Bone marrow stromal cells transduced with human hemangiopoietin gene support hematopoiesis *in vitro*

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Background and Objectives. The aim of this study was to construct a eukaryotic expression vector containing human hemangiopoietin (*hHAPO*) gene and express it in mouse bone marrow stromal cell line HESS-5, then support hematopoiesis *in vitro* with gene-modified HESS-5 (hHAPO-HESS-5).

Design and Methods. The polymerase chain reaction (PCR) products of *HAPO* were digested with *BamH*I and *Bg*II. Then the *HAPO* gene segment obtained was cloned into pIRES2-EGFP to construct the recombinant eukaryotic expression vector HAPO-pIRES2-EGFP. The recombinant vector was identified by enzyme digestion analysis, PCR, and sequencing. HESS-5 cells were transfected by recombinant vector and positive clones were selected with G418. The expression of *HAPO* gene in the transformed cells was detected by studying EGFP expression, reverse transcription (RT)-PCR, and Western-blot analysis. Support of human hematopoiesis by hHAPO-HESS-5 cells was evaluated in co-culture experiments with human $CD34^+$ cells.

Results. Enzyme digestion analysis and sequencing showed that the target gene had been cloned into the recombinant vector. The expression of *HAPO* gene in the transformed stromal cells was demonstrated by fluoro-microscopy and RT-PCR analysis. HAPO protein was also detected in the supernatant of hHAPO-HESS-5 by Western blot analysis. As expected, stably transfected hHAPO-HESS-5 cells significantly increased in both relative and absolute numbers of $CD34^+$ cells after 14 days of culture. The PKH26 study demonstrated that cell division was faster in $CD34^+$ cells co-cultured with hHAPO-HESS-5 cells than in cells cocultured with vector-HESS-5 cells. The hHAPO-HESS-5 cells also supported human hematopoiesis *in vitro* more efficiently than did control vector-HESS-5 cells.

Interpretations and Conclusions. A recombinant eukaryotic expression vector has been constructed and expressed successfully in transformed cells. The hHAPO-HESS-5 cells support rapid generation of primitive progenitor cells and maintain reconstituting ability of hematopoietic stem cells *in vitro*. Therefore, it would be possible to use stromal cells expressing *HAPO* gene as seed cells in bone marrow transplantation.

Key words: hemangiopoietin, eukaryotic expression vector, bone marrow stromal cell, hematopoiesis, stem cells.

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The production of mature blood cells from hematopoietic stem cells (HSC) is controlled by the microenvironment constituted by bone marrow stromal cells. Stroma comprises various types of cells including fibroblasts, adipocytes, endothelial cells and macrophages. These cells interact with hematopoietic cells through direct contact, mediated by adhesion molecules, and by soluble growth factors. Stromal functions can be altered in hematopoietic pathologies. In malignant diseases, stromal dysfunction can result from alterations in the expression of adhesion molecules or growth factor production by stromal cells.[1] Many data indicate that immunodeficiency virus infection of bone marrow stromal cells *in vitro* lessens the cells' ability to support hematopoiesis.[2,3] Stromal cells are now proposed as targets for gene therapy of some hematologic disorders.[4] For example, transgenic stromal cells could deliver hematopoietic growth factors directly to the bone marrow over long periods of time, since at least some of them are thought to be long-lived, unlike most hematopoietic cells. In the case of accidental radiation-induced bone marrow aplasia, local production of growth factors could provide an alternative to systemic administration of growth factors, a procedure often complicated by severe side-effects when high doses are injected into patients.

Hemangiopoietin (HAPO) is a novel cytokine recently identified and characterized by our research group.[5] This cytokine is able to support the proliferation and survival of human and murine hematopoietic stem/progenitor cells and endothelial cells *in vitro*. The aim of the

present study was to evaluate the ability of bone marrow stromal cells, engineered to produce human HAPO, to support hematopoiesis *in vitro*. HESS-5, a murine bone marrow stromal cell line that supports human hematopoiesis,[6] was engineered by gene transfer to produce human HAPO (hHAPO-HESS-5). Co-culture experiments with human $CD34^+$ cells were used to evaluate to what extent hHAPO-HESS-5 and vector-HESS-5 cells, which were transfected with plasmid vector control, supported human hematopoiesis.

Design and Methods

Cells

HESS-5, a murine marrow stromal cell line, was maintained in RPMI1640 (GIBCO) supplemented with 10% (v/v) heat-inactived fetal bovine serum (FBS) and 1% penicillin-streptomycin solution at 37°C in 5% CO_2 humidified air. The adherent layer reached confluence within 1 week. Human $CD34^+$ cells were positively selected from cord blood using MACS Cell Isolation Kits (Miltenyi Biotech, Germany) as previously described.[7] Flow cytometric analysis showed that 85-98% of the cells separated were CD34-positive.

