ORIGINAL ARTICLE

Efficient retroviral transduction of human B-lymphoid and myeloid progenitors: marked inhibition of their growth by the *Pax5* transgene

Rieko Sekine · Toshio Kitamura · Takashi Tsuji · Arinobu Tojo

Abstract We applied a coculture system for the genetic manipulation of human B-lymphoid and myeloid progenitor cells using murine bone marrow stromal cell support, and investigated the effects of forced Pax5 expression in both cell types. Cytokine-stimulated cord blood CD34$^+$ cells could be transduced at 85% efficiency and 95% cell viability by a single 24-h infection with RD114-pseudotyped retroviral vectors, produced by the packaging cell line Plat-F and bicistronic vector plasmids pMXs-Ig, pMYs-Ig, or pMCs-Ig, encoding EGFP. Infected CD34$^+$ cells were seeded onto HESS-5 cells in the presence of stem cell factor and granulocyte colony-stimulating factor, allowing the extensive production of B progenitors and granulocytic cells. We examined the cell number and CD34, CD33, CD19, and CD20 lambda and kappa expressions by flow cytometry. Ectopic expression of Pax5 in CD34$^+$ cells resulted in small myeloid progenitors coexpressing CD33 and CD19 and inhibited myeloid differentiation. After 6 weeks, the number of Pax5-transduced CD19$^+$ cells was 40-fold lower than that of control cells. However, the expression of CD20 and the κ/λ chain on Pax5-transduced CD19$^+$ cells suggests that the Pax5 transgene may not interfere with their differentiation. This report is the first to describe the effects of forced Pax5 expression in human hematopoietic progenitors.

Keywords B-cell differentiation · Pax5 · CD19 · Stromal cell · Retroviral vector

1 Introduction

The entry of lymphoid progenitors into the B-cell pathway depends on the transcription factors E2A, EBF, and Pax5 [1]. B-cell development is closely associated with the coordinated expression of phenotypic markers, such as B-cell receptors, cytokine receptors, and their signal mediators [2–5]. Most of our knowledge on B-cell development has come from extensive studies in mice; the examination of human B-cell lymphopoiesis has been hampered by a lack of appropriate experimental systems, although some studies have been performed on tumor cells. Recently, human B lymphopoiesis was partially reconstituted in immuno-deficient mice, and, several weeks after transplantation, the major B-cell populations consisted of immature CD19$^+$IgM$^-$ B-cells [6]. However, such an in vivo system is not amenable to the sequential observation of B-cell differentiation, especially in the early stages.

For manipulating and observing human B-lymphoid progenitors, the in vitro simulation of human B lymphopoiesis by cultivating cord blood CD34$^+$ cells (CBCD34$^+$ cells) on a layer of murine, bone marrow stromal cells has been described [7]. Studies have also reported the molecular characterization of human hematopoietic progenitors [8] and early T/NK and B-lymphoid progenitors [9], derived from coculturing CBCD34$^+$ cells with murine stromal cells. This culture method offers a limited, but

R. Sekine · A. Tojo (✉)
Division of Molecular Therapy,
Advanced Clinical Research Center,
Institute of Medical Science, University of Tokyo,
4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
e-mail: a-tojo@ims.u-tokyo.ac.jp

T. Kitamura
Division of Cellular Therapy, Institute of Medical Science,
University of Tokyo, Tokyo, Japan

T. Tsuji
Department of Industrial Science and Technology,
Science University of Tokyo, Noda, Japan

useful experimental system for investigating the transcriptional regulation of both B-cell and granulocyte differentiation via the ectopic expression or silencing of lineage-specific transcription factors, including EBF, E2A, Pax5, and C/EBP family members [10].

Retro- and lentiviral vectors have been widely used for gene delivery to human hematopoietic stem/progenitor cells [11]. A major problem with Moloney murine leukemia virus (MoMLV)-based vectors is their low transduction efficiency, due to the reduced expression of the corresponding cellular receptors. This problem was resolved by the application of a pseudo-typed envelope protein from RD114, a feline endogenous retrovirus [12]. Another problem involves silencing the promoter activity of its long terminal repeat (LTR) in primitive stem cells [13]. The use of the LTR from murine stem cell virus (MSCV) [14] or myeloproliferative sarcoma virus (MPSV) [15] facilitated a more stable expression of foreign genes, both in vitro and in vivo. Eventually, bicistronic, MSCV-based retroviral vectors expressing enhanced green fluorescent protein (EGFP) were developed that could effectively transduce murine [16] and human [17] stem/progenitor cells. One report described a series of retroviral vectors, including pMXs-Ig, pMYs-Ig, and pMCs-Ig, together with a novel packaging cell line, Plat-F, which generates the RD114 envelope protein [18].

In the present study, we assessed these three retroviral vectors for their ability to transduce human CBCD34^{+} cells and to stably express a foreign gene during differentiation toward B-lymphoid and myeloid lineages using a coculture system with HESS-5 cells. We showed that a single 24-h infection was sufficient for these vectors to transduce CBCD34^{+} cells and to achieve stable expression of the foreign gene. Furthermore, we examined the effects of the ectopic expression of Pax5 in early B-lymphoid and myeloid progenitors.

2 Materials and methods

2.1 Preparation of CD34^{+} cells from human umbilical cord blood (CB)

Umbilical CB units were collected from single-birth, full-term, normal deliveries following the standards for CB collection developed by the Tokyo Cord Blood Bank and the Institute of Medical Science, University of Tokyo. CD34^{+} cells were purified from mononuclear cells using a CD34^{+} Progenitor Cell Isolation kit and a MiniMACS separation unit (Miltenyi Biotec GmbH, Glandbach, Germany) and were stored frozen until use. Samples with CD34^{+}-cell purity and viability of at least 95% were used for experiments.

2.2 Cell culture

The murine stromal cell line, HESS-5, was maintained in DNA, RNA-free alpha Modified Eagle's Medium (a-MEM; 12561-056; Gibco, Grand Island, NY) supplemented with 10% (v/v) horse serum. Only HESS-5 cells below passage 30 were used in coculture experiments. Plat-F cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), containing puromycin (1 mg/mL) and brastcidine (10 mg/mL). All cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

2.3 Culturing CBCD34^{+} cells on a murine stromal layer

HESS-5 cells were prepared at 2×10^4 cells/well in a 6-well plate, 1 week prior to seeding CD34^{+} cells, and were allowed to reach confluence without irradiation. CD34^{+} cells were plated at a density of 3×10^3 cells/well onto the HESS-5 cell layer in 3 mL of a-MEM containing 10% heat-inactivated FBS with recombinant human stem cell factor (SCF, 20 ng/mL) and recombinant human granulocyte colony-stimulating factor (G-CSF, 10 ng/mL). The coculture medium was removed and replenished with fresh medium every week for up to 9 weeks. Non-adherent cells in the medium were collected. Each week, one well of cocultures was harvested, separating non-adherent and migrating cells beneath the HESS-5 cell layer for RNA extraction and flow cytometry (FCM).

