Modeling the Initiation and Progression of Human Acute Leukemia in Mice

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Our understanding of leukemia development and progression has been hampered by the lack of in vivo models in which disease is initiated from primary human hematopoietic cells. We showed that upon transplantation into immunodeficient mice, primitive human hematopoietic cells expressing a mixed-lineage leukemia (MLL) fusion gene generated myeloid or lymphoid acute leukemias, with features that recapitulated human diseases. Analysis of serially transplanted mice revealed that the disease is sustained by leukemia-initiating cells (L-ICs) that have evolved over time from a primitive cell type with a germline immunoglobulin heavy chain (Igh) gene configuration to a cell type containing rearranged Igh genes. The L-ICs retained both myeloid and lymphoid lineage potential and remained responsive to microenvironmental cues. The properties of these cells provide a biological basis for several clinical hallmarks of MLL leukemias. Over 50% of infant acute leukemias exhibit rearrangements of the mixed-lineage leukemia gene (MLL), also termed ALL-1 and HRX) at human chromosome 11q23 (4). Translocations of MLL to >40 different partner genes have been identified, and the resulting fusion proteins are strong transcriptional activators that drive the aberrant expression of homeobox family genes (5). In view of the potent oncogenic properties of MLL fusion genes, we tested the leukemogenic potential of MLL–eleven-nineteen
leukemia (ENL), the product of t(11;19) that is found in both acute myeloid leukemias (AMLs) and acute lymphoid leukemias (ALLs). A lineage-depleted fraction of human umbilical cord blood enriched for stem and progenitor cells (Lin− CB) was infected with either a retrovirus encoding MLL-ENL and an enhanced green fluorescent protein (EGFP) marker gene or a control retrovirus encoding EGFP only and then injected into sublethally irradiated immunodeficient mice (6).

The recipients of cells transduced with the control retrovirus (hereafter referred to as control mice) displayed no overt pathology. In contrast, >75% of the mice receiving MLL-ENL–transduced cells (hereafter referred to as MLL-ENL mice) were dying within 135 days of transplantation, appearing pale and lethargic (Fig. 1A). Postmortem analysis revealed splenomegaly and generalized lymphadenopathy (fig. S1), and in 26 out of 29 MLL-ENL mice, L1 type lymphoblasts were found in the blood and bone marrow (BM), which is consistent with the development of ALL. As in the human disease, these cells also infiltrated the liver, lungs, kidneys, brain, and testes (Fig. 1, B and C, and fig. S2A). To assess the lineage and maturation stage of the leukemic blasts, human grafts were studied by flow cytometry (Fig. 1, D and E, and fig. S3). In control mice, the human graft made up 76 ± 15% of the BM and consisted mainly of leukocytes expressing the pan-B cell marker CD19, with a minor population of myeloid cells (CD33+CD19−), as previously described (7). In contrast, in MLL-ENL mice, the human graft made up 96 ± 2% of the BM and consisted almost exclusively of B cells. The human B cell population in control mice showed evidence of normal terminal differentiation, whereas in MLL-ENL mice, these cells were arrested at the pro-B cell stage of differentiation (CD19+, CD20−, IgM+ and IgD+) (8). Thus, the cell surface phenotype and tissue-infiltration pattern of the leukemic blasts seen in this model resemble those observed in B-ALL patients with this translocation (9, 10). B-precursor ALL has rarely been observed in previous work where various MLL fusions were studied in transgenic mice or by murine BM transplantation (11–17).

We next tested the leukemogenic potential of MLL-AF9, a different fusion gene predominantly associated with AML (5, 10). Clinically, different MLL fusions are associated with distinct frequencies of myeloid and lymphoid leukemias. In contrast to our findings with MLL-ENL,
Lin^+ CB cells expressing MLL-AF9 initiated both B-lymphoid and myeloid disease upon transplantation (Fig. 1, B, D, and E; figs. S2B, S3, and S4; and table S1). Eight of 16 MLL-AF9 mice developed B-precursor ALL with features identical to those noted with MLL-ENL. Two mice developed AML, characterized by myelomonocytic blasts in the blood and BM with diffuse organ infiltration. This disease corresponds phenotypically to the myelomonocytic or monoblastic leukemias seen in the majority of patients with MLL-AF9 translocations. A single MLL-AF9 mouse developed a mixed leukemia composed of both B-lineage lymphoblasts and myeloblasts. Thus, as seen in mouse models of MLL leukemias and in patients, the identity of the fusion partner plays an instructive role in determining leukemia lineage in our model (18).