Construction of hHAPO-expressing vectors

Human *HAPO* (*hHAPO*) was subcloned into the bi-cistronic expression vector pIRES2-EGFP to make hHAPO-pIRES2-EGFP. To ensure correct orientation of this DNA insert into pIRES2-EGFP, polymerase chain reaction (PCR) amplification primers of hHAPO were designed with 5' *Bgl*II and 3' BamHI restriction enzyme overhangs: forward 5'CCC AGA TCT ATG GCA TGG AAA ACA CTT3'and reverse 5'CGG GGA TCC TTA GGT GGG TGC AGA CTT 3'. The PCR amplification reaction mixtures (50 μL) contained cDNA kindly provided by Dr. Herui, forward/reverse primers synthesized in Shanghai Sangon Company, and pfu Polymerase (Shanghai Sangon Company). Thermal cycling conditions included holding the reactions at 50°C for 2 minutes and at 95°C for 10 minutes and cycling for 35 cycles at 95°C for 15 seconds and 60°C for 1 minute. Restriction enzyme digestion of 1 μg pIRES2-EGFP and 1 μg *hHAPO* was performed in a commercial universal buffer purchased from Takara Biotechnology Co., Ltd. (Dalian, China) with 20 U/μL BamHI and 5 U/μL *Bgl II* for 2 hours at 37°C. Digestion was confirmed by gel electrophoresis. Ligation was performed with a 1:3 molar ratio of plasmid to insert for 12 hours at 16°C. *E. coli DH5a* was transfected with the *hHAPO*-pIRES2-EGFP ligation mix and grown on kanamycin (50 μg/mL) Luria broth agar. The hHAPO-pIRES2-EGFP plasmid was purified with a kit (Endofree Plasmid Maxi Prep Kit; Qiagen, Inc.). Purified products were subsequently analyzed by direct sequencing (Prism 3730 DNA sequencer, Shanghai BioAsia Biotechnology Co., Ltd.), PCR, and digested with *Bam*HI and *BglII*, to confirm full CDS for human HAPO. The plasmid was resuspended at 500 ng/μL in endotoxin-free water and stored in aliquots at -20°C.

DNA transfection and selection of cells

HESS-5 cells were either transfected with the hHAPO-pIRES2-EGFP plasmid or pIRES2-EGFP vector control using Lipofectamine™ 2000 (LF2000) according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD, USA). Briefly, 1×10^5 HESS-5 cells were seeded into an individual well of a 24-well plate (Becton Dickinson, San Jose, CA, USA). For cells of each well to be transfected, 1 μg of DNA and 2 μL LF2000 were diluted into 50 μL OPTI-MEM I medium (Life Technologies), respectively. Once the LF2000 had been diluted, it was incubated with the DNA at room temperature for 30 minutes to allow the DNA-LF2000 complex to form, and then the complex was directly added to each well containing cells. Transfected cells were named as hHAPO-HESS-5 and vector-HESS-5 respectively, and cultured in DMEM (GIBCO) supplement with 10% FBS. Transduction efficiency was evaluated 12-24 hours after transfection by flow cytometry analysis of GFP expression. Forty-eight hours after the transduction, the supernatant of cells was collected for hHAPO protein expression analysis by Western blotting. The stromal cells were then trypsinized and split between two 75 cm^2 flasks (Becton Dickinson, San Jose, CA, USA) in stromal medium. The selective agent G418 (Geneticin, GIBCO BRL) was added at a concentration of 600 μg/mL active drug, and selection was allowed to proceed for seven days. Nontransduced stromal cells were used as a control, to ensure complete killing. After 10 days, G418-resistant cells were transferred to a 25 cm^2 flask (Becton Dickinson, San Jose, CA, USA) and expanded.

Expression of enhanced GFP

HESS-5 cells with the bi-cistronic hHAPO-pIRES2-EGFP were examined for expression of GFP and photographed under an inverted fluorescence microscope (Olympus BX60; Olympus, Tokyo, Japan) equipped with a digital camera (Olympus, Tokyo, Japan).

Measurement of hHAPO RNA expression by RT-PCR

Total RNA was extracted from cell cultures with

RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA was used as a template for cDNA synthesis in a 20 μL volume containing 2.5 μM Oligo dT, 1 unit/μL RNase inhibitor (Perkin-Elmer, Foster City, CA, USA), 500 μM deoxynucleotide triphosphate mix (Life Technologies, Inc.), 10 μM DTT, 5× buffer, and 200 units of M-MuLV RNase reverse transcriptase (Promega, USA). The reaction was incubated at 42°C for 60 min. PCR analysis for human *HAPO* expression was performed using primers that amplified a fragment of 1000 bp. The PCR reaction mixture (final volume of 50 μL) contained 200 μM deoxynucleotide triphosphate mix, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Shanghai Sagon, Inc.), and 1 μL of cDNA template. A denaturation step of 95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min, was used. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.

hHAPO protein expression analysis by Western blot

hHAPO protein expression in the supernatant of hHAPO-HESS-5 cells was determined by electrophoresis (15% SDS-PAGE) and the immunoblot methodology. In brief, proteins from cell supernatants were concentrated using a 10,000 molecular weight concentration column (Millipore, Bedford, MA, USA). Equal loading of protein samples was confirmed using Coomassie blue stain. Protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) by means of a wet transfer system with a constant current of 200 A for 2 hours at 4°C. The membranes were blocked in 5% bovine serum albumin (BSA)/PBS-1% Tween 20 at 4°C overnight. The membranes were incubated on an orbital shaker for 1 hour at 4°C with mouse monoclonal anti-HAPO (provided by another team in our laboratory) antibody (1:2000) and then underwent three 15-minute washes with PBS+0.1% Tween 20, and then horseradish peroxidase coupled with anti-mouse immunoglobulin (IgG) antibodies (1:7000, Beijing Zhongshan Biotechnology, Inc.) was added for the same incubation period and the wash was repeated. The DAB detection system was used to visualize the presence of proteins on the PVDF blots.

