Notch-Induced hIL-6 Production Facilitates the Maintenance of Self-Renewal of hCD34⁺ Cord Blood Cells Through the Activation of Jak-PI3K-STAT3 Pathway

Bongkum Choi,^{*†} Eunyoung Chun,[‡]
So Yong Kim,[‡] Miyoung Kim,[‡] Ki-Young Lee,[‡] and
Sung Joo Kim^{*†}

From the Departments of Molecular Medicine* and Surgery,[†]
Samsung Medical Center, Sungkyunkwan University School of
Medicine, Seoul; and the Department of Molecular Cell Biology,[‡]
Samsung Biomedical Research Institute, Sungkyunkwan University
School of Medicine, Suwon, South Korea

Ex vivo expansion of CD34⁺ stem cells in contact culture between hCD34⁺CD38[−]Lin[−] cord blood stem cells and human delta-like-expressing AFT024 feeder cells revealed increased amounts of stemness-related proteins such as HoxB4, GATA2, Bmi-1, and p21 and anti-apoptotic proteins such as Bcl-2, Bcl-xL, Mcl-1, and phospho-Bad, when compared with control or noncontact culture. Production of human IL-6 (hIL-6) was markedly elevated in the culture, but was profoundly inhibited by treatment with γ -secretase inhibitor. In addition, Notch-induced activation of STAT3 was directly involved in gene expression of hIL-6 and soluble hIL-6R α , indicating the close linkage between Notch signaling and hIL-6 production. Furthermore, depletion of soluble hIL-6 (with hIL-6-specific antibodies) and inhibition of IL-6-mediated signals (with a Jak1 inhibitor and wortmannin) severely affected the maintenance of self-renewal of hCD34⁺ cord blood cells. It was also observed that the *ex vivo* expanded CD34⁺ cord blood cells were induced to reconstitute human immune cells in nonobese diabetic mice with severe combined immunodeficiency when compared with freshly isolated CD34⁺ cord blood cells. Together, these results strongly demonstrate that Notch signaling in the “cell-to-cell contact” between hCD34⁺ cord blood and delta-like-expressing AFT024 feeder cells facilitates maintenance of self-renewal of hCD34⁺ cord blood cells through direct regulation of hIL-6 production. (*Am J Pathol* 2012, 180:351–364; DOI: 10.1016/j.ajpath.2011.09.030)

Hematopoietic progenitor CD34⁺ cells derived from umbilical cord blood are a feasible source of stem cells for allogeneic stem cell transplantation, primarily in patients who lack an HLA-matched marrow donor. The potential advantages of this technique include time saving, ease of treatment, decreased risk, and ease of collection.^{1,2} If a child's umbilical cord blood has been stored, there is immediate availability of matching stem cells for that child, eliminating the need to search for a suitable bone marrow donor. If another family member becomes ill, the disease can be treated using cord blood stem cells, and, moreover, there is also a chance that the child's sample may be a perfect match for the patient. In addition, stem cells derived from umbilical cord blood have a better chance of being an acceptable match because they are immunologically immature and undeveloped, in particular, in comparison with other sources of adult stem cells such as bone marrow or peripheral blood. Despite these advantages, it remains difficult to proceed with many biomedical and clinical studies because of the small number of nucleated cells and CD34⁺ cells in a single cord blood collection. Thus, strategies to expand self-renewing CD34⁺ stem cells from umbilical cord blood is an area of active research. Some earlier studies have

Supported by the Mid-Career Researcher Program through National Research Foundation of Korea grants 2009-0084573 (K.-Y.L.) and 2009-0083790 (S.J.K.) funded by the Ministry of Education, Science, and Technology.

Accepted for publication September 12, 2011.

B.C. and E.C. contributed equally to this work.

Supplemental material for this article can be found on <http://ajp.amjpathol.org> or at doi: 10.1016/j.ajpath.2011.09.030.

Current address of E.C.: Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts.

Address reprint requests to Ki-Young Lee, Ph.D., Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea, or Sung Joo Kim, M.D., Ph.D., Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea, E-mail: thylee@skku.edu or kmhyj111@skku.edu.

pointed out the requirement for cytokines such as human thrombopoietin, human FMS-like tyrosine kinase-3 (hFlt-3) ligand, human erythropoietin, human granulocyte macrophage–colony-stimulating factor (hGM-CSF), human IL-3 (hIL-3), and human IL-6 (hIL-6) for *ex vivo* expansion of CD34⁺ cord blood cells.^{3–8} In addition, several distinct molecular pathways such as Notch,^{9,10} Wnt,^{11,12} multifunctional bone morphogenetic protein,¹³ and the receptor tyrosine kinase Tie2¹⁴ are involved in expanding the stem cell niche in cultures, eventually regulating the cell proliferation and maintenance of stemness. Despite increased knowledge of the requirements for providing efficient *ex vivo* expansion of CD34⁺ cord blood stem cells, substituting a hematopoietic niche environment seems to be insufficient for promoting self-renewal and progenitor cell expansion without differentiation. Thus, understanding the detailed cellular and molecular mechanisms related to the cytokines and the aforementioned signaling pathways might be critical for optimizing conditions for hematopoietic stem cell culture.

To enhance the *ex vivo* expansion of CD34⁺ cord blood stem cells, we used an *ex vivo* culture condition called “cell-to-cell contact.” To provide a Notch signal to the stem cells, we chose a murine fetal liver stromal cell line (AFT024) that preferentially expresses human delta-like (hdk) protein in the form of Notch ligand and is also a regulator of positive stem cells and human primitive myeloid and lymphoid progenitor cells.^{15–17} The AFT024 feeder cells were also used for the study, separately from the stem cells, but in a manner in which they can interact with each other via high pore density polyethylene terephthalate track-etched membranes. We observed that cell-to-cell contact can induce activation of Notch-mediated signaling and production of IL-6, thereby facilitating proliferation and self-renewal of CD34⁺ cord blood stem cells. It was also observed that hCD34⁺ cord blood cells *ex vivo* expanded in cell-to-cell contact culture have the ability to reconstitute human immune cells *in vivo*. The results strongly suggested that cell-to-cell contact can improve current *ex vivo* expansion protocols and that the Notch signal provided in the culture has an essential role in maintenance of self-renewal of CD34⁺ cord blood stem cells via production of hIL-6.

Materials and Methods

Isolation of Human CD34⁺CD38[−]Lin[−] Cord Blood Cells and *ex Vivo* Expansion

According to the guidelines established by Samsung Medical Center (Seoul, Korea), human cord blood samples were acquired from normal full-term deliveries after obtaining informed parental consent. MNCs were isolated via Ficoll-Hypaque density gradient centrifugation. Subsequently, hCD34⁺ cells were purified from total MNCs using the MACS hCD34 MicroBead Kit in an autoMACS Cell Separator (both from Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. The purity of the sample was estimated at flow cytometry using antibodies specific for

hCD34 (BD Pharmingen, BD Biosciences, Franklin Lakes, NJ), hCD38 (eBiosciences, Inc., San Diego, CA), and hLin (Becton Dickinson & Co., Franklin Lakes, NJ). A purity index greater than 98% pure hCD34⁺CD38[−]Lin[−] cells was used for the *ex vivo* expansion culture in the present study (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). For *ex vivo* expansion culture, hdk-expressing AFT024 cells were seeded on the back side of high pore density polyethylene terephthalate track-etched 12-well membranes (1.0 × 10⁸/cm² density, 0.4-μm pore size) of the insert chamber plate (the “contact culture”) or the bottom of 12-well plates (Multiwell; BD Biosciences, Discovery Labware, Franklin Lakes, NJ) (the “noncontact culture”). The seeded AFT024 cells were cultured for 2 days at 33°C under 5% CO₂ atmosphere. As a confluent feeder layer, the cells were treated with 10 μg/mL mitomycin C (Sigma-Aldrich Corp., St. Louis, MO) for 2 hours at 37°C. The cells were then washed more than five times using 1X PBS (HyClone Laboratories, Inc., Logan, UT). Purified hCD34⁺CD38[−]Lin[−] cord blood cells (2 × 10⁴) were plated into the inserted chamber plates and cultured using α-minimal essential medium supplemented with 20% fetal bovine serum (both from HyClone Laboratories), 1% bovine serum albumin (Sigma-Aldrich Corp.), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco-BRL, Invitrogen Corp., Carlsbad, CA). The recombinant human cytokine cocktails contained 50 ng/mL stem cell factor, 50 ng/mL thrombopoietin, 50 ng/mL Flt-3 ligand, 10 ng/mL GM-CSF, 3 U/mL erythropoietin, and 20 ng/mL IL-3. These cytokines were purchased from R&D Systems, Inc. (Minneapolis, MN). The cultures were incubated for both 7 and 14 days.