2.4 Microscopic and flow cytometric analysis

Non-adherent cells were harvested from the culture medium. After removing the non-adherent cells, adherent cells were separated in phosphate-buffered saline (PBS) containing 5 mM EDTA. Cells were resuspended in PBS (−) containing 2 mM EDTA and 2% FBS and counted by trypan blue dye exclusion. Non-adherent cells (myeloid cells) from the culture medium or adherent cells (B-lymphoid cells) were centrifuged on glass slides using a cytospin centrifuge and stained for 5 min in May–Grunwald solution and 20 min in Giemsa solution before being analyzed under a microscope (ECLIPSE E600; Nikon, Tokyo, Japan). Photographs of cytospin smears were captured with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a 60× objective lens, Olympus DP70 CCD camera, and Sysmex LAFIA picture filing system (Sysmex Corp., Kobe, Japan). Cells were then passed through a 40-mm nylon-mesh filter and labeled with a combination of FITC-CD19 and PE-CD34, FITC-CD20 and PE-CD19, FITC-lambda- and PE-kappa-labeled monoclonal antihuman antibodies (BD Pharmingen, San Diego,

CA, USA). The monoclonal antihuman antibodies were first tested for the absence of detectable cross-reactivity with HESS-5 cells. Two-color FCM was performed using a FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest acquisition software (BD Pharmingen). List mode files were analyzed with the FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.5 Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from CBCD34$^+$ cells and non-adherent and adherent cells cocultured for 1–8 weeks using the RNeasy micro kit (Qiagen, Valencia, CA, USA). Total RNA (200 ng) from each sample was reverse-transcribed using the SuperScriptII First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primer, resulting in 20 μL of RT products. Full-length human E2A, EBF, Pax5 and RAG2, the N-terminal fragment of RAG1, and the C-terminal fragment of RAG1 cDNAs were amplified from 2 μL of the RT products, using a high fidelity PCR kit (Takara Syuzo, Otsu, Japan) and the following primers:

E2A (forward 5′-GAGAATGAACCAGCCGCAGAG GATG-3′ and reverse 5′-TTCACATGTGCCCGGCGGGG TTGTG-3′); EBF (forward 5′-TTTCATGTTTGGGATT CAGGAAGC-3′ and reverse 5′-TTCACATAGGAGGAA CAATCATGCC-3′); Pax5 (forward 5′-ATGGATTTAGA GAAAAATTATCCG-3′ and reverse 5′-TCAGTGACGG TCATAGGCAGTG-3′); N-RAG1 (forward 5′-TGCCGCA TCTGTGGGAATTCTTTT-3′ and reverse 5′-CTGAGCT CCTTCTCTTGCGCTGACG-3′); and C-RAG1 (forward 5′-CCAGCTGTTTGCTTGGCCATGCGT-3′ and reverse 5′-TTGGGATCTCATGCCTTCCAAGAT-3′). The resulting PCR products [E2A 1,970 base pairs (bp), EBF 1,776 bp, Pax5 1,470 bp, RAG2 1,561 bp, N-RAG1 154 bp, C-RAG1 331 bp] were cloned into the pCR-BluntII cloning vector (Invitrogen), and sequenced using the BigDye terminator cycle sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems,

Foster City, CA, USA). These plasmids were then used as controls in the quantitative, real-time PCR.

2.6 Quantitative RT-PCR (QR-PCR) analysis

Quantitative analysis of mRNA expression was performed using 2 μL of the RT product per reaction, the TaqMan Universal PCR Master Mix (Applied Biosystems), human gene-specific primers, and 5′-FAM- and 3′-TAMRA-labeled probes (Table 1), according to the manufacturer's instructions. Each reaction was performed in duplicate. The specified genes were amplified for 50 cycles, and PCR reactions were analyzed with an ABI Prism 7700 (Applied Biosystems). Quantification of E2A, EBF, Pax5, and RAG1 gene expression in each sample was accomplished by the relative standard curve method, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal RNA standard [19]. We confirmed the efficiency of the PCR reactions and the threshold cycle, calculated with a standard curve generated from serially diluted (10-fold diluted from 10^6 to 10^1 copies) control plasmids.

2.7 Construction and production of, and infection with, retroviral vectors

Plat-F packaging cells were transiently transfected with pMXs-Ig, pMYs-Ig, and pMCs-Ig using the FuGENE 6 transfection reagent (Roche, Basel, Switzerland). Retroviral supernatants were harvested 72 h after transfection. The viral titer of each supernatant, determined by terminal dilution with CBCD34$^+$ cells, was nearly 1.5×10^6 infectious units/mL.

CBCD34$^+$ cells were precultured for 24 h in the conditioning medium: Iscove's Modified Dulbecco's Medium (IMDM) with 20% FBS, SCF (50 ng/mL), Flt-3 ligand (Flt-3L, 50 ng/mL), thrombopoietin (TPO, 10 ng/mL), and interleukin-3 (IL-3, 10 ng/mL). Infection of CBCD34$^+$ cells was performed in Retronectin (CH-296; Takara Shuzo)-coated, 24-well plates. An aliquot of 2×10^5

Table 1 Primers and probes for quantitative RT-PCR

NCBI-GeneID	Forward primer (5′–3′)	Probe (5′–3′)	Reverse primer (5′–3′)	Amplicon (bp)
E2A 6929	AGGGCACCCACTTCACTGAG	CGCACAGCAGCCTCTCTTCATCCA	GAGTCCCGGTCCCAGGAAT	65
EBF 1879	GATACGGCTCTGCCGCAAT	TCCAATTTGGGCGGCTCCCC	CAGCTGAGCCGTTGAGGAAG	63
RAG1 5896	TTTACTTCCCGAGGAACGTGA	ATGGAGTGGCACCCCCACACACC	GGGCAGTGTTGCAGATGTCA	72
Pax5 5079	TACTCAGCACCGACGC	ATTCCTCGTACAACGACTCCTGGAGGTTCC	GCGGCAGCGCTATAAT	149
GAPDH 2597	GAAGGTGAAGGTCGGAGTC	CAAGCTTCCCGTTCTCAGCC	GAAGATGGTGATGGGATTTC	226

precultured CD34$^+$ cells was cultured in 1.3–1.5 mL of viral supernatant for 24 h. The cytokine conditions corresponded to the preculture medium, except for the presence of additional protamine (5 mg/mL). For experiments with the pMCs-Ig and Pax5 vectors, fresh viral supernatant was replaced 4× during the 48-h infection with cytokines and protamine (5 mg/mL) at the same concentration.

2.8 Cell sorting, coculture, and colony-forming assay

Infected CBCD34$^+$ cells were sorted into EGFP-positive and -negative fractions using a FACS Aria cell sorter (BD Pharmingen). Then, 9×10^3 EGFP$^+$ cells were subjected to a coculture assay, and 9×10^2 EGFP$^+$ cells were used in a colony-forming assay. EGFP$^+$ cells were seeded onto a HESS-5 monolayer in triplicate and cultured for 6–9 weeks, as mentioned above. Cocultures were observed every week under an inverted Nikon TMD300 microscope with a fluorescence light unit. Photomicrographs were captured using the same microscope equipped with a 20× objective lens and a Nikon DXM1200 digital camera system. The total medium volume was changed at weekly intervals, and all non-adherent cells were collected and subjected to cell count and flow cytometric analysis. On day 42, all cocultured cells were harvested with PBS (−) containing 0.5% BSA and 5 mM EDTA and subjected to similar analysis. Cells were stained with a combination of PE-CD33 and APC-CD19, PE-CD20 and APC-CD19, and, PE-kappa and APC-CD19, and analyzed by three-color FCM. The colony-forming assay was carried out in triplicate in 35-mm Petri dishes by incubating 3×10^2 EGFP-positive cells in 1 mL METHOCULT H4435 (Stem Cell Technologies, Vancouver, BC, Canada) containing erythropoietin (EPO, 3 U/mL), SCF (50 ng/mL), GM-CSF (20 ng/mL), IL-3 (20 ng/mL), interleukin-6 (IL-6, 20 ng/mL), and G-CSF (20 ng/mL). The colony number was calculated on days 12–14 of culture. Pax5 experiments were independently performed 3×.

