

# Hair follicle-derived mesenchymal cells support undifferentiated growth of embryonic stem cells

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**Abstract.** The aim of the present study was to investigate whether feeder layers composed of human hair follicle-derived mesenchymal stem cells (hHFDCs) are able to support human embryonic stem cells (hESCs). hHFDCs and mouse embryonic fibroblasts (MEFs) were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 and low-glucose DMEM, respectively. hHFDCs were passaged three times and subsequently characterized. hHFDCs and MEFs were mitotically inactivated with mitomycin C for 3 h prior to co-culture with H9-hESCs. hESCs were initially established on a mouse feeder layer, subsequently transferred onto a human feeder layer and split every 5 days. Cell morphology, expression of specific 'undifferentiation' markers and growth factors, and the differentiation capacity of hESCs grown on the hHFDC feeder layer were analyzed. hHFDCs are adherent to plastic, possess the classic mesenchymal stem cell phenotype [they express cluster of differentiation (CD)90, CD73 and CD105] and are able to differentiate into adipocytes, chondroblasts and osteocytes, indicating that these cells are multipotent. Population-doubling time analysis revealed that hHFDCs rapidly proliferate over 34.5 h. As a feeder layer, hHFDC behaved similarly to MEF in maintaining the morphology of hESCs. The results of alkaline phosphatase activity, reverse transcription-quantitative polymerase chain reaction analysis of the expression of pluripotency transcription factors [octamer-binding transcription factor 4 (Oct4), Nanog and sex determining region Y-box 2], and immunofluorescence assays

of markers (stage-specific embryonic antigen-4 and Oct4) in hESCs co-cultured over hHFDC, indicated that the undifferentiated state of hESCs was preserved. No change in the level of growth factor transcripts (bone morphogenetic protein 4, fibroblast growth factor-2, vascular endothelial growth factor, Pigment epithelium-derived factor and transforming growth factor- $\beta$ 1) was detected for either feeder layer prior to or following inactivation. Similar phenotypes of embryoid body formation, size and morphology were observed in the hHFDC and MEF feeders. In conclusion, hHFDC maintained hESCs in an undifferentiated state comparable to MEF in standard conditions, which may be an important finding regarding the establishment of stem cell-based translational applications.

## Introduction

Human embryonic stem cells (hESC) were originally derived and maintained in culture systems containing inactivated mouse embryonic fibroblast (MEF) feeder layers to permit them to grow continuously in an undifferentiated state (1,2). Induced pluripotent stem cells (iPSCs) generated from somatic cells were also cultured on MEF (3). The possibility of differentiating these pluripotent cells into cells with a defined phenotype is of great importance in scientific research. However, effective alternatives to xenogenic-based culture systems must be developed if therapies using cell types derived from pluripotent cells are to become a clinical reality (4,5). In this clinical scenario, feeder-free culture systems have been developed to avoid the use of MEF (4-7). However, despite the availability of these alternative techniques, feeder layers are still considered to be the best culture method owing to their ability to preserve certain critical properties such as cell growth and viability, stemness phenotype and clonogenic potential (8). Several previous studies have reported that human-derived cells and decellularized cellular matrices are feasible as feeder layers to support hESCs in culture (9-13); however, there is no consensus on the best cell type to use. Therefore, human hair follicle-derived mesenchymal stem cells (hHFDCs) have emerged as a promising source of feeder-layer cells for hESCs as they can be obtained from both males and females, they have the capability of long-term proliferation, there is a plentiful supply of starting material and the possibility of

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autologous matching, and they are compatible with clinical applications (14).

In the present study, hHFDCs were isolated, cultured and characterized in order to evaluate whether they can be used as a substitute for MEFs as a feeder layer for hESCs.

## Materials and methods

**Isolation and culture of mouse embryonic fibroblasts.** The present study was approved by the Ethics Committee of the Health Sciences Center of the Federal University of Rio de Janeiro (Protocol 026; Rio de Janeiro, RJ, Brazil) and was conducted in compliance with National Institutes of Health policies (15).

A total of 5 8-week-old pregnant female C57/BL6 mice (20–30 g) were obtained from the Institute of Biophysics Carlos Chagas Filho (Rio de Janeiro, Brazil). The animal protocol was designed to minimize pain or discomfort to the animals. Animals were housed at a controlled temperature (23°C) and humidity (55%) under a 12:12 h light-dark cycle and received standard mouse chow and water *ad libitum*.

MEFs were derived from C57Bl/6 mouse strain embryos. Pregnant females were euthanized by barbiturate overdose (150 mg/kg thiopental; Thiopentax; Cristália Produtos Químicos Farmacêuticos, Itapira, São Paulo, SP, Brazil) at day 13.5 of gestation. The embryos were placed in 100-mm plastic culture dishes containing sterile ice-cold PBS (LGC Biotecnologia, Cotia, São Paulo, SP, Brazil). Using a magnifying glass and tweezers, the viscera were removed and the remaining cells were transferred to culture dishes containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 0.25% trypsin-EDTA solution (Gibco; Thermo Fisher Scientific, Inc.) for enzymatic dissociation. All material was poured into 15 ml conic tubes, and 3 ml DMEM-F12 supplemented with 200 ml/l fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 50 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) were added to inactivate trypsin. The solution was subsequently centrifuged at 300 x g for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in 10 ml of the same medium. Finally, cells were seeded at a density of approximately 10<sup>5</sup> cells/ml in 75 cm<sup>2</sup> culture flasks (Corning Incorporated, Corning, NY, USA) and placed in an incubator for 3–4 days at 37°C with an atmosphere containing 5% CO<sub>2</sub> and 95% humidity.

