

# Self-Renewing Hematopoietic Stem Cell Is the Primary Target in Pathogenesis of Human Chronic Lymphocytic Leukemia

Yoshikane Kikushige,<sup>1,2</sup> Fumihiko Ishikawa,<sup>1,3</sup> Toshihiro Miyamoto,<sup>1</sup> Takahiro Shima,<sup>2</sup> Shingo Urata,<sup>1</sup> Goichi Yoshimoto,<sup>1</sup> Yasuo Mori,<sup>1</sup> Tadafumi Iino,<sup>2</sup> Takuji Yamauchi,<sup>1</sup> Tetsuya Eto,<sup>4</sup> Hiroaki Niino,<sup>1</sup> Hiromi Iwasaki,<sup>2</sup> Katsuto Takenaka,<sup>1</sup> and Koichi Akashi<sup>1,2,\*</sup>

<sup>1</sup>Medicine and Biosystemic Science

<sup>2</sup>Center for Cellular and Molecular Medicine

Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan

<sup>3</sup>Research Unit for Human Disease Model, RIKEN Center for Allergy and Immunology, Kanagawa 230-0045, Japan

<sup>4</sup>Department of Hematology, Hamanomachi Hospital, 810-8539 Fukuoka, Japan

\*Correspondence: [akashi@med.kyushu-u.ac.jp](mailto:akashi@med.kyushu-u.ac.jp)

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## SUMMARY

We report here that in chronic lymphocytic leukemia (CLL), the propensity to generate clonal B cells has been acquired already at the hematopoietic stem cell (HSC) stage. HSCs purified from patients with CLL displayed lymphoid-lineage gene priming and produced a high number of polyclonal B cell progenitors. Strikingly, their maturation into B cells was restricted always to mono- or oligo-clones with CLL-like phenotype in xenogeneic recipients. These B cell clones were independent of the original CLL clones because they had their own immunoglobulin VDJ genes. Furthermore, they used preferentially VH genes frequently used in human CLL, presumably reflecting the role of B cell receptor signaling in clonal selection. These data suggest that HSCs can be involved in leukemogenesis even in mature lymphoid tumors.

## INTRODUCTION

Malignant transformation can occur through a multistep acquisition of critical somatic mutations. Therefore, the precursor of malignant stem cells should have a long life span to accumulate such mutations. In human hematopoiesis, genetic abnormalities for transformation should be accumulated in self-renewing hematopoietic stem cells (HSCs). HSCs can continuously produce a number of progenitors with the same genetic alteration, which are also potential targets for additional mutations (Rossi et al., 2008). Such HSCs or downstream progenitors finally become leukemia stem cells that possess self-renewal but lack normal differentiation activity (Huntly et al., 2004; So et al., 2003). This notion of leukemia development has been well accepted to explain acute myeloid leukemia (AML) development, and AML-initiating cells capable of reconstituting human leukemias in

xenogeneic hosts have been purified (Bonnet and Dick, 1997) as a potential therapeutic target (Jin et al., 2006, 2009; Kikushige et al., 2010; Majeti et al., 2009; Saito et al., 2010). However, in lymphoid malignancies, leukemia or lymphoma cells usually have monoclonal immunoglobulin or T cell receptor gene rearrangements, suggesting that lymphoid malignant stem cells originate after cells have committed to the lymphoid lineage. Recent studies have shown that acute lymphoid leukemia (ALL)-initiating cells upon xenogeneic transplantation are composed of multiple genetically distinct subclones (Anderson et al., 2011; Notta et al., 2011). These data clearly show that lymphoid cells can easily accumulate genetic abnormalities, presumably because they can persist longer than myeloid cells, and are capable of clonal expansion simulating self-renewal (Luckey et al., 2006). Because of such property of lymphoid cells, the involvement of HSCs in lymphoid leukemogenesis has never been underscored.

## Significance

HSCs capable of self-renewal should be the main target for accumulating mutational events to develop hematological malignancies. This paper shows that HSCs play such a role also in mature lymphoid malignancies. Most human CLL cases have a precursor phase, called monoclonal B lymphocytosis (MBL), that is asymptomatic monoclonal or oligoclonal proliferation of B cells. HSCs from patients with CLL but not normal HSCs developed monoclonal or oligoclonal B cells simulating MBL after xenogeneic transplantation. Acquisition of chromosomal abnormalities appeared to be secondary events to transform MBL into clinical CLL. Thus, even in CLL, accumulation of oncogenic events starts at the HSC stage. Our xenograft model might be very useful to understand the pathogenesis of human CLL.

Chronic lymphocytic leukemia (CLL), the most common leukemia in adults in western countries, is a mature B cell malignancy (Chiorazzi et al., 2005). It is characterized by accumulation of clonal B cells in the blood, the bone marrow, and the lymphoid tissues. The consistent clonal expansion of mature B cells frequently expressing CD5 is the major phenotype of patients with CLL. Unfortunately, the development of its xenograft models by transplanting primary CLL cells into immunodeficient hosts has failed because the engraftment was extremely inefficient (Dürig et al., 2007; Hummel et al., 1996). Thus, the search for CLL-initiating cells has never been successful.

Human CLL cells have functional B cell receptors (BCRs) on their surface as a result of productive rearrangement of immunoglobulin genes (Caligaris-Cappio and Ghia, 2008; Chiorazzi et al., 2005; Stevenson and Caligaris-Cappio, 2004). CLL has been divided into two subgroups based on the presence of somatic hypermutations within the variable regions of immunoglobulin heavy-chain (IGHV) genes, which normally occurs in the germinal center during naive to memory B cell transition. The group of CLLs with mutated BCRs has a more favorable prognosis than those with unmutated BCRs (Hamblin et al., 1999). However, recent studies suggest that both types of CLLs originate from self-reactive B cell precursors and that the status of somatic hypermutations does not indicate their origin (Hervé et al., 2005; Klein et al., 2001; Rosenwald et al., 2001). Interestingly, CLL cells preferentially use the IGHV genes, such as VH1, VH3, and VH4 regions (Chiorazzi and Ferrarini, 2003; Fais et al., 1998), and express a restricted BCR repertoire including antibodies with quasi-identical complementarity-determining region 3 (CDR3) (Ghiotto et al., 2004; Messmer et al., 2004; Tobin et al., 2003, 2004; Widhopf et al., 2004), suggesting specific antigen recognition by CLL cells (Chiorazzi and Ferrarini, 2003; Stevenson and Caligaris-Cappio, 2004).

To trace the origin of genetic aberration in human CLL, it is important to note the fact that CLL cells are not always monoclonal, but more than one CLL clone is found in up to ~14% of patients with CLL (Sanchez et al., 2003). Furthermore, a recent cohort study has shown that 44 out of 45 patients with CLL have a precursor state such as monoclonal B lymphocytosis (MBL) for 6 months to 7 years (Landgren et al., 2009). MBL represents asymptomatic proliferation of clonal B cells whose numbers in circulation are below 5000/ $\mu$ l (Marti et al., 2005). Of note, human MBL is frequently (20%–70% of total cases) composed of more than one B cell clone (Dagklis et al., 2009; Lanasa et al., 2010; Nieto et al., 2009). More than a half of such MBL clones express CD5 (Scarfò et al., 2010), and patients with these CLL-like MBL clones frequently develop into clinical CLL (Rawstron et al., 2008). Furthermore, like CLL cells, CD5<sup>+</sup> MBL clones use a biased set of VH genes, including VH1, 3, and 4 (Rawstron et al., 2008). The usage of such biased BCR types found in CLL and its precedent MBL clones strongly suggests that the antigenic drive contributes to clonal expansion and/or cell survival also during the transition from MBL to clinical CLL (Pleyer et al., 2009).

The question is: If progression from MBL to CLL reflects stepwise leukemogenesis, at what stage does the first oncogenic event occur. The existence of oligoclonal B cell clones in patients with CLL and with those MBL strongly suggests that the first oncogenic event could at least be traced up to the

progenitor or HSCs that have not rearranged IGH genes. These data led us to search for CLL-initiating cells within the early hematopoietic stages utilizing an efficient xenotransplantation system.