2.9 Cell cycle analysis and apoptosis assay

To assess the cell cycle, collected cells were fixed with cooled 70% ethanol. The fixed cells were washed twice with PBS (−) and incubated with 0.25 mg/mL ribonuclease A for 30 min. The 4-week cultured cells were fixed with IntrePrep^{TM} Permeabilization Reagent 1 (Immunotech, Marseille, France) containing 5.5% formaldehyde and permeabilized with Reagent 2 containing 0.001% saponin and 0.25 mg/mL ribonuclease A for 30 min according to the instructions. Subsequently, propidium iodide (final concentration, 50 µg/ml) was added to each sample to stain DNA. For the detection of apoptosis, collected cells were stained with an Annexin V–PE Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the instructions. Each sample was analyzed by flow cytometry. The cell cycle and population of apoptotic cells were analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

3 Results

3.1 Cellular and molecular characteristics of B-cell differentiation in vitro

When CBCD34$^+$ cells were cultured on HESS-5 cells, the number of non-adherent myeloid cells increased after 2 weeks, reaching a maximum during the third week, and decreasing thereafter. In adherent cell layers, definite foci of small B-lymphoid cells beneath the stromal layer appeared during the second week and increased in size until the sixth week. At first, B-lymphoid progenitors developing in the coculture system were characterized by surface marker analysis and the quantitative measurement of B-cell-specific gene expression. Non-adherent or loosely adherent CD33$^+$ myeloid cells were washed out before harvesting cells from the culture. CD34$^+$CD33$^-$ cells could be observed as single cells beneath the stromal layer during the first week of culture, and formed colonies of 10–20 cells during the second week (data not shown). On day 21, CD34$^+$CD19$^-$ cells comprised 20% of the CD33$^-$ cell population, and a comparable proportion of CD34$^+$CD19$^+$ proB and CD34$^-$CD19$^+$ preB cells appeared in the culture. They differentiated into smaller CD34$^-$CD19$^+$CD20$^+$ late preB cells on day 28. On day 42, more than 10% of the adherent lymphoid cells expressed immunoglobulin (Ig) light chains, suggesting the appearance of immature B-cells (Fig. 1a).

Next, B lymphoid cells adherent to or beneath the HESS-5 layer were harvested weekly from days 1–49, and total RNA was extracted for QR-PCR analysis of B-cell-specific transcripts including E2A, EBF, Pax5, and RAG1. The vast majority of the initial cells were CD34$^+$CD19$^-$, and, at this stage, all transcripts could be detected. The expression levels of both E2A and RAG1 were relatively high throughout the culture period. In contrast, EBF was weakly expressed in the early stage and upregulated after 14 days of culture. Pax5 was initially expressed at a very low level, but was significantly upregulated after 14 days of culture, before which a transient down-regulation of E2A and RAG1 transcripts was observed. The expression levels of EBF and Pax5 were comparable in the middle and late phases of culture, but were still 10-fold lower than that of E2A (Fig. 1b).

Fig. 1 a Sequential phenotypic analysis of in vitro B-cell differentiation examined by flow cytometry of CD34, CD19, CD20, and light chain (kappa or lambda) expression. The quadrate gate of two-color analysis was determined by each isotype control. One representative result of four repeat experiments is shown. b Kinetic analysis of B-lymphopoiesis-associated gene expression by QR-PCR for the transcription factors E2A, EBF, Pax5, and RAG1 proteins. The results are the mean ± SD of duplicate experiments
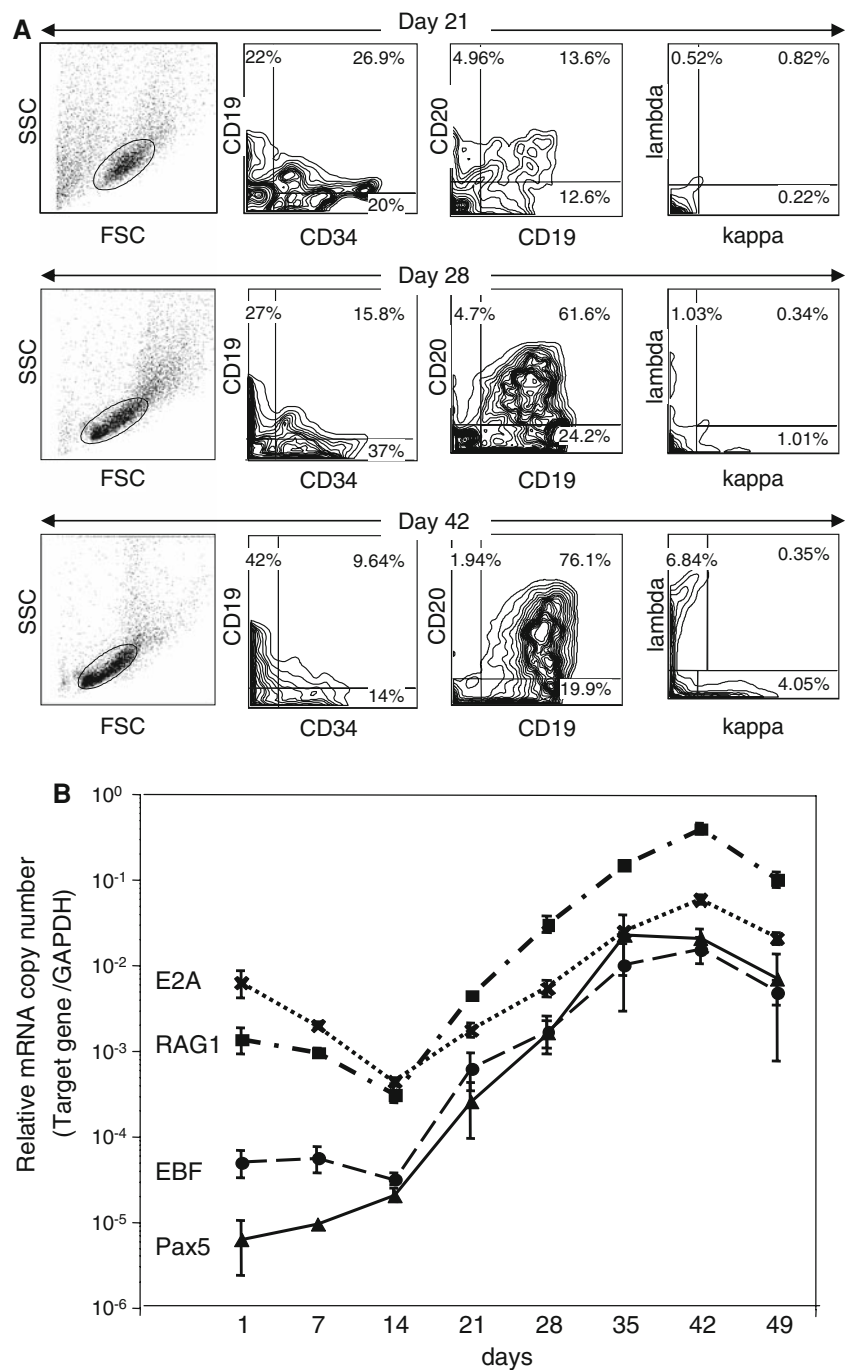
3.2 Retroviral transduction of CBCD34+ cells

The three retroviral vectors used in this study have been described elsewhere [18]; a schematic representation of the constructs is shown in Fig. 2. To compare the transduction efficiency of these vectors, we prepared >95% pure human CBCD34+ cells. Cytokine-stimulated CD34+ cells were infected for 24 h with pMXs-Ig, pMYs-Ig, or pMCs-Ig [multiplicity of infection (MOI) = 10]. The cell viability was typically >95%. The transduction efficiency was comparable among the three vectors, and was typically >60% at the end of infection. The percentage of CD34+ cells showed no change between before and after viral infection (Fig. 3a).

