzymes and regulatory proteins as potential AML therapeutics, with the already discovered inhibitors sub-grouped into the categories targeting either the "writing," "reading," or "erasing" function of epigenetic modulators (**Table 1**).

TARGETING CHROMATIN "WRITERS"

MLL Inhibitors (MLLi)

The *Mixed-Lineage Leukemia* gene (*MLL/MLL1/KMT2A*) encodes one of the KMT2 family of methyltransferase enzymes that contain multiple structural domains, including a C-terminal SET domain catalyzing methylation of histone H3, Lys4 (H3K4) (44–46). *MLL* rearrangement and translocation, which typically affect one allele, are responsible for about 70% of infant leukemias and 5–10% of childhood and adult AML cases (28, 29). Often, the leukemia-associated *MLL* gene rearrangement produces the MLL fusion oncoprotein that loses MLL's C-terminal SET domain and gains a partial sequence from its fusion partner such as AF4, AF9, AF10, or ENL, which recruits the DOT1L-associated transcription elongation complexes. MLL fusion oncoproteins still retain MLL's N-terminal domains, which mediate chromatin association and interaction with functional cofactors such as Menin. Previously, the remaining wild-type *MLL* allele in cancer cells was shown to be critical for leukemogenesis induced by MLL fusion (47); however, a recent study reported that MLL2/KMT2B, another trithorax family methyltransferase that is most closely related to MLL/KMT2A (48), sustains growth of *MLL*-rearranged leukemia and represents a more relevant drug target (49). While the transcription elongation activity acquired by MLL fusion remains as an attractive targeting strategy (see the section of DOT1Li), these studies have justified development of MLL1/2 inhibitors (MLLi) for the treatment of *MLL*-rearranged leukemias.

Using the structure-guided design, Cao et al. developed an MLLi termed MM-401 (**Figure 1A**, left and **Table 1**) to disrupt direct interaction of MLL1 with WDR5, a cofactor associated with the SET domain of MLL/KMT2 enzymes, and thus inhibit MLL1's methylase or "writer" function (50). *In vitro* biochemical assays showed that MM-401 specifically targets WDR5 interaction to MLL1, and not other MLL/KMT2 family enzymes. Treatment with MM-401 blocked proliferation and induced myeloid differentiation of *MLL*-rearranged leukemia cells while not significantly affecting normal blood stem/progenitor cells (50). A recent study reported that MLL2 represents a more relevant therapeutic target in a range of *MLL*-rearranged leukemia

TABLE 1 | Epigenetic therapies in acute myeloid leukemia (AML): targets, compounds, and clinical development.

Targets	Role in epigenetic regulation	Representative compounds	Indications	Clinical development
Writers				
MLL protein complex	H3K4 methyltransferase	MM-401 MIV-6R[a] MI-503[a]	MLL-rearranged AML	Preclinical
G9A	H3K9 methyltransferase	UNC0648	HOXA9-overexpressed AML	Preclinical
EZH2	H3K27 methyltransferase	GSK126 UNC1999 EPZ005687 Tazemetostat	MLL-rearranged AML	Preclinical
DOT1L	H3K79 methyltransferase	SGC0946 EPZ-5676	MLL-rearranged AML, and others	Phase I
PRMT1	H4R3 methyltransferase	AMI-408	MLL-EEN/GAS7, MOZ-TIF2 and AML1-ETO AML	Preclinical
Readers				
Bromodomain proteins	Histone acetylation readers	JQ1 I-BET151 I-BED762 CPI-0610 OTX015 TEN-01 FT-1101 GSK525762	MLL-rearranged AML, and others	Phase I and Phase II
NUP98-PHF23 or NUP98-JARID1A	H3K4me3 readers	Disulfiram	AMLs with NUP98-PHF23 or NUP98-JARID1A	Preclinical
Erasers				
Histone deacetylases	Histone deacetylases	Vorinostat Romidepsin Panobinostat Givinostat Mocetinostat Ricolinostat AR-42 CUDC-907	AML	Phase I and Phase II for AML; FDA approved for T cell lymphoma and multiple myeloma
LSD1	H3K4 demethylase	GSK2879552 ORY-1001	MLL-rearranged AML, and others	Phase I
KDM4C	H3K9 demethylase	SD70	MLL-EEN/GAS7 and MOZ-TIF2 AML	Preclinical

[a]*MLL/Menin inhibitor is likely to act through inhibiting MLL fusion and not wild-type MLL proteins and probably should not be listed among the "writer" inhibitor category.*

models and that MLL2 and MLL1 collaborate to maintain oncogenesis *via* regulating distinctive gene-expression pathways (49). Therefore, dual inhibitors of MLL2 and MLL1 or a specific one against MLL2 need to be developed and may provide a more effective treatment strategy.

Menin, a cofactor associated with the N-terminal region of both MLL fusion and wild-type MLL1/2 proteins, is required for MLL- and MLL fusion-mediated target gene activation and for leukemic transformation caused by *MLL* rearrangement (51–55). Menin is required for association and/or recruitment of MLL and MLL fusion proteins to their gene targets and represents a validated drug target of *MLL*-rearranged leukemia. Recently, through high-throughput screening and structure-based development, a series of MLLi, including MIV-6R (56), MI-463, and MI-503 (57), were discovered and optimized to disrupt MLL–Menin interaction, with some achieving *in vitro* inhibition in the nanomole range (**Figure 1A**, right; **Table 1**). These MLL–Menin inhibitors efficiently suppressed growth of *MLL*-rearranged leukemia cells *in vitro*/vivo and did not affect that of non-*MLL*-rearranged leukemias. Treatment with these MLLi led to down-regulation of gene-expression programs enforced by MLL fusion, such as *HOXA9* and *MEIS1*, in the leukemia cells. The effect of MLL–Menin inhibitors on steady-state normal hematopoiesis appears to be small (57), suggesting that their anti-leukemia effect is mainly through inhibiting Menin interaction to MLL fusion and not wild-type MLL1 proteins. For this reason, MLL–Menin inhibitors should not be categorized as the "writer" inhibitor. However, it is worthy noting that, besides MLL1/KMT2A, Menin also interacts with MLL2/KMT2B through conserved interfaces (46, 51, 53). It remains to be determined whether the above MLLi also targets MLL2, a recently validated oncoprotein that sustains *MLL*-rearranged leukemias (49). For convenience, we decide to list the MLL–Menin inhibitors as MLLi and "writer" inhibitors (**Table 1**).

G9A Inhibitors (G9Ai)

Euchromatic histone lysine methyltransferase 2 (*EHMT2*, also known as *G9A/KMT1C*) encodes a methyltransferase that catalyzes mono/di-methylation of histone H3, Lys9 (H3K9me1/2), a histone modification correlated with gene silencing. Knockout

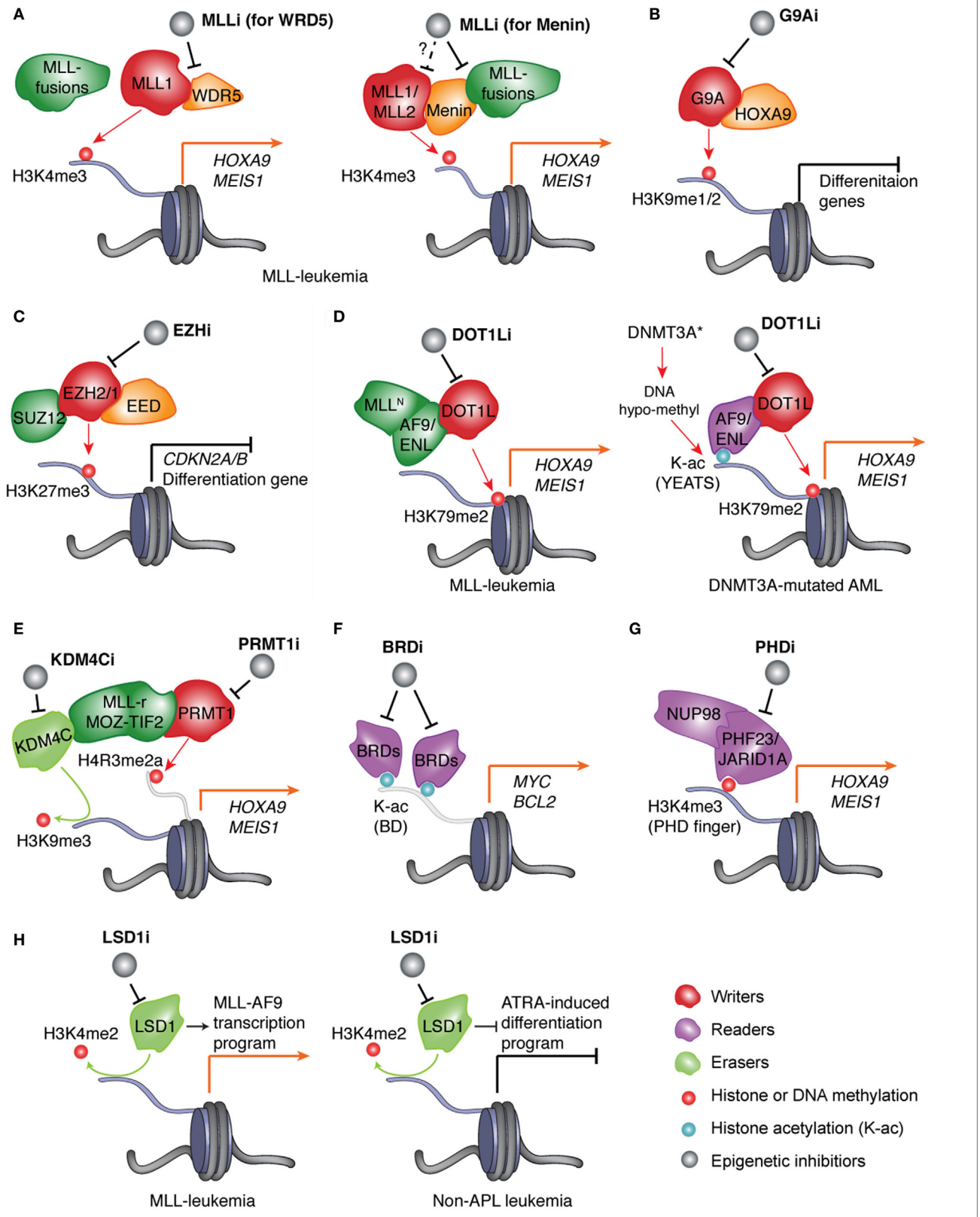

FIGURE 1 | Continued

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Pharmacological inhibition of the epigenetic "writers," "readers," or "erasers" responsible for deregulation of chromatin modification and gene expression in AMLs. **(A)** In leukemias with *MLL* rearrangement (MLL-r), protein complexes assembled by the wild-type MLL and aberrant MLL fusion proteins induce H3K4me3 and H3K79me2, respectively, to cooperatively mediate activation of MLL targets such as "stemness" genes *HOXA9* and *MEIS1*. Inhibitor of MLL (MLLi) disrupts physical association of MLL (MLL1 or MLL2) and MLL-fusion to its interacting partner, either WDR5 (left) or Menin (right), thereby preventing target gene activation and AML development. **(B)** HOXA9, a transcription factor (TF) found overexpressed in ~50–70% of AML patients, promotes leukemogenesis partly through recruiting G9A, an H3K9me1/2-specific "writer" enzyme, to suppress gene-expression programs crucial for myeloid differentiation. Inhibitor of G9A (G9Ai) targets this differentiation-arrest mechanism in AMLs with HOXA9 overexpression. **(C)** In AMLs, treatment with inhibitor of EZH2 and/or EZH1 (EZHi) results in suppression of H3K27me3 and de-repression of polycomb repressive complex 2 (PRC2) target genes, which include tumor suppressor genes (such as CDKN2A/B) and myeloid differentiation-associated genes. **(D)** Left panel: in *MLL*-rearranged leukemias, MLL fusion partners such as AF9 and ENL recruit DOT1L, an H3K79me2-specific "writer" enzyme, to maintain high expression of target genes such as *MEIS1* and *HOXA9*. Right panel: in normal-karyotype AMLs with *DNMT3A* mutation, focal decrease of DNA methylation (i.e., hypo-methylation) results in increase of histone acetylation (K-ac) and binding of the YEAST domain-containing K-ac "reader" proteins AF9 and ENL, which subsequently recruit DOT1L to promote H3K79me2 and transcriptional activation/elongation of "stemness" genes. In both genetically defined AML subtypes, inhibitor of DOT1L (DOT1Li) blocks the above oncogenic program and leukemia progression. **(E)** In leukemias with aberrant fusion of MLL or MOZ-TIF2, PRMT1, an H4R3-specific methyltransferase/"writer," and KDM4C, an H3K9-specific demethylase/"eraser," are recruited by leukemic fusion oncoproteins to modulate histone methylation and promote target gene activation. Blockage of PRMT1 or KDM4C provides a new treatment strategy. **(F)** In AMLs, inhibitor of bromodomain (BRD)-containing K-ac "readers" (BRDi) selectively blocks interaction of bromodomain proteins (BRD4 and related BRD2/3) with K-ac and represses expression of vital oncogenes such as *MYC* and *BCL2*, thus suppressing leukemic growth. **(G)** In AML patients, aberrant rearrangement of the gene encoding the H3K4me3-"reading" proteins JARID1A/KDM5A and PHF23 produces the leukemogenic fusion protein NUP98-JARID1A and NUP98-PHF23, respectively, which rely on their H3K4me3-"reading" plant homeodomain (PHD) finger domains to maintain high expression of AML-associated genes. Inhibitor of PHD fingers (PHDi) shall provide an attractive therapeutic method for these AML patients. **(H)** Left: in MLL-rearranged leukemia, inhibitor of LSD1 (LSD1i) downregulates MLL target genes and inhibits leukemia development. Right: in non-acute promyelocytic leukemia (APL) leukemia, LSD1i promotes all-trans retinoic acid (ATRA)-induced cell differentiation thereby suppressing leukemogenesis.

of *G9A* in hematopoietic systems led to decreased proliferation of myeloid progenitors without affecting the function of long-term repopulating hematopoietic stem cells (58). In mouse AMLs induced by *HOXA9*, a homeodomain TF gene found over-expressed in about 50–70% of human AMLs, loss of *G9A* suppressed leukemogenesis. Mechanistically, G9A physically interacts with HOXA9. Inhibition of G9A led to de-repression of HOXA9 target genes (58). UNC0638 (59), a recently developed G9Ai, demonstrated similar AML therapeutic effect (**Figure 1B**; **Table 1**). While no method is currently available for directly targeting HOXA9 oncoprotein, the above studies provide an alternative strategy.

EZH Inhibitors (EZHi)

EZH2/KMT6A serves as the catalytic subunit of the polycomb repressive complex 2 (PRC2) mediating transcriptional repression through tri-methylation of H3K27 (H3K27me3) (60). EZH1, an EZH2-related methylase, can partially compensate EZH2's functions on a subset of gene targets when assembled in a separate complex with the same set of PRC2 components such as SUZ12 and EED (60, 61). Genomic deletion and loss-of-function mutations of *EZH2/KMT6A* were frequently found in MDS and other myeloid malignancies (62), whereas its gain-of-function mutations occur in 10–20% of B-cell lymphoma patients (63–65). Such *EZH2/KMT6A* somatic mutation is rare among AMLs (66). Recent investigation of animal blood cancer models, however, has shown that complete loss of *EZH2* promotes MDS development but prevents AML transformation (67). MDS induced by *EZH2* loss requires EZH1 for disease progression (68), indicating a context-dependent role of these PRC2 enzymatic complexes in development of MDS and blood malignancy. Furthermore, several studies demonstrated that the *MLL*-rearranged leukemias require functionality of EZH2 and/or EZH1 to maintain leukemogenecity (69–74). Mechanistically, these PRC2 enzymes suppress genes related to tumor suppression (such as *Cdkn2a/b*) and cell differentiation (such as *Egr1*) through inducing gene-repressive H3K27me3/2 (**Figure 1C**). Additionally, PRC2 was found to promote expression of MYC-associated gene signatures probably *via* an indirect mechanism. Furthermore, about 5–10% of AML patients carry the inactivating mutation of the *WT1* gene, which was shown to induce a DNA hyper-methylation phenotype through interfering with WT1-mediated recruitment of TET DNA demethylases (75, 76). The induced DNA hyper-methylation sites were found enriched in myeloid differentiation genes and PRC2 targets, and *EZH2* is highly expressed in *WT1*-mutated AMLs to maintain repression of genes with DNA hyper-methylation, leading to cell differentiation block (77). Importantly, in cellular and murine models of *MLL*-rearranged (69, 70, 72) or *WT1*-mutated AMLs (77), knockdown or knockout of PRC2 inhibited cell proliferation and restored gene-expression programs involved in myeloid differentiation. These studies unveiled the oncogenic function of PRC2 and EZH2 in these genetically defined AMLs, supporting PRC2 as a drug target of AML.

