Effect of testosterone and hypoxia on the expansion of umbilical cord blood CD34$^+$ cells *in vitro*

LIPING ZHOU[1,2], XIAOWEI ZHANG[2], PANPAN ZHOU[1], XUE LI[1], XUEJING XU[1], QING SHI[1], DONG LI[1] and XIULI JU[1]

[1]Department of Pediatrics, Qilu Hospital, Shandong University, Jinan, Shandong 250012; [2]Department of Pediatrics, The Sixth People's Hospital of Jinan, Jinan, Shandong 250200, P.R. China

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Abstract. Successfully expanding hematopoietic stem cells (HSCs) is advantageous for clinical HSC transplantation. The present study investigated the influence of testosterone on the proliferation, antigen phenotype and expression of hematopoiesis-related genes in umbilical cord blood-derived cluster of differentiation (CD)34$^+$ cells under normoxic or hypoxia conditions. Cord blood (CB) CD34$^+$ cells were separated using magnetic activated cell sorting. A cytokine cocktail and feeder cells were used to stimulate the expansion of CD34$^+$ cells under normoxic (20% O_2) and hypoxic (1% O_2) conditions for 7 days and testosterone was added accordingly. Cells were identified using flow cytometry and reconstruction capacity was determined using a colony-forming unit (CFU) assay. The effects of oxygen concentration and testosterone on the expression of hematopoietic-related genes, including homeobox (HOX)A9, HOXB2, HOXB4, HOXC4 and BMI-1, were measured using reverse transcription-quantitative polymerase chain reaction. The results indicated that the number of CFUs and total cells in the testosterone group increased under normoxic and hypoxic conditions compared with the corresponding control groups. Furthermore, the presence of testosterone increased the number of CFU-erythroid colonies. In liquid culture, the growth of CD34$^+$ cells was rapid under normoxic conditions compared with under hypoxic conditions, however CD34$^+$ cells were maintained in an undifferentiated state under hypoxic conditions. The addition of testosterone under hypoxia promoted the differentiation of CD34$^+$ cells into CD34$^+$CD38$^+$CD71$^+$ erythroid progenitor cells. Furthermore, it was determined that the expression of hematopoietic-related genes was significantly increased ($P<0.05$) in the hypoxia testosterone group compared with the other groups. Therefore, the results of the current study indicate that a combination of hypoxia and testosterone may be a promising cultivation condition for HSC/hemopoietic progenitor cell expansion *ex vivo*.

Correspondence to: Professor Xiuli Ju or Professor Dong Li, Department of Pediatrics, Qilu Hospital, Shandong University, 107 Wenhua West Road, Jinan, Shandong 250012, P.R. China
E-mail: juxiulimed@163.com
E-mail: lidong73@sdu.edu.cn

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Introduction

Hematopoietic stem cell (HSC) transplantation is a potentially life-saving procedure used to treat a broad spectrum of disorders, including hematological, immune and genetic diseases (1). It has been demonstrated that bone marrow reconstituting HSCs reside within a small subpopulation of bone marrow or blood-derived mononuclear cells that express the surface antigen cluster of differentiation (CD)34. The efficacy of cord blood (CB) transplantation is limited by the low cell dose available. Low cell doses at transplant are correlated with delayed engraftment, prolonged neutropenia and thrombocytopenia and elevated risk of graft failure. The successful *ex vivo* culture and amplification of blood-derived CD34$^+$ progenitor cells offers the possibility of HSC transplantation (2). CB is used as an alternative for bone marrow or mobilized peripheral blood grafts, particularly when no matched human leukocyte antigen-related or unrelated donors are available (3). Under similar conditions, recipients of CB transplants exhibit a lower incidence of acute and chronic graft-versus-host disease compared with recipients of bone marrow transplants (4). The use of CB as a source of HSCs utilized for transplantation has increased and >3,000 CB transplants are conducted annually (5).

Allogeneic transplantation with human umbilical cord blood (hUCB) in adult recipients is mainly limited by a low CD34$^+$ cell dose (6). Thus, CBT is generally only used in children and low-weight adults. Multiple strategies have been investigated to try to overcome these limitations, one of which involves the *ex vivo* expansion of CB units prior to transplantation (7). Previous studies have demonstrated that HSCs may expand, suggesting that *in vitro* HSCs should be exposed to specific factors and signals that promote self-renewal and amplification (8-10). Furthermore, it has been demonstrated that cell survival and proliferation *in vitro* may be efficiently stimulated by several cytokines, particularly stem cell growth factor and thrombopoietin (TPO) (11). It has also been indicated that the fate of HSCs may be chemically modulated by adding small biological and chemical molecules to the culture

media *in vitro* to induce cell survival and division, while simultaneously preventing stem cell differentiation (1). Small molecules, including all-trans retinoic acid copper chelator, tetraethylenepentamine, prostaglandin E2 and 6-bromoindirubin-3'-oxime (BIO) all serve a role in HSC (12); for instance, all-trans retinoic acid serves a role in stem cell differentiation to several lineages, including myeloid differentiation, and BIO is the first pharmacological agent demonstrated to maintain self-renewal in embryonic stem cells (12).

The present study evaluated the effects of three small-molecule steroid hormones, testosterone, norepinephrine and epinephrine, on HSCs. As HSCs all express the surface antigen CD34, $CD34^+$ cells were selected for subsequent experiments.

Oxygen concentration is an important influence on the growth of HSCs. *In vivo*, HSCs are found in microenvironments, known as niches, in the bone marrow. Throughout the bone marrow, physiological oxygen concentrations are <4% and almost 0% in certain areas (13). It has been hypothesized that the hypoxic environment maintains the characteristics of HSCs and numerous *in vitro* studies investigating the cultivation of HSCs under hypoxic conditions have been performed (14-17). Thus, the present study included hypoxia as a condition in the study design, in order to determine the effects of the three small molecules on $CD34^+$ cell amplification under hypoxic conditions.

