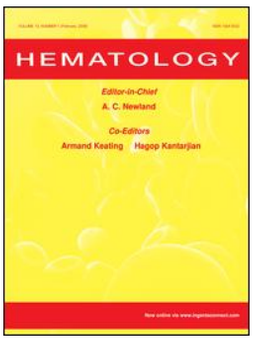

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Over expression of HIF-1α in human mesenchymal stem cells increases their supportive functions for hematopoietic stem cells in an experimental co-culture model

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Introduction: Bone marrow transplantation is a critical approach for the treatment of many hematological disorders. Success of this approach is dependent on many factors the most important of which is the number of hematopoietic stem cells along with an efficient stroma. Co-transplantation of efficient mesenchymal stem cells can greatly improve the outcome of transplantations. Current researches assign a critical role for hypoxia inducible factor (HIF)-1α in protection of various cells and tissues probably through induction of cytokines. To make this feature applicable to human bone marrow-derived mesenchymal stem cells, we manipulated these cells to over express HIF-1α gene.

Materials and methods: Full-length cDNA of human HIF-1α was inserted into human bone marrow mesenchymal stem cells by pcDNA.3.1 non-viral plasmid vector, and the effect of this over expression on production of some hematopoietic growth factors was explored. Moreover, using a co-culture system, the interactive impact of HIF-1α-overexpressed mesenchymal stem cells on hematopoietic stem cells was evaluated.

Results: Over expression of HIF-1α in mesenchymal stem cells in normoxia increased production of one of the most important hematopoietic growth factors, Stem cell factor (also known as Steel factor or c-kit ligand). HIF-1α overexpression had no effect on production of other hematopoietic growth factors. In co-culture of mesenchymal stem cells-HIF-1α with hematopoietic stem cells, enhancement of colony formation and reduced differentiation of hematopoietic stem cells were observed.

Conclusion: Over expression of HIF-1α in human bone marrow-derived mesenchymal stem cells can augment the production of some hematopoietic growth factors, and we suggest this response of mesenchymal stem cells could help to improve the outcome of bone marrow transplantation.

Keywords: HIF-1α, Human mesenchymal stem cells, Hematopoietic stem cells, Stem cell factor, Co-culture

Introduction

Mesenchymal stem cell (MSC) therapy is now used in treatment of many disorders. Death of most cells after transplantation caused by several mechanisms such as mechanical damage, free radicals, lack of growth factors, and time length passed after isolation from the body is the most challenging issue in therapeutic use of MSCs. Herein, many researchers have been struggling to to maintain survival of these cells for transplantation puposes. MSCs are a suitable source of cytokines and molecules involved in the development of hematopoietic cells, and they play important roles in improvement and efficiency of bone marrow transplantation.[1–4] It has been observed that the hematopoietic system in patients receiving hematopoietic

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stem cells (HSCs) along with MSCs after high-dose chemotherapy has been much more successful than patients who only received HSCs.[5]

Autologous HSC infusion together with experimentally expanded MSCs has considerably improved the hematopoietic process.[6] Of note, due to weak expression of HLA antigens on MSCs, compatibility could not be concerning hurdle.[7]

It is now known that all tissue-specific stem cells including hematopoietic, mesenchymal, neural, and embryonic, spend most of their lifetime in an environment with oxygen pressure ranging from 14 to 65 mmHg (amounting to 2–9%),[8–10] which is interestingly accepted as physiological normoxia by some researchers.[11]

Hypoxia inducible factor (HIF) is one of the most highly expressed genes in above hypoxic conditions (physiological normoxia). HIF has two sub-units: HIF-1α and HIF-1β. HIF-1α is quickly destroyed in normoxia by Von Hippel–Lindau protein (pVHL), but it is more stable in hypoxia.[12] HIF-1α has high activity in response to hypoxia in nearly all types of cells[13] and plays a key role in controlling vital cell mechanisms such as apoptosis, metabolism, and cell proliferation[14] and it is a cytoprotective protein against environmental stresses.[15]

It has also been reported that HIF controls at least 150 genes involved in controlling cell metabolism, motility, and survival, hematopoiesis, angiogenesis, and cell membrane integrity.[16]

Indeed above mentioned functions of HIF-1α will also apply to cells in the bone marrow environment including MSCs, thus we were prompted to increase production of HIF protein in MSCs, as level of this protein is reportedly low.[17]

In this study, we tried to explore whether HIF-1α overexpression in human bone marrow-derived MSCs would modify production of some hematopoietic growth factors by these cells in normal and hypoxic conditions.

Additionally, we co-cultured the HIF-1α transfected MSCs (MSCs-HIF-1α) with hematopoietic stem cells, and evaluated the role of this co-culture on colony formation and differentiation of HSCs.

Materials and methods

Isolation, culture, and identification of mesenchymal stem cells

MSCs were isolated from the bone marrow of healthy donors after obtaining informed consent provided by the local ethics committee. The method for MSCs isolation was adopted from Halabian *et al.*[18] MSCs were identified based on morphology using an inverted microscope and based on expression pattern of a panel of surface markers, CS105, CD90, CD73 and CD166, CD14, CD34, and CD45 using fluorescence-activated cell sorting (FACS). In brief, the cells were detached using 0.25 trypsin (Sigma, Munich, Germany), washed with cold FACS buffer (phosphate buffered saline (PBS) + 0.5% bovine serum albumin (BSA) + 0.01% sodium azide) and incubated with fluorescent-conjugated antibodies against the above markers. The samples were left for 30 minutes in a fridge (4°C). After washing with FACS buffer, the samples were applied to FACS analysis using Flowmax software. For each of the above antibodies, an isotype control was also used.

HIF-1α gene cloning

Naturally, MSCs express HIF-1α at low levels. They were subject to mRNA extraction using an RNA isolation kit (Qiagene, Düsseldorf, Germany) according to the manufacturer's instruction. cDNA was synthesized using a cDNA synthesis kit (Bioneer, Korea) according to manufacturer's protocol. Forward and reverse primers containing Kozak sequence, NHeI (forward) and BamHI (reverse) restriction enzyme recognition sites were designed for amplification of full-length human HIF-1α cDNA. Primer sequences are given in Table 1. Using Platinum® Taq DNA Polymerase High Fidelity enzyme (Invitrogen, Carlsbad, CA, USA), the full-length human HIF-1α cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) (Gene Amp PCR system 9600; Perkin Elmer Life and Analytical Sciences, Wellesley, MA, USA).

PCR conditions included a denaturation step of 5 minutes at 95°C, followed by 33cycles of 30 seconds at 95°C, 30 seconds at 55°C and 155 seconds at 72°C, and a final extension step of 7 minutes at 72°C.

The HIF-1α amplified cDNA was inserted into the mammalian expression vector pcDNA3.1 (þ) (Invitrogen, Carlsbad, CA, USA) in the sense orientation, and was used for transformation of competent DH5 α *Escherichia coli* cells. Then, the LB agar medium containing 100 μg/ml ampicillin was used to screen the recombinant bacteria. Finally, using PCR,

Table 1 Oligonucleotide sequences of primers designed for HIF-1α and β-actin

Primer set	Sequence	Feature
Hif-1α, for amplification of the gene	5′-TTG CTA GCA TGG AGG GCG CCG GCG GCG C-3′ 5′-AGG GAT CCT CAG TTA ACT TGA TCC AAA GCT C-3′	NheI site BamHI site
Hif-1α, for evaluation of expression	5′-CGG CGC GAA CGA CAA GAA AAA GA-3′ 5′-ATG TGG AAG TGG CAA CTG ATG AGC A-3′	
Beta-actin	5′-GCACAGAGCCTCGCCTTT-3′ 5′-GAAGCCGGCCTTGCACAT-3′	

the presence of the insert was established and the fidelity of the HIF-1α-cloned sequence was confirmed with DNA sequencing.

HIF-1α cDNA transfection

MSCs were transfected with pcDNA-HIF-1α using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, total cellular mRNA was extracted from transfected cells (**MSCs-HIF-1α**) on days 1–14, and used at a concentration of 1 μg/μl to construct cDNA. This cDNA was then applied to amplification by RT-PCR and real-time PCR for monitoring the HIF-1α expression. Beta-actin was also used as a housekeeping gene for normalization. Characteristics of the PCR products of HIF-1α and β-actin are given in Table 1.

As control, we also transfected the MSCs with empty pcDNA plasmids (**MSCs-pcDNA**). The HIF-1α baseline expression was also down-regulated with a human HIF-1α unique 29mer shRNA produced by pGFP-V-RS plasmid (Origen, Austin, TX, USA) with the sequence of GCCACATTCACGTA TATGATACCAACAGT (**siRNA-HIF-1α**).

