ARTICLE
Journal of Cellular Biochemistry 112:1353–1363 (2011)

Journal of Cellular Biochemistry

Co-Culture of Mesenchymal-Like Stromal Cells Derived From Human Foreskin Permits Long Term Propagation and Differentiation of Human Embryonic Stem Cells

Murali Krishna Mamidi,[1] Rajarshi Pal,[1] Nor Azah Binti Mori,[2] Greetha Arumugam,[1] Saratha Thevi Thrichelvam,[1] Puteri J Noor,[2] Hj. Mohamad Farouk Abdullah,[3] Pawan Kumar Gupta,[4] Anjan Kumar Das,[1] Zubaidah Zakaria,[2*] and Ramesh Bhonde[1*]

[1] *Stempeutics Research Malaysia Sdn. Bhd., Technology Park Malaysia, 57000 Kuala Lumpur, Malaysia*

[2] *Hematology Unit, Cancer Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia*

[3] *Department of Obstetrics & Gynaecology, Hospital Tengku Ampuan Rahimah, 41200 Selangor, Malaysia*

[4] *Manipal Institute of Regenerative Medicine, Manipal University Branch Campus, Domlur Layout, Bangalore 560 071, India*

ABSTRACT

Among the different parameters governing the successful derivation and expansion of human embryonic stem cells (hESC), feeder layers play the most important role. Human feeders in form of human mesenchymal stromal cells (hMSCs) and human foreskin fibroblasts (HFFs) lay the foundation for eradication of animal-derived hESC culture system. In this study we explored the potential of human foreskin derived mesenchymal like stromal cells (HF-MSCs) to support self renewal and pluripotency of hESC. The MSCs isolated from human foreskin were found to be resistant to standard concentrations and duration of mitomycin-C treatment. Growth pattern, gene profiling (Oct-4, Nanog, Sox-2, Rex-1), cytoskeletal protein expression (vimentin, nestin) and tri-lineage differentiation potential into adipocytes, chondrocytes and osteocytes confirmed their mesenchymal stromal cell status. Further, the HF-MSCs were positive for CD105, CD166, CD73, CD44, CD90, SSEA-4, and negative for CD34, CD45, HLA-DR cell-surface markers and were found to exhibit BM-MSC-like characteristics. hESC lines co-cultured with HF-MSC feeders showed expression of expected pluripotent transcription factors Oct-4, Nanog, Sox-2, GDF-3, Rex-1, STELLAR, ABCG2, Dppa5, hTERT; surface markers SSEA-4, TRA-1-81 and maintained their cytogenetic stability during long term passaging. These novel feeders also improved the formation of embryoid bodies (EBs) from hESC which produced cell types representing three germ layers. This culture system has the potential to aid the development of clinical-grade hESCs for regenerative medicine and drug screening. Further, we envisage foreskin can serve as a valuable source of alternative MSCs for specific therapeutic applications. J. Cell. Biochem. 112: 1353–1363, 2011.

KEY WORDS: HUMAN EMBRYONIC STEM CELLS (hESC); EMBRYOID BODY (EB); HUMAN FORESKIN; MESENCHYMAL STROMAL CELLS; PLURIPOTENCY; DIFFERENTIATION

Postnatal stem cells are progenitors that reside in adult tissue and have the ability to replace the injured and dead somatic cells to maintain tissue homeostasis. Several studies demonstrated that even skin could serve as a source of adult stem cells apart from the conventional source of stem cells [Fernandes et al., 2004; Blanpain and Fuchs, 2006]. Epidermis of skin contains subpopulations of progenitors such as the cells isolated from hair follicles, bulge and precursors derived from sweat and subcutaneous glands [Fu et al., 2005; Ohyama et al., 2006; Tiede et al., 2007]. Recent studies revealed that the skin-derived precursors possess broad differentiation potential towards smooth muscle cells, melanocytes, neuronal cells, and glial cells [Toma et al., 2001; Crigler et al., 2007].

Murali Krishna Mamidi and Rajarshi Pal contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Institute for Medical Research (IMR), Ministry of Health, Malaysia; Grant number: JPP-IMR-08-019.

*Correspondence to: Zubaidah Zakaria and Ramesh Bhonde, Lot 3-I-7, Enterprise 4, Technology Park Malaysia, Bukit Jalil, Kuala Lumpur 57000, Selangor, Malaysia. E-mail: Zubaidah@imr.gov.my, rrbhonde@stempeutics.com.my

Received 11 August 2010; Accepted 20 January 2011 • DOI 10.1002/jcb.23052 • © 2011 Wiley-Liss, Inc.
Published online 17 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

Further it has been shown that dermal and hair follicle precursor cells are able to differentiate into neuronal, smooth muscle, melanocyte, chondrocyte, and Schwan cells in vitro [Toma et al., 2005]. Interestingly the application of these skin derived progenitors has been evaluated in the treatment for bone repair [Lavoie et al., 2009]. Thus, it may be promising to establish a generic protocol for isolation and characterization of skin-derived MSC in order to explore their role in cell replacement therapy as an alternative to conventional source of BM-MSCs.

A co-existence and co-operation between BM-MSC and BM-haematopoietic stem cells (BM-HSC) as well as dermis and epidermis of skin is observed both in vivo and ex vivo conditions [Durig et al., 2000]. Likewise, dermal equivalents or feeder layers are required for proliferation and differentiation of epidermal keratinocytes in vitro [Xiao et al., 2008]. This phenomenon is conserved in bone marrow niche which permits proliferation and differentiation of BM-HSC. The mesenchymal and epithelial interaction is commonly involved in normal development as well as in carcinogenesis or tumorogenesis [Raimondi et al., 2010]. Even the proliferation and differentiation of hESC also depends on the support of feeder layers. Therefore, different types of human feeder layers have been evaluated for derivation and culturing of hESCs. This also helps in getting rid of the potential zoonotic pathogen and xeno-antigen transmission; hESCs have been cultured on various human feeder layers such as fetal muscle, skin, adult fallopian tubal epithelial cells [Richards et al., 2002]; adult marrow cells [Cheng et al., 2003]; adult lung fibroblasts [Richards et al., 2003]; uterine endometrium, breast parenchyma, and aborted embryonic fibroblasts [Lee et al., 2004]. More recently hESCs have been co-cultured with human mesenchymal stromal cells (hMSCs) or hMSC-conditioned media [Cortes, 2009; Montes et al., 2009]. Among different human derived feeder layers, hMSCs have been shown to be superior to human foreskin fibroblasts (HFF) in supporting the proliferation of hESC [Zhu et al., 2009]. Moreover, previous reports not only support the ubiquitous presence and functional heterogeneity of fibroblasts but an increasing body of present evidence also unravels an intimate relationship between fibroblasts and hMSCs [Haniffa et al., 2009].

Owing to their ability of unlimited self renewal and differentiation capacity into more than 200 cell types, hESCs hold tremendous promise for regenerative medicine and drug discovery [Odorico et al., 2001]. However the risk of unknown components and xeno-protein transmission has emerged as major limitations of the hESC culture system in presence of mouse embryonic fibroblasts (MEF) [Cheng et al., 2003; Richards et al., 2003]. Although there have been several reports on the development of xeno-free systems for hESC, the long-term safety and efficacy of these systems has not been evaluated. The use of foreskin-derived mesenchymal-like stromal cells (HF-MSC) has several advantages compared with other sources; it is abundant, inexpensive and can be obtained through non-invasive procedures that do not pose any ethical concern.

