RAPID COMMUNICATION

The Fetal Origin of B-Precur sor Leukemia in the Eµ-ret Mouse
By Xiang-Xing Zeng, Haige Zhang, Richard R. Hardy, and Robert Wasserman

Before the clinical onset of B-precursor lymphoblastic leukemia, Eµ-ret mice have an expansion of late pro-B cells (CD45R<sup>+</sup>CD43<sup>-</sup>CD24<sup>-</sup>BP-1<sup>+</sup>) within the bone marrow. To characterize the early effects of the transgene product on lymphopoiesis, we initially sequenced the Ig heavy chain (IgH) rearrangements within the late pro-B cells in 24-day-old Eµ-ret and transgene negative mice. In both mouse populations, the IgH rearrangements were polyclonal, predominately nonproductive, and exhibited similar V, D, and J gene usage. However, the frequency of N regions, a marker of postnatal lymphopoiesis, was notably different. At the VD junction, N regions were found in 25 of 25 (100.0%) rearrangements from transgene-negative mice compared with 12 of 36 (33.3%) rearrangements from Eµ-ret mice. At the DJ junction, N regions were found in 21 of 25 (84.0%) rearrangements from transgene negative mice compared with 4 of 36 (11.1%) rearrangements from Eµ-ret mice. Subsequently, we sequenced the clonal IgH rearrangements from 9 leukemias that developed in 10- to 38-week-old mice and found that 7 leukemias had a least 1 rearrangement that lacked N regions at the DJ junction. In addition, V replacement events were observed in the 1 leukemia studied in detail. Terminal deoxynucleotidyl transferase, the enzyme responsible for N region addition, was expressed at markedly lower levels in late pro-B cells from 7- to 10-day-old Eµ-ret mice compared with transgene-negative mice. Examination of fetal lymphopoiesis in Eµ-ret mice identified a relative increase in early (CD45R<sup>+</sup>CD43<sup>-</sup>CD24<sup>-</sup>BP-1<sup>+</sup>) and late pro-B cells and a decrease in more differentiated CD43<sup>-</sup> B-lineage cells. Fetal early pro-B cells from Eµ-ret mice proliferated threefold to fivefold greater but differentiated to a lesser extent than those from transgene negative mice when cultured in vitro with interleukin-7. These data suggest that the B precursor leukemias in adult Eµ-ret mice arise from the progeny of pro-B cells generated in utero.

MATERIALS AND METHODS

Mice and cell preparation. Eµ-ret mice were generated as previously described. A single-cell suspension of bone marrow (femur and tibia) was prepared by injecting ice-cold staining medium (deficient RPMI [Irvine Scientific, Santa Ana, CA] containing 10 mmol/L HEPES, 3% fetal calf serum [FCS], and 0.1% NaN<sub>3</sub]) into the bone to flush out cells, followed by gentle mixing with a 1-mL syringe. Fetal liver was obtained from timed matings of an Eµ-ret male with a BALB/c female. Fetal liver cells were prepared by dissociation between frosted...
glass slides and a single-cell suspension was prepared in staining medium. Bone marrow and fetal liver cells were treated with 0.165 mol/L NH₄Cl to eliminate erythrocytes before staining with antibodies. **Cell surface staining and flow cytometry.** Bone marrow and fetal liver cells were stained as described previously with a four-color combination of fluorescent monoclonal antibodies: anti-CD45R (B220) (allophtocyanin-696-B2), anti-CD4 (fluorescein-S7), anti-CD19 (phycoerythrin-605-B1), and anti-CD24/HSATA (biotin-30F1/Texas Red [or Red-613]-avidin). Cultured fetal livers cells were stained simultaneously with the above-noted four-color combination or a two-color combination of anti-CD45R and anti-IGM (fluorescein-331-D12). Four-color flow cytometry analysis and sorting of bone marrow was performed using a dual-laser (FACSvantage; Becton Dickinson Immunocytometry Systems, San Jose, CA) or a dual-laser/dye-laser flow cytometer (FACStarPLUS; Becton Dickinson) equipped with appropriate filters for four-color immunofluorescence. Reanalysis of sorted fractions consistently showed purities in excess of 98%. Two-color analyses were performed on a FACSCaliber (Becton Dickinson).