## RESULTS

### Clonal Selection of CLL B Cells Occurs at the Mature B Cell Stage in Human CLL

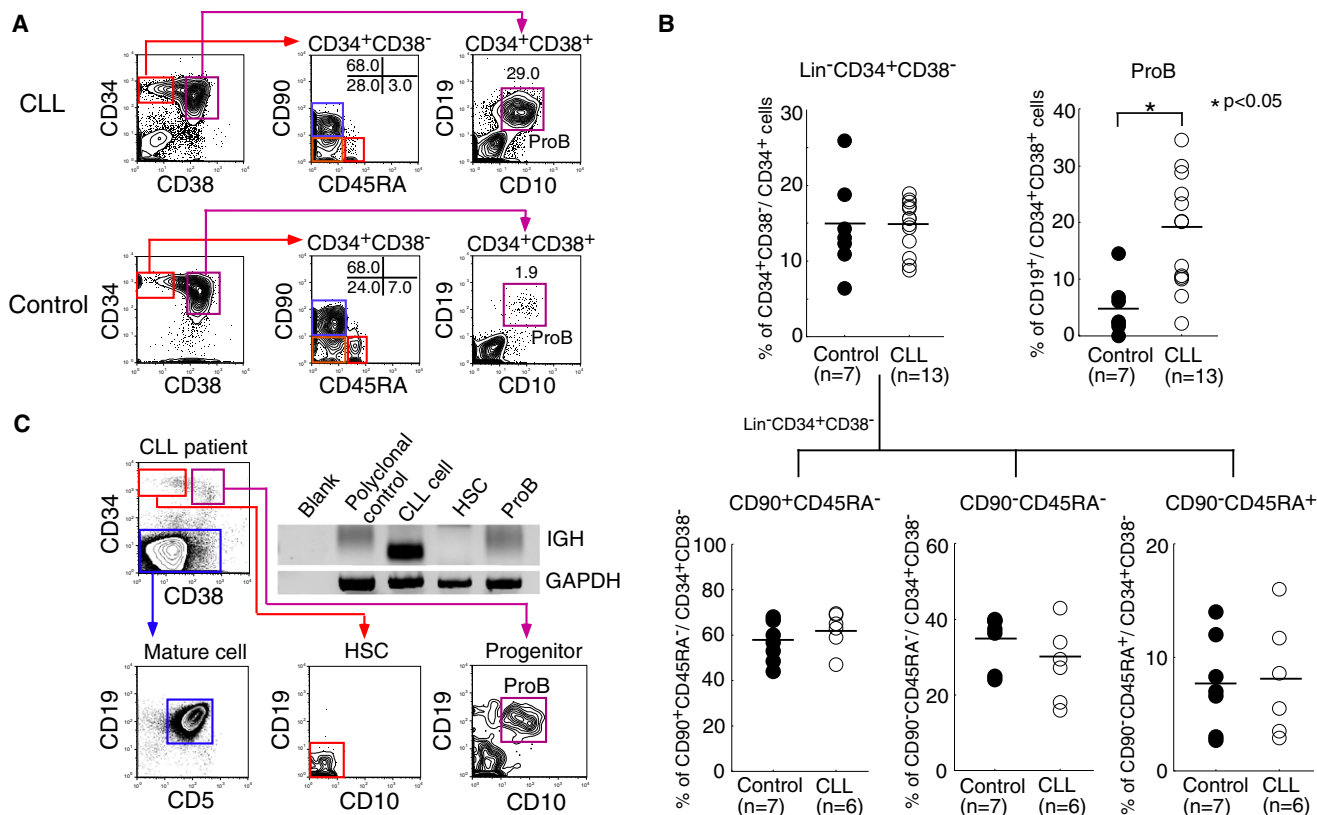
To search for the cell population with CLL-initiating activity in human CLL, we first tried to locate the developmental stage at which CLL B cell clones appear. Patients' characteristics are shown in Table S1 available online.

Figure 1A shows the FACS analysis of the bone marrow of a patient with CLL. The bone marrow contained CD34<sup>+</sup>CD38<sup>−</sup> HSCs (Bhatia et al., 1997), and the CD34<sup>+</sup>CD38<sup>+</sup> progenitor fraction that contains myeloid and lymphoid progenitors (Manz et al., 2002). Interestingly, percentages of CD10<sup>+</sup>CD19<sup>+</sup> proB cells in the bone marrow of patients with CLL were high in most patients: in 12 out of 13 patients with CLL, proB cell frequency was higher than the average of 7 normal controls, and the average proB cell frequency in patients with CLL was higher than that in normal controls by ~5-fold (Figure 1B). In contrast, frequencies of the CD34<sup>+</sup>CD38<sup>−</sup> HSC population were equal (Figure 1B). Recent reports have shown that the CD34<sup>+</sup>CD38<sup>−</sup> HSC population can further be divided into subpopulations including CD90<sup>+</sup>CD45RA<sup>−</sup>, CD90<sup>−</sup>CD45RA<sup>−</sup>, and CD90<sup>−</sup>CD45RA<sup>+</sup> that mainly contain long-term HSCs (LT-HSCs), multipotent progenitors (Majeti et al., 2007), and early lymphoid/myeloid progenitors (Doulatov et al., 2010; Goardon et al., 2011), respectively. We performed the HSC subpopulation analysis in six CLL cases, and found that the distribution of these HSC subpopulations did not differ in normal and CLL bone marrow, and the majority (~60%) of CD34<sup>+</sup>CD38<sup>−</sup> cells were the most primitive CD90<sup>+</sup>CD45RA<sup>−</sup> population (Figure 1B). Thus, we tested whether the expansion at the proB stage reflects clonal proliferation of CLL precursors by analyzing the rearrangement status of the IGH gene.

As shown in Figure 1C, the purified CD34<sup>+</sup>CD38<sup>−</sup> HSC population in patients with CLL (CLL-HSCs) presented the germline configuration, and CD34<sup>+</sup>CD19<sup>+</sup> CLL cells had a clonal IGH rearrangement. Of note, proB cells in CLL bone marrow exhibited polyclonal rearrangement of IGH genes, suggesting that CLL clones are selected in vivo among such expanded polyclonal B cells. These data clearly show that CD34<sup>+</sup>CD38<sup>−</sup> CLL-HSC populations do not rearrange the IGH gene, and therefore, are not contaminated with detectable CLL clones. However, CLL-HSCs are able to develop a higher number of polyclonal B cells as compared to normal HSCs, suggesting that developmental potential of CLL-HSCs is skewed toward B cell lineage probably reflecting their cell-intrinsic abnormality.

### Purified HSCs from Patients with CLL Are Able to Generate Clonal B Cells with CLL-like Phenotype after Xenogeneic Transplantation

We then tried to identify the CLL-initiating cell population by transplanting subpopulations of CLL cells into immunodeficient mice. In these experiments, NOD/SCID/IL2rg<sup>null</sup> (NSG) (Ishikawa et al., 2005) newborns or NOD/RAG-1<sup>−/−</sup>IL2rg<sup>null</sup>



**Figure 1. Hematopoietic Stem and Progenitor Cells in the Bone Marrow of Patients with CLL**

(A) Stem and progenitor FACS analysis of the bone marrow from a patient with CLL and a normal control. A representative analysis is shown.

(B) Frequencies of CD34<sup>+</sup>CD38<sup>-</sup> HSCs and CD34<sup>+</sup>CD38<sup>+</sup>CD19<sup>+</sup> pro B cells in 13 patients with CLL and 7 normal controls (upper panels), and frequencies of HSC subpopulations including CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>CD45RA<sup>-</sup>, CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>-</sup>CD45RA<sup>-</sup>, and CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>-</sup>CD45RA<sup>+</sup> fractions in 6 patients with CLL analyzed (lower panels) are shown. Note that the CLL bone marrow contains a higher number of CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>+</sup>CD19<sup>+</sup> proB cells than the normal bone marrow does ( $p < 0.05$ ) (see also Table S1), whereas percentages of HSC fractions do not differ in normal and CLL bone marrow. The distribution of these HSC subfractions is unchanged in patients with CLL.

(C) IGH rearrangement status of HSC, proB, and B cell fractions in the bone marrow of a patient with CLL. HSCs did not rearrange IGH (germline), whereas proB cells showed polyclonal IGH rearrangement.

(NRG) (Pearson et al., 2008) adult mice were used as recipients (Table 1).

CD19<sup>+</sup> CLL cells were purified from the blood or the bone marrow of patients 1–8, and  $0.2$  to  $1 \times 10^7$  cells were transplanted. However, even until 6 months after transplantation, human CD45<sup>+</sup> cells were never found in any of the 15 recipients analyzed (Figure S1). These data strongly suggest that CLL cells are incompetent for expansion to recapitulate human CLL in immunodeficient mice. We also transplanted  $10^4$  CD34<sup>+</sup>CD38<sup>+</sup>CD10<sup>+</sup>CD19<sup>+</sup> proB cells in these patients, but none of ten recipients was engrafted 12 weeks after transplantation (not shown). These data led us to analyze the engraftment potential of CLL-HSCs in the xenogeneic transplantation system. Purified  $3.3 \times 10^3$  to  $6.5 \times 10^4$  CD34<sup>+</sup>CD38<sup>-</sup> HSCs or  $5.0 \times 10^3$  to  $1 \times 10^4$  CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup> LT-HSCs from 16 independent patients with CLL were transplanted into 25 mice (Table 1; Table S2), and  $\sim 10^4$  CD34<sup>+</sup>CD38<sup>-</sup> cells from 11 normal controls were transplanted into 29 mice.