Infected CD34+ cells were immediately seeded onto confluent HESS-5 cells. Four days later, the EGFP+ cell population in CD33+ cells increased to 82.6% (pMXs), 88.2% (pMYs), and 88.5% (pMCs), and the mean fluorescence intensity (MFI) of EGFP was 209, 760, and 537, respectively. Repeat experiments confirmed that the

intensity of EGFP expression in CD33^{+} cells was in this order (Fig. 3b). After 28 days of culture, most of the non-adherent myeloid cells resembled mature neutrophilic granulocytes, and small B-lymphoid cells formed numerous cobblestone areas beneath the HESS-5 layer. Up to this
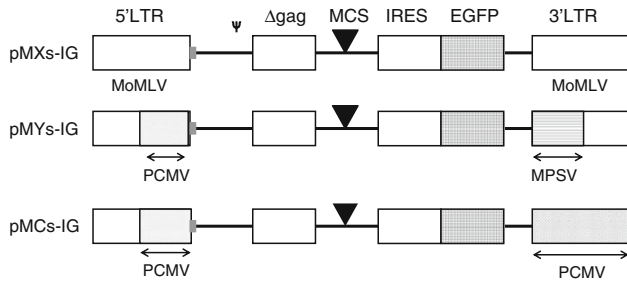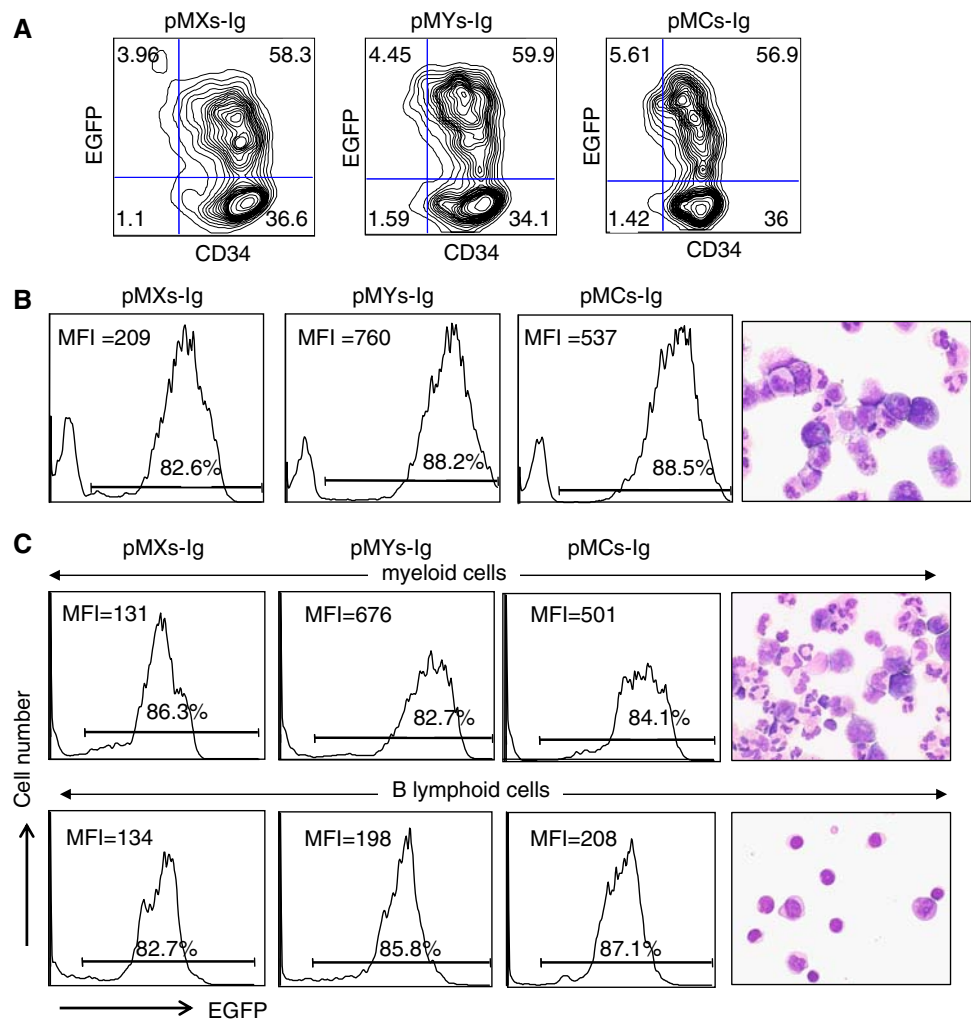
stage, the percentage of EGFP^{+} cells was always over 80% throughout the culture, regardless of the cell lineage or type of retroviral vector. The MFI of EGFP in granulocytes transduced with pMXs, pMYs, and pMCs was 131, 676, and 501, respectively (Fig. 3c, upper panel). The MFI of EGFP in late preB cells transduced with pMXs, pMYs, and pMCs was 134, 198, and 208, respectively (Fig. 3c, lower panel). Thus, the promoter activity of each retroviral vector was sensitive to the developmental stage and/or cell lineage.

3.3 Sustainability of EGFP expression during B-cell and granulocytic differentiation in vitro

Based on the comparative data between the three retroviral vectors, further experiments were conducted using pMCs-Ig, as it provided adequate expression of the foreign genes in both B-lymphoid, myeloid, and progenitor cells. To improve the transduction efficiency of CD34^{+} cells, cytokine-treated CD34^{+} cells were infected with fresh viral supernatant at 12-h intervals during the 48 h. The

Fig. 2 Structure of pMXs-Ig, pMYs-Ig, and pMCs-Ig retrovirus vectors. ψ packaging signal, Δ gag truncated gag sequence, LTR long terminal repeat, MCS multi-cloning site, IRES internal ribosomal entry site, EGFP enhanced green fluorescent protein, white box Mo-MLV LTR, gray box PCMV LTR, hatched box MPSV LTR

Fig. 3 a The relationship between EGFP expression and the surface marker CD34 at the end of infection. Cells were stained with PE-conjugated anti-CD34 and analyzed by two-color FCM. b After a 4-day coculture, EGFP expression levels in CD33^{+} cells were compared between the three vectors by FCM, and cytospin preparations revealed an immature myeloid cell morphology. The photograph of pMCs-Ig cells shown is representative (×600). c Coculture at 28-days. Constitutive EGFP expression by pMXs, pMYs, and pMCs in mature myeloid cells (upper panels), and B-lymphoid cells (lower panels). The photograph of pMCs-Ig cells is representative (×600)

transduction efficiency, evaluated by the percentage of EGFP$^+$ cells after final infection, increased to 80.4 ± 6%; meanwhile, the percentage of CD34$^+$ cells decreased to 75.8 ± 2.7%. The EGFP$^+$ cells represented 86.3 ± 4.6% of CD34$^+$ cells at the end of the infection procedure ($n = 3$; Fig. 4a).

Next, EGFP$^+$ cells were sorted on a FACS (purity > 98%) and seeded onto the HESS-5 layer. An aliquot of sorted cells was subjected to a methylcellulose colony-forming assay. Cloning efficiency was 147.9 ± 15

colonies/300 cells ($n = 3$). In coculture experiments, we monitored EGFP expression by FCM at 1, 6, and 9 weeks of culture to assess the sustainability of pMCs-mediated gene expression. After 1 week, almost all non-adherent myeloid cells were positive for EGFP. Furthermore, adherent CD19$^+$ small preB cells stably expressed EGFP after 6 and 9 weeks, while a two-peaked expression pattern was noted on day 42. Accordingly, pMCs-mediated gene expression was maintained at a high level over 9 weeks of culture (Fig. 4b).

