

# V(D)J recombinatorial repertoire diversification during intracлонаl pro-B to B-cell differentiation

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**The initial B-cell repertoire is generated by combinatorial immunoglobulin V(D)J gene segment rearrangements that occur in a preferential sequence. Because cellular proliferation occurs during the course of these rearrangement events, it has been proposed that intracлонаl diversification occurs during this phase of B-cell development. An opportunity to examine this hypothesis directly was provided by the identification of a human acute lym-**

**phoblastic leukemic cell line that undergoes spontaneous differentiation from pro-B cell to the pre-B and B-cell stages with concomitant changes in the gene expression profile that normally occur during B-cell differentiation. After confirming the clonality of the progressively differentiating cells, an analysis of immunoglobulin genes and transcripts indicated that pro-B cell members marked by the same DJ rearrangement generated daugh-**

**ter B cells with multiple V<sub>H</sub> and V<sub>L</sub> gene segment rearrangements. These findings validate the principle of intracлонаl V(D)J diversification during B-cell generation and define a manipulable model of human B-cell differentiation. (Blood. 2003;101:1030-1037)**

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## Introduction

B-cell development normally begins within specialized microenvironments in the hematopoietic fetal liver and bone marrow. Therein precursor cells undergo proliferation during the course of a series of differentiation events featuring the ordered rearrangement of variable (V), diversity (D), and joining (J) gene segments to generate a diverse antibody repertoire.<sup>1</sup> The D→J<sub>H</sub> gene rearrangements in progenitor B (pro-B) cells are followed by V→DJ<sub>H</sub> rearrangements that allow precursor B (pre-B) cells to produce μ heavy chains (HCs).<sup>2,3</sup> The μHCs associate with the surrogate light chains (SLCs), composed of VpreB and λ5/14.1 proteins, and Igα/β heterodimers to form the pre-B cell receptor (pre-BCR; Karasuyama et al<sup>4</sup> and Burrows et al<sup>5</sup> and references therein). Signaling initiated via this receptor complex is essential for pre-B cell survival, proliferation, and transient down-regulation of recombination-activating genes (*Rag1/2*) to terminate V(D)J rearrangements in the immunoglobulin (Ig) HC locus.<sup>6,7</sup> The subsequent extinction of SLC gene expression is temporally related to an exit from the cell cycle, *Rag1/2* up-regulation, and Ig light chain (LC) V-J gene rearrangement.<sup>8-11</sup> Immature IgM<sup>+</sup> B cells generated by productive V<sub>L</sub>-J<sub>L</sub> rearrangements may then undergo positive or negative selection by encounter with self-antigens.<sup>12,13</sup>

B-cell clonality is usually defined operationally by the shared expression of a unique BCR specificity, although intracлонаl antigen receptor diversity may be generated by several mechanisms. Antibody diversity can be generated by receptor editing, a process by which the specificity of an autoreactive BCR is changed thorough a secondary V<sub>L</sub>-J<sub>L</sub> rearrangement at an immature B-cell stage.<sup>14,15</sup> Additional intracлонаl BCR diversification can be generated by point mutations in the

variable region during antigen (Ag)-driven clonal expansion in germinal centers.<sup>1,16,17</sup> B-cell malignancies, including follicular B-cell lymphoma, chronic lymphocytic leukemia, and Hodgkin disease, may exhibit this type of clonal diversification as a manifestation of their germinal center (GC) origin.<sup>18</sup>

It has been proposed that intracлонаl diversity may also be generated as a consequence of the cellular proliferation that accompanies the normal stepwise V(D)J rearrangement events in pro-B and pre-B cells.<sup>19</sup> According to this hypothesis, a pro-B cell undergoing the initial DJ<sub>H</sub> rearrangement can give rise to daughter cells that select different V<sub>H</sub> genes for subsequent V-DJ<sub>H</sub> rearrangement. In turn, the pre-B cell progeny would have the opportunity to select different V<sub>L</sub> and J<sub>L</sub> gene segments for VJ<sub>L</sub> pairing, thereby providing additional intracлонаl BCR diversity. Because a clonal model of early B-lineage differentiation would allow testing of this hypothesis, we sought a stable cell line that recapitulates this developmental process. Here, we report the characterization of a human acute lymphoblastic leukemic (ALL) cell line, EU12, that undergoes continual B-lineage differentiation from the pro-B to the pre-B and B-cell stages. Analysis of this clonal model of human B-lineage differentiation validates the principle of intracлонаl V(D)J recombinatorial diversification during B-cell genesis.

## Materials and methods

### Cells and antibodies

The EU12 cell line was established from bone marrow cells obtained from a child with relapsed B-cell precursor ALL.<sup>20-22</sup> The EU12 cell line was found

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to be negative for Epstein-Barr virus-associated nuclear antigen (EBNA) and to represent the leukemic clone by immunophenotypic analysis. The Nalm16 pro-B, OB5 pre-B, and EU12 ALL cell lines were cultured in Iscove medium supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 10% heat-inactivated fetal calf serum (FCS), and 2 mM L-glutamine. (Life Technologies, Grand Island, NY). Immunofluorescence assays used the following monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-conjugated CD2, CD3, CD5, CD10, CD11a, and CD34 (Becton Dickinson, Mountain View, CA); phycoerythrin (PE)-conjugated CD19, CD20, CD21, CD23, and HLA-DR (Becton Dickinson); FITC-conjugated CD45RA and antihuman  $\lambda$ LC; PE-conjugated CD32, CD33, CD69, and VpreB8 (Pharmingen, San Diego, CA); and the CY5-labeled VpreB8 mAb as described previously.<sup>23</sup> Polyclonal antibodies included FITC-labeled anti-terminal deoxynucleotidyl transferase (TdT; Supertechs, Bethesda, MD), FITC-labeled goat anti- $\mu$ HC, biotin-conjugated goat anti- $\mu$ HC, and PE-labeled goat anti-IgD. Allophycocyanin (APC)-conjugated streptavidin (Southern Biotechnology, Birmingham, AL) was used as a second-step reagent for the biotin-labeled antibodies.

### Immunofluorescence analysis and cell sorting

Viable cells were incubated with conjugated mAbs for immunofluorescence analysis and for fluorescence-activated cell sorting (FACS). Subpopulations of the EU12 cells were purified by 2 rounds of sorting using FACSTAR (Becton Dickinson) and MoFlow (Cytomation, Fort Collin, CO) instruments. For intracellular staining, the purified subpopulations of cells were fixed in 0.05% paraformaldehyde solution at 4°C for 1 hour, permeabilized with 0.2% Tween-20 in phosphate-buffered saline at room temperature for 20 minutes, and blocked with mouse serum for 10 minutes before incubation with fluorochrome-conjugated antibodies.

### RT-PCR assays

Twice-sorted subpopulations of EU12 cells were lysed in TRIzol reagent (Gibco, Grand Island, NY), and total cellular RNA prepared following procedures recommended by the manufacturer. The synthesis of first-strand cDNA was performed as previously described.<sup>23</sup> For each cDNA preparation, a control synthesis reaction was performed without reverse transcriptase (RT) to test for genomic DNA (gDNA) contamination. The protocols for polymerase chain reaction (PCR) included denaturing at 94°C for 3 minutes amplification by 30 cycles of 94°C for 1 minute, annealing for 30 seconds at 60°C for interleukin 7 receptor (*IL-7R*) and *mb-1*; 65°C for *C $\kappa$* ; and 72°C for *Bcl-2*; and extension at 72°C for 5 minutes. The primers for PCR amplification were *IL-7R*, 5'-GTCGCTCTGTTGGTCATCTTG-3' and 5'-TTTTG TCTTCTCTGTGCTGTG-3'; *mb-1*, 5'-GCTCCCCTAGAG-GCAGTAAAG GC-3' and 5'-AGGGTAACTCACTGTTAGGCCAG-GC-3'; *C $\kappa$* , 5'-TGGCTGCACATCTGTCATCA-3' and 5'-TTGAAGCTC-TTTGTGACGGGC-3'; *Bcl-2*, 5'-TGCACCTGACG CCCTTAC-3' and 5'-AGACAGCCAGGAGAAATCAAACAG-3'. The protocols and primers used for PCR amplification of *Rag1*, *Rag2*, *TdT*, *B29*, *C $\mu$* , and  $\beta$ -*actin* have been described.<sup>11</sup>