Materials and methods

Collection and purification of $CD34^+$ cells. Human CB (n=12; male newborns) was obtained from mothers undergoing full-term deliveries between January and May 2015 in the Department of Obstetrics in Qilu Hospital of Shandong University (Jinan, China) after informed written consent was obtained. Maternal age was between 20 to 40 years old (mean, 26±2), and there was no history of acute, chronic or infectious disease, neonatal apnea, edema or jaundice. The present study was approved by the Ethics Committee of Qilu Hospital of Shandong University. Within 12 h of harvesting the CB. Mononuclear cells (MNCs) were separated using Ficollpaque medium (density 1.077±0.001 g/ml; HaoYang company, Tianjin, China) and centrifuging at 1,726 x g at 12˚C for 25 min. Isolated MNCs were collected and washed twice in RPMI 1640 (Gibco, Los Angeles, USA) plus 5% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cord blood MNCs were incubated with 100 μl of $CD34^+$ micro beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; cat. no. 130046703) at 4˚C for 30 min. Cells were subsequently passed through an LS MACS column (Miltenyi Biotec GmbH) and enriched $CD34^+$ cells were collected in 15 ml tubes by flushing the column. Cells were subsequently suspended in 0.1 M phosphate buffered saline (PBS; pH 7.4). The purity of $CD34^+$ cells was detected using a fluorescence-activated cell sorting system (Guava easyCyte8HT; EMD Millipore, Billerica, MA, USA) and data were analyzed with Guava Incyte version 2.8 (EMD Millipore).

Hormone screening test. $CD34^+$ cells at a density of $2x10^3$ cells/well were seeded into 24-well plates with methylcellulose semisolid medium (MethoCult™ GF H4434) supplemented with stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor, erythropoietin and interleukin (IL)-3 (all Stem Cell Technologies, Inc., Vancouver, BC, Canada) and used to perform colony-forming unit (CFU) assays following the manufacturer's instructions. Cells were cultured under either normoxic or hypoxic conditions. $CD34^+$ cells were divided into control, testosterone ($4.6x10^{-8}$ mol/l; XianJu company, Zhejiang, China), norepinephrine ($5.9x10^{-5}$ mol/l; ShuangHe Company, Beijing, China) and epinephrine groups ($2.7x10^{-6}$ mol/l; YongKang company, Beijing, China). The control groups were treated without hormones and at the same oxygen concentration as other groups. The concentrations of these hormones were determined as previously described (18-23). Cells were incubated in an atmosphere containing 20% O_2 (normoxic conditions) using a Heal Force Tris-gas incubator (HF240; Heal Force Bio-meditech Holdings Limited, Shanghai, China) or 1% O_2 (hypoxic conditions; HF100; Heal Force Bio-meditech Holdings Limited) containing 5% CO_2 at 37˚C. Following 2 weeks culture, the number and type of CFUs were determined using an inverted microscope (IX71 Olympus Inverted Microscope; Olympus Corporation; Tokyo, Japan). The types of colonies identified included colony-forming units-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte/macrophage (CFU-GM) and colony forming units-mixed (CFU-Mix; >50 cells) as previously described (24).

Preparation of feeder. Umbilical cord tissue was also obtained from the healthy donor mothers who donated CB for the present study. Informed written consent was received. Umbilical cords were dissected following thorough washing and blood vessels were removed. Small fragments (1-2 mm^3) were cut and placed in plates containing low glucose-Dulbecco's modified Eagle's medium (L-DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (All Gibco; Thermo Fisher Scientific, Inc.). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2, as previously described (25,26). The medium was replenished every 3-4 days. Following 7 days culture, adherent cells were observed growing from the individual tissue explants. Adherent fibroblast-like cells became confluent following 2-3 weeks culture. Subsequently, cells were treated with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and passaged at $1x10^4$ cells/cm^2 in L-DMEM. Cells at the 5 and 7th passage were used following γ-irradiation at a dose of 15 Gy following a previously described protocol (25). A total of $5x10^5$ umbilical cord-mesenchymal stem cells were seeded in a 25-cm^2 culture flask and served as the feeder layer for subsequent experiments.

Co-cultivation of $CD34^+$ cells with feeder. CB $CD34^+$ cells ($1.1x10^5$ cells/ml) were co-cultured with feeder in HSC expansion medium (Stem Cell Technologies, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 70 ng/ml SCF, 30 ng/ml IL-3, 30 ng/ml FMS-like tyrosine kinase 3 ligand, 20 ng/ml of IL-6, 20 ng/ml bone morphogenetic protein-2 and 20 ng/ml TPO (all R&D Systems, Inc., Minneapolis, MN, USA). Cultures were maintained at 37˚C in an atmosphere containing 5% CO_2 for 7 days. Cytokine concentrations were determined as described previously (11,27). $CD34^+$ cells were divided into 4 groups: i) A normoxia

testosterone group, consisting of a co-culture of $CD34^+$ cells and feeder plus testosterone undecanoate ($4.6x10^{-8}$ mol/l) in 20% O_2; ii) a normoxia control group, consisting of co-culture of $CD34^+$ cells and feeder without hormone in 20% O_2; iii) a hypoxia testosterone group, consisting of a co-culture of $CD34^+$ cells and feeder plus testosterone undecanoate in 1% O_2 and iv) a hypoxia control group, consisting of a co-culture of $CD34^+$ cells and feeder without hormone in 1% O_2. The medium was replenished every 3-4 days. On day 7, the total cell suspensions were harvested for use in subsequent experiments.