The present study was therefore aimed at selective growth of MSC-like cells from non-controversial and often discarded source of human foreskin and explores their potential to support hESC. Detailed characterization have demonstrated that human foreskin derived mesenchymal like stromal cells (HF-MSC) can differentiate into adipogenic, osteogenic and chondrogenic lineages in vitro and share a transcription factor and surface marker profile similar to those of BM-MSC. In order to adapt these highly proliferative mitomycin-C resistant cells [Nieto et al., 2007] as alternative feeders to culture hESC, we optimized the method of inactivation and finally showed that HUES-7 cells can be grown on these HF-MSCs for over 30 passages similar to hESCs grown on conventional MEFs.

MATERIALS AND METHODS

ISOLATION OF MESENCHYMAL-LIKE STROMAL CELLS FROM CIRCUMCIZED HUMAN FORESKIN SAMPLES

Foreskin samples were obtained from Hospital Tengku Ampuan Rahimah, Klang (HTAR) (with prior approval from Medical Research Ethics Committee (MREC), Ministry of Health, Malaysia) after obtaining written informed consent from 7- to 22-year-old donors or their legally authorized representatives (LARs). To isolate desired cells, circumcised foreskin pieces were washed in DPBS with antibiotic and anti-mycotic solution (Gibco) and then rinsed with 70% alcohol for a minute. The skin was slowly peeled and the dermis was separated from epidermis, cut into small pieces and incubated at 37°C and 5% CO_2 in air (Binder, USA) with 0.25% trypsin-EDTA (Gibco) and 0.05% collagenase (Type II; Sigma–Aldrich). FBS (Hyclone) was used to neutralize the enzymatic action and the cell suspension was passed through cell strainer (BD Biosciences). The resulting cell suspension was plated onto a T-75 flask (Nunc) along with growth media comprising 89% Dulbecco's modified Eagles medium (DMEM)-high glucose (Gibco) supplemented with 10% (v/v) FBS (Hyclone), 1 mM L-glutamine (Gibco), 0.1 mM b-mercaptoethanol (Sigma), 1% nonessential amino acid (Gibco) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Once the cultures reached 80–90% confluence the cells were trypsinized and expanded further; another half of the cells were cryopreserved in 10% dimethylsulphoxide (DMSO; Sigma) at a density of 2×10^6 cells/vial. Each batch of cells was tested for post-thaw viability, endotoxin, mycoplasma and microbes (bacteria, viruses, etc.) before using them as feeders for hESCs.

GROWTH KINETICS

HF-MSCs were plated at a density of 1,000, 2,000 and 5,000 cells/cm^2 in T25 flask (BD Pharmingen). Cells were trypsinized, counted, and re-plated once they reached 70–80% confluency. Culture was terminated when the cell population failed to double after 2 weeks in culture. Population doubling (PD) time was calculated using the formula PD = tplg2/(lgNH-lgNI). NI: the inoculum cell number; NH is the cell harvest number and t is the time of the culture (in hours).

EVALUATION OF HUMAN FORESKIN-DERIVED CELLS FOR MESENCHYMAL-LIKE STROMAL CELL PROPERTIES

Expression of surface markers. Immunophenotyping of the cultured HF-MSCs was performed using flow cytometry with an objective to identify the presence of specific cell surface antigens. Directly conjugated antibodies CD166-PE, CD73-PE, CD44-PE, CD90-PE, CD45-FITC, CD34-FITC, HLA-DR-FITC (BD Biosciences) were used to characterize these cells. Briefly HF-MSCs were dissociated with 0.25% trypsin-EDTA and re-suspended in DPBS

(Invitrogen) at a concentration of 1×10^6 cells/ml. Cell viability was measured using 7-amino actinomycin D (7AAD, which can penetrate through cell membrane of dead cells). Two hundred microliters of cell suspension was incubated in the dark for 30 min at 4°C with saturating concentrations of fluorescein isothiocyanate (FITC) or phycoerythrin-(PE), conjugated antibodies (Supplementary material; Table-1). Appropriate isotype matched controls were used to set the instrument parameters. After incubation, cells were washed thrice with DPBS and re-suspended in 0.5 ml of DPBS for analysis. Flow cytometry was carried out on a Guava EasyCyte Plus (Millipore). Cells were identified by light scatter for 10,000 gated events and analyzed using Cyto Soft v5.2 software.

DNA cell cycle analysis by propidium iodide (PI) staining. HF-MSCs and MEF cells were fixed overnight using 70% ethanol at 4°C and stained with PI/RNase staining buffer (BD Pharmingen) for 15 min at room temperature in darkness. Cell cycle distribution was analyzed by flow cytometry using Cytosoft, Version 5.2, Guava Technologies software. Doublet and cell aggregations were discriminated by adjusting the gates on the unstained control to surround only the singlet's in the forward scatter versus side scatter plot [Kang et al., 2001].

DIFFERENTIATION STUDIES

To assess the mesodermal differentiation potential of the HF-MSCs, the cultures were initiated at a density of 1,000 cells/cm^2 in six-welled plates (Nunc) and were grown to confluence. Thereafter cells were induced for differentiation. (a) Osteogenesis: Cells grown in DMEM-KO (Gibco) supplemented with 10% FBS (Hyclone), 200 mM glutamax (Invitrogen), 10^{-8} M dexamethasone (Sigma–Aldrich), 30 μg/ml ascorbic acid (Sigma–Aldrich) and 10 mM β-glycerophosphate (Sigma–Aldrich) for 3 weeks. To assess the mineralization, osteogenic medium-treated cells were then applied for Von Kossa staining to observe calcium deposits. (b) Chondrogenesis: Briefly, cells were cultured in media supplemented with 1XITS (Sigma–Aldrich), 50 μM L-ascorbic acid, 55 μM sodium pyruvate (Invitrogen), 25 μM L-proline (Sigma–Aldrich) and 10 ng/ml of Transformation Growth Factor-beta (TGF-b) (Sigma–Aldrich). After 3 weeks, accumulation of proteoglycans was evaluated by Alzarin Blue staining (Sigma–Aldrich). (c) Adipogenesis: To induce adipogenic differentiation, HF-MSCs were cultured for up to 3 weeks in DMEM-KO supplemented with 10% FBS, 200 mM glutamax, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 μg/ml insulin and 100 μM indomethacin (all Sigma–Aldrich). Build up of lipid droplets in the generated adipocytes were visualized by Oil Red O staining (Sigma–Aldrich). For (a), (b), and (c) the images were captured using Nikon Eclipse 90i microscope (Nikon) and Image-Pro Express software (Media Cybernetics).

Optimal inactivation of HF-MSCs for supporting hESC proliferation. Semi-confluent HF-MSCs were inactivated using mitomycin C (Sigma–Aldrich). Different concentrations of mitomycin C starting from 10 μg/ml up to 15 μg/ml for various time points including 2–5 h was tested to standardize effective inactivation of HF-MSCs. After incubation with mitomycin C, the cells were washed for 6–8 times with DPBS (Invitrogen) to remove any traces of mitomycin C. The monolayer of cells was then dispersed using 0.25% trypsin EDTA (Gibco) followed by mechanical disruption. Resuspended cells were counted using a hemocytometer and thereafter plated on 0.2% gelatin (Sigma) coated 35 mm tissue culture dishes (Falcon) at a concentration of 1.5×10^5 cells/ml. Further, different seeding densities of these inactivated HF-MSCs were plated ranging from 5.5×10^5 to 1.0×10^5 cells/ml. Finally HF-MSCs inactivated with 15 μg/ml of mitomycin C for 4 h, seeded at a density of 1.5×10^5 cells/ml was found to be most suitable to support proliferation of hESC.

