Cell Prolif. 2007, **40**, 75–90

Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells

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Received 19 *May* 2006*; revision accepted* 11 *October* 2006

Abstract. *Objectives*: To characterize mesenchymal stem cell-like cells isolated from human amniotic fluid for a new source of therapeutic cells. *Materials*: Fibroblastoid-type cells obtained from amniotic fluid at the time of birth. *Methods*: The ability of *ex vivo* expansion was investigated until senescence, and stem cell-like characteristics were analyzed by examining differentiation potential, messenger RNA expression and immunophenotypes. *Results and Conclusions*: A morphologically homogenous population of fibroblastoid-type (HAFFTs) cells, similar to mesenchymal stem cells from bone marrow (BM-MSCs), was obtained at the third passage. The cells became senescent after 27 passages over a period of 8 months while undergoing 66 population doublings. Under appropriate culture conditions, by the 8th passage they differentiated into adipocytes, osteocytes, chondrocytes and neuronal cells, as revealed by oil red O, von Kossa, Alcian blue and anti-NeuN antibody staining, respectively. Immunophenotype analyses at the 17th passage demonstrated the presence of TRA-1–60; SSEA-3 and-4; collagen types I, II, III, IV and XII; fibronectin; α-SMA; vimentin; desmin; CK18; CD44; CD54; CD106; FSP; vWF; CD31; and HLA ABC. Reverse transcriptase–polymerase chain reaction analysis of the HAFFTs from passages 6–20 showed consistent expression of Rex-1, SCF, GATA-4, vimentin, CK18, FGF-5 and HLA ABC genes. Oct-4 gene expression was observed up to the 19th passage but not at the 20th passage. HAFFTs showed telomerase activity at the 5th passage with a decreased level by the 21st passage. Interestingly, BMP-4, AFP, nestin and HNF-4α genes showed differential gene expression during *ex vivo* expansion. Taken together, these observations suggest that HAFFTs are pluripotent stem cells that are less differentiated than BM-MSCs, and that their gene expression profiles vary with passage number during *ex vivo* expansion.

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INTRODUCTION

Stem cells have an extensive capacity to self-renew and can maintain their population numbers throughout the life of an organism. Under the influence of specific biological signals, they can differentiate into specialized cells that are phenotypically distinct from their precursors. Stem cells are generally classified into two groups: embryonic stem cells (ESCs) if isolated from the inner cell mass of a blastocyst, and adult stem cells if isolated from other tissues.

In the last decade, success in the isolation and culture of human ESCs has created new opportunities with respect to exploring the biological control of these cells and evaluating their potential for use in cell-based therapies for human disease (Thomson *et al*. 1998). However, the use of ESCs for research or therapeutic purposes has been constrained by complex social and ethical considerations. In addition, maintaining ESCs *in vitro* presents significant technical challenges. ESCs require either feeder cells or expensive cytokines to support their growth (Thomson *et al*. 1998), and they frequently undergo genomic alterations and/or chromosomal aberrations during maintenance *in vitro* (Hanson & Caisander 2005; Maitra *et al*. 2005). Most importantly, elimination of undifferentiated cells that could develop malignancy after transplantation of them into the human body, is not yet possible (Fujikawa *et al*. 2005).

The use of adult stem cells for research or therapy is much less controversial. These cells have the capacity to differentiate into several different cell types, although their differentiation potential is limited compared to that of ESCs. Thus, adult stem cells are regarded as an exciting source for new cell therapies. In particular, human bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent cells capable of differentiating into diverse lineages, including osteocytes, chondrocytes, adipocytes and cardiomyocytes (Prockop 1997). Morphologically, they are spindle-shaped and resemble fibroblasts. BM-MSCs were initially identified in adult bone marrow, but cells resembling BM-MSCs have also been found in many other tissues, including adult and foetal peripheral blood, foetal liver, foetal spleen, placenta, umbilical cord, umbilical cord blood, amniotic membrane and synovial fluid (Okita *et al*. 1983; Campagnoli *et al*. 2001; Hu *et al*. 2003; Romanov *et al*. 2003; Bilic *et al*. 2004).

Bone marrow-derived mesenchymal stem cells have been widely used in clinical applications. For example, they have been transplanted into children with osteogenesis imperfecta. Representative specimens of trabecular bone taken 3 months after osteoblast engraftment revealed formation of new dense bone, and all patients had increases in total body bone mineral content (Horwitz *et al*. 1999). Some metabolic diseases, such as Hurler syndrome and metachromatic leukodystrophy, have been corrected by infusion of allogenic and multipotent BM-MSCs (Koc *et al*. 2002). The use of adult BM-MSCs does have some disadvantages, however. In particular, the number of MSCs in adult bone marrow is low, and harvesting them from a patient is an invasive procedure. Therefore, finding alternative sources of MSCs that are useful in clinical applications is an important research goal.

Human amniotic fluid (HAF) obtained during the process of amniocentesis contains a variety of stem cells originating from embryonic and extra-embryonic tissues (Gosden 1983). Although these cells are routinely used for prenatal diagnosis of a wide range of foetal abnormalities caused by genetic defects, the cell type subsets present in HAF have not been thoroughly characterized. The types and properties of amniotic fluid cells vary with gestational age and if there is foetal pathology.

Based on their morphological and growth characteristics, amniotic fluid cells can be classified into three types: epithelioid, amniotic fluid-specific and fibroblastoid (Milunsky 1979). Fibroblastoid-type cells usually appear late during *in vitro* primary culture and exhibit phenotypes

and multilineage differentiation potentials similar to those of BM-MSCs (In't Anker *et al*. 2003). Interestingly, HAF has been shown to contain cells expressing Oct-4 antigen, a specific marker of pluripotent stem cells (Prusa *et al*. 2003), and these cells display multilineage differentiation potential; depending on the specific culture conditions, they can differentiate into adipocytes, osteocytes or neuronal cells (Tsai *et al*. 2004). Thus, HAF is intriguing as a possible source of pluripotent stem cells for cell-based therapeutics, and does not raise the ethical concerns associated with use of ESCs.

