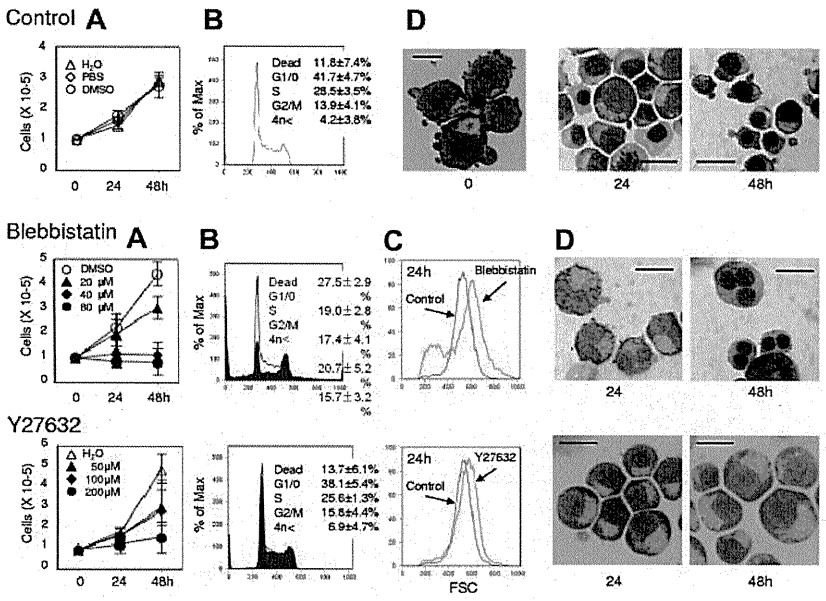

Figure 1. Myosin inhibitors block cell division of human CFU-E. Human CFU-E generated from purified CD34$^+$ cells were cultured for the indicated periods in the presence of EPO with or without various concentrations of inhibitors for non-muscle myosin II ATPase (blebbistatin) and a myosin activator Rho kinase (Y27632). (A) Effects of inhibitors and vehicle on the proliferation of CFU-E. Results are presented as the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red areas) or without (solid lines) 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown and is presented as the mean ± SD. (C) Size distribution analysis of CFU-E before (0 hours) and after 24 hours culture (red) with 80μM blebbistatin (green, middle panel) and 200μM Y27632 (green, bottom panel). A representative result of 3 independent experiments is shown. (D) May-Grünwald-Giemsa staining of cells cultured for 24 and 48 hours with or without 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

Confocal microscopy

Fluorescence staining was imaged using a Confocal Laser Scanning Microscope 510 (LSM510; Carl Zeiss Microscope Systems) equipped with a 100× objective lens and a 10× camera lens (Carl Zeiss Microscope Systems) at zoom 3, as reported elsewhere.[29] Fluorochromes were excited using an argon laser at 488 nm for Alexa 488. Detector slits were configured to minimize cross talk between channels and processed using a software package (LSM510 Version 3.2) and Adobe Photoshop (Adobe Systems).

Construction of plasmid DNA

The myosin II tailpiece determines its paracrystal structure, filament assembly properties, and cellular localization.[30] To study the role of non-muscle myosin II isoforms in enucleation of human erythroblasts by the exogenous expression of their rod fragments, we constructed the plasmids encoding the rod fragments of the 2 NMHC-II isoforms as N-terminal GFP-fused protein. DNA fragments encoding Leu 1666 - Glu 1961 of NMHC IIA (ARF296) and Phe 1672–Glu 1976 of NMHC IIB (BRF305) were amplified by PCR as described elsewhere.[31,32] Each of them was subcloned into the *Hin*dIII-BamH1 sites of pEGFP-C3 (Clontech) to generate the pEGFP-ARF296 and pEGFP-BRF305, respectively.

Transfection

Transfected cells were obtained with the Amaxa nucleofection method. The Amaxa Nucleofector system (Lonza Cologne AG) was used as described by the manufacturer. Briefly, a pellet of 2-5 × 10^6 cells in 100 μL of Amaxa Human CD34$^+$ Cell Nucleofector solution wasmixed with 5 μg of pEGFP plasmid and subjected to nucleofection with a specific predefined program (Program U-008). After 24 hours of incubation, the cells were harvested and washed twice with IMDM containing 0.3% BSA. The dead cells were removed using an Annexin V MicroBead Kit (Miltenyi Biotec) and then incubated in the erythroid medium. GFP-positive cells were collected using a cell sorter (Dako Cytomation MoFlo).

Western blot analysis

CD34$^+$ cells were incubated in erythroid medium for the indicated days (day 7, 10-12 cells). Western blot analysis was carried out according to the manufacturer's protocol (Invitrogen).

Statistical analysis

Statistical analysis was performed using the Student *t* test for parametric data and the Mann-Whitney test for nonparametric data. A 2-tailed *P* value < .05 was accepted as statistically significant.

Results

Myosin inhibitors block cell division of human CFU-E

In this study, efficient inhibitors of cell division in human primary erythroid cells were selected using human CFU-E generated from purified CD34$^+$ cells (Figures 1-2). As cellular division consists of both mitotic and cytokinetic events, and given that the inhibition of either step should block cell proliferation, efficient inhibitors were defined as those that blocked the proliferation of CFU-E.

Figure 1A presents a time course analysis of the total cell number when human CFU-E were cultured with or without various concentrations of blebbistatin and Y27632. Blebbistatin and Y27632 were found to inhibit CFU-E proliferation in a dose-dependent manner. Cell cycle analysis demonstrated that blebbistatin decreased the number of cells in the G1/G0/S phase and resulted in the accumulation of cells in the G2/M phase, a finding that was accompanied by an increase in dead cells (Figure 1B). In addition, an increase in cells with high DNA content (Figure 1B) and an increase in larger erythroid progenitors compared with control cells (Figure 1C) were observed, suggesting the presence of multinucleated cells. This finding was confirmed by morphologic analysis (Figures 1D and 3). These data indicate that non-muscle myosin II ATPase is essential for the cellular division of CFU-E.

In contrast, Y27632 slightly increased the number of cells in the G2/M phase, generated larger cells compared with the control cells (Figure 1C) and produced multinucleated cells from 0.7 ± 1.2% to 5.7 ± 1.2% during 48 hours of incubation (n = 3, $P < .01$; Figures 1D and 3), albeit to a lesser extent than that observed in the presence of blebbistatin (from 0.7 ± 1.2% to 71.0 ± 6.1%; n = 3, $P < .01$; Figure 3). Western blot analysis showed that Y27632 does not affect the myosin light chain 2 (MLC2) expression, a downstream molecule of ROCK (a regulator of myosin phosphorylation[23,24]), but reduces the phosphorylation of MLC2 at Thr18/Ser19 in CFU-E, with a statistical significance (supplemental Figure 2). Given that the blocking of non-muscle myosin II ATPase and ROCK showed complete inhibition of the proliferative capacity of