Fig. 4 a Transduction rates evaluated by the percentage of EGFP$^+$ cells and percentage of CD34$^+$ cells after 4× repeat infection. The mean transduction rate and SD were 80.4 ± 6%, the mean percentage of CD34$^+$ cells decreased to 75.8 ± 2.7%, and the percentage of EGFP$^+$ cells in the CD34-positive gate was 86.3 ± 4.6% ($n = 3$). The data shown are from one representative experiment of three. **b** The stability of EGFP expression in pMCs-Ig-transduced cells was determined after 1, 6, and 9 weeks of coculture. Non-adherent cells in the culture medium on day 7 (*upper*). Almost all cells were developing into CD33$^+$ myeloid cells. All of the cocultured cells were trypsinized on day 42 (*middle*) and day 63 (*lower*), developing into CD19$^+$ B-lymphoid cells. In each case, the percentage of EGFP$^+$ cells determined by FACS was above 98%
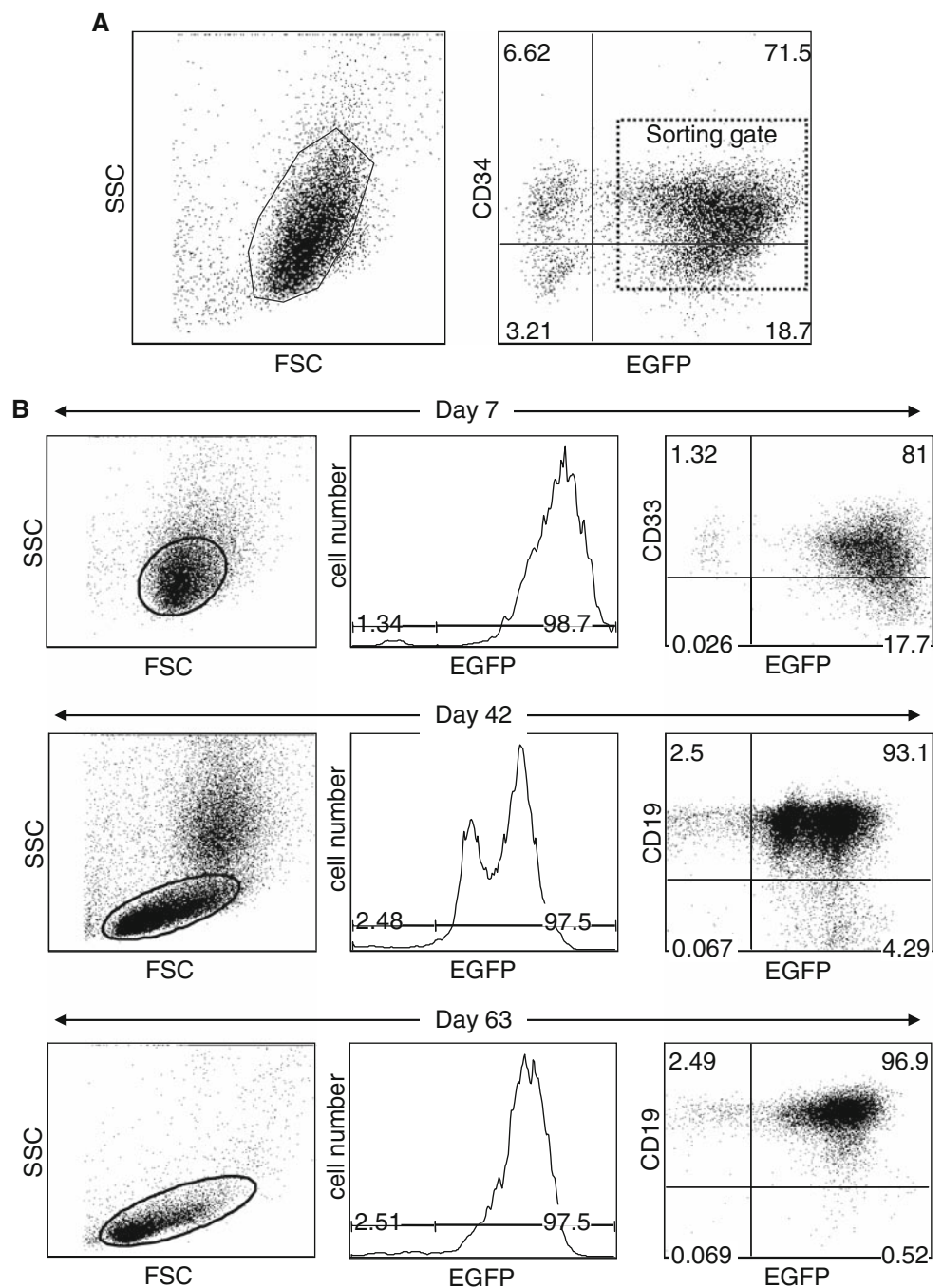
3.4 Disruption of B lymphopoiesis and myelopoiesis by the Pax5 transgene

A combination of retroviral gene transfer to CBCD34^{+} cells with the HESS-5 coculture system allowed us to simultaneously engineer both B-cell and myeloid progenitors. Pax5 is essential for B-cell-specific gene expression such as CD19, and is indispensable throughout the early stages of B-cell differentiation [5]. In addition, a subset of acute myeloid leukemia cells harboring t(8;21) translocation frequently express both Pax5 and CD19, suggesting the possible involvement of ectopic Pax5 expression in myeloid leukemogenesis [20, 21]. Therefore, we focused on the impact of the Pax5 transgene on the growth and bilineage differentiation of CBCD34^{+} cells.

After infection with pMCs–Pax5-Ig or pMCs-Ig, sorted EGFP^{+} cells were subjected to the standard colony-forming and coculture assays with HESS-5 cells. Colony-forming assays showed that total plating efficiency of control progenitors was $147.9 \pm 15/300$ cells, as described above, but that Pax5-transduced progenitors produced only 33 ± 1.8 colonies/300 cells. CFU-GM-derived colony formation was inhibited by up to 50% compared to the control, and, surprisingly, CFU-Mix/BFU-E-derived colony formation was almost completely inhibited by the Pax5 transgene (Fig. 5a).

In the coculture system, the entire medium containing non-adherent and loosely attached cells was harvested weekly for cell counting, and an equal volume of fresh medium was added. Changes in the non-adherent cell number are shown in Fig. 5b. In the control culture, the number of non-adherent cells reached a maximum during the third week and gradually decreased thereafter. In the Pax5-transduced culture, the cell number was markedly reduced and almost negligible after 4 weeks. Non-adherent cells after 1 week in the control culture consisted of various differentiation stages of myeloid cells with a normal appearance. In contrast, Pax5-transduced cells consisted primarily of immature, dysplastic myeloid cells and also contained slightly dysplastic neutrophils (Fig. 5c).

On day 7, Pax5-transduced adherent cells were fewer in number and smaller in size than control cells. After 6 weeks of coculture, the difference in the adherent cell number became much larger. In the control culture, small B-lymphoid cells vigorously proliferated and formed massive cobblestone areas beneath the HESS-5 layer, whereas, in the Pax5-transduced culture, adherent cells were sparse and did not form any definite cobblestone area (Fig. 5d). The total number of adherent cells was $15.5 \pm 0.65 \times 10^5$ cells in the control culture and $0.65 \pm 0.26 \times 10^5$ cells ($5.2 \pm 3.8\%$ of the control) in the Pax5-transduced culture (Fig. 5e). However, the remaining Pax5-transduced cells expressed CD20 and the Ig light chain at rather higher levels than control cells, suggesting intact maturation (Fig. 5f).