Co-culture of hHAPO-HESS-5 and CD34⁺ cells

Twenty-four well plates were precoated with 0.5%BSA at 37°C, in 5% CO_2 in air. The wells were then rinsed twice with PBS before seeding 2×10^4 vector-HESS-5 or hHAPO-HESS-5 cells into 0.5 mL in each well. Twenty-four hours later 10^4 $CD34^+$ cells suspended in 0.5 mL were added to each well as previously described.[8] Briefly, human cord blood $CD34^+$ cells were incubated with or without a cocktail of recombinant human (rh) cytokines (rh interleukin-3 10 ng/mL, rh granulocyte-macrophage colony-stimulating factor 10 ng/mL, and rh stem cell factor 50 ng/mL) in the upper compartment of transwell inserts placed on top of stromal cell layers prepared in the lower compartment of a 24-well plate (#3421; Costar, Cambridge, MA, USA). The recombinant human cytokines were purchased from R&D Systems (Minneapolis, MN, USA). The transwell microporous membrane of the insert cultures was a 0.4-μm microporous filter (Costar). Stroma-free cultures were established by seeding cells in the upper compartment of the transwell insert placed in empty wells. The same volume of culture medium was added to wells on day 3, and half the amount of growth medium was exchanged every 3 days. All the plates were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Nonadherent hematopoietic cells were harvested at intervals, counted and examined under the microscope after staining using a modified Wright-Giemsa method, and immunophenotyped by flow cytometric analysis.

Flow cytometric analysis

Cell surface molecule expression was determined on $CD34^+$ cells after isolation and on harvested cells after 7 days in culture. Cells were stained with isothiocyanate (FITC)-labeled anti-CD34 (anti-HPCA-2) or fluorescein phycoerythrin (PE)-labeled anti-CD38 monoclonal antibodies or anti-CD33-FITC. Simultest control (FITC IgG1 + PE IgG_{2a}) served as a negative control. All monoclonal antibodies were purchased from Becton Dickinson Immunocytometry Systems (Becton Dickinson, San Josè, CA, USA). Acquisition and analysis were performed on a FACSort (Becton Dickinson) flow cytometer equipped with an air-cooled argon-ion laser tuned at 488 nm. The CELLQuest and Attractors (Becton Dickinson) software packages were used for analyses. At least 10^4 events were acquired for each analysis.

PKH26 study

Purified $CD34^+$ cells were stained with PKH26 (Sigma ImmunoChemicals, St Louis, MO, USA), the red fluorescent cell linker, before culture according to the manufacturer's instruction with slight modifications. Briefly, aliquots of cells suspended in 1 mL of Diluent C (Sigma ImmunoChemicals) were transferred into a polypropylene tube containing 1 mL of 4×10^{-6} M PKH26 dye in Diluent C. After incubation for 5 min at room temperature, 2 mL of 1% BSA/PBS were added. After 1 min, the total volume was brought up to 8 mL using the serum-free medium, and the cells were washed three times. Then the cells were co-cultured on hHAPO-HESS-5 or vector-HESS-

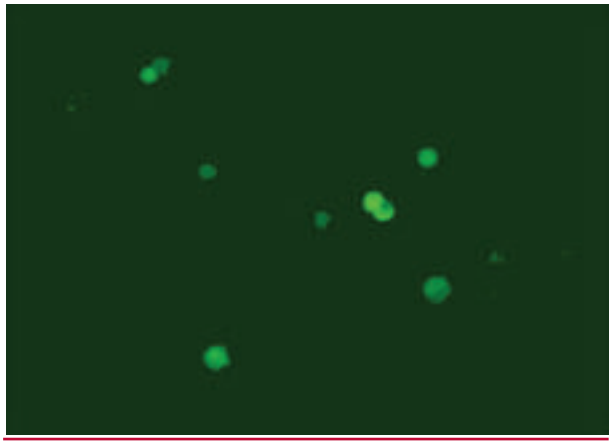

Figure 1. Expression of enhanced GFP. The hHAPO-HESS-5 cells transfected with the bicistronic hHAPO-pIRES2-EGFP were examined for expression of GFP and photographed with an inverted fluorescent microscope.