CFU assay following liquid culture. $CD34^+$ cells were amplified 7 days after co-cultivation with feeder and were seeded into 24-well plates in the MethoCult GF H4434 medium. The cells were seeded at a density of $2x10^3$ cells/well following the manufacturer's instructions for the CFU assay. Each cell group was plated in 6 replicate wells and cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2 and 20% O_2. Following 2 weeks culture, the number and type of CFUs were determined using an inverted microscope (magnification, x20; IX71 Olympus Inverted Microscope; Olympus Corporation).

Immunophenotypic analysis. Following co-culture of $CD34^+$ cells for 7 days, cells were confirmed by four-color flow cytometry using a fluorescence-activated cell sorting Calibur analyzer (Guava Cyte 8HT; EMD Millipore). The cells ($1x10^6$) were suspended in 100 μl PBS containing fluorescein isothiocyanate-conjugated anti-CD34 antibody 10 μl (BioLegend, San Diego, USA; cat. no. 343604), phycoerythrin-conjugated anti-CD71 (BD Biosciences, San Jose, CA, USA; cat. no. 560981), and phycoerythrin-71-conjugated anti-CD38 antibodies (BD Biosciences; cat. no. 555537; 1:10) for 15 min at room temperature, following the manufacturer's instructions. Cells were subsequently washed with PBS and the data was examined using Guava Incyte (Version 2.8, EMD Millipore).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). $CD34^+$ cells were underwent RT-qPCR following 7 days co-culture *in vitro*. Total RNA was extracted using TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using the ReverTra Ace QPCR RT Master mix kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. Levels of homeobox (HOX)A9, HOXB2, HOXB4, HOXC4, BMI1, GATA-1, C-MYB, HOXB6, NFE2 and hypoxia inducible factor α (HIF-1α) were analyzed using qPCR (95˚C for 1 min, 95˚C for 15 sec, and 60˚C for 1 min for a total of 40 cycles) on an ABI 500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR green I dye (Toyobo Co., Ltd.). Table I presents the primer sequences used in RT-qPCR and all primers were purchased from BioSun Technology Co. Ltd. (Shanghai, China). GAPDH was used as an internal control. The expression of each gene was determined using the $2^{-\Delta\Delta Cq}$ method (28) and data were analyzed using Sequence Detection software (version 1.4; Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of mRNA is presented as the fold difference with respect to the untreated control groups and the control group values were set at a fold change equal to one.

Statistical analysis. Data were analyzed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± standard deviation. Two-way analysis of variance with Fisher's least significant difference as a post hoc analysis was used for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Primer sequence (5'-3')
HOXB4	Forward	AGCACGGTAAACCCCAATTACG
	Reverse	GTGTCAGGTAGCGGTTGTAGTG
HOXB6	Forward	TCGTGCAACAGTTCCTCCTT
	Reverse	CGCGTCAGGTAGCGATTGTA
HOXA9	Forward	CCACGCTTGACACTCACACT
	Reverse	GGGTTATTGGGATCGATGGGG
GATA1	Forward	GACACTCCCCAGTCTTTCAGG
	Reverse	CAGTTGAGGCAGGGTAGAGC
NFE2	Forward	ACTCTGGCCCAGTAGGATGT
	Reverse	TTGGAGCATTCAGACCCTGC
HIF-1α	Forward	TTCCTTCTCTTCTCCGCGTG
	Reverse	AACTTATCTTTTTCTTGTCGTTCGC
HOXB2	Forward	CTAGCCTACAGGGTTCTCTC
	Reverse	CACAGAGCGTACTGGTGAAAAA
BMI-1	Forward	TGGACTGACAAATGCTGGAGA
	Reverse	GAAGATTGGTGGTTACCGCTG
C-MYB	Forward	GAGGTGGCATAACCACTTGAA
	Reverse	AGGCAGTAGCTTTGCGATTTC
HOXC4	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA

HOX, homeobox; HIF-1α, hypoxia inducible factor α.

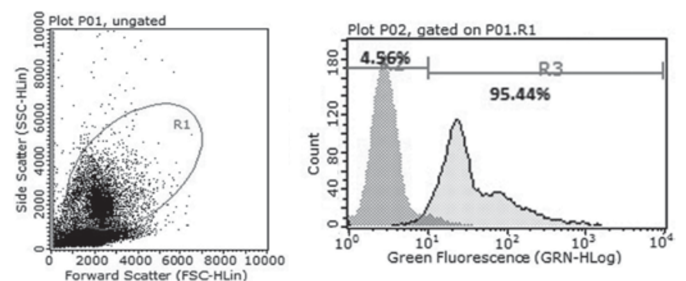

Figure 1. Flow cytometric analysis determined the magnetic activated cell sorting-enriched $CD34^+$ cell purity. Flow cytometry indicated that >95% magnetic activated cell sorting-enriched cells expressed $CD34^+$. CD, cluster of differentiation.

Results

Effects of hormone and oxygen concentrations on CFU. Magnetic activated cell sorting and flow cytometric analysis demonstrated that >95% cells expressed $CD34^+$ (Fig. 1).

