MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice

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Summary

A specific association with mixed lineage leukemias suggests that MLL oncoproteins may selectively target early multipotent hematopoietic progenitors or stem cells. We demonstrate here that a representative MLL fusion protein, MLL-GAS7, impairs the differentiation and enhances the in vitro growth of murine hematopoietic cells with multipotent features. The multilineage differentiation potential of these cells was suggested by their immuno-phenotypes and transcriptional programs and confirmed by their ability to induce three pathologically distinct leukemias in mice, including an acute biphenotypic leukemia (ABL) that recapitulates the distinctive hallmark features of many MLL-associated leukemias in humans. This experimental modeling of ABL in mice highlights its origin from multipotential progenitors that arrest at a bipotential stage specifically targeted or induced by MLL oncogenes.

Introduction

Chromosomal rearrangements of the Mixed Lineage Leukemia (MLL) gene are associated with a subset of de novo and secondary acute leukemias of both children and adults (Djabali et al., 1992; Gu et al., 1992; Tkachuk et al., 1992). As a consequence, MLL undergoes fusions with a wide variety of partner genes to yield a plethora of chimeric proteins in leukemias of either myeloid or lymphoid derivation (AytIon and Cleary, 2001 for review). The remarkable variety of MLL fusion partners (up to 50) and their diverse lineage associations contrast with most other chimeric leukemia oncoproteins, which are not as variable and tend to be specific for leukemias of a single lineage. Furthermore, a hallmark feature of many MLL-associated leukemias is the co-expression of both myeloid and lymphoid antigens representative of a biphenotypic or mixed lineage derivation (Chen et al., 1993), hence the name MLL (McCabe et al., 1992). In addition to their unique phenotypes, MLL biphenotypic leukemias also display transcriptional programs distinct from those displayed by myeloid or lymphoid acute leukemias, respectively (Armstrong et al., 2002; Yeoh et al., 2002).

Two alternative, but not mutually exclusive, mechanisms have been proposed to account for mixed lineage features. MLL oncoproteins may transform early progenitors of either the myeloid or lymphoid lineages, but leukemias with unusual biphenotypic (ABL) features may result from so-called “lineage infidelity” representing aberrant expression of lineage-associated genes (McCulloch, 1983). Alternatively, MLL oncoproteins may target hematopoietic cells with multilineage differentiation potential, such as hematopoietic stem cells (HSC) (Morrison and Weissman, 1994), their immediate downstream progeny, the multipotent progenitor (MPP) (Morrison et al., 1997), or progenitors with multilineage potential (Cumano et al., 1992; Montecino-Rodriguez et al., 2001). In this scenario, ABL may recapitulate otherwise normal phenotypes of early progenitors expressing dual lineage markers, a phenomenon called “lineage promiscuity” (Greaves et al., 1986). Elucidation of their origin will further an understanding of the unique biology and etiology of mixed lineage leukemias, which are generally associated with a poor clinical prognosis (Chen et al., 1993; Pui et al., 2002).

Several animal models have been employed to study the biological roles of MLL oncoproteins in leukemogenesis. Knockin of AF9 into the Mll locus by homologous recombination resulted in acute leukemias preceded by myeloproliferation that mimics the corresponding human disease (Corral et al., 1996; Dobson et al., 1999). Similarly, retroviral transduction/trans-
yielded leukemias with myeloid/lymphoid biphenotypic features. It is therefore still largely unknown how MLL oncoproteins result in multilineage leukemias in humans.

We report here that a representative MLL fusion protein (MLL-GAS7) impairs the differentiation and enhances the in vitro growth of murine hematopoietic cells with features of MPP. Transformed MPP-like cells induced leukemias of multiple lineages in syngeneic mice including an acute biphenotypic leukemia (ABL) expressing both myeloid and lymphoid antigens and genes. Biphenotypic progenitors were also generated in vitro from HSC transduced with MLL-GAS7. This represents a successful modeling of ABL in mice and highlights its origin from a previously undefined biphenotypic progenitor that may be specifically affected by MLL oncogenes.