Real-time PCR and RT-PCR

Real-time PCR was used to monitor changes in the expression of HIF-1α gene. In brief, real-time PCR was carried out using a Rotor – Gene 3000 system (Corrbet, Germany) with the mentioned primers. Amplification was conducted using Takara solute Syber green ROX mix (Takara, Japan) according to the manufacturer's instruction. PCR conditions consisted of initial denaturation at 94°C for 5 minutes followed by 40 amplification cycles consisting of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds. Threshold cycle values were normalized to β-actin expression.

RT-PCR conditions included a primary denaturation step of 5 minutes at 94°C, followed by 33 cycles of 30 seconds at 94°C, 30 seconds annealing temperatures including 59°C and 20 seconds at 72°C both for human HIF-1α and β-actin, and a final extension step of 7 minutes at 72°C. Finally, each PCR product was analyzed by 2% agarose gel electrophoresis.

Western blotting

Western blot was used to evaluate the expression of HIF-1α protein. Total protein was extracted using Complete Lysis M reagent (Roche, Germany) according to the method described in the kit. Then, the samples were boiled for 5 minutes in loading buffer containing 4% sodium dodecylsulfate (SDS), 20% glycerol, and bromophenol blue. Then, the proteins were resolved on 10% SDS-PAGE and transblotted onto polyvinylidene fluoride membranes (Roche, Germany). Membrane were blocked followed by overnight incubation at 4°C with specific primary antibodies, i.e. β-actin (Sigma, Munich, Germany) or monoclonal anti-HIF-1α antibodies (Abcam, Cambridge, UK). Thereafter, membranes were washed with tris-buffered saline including 0.1% Tween 20 and incubated with HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK) secondary antibody. Finally, signals were developed using DAB solution (Sigma, Munich, Germany).

Culture and expansion of MSCs

Normal MSCs and pcDNA-HIF-1α transfected MSCs (***MSCs-HIF-1α***) were studied in separate experimental settings. Twenty thousand MSCs were suspended in 2 ml DMEM-LG (Sigma, Munich, Germany) medium containing 10% fetal bovine serum (FBS) (Sigma, Munich, Germany) and seeded in each well of 6-well plates. The cells were incubated for 3–4 days in a 37°C incubator with 5% CO_2.

Analysis of hematopoietic growth factors mRNA expression

we selected cytokines for analysis based on their various effects on cells lines as reported in other studies.[19–22] Accordingly, GM-CSF, G-CSF, Kit ligand (SCF), and FLT3Lwere studied. The sequences of gene-specific primers used for these growth factors are given in Table 2. Conditions for real-time PCR and RT-PCR were the same as described above.

As the highest expression of HIF-1α gene was detected on the third day, total RNA of the cells was isolated in the end of that day according to protocols mentioned above. Then, cDNA of these cells was constructed and studied for the expression of hematopoietic growth factor genes by RT-PCR and real-time-RT-PCR. Another group of cells, including normal MSCs and *MSCs-HIF-1α*, were cultured for three days after culture for 12 hours in hypoxia (1% oxygen) in Galaxy 48 R Incubator (New Brunswick,

Table 2 Oligonucleotide sequences of primers used for RT-PCR and real-time PCR reactions of hematopoietic growth factors

Gene name	Sequence	TM	Product length
GMCSF	CCATGATGGCCAGCCACTAC CAGCAGTCAAAGGGGATGACA	60	133
Flt3	TGTCGCTGGGTCCAAGATG TCTGGACGAAGCGAAGACAG	59	110
KITL	CGGAGTCGCCACACCACTGTTT CACACGATTCCTGCAGATCCCTTCA	61	149
GCSF	AGAGCTTCCTGCTCAAGTGC AGCTTGTAGGTGGCACACTC	59	100

Germany). Afterward total RNA was collected and applied to expression analysis of cytokine genes.

Enzyme-linked immunosorbent assay

Twenty thousand cells from normal MSCs and *MSCs-HIF-1α* in 1 ml DMEM-LG medium containing 10% FBS were seeded in each well of a 6-well plate. Culture supernatants were collected at the first day of transfection (days 0) to 4 and cytokines levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Germany). In separate experiments, cells cultured in hypoxic conditions for 24 hours were also studied for cytokine production. Detection limit of SCF kit was 5.0 pg/ml. Determinations were performed in duplicate and results were expressed as pg/ml.

Isolation of hematopoietic stem cells from cord blood

After obtaining written consent from parents, cord blood samples were collected in specific bags containing CPD-A anticoagulant in sterile conditions, and were carried to the laboratory in 16–24°C temperature. The samples were mixed in 3:1 ratio with MACS

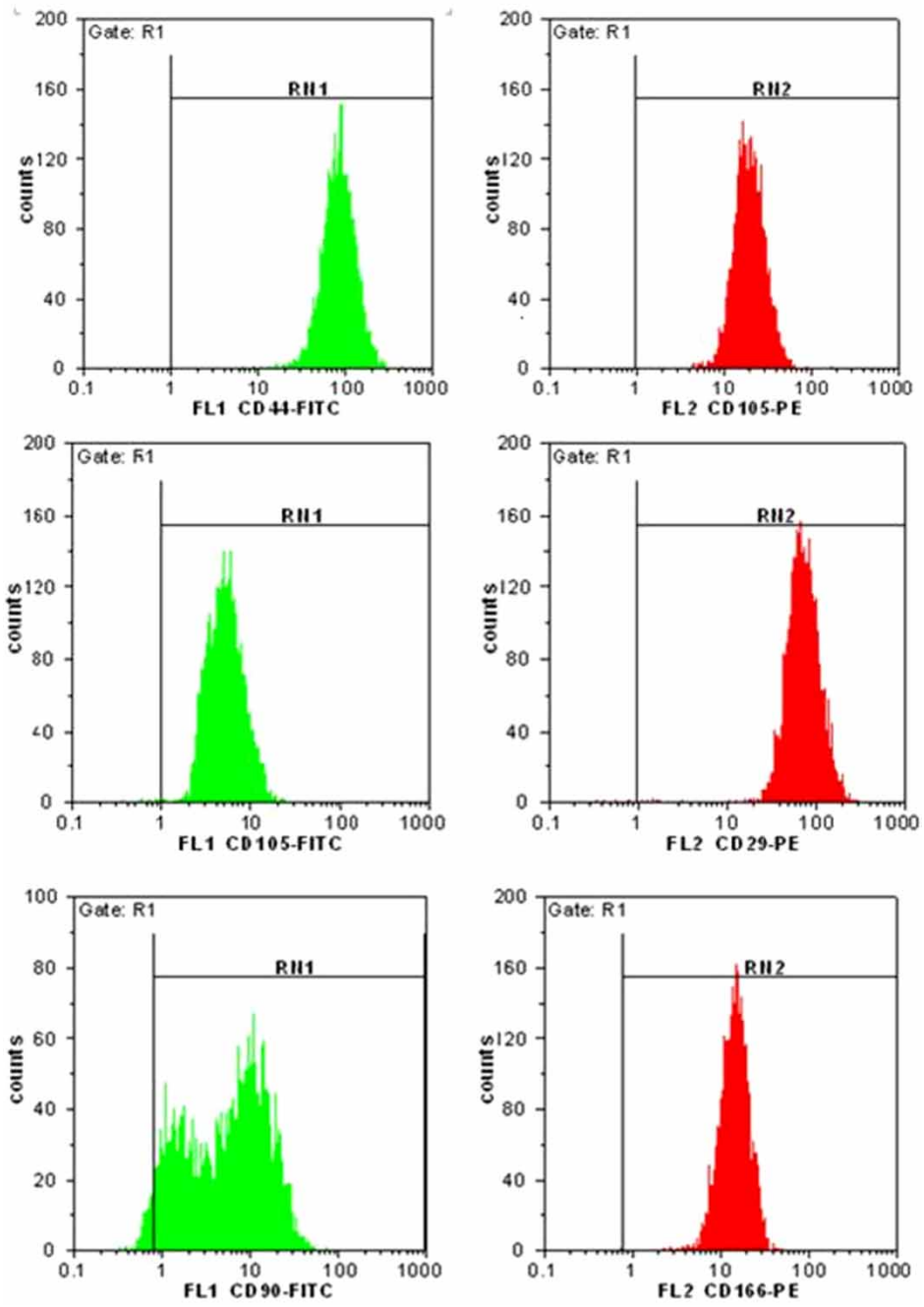

Figure 1 Immunophenotypic analysis of human MSCs by flowcytometry. The results indicate that MSCs are positive for the antigens CD166, CD105, CD90, CD44, and CD29, but negative for CD34 and CD38.

buffer (PBS containing 0.5% BSA and 2 mM EDTA) within less than 4 hours after sampling. Then, samples were mixed with hydroxy ethyl starch in 7:1 ratio to precipitate the red blood cells. The upper fraction was washed with MACS and mononuclear cells were isolated using Ficoll density gradient centrifugation.[23]

For each 10^8 mononuclear cells, 100 µl of FC receptor blocker (Miltenyi Biotec, Germany) was added and cell suspension was incubated for 10 minutes at 4°C. Next, 100 µl of Anti-CD34 Micro Bead Antibody (Miltenyi Biotec, Germany) was added to the cell suspension and incubation extended for 1 hour at 4°C. Cells were washed with MACS buffer and passed through a 30-micron filter to remove cell clumps. To isolate $CD34^+$ hematopoietic stem cells; the suspension was passed through MACS columns (Miltenyi Biotec, Germany). After flushing the columns with MACS buffer, the flow-through containing desired cells was washed twice with buffer and used for downstream experiments. To assess the purity of $CD34^+$ population, cells were analyzed by FACS using antibodies against CD38-PE and CD34-FITC antibodies. The above approach yielded almost $1.5–2 \times 10^6$ $CD34^+$ cells out of each blood unit (~100 ml) with a cell viability of more than 88% as confirmed with trypan blue.