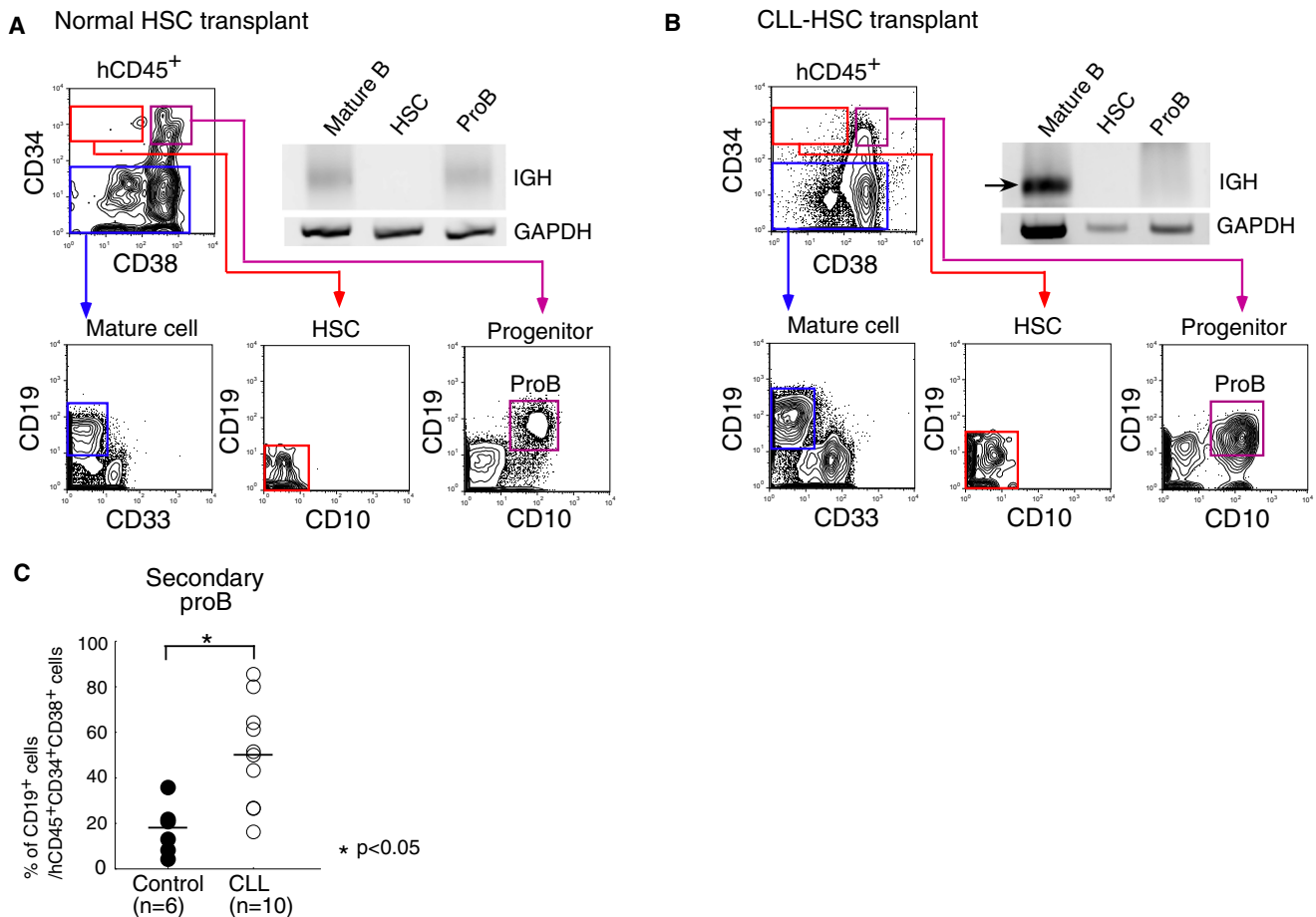
Previous xenogeneic transplantation studies have shown that normal HSCs are able to reconstitute multilineage hematopoietic

cells, and polyclonal B cells are normally developed in NOD-SCID or NSG mouse bone marrow and spleen (Hiramatsu et al., 2003; Ishikawa et al., 2005; Kolar et al., 2004; Matsumura et al., 2003; Rossi et al., 2001). As shown in Figures 2A and 2B, both CLL-HSCs and normal HSCs gave rise to secondary CD34<sup>+</sup>CD38<sup>-</sup> HSCs, CD34<sup>+</sup>CD38<sup>+</sup> progenitor cells, CD34<sup>-</sup>CD19<sup>+</sup> B cells, and CD34<sup>-</sup>CD33<sup>+</sup> myeloid cells in the bone marrow. Of note, the percentage of CLL-HSC-derived human proB cells was significantly higher than that of normal HSC-derived ones (Figure 2C), as we found in the bone marrow analysis of patients with CLL and normal controls (Figure 1B), suggesting again that differentiation of CLL-HSCs skews toward B cell lineage. Interestingly, CLL-HSC-derived CD19<sup>+</sup> B cells in the bone marrow frequently coexpressed CD5 (Figure 3B and Table 1), which is a characteristic of de novo human CLL cells. Normal human HSCs generated mainly CD5<sup>-</sup> and very rare ( $<1\%$ ) CD5<sup>+</sup> B cells in the bone marrow in all 29 recipients. In total, 5 out of 25 mice transplanted with CLL-HSCs developed both CD5<sup>+</sup> and CD5<sup>-</sup> B cell clones, 9 mice developed only CD5<sup>+</sup> B cell clones, and the remaining 11 mice developed only CD5<sup>-</sup> B cell clones

**Table 1. Results of Xenogeneic Transplantation Assays of CLL-HSCs**

				No. of Cells					CD5 <sup>-</sup> B Cell		CD5 <sup>+</sup> B Cell	
Patient No.	Mouse		Weeks after Transplant	Transplanted Cells	Transplanted (×10 <sup>3</sup> cells)	hCD45 <sup>+</sup> Cells (%)	hCD19 <sup>+</sup> in hCD45 <sup>+</sup> (%)	hCD33 <sup>+</sup> in hCD45 <sup>+</sup> (%)	Cells in Total B Cells (%)	No. of Clones	Cells in Total B Cells (%)	No. of Clones
1	1-1	NRG	16	CD34 <sup>+</sup> CD38 <sup>-</sup>	20	0.1	57.9	NA	100	1	–	–
	1-2	NRG	18	CD34 <sup>+</sup> CD38 <sup>-</sup>	40	0.5	32.6	52.8	93.6	P	6.4	3
2	2	NRG	12	CD34 <sup>+</sup> CD38 <sup>-</sup>	65	0.1	33.3	NA	100	1	–	–
3	3	NRG	16	CD34 <sup>+</sup> CD38 <sup>-</sup>	14	1.6	92.3	NA	100	1	–	–
4	4	NSG	5	CD34 <sup>+</sup> CD38 <sup>-</sup>	3.3	22.4	5.14	40.4	100	2	–	–
5	5	NSG	11	CD34 <sup>+</sup> CD38 <sup>-</sup>	7.6	11.4	83	7.1	89.2	2	10.8	2
6	6	NSG	12	CD34 <sup>+</sup> CD38 <sup>-</sup>	7.0	18.7	7.54	89	100	2	–	–
7	7-1	NSG	24	CD34 <sup>+</sup> CD38 <sup>-</sup>	30	31.6	53.2	32.5	95.0	P	5.0	3
	7-2	NSG	24	CD34 <sup>+</sup> CD38 <sup>-</sup>	7.0	1.8	17.6	58.1	65.7	2	34.3	2
	7-3	NSG	24	CD34 <sup>+</sup> CD38 <sup>-</sup>	16	4.3	63.3	31.3	89.8	P	10.2	2
8	8	NSG	24	CD34 <sup>+</sup> CD38 <sup>-</sup>	18	4.1	17.5	60.2	89	1	11	3
9	9-1	NSG	13	CD34 <sup>+</sup> CD38 <sup>-</sup>	4.0	2.0	72.3	19.4	97.5	P	2.4	1
	9-2	NSG	13	CD34 <sup>+</sup> CD38 <sup>-</sup>	5.0	14.0	10.2	51.8	100	1	–	–
10	10-1	NSG	13	CD34 <sup>+</sup> CD38 <sup>-</sup>	15	18.1	88.3	2.9	100	1	–	–
	10-2	NSG	13	CD34 <sup>+</sup> CD38 <sup>-</sup>	10	11.0	68.5	20.1	100	1	–	–
	10-3	NSG	30	CD34 <sup>+</sup> CD38 <sup>-</sup>	5.0	18.5	63.3	24.1	96.9	3	3.1	1
11	11-1	NSG	33	CD34 <sup>+</sup> CD38 <sup>-</sup>	10	0.5	50.1	NA	100	1	–	–
	11-2	NRG	14	CD34 <sup>+</sup> CD38 <sup>-</sup>	18	0.1	28.6	50	100	2	–	–
12	12	NRG	12	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	6.0	0.1	47.5	31.9	89.6	P	10.4	2
13	13-1	NRG	14	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	8.0	0.5	87.1	2.5	94.7	P	5.3	1
	13-2	NRG	14	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	8.0	3.7	88.1	1.1	96	P	3.9	1
	13-3	NRG	17	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	6.0	1.0	86.1	5.2	99	P	1.0	2
14	14	NRG	9	CD34 <sup>+</sup> CD38 <sup>-</sup>	60	1.0	75.1	17.1	99	2	0.9	2
15	15	NRG	9	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	10	0.2	67.7	26	100	1	–	–
16	16	NRG	21	CD34 <sup>+</sup> CD38 <sup>-</sup> CD90 <sup>+</sup>	5.0	1.8	92.1	1.9	98	P	1.7	1

NA, not analyzed; P, polyclonal.