However, therapeutic use of HAF-derived cells will require a much more thorough understanding of their biology. Some important goals include determining the replicative lifespan and cell production potential of the component cell types in culture, characterizing the gene expression profile of these cells, and determining whether the profile changes during *ex vivo* expansion. In addition, the issue of whether HAF-derived cells can differentiate into chondrocytes, which are typical mesodermal lineage cells, has not been resolved.

In the present study, we have addressed these issues by further characterizing the *in vitro* growth kinetics, replicative lifespan and biological properties of HAF-derived BM-MSC-like cells throughout their existence. The techniques used here have included reverse transcriptase–polymerase chain reaction (RT-PCR), immunocytochemistry, telomerase activity assays and differentiation potential assays.

MATERIALS AND METHODS

Cell isolation and culture

Five millilitres of HAF samples were obtained from patients undergoing amniocentesis for routine prenatal diagnosis at 14–16 weeks of pregnancy. Cells were isolated from the HAF no more than 12 h prior to use in experiments. HAF samples were centrifuged at 300 ***g*** for 15 min, and the resulting pellets were washed twice with low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) to remove blood and cell debris. All of the cells isolated from the 5-ml sample were plated in a 25-cm^2 culture flask (Nunc, Rochester, MN) containing DMEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco), 3.7 mg/ml sodium bicarbonate, 10 ng/ml epidermal growth factor (EGF) (Peprotech, Princeton, NJ) and 10% foetal bovine serum (FBS) (Gibco). Seven days after the initiation of culture, the medium was replaced with fresh ones, and was subsequently replaced twice a week.

When the cells reached confluence, they were treated with 0.125% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA) for 3 min. The released cells were collected and re-plated for subculture. A morphologically homogeneous population of fibroblast-like cells was obtained after two rounds of subculture. These HAF-derived fibroblastoid-type cells were maintained in a humidified atmosphere in an incubator under 5% CO_2 at 37 °C.

Informed consent had been obtained from the subjects, and the study protocol was approved by the ethics committee of Ajou University, Korea.

Differentiation potential assay

For differentiation experiments, 8th-passage cells were cultured in various media with replacement of the medium twice a week. Cells were examined after 6 days in neurogenic medium, after 2 weeks in adipogenic or osteogenic differentiational medium, and after 3 weeks in chondrogenic medium. The adipogenic medium was DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, 0.05 mg/l human insulin, and 200 μM indomethacin.

After culture, presence of intracellular lipid droplets indicative of adipocyte differentiation was assessed by staining cells with oil red O. The osteogenic medium was DMEM supplemented with 10% FBS, 0.1 μM dexamethasone, 100 mM β-glycerol phosphate and 50 μM ascorbic acid-2-phosphate. Mineralized calcium indicating osteogenic differentiation was assessed by von Kossa staining.

Chondrogenesis was induced by culturing the cells in chondrogenic medium consisting of high-glucose DMEM supplemented with 0.1 μM dexamethasone, 50 μg/ml ascorbic acid-2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline, 10 ng/ml transforming growth factor-β1 (TGF-β1) (R&D Systems, Minneapolis, MN) and 50 mg/ml ITS premix (insulin, transferrin and selenious acid at 6.25 μg/ml each, 1.35 mg/ml BSA and 5.35 mg/ml linoleic acid; Becton Dickinson, San Jose, CA). Chondrogenic differentiation was assessed by staining with Alcian blue.

For neurogenic differentiation, cells were initially treated overnight with 20% FBS, 20 ng/ml basic fibroblast growth factor (bFGF) (Peprotech) and 20 ng/ml EGF. Neuronal differentiation was then induced by treatment with 2% dimethyl sulfoxide, 200 μM butylated hydroxyanisole, 25 mM KCl, 2 mM valproic acid and 1 μM hydrocortisone in N2 medium (Gibco) plus 1 × N2 supplement (Gibco) for 5 days. Neuronal differentiation was assessed by immunocytochemical staining with mouse monoclonal antihuman Neu N antibody (Chemicon, Temecula, CA).

Immunocytochemistry

HAFFTs at the 17th passage were plated on a Laboratory-Tek chamber slide (Nunc), fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (Gibco) at 4 °C for 2 h, and were rinsed with PBS. They were then permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After several washes with PBS, the cells were incubated in 3% hydrogen peroxidase for 15 min to quench endogenous peroxidase activity. They were then rinsed with PBS and incubated in blocking solution consisting of 2% BSA in PBS for 1 h at room temperature. They were then incubated with a mouse monoclonal or rabbit polyclonal primary antibody for 17 h at 4 °C. The monoclonal antibodies were specific for collagen types I (1 : 50), II (1 : 100), IV (1 : 100) and XII (1 : 500); fibronectin (1 : 100); CD31 (1 : 40); CD44 (1 : 50); CD54 (1 : 200); CD106 (1 : 100); von Willebrand factor (vWF; 1 : 100); CK18 (1 : 50); desmin (1 : 50); vimentin (1 : 200); α-smooth muscle actin (α-SMA); TRA-1–60 (1 : 50); stage-specific embryonic antigen 3 (SSEA)-3 and -4 (both 1 : 100); fibroblast surface protein (FSP; 1 : 500); HLA ABC (1 : 25); and HLA DR (1 : 50).

After incubation with primary antibody, slides were rinsed three times with PBS and then incubated in biotinylated goat antimouse or antirabbit IgG (Dinona, Seoul, Korea) for 20 min at room temperature. The slides were rinsed with PBS and then incubated with horseradish peroxidase-conjugated streptavidin (Dinona) for 20 min at room temperature. Immunoreactivity for each protein was visualized using 3,3′-diaminobenzidine tetrahydrochloride (Dinona) and counterstaining was performed with Mayer's haematoxylin. Finally, the slides were photographed under a Zeiss LSM410 microscope using bright-field illumination (Carl Zeiss, Oberkochen, Germany).

Telomerase activity assay

Telomerase activity was measured using a telomeric repeat amplification protocol (TRAP) assay kit (Chemicon) with PBS-washed HAFFT cell pellets that were stored at −70 °C until use. A total of 1×10^6 HAFFTs were resuspended with 200 μl of CHAPS lysis buffer and were incubated on ice for 30 min. The suspension was centrifuged at 12 000 ***g*** for 20 min at 4 °C, and 160 μl of the supernatant fraction was removed for the telomerase activity assay. Ten-microlitre

portions of the extract were heat-treated at 85 °C for 10 min to inactivate telomerase, and the heat-treated extracts were used in control reactions.