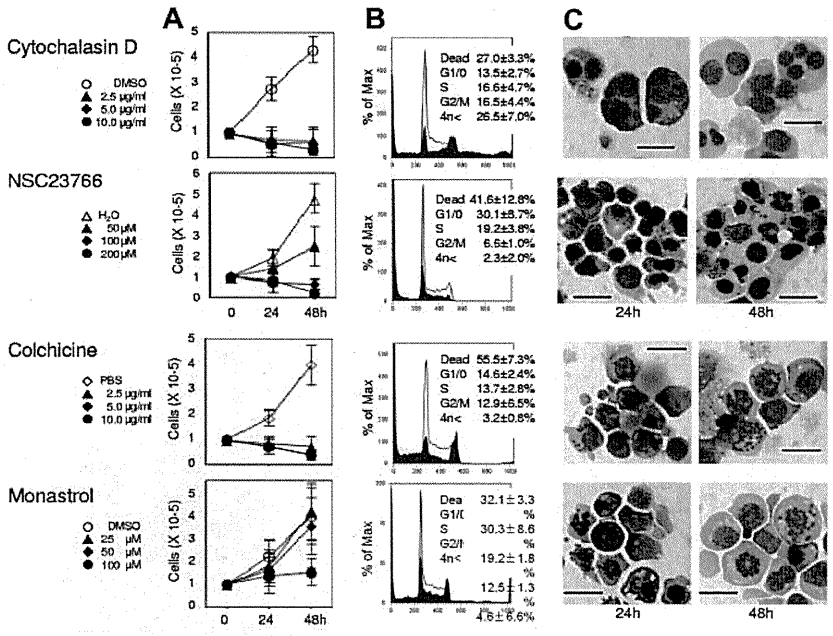

Figure 2. Actin, tubulin, and Eg5 inhibitors block cell division of human CFU-E. Human CFU-E were cultured for the indicated periods in the presence of EPO with or without various concentrations of inhibitors for actin polymerization (cytochalasin D and NSC23766), tubulin (colchicine) and Eg5 (monastrol). (A) Effects of inhibitors and vehicle on the proliferation of CFU-E. Results presented are the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red areas) or without (solid lines) 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown and is presented as the mean ± SD (C) May-Grünwald-Giemsa staining of cells cultured for 24 and 48 hours with or without 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

CFU-E, non-muscle myosin II is suggested to be involved in cell division of human erythroid progenitor cells.

Actin, tubulin, and Eg5 inhibitors block cell division of human CFU-E

Although Koury et al have clearly shown that F-actin plays an important role in enucleation in murine FVA cells while microtubules do not,[5] the efficacy of inhibitors for cell division often depends on the species of the cell observed and their redundancy in the cells themselves.[19] We therefore reevaluated the efficacy of actin and tubulin/kinesin inhibitors on human CFU-E and erythroblasts. As illustrated in Figure 2, cytochalasin D, an inhibitor of actin polymerization, NSC23766, a specific inhibitor of Rac1 GTPases[21] that regulate actin polymerization, colchicine, an inhibitor of microtubules,[22] and monastrol, an inhibitor of kinesin Eg5, completely blocked CFU-E proliferation. Cytochalasin D decreased the number of cells in the G1/G0/S phase resulting in the accumulation of cells in the G2/M phase (Figure 2B), and produced multinucleated cells (Figures 2C and 3). Interestingly, NSC23766 dramatically increased the total number of dead cells and decreased the number of cells in the mitotic fraction (Figure 2B), and did not produce multinucleated cells (Figures 2C and 3). These data indicate that cytochalasin D and NSC23766 block the cell division of human CFU-E.

Colchicine and monastrol decreased the number of cells in the G1/G0/S phase, while cells accumulated in the G2/M phase, a finding that was accompanied by an increase in the number of dead cells (Figure 2B). Morphologic analysis showed that these inhibitors increased the number of cells undergoing mitosis (Figures 2C and 3). These data indicate that colchicine and monastrol block mitosis, and are efficient inhibitors of cell division in human CFU-E.

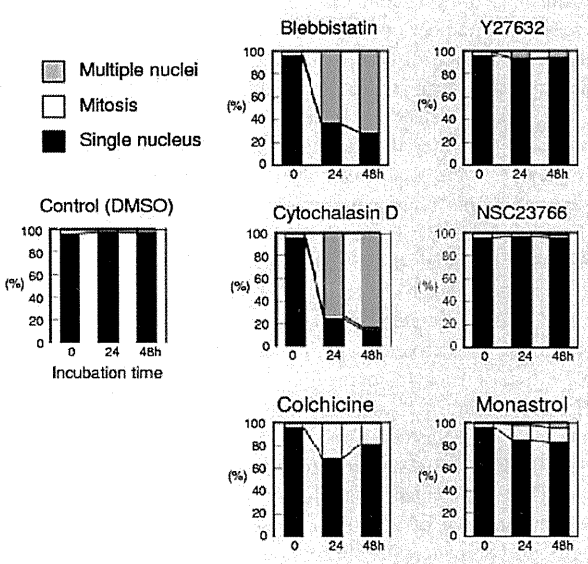

Figure 3. Morphologic analysis of cells cultured with or without inhibitors. Differential counts of cells with a single nucleus (closed bars), multiple nuclei (shaded bars), and cells in mitosis (open bars) are shown. Results presented are the mean of 3 independent experiments.

Myosin inhibitors block enucleation of human erythroblasts

Figure 4A presents a time course analysis of the enucleation ratio when day 11 human mature erythroblasts were cultured with or without various concentrations of blebbistatin and Y27632. During the 72-hour culture period, mature erythroblasts differentiated and started the enucleation process (Figure 4A and C top panel). After beginning the incubation, enucleation was rapidly initiated within 24 hours and continued until 72 hours. Both blebbistatin and Y27632 caused an immediate and significant inhibition of enucleation, in a dose dependent manner (Figure 4A and C middle and bottom panels). Western blot analysis showed that Y27632 reduces the phosphorylation of MLC2 at Thr18/Ser19 in these cells, with a statistical significance (supplemental Figure 2). In contrast to the significant effect on CFU-E (Figure 1B), neither blebbistatin nor Y27632 affects the cell cycle of day 11 mature erythroblasts (Figure 4B). These inhibitors did not affect the level of hemoglobinization of day 11 cells during 72 hours incubation (supplemental Figure 3). These results indicate that most of day 11 erythroblasts

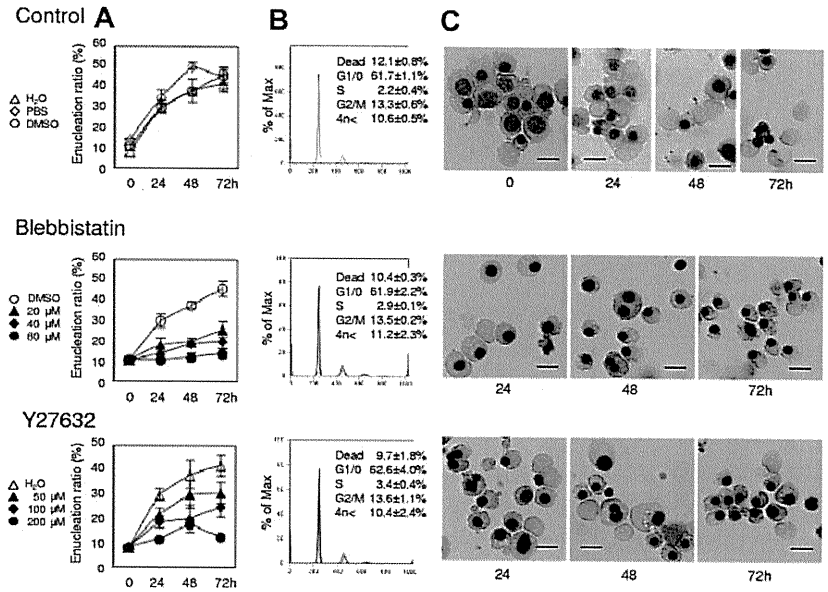

Figure 4. Myosin inhibitors block enucleation of human erythroblasts. Human CFU-E were cultured with EPO for an additional 4 days (day 11 cells) and differentiated to the level of mature erythroblasts incipient of enucleation. Mature erythroblasts were then cultured in the presence of EPO with or without various concentrations of inhibitors for non-muscle myosin II ATPase (blebbistatin) and a myosin activator Rho kinase (Y27632). (A) Effects of inhibitors and vehicle on the enucleation of day 11 mature erythroblasts. Results are presented as the mean ± SD of 3 independent experiments. (B) Cell cycle analysis of cells cultured for 24 hours with (red lines) or without (black lines) 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown and is presented as the mean ± SD. (C) May-Grünwald-Giemsa staining of day 11 cells cultured for 24, 48 and 72 hours with or without 80μM blebbistatin and 200μM Y27632. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

consist of mature erythroblasts just before the stage of enucleation and that non-muscle myosin II is essential for the enucleation of human erythroblasts.

