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Chromatin Complexes Maintain Self-Renewal of Myeloid Progenitors in AML: Opportunities for Therapeutic Intervention

Hannah J. Uckelmann[1] and Scott A. Armstrong[1,*]
[1]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Division of Hematology/Oncology, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA
*Correspondence: scott_armstrong@dfci.harvard.edu
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Specific subgroups of acute myeloid leukemia (AML), including those containing *MLL* rearrangements and *NPM1*c mutations, possess characteristic stem cell-like gene expression profiles. These expression programs are highly dependent on components of the MLL histone methyltransferase complex, including Menin and DOT1L. Understanding the chromatin-based mechanisms through which cancer cells subvert certain aspects of normal stem cell biology helped identify specific vulnerabilities and translate them into targeted therapy approaches. Exciting progress has been made in the development of small-molecule inhibitors targeting this epigenetic machinery in leukemia cells and prompted the development of clinical trials in patients with hematologic malignancies.

From Stem Cells to Leukemia Stem Cells

Our understanding of the differentiation from a hematopoietic stem cell to mature myeloid and lymphoid progeny has been traditionally defined by isolating bone marrow cells using cell surface markers and analyzing their output upon transplantation into irradiated recipient mice (Akashi et al., 2000; Spangrude et al., 1988). Using additional cell surface markers to separate specific cell types, multiple studies defined more and more restricted subpopulations that have well-delineated lineage outputs and, in some cases, reached single-cell resolution (Yamamoto et al., 2013). With the help of new lineage tracing technologies and gene expression analysis of individual cells, it is becoming more apparent that hematopoietic differentiation is a continuum of cells that gradually shift toward a specific lineage instead of passing through distinct intermediate stages and that defined subpopulations represent merely a snapshot of a mixture of cells going through transitions. The intricate balance between the various blood cell lineages can be acutely altered by environmental factors, such as stress, inflammation, or blood loss, to provide an emergency supply of certain blood and immune cells (Essers et al., 2009; Haas et al., 2015; King and Goodell, 2011; Walter et al., 2015). Differentiation short cuts have also been uncovered, such as differentiation from long-term stem cells to megakaryocytes, which allows for a rapid recovery of platelets on demand (Haas et al., 2015; Rodriguez-Fraticelli et al., 2018).

With increased age, a number of mutations can occur in the hematopoietic stem cell compartment that may perturb their differentiation or self-renewal and result in expansion of stem cell clones with a proliferative/survival advantage. This clinical phenomenon is often described as clonal hematopoiesis of indeterminate potential (CHIP). Mutations found in CHIP, such as those in *DNMT3A*, *TET2*, and *ASXL1* can be present in otherwise healthy adults and accumulate with increasing age. Additional events, in most cases mutations, are required to drive progression to leukemia (Abelson et al., 2018). Some CHIP mutations, such as *TP53* and *IDH* mutations, carry a higher risk of such progression to acute myeloid leukemia (AML) while others may be present for decades without giving rise to leukemia (Desai et al., 2018; Jaiswal et al., 2017). As a result, we are now able to identify high-risk populations and monitor them for potential progression to leukemia. The presence of CHIP that precedes development of myeloid malignancies, such as AML, provides a potential opportunity for early intervention, but therapies that target these preleukemic clones are still lacking.

Self-Renewal of Myeloid Progenitors and Progression to AML

Hematopoietic stem cells represent a small fraction of the bone marrow and are defined by their ability to renew themselves, as well as give rise to specialized mature cells over long periods of time. In the course of differentiation, cells downregulate gene expression programs involved in self-renewal and eventually commit irreversibly to a distinct lineage fate. However, limitless self-renewal also represents one of the hallmarks of cancer cells in combination with an increase in proliferative potential and the inability to properly differentiate leading to an expansion of immature cells (Hanahan and Weinberg, 2000). Stem cells that accumulate mutations can represent the cell of origin for various leukemias (Warner et al., 2004). This holds true for mutations in genes, such as *DNMT3a* and *BCR-ABL* (Huntly et al., 2004; Yang et al., 2016). The concept that a committed progenitor cell with higher proliferation potential could gain self-renewal capacity and act as a cell of origin for AML was first demonstrated in the early 2000s using overexpression of fusion oncogenes, such as *MLL-AF9*, *MLL-ENL*, and *MOZ-TIF2* in myeloid progenitors (Cozzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006). Furthermore, a fully developed leukemia