Immunofluorescence Staining

The AFT024 cells were plated on chamber slides (Lab-Tek II; Nalge Nunc International, Rochester, NY), cultured for 2 days at 33°C, fixed with 4% paraformaldehyde, permeabilized with ice-cold conditioned ethanol and acetic acid buffer, and further incubated at −20°C for 30 minutes. After the cells were washed with 1X PBS containing 0.1% Triton X-100, the slides were incubated using goat polyclonal anti-hdk antibody (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. The slides were washed, and subsequently FITC-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Inc.) was added, and the slides were incubated in the dark for 1 hour. After washing, the slides were mounted and observed under a light microscope (Olympus BX40; Olympus Corp., Tokyo, Japan) with ×10/22 numeric aperture and ×40/0.75 numeric aperture objective.

Flow Cytometric Analysis

Cells were prepared and stained with FITC-conjugated anti-human lineage cocktail 1 (Becton Dickinson & Co.), phycoerythrin (PE)–conjugated anti-hCD34 (Becton Dickinson & Co.), and PE-cyanine 5–conjugated or PE-cyanine 7–conjugated anti-hCD38 (eBiosciences, Inc.,

San Diego, CA) monoclonal antibodies at 4°C for 30 minutes. To ensure specificity, isotype-negative control was prepared, and cells were stained with FITC-, PE-, and PE-cyanine 5- or PE-cyanine 7 mouse IgG1k antibodies. The unbound antibodies were removed by washing, and the cells were resuspended in 1X PBS buffer supplemented with 2 mmol/L EDTA and 2% fetal bovine serum. The cells were acquired using a FACSAria cell counter and analyzed using FACSDiva software (both from Becton Dickinson & Co.).

Clonal Culture Assay

Ex vivo expanded hCD34⁺ cord blood cells were harvested and aliquoted into Human Methylcellulose Complete Media (R&D Systems, Inc.) at a concentration of 5×10^2 cells/mL. One milliliter culture mixture contained cells and recombinant human cytokines: 50 ng/mL stem cell factor, 10 ng/mL GM-CSF, 10 ng/mL IL-3, and 3 U/mL erythropoietin. The mixture was cultured in 35-mm culture dishes with 2 × 2-mm grids (Nalge Nunc International) at 37°C under 5% CO₂ for 14 days. All cultures were set up in triplicate. Total colony-forming units in culture and mixed colonies containing erythroid and myeloid cells and megakaryocytes consisting of 40 or more cells were scored under an inverted microscope. To assess the accuracy of *in situ* identification of the colonies, individual colonies were lifted using an Eppendorf micropipette under direct microscopic visualization, spread on glass slides using a cytocentrifuge, and stained with May-Grünwald-Giemsa.

Screening of Secreted Cytokines and Growth Factors Using ELISA

After 14 days of culture, supernatants were harvested from *ex vivo* expanded cultures and stored at -70°C. For analysis using an enzyme-linked immunosorbent assay (ELISA), samples were thawed, and various cytokines and growth factors were measured according to the manufacturer's instructions: Human Stem Cell Factor ELISA Development Kit (PeproTech Asia, Rehovot, Israel); hIL-6 and human interferon- γ ELISA Ready-SET-Go (eBiosciences, Inc.); hIL-6-soluble receptor, human erythropoietin, human thrombopoietin, hIL-3, hFlt-3 ligand, hGM-CSF, human tumor growth factor- β , and human fibroblast growth factor DuoSet ELISA Development System (R&D Systems, Inc.). In brief, each capture antibody was diluted using 1X PBS and coated in 96-well immunoplates (Nunc A/S, Roskilde, Denmark), and incubated overnight at either room temperature or 4°C. The plates were washed five times with washing buffer (1X PBS containing 0.05% Tween 20) and blocked using blocking buffer (1% bovine serum albumin in 1X PBS) for 1 hour at room temperature. Cultured supernatants were diluted 10-fold in blocking buffer and were loaded onto the immunoplates for 2 hours at room temperature. After incubation, plates were washed using washing buffer, and diluted antibodies were loaded into each well of the immunoplate and incubated at room temperature for 1 to 2 hours. After

washing five times, avidin-horseradish peroxidase was added to the wells of the immunoplate, and the plate was incubated at room temperature for 30 minutes. The reaction plates were washed, and tetramethylbenzidine was added as a substrate solution. The reaction was stopped via addition of 2N H₂SO₄. The plates were read at 450 nm using a multiwell plate reader. Measurements were performed in triplicate.

Western Blot Analysis

At the end of *ex vivo* expansion under various culture conditions, cells were harvested and lysed using lysis buffer [50 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing 20 mmol/L NaH₂PO₄, 1 mmol/L Na₃VO₄, 4 mmol/L benzamide, 0.2 mmol/L AEBSF (Pefabloc; Sigma-Aldrich Corp.), 50 mmol/L β -glycerol phosphate, 5 μ g/mL aprotinin, and 5 μ g/mL leupeptin at 4°C for 20 minutes. Protein concentrations were measured using a spectrophotometer (NanoDrop; Thermo Scientific, Inc., Waltham, MA). For Western blot analysis, equal amounts of extracted protein (50 μ g) were subjected to 8% to 12% SDS-PAGE, and transferred to polyvinylidene difluoride or 0.45- μ m pure nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (all from Bio-Rad Laboratories, Inc., Hercules, CA). The membranes were blocked using 5% bovine serum albumin in 1X Tris-buffered saline solution [20 mmol/L Tris HCl (pH 7.6) plus 137 mmol/L NaCl] containing 0.1% Tween 20 for 1 hour at room temperature, and were incubated using primary antibodies in antibody dilution buffer (5% bovine serum albumin in Tris-buffered saline solution-Tween 20) overnight at 4°C with gentle shaking. For Western blot analysis, the following human-specific primary antibodies were used: mouse monoclonal anti-gp130 (Santa Cruz Biotechnology, Inc.), mouse polyclonal anti-HoxB4 (Novus Biologicals LLC, Littleton, CO), and anti-PI3 kinase (BD Biosciences); rabbit polyclonal anti-GATA2, anti-Bmi-1, anti-Hes-1 (all three from Abcam, Ltd., Cambridge, England); rabbit polyclonal anti- β -actin (BioLegend, Inc., San Diego, CA); rabbit polyclonal anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1, anti-phospho-Bad (Ser136), anti-Jak1, anti-STAT3, anti-Akt, anti-mTOR, anti-p70S6 kinase, anti-Notch1, anti-cleaved Notch1 (Val1744), anti-phospho-PI3 kinase (Tyr458), anti-phospho-mTOR (Ser2448), and anti-phospho-p70S6 kinase (Ser371) (Cell Signaling Technology, Inc., Beverly, MA); rabbit polyclonal anti-PDK1, anti-phospho-PDK1 (Ser241), anti-phospho-Jak1 (Tyr1022), anti-phospho-Akt (Ser473), and phospho-STAT3 (Tyr705) (Signalway Antibody, Inc., Pearland, TX); rabbit polyclonal anti-phospho-gp130 (Ser782) (Santa Cruz Biotechnology, Inc.); and mouse/human/rat polyclonal anti- β -catenin and p21^{WAF1/Cip1} (eBiosciences, Inc.). Immunostained membranes were developed using Amersham ECL Plus Western blotting detection reagents (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, England), and were exposed to x-ray film (Agfa HealthCare NV, Mortsel, Belgium).

Ex Vivo Expanded Cell Viability Assay

The cell proliferation assay was performed using a Cell Death Detection ELISA Kit (Roche Diagnostics, Ltd., Basel, Switzerland) according to the manufacturer's instructions.

hIL-6 Neutralization Assay

For neutralization assay, cells were cultured without or with monoclonal anti-hIL-6 antibody (R&D Systems, Inc.) at concentrations of 0.001, 0.01, 0.1, 1, and 10 $\mu\text{g/mL}$ in cultured media. After expansion, cells were harvested and replated into 96-well plates (2×10^4 cells per well). Ten microliters Premixed WST-1 cell proliferation reagent (CloneTech Laboratories, Inc., Mountain View, CA) was added to each well, and the plates were incubated at 37°C for half an hour. The plates were read at 450 nm using a multiwall plate reader. For Western blot analysis, cells were harvested and lysed using lysis buffer.