**Cell culture conditions.** Sorted fetal early pro-B cells were initially cultured in 24-well plates for 4 days in 1 mL of standard medium (RPMI-1640 supplemented with 5% FCS, 2 mmol/L L-glutamine, 25 mmol/L [HEPES, and 50 µmol/L 2-mercaptoethanol). Interleukin-7 (IL-7; Genzyme, Cambridge, MA) at 100 U/mL was added at time 0. The cultures maintained for 17 days were split approximately every 4 days with IL-7 added.

**Pro-B culture and VDJ-PCR.** For the first PCR round with IgH V DNA preparation and VDJ-PCR. Pro-B cells were sorted directly into microcentrifuge tubes containing 350 µL of RNA lysis buffer for RNA extraction and cDNA synthesis following our previously published protocol.9 PCR reactions were performed in a 50 µL reaction mixture containing 1 to 2 µL of cDNA with final concentrations of 1.3 mol/L NH₄Cl to eliminate erythrocytes before staining with antibodies. **Cell surface staining and flow cytometry.** Bone marrow and fetal liver cells were treated as described previously with a four-color combination of fluorescent monoclonal antibodies: anti-CD45R (B220) (allophtocyanin-696-B2), anti-CD4 (fluorescein-S7), anti-CD19 (phycoerythrin-605-B1), and anti-CD24/HSATA (biotin-30F1/Texas Red [or Red-613]-avidin). Cultured fetal livers cells were stained simultaneously with the above-noted four-color combination or a two-color combination of anti-CD45R and anti-IGM (fluorescein-331-D12). Four-color flow cytometry analysis and sorting of bone marrow was performed using a dual-laser (FACSvantage; Becton Dickinson Immunocytometry Systems, San Jose, CA) or a dual-laser/dye-laser flow cytometer (FACStarPLUS; Becton Dickinson) equipped with appropriate filters for four-color immunofluorescence. Reanalysis of sorted fractions consistently showed purities in excess of 98%. Two-color analyses were performed on a FACSCaliber (Becton Dickinson).

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**RESULTS**

To characterize the expanded late pro-B–cell population in the Eµ-ret mouse, we examined the structure of the IgH rearrangements. We sorted CD45R⁻/CD43⁺/CD24⁺/BP-1⁻ late pro-B cells (as defined in Hardy et al13) from the bone marrow of 2 Eµ-ret and 3 transgene-negative 24-day-old mice. In both populations, the majority of VDJ rearrangements were nonproductive: 17 of 25 (68.0%) of the VDJ joinings in the transgene-negative mouse compared with 29 of 36 (80.6%) of the VDJ joinings in the Eµ-ret mice. In both populations, the most frequent cause for a nonproductive rearrangement was that the J gene was out-of-frame.

We next quantitated the proportion of VDJ joinings containing N regions in the two late pro-B–cell populations (Fig 1, upper panel). We found a striking difference in the incidence of N regions at both the VD and DJ junction in the two populations. Specifically, at the VD junction, 25 of 25 (100.0%) joinings had N regions in the transgene-negative late pro-B cells, whereas only 12 of 36 (33.3%) VDJ joinings from the Eµ-ret late pro-B cells had N regions. At the DJ junction, 21 of 25 (84.0%) joinings had N regions in the transgene-negative late pro-B cells, whereas only 4 of 36 (11.1%) DJ joinings from the Eµ-ret late pro-B cells had N regions. In the VDJ joinings that lacked N regions in the Eµ-ret mice late pro-B cells, nucleotides that could be derived from either adjoining V, D, or J segments were frequently found at both the VD (11/24 [45.8%]) and DJ junction (27/32 [84.3%]). These rearrangements likely occurred by homology-directed recombination, the process by which rearrangement is targeted to short stretches of sequence homology near the ends of the segments to be joined, as is commonly observed in the Ig rearrangements of fetal and newborn mice.11,19

We also analyzed the V, D, and J gene usage in the VDJ joinings of the two late pro-B–cell populations (Fig 1, bottom panel). The only notable differences were that the DQ52 gene was found less frequently in the rearrangements from the transgene-negative mouse (1/25 [4.0%] v 7/36 [19.4%]) and that J2 (13/25 [52.0%]) was the predominant J gene found followed by J4 (7/25 [28.0%]) in the transgene-negative mice, whereas J4 was the predominant gene (14/36 [38.9%]) used followed by J2 (13/46 [36.1%]) in the Eµ-ret mice.