**Isolation and culture of human hair follicle derived cells.** Human skin tissue from the temporal region of the scalp was obtained during rejuvenation facial plastic surgery on 3 patients between 40 and 60 years old from the Luiz Pimentel Plastic Surgery Clinic in Niterói, Rio de Janeiro in 2008, with approval of the Institutional Ethics and Research Committee. All patients gave informed consent prior to the study. Skin tissue was washed extensively in cold PBS, and fat was removed using scissors, forceps and a magnifying glass in a 35 mm culture dish (Corning Incorporated) containing cold PBS. The tissue was sectioned into 2 mm slices, transferred to an Erlenmeyer flask and subjected to enzymatic dissociation

with collagenase type II (360 U/mg; Worthington Biochemical Corporation, Lakewood, NJ, USA) diluted in DMEM, with gentle agitation overnight at 37°C. The digested material was poured into 50 ml conical tubes and centrifuged at 380 x g for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in low-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 150 ml/l FBS, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were seeded in 25 cm<sup>2</sup> culture flasks (Corning Incorporated) coated with 10 ml/l gelatin (Sigma-Aldrich; Merck kGaA, Darmstadt, Germany) and cultured for 5–7 days in an incubator at 37°C, in an atmosphere containing 5% CO<sub>2</sub> and 95% humidity. The medium was replenished every 2 days.

**Feeder layer preparation.** hHFDC (3–10 passages) and MEF (3 passages) were used as feeder cells. These cells were mitotically inactivated using 10 µg/ml mitomycin C (Sigma-Aldrich; Merck kGaA) for 3 h and washed three times with PBS. Inactivated hHFDC and MEF were then seeded on 100 ml/l gelatin (Sigma-Aldrich; Merck kGaA) coated 6-well plates (Corning Incorporated) at 8x10<sup>4</sup> cells/well (35 mm). Feeder cells were grown to confluence in their respective growth media (as described above) and then the medium was changed to hESC medium.

**hESC culture on MEF and hHFD feeder layers.** The hESC line H9 (2) was donated to the Federal University of Rio de Janeiro by Dr James Thomson from the University of Wisconsin (Madison, WI, USA). At passages 56–65, all the colonies of H9-hESC, which had until then been cultured on MEF, were subsequently sub-cultured on inactivated feeder cells (hHFDC and MEF) at 37°C for 4–5 days. Morphologically undifferentiated, colony-forming cells were selected for each passage and dissociated mechanically into small clumps with a needle and micropipette tip, visualized using light microscopy. The culture medium for H9 cells consisted of DMEM/F12 supplemented with 200 ml/l knockout serum (KSR; Gibco; Thermo Fisher Scientific, Inc.), 10 ml/l non-essential amino acids (Gibco; Thermo Fisher Scientific, Inc.), 0.1 mmol/l β-mercaptoethanol (Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l L-glutamine, 55 µg/ml garamycin (Schering-Plough Corporation, Kenilworth, NJ, USA) and 10 ng/ml basic fibroblast growth factor (bFGF; Gibco; Thermo Fisher Scientific, Inc.). The culture medium was replenished daily. Cells were then frozen in liquid nitrogen using a freezing solution consisting of 100 ml/l dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck Millipore) and 900 ml/l FBS.

**Flow cytometric analysis.** hHFDCs at passage 3 were dissociated with 0.25% trypsin-EDTA and counted for immunophenotypic analysis. Samples were blocked with PBS supplemented with 50 ml/l FBS for 20 min at 4°C. Then, 0.5–1x10<sup>6</sup> cells were stained using monoclonal antibodies (dilution according to the manufacturer's protocol) for 20 min in the dark at 4°C. The following monoclonal antibodies were used, all at a dilution of 1:30: Cluster of differentiation (CD)45-APC (cat. no. 340942), CD117-PerCP-Cy5.5 (cat. no. 333947), CD73-PE (cat. no. 550257), CD31-FITC (cat. no. 555445), CD54-PerCP-Cy5.5 (cat. no. 555512), CD166-PE (cat. no. 559263), CD44-FITC (cat. no. 347943), CD146-PE

(cat. no. 550315), CD19-FITC (cat. no. 347543), CD14-PE (cat. no. 555398), human leukocyte antigen-antigen D related (HLA-DR)-PercP-Cy5.5 (cat. no. 551375), CD90-PE-Cy5 (cat. no. 555597; all BD Pharmingen, San Diego, CA, USA), CD34-PE-Cy7 (cat. no. 348801; BD Biosciences, Franklin Lakes, NJ, USA), CD105-FITC (cat. no. 2CHF11; Immunostep, Salamanca, Spain), and CD133-PE (cat. no. 29303; Miltenyi Biotec, Inc., Cambridge, MA, USA). Following staining, the cells were washed with PBS, centrifuged at 4°C and 250 x g for 5 min and resuspended in PBS in preparation for processing. Samples were processed in a BD FACSaria II instrument (BD Biosciences) and the results were analyzed using BD FACS-Diva 6.0.1 software (BD Biosciences). These characterization experiments were repeated in three independent hHFDC lines.

**Population-doubling time (PDT) assay.** hHFDCs at passage 3 were seeded at a density of  $\sim 2 \times 10^4$  cells in 35 mm plates with a 2 mm grid (Nalge Nunc International, Penfield, NY, USA). The next day, 4 quadrants were randomly chosen and cells were counted each day for 7 days, when the cells reached confluence and an exponential curve of cell growth was constructed. By applying a base 2 logarithm to the cell/mm<sup>2</sup> axis a linear regression was performed and the inverse of the angular coefficient  $\alpha$  was used to calculate the PDT.