**Figure 2. The Bone Marrow Cell Analysis in Mice Transplanted with the CD34<sup>+</sup>CD38<sup>−</sup> HSC Population Purified from Normal Controls and Patients with CLL**

(A and B) IGH rearrangement status of HSC, proB, and B cell fractions and in the bone marrow of mice transplanted with normal HSCs (A) and CLL-HSCs (B). In all analysis, secondary HSCs and proB cells showed germline and polyclonal rearrangement of IGH genes, respectively. However, secondary mature B cells had clonal IGH only in mice reconstituted with CLL-HSCs but not in those transplanted with normal HSCs. These results suggest that B cell clones derived from CLL-HSCs were selected in vivo.

(C) Frequencies of proB cells in the bone marrow of mice transplanted with CLL-HSCs and normal HSCs. A representative xenogeneic transplantation result of CD19<sup>+</sup> CLL cells is shown in Figure S1.

(Table 1). These CD5<sup>+</sup> B cells derived from CLL-HSCs expressed surface IgM, CD20, and CD23 (Figure 3C) but lacked CD10, like original CLL cells in patients.

#### CLL-HSC-Derived B Cell Clones Had IGH-VDJ Combination Independent of the Original CLL Clones, and Used Preferentially the VH1, VH3, and VH4 Genes

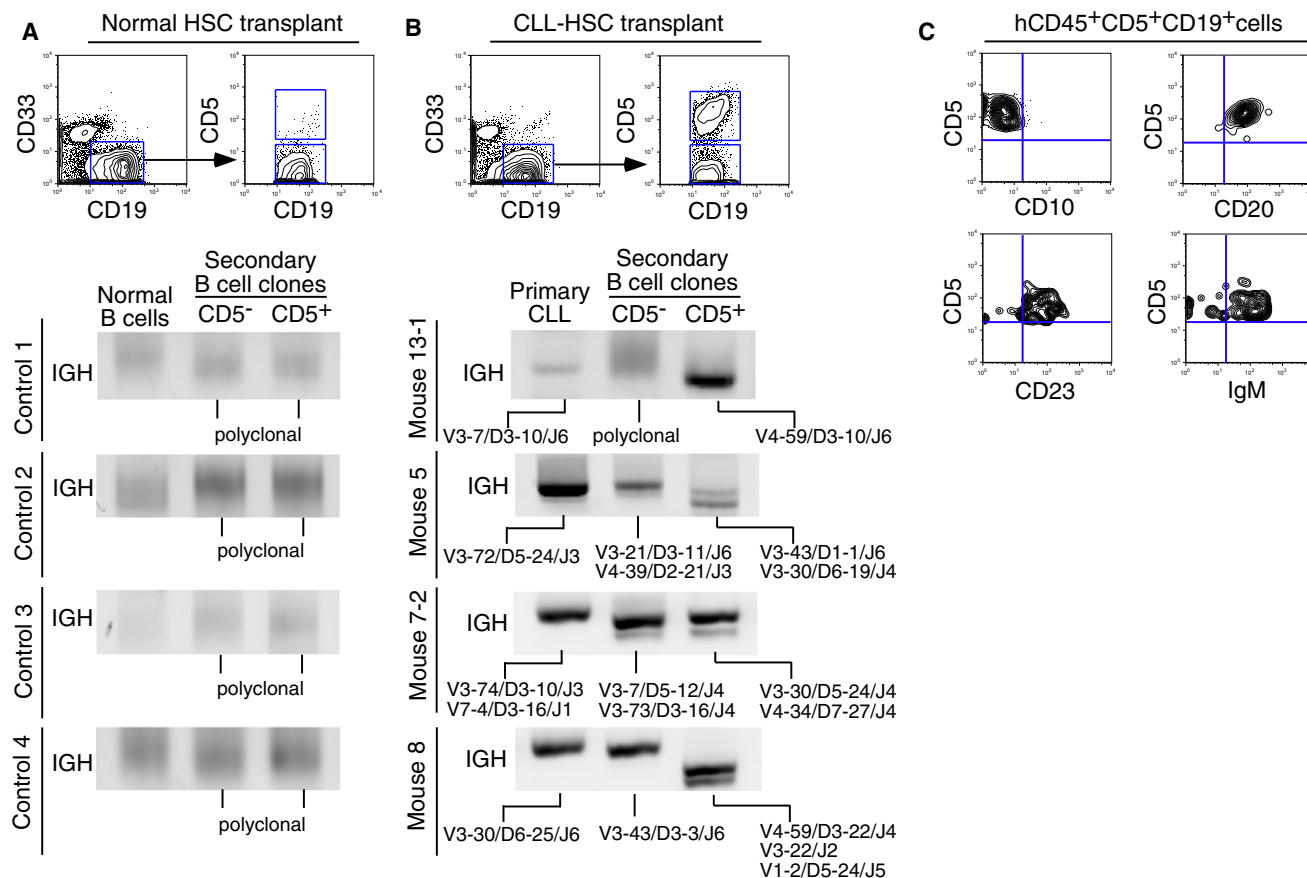
IGH rearrangement status of CLL-HSC-derived B cells was then tested by PCR analysis. In the mouse bone marrow transplanted with normal HSCs (Figure 2A), secondary CD34<sup>+</sup>CD38<sup>−</sup> HSCs did not rearrange IGH, and both proB and CD5<sup>−</sup> mature B cells had polyclonal rearrangement, indicating that control HSCs normally develop polyclonal B cells in this system. Similarly, in mice reconstituted with CLL-HSCs, secondary HSCs retained the germline, and the expanded proB cell population displayed polyclonal IGH rearrangement (Figure 2B). However, to our surprise, mature B cell progeny appeared to have monoclonal or oligoclonal IGH rearrangement, suggesting that clonal selection of

B cells occurred even in xenogeneic recipients (Figures 2B and 3B).

We then analyzed the usage of the VDJ genes in B cell progeny to evaluate clonal relationships between patients' original CLL cells and B cell clones developed in mice from CLL-HSCs. When we found clonal bands in the IGH rearrangement analysis, we evaluated the frequency of B cell clones with specific VDJs by TA cloning of the IGH gene PCR products (Landgren et al., 2009). The PCR products were ligated into the vector, transformed in *Escherichia coli*, picked up randomly ~35 colonies per CD5<sup>+</sup> or CD5<sup>−</sup> B cell samples on average, and they were sequenced to confirm the clonality of BCRs. This analysis was performed in 25 mice reconstituted with 16 patients' CLL-HSCs (Table S2).

Figure 3 shows the representative VDJ recombination analysis of B cell progeny in mice reconstituted with normal HSCs from healthy donors (Figure 3A), or with CLL-HSCs from patients 5, 7, 8, and 13 (Figure 3B). Strikingly, in mice transplanted with CLL-HSCs from these patients, both CD5<sup>+</sup> and CD5<sup>−</sup> B cells





**Figure 3. CLL-HSCs Give Rise to Monoclonal or Oligoclonal B Cells with CLL-like Phenotype after Xenogeneic Transplantation**

(A) FACS and IGH rearrangement analysis of mice transplanted with normal HSCs. CD5<sup>+</sup> B cells were rare, and both CD5<sup>+</sup>CD19<sup>+</sup> and CD5<sup>-</sup>CD19<sup>+</sup> B cell fractions displayed polyclonal IGH rearrangement.

(B) FACS and IGH rearrangement analysis of mice transplanted with CLL-HSCs. Development of CD5<sup>+</sup>CD19<sup>+</sup> B cells was frequently seen in these mice (as summarized in Table 1). In mouse 13-1, CD5<sup>-</sup> B cells were polyclonal, but CD5<sup>+</sup> B cells were monoclonal. In other mice shown here, both CD5<sup>-</sup> and CD5<sup>+</sup> B cells are composed of one to three B cell clones. The B cell clones developed in mice always had VDJ genes different from those of the original CLL cells and, therefore, were independent of the original patients' CLL clone. VH gene usage and similarity of CDR3 amino acid sequences of these independent B cell clones are shown in Figure S2.

(C) The CLL-HSC-derived B cell clones expressed CD20, CD23, and IgM. Representative data are shown.