Due to frequent overexpression and gain-of-function mutation of *EZH2* in solid cancer and lymphoma, several pharmaceutical companies have embarked on high-throughput screening campaigns leading to discovery of a series of small-molecule compounds (**Table 1**) that compete binding of S-adenosyl-methionine (SAM), the methyl donor of PRC2, thereby suppressing PRC2's methyltransferase activity (78–82). These EZHi compounds demonstrate high selectivity and high potency toward EZH2 and/or EZH1. In *MLL*-rearranged AML models, dual inhibition of EZH2 and EZH1 by an EZHi, UNC1999, derepressed PRC2 target genes and significantly suppressed AML malignant growth *in vitro* and *in vivo* (74) (**Figure 1C**). Treatment of *WT1*-mutated AML cells with GSK126 (79), an EZH2-selective inhibitor, had similar anti-cancer effect (77). Currently, several EZHis show

drug-like properties and are used in clinical trials of lymphoma treatment. Their potential therapeutic effect in AMLs remains to be determined in clinical settings.

DOT1L Inhibitors (DOT1Li)

Disruptor of telomeric silencing 1-like (DOT1L/KMT4) is a histone H3 Lys79 (H3K79)-specific methyltransferase that regulates gene transcriptional elongation, telomeric silencing, and DNA damage response (83). Biochemical interaction studies found that DOT1L interacts with transcriptional elongation factors including AF4, AF9, AF10, and ENL, which are also common fusion partners of *MLL*-rearrangement in AMLs (29, 84–86). DOT1L loss-of-function studies in *MLL*-rearranged leukemias support its crucial role in leukemogeneicity, possibly through maintaining expression of target transcripts of MLL-fusion such as *HOX* cluster genes and *MEIS1* (84, 87–91).

Structure-based design has led to development of several DOT1Li (**Table 1**) that specifically targets the SAM-binding pocket of DOT1L enzymes (92, 93). Consistent with DOT1L loss-of-function studies, these DOT1Li also selectively inhibited expression of MLL-fusion target genes such as *HOXA9* and *MEIS1* and selectively killed *MLL*-rearranged leukemia cells and xenografted tumors (90–92, 94). Furthermore, recent investigation supports that DOT1L can potentially serve as a therapeutic target of other genetically defined AMLs, which include the subtype with translocation of *NUP98-NSD1* (95), somatic mutation of *DNMT3A* (96, 97), *NPM1* (98) or *IDH1/2* (99), or overexpression of *MN1* (100). While *NUP98-NSD1* induced leukemic transformation through direct targeting and epigenetic modulation of AML-promoting "stemness" genes (*HOX* gene clusters and *MEIS1*) (30), a *DNMT3A* hotspot mutation ($DNMT3A^{R882H}$) was recently found to focally suppress DNA methylation at cis-regulatory elements of these genes thereby promoting their transcription activation (96). In addition, aberrant over-expression of *HOX* cluster genes is a hallmark of AMLs that harbor *NPM1* mutation (98), and overexpression of *MN1* was found to induce an aggressive myeloid leukemia that strictly relies on the same "stemness" genes-expression program in the leukemia-initiating cells (100). Leukemia cells from the above AML subtypes were found generally sensitive to DOT1Li, and DOT1Li treatments repressed "stemness" gene-expression programs, supporting a broader role of DOT1L and "stemness" TF nodes in AML biology (**Figure 1D**). EPZ-5676 (94) represents the first DOT1Li used for clinical trials for *MLL*-rearranged leukemia; however, drug-like properties of the disclosed DOT1Li such as half-life *in vivo* are generally poor and need to be improved.

PRMT1 Inhibitors (PRMT1i)

Protein arginine methyltransferase 1 (*PRMT1*) encodes a methyltransferase for histone H4 arginine-3 (H4R3) and associates with gene activation. PRMT1 was shown to interact with AML1-ETO, a gene fusion product defining AML with t(8;21) translocation, activate the downstream target genes of AML1-ETO, and promote progression of AML1-ETO-asssociated leukemia (101). Recent studies have additionally demonstrated specific requirement of PRMT1 in leukemogenesis induced by *MLL*-rearrangement (such as *MLL-GAS7*) or the *MOZ-TIF2* translocation (102, 103). Similar to what was found in t(8;21) AMLs, PRMT1 physically associates with these leukemic fusion oncoproteins and is required for high expression of their target genes such as *HOX* and *MEIS1*, supporting targeting PRMT1 as new AML therapeutics. Indeed, in various leukemia cell lines and animal models with *MLL* fusion or *MOZ-TIF2*, AMI-408 (104), a PRMT1i, suppressed AML growth (103) (**Figure 1E**; **Table 1**). These works have established a foundation for further validation of PRMT1i's therapeutic effect in clinical settings.

TARGETING CHROMATIN "READERS"

Epigenetic or chromatin "readers" are a subclass of factors that specifically recognize DNA or histone modification to induce subsequent events and elicit functional readout of the modification (1, 2, 105–107). Compared to a generally high druggability of chromatin-modifying "writer" or "eraser" enzymes, that of various epigenetic "reader" families varies (108). Despite challenges, targeting chromatin "reader" function is increasingly considered as promising partly due to recent success in discovery of bromodomain (BRD) protein inhibitors.

BRD Inhibitors (BRDi)

BRD-containing proteins BRD4 and related BRD2/3 recognize histone lysine acetylations subsequently recruiting pTEFb, a CDK9/Cyclin-T kinase complex, to activate RNA polymerase II and target gene expression (109). Originally, these BRD genes were found aberrantly rearranged in malignant NUT midline carcinomas. A pioneering functional genomics screening of chromatin regulators in *MLL*-rearranged leukemia unveiled a role for BRD4 in maintenance of *C-MYC* expression and leukemia oncogenicity (110). Since advent of JQ1, the first highly selective and highly potent BRDi (showing a nano-molar range inhibition of BRDs) that competes BRD4 off acetylated histone ligands (111), multiple BRDis have been developed and their therapeutic effect seen in a wide range of human diseases including AML and other cancers (109). Mechanistically, BRDi such as JQ1 and I-BET151 repressed expression of a number of key oncogenic nodes including *C-MYC* and *BCL2* in mouse and human leukemia models carrying *MLL*-rearrangement (110, 112) (**Figure 1F**). BRDi was also found effective in treating non-*MLL*-rearranged AMLs such as those with *NPM1* mutation (113) or deletion of chromosome 7 and 7q [−7/del(7q)] (114), supporting their broader application in AML therapeutics. Even more potent BRDi, including a degrader derivative that can both inhibit BRD's "reading" function and induce its proteasome-mediated degradation (115), have been developed, with several currently under clinical evaluation for the treatment of refractory AMLs (109). Following these encouraging advances, inhibitors of other RNA Pol-II activators such as the CDK7 and CDK9 kinases are on the horizon becoming a strategy to target transcriptional addiction to vital oncogenes seen in cancer (116, 117).

Plant Homeodomain (PHD) Finger Inhibitors (PHDi)

The PHD finger-containing proteins comprise a large class of chromatin-associated proteins, some of which harbor the "reading" specificity toward H3K4 methylation (2, 106). In human AMLs, genes encoding the PHD finger-containing protein JARID1A (also known as KDM5A, a PHD finger-containing histone demethylase) and PHF23 were altered due to chromosomal abnormalities, resulting in in-frame fusion of their C-terminal H3K4me3-"reading" PHD finger to NUP98, a promiscuous gene translocation partner in human AMLs (32, 106). Despite generally low frequency of these genetic abnormalities in AMLs, the *NUP98-JARID1A/KDM5A* translocation was reported to be recurrent and detected in ~10% of the pediatric acute megakaryoblastic leukemia subtype (33). The produced NUP98-JARID1A or NUP98-PHF23 oncoproteins were highly potent in inducing AML transformation *in vitro*/vivo and rely on their H3K4me3-"reading" PHD finger domain to maintain high expression of "stemness" nodes, notably *HOX* and *MEIS1* (32, 118). Disulfiram, a previously FDA-approved drug, was found to carry the ability to inhibit binding of these PHD fingers to H3K4me3 possibly through structural alteration (119) and to selectively kill the leukemic cells transformed by *NUP98-PHF23* or *NUP98-JARID1A/KDM5A* (118) (**Figure 1G**). However, the potency and selectivity of disulfiram appear poor and the ligand-competitive inhibitors still remain to be developed for these PHD fusion oncoproteins.

TARGETING CHROMATIN "ERASERS"

HDAC Inhibitors (HDACi)

Histone deacetylases (HDACs) remove acetylation off histones to influent gene expression. HDACi (**Table 1**) including Vorinostat (also known as SAHA) and Panobinostat are the earliest inhibitors of epigenetic "erasers" approved by FDA for treatment of cutaneous T cell lymphoma and, recently, multiple myeloma. Currently, HDACi is under phase I/II trials of relapsed AML patients. As HDACs also deacetylate numerous non-histone substrates, effect of HDACi remains controversial as of the detailed mechanisms, especially through targeting histone versus non-histone proteins.

LSD1 Inhibitors (LSD1i)

Lysine-specific demethylase 1 (LSD1/KDM1A) is the first identified histone demethylase with specificity toward H3K4 mono/di-methylation (H3K4me1/2) (120). Several LSD1i have been developed. In the *MLL*-rearranged leukemias, terminal differentiation arrest was partially enforced by LSD1, and LSD1i treatment induced myeloid differentiation and suppressed leukemogenesis *in vivo* (**Figure 1H**) (121). Mechanistically, LSD1i may perturb the H3K4me3/H3K4me2 ratio at MLL target genes thus reducing their transcription (121). Also, therapeutic effect of LSD1i was reported in AMLs without *PML-RARA* (i.e., non-APL AML), where LSD1i sensitized the pro-differentiation effect of ATRA, an agent only for *PML-RARA*-positive APLs (**Figure 1H**) (122). Here, combinational treatment of non-APL human AMLs with ATRA and LSD1i showed a potent anti-leukemic effect, with increased H3K4me2 and expression found at the myeloid differentiation genes (122). Several LSD1i are now in clinical trials in refractory AMLs.

KDM4C Inhibitors

KDM4C (also known as *JMJD2C/GASC1*) encodes an "eraser" carrying the H3K9-demethylating and gene-activating activities. Like PRMT1, KDM4C was also found to interact with various AML oncoproteins including MLL-GAS7 and MOZ-TIF2 (103). Knockdown of *KDM4C* partially reversed target gene activation mediated by these AML fusion proteins. Moreover, pharmacological inhibition of KDM4C can be achieved by an inhibitor SD70 and proposed to be a potentially new AML treatment strategy (103) (**Figure 1E**).

PERSPECTIVES

In short, epigenetic modulators emerge rapidly as potential drug targets for the treatment of currently incurable AMLs. With many showing high selectivity, high potency and/or promising drug-like properties, the already developed epigenetic inhibitors shall provide potential alternatives or adjuvants to current therapeutic arsenal that frequently relies on non-specific cytotoxic agents. While the area is in its infancy, we wish to pinpoint several directions that may broaden application of epigenetic inhibitors.

Newly Validated Epigenetic Factors and Cancer Cell Dependency Pathways Remain to Be Targeted

An existing advance in understanding the biology of gene activation is recent identification of a YEATS family of protein domains as a new "reader" class of histone acylation such as acetylation and crotonylation (123, 124). In the *MLL*-rearranged AML cells, a YEATS domain harbored in ENL was recently shown to be crucial for tethering/stabilizing the MLL fusion proteins at sites with histone acetylation to induce downstream gene activation (125, 126). Similar mechanisms might be also functional among *DNMT3A*-mutated leukemias (**Figure 1D**, right) where *DNMT3A* mutations perturb efficient CpG methylation at cis-regulatory sites leading to elevated histone acetylation and increased binding of DOT1L-associated complexes that harbor YEATS-containing AF9 and ENL (96). Furthermore, LEDGF (lens epithelium-derived growth factor), a protein that mediates chromatin association of the MLL complex, was previously found to be essential for *MLL*-rearrangement-induced leukemic transformation (55). A recent work reports that the PWWP domain of LEDGF recognizes and "reads" H3K36 methylation added by the ASH1L methyltransferase at proximal promoter chromatin, and this event was found critical for recruiting/stabilizing MLL fusions onto target sites to activate gene expression in leukemia cells (127). Additionally, NSD1 and NSD3, two related H3K36 methyltransferases, were previously found to be aberrantly rearranged in ~15% of pediatric AMLs (31) and their "writing" SET domains represent the validated site that remains to be

pharmacologically targeted (30). Thus, these discovered circuits should offer additional therapeutic opportunities, both in the "reading" domains (YEATS of AF9 or ENL; PWWP of LEDGF) and the catalytic "writing" domains (SET of ASH1L and NSD1/3), for AML treatment.

Identification of BRD4 as a novel AML dependency was achieved through shRNA-based functional screening of epigenetic factors (110). Small-guide RNA-based CRISPR/Cas9 technology has provided an alternative system to perform screening in human AML cell lines, which recently led to identification of the histone acetyltransferase KAT2A/GCN5 as an AML dependency gene (128). In future, functional genomics studies using a range of AML cell lines that represent various genetically defined AML subtypes, as well as validation with primary human AML samples, are likely to produce useful information for subtype-specific dependencies on epigenetic modulators, which would guide drug discovery efforts aiming to developing the personalized AML treatment.

Implication in the Treatment of Pre-leukemic Disease

Somatic mutations of several epigenetic modulators (DNMT3A, TET2, IDH1/2) occur frequently among patients with pre-leukemia diseases such as MDS and apparently healthy individuals with clonal hematopoiesis or CHIP, an aging-related phenotype associated with increased risk of AML (21, 22, 25, 26). These mutations and resultant epigenetic deregulations are likely to be the "founder" lesion initiating pre-malignant disease and shaping subsequent malignant formation. Identification of the epigenetic vulnerabilities associated with these gene mutations in the context of AML shall provide useful information on how to treat premalignant diseases. For example, using a murine AML model harboring the coexisting kinase and *DNMT3A* mutations, a recent study demonstrated that *DNMT3A* mutation induced epigenetic dysregulation to promote "stemness" gene-expression programs, a process that can be reversed by DOT1L inhibitors (**Figure 1D**, right) (96). We speculate that the same mechanism/pathways act among premalignant diseases, and if so, the similar epigenetic inhibitors could reverse the premalignant alternations thus preventing malignant development in individuals with MDS or CHIP. In support, the epigenetic inhibitors and hypomethyating agents such as 5-Aza delay malignant transformation of MDS and are FDA-approved drugs for its treatment. However, as a life-threatening disease with a risk of conversion into AML, MDS has additional immediate needs to treat other complications such as anemia and transfusion associated iron overload, bleeding and infectious risk associated with the cytopenias. Currently, the definitive cure of MDS-associated leukemia risk is still allogeneic HSC transplantation. As for CHIP, there is consensus in the field that the relatively low risk of transformation of CHIP does not warrant the targeted therapies. Potential application of targeted epigenetic inhibitors in the treatment of pre-AML diseases such as MDS and myeloproliferative neoplasms warrants further investigation.