Inhibition Assays Using Jak1 Inhibitor, Wortmannin, and γ -Secretase Inhibitor

Jak1 Inhibitor Assay

In the cell-to-cell contact culture, purified hCD34⁺CD38⁻Lin⁻ cord blood cells (2×10^4) were cultured without or with Jak1 inhibitor (Calbiochem, Darmstadt, Germany) at concentrations of 0.25, 0.5, 1, 5, 15, 25, and 35 nmol/L. After 14 days, hIL-6 and hslL-6R α concentrations were measured in the supernatants using an ELISA, as described previously. After 14 days, hCD34⁺ cord blood cells from ex vivo expansion culture were purified using an autoMACS Cell Separator (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer's instructions, and further incubated in the presence of 25 pg/mL recombinant hIL-6 and 200 pg/mL recombinant hslL-6R α (ProSpec-Tany TechnoGene, Ltd., Rehovot, Israel) for 24 hours. Cell lysates were subjected to Western blot analysis and probed using antibodies specific for STAT3 and phospho-STAT3 (Tyr705). For phenotypic analysis, purified hCD34⁺CD38⁻Lin⁻ cord blood cells (2×10^4) were cultured without or with 15 nmol/L Jak1 inhibitor in contact culture. After ex vivo expansion for 14 days, cultured cells were harvested, stained with antibodies specific for hCD34, hCD38, and hLin molecules, and analyzed using flow cytometry (see *Flow Cytometric Analysis*).

Wortmannin Treatment Assay

A concentration of 10 nmol/L wortmannin (Calbiochem) was added to culture media during ex vivo expansion in cell-to-cell contact culture. After 14 days of culture, the concentrations of hIL-6 in supernatant and cell proliferation were measured using an ELISA and PreMix WST-1 cell proliferation reagent (CloneTech Laboratories, Inc.), respectively, as previously described. For Western blot analysis, cultured cells were harvested and lysed using the lysis buffer. Cell lysates were subjected to Western blot analysis using antibodies specific for Akt, phospho-Akt, STAT3, and phospho-STAT3 (Tyr705). For phenotypic analysis, purified

hCD34⁺CD38⁻Lin⁻ cord blood cells (2×10^4) were cultured without or with 10 nmol/L wortmannin in contact culture. After ex vivo expansion for 14 days, cultured cells were harvested, stained with antibodies specific for hCD34, hCD38, and hLin, and analyzed using flow cytometry (see *Flow Cytometric Analysis*).

γ -Secretase Inhibitor Assay

γ -Secretase inhibitor (Calbiochem) at a concentration of 50 $\mu\text{mol/L}$ was added to the culture medium during ex vivo expansion in contact culture. After ex vivo expansion for 14 days, the concentrations of hIL-6 and hslL-6R α in supernatant and the cell proliferation were measured using an ELISA and PreMix WST-1 cell proliferation reagent (CloneTech Laboratories, Inc.), respectively. For Western blot analysis, cultured cells were harvested and lysed using lysis buffer. Cell lysates were subjected to Western blot analysis using antibodies specific for cleaved Notch1 (Val1744), Hes-1, STAT3, phospho-STAT3 (Tyr705), HoxB4, GATA2, and Bmi-1 proteins.

NOD-SCID Repopulating Cell (SRC) Assay

Nonobese diabetic mice with severe combined immunodeficiency (NOD-SCID mice) were purchased from Jackson Laboratory (Bar Harbor, ME) and were bred and maintained under specific pathogen-free conditions in accordance with the ethical guidelines for the care of these animals at the Laboratory Animal Research Center at Samsung Biomedical Research Institute (Seoul, Korea). NOD-SCID mice, approximately 6 to 8 weeks old, received 350 cGy total-body irradiation. hCD34⁺ cells at a density of 2×10^5 were purified from fresh cord blood cells or ex vivo expanded cells were transplanted into the irradiated mice via injection into the tail vein ($n = 6$ per group). The mice were sacrificed at 8 weeks after transplantation, and bone marrow from two femurs and two tibias of each mouse were flushed into RPMI 1640 medium containing 10% fetal calf serum. The cells were stained with the following antibodies: FITC-conjugated anti-hCD45, phycoerythrin-conjugated anti-hCD20, phycoerythrin-conjugated anti-CD34, phycoerythrin-cyanine 7-conjugated anti-hCD11c, and allophycocyanin-conjugated anti-hCD3 (all from eBiosciences, Inc.). Stained cells were analyzed using FACS Aria (Becton Dickinson & Co.). Ten thousand to one million events were acquired per sample, and were analyzed using a flow cytometry analysis program (FACSDiva software version 5.0.2; Becton Dickinson & Co.).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from each ex vivo expanded culture using a Nuclear Extraction Kit (Panomics, Inc., Fremont, CA). In brief, the cells were harvested and washed with ice-cold 1X PBS. The cell pellets were immediately resuspended in ice-cold buffer "A" working reagent (10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and phosphatase inhibitors), and the reaction was performed at 4°C on a rocking platform at 200 rpm for 10 minutes. Cells were

centrifuged and resuspended in supplied ice-cold buffer “B” working reagent (20 mmol/L HEPES, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and phosphatase inhibitors). Samples were incubated in an ice bucket on a rocking platform at 200 rpm for 2 hours and then centrifuged. The extracted nuclear proteins were used for the electrophoretic mobility shift assay. For the protein-DNA binding assay, 10 μ g extracted nuclear proteins were incubated with 1 μ g poly d(I-C) at room temperature for 5 minutes in 5X binding buffer (50 mmol/L HEPES, 50 mmol/L KCl, 5 mmol/L MgCl₂, 5 mmol/L dithiothreitol, 5 mmol/L EDTA, and 25% glycerol). Subsequently, 1 μ L biotin-labeled hSTAT3-specific probe was added, and the sample was incubated at 15°C for 30 minutes. After the reaction, loading dye was added, and the samples were loaded on 6% nondenaturing polyacrylamide gel in pre-chilled 0.5X TBE buffer (50 mmol/L Tris, 45 mmol/L boric acid, and 0.5 mmol/L EDTA). The gel was transferred onto the pre-shocked Biodyne B nylon membrane (Pall Corp., Port Washington, NY) at 300 mA for 30 minutes in 0.5X TBE, then fixed via UV cross-linking for 3 minutes. The biotin-labeled STAT3-specific probe was detected using streptavidin-horseradish peroxidase and chemiluminescent detection reagent, and the membrane was exposed to x-ray film (Agfa HealthCare NV).

Chromatin Immunoprecipitation and Quantitative RT-PCR

The chromatin immunoprecipitation assay was performed using the ExactaChIP human/mouse STAT3 chromatin immunoprecipitation kit (R&D Systems, Inc.) according to the manufacturer's instructions. In brief, 5 $\times$ 10⁶ cells were fixed using 1% formaldehyde at room temperature for 15 minutes, and then the cross-linking was stopped via addition of 125 mmol/L glycine. After fixing, cells were harvested, washed with 1X PBS, lysed using lysis buffer [50 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 1% Triton X-100, and 0.5% NP-40] containing protease inhibitors (10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride), and incubated on ice for 10 minutes. Cell lysates were sonicated and centrifuged at 12,000 $\times$ g for 10 minutes. The supernatants of the sonicated samples were diluted using dilution buffer [20 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.01% SDS, and 1% Triton X-100] supplemented with protease inhibitors (10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride) and incubated using biotin-labeled sheep polyclonal anti-human/mouse STAT3 antibody in an ultrasonic bath for 15 minutes. The supernatants were incubated at 4°C overnight using pre-cleared streptavidin agarose beads. The immunoprecipitated DNA was eluted using chelating resin solution (100 mmol/L NaHCO₃ and 1% SDS) and subjected to boiling. DNA samples were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and used as a template for RT-PCR. RT-PCR

was performed using the 7300 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). PCR primers were specifically designed as follows: for hIL-6, forward, 5'-AAGCCAGAGCTGTGCAGATGAGTA-3', and reverse, 5'-TGTCTCTGCAGCCACTGGTTC-3' (accession No. NM000600); for hIL-6R α , forward, 5'-TGAGATCAGCCTGGGCAATG-3', and reverse, 5'-GGACTTCAGGCACACTCTACCACA-3' (accession No. NM000565); and for human glyceraldehyde-3-phosphate dehydrogenase, forward, 5'-GCACCGTCAAGGCTGAGAAC-3', and reverse, 5'-TGGTGAAGACGCCAGTGGA-3' (accession No. NM002046). Total volume of 20 μ L RT-PCR mixtures contained 10 μ L SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Shiga, Japan), gene-specific primers (10 μ mol/L each), and dye. Purified DNA was plated in 96-well RT-PCR reaction plates. RT-PCR was performed using the following cycles: 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds and 56°C for 30 seconds. The samples were analyzed in triplicate, and the hIL-6 and hIL-6R α levels were normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase expression levels. Amplified products were separated on 1% agarose gels and stained with ethidium bromide. The stained gels were visualized using a Chemidoc UV Trans Illuminator (Bio-Rad Laboratories, Inc.).