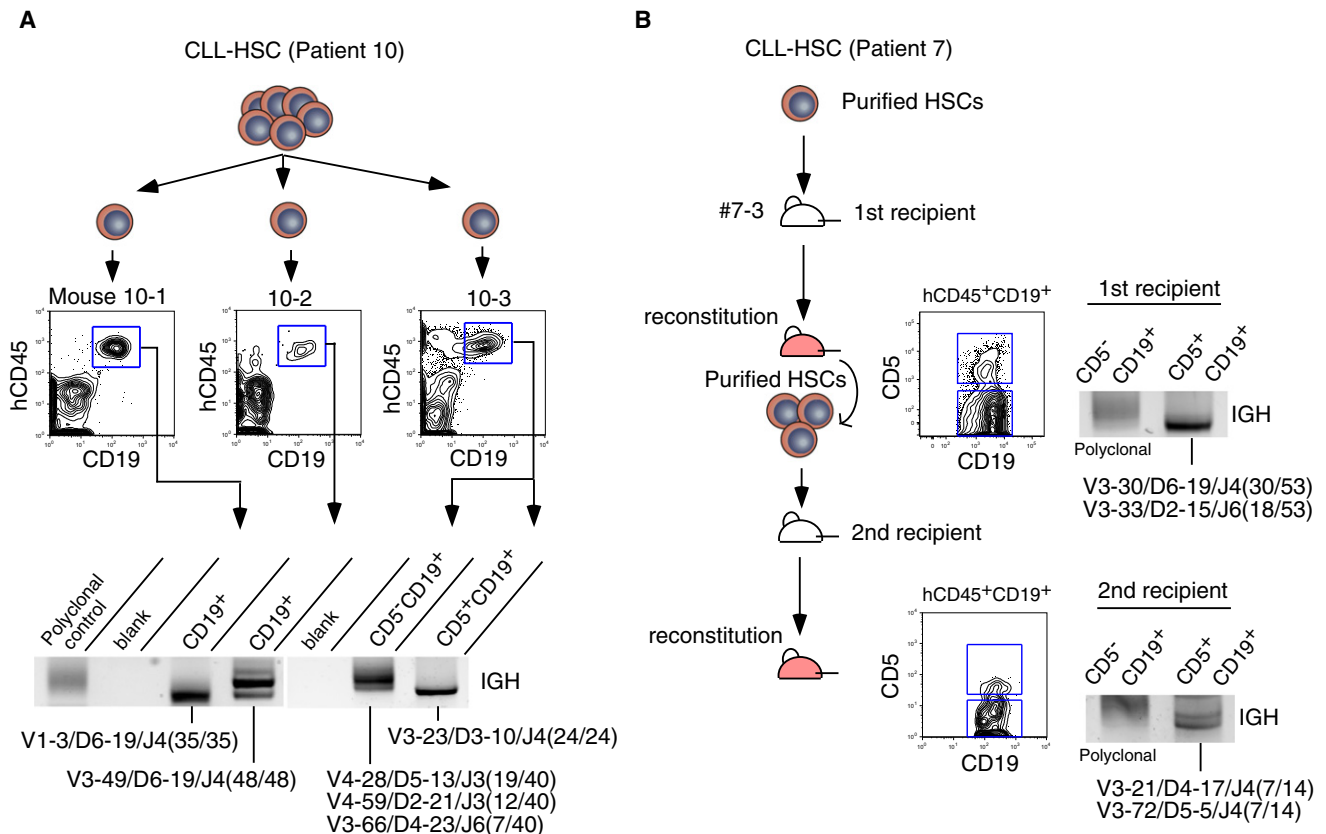
were developed, and each of them was composed of one to three B cell clones. Importantly, these B cell clones developed in recipients possessed the VDJ combinations different from those used in CLL clones in original patients (Figure 3B). In summary, CD5<sup>+</sup> B cells were developed in 14 out of 25 mice transplanted with CLL-HSCs, and these CD5<sup>+</sup> B cells consisted always of mono- or oligo-clones (Table 1). Clonal B cell populations were also found in CD5<sup>-</sup> B cell progeny in 16 out of 25 mice transplanted with CLL-HSCs (Table 1). As a result, in all patients analyzed, mice transplanted with CLL-HSCs developed B cell clones either of CD5<sup>+</sup> phenotype, CD5<sup>-</sup> phenotype, or both (Table 1), whose VDJs were always independent of those in original CLL cells (Table S2).

Furthermore, when we transplanted CLL-HSCs from single patients (patients 7, 9, 10, and 13) into more than two mice simultaneously, the B cell progeny of each mouse was again composed of independent clones with different VDJ recombination (Table S2). Representative data of patient 10 are shown in

Figure 4A. These data suggest that the clonal selection occurs within polyclonal B cell progeny in each recipient somewhat in a stochastic manner.

Table S2 summarized VDJ recombination and amino acid sequences of CDR3 in CLL-HSC-derived B cell clones. It has been shown that de novo CLL cells preferentially used VH1, VH3, and VH4 for IGH rearrangement (Chiorazzi and Ferrarini, 2003; Fais et al., 1998). Interestingly, frequency of VH1, VH3, and VH4 usage is higher in B cell clones derived from CLL-HSCs (48 out of 50 clones), as compared to polyclonal B cells developed from normal HSCs (197 out of 233 clones) (Figure S2). The difference was statistically significant on Fisher's exact test ( $p < 0.05$ ).

The status of SHM was also evaluated. Sequencing results with less than 98% germline identity were judged as mutated, whereas those with >98% germline identity were regarded as unmutated (Damle et al., 1999; Hamblin et al., 1999). The majority (45 out of 50) of B cell clones after transplantation



**Figure 4. CLL-HSCs Are Capable of Self-renewal, and Their B Cell Progeny Is Clonally Selected in Xenogeneic Recipients**

(A) CLL-HSCs from patient 10 were purified and transplanted into three recipients simultaneously. All recipients developed monoclonal or oligoclonal B cell clones. Note that the B cell clones of each mouse were independent and used different VDJ genes.

(B) HSCs were harvested from the bone marrow of a mouse transplanted with CLL-HSCs of patient 7 and retransplanted into the second recipient. B cell clones in the second recipient were independent of that in the first recipients with different VDJ gene recombination, indicating that CLL-HSCs are capable of self-renewal. See also Table S3.

possessed mutated IGHVs, regardless of the SHM status of the original CLL cells.

#### B Cell Clones Are Derived from CLL-HSCs Capable of Self-renewal

To confirm that CLL-HSCs that generate clonal B cells in mice are capable of self-renewal, we performed a serial transplantation assay in patients 7 and 16 (Table S3). Experiments of patient 7 are shown in Figure 4B. The primary recipient (mouse 7-3 in Table 1 and Tables S2 and S3) developed two CD5<sup>+</sup> B cell clones. We then purified CD34<sup>+</sup>CD38<sup>−</sup> HSCs from the bone marrow of the primary recipient and retransplanted into the secondary recipient. The secondary recipient again developed two CD5<sup>+</sup> B cell clones, indicating that CLL-HSCs are capable of self-renewal. The VDJ recombination analysis showed that all four B cell clones were independent and had their own VDJ combination different from the original CLL clone. The serial transfer experiment was performed also in patient 16, and the secondary recipient gave rise to two clones independent of the one developed in the primary recipient (Table S3). These data collectively suggest that self-renewing CLL-HSCs but not normal HSCs are able to develop monoclonal or oligoclonal B cells as

a result of in vivo selection, and that the pathogenesis of CLL could be traced up to the self-renewing HSC stage.

#### CLL-HSCs Do Not Have Chromosomal Abnormalities Related to CLL Pathogenesis

CLLs frequently have aberrations in a few chromosomal regions, including del13q14, del11q23, trisomy 12, and del17p (Döhner et al., 2000), and some of these appear to be directly involved in pathogenesis of CLL (Cimmino et al., 2005; Klein et al., 2010; Ouillette et al., 2008). Therefore, we tested whether CLL-HSCs have such abnormal karyotypes. Results are shown in Table 2. Purified CD19<sup>+</sup> CLL cells in patients 2 and 11 possessed del13q14, and patients 1 and 3 had both del13q14 and del11q23 by FISH analysis. However, purified CD34<sup>+</sup>CD38<sup>−</sup> CLL-HSCs and CD33<sup>+</sup> myeloid cells did not have such abnormalities in any patients, suggesting that these chromosomal abnormalities are acquired at the mature B cell stage.

To exclude the possibility that the very minor population having such abnormal karyotypes within the CD34<sup>+</sup>CD38<sup>−</sup> CLL-HSC fraction gave rise to CLL cells, we evaluated the karyotype of B cell clones developed from purified CLL-HSCs. Purified CLL-HSCs in patients 1–3 and 11 were transplanted into

**Table 2. FISH Analyses of Purified CLL Fractions and Secondary B Cell Clones**

Patient No.	Patients' CLL Bone Marrow					CLL-HSC-Derived B Cell Clones		
	VDJ Gene of CLL Clone	FISH Target	Abnormal Karyotypes (%)			VDJ Genes of B Cell Clone	FISH Target	Abnormal Karyotype (%) hCD45+ Cell
			B Cell	Myeloid	HSC			
1	V3-66/D3-22/J4	del13q14	77.7	<2.0	<2.0	V1-2/D3-10/J6	del13q14	<2.0
		del11q23	77.0	<2.0	<2.0		del11q23	<2.0
2	V2-5/D6-19/J4	del13q14	59.9	<2.0	<2.0	V4-59/D3-16/J4	del13q14	<2.0
3	V3-23/D5-12/J4	del13q14	95.2	<2.0	<2.0	V3-48/D6-13/J6	del13q14	<2.0
		del11q23	12.5	<2.0	<2.0		del11q23	<2.0
11	V3-20/D1-26/J6	del13q14	92.6	<2.0	<2.0	V5-51/D3-9/J5	del13q14	<2.0

immunodeficient mice. In all cases, recipients again developed clonal B cell populations with VDJ recombination independent of original CLL cells, but such B cell clones have normal karyotypes: they were free from any abnormal karyotypes that original CLL cells had (Table 2). Thus, oncogenic events resulting from these chromosomal abnormalities are not required for CLL-HSCs to generate clonal B cells, suggesting that these abnormalities are acquired at the mature B cell stage as an additional leukemogenic event to transform into clinical CLL.