Culture of hematopoietic stem cells ($CD34^+$ cells)

Ten thousand cells from freshly isolated $CD34^+$ cells were suspended in 1 ml of serum-free Stem span medium (Stem Cell Technologies, Atlanta, GE, USA) containing the cytokine mixture Flt3 ligand (50 ng/ml), thrombopoietin (100 ng/ml) and SCF (100 ng/ml). Cell suspension was added to each well of a 6-well plate which was then incubated in a 37°C incubator with 5% CO_2. On days 2, 4, 6, 8, and 10, the medium was refreshed in such a way that half of the supernatant was gently removed and the same amount of fresh medium was added.

Co-culture of $CD34^+$ cells with MSC layer

MSCs were studied in two separate groups: normal MSCs and *MSCs-HIF-1α*. One thousand/cm^2 MSCs were seeded onto each well of 4-well plates containing 2 ml of DMEM-LG medium supplemented with 10% FBS. The cells were incubated at 37°C and 5% CO_2 for 2 days, and were washed using PBS. Then, 1 ml of Stem span medium containing the above-mentioned cytokine cocktail together with 1×10^4 $CD34^+$ cells/cm^2 was added to confluent MSC monolayer in two wells, one containing normal MSCs and the other *MSCs-HIF-1α*. This method was also repeated with Stem span medium without

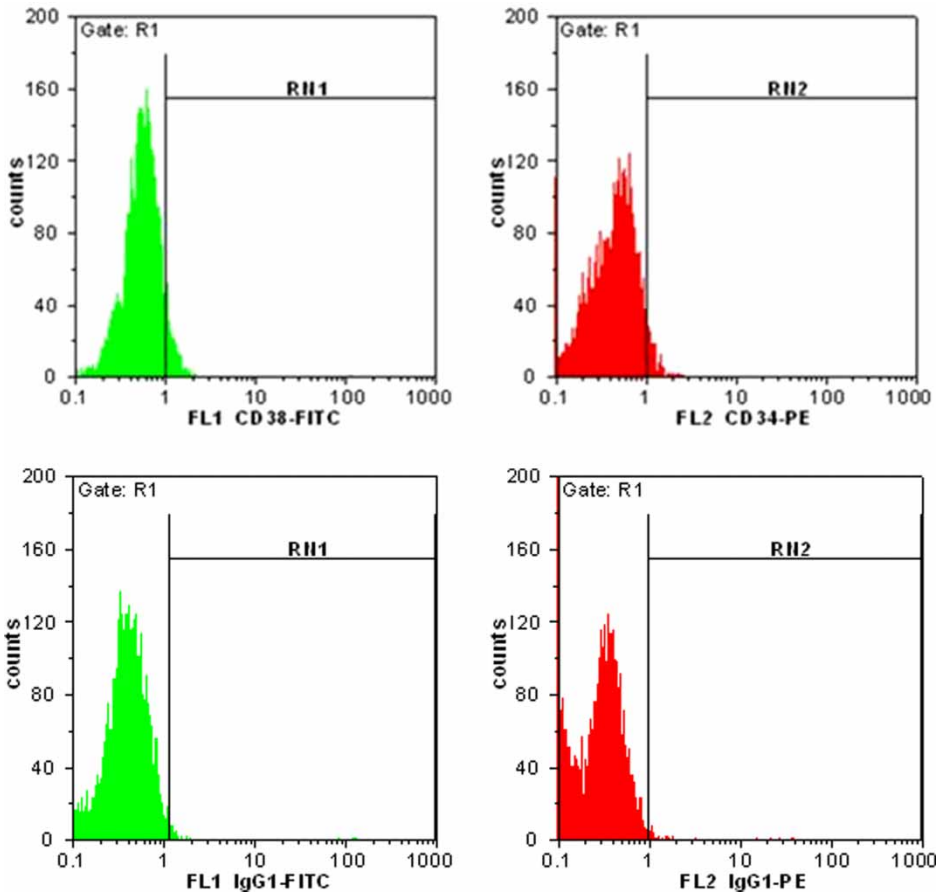

Figure 1. Continued

cytokines. On days 2, 4, 6, 8, and 10, the medium was changed, so that the half of the supernatant was gently removed and the same amount of fresh medium was added.[24,25]

To calculate the expansion rates of $CD34^+$ cells in mono- or co-culture conditions, we then determined percentage $CD34^+$ cells using flowcytometry and their absolute counts using a hematologic cell counter (Sysmex K 1000, Japan) at days 2, 4, 6, 8, 10, and 14.

First we used Trypan blue exclusion assay to determine the number of viable total nucleated cells (TNC) for day 'X'. The obtained TNC was multiplied by 2n, where n was the number of times we refreshed the culture medium before day 'X' (as explained above, in each time half of the medium was aspirated and replaced with fresh medium). Likewise, for calculating expansion fold of $CD34^+$ cells at day 'X', TNC was multiplied by the percentage of $CD34^+$ cells in flowcytometric analysis.

In vitro clonal assay

One thousand $CD34^+$ cells from different conditions (freshly isolated, day 0) cells maintained in culture medium in the presence or absence of cytokines and cultured with mesenchymal layer (normal MSCs or *MSCs-HIF-1α*) at day 5, or cells cultured alone in the presence or absence of cytokines at day 5, were added to Methocult medium (H4435, Stem cell Technology, Vancover, Canada) and homogenized. Afterwards, 1.1 ml of the cell suspension was gently added to 35 mm plates by using 1.5-inch-16 G needle syringes. Plates were incubated at 37°C and 5% CO_2 and after14 days, the colony-forming units in culture (CFU-C) were counted using stereomicroscope and a 100-mm measured plate, as already described.[26]

Statistical analysis

As required, Student's *t*-test or analysis of variance test was applied to statistical analyses. In all tests, $P < 0.05$ was considered significant.

Results

Flowcytometric analysis of bone marrow-derived MSCs

Microscopic evaluation of MSCs demonstrated a heterogeneous cell population with a dominant spindle-shaped morphology. Flow cytometric analysis of cell-surface markers authenticated the expression of specific non-hematopoietic surface markers, such as CD105, CD166, CD90, CD29, and CD44, while hematopoietic markers including CD34 and CD38 were not detected (Fig. 1). These findings conform to what have already been reported.[27]

Isolation and cloning of HIF-1α

Using total cell RNA, HIF-1α cDNA was amplified as template and specific primers. A single band of about 2496 bp on agarose gel authenticated the amplified fragment (Fig. 2). Thereafter, the PCR product was cloned into the *Bam*HI and *Nhe*I sites of pcDNA3.1 plasmid (Fig. 3). Accuracy of the cloning was first confirmed by PCR analysis and restriction enzyme digestion of the recombinant plasmid. Eventually, the fidelity of the cloned sequence was confirmed by DNA sequencing. The verified plasmid was designated as pcDNA3.1-HIF-1α.

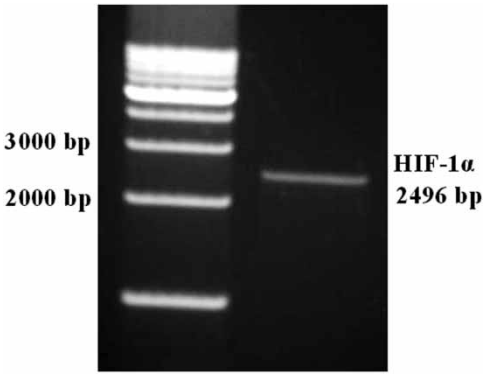

Figure 2 Full length HIF-1α mRNA.