Results

Induction of mixed lineage leukemias by MLL-GAS7

Primary murine hematopoietic cells enriched for HSC and early progenitors (c-kit+ fraction) were transduced with recombinant retroviruses encoding MLL-GAS7 and then directly transplanted into lethally irradiated, syngeneic mice (Figure 1A). All mice succumbed to leukemias within 5 months (Figure 1B). The leukemias were oligoclonal as judged by proviral integration status (Figure 1C), suggesting that MLL-GAS7 required few additional mutations to induce leukemia in mice. Most (80%) of the leukemias were AMLs based on phenotypic (c-kit+/Mac1+/Gr-1+/CD19+/B220-) and cytologic features. One was ALL as evidenced by its immunophenotype (c-kit+/CD19+/B220+/Mac1+-Gr-1-) and presence of Ig heavy chain gene rearrangements (Figure 1C and data not shown). Notably, one of the leukemias appeared to be of mixed lineage containing a subset of cells in the bone marrow that displayed bilineage features (c-kit+/B220+/Mac1+/CD19-/Gr-1-) as well as cells expressing exclusively myeloid antigens. At several extramedullary sites of this mouse, only monoclonal myeloid leukemic cells were present, suggesting that monoclonal myeloid leukemic cells were present, suggesting that monoclonal leukemias arose in the bone marrow from which individual leukemic clones disseminated to peripheral sites (Figure 1C). These observations suggested that MLL-GAS7 is directly capable of inducing leukemias of different lineages in vivo, consistent with the early immature and lineage promiscuous features of human leukemias associated with MLL mutations.

MLL-GAS7 enhances the growth of hematopoietic cells with features of multipotent progenitors

In vitro studies were conducted to investigate the possible oncogenic effects of MLL-GAS7 in hematopoietic cells with multilineage differentiation potential. Primary murine hematopoietic stem cells and progenitors (from 5-FU treated BM) were transduced with recombinant retroviruses encoding MLL-GAS7, or the respective portions of MLL (5’-MLL) or GAS7 (GAS7) (Figures 1A and 2A). Plating of transduced cells in methylcellulose yielded similar numbers of G418-resistant colonies for each of the constructs, indicating comparable transduction efficiencies. Cells transduced with MLL-GAS7 showed enhanced growth potential as evidenced by continued ability to generate compact, immature CFU-blast colonies upon serial replating in...
methylcellulose culture (Figure 2A). Cells harvested from second or third round methylcellulose cultures (200–400 pooled colonies) gave rise to long-term growth of clonal populations in liquid medium. Interestingly, their immunophenotypes showed high level co-expression of c-kit and Sca-1 (Figure 2B), a characteristic phenotype of HSC and MPP (Morrison and Weissman, 1994; Morrison et al., 1997). They were negative for most lineage-specific markers tested (Gr-1, CD19, Ter119, CD8) except B220<sup>lo</sup> and Mac-1, which also can be expressed by MPP (Morrison et al., 1997). These data suggested that MLL-GAS7 enhanced the in vitro growth of cells with some features of MPP. We will therefore refer to them as MPP-like based on their phenotypic and functional (defined below) properties.