#### Single CLL-HSCs Prime Lymphoid Lineage-Related Genes

The fact that the CLL-HSC always generates monoclonal or oligoclonal B cell populations strongly suggests that the CLL-HSC possesses cell-intrinsic abnormalities to exhibit this phenotype. We and others have shown that priming of lineage-associated genes reflects the developmental potential of hematopoietic stem and progenitor cells (Akashi et al., 2003; Hu et al., 1997; Miyamoto et al., 2002). Therefore, we analyzed the expression profile of lineage-related transcription factors in CD34<sup>+</sup>CD38<sup>−</sup> CLL-HSCs. Conventional quantitative PCR of mRNA purified from 1000 cells showed that CLL-HSCs expressed IKZF1 (IKAROS), an early lymphoid transcription factor (Georgopoulos et al., 1992), and early B lymphoid ones including TCF3 (E2A) and IRF8 at significantly higher levels, as compared to normal CD34<sup>+</sup>CD38<sup>−</sup> HSCs (Figure S3). Other relatively late B lymphoid-related genes including EBF, PAX5, IGLL1, DNMT, and VPBEB3 were not detected in either CLL-HSCs or normal HSCs (data not shown). In contrast the expression levels of myeloid-related RUNX-1 and CEBPA, myeloid/B lymphoid-related PU.1, and T lymphoid-related NOTCH1 were not different between CLL-HSCs and normal HSCs (Figure S3). Thus, transcription factors required at a very early stage of B cell development appeared to be primed in the CLL-HSC.

To directly assess the frequency of lymphoid-primed CLL-HSCs within the CD34<sup>+</sup>CD38<sup>−</sup> fraction of patients with CLL, we performed the single-cell gene expression assay of CLL-HSCs, as well as of HSCs, common lymphoid progenitors (CLPs) (Galy et al., 1995), and proB cells from normal controls. Figure 5A shows the representative PCR data of CLL-HSCs from two patients and of normal HSCs from a control. The summary of data of six patients with CLL and normal controls is shown in Figure 5B. The data showed that only ~15% of normal HSCs expressed IKAROS, whereas ~60% of single CLL-HSCs expressed IKAROS at a detectable level in

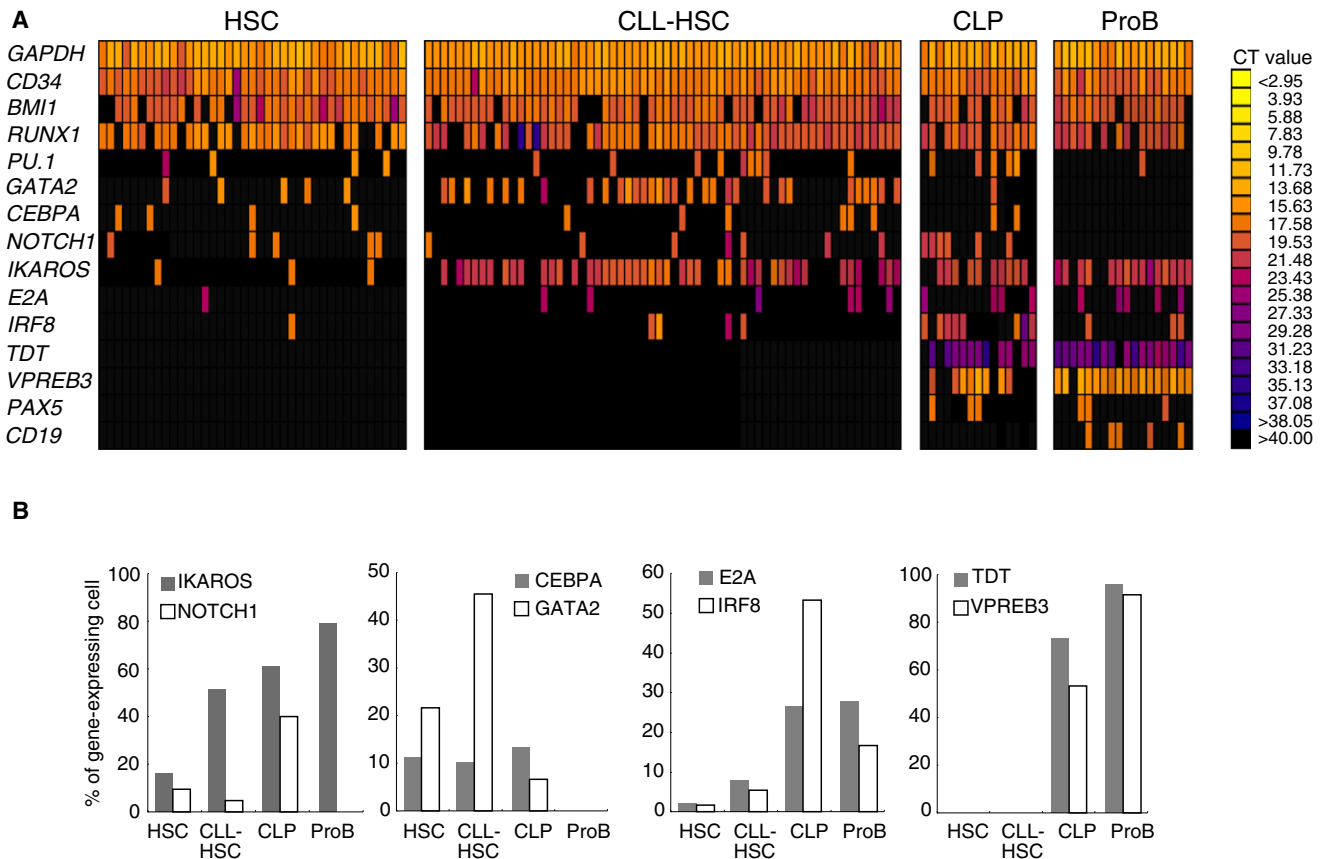
this assay system. The frequency of IKAROS-expressing cells gradually increased as normal HSCs differentiated into CLPs and then into proB cells. Similarly, cells expressing E2A and IRF8 began to appear at the CLL-HSC stage, but frequencies of cells expressing these molecules increased in CLP and proB cells. Cells expressing TDT, VPBEB3, and PAX5 appeared on and after the CLP stage. In contrast, IKAROS expressing single CLL-HSCs frequently coexpressed early myeloid transcription factors such as GATA-2 and CEBPA that were progressively shut off in CLP or proB cells, reflecting their multipotency (Figures 5A and 5B). These data suggest that a considerable fraction of CLL-HSCs has activated early lymphoid transcription factors, presumably reflecting their cell-intrinsic priming into the lymphoid lineage.

#### DISCUSSION

In the present study, we showed evidence that self-renewing HSCs are involved in pathogenesis of CLL, a mature B cell neoplasm. In the xenogeneic transplantation system, both CLL-HSCs and normal HSCs showed multilineage differentiation, but only the former gave rise to clonal B cells. Such B cell clones frequently expressed CD5 and CD23 surface antigens, which are the typical phenotypic characteristics of de novo CLL. These CLL-HSC-derived B cells were monoclonal or oligoclonal but were independent of the original patients' CLL clones confirmed by VDJ recombination analyses. In contrast, normal HSCs always produced polyclonal B cells. Furthermore, patients with CLL had ~5-fold higher numbers of polyclonal proB cells as compared to normal individuals, and CLL-HSCs frequently displayed the primed expression of early lymphoid transcription factors including IKAROS and E2A at the single-cell level. After transplantation into xenogeneic recipients, CLL-HSCs produced higher numbers of polyclonal proB cells than normal HSCs. CLL-HSCs did not have abnormal karyotypes frequently detected in CLL (Table 2). These data suggest that the CLL-HSC possesses cell-intrinsic abnormalities for enhanced production of polyclonal B cell progenitors, and among whose progeny, B cell clones with CLL or MBL phenotype selectively expand in vivo.