Statistical Analysis

Data from the experiments are given as mean $\pm$ SD. The Student's *t*-test was used to calculate statistical differences, which were considered significant at *P* < 0.05 or *P* < 0.01. All statistical analyses were conducted using PRISM version 5.01 software (GraphPad Software, Inc., San Diego, CA).

Results

Cell-to-Cell Contact between hCD34⁺CD38⁻Lin⁻ Cord Blood Stem Cells and hdlk-Expressed AFT024 Feeder Cells Induces Cell Proliferation

To investigate whether cell-to-cell contact, which provides the Notch signal, can facilitate *ex vivo* expansion of CD34⁺ cord blood stem cells with self-renewal properties, we modified a previous culture system⁶ (Figure 1A). AFT024 feeder cells were attached on the opposite side of the insert membrane (contact culture) or on the bottom of the culture plate (noncontact culture). Inasmuch as many previous reports have demonstrated that Notch might be critically involved in maintenance of the hematopoietic stem cell pool,^{9,10,18} we used hdlk-expressing AFT024 feeder cells to provide the Notch signal to hCD34⁺ cord blood stem cells (Figure 1B). CD34⁺CD38⁻Lin⁻ cord blood cells were purified from umbilical cord blood, and their purity was verified to be >98% (Figure 1C; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). Using electron microscopy, we observed direct contact between CD34⁺ cord blood stem cells and cytoplasmic villi of AFT024 feeder cells, which had passed through the pores of

Figure 1. *Ex vivo* expansion of hCD34⁺CD38⁻Lin⁻ cord blood cells in cell-to-cell contact culture. **A:** Illustrations of *ex vivo* expansion conditions used in the present study. **B:** hdlk expression was analyzed in the AFT024 cells as described (see *Materials and Methods*). **C:** hCD34⁺CD38⁻Lin⁻ cord blood cells were isolated from human umbilical cord blood as described (see *Materials and Methods*). **D:** Electron microscopic views of the contact culture. Photographs represent views of cultured hCD34⁺CD38⁻Lin⁻ cord blood cells on the upper side of the membrane insert (**upper panel**) and of hdlk-expressing AFT024 cells on the back side of the membrane (**lower panel**). **E:** Purified hCD34⁺CD38⁻Lin⁻ cord blood cells at a density of 2×10^4 were cultured as described (see *Materials and Methods*). On days 7 and 14 after culture, MNCs were harvested and counted (**right panel**), and the fold increase was calculated (**left panel**). Bars represent mean $\pm$ SEM from three independent experiments. **F** and **G:** On days 7 and 14 after culture, cells were harvested, stained with antibodies specific for hCD34, hCD38, and hLin, and analyzed using flow cytometry. Each dot plot is representative of analysis of three independent cultures. The percentage of cells within each quadrant is indicated (representative of analysis of three independent cultures) (**F**). **G:** Fold increase and absolute number of hCD34⁺CD38⁻ cells are presented. Bars represent mean $\pm$ SEM from three independent experiments.

the membrane in the cell culture (Figure 1D). Purified CD34⁺CD38⁻Lin⁻ cord blood cells were co-cultured with soluble factors (see *Materials and Methods*) for 14 days under three different culture conditions: control culture (CD34⁺CD38⁻Lin⁻ cord blood cells alone), noncontact culture, and contact culture. After 7 and 14 days of culture, total MNCs in the upper insert membrane were harvested and counted. No significant differences in the number of total MNCs were detected between the various cultures (Figure 1E). However, when subset analysis was performed using antibodies specific for hCD34, hCD38, and hLin molecules, marked increases in the percentage of hCD34⁺Lin⁻ or hCD34⁺CD38⁺ cells were detected after 14 days in contact culture compared with other cultures: hCD34⁺Lin⁻ cells, 87% in contact culture versus 33% in noncontact culture or 29% in control culture; and hCD34⁺CD38⁻ cells, 49% in contact culture versus 12% in noncontact culture or 10% in control culture (Figure 1F). Moreover, significant increases in both fold increase and absolute number of hCD34⁺CD38⁻ cells were detected in the contact culture (Figure 1G). These results demonstrate that the contact culture between CD34⁺CD38⁻Lin⁻ cord blood cells and hdlk-expressing AFT024 feeder cells induces cell proliferation, although it is unclear whether the *ex vivo* expanded cells possess the self-renewal property.

Secreted hIL-6 Enhances Proliferation of hCD34⁺ Cord Blood Cells Through Activation of Jak1 and PI3K

Numerous previous studies have suggested important roles for soluble mediators capable of maintaining self-renewal properties including cell proliferation and *ex vivo* expansion of hematopoietic stem cells including CD34⁺ cord blood stem cells.^{3–8} Therefore, we wondered which kinds of soluble mediators existed in the cultures that contributed to proliferation of hCD34⁺ cord blood cells. To examine these issues, we first screened secreted cytokines in the culture supernatants. There were no significant changes in the levels of cytokines such as human thrombopoietin, hFlt-3 ligand, human erythropoietin, hGM-CSF, human fibroblast growth factor, human transforming growth factor- β , and human interferon- γ (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). In contrast, hIL-3 was significantly elevated in both noncontact and contact cultures with feeder cells but not in control culture (Figure 2A). Levels of hIL-6 (Figure 2B) and hIL-6R α (Figure 2C) were markedly increased in contact culture but were unchanged in noncontact and control cultures, indicating that hIL-6 may be strongly produced

Figure 2. Secreted hIL-6 enhances proliferation of hCD34⁺ cord blood cells. Secreted hIL-3 (**A**), hIL-6 (**B**), and hIL-6Rα (**C**) were measured in the supernatants of three different cultures (Figure 1A) using ELISA (see *Materials and Methods*). Data represent the mean $\pm$ SEM of three independent experiments (* $P < 0.01$). **D:** hIL-6 monoclonal antibodies were co-cultured in contact culture at the indicated concentrations. After *ex vivo* expansion for 14 days, expanded cells were harvested, and the proliferation assay was performed as described (see *Materials and Methods*). Cell lysates were subjected to Western blot analysis and probed with antibodies specific for Jak1, phospho-Jak1 (Tyr1022), PI3K, and phospho-PI3K (Tyr458).

by hCD34⁺ cord blood cells under the contact culture condition and, thereby, may be involved in proliferation of hCD34⁺ cord blood cells. To directly verify the role of secreted hIL-6, we performed hIL-6 depletion assays in contact culture. Depletion of hIL-6 with anti-hIL-6 monoclonal antibodies severely inhibited cell proliferation and activation of Jak1 and PI3K in a dose-dependent manner, indicating that secreted hIL-6 is involved in cell proliferation via the Jak1 and PI3K pathways (Figure 2D).

