**Endothelial progenitor cells promote efficient *ex vivo* expansion of cord
blood-derived hematopoietic stem/progenitor cells**

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Abstract

Background aims. Cord blood (CB) hematopoietic stem cell transplantation has often been limited by the scarcity of stem cells. Therefore, the number of CB hematopoietic stem/progenitor cells (HSPCs) should be increased while maintaining the stem cell characteristics. *Methods.* We designed an *ex vivo* culture system using endothelial progenitor cells (EPCs) as stroma to determine the capacity of expanding CB-HSPCs in a defined medium, the effect on engraftment of the expanded cells in a mouse model and the underlying mechanism. *Results.* After 7 days of culture, compared with those cultured with cytokines alone (3.25 ± 0.59), CD34⁺ cells under contact and non-contact co-culture with EPCs were expanded by 5.38 ± 0.61 ($P = 0.003$) and 4.06 ± 0.43 ($P = 0.025$) fold, respectively. Direct cell-to-cell contact co-culture with EPCs resulted in more primitive CD34⁺CD38⁻ cells than stroma-free culture (156.17 ± 21.32 vs. 79.12 ± 19.77 fold, $P = 0.010$). Comparable engraftment of day 7 co-cultured HSPCs with respect to HSPCs at day 0 in NOD/SCID mice was measured as a percentage of chimerism ($13.3\% \pm 11.0\%$ vs. $16.0\% \pm 14.3\%$, $P=0.750$). EPCs highly expressed IL-6 and angiopoietin-1, the hematopoietic related cytokines. A higher transcriptional level of Wnt5A genes in EPCs and co-cultured HSPCs suggests that the activation of Wnt signaling pathway may play a role in HSPCs expansion *ex vivo*. *Discussion.* These data demonstrated that EPCs improve the CD34⁺ population but not compromise the repopulating efficacy of the amplified HSPCs, possibly via cytokine secretion and Wnt signaling pathway activation.

Key Words: *endothelial progenitor cells; cord blood transplantation; non-obese diabetic/severe combined immunodeficient mice; hematopoietic engraftment*

Abbreviations

APC, allophycocyanin

AGM, aorta-gonad-mesonephros

BM, bone marrow

BFU-E, burst-forming unit-erythrocytes

CFU, colony-forming unit

CFU-GM, colony-forming unit granulocyte-macrophages

CFU-GEMM, colony-forming unit granulocytes, erythrocytes, monocytes and megakaryocytes

CFU-E, colony-forming unit-erythrocytes

CBT, cord blood transplantation

EBM, endothelial basal medium

ECs, endothelial cells

ECFCs, endothelial colony-forming cells

EGM, endothelial growth medium

EPCs, endothelial progenitor cells

ELAM-1, endothelial-leukocyte adhesion molecule 1

FBS, fetal bovine serum

FL, Flt-3 ligand

FITC, fluorescein isothiocyanate

FACS, Fluorescence activated flow cytometry

GVHD, graft-versus-host disease

GM-CSF, granulocyte-macrophage colony-stimulating factor

HSCT, hematopoietic stem cell transplantation

HSPCs, Hematopoietic stem/progenitor cells

HLA, human leukocyte antigen

HUVECs, Human umbilical vein endothelial cells

IGF, insulin-like growth factor

IL, interleukin

KDR, kinase insert domain receptor

MSCs, Mesenchymal stromal cells

MPB, mobilized peripheral blood

MNCs, mononuclear cells

PBS, phosphate buffered saline

PE, phycoerythrin

RT-qPCR, real-time quantitative polymerase chain reaction

rh EGF, recombinant human epidermal growth factor

rhFGF- β , recombinant human fibroblast growth factor- β

Shh, sonic hedgehog

SCF, stem cell factor

TPO, thrombopoietin

Tie, tyrosine kinase with immunoglobulin-like and epidermal growth factor homology domains

UCB, umbilical cord blood

VEGF, vascular endothelial growth factor

Introduction

Hematopoietic stem/progenitor cells (HSPCs) obtained from umbilical cord blood (UCB) have been extensively studied in stem cell research for cellular therapies during the past two decades [1, 2]. In particular, the advantages of using CB over bone marrow (BM) and mobilized peripheral blood (MPB) as source of hematopoietic stem cell transplantation (HSCT) include easy availability, tolerance to human leukocyte antigen (HLA) mismatch, and low incidence of acute graft-versus-host disease (GVHD)[3] as well as noninvasive collection procedures. However, the application of cord blood transplantation (CBT) is limited by the dose of HSPCs, particularly in adults, though their concentration is much higher than BM or MPB. Further, the hematopoietic recovery is slower. Thus, efforts to expand the CB-HSPCs population without damaging their repopulating ability are required for successful clinical transplantation.

Until now, the emphasis has been on different cytokines[4], feeder cells[5, 6] and a combination of the two methods[7-9] to achieve *ex vivo* expansion of HSPCs. Basically, the stem cell niches compromise hematopoietic microenvironment and provide a rich milieu of molecular signals and growth factors. Cell-to-cell adhesion, extracellular matrix deposition and a combination of cytokines contribute to HSPCs self-renewal and differentiation [10].

Vascular niches act as important reservoir of BM hematopoietic microenvironment.

