Runx1 deficiency predisposes mice to T-lymphoblastic lymphoma

(Running title: Runx1 deficiency and lymphoma)

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(MK designed and performed the experiments, analyzed the data, and wrote the manuscript; SC performed the experiments and participated in analyzing the data; LG performed the experiments; TS generated and contributed the Runx1lacZ/lacZ ES cell line; MS and ME analyzed the data; NAS contributed the ES cell line and participated in writing the manuscript; PPL designed and performed the experiments, analyzed the data, and wrote the manuscript)
ABSTRACT

Chromosomal rearrangements affecting RUNX1 and CBFB are common in acute leukemias. These mutations result in the expression of fusion proteins that act dominantly-negatively to suppress the normal function of the RUNX/CBFβ complexes. In addition, loss-of-function mutations in RUNX1 have been identified in sporadic cases of acute myeloid leukemia (AML) and in association with the familial platelet disorder with propensity to develop AML (FPD/AML). In order to examine the hypothesis that decreased gene dosage of RUNX1 may be a critical event in the development of leukemia, we treated chimeric mice generated from RunxlacZ/lacZ embryonic stem (ES) cells that have homozygous disruption of the Runx1 gene with N-ethyl-N-nitrosurea (ENU). We observed an increased incidence of T-lymphoblastic lymphoma in RunxlacZ/lacZ compared to wildtype chimeras, and confirmed that the tumors were of ES cell origin. Our results therefore suggest that deficiency of Runx1 can indeed predispose mice to hematopoietic malignancies.
Introduction

*AML1-ETO*, the fusion gene resulting from t(8;21) in acute myeloid leukemia (AML) subtype M2 and *CBFB-MYH11*, the fusion gene resulting from inv(16) in AML subtype M4 Eo, are both capable of blocking hematopoiesis in mouse models\(^1,2\). Runx1\(^+/ETO\) and Cbfb\(^+/MYH11\) heterozygous embryos die at E12.5-13.5 from hemorrhages and defective hematopoiesis, similar to Runx1\(^+/−\) and Cbfb\(^+/−\) embryos\(^3-5\). In vitro studies have demonstrated that the proteins encoded by these two fusion genes are capable of repressing the expression of Runx1/Cbfβ target genes\(^6,7\). These observations have led to the hypothesis that the fusion genes block hematopoietic differentiation and induce leukemia by inhibiting Runx/Cbfβ activity in a dominant negative manner. More recently, sporadic loss of function mutations – both mono-allelic and bi-allelic – have been found in patients with AML\(^8,9\). In addition, the disease familial platelet disorder with propensity to develop AML (FPD/AML) has been linked to heterozygous mutations in *RUNX1*\(^10,11\). Together, these studies have led to the hypothesis that inhibition of Runx1/Cbfβ function is a critical step in the development of hematopoietic malignancies.

In this study, we used a mouse model to determine whether disruption of Runx1 expression predisposes mice to the development of hematopoietic malignancies. Chimeras generated with Runx1\(^{lacZ/lacZ}\) ES cells developed lymphoma at increased incidence after treatment with N-ethyl-N-nitrosourea (ENU) to induce additional genetic changes. This finding provides strong evidence that Runx1-deficient cells are susceptible to malignant transformation.
**Materials and Methods**

**Transgenic mice.** Runx1<sup>lacZ/lacZ</sup> ES cells were generated from Runx1<sup>+/lacZ</sup> ES cells<sup>12</sup> (Runx1<sup>tm2Spe/+</sup>) by growth in 4.0 mg/ml G418<sup>13</sup>. Runx1<sup>lacZ/lacZ</sup> (LZD) and wild-type 129 (TC1) ES cells were used to generate chimeric mice using standard protocols. One month old mice were given a single dose of ENU (100mg/kg) by intra-peritoneal injection<sup>14</sup>.

**Immunohistochemistry.** Liver sections were stained with an anti-CD3 antibody and a Peroxidase staining kit (DAKO, Carpinteria, CA). Spleen sections were stained with a rabbit anti-β-galactosidase antibody and a DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA).

**Flow cytometry.** Thymocytes were stained with PE-conjugated anti-CD4 and Cy-chrome-conjugated anti-CD8 (BD Pharmingen, San Diego, CA) and analyzed using FACSCalibur (BD Biosciences, San Diego, CA).

**Southern blot analysis.** Southern blot analysis was performed on DNA isolated from bone marrow cells, using a 400bp Runx1 intron 8 probe as described previously<sup>12</sup>.