Based on these results, we asked two further questions. First, do the cultured supernatants derived from

the contact culture or does addition of exogenous hIL-6 itself have potential effects on proliferation and maintenance of hCD34⁺ cord blood cells? Second, is expression of hdlk on feeder cells critical for maintenance of hCD34⁺hCD38[−] cord blood cells. To initially verify the effects of soluble factors in *ex vivo* expansion of hCD34⁺ cord blood cells, the cultured supernatants were harvested from the cell-to-cell contact culture (Figure 1A) and then added to various noncontact conditions such as feeder-free hdlk-negative feeder cells (2018 cell line),^{19,20} or hdlk-expressing AFT024 feeder cells. After 7 days of culture, total MNCs were harvested and counted. No significant differences in terms of fold increases and absolute numbers of MNCs were detected (Figure 3, A and B). Moreover, no significant differences in hCD34⁺hCD38[−] cord blood cells after culture could be detected under these culture conditions (Figure 3, C and D; see also Supplemental Figure S3 at <http://ajp.amjpathol.org>), indicating that the cultured supernatants might be insufficient for inducing proliferation and maintenance of hCD34⁺ cord blood cells under the noncontact culture condition. Under the feeder-free culture condition, supplied with conventional culture media, significant increases in MNC levels could be detected, and was enhanced by addition of exogenous hIL-6 in the cultures (Figure 3, A–D). However, there were no observable significant effects that induced an increase in hCD34⁺hCD38[−] cells when compared with the culture without exogenous hIL-6 (Figure 3, C and D), indicating that exogenous hIL-6 in part induces proliferation of hCD34⁺ cord blood cells but not maintenance of hCD34⁺ cord blood cells. We next evaluated the effect of feeder cells. In the noncontact cultures with feeder cells such as Notch-negative 2018 and Notch-positive AFT024 cell lines, exogenous hIL-6 induced a significant increase in MNCs when compared with the cultures without exogenous hIL-6 (Figure 3, A and B). The pattern was similar to that of feeder-free cultures supplied with conventional culture media and/or exogenous hIL-6. However, a significant increase in the number of hCD34⁺hCD38[−] cells could be detected in the noncontact culture with AFT024 feeder cells but not in 2018 feeder cells (Figure 3, C and D). Similarly as in Figure 1G, the effect was markedly increased in the contact culture with hdlk-expressing AFT024 cells, whereas little effect could be detected in the contact culture with hdlk-negative 2018 feeder cells (Figure 3, C and D), indicating an important role of the Notch-mediated signal in maintenance of hCD34⁺hCD38[−] cord blood cells. These results suggest that soluble mediators alone may be insufficient for effective *ex vivo* expansion of hCD34⁺ cord blood cells, in particular for maintenance of hCD34⁺hCD38[−] cord blood cells, and that the cell-to-cell contact culture providing the Notch-mediated signal and production of hIL-6 may have potential effects on *ex vivo* expansion of hCD34⁺ cord blood cells, although more biochemical and molecular studies are needed to examine self-renewal properties of hCD34⁺ cord blood cells expanded under the culture conditions.

Figure 3. Effects of exogenous hIL-6 and cultured supernatants derived from contact culture in *ex vivo* expansion of hCD34⁺ cord blood cells. **A** and **B**: For collection of the cultured supernatants, cell-to-cell contact culture, as indicated in Figure 1A, was performed. After 7 days, the supernatants were harvested and used. Under the noncontact culture condition, 2×10^4 purified hCD34⁺CD38⁻Lin⁻ cord blood cells were cultured with the cultured supernatants harvested from the cell-to-cell contact culture. Purified hCD34⁺CD38⁻Lin⁻ cord blood cells at a density of 2×10^4 were cultured using conventional culture medium (recombinant human cytokine cocktails) supplied without or with human IL-6 (25 pg/mL) under the feeder-free condition. Under the noncontact culture condition, hdlk-expressing AFT024 or hdlk-negative 2018 feeder cells were plated, and 2×10^4 purified hCD34⁺CD38⁻Lin⁻ cord blood cells were cultured using conventional culture medium (recombinant human cytokine cocktails) supplied without or with human IL-6 (25 pg/mL). Under the contact culture condition, AFT024 or 2018 feeder cells were plated, and 2×10^4 purified hCD34⁺CD38⁻Lin⁻ cord blood cells were cultured using conventional culture medium (recombinant human cytokine cocktails). On day 7 after culture, MNCs were harvested and counted, and the fold increase was calculated. Bars represent mean $\pm$ SEM from three independent experiments. **C** and **D**: On day 7 after culture, cells were harvested, stained with antibodies specific for hCD34 and hCD38, and analyzed using flow cytometry. The fold increase and absolute number of hCD34⁺CD38⁻ cells are presented. Bars represent mean $\pm$ SEM from three independent experiments.

The Notch Signal in Contact Culture Is Important for Self-Renewal of *ex Vivo* Expanded hCD34⁺ Cord Blood Cells

Self-renewal of stem cells requires stemness and cell proliferation. To test whether *ex vivo* expanded cells exhibit self-renewal properties, we analyzed the expression of transcriptional regulators related to self-renewal programs of stem cells such as HoxB4, GATA-2, and Bmi1.

Expression of GATA-2 and Bmi1 was significantly increased in CD34⁺ cells isolated from contact culture when compared with the levels in control and noncontact cultures, whereas HoxB4 was equally expressed in the three cultures (Figure 4A), which indicates that Notch signaling may be responsible for GATA-2 and Bmi1 expression in contact culture. Furthermore, cell cycle progression and levels of anti-apoptotic proteins such as p21, Bcl-2, Bcl-xL, Mcl-1, and phospho-Bad were also

Figure 4. Notch signal in the maintenance of self-renewal of *ex vivo* expanded hCD34⁺ cord blood cells. **A** and **B**: After *ex vivo* expansion for 14 days, expanded cells were harvested, and cell lysates were subjected to Western blot analysis using antibodies specific for HoxB4, GATA2, Bmi-1, p21, Bcl-2, Bcl-xL, Mcl-1, phospho-Bad (Ser136), and β -actin. **C** and **D**: Assessment of clonogenic progenitor cell capacity of the expanded hCD34⁺ cord blood cells after 14 days under various culture conditions. Data are given as mean $\pm$ SEM of the number of colonies from three independent triplicate experiments (* $P < 0.05$). **E**: After *ex vivo* expansion for 14 days, cell lysates were subjected to Western blot analysis using antibodies specific for Notch1, cleaved Notch1, Hes-1, and β -actin. **F**: γ -Secretase inhibitor was co-cultured in the contact culture. After *ex vivo* expansion for 14 days, cell lysates were subjected to Western blot analysis using antibodies specific for HoxB4, GATA2, Bmi-1, and β -actin. **G**: After *ex vivo* expansion for 14 days in the presence or absence of γ -secretase inhibitor, cell lysates were subjected to Western blot analysis using antibodies specific for Hes-1, cleaved Notch1, STAT3, and phospho-STAT3 (Tyr705).

Figure 5. Notch signal induces self-renewal of hCD34⁺ cord blood cells through direct regulation of hIL-6 production. **A:** After *ex vivo* expansion for 14 days, expanded cells were harvested, and cell lysates were subjected to Western blot analysis using antibodies specific for STAT3 and phospho-STAT3 (Tyr705). The expression level was assessed using densitometry analysis. **B:** Electrophoretic mobility shift assay was performed as described (see *Materials and Methods*). **C and D:** Chromatin immunoprecipitation and quantitative real-time PCR assays were performed as described (see *Materials and Methods*) (**P* < 0.05). **E and F:** γ -Secretase inhibitor was co-cultured in contact culture. After *ex vivo* expansion for 14 days, the levels of hIL-6 and hIL-6R α were measured using an ELISA as described (see *Materials and Methods*). Data represent the mean $\pm$ SEM of three independent experiments (***P* < 0.01). **G:** Various concentrations of Jak1 inhibitor were co-cultured in contact culture as indicated. After *ex vivo* expansion for 14 days, the levels of hIL-6 and hIL-6R α were measured using an ELISA as described (see *Materials and Methods*). hCD34⁺ cells were purified from *ex vivo* expanded cells and treated with various concentrations of Jak1 inhibitor in the presence of hIL-6 and rhIL-6R α for 24 hours. Cell lysates were subjected to Western blot analysis using antibodies specific for STAT3, phospho-STAT3 (Tyr705), and phospho-STAT3 (Tyr727). **H:** hCD34⁺ cord blood cells were *ex vivo* expanded with or without 15 nmol/L Jak1 inhibitor under the contact culture condition. After *ex vivo* expansion for 14 days, phenotype analysis was performed as described (see *Materials and Methods*). The percentage of each cell represents the mean $\pm$ SEM of three independent experiments.

dramatically elevated (Figure 4B). To further confirm the self-renewal property, we performed analyses of colony-forming units in culture (Figure 4C) and mixed colonies containing erythroid and myeloid cells and megakaryocytes (Figure 4D). The number of colonies was markedly higher in CD34⁺ cells expanded in contact culture than in cells expanded in control or noncontact culture (Figure 4, C and D), which indicates that the cell-to-cell contact culture may facilitate maintenance of self-renewal of *ex vivo* expanded CD34⁺ cord blood cells. We next examined whether the effects are dependent on the Notch-mediated signal between hdk-expressing AFT024 and hCD34⁺ cord blood cells. *Ex vivo* expanded CD34⁺ cord blood cells in contact culture exhibited increased levels of cleaved Notch1 and Hes-1 proteins (Figure 4E). When contact culture was treated with γ -secretase inhibitor, the expression levels of HoxB4 and GATA-2 were significantly decreased when compared with culture without γ -secretase inhibitor (Figure 4F). Moreover, the treatment abolished the levels of cleaved Notch1 and Hes-1 proteins and led to decreased STAT3 activation (Figure 4G), indicating that the Notch signal may have a key role in maintaining the self-renewal property of hCD34⁺ cord blood cells.