**Transformed MPP-like cells induce leukemias of diverse lineages**

Transduced cells clonally expanded from in vitro cultures described above were assessed for their leukemogenic potential in sub-lethally irradiated, syngeneic C57BL/6 mice. Within a relatively short latency period (mean 3 months) similar to that observed above for in vivo experiments, all animals succumbed to acute leukemias that expressed the MLL fusion protein (Figures 3A and 3B). The leukemias exhibited diverse pathologic and phenotypic features (Table 1 and Figure 3C) that defined three specific subtypes. These were: (1) AML with features of early myeloid precursors (c-kit<sup>−/−</sup>/Mac-1<sup>−/−</sup>/Gr-1<sup>−/−</sup>/B220<sup>−/−</sup>/CD19<sup>−/−</sup>); (2) ALL with features of early B-lymphoid precursors (c-kit<sup>−/−</sup>/CD43<sup>−/−</sup>/B220<sup>−/−</sup>/BP-1<sup>−/−</sup>/CD19<sup>−/−</sup>/Mac-1<sup>−/−</sup>/Gr-1<sup>−/−</sup>); and (3) acute bi-phenotypic leukemia (ABL) comprised of blasts co-expressing both myeloid and lymphoid markers (c-kit<sup>−/−</sup>/Mac-1<sup>−/−</sup>/B220<sup>−/−</sup>/CD19<sup>−/−</sup>/Gr-1<sup>−/−</sup>). The leukemia subtypes were similar to those resulting from the direct transplant experiments (Figure 1) indicating that this was a reproducible feature of MLL-GAS7-induced leukemias. Although the leukemic cells from all three subtypes lacked expression of Sca-1, which distinguished them from the injected MPP-like cells, all subtypes displayed identical genomic configurations of the integrated MSCV/MLL-GAS7 provirus on Southern blot analysis (Figure 3C), indicating that they derived from a single clone of cells and not from rare, genetically distinct subclones that may have been present in the original culture.

The different lineage derivations of the leukemias were further confirmed by immunoglobulin (Ig) gene rearrangement status. AML cells maintained germline configurations for IgH genes whereas ALL cells displayed IgH gene rearrangements in the absence of light chain gene rearrangements (Figure 3C and data not shown), suggesting an arrest at a late pro-B (fraction C)

**Figure 2.** Cells transformed in vitro by MLL-GAS7 display phenotypic features of multipotential progenitors

A: Schematic diagram of constructs used in hematopoietic progenitor transformation assays (left). Bar graph (right) shows numbers of colonies obtained after each round of plating in methylcellulose (average of three independent assays) following transduction of BM cells enriched for stem/progenitor cells following 5-FU treatment. Similar results were obtained with c-kit enriched BM cells. Insert shows the typical morphology of colonies generated in methylcellulose by bone marrow cells transduced with MLL-GAS7.

B: Phenotypic analysis of cells transformed by MLL-GAS7 in vitro. Shadow profiles represent FACS staining obtained with antibodies specific for the indicated cell surface antigens. Red lines represent staining obtained with isotype control antibodies.
Figure 3. Progenitors transformed by MLL-GAS7 induce leukemias of different lineage with short latency
A: Survival curves are shown for cohorts (n = 10) of mice injected with cells transformed by different MLL fusion proteins (So and Cleary, 2002, 2003) or mock injected controls (control). MLL-GAS7 results in leukemias with shorter latencies than MLL-AFX or MLL-FKHRL1.
B: Detection of MLL-GAS7 fusion protein in the blasts of leukemic mice. Western blot analysis shows MLL-GAS7 and wt MLL in leukemia cells. By comparison, Ba/F3 pro-B cells contain only wild-type MLL.
C: Phenotypic and clonal analysis of leukemic blasts from MLL-GAS7 mice. Upper panels show typical FACS profiles of leukemic cells from mice with AML, ABL, and ALL, respectively. Shadow profiles represent FACS staining obtained with antibodies specific for the indicated cell surface antigens. Gray lines represent staining obtained with isotype control antibodies. Lower panels show the results of Southern blot analysis for immunoglobulin gene (IG-JH4) and MSCV proviral integration status of leukemic cells. gl, germline configuration of IG-JH4 gene.