Actin inhibitors, but not tubulin/Eg5 inhibitors, block enucleation of human erythroblasts

Both cytochalasin D and NSC23766, which are known to inhibit enucleation,[5,6] caused an immediate and complete inhibition of enucleation of day 11 human mature erythroblasts (Figure 5 top 2 panels), indicating that actin polymerization is essential for the enucleation of human erythroblasts. In contrast, colchicine, an inhibitor of microtubules that are known to not be directly involved in enucleation,[5] did not block enucleation (Figure 5 bottom 3rd panel), indicating that day 11 human mature erythroblasts consist of postmitotic cells just before the stage of enucleation. In addition to the current confirmation of previous reports, we found for the first time that monastrol, an inhibitor of kinesin Eg5 which is a microtubule-based motor that plays a critical role in mitosis,[25] does not block enucleation of human erythroblasts (Figure 5 bottom panel).

Human erythroblasts express non-muscle myosin IIA and IIB

Mammalian cells have 3 isoforms of NMHC, termed IIA, IIB and IIC.[15,33,34] Western blot analysis showed that human CFU-E possess both NMHC IIA and IIB. The relative expression levels of NMHC IIA and IIB appeared to decrease as they matured, but the decreases were not statistically significant (Figure 6A). NMHC IIB was extremely low or undetectable in human PBMCs and NMHC IIC was not detected in either human erythroblasts or PBMC (data

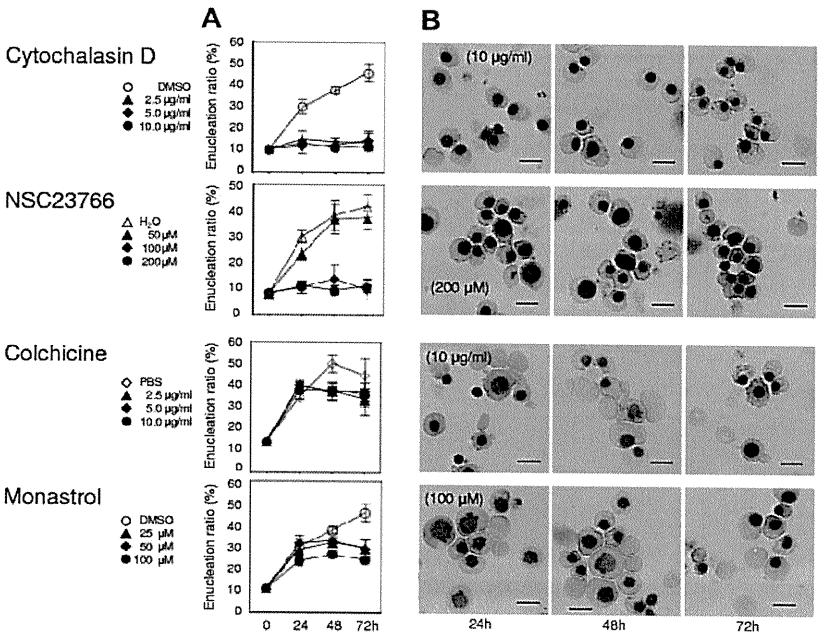

Figure 5. Actin inhibitors, but not tubulin/Eg5 inhibitors, block enucleation of human erythroblasts. Mature erythroblasts were cultured in the presence of EPO with or without various concentrations of inhibitors for actin polymerization (cytochalasin D and NSC23766), tubulin (colchicine) and Eg5 (monastrol). (A) Effects of inhibitors and vehicle on the enucleation of mature erythroblasts. Results are presented as the mean ± SD of 3 independent experiments. (B) May-Grünwald-Giemsa staining of cells cultured for 24, 48 and 72 hours with or without 10 μg/mL cytochalasin D, 200μM NSC23766, 10 μg/mL colchicine and 100μM monastrol. A representative result of 3 independent experiments is shown. Scale bar = 10 μm.

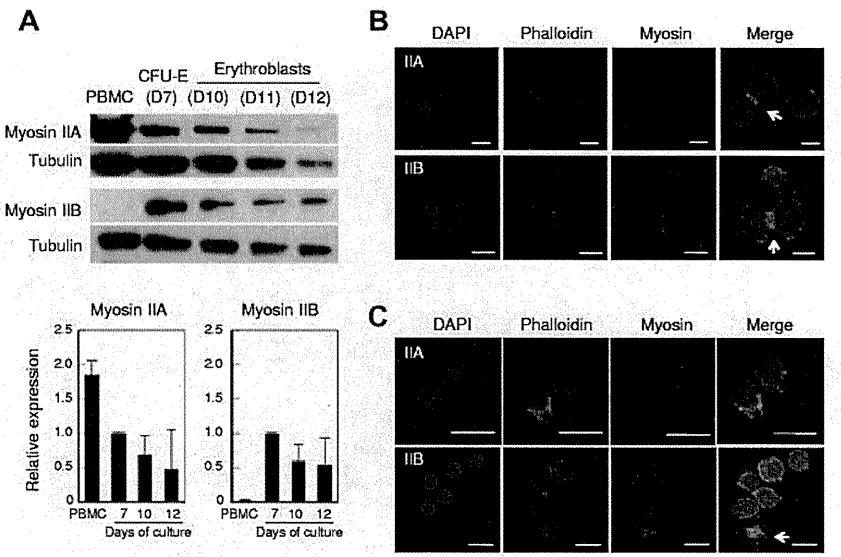

Figure 6. Human erythroblasts express non-muscle myosin IIA and IIB. (A) Western blotting of human PBMCs, CFU-E (day 7 cells, D7) and erythroblasts (D10-D12). D7-D12 indicates days of culture of purified human CD34$^+$ cells to induce erythroid differentiation. At indicated days, the cells were harvested and the protein obtained from 1×10^5 cells was applied to each lane. The relative expression levels of myosin II were normalized with tubulin expression and are the mean ± SD of 3 independent experiments. (B-C) Confocal microscopy of CFU-E (B) and day 12 enucleating mature erythroblasts (C) stained by DAPI, phalloidin and myosin IIA or IIB. NMHC IIA and IIB colocalized with actin (B arrows). In enucleating erythroblasts, NMHC IIB was present on the enucleated reticulocytes in the shape of a small ring colocalized with actin (C arrow).

not shown). The localizations of NMHC IIA and IIB were similar in CFU-E (Figure 6B), although IIB appeared to be more prominent in the contractile ring and colocalized with actin (Figure 6B arrow). In enucleating erythroblasts, NMHC IIA and IIB appeared to localize between the expelling nuclei and the reticulocytes (Figure 6C). Interestingly, NMHC IIB was present on the enucleated reticulocytes in the shape of a small ring that was colocalized with actin, which might indicate the expulsion route of the nuclei expelled from erythroblasts (Figure 6C arrow).