**In vitro hHFDC differentiation.** hHFDCs were seeded at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> in six-well plates. The culture medium was then replaced by the specific differentiation medium. For adipogenic differentiation, cells were cultured at 37°C in adipogenic medium consisting of 4.5 g/l DMEM-high glucose (Gibco; Thermo Fisher Scientific, Inc.) with 200 ml/l FBS, 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin,  $10^{-7}$  M dexamethasone (Sigma-Aldrich; Merck kGaA), 2.07 µM insulin (Sigma-Aldrich; Merck kGaA) and 0.45 mmol/l 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck kGaA) for 3 weeks. Differentiated cells were stained with 20 ml/l Oil Red O (Sigma-Aldrich; Merck kGaA) to detect cytoplasmic lipid vacuoles. For osteogenic differentiation, cells were cultured in osteogenic medium consisting of DMEM-High glucose 4.5 g/l with 200 ml/l FBS, 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin,  $10^{-7}$  M dexamethasone, 0.5 µM ascorbic acid (Sigma-Aldrich; Merck kGaA) and 10 mmol/l Na-β-glycerolphosphate (Sigma-Aldrich; Merck kGaA) for 3 weeks. Differentiated cells were stained with 10 ml/l Alizarin Red (Sigma-Aldrich; Merck kGaA) to detect extracellular calcium deposits. For chondrogenic differentiation, cells were cultured at 37°C in chondrogenic medium consisting of DMEM-high glucose 4.5 g/l with 200 ml/l FBS, 10 ng/ml transforming growth factor β (TGF-β; Sigma-Aldrich; kGaA), 0.5 µg/ml insulin, 50 µM ascorbic acid and 10 ml/l goat serum for 3 weeks. Differentiated cells were processed for histology and stained with 10 ml/l toluidine blue, which stains connective tissue.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from cells using an RNeasy micro kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA using a High-Capacity Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the

manufacturer's protocol. Genomic DNA contamination was removed using the gDNA Eliminator Spin Column that was part of the kit. Aliquots (500 ng) of each DNA sample were amplified in a Peltier Thermal Cycler PTC-200 (MJ; Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a 20-µl reaction mixture containing 1X PCR Buffer (Promega Corporation, Madison, WI, USA), 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 mM each of sense and antisense primers, and 1.25 units of Go TaqR DNA Polymerase (Promega Corporation). PCR was performed using the following parameters: Denaturation at 95°C for 5 min, 30 cycles at 95°C for 1 min, primer-specific annealing temperature at 56-62°C (Tables I and II) for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min. Negative control RT-PCRs were conducted under the same conditions without reverse transcriptase. Internal control PCRs were conducted using primers for GAPDH or β-actin. The sequences of primers and sizes of the expected products are listed in Tables I and II. The PCR product (n=3) was electrophoresed using a 2% agarose gel (Roche Diagnostics, Basel, Switzerland) and stained with ethidium bromide (E1510; Sigma-Aldrich; Merck kGaA). Gels were visualized on a UV transilluminator (UVVIS-20 Mighty Bright; Hoefer, Inc., Holliston, MA, USA).

**Alkaline phosphatase activity.** hESCs at passages 4 and 10 were fixed in 40 ml/l paraformaldehyde for 2 min at room temperature, permeabilized with 50 ml/l Triton™ X-100 (T9284; Sigma-Aldrich, Merck kGaA) and stained using an alkaline phosphatase detection kit (SCR004; Merck kGaA), containing Fast Red Violet (0.8 g/l), Naphthol phosphate solution (4 mg/ml) and water in a 2:1:1 ratio at room temperature away from light for 15 min. Red color reactions in cells were visualized using phase contrast microscopy (Olympus Corporation, Tokyo, Japan).

**Immunohistochemistry.** For immunofluorescence analysis, hESCs and hHFDCs were washed with PBS and fixed with 40 ml/l paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, permeabilized with three washes with 30 ml/l Triton™ X-100 in PBS for 10 min and then blocked with 20 ml/l bovine serum albumin (Sigma-Aldrich; Merck kGaA) in PBS for 30 min at room temperature to prevent non-specific binding. Incubation was carried out overnight at 4°C with the following primary antibodies: Rabbit anti-human octamer-binding transcription factor (Oct)4 polyclonal antibody (1:200; ab-19857; Abcam, Cambridge, MA, USA) and mouse anti-human stage-specific embryonic antigen (SSEA)-4 monoclonal antibody (1:100; MAB4304; Merck kGaA). Cells were subsequently washed three times with PBS for 10 min and incubated with the following secondary antibodies for 1 h at room temperature: Alexa Fluor 488 conjugate goat anti-mouse polyclonal antibody (1:400; A-21151; Thermo Fisher Scientific, Inc.) and Cy3-AffinePure donkey anti-rabbit polyclonal antibody (1:1,000; 711-165-152; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The specificity of each antibody was verified by negative controls included in each experiment. In addition, nuclear DNA was stained with 4'-6-diamidino-2-phenylindole (DAPI; D9542; Sigma-Aldrich; Merck kGaA) and coverslips were mounted in an anti-fade



Table I. Primer sequences and polymerase chain reaction conditions for hESC pluripotency and differentiation gene expression.

Gene product	Sense primer sequence Orientation: 5'-3'	Anti-sense primer sequence Orientation: 3'-5'	Annealing temperature (°C)	Size (base pairs)
<i>Oct3/4</i>	AGCCTGAGGGCGAAGCAGGA	CCCCAGGGTGAGCCCCACAT	56	236
<i>Sox2</i>	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA	56	126
<i>Klf4</i>	TCTCAAGGCACACCTGCGAA	TAGTGCCTGGTCAGTTCATC	56	105
<i>Nanog</i>	CAGCCCTGATTCTTCCACCAGTCCC	TGGAAGGTTCCCAGTCGGGTTTACC	56	391
<i>Afp</i>	GAATGCTGCAAAGTACCACGCT GGAAC	TGGCATTCAAGAGGGTTTTCAGTCT GGA	62	281
<i>Gata6</i>	GCCAACTGTCACACCACAAC	TGGGGGAAGTATTTTTGCTG	60	265
<i>Brachyury</i>	GCCCTCTCCCTCCCCTCCACGCACAG	CGGCGCCGTTGCTCACAGACCACAGG	62	274
<i>Msx1</i>	CGAGAGGACCCCGTGGATGCAGAG	GGCGGCCATCTTCAGCTTCTCCAG	62	305
<i>Nestin</i>	CACCTCAAGATGTCCCTCAG	AGCAAAGATCCAAGACGCC	59	176
<i>Gapdh</i>	ACCATGGGGAAGGTGAAGGT	CATGGGTGGAATCATATTGG	62	163

hESC, human embryonic stem cell; Oct, octamer-binding transcription factor; Sox2, sex determining region Y box 2; Klf, Kruppel-like factor; Afp, alpha fetoprotein; Msx 1, Msh homeobox 1.