Polymerase chain reaction amplification was performed in a 50-μl reaction volume containing 5 μl of 10 × TRAP reaction buffer, 1 μl of 50 × dNTPs, 1 μl of TS primer, 1 μl of TRAP primer mix, 0.4 μl (2 U) *Taq* polymerase, 39.6 μl dH_2O and 2 μl of HAFFT cell extract. Reactions were incubated at 30 °C for 30 min and then placed in a thermocycler (Perkin Elmer, Boston, MA). Amplification was performed with 30 cycles of a two-step PCR protocol consisting of 94 °C for 30 s and 59 °C for 30 s. When PCR reactions were complete, the products were mixed with 5 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 50 mM EDTA) and separated on a 10% non-denaturing polyacrylamide gel in 0.5 × TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8.0) at 40 V. After electrophoresis, the gel was stained with SYBR® Gold Nucleic Acid Stain (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. DNA bands in the gel were then visualized under ultraviolet light using a Bioprofile image analysis system (Viber Lourmat, Mame la Vallee, France).

Total RNA isolation and RT-PCR

All solutions were prepared using distilled water treated with 0.1% diethylpyrocarbonate. HAFFT cell pellets were washed with Ca^{2+}- and Mg^{2+}-free PBS and then transferred to a chilled Eppendorf microcentrifuge tube on ice. Immediately thereafter, 500 μl of Tri-reagent (Sigma Chemical Co., St. Louis, MO) were added to the tube, which was then stored at −20 °C until use. Total RNA was isolated from the HAFFT cell pellets according to the manufacturer's instructions. The RNA was allowed to stand at 65 °C for 5 min in a heating block, chilled on ice and then quantified spectrophotometrically. Purity of the RNA was assessed by determining the ratio of absorbance at 260 nm to that at 280 nm (> 1.8).

For each sample, 5 μg of total RNA was reverse-transcribed in a 20-μl reaction containing 1 × reaction buffer, 1 mM dNTP mixture, 0.5 μg/μl oligo(d)T15, 20 U RNase inhibitor (Takara, Japan) and 20 U M-MuLV reverse transcriptase (Fermentas, Canada). Reactions were allowed to proceed for 60 min at 42 °C, and the RT products (cDNAs) were used directly in PCR reactions.

Polymerase chain reaction amplification of HAFFT cell cDNA was performed in a GeneAmp PCR system 2400 (Perkin Elmer). The 10-μl reaction mixtures contained 2 mM $MgCl_2$, 1 × *Taq* buffer, 0.25 U *Taq* polymerase (Takara) and 10 pM sense and antisense gene-specific primers (Table 1). Amplification was performed for 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s. Annealing temperatures were dependent on the primers used and are shown in Table 1. Upon completion of the reactions, the PCR products were mixed with 6 × loading buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose and then separated on a 2% agarose gel. The gel was stained with ethidium bromide, and DNA in the gel was imaged under ultraviolet light using a Bioprofile image analysis system (Viber Lourmat).

Antibodies and reagents

Mouse monoclonal antibodies specific for the following human proteins were used: CD31, CK18 and desmin (Dako, Carpinteria, CA), TRA-1–60 and SSEA-4 (Chemicon), FSP (Abcam, Cambridge, UK), CD44, CD106, CD54, collagen type II, collagen type IV, vimentin, fibronectin, vWF, α-SMA, HLA ABC and HLA DR (Novo Castra; Newcastle, UK), collagen type I (Acris Antibodies, Hiddenhausen, Germany), collagen type XII (Kamiya, Seattle, WA) and SSEA-3 (R&D Systems). A rabbit polyclonal antibody specific for collagen type III was purchased from Abcam. All other reagents that are not specified elsewhere were obtained from Sigma Chemical Co.

Table 1. RT-PCR primer sequences and products size

Gene	Primer sequence (5′→3′)	Accession number	Size (bp)	Annealing temp. (°C)
GAPDH	aca act ttg gta tcg tgg aa aaa ttc gtt gtc ata cca gg	NM_002046	456	53
Oct-4	cgt gaa gct gga gaa gga gaa gct g caa ggg ccg cag ctc aca cat gtt c	AF268617	245	55
Rex-1	atg gct atg tgt gct atg agc cct caa ctt cta gtg cat cc	NM_174900	449	57
SCF	cca ttg atg cct tca agg ac ctt cca gta taa ggc tcc aa	M59964	275	55
GATA-4	ttc ctc ttc cct cct caa at tca gcg tgt aaa ggc atc tg	NM_002052	194	60
Vimentin	cct tcg tga ata cca cg acct gc taa tat atc gcc tgc cac tga g	Z19554	321	56
CK18	gag atc gag gct ctc aag ga caa gct ggc ctt cag att tc	NM_00024	357	57
HLA ABC	gta ttt ctt cac atc cgt gtc ccg gtc cgc cgc ggt cca aga gcg cag	L18898	394	70
FGF-5	gct gtg tct cag ggg att gta gga ata tat cca aag cga aac ttg agt ctg ta	NM_004464	434	55
Brachyury	gag ctc acc aat gag atg at ggc tca tac tta tgc aag ga	NM_002052	335	57
Pax-6	aga ttc aga tga ggc tca aa aat tgg ttg gta gac act gg	AY707088	313	57
NCAM	gag ggg gaa gat gcc gtg atg tg ata ttc tgc ctg gcc cgg atg gta g	NM_000615	269	60
BMP-2	ttg cgg ctg ctc agc atg tt ttg cga gaa cag atg caa gat g	BC069214	315	55
HLA DR	ctg atg agc gct cag gaa tca tgg gac tta ctt cag ttt gtg gtg agg gaa g	X06079	220	60
BMP-4	agc cat gct agt ttg ata cc tca ggg atg ctg ctg agg tt	D30751	383	55
AFP	gtg ctg cac ttc ttc ata tgc tga cag cct caa gtt gtt cc	NM_001134	218	54
Nestin	cca gaa act caa gca cca c ttt tcc act cca gcc atc c	X65964	398	54
HNF-4α	gag cag gaa tgg gaa gaa tg ggc tgt cct ttg gga tga ag	NM_178849	205	62

RESULTS

Ex vivo expansion of HAFFTs

When the cells isolated from HAF were observed during primary culture, they were somewhat heterogeneous but consisted primarily of two types of cells: one was similar to fibroblasts and the other was flat and circular, resembling epithelial cells. Fibroblast-like cells sometimes appeared as colonies in the culture flask during the primary culture (Fig. 1a,b). After 16 days of culture, the cells were confluent and heterogeneous (Fig. 1c,d).