HSPCs reside in intimate anatomic proximity to ECs in the BM microenvironment and this association contributes to the self-renewal and differentiation of HSPCs pool *in vivo* [11, 12]. On the other hand, endothelial progenitor cells (EPCs), which are also designated as endothelial colony-forming cells (ECFCs), represent the precursors of vascular endothelial cells (ECs). During embryonic development, EPCs and hematopoietic cells are derived from a common precursor cell, the hemangioblast [13], and are adjacent to aorta-gonad-mesonephros (AGM) region. In addition to the earliest stages of embryonic development, the developmental process of HSPCs' migration to the fetal liver and ultimately to the BM compartment in adults occurs closely within EPCs/ECs[14]. EPCs were first reported in 1997 by Asahara et al[15] in peripheral blood, and were also isolated in CB[16] at a higher concentration.

Until now, the ability of EPCs to support the *in vitro* expansion of HSPCs remains unambiguous. In this study, we isolated EPCs from CB, a rich source of stem cells, and investigated the potentiality of CB-EPCs to support *in vitro* expansion of CB-HSPCs and maintain pluripotency as well as capacity for BM reconstitution.

Materials and Methods

Isolation and culture of CB-EPCs

Fresh human umbilical cord blood (40ml) was donated by healthy full-term deliveries after maternal written informed consent according to the Ethics Committee of the First

Affiliated Hospital of Soochow University. Within 8 hours of collection, the CB was diluted 1:2 with phosphate buffer saline and overlaid onto LymphoprepTM (Axis-Shield, Norway) density gradient media. Cells were then centrifuged at 600g for 20 min. Buffer coat mononuclear cells (MNCs) were collected and washed 3 times in PBS. For further culture of CB-EPCs, MNCs were resuspended in endothelial growth medium (EGM)-2 medium (Lonza, Switzerland), which comprises endothelial basal medium (EBM) and EGM-2 MV SingleQuot Kit Suppl. & Growth Factors [(recombinant human fibroblast growth factor- β (rhFGF- β), recombinant human epidermal growth factor (rh EGF), R3-insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), ascorbic acid and GA-100)] and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), and seeded on fibronectin (Merck Millipore, USA)-coated 75 cm² flask. After 3 days of culture, nonadherent cells were eliminated by media replacement, and the media were replaced every 3 days. The outgrowth of ECFCs occurred between 21 and 28 days of culture. ECFCs were identified by their cobblestone-like structure. The cells used in the experiments were obtained from passage 1 to passage 5. Human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Institute of Life Science & Cell resource center, and cultured with EGM-2 medium. All cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Mesenchymal stromal cells (MSCs) were obtained with the donor's consent and gifted from cell therapy center of the First Affiliated Hospital of Soochow University.

Fluorescence activated flow cytometry (FACS) analysis of CB-EPCs phenotype

CB-EPCs or HUVECs were detached using 0.25% Trypsin-EDTA (Life Technologies, USA) when 90% cells were confluent. The cells were labeled with phycoerythrin (PE)-conjugated mouse anti-human CD34 antibody (BD Bioscience, USA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45 antibody (BD Bioscience, USA), allophycocyanin (APC) conjugated mouse anti-human CD133 antibody (Miltenyi Biotech Inc, Bergish Gladbach, Germany), PE-CY5-conjugated mouse anti-human kinase insert domain receptor (KDR) antibody (Biolegend, San Diego, CA, USA), PE-conjugated mouse anti-human CD31 antibody (BD Bioscience, USA) and FITC-conjugated mouse anti-human CD14 antibody (BD Bioscience, USA). The mouse isotype IgG conjugated with the above fluorescence served as negative control separately for 30min at 4°C in PBS. FACS was performed with Beckman coulter Cyan ADP Analyzer and a minimum of 10,000 events were analyzed for each sample. The data were analyzed with Summit (version 4.3) software.

Cell proliferation assay

Cells were plated in 96-well plates at 1×10^4 /ml per well in a final volume of 100µl in triplicate, and cultured for 7 days. Cells were incubated with 10% CCK-8 (Dojindo, Kumamoto, Japan), which was diluted in normal medium at 37°C and 5% CO₂ culture

conditions until the visual color conversion occurred. After incubation, the absorbance at 450nm was analyzed by an enzyme-linked immunosorbent assay plate reader.

In vitro angiogenesis assay

Capillary tube formation was performed by laying Matrigel (BD science, USA) into a 48-well plate. The gel was allowed to polymerize in the 37°C CO₂-incubator for at least 30 min. CB-EPCs and HUVECs were seeded on Matrigel (3×10^3 cells/well) and suspended in EGM-2 medium for 12 hours at 37°C with 5% CO₂. Capillary tube formation in Matrigel was observed under an inverted microscope and capillary tube numbers were calculated in at least 3 scope (40×).

Migration assay

The migration assay of CB-EPCs and HUVECs was performed in a 24-well Transwell chamber with 8µm pore diameter (Corning Incorporated, USA). The EGM-2 medium was added to the lower chamber, while 2×10^4 cells resuspended in EBM-2 medium without fetal bovine serum or growth factor were seeded in the upper chamber. After incubation for 18 hours at 37°C with 5% CO₂, the cells on the upper surface of the membrane filter were removed by wiping with a cotton swab. The membranes were mounted, Wright-stained and photographed through an inverted microscope coupled to a camera. The number of cells per field (100×) were counted. Results were shown

as the mean of 3 to 6 areas from 3 pictures.

CD34+ hematopoietic stem/progenitor cells (HSPCs) purification

Human CD34+ cells were purified from umbilical CB samples, which were collected from different donors for CB-EPCs culture. Mononuclear cells were isolated by LymphoprepTM (Axis-Shield, Norway) density gradient media as described above. CD34+ cells were enriched by immunomagnetic beads using the MACS CD34 Progenitors Cell Isolation Kit (Miltenyi Biotech Inc, Bergish Gladbach, Germany) following the manufacturer's instructions. The purity of CD34 cells was analyzed by FACS up to more than 87%.