solution, VECTASHIELD H-1000 (Vector Laboratories, Inc., Burlingame, CA, USA). Fluorescence was observed using an inverted fluorescence Zeiss Axiovert 130 microscope (Carl Zeiss Microscopy, GmbH, Germany) coupled to an AxioVision version 4.6 software system (Carl Zeiss AG, Oberkochen, Germany) and digital photomicrographs were captured.

**Embryoid body formation.** For embryoid body (EB) formation, cell aggregates of hESCs were grown in suspension in non-adherent plates without feeder layers in medium without bFGF. After 1 week, EBs derived from hESCs cultured over hHFDC or MEF were collected and transferred to 2% gelatin-coated dishes for 2 more weeks and subsequently analyzed using RT-PCR (as described above). The markers used to test hESC differentiation into embryonic germ layers were: Ectodermal (Nestin), mesodermal (Msx1 and Brachyury) and endodermal (alpha fetoprotein and Gata 6). The sequences of primers used are listed in Table I.

## Results

**Characterization of hHFDC.** For the long-term use of these cells as feeder layers, primary cultured cells were cryopreserved at early passages and maintained under the same culture conditions as MEF. hHFDCs were efficiently cultured *in vitro* with high proliferation rates and exhibiting elliptical nuclei and fibroblast-like morphology (thin and elongated adherent cells) similar to MEF (Fig. 1A). Immunophenotypic analysis detected mesenchymal stem cells markers (CD105, CD90, CD73, CD44, CD146 and CD166). Furthermore, hHFDCs exhibited low expression of hematopoietic surface markers (<2%), such as CD14, CD45, CD19 and HLA-DR, and endothelial or progenitor markers including CD31, CD34, CD133 and CD117 (Fig. 1B). To further characterize the cells and to assess the time to confluence during routine culture, the doubling time of hHFDCs was measured. These cells exhibited rapid proliferation, with a mean PDT of  $34.5 \pm 0.02$  h

( $n=3$ ; Fig. 1C). To evaluate differentiation, hHFDCs were induced to differentiate into adipogenic, osteogenic and chondrogenic lineages (Fig. 1D). Adipogenic differentiation of hHFDC was apparent following 3 weeks of incubation in adipogenic medium. The formation of lipid vacuoles was evident, as detected by positive Oil Red O staining. Similarly, differentiation in osteogenic medium induced an osteoblastic phenotype, with the cells being strongly stained by Alizarin Red, which indicates the deposition of calcium in the extracellular matrix. Connective tissue was observed in hHFDCs submitted to chondrogenic induction via staining with toluidine blue. Non-treated control cultures did not show spontaneous adipocyte, osteoblast or chondroblast formation following 3 weeks of culture.

**hESC morphology.** hESC colonies maintained on hHFDC feeder layers exhibited typical morphology of undifferentiated hESC, including a high nucleus-to-cytoplasm ratio, 1 to 3 nucleoli, rounded shape and typical spacing between the cells, similar to hESCs cultured on MEF (Fig. 2A and B). Continuous proliferation of hESCs on the human feeder layer was normally maintained for >10 passages with preservation of the undifferentiated state (data not shown).

**Pluripotency maintenance of hESC on hHFDC.** RT-PCR demonstrated that hESC colonies cultured on hHFDC expressed pluripotency-associated genes, including transcription factors Oct3/4, Sox2 and Nanog at passages 4 and 10, similar to what is observed when these cells are cultured on MEF (Fig. 2C). Inactivated hHFDCs were used as a negative control for the PCR. Positive staining for alkaline phosphatase was strongly detected in hESCs cultured on both MEF and hHFDC feeders at passages 4 and 10 (Fig. 2D). Additionally, immunofluorescence staining demonstrated the nuclear localization of Oct3/4 protein and cytoplasmic localization of SSEA-4 in hESC colonies cultured on hHFDC or MEF layers (Fig. 3).

Table II. Primer sequences and polymerase chain reaction conditions for growth factor expression in MEF and hHFDC feeders.

Gene product	Sense primer sequence Orientation: 5'-3'	Anti-sense primer sequence Orientation: 3'-5'	Anneling temperature (°C)	Size (base pairs)
<i>qmBmp4</i>	ATTGCAGCTTTCTAGAGGTCC	GGGAGCCAATCTTGAACAAAC	59	149
<i>qhBmp4</i>	GCACTGGTCTTGAGTATCCTG	TGCTGAGGTTAAAGAGGAAACG	59	135
<i>qmFgf2</i>	GTCTATCAAGGGAGTGTGTGC	CTGGAGTATTTCCGTGACCG	58	150
<i>qhFgf2</i>	ACCCTCACATCAAGCTACAAC	AAAAGAAACACTCATCCGTAACAC	58	141
<i>qmTgfb</i>	CCTGAGTGGCTGTCTTTTGA	CGTGGAGTTTGTATCTTTGCTG	61	124
<i>qhTgfb</i>	GCCTTTCCTGCTTCTCATGG	TCCTTGCGGAAGTCAATGTAC	61	150
<i>qmPedf</i>	ACATCCACAGCACCTACAAG	TCCCATAGGACTTCTCCAGAG	59	141
<i>qhPedf</i>	AGCTGAGTTATGAAGGCGAAG	ATCCTCGTTCCACTCAAAGC	59	149
<i>qmVegf</i>	GAACCTTCTGCTCTCTTGGG	CTTCGCTGGTAGACATCCATG	60	144
<i>qhVegf</i>	AGGGCAGAATCATCACGAAG	GGATGGCTTGAAGATGTACTCG	60	130
<i>Gapdh</i>	ACCATGGGGAAGGTGAAGGT	CATGGGTGGAATCATATTGG	62	163
<i>β-actin</i>	CATCACTATTGGCAACGAGCG	ATGGATGCCACAGGATTCCA	58	85