### DNA blotting

gDNA samples from sorted EU12 subpopulations and placenta (germline configuration control) were subjected to restriction enzyme digestion, electrophoresis, transfer, and hybridization with <sup>32</sup>P-labeled DNA probes for analysis of Ig HC and LC gene configuration as described.<sup>24</sup> DNA probes, including a 3.6 kilobase (kb) *Bg*III J<sub>H</sub> probe, a *Hind*III J<sub>K</sub> probe, and an *Eco*RI C <sub>$\lambda$</sub>  probe, were used as described.<sup>24</sup>

### Genomic PCR assay

gDNA samples (0.5  $\mu$ g) from EU12 subpopulations and the control HepG2 cells were used as templates. The protocol for PCR amplification of IgH gene segments involved denaturing at 95°C for 5 minutes, amplifying by 36 cycles of 95°C for 1 minute, annealing at 65°C for 30 seconds and at 72°C for 30 seconds, and extending at 72°C for 5 minutes. The primers for amplification, DXP'1, 5'-ATTACTATGGTTCGGGGAGTT-3'; DXP'1ext, 5'-GGTGAGGTCTGTGTCAC-3'; J<sub>H</sub>5, 5'-GTCGAACCAAGTTGTCAC-

CATTG TG-3'; J<sub>H</sub>6, 5'-ACCTGAGGAGACGGTGACC-3', and Pan-V<sub>H</sub>FR1, and PanV<sub>H</sub>FR3 were as described.<sup>25</sup> The PCR products were separated by electrophoresis, transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH). After standard prehybridization, the blots were hybridized at 42°C with oligonucleotide DXP'1 or J<sub>H</sub>5 probes labeled with digoxigenin (DIG) using the DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. After washing at 42°C, hybridization signals were revealed using the DIG luminescence detection kit for nucleic acids (Boehringer Mannheim) according to the manufacturer's instructions. To detect the V <sub>$\lambda$</sub> J <sub>$\lambda$</sub>  rearrangements in each of the EU12 subpopulations, the protocol of PCR amplification using a set of V <sub>$\lambda$</sub>  and J <sub>$\lambda$</sub>  primers was used as described.<sup>26</sup>

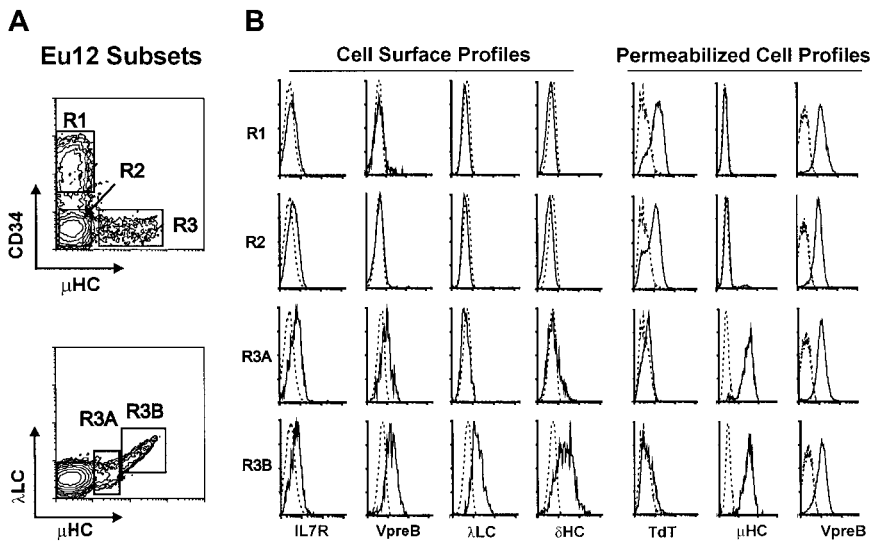
### Ig gene sequencing and single-cell PCR assay

To determine  $\mu$ HC and  $\lambda$ LC transcript sequences, cDNA of the test cells was synthesized for PCR amplification as previously described.<sup>23</sup> Pan-V<sub>H</sub>FR1 and C <sub>$\mu$</sub>  primers were used for amplification of the  $\mu$ HC cDNAs, and the set V <sub>$\lambda$</sub>  and J <sub>$\lambda$</sub>  primers and PCR protocol for  $\lambda$ LC cDNA amplification were performed as above. The PCR products of  $\mu$ HC or  $\lambda$ LC transcripts were isolated and subjected to the TA cloning reaction as described by the manufacturer (Invitrogen, Carlsbad, CA). Plasmids carrying  $\mu$ HC or  $\lambda$ LC transcripts were purified from randomly selected *Escherichia coli* colonies for sequencing reactions. For analysis of single-cell V<sub>H</sub> usage, individual  $\mu$ HC<sup>+</sup> EU12 cells were deposited into tubes containing 20  $\mu$ L PCR buffer with 0.25 mg/mL proteinase K. Reactions were incubated at 50°C for 1 hour and used as templates in a single-cell PCR assay. In the first-round PCR reaction, a combination of V<sub>H</sub>1,2,3-specific oligonucleotides was used as forward primer, and an oligonucleotide sequence common to J<sub>H</sub>1,2,4,5 was used as the reverse primer. In the second round of a nested PCR, 5  $\mu$ L of the first PCR reaction product was used as the template, individual V<sub>H</sub> oligonucleotides were used as the forward primer, and the J<sub>H</sub>1,4,5 oligonucleotide was used as the nested reverse primer. The primer sequences have been described<sup>27</sup> and the protocol included first-round V<sub>H</sub> gene amplification at 95°C for 5 minutes, 59°C for 4 minutes, and 72°C for 3 minutes (cycle 1); 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 80 seconds (cycles 2-36); then extension at 72°C for 5 minutes; and maintenance at 4°C. For second-round PCR, the protocol for amplification of the first round PCR product included 95°C for 3 minutes, 61°C for 1 minute, 72°C for 1 minute (cycle 1), 95°C for 1 minute, 61°C for 30 seconds, 72°C for 50 seconds (cycles 2-40), and extension at 72°C for 5 minutes, and then maintenance at 4°C. PCR products were separated by electrophoresis on a 1.5% agarose gel and purified for the sequencing reaction by using a gel extraction kit (Qiagen, Valencia, CA). Purified PCR products were sequenced directly using V<sub>H</sub>-specific primers. All sequences were analyzed with the DNAPlot program (<http://www.dnplot.de/>).

## Results

### Phenotypic and karyotypic characterization of the EU12 leukemic cell line

EU12 was unique among a panel of 11 ALL cell lines in that phenotypic analysis identified cells at multiple stages of B-lineage differentiation. All of the EU12 cells expressed the CD19, CD20, CD22, CD23, CD32, CD33, CD38, CD40, and HLA-DR antigens, but subpopulations of this cell line were found to be either positive or negative for CD34, CD10, and  $\mu$ HC (Figure 1). Differential immunofluorescence analysis indicated that the EU12 cell line is composed of 3 distinctive subpopulations: CD34<sup>+</sup> $\mu$ HC<sup>-</sup> (R1), CD34<sup>-</sup> $\mu$ HC<sup>-</sup> (R2), and CD34<sup>-</sup> $\mu$ HC<sup>+</sup> (R3). The latter subpopulation could be subdivided into  $\mu$ HC<sup>low</sup>SLC<sup>+</sup> (R3A) and  $\mu$ HC<sup>high</sup> $\lambda$ LC<sup>+</sup> (R3B) subsets (Figure 1). Cells in both the R3A and R3B subsets expressed cell surface SLCs, and the cells in the R3B subset also expressed  $\lambda$ LCs and  $\delta$ HCs. The  $\mu$ HC<sup>low</sup> R3A cells thus



**Figure 1. Phenotypic characterization of EU12 subpopulations.** (A) Cell surface immunofluorescence analysis of EU12 cells reveals 4 subpopulations (R1, R2, R3A, and R3B) on the basis of CD34,  $\mu$ HC, and  $\lambda$ LC expression. (B) FACS-sorted EU12 cells were counterstained with anti-IL-7R, anti-VpreB, anti- $\lambda$ LC, or anti- $\delta$ HC mAbs for cell surface profile analysis or fixed and permeabilized before analysis with anti-TdT,  $\mu$ HC, and VpreB Abs.

bear pre-BCR, whereas the  $\mu$ HC<sup>high</sup> R3B cells have pre-BCR and BCR. IL-7R, barely detectable on the R1 and R2 subpopulations, was present at higher levels on the R3A and R3B subsets.