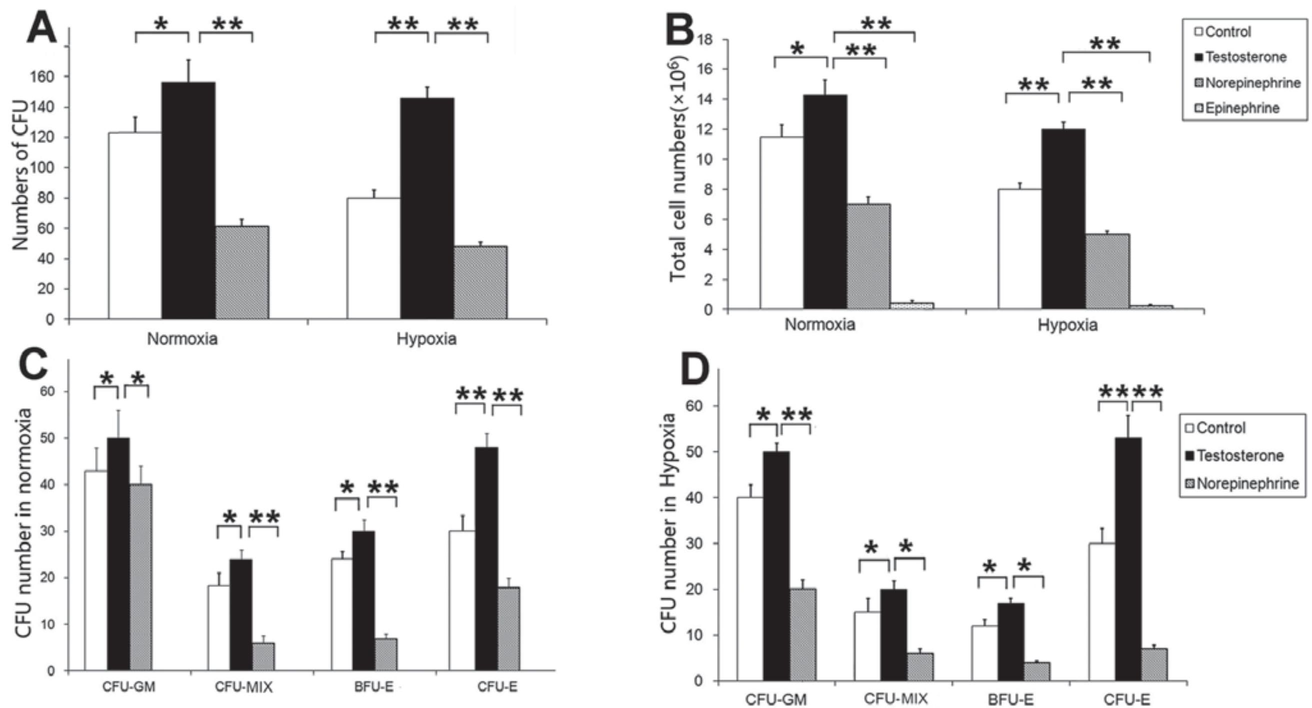

Figure 2. Effects of different hormones and different oxygen concentrations on CFU formation (A) Comparison of the number of CFUs in different groups. (B) Comparison of the total number of cells in different groups. (C) Comparison of the colony types in different normoxia groups. The presence of testosterone significantly increased the numbers of CFU, particularly CFU-Es. (D) Comparison of the colony types among different hypoxia groups. The presence of testosterone significantly increased CFU numbers, particularly CFU-Es. Norepinephrine significantly inhibited colony formation and cell amplification under normoxic and hypoxic conditions. Data are presented as the mean + standard deviation. *P<0.05 and **P<0.01. CFU, Colony-forming unit; CFU-GM, colony-forming unit-granulocyte/macrophage; CFU-MIX, colony forming units-mixed; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming units-erythroid.

Subsequently, the effects of testosterone, norepinephrine and epinephrine on the colony formation function of CD34+cells were investigated. The colony number of the testosterone groups were significantly increased compared with the control and norepinephrine groups under normoxia (P<0.05 and P<0.01, respectively) and hypoxia (both P<0.01; Fig. 2A). There was no evidence of colony formation in the epinephrine group under normoxic and hypoxic conditions, thus the epinephrine group was excluded from the analysis. In addition, the total number of cells in the testosterone group was significantly increased compared with the control, norepinephrine and epinephrine groups under normoxia (P<0.05, P<0.01 and P<0.01 respectively) and hypoxia (all P<0.01; Fig. 2B).

Regarding colony types, the presence of testosterone significantly increased the number of different CFUs (Fig. 2C). In the testosterone groups, under normoxic conditions the number of CFU-GMs was significantly increased (50±8) compared with the control group (43±6; P<0.05) and the norepinephrine group (39±4; P<0.05). Furthermore, the number of CFU-MIXs was significantly increased (22±2) compared with the control group (18±3; P<0.05) and the norepinephrine group (5±1; P<0.01). In addition, the number of BFU-Es was significantly increased (30±4) compared with the control group (26±5; P<0.05) and the norepinephrine group (6±1; P<0.01). The number of CFU-Es was significantly increased (48±5) compared with the control group (30±6; P<0.01) and the norepinephrine group (18±2; P<0.01; Fig. 2C). In the testosterone groups under hypoxic conditions, the number of CFU-GM colonies was significantly increased (50±2) compared with the control group (40±4; P<0.05) and the norepinephrine group (20±2; P<0.01). The number of CFU-MIX colonies was significantly increased (19±2) compared with the control group (18±3; P<0.05) and the norepinephrine group (5±2; P<0.05), and the number of BFU-E colonies was significantly increased (17±2) compared with the control group (14±3; P<0.05) and the norepinephrine group (3±1; P<0.05). Similarly, the number of CFU-E colonies was significantly increased (54±8) compared with the control group (28±5; P<0.01) and the norepinephrine group (5±1; P<0.01; Fig. 2D). The results demonstrated that norepinephrine and epinephrine may inhibit cell amplification under normoxic and hypoxic conditions and were therefore not used in subsequent experiments.