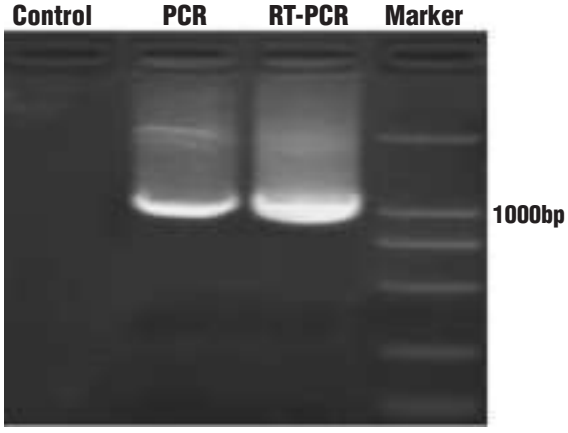

Figure 2. Detection of the hHAPO-pIRES2-EGFP plasmid and its expression in hHAPO-HESS-5 cells. HESS-5 cells were transfected with hHAPO-pIRES2-EGFP, selected during 10 days in 600 μg/mL G418, then expanded. A 1002-bp PCR product shows the presence of the hHAPO-pIRES2-EGFP plasmid (PCR) and its expression (RT-PCR).

5 cell layers. Before and after 7 days of culture, the labeled cells were subjected to flow cytometry, and the cell division history was assessed based on the fact that fluorescence intensity of PKH26 is reduced by one half with each cell division.[9-11]

Clonal cell culture

Assays for hematopoietic progenitors were performed by slight modification of an established method.[12] Briefly, hematopoietic cells from co-cultures were plated in triplicate in 35-mm dishes (Becton Dickinson, San Jose, CA, USA) at 10^3 cells/mL in methylcellulose medium, with a cocktail of recombinant human cytokines including interleukin-3 (IL-3) (10 ng/mL), granulocyte-macrophage colony stimulating factor (GM-CSF) (10 ng/mL), erythropoietin (EPO) (3 U/mL) and stem cell factor (SCF) (50 ng/mL). Cultures were incubated at 37°C in humidified 5% CO_2 in air. Colonies were defined as greater than 50-cell aggregates and counted using an inverted microscope twice a week. All cultures were performed in triplicate.

Assays of long-term culture-initiating cells (LTC-IC)

For enumeration of LTC-IC, the $CD34^+$ cells to be tested were placed in 35-mm tissue culture dishes that already contained a feeder layer of irradiated (8000cGy) HESS-5 stromal cells engineered to expressed HAPO, with or without a cocktail of cytokines including rhIL-3 (10 ng/mL), rhGM-CSF (10 ng/mL), and rhSCF (50 ng/mL), plus 2.5 mL of LTC-IC medium which contains 12.5% horse serum, 12.5% FBS, and 10^{-4} M 2-ME, and 10^{-6} M hydrocortisone. The cultures were then incubated for 6 weeks at 37°C with weekly replacement of half the medium and removal of half of the non-adherent cells.[13] After 6 weeks, all the remaining non-adherent cells from each assay culture were removed and added to the corresponding trypsinized adherent cells; the combined pool was then washed and assayed for colony-forming units (CFU) as described above. The counts obtained were used to calculate the total yield of CFU from each input inoculum tested as this value provides a relative, but nevertheless, quantitative measure of the number of LTC-IC initially present.[14]

Statistical analysis

The results are presented as the mean ± SD of the data obtained from three or more experiments performed in duplicate. Statistical significance was determined using the Student's t-test. A p value of <0.05 was considered statistically significant.

Results

HESS-5 cells expressing HAPO gene and producing human HAPO

The 1002-bp *HAPO* insert amplified by PCR was cloned into the BgI II and the BamH I site of the eukaryotic expression vector, pIRES2-EGFP. The orientation of *HAPO* constructs was confirmed by means of restriction enzyme mapping and DNA sequencing. Following transfection of HESS-5 cells with the *HAPO* construct (or vector alone as a control) and subsequent antibiotic selection, G418-resistant cells were transferred to a 25 cm^2 flask and expanded. These cells were positive for GFP expression under fluoromicroscopy (Figure 1) and the

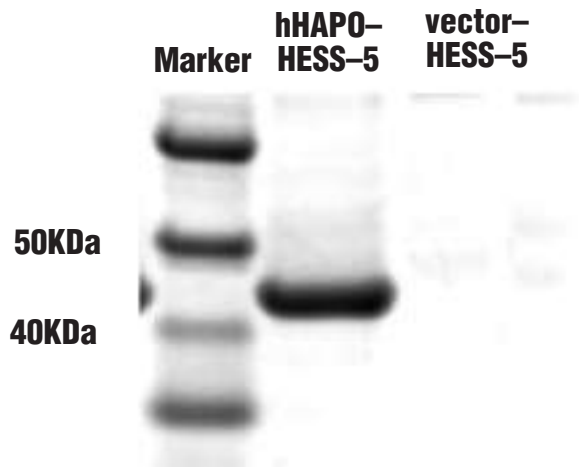

Figure 3. The hHAPO protein expression in hHAPO-HESS-5 cell supernatant. The hHAPO production in the culture supernatant was assayed by Western blot analysis, described in the Design and Methods section, after 48 hours of transfection. Protein was confirmed to be present in hHAPO-HESS-5 cell supernatant, but not in control vector-HESS-5 supernatant.