Co-culture of CD34+ cells with CB-EPCs

CB-EPCs were plated at 5×10^5 cells/well in fibronectin-coated 24-well plates. After 48 hours of culture, the adherent CB-EPCs monolayers were washed with PBS, and 5×10^4 CD34+ HSPCs/well suspended in serum-free StemSpan^R medium (Stem cell technologies, Vancouver, BC, Canada), and a standard cytokine combination of 100ng/mL stem cell factor (SCF), 100ng/mL Flt-3 ligand (FL), and 20ng/mL thrombopoietin (TPO) (all three cytokine were purchased from Peprotech, Rocky Hill, NJ, USA) was directly added. After 7 days of direct contact co-culture at 37°C and 5% CO₂, the monolayers were washed to remove all nonadherent cells and viable

collected cells were counted using trypan blue exclusion dye. The single culture of CD34⁺ cells served as a control and cells were seeded in stroma-free and cytokine cocktail-containing conditions.

For noncontact co-culture, 2×10^6 cells/well CB-EPCs were seeded in fibronectin-coated lower Transwell chamber with 0.4 μ m pore diameter (Corning Incorporated, USA). After incubation for 48 hours, 2×10^5 CD34⁺ cells/well were added to the upper chamber and co-cultured for 7 days and then counted as mentioned above.

Phenotype and cell cycle analysis of the expanded cells

Monoclonal antibodies conjugated with FITC-mouse anti-human CD38 antibody and with PE mouse anti-human CD34 antibody (BD Biosciences, USA) were used to characterize hematopoietic cell populations before and after *in vitro* expansion. Appropriate isotype controls for non-specific binding were used for each antibody. A minimum of 10,000 events were acquired for each sample by Beckman coulter Cyan ADP Analyzer and analyzed with Summit (version 4.3) software. Cells harvested before and after expansion, were fixed in 70% ethanol for more than 2 hours at 4°C, and washed with PBS and resuspended in 500 μ l dyeing buffer. Cells were then supplemented with 0.2 mg/mL RNase A and 20 μ g /mL propidium iodide using Cell Cycle Analysis Kit (Beyotime Biotechnology, Jiangsu, China) for 30min at 37°C. Cells were acquired using Beckman Coulter FC500 Flow Cytometer and analyzed by

DNA cell cycle analysis software (phoenix flow system, San Diego, USA).

Clonogenic assay of hematopoietic stem/progenitor cells

Quantification of colony-forming units was performed before and after expansion. Cells (2×10^3) were resuspended in 1mL of the methylcellulose-based medium MethoCult H4434 Classic (Stem cell technologies, Vancouver, BC, Canada) to detect and quantify human hematopoietic progenitors in colony-forming unit (CFU) assays. Cells were seeded in duplicate in 35-mm culture dishes and colonies containing greater than 50 cells were counted after 14 days of culture. CFUs and lineage-specific colonies including burst-forming unit-erythrocytes (BFU-E), colony-forming unit-erythrocytes (CFU-E), colony-forming unit granulocyte-macrophages (CFU-GM) and colony-forming unit granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM) were calculated according to the manufacturer's instructions.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted after 7 days of culture of hematopoietic stem/progenitor cells. Each sample (1×10^6 cells) was harvested and treated by RNAsimple Total RNA Kit (Tiangen, Beijing, China). The mRNA was reverse-transcribed (RT) into the first-strand cDNA using oligo (dT) primer. The RT reaction was performed for 15min at 37°C and stopped by heat inactivation for 5

seconds at 85°C using PCR amplifier (Applied Biosystems 2720 thermal cycler). The DNA was subjected to RT-qPCR amplification with specific primers under linear conditions, using a kit (SYBR Premix EX Taq, Takara) and the Applied Biosystems 7500 Real-Time PCR system. RT-qPCR conditions were as follows: at 95°C for 30 seconds, 40 cycles at 95°C for 5 seconds followed by 34s at 60°C. β -actin was used as a housekeeping gene. The primers used in the study are listed in Table 1. The $2^{-\Delta\Delta C_t}$ method was used and results were expressed as gene expression fold increase relative to the controls.

Engraftment of NOD/SCID mice after transplantation of expanded hematopoietic/progenitor cells

NOD/SCID mice were purchased from the Animal Center of Chinese Medical Academy of Science and housed in the animal facilities of the Institute of Zoology, the First Affiliated Hospital of Soochow University. Mice were housed in filter-top cages under specific pathogen-free conditions and provided with sterilized food and drinking water. The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of the First Affiliated Hospital of Soochow University.

Six to twelve hours prior to transplantation, 8 to 10-week-old female NOD/SCID mice were sublethally irradiated with 300cGy total body irradiation (200cGy/min). Positive control mice of group A were injected with 5×10^5 CD34+ fresh cells intravenously via tail vein. The mice in group B received 5×10^5 progeny of day-7 single culture. Group C mice received 5×10^5 progeny of day-7 co-culture. Negative control mice of group D were injected intravenously with identical volume of PBS.

Blood cell counts were analyzed by automatic blood cell counter (Sysmex, Japan) weekly by collecting blood from tail vein. The mice were sacrificed by cervical dislocation 8 weeks after transplantation, and BM from the femur and tibia were harvested, pooled and lysed to eliminate the contaminated red blood cells. The cells were then stained with FITC-conjugated anti-human CD45 antibody and analyzed by flow cytometry.

Statistical analysis

The data were presented as mean $\pm$ SD from at least three separate experiments. Statistical comparisons were performed using a two-tailed Student's t-test and χ^2 test. Survival data were analyzed by means of the log-rank test, and the survival curves were obtained using the Kaplan-Meier method. Values were considered statistically significant when $P < 0.05$.