Expansion of CD34+ cells and CFU assay. Fresh enriched CB CD34+ cells were co-cultured for 7 days with feeder. Under normoxic conditions, during the first 3 days the cells rapidly entered the logarithmic growth period (testosterone group, $41.53\pm4.31\times10^5$; control group, $30.65\pm2.74\times10^5$; P>0.05), whereas cells under hypoxia grew more slowly (testosterone group, $15.22\pm3.41\times10^5$; control group, $13.37\pm2.10\times10^5$; P>0.05; Fig. 3A and B). However, there was an acceleration in the growth of cells in the hypoxia groups after the first 3 days (testosterone group, $118.44\pm17.72\times10^5$; control group, $79.04\pm10.54\times10^5$; P<0.05 Fig. 3B). Following 7 days culture, total cell numbers in the two normoxia groups were

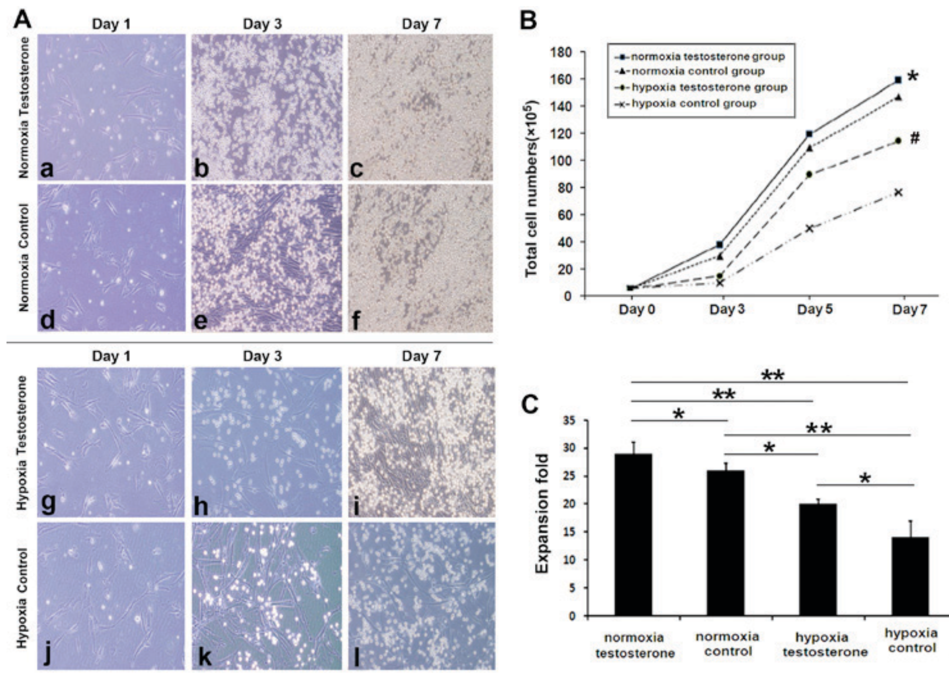

Figure 3. Cluster of differentiation CD34+ cell expansion with cytokine cocktail and feeder (magnification, x200). (A) Cells in the (a-f) normoxia or (g-l) hypoxia testosterone and control groups were cultured for (a,d,g,l) 1, (b,e,h,j) 3 and (c,f,i,l) 7 days. Cell proliferation was markedly higher in the normoxia groups compared with the hypoxia groups and addition of testosterone promoted cell expansion in the normoxia and hypoxia groups. (B) Comparison of the total cell growth rate among all groups. The amplification efficiency was significantly increased in the normoxia groups compared with the hypoxia groups and the presence of testosterone increased cell expansion, particularly during the final 4 days under hypoxia. *P<0.05 vs. normoxia control, hypoxia testosterone and hypoxia control groups. #P<0.05 vs. hypoxia control group. (C) Comparison of total cell numbers. Cell numbers were significantly increased in the normoxia groups compared with the hypoxia groups. The addition of testosterone increased amplification in normoxia and hypoxia groups. Data are presented as the mean + standard deviation. *P<0.05 and **P<0.01.

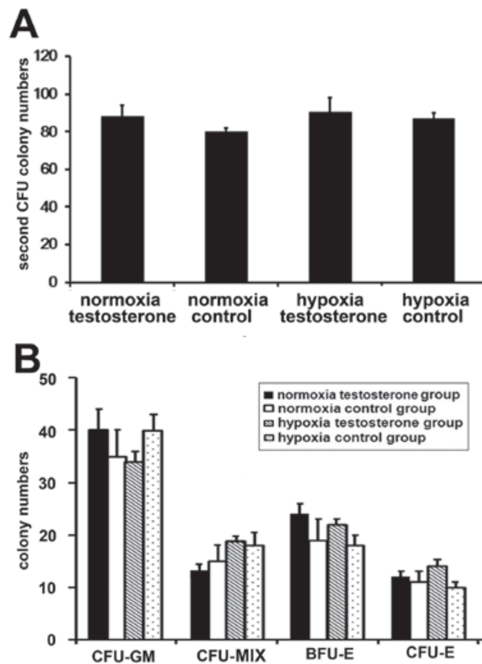

Figure 4. CD34+ cells were amplified 7 days later and transferred into the methylcellulose medium for CFU assay. Similar CFU colony numbers were observed among all groups, indicating that the ability of CD34+ cells to differentiate was not affected. (A) Comparison of CFU numbers in all groups indicated that there was no significant difference among any of the groups. (B) Comparison of CFU types in all groups. No significant differences were observed among groups. Data are presented as the mean + standard deviation. CD, cluster of differentiation; CFU, colony-forming unit; CFU-GM, colony-forming unit-granulocyte/macrophage; CFU-MIX, colony forming units-mixed; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming units-erythroid.

significantly increased compared with the two hypoxia groups (P<0.05; Fig. 3C). In addition, the highest amplification efficiency of the cells was observed in the normoxia testosterone group. Subsequently, the expanded CD34+ cells were cultured in methylcellulose medium for the CFU assay to observe the differential potential of HPCs. No significant differences were observed in the number (P>0.05; Fig. 4A) and type (P>0.05; Fig. 4B) of CFUs among the 4 groups.