Dr. Armstrong is the Chairman of Pediatric Oncology at the Dana Farber Cancer Institute, Associate Chief of the Division of Hematology and Oncology at Boston Children's Hospital and the David G. Nathan Professor of Pediatrics at Harvard Medical School

He was previously the Director of the Center for Epigenetics Research at Memorial Sloan Kettering Cancer Center and Professor of Pediatrics at the Weill Cornell Medical College where he initiated a research program in cancer epigenetics. Armstrong obtained his MD and PhD degrees from the University of Texas Southwestern Medical School, where he trained with Nobel Laureates Michael Brown and Joseph Goldstein. He completed an internship and residency at Boston Children's Hospital and clinical and research fellowships at Dana-Farber Cancer Institute under the direction of Dr. Stanley Korsmeyer. He then began his independent research career at Boston Children's Hospital and Dana-Farber as a faculty member and attending physician in pediatric oncology. The major focus of his career has been on delineating the biology of childhood cancers, particularly leukemia. His group has made seminal discoveries into the relationship between normal hematopoietic stem cells and leukemia and identified specific epigenetic mechanisms as therapeutic opportunities. This work has led to the development of several new classes of therapeutic agents that target epigenetic mechanisms, with many already being tested in clinical trials for both children and adults. Dr. Armstrong's work has been recognized by multiple awards including the Till and McCulloch Award from the International Society of Experimental Hematology, the Paul Marks Prize for Cancer Research from Memorial Sloan Kettering Cancer Center, the E. Mead Johnson Award from the Society for Pediatric Research, and the Dameshek Prize from the American Society of Hematology. He is an elected member of the Association of American Physicians (AAP), is a fellow of the Association for the Advancement of Science and a Member of the National Academy of Medicine. Dr Armstrong delivered the ISSCR Tobias Award Lecture at the 2019 Annual Meeting. The ISSCR Tobias Award Lecture was established in 2015 and is supported by the Tobias Foundation. The award recognizes original and promising basic hematology research as well as direct translational or clinical research related to cell therapy in hematological disorders.

stem cell may often be derived from a myeloid progenitor that aquires self-renewal properties and a limited stem cell-like gene expression program (Krivtsov et al., 2006). The commonality between these fusion oncogenes and model systems is the induction of a stem cell-associated gene expression program in myeloid progenitor cells that includes expression of the *HOXA* cluster genes and *MEIS1* and which disrupts myeloid differentiation at different stages of commitment (Figure 1). *DNMT3a* mutations, on the other hand, are not able to induce sufficient self-renewal capacity in non-stem cells to result in leukemic transformation. The overall phenotype of progenitor-derived leukemias seems to be less aggressive *in vivo* than their stem cell-derived counterparts (George et al., 2016; Krivtsov et al., 2013). This might be explained by the fact that *MLL-AF9* and other oncogenes can only reconstitute parts of the stem cell machinery, but cannot completely reprogram myeloid progenitor cells to be indistinguishable from a stem cell-derived clone. Indeed, we were able to demonstrate that the cell of origin can be identified by a distinct expression pattern, including high expression of *Evi-1* and increased tolerance to cytarabine and doxorubicin chemotherapy treatment in stem cell-derived AML cells (Krivtsov et al., 2013). While these findings were based on mouse studies, there is emerging evidence suggesting that human *MLL*-rearranged AML cases can be separated into high and low *EVI-1*-expressing groups and that the *EVI-1* high expression is indicative of an adverse outcome (Gröschel et al., 2013; Ho et al., 2013). Further studies are required to understand the differences in stem cell- versus progenitor-derived leukemias to develop more effective and targeted treatment approaches that might differ depending on the cell type from which the leukemia was derived. These studies demonstrate that multiple hematopoietic cell types can be the cell of origin of leukemia. Also, a combination of the cell of origin and the genetic mutation work in concert to define the phenotype of the resultant leukemia.