Results

Derivation and phenotypic characterization of CB-EPCs

We harvested MNCs from the CB of full-term infants to isolate EPCs, and cultured in the presence of EGM-2 on fibronectin-coated plates. Small EPC colonies emerged after 21 to 28 days of culture. At confluence, the EPC exhibited typical endothelial cobblestone morphology with a high proliferation potential (Figure 1A). The expanded EPCs could be cultured up to 10 passages.

The phenotype of the expanded EPCs was characterized by measuring the expression of KDR and CD31 as endothelial markers; CD34 as both endothelial and hematopoietic progenitor markers [17]; CD133 as progenitor cell marker; CD14 as a monocyte/macrophage antigen and CD45 as a common leukocyte antigen. HUVECs were included as mature endothelial cell controls. As shown in Figure.1B, both CB-EPCs and HUVECs expressed the endothelial markers such as KDR and CD31, and CB-EPCs manifested a higher level of progenitor cell markers including CD34 and CD133, but very weakly positive for CD45 and CD14, indicating that the EPCs were not of hematopoietic origin and nearly not contaminated with other cell types. To further identify the endothelial characteristics, the expression of endothelial-specific genes including VE-cadherin, vWF, VEGF, tyrosine kinase with immunoglobulin-like and epidermal growth factor homology domains (Tie-1), Tie-2 and E-selectin of EPCs was estimated by RT-qPCR. Compared with HUVECs, VE-cadherin mRNA showed a 1.64 ± 0.32 fold increase ($P = 0.025$). The

transcriptional levels of vWF and VEGF, which were encoded and secreted by endothelial cells were increased by 4.49 ± 0.50 ($P = 0.000$) and 1.76 ± 0.12 ($P = 0.008$) fold in CB-EPCs than HUVECs respectively. Tie-1 and Tie-2 played a role as important angiopoietin receptors, and CB-EPCs expressed 2.08 ± 0.28 ($P = 0.021$) and 2.25 ± 0.26 ($P = 0.014$) fold when compared with HUVECs. Further, E-selectin, which was also known as endothelial-leukocyte adhesion molecule 1 (ELAM-1) and expressed only on endothelial cells, was also expressed at a much higher transcriptional level of 7.34 ± 0.61 fold compared with the mature endothelial cells ($P = 0.000$) (Figure 1C).

Functional characteristics of CB-EPCs

Before testing the functional ability of CB-EPCs, we investigated the replicative potential of the expanded cells, and growth curves were generated after recording the absorbance following addition of CCK8 reagent. As expected for progenitors, the CB-EPCs showed considerably enhanced proliferative potential when compared with HUVECs, the differentiated mature counterparts. Further, the CB-EPCs used within 5 passages in our experiment had comparatively high proliferation potential (Figure 2A).

CB-EPCs possessed tube formation potential. However, the number of tubes formed by CB-EPCs (F1, 28.67 ± 4.51 ; F3, 29.33 ± 4.51 ; F5, 31.33 ± 4.04 , were observed at 40x) was less than HUVECs (71.67 ± 7.64 , 40x), as described in Figure 2B. In

addition, the tubes formed by different passages of CB-EPCs were similar ($P = 0.749$).

The lower tube formation capacity of CB-EPCs might indicate the progenitor cell stage and relative immaturity of endothelial characteristics.

Since migration capacity is also one of the endothelial cell features, we further used the Transwell chamber to identify the migration characteristics of CB-EPCs. After a brief incubation, several more CB-EPCs (F1, 208.67 ± 13.05 ; F3, 204.33 ± 16.17 ; F5, 203.00 ± 16.37 , were observed at $100 \times$ under the chamber than HUVECs (71.67 ± 3.79), without any difference among CB-EPCs passages ($P = 0.925$) (Figure 2C), indicating a more rapid migration capacity of CB-EPCs. Thus, the increased migration expedited CB-EPCs repair of the endothelial injury.

CB-EPCs enhanced ex vivo expansion of HSPCs

The enriched CD34⁺ cells were co-cultured with CB-EPCs or cultured alone with cytokine- conditioned medium. After seven days of incubation, the cells cultured alone with cytokines manifested a 3.76 ± 0.60 fold expansion. By contrast, the contact of HSPCs on CB-EPCs supported *in vitro* expansion of HSPCs 6.75 ± 0.84 fold ($P = 0.003$) (Figure 3A, B). The noncontact co-culture of HSPCs contributed to a significant (7.56 ± 0.80 fold) increase in total cell numbers compared with HSPCs cultured alone ($P = 0.001$), and displayed an approximate cell expansion similar to direct contact co-culture system ($P = 0.239$), suggesting that the improved hematopoietic cell expansion was mainly mediated by EPCs-secreted factors rather

than HSPCs-EPCs physical interaction.