Analysis of cell phenotypes. Following culture of CD34+ cells for 7 days, CD34, CD38 and CD71 antigen phenotypes were analyzed using flow cytometry (Fig. 5). CD34 and CD38 are the surface markers currently used to identify HSC/HPC and CD71 is the surface marker of erythroid progenitor cells (29,30). The results demonstrated that the proportion of CD34+CD38+CD71+ cells in the normoxia testosterone and control groups were similar (74.98±8.79% vs. 75.82±9.50%, respectively; P>0.05; Fig. 5). However, there were significant differences between the hypoxia testosterone and control groups (87.15±10.13% vs. 60.45±6.58%; P<0.05; Fig. 5). Furthermore, the proportion of CD34+CD38+CD71+ cells in the hypoxia testosterone group was significantly higher compared with the other groups (87.15±10.13% vs. 74.98±8.79%, 75.82±9.50% and 60.45±6.58%; P<0.05). The proportion of CD34+CD38+CD71+ cells in the hypoxia control group was lowest compared with the other groups (60.45±6.58%; P<0.05; Fig. 5). These results suggest that hypoxia may be beneficial in maintaining CD34+ cells in an undifferentiated state and the addition of testosterone may promote the differentiation

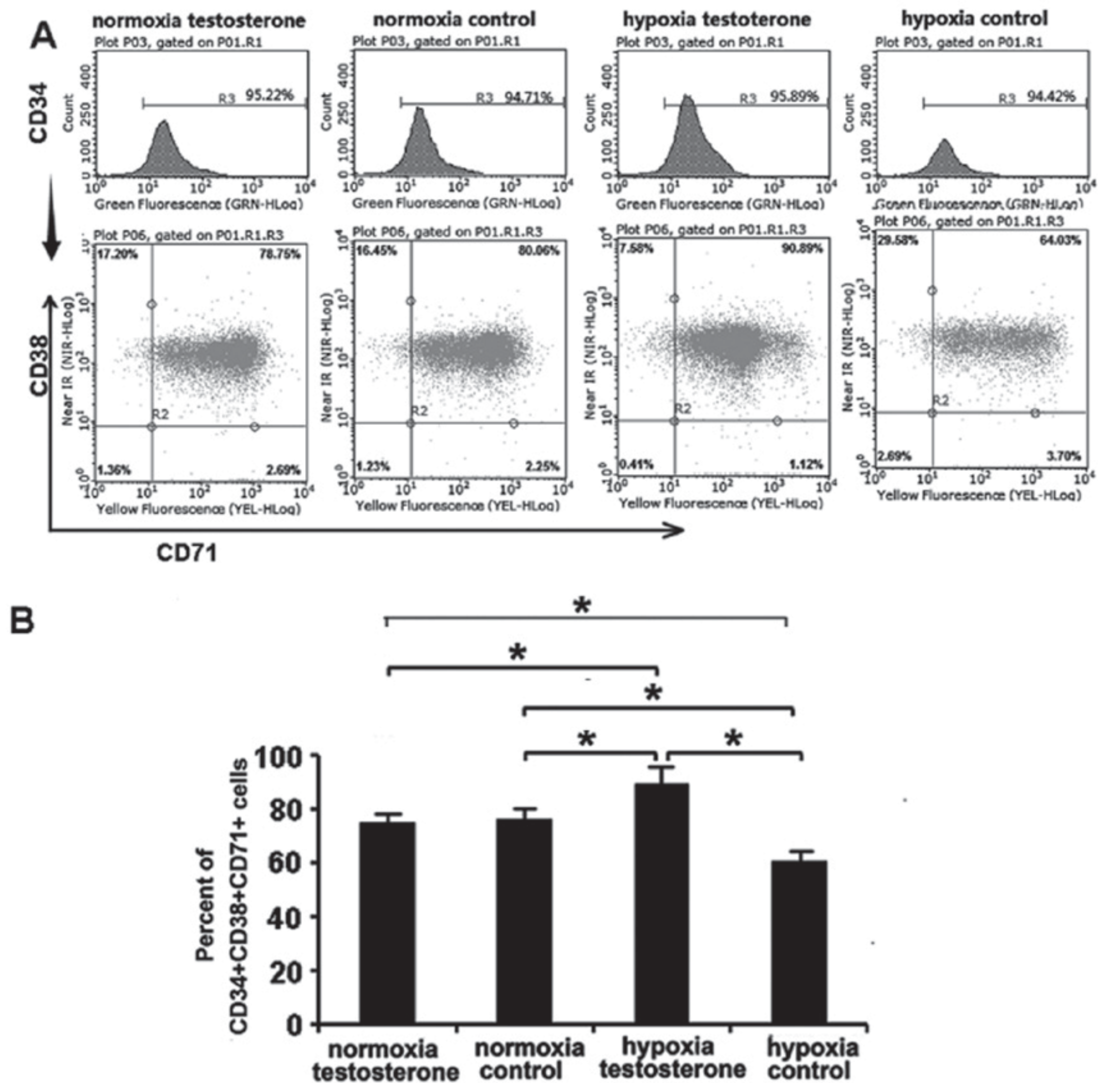

Figure 5. Detection of the hematopoietic stem cell antigen phenotype. (A) Flow cytometry graphs of the normoxia testosterone, normoxia control, hypoxia testosterone and hypoxia control groups. (B) The proportion of $CD34^+CD38^+CD71^+$ cells in the groups were compared and it was determined that the hypoxic testosterone group exhibited a significantly increased percentage of such cells compared with the other groups. The proportion of $CD34^+CD38^+CD71^+$ cells was lowest in the hypoxic control group. Data are presented as the mean + standard deviation. $^*P<0.05$. CD, cluster of differentiation.

of $CD34^+$ cells into erythroid hematopoietic progenitor cells (HPCs).