Notch Signal Induces Self-Renewal of hCD34⁺ Cord Blood Cells Through Regulation of hIL-6 Production

Our results demonstrate that the Notch signal and hIL-6 may have key roles in the maintenance of self-renewal properties of *ex vivo* expanded hCD34⁺ cord blood cells in contact culture. Furthermore, we observed that treatment with γ -secretase inhibitor significantly inhibited the activation of STAT3 (Figure 4G), which suggests that STAT3 connects the Notch signal and hIL-6 in the maintenance of self-renewal properties. When we measured the activation of STAT3 using Western blot analysis and the electrophoretic mobility shift assay with a radiolabeled probe, it was observed that the levels of phospho-STAT3 and STAT3 DNA-binding activity were significantly increased in the hCD34⁺ cord blood cells *ex vivo* expanded in the contact culture as compared with that in the control and noncontact cultures (Figure 5, A and B, respectively). In addition, the chromatin immunoprecipitation assay revealed that STAT3 strongly binds to the hIL-6 gene promoter in cells expanded in contact culture when compared with cells expanded in

Figure 6. hCD34⁺ cord blood cells expanded from the cell-to-cell contact system exhibit activation of Jak/STAT and PI3K pathways. On day 14 after *ex vivo* expansion, cells were harvested and lysed using lysis buffer [50 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing 20 mmol/L NaH₂PO₄, 1 mmol/L Na₃VO₄, 4 mmol/L benzamide, 0.2 mmol/L AEBSF (Pefabloc), 50 mmol/L β-glycerolphosphate, 5 μg/mL aprotinin, and 5 μg/mL leupeptin at 4°C for 20 minutes. Protein concentrations were measured using a spectrophotometer. For Western blot analysis, equal amounts of extracted protein (50 μg) were subjected to 8% to 12% SDS-PAGE and then transferred to polyvinylidene difluoride or 0.45 μm pure nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell. Immunoblotting was performed using antibodies specific for gp130 and phospho-gp130 (A), Jak1 and phospho-Jak1 (B), PI3K and phospho-PI3K (C), PDK1 and phospho-PDK1 (D), Akt and phospho-Akt (E), mTOR and phospho-mTOR (F), and p70S6k and phospho-p70S6k (G) molecules. Bar graphs represent relative intensities of phosphorylated proteins compared with those of control culture and mean ± SEM from three independent experiments.

control or noncontact culture (Figure 5, C and D, respectively). Furthermore, treatment with γ-secretase inhibitor in the culture profoundly inhibited secretion of hIL-6 and hsIL-6Rα (Figure 5, E and F). These results strongly suggest that the Notch signal can directly regulate the expression of hIL-6 and hsIL-6Rα through activation of STAT3.

Based on these results, we further explored whether hIL-6 expression induced by the Notch signal affects the maintenance of self-renewal properties of hCD34⁺ cord blood cells via hIL-6-mediated signaling. We observed that the activation of hIL-6-related signaling molecules such as gp-130, Jak1, PI3K, PDK1, Akt, mTOR, and p70S6K were profoundly enhanced in the *ex vivo* expanded CD34⁺ cord blood cells derived in contact culture compared with those in control or noncontact culture (Figure 6), which indicates that the effects may be due to soluble hIL-6 induced by Notch. To verify the roles of hIL-6-mediated signaling, we used two specific inhibitors, Jak1 inhibitor and wortmannin, to block the Jak1 and PI3k-Akt signaling pathways, respectively. Treatment with Jak1 inhibitor in *ex vivo* expanded hCD34⁺ cord blood cells resulted in inhibition of STAT3 activation in the presence of hIL-6 and rhIL-6Rα (Figure 5G). Furthermore, production of hIL-6 and hsIL-6Rα was gradually decreased in a dose-dependent manner (Figure 5G). More interesting, when the Jak1 inhibitor was co-cultured in the contact culture for 14 days, the phenotypical main-

tenance of hCD34⁺hCD38[−]hLin[−] cells was severely affected; levels of hCD34⁺hLin⁺ or hCD34⁺hCD38⁺ cells were markedly decreased in the culture with the Jak1 inhibitor, whereas levels of hCD34[−], hCD34[−]hLin⁺, and hCD34[−]hCD38⁺ cells were increased (Figure 5H). Similarly, compared with the culture without wortmannin, treatment with wortmannin, a specific PI3K inhibitor, resulted in inhibition of cell proliferation, decrease in hIL-6 production, suppression of Akt and STAT3 phosphorylation, and a change in the phenotypical maintenance of hCD34⁺hCD38[−]hLin[−] cells (Figure 7). These results strongly demonstrate that hIL-6 signaling has an essential role in both cell proliferation and maintenance of stemness of hCD34⁺ cord blood cells. In Figure 7F, our results are summarized and a possible pathway is proposed by which Notch and hIL-6 facilitate maintenance of self-renewal of hCD34⁺ cord blood cells. Under the cell-to-cell contact condition between hdlk-expressing AFT024 and hCD34⁺hCD38[−]hLin[−] cells, the Notch signal induces activation of STAT3 in the hCD34⁺ cord blood cells through the Jak1-PI3K-Akt pathway, and subsequently, the activated STAT3 enhances the gene expression of hIL-6 and hsIL-6Rα. In turn, hIL-6 and hsIL-6Rα are secreted and interact with hIL-6R, and eventually the complex induces activation of STAT3. Through both Notch signaling and the positive feedback effect of hIL-6, the self-renewal property of hCD34⁺

Figure 7. Wortmannin inhibits maintenance of self-renewal of hCD34⁺ cord blood cells in *ex vivo* expansion. **A:** hCD34⁺ cord blood cells were *ex vivo* expanded with or without 10 nmol/L wortmannin under the contact culture condition. After *ex vivo* expansion for 14 days, cells were harvested, and the proliferation assay was performed as described (see *Materials and Methods*). **B:** The level of hIL-6 was measured in the supernatants using an ELISA as described (see *Materials and Methods*). **C** and **D:** Phenotype analysis was performed as described (see *Materials and Methods*). The percentage of each cell represents the mean $\pm$ SEM of three independent experiments. **E:** *Ex vivo* expanded cells were harvested, and cell lysates were subjected to Western blot analysis using antibodies specific for Akt, phospho-Akt (Ser 473), STAT3, and phospho-STAT3 (Tyr705). **F:** Model for cooperation between Notch- and IL-6-mediated signals for maintenance of self-renewal of *ex vivo* expanded hCD34⁺ cord blood cells.

cord blood cells may thus be maintained in the *ex vivo* expansion condition.

hCD34⁺ Cord Blood Cells Derived *ex Vivo* Expansion in Contact Culture Potently Induced Reconstitution of Human Immune Cells in NOD-SCID Mice

We examined whether hCD34⁺ cord blood cells cultured in the cell-to-cell contact culture system are capable of long-term multilineage reconstitution *in vivo*. hCD34⁺ cord blood cells were isolated from the culture and transplanted into NOD-SCID mice. As controls, uncultured hCD34⁺ cord blood cells were freshly isolated from human cord blood and transplanted into other NOD-SCID mice. Eight weeks after transplantation, hCD45⁺ cells were observed in the bone marrow cells of mice that received the *ex vivo* expanded hCD34⁺ or freshly isolated hCD34⁺ cells (Figure 8A). Both the percentage and absolute number of hCD45⁺ cells were markedly higher in mice that received expanded hCD34⁺ cells than in mice that received freshly isolated hCD34⁺ cells. There was also a quantitative increase in the percentage of human hematopoietic cells represented by hCD45⁺hCD34⁺ in the murine bone marrow (Figure 8E). hCD45⁺ cells in the murine bone marrow were further analyzed using flow cytometry to determine multilineage reconstitution. Both the percentage and absolute number of human B, T, and dendritic cells were markedly increased in the mice that received expanded hCD34⁺ cells (Figure 8, B–D). The results indicate that hCD34⁺ cord blood cells *ex vivo* expanded in cell-to-cell contact culture are able to reconstitute human immune cells *in vivo*.