MEF, mouse embryonic fibroblast; hHFDC, human hair follicle-derived mesenchymal cell; Bmp, bone morphogenetic protein; Fgf, fibroblast growth factor; Tgf, transforming growth factor; Pedf, pigment epithelium-derived factor; Vegf, vascular endothelial growth factor.

**Growth factor analysis.** Growth factor analysis revealed similar amplification of transcripts for bone morphogenetic protein (BMP)-4, FGF-2, vascular endothelial growth factor (VEGF), pigment epithelial-derived factor and TGF- $\beta$  in hHFDCs and MEFs prior to and following inactivation. Expression of all of these growth factors was maintained when hESCs were cultured on both feeder layers (Fig. 4).

**Differentiation capacity of hESC.** To determine the differentiation capacity of pluripotent hESC *in vitro*, EBs were cultured in suspension. Similar to EBs grown on MEF feeders, hESCs on hHFDC underwent spontaneous differentiation into the three germ layers when cultured without a feeder layer and without bFGF *in vitro* (Fig. 5A). The EBs cultured for 1 and 2 weeks expressed markers for ectoderm (Nestin), mesoderm (Brachyury and Msx1) and endoderm (alpha fetoprotein and GATA 6), confirming the pluripotent state of hESC grown on hHFDC and MEF (Fig. 5B). Notably, Oct3/4 was detected in EBs cultured on both feeder layers (Fig. 5B).

## Discussion

Mesenchymal stem cells show great potential for use in cellular therapies. In hair follicles, populations of transit amplifying cells form bulbs that encase clusters of mesenchymal cells known as dermal papilla (DP) cells (16). DP cells are the focus of intense interest as they regulate hair follicle development and growth, and are also thought to be a reservoir of multipotent stem cells (17). Previous studies have demonstrated that the hair follicle DP contains mesenchymal stem cells (14,18-20); however, the multipotentiality of hHFDCs has not been fully investigated.

In the present study, a population of hHFDCs from the scalp of patients undergoing facelift surgery were isolated and characterized, and found to have features of mesenchymal stem cells. Immunophenotypic analysis of hHFDCs cultured

*in vitro* was performed, and the results demonstrated that >95% of cells expressed surface molecules characteristic of mesenchymal stem cells (CD90, CD73, CD105), whereas <2% expressed hematopoietic markers (CD45, CD34, CD14, CD19 and HLA-DR). These results are similar to those of Liu *et al* (18), who also investigated human hair follicle cells; however, a lower expression of CD105 (33.77%) was reported than in the present study (99%). This difference may be due to the cell type used (only cells that migrated from specific regions of the hair follicle) and/or the shorter time (4 h) used in the previous study for collagenase digestion (18). The results of the present study demonstrates that this culture method is effective for expanding a homogeneous population of mesenchymal stem cells, as prescribed by the International Society for Cellular Therapy in 2006 (21). Furthermore, the adhesion molecules CD44 and CD166 were expressed, which confirms that the cells retained mesenchymal characteristics, again in consonance with the results of Liu *et al* (18). The present study also confirmed the presence of CD146, which is considered a pericyte marker present in mesenchymal cells (22,23). The results demonstrated a low expression of the molecules commonly found in endothelial cells (CD31 and CD133) and progenitor cells (c-kit), corroborating the results of a previous study by Wang *et al* (24) investigating umbilical cord cells.

The PDT recorded in the present study was also consistent with the characteristics of stem cells, specifically a high proliferation potential of 34.5 h. Similar results have been demonstrated in previous studies utilizing cells of the outer root sheath of the human hair follicle (PDT=33 h) (25) and human hair follicle-derived mesenchymal cells (PDT=36 h) (14). In addition, several studies have reported similar results for mesenchymal cells from other sources, including menstrual blood cells (PDT=24-37 h) (26,27) and umbilical cord blood cells (PDT=36 h) (28).

Furthermore, the present study determined that hHFDCs are able to differentiate into several mesenchymal lineages