Adaptation of hESCs on in-house derived HF-MSC feeders. HUES-7 cell line routinely cultured on MEF cells [Pal et al., 2009] was transferred to HF-MSC feeders in ES culture medium consisting of 79% KO-DMEM (Gibco), 20% Knockout serum replacement (Gibco), 1 mM L-Glutamine (Gibco), 1% non-essential amino acid solution (Gibco), 0.1 mM mercaptoethanol (Sigma), 4 ng/ml human basic growth factor (bFGF; Sigma), 50 IU/ml pencicillin and 50 μg/ml streptomycin (Gibco). During the initial stages of transition (1/2 passages), the rate of cell proliferation and morphology of the colonies was not satisfactory; however the cell quality recovered completely over time. HUES-7 has been successfully propagated for 30 passages on HF-MSC feeders and is still ongoing. At every five passages, hESC were cryopreserved in clumps of about 200–250 cells by conventional slow-freezing method.

Induction of embryoid body (EB) formation. hESC colonies were manually cut into clumps and plated on 60 mm bacteriological petridishes (BD Biosciences) in EB formation media consisting of 79% KO DMEM (Gibco), 20% Knockout serum replacement (Gibco), 1 mM L-Glutamine (Gibco), 1% non-essential amino acid solution, 0.1 mM mercaptoethanol (Sigma), 50 IU/ml pencicillin and 50 μg/ml streptomycin (Gibco). EB's collected from four time points (days 5, 10, 15, and 20) were stored in −80°C as cell pellet to perform gene expression analysis by RT-PCR.

CHARACTERIZATION OF HUES-7 CELL LINE GROWN ON HF-MSC

Gene expression analysis by RT-PCR. Total RNA was isolated from hESCs, EB's using TRIZOL reagent (Invitrogen) as per the manufacturer's protocol and was quantified using Nanodrop (Agilent). cDNA was synthesized from 1 μg of RNA with Superscript II First Strand Synthesis system (Invitrogen) as per the manufacturer's instructions. PCR was performed for 35 cycles consisting of initial denaturation at 94°C for 5 min followed by 35 amplification cycles of denaturation at 94°C for 30 s, annealing temperature of respective primer for 45 s and extension at 72°C for 45 s and final extension at 72°C for 10 min. In this study, we checked the expression of a set of pluripotent and lineage-specific markers (Supplementary material; Table-2) in undifferentiated stem cells and EBs.

Indirect immunofluorescence. We performed immunochemistry to demonstrate that HUES-7 cells in situ express both surface and nuclear proteins concomitant to various stages of embryogenesis. hESCs, hEBs, and HF-MSCs were plated on two-well chamber slides (BD Biosciences) and allowed to grow for 2 days. Attached cells were washed with PBS and then fixed in 4% paraformaldehyde at4°C for 30 min; permeabilized with 0.2% Triton X-100 for 10 min at 25°C and probed with primary antibodies overnight at 4°C. The primary antibodies used in the present study were Oct-4, Sox-2, GATA-4, brachyury, MAP-2 (all Abcam); Nanog, β-III-tubulin, GFAP, SSEA-4, TRA-1-81, vimentin, desmin, α-fetoprotein, cytokeratin-18 (all

Millipore), and Sox-17 (Santa Cruz) (Supplementary material; Table-1). Primary antibodies were diluted in 1× PBS (Invitrogen) containing 1 mg/ml BSA (Sigma) to block nonspecific reactivity. Antibody localization was confirmed using appropriate secondary antibodies conjugated to flourescein isothiocyanate or rhodamine (Millipore) and incubated at room temperature for 2 h. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 μg/ml; Sigma) was used as a counter stain; cells were then mounted and examined under the microscope (Olympus; BX51TR). Negative controls without primary antibodies were taken for all markers in the study.

Flow cytometry. For FACS analysis, hESCs were carefully dissected around the periphery of the colony followed by collagenase treatment. Briefly, single cell suspensions were washed with DPBS (Invitrogen) and fixed with 4% paraformaldehyde at 4°C for 30. The hESC was incubated with primary antibodies including SSEA-1, SSEA-4, and TRA-1-81 (Millipore) (Supplementary material; Table-1) for 1 h at room temperature. Thereafter, cells were washed twice with DPBS and incubated with secondary FITC-conjugated antibodies along with a separate mouse isotype control in the dark. FACS was performed on Guava EasyCyte Plus (Millipore) and data were analyzed by Cyto Soft v5.2 software.

Karyotyping by G-banding. HF-MSC and hESC were incubated in growth medium in presence of colcemid (Invitrogen) for 3–4 h at 37°C in a 5% CO_2 incubactor (Binder) to arrest cells at metaphase stage. After incubation cells were trypsinized and incubated with 0.075 M potassium chloride for 30 min at 37°C. Followed by hypotonic treatment, cells were fixed with 3:1 methanol/glacial acetic and dropped onto pre-cleaned chilled glass slides. Chromosome spreads were visualized by standard Giemsa-banding procedure. At least 20 metaphase spreads and five banded karyotypes were evaluated for chromosomal rearrangements.

Determining levels of extracellular proteins by biochemical assays. Supernatants of hESC and EB cultures were collected at important time points and estimated for levels of extracellular proteins by biochemical methods. In this study, secreted AFP and albumin concentrations were determined. The analysis of AFP was done using electrochemiluminescence immunoassay (ECLIA) with Roche Elecsys Cobas diagnostic kit. For albumin, bromocresol green was used as the anionic dye for binding with albumin which was standardized against the reference preparation on Roche/Hitachi Cobas-C system.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Results were analyzed by one-way ANOVA with Tukey's multiple comparison post-test for more than two groups. Differences were considered statistically significant when $P < 0.05$.

RESULTS

CHARACTERIZATION OF HUMAN FORESKIN-DERIVED CELLS REVEAL THEIR MESENCHYMAL STROMAL-LIKE FEATURES DURING IN VITRO PROPAGATION

Cells isolated from human foreskin tissues showed spindle-shaped morphology at early (P3) and late passages (P20) (Fig. 1A,B) and also displayed other characteristics typical of mesenchymal stromal cells including high proliferative capacity, expression of cell surface antigens, cytoskeletal proteins, transcription factors, and tri-lineage differentiation potential.

MARKER EXPRESSION

Flow cytometry analysis revealed that the expression pattern of key surface markers like CD73, CD166, CD105, CD44, CD90, Vimentin, SSEA-4 (positive), CD34, CD45, HLA-DR, 7-AAD (negative) (Fig. 1N–Z) is consistent and conforms to the minimum criteria for MSC as recommended by International Society for Cellular Therapy (ISCT) [Dominici et al., 2006]. Further, these cells stained positive for cytoskeletal proteins Vimentin (Fig. 1C–E), the embryonic cell surface antigen SSEA-4 (Fig. 1F–H) and Nestin (Fig. 1J). Interestingly, upon immunocytochemistry we found that these cells not only expressed Nestin but also co-expressed Vimentin (Fig. 1I–L). These findings were supported by our RT-PCR data which revealed upregulation of pluripotent markers including Oct-4, Nanog, Sox-2, Rex-1, and TDGF-1 compared to BM-MSC (Fig. 2A). Moreover presence of early stage lineage specific markers such as Nestin, B-III tubulin, HAND1, BMP-4, AFP, GATA-4, and HNF-3B were also found to be expressed in HF-MSC (Fig. 2A).