Enucleation of human erythroblasts involves non-muscle myosin IIB

Figure 7A illustrates the rod fragments, ARF296 and BRF305, which are encoded by Leu 1666–Glu 1961 of NMHC IIA and Phe 1672–Glu 1976 of NMHC IIB, respectively.[31,32] When ARF296 or BRF305 was exogenously expressed to CFU-E, these rods localized in cytoplasm (Figure 7B) and blocked the proliferation of CFU-E (Figure 7C). To investigate the function of myosin IIA and IIB in the enucleation of erythroblasts, mature erythroblasts (day 10 cells) were transfected with ARF296 and BRF305. The transfected cells were sorted by flowcytometry and cultured for an additional 72 hours (Figure 7D). Confocal microscopy showed that transfected cells maintain GFP-expression during the culture period (Figure 7D). As illustrated in Figure 7E, the enucleation of mature erythroblasts was inhibited by the exogenous expression of BRF305, with statistical significance, while only a slight inhibitory effect of ARF296 was apparent. These data indicate that enucleation of human erythroblasts involves non-muscle myosin IIB.

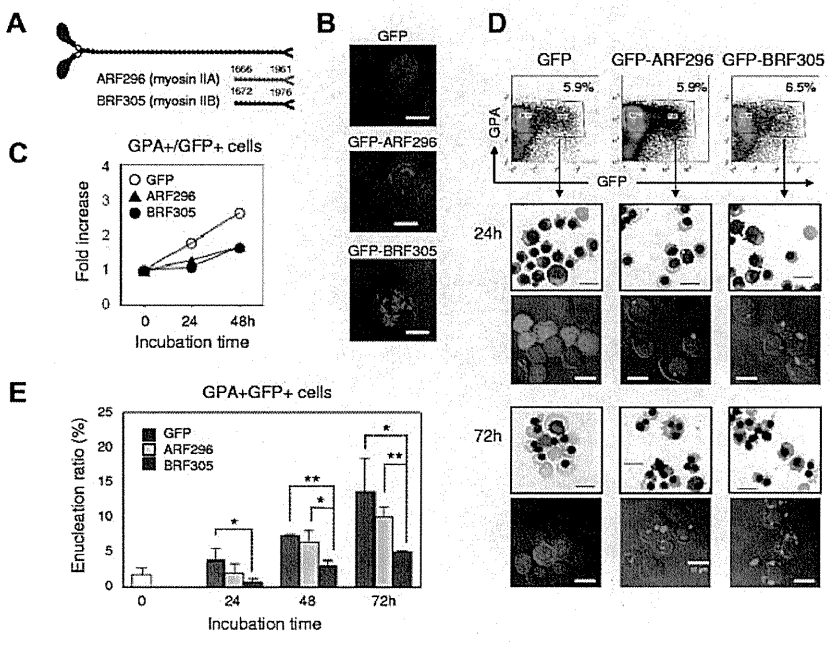

Figure 7. Enucleation of human erythroblasts involves non-muscle myosin IIB. (A) A schematic presentation of rod fragments: ARF296 (for myosin IIA) and BRF305 (for myosin IIB). (B) Confocal microscopy of CFU-E transfected by pEGFP-ARF296 and pEGFP-BRF305. Scale bar = 10 μm. (C) CFU-E were transfected with pEGFP-ARF296 and pEGFP-BRF305. After 24 hours of incubation, the dead cells were removed and then incubated in the erythroid medium. After culture for the indicated period, the cells were harvested, counted and GPA$^+$/GFP$^+$ cells were analyzed by flowcytometry. Representative data for 2 independent experiments are shown. (D) Mature day 10 erythroblasts were transfected with pEGFP-ARF296 and pEGFP-BRF305. GPA$^+$/GFP$^+$ cells were sorted and cultured in the erythroid medium. After culture for the indicated period, the cells were harvested and stained with May-Grünwald-Giemsa. An aliquot of the cells were observed by confocal microscopy. Scale bar = 10 μm. (E) GPA$^+$/GFP$^+$ cells were cultured in the erythroid medium. At the indicated period, the cells were harvested and counted. Results are presented as the mean ± SD of 3 independent experiments. *$P < .05$ and **$P < .01$.

Discussion

In this study, we demonstrate for the first time that blebbistatin and Y27632 inhibit the enuclation of human erythroblasts. Blebbistatin is a small molecule inhibitor that shows a high affinity and selectivity toward non-muscle myosin II ATPase,[20] while Y27632 is well-established inhibitor of ROCK.[23,24] Collectively, these data suggest that the enucleation of human erythroblasts involves non-muscle myosin II. This is of particular interest given that the precise role of myosin in enucleation has remained unclear, even though the formation of the contractile actin ring (CAR) and furrow formation are known to occur on the plasma membrane of enucleating erythroblasts.[5,6] As non-muscle myosin II is one of the central mechanisms involved in cytokinesis and is required for furrow formation,[22,24,35] our study also suggests that myosin shares a similar mechanistic function between cytokinesis and the enucleation of human erythroblasts.

Although we confirmed that Y27632 inhibits the phosphorylation of MLC2 at Thr18/Ser19, several lines of evidence indicate that activation of myosin II by ROCK is redundant with the action of other kinases such as citron kinase and that Y27632 blocks neither the initiation nor completion of cytokinesis of Hela cells, although it slows cleavage contraction.[36,37] Reportedly, ROCK-dependent phosphorylation of the mDia2 is an important determinant of mDia2 activity and this signaling mechanism affects actin polymerization.[38] Although further studies are needed, considering that Y27632 produces a limited number of multinucleated cells in the culture of CFU-E, the main mechanism of Y27632 in inhibiting cell division and enucleation may involve the Rac 1 GTPases and their downstream effector mDia2.

In addition to the previous report showing that human erythroblasts express both NMHC IIA and IIB,[39] our study demonstrates that human erythroid progenitor cells such as CFU-E also express both NMHC IIA and IIB. As shown in Figure 6A, PBMC that comprise monocytes and lymphocytes also exclusively express the IIA isoform. Platelets and granulocytes exclusively express the IIA isoform.[18] Taken together, the presence of IIB isoform may be restricted to the erythroid lineage among hematopoietic cells, which may be an intriguing distribution of myosin II isoforms considering the characteristic feature of enucleation of erythroblasts.

We demonstrated that the proliferation of CFU-E was inhibited by the exogenous expression of the rod fragments for NMHC IIA or IIB, which suggests that the expressions of the rod fragments of NMHC IIA and IIB are functional in the inhibition of cell division of CFU-E. We further showed that the enucleation of mature erythroblasts was inhibited by the transfection of the rod fragment for myosin IIB. Because filament formation is necessary for myosin II to function, exogenous expression of rod fragments containing the critical regions for assembly could exhibit a dominant negative effect, preventing the normal assembly of endogenous myosin II. In fact, it was shown that a 72-kDa rod fragment of NMHC IIB acts as a dominant-negative form and induced aberrant cell shape.[40] We also demonstrated that the myosin IIB rod fragment, BRF305 (Phe 1672–Glu 1976), can inhibit the function of endogenous myosin IIB by inhibiting normal filament assembly of MRC-5 SV1 TG1 cells, the SV40-transformant of human embryonic lung fibroblast MRC-5.[32] Thus, our findings indicate that enucleation of human erythroblasts involves non-muscle myosin IIB. Information concerning the specific functions of myosin IIB has been increased by the studies of cells isolated from NMHC IIB knockout mice. Ablation of NMHC IIB resulted in survival to E14.5, but with marked abnormalities in the heart including a ventricular septal defect, abnormal positioning of the aorta, and abnormalities including hydrocephalus and the abnormal migration of certain groups of neurons.[41-43] A defect in cytokinesis was observed in NMHC IIB$^{-/-}$ cardiac myocytes,[42] possibly because myosin IIA is absent in these cells.