Table S1 summarizes the data corresponding to all primary mice from six independent transplantation experiments. Using the clinical definition of human leukemia (≥20% blasts in the BM), we found that 78% of mice (35 out of 45) injected with cells expressing MLL fusion genes developed leukemia in less than 19 weeks, and two mice were preleukemic when they were killed (<20% blasts in the BM, but present in blood and peripheral organs). Among mice that were engrafted with transduced human cells, 93% developed disease (8). This high penetrance of leukemia and the short latency to disease suggest that MLL fusion genes efficiently initiate leukemogenic programs and that minimal cooperating events are required for the development of fully transformed leukemic stem cells that are capable of sustaining aggressive disease. These results clearly contrast with models of solid tumors that required at least three different oncogenes to transform primary human cells (19–21).

For L-ICs to sustain and propagate disease, they must self-renew. We assessed the self-renewal of MLL-derived L-ICs by serial transplantation. BM cells from control, MLL-ENL, and MLL-AF9 primary mice were transplanted into 34 secondary recipients (table S2). After 15 weeks, the BM from controls generated no detectable human grafts in secondary mice. In contrast, recipients of BM from MLL-ENL and MLL-AF9 mice were engrafted with human cells and developed leukemia of the same phenotype (cell surface markers and organ involvement) as the corresponding primary mouse, but with a shorter latency (fig. S5). Secondary transplantation of limiting doses of leukemic blasts from primary mice allowed us to estimate that the L-IC frequency was >1/2000 cells (table S3).

To investigate self-renewal at a clonal level, retroviral insertion sites were analyzed by Southern blotting. The transduced human graft in the BM of control primary mice was derived from two to five clones (Fig. 2A), as previously reported (7). In all MLL primary mice, the leukemic grafts were composed of either one or two major clones with variable numbers of minor contributors (Fig. 2, A and C, and fig. S6). In general, the predominant clone from a primary mouse persisted to generate monoclonal disease in its corresponding secondary mice. However, in two instances, a novel clone that was below the detection limit in primary mice made a substantial contribution in secondary recipients (Fig. 2C). One interpretation of this finding, supported by clonal tracking studies in human AML (3), is that such clones arose from L-ICs that were quiescent in primary mice and became activated after secondary transplantation.

To determine the type of cell transformed by MLL fusion genes, Southern blotting was used
to study immunoglobulin heavy chain (IgH) gene rearrangement, an event that occurs at a stage of B-lymphoid differentiation between the common lymphoid progenitor (CLP) and the pro-B cell (22). Although control and MLL-AF9 mice with AML displayed only the germline arrangement (fig. S6), all primary mice with B-precursor ALL displayed varying rearrangements of the IgH gene, in addition to the germline band (Fig. 2B and fig. S6). In ~40% of the primary mice, the germline configuration accounted for >50% of all the IgH gene configurations. These data allow us to exclude the possibility that, in these cases, transformation occurred in a committed B cell with a rearranged IgH gene. Instead, they indicate that a more primitive cell type that had not initiated IgH gene rearrangement (such as the hematopoietic stem cell, CLP, or the early B cell) was the target of transformation.

A comparison of the IgH gene-rearrangement patterns in secondary mice and their corresponding primary mice showed a consistent decrease in the intensity of the germline configuration, accompanied by a greater contribution by clones with rearranged IgH alleles. The same rearrangements were often present in multiple secondary mice derived from the same primary animal (Fig. 2B and fig. S6), suggesting that the L-ICs in primary mouse underwent self-renewal divisions and generated daughter L-ICs capable of initiating ALL upon transplantation. Thus, in leukemias where a primitive cell type was deemed to be the target of transformation, we can conclude that the L-ICs present at later time points were not identical to those initially generated, though they shared the same retroviral integration. Instead, it appears that the phenotype of the L-ICs evolved over time from a primitive (germline IgH) cell type to a cell type with rearranged IgH genes. This clonal evolution indicates that analysis of L-IC properties and phenotype at a single time point may not provide a complete picture of L-IC biology or insights into the identity of the cell of origin.