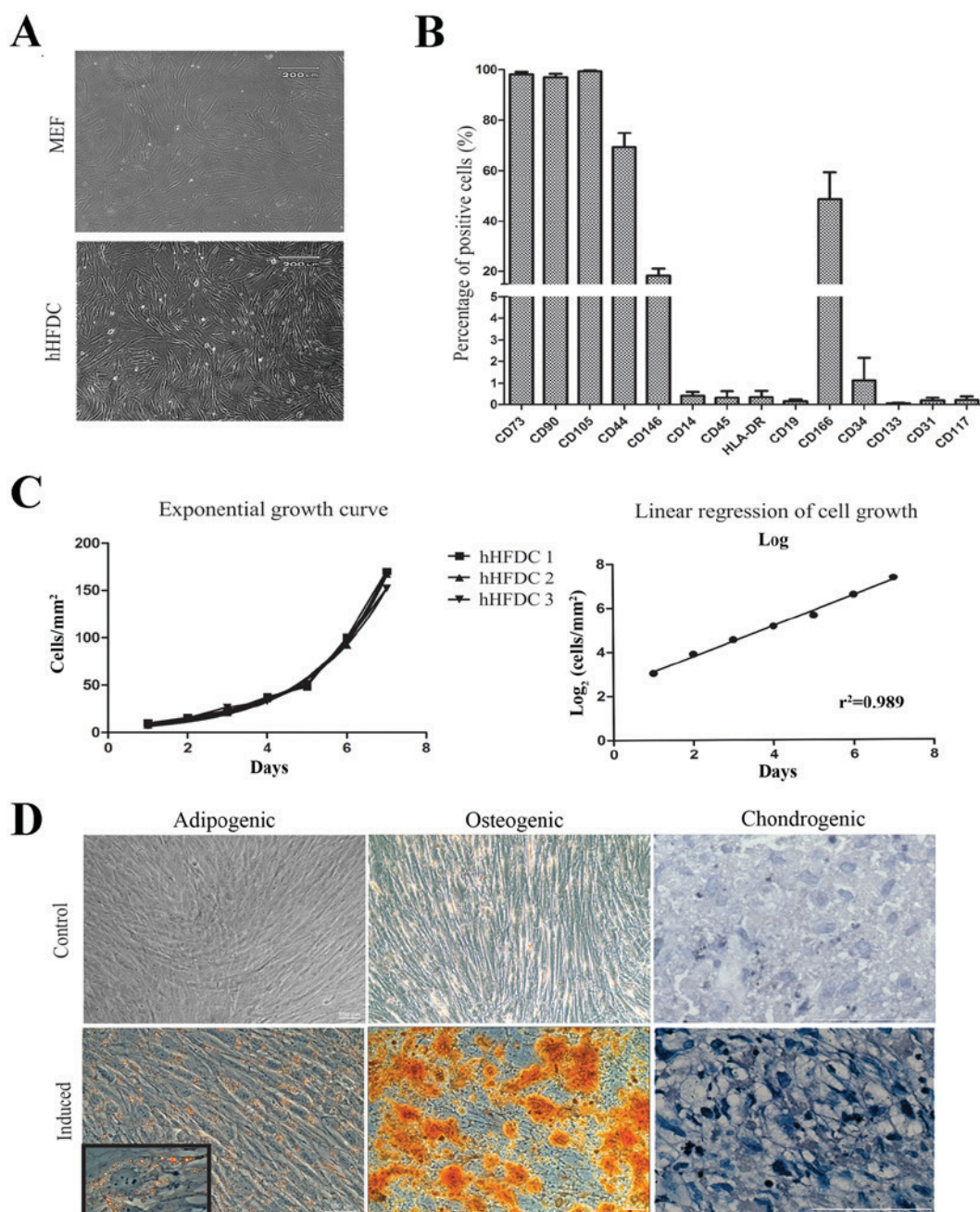


Figure 1. hHFDC characterization. (A) Morphology of MEF and hHFDC feeder layers. (B) Immunophenotypic profile of hHFDC: Percentage of cells that express molecular characteristics of mesenchymal, hematopoietic, endothelial and progenitor cells, respectively. (C) PDT: hHFDCs show rapid proliferation, with an average PDT of  $34.5 \pm 0.02$  h ( $n=3$ ). (D) Differentiation of hHFDCs into adipocytes, osteoblasts and chondroblasts. Staining with Oil Red O for fat vacuoles demonstrated differentiation into adipogenic tissue in induced and non-induced controls (inset magnification,  $\times 40$ ); staining with Alizarin Red in induced cells revealed calcium deposits compared with non-induced controls. Staining with toluidine blue for sulfated proteoglycans demonstrated the differentiation into chondrogenic tissue types in induced compared with non-induced hHFDC controls. Scale bar:  $100 \mu\text{m}$ . hHFDC, human hair follicle-derived mesenchymal stem cell; MEF, mouse embryonic fibroblast; PDT, population doubling time; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related.

including osteoblasts, adipocytes and chondroblasts (14,18,20). However, Bajpai *et al* (14) reported a faster rate of adipogenic differentiation (14 days) than that observed in the present study, in which lipid vacuoles in the cytoplasm of hHFDCs were only observed 21 days following adipogenic differentiation. This difference may be due to the supplementation of bFGF in the culture medium used by Bajpai *et al* (14). Notably, the culture method used in the present study was effective at producing human hair follicle mesenchymal stem cells that

maintained the expression of surface molecules characteristic of mesenchymal cells and were able to differentiate into three mesodermal lineages.

The hHFDC culture was established and it was subsequently evaluated whether these cells could replace MEF as a feeder layer for hESCs. hHFDCs are an attractive cell source in regenerative medicine for several reasons: Cell isolation is feasible, cells may potentially be used in both males and females and they may allow autologous matching compatible