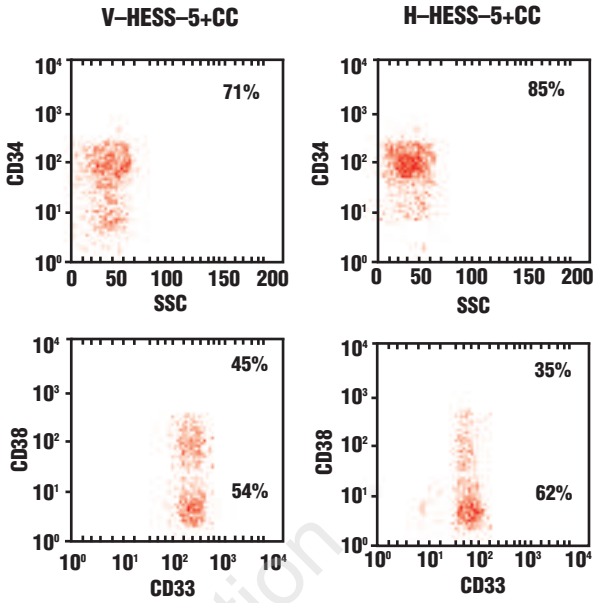

Figure 4. Dot plot of CD34⁺ cells from a typical experiment with a cocktail of cytokines (CC) in the presence of hHAPO-HESS-5 stromal layers or vector-HESS-5 stromal layers. Representative flow cytometric analysis of CD34 expression versus side scatter (SSC) and within the CD34⁺ cell population for expression of CD38 and CD33 on subpopulations of viable (PI-) cells generated from input CD34⁺ cells after 7 days of culture supplemented with the indicated CC in the presence of hHAPO-HESS-5 stromal layers or vector-HESS-5 stromal layers. The lower left-hand quadrant in each plot represents boundaries set by 99.9% of the unstained and isotype-labeled antibody controls. All plots are gated in forward and side scatter on viable cells, showing a total of 45,000 (hHAPO-HESS-5 group), and 36,000 (vector-HESS-5 group) events; the total number of events analyzed for both analyses was 50,000.

expression of *HAPO* cDNA insert was confirmed by PCR (Figure 2). The expected 1002-bp band of the PCR product was observed in hHAPO-HESS-5 cell extracts. With respect to *HAPO* transgene expression, a similar observation was made using RT-PCR. Selection of the *hHAPO* transgene product in selected cells was demonstrated by detection of human *HAPO* in the cell culture supernatant. The results showed that hHAPO production could be measured in culture supernatant of hHAPO-HESS-5 cells after 48 hours of transfection (Figure 3). The hHAPO was not detectable in control vector-HESS-5 cell supernatant.

Effect of hHAPO-HESS-5 cells on cultivating CD34⁺ cells

The hHAPO-HESS-5 cells had a profound effect on the cultured CD34⁺ cells. This is demonstrated in Figure 4, which illustrates the surface antigen profiles of the cells generated in short-term (7 days) culture with the indicated cocktail of cytokines in the presence of hHAPO-HESS-5 or vector-HESS-5 stromal layers.

Table 1 shows differential counts of the human hematopoietic cells types which appeared in the culture medium when human CD34⁺ cells were co-cultured with hHAPO-HESS-5 or vector-HESS-5 stromal layers in the absence of the cocktail of cytokines. The proportion of differentiated cells (eosinophils, neutrophils and monocytes) present at day 7 was higher in the co-cultures with hHAPO-HESS-5 stromal layers than in the co-cultures established with vector-HESS-5 cells. However, the total cell number in the cultures containing the cocktail of cytokines increased 31.7±0.4-fold in the presence of hHAPO-HESS-5 and 25.4±0.2-fold in the presence of vector-HESS-5 cells (n=3) (Table 2). Among

Table 1. Differential counts of CD34⁺ cells cultured on stromal layers.

Stromal layer	Type of cells: Undifferentiated cells	MB/MPL	ML/MML	EO/NP	MONO
HAPO-HESS-5	14±5%	22±3%	17±6%	25±4%	20±4%
Vector-HESS-5	49±6%	33±8%	11±5%	2±2%	4±2%

CD34+ cord blood cells were cultured without a cocktail of cytokines for 7 days on stromal layers. Cultured cells were collected and cytocentrifuged on to microscope slides, stained by a modified Wright-Giemsa method, and analyzed by light microscopy. Numbers indicate the mean percentage ±SD of each cell type observed in five fields. Data are from two experiments. MB/PML, myeloblasts/promyelocytes; ML/MML, myelocytes/metamyelocytes; EO/NP, eosinophils/neutrophils; MONO, monocytes.