During the first 3 weeks of coculture, cell cycle analysis of the infected cells, most of which had differentiated into myeloid cells, showed that the G2/M population of the Pax5-transduced culture was nearly equal in size to that of the control. Meanwhile, the ratio of apoptotic cells in the Pax5-transduced culture gradually increased compared with that in the control culture (Fig. 6a). In spite of the sorting procedure prior to coculture, contaminated, nontransduced cells became dominant after 4 weeks. The percentage of EGFP^{+} cells in the Pax5-transduced culture was 80% in the first week, but decreased to 50% in the fourth week (data not shown). Consequently, we conducted two-color analysis of the 4-week cultured cells using IntraPrep[TM] Permeabilization Reagent, which allows the retention of cytosolic EGFP and PI-based DNA staining. Cell cycle analysis of developing lymphoid cells showed that the G2/M population in the Pax5-transduced culture was nearly equal in size to that of the control. However, the ratio of apoptotic cells in the Pax5-transduced culture increased slightly as compared with that in the control culture (Fig. 6b).

4 Discussion

The detection and tracking of genetically manipulated human B-lymphoid progenitors in vitro not only promotes our understanding of the physiological mechanisms underlying human B-cell development, but will also aid in elucidating the pathological processes involved in congenital and acquired disorders. Information on early B lymphopoiesis in humans substantially lags behind that in mice, and important differences exist between the two species. For example, interleukin-7 can support this process in mice [22, 23], but not in humans [24, 25]. No combination of cytokines has been identified that stimulates human B-cell production in culture. Thus, the most effective models for human B lymphopoiesis employ murine stromal cells, which provide, as yet, uncharacterized stimuli [26].

CBCD34^{+} cells are heterogeneous, and most of them express CD33. Early B-lymphoid progenitors are included in a CD34^{+}CD33^{−}CD19^{−} minor fraction. Because E2A is commonly expressed in early B-lymphoid [27] and myeloid progenitors of rats [28] and mice [29], its high-level expression on day 1 in CD34^{+} cells is reasonable. In murine experiments, EBF is primarily expressed around the proB cell stage, and Pax5 can be seen from the late proB to mature B-cell stage [30]. Their low level of expression during the first 2 weeks of culture seems to reflect the quite rare population in this stage among CB progenitors.

Fig. 5 a *White box* total number of control colonies, *black box* total number of Pax5 colonies, *slanted box* BFU-E colony plus mixed colony numbers, *gray box* CFU-GM colony number. Three hundred control and Pax5 cells were seeded, and the colony number was counted 14 days later (the data shown are representative of four independent experiments). **b** The total weekly number of non-adherent cells, control cells (*open circles*) versus Pax5 cells (*filled circles*). The number of Pax5 cells was consistently lower than that of control cells. **c** Surface marker and morphology of myeloid cells after 1 week of coculture. EGFP⁺ myeloid cells were stained with PE-CD33 and APC-CD19 and examined by three-color FCM. A minor but significant fraction of non-adherent cells could be detected in the lymphoid gate of the cell population transduced with Pax5 vector, but not the control vector. M-G-stained cytospin preparations revealed the myelogenetic maturation arrest of Pax5 cells (×600). One representative result of three repeat experiments is shown. **d** Image capture with an inverted Nikon TMD300 microscope after 1 and 6 weeks of coculture (×400). The *cobblestone area* generated by Pax5 cells was markedly reduced. **e** Total cell number of control and Pax5 cells on day 42 ($n = 3$). **f** Living EGFP⁺ cells from the Pax5-transduced CD34⁺ cells expressed CD20 at a higher level than the control. A small number of EGFP⁺ cells also expressed the kappa light chain (these data are representative of three independent experiments)
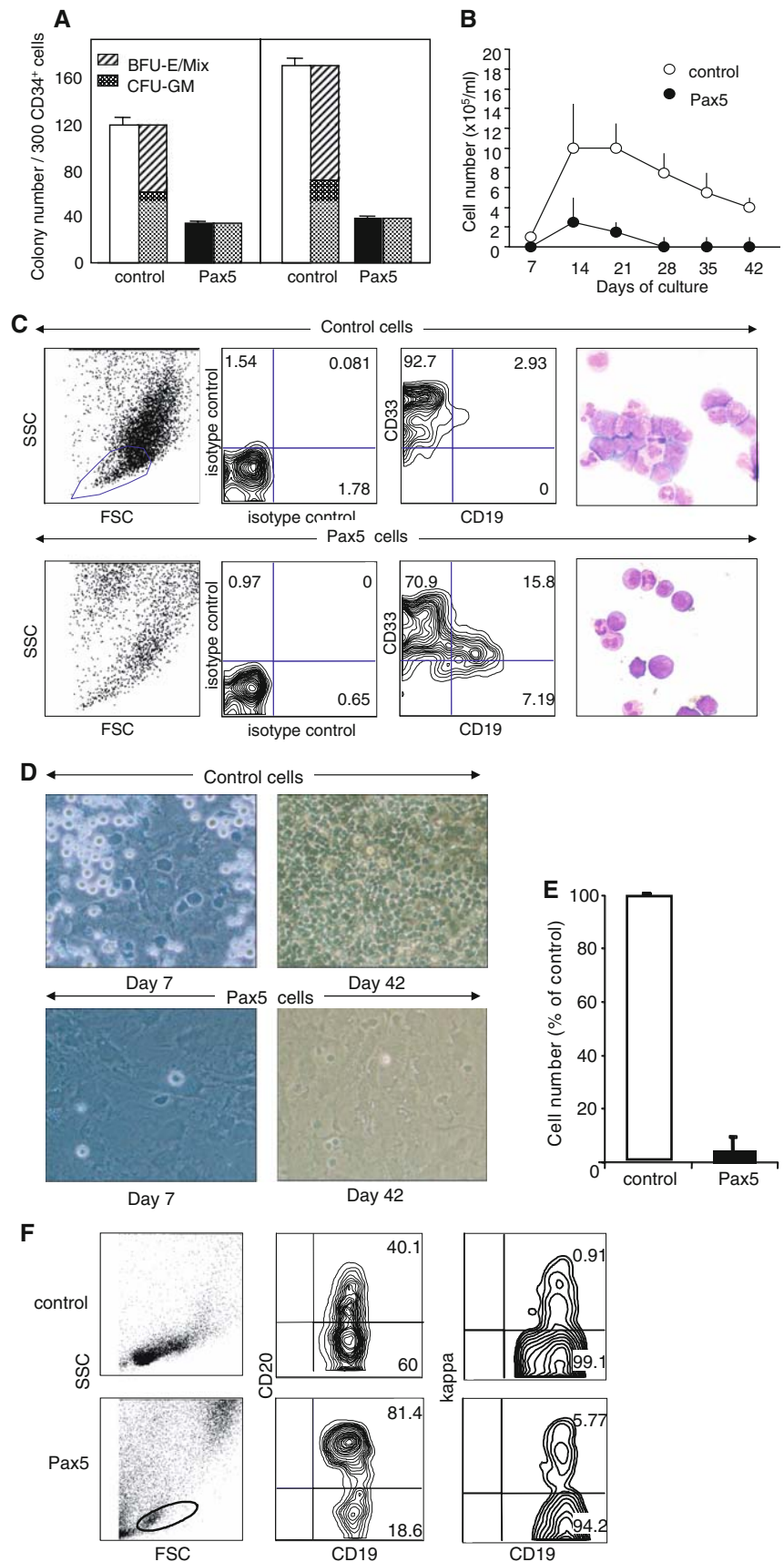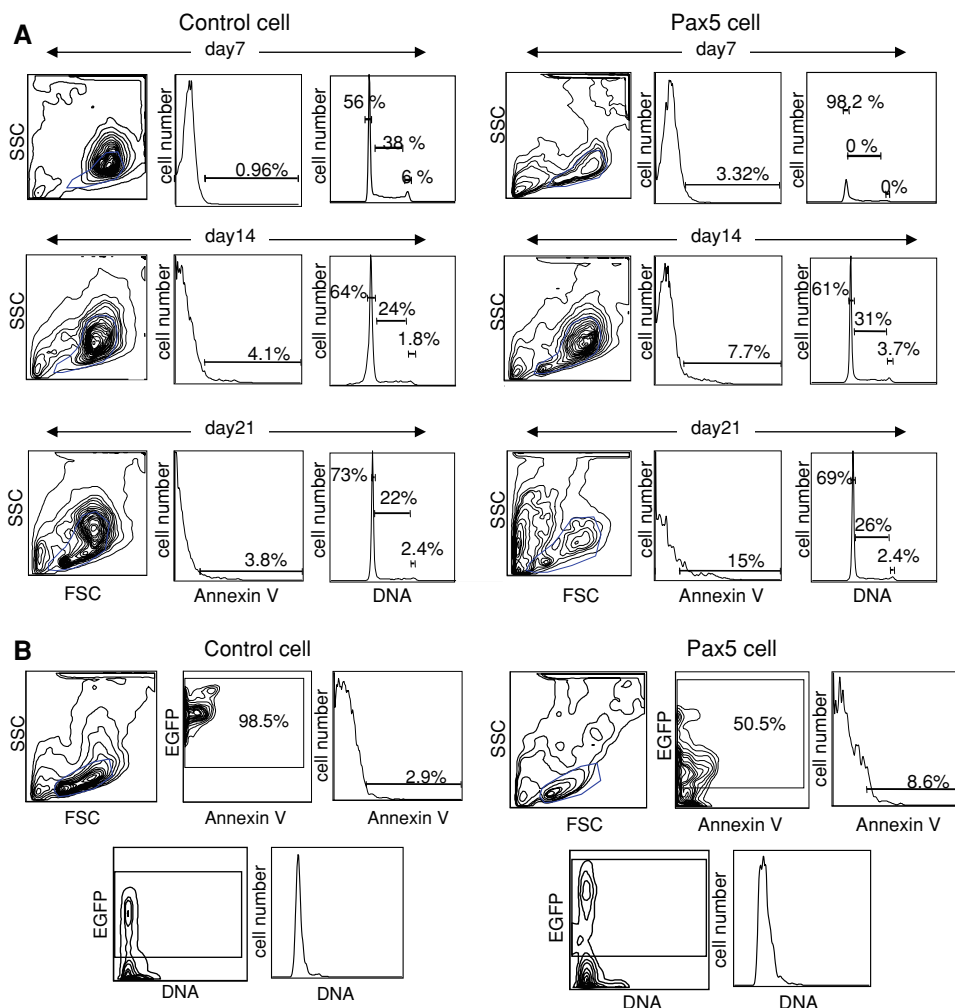
Fig. 6 a Cell cycle analysis
using 70% ethanol fixation and
the apoptosis assay, comparing
Pax5 cells with control cells on
days 7, 14, and 21. **b** Cell cycle
analysis of EGFP-positive cells
using formaldehyde fixation and
the apoptosis assay, comparing
Pax5 cells with control cells on
day 28