At 80% confluence, cells were treated with EDTA for 2 or 5 min, but no cells were isolated. Cells were then treated with both trypsin and EDTA for 5 min, which resulted in the detachment

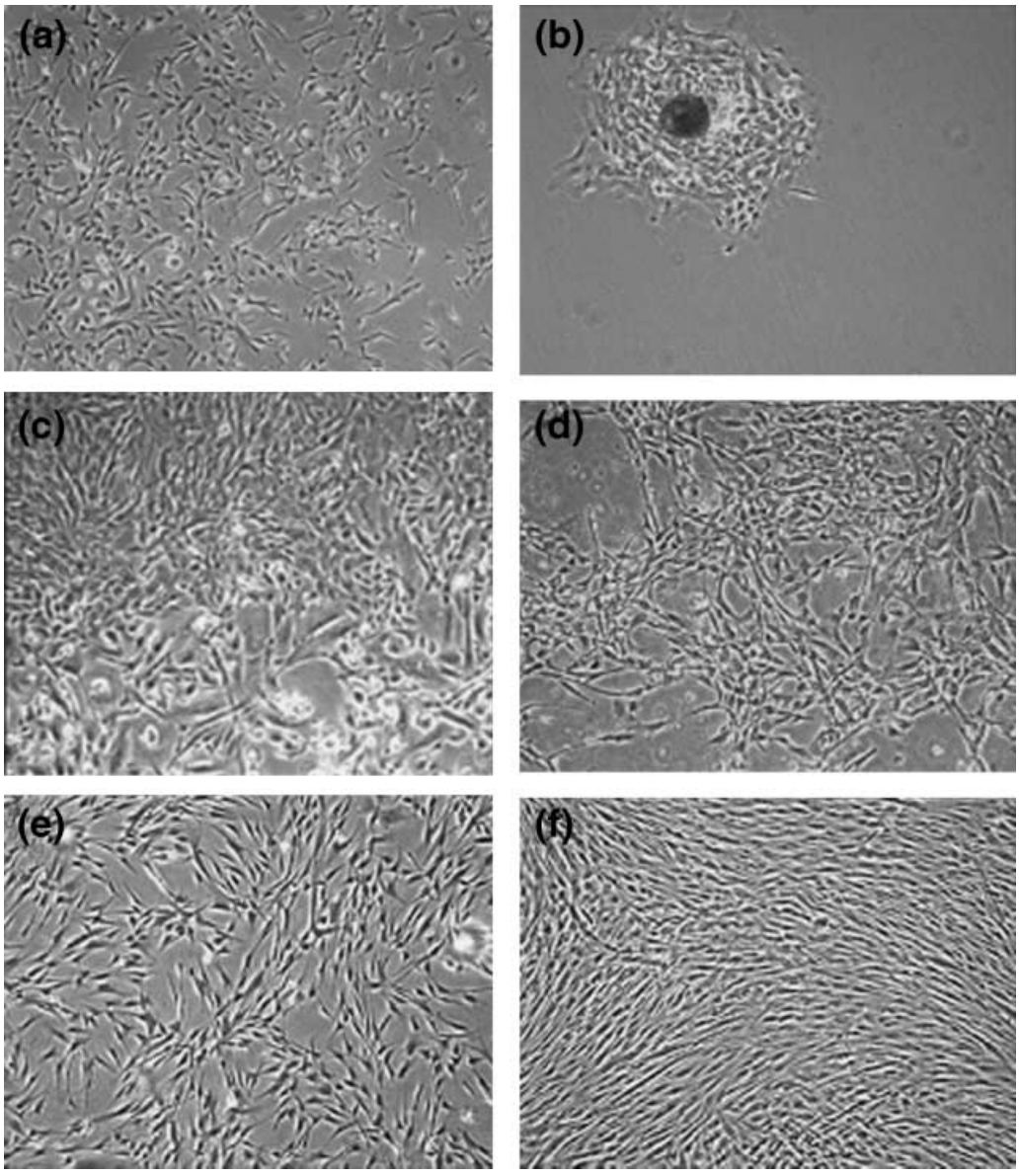

Figure 1. Morphological appearance of HAFFTs during culture. (a) Seven days after initiation of the primary culture, many isolated cells were visible. (b) A cell colony in the same culture flask as in (a). (c) At 16 days, a heterogeneous population of cells was present in the same culture flask as in (a). (d–f) The same culture flask as in (a) is shown at 27 days (d), 51 days (at third passage; e), and 123 days (at 10th passage; f) after initiation of the primary culture, respectively. Magnification: × 40.

of a group of cells from the bottom of the flasks. These separated cells were designated as HAFFTs and were used in all subsequent experiments. Other cells that remained attached after this treatment were not used as they were phenotypically heterogeneous and grew slowly, not stem cell characteristics.