After seven days of culture, the percentages of CD34⁺ and CD34⁺CD38⁻ cells were compared. Contact co-cultured CD34⁺ cells exhibited 5.38 ± 0.61 fold expansion, and cells cultured with cytokines alone contributed to a 3.25 ± 0.59 increase in CD34⁺ cell expansion ($P = 0.003$). Further, CD34⁺ cells also showed significant increase (4.06 ± 0.43) under non-contact conditions compared with cytokine-cultured cells ($P = 0.025$). Further hematopoietic subtype analysis revealed that the brief contact co-culture of HSPCs on CB-EPCs induced 156.17 ± 21.32 fold expansion of the much earlier subtype of CD34⁺CD38⁻ population compared with day 0, which was higher than that of the HSPCs cultured alone (with 79.12 ± 19.77 fold increase, $P = 0.010$) and with non-contact co-culture (with 95.43 ± 33.00 fold increase, $P = 0.026$). Further, the percentage of CD34⁺CD38⁻ cells increased from $2.13\% \pm 0.58\%$ of the total population on day 0 to $44.29\% \pm 7.30\%$, $48.38\% \pm 5.07\%$ and $24.58\% \pm 3.02\%$ under cytokines alone, contact co-culture and non-contact co-culture conditions, respectively (Table 2) (Figure 3C). The non-contact co-cultured cells showed a decrease in HSPC number and fraction compared with cells under contact co-culture conditions. Though cellular expansion was mainly driven by factors secreted by CB-EPCs, the direct cell-cell interaction under contact culture conditions may be necessary to maintain the phenotype of the progenitors or precursors, and inhibition of excessive or uncontrolled differentiation.

Co-culture with CB-EPCs retained hematopoietic stem cell pool

On day 0, $90.77\% \pm 2.35\%$ of HSPCs resided in G0/G1 phase, $4.67\% \pm 0.47\%$ in S and $5.56\% \pm 0.29\%$ in G2/M phase. HSPCs cultured without CB-EPCs showed a decrease of cells in G0/G1 phase ($60.67\% \pm 1.68\%$, $P = 0.000$), an increase of cells in S phase ($31.40\% \pm 3.50\%$, $P = 0.000$), and comparable cells in G2/M phase ($5.94\% \pm 0.17\%$, $P = 0.120$) compared with HSPCs on day 0, which suggested that more hematopoietic stem cells entered cell division after *in vitro* culture. Similarly, CB-EPCs contact co-culture induced $59.77\% \pm 1.94\%$ ($P = 0.000$) of HSPCs into G0/G1 phase, $35.17\% \pm 2.75\%$ into S phase ($P = 0.000$) and $5.62\% \pm 0.39\%$ into G2/M phase ($P = 0.851$) compared with HSPCs on day 0 (Figure 4A). Thus, more cells entered cell division than cells in day 0 under *in vitro* single culture or co-culture condition and resulted in cell expansion. Notably, though more obvious expansion was achieved under co-culture conditions than in single HSPCs culture, a comparable proportion of cells existed in G0/G1 phase, which indicated that the co-culture of HSPCs with CB-EPCs retained considerable quiescent stem cells (nearly 60%) to guarantee the long-term expansion and hematopoietic development. The HSPCs under co-culture conditions were not amplified at the expense of the characteristic loss of stem cells.

Co-culture with CB-EPCs heightened the pluripotent differentiation of HSPCs in vitro

The clonogenic potential of the HSPCs under contact co-culture for 7 days was then examined for CFU potential. Significantly higher CFU counts were observed at 1 week in HSPCs co-cultured with CB-EPCs (446.33 ± 6.66) than in those cultured without CB-EPCs (257.33 ± 6.67) ($P = 0.000$), and were also higher than those of freshly isolated HSPCs (124.67 ± 9.71 , $P = 0.000$) (Figure 4B). Further, the cell components of the CFU-C differed, and a larger number of lineage-committed CFU-C were detected, with higher amounts of CFU-GM (63.67 ± 10.60 vs. 25.33 ± 4.73 , $P = 0.001$), BFU-E (196.00 ± 14.93 vs. 60.67 ± 5.69 , $P = 0.000$) and CFU-E (174.67 ± 16.26 vs. 27.33 ± 5.69 , $P = 0.000$) in co-cultured HSPCs than fresh isolated HSPCs, while the amounts of CFU-GEMM were similar (12.00 ± 3.00 vs. 11.33 ± 4.73 , $P = 0.816$) (Figure 4B). Higher component amounts of CFU were also observed in co-cultured HSPCs than in single culture HSPCs with respect to CFU-GM (14.67 ± 4.51 , $P = 0.000$), CFU-GEMM (3.33 ± 1.53 , $P = 0.019$), BFU-E (104.00 ± 14.53 , $P = 0.000$) and CFU-E (135.33 ± 13.87 , $P = 0.009$).

CB-EPCs co-cultured cells engrafted in NOD/SCID mice

To determine the long-term reconstitution potential of *ex-vivo* expanded human HSPCs, the NOD/SCID model system was used to explore the engraftment of

SCID-repopulating cells (SRCs) in NOD/SCID mice. As shown in Figure 5A, mice transplanted with progeny of HSPCs after contact co-culture in group C ($n = 10$) manifested a similar recovery **trend** of blood cell count as those with freshly isolated HSPCs in group A ($n = 10$), while mice transplanted with single culture HSPCs in group B ($n = 7$) showed mild recovery around 3 to 4 weeks but declined and died soon within 5 weeks. As a control, mice in group D ($n = 3$) without HSPCs infusion only showed one-week decreased blood cell count as they died within 2 weeks. No significant difference in survival was observed between group A and group C ($P = 0.892$), with a median survival of 47 days for group A (Figure 5B). The survival of group B, however, was significantly lower than in group A ($P = 0.008$) and group C ($P = 0.007$) with a median survival time of 20 days. Mice in group D died in 7, 10, and 13 days, respectively.

The engraftment was determined after 8 weeks from transplantation as the percentage of human CD45⁺ cells out of BM cells. Mice with more than 0.1% CD45⁺ cells were considered positive. There were 5 mice in group A, 6 mice in group C and no mouse in group B or group D that survived for 8 weeks. Therefore, engraftment analysis was carried out between group A and group C as shown in Figure 5C. All mice analyzed in group A and 5 out of 6 mice in group C were engrafted with human HSPCs. The median chimerism percentage of group A was $16.0\% \pm 14.3\%$, and when compared with group C ($13.3\% \pm 11.0\%$), no difference was observed ($P = 0.750$).