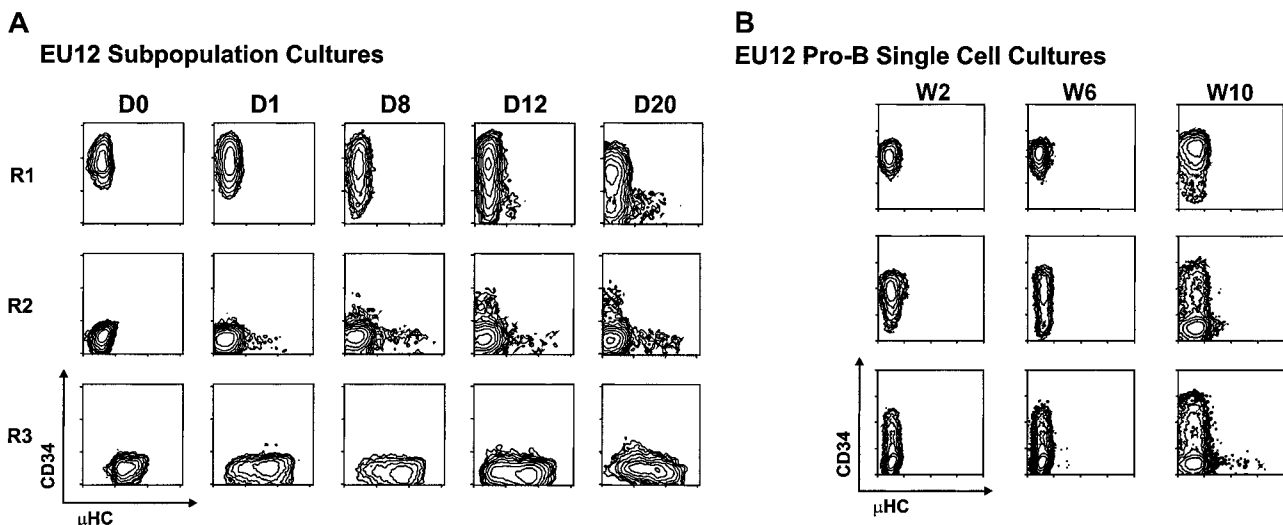
To characterize further the developmental stages of the EU12 cell line, each subpopulation was purified for intracellular immunofluorescence analysis (Figure 1). VpreB was found in permeabilized cells of all 3 subpopulations, whereas  $\mu$ HC was observed only in R3 cells and rarely in R2 cells (< 1%). In contrast, TdT expression was evident in most R1 and R2 cells, but not in the R3 subpopulation. The composite data from this analysis indicate that the EU12 cell line contains cells of pro-B (R1 and R2), pre-B (R3A), and B-cell (R3B) phenotypes, although the B-cell subpopulation is unusual in its IL-7R expression and simultaneous expression of surrogate and conventional  $\lambda$ LCs.

The distinctive phenotype of EU12 cell line prompted a detailed cytogenetic analysis, and the results indicated the EU12 cell line is nearly tetraploid and has complex abnormalities involving chromosomes 2, 3, 4, 5, 6, 7, 11, 12, 17, and 21. Detailed description of its chromosome complement is as follows: 88, XXYY, +2, del(2)(q13) ×

2, del(3)(q26.2), -4, der(4)t(2;4) (q13;q21), inv(5)(q15q33) × 2, del(6)(q23) × 2, t(7;21) (p13;q11) × 2, -8, -9, -11, del(11)(p13), -12, add(12)(p13), +13, -14, -14, +16, del(17)(p11) × 2, add(17)(p13), -18, +20, -21, der(21)t(7;21)(p13;q11), +mar.

#### EU12 cells spontaneously undergo pro-B to B-cell differentiation

The representation of multiple stages of B-lineage differentiation among the EU12 cells suggested this cell line either is undergoing differentiation or represents a multiclonal mixture of cells. To discern between these possibilities, each subpopulation was purified by 2 rounds of FACS before reculture and phenotypic monitoring. Serial immunofluorescence analysis indicated that isolated cells of pro-B cell phenotype (CD34<sup>+</sup> $\mu$ HC<sup>-</sup>) gave rise to cells of pre-B phenotype (CD34<sup>+</sup> $\mu$ HC<sup>low</sup>) by day 12 and cells of B-cell phenotype (CD34<sup>-</sup> $\mu$ HC<sup>high</sup>) by 3 weeks in culture. Purified cells of the R2 subset (CD34<sup>-</sup> $\mu$ HC<sup>-</sup>) likewise gave rise to cells of pre-B and B-cell phenotypes, whereas the R3 subpopulation



**Figure 2. Analysis of the differentiation potential of EU12 cells.** (A) Subpopulations of EU12 cells were purified by 2 rounds of FACS on the basis of their cell surface expression of CD34 and  $\mu$ HC. The purified EU12 subpopulations were cultured and their cell surface profiles reanalyzed on day 0 (D0), day 1 (D1), day 8 (D8), day 12 (D12), and day 20 (D20). (B) Single EU12 pro-B cells (CD34<sup>+</sup> $\mu$ HC<sup>-</sup>) were cultured in individual wells and their progeny were characterized by immunofluorescence analysis at week 2 (W2), week 6 (W6), and week 10 (W10).

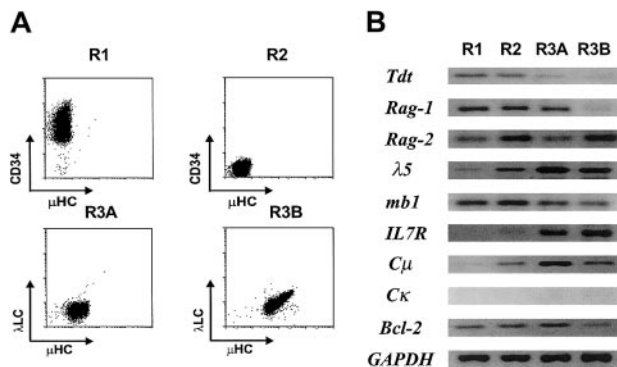
retained cells of pre-B and B phenotypes (Figure 2A). These results indicated that EU12 cells can undergo continual differentiation from pro-B cells to B cells. To verify their developmental potential at a clonal level, single R1 pro-B cells were placed in separate culture wells. Serial examination of their cellular progeny indicated that individual EU12 cells of pro-B cell phenotype are capable of giving rise to mature B-cell progeny (Figure 2B), although a longer time interval was required for this progression when the cultures are initiated with single cells, and B-cell differentiation was not seen in every subclone over the 10-week observation period. These results document the spontaneous differentiation capabilities of the EU12 cell clone and suggest variability in the progression of individual cells along the B-cell differentiation pathway.