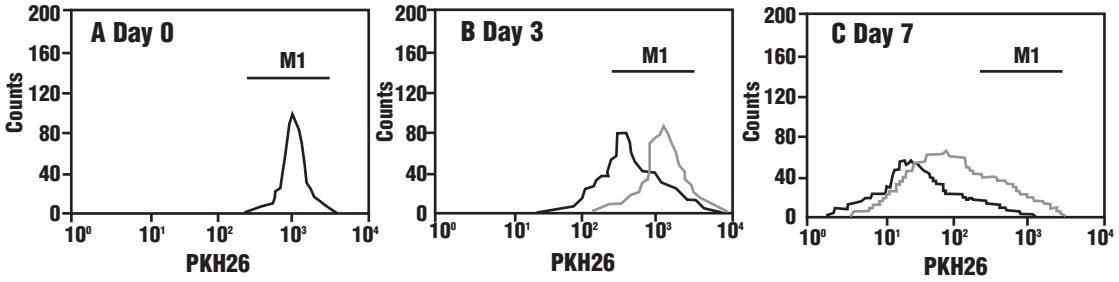

Figure 5. Effects of hHAPO-HESS-5 cells on CD34⁺ cell kinetics during co-culture. CD34⁺ cells purified from cord blood were stained with PKH26 and cultured for 7 days on the hHAPO-HESS-5 cell layers. (A) PKH26 fluorescence of initial CD34⁺ cells. The M1 fraction was 98.1%. (B) PKH26 fluorescence of the CD34⁺ fraction of cells after 3 days of co-culture with hHAPO-HESS-5 cells (thick line) or vector-HESS-5 cells (thin line). The M1 fraction was 82.1% and 94.3%, in the hHAPO-HESS-5 cell and vector-HESS-5 cell group, respectively. (C) PKH26 fluorescence of the CD34⁺ fraction of cells after 7 days of co-culture with hHAPO-HESS-5 cells (black line) or vector-HESS-5 cells (gray line). The M1 fraction was 23.6% and 37.3% in the hHAPO-HESS-5 cell and vector-HESS-5 cell group, respectively. The data presented are representative of three independent experiments.

Table 2. Effects of stromal cells on the fold expansions of CD34⁺ cells.

Cell population	*HAPO-HESS-5*		*Vector-HESS-5*		*Stroma-free*	
	G/3/E/S	*Cytokine(⁻)*	*G/3/E/S*	*Cytokine(⁻)*	*G/3/E/S*	*Cytokine(⁻)*
Total number of cells	31.7±0.4	9.6±0.2	25.4±0.2	1.5±0.1	4.5±0.2	0.7±0.1
CD34⁺ cells	27.3±0.3	3.5±0.2	18.1±0.3	0.8±0.1	2.2±0.1	0.4±0.1
CD34⁺/CD38⁻ cells	16.9±0.3	1.2±0.2	9.8±0.2	0.8±0.1	7.6±0.2	1.1±0.1

CD34⁺ cord blood cells were cultured for 7 days on stromal layers or stroma-free, with or without a cocktail of cytokines. Fold expansion is given as the mean ± SD (n=5). GM-CSF (G), IL-3 (3), EPO (E), SCF (S).

these cells, 81±9% and 68±11%, respectively, were CD34⁺. Within these CD34⁺ cell populations, 64±15% (H-HESS-5 group) and 53±14% (V-HESS-5 group) of the cells were CD38⁻ (Figure 4). Thus, the total number of CD34⁺ CD38⁻ cells present in the hHAPO-HESS-5 and vector-HESS-5 co-cultures with a cocktail of cytokines increased by about 17- and 10-fold, respectively. Interestingly, most of the CD34⁺ cells present after 7 days were positive for CD33.

Assessment of cell kinetics during co-culture with hHAPO-HESS-5 cells

We then assessed cell kinetics during culture by using PKH26 staining. On day 0, all CD34⁺ cells labeled with PKH26 dye were PKH26^{high} (Figure 5). Aliquots of the PKH26^{high} cells were subjected to co-culture in the presence of hHAPO-HESS-5 or vector-HESS-5 cells. After 3 days of co-culture, the PKH26^{high} proportion (M1 fraction) was significantly lower in the presence of hHAPO-HESS-5 cells than in the presence of vector-HESS-5 cells (81.19±2.55 vs 94.55±4.6%, $p<0.01$). After 7 days of co-culture, the majority of the CD34⁺ cells cultured in the presence of hHAPO-HESS-5 cells became PKH26^{low}, indicating that they had experienced cell division. Therefore, cell division and generation from CD34⁺ cells were faster in cells co-cultured with hHAPO-HESS-5 cells than in cells co-cultured with vector-HESS-5 cells. These results indicate that the cell cycle progression of CD34⁺ cells was accelerated by the presence of hHAPO-modified stromal cells.

The hHAPO-HESS-5 cells support human hematopoiesis in vitro

We analyzed the capacity of hHAPO-HESS-5 cells, as compared to control vector-HESS-5 cells, to support the growth of human progenitor cells in a methylcellulose culture system. CD34⁺ cord blood cells were seeded onto hHAPO-HESS-5 and control stromal layers, and then transferred to a methylcellulose culture system. Hematopoietic cell numbers and CFU-Mix present in the cultures were counted twice a week: there was a significant increase in the proliferation of hematopoietic cells and the production of CFU-Mix (Figure 6) in hHAPO-HESS-5 co-cultures as compared to in the co-cultures with vector-HESS-5 as well as in the control group.