GROWTH KINETICS, CYTOGENETIC STABILITY, AND CELL CYCLE DISTRIBUTION

Fibroblast like cells isolated from samples 1 to 4 exhibited similar proliferation rate in culture, whereas sample five required additional time to attain confluence indicating slightly lower self-renewal capacity (data not shown). PD time of HF-MSC was found to be 30 h over the period of 10 passages (Fig. 1M) which was comparable to standard BM-MSC. However, not much difference was observed among various seeding densities. HF-MSCs exhibited stable normal karyotype at passage 4 which was conserved beyond 20 passages without any chromosomal aberrations (Fig. 2B,C). Further the DNA content of HF-MSC was analyzed in comparison with MEF wherein 85% of HF-MSCs were observed in G0/G1 phases compared to 64.3% of MEFs in the same phase; almost similar population of cells was found in S-phase for both the cell types. However, the percentage of MEF cells in G2/M phases (32.9%) increased when compared to HF-MSCs (12.48%) (Fig. 2J,K). It is clear that HF-MSC not only has a higher rate of cell proliferation compared to other cell types but also constitutes a higher proliferation of haploid cells undergoing cell division.

HF-MSCs EXHIBIT TRI-LINEAGE DIFFERENTIATION CAPACITY

The hallmark of MSC is its ability to differentiate into mesodermal lineage. We therefore undertook studies to induce differentiation of HF-MSC into adipogenic, chondrogenic, and osteogenic lineage by using standard MSC differentiation protocols. Although not extensive, adipogenic differentiation was observed after 3 weeks in culture, displayed by intracellular lipid accumulation verified by Oil Red O staining (Fig. 2D) as compared to non-induced controls. The chondrogenic potential of HF-MSC was confirmed by formation of sulfated proteoglycans verified by Alcian blue staining (Fig. 2E)

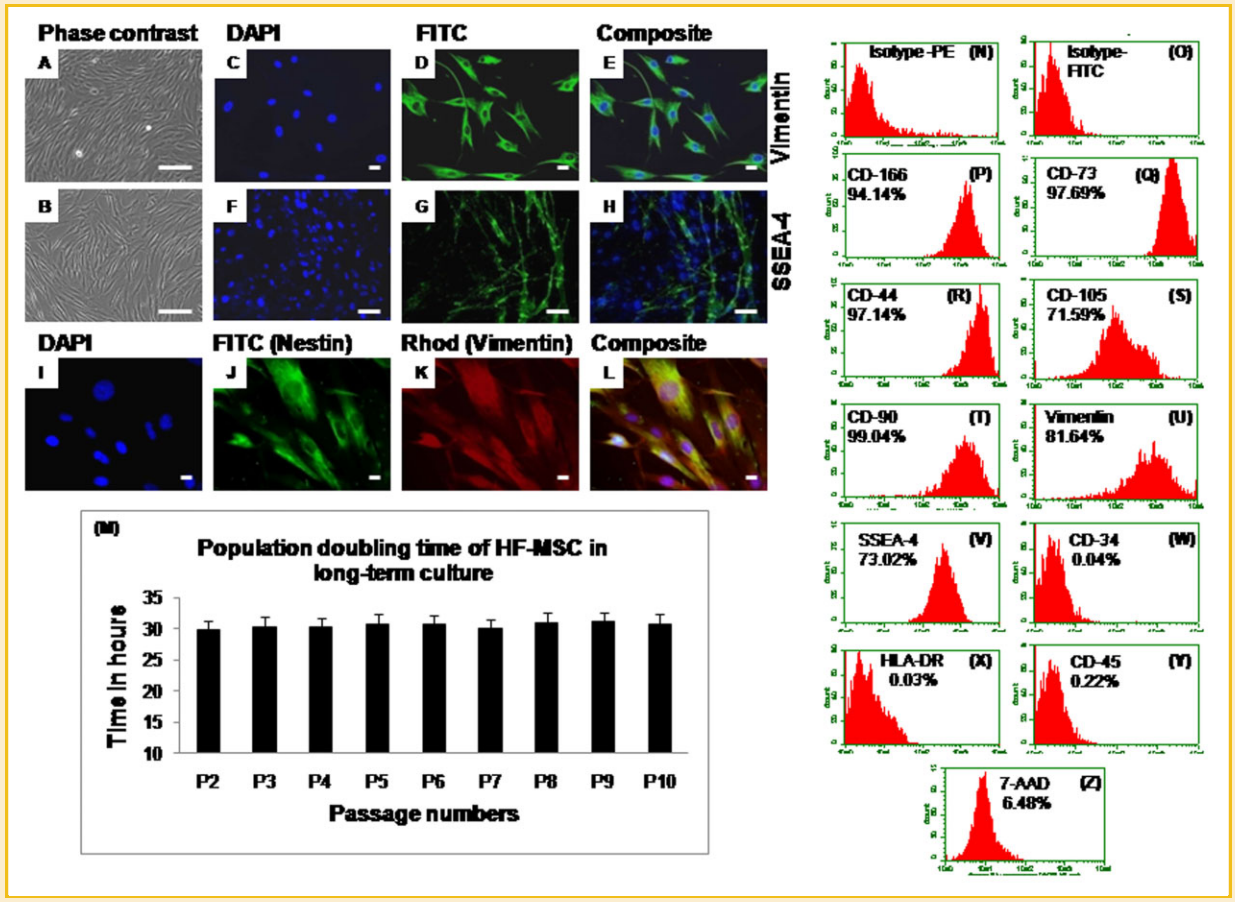

Fig. 1. Isolation, propagation and characterization of foreskin-derived mesenchymal-like stromal cells. Morphology of HF-MSC at early (P3) and late passages (P21) respectively (A,B). Immunofluorescence analysis of HF-MSC cultures using anti-vimentin (C–E), anti-SSEA-4 (F–H) antibodies. I–L: Co-expression of cytoskeletal markers nestin (FITC) and vimentin (Rhodamine). DAPI (4′,6-diamino-2-phenylindole) was used for each sample as a nuclear stain. M: Population doubling time of HF-MSC as determined over a period of 10 passages. Experiments were carried out in triplicates and data expressed as mean of standard deviation (SD). N–Z: Flow cytometry analysis demonstrates that human foreskin-derived cells express key markers associated with MSCs; percentage of positive cells is indicated for each antigen studies. These experiments were repeated at least three times and similar results were observed. Scale bars range from 50 to 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

as compared to non-induced controls after 28 days in culture. When subjected to osteogenic differentiation, HF-MSC underwent morphologic changes and formed cell piling up eventually demonstrating abundant amounts of calcium deposits typical of bone formation detected by von Kossa staining (Fig. 2F). All together, these data clearly demonstrate that HF-MSCs are easily differentiated into mesodermal lineage. Moreover HF-MSCs showed the formation of cell aggregates when they were cultured in hanging drop method (Fig. 2G–I).