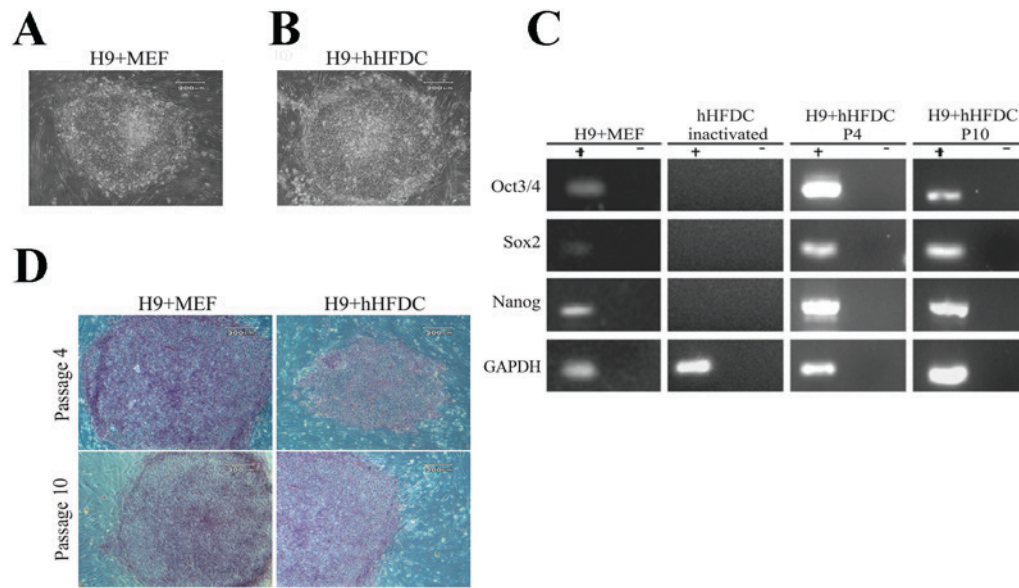


Figure 2. Morphology of cells and colonies of hESCs showing the expression of AP, transcription factors characteristic of undifferentiated cells and pluripotency makers. H9 cells grown over (A) MEF and (B) hHFDC. (C) Expression of transcription factors characteristic of undifferentiated hESCs. MEF as a positive control, inactivated HFDCs as a negative control, and H9 cells in P4 and P10 grown over hHFDC. (D) Expression of AP in hESCs grown over MEF and hHFDC in P4 and P10. Scale bar: (A-C) 200  $\mu$ m. hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; hHFDC, human hair follicle-derived mesenchymal stem cell; AP, alkaline phosphatase; P, passage; Oct, octamer-binding transcription factor; Sox2, sex determining region Y box 2.

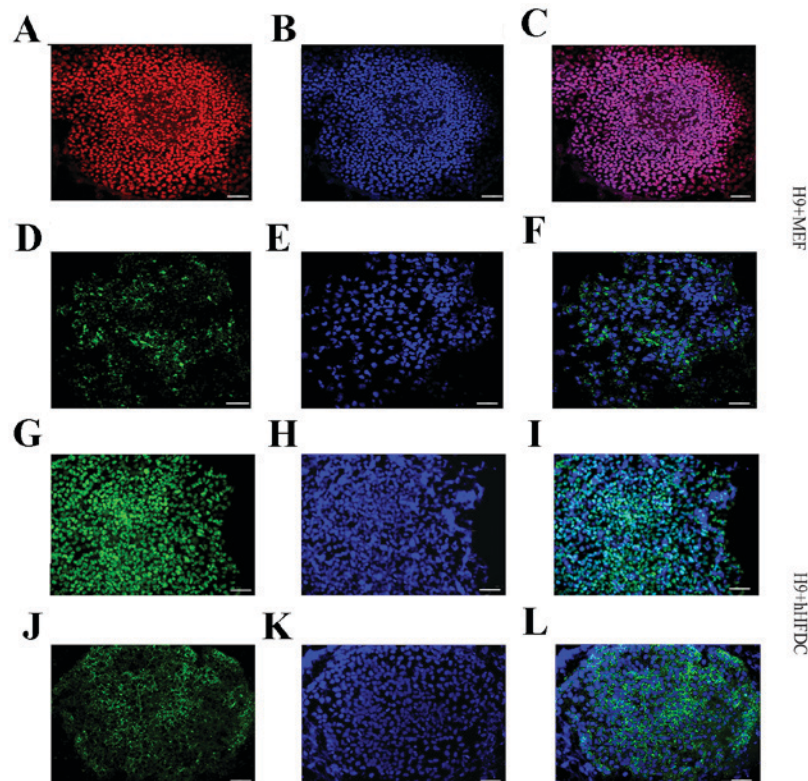


Figure 3. Expression of pluripotency molecules in undifferentiated hESCs grown over MEF and hHFDC. The cells were positive for Oct3/4 (red and green; A and G, respectively) and SSEA-4 (green; D and J). The nuclei were labeled with 4',6-diamidino-2-phenylindole (blue; B, E, H and K). Merged images (C, F, I and L). Scale bar: 20  $\mu$ m. hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; hHFDC, human hair follicle-derived mesenchymal stem cell; P, passage; SSEA, stage-specific embryonic antigen; Oct, octamer-binding transcription factor.

with clinical applications. hESCs grown over MEF and hHFDC layers were maintained under the same culture conditions and

hHFDCs displayed a fibroblast-like morphology similar to MEF, as described in the literature (14,18). No morphological

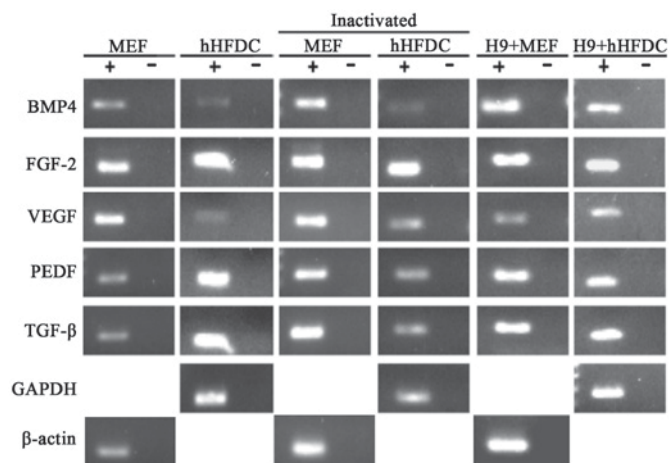


Figure 4. Expression of growth factors in feeder cells. Reverse transcription polymerase chain reaction revealed amplification of transcripts in MEFs, hHFDCs, inactivated MEFs, inactivated hHFDCs, H9 grown over MEF and H9 grown over hHFDC. GAPDH and  $\beta$ -actin were used as internal controls. MEF, mouse embryonic fibroblast; hHFDC, human hair follicle-derived mesenchymal cell; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor; TGF, transforming growth factor.