Assessment of transient overexpression of HIF-1α in MSCs

The confirmed MSCs were transfected with pcDNA3.1-HIF-1α plasmids. To monitor the expression of HIF-1α mRNA, RT-PCR and real-time PCR were performed at various time points including 1, 2, to 14 days after transfection. Initial overexpression of HIF-1α mRNA was detected 1 day post-transfection, reached the highest level on day 3, and then decreased until day 6, and no overexpression was detected on day 7. As shown in Fig. 4 (lane 10) and Fig. 5, the expression of HIF-1α

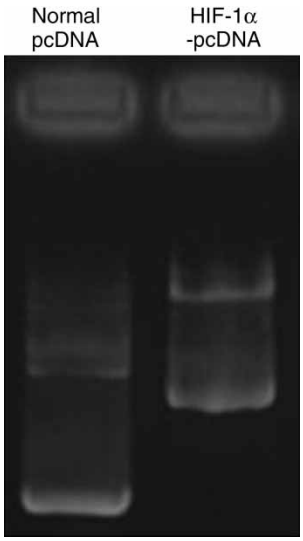

Figure 3 Comparison of plasmids containing HIF-1α with the plasmid lacking HIF-1α: it is obvious that pcDNA-HIF-1α is larger (in bp) than empty pcDNA 3.1 as indicated by slower mobility on the gel.

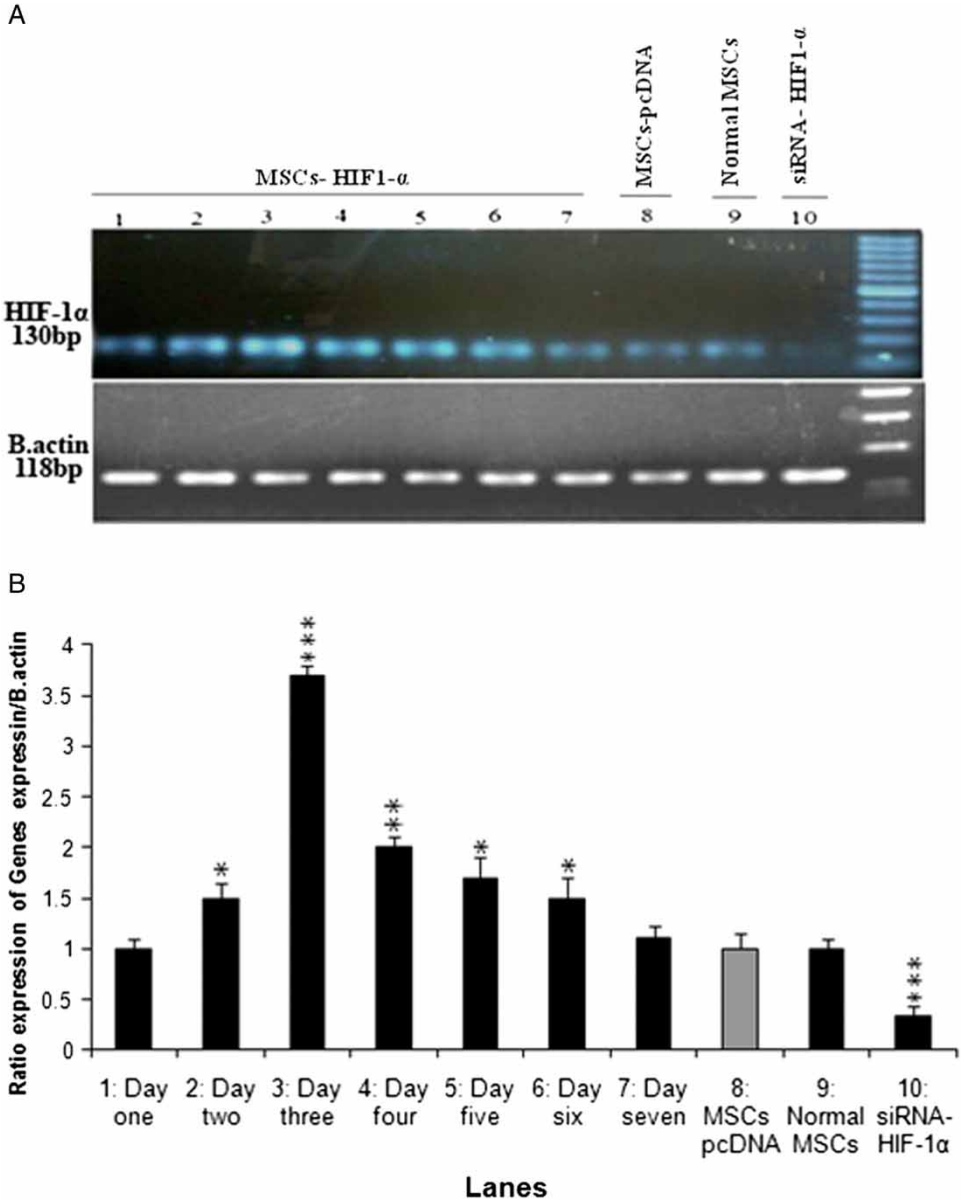

Figure 4 (A) Assessment of transient expression of HIF-1α in MSCs from day 1 to 7 post-transfection (lanes 1–7). The highest expression level was observed on the third day, and declined to the basal expression level until seventh day. The expression of baseline HIF-1α was also down-regulated using HIF-1α siRNA (lane 10). No difference was observed in HIF-1α expression between the MSCs and MSCs-pcDNA. (B) Analysis of PCR band density using UVIdoc Gel Documentation System (Avebury House 36a Union Lane Cambridge CB4 1QB-uk). Results indicate significant increase in HIF-1α gene expression at day 3 post-transfection and also down-regulation of HIF-1α after HIF-1α siRNA silencing (mean ± SD); figure represents three independent experiments, and data were compared to day 0. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mRNA was decreased by the HIF-1α siRNA. Furthermore, western blot analysis detected a transient up-regulation of HIF-1α protein in MSC-HIF-1α cells but not in normal MSCs (Fig. 6). Moreover, despite up-regulation of the HIF-1α, most *MSC-HIF-1α* cells showed no morphological changes and remained adherent to tissue culture surfaces (data not shown).

Over expression of HIF-1α enhances the expression of kit ligand (SCF) gene in MSCs and increases the SCF production in culture medium

The expression of hematopoietic growth factors mRNAs was evaluated by RT- PCR and real-time PCR at the end of day 3 after HIF-1α transfection. The results showed that over expression of HIF-1α in MSCs only significantly increased mRNA expression of SCF (Figs. 7 and 8), and had no significant effect on mRNA expression of other growth factors such as GM-CSF, G-CSF, and Flt3L (data not shown). Regarding increased expression of SCF gene, we then used ELISA to analyze SCF protein in culture supernatants collected from day 0 (first day of transfection) until day 4. We found that a high level of SCF protein was produced in HIF-1α – MSCs, but only a low amount by normal MSCs in normoxia and a non significant increase after 24-hour hypoxia (Table 3).

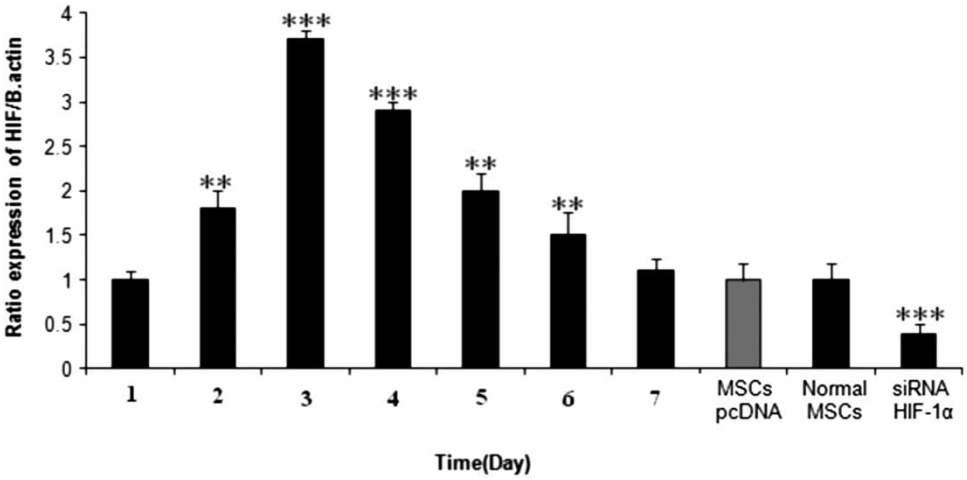

Figure 5 Expression of HIF-1α gene in MSCs by real-time PCR. Expression of HIF-1α mRNA at various time points post-transfection (data represents mean ± SD; number of replicates = 3, **$P < 0.01$, ***$P < 0.001$). All data were compared to day 1. At day 3, the highest level of HIF-1α expression was detected and expression declined gradually afterwards. However, after 7 days, the expression level of HIF was the same as control. The transfection of siRNA-HIF-1α could also reduce the basal expression of HIF-1α in MSCs. No difference was observed in HIF-1α expression level between normal MSCs and MSCs-pcDNA.