The LTC-IC content of the input CD34⁺ cells harvested from each of the different culture systems was measured by assessing the total CFU output for a fixed number of original CD34⁺ cells. The validity of this approach, rather than using limiting dilution analyses for quantifying LTC-IC in each suspension, is based on previous data showing that the numbers and types of CFU generated from individual LTC-IC, although highly variable, remain on average the same when freshly isolated and cultured LTC-IC are compared.[15,16] From 1000 input CD34⁺ cells, 71±8 CFU were generated and harvested from 7-day liq-

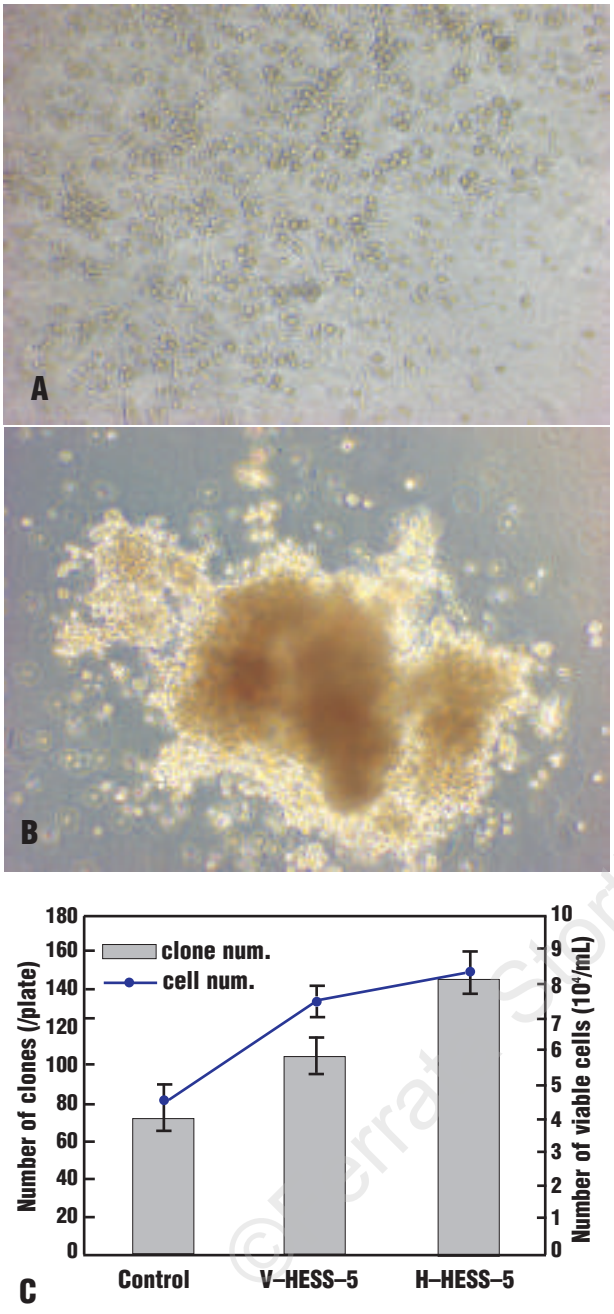

Figure 6. Support of human hematopoiesis by hHAPO-HESS-5 cells *in vitro*. CD34⁺ cells purified from human cord blood were co-cultured in the presence of hHAPO-HESS-5 or vector-HESS-5 stromal layers. (A) Appearance of cobblestone-like colonies generated in the co-culture of human cord blood CD34⁺ cells with hHAPO-HESS-5 cells at day 7 of culture. (B) Appearance of a part of a mixed hematopoietic colony produced by the cells harvested from the co-culture of CD34⁺ cells with hHAPO-HESS-5 cells at week 2 of culture. (C) Hematopoietic cell proliferation was estimated by counting viable cells after trypan blue exclusion. Maintenance of hematopoietic progenitors was evaluated by clonogenic assays; 10^3 cells from the co-culture supernatant were cultured in methylcellulose. The data represent cumulative numbers of CFU-Mix generated during 14 days of co-culture in two independent experiments, and are the mean of triplicate determinations ± SD. In the control group experiments 71±8 CFU were generated from 1000 input CD34⁺ cells, harvested from 7-day liquid suspension culture without stromal layers.

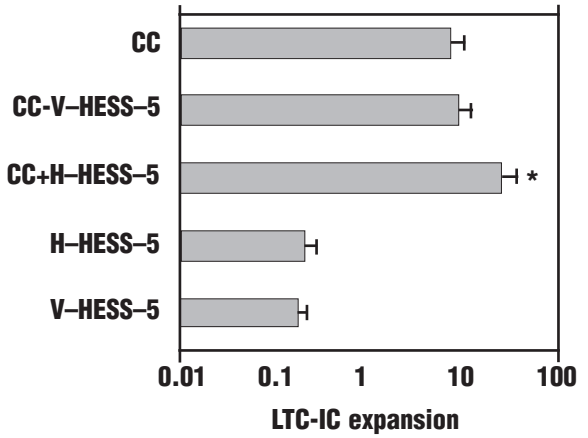

Figure 7. Variable LTC-IC expansion from CD34⁺ cells incubated for 6 weeks in a stromal layer with or without a cocktail of cytokines (CC) as shown: 71±8 CFU were generated from 1000 input CD34⁺ cells harvested from 7-day liquid suspension culture without stromal layers. The data (bars) represent the increases of CFU in 6-week long-term culture conditions relative to that in 7-day liquid suspension culture, and are the mean of triplicate determinations ±SD (*) Significant differences were found versus the CC and CC+V-HESS-5 groups ($p<0.05$) or versus the vector-HESS-5 group ($p<0.01$) as determined by a two-tailed t test.