Role of hematopoiesis-related factors and Wnt signaling pathway in HSPCs expansion

After successful expansion and evaluation of HSPCs co-cultured with CB-EPCs, we further explored the underlying mechanism. The gene expression of hematopoietic factors of MSCs was assayed and identified by many other reports [18-20]. MSCs acted as effective feeder cells for HSPCs expansion and served as a positive control in our study. RT-qPCR analysis revealed that granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and FLT3-L were lower in CB-EPCs than MSCs, with 3.38 ± 1.36 ($P = 0.031$), 1.02 ± 0.74 ($P = 0.009$) and 0.77 ± 0.52 ($P = 0.027$) fold decrease, respectively. The levels of SCF and TPO were comparable. Nevertheless, the transcription of IL-6 (4.24 ± 1.42 , $P = 0.017$), angiopoietin-1 (7.75 ± 0.90 , $P = 0.000$) and SDF-1 (8.25 ± 2.03 , $P = 0.025$) was elevated in CB-EPCs compared with MSCs (Figure 6A).

The expression of key signaling molecules relevant to hematopoietic stem cell proliferation was also explored. As shown in Figure 6B, CB-EPCs showed a higher transcriptional level of Wnt5A, the key ligand in Wnt signaling pathway, with an 11.76 ± 2.11 fold increase compared with MSCs. Other signaling molecules including sonic hedgehog (Shh) and Jagged-1 of CB-EPCs exerted similar transcriptional level with MSCs with $P > 0.05$. Further, the co-cultured HSPCs exhibited a higher level of Wnt5A transcription as well, with a 3.49 ± 0.54 fold increase ($P = 0.001$) than cells before co-culture, while other molecules (Wnt1, Wnt3A, Wnt10B, Shh and Jagged-1)

showed similar expression level. It might indicate that Wnt5A mediated Wnt signaling controls HSPCs expansion under CB-EPCs co-culture conditions (Figure 6C).

Discussion

The need for contact between HSPCs and AGM subregion stromal cells in HSPCs expansion has been demonstrated[21]. It is well known that hematopoietic and endothelial cells are located adjacent to each other in AGM region and originate from the same progenitor during embryonic development [13], which suggests a close association of EPCs with HSPCs. EPCs also reside in BM microenvironment[22], and act as endothelial precursors and important cell components involved in self-renewal and differentiation of HSPCs. Here we report an effective method to expand CB-HSPCs using EPCs as new feeder cells. We isolated and cultured EPCs from CB as feeder cells for *in vitro* HSPCs expansion, due to the non-invasive harvesting procedure, high concentration and high proliferative ability compared with adult BM and PB[23]. CB-EPCs were successfully obtained using endothelial-specific medium and presented as forming colonies and a typical endothelial morphology. As studied by Ashara T [15] and other researchers [24-26], the EPCs phenotypes were mainly defined by the expression of progenitor cell markers (CD34 and CD133) and endothelial cell surface markers (CD31 and KDR), and negative for hematopoietic

surface markers (CD45 and CD14). The expression of CD133 in EPCs was distinct from mature endothelial cells. A higher expression of endothelial genes was identified, further consolidating the endothelial characteristics of EPCs. The MNCs from CB were cultured on fibronectin for more than 14 days to obtain EPCs, which were also reported as ECFCs with a higher proliferative capacity and spontaneously form human blood vessels *in vivo* [25, 27]. In our study, it also revealed a higher proliferative ability of EPCs than mature endothelial cells, and the number of cultured EPCs was guaranteed for large scale clinical use. As a precursor, EPCs possessed immature features such as relative weak tube formation. On the other hand, however, they had stronger migration potential.

The expanded EPCs were ready for use with different donors from CB for *ex vivo* expansion. The combination of cytokine factors including SCF, FLT3-L and TPO has been widely used for CD34⁺ cells expansion in serum-containing medium [19, 28]. Using EPCs as feeder cells for CB-derived CD34⁺ cells *ex vivo* expansion, we found a greater total cell and CD34⁺ cell expansion than those cultured with cytokines alone, indicating that EPCs act as potential feeder cells. Direct contact co-culture exhibited a similar total cell and CD34⁺ cells expansion with non-contact co-culture, suggesting that the effect of EPCs on HSPCs expansion was mostly mediated by the presence of soluble factors other than cell-to-cell contact. Further, the *ex vivo* pluripotency was strengthened by co-culture with EPCs, and cell cycle analysis demonstrated that co-culture promoted cell division and simultaneously retained considerable quiescent cells.

Notably, increased CD34⁺ cell number and CFUs are not quantitative indicators of *in vivo* repopulating potential [29]. A few *ex vivo* HSPCs culture system showed disappointing results owing to insufficient activation of primitive CD34⁺CD38⁻ cells differentiation and a loss of repopulating capacity following short-term culture[30]. Further, SRCs represent cell subsets with long-term reconstitution potential in NOD/SCID model system and are most highly concentrated within CD34⁺CD38⁻ population[29]. By contrast, CFUs and long-term culture-initiating cells (LTC-ICs) are found in both the CD34⁺CD38⁺ and CD34⁺CD38⁻ populations and contributed little to NOD/SCID engraftment[31, 32]. Therefore further phenotypic analysis of CD34⁺CD38⁻ cells by flow cytometry revealed an average fold expansion of 156.17 under direct contact co-culture condition, higher than those cultured with cytokines alone (79.12) and under non-contact co-culture condition (95.43). It indicated that using EPCs as feeder cells promoted stem cells proliferation and division as well as maintained stem cell repopulating capacity for successful engraftment. More interestingly, direct contact co-culture resulted in a higher percentage of CD34⁺CD38⁻ cells with an average of 48.38% than that of non-contact co-culture (24.58%), which suggested that cytokine secretion was unlikely to be the sole mechanism for positive effect of EPCs on HSPCs expansion, and the requirement of physical contact between HSPCs and EPCs prevented the loss of long-term reconstitution capacity after short-term *ex vivo* culture. A previous study using MSCs as feeder cells to expand CB also reported the necessary effect of cell-to-cell contact [33, 34]. Studies using mature human brain endothelial cell to support CD34⁺ cells,