**Differential gene expression profiles in the EU12 subpopulations**

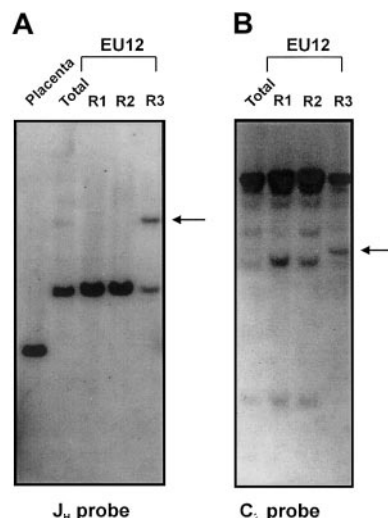
Cells representative of each definable stage in EU12 differentiation were purified by 2 rounds of FACS before RT-PCR analysis of their gene expression profile (Figure 3). As anticipated from its protein expression profile (Figure 1), the *Tdt* lymphoid cell-specific gene was down-regulated in the R3A subset and extinguished in the R3B subset. *Rag1* was found to follow a similar expression pattern, whereas *Rag2* transcripts were expressed in all of the subpopulations, as were transcripts for the *mb1*, *B29*,  $\lambda 5$ , and *VpreB* B-lineage genes. In keeping with results of the immunofluorescence analysis (Figure 1), *IL-7R* and *C $\mu$*  transcripts were not detected in the R1 pro-B cells, but were expressed in subpopulations representing later stages in differentiation. Interestingly, *Bcl-2* transcription was noticeably down-regulated in the R3B B-cell subpopulation, possibly suggesting that the most mature EU12 cells are more susceptible to programmed cell death. These data support the conclusion that the EU12 cells undergo many of the changes in gene expression that characterize normal B-cell differentiation.

**Analysis of Ig HC and LC gene configuration in the EU12 cells**

Southern blot analysis was initially used to characterize the Ig gene rearrangement status at the different EU12 differentiation stages. Restriction enzyme-digested gDNA samples of each EU12 subpopulation, and of placenta as a germline control, were hybridized with  $J_H$ ,  $C_\kappa$ , and  $C_\lambda$  probes (Figure 4 A-B; data not shown). Germline  $J_H$ -containing fragments were not detectable in the unfractionated EU12 cells; instead, 2 rearranged bands were observed, a dominant



**Figure 3. Expression of B lineage-specific genes in the EU12 subpopulations.** (A) Each subpopulation of EU12 cells was purified by 2 rounds of FACS. (B) RNA obtained from each subpopulation was used in RT-PCR amplification of B lineage-specific genes as described in "Material and methods." PCR products were visualized by ethidium bromide staining of agarose gels.



**Figure 4. Southern blot analysis of Ig gene configuration in the EU12 subpopulations.** gDNA from placenta and sorted EU12 subpopulations was digested with *Bgl*II before hybridization with a  $J_H$  probe for IgH chain gene analysis (A), or with *Eco*RI before hybridization with a  $C_\lambda$  probe for Ig $\lambda$  L chain gene analysis (B). Arrows indicate HC and LC gene rearrangements in the R3 subpopulation.

6.2-kb band and a minor 12-kb band (Figure 4A, lane 2). Of these, the R1 and R2 subpopulations contained only the 6.2-kb band (Figure 4A, lanes 3 and 4), whereas the  $\mu$ HC<sup>+</sup> R3 subpopulation contained both bands of similar intensity (Figure 4A, lane 5). These findings, together with a karyotypic analysis demonstrating 2 copies of chromosome 14 (data not shown), suggest that the least differentiated R1 cells of EU12 contain 2 very similar  $J_H$  rearrangements, but are incapable of producing  $\mu$ HCs because the rearrangements either are incomplete DJ<sub>H</sub> or nonproductive VDJ<sub>H</sub>. The occurrence of an additional rearrangement event on one HC allele in the R3 cells coincides with the expression of  $\mu$ HC.

No  $J_\kappa$  hybridizing bands were detected in a DNA blot analysis of the  $\kappa$ LC locus (data not shown) in accordance with the absence of  $\kappa$ LC transcripts in EU12 cells (Figure 3). These findings suggest that the EU12 clone has deleted the  $\kappa$ LC locus, although chromosome 2 appeared intact at the level of resolution provided by karyotypic analysis. When *Eco*RI-digested DNA samples from each subpopulation were hybridized with the  $C_\lambda$  probe, a rearranged  $J_\lambda C_\lambda$  segment was evident among the 7  $J_\lambda C_\lambda$  gene pairs in the R3 subpopulation (Figure 4B). The finding demonstrates  $V_\lambda \rightarrow J_\lambda C_\lambda$  gene rearrangement in the  $\mu$ HC<sup>+</sup> R3 subpopulation, but not in the earlier fractions, and is consistent with the proposed developmental sequence R1  $\rightarrow$  R2  $\rightarrow$  R3. The cDNA sequence analysis (Figure 5) indicates that multiple rearrangements using the same  $J_\lambda C_\lambda$  gene segments have occurred during the in vitro differentiation of the EU12 cells.

**V<sub>H</sub> and V<sub>L</sub> gene usage in EU12 cells**

To examine the extent of V(D)J recombinatorial diversification in the EU12 cells, the  $\mu$ HC<sup>+</sup> R3 subpopulation of cells was purified and RNA extracted for cDNA synthesis and PCR amplification of  $\mu$ HC transcripts using PanV<sub>H</sub>FR3 and  $C_\mu$  primers. The resultant  $\mu$ HC PCR products were cloned and the analysis of sequences obtained from 17 randomly selected clones identified the same DXP1/J<sub>H</sub>4 gene segment rearrangement with no nucleotide addition in this D-J<sub>H</sub> joint region. This analysis also demonstrated the use of multiple V<sub>H</sub> gene segments by EU12 cells in that the V<sub>H</sub>3-7 gene segment was found in 12 of the 17 cDNA clones, V<sub>H</sub>1-8 in 3

**A cDNA Clones**

	V <sub>H</sub>	N	DXP'1	J <sub>H4</sub>	Number of Sequences
IgVH3-7:	TGT GCG AGA GA		G TAT TAC TAT GGT TCG GGG AGT TAT AC TAC		4
Clonetype1	---	CC CC	---	---	7
Clonetype2	---	TG GAC TCA CA	---	---	1
Clonetype3	---	CT CAC	---	---	
IgVH1-8:	TGT GCG AGA G		G TAT TAC TAT GGT TCG GGG AGT TAT AC TAC		2
Clonetype4	---	CT CAA AAA ACT AC	---	---	1
Clonetype5	---	CG AGA GTG GAC TCA CA	---	---	
IgVH3-11:	TGT GCG AGA G		G TAT TAC TAT GGT TCG GGG AGT TAT AC TAC		2
Clonetype6	---	TCA AAA GTG GAC TCA CA	---	---	

**B Single Cell Genomic PCR**

	V <sub>H</sub>	N	DXP'1	J <sub>H4</sub>	
IgVH3-7	TGT GCG AGA GA		G TAT TAC TAT GGT TCG GGG AGT TAT AC TAC		15
Clonetype1	---	CC CC	---	---	12
Clonetype2	---	TG GAC TCA CA	---	---	
IgVH2-5	TGT GCA CAC AGA		GT ATT ACT ATG GTT CCG GGA GTT ATT ACT AC		13
Clonetype3	---		---	---	

**C cDNA Clones**

	V <sub>λ</sub>	N	J <sub>λ2</sub>	
Vλ3-10:	AGT GGT AAT CAT AGC		T GTG GTA TTC GGC	1
Clonetype1	---	GG	---	1
Clonetype2	---	AGC	---	
Vλ3-19:	AGC AGT GGT AAC CAT CT		T GTG GTA TTC GGC	4
Clonetype3	---	CC	---	1
Clonetype4	---	G GCC	---	1
Clonetype5	---	C TAC ACA GAG GAG	---	1

cDNA clones, and V<sub>H</sub>3-11 in the other 2 cDNA clones (Figure 5A). In addition, different nucleotide additions in μHC transcripts using the same V<sub>H</sub> gene provided further evidence of intraclonal V(D)J diversification.