HIGH CONCENTRATION OF MITOMYCIN C IS REQUIRED FOR HF-MSC FEEDER WHICH SUPPORTS LONG TERM PROPAGATION OF hESC

Initially we followed the conventional method and plated hESCs on HF-MSCs (as feeders) which were inactivated with 10 μg/ml mitomycin C for 2.5 h. However, we noticed that HF-MSC started growing after 5–6 days and eventually overwhelmed the cultures with hESC colonies getting engulfed inside the feeders and showing extensive differentiation (Fig. 3A,B). This clearly indicated that this method is inadequate for efficient inactivation of our newly derived HF-MSC. Hence we adopted different concentrations and regimes of mitomycin C treatment, seeding density and checked their capacity to sustain hESC proliferation. HF-MSC exposed to 15 μg/ml mitomycin C for 4 h with a seeding density of 1.5×10^5 cells/ml worked best in our hands in terms of supporting long term hESC growth. Even after complete inactivation of HF-MSC feeders, hESC required few passages to become accustomed on the new feeder layer since they were transferred from MEF (Fig. 3C). However, HUES-7 cells were quickly adapted onto HF-MSC feeders and thereafter demonstrated healthy morphology marked by high nucleus to cytoplasm ratio, well-defined boundaries, and compact colonies. Interestingly we have been successful in maintaining these hESCs in undifferentiated state (completely devoid of differentiation) for a period of 7–10 days (Fig. 3D), compared to hESCs grown on MEF showed signs of spontaneous differentiation right from day 5 onwards. Further, 60–70% viability could be obtained post-freeze-thaw of the same batch of hESCs.

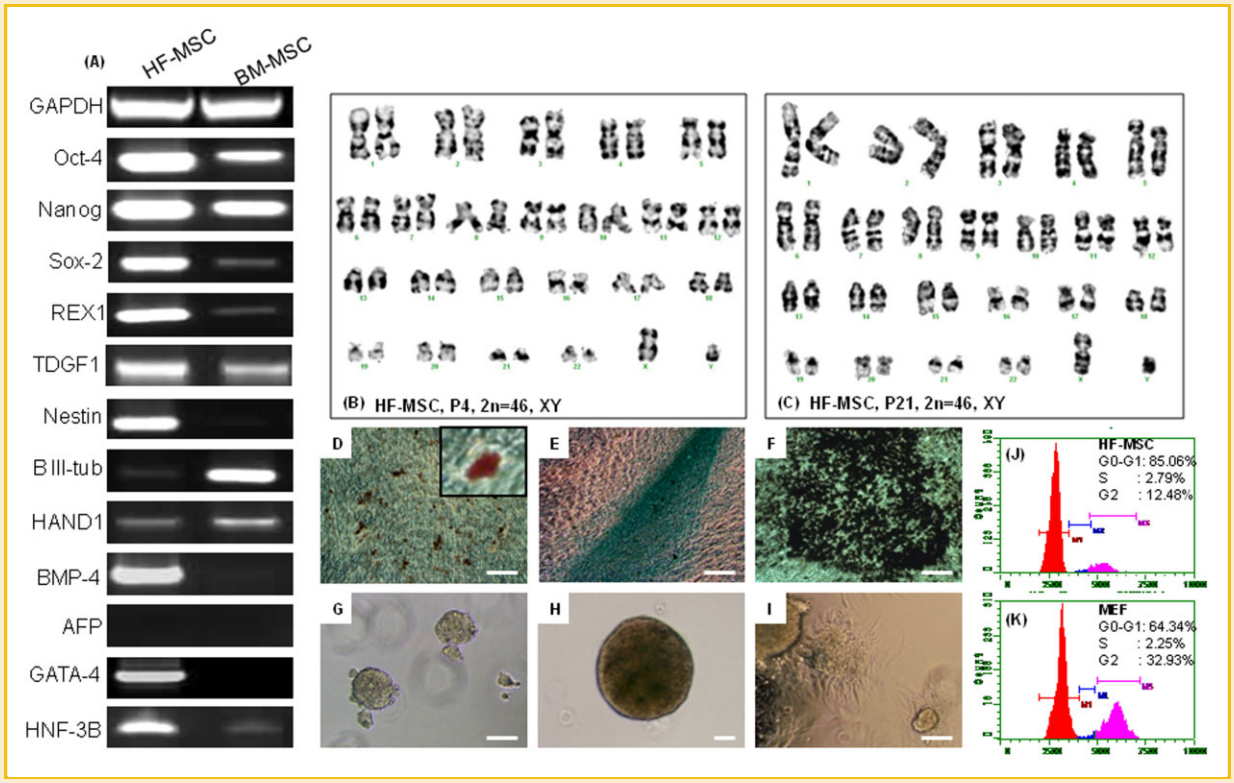

Fig. 2. In vitro differentiation potential of HF-MSC. RT-PCR analysis of HF-MSCs when compared to BM-MSCs shows upregulation of pluripotency and germ-layer specific markers (A). Normal karyotype of HF-MSC at passage 4 (B) and passage 21 (C) establishes the genetic stability of these cells over long-term propagation. HF-MSC upon induction showed multilineage differentiation potential. D: Adipogenic induction formed lipid droplets which were stained with Oil Red O. E: Chondrogenic induction was detected with Alcian blue staining for sulfated proteoglycans. F: Calcium deposits were formed as a result of guided osteogenic differentiation was stained with silver nitrate. In addition HF-MSC exhibited the formation of aggregates when cultivated in hanging drop containing (G) 20 μl and (H) 10 μl of cell suspension in medium; (I) further they retained their adherent property when re-plated on tissue culture coated dishes. Flow cytometry analysis of PI stained HF-MSC and MEF showed difference in population of cells in different stages of cell cycle (G1/S/G2) as indicative of their DNA content (J,K). Scale bars represent 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

ESTABLISHMENT OF PLURIPOTENCY BY EXPRESSION OF A CANDIDATE SET OF MOLECULAR, PROTEIN MARKERS, AND LONG TERM STABILITY

It was important to authenticate the quality of hESC that were grown on HF-MSC employing standard methods of characterization. We examined the expression of pluripotent stem cell markers at mRNA transcript and protein levels at every five passages; however the data reported belongs to passage 20. RT-PCR analysis confirmed unambiguous expression of a key set of pluripotent genes such as Oct-4, Nanog, Sox-2, Rex-1, hTERT, GDF-3, TDGF-1, Thy-1, Dppa-5, STELLAR, and ABCG-2 including tight junction molecules connexcin-45 and 43 in HUES-7 grown on HF-MSC in comparison to HUES-7 grown on MEF (Fig. 3U). Next, immunofluorescence studies showed the localization of transcription factors Oct-4, Nanog, and surface markers SSEA-4 and TRA-1-81 distributed throughout the hESC colonies (Fig. 3E–P). Tri-color immunostaining revealed that entire hESC colonies were positive for Oct-4 (green) and the margin of the colony was positive for vimentin (red) which indicates the presence of stromal cells towards the periphery of the colonies (Fig. 3Q–T). Further, the presence of cell surface markers by immunochemistry was confirmed by flow cytometry where we observed 83%, 84%, and 2.8% immunoreactivity against SSEA-4, TRA-1-81, and SSEA-1 (Fig. 4A–D). Importantly, HUES-7 cells after being expanded on HF-MSC retained their genetic stability witnessed by demonstration of stable karyotype for more than 30 passages (over 9–10 months; Fig. 4E,H). Moreover, cells collected for karyotype analysis at respective passages demonstrated morphological features typical of healthy hES cells (Fig. 4F,G).