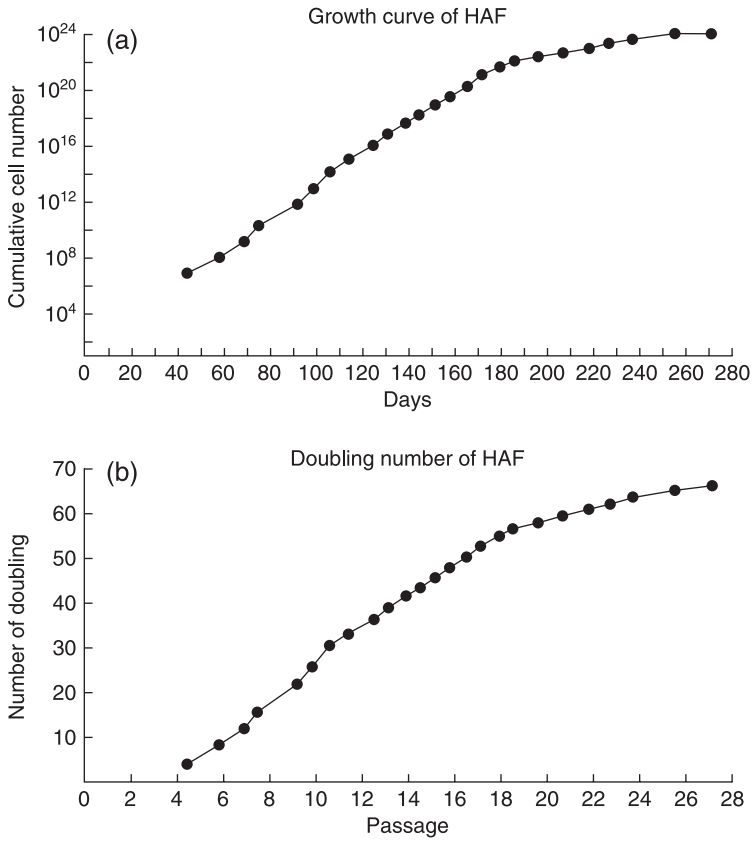

Figure 2. Growth curve and doubling number of HAFFTs. (a) HAFFT growth curve observed over 271 days of culture. (b) HAFFT population doubling number during the same culture period.

At the 3rd passage, the HAFFT cells became morphologically homogeneous, and they consistently exhibited fibroblast-like morphology until the end of the culture period (Fig. 1e,f). At the 27th passage, the HAFFTs ceased proliferating and became large and flat, as is typical for senescent cells. Expansion factor and doubling number of the HAFFTs were calculated according to the period of culture (Fig. 2a,b). Average doubling time from passages 3–26 was 3.6 days. Over the course of the 8-month experiment, the HAFFTs were successively passaged 27 times, undergoing around 66 cell doublings. The cumulative cell number was thus estimated as 7.7×10^{23}.

Differentiation potential of HAFFTs

We examined the ability of HAFFTs to differentiate into mesodermal lineage cells (adipocytes, osteocytes and chondrocytes) and into ectodermal lineage cells (neuronal cells). When HAFFTs at the 8th passage (Fig. 3a) were cultured under adipogenic conditions for 2 weeks, they exhibited intense cytoplasmic staining with oil red O, signifying the accumulation of lipid vacuoles (Fig. 3b). When cultured in osteogenic medium for 2 weeks, the HAFFTs differentiated into osteoblasts, as shown by positive von Kossa staining (Fig. 3c) and when the cells were cultured under chondrogenic conditions for 3 weeks, they exhibited positive Alcian blue staining (Fig. 3d). When cultured for 5 days in neurogenic induction medium, the cells expressed distinct immunoreactivity for the neuronal marker NeuN (Fig. 3c,d).