Proliferation of $CD34^+$ cells is increased in co-culture with MSCs-HIF-1α

The percentage of $CD34^+$ cells on days 2, 4, 6, 8, 10, and 14 in different culture conditions was determined by flow cytometric analysis (Fig. 9). As Fig. 10 shows, co-culture of hematopoietic stem cells with *MSCs-HIF-1α* without cytokines, could significantly increase the percentage of $CD34^+$ cells at fourth and sixth days compared to co-culture with normal MSCs. There was no significant difference between the percentage of $CD34^+$ cells in co-culture with normal MSCs and MSCs–pcDNA. Furthermore, as depicted in Fig. 11,

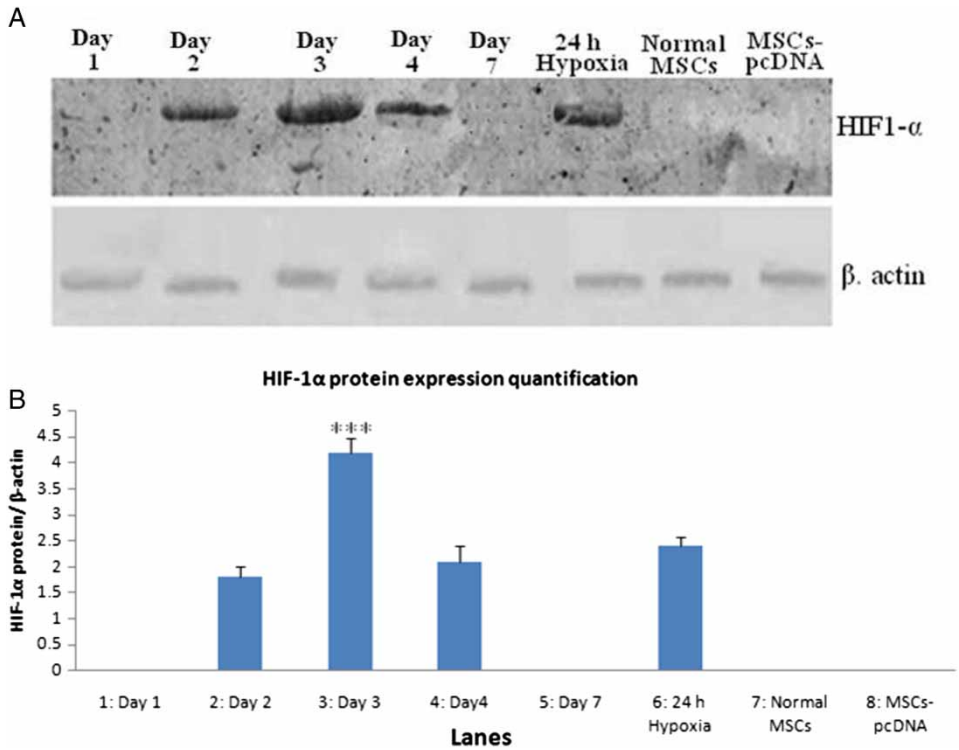

Figure 6 (A) Western blot analysis of HIF-1α protein expression in MSCs transfected with pcDNA-HIF-1α from day 1 to 7.The highest protein expression level was observed at day 3, and decreased to the basal level at day 7. HIF-1α protein expression in normal MSCs was not detected by DAB staining in normoxic conditions. No difference was observed in HIF-1α protein expression level between the MSCs and MSCs-pcDNA. (B) Analysis of HIF-1α protein band densities using UVIdoc Gel Documentation System (Avebury House, 36a Union Lane, Cambridge CB4 1QB, UK). The highest level of HIF-1α protein was detected at day 3 post-transfection (*$P < 0.001$).**

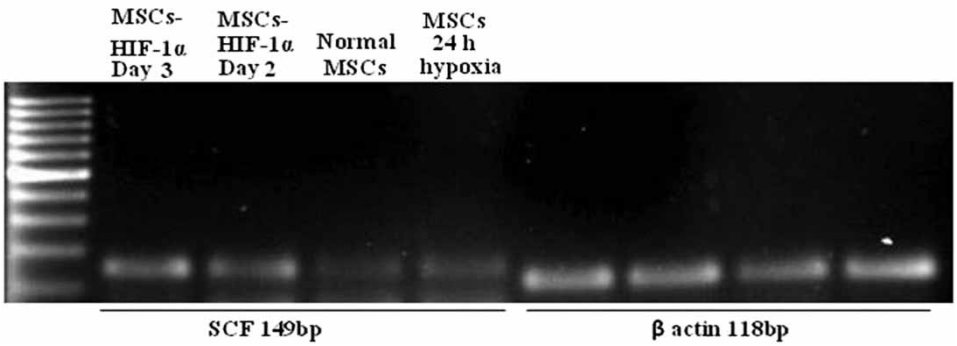

Figure 7 Analysis of SCF mRNA expression with RT-PCR following HIF-1α overexpression. The highest expression level was observed at day 3. SCF expression in normal MSCs was slightly enhanced in hypoxic condition.

$CD34^+$ cells displayed the highest expansion rate (folds) on day 10 in co-culture with *MSCs-HIF-1α* without cytokines compared to co-culture with normal MSCs. Interestingly, co-culture of $CD34^+$ cells with *MSCs-HIF-1α* in the presence of cytokines yielded high expansion folds in all conditions. Likewise, there was no significant difference between expansion fold of $CD34^+$ cells in co-culture with normal MSCs and *MSCs-pcDNA*.

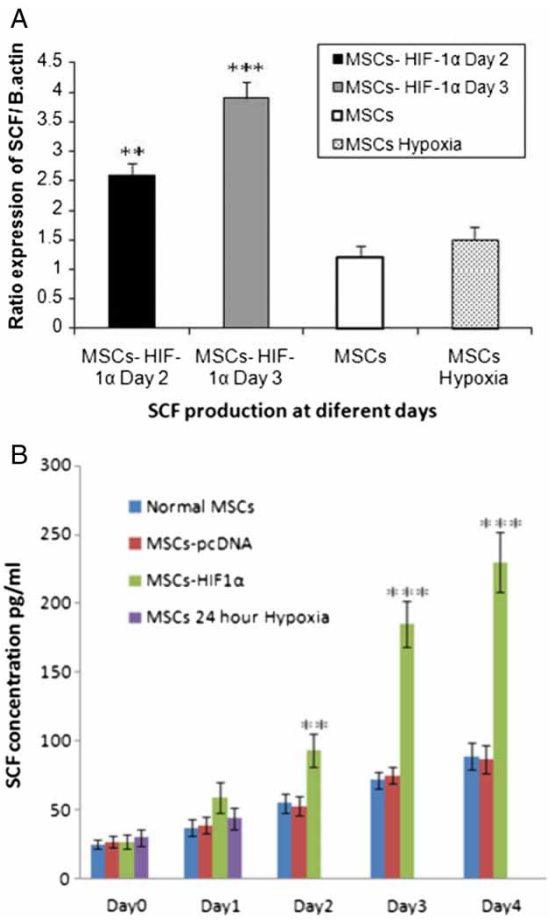

Figure 8 (A) Analysis of SCF expression level with real-time RT-PCR at various time points post-transfection (data represent mean ± SD; number of replicates = 3, **$P < 0.01$, ***$P < 0.001$. (B) ELISA evaluation of SCF cytokine from day 0 to 4 post-transfection. Experiments were performed on culture supernatants from MSCs-HIF-1α in co-culture with normal MSCs in normoxia and 24 hours hypoxia (data represent mean ± SD; number of replicates = 3, ***$P < 0.001$).

Co-culture with MSCs-HIF-1α induces the clonogenic potential of HSCs

The clonogenic potential of freshly isolated $CD34^+$ cells and those expanded in different culture conditions were assessed (using colony counting) after 14 days culture in Metocult medium. As depicted in Fig. 12, the number of colonies for $CD34^+$ cells in different conditions is as follows: freshly isolated $CD34^+$ cells 60 ± 9, $CD34^+$ cells with cytokines 78 ± 8, $CD34^+$ cells in co-culture with normal MSCs and cytokines 82 ± 10, $CD34^+$ cells in co-culture with normal MSCs without cytokines 71 ± 8, $CD34^+$ cells in co-culture with *MSCs-HIF-1α* without cytokines 75 ± 12, and finally $CD34^+$ cells in co-culture with *MSCs-HIF-1α* with cytokines 90 ± 9. Furthermore, there was no significant difference in clonogenic potential of $CD34^+$ cells in co-culture with normal MSCs and *MSCs-pcDNA* (Fig. 13).