Table 1. Features of leukemic mice injected with MLL-GAS7 transformed cells

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>AML</th>
<th>ABL</th>
<th>ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice with disease (N = 15)</td>
<td>n.a.</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Disease latency (days)*</td>
<td>n.a.</td>
<td>115 ± 37</td>
<td>100 ± 42</td>
<td>90 ± 35</td>
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<tr>
<td>WBC (10⁶/µl)*</td>
<td>6.9 ± 1.2</td>
<td>100 ± 84</td>
<td>15 ± 3</td>
<td>19 ± 4</td>
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<tr>
<td>Blasts in peripheral blood (%)*</td>
<td>0</td>
<td>56 ± 7</td>
<td>24 ± 6</td>
<td>26 ± 12</td>
</tr>
<tr>
<td>Liver weight (mg)*</td>
<td>0.45 ± 0.12</td>
<td>4.3 ± 3.2</td>
<td>2.2 ± 0.3</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Spleen weight (mg)*</td>
<td>0.12 ± 0.03</td>
<td>0.8 ± 0.5</td>
<td>0.7 ± 0.3</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Mice with lymphoid organ involvement (%)</td>
<td>n.a.</td>
<td>75</td>
<td>100</td>
<td></td>
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</table>

*Values shown as mean ± standard deviation.
 n.a., not applicable.
GAS7 with MPP-like features were capable of inducing three different leukemias in vivo.

**AML, ALL, and ABL display distinct gene expression profiles**

Hematopoietic lineage commitment is regulated in part by the functional balance of transcription factors, which turn on/off specific lineage-defining genes. Thus, it was of interest to assess the definitive gene expression profiles of the various leukemias in comparison with transduced MPP-like cells. RT-PCR analysis showed that AML cells expressed myeloid-specific genes including myeloperoxidase (MPO), G-CSF-R, M-CSF-R, and GM-CSF-R, but not genes normally expressed in early lymphoid progenitors (Figure 5). Conversely, ALL cells expressed an entirely different set of genes including GATA-2, GATA-3, and SCL, but were notable for their lack of expression of the above myeloid genes. Interestingly, ABL cells expressed genes common to both myeloid and lymphoid progenitors. While the contrasting expression profiles exhibited by AML and ALL reflected their lineage-specific origins, and expression of both lymphoid and myeloid genes in ABL suggested maturation arrest at a more uncommitted stage of differentiation consistent with myeloid/lymphoid bipotential progenitors. The expression profile in MPP-like cells was even broader than that of ABL, consistent with a model in which the most primitive progenitors express the widest array of genes, many of which are silenced during subsequent lineage commitment (Hu et al., 1997; Enver and Greaves, 1998).
AML, ALL, and ABL cells exhibit distinct gene expression profiles

Results of RT-PCR analyses are shown for expression of transcripts from the genes indicated to the left of the respective panels. Total RNA was isolated from short-term (7 day) explants of leukemic BM cells of different leukemia types (listed at the tops of lanes) or cultured MPP-like cells. RNA isolated from unfractionated bone marrow cells (WBM) served as a positive control.

Identification of a leukemogenic, biphenotypic progenitor transformed by MLL-GAS7

Biphenotypic (B220⁺/Mac-1⁻) progenitors were also transiently generated in vitro from MPP-like cells. When cultured under myeloid conditions (RPMI containing 20% FCS and 20% WEHI-conditioned medium as a source of IL-3), MPP-like cells progressively changed their phenotype by first downregulating Sca-1, then upregulating B220 and Mac-1, and finally downregulating B220 (Figure 6A). These results suggested that Sca-1⁺/c-kit⁺ MPP-like cells could directly give rise to B220⁺/Mac-1⁺/c-kit⁺ biphenotypic cells, which further differentiated into myeloid precursors (Mac-1⁺/c-kit⁻). Since the in vitro culture conditions favored myeloid differentiation, the differentiation potential of biphenotypic cells was also examined in vivo. B220⁺/Mac-1⁻ cells were purified by two rounds of FACS (Figure 6A) and then injected (10⁵/mouse) into sub-lethally irradiated, syngeneic recipient mice. Acute leukemias developed in 4 of 10 transplanted mice after a latency period of approximately 7 months. Immunophenotype and genotype analyses revealed that all the mice had AML and half had ALL (Figure 6B). All leukemias displayed configurations of proviral integration sites that were identical to the injected biphenotypic cells as well as the parental MPP-like cells, indicating that they were derived from a single clone of transduced cells. These studies suggested that transformed MPP-like cells were capable of sequentially differentiating into biphenotypic progenitors with the capacity to further differentiate along either the myeloid or lymphoid lineages to induce AML or ALL, respectively.