Fig. 3. MLL-ENL and MLL-AF9 cells cultured in myeloid-promoting conditions generate different graft phenotypes upon injection into immunodeficient mice. (A) In vitro growth curves of control, MLL-AF9, and MLL-ENL cells under conditions that promote myeloid differentiation. At the indicated time points, cultured cells were injected into immunodeficient mice. IL-3, interleukin-3; SCF, stem cell factor. (B) Types of human grafts generated in mice. Contour plots are gated on live cells, and BM cytospins (MGG staining) illustrate the morphology of the indicated leukemias. Scale bars, 25 μm.
Because a primitive cell was the target for the transformation in ~40% of our experimental leukemias, we investigated the differentiation potential of MLL-derived L-ICs. Leukemic cells were harvested from the BM of primary mice and cultured in vitro under conditions supportive of both B-lymphoid and myeloid cells. Both AML and B-precursor ALL grew for >100 days (fig. S7, A and B), and in 10 out of 14 cultures initiated from mice with MLL-ENL B-ALL, a CD33<sup>+</sup>CD19<sup>+</sup> population; in one instance, this was followed by a reversion back to B-lineage cells (Fig. 2D). Retroviral integration analysis showed that clonality was maintained during these lineage switches, but cells with either rearranged IgH alleles or strictly germline during these lineage switches, but cells with either rearranged IgH alleles or strictly germline status were competent to switch lineages (Fig. 2E and fig. S7E). Consistent with these observations, intracanalonal lineage switching has been documented in patients with MLL leukemias upon relapse (23–25) and was also found in our in vivo studies (fig. S8). Together these in vitro and in vivo data indicate that MLL-derived L-ICs, including those with rearranged IgH genes, retain both lymphoid and myeloid potential, a finding also observed in murine models (17).

Finally, to investigate the influence of microenvironment on MLL L-ICs, we seeded cells expressing MLL-ENL or MLL-AF9 into myeloid-promoting suspension cultures immediately after transduction. Under these conditions, cells expressing MLL fusions outgrew controls, generating populations of monoblastic cells (MLL-ENL) and myelomonoblastic cells (MLL-AF9) (Fig. 3A and fig. S9). Analysis of the leukemia-initiating capacity of cells from different times in culture showed that at the earliest time point, cultured MLL-ENL cells generated B-precursor ALL in vivo; however, at later passages the phenotype of the human grafts shifted toward the myeloid lineage, with some mice showing monoclonal mixed-lineage leukemias and AML (Fig. 3, fig. S10, and table S4). Thus, exposure to myeloid-supportive conditions was permissive for the development of clones capable of generating myeloid disease in vivo. In contrast to MLL-ENL, the injection of MLL-AF9 cells from culture resulted in strictly myeloid outgrowths in all but one engrafted mouse, again highlighting an instructive role for the MLL fusion partner in determining leukemia lineage.

Our data show that MLL fusion genes can initiate both myeloid and lymphoid leukemogenic programs in primary human hematopoietic cells. Which program is ultimately followed is influenced by the identity of the fusion partner, as well as by microenvironmental signals. The respon- siveness of MLL-derived L-ICs to extrinsic cues, coupled with their lympho-myeloid potential, provides a biological basis for several hallmarks of MLL leukemias, including lineage switching at relapse and the high incidence of B-ALL in infants (26), where the intrinsic and extrinsic determinants of neonatal hematopoiesis favor B cell development (27). The finding that L-ICs undergo clonal evolution indicates that disease progression is linked to the biological properties of the leukemia stem cells that underlie the disease, rather than to the evolution of leukemic blasts. Thus, it will be essential to understand the cellular and molecular properties of L-ICs at all stages, from leukemic initiation to disease progression, in order to devise therapies to target their eradication.

References and Notes
6. Materials and methods are available as supporting online content.
8. Details pertaining to engagement and leukemogenic phenotypes are available as supporting material on Science Online.
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Materials and Methods
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Regulation of the Germinal Center Response by MicroRNA-155

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MicroRNAs are small RNA species involved in biological control at multiple levels. Using genetic deletion and transgenic approaches, we show that the evolutionarily conserved microRNA-155 (miR-155) has an important role in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell–dependent antibody response. miR-155 exerts this control, at least in part, by regulating cytokine production. These results also suggest that individual microRNAs can exert critical control over mammalian differentiation processes in vivo.

MicroRNAs are emerging as key players in the control of biological processes, and the stage-specific expression of certain microRNAs in the immune system suggests that they may participate in immune regulation. One such microRNA is miR-155, produced from the non–protein-coding transcript of the bic gene. bic was discovered as a recurrent integration site of avian leukemia virus in chicken lymphoma cells (I). The hairpin from which miR-155 is processed represents the only evolutionarily conserved sequence of the bic gene, indicating that miR-155 mediates bic function (2–4). bic/miR-155 has been shown to be highly expressed in a variety of human B cell lymphomas, including the Hodgkin-Reed

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