To substantiate whether the leukemias that emerge in older
Eµ-ret mice might arise from this abnormal pool of polyclonal late pro-B cells with infrequent N regions, we sequenced the IgH rearrangements from 9 different leukemias obtained from mice who developed lymphadenopathy at 10 to 38 weeks of age. The repetitive VDJ joinings sequenced from these bands are shown in Table 1. Of note, N regions were found at the VD junction in 11 of 13 (84.6%) of the IgH rearrangements with identifiable D regions, but in only 3 of 13 (23.1%) DJ joints. Homology-directed recombination in the VDJ joinings that lacked N regions was found in 1 of 2 (50%) VD and 7 of 10 (70%) DJ joints. Most strikingly, the oldest mouse (5/9) to develop leukemia, at 38 weeks of age, had two VDJ rearrangements identified, both of which lacked N regions at the DJ joining and 1 that lacked an N region at the VD joint. In addition, 8 of the 9 leukemias were composed of IgH rearrangements that were nonproductive, consistent with their origin from late pro-B cells.

The marked disparity in N region frequency between the VD

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**Table 1. IgH Rearrangements in the B-Precursor Leukemias From Eµ-ret Mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age (wks)</th>
<th>V Family</th>
<th>3’ V</th>
<th>pNp</th>
<th>D or Unknown</th>
<th>pNp</th>
<th>5’ J</th>
<th>J #</th>
<th>D Family</th>
<th>P/NP</th>
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<tr>
<td>2/19</td>
<td>20</td>
<td>J 558</td>
<td>TGTGCCAGAC</td>
<td>AG</td>
<td>ACT*AC</td>
<td>*TACTTT</td>
<td>2</td>
<td>SP or FL</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>6/26</td>
<td>18</td>
<td>Q52</td>
<td>TGTGCC</td>
<td>CCC</td>
<td>CTAAGGAACCT*AC</td>
<td>*TACTTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7183</td>
<td>TGTGCCAGAC</td>
<td>?</td>
<td>GCGGAGGG</td>
<td>?</td>
<td>CCGTGT</td>
<td>3</td>
<td>?</td>
<td>NP</td>
</tr>
<tr>
<td>5/9</td>
<td>38</td>
<td>Q52</td>
<td>TGTGCCAGAAA</td>
<td>A</td>
<td>ATA*GGT</td>
<td>*TTGGTT</td>
<td>3</td>
<td>SP</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>6/14</td>
<td>24</td>
<td>Q52</td>
<td>TGTGCCAGAC</td>
<td>CATAA</td>
<td>ATTACAGGTAGTAG*CT</td>
<td>*GCGGGG</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>5/58</td>
<td>38</td>
<td>Q52</td>
<td>TGTGCCAGAC</td>
<td>?</td>
<td>GCGGAGGG</td>
<td>?</td>
<td>CCGTGT</td>
<td>3</td>
<td>?</td>
<td>NP</td>
</tr>
<tr>
<td>7/1</td>
<td>10</td>
<td>7183</td>
<td>TGTGCCAGAC</td>
<td>tAG</td>
<td>TATGATTACG</td>
<td>CCGTGT</td>
<td>3</td>
<td>SP</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>2/28</td>
<td>22</td>
<td>7183</td>
<td>TGTGCCAGAC</td>
<td>GG</td>
<td>A*ACTAC</td>
<td>*TGAGGG</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>2/17</td>
<td>22</td>
<td>7183</td>
<td>TGTGCCAGAC</td>
<td>AG</td>
<td>ATAGTAAGTCAT</td>
<td>TTC</td>
<td>ACTGGA</td>
<td>3</td>
<td>SP</td>
<td>NP</td>
</tr>
<tr>
<td>2/22</td>
<td>16</td>
<td>7183</td>
<td>TGTGCCAGAC</td>
<td>GGG</td>
<td>AGCTAGTAG</td>
<td>AGCTAGT</td>
<td>1</td>
<td>FL</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>7/8</td>
<td>14</td>
<td>3660</td>
<td>TGTGCCAGAC</td>
<td>GGA</td>
<td>ACTACG*CT</td>
<td>*TACTG</td>
<td>3</td>
<td>FL</td>
<td>NP</td>
<td></td>
</tr>
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</table>