Potential Drug Resistance and Combinational Therapy

Resistance to drug remains a challenge in achieving durable remissions in cancer and epigenetically targeted drugs are no exception. The molecular understanding of resistance in epigenetic therapy is just at its beginning. For example, *MLL*-rearranged leukemias with PRC2 loss, either pre-existing or acquired, are resistant to BRDi presumably due to enhanced transcription of oncogenes such as *MYC* (129); furthermore, recent reports documented acquisition of somatic mutation by blood cancer cells during resistance to BRDi or EZHi (129, 130). Conceptually, combinational treatment using two or more drugs that target multiple cancer cell dependencies should help overcome treatment resistance. Furthermore, regardless of drug resistance, combinational therapy should improve treatment when their potential toxic effect can be mitigated. As mentioned above, a good example is that LSD1i sensitizes non-APL AML cells to ATRA treatment (122). In addition, DOT1Li and BRDi are shown to be synergistic in treating *MLL*-rearranged leukemia, possibly due to functional collaboration between DOT1L and BRD4 at the highly transcribed super-enhancer genes (131). Future studies of drug resistance, toxicity, and combinational treatment strategies would be necessary to further develop and optimize the existing leads into those useful compounds for clinical trials.

AUTHOR CONTRIBUTIONS

RL and GW wrote the manuscript and generated the figures/tables.

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REVIEW
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Leukemia-Initiating Cells in T-Cell Acute Lymphoblastic Leukemia

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T-cell acute lymphoblastic leukemia (T-ALL) is a hematological malignancy characterized by the clonal proliferation of immature T-cell precursors. T-ALL has many similar pathophysiological features to acute myeloid leukemia, which has been extensively studied in the establishment of the cancer stem cell (CSC) theory, but the CSC concept in T-ALL is still debatable. Although leukemia-initiating cells (LICs), which can generate leukemia in a xenograft setting, have been found in both human T-ALL patients and animal models, the nature and origin of LICs are largely unknown. In this review, we discuss recent studies on LICs in T-ALL and the potential mechanisms of LIC emergence in this disease. We focus on the oncogenic transcription factors *TAL1*, *LMO2*, and *NOTCH1* and highlight the significance of the transcriptional regulatory programs in normal hematopoietic stem cells and T-ALL.

Keywords: T-cell acute lymphoblastic leukemia, leukemia initiating cells, TAL1, NOTCH1, core regulatory circuit

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INTRODUCTION

Since the establishment of functional repopulation assays in the late 1990s, accumulating studies have demonstrated the existence of cancer stem cells (CSCs) that possess self-renewal capability and the potential to generate differentiated daughter cells (1–4). Purification of a unique cell population based on the expression of specific cell surface markers enabled the prospective isolation of CSCs in various types of cancers. A prime example is acute myeloid leukemia (AML), which has been extensively studied as a model disease for the establishment of the CSC theory. Although T-cell acute lymphoblastic leukemia (T-ALL) has many similarities in pathophysiological features to AML, the CSC concept in T-ALL has not been firmly established. Leukemia-initiating cells (LICs), which can generate leukemia in a xenograft setting, have been confirmed in both human T-ALL patients and mouse models (5–12), but common stem cell markers have not been identified in this disease.

Unlike AML, which arises from the bone marrow, T-ALL clones originally emerge in the thymus, which does not provide a niche for hematopoietic stem cells (HSCs) (13–15). In many T-ALL cases, oncogenes are driven by a chromosomal translocation involving the *T-cell receptor* (*TCR*) gene locus, which is associated with somatic recombination in immature thymocytes (16). This suggests that T-ALL arises from committed T-cell precursors, but not from multi-potent HSCs. It is likely that developing thymocytes acquire stemness capability as a consequence of genetic and epigenetic abnormalities. On the other hand, recent studies have shown that early thymocytes can self-renew under certain condition (17, 18). Therefore, it is also possible that T-ALL arises from thymocytes that already possess self-renewal potential.

GENETIC ABNORMALITIES IN T-ALL: OVERVIEW

Acute lymphoblastic leukemia (ALL) is the most common type of childhood malignancy (19). Approximately 20% of ALL cases are classified as T-ALL. T-ALL is an aggressive malignancy characterized by the clonal proliferation of immature T-cell precursors that arise from the thymus and infiltrate into the bone marrow and peripheral blood (13–16). Enormous progress has been made in

the treatment of T-ALL in the past few decades, with long-term remission observed in approximately 80% of children and 60% of adult patients (20, 21). However, a substantial fraction of T-ALL patients fail to respond to induction therapy or relapse within 2 years of diagnosis. The prognosis for this group of patients is very poor, with a 5-year survival rate of less than 25% (22).

T-ALL development requires multi-step genetic alterations of crucial oncogenes and tumor suppressors *via* different recurrent mechanisms, such as chromosomal translocations, intrachromosomal rearrangements, and mutations in protein-coding genes or enhancer elements, as well as epigenetic abnormalities (13–16). These alterations commonly affect genes that are required for cell growth, survival, and differentiation during normal T-cell development (14, 16). Results from recent genome-wide sequencing studies across different types of cancers indicate that ALL exhibits the fewest genomic abnormalities compared with other hematological malignancies and solid tumors (23, 24). This suggests that relatively few molecular alterations are crucial and significant enough to hijack the normal developmental program and promote malignant transformation.

Molecular Abnormalities That Delineate the T-ALL Subgroups

Chromosomal translocation is a hallmark of T-ALL (16, 25). The most commonly observed translocations involve the *TCR* loci on chromosome 14q11.2 (*TCR alpha/delta*), 7q34 (*TCR beta*), and 7p14 (*TCR gamma*). They are often fused to a range of oncogenic transcription factors that are important during different stages of normal hematopoiesis and lymphocyte development (13–16), resulting in constitutive and ectopic expression of these factors. The affected genes include transcription factors from the basic helix-loop-helix family, including *TAL1*, *TAL2*, and *LYL1*; the homeobox family, including *TLX1*, *TLX3*; the *HOXA* genes; *NKX2-1*; *MYB*; and the LIM domain-only (LMO) genes *LMO1* and *LMO2*.

Cytogenetic analysis coupled with gene expression profiling has been used to classify T-ALL into several subgroups: *TAL1/LMO1/2-*, *TLX1/3-*, *HOXA/MEISI-, LMO2/LYL1*, and *NKX2-1*-positive T-ALL cases (25–27). Briefly, *TAL1*, *LMO2*, and *LYL1* are essential regulators of hematopoiesis (28–33). Those factors can be oncogenic when abnormally or ectopically overexpressed in immature T-cells (8, 34, 35), as we discuss later. Besides translocation, *TAL1* is aberrantly induced by intrachromosomal rearrangement or mutations in the enhancer (36–38). *TLX* genes are expressed during embryogenesis and required for normal development of the spleen (39). Overexpression of *TLX1* leads to T-ALL and exhibits aneuploidy in a mouse model (40). The *HOX* genes are a family of homeodomain containing transcription factors, which are expressed in HSCs and immature progenitors compartments (41). HOX cofactors such as MEIS1 which is important to improve binding selectivity and specificity of HOX proteins are also found to be overexpressed in T-ALL (42). Notably, these subgroups are mutually exclusive to each other and reflect the arrest of T-cell differentiation at different stages, including (a) early blockage at the $CD4^{-}CD8^{-}$ double-negative (DN) stage of thymocyte development for the *LMO2/LYL1* group, (b) early cortical T-ALL ($CD1a^{+}$, $CD4^{+}$, and $CD8^{+}$) with expression of *TLX1/3 or NKX2-1*, and (c) late cortical T-ALL ($CD3^{+}$, $CD4^{+}$, and $CD8^{+}$) with expression of *TAL1* (26, 43). More recently, the early T-cell precursor (ETP) subtype has been defined based on cell surface markers and gene expression profiles (43). ETP is enriched in the *LMO2/LYL1* group but can be also found in other subgroups (27).

Activation of the NOTCH1 Pathway

Another major molecular abnormality in T-ALL is the mutations that affect the *NOTCH1* pathway (13–16). *NOTCH1* signaling is essential for normal T-cell precursor development and is strictly regulated in a ligand-dependent manner. Remarkably, activating mutations affecting *NOTCH1* are observed in more than 50% of T-ALL cases (44). Aberrant activation of *NOTCH1* was originally identified in T-ALL cases harboring the t(7;9)(q34;q34.3) chromosomal translocation, through which the intracellular form of NOTCH1 (ICN1) gene fuses to the *TCR beta* regulatory element, leading to expression of a constitutively active, truncated form of NOTCH1 (45). However, the majority of aberrant *NOTCH1* activation observed in T-ALL occurs due to mutations in its heterodimerization (HD) domain and/or the PEST domain (44). Mutations in the HD domain cause the NOTCH1 receptor to be susceptible to proteolytic cleavage and release of the ICN1 protein, while the PEST domain mutations inhibit the proteasomal degradation of ICN1 by the FBXW7 ubiquitin ligase, thus lengthening its half-life in T-ALL cells. Additionally, deletions or inactivating mutations of *FBXW7* are frequently observed in T-ALL (46, 47).

The oncogenic roles of NOTCH1 signaling in T-ALL have been extensively studied both in humans and in animal models. Overexpression of ICN1 protein in mouse hematopoietic progenitor cells leads to very rapid onset of T-ALL (48). Subsequent studies have identified the direct transcriptional targets of NOTCH1 in T-ALL, which are enriched in genes responsible for cell proliferation, metabolism, and protein synthesis, including *MYC* and *HES1* (49–53). These studies implicated *NOTCH1* as a driver oncogene in T-ALL.

Epigenetic Regulators and Other Molecular Abnormalities

Alterations in genes that encode for epigenetic regulators such as *EZH2*, *SUZ12*, and *EED* have been also identified in T-ALL (54–57). These genes make up the core components of the polycomb repressor complex 2 that mediates the repressive histone mark H3 lysine 27 trimethylation (H3K27me3). Loss-of function mutations in these genes can lead to accelerated leukemia onset in mice (54, 55), suggesting that they act as tumor suppressors in T-ALL. Recent studies have shown that the KDM6A/UTX, which is responsible for demethylating H3K27me3, have cases of inactivating lesions and downregulation of this gene accelerates NOTCH1-driven leukemia in mice (55, 56). In contrast, another study showed that KDM6A/UTX acts as a pro-oncogenic cofactor when it is recruited by TAL1 in T-ALL to activate target gene expressions (57).

Other recurrent molecular abnormalities include genes that encode for proteins involved in the JAK-STAT signaling pathway, such as *IL7R*, *JAK1*, *JAK3*, and *STAT5B*; genes that are

involved in PI3K-AKT signaling pathways, such as *PI3K* and *PTEN*; and genes involved in RAS-MAPK signaling pathways, such as *HRAS*, *KRAS*, and *PTPN11* (13–16). Additionally, recent sequencing studies discovered several new alternations including mutations in *CCND3, CTCF*, and *MYB* genes (27), and *SPI1/PU.1* fusions (58).

CSC AND LIC CONCEPTS

The concept of CSCs originates from the observation that tumors consist of a hierarchically organized, heterogeneous population of cells with a minority of biologically distinct subsets capable of self-renewing and giving rise to clonal daughter cells (1–4). A number of studies have shown the existence of CSCs in various types of cancers. The CSC model also indicates that this rare cell population is able to tolerate therapeutic agents such as chemotherapy and radiation that eradicate the bulk of the rapidly proliferating tumor cells, thus resulting in inevitable cancer relapse in the long term (1–4, 59).

The most definitive property of stem cells lies in their self-renewal ability (1–4). Self-renewal in normal cells or CSCs gives rise either to one stem and one differentiated daughter cell *via* asymmetric division or to two stem cells *via* symmetric division. The general consensus in stem cell research is that CSCs are able to initiate and maintain clonal growth in long-term repopulation assays where the cancer cells are serially transplanted into immunodeficient recipient mice. The purification of a unique cell population based on the expression of specific cell surface markers has allowed researchers to isolate CSCs in various cancers, including AML and breast cancer (60–62). However, such populations have not been well characterized in many other cancers, including T-ALL. Hence, other terms, such as "tumor-initiating cells (TICs)" or "LICs," have been coined to refer to the ability of transplanted cells to initiate tumor formation or leukemia in animals and are more preferentially used in experimental settings (1). Notably, the TIC/LIC concept is distinct from the "cell-of-origin" idea, as TIC/LIC strictly refers to cells in which tumorigenesis can be initiated (63), whereas the cell of origin that received the first oncogenic "hit" would progressively accumulate mutations during clonal evolution of the tumor. The acquisition of stem cell-like properties may occur at a much later stage of tumorigenesis in the evolved cells than the original cell that received only the first hit. In this regard, John Dick has proposed that TICs/LICs should be defined by their ability to (a) generate tumors in xenograft models that are representative of the parent tumors, (b) generate tumors upon serial passages in xenograft models, and, lastly, (c) give rise to daughter cells that can proliferate but might not be able to establish tumors after serial passages (1).

LICs IN HUMAN AML AND ALL: DISCOVERY AND CHALLENGES

The presence of LICs was first reported by Dick and his colleagues in the late 1990s in studies of AML (60, 61). In a series of seminal studies, they showed that a rare subset of $CD34^+CD38^-$ cells isolated from AML patients was able to initiate the disease when transplanted into severe combined immunodeficient (SCID) mice (60). Crucially, the more differentiated $CD34^+CD38^+$ cells were unable to generate leukemia. In the initial study, secondary transplant of leukemic cells from SCID mice failed to generate leukemia. However, using a more immunocompromised non-obese diabetic (NOD)/SCID mouse model, the authors demonstrated that $CD34^+CD38^-$ cells have self-renewal properties (61). Furthermore, this group has shown that the engrafted $CD34^+CD38^-$ cells were able to give rise to more differentiated leukemic cells (61). Thus, this study demonstrated the presence of a leukemic hierarchy, with the $CD34^+CD38^-$ LICs at the top of the pyramid.

These results have also been challenged by studies utilizing more immunocompromised mouse models. For example, in the NOD/LtSz-scid IL-2Rγchain^{null} (NSG) mouse model, AML LICs are not only present exclusively in $CD34^+CD38^-$ cells (64). Results from this model showed that LICs can also be found in more differentiated $CD34^-$ and $CD38^+$ cells. The concept of LICs was also challenged by a study in which leukemic cells from *Ras*-induced T-cell lymphoma or an *E*μ-*Myc* model of pre-B/B-cell lymphoma were shown to engraft in non-congenic animals regardless of the number of cells injected (65). The authors stressed the need to interpret data from serial transplantations more carefully, since failure to show engraftment could simply be due to the inability of the human cells to adapt to the microenvironment in the mouse.

The identification of LICs in ALL is even more challenging. To date, the identity and presence of LICs in human ALL has not been firmly established and is still debatable. Early studies in B-cell ALL (B-ALL) reported that the relatively immature $CD34^+CD19^-$ cells could contain LICs (66, 67). However, recent studies have found that more mature $CD34^+CD19^+$ leukemic blasts could initiate leukemia in *ETV6-RUNX1*- or *TEL-AML1*-positive B-ALL cases (68). In addition, a more recent study on *MLL-AF4*-positive infant ALL indicated that the LICs capable of reconstituting transplanted mice are exclusively $CD19^+$ but exhibit variable CD34 expression (69). These studies highlight the heterogeneity of LICs in B-ALL cases and suggest that different cytogenetic abnormalities might play a role in determining the type of LICs present.