uid suspension culture without stromal layers (Figure 6, control group). However, the same number of input CD34⁺ cells from 6-week co-culture with hHAPO-HESS-5 or vector-HESS-5 stromal layers with a cocktail of cytokines, yielded 1306±56 and 895±41 CFU, respectively. The extent of LTC-IC amplification in the presence of hHAPO-HESS-5 or vector-HESS-5 was 18±7 and 12±5-fold, respectively (Figure 7). Moreover, the magnitude of LTC-IC expansion in the presence of the hHAPO-HESS-5 layer was significantly higher than that in the presence of the vector-HESS-5 layer when CD34⁺ cells were cultured in the same cocktail of cytokines ($p<0.01$), suggesting that HAPO secreted from hHAPO-HESS-5 might stimulate the proliferation of LTC-IC, when combined with other cytokines.

Discussion

Molecular engineering of cells, either transiently or permanently, has become a mainstay of cell and molecular biology, leading to many exciting insights into the role of a given protein in cell metabolism both *in vitro* and *in vivo*. A wide variety of techniques and reagents are used to delivery nucleic acid into eukaryotic cells. DNA delivery by virus infection is the most commonly used technique for experiments because of its high transfection efficiency, but biosafety problems would arise in clinical applications. On the other hand, DNA delivery by lipo-

fection is less toxic and is a simple method requiring only a few reagents with the plasmid DNA containing the gene of interest under the control of a strong promoter. Recently, its simplicity and the numerous advances that have increased its level of expression efficiency have made lipofection a more popular method for nucleic acid delivery.[17] In this study, DNA delivery by lipofection was applied for a potential clinical use of cell therapy. Transfection efficiencies of the *hHAPO* gene by lipofection were satisfactory for stable protein expression able to support hematopoiesis.

HAPO is a novel growth factor, originally identified and purified from the urine of patients with aplastic anemia. Recently, human recombinant HAPO (rhHAPO) has been produced based on the N-terminal amino acid sequence of purified native HAPO. The rhHAPO supports survival and proliferation of primitive cells of both hematopoietic and endothelial cell lineages,[5] and efficiently protects cells from apoptosis through the PI3K-Akt pathway.[18] Concerning the stimulatory effect of HAPO on hematopoiesis, we hypothesized that stromal cells transduced with *HAPO* gene may have an increased effect in supporting hematopoiesis. In the present study, we successfully constructed a hHAPO expressing vector, hHAPO-HESS-5, in a hematopoietic-supportive murine stromal cell line.[19] We observed that hHAPO-HESS-5 cells have a greater capacity to sustain the differentiation of hematopoietic precursors *in vitro* than do vector-HESS-5 cells in the absence of other cytokines. A significant increase in cord blood $CD34^+CD38^-$ cells, which are considered to contain very immature stem cells,[20,21] was obtained after short-term (7 days) co-culture with hHAPO-HESS-5. It has been reported that the $CD34^+CD38^-$ cells from cord blood require FL and IL-6/sIL-6R for optimal amplification of long-term culture-initiating cells (LTC-IC).[22] Our results indicate that HAPO protein secreted by gene-modified stromal cells is also conducive to the amplification of LTC-IC.

PKH26, a fluorescent dye that stably inserts in the lipid bilayer cell membrane, was used for cell tracking and for identifying a group of cells that failed to respond to cytokine stimulation.

$CD34^+PKH26^{high}$ cells have been previously described in studies examining *ex vivo* expansion of bone marrow cells.[10,23,24] The PKH26 study in our system further confirmed that the cord blood $CD34^+$ cells entered the cell cycle more rapidly when co-cultured with *HAPO* gene-modified stromal cell layers. The decrease of the $CD34^+PKH26^{high}$ population and the amplification of LTC-IC support the possibility of self-renewal of stem cells processed in the hHAPO-HESS-5 culture, a phenomenon previously reported by Yasuhito *et al*.[25] In addition, the number of CFU-Mix was doubled. These results are agreement with our previous observation[5] that HAPO efficiently protects mice from radiation damage by stimulating hematopoiesis *in vivo*.

Overall, we have demonstrated by transduction of the *HAPO* gene into stromal cells that HAPO is a hematopoietic growth factor and that bone marrow stromal cells could serve as a powerful cellular system to deliver HAPO in order to support hematopoiesis. We believe that this approach will be useful not only as an alternative to systemic administration of growth factors, which is often complicated by severe side-effects, but also as a more general tool for modifying the microenvironment in bone marrow reconstitution after transplantation. These observations may also provide insights into HAPO biology and give indications for future cellular therapy or graft engineering.

Dr. Z-SX, Dr. Z-ZC and Dr. ZCH made substantial contributions to the conception and design of this study. Dr. Z-SX, Dr. Y-JL, Dr. RH, Dr. L-LL, Dr. BX, Mr. Z-BH, Ms. S-HL and Ms. TW were in charge of acquisitions, analysis and interpretation of data. All authors approved the version to be published. The authors declare that they have no potential conflicts of interest.

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