In human the CD34<sup>+</sup>CD38<sup>−</sup> population in the bone marrow contained most, if not all, of HSCs (Bhatia et al., 1997; Terstappen et al., 1991). In HSC subpopulation analysis (Figures 1A and 1B), more than 90% of the CD34<sup>+</sup>CD38<sup>−</sup> cells consisted of CD90<sup>+</sup>CD45RA<sup>−</sup> LT-HSCs (~60%) and CD90<sup>−</sup>CD45RA<sup>−</sup> multipotential progenitors (~30%) (Majeti et al., 2007), and the





**Figure 5. Lymphoid-Lineage Gene Priming in Single CLL-HSCs**

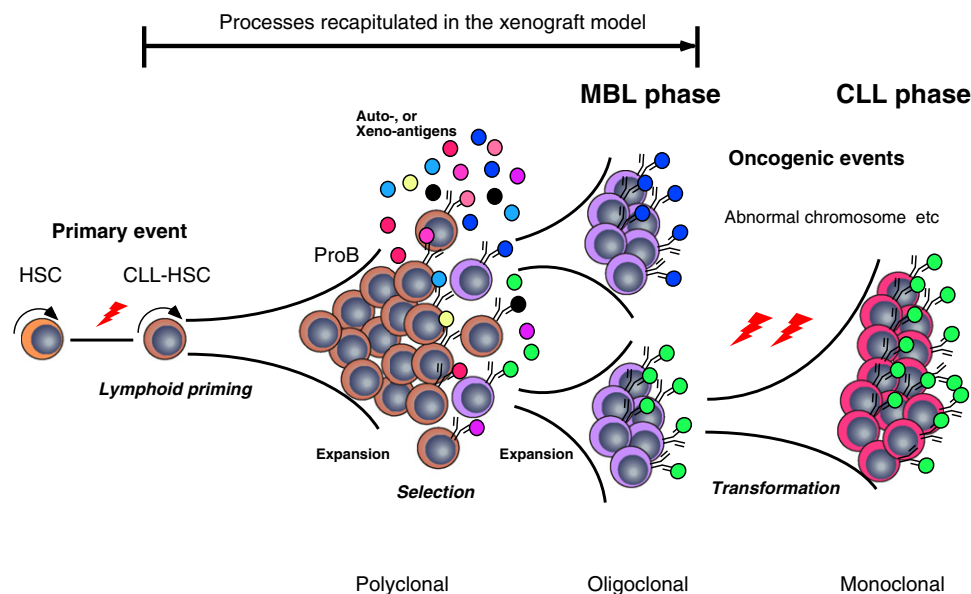
(A) Single-cell quantitative gene expression analyses of CLL-HSCs, and of normal HSCs, CLP, and proB cells. Each lane represents the analysis of single cells. IKAROS, E2A, and IRF8 were more frequently expressed in CLL-HSCs as compared to normal HSCs. IKAROS was expressed in >50% of single CLL HSCs, but only in 15% of single normal HSCs, suggesting that CLL-HSCs are primed to the lymphoid lineage. In contrast, other myeloid or T-lymphoid transcription factors including CEBPA, RUNX1, PU.1, and NOTCH1 did not differ between normal HSCs and CLL-HSCs. Representative results are shown. Conventional quantitative PCR analyses of lineage-related genes in CLL-HSCs and normal HSCs are shown in Figure S3.

(B) The summary of frequencies of cells expressing the listed genes in stem and progenitor cell fractions (control  $n = 6$ , CLL  $n = 6$ ).

CD90<sup>+</sup>CD45RA<sup>+</sup> population that was reported to initiate lymphomeloid differentiation (Doulatov et al., 2010; Goardon et al., 2011) constituted only a minor (<10%) population in both normal and CLL bone marrow. Furthermore, quantitative digital PCR analysis showed that the expression pattern of major transcription factors in single cells in the CD34<sup>+</sup>CD38<sup>−</sup> HSC or CLL-HSC fractions appeared to be homogeneous, and among >200 single cells analyzed, none of them expressed relatively late lymphoid molecules such as TDT, VPREB3, and PAX5 that were expressed in the majority of CLP and proB cells (Figure 5). Thus, the vast majority of the CD34<sup>+</sup>CD38<sup>−</sup> population is uncommitted stem or progenitor cells. However, it was still possible that the purified CD34<sup>+</sup>CD38<sup>−</sup> CLL-HSC population contained a few original CLL clones with recombined VDJ genes, from which the B cell clones were expanded to become visible after transplantation. This possibility was excluded based on results of the following experiments. First, CLL-HSCs as well as CLL-like B cell clones developed in xenogeneic recipients did not have karyotypic anomaly such as del13q14 and del11q23 that the original CLL cells had. Second, the CLL-HSC purified from

a single patient always produced independent B cell clones in multiple recipients (Figure 4A). Third, purified CLL-HSCs were capable of self-renewal as shown in the serial transplantation experiment (Figure 4B), and the secondary recipient developed B cell clones independent of those in the primary recipient (Table S3). Collectively, the CLL-HSC fraction is the self-renewing population not contaminated with B cell clones. Our hypothesis on development of CLL is schematized in Figure 6.

It has been shown that virtually all patients with CLL have a precursor state such as MBL before it develops into clinically evident CLL (Landgren et al., 2009). Around 20%–70% of patients with MBL have more than one B cell clone (Dagklis et al., 2009; Lanasa et al., 2010; Nieto et al., 2009), whereas only ~10% of patients with CLL have two or more CLL clones (Sanchez et al., 2003). Progression into CLL is seen in a fraction of patients with MBL. A previous cohort study reported that during this process, one of the MBL clones was selected to develop into CLL (Landgren et al., 2009). B cell clones that arose from CLL-HSCs in our system appeared to resemble MBL, rather than CLL: more than one B cell clone was present in 13 out of 25



**Figure 6. Schematic Presentation of Human CLL Development Based on the Xenogeneic Transplantation Model**

CLL-HSCs have accumulated genetic abnormalities that might play a role in amplified B cell differentiation, and produce a high number of polyclonal B cells carrying the same genetic aberrations. B cell clones are selected, and expanded in response to BCR signaling driven presumably by xeno-antigens, simulating progression of MBL. Additional abnormalities such as aberrant karyotypes might play a role in progression from MBL into human CLL. This final step was not recapitulated in the xenograft model.

(~50%) mice analyzed in our experiments (Table 1). Importantly, such B cell clones developed as short as 3 months after xenogeneic transplantation, and they did not have chromosomal abnormalities that original patients had. Somatic mutation status of B cell clones also implies their MBL-like characteristics. It has been shown that ~90% of MBL clones carry IGHV genes with somatic mutation, whereas ~60% of CLL clones have mutated IGHV genes. In the present study 13 out of 16 patients with CLL had CLL cells with mutated IGHVs, whereas after transplantation, 45 out of 50 B cell clones developed from CLL-HSCs had mutated IGHVs. The frequent usage of mutated IGHV in B cell clones again suggests that these B cell clones developed in mice might correspond to de novo MBL. Collectively, xenogeneic transplantation of CLL-HSCs in immunodeficient mice could recapitulate at least the progression into the MBL, suggesting that the primary genetic abnormality to cause MBL might be acquired already at the long-term self-renewing CLL-HSC level.

It is still unknown as to how such MBL clones are expanded, and are selected in vivo to become CLL. Interestingly, MBL clones that progress into CLL use a biased set of VH genes including VH1, 3, and 4, which de novo CLL cells preferentially use (Landgren et al., 2009; Rawstron et al., 2008). It is also known that CLL cells express a restricted BCR repertoire, including antibodies with quasi-identical CDR3 (Ghiotto et al., 2004; Messmer et al., 2004; Tobin et al., 2003, 2004; Widhopf et al., 2004). The striking degree of structural restriction of the entire BCR in CLL suggests that common or similar antigens are recognized by CLL cells, and supports the hypothesis that an antigen-driven process contributes to CLL pathogenesis (Zenz et al., 2010). Such antigens may include autoantigens, partly because

CLL clones frequently produce autoreactive antibodies (Borche et al., 1990; Bröker et al., 1988; Sthoeger et al., 1989). In this context it is possible that human CLL cells could not engraft into mice because the BCR of patients' CLL cells cannot recognize xeno-antigens in mice.

Similarly, in our xenogeneic transplantation analysis, CLL-HSC-derived B cell clones but not normal HSC-derived polyclonal B cells preferentially used the VH1, VH3, and VH4 (Table S2), indicating that propensity of biased usage of VH genes is preserved in CLL-HSCs, but not normal HSCs. The possible explanation for this phenomenon is that B cell clones with these VH genes were preferentially selected by BCR signals triggered by antigens, or that CLL-HSCs possess some cell-intrinsic defects in recombining other than these VH genes. Interestingly, CDR analysis of CLL-HSC-derived B cell clones showed that >65% of CDR3 amino acids between clonal B cells in mice 10-3 and 12 were identical, and three independent B cell clones in mice 3, 7-3, and 12 shared >60% of CDR3 amino acids (Figure S2 and Table S2). These independent B cell clones correspond to the moderate level of CDR3 homology defined by a previous study (Tobin et al., 2004), suggesting that the BCR of these B cell clones may recognize common xeno-antigen to expand, and antigen-driven process may play a critical role in clonal B cell development even in our xenogeneic transplantation model.