Similarly, the nature of LICs in human T-ALL has not been well characterized. An early study suggested that $CD34^+CD4^-$ and $CD34^+CD7^-$ cells, which make up a fraction of the leukemic cells from pediatric T-ALL patients, had leukemia-initiating properties when engrafted into NOD/SCID mice (5). A follow-up study investigating LIC activity in cortical/mature T-ALL patients reported that the $CD34^+CD7^-$ population from these patients contained normal hematopoietic cells that were able to differentiate into different lineages, while the $CD34^+CD7^+$ cells possessed LIC capability (6). Dick and Chiu et al. have also reported that the $CD7^+CD1a^-$ subset is enriched for LIC activity and exhibits glucocorticoid resistance (7).

LICs IN ANIMAL MODELS OF T-ALL

Although the findings on LICs in primary human T-ALL are limited, several studies have been performed on transgenic animal models of T-ALL.

LICs in the Tal1-Induced Mouse Models of T-ALL

One of the most commonly used T-ALL mouse models in the study of LICs is the *Tal1* transgenic mouse model; approximately 30% of these mice develop leukemia after a long latency period (8, 34, 35). Notably, tumor onset and progression can be accelerated by co-expressing the oncogene *Lmo1* or *Lmo2*. Tremblay and Hoang et al. have found that overexpression of *Tal1* and *Lmo1* resulted in a marked expansion of T-cells making up the $CD4^{-}CD8^{-}$ DN1, DN3, and DN4 populations and blocked differentiation into the $CD4^{+}CD8^{+}$ double-positive (DP) stage (8). The leukemia cells contain LICs that can generate leukemia in transplanted mice. Interestingly, they demonstrated that LICs are enriched in the DN population, especially DN3 and DN4, compared with the DP population and that these LICs could give rise to more differentiated leukemic cells (8). This study suggested that committed DN-stage T-cells with ectopic expression of *Tal1* and *Lmo1* exhibit self-renewal properties while retaining the potential to differentiate. A subsequent study by Kelliher and her colleagues utilizing the *Tal1/Lmo2* mouse model of T-ALL also showed that the DN3 and DN4 populations of leukemia cells possess LIC properties and drive T-ALL leukemogenesis (9, 10). In support of these data in double transgenic mice, McCormack and Curtis et al. demonstrated that *Lmo2* single transgenic mice show an increase in thymic progenitors in the DN3 subset while also displaying properties of LICs in serial transplantation experiments (11). Interestingly, several genes, such as *Hhex* and *Lyl1*, that are normally expressed in HSCs were expressed in the self-renewing cells. This suggests that an HSC-like transcriptional program might be induced in T-ALL cells. Taken together, these studies indicated that DN3 thymocytes gained self-renewal potential.

Significance of NOTCH1 Activation in Mouse Models of T-ALL

Notably, gain-of-function mutations of the *Notch1* gene are frequently found in the *Tal1*/*Lmo1* mouse model of T-ALL (8, 70), similar to observations in human T-ALL (44). Tremblay and Hoang et al. reported that *Notch1* mutations occurred mostly at the DN4 preleukemic stage and that the mutations could also be observed during overt leukemia in the same mice (8). Interestingly, leukemia development and *Notch1* mutations were abolished in the absence of *CD3e*. Similarly, Cui and Mackall have reported that forced expression of *TCR* during early stages of T-cell development caused T-ALL in 100% and all cases harbored *Notch1* mutations (71). These results suggested that pre-TCR and TCR signaling have a permissive role in the acquisition of *Notch1* mutations and that active NOTCH1 signaling confers clonal dominance upon leukemia development.

Importantly, Tremblay and Hoang et al. showed that *Notch1*/*Tal1*/*Lmo1* triple transgenic mice developed leukemia significantly faster than single or double transgenic animals (8). The DN1–DN2 and DN3–DN4 subsets from *Notch1*/*Tal1*/*Lmo1* triple transgenic mice were able to induce T-ALL in secondary hosts with high efficiency compared with *Tal1*/*Lmo1* double transgenic mice (8). A subsequent study from the same group further suggested that *Notch1* drives self-renewal of thymocytes from the *Tal1*/*Lmo1* mouse model *via* its target genes *Hes1* and *Myc* (12). Treatment of the leukemic cells before and throughout the transplantation period with γ-secretase inhibitor, which inhibits the catalytic cleavage of NOTCH1, completely abolished the LIC function of the leukemic T-ALL cells. Given the importance of active NOTCH1 signaling in primary human T-ALL patient samples, these studies support the hypothesis that *Notch1*-activating mutations are important for the cells to gain clonal dominance during disease development.

Notably, a recent study by Pear and his colleagues showed that LICs in T-ALL induced by the overexpression of a mutant form of *NOTCH1* in adult mouse bone marrow progenitor cells are enriched in a single-positive (SP) T-cell population consisting of the $CD8^{+}CD4^{-}HSA^{hi}$ fraction of cells (72). Thus, the types of LICs generated could be different from those found in the *Tal1*/*Lmo* transgenic mouse model.

LICs in Other Animal Models of T-ALL

Additionally, several other animal models of T-ALL have been used to analyze LICs. In studies of *Pten*-null mice, which develop T-ALL with 100% penetrance, LICs are identified as $cKit^{mid}CD3^{+}$ cells and often overexpress *Myc* due to a recurrent chromosomal translocation at t(14;15). The self-renewal properties of these LICs could also be abolished *via* targeting both the deregulated PI3K signaling pathway and *Myc* expression concurrently (73, 74).

Apart from studies in T-ALL mouse models, a T-ALL zebrafish model has also been employed to investigate the presence of LICs in T-ALL. Langenau and Look et al. reported that the *Myc*-induced T-ALL zebrafish model demonstrates very similar molecular characteristics to human T-ALL patients that overexpress *TAL1* and *LMO2* (75). More recently, Langenau and his colleagues used syngeneic clonal zebrafish that can be transplanted into hosts without prior irradiation to show that the proportion of LICs in the *Myc*-induced T-ALL zebrafish model is much higher than previously reported (76). Further studies by the same group demonstrated that abnormal activation of the AKT-mTORC1 signaling pathway is the main underlying cause of the acquisition of LIC potential (77). These results support the mouse studies on LICs in T-ALL.

THE ROLE OF MICROENVIRONMENT IN T-ALL PATHOGENESIS

Another important consideration in the study of LICs is the interaction between leukemia cells and non-leukemia cells in the microenvironment. Bone marrow niche is essential for the maintenance and regulation of normal HSCs (78, 79). AML and ALL cells also home and expand in the bone marrow. Several studies have shown that signals from the bone marrow niche can dictate the survival of LICs and their responses to various types of treatment administered (80, 81).

Notably, two recent studies have elucidated the roles of bone marrow niche in T-ALL pathogenesis and implicated the CXCL12-CXCR4 signaling axis in the maintenance and progression of T-ALL (82, 83). CXCL12 is a chemokine secreted from endothelial and mesenchymal cells in the bone marrow and binds to its G protein-coupled receptor CXCR4 (79). Pitt et al. showed that in the bone marrow, T-ALL cells reside in close contact with stroma cells that secrete Cxcl12 (82). Deletion of the *Cxcr4* receptor resulted in a reduction of leukemia burden and their infiltration into the bone marrow,

thymus, and spleen in mouse model of T-ALL (82). Treatment of patient-derived human T-ALL cells in xenografts with a CXCR4 antagonist also produced the same result. Importantly, the authors observed a reduction in LIC activity in the absence of *Cxcr4* in mice (82). Passaro et al. independently showed that depletion of *CXCR4* affected T-ALL cell migration and expansion (83). Furthermore, the authors reported that calcineurin regulates CXCR4 expression in a cortactin-dependent manner (83). Those studies demonstrated the roles of the bone marrow niche in the maintenance of T-ALL.

SELF-RENEWAL CAPABILITY OF T-ALL CELLS: DOES IT ALREADY EXIST IN THE THYMUS OR IS IT ACQUIRED?

One of the fundamental questions in LIC research is whether the LICs are derived from cells that already have self-renewal potential, such as HSCs, or whether they emerge from differentiated cells by newly acquiring stemness capability. T-ALL is derived from committed T-cell precursors in the thymus, which does not provide a niche for HSCs. The chromosomal translocation involving the *TCR* gene locus found in many T-ALL cases is associated with somatic recombination in immature thymocytes (16). These findings suggest that developing thymocytes likely acquire stemness capability as a consequence of genetic and epigenetic abnormalities. *Tal1* and *Lmo1/2* transgenic mice show an increased number of thymic progenitors that can generate leukemia, indicating that these oncogenic transcription factors are capable of inducing LIC ability in immature thymocytes.

On the other hand, recent studies have shown that normal thymocytes can self-renew in the absence of competitive precursor replacement (17, 18, 84). In general, HSCs differentiate into common lymphoid progenitor (CLP) cells in the bone marrow. CLPs migrate into the thymus and are committed to T-cell precursors that can differentiate into the DN to DP stage of thymocytes. In this well-accepted model, a continuous supply of lymphoid progenitor cells from the bone marrow is necessary to support T-cell development. Interestingly, Martins and Rodewald et al. recently reported that in *Rag2*$^{-/-}\gamma c^{-/-}Kit^{W/Wv}$ mice, which do not produce lymphoid progenitors from the bone marrow, a transplanted wild-type thymus sustained T-cell development for a long period of time (17). Similarly, Peaudecerf and Rocha et al. reported that in *Rag2*$^{-/-}\gamma c^{-/-}$*IL7 receptor*$^{-/-}$ mice engrafted with a wild-type thymus, persistent development of donor T-cells was observed (18). In this setting, host lymphoid progenitors can still migrate into the thymus and replace donor thymocytes but cannot differentiate after the DN2 stage, because IL7R signaling is required for the proliferation of early T-cell progenitors. Thus, competitive replacement by the host lymphoid progenitors is restricted to the DN1 and DN2 stages in this mouse model. This indicates that the donor thymus, which contains DN3 thymocytes, sustained T-cell development. Although this mechanism may be activated only when the competitive DN3 thymocytes are absent, these studies indicate that the thymus harbors cell populations with self-renewal potential that are capable of reconstituting the full diversity of T-cells.

Importantly, a large fraction of mice develop T-ALL in these settings (85). *Tal1* and *Lmo2* expression is strongly upregulated in these mouse T-ALL cells, and *Notch1* mutations are also frequently found. This is consistent with observations in *Tal1* and *Lmo2* transgenic mice, which exhibit LICs in the DN3 subset and acquire *Notch1* mutations (8). One possible mechanism is that differentiation arrest and expansion of DN3 thymocytes caused by overexpression of oncogenic transcription factors result in a loss of competitive replacement by bone marrow-derived progenitor cells, leading to activation of self-renewal machinery and malignant transformation. Alternatively, a loss of competitive replacement may result in the failure to silence the transcription factors that are normally expressed in stem and progenitor cells. Although the intrinsic mechanism of self-renewal in thymocytes is still unclear, these studies suggest that in T-ALL, LICs may arise from thymocytes that already have self-renewal potential *via* cellular competition.

TRANSCRIPTIONAL REGULATORY PROGRAMS IN HSCs AND T-CELL DIFFERENTIATION

Mouse studies have suggested that cellular competition potentially triggers the self-renewal capability of immature thymocytes, which may eventually lead to malignant transformation *via* the acquisition of genetic abnormalities such as *Notch1* mutations. In human T-ALL, a loss of competition may be caused by overexpression of oncogenic transcription factors such as *TAL1* and *LMO2*. Notably, these transcription factors themselves are also involved in the stem cell regulatory program during normal hematopoiesis.

In general, cellular differentiation of hematopoietic cells is associated with developmental restrictions that can be illustrated by the analogy of a "ball rolling down a hill" (86). During the differentiation process, HSCs lose their self-renewal and lineage potential. This process is regulated by an epigenetic and transcriptional network (87–89). A number of hematopoietic transcription factors are involved in this process. For example, TAL1 has been implicated as an essential regulator of hematopoiesis (33). TAL1 is expressed in normal HSCs, progenitor cells, and erythro-megakaryocytic lineages. Studies in knockout mouse models have revealed that this factor is required for hematopoietic specification and the genesis of hematopoietic cells (28, 29). In normal hematopoietic cells, TAL1 forms a large transcriptional complex with E-protein, LMO2, LDB1, and GATA (90). Several other transcription factors, including RUNX1 and the ETS family proteins, also frequently co-regulate downstream target genes (91).

Interestingly, these transcription factors co-occupy their own regulatory elements and positively regulate each other, thus forming an interconnected auto-regulatory loop (87, 88, 92). This structure is also termed a "core regulatory circuit" (CRC) and has been reported in other stem cells (93–95). For example, in embryonic stem cells, three key transcription factors that establish stem cell identity, OCT4, SOX2, and NANOG regulate each other (93, 94). This mechanism is thought to reinforce and stabilize downstream gene expression by "interlocking" the regulatory loop and is likely required for stem cell properties (92). Importantly, ectopic expression of these transcription factors can reprogram somatic cells back into stem cells, as has been established for the production of induced pluripotent stem cells (96). Similarly, recent studies have demonstrated that adult somatic fibroblasts can be reprogrammed into multi-potent hematopoietic stem progenitor cells by ectopic

overexpression of *TAL1*, *LMO2*, *RUNX1*, *GATA2*, and *ERG* ("iHSPCs") (97). This clearly indicates that a relatively small number of transcription factors are sufficient to control cell fate and identity.

In contrast to the regulatory circuit in HSCs, a very different type of transcriptional program is formed in developing thymocytes to regulate genes that are essential for T-cell differentiation (98). This process requires a number of transcription factors working in a cascade as well as the interactions in the microenvironment (**Figure 1**). Briefly, the NOTCH ligand expressed on thymic stromal cells induces expression of the transcription factors TCF7 and GATA3, which regulate other key transcription factors such as BCL11B and LEF1 (98). During this process, stem cell transcription factors such as TAL1 and LMO2 are gradually silenced, resulting in the loss of stem and progenitor cell potential. Meanwhile, E-proteins (E2A and HEB) are functionally and transcriptionally upregulated to induce *RAG1*, *RAG2*, and *PTCRA*, for example, which are required for somatic *TCR* recombination (99, 100). Such orchestrated stage-specific regulation of transcription factors mediates the T-cell differentiation process like a "ball rolling down a hill." TAL1 and LMO2 silencing and E-protein upregulation are crucial to controlling the reciprocal switch from self-renewal to lineage-specific genetic programs. In other words, ectopic expression of TAL1 and LMO2 in developing thymocytes may rewrite the internal regulatory program.

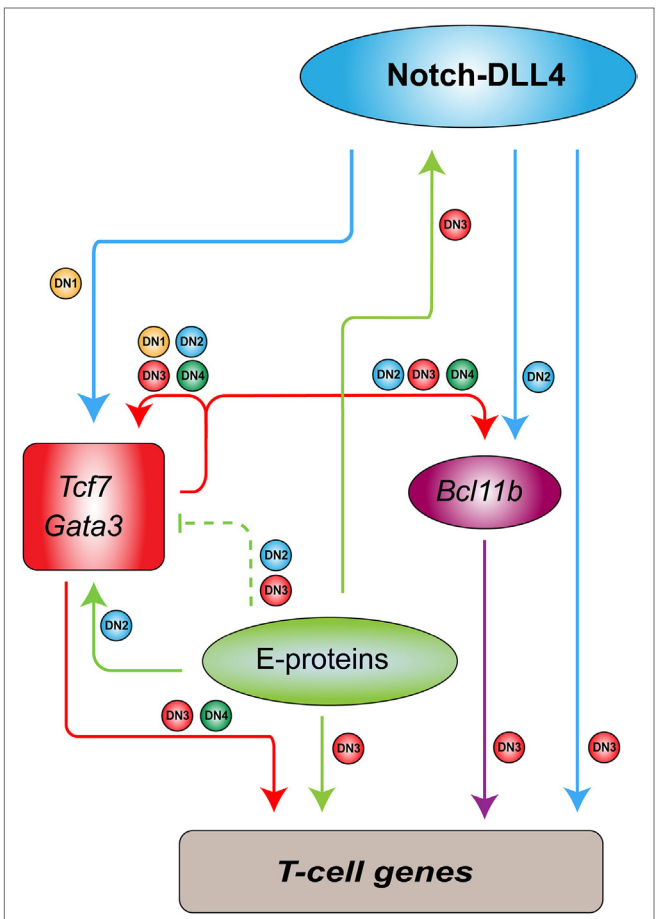

FIGURE 1 | Transcriptional regulatory program in developing thymocytes [adapted and modified from figures by Yui and Rothenberg (98)]. In mouse models, Notch-DLL4 ligand expressed on thymic stroma cells induces the expressions of Tcf7 and Gata3, which regulate additional transcription factors such as Bcl11b. These factors, together with E-proteins and Notch1, stimulate the expressions of T-cell genes in a differentiation stage-specific manner. Arrows show activation or positive regulation. Dashed lines indicate "soft repression" of the maximal activity of the target. Small circles beside the lines correspond to the differentiation stages [double-negative (DN1–4)] at which the regulation occurs.