DIFFERENTIATION CAPACITY OF HUES-7 GROWN ON HF-MSC IS CONGRUENT WITH ITS PROPENSITY TO FORM MESENDODERM LINEAGE IN VITRO

HUES-7 cell line maintained on HF-MSC feeders was differentiated through EB formation; only healthy EBs were cultured for 5, 10, 15, 20 days and harvested for lineage and tissue-specific marker expression analysis. Recently, we had reported that HUES-7 grown on MEF shows an intrinsic tendency towards the formation of mesoderm and endoderm lineage [Pal et al., 2009] compared to other cell lines. Therefore, we reasoned that HUES-7 grown on HF-MSCs when induced to differentiate in parallel to HUES-7 grown on MEF, followed by direct comparison of the gene and protein profiling data may be substantial for validation of our newly developed feeder. Here we have used a candidate set of five (5) markers for RT-PCR and three (3) markers for immunocytochemistry representing three germ

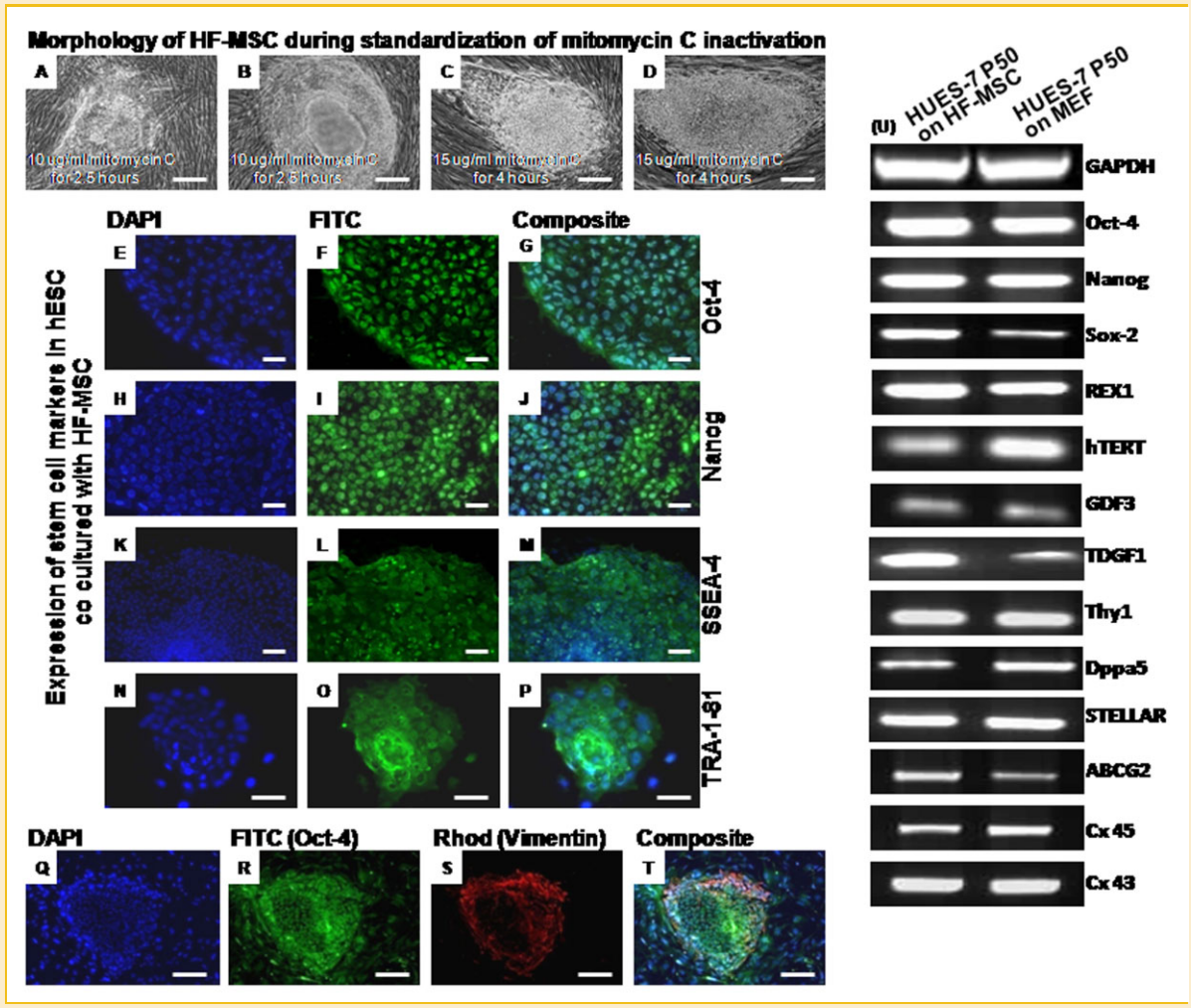

Fig. 3. HF-MSC enhances undifferentiated growth of hESC. Phase contrast microscopy of hESC at different stages during optimization of HF-MSC inactivation. A: hESC colony embedded within feeders indicating insufficient inactivation of HF-MSC; (B) hESC morphology showing thick opaque colony, crater formation and spontaneous differentiation on suboptimal HF-MSC feeders; (C) day 5 hESC colony showing prominent nucleoli and compactly arranged cells on optimized HF-MSC feeders; (D) typical morphology of hESC colony maintained up to day 8 in culture; it is noteworthy that the colony is organized in an elongated and elliptic shape which is characteristic of hESC grown on human feeders. hESC demonstrated uniform fluorescence distributed all over the colony when immunostained against (E–G) Oct-4, (H–J) Nanog, (K–M) SSEA-4, and (N–P) TRA-1-81 antibodies. Oct-4 and Nanog being transcription factors shows nuclear localization while SSEA-4 and TRA-1-81 being cell surface antigens demonstrates surface localization. Q–T: Co-expression of Oct-4 at the center and stromal marker vimentin towards the periphery of a hESC colony indicates the role of HF-MSC in maintaining undifferentiated status of hESC cultures. DAPI was used as a counter stain; green and red color represents FITC and rhodamine conjugates respectively. Scale bars range from 50 to 200 μm. U: RT-PCR analysis of hESC grown on HF-MSC in comparison with hESC grown on MEF showed similar levels in expression of Oct-4, Nanog, Sox-2, Rex1, hTERT, GDF-3, TDGF-1, Thy1, Dppa5, STELLAR, ABCG2, Connexin 45, and Connexin 43 (top to bottom), thereby establishing the quality of hESC grown on these new feeders. GAPDH was used as a housekeeping gene control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

layers including ecto-, meso-, and endo-derm. RT-PCR analysis showed a consistently strong expression of all the lineage- and tissue-specific markers corresponding to early, middle and late stages of differentiation including nestin, β-III tubulin, Nurr1, Keratin-15, NFH (ectoderm); brachyury, MEF-2, c-actin, GATA-2, Myf-5 (mesoderm); AFP, Sox-17, HNF-3β, HNF-4α, cytokeratin-19 (endoderm; Fig. 5A). The specific markers get expressed after 5 days and sustain their expression throughout 20 days in culture except brachyury (Fig. 5A) which showed a significant down regulation at day 20. Moreover the highest expression for most of these markers was detected at day 15 EBs. In addition, quantitative real time PCR analysis supported our semi-quantitative RT-PCR results reassuring the upregulation of genes associated with early meso- and endoderm lineage including c-actin and Sox-17 (Fig. 5B). Furthermore, indirect immunofluorescence demonstrated positive staining for antibodies against β-III-tubulin, GFAP, MAP-2, desmin, brachyury, GATA-4, AFP, Sox-17, and cytokeratin-18 antigens (Fig. 5C1–K3). Although these data confirmed the presence of all three germ layers in the EBs generated from HUES-7 cells co-cultured with HF-MSC; the expression of mesendoderm markers like GATA-2, c-actin, desmin, AFP, Sox-17, HNF-3β, HNF-4α, and cytokeratin-18/19 were stronger than ectoderm markers such as Nurr1, NFH, GFAP, and MAP-2 (Fig. 5). This finding is concomitant to our earlier report and thereby endorses our hypothesis. As a third criterion, we