There is a possibility that the relative expression level of the rod fragment, ARF296, against endogenous myosin IIA is not sufficient to show an inhibitory effect[32] in the enucleation of mature erythroblasts. We thus do not exclude the possibility that myosin IIA is also involved in enucleation of human erythroblasts. Interestingly, the clinical manifestations[12] of MYH9-related diseases involve the lens, causing cataracts.[18] The specific role of myosin IIA in maintaining transparency of the lens is unknown; however, Maddala et al[44] showed that inhibition of myosin light chain kinase resulted in the development of nuclear lens opacity and abnormal fiber cell organization, suggesting an association between myosin IIA and lens cell enucleation.

Keerthivasan et al previously reported that blebbistatin failed to block enucleation, and suggested that contraction of the actomyosin ring is not essential for the nuclear expulsion of murine erythroblasts.[10] However, we and others have demonstrated the presence of actomyosin contractile rings in the erythoblasts with incipient enucleation,[5,6] which strongly suggests that the actomyosin ring is functional in enucleation. The reason for this discrepancy in the effects of blebbistatin between mouse and human erythroblasts remains unclear. The efficacy of inhibitors for cell division often varies depending on the species of the cell observed.[19] Moreover, there are phenotypic differences in non-muscle myosin IIA deficiency between humans and mice. In humans, various mutations in the *Myh9* gene that encodes the NMHC IIA cause autosomal dominant disease, whereas in mice the complete deficiency is embryonic lethal but heterozygous mice are nearly normal.[18,45] One hypothesis might be that the degree of inhibition of non-muscle myosin II is more strictly required in mice to observe any effect of this molecule on the enucleation of erythroblasts.

Using human erythroblasts, we confirmed that the actin polymerization inhibitor cytochalasin D[5] and the Rac-specific inhibitor NSC23766[6] completely blocked enucleation. Tubulin/kinesin inhibitors blocked cell division of CFU-E but did not inhibit the enucleation, which suggests that the process of erythroblast enucleation is not entirely the same as that of cytokinesis. In addition, this study contributes the novel finding that the Eg5 inhibitor monastrol also does not inhibit the final step of enucleation in human erythroblasts. Monastrol is a reversible, cell-permeable, non-tubulin-interacting inhibitor of the mitotic kinesin Eg5 motor protein.[46,47] Eg5 is a member of the Kinesin-5 (BimC) subclass of kinesins and influences microtubules that form and organize the mitotic spindle in dividing cells.[46,47] Mammalian cells exposed to monastrol in vitro progress normally through the S and G2 phases of the cell cycle, but are temporarily delayed during mitosis. This delay is accompanied by faulty chromosome separation and the presence of a monoastral spindle composed of a radial array of microtubules surrounded by a ring of chromosomes.[48] Eg5 independent enucleation of human erythroblasts suggests that this is a process independent of the centrosome.

In conclusion, this study shows that the inhibition of ROCK and non-muscle myosin II ATPase completely blocked enucleation of human erythroblasts, demonstrating for the first time that non-muscle myosin II is required for human erythroblast enucleation. Further, we demonstrated that myosin IIB is involved in human

erythroblast enucleation. An increased body of mechanistic knowledge about non-muscle myosin II isoforms in enucleation may help us to explore the unknown NMHC abnormalities in hematologic disorders.

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Authorship

Contribution: K.U. designed and performed experiments, analyzed data, and wrote the manuscript; Y.-M.G., M.T., M.H., Y.M., M.N., H.T., N.T. and A.K. performed experiments and helped to write the manuscript; W.N. and Y.T. analyzed and interpreted the data and helped to write the manuscript; and K.S. designed the study, interpreted data and helped to write the manuscript.

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

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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/μl (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, $CD5^+$ 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 $CD34^+CD38^-$ HSCs (Bhatia et al., 1997), and the $CD34^+CD38^+$ progenitor fraction that contains myeloid and lymphoid progenitors (Manz et al., 2002). Interestingly, percentages of $CD10^+CD19^+$ 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 $CD34^+CD38^-$ HSC population were equal (Figure 1B). Recent reports have shown that the $CD34^+CD38^-$ HSC population can further be divided into subpopulations including $CD90^+CD45RA^-$, $CD90^-CD45RA^-$, and $CD90^-CD45RA^+$ 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 $CD34^+CD38^-$ cells were the most primitive $CD90^+CD45RA^-$ 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 $CD34^+CD38^-$ HSC population in patients with CLL (CLL-HSCs) presented the germline configuration, and $CD34^-CD19^+$ 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 $CD34^+CD38^-$ 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/$IL2rg^{null}$ (NSG) (Ishikawa et al., 2005) newborns or NOD/RAG-$1^{-/-}$IL2rg^{null}

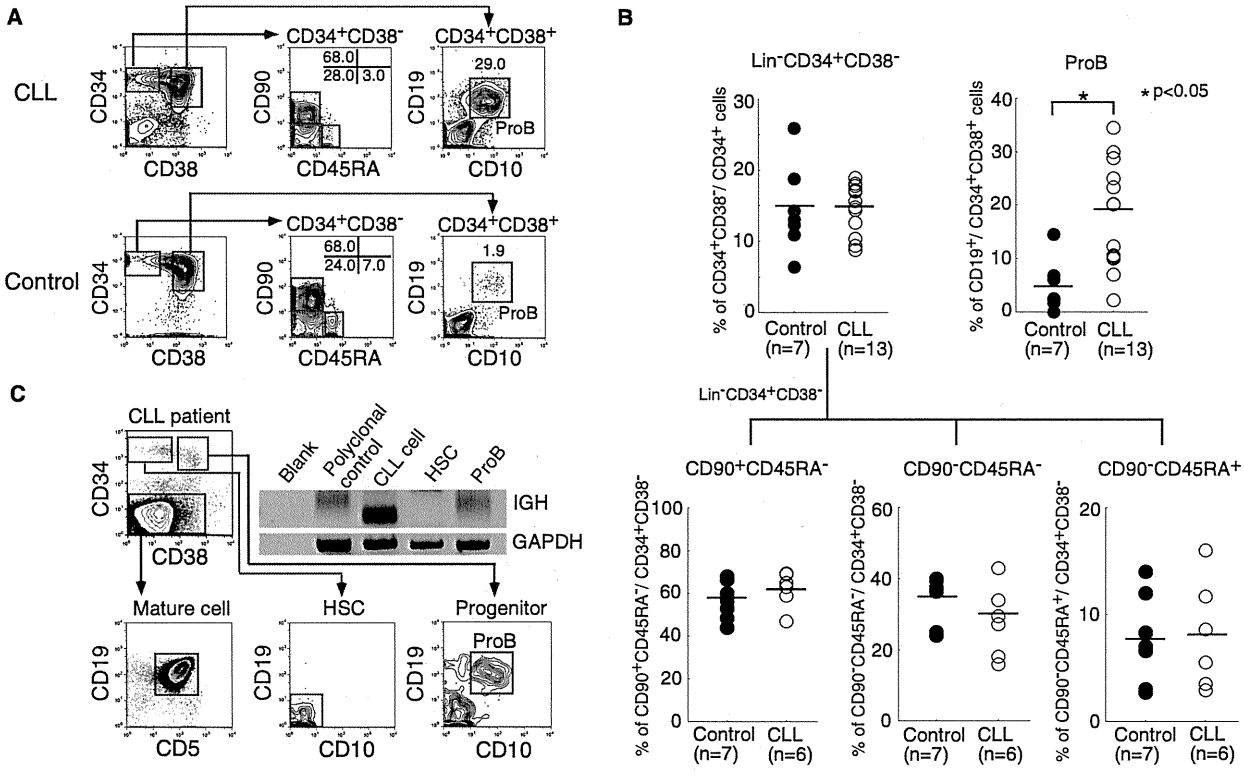

Figure 1. Hematopoietic Stem and Progenitor Cells in the Bone Marrow of Patients with CLL