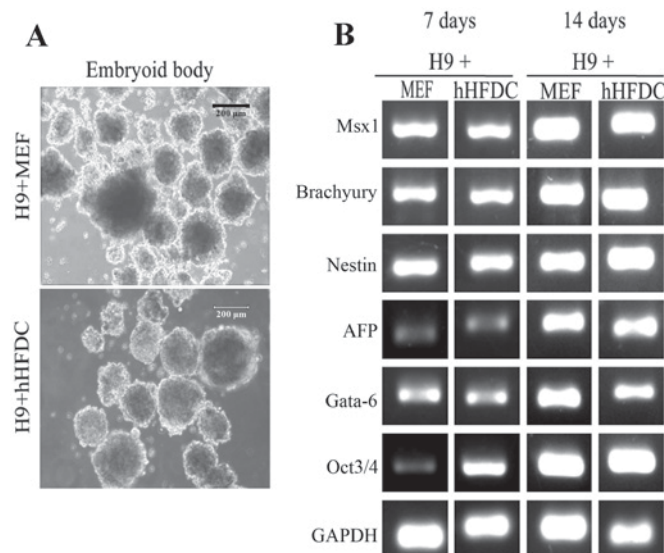


Figure 5. Differentiation capacity of hESCs. (A) Embryoid bodies formed from hESCs (H9) previously plated on MEF and hHFDC feeder layers had similar sizes and morphologies. (B) Expression of genes from the three germ layers; mesodermal (*Msx1* and *Brachyury*), endodermal (*Gata-6* and *AFP*), and ectodermal (*Nestin*). *Oct3/4* expression was still detected in embryoid bodies at 14 days. hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; hHFDC, human hair follicle-derived mesenchymal cell; *Msx1*, Msh homeobox 1; *AFP*, alpha fetoprotein; *Oct*, octamer-binding transcription factor.

changes were observed when these cells were inactivated with mitomycin C (data not shown), in agreement with the results of Lee *et al* (29). In previous studies the morphology of hESCs maintained on human feeder layers differed slightly from hESCs maintained on MEF layers (30,31). However, hESCs grown on hHFDC were found to have typical hESC morphology, with a high nucleus/cytoplasm ratio, prominent nucleoli and close spacing between cells that were maintained for 10 passages (1,2). Similar results were found when hESCs

were cultured on foreskin cells (30) and menstrual blood cells (9).

Many authors use enzymatic digestion for hESCs cultured on human feeder layers (30,32-34). However, the commonly used enzymes including trypsin, collagenase type IV and dispase (35), are animal derived. Additionally, it has been observed that manual dissection of colonies is better for maintaining genetic stability compared with enzymatic disaggregation as a method of passaging hESCs (36-39). To avoid genetic alterations and minimize the risk of contamination with products of animal origin, hESCs on hHFDC were passaged every 5 days by mechanical dissociation in the present study, as reported by Cho *et al* (40). Furthermore, an increase in the number of differentiated colonies was observed when the enzymatic digestion method was used (data not shown), as described in the literature (41).

The pluripotency of hESCs on hHFDC was confirmed by RT-PCR, which detected the expression of genes encoding transcription factors *Oct3/4*, *Sox2* and *Nanog* in passages 4 and 10. Inactivated HFDCs did not express these genes, demonstrating that expression only occurs in hESCs. Similar results were obtained by Lee *et al* (33), who reported that *Oct3/4* was expressed in hESCs grown over placental cells. Furthermore, hESCs grown over hHFDC expressed the surface protein SSEA-4, the transcription factor *Oct3/4*, and alkaline phosphatase, in accordance with results reported by Zhan *et al* (32) and Cho *et al* (40), who used umbilical cord cells as feeder layers. It should be noted that none of the feeder-layer cells expressed these proteins.

The results of the present study characterize these cells as pluripotent stem cells, demonstrating that hHFDCs readily retain the undifferentiated state of hESCs. It is important to mention that previous studies have not evaluated the gene expression or alkaline phosphatase of hESCs cultured on foreskin cells (30,42,43) or human umbilical cord blood cells (32).

Recently, several groups have demonstrated that growth factors produced by fibroblasts, including FGF2, TGF- $\beta$ , BMP-4 and PDGF (41,44-48), may serve a role in maintaining the undifferentiated growth of hESCs. The most commonly used fibroblasts are MEF (1,2). However, these primary cells senesce after 5 to 6 passages, thus limiting their continued use (49) and the need to derive new feeder cells may result in culture variation. In the present study, feeder cells were established from human hair follicles, which can be used for at least 16 passages as feeders for hESCs. Previous studies have demonstrated that TGF $\beta$  and BMP4 induce the catagen phase in hair follicles (50,51). Conversely, VEGF is thought to be important for anagen maintenance (52) and FGF-2 has been identified as the major regulator in determining the patterning of hair pigmentation (53). Growth factor analysis revealed that all these transcripts were amplified in hHFDCs before and after inactivation and when hESCs were grown on hHFDC and MEF. Future studies investigating longer-term cultures and the role and pathways for each of these factors in undifferentiated hESC growth may provide more information regarding clinical applications.

The differentiation capacity of hESC was confirmed when cells were grown in suspension following continuous culture on both types of feeder layers. The cells were able to differentiate, spontaneously, forming EBs containing the cell types



representative of the three germ layer; this is, similar to results found in studies using feeder layers of breast parenchymal cells, endometrial cells and human fibroblast cells (33), placental cells (54), foreskin cells (30), umbilical cord blood cells (32), and umbilical cord stromal cells (40). Following 14 days culture, the expression of pluripotency transcription factors was still detected, suggesting that 14 days was insufficient to induce the differentiation of all EBs. These results are consistent with those of a previous report in which undifferentiated cells grown on foreskin cells expressed Oct3/4 even after one month of the EB culture protocol (30).

In summary, the present study demonstrates that hHFDs are a suitable for use in feeder layers for human pluripotent cells due to their high proliferative capacity and availability, and the possibility of autologous use when used with iPSC derived from the same patient hHFDs are therefore a more effective as feeder layers compared with other types of human feeder cells.

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