Expression of hematopoiesis-related genes. RT-qPCR was performed to detect the expression of the stem cell-specific genes HOXB4, BMI-1 and C-MYB, the erythroid-specific genes HOXB2 and HOXB6, the lymphocyte lineage-related gene HOXC4, the granulocyte lineage-related gene HOXA9, the megakaryocyte lineage-related genes GATA-1 and NFE2, and the hypoxia-related gene HIF-α to determine the properties of self-renewal and multi-differentiation of $CD34^+$ cells. Following 7 days culture, the expression of the HOXA9, HOXB2, HOXB4, BMI1, HOXC4, GATA-1, HIF-1α and C-MYB genes were significantly increased in the hypoxia testosterone group compared with all other groups (P<0.05; Fig. 6). However, the expression of HOXB6 and NFE2 did not significantly differ among the hypoxia control group, normoxia testosterone group and normoxia control group (data not shown).

Discussion

To promote efficient HSC proliferation and to mimic the microenvironment in the bone marrow, small molecules were introduced in the culture medium. In the present study, testosterone, norepinephrine and epinephrine small-molecule steroid hormones were selected and the effects of these hormones on $CD34^+$ cells were investigated. The results demonstrated that the number of CFUs and total cells in the testosterone group were significantly increased under normoxic and hypoxic conditions compared with the corresponding control groups. Furthermore, the results indicated that norepinephrine and epinephrine significantly inhibited colony formation and cell amplification under normoxic and hypoxic conditions. As a result, testosterone was selected for use in subsequent experiments.

It has been reported that androgens significantly reduce the quiescence ratio and promote HSC proliferation (31). Previous studies have also demonstrated that testosterone

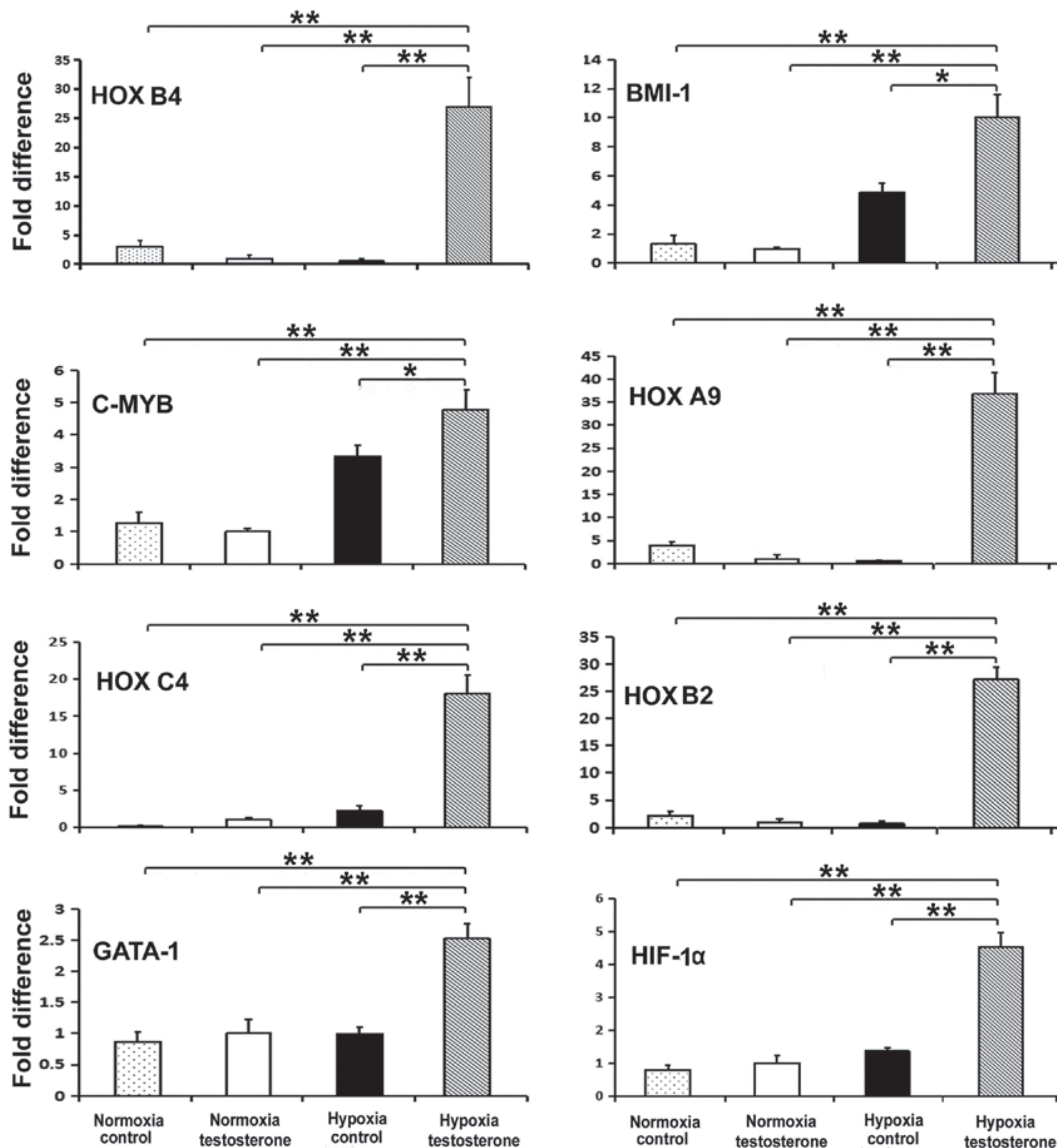

Figure 6. The expression of genes in co-cultured CD34+ cells and feeder after 1 week. The results demonstrated that the expression of the HOXA9, HOXB2, HOXB4, BMI1, HOXC4, GATA-1, HIF-1α, and C-MYB genes was significantly higher in the hypoxia testosterone group compared with all other groups. Data are presented as the mean + standard deviation. *P<0.05 and **P<0.01. HOX, homeobox; HIF-1α, hypoxia inducible factor α.

significantly enhances colony formation and the expansion of HSCs (32-34). Kim *et al* (20) revealed that androgens exhibit a modest growth- and survival-enhancing effect on CFU-E, but not on CFU-GM or BFU-E. Similarly, the present study demonstrated that testosterone significantly increased the number of different types of CFUs, particularly CFU-E, under normoxic and hypoxic conditions. These results indicate that androgens have an effect on HPC that is restricted to mature erythroid progenitors. Norepinephrine and epinephrine also affected HSCs. Norepinephrine signaling controls HSC/HPC mobilization and the sympathetic nervous system to regulate the attraction of stem cells to their niche (35). However, increasing the concentrations of norepinephrine and epinephrine may reduce HSC cloning, thereby inhibiting hematopoiesis (18,23,30).