Multilineage transformation arises from multipotential progenitors but not committed myeloid or lymphoid progenitors

To identify normal progenitors susceptible to MLL-induced multilineage oncogenic readouts, in vitro transformation assays were performed using purified populations of known phenotypically defined progenitor cells. HSC, common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte/
monocyte progenitors (GMP), and megakaryocyte/erythocyte progenitors (MEP) were isolated by FACS based on their surface phenotypes (Kondo et al., 1997; Akashi et al., 2000). Purified cells were transduced with retroviruses and subjected to serial replating analysis in methylcellulose medium containing cytokine cocktails that favored myeloid or lymphoid differentiation (Figure 1A). Under myeloid culture conditions, transduced HSC, CMP, and GMP generated colonies through the third round of serial plating (Table 2). The transformed cells had phenotypic features of myeloid precursors (c-kit+/Gr-1+/Mac-1+) corresponding to maturation arrest at a late stage of myeloid differentiation, most probably positioned developmentally downstream of GMP but prior to terminal differentiation as observed for another MLL oncoprotein (A. Cozzio et al., submitted). HSC yielded a minor fraction of Mac-1+ /B220- biphenotypic cells (Table 2 and data not shown) similar to those described in the preceding section above, while transformed CMP and GMP did not. Conversely, under lymphoid culture conditions, only transduced HSC resulted in the growth of third round colonies (Figure 7). These colonies were heterogenous in appearance, varying from densely compact to diffuse, and were composed of at least three phenotypically distinct subpopulations: (1) Mac-1+ cells with features of myeloid precursors; (2) B220+/CD19+ cells with features of B-lymphoid precursors; and (3) B220+/Mac-1+ cells with features of biphenotypic progenitors (Figure 7). Although transduced CLP gave lymphoid colonies in the second plating (Figure 7), they failed to replate (Table 2). These results indicate that committed myeloid or lymphoid progenitors are not the targets for multilineage readouts induced by MLL-GAS7, and only transduced HSC that are capable of differentiating into MPP can give rise to transformed biphenotypic progenitors.

### Discussion

In the present study, we demonstrate the induction of mixed lineage leukemias by an MLL oncogene. These leukemias appeared to result from a primary effect of MLL-GAS7 to impair the differentiation of cells with features of multipotent hematopoietic progenitors. MLL-GAS7 enhanced the in vitro clonogenic properties of Sca-1+ /c-kit-/lin-/ cells under conditions where progenitors normally exhaust their growth potential and undergo terminal differentiation. Previous studies have shown that the highly purified population co-expressing these antigens in mouse bone marrow is comprised of three functionally related hematopoietic progenitor sets with different self-renewal potentials: long-term self-renewing HSCs (lin- /thy1.1+/Flk2+/c-kit+/Sca-1+); short-term self-renewing HSCs (lin- /thy1.1+/Flk2+/c-kit+/Sca-1+); and MPP (lin- /thy1.1+/Flk2+/c-kit+/Sca-1+).
Mac1\(^{+}\)) without detectable self-renewal potential (Morrison and Weissman, 1994; Morrison et al., 1997; Christensen and Weissman, 2001). These represent the most primitive populations of defined progenitors with potential to differentiate into all known hematopoietic cell lineages (Morrison et al., 1997). The progenitors affected by MLL-GAS7 in vitro are most comparable (but not identical) phenotypically to MPP, the direct progeny of short-term HSC. The multilineage differentiation potentials of the transformed cells were suggested by their transcriptional programs and confirmed by an ability to induce leukemias of three different lineages in mice. MLL-GAS7 also induced multilineage readouts from purified HSC in vitro, indicating that both HSC and MPP are potential targets for the induction of mixed lineage leukemias by MLL oncogenes.