*P . . T enclose nucleotides that could be assigned to either gene segment and thus represent regions of overlapping homology. P/NP signifies whether the rearrangements were productive (P) or nonproductive (NP). P (p) nucleotides as defined by Lafaille et al. are shown in lowercase.
**Fig 2.** RT-PCR analysis of TdT expression in sorted early (B) and late (C) pro-B cells from neonatal Eµ-ret (Tg+) and transgene-negative (Tg–) mice. Shown are ethidium bromide staining patterns of 26 (β-actin) or 30 cycles (TdT) of amplification resolved on a 1.5% agarose gel.

and DJ junction in the leukemic IgH rearrangements led us to consider the possibility that the leukemias may have originated in fetal pro-B cells that subsequently upregulated TdT over time. In the late pro-B–cell population from the 24-day-old Eµ-ret mouse analyzed above and in the majority of leukemias, we found TdT expression to be comparable to the level expressed in transgene-negative late pro-B cells (data not shown). We then determined whether TdT expression was absent or reduced in the late pro-B cells of Eµ-ret mice younger than 24 days. In Fig 2, we sorted CD45R+CD43+CD24+B-1+ early pro-B cells (B) and CD45R+CD43+CD24+B-1+ late pro-B cells (C) from postnatal day-1 liver, day-7 liver and spleen, and day-10 bone marrow and measured TdT levels. In day-1 liver, TdT expression is detectable in early pro-B cells but not in late pro-B cells in both transgene-negative and Eµ-ret mice, likely indicating that newly generated early pro-B cells are upregulating TdT in this postnatal environment. By day 7 in the liver and spleen and day 10 in the bone marrow, TdT expression is prominent in both the early and late pro-B cells from the transgene-negative mice, as well as in the early pro-B cells from Eµ-ret mice. In contrast, TdT expression is markedly reduced in the late pro-B cells from Eµ-ret mice.

Another mechanism that could account for the higher N region frequency at the VD junction is V replacement, in which a 5’ unrearranged V gene replaces the downstream recombined V gene, as has been described in both mouse B-precursor lines and childhood ALL.20-22 Thus, a VDJ rearrangement that lacked N regions at the VD junction could have undergone a V replacement event in the presence of TdT. To determine whether V replacement events occur in the leukemias of the Eµ-ret mice, we analyzed the PCR products generated with the J558 V family-specific primer (and the 4 J gene primers) from the bone marrow DNA of mouse 6/26. The J558 V genes are upstream to the 7183 and the Q52 V genes used in the leukemic rearrangements.26 We observed a faint smear band after gel electrophoresis and subsequently cloned and sequenced 14 unique VDJ rearrangements. Five of these rearrangements appeared to be derived from the leukemic sequences, as shown in Table 2. V replacement events involving the Q52 rearrangement added new N nucleotides to the original N region (CCC) at the VD junction and left the D region unmodified. In regards to the 7183 rearrangement, V replacement events preserved the original nucleotides found between the V and J genes as well as the 3’ end of the 7183 V gene.

We then investigated whether fetal lymphopoiesis in Eµ-ret mice was abnormal compared with transgene-negative mice. We studied B-lineage development in the later stages of fetal development when both CD43+ and CD43– populations are present. As shown in Table 3, the mean percentage of residual CD45R+CD43+ cells (pro-B and early pre-B cells) was only slightly increased in the fetal livers from the Eµ-ret mice at days 18 and 19 of gestation. In addition, the mean percentage of CD45R+CD43– cells (pre-B, immature B, and mature B cells) was reduced in the fetal livers from the Eµ-ret mice at both days. Most notably, at day 18 of gestation, 5 of the 8 Eµ-ret fetal livers contained more CD45R+CD43+ cells than CD45R–CD43– cells, whereas all 5 transgene-negative fetal livers contained more CD45R–CD43– cells.

To determine whether the CD45R+CD43– population was different in the Eµ-ret fetal livers, we compared the BP-1 and CD24 expression patterns of these cells to distinguish early pro-B, late pro-B, and early pre-B subsets. Figure 3 provides a flow cytometry analysis of the fetal livers from the Eµ-ret mouse and the transgene littermate with the highest percentages of CD45R+CD43+ cells at day 19 of gestation. Specifically, the CD45R+CD43+ population (right upper quadrant, top panels) was gated for BP-1 and CD24 expression (lower panels). The increased percentage of CD45R+CD43+ cells in the fetal liver in the Eµ-ret mouse relative to the transgene-negative mouse is accounted for by an increase in both the BP-1+ early pro-B cell (1.6% vs. 0.7%) and BP-1+CD24+ late pro-B cell (1.9% vs.1.2%) population. The percentage of the more differentiated BP-1+CD24++ early pre-B cell population is the same (1.1%).