**Genomic PCR analysis.** Thymocytes were stained with a PE conjugated anti-CD4 and a FITC conjugated anti-CD8 antibody (BD Pharmingen, San Diego, CA) and sorted with FACS Aria (Becton Dickinson, San Jose, CA). Bone marrow cells were isolated and sorted after staining with a FITC-conjugated anti-c-kit antibody and PE-conjugated antibodies against CD3, B220, Mac-1, Gr-1, and TER119 (BD Pharmingen, San Diego, CA). The DNA was extracted from the subpopulations. PCR was performed using 200-400 ng DNA and the following PCR conditions: 94°C for 4 minutes; 40 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes; 72°C for 4 minutes. The PCR primers used were LacZ Forward, ACTGGCAGATGCACGGTTAC and LacZ Reverse, GTGGCAACATGGAAATCGCTG. Spleen sections effaced by lymphoma were treated with zylene and methanol to remove paraffin, and the genomic DNA was isolated using QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA).
Primers for markers D7Mit44 and D11Mit188 were used for PCR genotyping to distinguish 129 from C57BL/6J cells\textsuperscript{15}.
Results and Discussion

We generated chimeric mice using Runx1lacZ/lacZ ES cells and monitored for leukemia development. None of the mice developed any malignancy (Fig. 1A). We then treated them with a single injection of 100 mg/kg ENU. Chimeras generated with wild type ES cells were used as controls. Due to technical difficulties, only a relatively small number of chimeras (8 for the test and 9 for the control groups respectively) became available for the study. Four of the Runx1lacZ/lacZ chimeras developed lymphoblastic lymphoma within the first 12 months after ENU treatment. These four lymphomas were all derived from the ES cells (see below). Two mice died from unknown causes at 4 and 8 months after injection and were excluded from statistical calculation. During the same time period, one chimera generated with wild-type ES cells developed lymphoma. However, genotyping by PCR indicated that the lymphoma was derived from the C57BL/6J cells (data not shown). In addition, two other chimeras generated with wildtype ES cells died from unknown causes at 7 and 8 months after ENU injection. These three mice were excluded from the statistical analysis. Therefore, 4 of the 6 Runx1lacZ/lacZ chimeras developed lymphoma of ES cell origin, while none of the 6 wildtype ES cell chimeras did (Fig. 1A). This represents a significant increase (p=0.03, by Fisher exact test, one-sided) in the incidence of lymphoma in the Runx1lacZ/lacZ chimeras compared to wild-type chimeras.

Runx1lacZ/lacZ chimeras that developed lymphoblastic lymphoma (LZD #2, 3, 4, and 5) had enlarged thymuses that were completely effaced by the malignant cells (Fig 1B). There was neoplastic infiltration in the lungs, liver (Fig 1B), kidney, spleen (Fig 2A) and bone marrow (Fig 1B). Staining of liver sections with anti-CD3 antibody revealed that the lymphoma was of T-cell origin (Fig 1B). Flow cytometric analysis revealed co-expression of CD4 and CD8 (Fig 1C), a common phenotype for T-lymphoblastic lymphoma16.

To verify the ES cell origin of the lymphoblastic lymphomas that developed in the Runx1lacZ/lacZ chimeras, we stained spleen sections for β-galactosidase and performed southern
blot hybridization with DNA isolated from bone marrow cells. The spleens of Runx1$^{lacZ/lacZ}$ chimeras that developed lymphoma were completely effaced by β-galactosidase-positive tumor cells (Fig. 2A), indicating their ES cell origin. In LZD#2, 3, and 4, the bone marrow was completely effaced by lymphoma and only the targeted LacZ allele was detected by southern blot hybridization (Fig 2B), confirming that the malignant cells were derived from ES cells. On the other hand, only the wild-type allele was detected in the bone marrow of LZD#6 (unaffected) (Fig 2B). These results suggest that the four T-lymphoblastic lymphomas that developed within seven months were of ES cell origin.

Since there is an early block in hematopoiesis in Runx1$^{lacZ/-}$ embryos\textsuperscript{12}, it is somewhat puzzling that there are hematopoietic Runx1$^{lacZ/lacZ}$ cells in the adult chimeras that can give rise to lymphoma. It is likely that similar to Cbfb\textsuperscript{+/MYH11} chimeras\textsuperscript{14}, some Runx1$^{lacZ/lacZ}$ precursors are able to survive in the context of a chimeric animal. To address this possibility, we analyzed the contribution of Runx1$^{lacZ/lacZ}$ ES cells to the hematopoietic compartments in untreated healthy Runx1$^{lacZ/lacZ}$ chimeras. By genomic DNA PCR, we were able to detect the presence of ES cell-derived cells in all populations from the thymus (Figure 2C). ES cell-derived cells were also detected by PCR in the c-kit+/lin- and c-kit+/lin+ subpopulations in the bone marrow, but were barely or not at all detectable in the c-kit-/lin+ population (Figure 2D). However, the overall hematopoietic contribution from the ES cells must be small, since by southern blot we can only detect the wild-type allele in LZD#6, an ENU-treated Runx1$^{lacZ/lacZ}$ chimera that did not develop neoplasia (Fig 2B). In addition, there was no significant beta-galactosidase staining of the spleen sections from LZD#6 (Fig 2A), and two untreated healthy Runx1$^{lacZ/lacZ}$ chimeras (data not shown). The results suggest that Runx1$^{lacZ/lacZ}$ ES cells gave rise to hematopoietic stem cells and early progenitors, but later stages of differentiation were blocked.