MLL-GAS7 appears particularly potent at altering the growth properties of MPP-like cells compared to other MLL fusion proteins by mechanisms that remain undefined. We have observed that MLL-ENL is also capable of inducing the outgrowth of biphenotypic progeny from purified HSC under in vitro lymphoid culture conditions, but it is significantly less effective than MLL-GAS7 (data not shown). Recently, MLL-ENL was reported to transform chicken cells with phenotypic features of MPP (Schulte et al., 2002). Their differentiation potential, however, was restricted to the myeloid lineage and they failed to induce lymphoid leukemias, further suggesting that MLL-ENL strongly favors myeloid transformation in vivo consistent with our recent observations using purified progenitor subsets (A. Cozzio et al., submitted). Thus, despite the targeting of MLL oncogenes to multipotent progenitors, specific fusion partners appear to heavily influence the actual features of MLL-associated leukemias presumably through interactions with lineage-specific factors.

Although the in vitro growth and differentiation properties of MPP/HSC are perturbed by MLL oncogenes, the resultant leukemias typically display features of maturation arrest at a later stage of differentiation. This suggests that although multipotent hematopoietic progenitors are impaired in their differentiation potential, they retain an ability to generate more differentiated progeny. This hypothesis is consistent with the finding that forced expression of MLL-ENL in purified HSC yielded acute myeloid and not stem cell leukemias (A. Cozzio et al., submitted). Similarly, stem cell leukemia is not a feature of AML1-ETO positive patients in spite of expression of the fusion gene in the HSC compartments (Miyamoto et al., 2000). Taken together, these studies suggest a model in which MLL fusion genes might enhance the self-renewal potential of multipotent progenitors that otherwise retain an ability to differentiate into downstream progeny. The latter may be more susceptible to the differentiation blocking effects of MLL oncoproteins or more prone to develop the secondary mutations required for complete hematopoietic transformation (Figure 8).

Consistent with the above model, transduced Sca-1\(^{+}\)/c-kit\(^{+}\) progenitors gave rise to biphenotypic progeny in vitro and, when transplanted, to biphenotypic leukemias (ABL) in vivo (in addition to AML and ALL). Gene expression profiling studies of the leukemic cells were consistent with immunophenotype and Ig genotype analyses on defining three distinct lineage derivations of the leukemias. Genes required for myeloid development, such as GM-CSF-R, M-CSF-R, and G-CSF-R (Zhu and Emerson, 2002), were expressed by AML but not ALL cells. The latter expressed a different non-overlapping set of genes including GATA3, which is expressed by normal CLP but not myeloid progenitors (Akashi et al., 2000). Conversely, biphenotypic ABL cells and MPP-like cells expressed a broader range of genes common to both the myeloid and lymphoid lineages. These observations are consistent with the proposed multilineage priming feature of uncommitted progenitors, which express transcripts for most of the known key transcription factors, growth factor receptors, and other genes encoding lineage-exclusive functions (Hu et al., 1997; Enver and Greaves, 1998). Under this model of hematopoietic differentiation, unilineage commitment is achieved by specific upregulation of genes for one particular lineage and downregulation of genes for all other lineages. We hypothesize that activating mutations of MLL, which is genetically implicated in maintaining normal developmental gene expression, may oppose unilineage differentiation by preventing the downregulation of genes involved in maintaining the multipotentiality of HSC/MPP. Our limited transcriptional profiling studies are also consistent with recent studies of
human leukemias harboring MLL chromosomal translocations, which display a gene expression profile distinct from ALL and AML, respectively (Armstrong et al., 2002; Yeoh et al., 2002). Taken together, these results suggest that we have successfully modeled the distinctive biphenotypic human leukemias commonly associated with MLL mutations.