Table 3 SCE production by MSCs in different culture conditions

	Normal MSCs	MSCs-pcDNA	MSCs-HIF1α	MSCs 24-hour hypoxia
SCF concentration pg/ml (day 0)	25 ± 3	27 ± 4	27 ± 5	30 ± 6
SCF concentration pg/ml (day 1)	37 ± 6	39 ± 6	59 ± 11	44 ± 8
SCF concentration pg/ml (day 2)	55 ± 7	53 ± 7	93 ± 12	–
SCF concentration pg/ml (day 3)	72 ± 6	75 ± 6	185 ± 17	–
SCF concentration pg/ml (day 4)	89 ± 10	87 ± 10	230 ± 22	–

The cells cultured in hypoxia were also studied for cytokine production after 24 hours of hypoxia.

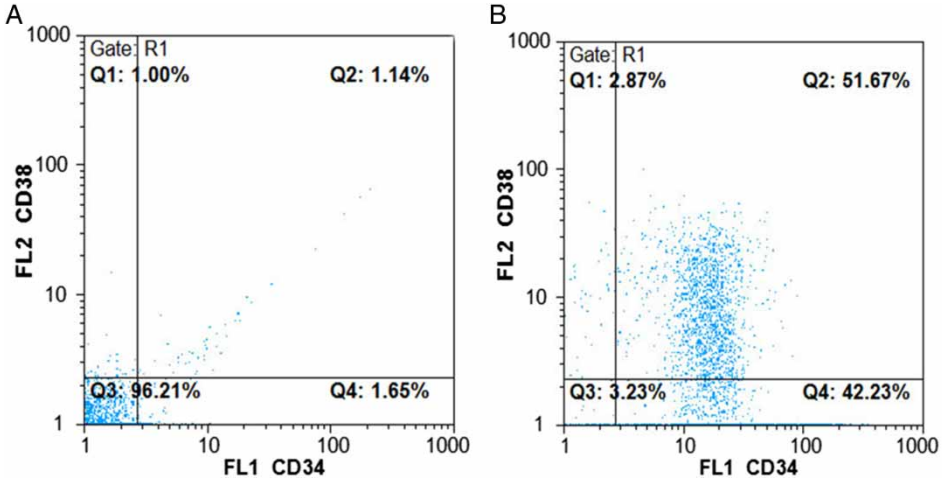

Figure 9 Flow cytometeric analysis of freshly isolated CD34$^+$ cells (day 0). The purity of CD34$^+$ cells was 94.25% of which 54.63% were CD38$^+$ cells.

Discussion

Human MSCs are basically endowed with the ability to migrate to injured sites,[28,29] and this possibly could explain to a large extent their potential in improving the cell therapy process. Hence, current researches aim for increasing their efficiency.

As a large number of MSCs are destroyed in isolation and propagation procedures *in vitro* within the first hours after transplantation.[30] Increased expression of HIF-1α can be effective in augmenting the resistance of these cells against extreme conditions.[31,32] On the other hand, if this increased expression is associated with stimulated production of some hematopoietic growth factors, the quality of transplantation can be rather improved.

One of the most challenging and novel approaches to achieve this, is gene manipulation of human MSCs to overexpress such genes as HIF-1α which is transiently expressed at a very low level. However, the worth of these approaches should be weighed against any complication of induced gene activity including development of tumors.[14,33–35]

In this study, a plasmid vector, pcDNA 3.1, was used to transiently enhance HIF-1α expression in human bone marrow-derived MSCs and it was shown that the maximum level of HIF-1α gene and protein expression was on day 3 (after trancfection) with gradual decrease afterwards.

Next, we assessed the effect of increased HIF-1α expression in MSCs on production of

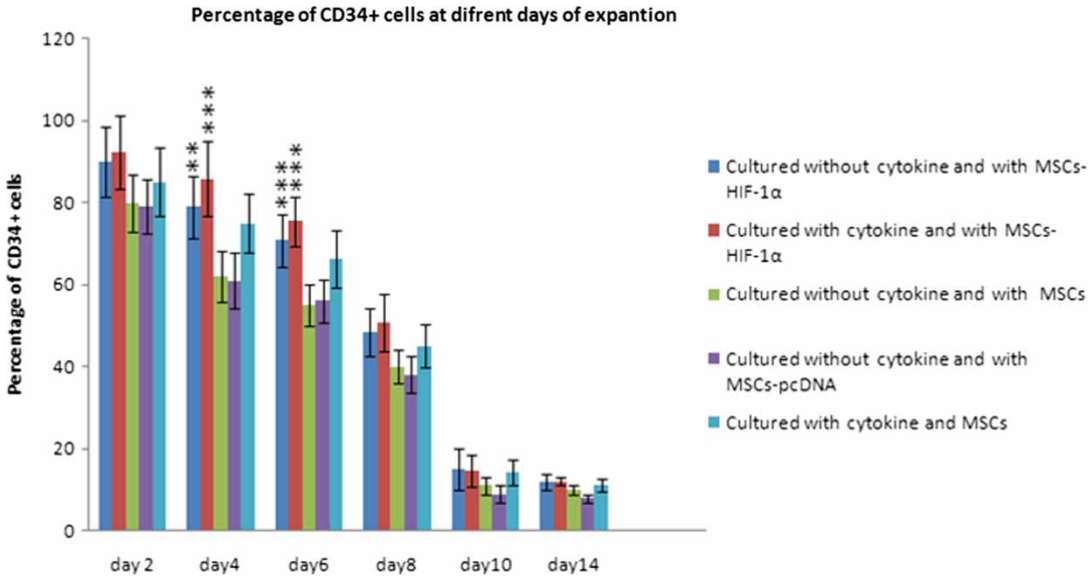

Figure 10 The percentage of CD34$^+$ cells in culture medium in different days and conditions of culture. The number of CD34$^+$ cells cultured with MSCs-HIF-1α without any cytokines at day 4 and day 6 showed a significant increase compared with similar culture conditions of CD34$^+$ cells with normal stroma, while in co-culture of CD34$^+$ cells with MSCs-HIF-1α in the presence of cytokine, no significant difference was found compared with CD34$^+$ co-culture with cytokines and normal stroma. No difference was observed between the MSCs and MSCs-pcDNA (mean ± SD; **$P < 0.01$ and ***$P < 0.001$; figure represents data analysis from three independent experiments).

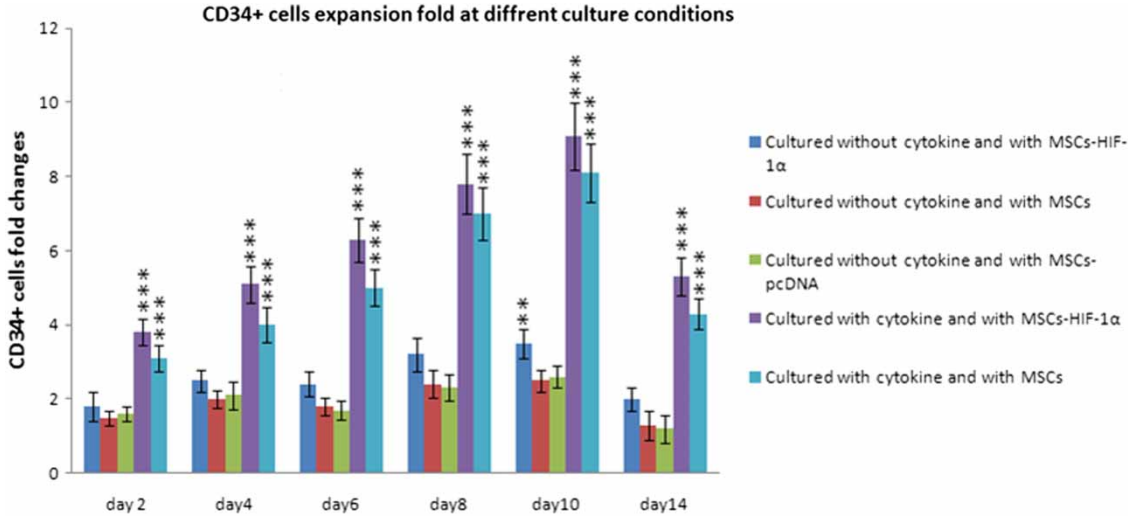

Figure 11 Comparison of CD34$^+$ cells expansion fold changes in different conditions. In the first few days of culture, the cells quickly proliferated and multiplied over time. The fold change in CD34$^+$ cells was assessed by flow cytometric analysis and by determining the percentage of these cells in total nucleated cells. In this figure, fold change of CD34$^+$ cells at days 2, 4, 6, 8, 10, and 14 is shown. The highest fold change of CD34$^+$ cells occurred during their culture with normal MSCs and MSCs-HIF-1α in presence of cytokine, but there was no significant difference between the two groups. The fold change inCD34$^+$ cells during culture with normal MSCs and MSCs-HIF-1α without cytokines was much lower compared with the same conditions in presence of cytokines. Significant difference between these two groups was only observed on the fourth day, and on other days, despite increased fold change of cultured cells with MSCs-HIF-1α, this difference was not significant. (mean ± SD; **$P < 0.01$ and ***$P < 0.001$; figure represents data analysis from independent experiments).