Discussion

Current strategies to expand hCD34⁺ stem cells with self-renewal properties from umbilical cord blood have focused on how to provide an *in vivo* niche environment to the hCD34⁺ stem cells under the *ex vivo* expansion culture condition. The reason seems to be due to the limitation of the small number of primitive hematopoietic stem cells. Earlier *ex vivo* expansion studies have revealed that a stroma-free culture system supplied with combinations of hematopoietic growth factors such as IL-1, IL-3, IL-6, G-CSF, GM-CSF, and stem cell factor can achieve a degree of amplification in the number of peripheral blood CD34⁺ cells.²¹ Since then, although several groups have developed more advanced stroma-free culture systems supplemented with multiple cytokines, most studies have detected limited expansion of cells capable of repopulating NOD-SCID mice.^{3,22} Nevertheless, a previous report has demonstrated more than 50-fold expansion of Src when umbilical cord blood CD34⁺ cells were cultured for 6 to 10 weeks in a human serum-containing system with fetal liver tyrosine kinase ligand, thrombopoietin, and/or stem cell factor and IL-6,²³ which indicates that CD34⁺ cord blood stem cells can be grown and expanded *in vitro* for an extremely long time when supplied with essential cytokines. Among the supplied cytokines for *ex vivo* expansion of CD34⁺ cord blood stem cells, IL-6 has an important role in both human hematopoiesis and *ex vivo* expansion of human hematopoietic stem cells.^{24–26} IL-6 receptor is composed of two functionally different chains, a ligand-binding chain referred to as IL-6R and gp130, both of which are required for signal transduction.²⁷ The IL-6 complex comprising IL-6, soluble IL-6R, and homodimeric gp130 initiates a downstream signaling cascade that includes rat sarcoma (ras) gene product (RAS)/mitogen-activated protein kinase

Figure 8. hCD34⁺ cord blood cells derived from *ex vivo* expansion in contact culture potently induce reconstitution of human immune cells in NOD-SCID mice. **A–E:** hCD34⁺ cord blood cells purified from fresh cord blood cells or *ex vivo* expanded cells were transplanted into irradiated mice via tail-vein injection ($n = 6$ per group) as described (see *Materials and Methods*). The mice were euthanized at 8 weeks after transplantation. Bone marrow cells were isolated from two femurs and two tibias of each mouse, counted, and stained using antibodies specific for hCD45, hCD20, hCD3, hCD11c, and hCD34 molecules. Stained cells were acquired using FACSARIA, and were analyzed using the FACSDiva software version 5.0.2 flow cytometry analysis program. Bars represent mean $\pm$ SEM from six mice per group.

and Janus kinase (JAK)/signal transducers and activators of transcription (STAT), leading to a cellular response related to hematopoiesis and proliferation of multipotent hematopoietic stem cells in both mouse and humans. However, these early culture systems are still associated with problems related to expansion of the number of long-term reconstituting cells.

Because current *ex vivo* culture systems are not equipped to enhance long-term reconstituting cells, it is necessary to understand and manipulate molecular pathways that expand the stem cell niche in culture. Notch

signaling is involved in cellular differentiation, proliferation, apoptosis, adhesion, and epithelial-to-mesenchymal transition.^{28–31} There are five Notch ligands in mammals, Jagged-1 and Jagged-2 and Delta-1, Delta-3, and Delta-4. Binding of a Notch ligand to a Notch receptor results in cleavage and release of the intracellular domain of the Notch receptor by a membrane-associated protease complex and the eventual regulation of the transcription of several Notch effector genes.³² Jagged-1 can induce expansion of early progenitor cells in *ex vivo* culture, and immobilized Delta-1 can induce ex-

pansion of h CD34⁺CD38[−] cord blood progenitor cells in the presence of fibronectin and growth factors,^{9,10,18} which indicates the importance of Notch signaling in expansion of CD34⁺ cord blood stem cells. IL-6-mediated signaling is closely related to the Notch signaling pathway via Jagged-1 and STAT3.^{27,33–35} Inasmuch as recent evidence has demonstrated that Notch signaling is indispensable for maintaining stem cells by enhancing their self-renewal capacity and by repressing differentiation into progenitor cells, the cooperation between IL-6 and Notch signaling may synergistically affect the proliferation of stem cells and maintenance of stemness.

Insofar as the technical aspects for *ex vivo* expansion of stem cells derived from umbilical cord blood, a mixed culture system with stromal cells that provide a rich environment including soluble mediators has been established.³⁶ Previous *in vitro* and *in vivo* studies have demonstrated that stromal cells provide a rich environment of signals induced by cytokines, extracellular matrix proteins, and adhesion molecules that control proliferation, survival, and differentiation of hematopoietic progenitor cells and stem cells.³⁶ A number of murine-derived stromal cell lines have been cloned that support not only murine repopulating cells but also primitive human hematopoietic progenitor cells.^{15,16,37} Recent studies have demonstrated that AFT024 cells derived from murine fetal liver can induce maintenance of murine stem cells and also of human CD34⁺CD38[−] progenitor cells *in vitro*.^{15,38} Furthermore, delta-like protein produced in AFT024 cells has a key role in inhibiting the terminal differentiation of hematopoietic stem cells in the AFT024 co-culture system.³⁹ These findings demonstrate a pivotal role of stroma cells in maintenance of the self-renewal property in *ex vivo* expansion of hematopoietic stem cells. However, it is still poorly understood how the stromal cells facilitate maintenance of the self-renewal property of *ex vivo* expanded hematopoietic stem cells in terms of cellular and molecular mechanisms.

Herein, we have used a culture system to provide an interaction between CD34⁺ cord blood cells derived from umbilical cord blood and hdlk-expressing AFT024 feeder cells, and examined whether this system can facilitate proliferation of hCD34⁺ cord blood cells with self-renewal properties. We initially expected that the cellular interaction between hCD34⁺ cord blood cells and hdlk-expressing AFT024 feeder cells would have a key role in the maintenance of stemness, in particular, through Notch signaling, and thereby facilitate cell proliferation and self-renewal of hCD34⁺ cord blood cells. In addition, as a result of the interaction, some soluble mediators may be induced from hCD34⁺ cord blood cells, eventually affecting cell proliferation and self-renewal of hCD34⁺ cord blood cells via a positive feedback loop. These expectations are based on previous results. First, Notch signaling is essential for hematopoietic stem cell formation during embryogenesis and is also thought to be required for hematopoietic stem cell maintenance.^{9,10,28–32} Second, some soluble cytokines also have key roles in maintenance of self-renewal properties of hematopoietic stem cells.^{3–8,24–26}

In the present work, we provide evidence for molecular and cellular interaction between Notch signaling and hIL-6 to maintain self-renewal of hCD34⁺ cord blood cells during *ex vivo* expansion. Consistent with the previously addressed role of Notch signaling in hematopoietic stem cell maintenance,^{9,10,18} Notch signaling was profoundly important for maintenance of self-renewal of hCD34⁺ cord blood cells through regulation of transcriptional regulators related to hematopoietic stem cell self-renewal programs such as HoxB4, GATA-2, and Bmi1. Furthermore, we observed that Notch signaling was also involved in gene expression of hIL-6 and hIL-6R α through activation of STAT3. By using a depletion assay of hIL-6 with anti-hIL-6 monoclonal antibodies and an hIL-6 signal assay with specific inhibitors, ie, Jak1 and PI3K, we verified the specific roles of hIL-6-mediated signaling in maintenance of self-renewal of hCD34⁺ cord blood cells in *ex vivo* expansion. Our results suggest that Notch and hIL-6 might facilitate maintenance of self-renewal properties through a synergistic molecular and cellular network (Figure 7). Although the present work does not clearly suggest whether self-renewal of hCD34⁺ cord blood cells in *ex vivo* expansion is maintained by the obligatory asymmetric replication in which a stem cell divides into one father cell that is identical to the original stem cell and another daughter cell that is differentiated or by the stochastic differentiation in which, when one stem cell develops into two differentiated daughter cells, another stem cell undergoes mitosis and produces two stem cells identical to the original. We suppose that the contact co-culture is required for maintenance of hematopoietic hCD34⁺ stem cells in an undifferentiated state and for enriching a population of stem cells in a mixture of stem cells and non-stem cells. Altogether, the present work may contribute to improvements in the conditions for *ex vivo* expansion of hematopoietic stem cells and enable a wider range of stem cell applications for therapeutic purposes.

