MLL-AF9 Leukemia Stem Cells: Hardwired or Taking Cues from the Microenvironment?

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MLL rearrangements in humans lead to both acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). While AML has been successfully produced in mice, modeling ALL has been more difficult. In this issue of *Cancer Cell*, Wei et al. (2008) describe generation of AML, ALL, and biphenotypic leukemia by manipulating the cytokine milieu of human progenitor cells expressing MLL-AF9. They demonstrate that both multipotent and lineage-restricted progenitors are targeted by MLL-AF9 fusion proteins and that Rac signaling is crucial for survival. This study demonstrates the heterogeneity of MLL-AF9 leukemic stem cells and the importance of the microenvironment in determining lineage outcome.

One of the most common chromosomal breakpoint regions in human leukemias occurs at chromosome 11q23. This results in balanced translocations that fuse the mixed-lineage leukemia (*MLL*) gene, in frame, to one of more than 50 partner genes, resulting in expression of chimeric oncogenic fusion proteins. *MLL* mutations arise de novo and are also commonly found in therapy-related leukemia following treatment with topoisomerase inhibitors. MLL fusion proteins upregulate expression of A cluster *Hox* genes and *Meis1*, which are critical for transformation (Hess, 2004).

MLL rearrangements give rise to both acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). For unclear reasons, some fusion partners are associated more commonly with AML than with ALL and vice versa. The t(4;11) and t(11;19) translocations that result in MLL-AF4 and MLL-ENL are most frequently found in ALL, while t(9;11) and t(6;11) that form MLL-AF9 and MLL-AF6 fusions are the most common genetic alterations of *MLL* in AML. While these findings suggest that the fusion partner provides an instructive signal for lineage commitment of the resulting leukemia (Daser and Rabbitts, 2004), MLL leukemias modeled in mice using knockin technology or bone marrow retroviral transduction raise questions about this model. For example, MLL-ENL transduction and transplantation consistently generates AML in mice despite being found equally in both AML and ALL in humans (Lavau et al., 1997). In addition, studies using MLL-GAS7, which produces a biphenotypic leukemia, suggest that leukemic stem cells (LSCs) (some prefer the term leukemia-initiating cell) are multipotent (So et al., 2003). Recently, ALL was exclusively generated when MLL-ENL was expressed in human hematopoietic progenitor cells (HPCs) and transplanted into mice (Barabe et al., 2007). These findings raise a number of questions about LSCs in MLL-associated leukemias. Are LSCs heterogeneous? Do human and mouse hematopoietic cells have intrinsic differences that lead to different leukemias? To what extent does the microenvironment affect leukemia phenotype? In this issue of *Cancer Cell*, Mulloy and colleagues (Wei et al. 2008) experiment with human cord blood transformed by MLL-AF9 and provide important insights into all of these questions.

Bone Marrow Microenvironment Affects Leukemia Phenotype

To test the importance of the microenvironment, Wei et al. (2008) employed retroviral transduction of human $CD34^+$ cord blood and transplantation into three types of mice: nonobese diabetic/severe combined immunodeficient (NOD/SCID [NS]), NS-$\beta_2M^{-/-}$ (NS-B2M), and NS mice that overexpress the human cytokines SCF, GM-CSF, and IL-3 (NS-SGM3). Retroviral transduction of MLL-AF9 readily immortalized $CD34^+$ cells that could be cultured to promote $CD11b^+CD13^+CD14^+CD15^+$ myelomonocytic cells. When cultured in conditions promoting B cell differentiation, lymphoid cells could be generated expressing CD19 and CD10, demonstrating the importance of culture conditions for determining lineage outgrowth.

When MLL-AF9 cells from the myeloid or lymphoid cultures were injected into NS-SGM3 mice, aggressive myelomonocytic leukemia ensued. Strikingly, when the same cells were injected into NS or NS-B2M mice, a mix of AML, acute B-lymphocytic leukemia (B-ALL), and acute biphenotypic leukemia (ABL) resulted. While B-ALL and ABL cells were present in the injected mice, a distinct population of AML was also detected, implying that separate LSCs had proliferated in the mice or that a single LSC could generate both AML and ALL. While it is likely that species differences between human and mouse progenitor cells play a role in disease outcome, these data suggest that the cytokine milieu of leukemic stem cells profoundly affects their lineage commitment.