hematopoietic growth factors, and we found that production of SCF was significantly increased in MSCs in normoxia and hypoxia. It is well established that SCF and its tyrosine kinase receptor, c-Kit, play critical roles in hematopoiesis including effect on different hematopoietic stem

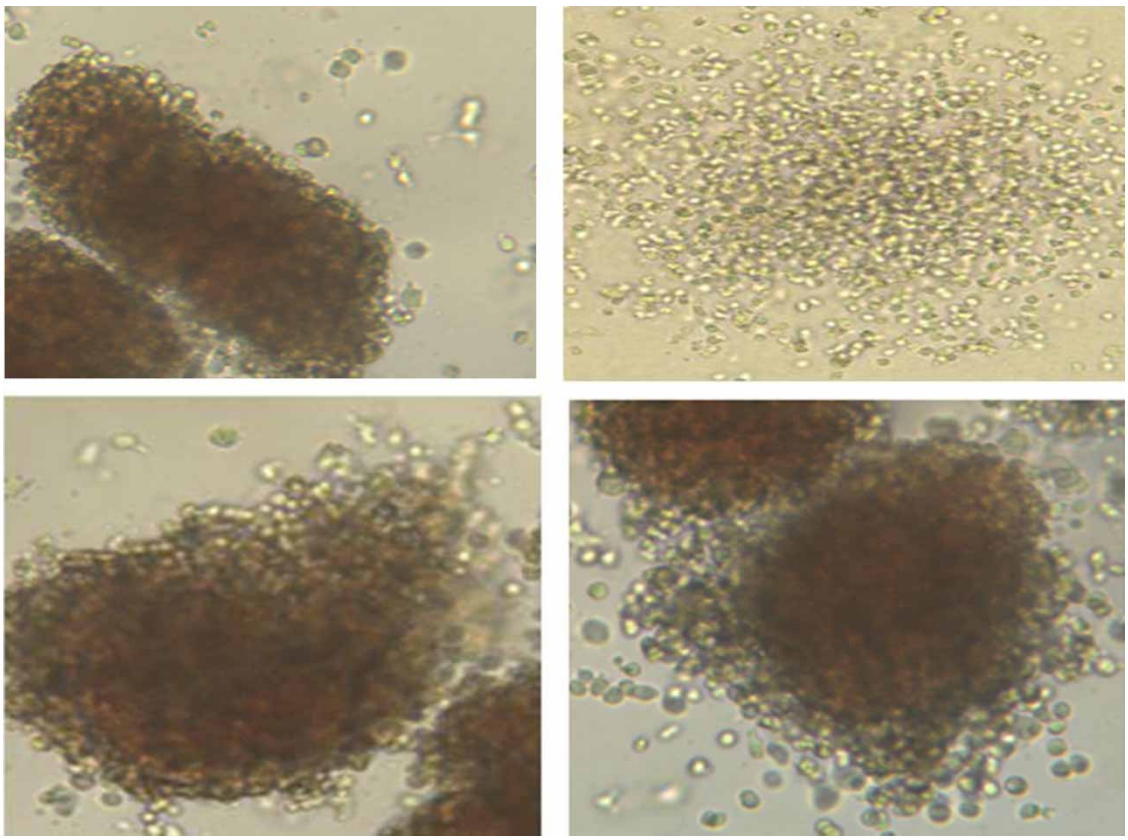

Figure 12 Some CFUs produced from freshly isolated CD34$^+$ cells and those expanded in different culture conditions after 14 days culture using Metocult medium.

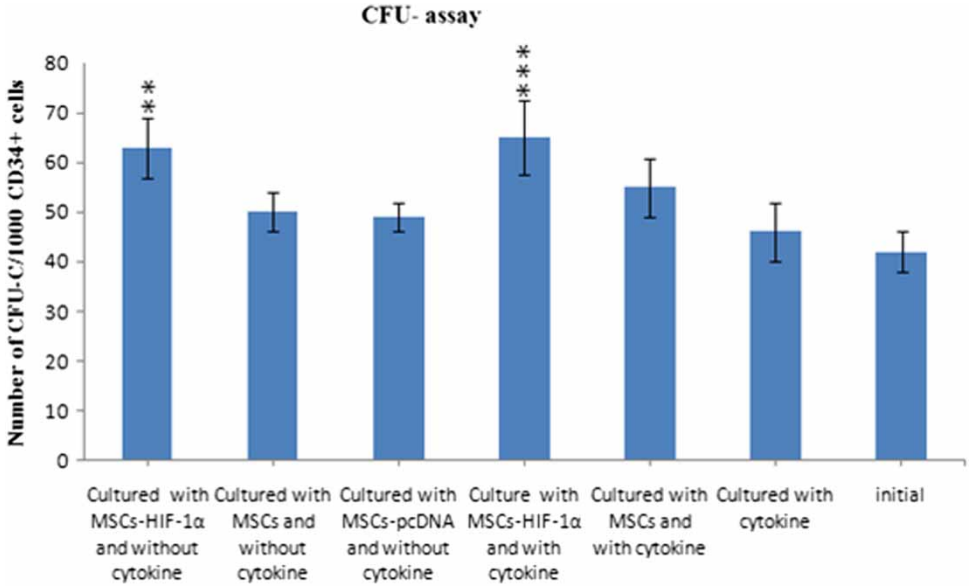

Figure 13 Assessment of the clonogenic potential (CFU-assay) of $CD34^+$ cells in different culture conditions. Clonogenic capacity of $CD34^+$ cells was assayed after 5 days of co-culture in different conditions. The highest numbers of colonies were observed in co-culture of $CD34^+$ and MSCs-HIF-1α with cytokines. The highest clonogenic potential of $CD34^+$ cells was obtained in their co-culture with MSCs-HIF-1α in the presence of cytokine, but was not significantly different from that of $CD34^+$ cells cultured with normal MSCs in the presence of cytokines. Clonogenic capacity of $CD34^+$ cells in co-culture with normal MSCs and MSCs-HIF-1α without cytokine was lower than the same conditions in the presence of cytokine. However, there was a significant difference in clonogenic potential of cells cultured with MSC-HIF-1α relative to normal MSCs layer (mean ± SD; $^{**}P < 0.01$ and $^{***}P < 0.001$; figure represents data analysis from three independent experiments).

cells and progenitors, promoting the survival of HSCs, and enhancement of CFU-S and CFU-C colony formation, indicating effect of SCF on primitive HSCs.[36,37]

Supporting our findings, some previous studies demonstrated the role of overexpressed HIF-1α on production of some growth factors such as erythropoietin,[38] VEGF,[39] and ILGF[14] in different tissues. Furthermore, Jeong *et al.*[40] showed that HIF-1α could stimulate the production of interleukin (IL) 6, IL8, and TNF-α in mast cells subsequent to stimulation by desferoxamine.

Hematopoietic stem cells in their early evolution express CD34 marker and lack the differentiation markers such as CD38. Therefore, in a population consisting of stem and progenitor cells, the higher number of $CD34^+$ cells indicates the more primitive and more effective population of hematopoietic stem cells.[41]

In this study, according to the Fig. 10, co-culture of MSCs-HIF-1α with hematopoietic stem cells could increase the percentage of $CD34^+$ cells and prevent the differentiation of hematopoietic stem cells. This indicates increase in the number of cells with higher expansion power. Moreover, according to the Fig. 11, co-culture of HSCs with MSCs-HIF-1α, increased $CD34^+$ cells fold expansion suggesting that overexpression of HIF-1α in MSCs and co-culture with HSCs could increase the proliferative potential of HSCs. However, the above effects of HIF-1α were significant only in the absence of cytokines.

In this study, it was also demonstrated that co-culture of MSCs-HIF-1α and $CD34^+$ cells could increase the number of $CD34^+$ colonies (in the presence of cytokines). These results indicate that the overexpression of HIF-1α in MSCs could increase their supportive potential in co-culture with HSCs and enhance the clonogenic capacity of latter cells (Fig.13).

Our findings on MSC-HSC co-culture are corroborated by others' who showed a higher level of expansion rate, colonogenicity, absolute number, and survival rate for HSCs in co-culture with.[42–43] And finally, Jing *et al.*[44] showed that co-culture with MSC greatly enhanced proliferation of human HSCs, especially of the more primitive $CD34^+$ fraction.