ABERRANT TRANSCRIPTIONAL REGULATORY PROGRAM IN *TAL1/LMO*-POSITIVE T-ALL

Interestingly, *TAL1* and *LMO2* function as oncogenes in T-ALL cells, similar to their behavior in normal HSCs (33). *TAL1* is expressed in 40–60% of T-ALL cases due to chromosomal translocation, intrachromosomal rearrangement, or mutations in non-coding elements (16, 36–38). These alterations replace an endogenous regulatory element controlling *TAL1* expression with a new, potent enhancer that drives ectopic expression of this oncogene. Similarly, *LMO2* or its related gene *LMO1* is ectopically expressed in T-ALL cells due to chromosomal translocation or mutations in the regulatory elements (16, 101, 102). *LMO1* or *LMO2* is often expressed together with *TAL1*. In T-ALL cells, TAL1 and LMO proteins form a transcriptional complex with E-proteins and GATA3 (103, 104). Their regulatory partners in normal HSCs, RUNX1, ETS1, and MYB are also endogenously expressed in T-cells (98). We previously reported that TAL1, GATA3, RUNX1, and MYB co-occupy their own regulatory elements and positively regulate each other, forming the interconnected auto-regulatory structure (**Figure 2**) (105). These factors coordinately regulate downstream target genes. All these mechanisms are essentially the same as the machinery observed in normal HSCs.

At the same time, TAL1 counteracts the function of E-proteins by sequestering them, thus preventing them from transcriptionally inducing genes required for T-cell differentiation (99, 100). In this context, E-proteins act as tumor suppressors, as several groups

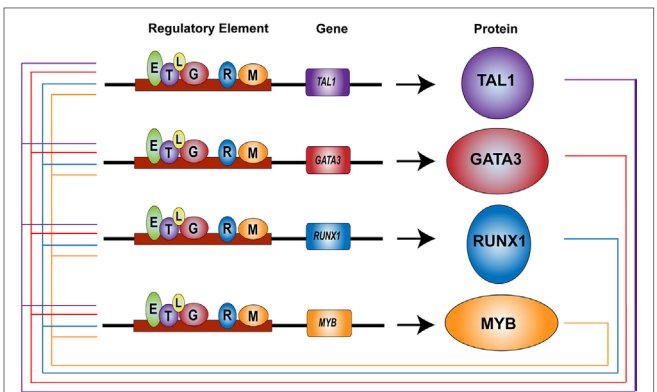

FIGURE 2 | Core regulatory circuit in T-cell acute lymphoblastic leukemia (T-ALL) (38, 105). TAL1, GATA3, RUNX1, and MYB proteins (circles) bind at their own regulatory elements (boxes) and positively regulate each other, thus forming an interconnected auto-regulatory loop structure in T-ALL cells. TAL1 (T), GATA3 (G), RUNX1 (R), MYB (M), E-protein (E), LMO1/2 (L).

have shown that *E2a*-deficient mice develop T-cell lymphoma and that this deficiency accelerates leukemia onset and progression in *Tal1*-transgenic mice (10, 106). Our recent study also revealed that in human T-ALL cells, TAL1 opposes the expression of E-protein target genes (105). Thus, the imbalance between the oncogenic TAL1 complex and E-protein is a primary determinant underlying the molecular pathogenesis of T-ALL (**Figure 3**) (107). Together, ectopic expression of *TAL1* and *LMO1/2* leads to the induction of HSC-like machinery and disruption of the T-cell differentiation program.

POTENTIAL STEM CELL SIGNATURE INDUCED BY TAL1 IN T-ALL

In this regard, it would be interesting to identify genes that are abnormally induced by the TAL1 complex in T-ALL cells. Recently, our group used a targeted approach to identify regulatory elements that are differentially controlled by TAL1 and E-proteins (108). From this analysis, we discovered an enhancer situated within a cluster of seven genes belonging to the *GTPase of Immunity Associated Protein* (*GIMAP*) family. This region is associated with active histone marks in T-ALL cells but not in the normal human thymus, suggesting that the *GIMAP* enhancer is aberrantly activated in T-ALL cells. Importantly, *GIMAP* genes are expressed in mouse HSCs and CD4 or CD8 SP mature T-cells, while they are downregulated in DN3-4 stage thymocytes where TAL1 is also silenced. Using an *in vivo* reporter system in zebrafish, we showed that the *GIMAP* enhancer can be activated in normal hematopoietic stem and progenitor cells but not in the thymus. In addition, a reporter assay in human T-ALL cell lines indicated that the *GIMAP* enhancer is activated by TAL1 and its regulatory partners (GATA3 and RUNX1) and is repressed by E-proteins (E2A and HEB). Although ectopic expression of human *GIMAP* genes in immature zebrafish thymocytes did not induce tumor formation, their overexpression accelerated leukemia development in the presence of the *MYC* oncogene. Thus, our results revealed that aberrant activation of the *GIMAP* enhancer contributes to T-cell leukemogenesis.

While *GIMAP* genes have been known to be involved in the development of mature T- and B-lymphocytes (109–112), another group has also implicated their importance in HSC survival and maintenance (113). The work of Chen et al. on *Gimap5*$^{-/-}$ mice demonstrated that Gimap5 regulates the survival of HSCs and other early hematopoietic progenitors by stabilizing the Mcl-1 protein, which is an anti-apoptotic Bcl-2 family member (113). The HSCs in *Gimap5*-deficient mice exhibited defective long-term repopulation capacity, as demonstrated by their impaired engrafting ability. This study provided insights into the critical roles of *GIMAP* genes in the survival of HSCs and early progenitor cells. Notably, NOTCH1 was also identified as a positive regulator of the *GIMAP* genes in T-ALL cells (114, 115). A functional study by Chadwick et al. showed that *Gimap5* mediates apoptosis protection in T-ALL cells upon its upregulation by NOTCH1 (114). Together with our findings, these studies suggest that as a consequence of *TAL1/LMO* overexpression and activation of the NOTCH1 pathway, the *GIMAP* genes could be reactivated in immature thymocytes in which they are normally repressed, possibly by E-proteins, thereby contributing to leukemogenesis.

Another gene that has been implicated in stem cells and is also aberrantly activated by the TAL1 complex in T-ALL is the *ALDH1A2* gene (105, 116). Based on our ChIP-seq and gene expression data, this gene was one of the top candidate genes directly regulated by TAL1 in human T-ALL cells (105). ALDH activity has been proposed to be a universal CSC marker, as demonstrated by the tumorigenic and self-renewal properties of ALDH^{+} cells isolated from leukemia and many solid tumors (117–119). Among the 19 isoforms in the ALDH family, only a few of them, including *ALDH1A2* are involved in retinoic acid signaling, which has been known to be associated with the stemness characteristics of CSCs. Another group and our recent study indicated that *ALDH1A2* is induced by TAL1 *via* an internal enhancer in T-ALL cells (116) (and Zhang and Tan et al., unpublished data). Although the role of *GIMAPs* and *ALDH1A2* in the self-renewal potential of malignant T-cells is yet to be elucidated, their ability to mark stem cells and T-ALL cells may be used as a signature of the aberrant transcriptional program induced by T-ALL oncogenes.

CONCLUSION AND FUTURE PROSPECTIVE

The transformation mechanism in T-ALL is very efficient. T-ALL oncogenes alter the intrinsic transcriptional regulatory program by disrupting the differentiation machinery and by introducing the stem cell-like properties into developing thymocytes. This may initiate or reactivate the self-renewal ability that potentially exists in thymocytes. This process is mediated by a relatively small number of oncogenic transcription factors and seems not require the accumulation of a large number of genetic and chromosomal abnormalities until it obtains the hallmarks of cancer.

In other words, this mechanism poses a potential severe risk hidden in the thymus. Thymocytes may always be "primed" to initiate leukemogenesis. As recently reported (17, 18, 84), the competitive replacement of thymocytes *via* a continuous supply of lymphoid progenitor cells from the bone marrow plays an important tumor suppressive role in homeostasis. Further investigation is necessary to elucidate the loss-of-competition mechanism in human T-ALL. In particular, it is of great interest to analyze whether T-ALL develops from a self-renewal pool prior to the *TCR* rearrangement or pre-leukemic clones, which harbor the *TCR* translocation newly acquire the self-renewal capability. Single cell sequencing analysis is ideal to detect the emergence of those clones. Another important consideration is the mechanism of self-renewal in the ETP subtype of T-ALL. ETP cases show a very different genomic landscape and gene expression signature as compared to non-ETP cases. For example, mutations of *NOTCH1* are less frequently found in ETP (27), thus suggesting that different oncogenic mechanisms are involved. Establishment of proper model systems is needed to analyze LICs in this particular subtype.

The mechanisms described above can be also therapeutic targets to eliminate LICs in T-ALL. Disruption of the transcriptional complex involving TAL1 would efficiently block the formation of the CRC and revert the functional imbalance between oncogenic TAL1 complex and E-protein tumor suppressors. Rabbit and his colleagues have developed a peptide and intracellular antibody targeting LMO2 protein to disassociate the TAL1–LMO2 complex

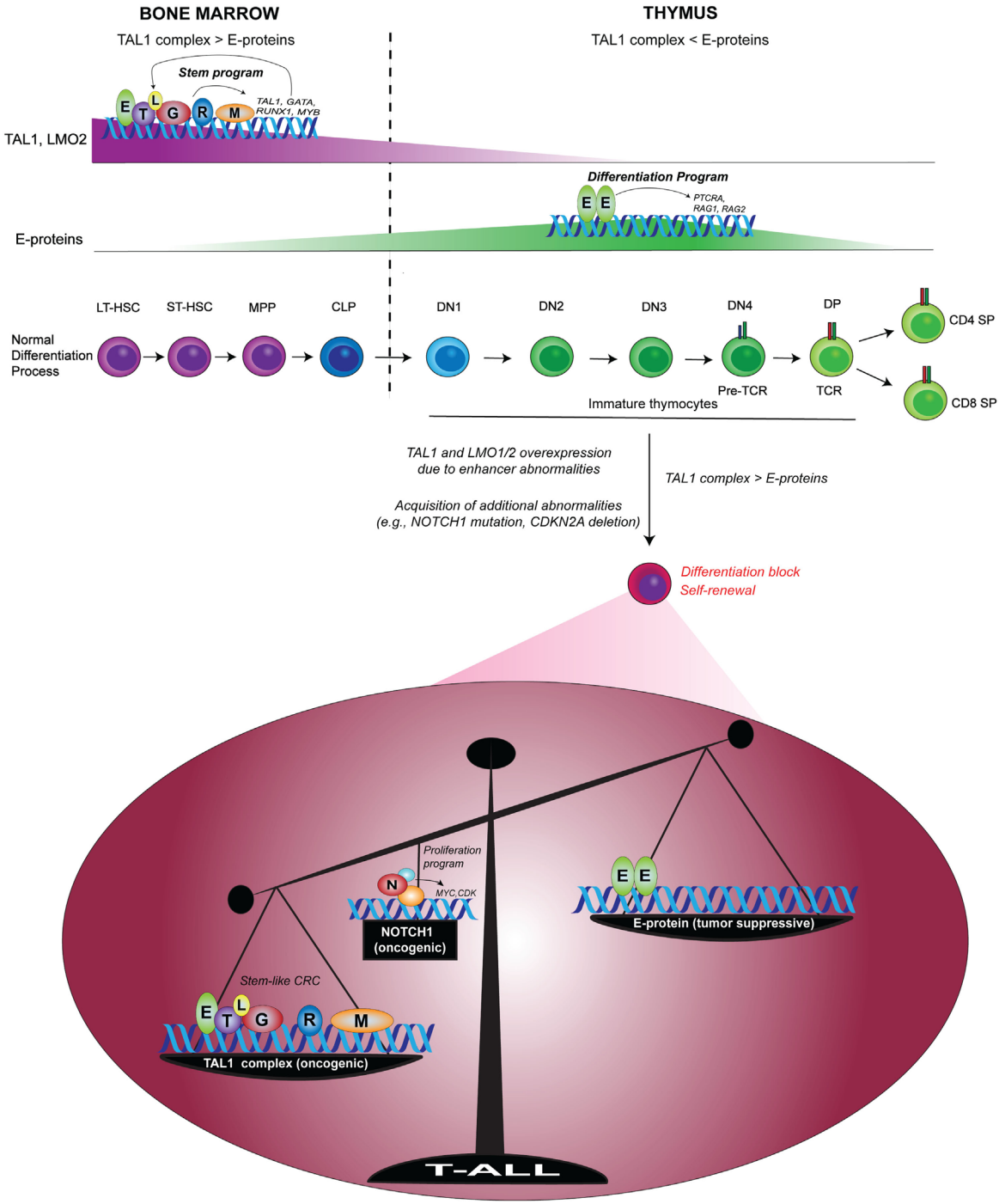

FIGURE 3 | Imbalance between the oncogenic TAL1 complex and E-protein tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL) [modified from a figure by Sanda and Leong (107)]. In normal hematopoiesis, TAL1 forms a transcriptional complex with E-protein, GATA2, RUNX1, MYB, and LMO2 to drive a regulatory program in HSCs and progenitor cells *via* the auto-regulatory loop. Upon the progression of T-cell commitment in the thymus, TAL1 and LMO2 expressions are silenced, while E-proteins are functionally and transcriptionally upregulated. E-protein dimers induce the expressions of *RAG1*, *RAG2*, and *PTRCA* to prompt the differentiation program of T-cells. In T-ALL, enhancer abnormalities (chromosomal translocation, intrachromosomal rearrangement or mutations in the enhancer) cause ectopic expressions of *TAL1* and/or *LMO1/2*, leading to the formation of TAL1 complex and the inhibition of E-protein dimers. T-ALL cells also acquire additional abnormalities such as genetic mutations of *NOTCH1* and deletion of *CDKN2A*. TAL1 and its regulatory partners form a stem cell-like core regulatory circuit (CRC) and NOTCH1 activates a different set of genes such as *MYC*. The functional imbalance between the oncogenic TAL1 complex and E-protein tumor suppressors possibly contributes to the induction of self-renewal program and the blockade of T-cell differentiation machinery. Mutated NOTCH1 boosts this oncogenic mechanism. LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; CLP, common lymphoid progenitor; DN, $CD4^{-}CD8^{-}$ double-negative; DP, $CD4^{+}CD8^{+}$ double-positive; SP, $CD4^{+}$ or $CD8^{+}$ single-positive; T, TAL1; E, E-protein; L, LMO1/2; G, GATA; R, RUNX1; M, MYB; N, NOTCH1.