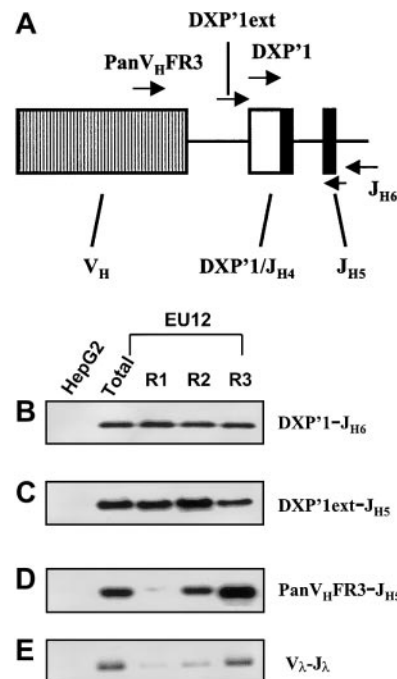
To avoid possible transcriptional bias in this analysis of V<sub>H</sub> gene usage, we performed a single-cell genomic PCR assay of sorted μHC<sup>+</sup> EU12 cells. The sequences derived from a panel of single-cell V<sub>H</sub> gene PCR products recaptured the 2 major V<sub>H</sub>3-7 gene rearrangements observed in the earlier analysis of μHC cDNA clones (Figure 5B). In addition, we identified a V<sub>H</sub>2-5 rearrangement with the same DXP'1/J<sub>H4</sub> sequence observed in the cDNA clones; however, this rearrangement was nonfunctional because of a reading frame shift. The resulting transcript would be targeted for destruction by nonsense-mediated mRNA decay,<sup>28</sup> thereby explaining why V<sub>H</sub>2-5 gene segment usage was not observed in the cDNA analysis. Consistent with the cDNA sequence analysis, 2 patterns of nucleotide additions were observed in the joint region between V<sub>H</sub>3-7 and DXP'1/J<sub>H4</sub>. The dominant joint region sequence featured a CCCC nucleotide addition, presumably added during the V<sub>H</sub>→DJ<sub>H</sub> recombination process. The other additions were longer and contained a consensus CACA sequence that was also found at the 3' end of V<sub>H</sub>2-5 gene segment.

With this information in hand, genomic PCR assays were performed to determine more precisely when the V(D)J rearrangements occurred during the EU12 cell differentiation process. Primers able to discriminate between DJ<sub>H</sub> and V<sub>H</sub>DJ<sub>H</sub> rearrangements were used in this analysis (Figure 6A). In a control experiment, the internal DXP'1 primer was used in conjunction with a downstream J<sub>H6</sub> primer, and genomic PCR products of the expected size were detected in each EU12 subpopulation after hybridization with a J<sub>H5</sub>-specific probe (Figure 6B). This result was as expected because the primer combination amplifies both VDJ<sub>H</sub> and DJ<sub>H</sub> rearrangements. To assay specifically for DJ<sub>H</sub> rearrangements, a primer based on the sequence upstream of the germline DXP'1 gene segment (DXP'1 ext) was used in conjunction with internal J<sub>H5</sub> primers and the PCR products were detected by a specific internal DXP'1 probe. The EU12 cells in each purified subpopulation were found to contain a DJ<sub>H</sub> segment rearrangement, albeit at apparently reduced levels in the R3 pre-B/B cell subpopulation (Figure 6C). Using PanV<sub>H</sub>FR3 and J<sub>H5</sub> primers in an assay that allows definition of the presence or absence of VDJ<sub>H</sub> rearrange-

**Figure 5. Sequence analysis of Ig HC and LC genes in μHC<sup>+</sup> EU12 cells.** The cDNA of sorted μHC<sup>+</sup> EU12 cells was used as template for PCR amplification at V<sub>H</sub>-C<sub>μ</sub> (A) and V<sub>L</sub>-C<sub>λ</sub> (C) transcripts using primer combinations described in "Materials and methods." (B) Single μHC<sup>+</sup> EU12 cells were sorted into PCR tubes with lysis buffer, and used for 2-round genomic PCR as described in "Materials and methods." PCR products were purified and directly sequenced with specific V<sub>H</sub> primers.

ments but does not identify unique rearrangements, VDJ<sub>H</sub> gene rearrangement was detected in the R2 subpopulation, and to a much greater extent in the R3 subpopulation (Figure 6D). The nonfunctional V<sub>H</sub>2-5 DXP'1/J<sub>H4</sub> rearrangement was not detected in this assay because the PanV<sub>H</sub>FR3 primer does not recognize the V<sub>H</sub>2-5 gene segment. However, using a V<sub>H</sub>2-specific primer, a genomic PCR product was detectable in the R1, R2, and R3 subpopulations (not shown).

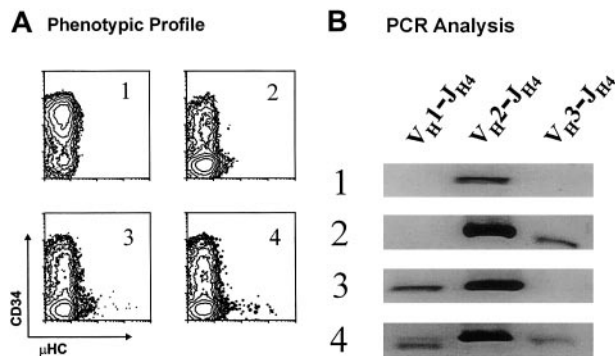
In an analysis of the V<sub>λ</sub> usage, cDNA derived from the μHC<sup>+</sup> R3 cells served as the template for PCR amplification of V<sub>λ</sub>-J<sub>λ</sub>



**Figure 6. Genomic PCR assay of Ig HC and LC genes in EU12 subpopulations.** The diagram (A) indicates the location of each oligonucleotide used in the PCR reactions. gDNA from unfractionated EU12 cells, each subpopulation of EU12 cells, and control HepG2 cells was used as the template for PCR amplification by primers DXP'1 (internal), and J<sub>H6</sub> (B), DXP'1 (external), and J<sub>H5</sub> (C), and PanV<sub>H</sub>FR3, and J<sub>H5</sub> (D) for Ig HC gene and a set of primers for Iga LC genes (E). PCR products were subjected to electrophoresis and blotted with probes J<sub>H5</sub> (B), and DXP'1 (internal) for panels C and D or revealed with ethidium bromide staining of agarose gels (E).

transcripts using a panel of  $V_{\lambda}$  and  $J_{\lambda}$  primers.<sup>26</sup> Analysis of the sequences obtained from 8 randomly selected cDNA clones indicated the use of at least 2  $V_{\lambda}$  gene segments each of which was recombined with the same  $J_{\lambda}2C_{\lambda}2$  gene segment (Figure 5C), data consistent with the observation of a single rearranged band on the Southern blots (Figure 4B).  $V_{\lambda}3-19$  gene segment usage appeared dominant, being seen in 6 of 8 clones, whereas  $V_{\lambda}3-10$  was used in the other 2 clones. Variations in the N nucleotide additions were also observed in  $\lambda$ LC transcripts with both  $V_{\lambda}$  gene segments, indicating the occurrence of multiple  $V_{\lambda} \rightarrow J_{\lambda}2C_{\lambda}2$  rearrangements in the R3 subpopulation. In a genomic PCR assay to detect  $V_{\lambda} \rightarrow J_{\lambda}$  rearrangements,  $VJ_{\lambda}$  rearrangements were detectable in R2 cells and to a much greater extent in the R3 cells (Figure 6E).