The lymphoid and myeloid lineages diverge at the level of very early progenitors (e.g., CLP versus CMP), but considerable evidence indicates a close developmental relationship between B-lymphoid and myeloid cells in normal definitive hematopoiesis (Borrello et al., 2001 for review). Although CMP and CLP have been shown to exhibit limited lineage plasticity in giving rise to lymphoid and myeloid progeny, respectively (Kondo et al., 1997, 2000; Akashi et al., 2000; Traver et al., 2000), they did not yield bi-lineage oncogenic readouts in response to MLL-GAS7. Rather, transduction of CMP (and GMP) resulted exclusively in transformed myeloid progeny, further confirming the ability of MLL fusion proteins to induce myeloid leukemias from committed progenitors (A. Cozzio et al., submitted). Biphenotypic progenitors that give rise to both B cells and macrophages at higher frequencies were first identified in murine fetal liver (Cumano et al., 1992). The existence of similar biphenotypic progenitors in adult hematopoiesis has recently been confirmed by isolation of a common B/macrophage precursor (c-kit+/CD45R-/CD19+) in postnatal bone marrow (Montecino-Rodriguez et al., 2001). While it is difficult to make a direct comparison between the common B/macrophage precursor identified in normal bone marrow with the biphenotypic ABL cells in our studies, the immunophenotypes (c-kit+/CD19+) and Ig genotypes (absence of DJ4 and VDJ4 rearrangements) of ABL cells suggest that they originate from more immature progenitors than the known B/macrophage precursors. Nevertheless, identification of B220+/Mac-1+ biphenotypic cells that induced both acute lymphoid and myeloid leukemias is most consistent with a “lineage promiscuity” model and strongly suggests that leukemogenic transformation of bipotential or multipotential progenitors by MLL mutations leads to multilineage leukemias.

**Experimental procedures**

**DNA constructs**

A full-length GAS7 cDNA was amplified by RT-PCR using primers based on the published sequence (accession number AB007854) (Megonigal et al., 2000). The resulting GAS7 cDNA was inserted into the NruI and XhoI sites of MSCV-neo-MLL5 (DiMartino et al., 2002) in-frame with MLL amino acids 1–3696 to yield an MSCV/MLL-GAS7 fusion construct, which was sequenced to exclude mutations introduced by PCR.

**In vitro and in vivo transformation assays**

In vitro hematopoietic progenitor transformation assays were performed as previously described (So and Cleary, 2002, 2003) except for differences in the preparation of enriched/purified hematopoietic stem/progenitors. Partial or highly purified hematopoietic cells were prepared from the bone marrow of 4- to 10-week-old Ly5.2 C57BL/6 mice by 1) pretreatment with 5-fluorouracil (5-FU) (100 mg/kg) 5 days before bone marrow harvesting; (2) positive selection for c-kit expression by magnetic activated cell sorting (MACS); or (3) fluorescent activated cell sorting (FACS) using specific anti-body to defined cell surface markers for various hematopoietic stem and progenitor cells as previously described (Morrison and Weissman, 1994; Kondo et al., 1997; Morrison et al., 1997; Akashi et al., 2000). Phenotypic populations were defined as HSC (Lin−/Thy1.1+/c-kit+/Sca-1+), CLP (Lin−/c-kit-/Sca-1+/IL-7R+/Thy1.1−), CMP (c-kit-/Sca-1−/CD34+/CD16/32+), GMP (Lin−/c-kit−/Sca-1−/CD34−/CD16/32+), and MEP (Lin−/c-kit−/Sca-1−/CD34+/CD16/32+). The properties of purified stem/progenitor cells were verified by in vitro cell culture assays and/or in vivo studies to establish appropriate lineage development.