However, RAG1 is expressed as early as in common lymphoid progenitors, transiently down-regulated after completion of the IgH gene rearrangement in the proB cell stage, and upregulated again at the preB cell stage, until completion of the IgL gene rearrangement [31, 32]. Based on our data from FCM and QR-PCR, human B lymphopoiesis simulated in vitro occurs as follows: before day 14, most B-lymphoid progenitors are in the pre-proB cell or earlier stages. Around day 21, mixed populations of proB and large preB cells occur. After day 28, small preB cells are increasing, and, on day 42, immature B-cells are developing. This is consistent with the report of Fluckiger et al. [33] who showed that the development of surface IgM$^+$ immature B-cells delimited the differentiation stage in a coculture system. Despite such a restriction, the present coculture system is a unique and suitable procedure for analyzing the early B lymphopoiesis in humans.

We verified the efficient retroviral transduction of both B-lymphoid and myeloid progenitors and stable expression of the foreign gene during their differentiation. We tested three types of vector containing different LTR sequences.

Although the expression level of EGFP mediated by each vector was comparable in primary CD34$^+$-cell populations, pMXs-mediated EGFP expression was more prominently reduced in myeloid and B-lymphoid cells compared to pMYs and pMCs. The use of Plat-F packaging cells facilitated a >85% transduction efficiency with a single 24-h infection protocol and >90% with a 4×-repeated infection protocol. Additionally, an adequate EGFP expression level was maintained in transduced cells for up to 9 weeks of culture. It has been reported that pMXs-mediated gene expression, and possibly pMYs (MSCV), was down-regulated in mature blood cells including erythroid, myeloid, and B-lymphoid cells after an in vivo, long-term BM repopulation [14]. Collectively, a combination of pMCs-Ig and Plat-F is valuable for genetically engineering human CD34$^+$ cells and their progeny in vitro.

Using this system, we examined whether ectopic Pax5 expression in CBCD34$^+$ cells would interfere with myeloid cell commitment and/or accelerate B-cell differentiation. Chiang and Monroe [34] showed that Pax5 inhibited the expansion of myeloid progenitors by suppressing their

response to myeloid growth factors and by directing multipotent progenitors toward the B-cell lineage in vitro. Cotta et al. [35] reported that Pax5 is not sufficient to block myeloid development in vivo when ectopically expressed in primitive, multipotent progenitors. Recently, Anderson et al. [36] demonstrated that the ectopic expression of Pax5 in multipotent stem/progenitor cells generated B220$^+$Gr1/ Mac1$^+$ biphenotypic progenitors, which sustained the long-term generation of myeloid progenitors in vitro and remained capable of myeloid differentiation. All of these studies were performed on murine stem/progenitor cells, and did not refer to the impact of the Pax5 transgene on inhibiting B-cell development. Our data indicated that Pax5 markedly inhibited myelopoiesis. Specifically, the clonal growth of common myeloid (CFU-Mix) and early erythroid (BFU-E) progenitors was almost completely inhibited. In the coculture assay, granulocytic differentiation during the first week was partially arrested. CD19 expression could be induced by Pax5 in a significant portion of immature myeloid cells, as was described in our previous report [37], and these cell populations rapidly disappeared from the culture. Assuming that these populations were human counterparts of B220$^+$Gr1/Mac1$^+$ biphenotypic progenitors, our culture conditions were not optimized to support their survival. Additionally, we examined the development of these biphenotypic progenitors in a lymphoid-directed cytokine condition [36] with HESS-5 support. The percentage of biphenotypic progenitors increased up to 70% in the second week, but we could not maintain them for more than 3 weeks (data not shown). We hypothesize that ectopic Pax5 may induce CD19 expression in myeloid progenitors and maintain its expression during the early phase of granulocytic differentiation. However, the differentiation of Pax5$^+$ myeloid cells seems to be blocked at the promyelocytic stage, and may undergo apoptosis instead of terminal differentiation. The results of cell cycle analysis and apoptosis assay support this hypothesis.