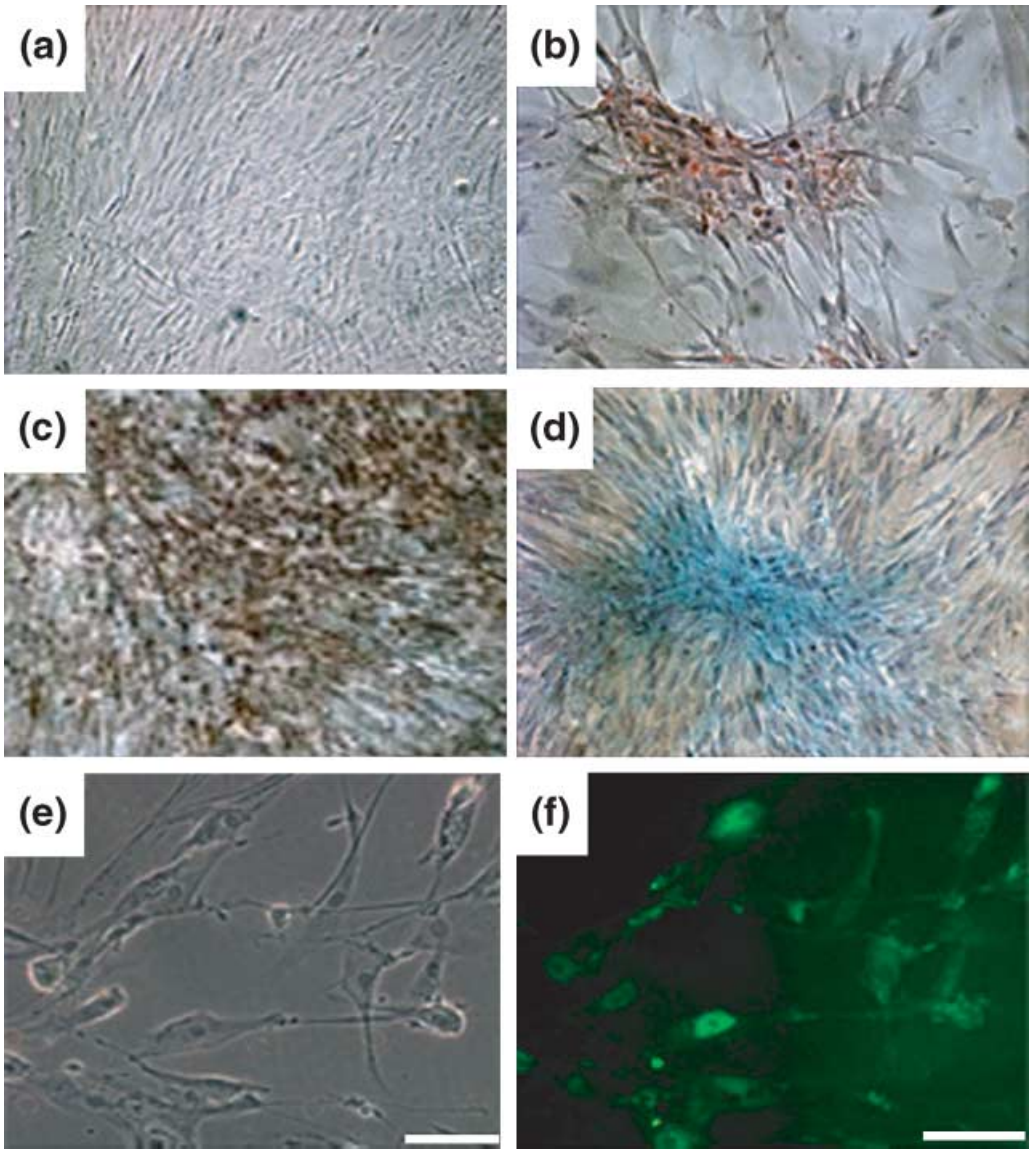

Figure 3. Mesodermal and neuronal differentiation of HAFFTs. HAFFTs at eighth passage were cultured in control (a), adipogenic (b), osteogenic (c) or chondrogenic (d) medium for 2–3 weeks. The differentiation capabilities of each group of HAFFTs were determined using tissue-specific staining and haematoxylin counterstaining. Magnification: × 100. (e) HAFFTs after culture in neurogenic medium. (f) Fluorescence image of cells shown in (e) after immunostaining with antibody against the neurone marker NeuN. Note the distinct staining of HAFFTs. Bar = 100 μm.

Immunocytochemical demonstration of HAFFTs expression profile

Immunocytochemical staining demonstrated that HAFFT cells at the 17th passage expressed collagen types I, II, III, IV and XII; fibronectin; CD44 (homing cell adhesion molecule, HCAM), CD54 (intercellular cell adhesion molecule-1, ICAM-1), CD31 (platelet/endothelial adhesion molecule-1, PECAM-1), CD106 (vascular cell adhesion molecule-1, VCAM-1),

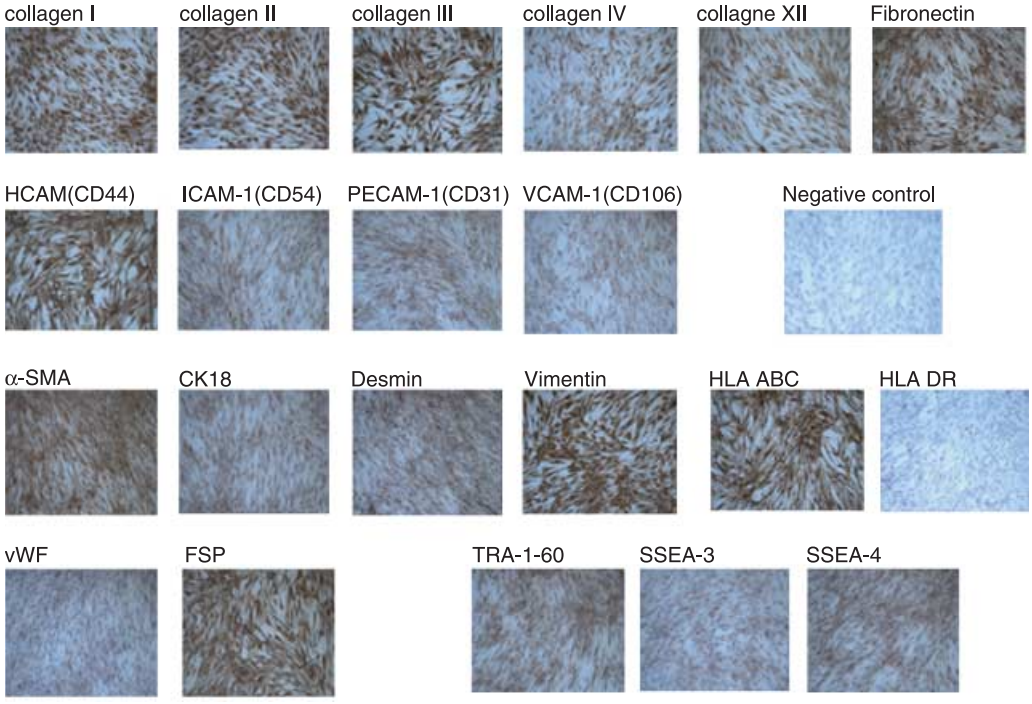

Figure 4. Immunocytochemical analysis of HAFFTs. HAFFTs at 17th passage exhibited distinct immunoreactivity with antibodies against collagen types I, II, III, IV and XII; fibronectin, HCAM (CD44); ICAM-1 (CD54); VCAM-1 (CD106); CK18; α-SMA; vimentin; FSP; desmin; TRA-1–60; SSEA-3 and -4; vWF; PECAM-1 (CD31); and HLA ABC. Note the absence of staining for the anti-HLA DR antibody and the negative control. Nuclei were counterstained with Mayers haematoxylin. Magnification: × 40.

α-SMA (alpha-smooth muscle actin), CK18, desmin, vimentin, vWF, TRA-1–60, SSEA-3 and -4, FSP and HLA ABC proteins. Staining was particularly strong with the anti-CD44, -vimentin, -FSP and -HLA ABC antibodies. Staining for collagen types I, II and III, fibronectin, α-SMA, TRA-1–60 and SSEA-4 was moderate, and no staining was observed with anti-HLA DR antibody. The immunocytochemical staining results are shown in Fig. 4.

Telomerase activity of HAFFTs

Telomerase is a ribonucleoprotein that synthesizes telomeric repeats and directs them onto the 3′ end of existing telomeres. Expression of telomerase activity is limited to immortal cells, such as malignant cells and germ cells. When the telomerase activity of HAFFTs was examined at passages 5 and 21, cells of both passages yielded distinct TRAP-ladder bands (Fig. 5, lanes 1 and 3), and disappearance of the uppermost band was observed only for HAFFTS at the 21st passage. When HAFFT cell extracts were heated before the TRAP assay reaction, the telomerase activity disappeared, confirming that the ladder bands indeed appeared as a result of the activity of heat-sensitive telomerase (Fig. 5, lanes 2 and 4).