It is somewhat unexpected that T-lymphoblastic lymphoma was induced in the Runx1$^{lacZ/lacZ}$ chimeras, since RUNX1 deficiency is more commonly associated with AML.
However, there is one report of a translocation involving RUNX1 in a 12-year old boy with T-cell acute lymphoblastic leukemia\textsuperscript{17}, suggesting that defects in RUNX1 expression can be associated with T-cell malignancies in humans as well as in mice. While our results may reflect some of the biological differences between human and mouse, it is also possible that Runx1 deficiency is capable of inducing both myeloid and lymphoid malignancies and myeloid leukemia would have been observed with a larger set of mice or higher dose of ENU. In one recent report, a transgenic mouse model was used to determine if expression of AML1-ETO can induce leukemia in mice\textsuperscript{18}. The AML1-ETO transgenic mice developed both AML and T-cell lymphoma (55\% and 45\% respectively) after treatment of ENU at a relatively high dose (300 mg/kg), while 100\% of the wildtype controls developed lymphoma.

Our results suggest that Runx1\textsuperscript{lacZ/lacZ}-derived cells are more susceptible than wild-type cells to developing lymphoblastic lymphoma after chemical mutagenesis. Although we cannot exclude the possibility that the Runx1-lacZ fusion gene exerts some novel activity, the phenotype of Runx1\textsuperscript{lacZ/c} embryos is identical to that of Runx1\textsuperscript{-/-} embryos with embryonic lethality between E12.5 and E13.5 from hemorrhages and absence of definitive hematopoiesis\textsuperscript{12}. Therefore, our results suggest that Runx1-deficiency can predispose to development of hematological malignancies in mice. Additional studies in Runx1\textsuperscript{+/-} chimeras or Runx1 conditional knockout mice will be needed to confirm the findings reported here.

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References


Figure legends

Figure 1. *Runx1*\textsuperscript{lacZ/lacZ} chimeras are more susceptible to developing T-lymphoblastic lymphoma than wild-type chimeras. (A) Kaplan-Meyer survival curves (lymphoma-free) are shown for untreated *Runx1*\textsuperscript{lacZ/lacZ} (n=6); and *Runx1*\textsuperscript{lacZ/lacZ} (n=6) and wild-type (n=6) chimeras treated with 100mg/kg ENU. Solid line with circles: untreated *Runx1*\textsuperscript{lacZ/lacZ} chimeras; solid line with squares: wildtype ES cell chimeras treated with ENU; dotted line with triangle: *Runx1*\textsuperscript{lacZ/lacZ} chimeras treated with ENU. (B) H&E staining of thymus, bone marrow (BM), and liver from LZD#2-5 (#2, #3, #4, and #5). Immunohistochemistry using an anti-CD3 antibody demonstrates that the malignant cells in the livers of these animals stained positively for CD3. (C) Flow cytometry of thymocytes from LZD#2 and LZD#4 using antibodies against CD4 and CD8 demonstrates that the majority of cells have an abnormal CD4\textsuperscript{+}/CD8\textsuperscript{+} phenotype.

Figure 2. T-lymphoblastic lymphoma is of ES cell origin in *Runx1*\textsuperscript{lacZ/lacZ} chimeras. (A) Immunohistochemistry using an anti-β-galactosidase antibody demonstrates that the malignant cells in the spleens of LZD #2-5 (#2, #3, #4 and #5) express β-galactosidase and are therefore derived from *Runx1*\textsuperscript{lacZ/lacZ} ES cells. The normal splenocytes from LZD#6 (which did not develop lymphoma) serve as negative controls. (B) Southern blot analysis detects presence of only the *Runx1-LacZ* knock-in allele in lymphoma-effaced bone marrow from LZD #2-4 (#2-#4), but only wild-type Runx1 allele from normal bone marrow of LZD#6. (C) Genomic PCR using LacZ gene primers and DNA from sorted thymic cells of a *Runx1*\textsuperscript{lacZ/lacZ} chimera. Lane 1: CD8+, lane 2: CD8+/CD4+, lane 3: CD4-/CD8-, lane 4: CD4+, lane 5: total thymus, lane 6: total thymus from a wildtype mouse, lane 7: no DNA. (D) Genomic PCR using LacZ gene primers and DNA from sorted bone marrow cells of a *Runx1*\textsuperscript{lacZ/lacZ} chimera. Lane 1: c-kit+/lin-, lane 2: c-kit+/lin+, lane 3: ckit-/lin+, lane 4: ckit-/lin-, lane 5: total bone marrow.
Fig. 1A
Fig. 1B
Fig. 1C
Fig. 2A
Fig. 2B
Fig. 2C and 2D
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