with maximum 5.4 fold of CD34⁺ cells and 212-fold of CD34⁺CD38⁻ cells increase[28, 31] used different cytokine combination and adult BM CD34⁺ cells for expansion, with no direct comparison. Further, the mature endothelial origin limited their use as feeder cells

Expansion and infusion of a higher proportion of hematopoietic progenitors owns potential to accelerate hematopoietic recovery. Though the overall hematopoietic recovery trend of mice transplanted with the progeny of co-cultured CD34⁺ cells was similar when compared with those with freshly isolated CD34⁺ cells, *in vivo* analysis still revealed a relatively faster hematopoietic recovery within the first 5 weeks after transplantation. It may be related to the release of mature cells by the expanded HSPCs faster than the non-expanded counterparts[8, 35], thereby protecting recipients against radiation and infection of early stage. Besides, it is necessary to verify the stem cell activity of expanded cells *in vivo*. When equal number of co-cultured cells and fresh isolated CD34⁺ cells were transplanted to NOD/SCID mice, comparable chimerism formation of BM were obtained, suggesting uncompromised ability to sustain long-term human hematopoiesis *in vivo* of the *ex vivo*-generated HSPCs. Though HSPCs cultured free from stroma also manifested expansion of CD34⁺ cells and CD34⁺CD38⁻ primitive cells, they were not successfully engrafted *in vivo* and the transplanted mice died within 5 weeks, which indicated that additional factors including disrupted homing and enhanced cell apoptosis affected engraftment [36].

The cytokines secreted by EPCs and cell-to-cell contact contributed to the *ex vivo* expansion of HSPCs. Higher gene expression of IL-6 and angiopoietin-1 in EPCs

played a role in the efficient expansion of HSPCs. IL-6 had a positive effect on primitive cell expansion, and the combination of FLT3-L, TPO, SCF and IL-6 has been shown to promote proliferation and expansion of human long-term *in vivo* repopulating cells[37, 38]. Angiopoietin-1 signaling via the receptor Tie-2 contributed to angiogenesis and hematopoiesis throughout development and in adults, played a key role in hematopoietic lineage differentiation and increased the long-term repopulation potential of HSPCs[39]. Higher expression of SDF-1 in EPCs might facilitate HSPCs homing to BM niches via its specific receptor CXCR4 [39]. EPCs may expand HSPCs via cytokine secretion and Wnt signaling pathway activation. The Wnt5A expressed higher both in EPCs and co-cultured HSPCs. **It could be one of the potential mechanism that Wnt5A participated in Wnt signaling pathway and increased short- and long-term HSPCs repopulation by maintaining HSPCs in a quiescent G0 state[40]. Wnt5A enhanced formation of multilineage colonies of HSPCs and SRCs of human CB cells [41]. Transduction of Wnt5A genes to feeder cells augmented their ability to support HSPCs[42], while removal of Wnt5A from conditioned medium would deplete the stimulatory activity to HSPCs' expansion[43]. However, mechanism study of Wnt5A effects on HSPCs expansion *in vitro* and repopulating capacity *in vivo* is limited and needs to be further elucidated.**

In summary, EPCs are able to expand the HSPCs *in vitro* and induce primitive cell expansion. EPCs provide a microenvironment promoting the proliferation of functional CB HSPCs and progenitor cells, by producing various growth factors and signaling molecules. Pluripotent HSPCs need to interact directly with EPCs in order

to maintain their stem-cell characteristics. The improved co-culture method reported here offers a possible and efficient way to expand CB HSPCs for transplantation clinically, although the ability of these cells for human engraftment has yet to be demonstrated.

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Figure legends

Figure 1. Characteristics of cultured EPCs. (A). Representative phase contrast photomicrograph of an ECFC colony (21 to 28 days after culture, left panel) and subsequent merged colonies (right panel) cultured from CB (100× magnification). (B) Cytometric analysis of cultured EPCs (lower panel) for endothelial cell markers KDR, CD31 and CD34, progenitor cell markers CD34 and CD133, and hematopoietic/monocytic markers CD45 and CD14, with matched isotype IgG as negative control. The mature endothelial cells HUVECs (upper panel) served as a phenotypic control. (C) RT-qPCR analysis of EPCs for endothelial markers VE-cadherin, vWF, VEGF, Tie-1, Tie-2 and E-selectin, with HUVECs as a control and is shown as $2^{-\Delta\Delta C_t}$ method. The asterisk indicates $P < 0.05$.

Figure 2. Functional analysis of EPCs. (A). Proliferation curve of different passages (P1, P3

and P5) of EPCs and compared with HUVECs by using CCK8 reagent analysis. (B). Both EPCs and HUVECs were plated in Matrigel for the formation of capillary-like structures (100× magnification). Capillary tube numbers were calculated in at least 3 scope (40×) and shown as mean ± SD. (C). Phase contrast photomicrograph of cells under the Transwell chamber (100× magnification). Data are shown as mean ± SD. The asterisk indicates $P < 0.05$.