(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 $CD34^+CD38^-$ HSCs and $CD34^+CD38^+CD19^+$ pro B cells in 13 patients with CLL and 7 normal controls (upper panels), and frequencies of HSC subpopulations including $CD34^+CD38^-CD90^+CD45RA^-$, $CD34^+CD38^-CD90^-CD45RA^-$, and $CD34^+CD38^-CD90^-CD45RA^+$ fractions in 6 patients with CLL analyzed (lower panels) are shown. Note that the CLL bone marrow contains a higher number of $CD34^+CD38^+CD10^+CD19^+$ 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).

$CD19^+$ CLL cells were purified from the blood or the bone marrow of patients 1–8, and 0.2 to 1 × 10^7 cells were transplanted. However, even until 6 months after transplantation, human $CD45^+$ 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 $CD34^+CD38^+$ $CD10^+CD19^+$ 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 × 10^3 to 6.5 × 10^4 $CD34^+CD38^-$ HSCs or 5.0 × 10^3 to 1 × 10^4 $CD34^+CD38^-CD90^+$ LT-HSCs from 16 independent patients with CLL were transplanted into 25 mice (Table 1; Table S2), and ~10^4 $CD34^+CD38^-$ 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 $CD34^+$ $CD38^-$ HSCs, $CD34^+CD38^+$ progenitor cells, $CD34^-CD19^+$ B cells, and $CD34^-CD33^+$ 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 $CD19^+$ 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 $CD5^-$ and very rare (<1%) $CD5^+$ B cells in the bone marrow in all 29 recipients. In total, 5 out of 25 mice transplanted with CLL-HSCs developed both $CD5^+$ and $CD5^-$ B cell clones, 9 mice developed only $CD5^+$ B cell clones, and the remaining 11 mice developed only $CD5^-$ B cell clones

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

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

NA, not analyzed; P, polyclonal.

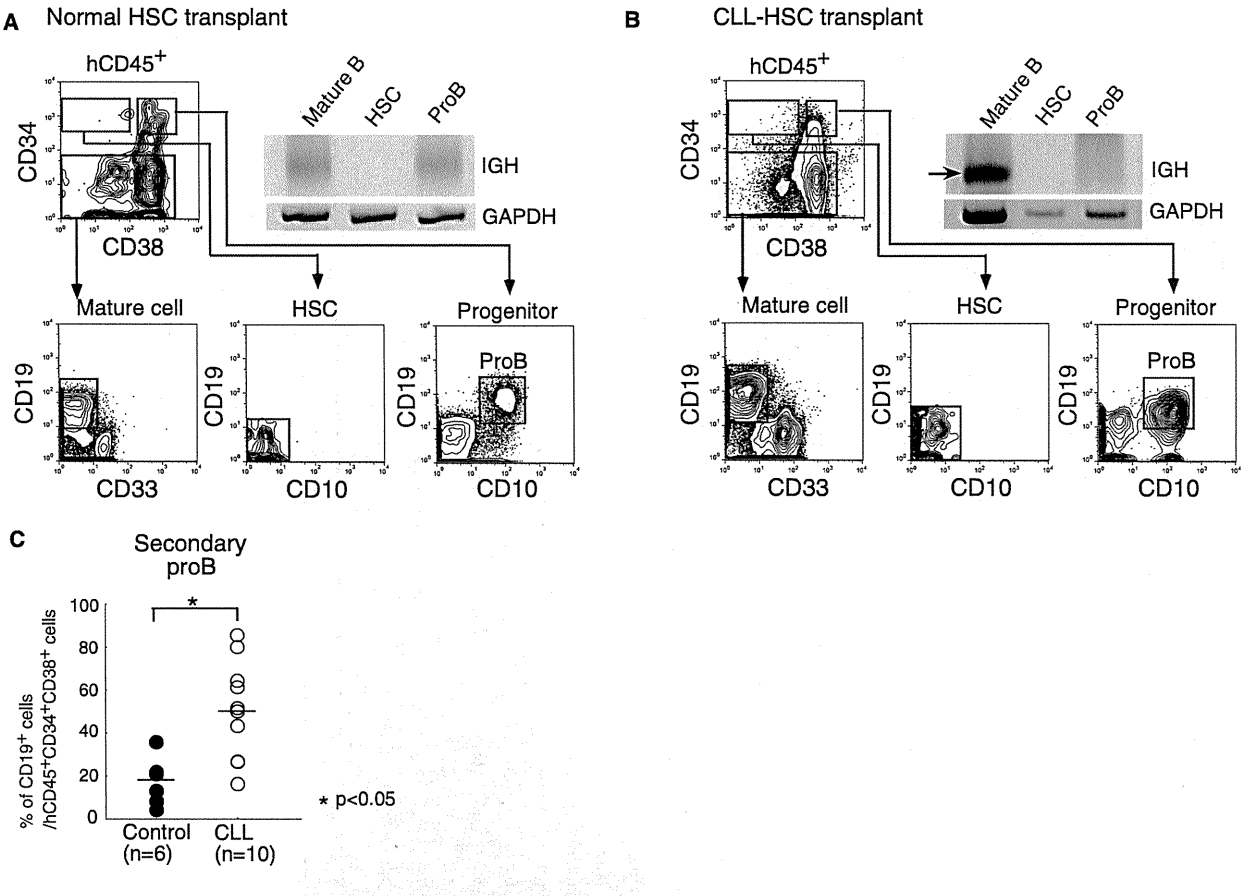

Figure 2. The Bone Marrow Cell Analysis in Mice Transplanted with the CD34$^+$CD38$^-$ HSC Population Purified from Normal Controls and Patients with CLL

(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$^+$ CLL cells is shown in Figure S1.