(32, 120, 121). Inhibition of transcriptional machinery by small-molecule inhibitors of CDK7 or BRD4 concurrently reduces expressions of multiple oncogenic transcription factors in T-ALL, thereby leading to cell death (122, 123). Moreover, targeting CXCR4/CXCL12 signaling is an ideal strategy to disrupt the interaction between T-ALL cells and stroma cells in the bone marrow niche, as recently reported (82, 83). Additionally, identification of specific cell surface markers associated with LIC capability in T-ALL is critical for developing better therapeutic strategy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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DNMT3A and TET2 in the Pre-Leukemic Phase of Hematopoietic Disorders

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In recent years, advances in next-generation sequencing (NGS) technology have provided the opportunity to detect putative genetic drivers of disease, particularly cancers, with very high sensitivity. This knowledge has substantially improved our understanding of tumor pathogenesis. In hematological malignancies such as acute myeloid leukemia and myelodysplastic syndromes, pioneering work combining multi-parameter flow cytometry and targeted resequencing in leukemia have clearly shown that different classes of mutations appear to be acquired in particular sequences along the hematopoietic differentiation hierarchy. Moreover, as these mutations can be found in "normal" cells recovered during remission and can be detected at relapse, there is strong evidence for the existence of "pre-leukemic" stem cells (pre-LSC). These cells, while phenotypically normal by flow cytometry, morphology, and functional studies, are speculated to be molecularly poised to transform owing to a limited number of predisposing mutations. Identifying these "pre-leukemic" mutations and how they propagate a pre-malignant state has important implications for understanding the etiology of these disorders and for the development of novel therapeutics. NGS studies have found a substantial enrichment for mutations in epigenetic/chromatin remodeling regulators in pre-LSC, and elegant genetic models have confirmed that these mutations can predispose to a variety of hematological malignancies. In this review, we will discuss the current understanding of pre-leukemic biology in myeloid malignancies, and how mutations in two key epigenetic regulators, *DNMT3A* and *TET2,* may contribute to disease pathogenesis.

Keywords: TET2, Dnmt3a, myelodysplastic syndromes, acute myeloid leukemia, pre-LSC, stem cell biology, epigenetic regulator, HSCs

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INTRODUCTION

One striking finding from NGS studies has been the wide range in the number of mutations that appear in different tumor types; while some tumors can contain thousands of changes in coding sequences, such as bladder adenocarcinoma and melanoma, other tumors have a paucity of genetic aberrations (1, 2). Hematological malignancies, such as acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), generally fall within this latter category, where it seems that only a handful of exome mutations are necessary and sufficient to drive these malignancies (1–5),

although these studies cannot rule out that mutations in regulatory elements and non-coding regions are the putative causal hits in these diseases. Nevertheless, while the low frequency of coding mutations in hematological malignancies would suggest that they would be simpler to manage clinically and more amenable to targeted therapies, clinical trials using selective inhibitors of supposed driver mutations in the bulk tumor population have been largely disappointing thus far and have not been able to achieve lasting remission (6–14). Intensive chemotherapy regimens with stem cell transplantation remain the standard of care (15, 16). Additionally, while clinical remission is achieved in a substantial proportion of AML cases, most patients will relapse and die from their disease. Determining why tumors with such low mutational burden are refractory to targeted therapy and why these patients relapse so frequently are therefore critical questions in the field.

Hematopoiesis is a complex and plastic differentiation system that requires the temporally coordinated expression of large cohorts of genes in progressively more differentiated hematopoietic stem and progenitor cells [HSPC; reviewed by Dick and Lapidot; Doulatov et al. (17, 18)]. These fate decisions have empirically been proven to result from the stoichiometry between master transcription factors (19–22). Global regulators of transcription, such as epigenetic and chromatin remodeling complexes, splicing factors, and the core transcription machineries, also play an important upstream role in establishing the correct transcriptional landscape to allow for the efficient activation or repression of target genes. Interestingly, a number of NGS studies have identified a growing list of mutations within these factors in MDS and AML (**Table 1**). Both MDS and AML are not single diseases, but rather collections of clinically related but phenotypically heterogeneous malignancies. MDS are classically thought of as a disease of hematopoietic stem cells (HSC), in which patients have either marked reductions in blood production, at times precipitating into bone marrow failure, significant dysplasia in the cells produced, or some combination of both. AML are at least eight separate morphological phenotypes [French–American–British (FAB) classification system] that involve a large expansion of immature blast populations. The clinical hallmark of these malignancies is a differentiation block and thus substantial defects in the generation of mature erythrocytes, platelets, and/or mononuclear cells such as lymphocytes, neutrophils, and monocytes. In both AML and MDS, there appears to be multiple parallel clones and subclones that undergo evolutionary competition during disease progression, producing a clonal hierarchy within each patient (5, 23–27). Importantly, these clones may have different susceptibilities to treatment options and therefore represent important reservoirs during disease relapse (28–30). Additionally, these diseases are not stagnant but continue to evolve over time; this is best exemplified by the finding that some patients with MDS will convert to AML over the course of their treatment.

One remarkable finding of AML and MDS has been the discovery of mutations that produce very different clinical phenotypes even when these mutations occur in the same cell. For instance, a mutation in some gene *X* in HSC could produce bone marrow failure as typified by conditions like aplastic anemia or MDS, or could produce a blastic like disease of more differentiated progenitor compartments as in AML. Furthermore, mutations occurring in the bulk tumor population can also frequently be found within supposedly "normal" HSPC that are contributing to multi-lineage differentiation (26, 28, 31). These findings have suggested the existence of a theorized pre-leukemic stem cells (pre-LSC). These pre-LSC are fundamentally distinct from the tumor initiating, $CD34^+$ $CD38^-$ leukemia stem cells (LSC or leukemia-initiating cells, LIC) described extensively over the past two decades (32–34). Pre-LSC, are clones within the hematopoietic hierarchy that are not proliferative or dysplatic, but are inherently more likely to transform into a frank leukemia at a higher rate than other HSPC clones. These pre-LSC contain a limited number of mutations in AML or MDS related genes, such as *TET2, DNMT3A,* or *ASXL1*, and have qualitative changes that make them leukemogenic, this is in stark contrast to non-pre-LSC clones in patients with clonal hematopoiesis of indeterminate potential (CHIP; see Discussion and Perspectives below). Importantly, pre-LSC are thought to contribute to normal hematopoiesis while slowly accumulating mutations until a critical number are reached to produce LSC. These LSC, having crossed some threshold, then give rise to MDS or AML while not contributing to normal hematopoiesis. Determining the identity of the genes required to cross this "leukemic threshold," knowing within which cell they arise, and knowing what order they occurred are the critical questions in the field.

TABLE 1 | Examples of somatic mutations identified in AML and MDS.

	AML	MDS	Protein names
Signal transduction (Class I)	*FLT3*	*FLT3*	Fms-related tyrosine kinase 3
	c-KIT		KIT proto-oncogene receptor tyrosine kinase
	N-Ras, K-Ras	*N-Ras, K-Ras*	Neuroblastoma and Kirsten Rat Sarcoma Viral (V-Ras) Oncogene Homolog
	JAK2	*JAK2*	Janus Kinase 2
		CBL	Casitas B-lineage lymphoma
Transcription (Class II)	*CEBP* α		CCAAT/enhancer-binding Protein alpha
	IKZF1		IKAROS family zinc finger 1
	RUNX1	*RUNX1*	Runt-related transcription factor 1 (or AML1)
	PHF6		PHD finger protein 6
Epigenetic regulation	*TET2*	*TET2*	Ten eleven translocation methylcytosine Dioxygenase 2
	IDH1/2	*IDH1/2*	Isocitrate dehydrogenase-1 and -2
	DNMT3A	*DNMT3A*	DNA methyltransferase 3A
	ASXL1	*ASXL1*	Additional sex combs like transcriptional regulator 1
	EZH2	*EZH2*	Enhancer of Zeste Homolog 2
RNA splicing	*U2AF1*		U2 small nuclear RNA auxiliary factor 1
	SF3B1	*SF3B1*	Splicing factor 3b, subunit 1
	SRSF2	*SRSF2*	Serine/arginine-rich splicing factor 2
		ZRSR2	Zinc finger (CCCH Type), RNA-binding motif, and serine-/arginine-rich 2
Tumor suppressor	*CDKN2A/B*		Cyclin-dependent kinase inhibitor 2A
	TP53	*TP53*	Tumor Protein p53
	WT1	*WT1*	Wilms Tumor 1
Other	*SMC1A*		Structural maintenance of chromosomes 1A
	NPM1	*NPM1*	Nucleophosmin

EVIDENCE FOR PRE-LSC IN HUMAN MYELOID MALIGNANCIES

The possibility of pre-leukemic HSC harboring leukemia-associated mutations is not novel. In 1987, Fialkow and colleagues first identified presumably leukemic clones contributing to normal erythropoiesis in acute non-lymphocytic leukemia (35). Building on seminal work by Lapidot and colleagues, which first established that $CD34^+$ $CD38^-$, but not blasts, from AML patients, constituted LSC and could initiate leukemia in xenotransplanted mice (36). Hope et al. (37) further demonstrated that these xenotransplanted LSC could undergo further clonal evolution in these mice, and later studies showed that these cells could even produce normal myeloid and lymphoid lineages (38). Recapitulating these findings in xenotransplantation models, Miyamoto et al. found that *AML1/ETO* transcripts, which are generated due to the leukemic translocation *t*(8:21) in AML, are detectable in mature blood cells of all lineages even after stable and complete remission (39). Then, in 2012, Jan et al. reported a pioneering study in the pre-LSC field using flow cytometry and single clone targeted re-sequencing (26). By following the frequency and co-occurrence of many mutations within the same patient longitudinally during therapy and at disease relapse, these authors constructed the most detailed maps to date describing clonal dynamics in human AML. Moreover, by isolating residual "normal" HSC from these patients (defined as $CD99^-$ $TIM3^-$ $CD34^+$ $CD38^-$ Lin^-), they identified a number of mutations in critical epigenetic regulators frequently mutated in AML and MDS. The authors proposed a model whereby the disease propagating LSC are derived initially from these residual, self-renewing HSPCs that harbored primary mutation(s) (**Figure 1A**). These mutations presumably maintained these pre-LSC in a "primed" state that was able to expand into AML LSC once a driving mutation was acquired. Importantly however, these residual pre-LSC but not putative LSC ($CD99^+$ $CD34^+$ $CD38^-$ Lin^- cells) were contributing to normal, multi-lineage hematopoiesis, again deviating significantly from prior cancer stem cell (CSC) models, whereby the CSC was incapable of generating normal tissue. Studies previously showing that LSC were capable of multilineage reconstitution were presumably assaying these same pre-LSC as those studies only used CD34 and CD38 to enrich for LSC (38).

In addition to providing the best evidence to date for pre-LSC, this work and studies by a number of other groups have since made the remarkable discovery that the mutations occurring in pre-LSC and the bulk tumor were categorically different: while early mutations in pre-LSC were frequently in epigenetic and chromatin remodeling regulators, driver mutations in myeloid transcription factors and signal transduction molecules such as tyrosine kinases tended to occur late in bulk blast cells (40, 41). This surprising finding not only helped explain why potent targeted therapies for some driver mutations failed to cure patients; it also suggested something fundamental about AML biology and the order in which mutations were acquired.

Based on these observations, a number of groups started investigating how mutation sequence affects clinical outcomes in myeloid malignancies. If leukemia did indeed arise from pre-LSC harboring mutations that primed cells for leukemogenesis, then one would predict that the order within which mutations were acquired might influence the clinical phenotype. Ortmann et al. (42) tested this hypothesis by determining the mutational order between the epigenetic regulator *Ten-Eleven Translocation 2* (*TET2*) and a putative driver *JAK2 V617F*. These genes have been reported to be mutated in both pre-LSC and fully transformed malignant disorders such as the Philadelphia chromosome negative myeloproliferative neoplasms (MPN) [such as primary myelofibrosis (PMF), essential thrombocythemia (ET), and polycythemia vera (PV)], AML, and MDS. In this study, the authors focused on the MPNs, ET and PV, where the same *JAK2* mutations are almost universal in both conditions despite very different clinical phenotypes, and sought to determine whether the order of *TET2* and *JAK2* mutations along the hematopoietic lineage and within malignant clones drove differences in the clinical phenotype of the MPN. They found that the mutation order of *TET2* and *JAK2 V617F* influenced the age when the MPN was diagnosed, the subclonal composition and proliferative capacity of flow cytometry defined HSPC in these patients, and the transcriptional profile of the malignant HSC (42). Similar observations were additionally reported for *DNA Methyltransferase 3 alpha* (*DNMT3A*) mutations in MPN (43). However, the most interesting finding from these studies was that the clinical manifestations of disease were also significantly influenced by mutational order: acquiring either *TET2* or *DNMT3A* mutation prior to the *JAK2* mutation resulted in a much higher frequency of ET rather than PV.

In recent years, a number of important sequencing studies have also established that while hematopoietic clonality can influence clinical outcomes, identifying clones with certain mutations carries much more prognostic information. Two whole-exome sequencing studies of peripheral blood mononuclear cells longitudinally tracked clonal hematopoiesis during aging to establish whether the presence of clonal hematopoiesis correlated with AML development (5, 27). In both studies, the authors found that clonal hematopoiesis became more common with older age, that patients with clonal hematopoiesis had slightly higher rates of AML, and that the most common mutations in these clones were in *ASXL1, TET2*, and *DNMT3A*. Consistent with these results, Yoshizato et al. found strong evidence in aplastic anemia patients that while the degree of clonal hematopoiesis was variable and itself not well correlated with overall survival, clones harboring mutations in the epigenetic modifiers *ASXL1* and *DNMT3A* expanded much more rapidly than other clones in the same patient and that patients carrying these types of clones had significantly poorer overall survival and higher rates of transformation to AML (44). Recently, another group described the presence of chemotherapy-resistant HSC in patients with AML after chemotherapy that appeared to expand rapidly upon depletion of the bulk tumor (28). Again, these clones harbored mutations in epigenetic modifiers commonly seen in AML patients and therefore may represent the expansion of leukemia primed pre-LSC in these patients. Finally, Ivey and colleagues found that minimal residual disease, which was detected by the presence of *Nucleophosmin* (*NPM1*)-mutated transcripts in normal mononuclear cells after achieving remission in AML,