As discussed, overexpression of HIF-1α in MSCs could be effective in increasing the supportive potential of these cells for hematopoietic stem cells in culture. This finding may be supported by following arguments:

1. Since HIF-1α is a protein that can increase cellular resistance against environmental stresses (ref), overexpression of HIF-1α protein in MSCs is likely to increase the number of these cells and their viability.
2. HIF-1α protein may induce the production of some cytokines other than those in this study, and even their slight enhancements could augment the supportive role of MSCs in culture medium.

3. Overexpression of HIF-1α increased SCF production, but such enhancement is negligible compared with the SCF added to culture medium. We believe that even this low amount could not be ineffective, and may play a role in increased supportive capacity of MSCs in culture medium
4. HIF-1α might have been effective upon MSCs surface molecules and perhaps exerted its effect by improving cell–cell interaction.

Taken all together, this study can present a new approach for increasing resistance and improving function of MSCs in unfavorable conditions before and after transplantation. Indeed, co-culture of gene-manipulated human MSCs with HSCs has not been performed to date, thus our study will be the first to introduce it to illuminate the way for future relevant investigations on other cell systems.

Conclusion

Establishing gene transfer systems with great performance along with overexpression of genes with high therapeutic potential in MSCs can obviate the need for long-term cultures resulting in aging and reduced quality of cell function, and can enable expected therapeutic effects with lower number of cells.

References

1 Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19(3):180–92.
2 Maitra B, Szekely E, Gjini K, Laughlin M, Dennis J, Haynesworth S, *et al.* Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. Bone Marrow Transplant. 2004;33(6):597–604.
3 Dazzi F, Horwood NJ. Potential of mesenchymal stem cell therapy. Curr Opin Oncol. 2007;19(6):650–5.
4 Jones E, McGonagle D. Human bone marrow mesenchymal stem cells in vivo. Rheumatology. 2008;47(2):126–31.
5 Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, *et al.* Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia. 2007;21(8):1733–8.
6 Tögel F, Westenfelder C. Adult bone marrow–derived stem cells for organ regeneration and repair. Developmental Dynam. 2007; 236(12):3321–31.
7 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005; 105(4):1815–22.
8 Brahimi-Horn MC, Pouysségur J. Oxygen, a source of life and stress. FEBS Lett. 2007;581(19):3582–91.
9 Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. Nature Reviews Molecular Cell Biol. 2008;9(4):285–96.
10 Panchision DM. The role of oxygen in regulating neural stem cells in development and disease. Journal of Cellular Physiol. 2009;220(3):562–8.
11 Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 2010;7(2):150–61.
12 Adams J, Difazio L, Rolandelli R, Lujan J, HaskÃ³ G, Csoka B, *et al.* HIF-1: a key mediator in hypoxia (Review). Acta Physiologica Hungarica. 2009;96(1):19–28.
13 Goda N, Dozier SJ, Johnson RS. HIF-1 in cell cycle regulation, apoptosis, and tumor progression. Antioxidants and Redox Signaling. 2003;5(4):467–73.
14 Semenza GL. Targeting HIF-1 for cancer therapy. Nature Reviews Cancer. 2003;3(10):721–32.
15 Zaouali MA, Mosbah IB, Boncompagni E, Abdennebi HB, Mitjavila MT, Bartrons R, *et al.* Hypoxia inducible factor-1α accumulation in steatotic liver preservation: Role of nitric oxide. World Journal of Gastroenterology: WJG 2010;16(28): 3499–509.
16 Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell. 2007;129(3):465–72.
17 Lee J-W, Bae S-H, Jeong J-W, Kim S-H, Kim K-W. Hypoxia-inducible factor (HIF-1) alpha: its protein stability and biological functions. Experimental & Molecular Med. 2004;36(1):1–12.
18 Halabian R, Mohammadi MH, Salimi Mohammad A, Mohammadi Roushandeh A, Aghaipour M, Amirizadeh N. Genetically engineered mesenchymal stem cells stably expressing green fluorescent protein. Iranian Journal of Basic Medical Sci. 2010;13(2):24–30.
19 McNiece IK, Briddell RA. Stem cell factor. Journal of Leukocyte Biol. 1995;58(1):14–22.
20 Gasson JC. Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood. 1991;77(6):1131–45.
21 Avalos BR, Gasson JC, Hedvat C, Quan S, Baldwin G, Weisbart R. Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. Blood. 1990;75(4):851–7.
22 Nicholls SE, Winter S, Mottram R, Miyan JA, Whetton AD. Flt3 ligand can promote survival and macrophage development without proliferation in myeloid progenitor cells. Experimental Hematol. 1999;27(4):663–72.
23 Soltanpour MS, Amirizadeh N, Zaker F, Oodi A, Nikougoftar M, Kazemi A. mRNA expression and promoter DNA methylation status of CDKi p21 and p57 genes in ex vivo expanded CD34+ cells following co-culture with mesenchymal stromal cells and growth factors. Hematology. 2013;18(1):30–8.
24 Zhang Y, Chai C, Jiang XS, Teoh SH, Leong KW. Co-culture of umbilical cord blood CD34þ cells with human mesenchymal stem cells. Tissue Eng. 2006;12(8):2161–170.
25 Jing D, Fonseca AV, Alakel N, Fierro FA, Muller K, Bornhauser M. Hematopoietic stem cells in co-culture with mesenchymal stromal cells-modeling the niche compartments in vitro. Haematologica. 2010;95(4):542–50.
26 Oodi A, Noruzinia M, Habibi Roudkenar M, Nikougoftar M, Soltanpour MS, Khorshidfar M. Expression of P16 cell cycle inhibitor in human cord blood CD34+ expanded cells following co-culture with bone marrow-derived mesenchymal stem cells. Hematology 2012;17(6):334–40.
27 Kazemnejad S, Allameh A, Soleimani M, Gharehbaghian A, Mohammadi Y, Amirizadeh N. Biochemical and molecular characterization of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells on a novel three-dimensional biocompatible nanofibrous scaffold. Journal of Gastroenterology and Hepatol. 2008;24(2):278–87.
28 Mouiseddine M, Francois S, Semont A, Sache A, Allenet B, Mathieu N. Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. British Journal of Radiol. 2007;80(Special Issue 1):S49–55.
29 Da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells 2008;26(9): 2287–99.
30 Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002;105(1): 93–8.
31 Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res. 2002;62(12):3387–94.
32 Piret JP, Mottet D, Raes M, Michiels C. Is HIF-1 [alpha] a pro- or an anti-apoptotic protein? Biochemical Pharmacol. 2002; 64(5–6):889–92.
33 Volm M, Koomägi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res. 2000;20(3A):1527–33.
34 Yatabe N, Kyo S, Maida Y, Nishi H, Nakamura M, Kanaya T. HIF-1-mediated activation of telomerase in cervical cancer cells. Oncogene 2004;23(20):3708–15.
35 Zhong H, Semenza GL, Simons JW, De Marzo AM. Up-regulation of hypoxia-inducible factor 1α is an early event in prostate carcinogenesis. Cancer Detect Prev. 2004;28(2):88–93.
36 Andrews RG, Knitter GH, Bartelmez SH, Langley KE, Farrar D, Hendren RW. Recombinant human stem cell factor, a c-kit

ligand, stimulates hematopoiesis in primates. Blood. 1991;78(8): 1975–80.
37 Migliaccio G, Migliaccio AR, Druzin ML, Giardina P, Zsebo KM, Adamson JW. Long-term generation of colony-forming cells in liquid culture of CD34+ cord blood cells in the presence of recombinant human stem cell factor. Blood 1992;79(10): 2620–7.
38 Grimm C, Wenzel A. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nature Med. 2002;8(7):718–24.
39 Dai Y, Xu M, Wang Y, Pasha Z, Li T, Ashraf M. HIF-1α induced-VEGF overexpression in bone marrow stem cells protects cardiomyocytes against ischemia. J Mol Cell Cardiol. 2007;42(6):1036–44.
40 Jeong HJ, Chung HS, Lee BR, Kim SJ, Yoo SJ, Hong SH, *et al.* Expression of proinflammatory cytokines via HIF-1α and NF-κB activation on desferrioxamine-stimulated HMC-1 cells. Biochem Biophys Res Commun. 2003;306(4): 805–11.
41 Bryder D, Rossi DJ. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol. 2006;169(2):338–46.
42 Zhang Y, Chai C, Jiang X-S, Teoh S-H, Leong KW. Co-culture of umbilical cord blood CD34þ cells with human mesenchymal stem cells. Tissue Eng. 2006;12(8):2161–70.
43 Hofmeister C, Zhang J, Knight K, Le P, Stiff P. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone Marrow Transplant. 2007;39(1):11–23.
44 Jing D, Fonseca A-V, Alakel N, Fierro FA, Muller K, Bornhauser M. Hematopoietic stem cells in co-culture with mesenchymal stromal cells-modeling the niche compartments in vitro. Haematologica. 2010;95(4):542–50.