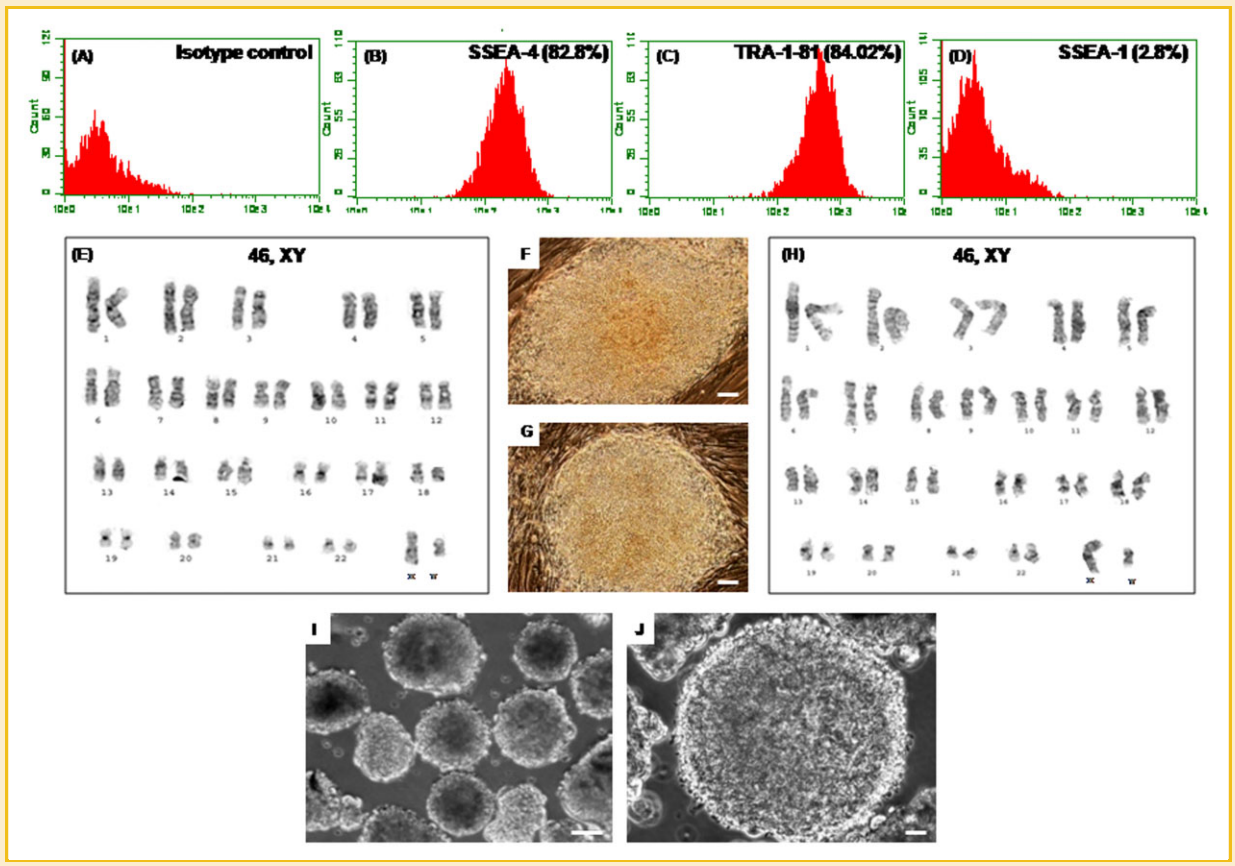

Fig. 4. Long-term propagation of hESC on HF-MSC feeder helps in retaining their pluripotent characteristics. Immunophenotyping of undifferentiated hESC at day 5 confirms the strong reactivity to (B) SSEA-4, (C) Tra-1-81, and absence of (D) SSEA-1. E,H: G-banding analysis depicting normal euploid karyotype of hESC cultured on HF-MSC at early passage 10 and late passage 30. F,G: Morphology of the hESC which was subjected to karyotype analysis at their respective passages. Long-term expansion of hESC on HF-MSC promotes in vitro differentiation via EB formation. I,J: Phase contrast micrograph of 14-day-old EBs at lower and high magnification. Scale bars range from 50 to 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

measured the levels of extracellular AFP and albumin secreted by liver cells. We observed increased levels of AFP in culture supernatants right from days 10 to 20 when compared with undifferentiated hESCs where albumin levels remained unchanged over time (Table I).

DISCUSSION

It has been reported that MSCs are present in almost all the organs of the body. The present investigation deals with the selective propagation of MSCs derived from human foreskin and their utility in supporting long term proliferation and differentiation of hESC. It is known that human foreskin-derived cells are a heterogeneous population of various progenitors inclusive of fibroblasts as well as skin resident mesenchymal stromal cells. Hence we adopted a twofold approach to isolate pure population of MSCs from the foreskin. In the first stage, we separated CD73 positive cells by flow cytometry and cultured them in vitro. In the second step we used high glucose media 4.5 g/L (DMEM) to propagate the CD73 positive cells to achieve selective growth of MSCs over fibroblasts. We could selectively grow the desired MSC population by using high glucose containing media thereby withering out fibroblasts without hampering the proliferation of MSCs. High glucose concentrations in media are known to have detrimental effects on different cell types including human skin fibroblasts [Lan et al., 2008]. However, high glucose contents in culture media do not affect human MSC proliferation [Weil et al., 2009]. Further these cells showed higher resistance towards inactivation by mytomycin C treatment when compared to fibroblasts which was in agreement with the earlier report [Nieto et al., 2007]. Therefore, we propose that mytomycin C treatment could be prospective criteria to distinguish between fibroblasts and MSCs.

Morphology, growth kinetics and cell surface marker profile data (Figs. 1 and 2) clearly demonstrated that we could successfully isolate and propagate MSCs from foreskin tissues as evidenced by the presence of CD105, CD166, CD73, CD44, CD90, SSEA4 which was similar to BM-MSC [Shih et al., 2005]. The mesenchymal phenotype of these cells was further strengthened by the expression of vimentin and nestin commonly found in BM-MSCs [Huang et al., 2010]. Further these HF-MSCs exhibited in vitro differentiation potential into adipocytes, chondrocytes, and oesteocytes (Fig. 2). Although human fibroblasts share some similarities in marker expression with HF-MSCs, neither they were capable of in vitro