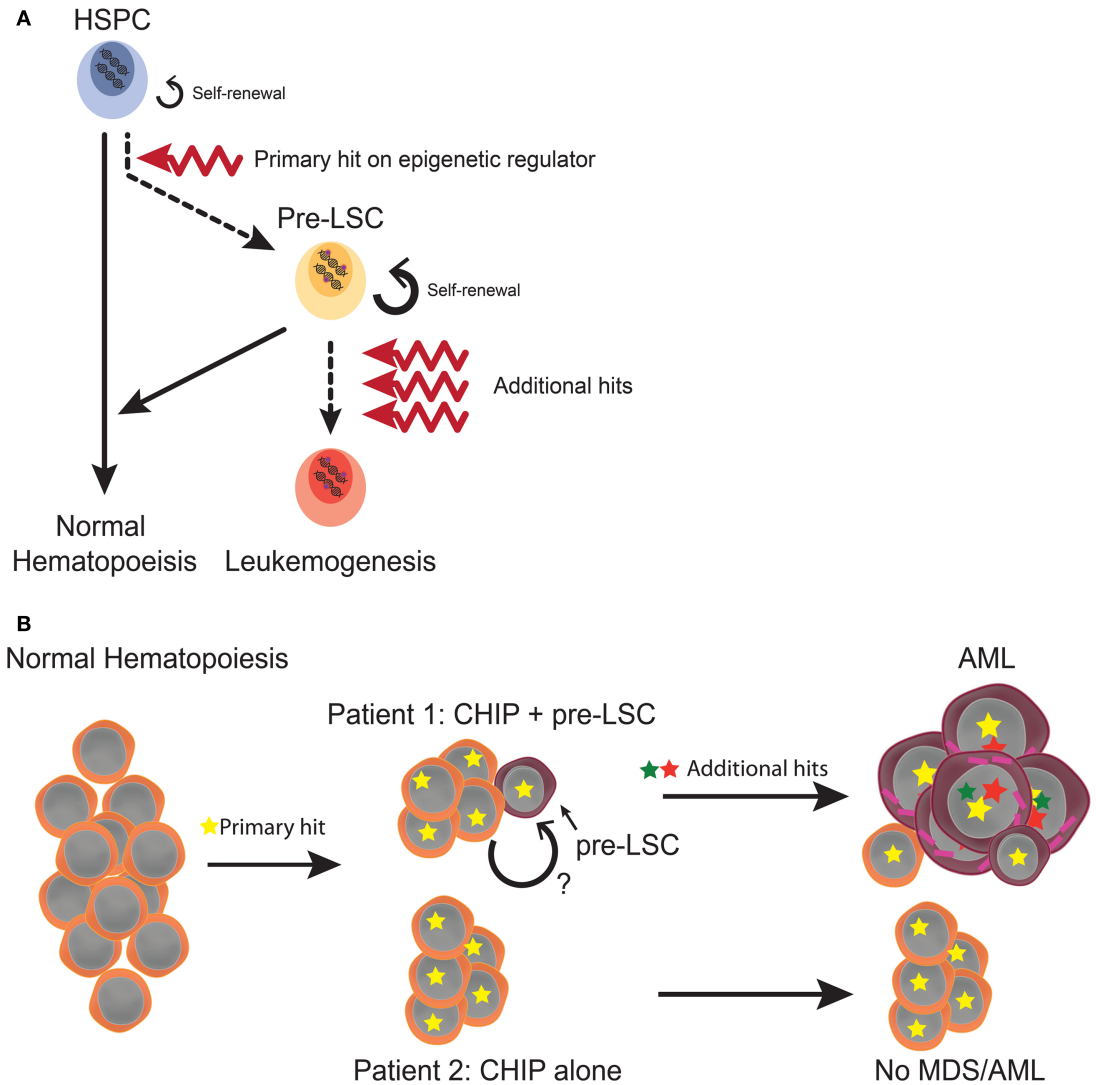

FIGURE 1 | Hypothetical model of leukemogenesis in *TET2* and *DNMT3A* mutations **(A)** Model of the stepwise mutation accumulation during pre-leukemic hematopoiesis and leukemogenesis. Numerous studies have suggested that mutations converting HSPC to pre-leukemic stem cell (pre-LSC) are in epigenetic regulators and may lead to aberrant transcriptional networks utilized in both HSC self-renewal and differentiation. When additional hits are then acquired in these pre-LSC, leukemia develops. Importantly, pre-LSC still contribute to normal hematopoiesis and self-renew similar to normal HSC (indicated in both by solid arrows) until malignant transformation (indicated with broken red arrow) in the pre-LSC model. **(B)** Hypothetical model of CHIP and pre-LSC. CHIP is defined as oligoclonal hematopoiesis in the presence of an AML or MDS mutation yet without cytopenia or dysplasia. Pre-LSC are phenotypically normal clones harboring mutations in AML and MDS genes, and can occur in CHIP or in non-CHIP patients. The major theoretical difference between pre-LSC and HSC participating in CHIP is the propensity to transform once additional hits are obtained. While these hits do not readily transform other CHIP clones, pre-LSC clones can rapidly progress to fully malignant state. The qualities that confer this "primed leukemic state" are currently unknown but presumably account for why not all patients with CHIP develop AML or MDS, as indicated in the figure. HSPC, hematopoietic stem and progenitor cell; CHIP, clonal hematopoiesis of indeterminate potential; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes.

was one of the strongest predictors of relapse and carried a poor overall survival. While this study was not addressing pre-LSC mutations *per se*, the fact that finding oncogenic transcripts in normal mature blood cells after AML remission carried a strongly poor prognosis clearly fits well with a model of pre-LSC being primed to transform rapidly into relapse AML despite contributing to normal hematopoiesis (45). Taken together, it appears that pre-LSC have qualitative changes that make them distinctly more prone to leukemia initiation than other HSC.

DISCRIMINATING CHIP FROM PRE-LEUKEMIA

Although the evidence for pre-LSC has garnered substantial support owing to the work described above, one question has plagued both clinical and translational studies: why do some patients with clonal hematopoiesis harboring mutations in MDS and AML-associated genes never develop disease? In the studies by Jaiswal et al. and Genovese et al. mentioned above, the

majority of patients with clonal hematopoiesis harboring mutations in canonical preleukemic mutations (e.g., *TET2*, *ASXL1*, or *DNMT3A*) never developed MDS or AML. This realization has complicated our understanding of pre-LSC as it suggests that preleukemic mutations are not fully sufficient to generate the "pre-LSC state" than primes for leukemic transformation. To help describe this scenario whereby a patient has limited hematopoietic clonality and harbors preleukemic mutations but does not have an increased risk of AML or MDS, a number of translational researchers and clinicians have described a clinical entity called "clonal hematopoiesis of indeterminate potential," or CHIP, which is analogous to monoclonal gammopathy of unknown significance (MGUS). CHIP is defined as oligoclonal hematopoiesis without morphological changes or cytopenia, where one or more genes typically associated with AML or MDS are mutated. CHIP patients have a very low rate of conversion to AML or MDS and therefore (and unlike in MDS) can be monitored clinically rather than proactively treated. A more detailed description of CHIP, the research leading to its characterization, and the diagnostic criteria separating it from MDS and AML are discussed extensively by Steensma and colleagues (46) and are beyond the scope of this review. While CHIP is clearly different from frank MDS or AML, discriminating CHIP from pre-leukemia is nuanced. CHIP is a risk classifier that describes, clinically, the probability of a patient to develop leukemia. Pre-LSC are cells that deterministically drive AML or MDS. While CHIP patients absolutely have an increased risk for these malignancies, the overall risk is still quite low. Pre-LSC, on the other hand, are fundamentally primed to contribute to leukemia initiation: according to the current model of leukemogenesis, all AML and MDS patients have pre-LSC that contribute to normal hematopoiesis, the bulk leukemia, and relapse. Therefore, all CHIP patients that develop AML had a resident pre-LSC clone in their CHIP and it does appear that CHIP seems to increase the risk of developing a pre-LSC. However, not all patients with CHIP will ever develop a pre-LSC and therefore will never develop MDS or AML. Moreover, not all AML patients originally had CHIP (**Figure 1B**). Importantly, the qualitative distinction that makes pre-LSC leukemogenic in patients with or without CHIP does not need to be genetic: epigenetic differences, metabolic rates, cell extrinsic influences, or the transcriptional context of that particular HSC may discriminate what is a primed pre-LSC from a normal HSC that happens to harbor an AML associated mutation. As such, exome capture alone is unlikely to fully capture why these clones are inherently more likely to transform. Unfortunately, as cell surface markers have not been discovered that unambiguously capture only pre-LSC from other HSC or CHIP clones, this model cannot be tested empirically at this time (for a more detailed description of the differences between HSC, pre-LSC, CHIP, and LSC, along with relevant references, see **Table 2**). Furthermore, we cannot exclude the possibility that all clones in CHIP would, if provided enough time, become pre-LSC and generate leukemia; it is completely possible (and indeed highly plausible) that this is an entirely stochastic process of trait acquisition. Nevertheless, irrespective of the difficulties in separating these clinical subtleties with current technology, the evidence outlined above clearly shows that pre-LSC are real biological entities. Moreover it is also certainly true that the mutations isolated from pre-LSC must have a role in transforming pre-leukemic hematopoiesis to AML and MDS, as the presence of these mutations absolutely increases the probability of leukemogenesis. As noted, studies have repeatedly shown that many of these candidate antecedent mutations in pre-LSC are in epigenetic regulators. Given these observations, a substantial amount of effort has been directed at understanding how mutations in epigenetic factors deregulate hematopoiesis and precipitate hematological malignancies.

MUTATIONS IN DNA METHYLATION REGULATORS

Since epigenetic modifications regulate genome wide transcriptional profiles and help establish cell-type specific gene expression profiles during cell differentiation (56), mutations in these genes in HSPC may have profound effects on normal hematopoiesis.

TABLE 2 | Cell types that associate with leukemoginesis and their cell surface markers.

Cell type	Hematopoietic lineage potential	Leukemogenic	Presence of AML/MDS mutations?	Cell surface markers
HSC	Yes Multilineage contribution to all mature blood populations, self-renewal	No	No	$Lin^-CD34^+CD38^-CD90^+$ (39)
Progenitors	Yes Restricted differentiation potential	No	No	Many, e.g., GMP: $Lin^-CD34^+CD38^+CD45RA^+CD123^+$ (47)
LSC	No	Yes	Yes	$Lin^-CD34^+CD38^-$. Many reported markers, CLL-1 (48), CD25 (49), CD32 (49), CD96 (50), TIM-3 (51, 52), CD99 (52), CD47 (53), IL3RA (54)
pre-LSC	Yes Multilineage contribution to all mature blood populations, self-renewal	Yes	Yes	Unclear. Reports suggest $Lin^-CD34^+CD38^-TIM3^-CD99^-$ (52) or $Lin^-CD34^+CD38^-IL1RAP^+$ (55) No definitive marker available
CHIP	Yes Multilineage contribution to all mature blood populations, self-renewal	Minimal risk	Yes	Unclear. Presumably same as HSC

GMP, granulocyte-macrophage progenitor; IL3RA, interleukin 3 receptor; TIM-3, T-cell immunoglobulin and mucin domain 3; IL1RAP, IL-1 receptor accessory protein.

Two of the best-characterized epigenetic mutations found in pre-leukemic HSPC are in *DNMT3A* and *TET2* (**Figure 2**). *DNMT3A* and *DNMT3B* are *de novo* methyltransferases that catalyze DNA methylation at target DNA, while *DNMT1* is responsible for maintenance methylation at the replication folk during DNA synthesis (57–59). *De novo* DNMTs are essential to mammalian development (60, 61) as these marks, particularly 5-methylcytosine (5-mC) in CpG islands, are correlated with transcriptional silencing. *TET2,* conversely, is an enzyme that plays a central role in DNA demethylation by catalyzing the conversion of 5-mC to 5-hydroxymethyl cytosine (5-hmC) (62–64). While first discovered in 1972 (65), the functional importance of 5-hmC was not clear until recently due to the high mutational frequency of *TET2* in myeloid malignancies (**Table 1**). We will now focus on mutations in these enzymes in MDS and AML to shed insight into the role these factors play in hematopoiesis and leukemia.

REGULATION OF *DNMT3A* AND ITS ROLE IN TRANSCRIPTIONAL CONTROL

DNMT3A is a *de novo* methyltransferase of DNA and mutations have been isolated in patients with AML (69, 71) and MDS (70, 75). *DNMT3A* mutations frequently co-occur with *NPM1, FLT3*, and *IDH1* (76), and overall approximately 20% of patients with AML (69, 71) and 8% of patients with MDS (70) carry a mutation in this factor. Patients harboring mutations in *DNMT3A* typically have a poorer overall prognosis, although this depends significantly on which cooperating mutations co-occur in that patient (69–71).

The importance of DNA methylation in determining cell identity is well documented, but how *de novo* methyltransferase activity is regulated and how methyltransferases are targeted to specific DNA sites remains poorly understood. Recent studies have suggested strong cross-talk between histone modifications, transcriptional activity, and prior DNA methylation status on DNMT localization. Highly conserved PWWP (proline–tryptophan–tryptophan–proline) domains in *DNMT3A* play an essential role in directing this factor to heterochromatic regions (77, 78), particularly those marked with histone 3 lysine 36 trimethylation (H3K36me3), which is a known repressive histone modification. Presence of this mark was also reported to increase the methyltransferase activity of DNMT3A (79). Protein–protein interactions with other factors also appear to play an important role in DNMT3A recruitment. *DNMT3A* has an ADD [ATRX-DNMT3- DNMT3-like (DNMT3L)] domain, which is a cysteine-rich (Cys-rich) zinc-finger DNA-binding domain, reported to interact with many transcription factors and chromatin remodeling factors such as HP1, SUV39H1 (80), EZH2 (81), HDAC1 (82), p53 (83), Myc (84), and PU.1 (85) (**Figure 2**). This domain in *DNMT3A* also shows high affinity for unmethylated histone H3 peptides but not H3 lysine 4 tri-methylated (H3K4me3) peptides (86–88), indicating that the ADD may also be involved in chromatin reading as well. Interestingly, based on the crystal structure of DNMT3A and after biochemical analyses, it was found that the ADD participates in an auto-regulatory capacity to effect changes in DNMT3A activity: in the absence of histone H3, ADD domain binds to the catalytic domain of DNMT3A leading to blocked enzymatic function; in the presence of unmethylated H3, the ADD binds this H3, allowing the catalytic domain to become accessible for *de novo* DNA methylation catalysis (89).

A final, important regulatory step controlling DNMT3A activity is tetramer formation. DNMT3A can exist in a variety of tetramer states composed of homo-dimers of DNMT3A or hetero-dimers with DNMT3L, which is a catalytically inactive protein that enhances the methyltransferase activity of DNMT3A (90). While all tetramers are catalytically active, each variant tetramer has marked differences in enzyme processivity (91). Therefore, understanding the regulation of tetramer formation, and specifically determining the mechanisms underlying homo- versus hetero-dimerization may be critical to understanding the regulation of DNMT3A function. Recent studies have also noted that these tetramers are sensitive to pH and decreasing pH disrupted the distribution of various tetramers of DNMT3A *in vitro* (91). As different cell types, and notably cancer cells, exist at slight variations in pH, destabilization of DNMT3A tetramers

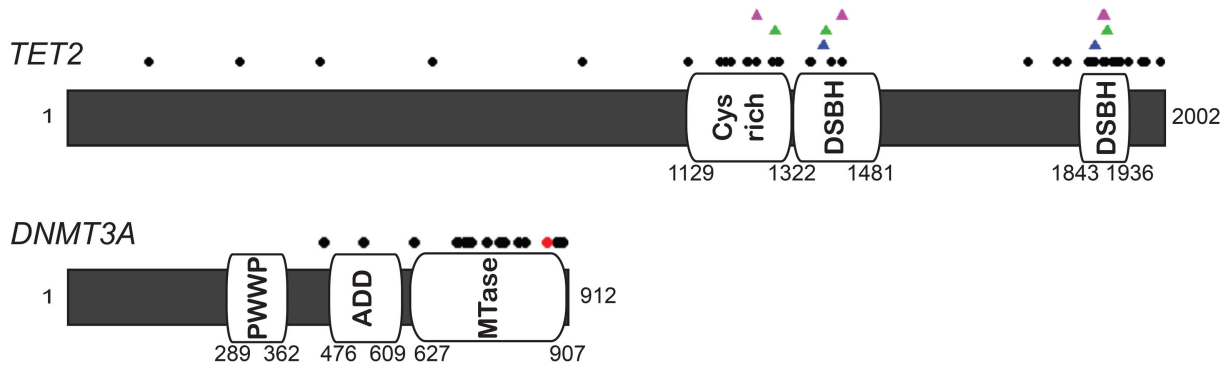

FIGURE 2 | Diagram of human *TET2* and *DNMT3A* and missense mutations in AML and MDS. The black dots indicate the missense mutation sites (66–71). The majority of mutations in *TET2* are identified in the two catalytic domains, shown as double-stranded β-helix (DSBH) and Cys-rich domain (64, 72). Magenda, green, and blue triangles on *TET2* diagram represent the site associated with *N*-oxalylglycine (NOG, a 2-OG analog), CpG recognition and Fe(II) binding based on the crystal structure and biochemical analysis (73, 74). Red dot on *DNMT3A* indicates the hot spot mutation at R882.

due to changes in intracellular pH may represent a relatively unexplored mechanism by which DNA methylation patterns are deregulated in tumors (92, 93).