The prevalent usage of a limited number of  $V_H$  gene segments and the occurrence of the same  $DJ_H$  rearrangements over a 2-year interval for EU12 subclones suggested a predisposition of the individual EU12 pro-B cells to undergo particular  $V_H \rightarrow DJ_H$  rearrangements. Alternatively, each pro-B cell might give rise to progeny that would use one of a limited number of  $V_H$  gene segments for rearrangement. To determine whether individual EU12 pro-B cells with the  $DXP'1/J_H4$  rearrangement could generate intracлонаl  $V_H$  gene diversity, DNA samples of 4 EU12 subclones were isolated for genomic PCR analysis of  $V_H$  gene utilization. Each of the subclones was derived from a single cell with the pro-B phenotype (R1 in Figure 2B) and the subclones were selected on the basis of their phenotypic profiles after 10 weeks in culture (Figure 7A). Whereas  $\mu$ HC<sup>+</sup> cells were not seen in subclone 1, subclone 2 progeny reached the pre-B cell stage, and subclones 3 and 4 contained both pre-B and B-cell subpopulations. In an assay of V(D)J rearrangements using the  $V_H$ -specific primers used for the single-cell PCR analysis described, all 4 subclones were found to undergo the  $V_H2-DJ_H$  rearrangement (Figure 7B). Subclone 2 also exhibited a  $V_H3-DJ_H$  rearrangement, subclone 3 had a  $V_H1-DJ_H$  rearrangement, and subclone 4 was the most diverse, manifesting all 3  $V_H$  gene segment rearrangements (Figure 7B). These results, which confirm the single-cell PCR sequence analysis, indicate that the progeny of individual EU12 pro-B cells can undergo productive VDJ rearrangements involving more than one  $V_H$  gene segment. These findings formally establish that individual EU12 pro-B cells can give rise to intracлонаl  $V_H$  diversity during B-cell generation.



**Figure 7. Analysis of  $V_H$  gene use in EU12 subclones.** (A) 4 EU12 subclones derived from sorted single cells with pro-B phenotype were selected on the basis of their phenotypic profiles after 10 weeks in culture. (B) gDNA was isolated and used as template for PCR reactions as described for single-cell PCR analysis. The PCR products were subjected to electrophoresis and revealed with ethidium bromide staining.

## Discussion

These studies define a clonal model of human B-cell development in which the pro-B cell subpopulation, as well as the individual pro-B cells, can spontaneously generate pre-B and B-cell progeny. The B lineage-specific gene expression profiles for each of these subpopulations resemble those observed during normal B-cell differentiation. These characteristics define the EU12 cell line as a novel model that recapitulates many of the central features of the human B-cell differentiation pathway.

Mouse pre-B cell hybridomas,<sup>2</sup> Abelson murine leukemia virus (AMuLV)-transformed cell lines,<sup>29</sup> human Epstein-Barr virus-transformed B-cell precursors,<sup>24</sup> and ALL-derived tumor cell lines<sup>30</sup> have been used in previous studies to gain insight into the sequential nature of Ig H and L chain gene rearrangements during B-lineage differentiation. Although most of these cell lines represent clonal populations frozen in a particular stage of B-lineage differentiation, the AMuLV-transformed cell line 300-19 was found to undergo pro-B to B-cell differentiation.<sup>29</sup> However, this proved to be an unstable phenotype because the AMuLV-transformed cell lines extinguish their *Rag1/2* expression with prolonged cultivation.<sup>31</sup> In humans, the search for an in vitro culture system or cell lines that recapitulate early B-lineage cell differentiation and self-renewal for cellular and molecular studies has been an ongoing challenge.<sup>32</sup> One leukemic cell line, BLIN-1, was shown to undergo pre-B to B-cell differentiation at low frequency,<sup>30</sup> and a more recently described cell line, BLIN-3, can progress from the pro-B to the pre-B cell stage in a stromal cell-dependent manner.<sup>33</sup> The EU12 cell line is unique in maintaining 4 distinguishable subpopulations,  $CD34^+s\mu$ HC<sup>-</sup> (R1),  $CD34^-s\mu$ HC<sup>-</sup> (R2),  $CD34^-s\mu$ HC<sup>low</sup> (R3A), and  $CD34^-s\mu$ HC<sup>high}s\deltaHC<sup>+</sup> $\lambda$ LC<sup>+</sup> (R3B), that recapitulate sequential stages in the normal B-cell differentiation pathway. Analysis of cell surface and intracellular SLC, TdT, and  $\mu$ HC expression indicates the pro-B cell nature of the R1 cells, while suggesting that the R2 cells represent a transitional pro-B to pre-B cell stage. The R3A cells express pre-BCR, whereas R3B cells express BCR indicating their B-cell status.</sup>

The sorted R2 subpopulation can give rise to occasional  $CD34^{low}\mu$ HC<sup>-</sup> cells, raising the possibility that this subset may contain rare precursors at an earlier stage of development than R1 cells. Although we cannot exclude the possibility of very rare pre-R1 cells, it seems unlikely that the R2 subset contains earlier progenitors. First, cells in all of the EU12 subpopulations express high levels of the B-lineage marker CD19, indicating that all R2 cells are B lineage-committed cells. Second, although sorted R2 cells can give rise to some  $CD34^{low}\mu$ HC<sup>-</sup> cells, these cells never express levels of CD34 antigen as high as those seen on the original R1 subpopulation, possibly suggesting they are in a transition state between the R1 and R2 subsets. The  $CD34^-\mu$ HC<sup>-</sup> cells represent a very minor proportion of B-lineage cells in normal bone marrow, yet R2 cells of this phenotype comprise a significant proportion among the EU12 cells. The explanation for this may lie in the fact that during normal B-lineage cell development, only a minor portion of the  $CD34^+CD19^+\mu$ HC<sup>-</sup> pro-B cells undergo  $V \rightarrow DJ_H$  gene rearrangement to produce  $\mu$ HC and become pre-B cells. Those that fail to make productive VDJ rearrangements may undergo apoptosis in vivo and therefore are eliminated. Although the R2 subset represents a pro-B to pre-B transitional stage because that is in the process of undergoing  $V \rightarrow DJ_H$  rearrangement, most of these cells fail to produce  $\mu$ HC. Nevertheless, the cells may not undergo immediate apoptosis due to the transformed characteristics

and in vitro location of the EU12 cells, thereby leading to an exaggerated representation of this differentiation stage.

Another notable departure from the normal B-cell differentiation scheme was observed for the EU12 cell line. SLC expression is normally down-regulated to extinguish pre-BCR expression at the late pre-B cell stage.<sup>11</sup> As a consequence, the receptorless pre-B cells exit the cell cycle and up-regulate their *Rag1* and *Rag2* expression to undergo V-J<sub>L</sub> gene segment rearrangements. EU12 B cells instead were found to coexpress pre-BCR and BCR, a feature noted previously for other "transitional pre-B/B" cell lines,<sup>34-36</sup> thus questioning why these transitional B cells fail to extinguish SLC gene expression and the possible role this failure may have in the leukemogenesis process. A complex locus control region, including the promoters and 5' enhancer for the *VpreB* and  $\lambda 5$  genes, has been identified in mice.<sup>37-40</sup> The promoter regions of both genes contain binding sites for Ikaros, EBF, E2A, and PAX5,<sup>41-43</sup> and these transcription factors are expressed throughout the pre-B cell differentiation process. Ikaros-mediated transcriptional silencing<sup>44-46</sup> may provide a mechanism for SLC gene down-regulation in normal late-stage pre-B cells. On the other hand, dominant-negative Ikaros isoforms, including Ikaros 6, have been found to be preferentially expressed in ALLs and could interfere with the usual transcriptional silencing of SLC genes.<sup>47,48</sup> The clonal EU12 cell line may provide a suitable model to test this hypothesis.