Similarly, when we compared the BP-1 and CD24 expression patterns from the Eµ-ret mouse and the transgene littermate with the lowest percentages of CD45R+CD43+ cells at day 19 of gestation (both with 1.5%), the Eµ-ret mouse had a higher percentage of early pro-B cells (0.5% vs. 0.3%) and late pro-B cells (0.7% vs. 0.5%), but had a lower percentage of early pre-B cells (0.3% vs. 0.6%). These data indicate that Eµ-ret mice retain

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**Table 2. V Replacements in the Clonal IgH Rearrangements of a B-Precursor Leukemia From an Eµ-ret Mouse**

<table>
<thead>
<tr>
<th>V Family</th>
<th>J V</th>
<th>N</th>
<th>D or Unknown</th>
<th>DJ Overlap</th>
<th>5’ J</th>
<th>j #</th>
<th>D Family</th>
<th>P/NP</th>
</tr>
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<tbody>
<tr>
<td>*Q52</td>
<td>TGTCGCG</td>
<td>CCC</td>
<td>CTATGGTAACT</td>
<td>AC</td>
<td>TACCTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
</tr>
<tr>
<td>J558</td>
<td>TGTCAGAAGA</td>
<td>GACC</td>
<td>CTATGGTAACT</td>
<td>AC</td>
<td>TACCTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
</tr>
<tr>
<td>J558</td>
<td>TGTCAGAAGA</td>
<td>GACC</td>
<td>CTATGGTAACT</td>
<td>AC</td>
<td>TACCTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
</tr>
<tr>
<td>J558*</td>
<td>TGTCAGAAGA</td>
<td>GACC</td>
<td>CTATGGTAACT</td>
<td>AC</td>
<td>TACCTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
</tr>
<tr>
<td>J558</td>
<td>TGTCAGAAGA</td>
<td>GACC</td>
<td>CTATGGTAACT</td>
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<td>TACCTT</td>
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<td>SP</td>
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</tr>
<tr>
<td>J558</td>
<td>TGTCAGAAGA</td>
<td>GACC</td>
<td>CTATGGTAACT</td>
<td>AC</td>
<td>TACCTT</td>
<td>2</td>
<td>SP</td>
<td>NP</td>
</tr>
</tbody>
</table>

* indicates the clonal VDJ joinings originally isolated from the leukemia of Eµ-ret mouse 6/26. The retained 3’ V nucleotides in the V replacement events involving the 7183 VDJ joining are underlined. P/NP signifies whether the rearrangements are productive (P) or nonproductive (NP).
a higher proportion of early and late pro-B cells among the residual CD43+ population late in gestation.

We did not previously observe notable differences in the proliferation and differentiation of the early pro-B cell population in the bone marrow of 3- to 5-week-old Eµ-ret mice compared with transgene-negative mice and unpublished observations. Moreover, from the TdT expression data given above, it appears that the early pro-B cells seen in the bone marrow of young Eµ-ret mice are predominately derived after birth. Thus, the RFP/RET transgene product might exert its effect preferentially on fetal-derived pro-B cells. Consequently, we sorted early pro-B cells (CD45R+CD43+CD24- BP-12) from day-17 fetal livers and cultured the cells in RPMI-based media supplemented with IL-7 and 5% FCS. After 4 days in culture, the Eµ-ret early pro-B cells proliferated threefold to fivefold greater (mean, 3.9; n = 3 livers) than the transgene-negative cells. However, the percentage of viable cells was only slightly greater in the Eµ-ret cultures (mean, 59.4%) compared with the transgene-negative cultures (mean, 51.3%). Although the cultures from both populations were composed of predominately sIg2 cells, the Eµ-ret cultures had less than 1% of the cells differentiating to the sIg1 stage compared with about 7% for the transgene-negative cultures (data not shown). Unlike the pro-B cells from transgene-negative mice, the Eµ-ret cells continued to proliferate in IL-7. As shown in Fig 4, the Eµ-ret cells recovered after 17 days are uniformly CD45R+CD43- and are composed of both an early pro-B (BP-1+ ~40%) and late pro-B (BP-1+ ~60%) population. Similarly, we found that Eµ-ret early pro-B cells sorted from day-16 fetal livers had a mean 4.5-fold greater proliferation (n = 3) and less differentiation than transgene-negative cells when cultured for 4 days in IL-7 (data not shown).