DNMT3A MUTATIONS IN AML AND MDS

About 40–60% of *DNMT3A* mutations in AML patients are a hotspot mutation in Arg882 (R882), which is located within the catalytic domain of the enzyme (69, 71) (**Figure 2**). In addition to presumably reducing the catalytic efficiency of DNMT3A, this hotspot mutation also appears to influence the ability of DNMT3A to homodimerize. Normally, DNMT3A functions as a tetramer, comprised of either two homodimers or heterodimers DNMT3L. While R882 mutations in *DNMT3A* are still able to undergo hetero-dimerization with DNMT3L (94), they are unable to homo-dimerize (95, 96), suggesting that the R882 mutation is a dominant-negative mutation, which interrupts tetramer formation leading to the reduction of methyltransferase activity. *DNMT3A* mutations probably were inducing AML by leading to passive demethylation of the genome, and some genes (e.g., *HOXB*) have been found to be differentially hypomethylated in *DNMT3A* mutant AML (71). One recent study in murine HSC has indicated that many genes deregulated in leukemia, including transcription factors, exist in sites termed methylation canyons that are prone to methylation loss in the absence of *DNMT3A* (97). These results, however, conflict with whole genome profiling using Methylated DNA IP (MeDIP)-chip analysis and gene expression profiling that have thus far found little impact of *DNMT3A* mutations on global methylation patterns and little correlation between changes in methylation status and differential gene expression (69). One possible interpretation of this finding is that *DNMT3A* mutations play a more important role in pre-LSC transcriptional changes in HSPC that allow for leukemia to develop in more differentiated blasts, and that these changes are in effect "averaged-out" with standard ensemble techniques. Consistent with this idea is the finding that in inducible mouse deletion models of *Dnmt3a*, HSPC have mild phenotypic changes such as impaired differentiation, increased self-renewal, and occasionally transform to a myeloproliferative disease, but do not show robust changes in DNA methylation patterns or correlation between methylation changes and gene expression profiles (98–101). Double knockouts for *Dnmt3a* and *Dnmt3b,* however, show synergism in their phenotype, suggesting that there may exist compensatory activity between the *de novo* DNMTs in murine HSC that reduces the impact of single gene loss (98, 101). Moreover, inducible overexpression of *Dnmt3b* in mice was able to significantly slow leukemia induction by both *Myc-Bcl* and *Mixed Lineage Leukemia* (*MLL*)*-AF9* (102). These findings in mice raise an important question as to why *DNMT3B* mutations are so rare in human AML and MDS, indicating that perhaps this compensatory pathway is not as robust in human HSC (76).

FUNCTIONAL ROLE OF TET2 IN TRANSCRIPTIONAL REGULATION

While 5-hmC-modified DNA was biochemically isolated decades ago, it was the recent discovery of *TET* mutations in AML and MDS that prompted further investigation of the functional role in these marks, and their writers the *TET* enzymes, play in transcriptional regulation. *TET1* was the first *TET* family member successfully isolated, originally found as a translocation partner of *MLL* gene in AML (103–105). While Ono and colleagues were the first to clone the gene and named it *LCX* (*leukemia-associated protein with a CXXC domain*) (106); Lorsbach et al. (107) cloned the same partner of the *MLL* translocation and named it *TET* for *Ten-Eleven Translocation* owing to its frequent *MLL* fusion [*t*(10;11)(q22;q23)] in AML. Three TET enzymes, TET1, 2, and 3, have since been identified (107).

All three TET enzymes convert 5-mC to 5-hmC, which is later converted to 5-formylcytosine (5-fC) and then 5-carboxylcytosine (5-caC) (62–64). While each enzyme is capable of catalyzing these reactions, expression profiling has shown cell type distribution differences between the different TET enzymes, indicating distinct functions or regulators (62). Classically, conversion of 5-mC to 5-hmC at promoters and transcription start sites (TSS) would be predicted to lead to transcriptional activation by eliminating DNA methylation, which is correlated with transcriptional repression at CpG islands. Williams et al. (108), however, reported an unexpected role for TET1 as a transcription repressor in embryonic stem cells. Moreover, other groups have found that TET1, but not TET2, interacts with the transcriptional repressive histone deacetylase SIN3A (108, 109). While TET2 is still typically believed to be involved in transcriptional activation, these non-canonical activities of other TET family members at least leaves open the possibility that TET2 may have as of yet unidentified regulatory roles in transcription. One recent finding is that TET2 can regulate histone *O*-acetylglucosaminylation (*O*-GlcNAcylation) of serine and threonine residues of histone 2B (H2B), which is reported to associate with active transcription at TSS (110). Chen et al. (111) found that TET2 regulates these levels indirectly by recruiting *via* its catalytic C terminus *O*-GlcNAc transferases (OGT) to target loci. Importantly, this interaction does not affect the 5-hmC catalytic activity of TET2 (111–113).

REGULATORS OF TET ACTIVITY

While different cell types seem to express different amounts of each TET enzyme, it has become clear that post-translational regulation is critical in controlling TET activity and targeting to genetic loci. All TET enzymes contain one Cys-rich domain and two double-stranded β-helix (DSBH) domains that display the core catalytic domains, which act in a Fe(II) and 2-oxoglutarate (2-OG, also called as α-ketoglutarate) dioxygenases-dependent manner (73). Mono ubiquitinylation at a conserved lysine residue (residue 1299 in TET2) (114, 115) or binding of ascorbic acid in this catalytic domain directly facilitates TET catalytic activity by stabilizing Fe(II) association with the enzyme (116, 117) (**Figure 2**).

Targeting of *TET2* to genomic regions was initially unclear as *TET2*, unlike *TET1* or *TET3*, does not possess a canonical CXXC domain that binds unmethylated CpG. Ko et al. (118) then found an ancestral variant of the CXXC domain, referred to as *IDAX* (a.k.a. *CXXC4*) 650 kb upstream of *TET2*, which appears to have

been separated from the *TET2* coding region by chromosome inversion during evolution. IDAX interacts with unmethylated CpG DNA *in vitro* similar to the canonical CXXC domain (118). Genomic distribution of IDAX as determined by Chromatin IP (ChIP) showed that about 40% of IDAX peaks were enriched in the promoter/TSS, which suggested that IDAX acted as a cofactor to recruit *TET2* to target sites. Unexpectedly, however, overexpression of IDAX actually reduced the global level of 5-hmC (118), despite finding no changes in the *TET2* mRNA levels. Additionally, while the variant CXXC domain of IDAX was able to directly associate with the catalytic domain of *TET2*, IDAX does not block TET2 enzymatic activity directly. Instead, it appears that IDAX destabilizes the TET2 protein, which is then degraded through caspase 3 and 8 (118).

An important regulatory control on TET activity has been recently discovered with the finding of *Isocitrate Dehydrogenase-1* and *-2* (*IDH1/2*) mutations in a variety of tumors, including AML and MDS. IDH1/2 are enzymes that play an important role in the tricarboxylic acid cycle (TCA). Heterozygous, gain of function mutations in these enzymes have been found in high frequency in myeloid malignancies (119, 120). These mutations cause *IDH1/2* to produce an oncometabolite, 2-hydroxyglutarate (2-HG), instead of 2-OG (121). This is a competitive antagonist of 2-OGT enzymes, which in turn leads to severely reduced TET enzyme activity. As predicted, Figueroa et al. (122) found that *IDH1/2* mutations in AML patients lead to genome wide DNA hypermethylation signatures. While the population of patients with genetic *IDH1/2* mutations does not overlap with *TET2* mutations, these different mutations have essentially synonymous hypermethylation signatures. This suggests that *IDH1/2* and *TET2* mutations phenocopy one another and therefore do not confer additional selective advantages during clonal evolution in these diseases.

TET2 MUTATIONS IN AML AND MDS

Loss of functional *TET2* has been extensively reported in both AML and MDS. In addition to translocation fusions with *MLL*, DNA FISH studies have shown that *TET2* is frequently deleted in both malignancies (123). *TET2* mutations have been identified in 12–24% of AML patients and 7–26% of MDS patients (66–68). Most mutations in *TET2* are heterozygous and the presence of mutations carries a poor prognosis in either malignancy (66). Missense mutations of *TET2* in AML and MDS patients are commonly located in the catalytic domain, spacer region, or the Cys-rich domain (**Figure 2**), or were nonsense or frameshift mutations. Notably, as many of these mutations can be found in flow cytometry defined HSC or early progenitor cells from patients with AML or MDS, *TET2* mutations are hypothesized to be possible pre-leukemic mutations (67, 68, 124).

A number of recent studies have focused on delineating the functional role TET2 plays in hematopoiesis. First, expression of *TET2* with mutations at its predicted Fe (II) and 2-OG binding residues led to decreased 5-hmC levels in cell lines compared to expression of wild-type enzyme, suggesting that the common mutations in these residues occurring in AML are loss of function. Loss of *TET2* has important phenotypic consequences in hematopoiesis. Transduction of *TET2* shRNA in bone marrow stem/progenitor cells impaired myelopoiesis (73), while both germline and conditional knockout of *Tet2* in mice in HSC leads to granulomonocytic (GM) lineage skewing at the expense of the erythroid and lymphoid lineages, as well as increased 5-mC level and decreased 5-hmC (125–127). Additionally, loss of *TET2* in human $CD34^+$ cord blood recapitulates findings in mice, with differentiation skewing along GM lineages in *ex vivo* culturing conditions, along with increased HSPC self-renewal (128). To summarize, in both mouse and human models, *TET2* loss appears to promote GM lineage skewing and increases the self-renewal capacity of HSPC with aberrant ratios between 5-mC and 5-hmC.

The detailed mechanism of how *TET2* mutations propagate leukemic and pre-leukemic states in myeloid malignancies remains poorly understood. As expected, many studies reported that *TET2* mutations or depletion resulted in decreased 5-hmC globally (73, 125, 128). While the functional importance of DNA methylation at CpG islands has been correlated with transcription silencing, it appears that demethylation reactions catalyzed by TET2 might be more nuanced. Specifically, it was found by Ko et al. (73) that DNA hypermethylation profiles in bone marrow samples from patients harboring *TET2* mutations was enriched predominantly in non-CpG sites, while CpG islands were actually hypomethylated. Other groups have confirmed that the hypermethylation phenotype of *TET2* mutations appears to be principally outside of CpG islands. Yamazaki and colleagues (129) reported also did not detect changes in DNA methylation in CpG islands caused by *TET* mutations but instead detected hypermethylation at non-CpG islands. Rasmussen and colleagues recently reported that depletion of *Tet2* in pre-leukemic hematopoietic cells in mice had little impact on the methylation status of CpG islands and promoters but rather led to progressive DNA hypermethylation at enhancer elements (130). While the failure to detect differential DNA methylation at CpG islands in the presence of *TET2* mutations could be due to the degradation of mutant TET2 by IDAX as described above, this is at best speculative to date given the lack of direct evidence available. Finally, as the functional role of DNA methylation in non-CpG sites such as enhancers and gene bodies are largely unknown, how *TET2* or *IDH1/2* mutations lead to leukemia promoting transcriptional changes through hypermethylation in these sites is unclear; while TET2 was found to be significantly enriched with H3K4me1 and transcription factor p300 at the enhancer regions (131), whether TET2 is required for establishing these enhancers marks, whether mutant *TET2* changes the behavior of these cis regulatory regions, and how this ultimately perturbs transcriptional networks is still unexplored.

DISCUSSION AND PERSPECTIVES

The high frequency of mutations in epigenetic regulators indicates that epigenetic deregulation may play a critical role in the pathogenesis of certain myeloid malignancies. The finding of these mutations both in malignant cells of AML and MDS as well as within the phenotypically normal HSC of patients indicates

that these mutations may play a critical role in the pre-malignant phase of oncogenesis and evidence from single cell sequencing studies suggest that these cells can serve as reservoirs for disease relapse. In light of the increasing evidence for pre-LSC in both primary and relapse AML and in MDS, it is now critical to develop a more comprehensive understanding of what these cells are, how they are separated from other clones in CHIP, and how mutations in epigenetic regulators prime these pre-LSC toward oncogenesis. While a causal role for *TET2* and *DNMT3A* mutations is likely given that they are some of the most frequently mutated genes found in pre-LSC of AML and MDS patients, how exactly these mutations lead to leukemogenesis is still far from understood. For one, it is not clear how these mutations and their associated effects on global and local DNA methylation drive gene expression aberrations that impair normal hematopoiesis. Recent work has begun to shed some light on transcriptional and cell biological mechanisms that play a role in the formation of pre-LSC and their progression (132); however, it is unclear how exactly these transcriptional changes prime cells to become leukemic after acquisition of another genetic hit. The major limitation in answering these questions is technical: at present there exist no reliable cell surface markers that unambiguously separate pre-LSC from non-leukemic HSC clones. Therefore, deciphering the deregulated transcriptional programs occurring in pre-LSC, and how they relate to changes in DNA methylation cannot be readily achieved using ensemble approaches. Second, normal HSC are already documented as transcriptionally and functionally heterogeneous (133). As such, even single-cell gene expression technology like single-cell RNA-seq may only be adequate for identifying these transcriptional programs if single cell NGS or MeDIP-seq is performed concomitantly. At the time of writing, this technique has yet to be reported and is likely to represent an enormous technological challenge. Therefore, identifying what transcriptionally constitutes a truly "normal" versus "pre-leukemic" HSPC will be challenging given the present technology. Second, why certain mutations significantly enrich with *TET2* and *DNMT3A* is not well understood. One possibility is that loss of *TET2* or *DNMT3A* specifically contributes to increased mutation rates at these cooperating hits. Another possibility is that these hits are randomly generated but are selectively able to complement *TET2* or *DNMT3A* to drive leukemic evolution. As the clinical and biochemical implications of these two models differ significantly, establishing which contributes to AML and MDS is critical to developing a full understanding of these conditions and possibly developing novel therapeutics. In either case, however, the preponderance of these mutations in pre-LSC strongly suggests an important pathogenic role as leukemia initiation. Third, while the importance of DNA methylation in transcriptional regulation is well documented, a detailed mechanism of how DNA methylation patterns are established and maintained is far from complete. How these processes are locally augmented during normal hematopoietic differentiation is similarly unknown. The fact that multiple studies looking at loss of *TET2* or *DNMT3A* reported similar phenotypic changes in HSC (namely GM skewed cell fate, increased self-renewal capacity, and global changes of DNA methylation status) seems to indicate that aberrant methylation patterns have robust effects on hematopoietic differentiation. The fact that hypo- and hyper-methylation patterns can have similar phenotypic consequences indicates that perhaps the marks *per se* are not as important as the appropriate ratio of these marks across many local regions of the genome in the same cell. Further complicating matters is the finding that these methylation patterns do not appear to correlate well with gene expression changes in AML and MDS samples, while mutations in both *TET2* and *DNMT3A* clearly have prognostic implications and participate in leukemogenesis. Given these points, deciphering the language of these methylation patterns and determining how they dictate hematopoietic differentiation is a major focus of current research.

A substantial amount of research will be still required to fully understand how these epigenetic factors behave in both normal and malignant hematopoiesis. With technological advancements, particularly in NGS and single cell techniques, many of the counterintuitive observations made regarding these enzymes may be elucidated. Given the inherent heterogeneity of normal HSPCs, it is quite likely that single cell transcriptomics and epigenomics may be ultimately required to fully understand how and when these factors become relevant in promoting LSC transformation. Despite the current technical limitations, however, the discovery of these mutations in pre-LSC has blossomed exciting new lines of research in both AML and MDS, diseases with classically poor prognoses and little therapeutic advances over the past few decades. Although it appears that the role epigenetic regulators play in leukemia initiation will be complex, those functions are likely to fundamentally alter current paradigms about how these myeloid malignancies develop and therefore may offer novel management avenues in the future.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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