Although the mechanism by which Pax5 inhibits myelopoiesis is unknown, cross-talk appears to occur between Pax5 transcription factors, including GATA and C/EBP family members [10], among which Pax5 is likely to be associated with PU.1 [38], a transcription factor essential for myelopoiesis. The entrapment of PU.1 by ectopic Pax5 in myeloid progenitors may interfere with their growth and differentiation.

One of the unexpected findings of this study was the marked inhibition of B lymphopoiesis by ectopic Pax5. In the cell cycle analysis and apoptosis assay, ectopic Pax5-transduced B-lymphoid cells generated similar results to control cells. Inhibition apparently occurred during the first week of culture, when the expression of endogenous Pax5 was barely detectable but E2A was strongly expressed. The reduced number of early B-cell progenitors may decrease

the production of B-cells without the impairment of differentiation. A defined hierarchy exists within B-cell-specific transcription factors, and endogenous Pax5 is induced by a coordinated action of E2A and EBF. Although we could not examine changes in the expression of endogenous transcription factors brought about by ectopic Pax5, negative feedback may occur from Pax5 to E2A, resulting in the impairment of further B-cell development. The conditional expression of Pax5, for example, by the use of estrogen receptor fusions during B-cell differentiation in combination with single cell sorting, will likely be required to examine the precise mode of action of ectopic Pax5. Nevertheless, this is the initial study to report the consequences of forced Pax5 expression in human hematopoietic progenitors.

References

1. Schebesta M, Heavey B, Busslinger M. Transcriptional control of B-cell development. Curr Opin Immunol. 2002;14:216–23.
2. Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature. 1999;401:556–62.
3. Okabe T, Watanabe T, Kudo A. A pre-B- and B cell-specific DNA-binding protein, EBB-1, which binds to the promoter of the VpreB1 gene. Eur J Immunol. 1992;22:37–43.
4. Schebesta M, Pfeffer PL, Busslinger M. Control of pre-BCR signaling by Pax5-dependent activation of the BLNK gene. Immunity. 2002;17:473–85.
5. Nutt SL, Urbanek P, Rolink A, Busslinger M. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. 1997;11:476–91.
6. Vormoor J, Lapidot T, Pflumio F, et al. Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficient mice. Blood. 1994;83:2489–97.
7. Rawlings DJ, Quan S, Hao QL, et al. Differentiation of human CD34+CD38− cord blood stem cells into B cell progenitors in vitro. Exp Hematol. 1997;25:66–72.
8. Vodyanik MA, Bork JA, Thomson JA, Slukvin II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood. 2005;105:617–26.
9. Haddad R, Guardiola P, Izac B, et al. Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. Blood. 2004;104:3918–26.
10. Hsu CL, King-Fleischman AG, Lai AY, Matsumoto Y, Weissman IL, Kondo M. Antagonistic effect of CCAAT enhancer-binding protein-alpha and Pax5 in myeloid or lymphoid lineage choice in common lymphoid progenitors. Proc Natl Acad Sci USA. 2006;103:672–7.
11. Guenechea G, Gan OI, Inamitsu T, et al. Transduction of human CD34+ CD38− bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. Mol Ther. 2000;1:566–73.

12. Gatlin J, Melkus MW, Padgett A, Kelly PF, Garcia JV. Engraftment of NOD/SCID mice with human CD34(+) cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. J Virol. 2001;75:9995–9.

13. Challita PM, Skelton D, el-Khoueiry A, Yu XJ, Weinberg K, Kohn DB. Multiple modifications in *cis* elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. J Virol. 1995;69:748–55.

14. Cheng L, Du C, Lavau C, et al. Sustained gene expression in retrovirally transduced, engrafting human hematopoietic stem cells and their lympho-myeloid progeny. Blood. 1998;92:83–92.

15. Pawliuk R, Eaves CJ, Humphries RK. Sustained high-level reconstitution of the hematopoietic system by preselected hematopoietic cells expressing a transduced cell-surface antigen. Hum Gene Ther. 1997;8:1595–604.

16. Persons DA, Allay JA, Allay ER, et al. Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors in vitro and identification of genetically modified cells in vivo. Blood. 1997;90:1777–86.

17. Dorrell C, Gan OI, Pereira DS, Hawley RG, Dick JE. Expansion of human cord blood CD34(+)CD38(−) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood. 2000;95:102–10.

18. Kitamura T, Koshino Y, Shibata F, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol. 2003;31:1007–14.

19. Yang AX, Mejido J, Luo Y, et al. Development of a focused microarray to assess human embryonic stem cell differentiation. Stem Cells Dev. 2005;14:270–84.

20. Gibson SE, Dong HY, Advani AS, Hsi ED. Expression of the B cell-associated transcription factors PAX5, OCT-2, and BOB.1 in acute myeloid leukemia: associations with B-cell antigen expression and myelomonocytic maturation. Am J Clin Pathol. 2006;126:916–24.

21. Tiacci E, Pileri S, Orleth A, et al. PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. Cancer Res. 2004;64:7399–404.

22. Grabstein KH, Waldschmidt TJ, Finkelman FD, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J Exp Med. 1993;178:257–64.

23. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med. 1995;181:1519–26.

24. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T(−)B(+)NK(+) severe combined immunodeficiency. Nat Genet. 1998;20:394–7.

25. LeBien TW. Fates of human B-cell precursors. Blood. 2000;96:9–23.

26. Dittel BN, LeBien TW. The growth response to IL-7 during normal human B cell ontogeny is restricted to B-lineage cells expressing CD34. J Immunol. 1995;154:58–67.

27. Bain G, Maandag EC, Izon DJ, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell. 1994;79:885–92.

28. Xin XQ, Nelson C, Collins L, Dorshkind K. Kinetics of E2A basic helix-loop-helix-protein expression during myelopoiesis and primary B cell differentiation. J Immunol. 1993;151:5398–407.

29. Voronova AF, Lee F. The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. Proc Natl Acad Sci USA. 1994;91:5952–6.

30. Kee BL, Quong MW, Murre C. E2A proteins: essential regulators at multiple stages of B-cell development. Immunol Rev. 2000;175:138–49.

31. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. 1990;248:1517–23.

32. Burrows PD, Stephan RP, Wang YH, Lassoued K, Zhang Z, Cooper MD. The transient expression of pre-B cell receptors governs B cell development. Semin Immunol. 2002;14:343–9.

33. Fluckiger AC, Sanz E, Garcia-Lloret M, et al. In vitro reconstitution of human B-cell ontogeny: from CD34(+) multipotent progenitors to Ig-secreting cells. Blood. 1998;92:4509–20.

34. Chiang MY, Monroe JG. BSAP/Pax5A expression blocks survival and expansion of early myeloid cells implicating its involvement in maintaining commitment to the B-lymphocyte lineage. Blood. 1999;94:3621–32.

35. Cotta CV, Zhang Z, Kim HG, Klug CA. Pax5 determines B- versus T-cell fate and does not block early myeloid-lineage development. Blood. 2003;101:4342–6.

36. Anderson K, Rusterholz C, Mansson R, et al. Ectopic expression of PAX5 promotes maintenance of biphenotypic myeloid progenitors coexpressing myeloid and B-cell lineage-associated genes. Blood. 2007;109:3697–705.

37. Sekine R, Kitamura T, Tsuji T, Tojo A. Identification and comparative analysis of Pax5 C-terminal isoforms expressed in human cord blood-derived B cell progenitors. Immunol Lett. 2007;111:21–5.

38. Linderson Y, Eberhard D, Malin S, Johansson A, Busslinger M, Pettersson S. Corecruitment of the Grg4 repressor by PU.1 is critical for Pax5-mediated repression of B-cell-specific genes. EMBO Rep. 2004;5:291–6.