**DISCUSSION**

We characterized the rearranged IgH chain in the expanded late pro-B–cell population of 24-day-old Eµ-ret mouse and found that the rearrangements were predominately nonproductive and polyclonal. Most notably, the rearrangements at both the VD and DJ junction had a low frequency of N regions when compared with those from transgene-negative mice. When we examined the rearranged IgH alleles in the leukemias from adult Eµ-ret mice, we found that the majority of VD junctions had N regions, whereas the majority of DJ junctions lacked N regions. Consequently, we hypothesize that the majority of leukemias

**Table 3. B-Lineage Cells in the Fetal Livers of Eµ-ret Mice**

<table>
<thead>
<tr>
<th>Gestational Day</th>
<th>Transgene</th>
<th>No. Analyzed</th>
<th>Mean Total Liver Cell No. ×10⁷</th>
<th>Mean CD45R:CD43+ % (range)</th>
<th>Mean CD45R:CD43- % (range)</th>
<th>No. With CD43+ Cells</th>
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<tbody>
<tr>
<td>18</td>
<td>-</td>
<td>5</td>
<td>1.4</td>
<td>3.2 (1.6-4.7)</td>
<td>5.7 (3.2-8.2)</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>8</td>
<td>1.7</td>
<td>3.4 (2.0-6.7)</td>
<td>3.2 (1.3-5.1)</td>
<td>5</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>5</td>
<td>2.1</td>
<td>2.5 (1.5-3.0)</td>
<td>6.9 (4.7-9.6)</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>3</td>
<td>2.1</td>
<td>3.2 (1.5-4.6)</td>
<td>5.5 (4.6-6.1)</td>
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**Fig 3.** Flow cytometry of day 19 fetal livers showing separation of B-lineage subsets in Eµ-ret (Tg+) and transgene-negative (Tg–) mice. In the upper diagrams, B-lineage cells (CD45R-) are resolved from total liver cells into less differentiated (CD43+) and more differentiated subsets (CD43-). The percentages of CD45R-CD43+ (left upper quadrant) and CD45R-CD43- (right upper quadrant) cells among total liver cells are shown in the corresponding quadrants. In the lower diagrams, CD45R-CD43+ cells are further resolved into BP-1- early pro-B (bottom region), CD24- BP-1+ late pro-B (upper left region), and CD24- BP-1+ early pre-B cells (upper right region) and their percentages are shown in their corresponding region. An arrow points to a population of late pro-B cells that expresses high levels of BP-1, as previously observed in the bone marrow of 3-to 5-week-old Eµ-ret mice.
occurred in fetal-derived pro-B cells with upregulated TdT, rather than from pro-B cells originating in the postnatal bone marrow, liver, or spleen. In support, we found normal levels of TdT gene expression in the late pro-B cells from the 24-day-old Eµ-ret mice, but late pro-B cells from 7- to 10-day-old Eµ-ret mice had markedly reduced levels of TdT compared with transgene-negative mice. In addition, early pro-B cells from the fetal liver of Eµ-ret mice upregulated TdT when cultured in vitro (data not shown), as has been recently observed for normal fetal multipotent hematopoietic cells and B-lineage–committed progenitors.27 In this latter study, N regions were found about fourfold more frequently at the VDJ junction compared with the DJ junction when these fetal B precursors were cultured for greater than 14 days.27 Thus, N regions in the Eµ-ret leukemic IgH alleles may have been generated during D to J joining, V to DJ joining, or V replacement in the presence of upregulated TdT activity. In childhood ALL, N regions are found less frequently at the DJ junction when these fetal B precursors were cultured for greater than 14 days.27 Thus, N regions in the Eµ-ret leukemic IgH alleles may have been generated during D to J joining, V to DJ joining, or V replacement events as well as ongoing D to J and V to DJ joining of the IgH chain have been observed.25-28,30

We next examined fetal lymphopoiesis in the Eµ-ret mouse. Unlike the bone marrow of young Eµ-ret mouse, where there is typically a 10- to 20-fold increase in the percentage of the late pro-B–cell population,9 we did not find such a marked difference in fetal liver. However, the Eµ-ret fetal livers retained a greater proportion of B-lineage cells in both the early pro-B– and late pro-B–cell stages than their transgene-negative counterparts. More notably, there was a marked difference in the growth of early pro-B cells from Eµ-ret and transgene-negative fetal livers when cultured in IL-7 alone. These later observations suggest a mechanism that could explain how an apparent mild quantitative abnormality in fetal lymphopoiesis could produce a marked abnormality within the first postnatal month. Consequently, we hypothesize that the residual fetal pro-B cells in the Eµ-ret mice are stimulated to proliferate and differentiate in the immediate postnatal period. During this time, fetal pro-B cells with nonproductive VDJ rearrangements fail to undergo apoptosis but continue to proliferate, and thus accumulate and expand in the late pro-B–cell pool.