Previous studies have reported that hypoxia is beneficial in maintaining the self-renewal properties of HSCs (36-38). Furthermore, a number of studies have suggested that hypoxia may promote HSC amplification (14,39,40). Ivanovic *et al* (14) identified that 3% was the lowest O_2 concentration that resulted in the same rate of colony-forming cell expansion as when O_2 concentration was 20%. In the present study, the number of CFUs and total cells in all hypoxia groups were significantly decreased compared with those in the normoxia groups. These results were consistent with the results of a study by Eliasson *et al* (16). However, in the present study, the addition of testosterone promoted CD34+ cell amplification in hypoxia and normoxia groups, indicating that hypoxia and testosterone may be important factors regulating the growth of CD34+ cells.

The results of the present study suggested that in the normoxia groups, CD34+ cells rapidly entered the logarithmic growth period within the first 3 days in liquid culture. Cell amplification in the normoxia testosterone group was increased, whereas cells grew slowly in the hypoxia groups. Notably, cell amplification in the hypoxia testosterone groups gradually

accelerated between days 3 and 7 in culture. Although hypoxia was not beneficial to $CD34^+$ cell proliferation, treatment with testosterone promoted $CD34^+$ cell proliferation in normoxic and hypoxic conditions. Flow cytometry determined that the proportion of $CD34^+CD38^+CD71^+$cells was lowest in the hypoxic control group, indicating that hypoxic conditions were beneficial in maintaining the characteristics of $CD34^+$ cells and delaying differentiation. Additionally, the highest proportion of $CD34^+CD38^+CD71^+$ cells was detected in the hypoxic testosterone group. These results suggest that a combination of hypoxia and testosterone may promote the differentiation of $CD34^+$ cells into erythroid HPCs. To further identify the ability of hematopoietic reconstitution, a CFU assay was performed. A variety of colonies formed, demonstrating that the multi-differentiation ability of the cells was unaffected by hypoxia or testosterone.

The expression of hematopoiesis-related genes in $CD34^+$ cells was detected using RT-qPCR and it was determined that there were significant differences in the levels of gene expression between the hypoxia testosterone group and the other groups. In the hypoxia testosterone group, the expression of hematopoiesis-related genes, including HSC-specific and differentiated, erythroid-specific, lymphocyte lineage-related granulocyte lineage-related, megakaryocyte lineage-related and hypoxia-related genes, were significantly higher compared with all other groups. This increase in the expression of genes may have been caused in part by the differentiation of $CD34^+$ cells into HPCs. The results of the secondary CFU assay and flow cytometry suggested that an increased number of $CD34^+$cells were differentiated into erythroid HPCs under the combined effects of testosterone and hypoxia. This indicates that the combination of hypoxia and testosterone was advantageous in promoting the expression of hematopoietic genes.

Androgens promote the amplification of HSCs but cannot effectively maintain the self-renewal characteristics of stem cells. Huang *et al* (41) demonstrated that bone marrow mesenchymal stem cells in androgen receptor-knockdown mice exhibited enhanced self-renewal ability. Nilutamidie is an anti-androgen agent that blocks the effects of androgen and promotes the self-renewal of ESCs (42). Given the common characteristics of stem cells, it was speculated that androgens may promote HSC proliferation rather than maintain the self-renewal of HSCs. Hypoxia (1%) is conducive to maintaining the undifferentiated HSC state but does not promote amplification (16). The results of the present study demonstrated that a combination of hypoxia and testosterone *in vitro* may increase $CD34^+$ cell differentiation and prolong the HPC stage. This phenomenon may be caused by a number of mechanisms. Firstly, hypoxia may not be conducive to HSC expansion, potentially due to the action of HIF-1α, which may decrease HSC proliferation and block cells in the G0 phase (16,32). The results of the present study indicated that there was a significant increase in the expression of HIF-1α in the hypoxia testosterone compared with the control groups. Another potential mechanism to consider is that hypoxia may directly suppress the proliferation and differentiation of erythroid progenitor/precursor cells. These effects may be reduced or counter-balanced by increasing erythropoietin (EPO) levels (43) and one acknowledged mechanism involved in the hematological effects of androgens is the promotion of erythroid progenitor expansion by increasing EPO levels (44). Furthermore, androgen and androgen receptor signals may promote the proliferation of HSCs/HPCs and stimulate hematopoietic lineage differentiation (45). Another potential mechanism may be that the addition of testosterone may enhance the G1-S transition rate in the cell cycle and the survival of HSCs (32). Additionally, androgens may stimulate telomerase-related gene expression and enzymatic activity in bone marrow $CD34^+$ cells and extend the lifespan of the $CD34^+$ stem/progenitor cells (46).

In conclusion, CB *ex vivo* expansion is a promising approach to deliver high doses of cells and improve the outcomes of CBT. Careful selection of optimal CB units for transplantation may improve the efficiency of the source of HSCs and HPCs in adult transplantation and reduce the cost of processing (47). The present results may provide a means to improve HSC/HPC culture conditions *in vitro*. Future studies investigating this technique may require a larger sample size, however the present study demonstrated that the combination of hypoxia and androgen *in vitro* may be a promising condition of cultivation.

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