Heterogeneity of the MLL-AF9 LSCs

Previous studies of the frequency and origin of MLL fusion LSCs in diseased mice suggest that both hematopoietic stem cells (HSCs) and lineage-restricted progenitors can function as LSCs when targeted by MLL fusions. Furthermore, it appears that the fusion proteins are capable of reinitiating a self-renewal program (Cozzio et al., 2003; Krivtsov et al., 2006; Somervaille and Cleary, 2006). In contrast to these previous studies, which utilized murine cells, Wei et al. (2008) tested whether human MLL-AF9 LSCs are derived from multipotent or lineage-restricted progenitors. Cord blood was transduced with MLL-AF9 and cultured under either

myeloid or lymphoid conditions. When injected into NS-B2M mice, these produced AML and ALL, respectively. Intriguingly, B-ALL and AML cells from separate diseased transplant recipients were found to be clonally related as determined by Southern blot analysis, demonstrating the multipotency of the LSCs and the potential for both AML and ALL generation from a single LSC. To determine the plasticity of more committed progenitors and whether they are also targeted by MLL fusion proteins, myeloid ($CD19^-CD33^+$) and lymphoid ($CD19^+CD33^-$) cells were isolated and cultured for 4 weeks. $CD19^+CD33^-$ cells were able to regenerate $CD19^-CD33^+$ cell types, and clonal analysis demonstrated that these cells originated from the $CD19^+$ LSCs. $CD19^-CD33^+$ cells did not generate $CD19^+$ cells in cell culture but in some cases produced both $CD33^+$ AML and $CD19^+$ ALL in mice. These data highlight the heterogeneity of MLL-AF9 LSCs, as both lineage-restricted and multipotent progenitors are targeted by MLL-AF9 and these cells retain at least some differentiation capacity along the myeloid and lymphoid lineage (Figure 1).

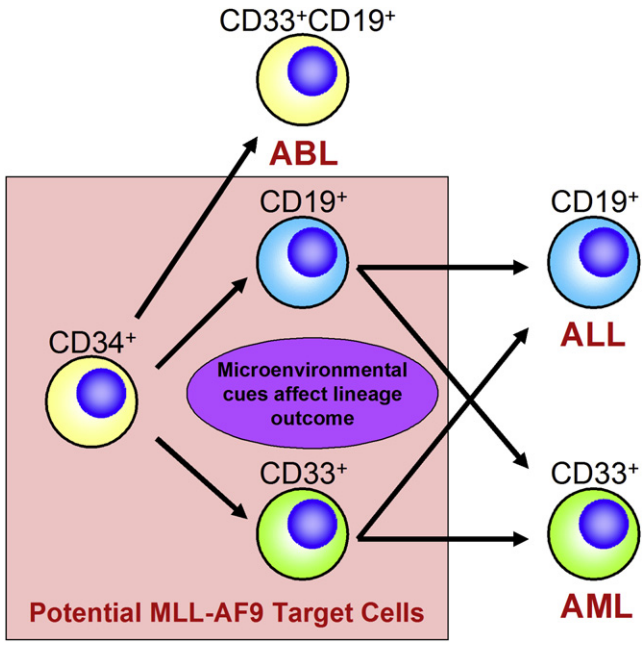

Figure 1. Microenvironment and Translocations Determine Lineage Outcome

Several cells are capable of immortalization by MLL-AF9 (shaded in red) leading to acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), and acute biphenotypic leukemia (ABL) (with characteristics of both myeloid and lymphoid cells). Microenvironmental cues can cooperate with MLL fusion proteins to determine lineage outcome in leukemia of multipotent progenitors ($CD34^+$) as well as progenitors that have partially differentiated ($CD19^+$ and $CD33^+$) (denoted by arrows).

Rac Signaling Is Important in MLL-AF9 AML

Although the cell signaling pathways in MLL-associated leukemias are largely undefined, increased activity of Rac1 has recently been reported in MLL-AF9 cells (Somervaille and Cleary, 2006). Wei et al. (2008) used shRNA and a Rac1-selective small molecule to inhibit Rac1 function and test the importance of this signaling pathway in MLL-AF9 leukemic cells. Treatment of MLL-AF9 cells with a Rac1 inhibitor resulted in cell-cycle arrest and apoptosis, while treatment of control cord blood or cells containing a different fusion protein (AML-ETO) were not affected. This effect was linked to regulation of levels of antiapoptotic Bcl-x_L, a downstream target of Rac1 signaling. Bcl-x_L was degraded in MLL-AF9 cells but remained stable in cord blood or AML-ETO cells. These experiments identify the Rac1 signaling pathway as essential and specifically required for growth of MLL-AF9 cells and uncover a potential therapeutic target for treatment of MLL fusion leukemia.

The results presented by Wei et al. (2008) strongly suggest a dynamic model for determining leukemic lineage outcome in which MLL fusion proteins drive the survival and proliferation of target cells and surrounding cytokines instruct cell lineage decisions (Wei et al., 2008). This does not, of course, exclude the possibility that fusion partners also provide an instructive signal or that translocation frequencies vary between cell types because of the expression levels at the involved loci. The MLL-AF9 target cell appears to include both the stem and progenitor cell compartments, and, given the regeneration of myeloid cells from lymphoid cultures, these target cells remain plastic and receptive to environmental cues. Recent data support greater plasticity within the hematopoietic system (Iwasaki et al., 2006). However, further complexity is added by the recent description of the effects of gene dosage on leukemic potential (Chen et al., 2008). *Mll-AF9* expressed at physiologic levels by knockin was found to be expressed at much lower levels than retrovirally transduced MLL-AF9. Furthermore, the fusion protein was expressed at higher levels in stem cells than in more differentiated progenitor cells. This translated to a significantly greater frequency of transformable cells in the stem cell compartment compared to more differentiated progenitors.