Figure 3. EPCs efficiently expanded CB-HSPCs in vitro. (A). Pictures of HSPCs in day 0 and day 7 under single culture or EPCs co-culture condition. Co-cultured HSPCs were suspended over the adherent EPCs (B). Expansion fold of total nucleated cells, CD34⁺ cells (left panel) and CD34⁺CD38⁻ cells (right panel) after 7 days culture in vitro. Values are reported as mean ± SD. The asterisk indicates $P < 0.05$ (C). Phenotypic analysis of CD34 and CD38 expression on HSPCs on day 0 and following single culture and EPCs co-culture for 7 days. Representative flow cytometry is shown.

Figure 4. Co-culture with EPCs retained hematopoietic stem cell pool and heightened the differentiation potential of HSPCs in vitro (A). Representative propidium iodide-stained cell cycle analysis of cells under different culture condition, with fresh isolated HSPCs as a control. (B). Count of different colony types of methylcellulose-based clonogenic assay performed on HSPCs plated after 7 days of culture under single culture and co-culture condition. HSPCs isolated at day 0 serve as control. The asterisk indicates $P < 0.05$.

Figure 5. The progeny co-cultured with EPCs engrafted in NOD/SCID mice. (A). Peripheral blood cells count of mice in different groups. (B). Survival analysis of different treatment groups (A vs. B, $P=0.008$; A vs. C, $P=0.892$; B vs. C, $P=0.007$). (C). Human cell engraftment in NOD/SCID mice receiving transplants of CD34⁺ cells (n=5) and co-cultured progeny (n=6). Percentage of human CD45 cells is shown.

Figure 6. Hematopoietic related cytokines and signaling molecules contribute to supportive effect of EPCs. (A). RT-qPCR analysis revealed the level of hematopoietic related genes of EPCs, with MSCs as a control. (B). The gene transcriptional levels of molecules related to

different signaling pathways of EPCs are shown, with MSCs as a control. (C).Molecules of signaling pathways are compared between HSPCs at day 0 and at day 7 after co-cultured with EPCs. All data are shown as mean $\pm$ SD. The asterisk indicates $P < 0.05$

Tables:

Table 1. The sequences of primers used for RT-qPCR

Genes	Primers (Forward)	Primers (Reverse)
VE-cadherin	GGACATAACACCACGAAACG	CGGTCAAACCTGCCCATACTT
vWF	TCGGACCCTTATGACTTTGC	CCAGAGGAGATGTTGCATGA
VEGF	CTACCTCCACCATGCCAAGT	AGCTGCGCTGATAGACATCC
Tie-1	GACCCACACTGACCAACACA	GCCAGACCATACTCCAGAGG
Tie-2	TGTGACCAGAGAATGGGCTA	TGATTGCCTCTCCTCGAACT
E-selectin	TGGCTAATGAAGGGAAAGGA	TACAGCGAGCAAGGGAGAGT
GM-CSF	CCAGCCACTACAAGCAGCAC	GGGATGACAAGCAGAAAGTCC
IL-3	GGACCAGAAGCATTTCACCT	ATGAGAACACAACCGCACAA
FLT3-L	GCTTGTGGAGCACTGACCT	CATCCTCTGGCTGGTGACT
SCF	TCATTCAAGAGCCCAGAACC	TTCAGATGCCACTACAAAGTCC
TPO	CTTCGTGACTCCCATGTCCT	CCCAAGCTAAAGTCCACAGC
IL-6	CTTCGGTCCAGTTGCCTTCT	GTGAGTGGCTGTCTGTGTGG
Angiopoietin-1	CGTGGAACCGGATTTCTCT	GCCATCTCCGACTTCATGTT
Wnt5A	CACTTTGTGGAGAGGGCATT	TAGGCATGGGTTTCCATTCT

EPCs as stroma to expand cord blood derived HSPCs

SDF-1	GGCTCCCTGTAACTCTTCA	CAGACTCAATCCCAACACACA
Wnt1	GGTCCTCCTAAGTCCCTTCC	CCCTGTAACTCCTGCTTCA
Wnt3A	AGATGGTGGTGGAGAAGCAC	GTTGGGCTCGCAGAAGTTG
Wnt10B	GGACTCTGGGATGTGTAGCC	TGAGAAGTGGGAGGAGCAAT
Shh	AGTAGCCCTAACCGCTCCAG	GCCAGGTGCCTATTTGGTAG
Jagged-1	CGAGGACTATGAGGGCAAGA	TTCAGGTGTGTCGTTGGAAG
β -actin	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT

Table 2. CB-EPCs co-culture promoted the expansion of CB CD34⁺ subsets (mean $\pm$ SD, $\times 10^5$ /ml)

Culture conditions	Total	CD34 ⁺	CD34 ⁺ CD38 ⁻	CD34 ⁺ CD38 ⁻ (%)
Input	5.00 $\pm$ 0.00	4.44 $\pm$ 0.29	0.11 $\pm$ 0.03	2.13 $\pm$ 0.58
Stoma-free	18.82 $\pm$ 3.02	15.21 $\pm$ 2.31	8.19 $\pm$ 0.67	44.39 $\pm$ 7.30
CB-EPCs contact	33.77 $\pm$ 4.20	24.27 $\pm$ 1.74	16.40 $\pm$ 3.10	48.38 $\pm$ 5.07
CB-EPCs non-contact	37.80 $\pm$ 4.01	18.32 $\pm$ 1.22	10.45 $\pm$ 0.40	24.48 $\pm$ 3.02

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6