(Table 1). These CD5$^+$ 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$^+$CD38$^-$ HSCs did not rearrange IGH, and both proB and CD5$^-$ 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$^+$ or CD5$^-$ 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$^+$ and CD5$^-$ B cells

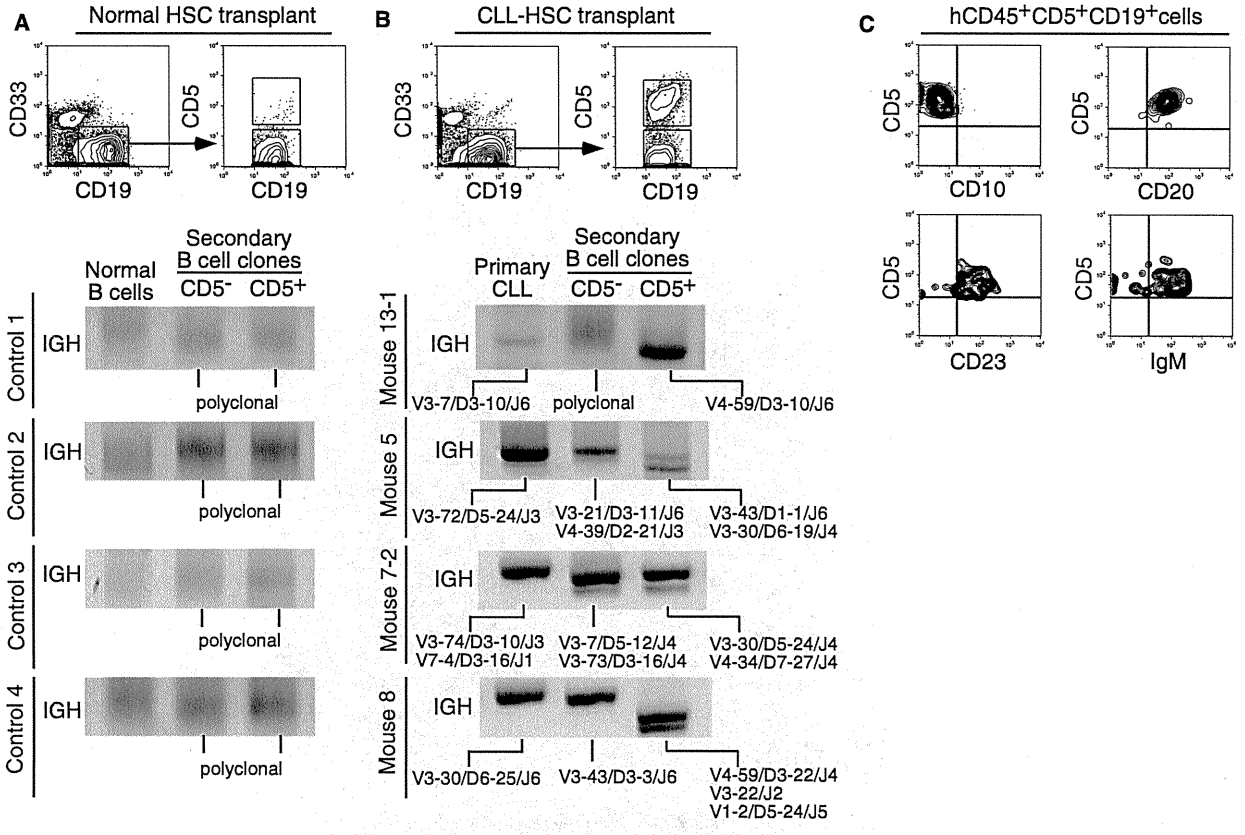

Figure 3. CLL-HSCs Give Rise to Monoclonal or Oligoclonal B Cells with CLL-like Phenotype after Xenogeneic Transplantation

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

(B) FACS and IGH rearrangement analysis of mice transplanted with CLL-HSCs. Development of $CD5^+CD19^+$ B cells was frequently seen in these mice (as summarized in Table 1). In mouse 13-1, $CD5^-$ B cells were polyclonal, but $CD5^+$ B cells were monoclonal. In other mice shown here, both $CD5^-$ and $CD5^+$ 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, $CD5^+$ B cells were developed in 14 out of 25 mice transplanted with CLL-HSCs, and these $CD5^+$ B cells consisted always of mono- or oligo-clones (Table 1). Clonal B cell populations were also found in $CD5^-$ 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 $CD5^+$ phenotype, $CD5^-$ 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

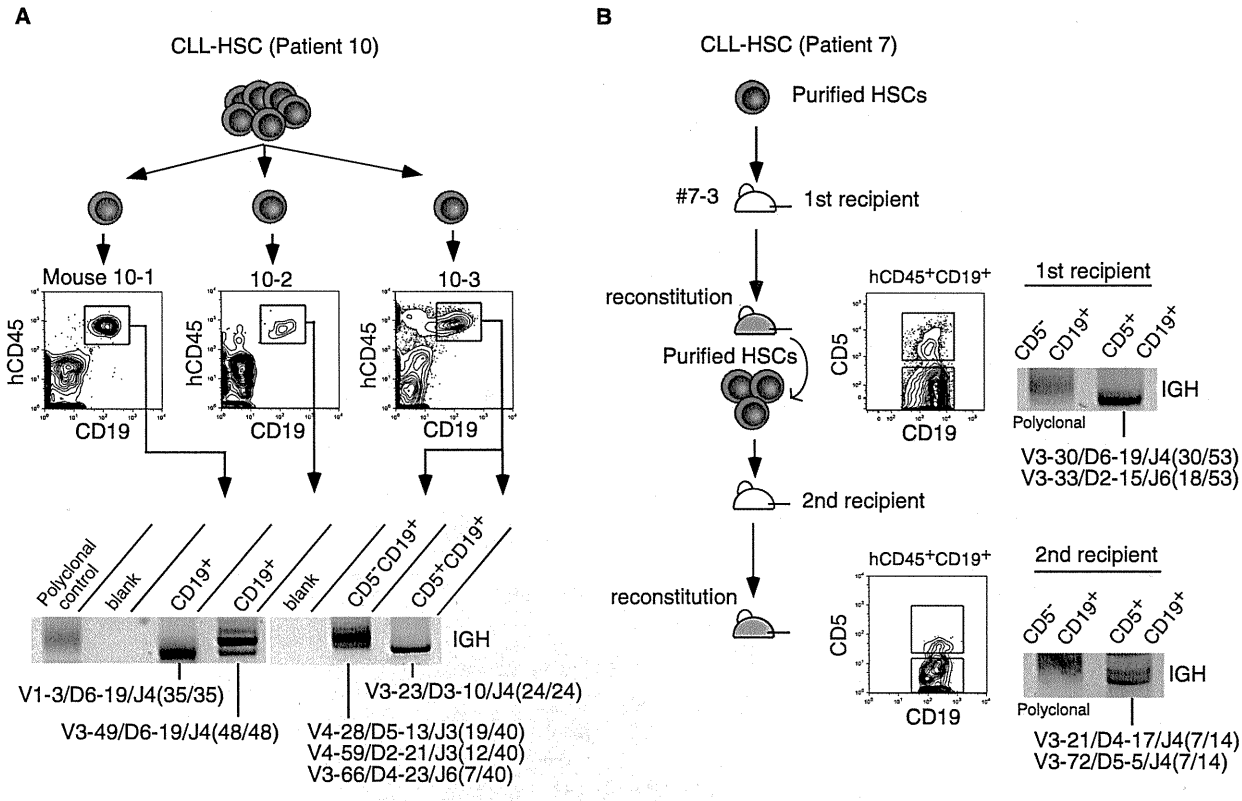

Figure 4. CLL-HSCs Are Capable of Self-renewal, and Their B Cell Progeny Is Clonally Selected in Xenogeneic Recipients
(A) CLL-HSCs from patient 10 were purified and transplanted into three recipients simultaneously. All recipients developed monoclonal or oligoclonal B cell clones. Note that the B cell 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 $CD5^+$ B cell clones. We then purified $CD34^+CD38^-$ HSCs from the bone marrow of the primary recipient and retransplanted into the secondary recipient. The secondary recipient again developed two $CD5^+$ 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 $CD19^+$ CLL cells in patients 2 and 11 possessed del13q14, and patients 1 and 3 had both del13q14 and del11q23 by FISH analysis. However, purified $CD34^+CD38^-$ CLL-HSCs and $CD33^+$ 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 $CD34^+CD38^-$ 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