Purified hematopoietic cells were transduced by spinoculation with viral supernatants collected 3 days after transfection of phoenix cells. For in vivo assays, 105 transduced Ly5.2 cells were transplanted together with 2 × 105 Ly5.1 total bone marrow cells into the retro-orbital venous sinus of 6-week-old syngeneic, lethally irradiated [10.5 Gy total body γ irradiation (105Cs)] Ly5.1 C57BL/6 mice. For in vitro assays, transduced cells were plated in 1% methylcellulose (StemCell Technologies, Vancouver, BC, Canada) supplemented with either myeloid or lymphoid cytokines in the presence or absence of 1 mg/ml G418. Myeloid conditioned methylcellulose contained an Iscove’s Modified Dulbecco’s Medium (IMDM)-based Methocult (Methocult M3231; StemCell Technologies) supplemented with 20 ng/ml stem cell factor (SCF) and 10 ng/ml each of IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis, Minnesota). Lymphoid conditioned methylcellulose consisted of IMDM-based Methocult M3630 (StemCell Technologies) containing 10 ng/ml human IL-7 supplemented with 20 ng/ml each of SCF and Flt-3 ligand. After 7 days culture, colonies were counted to calculate the transduction efficiency. Single-cell suspensions (106 cells) of G418-resistant colonies were then replated in methylcellulose medium supplemented with growth factors without G418. Plating was repeated every 7 days. In each round of replating, single cell suspensions were also expanded in RPMI liquid medium containing 20% FCS plus 20% WEHI-conditioned medium. For tumorigenicity assays, 105 or 106 immortalized cells were injected into the retro-orbital venous sinus of 6-week-old syngeneic Ly5.2 C57BL/6 mice, which had received a sublethal dose (5.25 Gy) of total body γ irradiation (105Cs). Mice were maintained on antibiotic water to avoid infection and monitored for development of leukemia by complete blood count, blood smear, and FACS analysis. Tissues were fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis.

**Cell sorting and immunophenotypic analysis by FACS**

Immunophenotypic analysis was performed by FACS using fluorochrome-conjugated monoclonal antibodies to Sca-1 (D7 clone), c-kit (2B8 clone), CD43 (S7 clone), Mac-1 (M1/70 clone), Gr-1 (RB6-8C5 clone), CD19 (1D3 clone), B220 (RA3-6B2 clone), CD3e (145-2C11), CD4 (RM 4–5), CD8a (53-67), Thy1.2 (53-2.1), and Ter-119 (TER-119) (Pharmingen Inc, San Diego, California). Genomic configurations of MLL fusion construct, which was sequenced to exclude mutations introduced by PCR.

**Genotype analyses**

Genomic configurations of Ig genes and retroviral integrations were determined by Southern blot analyses. Genomic DNAs were extracted from tumor tissues/cells and digested with appropriate restriction enzymes (XbaI for Ig gene rearrangement; BamHI or BamHI/ScaI for viral integration studies). MSCV/MLL-GAS7 contains one internal BamHI site and no ScaI sites. Diagnosed DNAs were separated in 0.8% agarose, transferred to nylon membranes, and probed with radiolabeled DNA probes. JH4- and J (H4)-specific probes were used for Ig heavy and light chain gene assessment, respectively. For MSCV/MLL-GAS7 retroviral integration studies, a human MLL cDNA probe (Takachuk et al., 1992) spanning nucleotides 3295–3726 (accession number NM_005933) was employed.

**Western blot and RT-PCR analyses**

Lysate proteins (30 μg) were fractionated in 5% polyacrylamide gel and transferred to ECL membranes (Amersham Pharmacia Biotech) using Tris-glycine-SDS transfer buffer. After blocking, membranes were probed with monoclonal antibody N4.4 directed against an MLL amino-terminal epitope as previously described (So and Cleary, 2002, 2003). Total RNAs purified
from bone marrow cultures using Trizol (Life Technologies) were amplified by RT-PCR using gene-specific primers as previously described (Akashi et al., 2000; Kondo et al., 2000). The details of primer sequences and PCR conditions are available by request.

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