Gene expression profiles of HAFFTs

Results of RT-PCR analysis showed that HAFFT cells at passages 6, 9, 16, 19 and 20 consistently expressed the *Rex-1*, *SCF*, *GATA-4*, *vimentin*, *CK18*, *HLA ABC* and *FGF-5* genes. In contrast,

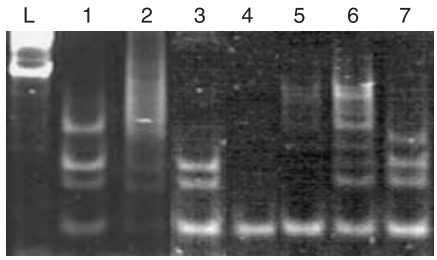

Figure 5. Telomerase activity in HAFFT extracts at the 5th and 21st passages. L, DNA ladder; lane 1, HAFFT extract at 5th passage; lane 2, heat-inactivated HAFFT extract (control for lane 1); lane 3, HAFFT extract at 21st passage; lane 4, heat-inactivated HAFFT extract (control for lane 3); lane 5, negative control; lane 6, positive control; lane 7, TSR8 (control template). Note the disappearance of the uppermost band in lane 3 compared to lane 1.

Table 2. RT-PCR analysis of gene expression by HAFFTs

	Passage number				
Gene	6	9	16	19	20
Oct-4	+	+	+	+	–
Rex-1	+	+	+	+	+
SCF	+	+	+	+	+
GATA-4	+	+	+	+	+
Vimentin	+	+	+	+	+
CK18	+	+	+	+	+
HLA ABC	+	+	+	+	+
FGF-5	+	+	+	+	+
Brachyury	–	–	–	–	–
Pax-6	–	–	–	–	–
NCAM	–	–	–	–	–
BMP-2	–	–	–	–	–
HLA DR	–	+	–	–	–

+, expressed; –, not expressed.

expression of genes coding for Brachyury, Pax-6, NCAM, BMP-2 and HLA DR was not observed throughout the culture period (Table 2). The *Oct-4* gene, coding for the transcription factor unique to pluripotent stem cells, was expressed until the 19th passage and was not expressed at the 20th passage. Interestingly, the expression patterns of some of the genes varied with passage number (Fig. 6). *BMP-4*, *AFP* and *nestin* genes were not expressed at the 6th passage but were expressed at the 16th and 20th passages, whereas the *HNF-4*α gene was not expressed at the 16th passage but was expressed at the 20th.

DISCUSSION

Previous studies have shown that second trimester HAF contains cells expressing the gene for Oct-4, an embryonic stem cell marker (Tsai *et al*. 2004), and that HAF is a source of foetal

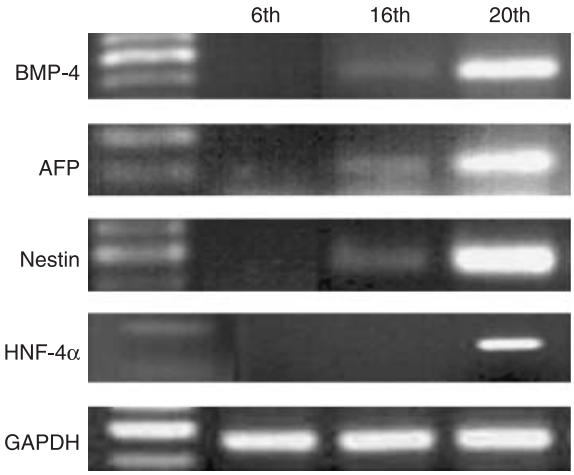

Figure 6. Expression profile of selected HAFFT genes. HAFFTs were collected at 6th, 16th and 20th passages, and their expression levels of BMP-4, AFP, nestin and HNF-4α mRNAs were compared to the level of GAPDH as a control using RT-PCR. Right column represents a part of DNA ladder.

MSCs that are phenotypically similar to BM-MSCs (In't Anker *et al*. 2003). Oct-4-expressing MSCs of HAF were subsequently shown to differentiate into adipocytes, osteocytes and neuronal cells (Prusa *et al*. 2004; Tsai *et al*. 2006). In the present study, we have demonstrated that HAFFTs possess immunophenotypes and gene expression profiles that are largely characteristic of undifferentiated cells. Hence, HAFFTs may have therapeutic potential that is even greater than that of BM-MSCs. In addition, we have shown that HAFFT cells exhibit gene expression profiles that change with passage number during *ex vivo* expansion.

HAFFTs share many antigenic properties and gene expression profiles with BM-MSCs. Of the immunophenotypes expressed by HAFFTs, HCAM-1, ICAM-1, VCAM-1, vWF and α-SMA (Conget & Minguell 1999); HLA-ABC (Majumdar *et al*. 2003); collagen types I, III and IV and fibronectin (Chichester *et al*. 1993); and vimentin (Kadner *et al*. 2002) are also expressed by BM-MSCs. The RT-PCR analyses performed in the present study showed that the genes for stem cell factor (SCF) (Majumdar *et al*. 2000), CK18 (Lee *et al*. 2004), nestin (Vogel *et al*. 2003) and HLA-ABC are expressed in HAFFTs, as they are in BM-MSCs. Our immunocytochemical and RT-PCR analyses showed no expression of HLA-DR in HAFFTs in common with BM-MSCs (Majumdar *et al*. 2003). Indeed, we have shown that HAFFTs, like BM-MSCs, exhibited the potential to differentiate into a variety of cell types, including neuronal cells and three typical mesodermal-lineage cells (adipocytes, chondrocytes and osteocytes).

Despite these similarities, many genes that are expressed in HAFFTs have not been shown to be expressed in BM-MSCs. Our immunophenotype analysis showed that HAFFTs express antigens of desmin, collagen type II, TRA-1–60, SSEA-3 and SSEA-4 that are not expressed in BM-MSCs (Ogueta *et al*. 2002; Xu *et al*. 2004). Furthermore, our RT-PCR analyses showed that HAFFTs also express genes for Rex-1, GATA-4 and FGF-5 throughout the culture period, and they express genes for BMP-4, nestin, AFP and HNF-4α at later passages; expression of these genes has not been reported in BM-MSCs (Lee *et al*. 2004).