MLL Fusions Drive Stem Cell Programs in Leukemic Cells through Recruitment of Chromatin-Associated Complexes

The *mixed lineage leukemia* (*MLL*) gene on chromosome 11q23 is a target for recurrent translocations leading to AML or acute lymphoblastic leukemia (ALL). Often, these genetic events are predictive of a poor prognosis. *MLL* rearrangements are found in >70% of leukemias in infants and are less frequent in adult AML (~5%–10%) (Charles and Boyer, 2017). While over 70 fusion partners have been

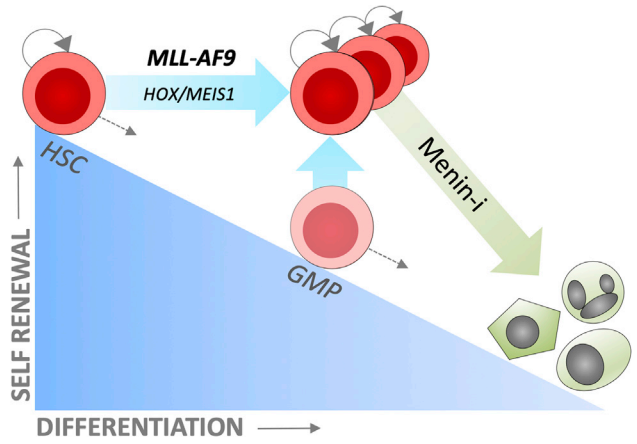

Figure 1. Hematopoietic Stem Cells Are at the Apex of the Hematopoietic Hierarchy and Possess the Highest Level of Self-Renewal Capacity

During differentiation, this self-renewal potential is sequentially lost as cells specialize toward a specific lineage until they reach a terminally mature cell stage. Oncogenes, such as MLL-AF9, induce high levels of stemness genes, including HOXA and MEIS1, and bestow an artificial self-renewal capacity on stem and progenitor cells, which prevents them from forming differentiated progeny and leads to an accumulation of immature leukemic cells. Inhibition of components of the epigenetic machinery, such as DOT1L and Menin, can reverse self-renewal gene expression programs and lead to differentiation of leukemia cells.

identified, the majority of *MLL* fusions occur with *AF4*, *AF9*, *AF10*, and *ENL* (Meyer et al., 2018). Fusion partners AF4, AF9, and ENL are all components of the super elongation complex (SEC), which also contains the elongation factor ELL (also a fusion partner in *MLL*-rearranged leukemia) and positive transcription elongation factor b. While wild-type MLL1 is thought to positively regulate transcription in part by methylating histone H3 on lysine 4 at the promoters of its target genes, the translocation leads to a loss of the C-terminal histone methyl transferase domain. The resulting AML cells, however, retain their H3K4 methylation at promoter regions suggesting that one of the multiple MLL family proteins remains present at target genes to maintain this modification. Surprisingly, wild-type *MLL1* seems to be dispensable not only for normal hematopoiesis but also for *MLL*-fusion cells, and recent data suggest that *MLL2* might be a particularly important H3K4 methyltransferase in *MLL*-rearranged AML (Chen et al., 2017; Mishra et al., 2014). The fusion of *MLL* with components of the SEC has been proposed to deregulate the release of paused Pol II and constitutively enhance the transcription of *MLL* target genes, such as *HOXA/MEIS1* (Lin et al., 2010). An overlapping group of *MLL*-fusion partners are components of the *disruptor of telomere silencing 1* (*DOT1L*) complex. *DOT1L* is the only known histone methyltransferase that catalyzes H3K79 mono-, di-, and trimethylation, which is associated with actively transcribed genes (Feng et al., 2002). *DOT1L* forms multiprotein complexes with *AF9*, *AF10*, and *ENL*, which are commonly found as fusion partners of *MLL*. The recuitment of the DOT1L complex causes an aberrant H3K79 dimethylation (H3K79me2) pattern at *MLL* target loci, such as *HOXA/MEIS1* (Deshpande et al., 2014; Guenther et al., 2008; Krivtsov et al., 2008). The deposition of H3K79 methyl marks at these loci is essential for maintaining high expression of these self-renewal genes by preventing gene silencing mediated by *SIRT1* and H3K9 methyltransferase *SUV39H1* (Chen et al., 2015). Genetic inactivation of *Dot1l* in mouse models has shown that *MLL*-fusion leukemia cells are highly dependent on *Dot1l* for the initiation and maintenance of leukemia (Bernt et al., 2011; Chang et al., 2010; Chen et al., 2013; Deshpande et al., 2013; Jo et al., 2011; Nguyen and Zhang, 2011).