The current study provides important insights, but a number of intriguing questions about MLL cancer biology remain. Why are specific fusion partners associated with particular lineages? It will be interesting to test MLL-AF4 or MLL-ENL cells (commonly associated with ALL) in the NS-SGM3 mice that readily formed AML. Can MLL fusion proteins transform committed myeloid or lymphoid cells at physiological expression levels? What is the basis for the selective "addiction" of MLL fusion protein-transformed cells to Rac? And, perhaps most importantly, is Rac signaling the long sought-after "Achilles' heel" that can be targeted for more selective and effective therapies for leukemias with *MLL* rearrangements?

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Linking miRNA Regulation to BCR-ABL Expression: The Next Dimension

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The introduction of tyrosine kinase inhibitors in the treatment of *BCR-ABL1*-rearranged malignancies has revolutionized therapy, but the prognosis for acute leukemias remains suboptimal. In this issue of *Cancer Cell*, Bueno et al. (2008) add a new dimension to the regulation of ABL1 expression. The authors demonstrate that ABL1 is a direct target of miR-203, miR-203 is silenced by genetic and epigenetic mechanisms in hematopoietic malignancies expressing either ABL1 or BCR-ABL1, and restoration of miR-203 expression reduces ABL1 and BCR-ABL1 levels and inhibits cell proliferation. These findings may have broad implications for mechanisms underlying malignant transformation in hematopoietic and other malignancies.

MicroRNAs (miRNAs) are noncoding RNAs that regulate many cellular functions including cell proliferation, differentiation, and apoptosis by silencing specific target genes through translational repression or direct mRNA degradation (Ambros, 2004). Although the detailed functions of the growing number of miRNAs identified in the mammalian genome are far from being completely characterized, recent studies have indicated that deregulated expression of specific miRNAs that modulate expression of oncogenes and tumor suppressors is associated with the development of malignancies, and specific miRNA expression signatures can be used to effectively classify human tumors (Lu et al., 2005). Although genome copy-number changes are associated with altered levels of miRNA expression in various human malignancies (Zhang et al., 2006), recent data suggest that miRNA inactivation by epigenetic mechanisms plays an important role as well, and re-expression of certain miRNAs by drugs that modulate epigenetic changes can lead to downregulation of target oncogenes (Fazi et al., 2007).

More than 40 years ago, the Philadelphia (Ph) chromosome was identified by Nowell and Hungerford. The Ph chromosome is a product of the t(9;22), which fuses the Abelson kinase gene (*ABL1*) from chromosome 9 with the breakpoint cluster region (BCR) from chromosome 22 that expresses the BCR-ABL1 fusion protein: a constitutively active tyrosine kinase. The BCR-ABL1 fusion oncoprotein is a hallmark of chronic myelogenous leukemia (CML) and is also present in a fraction of B progenitor acute lymphoblastic leukemia (ALL) cases that have a particularly poor prognosis. Aberrant expression of the wild-type *ABL1* oncogene may also be associated with the development of hematopoietic malignancies including T cell lymphomas (Ren, 2005). While the development of tyrosine kinase inhibitors (TKIs) like imatinib mesylate have revolutionized treatment of *BCR-ABL1*-rearranged leukemias, it has become increasingly clear in recent years that TKI treatment alone will not be curative in many cases, particularly in acute leukemias with *BCR-ABL1* rearrangement. Thus, further dissection of the regulatory networks that drive BCR-ABL1-induced malignant transformation may help to identify other novel therapeutic approaches that complement TKI treatment.

In this issue of *Cancer Cell*, a study by Bueno et al. (2008) begins to elucidate the role of silenced miRNA expression in the regulation of *BCR-ABL1*-rearranged leukemias and T cell lymphomas expressing ABL1. Using comparative genomic hybridization (CGH) analysis of murine γ radiation-induced lymphomas, the authors identify loss of heterozygosity of a fragile 7 Mb chromosomal region on murine chromosome 12, a region coding for approximately 12% (52 miRNAs) of the mammalian miRNAome known to date. miRNA profiling revealed decreased expression of one of the region's miRNAs: miR-203. Analysis of the miR-203 promoter demonstrated that miR-203 is silenced not only by genetic loss of one allele but also epigenetically by promoter CpG hypermethylation in the remaining DNA copy (Figure 1). Next, the authors assessed putative miR-203 targets and identified the *ABL1* tyrosine kinase