References

1. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, Cooper S, English D, Kurtzberg J, Bard J, Boyse EA: Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* 1989, 321:1174–1178
2. Gluckman E, Rocha V, Chevret S: Results of unrelated umbilical cord blood hematopoietic stem cell transplant. *Transfus Clin Biol* 2001, 8:146–154
3. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE: Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term *ex vivo* culture. *J Exp Med* 1997, 186:619–624
4. Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, Mitsui T, Manabe A, Tanaka R, Kobayashi K, Ito M, Yasukawa K, Nakahata T: Expansion of human NOD/SCID repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest* 2000, 105:1013–1021
5. Sauvageau G, Thorsteinsdottir U, Eaves CJ, Lawrence HJ, Largman C, Lansdorp PM, Humphries RK: Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations *in vitro* and *in vivo*. *Genes Dev* 1995, 9:1753–1765

6. Nakamura Y, Ando K, Chargui J, Kawada H, Sato T, Tsuji T, Hotta T, Kato S: Ex vivo generation of CD34⁺ cells from CD34⁺ hematopoietic cells. *Blood* 1999, 94:4053–4059
7. Ando K, Nakamura Y, Chargui J, Matsuzawa H, Tsuji T, Kato S, Hotta T: Extensive generation of human cord blood CD34⁺ stem cells from Lin[−]CD34⁺ cells in a long-term in vitro system. *Exp Hematol* 2000, 28:690–699
8. Hess DA, Levac KD, Karanu FN, Rosu-Myles M, White MJ, Gallacher L, Murdoch B, Keeney M, Ottowski P, Foley R, Chin-Yee I, Bhatia M: Functional analysis of human hematopoietic repopulating cells mobilized with granulocyte colony-stimulating factor alone versus granulocyte colony-stimulating factor in combination with stem cell factor. *Blood* 2002, 100:869–878
9. Varnum-Finney B, Purton LE, Yu M, Brashem-Stein C, Flowers D, Staats S, Moore KA, LE Roux I, Mann R, Gray G, Artavanis-Tsakonas S, Bernstein ID: The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* 1998, 91: 4084–4091
10. Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M: The Notch ligand Jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* 2000, 192: 1365–1372
11. Austin TW, Solar GP, Ziegler FC, Liem L, Matthews W: A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood* 1997, 89:3624–3635
12. Van Den Berg DJ, Sharma AK, Bruno E, Hoffman R: Role of members of the Wnt gene family in human hematopoiesis. *Blood* 1998, 92: 3189–3202
13. Ying QL, Nichols J, Chambers I, Smith A: BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003, 115:281–292
14. Arai F, Hirao A, Ohmura M, Sato H, Matsuo S, Takubo K, Ito K, Koh GY, Suda T: Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004, 118:149–161
15. Moore KA, Ema H, Lemischka IR: In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood* 1997, 89: 4337–4347
16. Punzel M, Wissink SD, Miller JS, Moore KA, Lemischka IR, Verfaillie CM: The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. *Blood* 1999, 93:3750–3756
17. Moore KA, Pytowski B, Witte L, Hicklin D, Lemischka IR: Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. *Proc Natl Acad Sci USA* 1997, 94:4011–4016
18. Ohishi K, Varnum-Finney B, Bernstein ID: Delta-1 enhances marrow and thymus repopulating ability of human CD34⁺CD38[−] cord blood cells. *J Clin Invest* 2002, 110:1165–1174
19. Chateauvieux S, Ichanté JL, Delorme B, Frouin V, Piéti G, Langonné A, Gallay N, Sensebé L, Martin MT, Moore KA, Charbord P: Molecular profile of mouse stromal mesenchymal stem cells. *Physiol Genomics* 2007, 29:128–138
20. Hackney JA, Charbord P, Brunk BP, Stoeckert CJ, Lemischka IR, Moore KA: A molecular profile of a hematopoietic stem cell niche. *Proc Natl Acad Sci USA* 2002, 99:13061–13066
21. Haylock DN, To LB, Dowse TL, Juttner CA, Simmons PJ: Ex vivo expansion and maturation of peripheral blood CD34⁺ cells into the myeloid lineage. *Blood* 1992, 80:1405–1412
22. Conneally E, Cashman J, Petzer A, Eaves C: Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-SCID/SCID mice. *Proc Natl Acad Sci USA* 1997, 94:9836–9841
23. Piacibello W, Sanavio F, Severino A, Danè A, Gammaitoni L, Fagioli F, Perissinotto E, Cavalloni G, Kollet O, Lapidot T, Aglietta M: Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. *Blood* 1999, 93:3736–3749
24. Wells JA, de Vos AM: Hematopoietic receptor complexes. *Annu Rev Biochem* 1996, 65:609–634
25. Bordeaux-Rego P, Luzo A, Costa FF, Olalla Saad ST, Crosara-Alberto DP: Both interleukin-3 and interleukin-6 are necessary for better ex vivo expansion of CD133⁺ cells from umbilical cord blood. *Stem Cells Dev* 2010, 19:413–422
26. Rofani C, Luchetti L, Testa G, Lasorella R, Isacchi G, Bottazzo GF, Berardi AC: IL-16 can synergize with early acting cytokines to expand ex vivo CD34⁺ isolated from cord blood. *Stem Cells Dev* 2009, 18:671–682
27. Bravo J, Heath JK: Receptor recognition by gp130 cytokines. *EMBO J* 2000, 19:2399–2411
28. Milner LA, Bigas A: Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood* 1999, 93:2431–2448
29. Sambandam A, Maillard I, Zediak VP, Xu L, Gerstein RM, Aster JC, Pear WS, Bhandoola A: Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol* 2005, 6:663–670
30. Ohishi K, Varnum-Finney B, Flowers D, Anasetti C, Myerson D, Bernstein ID: Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand. Delta-1. *Blood* 2000, 95:2847–2854
31. Masuya M, Katayama N, Hoshino N, Nishikawa H, Sakano S, Araki H, Mitani H, Suzuki H, Miyashita H, Kobayashi K, Nishii K, Minami N, Shiku H: The soluble Notch ligand, Jagged-1, inhibits proliferation of CD34⁺ macrophage progenitors. *Int J Hematol* 2002, 75:269–276
32. Schroeter EH, Kisslinger JA, Kopan R: Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* 1998, 393:382–386
33. Ancey C, Küster A, Haan S, Herrmann A, Heinrich PC, Müller-Newen G: A fusion protein of the gp130 and interleukin-6Rα ligand-binding domains acts as a potent interleukin-6 inhibitor. *J Biol Chem* 2003, 278:16968–16972
34. Sansone P, Storci G, Tavoroli S, Guarnieri T, Giovannini C, Taffurelli M, Ceccarelli C, Santini D, Paterini P, Marcu KB, Chieco P, Bonafè M: IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest* 2007, 117:3988–4002
35. Gao SP, Mark KG, Leslie K, Pao W, Motoi N, Gerald WL, Travis WD, Bornmann W, Veach D, Clarkson B, Bromberg JF: Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J Clin Invest* 2007, 117:3846–3856
36. Verfaillie CM: Hematopoietic stem cells for transplantation. *Nat Immunol* 2002, 3:314–317
37. Robin C, Pflumio F, Vainchenker W, Coulombel L: Identification of lymphomyeloid primitive progenitor cells in fresh human cord blood and in the marrow of nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice transplanted with human CD34(+) cord blood cells. *J Exp Med* 1999, 189:1601–1610
38. Thiemann FT, Moore KA, Smogorzewska EM, Lemischka IR, Crooks GM: The murine stromal cell line AFT024 acts specifically on human CD34⁺CD38[−] progenitors to maintain primitive function and immunophenotype in vitro. *Exp Hematol* 1998, 26:612–619
39. Nolte JA, Thiemann FT, Arakawa-Hoyt J, Dao MA, Barsky LW, Moore KA, Lemischka IR, Crooks GM: The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. *Leukemia* 2002, 16:352–361