The TRA-1–60, SSEA-3 and SSEA-4 antigens are typical markers of embryonal carcinoma cells and ESCs (Andrews *et al*. 1987; Xu *et al*. 2001). Oct-4 and Rex-1 expression is also a marker of these cells (Peter & Donovan 2001). Our finding that these markers of undifferentiated

cells are also expressed in HAFFTs leads us to conclude that HAFFTs may be less differentiated than most BM-MSCs, and may more closely resemble pluripotent ESCs. This hypothesis is supported by the superior replicative lifespan of HAFFTs. In our experiment, they exhibited an average doubling time of 3.6 days from passages 3–26. Over the 8 months before the onset of senescence, the HAFFTs doubled their numbers 66 times, with a cumulative yield of approximately 7.7×10^{23} cells. Typical BM-MSCs usually achieve 40–50 doublings during their lifespans (Stenderup *et al*. 2003), and reach senescence after 197.4 days and 10 passages, when they are obtained from subjects 0–18 years old (Baxter *et al*. 2004). HAFFTs could therefore generate more cells during *ex vivo* expansion than could BM-MSCs, which is an advantage in cell-based therapeutics.

D'Ippolito *et al*. (2004) have reported that BM-MSCs contain a unique population of cells that, like HAFFTs, express Oct-4, Rex-1 and telomerase. The population doubling time for these BM-MSCs was 36–72 h, and they have been expanded in culture for more than 50 doublings. They have been differentiated into bone-forming osteoblasts, cartilage-forming chondrocytes, fat-forming adipocytes and neural cells. An intriguing possibility is that these BM-MSCs, termed human marrow-isolated adult multilineage-inducible cells, are the same type of cells as HAFFTs.

In adult organisms, AFP is normally produced by the foetal gut, yolk sac and liver, and is known as a hepatocyte marker (Engelhardt *et al*. 1984). HNF-4α is well known for its role in liver development (Parviz *et al*. 2003), and its expression has been observed in other tissues as well, including the kidney, intestine and endocrine cells of the pancreas (Miquerol *et al*. 1994). *CK18* is one of the endodermal lineage-specific genes and a marker of hepatic differentiation (Wells *et al*. 1997). Therefore, together with the consistent expression of *CK18*, the onset of expression of the *AFP* and *HNF-4α* genes at later passages suggests that as HAFFTs age *in vitro*, they seem to differentiate easily into hepatocyte-like cells. BM-MSCs also exhibit spontaneous changes in their gene expression pattern during *ex vivo* expansion, but their progressive ageing leads to a commitment to osteogenic differentiation (Banfi *et al*. 2002).

HAFFTs expressed the *Oct-4* gene throughout the culture period until the 19th passage, ceasing to express it at the 20th passage. Because HAFFTs reached senescence at the 27th passage, the disappearance of *Oct-4* expression at the 20th passage may be related to the onset of ageing at this time, resulting in senescence by the 27th passage. Furthermore, that telomere shortening plays an important part in the molecular ageing process has been well established (Wright & Shay 2002). When we compared telomerase activity of HAFFTs at the 5th and 21st passages, we found that its activity significantly decreased by the 21st passage. These results coincide with a previous report that HAF-derived cells exhibit a decrease in both telomerase activity and telomere length during ageing *in vitro* (Mosquera *et al*. 1999). Similarly, Oct-4 and telomerase expression in ESCs decreases during differentiation *in vitro* (Lebkowski *et al*. 2001). In contrast, most BM-MSCs do not exhibit telomerase activity at all, and their telomere length decreases during *in vitro* expansion (Banfi *et al*. 2002).

Of the proteins expressed by HAFFTs, desmin is well known to play an important role in cardiac and skeletal muscle function (Goldfarb *et al*. 1998), and GATA-4 is a cardiac-specific member of the GATA family of zinc-finger transcription factors (Grepin *et al*. 1997). FGF-5 regulates neurone differentiation and survival (Lindholm *et al*. 1994), and is also involved in cardiac function (Suzuki *et al*. 2005). Nestin, a specific marker for neural stem cells (Lendahl *et al*. 1990), is also found in other differentiating cells, including muscle and myocardium (Sjoberg *et al*. 1994). Knockout of BMP-4 by homologous recombination results in embryonic lethality, and BMP-4 appears to play a role in chondrogenesis and articular cartilage repair as well as in bone formation (Kuroda *et al*. 2006), and collagen type II is a marker of cartilaginous tissue (Aigner *et al*. 1993). Thus, the spontaneous expression of all of these genes and their

proteins suggests that HAFFTs have the potential to readily differentiate into cardiomyocytes as well as other mesodermal derivatives, in addition to neural cells. In BM-MSCs, only prolonged treatment with hepatocyte growth factor induces the expression of the cardiac-specific markers GATA-4, MEF2C, TEF1, desmin, α-MHC, β-MHC and nestin (Forte *et al*. 2006).

Platelet/endothelial adhesion molecule-1 is a member of the immunoglobulin superfamily that is expressed by leucocytes, platelets and endothelial cells, and it primarily participates in homophilic binding between adjacent cells (Newton *et al*. 1997). VCAM-1 regulates leucocyte migration from the blood circulation into tissues; its expression is induced on endothelial cells during inflammatory bowel disease, atherosclerosis, allograft rejection, infection and asthmatic responses (Matheny *et al*. 2000). The endothelial cell marker vWF is a multimeric adhesive protein that has an important function in primary haemostasis and, as a carrier of factor VIII, it plays a pivotal role in thrombogenesis (Nieswandt & Watson 2003). The presence of these antigens as well as ICAM-1 leads to the intriguing speculation that HAFFTs share their origin with haematopoietic stem cells. However, the discrepancies between the antigenic profiles of HAFFTs and the previously described HAF-derived cells (In't Anker *et al*. 2003; Fauza 2004) might be explained by different origins for the two cell types. Because amniotic fluid contains a heterogeneous population of cells originating from embryonic and/or extra-embryonic tissues (Gosden 1983), this explanation seems plausible.

The results of the experiments described in this report demonstrate that HAFFTs can differentiate into adipocytes, osteocytes, chondrocytes and neuronal cells, can express many pluripotent stem cell-specific genes, exhibit telomerase activity and proliferate well during *ex vivo* expansion. In particular, we have found that HAFFT gene expression profiles change with the cells ages *in vitro*. Based on these unique properties, we conclude that HAFFTs are pluripotent stem cells that are probably less differentiated than BM-MSCs, and that thus, they have considerable potential for use in cell-based therapeutics.

ACKNOWLEDGEMENTS

This work was supported by the grant from the 2005 Research Program of the HurimBioCell Inc Seoul, Korea.

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