	Patients' CLL Bone Marrow					CLL-HSC-Derived B Cell Clones		
	VDJ Gene		Abnormal Karyotypes (%)			VDJ Genes		Abnormal Karyotype (%)
Patient No.	of CLL Clone	FISH Target	B Cell	Myeloid	HSC	of B Cell Clone	FISH Target	hCD45+ Cell
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 $CD34^+CD38^-$ 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 $CD34^+CD38^-$ HSCs (Figure S3). Other relatively late B lymphoid-related genes including EBF, PAX5, IGLL1, DNTT, and VPREB3 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 $CD34^+CD38^-$ 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, VPREB3, 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 $CD34^+CD38^-$ 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 $CD34^+CD38^-$ cells consisted of $CD90^+CD45RA^-$ LT-HSCs (~60%) and $CD90^-CD45RA^-$ multipotential progenitors (~30%) (Majeti et al., 2007), and the

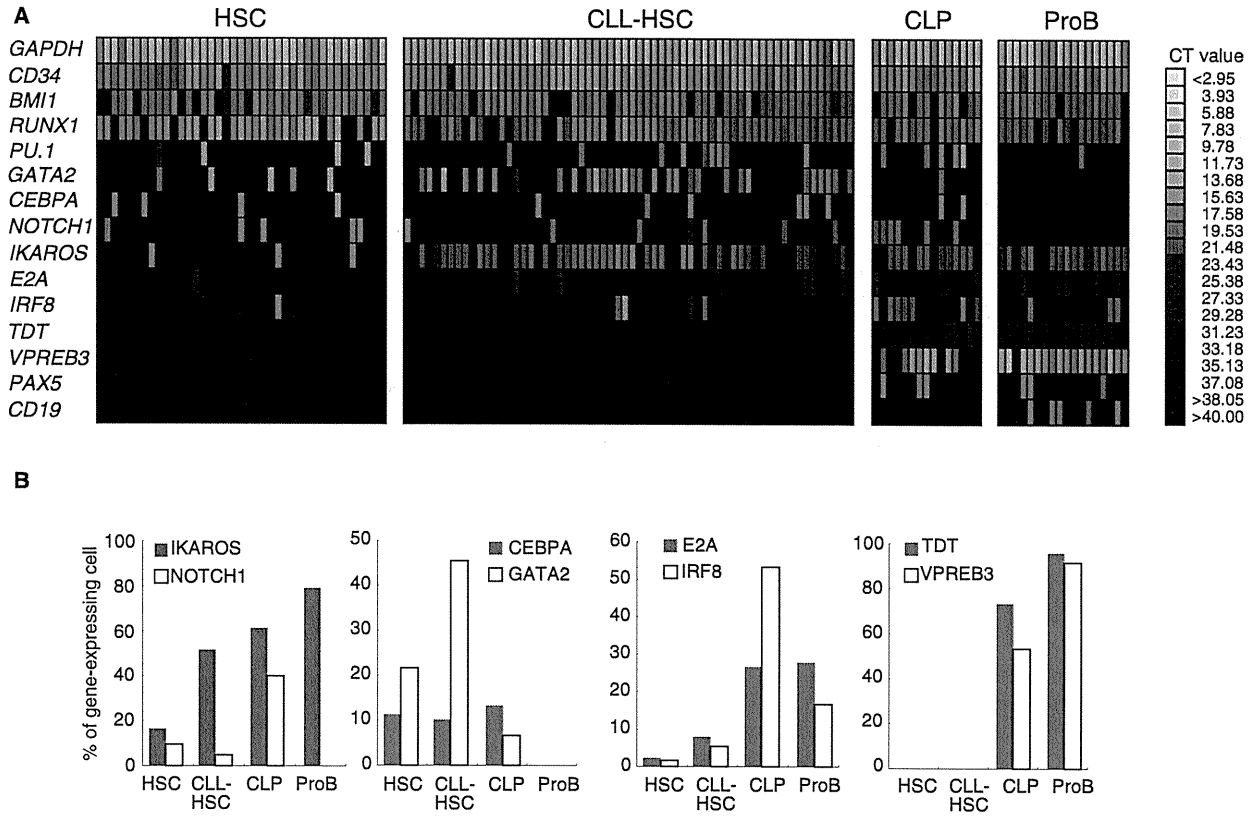

Figure 5. Lymphoid-Lineage Gene Priming in Single CLL-HSCs

(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).

$CD90^{-}CD45RA^{+}$ population that was reported to initiate lymphomyeloid 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 $CD34^{+}CD38^{-}$ 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 $CD34^{+}CD38^{-}$ population is uncommitted stem or progenitor cells. However, it was still possible that the purified $CD34^{+}CD38^{-}$ 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

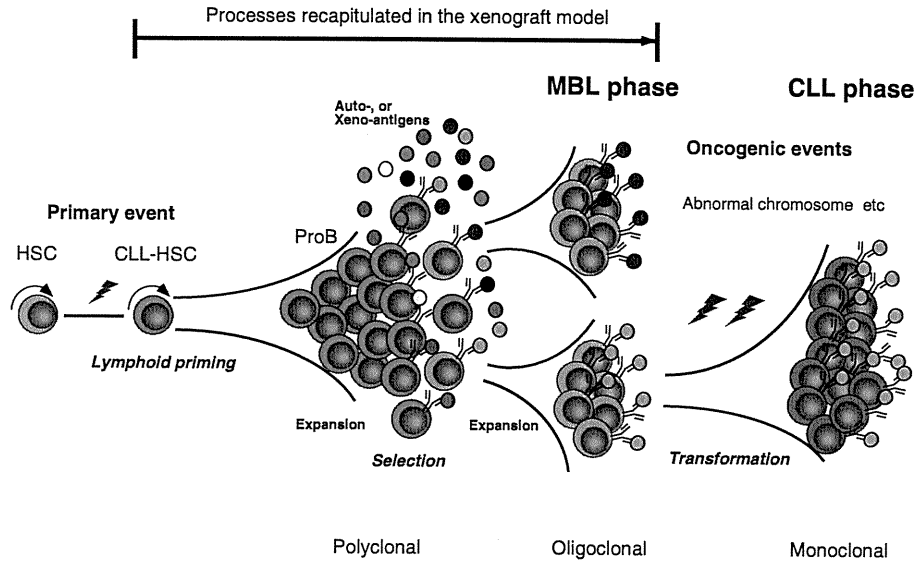

Figure 6. Schematic Presentation of Human CLL Development Based on the Xenogeneic Transplantation Model
CLL-HSCs have accumulated genetic abnormalities that might play a role in amplified B cell differentiation, and produce a high number of polyclonal B cells carrying the same genetic aberrations. B 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 $CD34^+$ 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 $CD34^+CD38^-$ cells from the fraction does not express lineage antigens as described below. In some cases $CD34^+CD38^-CD90^+$ 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 (UCTH2), 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^{scid}IL-2rg^{tm1Wjl}/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 $CD34^+CD38^-Lin^-$HSC, $CD34^+CD38^+CD10^+CD19^-Lin^-$CLP (Galy et al., 1995), or $CD34^+CD38^+CD10^+CD19^+Lin^-$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 ± 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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