We previously found that early pro-B cells from the bone marrow of 3- to 5-week-old Eµ-ret mice proliferated and differentiated similarly to transgene-negative controls in culture media supplemented with IL-7.7 These observations were surprising considering the inhibition in differentiation that was seen in the late pro-B cells from these Eµ-ret mice. Most notably, the early pro-B cells differentiated to a greater extent than the late pro-B cells sorted from the same Eµ-ret mice. However, we have now observed that TdT levels are normal in the early pro-B cells from 7- to 10-day-old Eµ-ret mice, in contrast to the reduced levels observed in the late pro-B cells from the same mice. Thus, it appears that the majority of early pro-B cells in the previously studied Eµ-ret mice were derived in the postnatal bone marrow, whereas the late pro-B cells were of fetal origin. Consequently, we propose that the RFP/RET transgene product preferentially affects fetal pro-B cells, perhaps by interacting with a pathway accessible primarily in fetal B-lineage cells.

The chimeric protein, RFP/RET, produced from the Eµ-ret transgene is predominately membrane bound and has constitutive tyrosine kinase activity.31,32 The in vitro culture results suggest that the transgene product may substitute for a stromal cell signal, because early pro-B cells from the fetal livers of Eµ-ret mice could be maintained long-term in IL-7 alone.33 It is unknown whether RET plays a role in human ALL. In one study, RET expression was detected in only 2 of 7 cases of B-precursor ALL.34 An increase in ALL has not been reported in patients with constitutional activating mutations in RET (multiple endocrine neoplasia 2 syndromes), but the malignancies develop in tissues with the highest levels of RET expression.35-37 The RET tyrosine kinase has the most homology with the fibroblast growth factor receptor (FGFR) family. Mutations and translocations involving members of the FGFR family have been found in hematopoietic abnormalities including T- and B-cell lymphoid malignancies.38-40 However, patients with skeletal abnormalities carrying constitutional activating mutations in FGFR members are not at increased risk for hematopoietic malignancies, suggesting that the level of tyrosine kinase activity in the hematopoietic cells of these patients is insufficient to induce transformation.39 Thus, the ability of the RFP/RET product to transform B-precursor cells in Eµ-ret mice likely reflects its high kinase activity and could occur through the constitutive activation of signaling pathways normally stimulated by other related tyrosine kinases. As an additional mechanism, the RFP/RET chimeric protein could bind the normal RFP transcriptional activator and inhibit its action.41

Greaves’ originally proposed a two-step model of childhood
ALL. During the initial expansion of B-lineage cells in utero, a spontaneous mutation arises in a B-precursor cell. Subsequently, as a result of the proliferative drive to make antibody-producing B cells against exogenous antigens in infancy and early childhood, a leukemia-inducing secondary mutation occurs in a fetal-derived B-precursor cell carrying the initial mutation. In the Eµ-ret mouse, the RFP/RET transgene acts as the first mutation in utero, producing only a mild perturbation in fetal lymphopoiesis. However, the expansion of the residual fetal pro-B cells in the immediate postnatal period does not appear to be leukemogenic, but may serve to produce a sufficient number of target cells in which leukemia-inducing secondary mutations will subsequently occur. In support, we previously did not observe changes in CYCLIN D1 and CASPASE-1 expression in healthy Eµ-ret mice until several months after this initial expansion.35 Surprisingly, BCL-X1 transgenic mice do not develop B-lineage malignancies despite markedly expanding the bone marrow with late pro-B cells.36 Thus, the RET/RFP product may also play a role in inducing secondary mutations as a consequence of its ability to enhance both proliferation and survival. In conclusion, with its predisposition for developing leukemia with features similar to childhood B-precursor ALL, the Eµ-ret mouse provides a model of an in utero initiating event in the pathogenesis of leukemia.

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