Identification of Targeted Therapy Approaches for *MLL*-Rearranged Leukemias

Since *DOT1L* is an enzyme that is important for MLL-fusion function, it represents an attractive therapeutic target. Small-molecule inhibitors developed to target the enzymatic activity of DOT1L suppress MLL-fusion-driven gene expression *in vitro* and *in vivo* with little toxicity. In clinical trials, the DOT1L inhibitor EPZ-5676 (Pinometostat) was well tolerated and clearly induced differentiation of leukemia cells in multiple patients. Complete remission was observed in two patients (Stein et al., 2018). The complete response in a small number of patients suggest that there might be bypass mechanisms present in leukemia cells that allow leukemia cells to adapt to the loss of H3K79 methylation. Therefore, combination therapy options may increase the efficacy of DOT1L inhibition.

One candidate target for combination treatment is Menin, a protein encoded by the *multiple endocrine neoplasia 1* (*Men1*) genes (Dafflon et al., 2017). *Menin* interacts with the N-terminal region of MLL that is common between wild-type and MLL-fusion proteins. It is thought to act as an adaptor protein since it lacks any known protein motifs, but has been shown to interact with different components of the MLL complex, such as LEDGF, and also binds DNA without known sequence specificity (La et al., 2004; Milne et al., 2005; Schnepp et al., 2004). Deletion of *Menin*, or mutation of the Menin-binding domain in MLL abrogates the leukemic transformation potential of *MLL*-fusion oncogenes (Chen et al., 2006; Yokoyama et al., 2005). It seems, however, that the requirement for Menin to maintain self-renewal is somewhat specific to leukemic cells since normal hematopoietic stem cells can tolerate its loss (Li et al., 2013). Therefore, Menin-MLL interaction inhibitors are another promising possibility to specifically target

leukemia cells. Initial studies using small molecules that inhibit the Menin-MLL interaction showed promising results, such as reduced expression of *MLL* target genes resulting in reduced tumor growth *in vivo* and a moderate increase in survival of mice transplanted with *MLL-AF9* leukemic cells (Borkin et al., 2015). Newly developed Menin inhibitors with improved drug-like properties and oral bioavailability demonstrate selective effects on chromatin and show dramatic results in patient-derived xenograft models of *MLL*-rearranged AML and B-ALL. Due to these promising results, these Menin inhibitors have recently entered phase I clinical trials (NCT04065399 and NCT04067336) (Klossowski et al., 2020; Krivtsov et al., 2019).

DOT1L inhibitors block only one part of the oncogenic fusion complex, which could be circumvented, for example, by relying mainly on the SEC for transcriptional activation. Menin-MLL interaction inhibitors, on the other hand, are thought to act by dissociating the entire MLL complex from chromatin, which might lead to greater compromise of MLL-fusion function since the complex is disrupted, and complex components may be less stable once dissociated (Wu et al., 2019). Complex disruption or degradation might offer a promising new direction of targeting *MLL*-fusion leukemias as it would likely make it more difficult for cells to adapt and develop resistance mechanisms due to the rapid loss of the oncogenic complex.