Previous data have shown that chromosomal abnormalities often found in patients with CLL, such as del13q14 and del11q23, are directly linked to the leukemogenesis of CLL. For example deletion of 13q14 causes loss of miR15a and miR16-1 that target Bcl-2, resulting in the upregulation of Bcl-2 (Cimmino et al., 2005) and proliferation of CLL cells (Klein

et al., 2010). Our data clearly show that expansion of B cell clones does not require such signaling caused by chromosomal aberration (Table 2). Thus, the acquisition of abnormal karyotypes is not necessary for MBL-like clonal B cell development but might play a role in progression from MBL into clinical CLL (Figure 6).

Thus, the propensity to progress into CLL is acquired already at the HSC level. HSCs in patients with CLL are able to produce a high number of B cells. Such B cells should carry the genetic abnormality identical to HSCs, which might play a role in clonal expansion after they differentiate into B cells presumably collaborating with BCR signaling in response to auto-antigens. Further accumulation of genetic alteration(s) such as chromosomal abnormalities might cause transformation of a fraction of MBL clones into clinical CLL. Accordingly, our results suggest that the blockage of BCR signaling, by Syk inhibitors (Friedberg et al., 2010; Suljagic et al., 2010), for example, might be useful to inhibit development of human MBL, or its progression into CLL. Our xenogeneic transplantation experiments may not recapitulate the full picture of CLL progression, but they do recapitulate the development of MBL starting from human HSCs of patients with CLL (Figure 6). Our data suggest that even in human CLL, the primary leukemogenic event involves multipotent, self-renewing HSCs. Identification of the intrinsic abnormality of HSCs in patients with CLL should be the key to finding the ultimate therapeutic target in human CLL.

## EXPERIMENTAL PROCEDURES

### Clinical Samples

Diagnostic and follow-up bone marrow or blood samples of 16 patients with CLL were used in this study. All cases were immunophenotyped as previously described (Chiorazzi et al., 2005) and met the diagnostic criteria of the National Cancer Institute Working Group (NCI-WG) (Hallek et al., 2008). Table S1 lists the patient characteristics. Human age-matched adult bone marrow and peripheral blood cells were obtained from healthy donors or purchased from AllCells Inc. (Emeryville, CA, USA). Informed consent was obtained from all patients and controls in accordance with the Helsinki Declaration of 1975 that was revised in 1983. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects.

### Antibodies, Cell Staining, and Sorting

Human HSCs, progenitors, and other hematopoietic cells were stained and sorted by FACS Aria (BD Biosciences, San Jose, CA, USA). The bone marrow mononuclear cells (MNCs) were concentrated by standard gradient centrifugation, and the CD34<sup>+</sup> cells were enriched from MNCs by using the Indirect CD34 MicroBead Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). The HSC population used for xenotransplant or PCR analyses purified as CD34<sup>+</sup>CD38<sup>−</sup> cells from the fraction does not express lineage antigens as described below. In some cases CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup> cells were used for the xenotransplantation assay (Table 1). Briefly, for the FACS analysis or sorting of human bone marrow cell fractions, cells were stained with a Cy5-PE- or PC5-conjugated lineage cocktail, including anti-CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD10 (HI10a), CD19 (HIB19), CD20 (2H7), CD11b (ICFR44), CD14 (RMO52), CD56 (NKH-1), and GPA (GA-R2). Cy5-PE-conjugated CD10, CD19, and CD20 monoclonal antibodies were excluded from lineage cocktail in the B-lymphoid progenitor assay. Cells were further stained with FITC-conjugated anti-CD10 (SS2/36), anti-CD34 (8G12) or anti-CD90 (5E10), PE-conjugated, anti-CD19 (HIB19), APC-conjugated anti-CD34 (8G12) or anti-CD38 (HIT2), PE-Cy7-conjugated anti-CD5 (L17F12), anti-CD19 (SJ25C1), anti-CD34 (8G12) or anti-CD38 (HIT2), Pacific Blue-conjugated anti-CD45RA (HI100), and biotinylated anti-CD38 (HIT2). For analysis of human cells developed in the immunodeficient mice, FITC-conjugated anti-CD5 (UCHT2), anti-CD33 (HIM3-4) or anti-human IgM (G20-127), PE-conjugated

anti-CD5 (UCHT2), anti-CD20 (L27), anti-CD23 (EBVCS-5), or anti-CD45 (HI30), APC-conjugated anti-CD45 (J.33) monoclonal antibodies were used. Streptavidin-conjugated APC-Cy7 or PE-Cy7 was used to visualize biotinylated antibodies (BD Pharmingen, San Jose, CA, USA). Nonviable cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control monoclonal antibodies were used to determine the level of background staining. The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets. For single-cell assays an automatic cell-deposition unit system (BD Biosciences, San Jose, CA, USA) was used.

### FISH Analysis

FISH analysis was performed on interphase nuclei from the bone marrow or blood cells. The probe sets detect 13q- (D13S319 at 13q14 and LAMP1 at 13q34), 12 (D12Z3 at CEN12), and 11q- (ATM at 11q23 and D11Z1 at CEN11). The specimens in this study were analyzed in a random order, by blinded observers. Intact, nonoverlapping nuclei were scored. A total of 1000 nuclei were analyzed for each probe set for each patient.

### Xenogeneic Transplantation

NRG mice (stock #7799) (Pearson et al., 2008) (purchased from The Jackson Laboratory) and NOD.Cg-Prkdc<sup>scid</sup>IL-2rg<sup>tm1Wjl</sup>/Sz (NSG) mice (Shultz et al., 2005; Ishikawa et al., 2005) were used for xenogeneic transplantation assays. Mice were housed in a specific pathogen-free facility in micro-isolator cages at the Kyushu University (Fukuoka, Japan) or RIKEN Center for Allergy and Immunology (Kanagawa, Japan). Animal experiments were performed in accordance with institutional guidelines approved by the animal care committee of each institute. For the reconstitution assays, sorted cells were transplanted into irradiated (100 cGy) NSG newborns via a facial vein within 48 hr of birth (Ishikawa et al., 2005) or into sublethally irradiated NRG adult mice (4.8 Gy) via a tail vein as previously reported (Kikushige et al., 2010).

### IGH Gene Rearrangement Analysis and Subcloning of PCR Products

Genomic DNA was extracted by Micro Kit (QIAGEN) according to the manufacturer's instructions. Multiplex PCR assays were employed to detect clonal B cell population (van Dongen et al., 2003). To evaluate the IGH gene rearrangement of a small number of sorted cells, semi-nested PCR assays were performed (d'Amore et al., 1997; Ramasamy et al., 1992; Reed et al., 1993). The clonal PCR product was excised from gel, purified by QIAquick Spin (QIAGEN), and directly sequenced with the heavy-chain primer by ABI 3730 Genetic analyzer (Applied Biosystems).

Subcloning was performed to detect clonal bands within polyclonal background, by using the TOPO TA Cloning kit (Invitrogen). The PCR products were ligated into the vector and transformed in *Escherichia coli* cells according to the manufacturer's recommendation. At least 12 colonies were selected and sequenced to confirm clonal expansion. The sequence results were analyzed on the IMGT tools (Giudicelli et al., 2004) and IgBLAST, and aligned to the closest match with the germline IGHV segment. Sequencing results with a germline identity of less than 98% were regarded as mutated, whereas those with a germline identity of 98% or more were regarded as unmutated according to previous studies (Damle et al., 1999; Hamblin et al., 1999).

### Single-Cell Quantitative PCR

For single-cell quantitative PCR analysis, single CD34<sup>+</sup>CD38<sup>−</sup>Lin<sup>−</sup>HSC, CD34<sup>+</sup>CD38<sup>−</sup>CD10<sup>+</sup>CD19<sup>−</sup>Lin<sup>−</sup>CLP (Galy et al., 1995), or CD34<sup>+</sup>CD38<sup>−</sup>CD10<sup>+</sup>CD19<sup>+</sup>Lin<sup>−</sup>proB cell was sorted directly into the mixture of CellsDirect 2× Reaction Mix (CellsDirect™; Invitrogen), 0.2× TaqMan Assay Mix (Applied Biosystems), and SuperScript™ III RT/Platinum Taq Mix (Invitrogen) according to the protocol of BioMark™ Dynamic Array (Fluidigm, CA, USA). After sorting single cells into 96-well plates, reverse transcription (RT) and specific target amplification (STA) were performed. Temperature setting for RT was 15 min at 50°C, and after RT reaction, samples were incubated for 2 min 95°C. Thermal-cycling settings for STA were 22 cycles of 95°C for 15 s and 60°C for 4 min. After RT and STA reaction, preamplified cDNA was diluted with TE buffer (1:5). Single-cell quantitative PCR was performed using BioMark™ 48 × 48 or 96 × 96 Dynamic Array. Data were analyzed by BioMark™ Real-Time PCR Analysis Software v2.0 (Fluidigm, CA, USA). TaqMan Gene

Expression Assay Mixes for all the genes analyzed in this study were purchased from Applied Biosystems.

### Statistical Analysis

Data were presented as mean  $\pm$  standard deviation. The significance of the differences between groups was determined by using Student's *t* test. *p* values  $<0.05$  were considered statistically significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at doi:10.1016/j.ccr.2011.06.029.

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