B-cell leukemias and lymphomas are considered clonal diseases derived from a single transformed precursor, based on the analysis of their karyotypes, glucose-6-phosphate dehydrogenase (G6PD) isoenzymes, and BCR idiotypes.<sup>49-51</sup> However, 15% to 45% of ALLs of B lineage represent oligoclonal malignancies according to the diversity of their HC gene expression.<sup>52-57</sup> Analysis of these patients has indicated that the leukemic population may diversify by generating multiple IgH gene rearrangements during the process of tumor progression.<sup>55-57</sup> Sequence analysis of the IgH genes has suggested that both V<sub>H</sub>→DJ<sub>H</sub> and V<sub>H</sub>→VDJ<sub>H</sub> recombinational events can occur during the clonal evolution of B cell leukemias.<sup>55</sup>

All of the cells in the clonal EU12 cell line, established from a childhood B-lineage ALL, apparently contain the same DXP'1/J<sub>H</sub>4 rearrangement with no N nucleotide addition in the DJ<sub>H</sub> join. This suggests that the formative transformational event may have occurred during fetal life in a pro-B cell that had undergone D→J<sub>H</sub> rearrangement.<sup>58</sup> Interestingly, overrepresentation of the DXP'1 and J<sub>H</sub>4 gene segments has been observed in other ALL samples.<sup>57,59</sup> Sequence analysis of the V<sub>H</sub> gene rearrangements in the EU12 B cells indicated the use of 4 gene segments, V<sub>H</sub>2-5, V<sub>H</sub>3-7, V<sub>H</sub>3-11,

and V<sub>H</sub>1-8, which are clustered in the D<sub>H</sub> proximal region of the HC locus. Preferential usage of the D<sub>H</sub> proximal V<sub>H</sub> gene segments has been noted previously in mice<sup>60</sup> and in human ALL.<sup>61</sup> The nontemplated nucleotide additions observed at the site of V-DJ joining include a dominant CCCC tetranucleotide, and other unusually long sequences that contain a consensus CACA sequence identical to the 3' end of the V<sub>H</sub>2-5 gene segment, located just 3' to the conserved internal cryptic RSS heptamer (TACTGTG). These results suggest that the onset of  $\mu$ HC expression in EU12 B cells could result from a V<sub>H</sub>→DXP'1/J<sub>H</sub>4 recombination and by V<sub>H</sub>→nonfunctional V<sub>H</sub>2-5-DXP'1/J<sub>H</sub>4 replacement events. The occurrence of V<sub>H</sub> gene replacement was initially suggested by studies of murine cell lines as a mechanism that could generate a functional  $\mu$ HC gene from a nonproductive VDJ<sub>H</sub> rearrangement.<sup>62,63</sup> The possible occurrence of V<sub>H</sub> gene replacement has been suggested by analysis of the V<sub>H</sub> repertoire of human B-lineage ALL<sup>55,64</sup> and in a transgenic mouse carrying a VDJ<sub>H</sub> rearrangement targeted to the endogenous IgH locus.<sup>65</sup> Confirmation of ongoing V<sub>H</sub> gene replacement as a mechanism to generate the unique EU12 CDR3 regions would make this cell line a valuable model to define the mechanism of the V<sub>H</sub> gene replacement reaction.

The discovery of sequential rearrangement and expression of Ig HC and LC genes in the immediate precursors of mammalian B cells led to the idea that intracлонаl V(D)J recombinatorial diversification might occur during B-cell genesis.<sup>19</sup> The present studies provide direct evidence for the validity of the principle of intracлонаl V(D)J diversification, in that individual EU12 pro-B cells marked with a DXP'1/J<sub>H</sub>4 gene segment give rise to a diverse repertoire during their progression from the pro-B to B-cell stage. Our analysis of the EU12 cell line model also suggests that V<sub>H</sub>→DJ<sub>H</sub> recombination and V<sub>H</sub>→VDJ<sub>H</sub> replacement events can occur contemporaneously in early B-lineage cells to generate intracлонаl V(D)J recombinatorial diversity independent of the bone marrow microenvironment. The EU12 cell line thus provides a useful model for analysis of the progression of human B-cell development.

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## References

- Rajewsky K. Clonal selection and learning in the antibody system. *Nature*. 1996;381:751-758.
- Burrows P, LeJeune M, Kearney JF. Evidence that murine pre-B cells synthesise  $\mu$  heavy chains but no light chains. *Nature*. 1979;280:838-840.
- Alt FW, Yancopoulos GD, Blackwell TK, et al. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO J*. 1984;3:1209-1219.
- Karasuyama H, Rolink A, Melchers F. Surrogate light chain in B cell development. *Adv Immunol*. 1996;63:1-41.
- Burrows PD, Stephan RP, Wang YH, Lassoued K, Zhang Z, Cooper MD. The transient expression of pre-B cell receptors governs B cell development. *Semin Immunol*. 2002;382:1-7.
- Ma A, Fisher P, Dildrop R, et al. Surface IgM mediated regulation of RAG gene expression in E  $\mu$ -N-myc B cell lines. *EMBO J*. 1992;11:2727-2734.
- Melchers F. Fit for life in the immune system? Surrogate L chain tests H chains that test L chains. *Proc Natl Acad Sci U S A*. 1999;96:2571-2573.
- Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med*. 1991;173:1213-1225.
- Grawunder U, Leu TM, Schatz DG, et al. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity*. 1995;3:601-608.
- Loffert D, Ehlich A, Muller W, Rajewsky K. Surrogate light chain expression is required to establish immunoglobulin heavy chain allelic exclusion during early B cell development. *Immunity*. 1996;4:133-144.
- Wang YH, Stephan RP, Scheffold A, et al. Differential surrogate light chain expression governs B-cell differentiation. *Blood*. 2002;99:2459-2467.
- Nemazee D, Weigert M. Revising B cell receptors. *J Exp Med*. 2000;191:1813-1817.
- King LB, Monroe JG. Immunology. B cell receptor rehabilitation—pausing to reflect. *Science*. 2001;291:1503-1505.
- Nemazee D. Receptor editing in B cells. *Adv Immunol*. 2000;74:89-126.
- Meffre E, Casellas R, Nussenzweig MC. Antibody regulation of B cell development. *Nat Immunol*. 2000;1:379-385.
- Clarke SH, Huppi K, Ruezinsky D, Staudt L, Gerhard W, Weigert M. Inter- and intracлонаl diversity in the antibody response to influenza hemagglutinin. *J Exp Med*. 1985;161:687-704.
- Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intracлонаl generation of antibody mutants in germinal centres. *Nature*. 1991;354:389-392.

18. Kuppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. *N Engl J Med*. 1999;341:1520-1529.
19. Cooper MD. Ontogeny of antibody-producing cells. In: Milgrom F, Abeyounis CJ, Albine B, eds. *Antibodies: Protective, Destructive and Regulatory Role: Proceedings of the Ninth International Convocation on Immunology*. Amherst, NY: Karger, Basel 1985; 1984:37-41.
20. Zhou M, Gu L, James CD, et al. Homozygous deletions of the CDKN2 (MTS1/p16ink4) gene in cell lines established from children with acute lymphoblastic leukemia. *Leukemia*. 1995;9:1159-1161.
21. Findley HW, Gu L, Yeager AM, Zhou M. Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. *Blood*. 1997;89:2986-2993.
22. Zhou M, Gu L, Yeager AM, Findley HW. Sensitivity to Fas-mediated apoptosis in pediatric acute lymphoblastic leukemia is associated with a mutant p53 phenotype and absence of Bcl-2 expression. *Leukemia*. 1998;12:1756-1763.
23. Wang YH, Nomura J, Faye-Petersen OM, Cooper MD. Surrogate light chain production during B cell differentiation: differential intracellular versus cell surface expression. *J Immunol*. 1998;161:1132-1139.
24. Kubagawa H, Cooper MD, Carroll AJ, Burrows PD. Light-chain gene expression before heavy-chain gene rearrangement in pre-B cells transformed by Epstein-Barr virus. *Proc Natl Acad Sci U S A*. 1989;86:2356-2360.
25. Gokmen E, Raaphorst FM, Boldt DH, Teale JM. Ig heavy chain third complementarity determining regions (H CDR3s) after stem cell transplantation do not resemble the developing human fetal H CDR3s in size distribution and Ig gene utilization. *Blood*. 1998;92:2802-2814.
26. Farmer NL, Dörner T, Lipsky PE. Molecular mechanisms and selection influence the generation of the human V lambda J lambda repertoire. *J Immunol*. 1999;162:2137-2145.
27. Kuppers R, Zhao M, Hansmann ML, Rajewsky K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J*. 1993;12:4955-4967.
28. Byers PH. Killing the messenger: new insights into nonsense-mediated mRNA decay. *J Clin Invest*. 2002;109:3-6.
29. Reth MG, Ammirati P, Jackson S, Alt FW. Regulated progression of a cultured pre-B-cell line to the B-cell stage. *Nature*. 1985;317:353-355.
30. Wormann B, Anderson JM, Liberty JA, et al. Establishment of a leukemic cell model for studying human pre-B to B cell differentiation. *J Immunol*. 1989;142:110-117.
31. Rathbun G, Oltz EM, Alt FW. Comparison of RAG gene expression in normal and transformed precursor lymphocytes. *Int Immunol*. 1993;5:997-1000.
32. LeBien TW. Fates of human B-cell precursors. *Blood*. 2000;96:9-23.
33. Bertrand FE, Vogtenhuber C, Shah N, LeBien TW. Pro-B-cell to pre-B-cell development in B-lineage acute lymphoblastic leukemia expressing the MLL/AF4 fusion protein. *Blood*. 2001;98:3398-3405.
34. Paige CJ, Kincade PW, Ralph P. Murine B cell leukemia line with inducible surface immunoglobulin expression. *J Immunol*. 1978;121:641-647.
35. Briskin M, Damore M, Law R, et al. Lipopolysaccharide-unresponsive mutant pre-B-cell lines blocked in NF-kappa B activation. *Mol Cell Biol*. 1990;10:422-425.
36. Martin D, Huang RQ, LeBien T, Van Ness B. Induced rearrangement of kappa genes in the BLIN-1 human pre-B cell line correlates with germline J-C kappa and V kappa transcription. *J Exp Med*. 1991;173:639-645.
37. Yang J, Gluzak MA, Blomberg BB. Identification and localization of a developmental stage-specific promoter activity from the murine lambda 5 gene. *J Immunol*. 1995;155:2498-2514.
38. Tian J, Okabe T, Miyazaki T, Takeshita S, Kudo A. Pax-5 is identical to EBB-1/KLP and binds to the VpreB and lambda5 promoters as well as the KI and KII sites upstream of the Jkappa genes. *Eur J Immunol*. 1997;27:750-755.
39. Sabbattini P, Georgiou A, Sinclair C, Dillon N. Analysis of mice with single and multiple copies of transgenes reveals a novel arrangement for the lambda5-VpreB1 locus control region. *Mol Cell Biol*. 1999;19:671-679.
40. Sigvardsson M. Overlapping expression of early B-cell factor and basic helix-loop-helix proteins as a mechanism to dictate B-lineage-specific activity of the lambda5 promoter. *Mol Cell Biol*. 2000;20:3640-3654.
41. Reya T, Grosschedl R. Transcriptional regulation of B-cell differentiation. *Curr Opin Immunol*. 1998;10:158-165.
42. Gisler R, Jacobsen SE, Sigvardsson M. Cloning of human early B-cell factor and identification of target genes suggest a conserved role in B-cell development in man and mouse. *Blood*. 2000;96:1457-1464.
43. Gisler R, Sigvardsson M. The human V-PreB promoter is a target for coordinated activation by early B cell factor and E47. *J Immunol*. 2002;168:5130-5138.
44. Brown KE, Guest SS, Smale ST, Hahn K, Merckenschlager M, Fisher AG. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell*. 1997;91:845-854.
45. Lundgren M, Chow CM, Sabbattini P, Georgiou A, Minaae S, Dillon N. Transcription factor dosage affects changes in higher order chromatin structure associated with activation of a heterochromatic gene. *Cell*. 2000;103:733-743.
46. Sabbattini P, Lundgren M, Georgiou A, Chow C, Warnes G, Dillon N. Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. *EMBO J*. 2001;20:2812-2822.
47. Sun L, Heerema N, Crotty L, et al. Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 1999;96:680-685.
48. Tonnelle C, Bardin F, Maroc C, et al. Forced expression of the Ikaros 6 isoform in human placental blood CD34(+) cells impairs their ability to differentiate toward the B-lymphoid lineage. *Blood*. 2001;98:2673-2680.
49. Zuelzer WW, Inoue S, Thompson RI, Ottenbreit MJ. Long-term cytogenetic studies in acute leukemia of children; the nature of relapse. *Am J Hematol*. 1976;1:143-190.
50. Levy R, Warnke R, Dorfman RF, Haimovich J. The monoclonality of human B-cell lymphomas. *J Exp Med*. 1977;145:1014-1028.
51. Dow LW, Martin P, Moehr J, et al. Evidence for clonal development of childhood acute lymphoblastic leukemia. *Blood*. 1985;66:902-907.
52. Bertoli LF, Kubagawa H, Borzillo GV, et al. Bone marrow origin of a B-cell lymphoma. *Blood*. 1988;72:94-101.
53. Bird J, Gallili N, Link M, Stites D, Sklar J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukemia. *J Exp Med*. 1988;168:229-245.
54. Beishuizen A, Hahlen K, Hagemeijer A, et al. Multiple rearranged immunoglobulin genes in childhood acute lymphoblastic leukemia of precursor B-cell origin. *Leukemia*. 1991;5:657-667.
55. Wasserman R, Yamada M, Ito Y, et al. VH gene rearrangement events can modify the immunoglobulin heavy chain during progression of B-lineage acute lymphoblastic leukemia. *Blood*. 1992;79:223-228.
56. Steenbergen EJ, Verhagen OJ, van Leeuwen EF, dem Borne AE, van der Schoot CE. Distinct ongoing Ig heavy chain rearrangement processes in childhood B- precursor acute lymphoblastic leukemia. *Blood*. 1993;82:581-589.
57. Choi Y, Greenberg SJ, Du TL, et al. Clonal evolution in B-lineage acute lymphoblastic leukemia by contemporaneous VH-VH gene replacements and VH-DJH gene rearrangements. *Blood*. 1996;87:2506-2512.
58. Wasserman R, Gallili N, Ito Y, Reichard BA, Shane S, Rovera G. Predominance of fetal type DJH joining in young children with B precursor lymphoblastic leukemia as evidence for an in utero transforming event. *J Exp Med*. 1992;176:1577-1581.
59. Steenbergen EJ, Verhagen OJ, van Leeuwen EF, et al. B precursor acute lymphoblastic leukemia third complementarity-determining regions predominantly represent an unbiased recombination repertoire: leukemic transformation frequently occurs in fetal life. *Eur J Immunol*. 1994;24:900-908.
60. Malynn BA, Yancopoulos GD, Barth JE, Bona CA, Alt FW. Biased expression of JH-proximal VH genes occurs in the newly generated repertoire of neonatal and adult mice. *J Exp Med*. 1990;171:843-859.
61. Mortuza FY, Moreira IM, Papaioannou M, et al. Immunoglobulin heavy-chain gene rearrangement in adult acute lymphoblastic leukemia reveals preferential usage of J(H)-proximal variable gene segments. *Blood*. 2001;97:2716-2726.
62. Kleinfeld R, Hardy RR, Tarlinton D, Dangl J, Herzenberg LA, Weigert M. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1+ B-cell lymphoma. *Nature*. 1986;322:843-846.
63. Reth M, Gehrmann P, Petrac E, Wiese P. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. *Nature*. 1986;322:840-842.
64. Mori H, Kiyoi H, Horibe K, Ohno R, Naoe T. Comparison of the immunoglobulin gene transcripts between immature B lineage acute lymphoblastic leukemia and the normal phenotypic counterparts in the bone marrow. *Leukemia*. 1997;11:1274-1280.
65. Chen C, Nagy Z, Prak EL, Weigert M. Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing. *Immunity*. 1995;3:747-755.