Disruption of Chromatin Complexes Inhibits Self-Renewal in Other AML Subtypes

Large-scale gene expression profiling of AML samples revealed that *MLL*-rearranged AML are not the only leukemia subtype with a characteristic pattern of *HOXA* cluster genes and *MEIS1* expression (Spencer et al., 2015). AMLs containing *NPM1c* mutations, showed a similar *HOXA/MEIS1* profile as *MLL*-fusion leukemias with the addition of aberrant *HOXB* cluster upregulation, which is also a hallmark of normal hematopoietic stem cells. Using *Npm1c* mutant knockin mice, we further demonstrated that *NPM1c* can induce leukemia from myeloid progenitors as well as stem cells, which is preceded by a prolonged preleukemic expansion of mutant clones (Uckelmann et al., 2020). *NPM1* gene mutations lead to cytoplasmic mislocalization of *NPM1* (*NPM1c*) usually through small insertions that corrupt the C-terminal nucleolar localization signal and convert it to a nuclear export signal (Falini et al., 2005). *NPM1c* mutations are among the most common types of aberrations in AML, and they often co-occur with other mutations, such as *FLT3-ITD* mutations in the FLT3 tyrosine kinase, which predicts an adverse prognosis (Döhner et al., 2015). The high prevalence of these leukemia cases and their often devastating outcome underscores the need for more efficient and targeted therapy options.

The above-mentioned similarities of *HOX/MEIS1* expression between *MLL*-fusion and *NPM1c* AML cells led us to determine if the chromatin complexes involved in the regulation of these stem cell genes are similar in both cancer types. Despite their lack of *MLL* rearrangements, we found that *NPM1c* AML cells were highly sensitive to DOT1L and Menin inhibition (Kühn et al., 2016). Furthermore, wild-type MLL1 and, more specifically, its Menin-binding domain, were shown to be essential in *NPM1c* AML. Similar to their *MLL*-rearranged counterparts, *NPM1c* AML cells also show a reversal of their aberrant self-renewal properties in response to novel Menin inhibitors, and patient-derived xenograft models demonstrate dramatic responses to this approach (Uckelmann et al., 2020). In addition to treating frank *NPM1c* AML, Menin-MLL inhibition can prevent the development of leukemia from preleukemic cells and rapidly eradicate preleukemic clones in a model of *Npm1c* mutant leukemia development (Uckelmann et al., 2020). These promising results demonstrate that therapeutic approaches designed to target MLL-fusion complexes can also be used in wild-type MLL-dependent cancers and premalignant cells, as they depend on similar epigenetic machinery for the regulation of neoplastic gene expression programs. Advances in the clinical development of DOT1L and Menin inhibitors could potentially benefit other leukemia types or even other cancer types that share similar dependencies.

Conclusion

Over the last two decades, our understanding of leukemia development has been reshaped by genome-scale and single-cell technologies. Many leukemias are believed to be derived from highly potent self-renewing stem cells through the acquisition of mutations that increase stem cell fitness or interfere with their ability to properly differentiate. While this holds true in many cases, our studies have revealed that certain oncogenes, such as *MLL* fusions and *NPM1c* mutations, have the ability to induce leukemia development at different stages of hematopoietic differentiation through upregulation of a network of stemness genes. The transcriptional programs, as well as the epigenetic complexes involved in maintaining these expression programs, were found to be highly similar in these leukemias. Insight into the mechanism of leukemogenesis has allowed us to examine several different approaches to disrupt this oncogenic machinery by interfering with either the enzymatic activity or assembly of the *MLL* complex on chromatin at various stages of AML development. DOT1L and Menin inhibitors have proven to be highly effective in model systems against *MLL*-rearranged, *NPM1c* mutant AMLs, and preleukemic cells which depend

on the wild-type Menin-*MLL* interaction for their stem cell gene expression programs. The rewiring of the chromatin landscape in these leukemia cells leads to rapid differentiation and eventually depletion of the leukemia stem cell pool. The discovery of effective targeted approaches that reverse oncogenic gene expression programs represent a critical step toward therapeutic development. Remarkably, they also seem to have little effect on normal stem cells. With the discovery of clonal hematopoiesis and long-term follow-up of patients with high-risk mutations, we are now entering an era where we might someday be able to prevent the development of leukemia through early detection of second hits that could be combated with targeted therapies, including those that target epigenetic mechanisms.

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