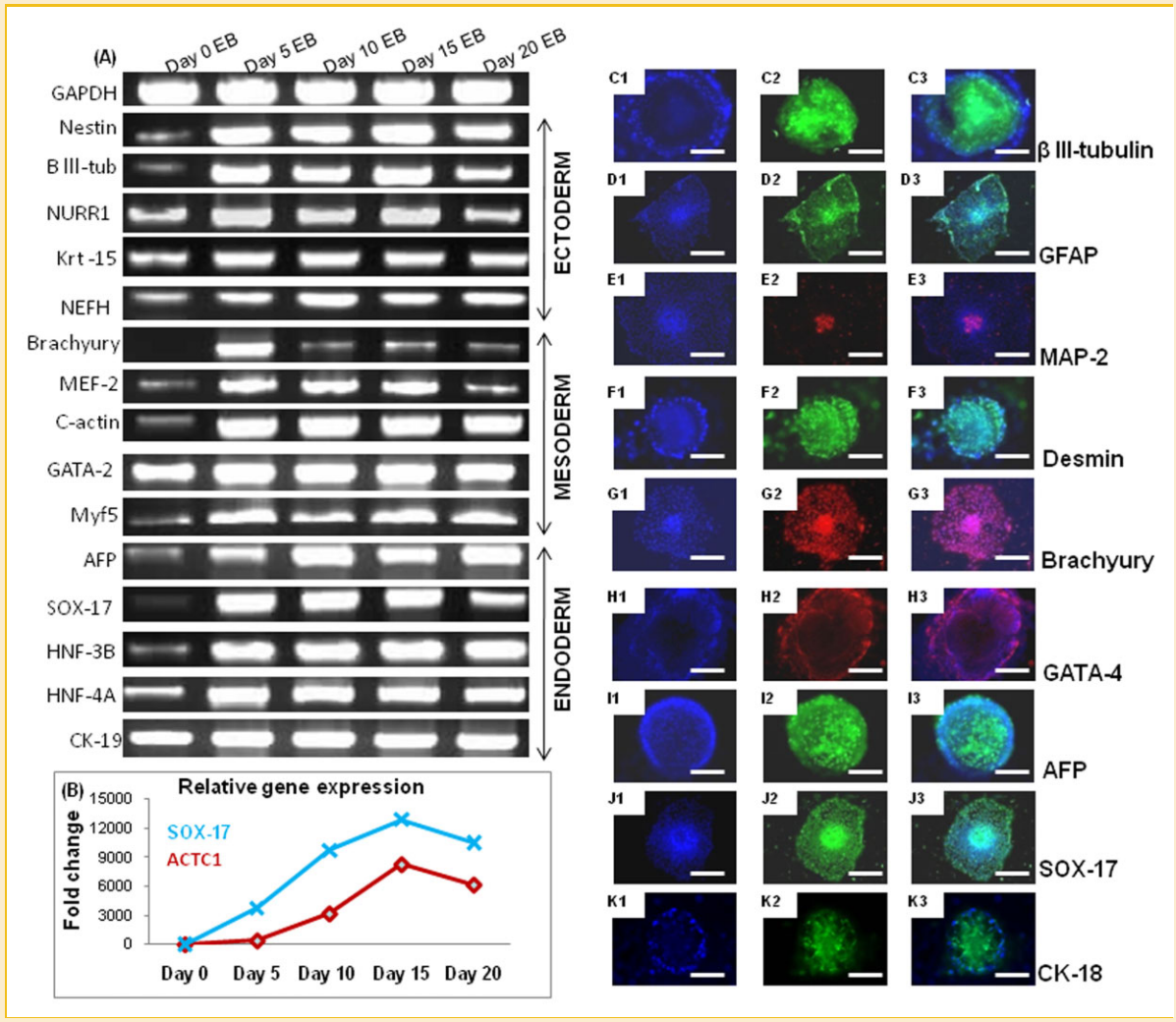

Fig. 5. hESC co-cultured on HF-MSC exhibits an increased potential for germ-lineage specific differentiation. A comprehensive gene expression profiling of differentiated hESCs derived from HUES-7 by RT-PCR. A: Days 0, 5, 10, 15, and 20 EBs showing differential expression of a candidate set of lineage-specific markers including nestin, β-III-tubulin, Nurr-1, Keratin 15, NFH (ectoderm); Brachyury, MEF-2, cardiac-actin, GATA-2, Myf5 (mesoderm); AFP, SOX-17, HNF-3β, HNF-4α, cytokeratin-19 (endoderm). Owing to the propensity of HUES-7 towards mesendoderm, (B) the expression of c-actin and SOX-17 gene transcripts in these cells were further evaluated by real-time quantitative RT-PCR assays; the maximum expression levels were observed at day 15 EB's for both these markers. Immunological characterization of HUES-7 EBs with a battery of markers closely associated with ecto-, meso-, and endo-derm. C1–C3: Fourteen-day EBs showing positive immunofluorescence for β III-tubulin; (D1–D3) GFAP, (E1–E3) MAP-2 (all ectoderm); (F1–F3) Desmin, (G1–G3) Brachyury, (H1–H3) GATA-4 (all mesoderm); (I1–I3) AFP, (J1–J3) Sox-17, and (K1–K3) Cytokeratin-18 (all endoderm). Scale bars represent 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

differentiation nor could proliferate more than 6–8 passages. On the other hand HF-MSCs could be easily propagated for 20–25 passages and retained their characteristics over the time (data not shown). Finally HF-MSCs also showed the formation of aggregates (floating spheres) that is characteristic of early stage stem cells which is in line with previous reports [Toma et al., 2005; Riekstina et al., 2008]. Interestingly our data is supported by the recent findings by Huang et al. [2010] showing multilineage differentiation potential of fibroblast-like stromal cells derived from human foreskin. We predict that these MSCs can be used as an alternative source to BM-MSCs for biological dressings and male genital dysfunctions purely based on their origin.

Upon confirming the MSC nature of these cells, we further exploited the potential of HF-MSCs for growing hESC in a xeno-free setting. Amplified hESC on HF-MSCs displayed unique morphology, molecular marker expression such as Oct-4, Nanog, Sox-2, hTERT, Rex-1, TDGF-1, Thy-1, Dppa-5, GDF-3, STELLAR, ABCG-2, connexcin-43 and 45 and cell surface markers SSEA-4, TRA-1-81 as good as hESC cultured on MEFs. hESCs co-cultured with HF-MSC exhibited a typical morphology of a high nucleus-to-cytoplasm ratio, prominent nucleoli and close spacing between cells (Figs. 3–5). Further, these hESC could survive repeated freeze/thaw cycles and showed in vitro differentiation potential into derivates from the three embryonic germ layers via embryoid body (EB) formation thus demonstrating successful preservation of stemness.

There are reports showing the propagation of human feeders such as fetal liver stromal cells, adult bone marrow cells and fetal muscle and adult skin cells having the ability to support hESC growth

TABLE I. Secretion Levels of Extracellular Proteins From HUES-7 Derived EBs on Days 5, 10, 15, and 20 Compared to Undifferentiated hESC Along With Their Reference Values

Protein	Undiff. hESC	Day 5 EB	Day 10 EB	Day 15 EB	Day 20 EB	Measuring units	Reference range
α-Feto protein	<0.54	1.59	1,840	1,185	548.7	ng/dl	Adult: <15 ng/dl; Fetal: 20–40 ng/dl
Albumin	1	0.9	1	1	1	gm/dl	3–5 g/dl

[Cheng et al., 2003; Richards et al., 2003; Ji et al., 2009]. On this background our choice of foreskin derived MSCs not only eases ethical issues but also supports the maintenance of hESC beyond 30 passages without compromising their pluripotency (Figs. 3–5). The hESCs maintained on HF-MSC retained undifferentiated status for 8–10 days compared to 4–5 days conventional culture methods and for several passages (>30). This could be due to the fact that hESCs respond by generating their own support cells (niche) and these support cells in turn permit the proliferation of hESC. Since paracrine effects and cross-talk between these cells are critical elements for their survival, hESC niche could be instrumental to preserve their self renewal capacity for longer time points [Jones and Wagers, 2008; Stewart et al., 2008]. Moreover, we have shown earlier that FGF2 expressing germ layer-derived fibroblast cells (GLDF) support the long term pluripotency of hESCs without exogenous FGF2 [Saxena et al., 2008].

There are several advantages of using MSCs as feeders over human fibroblasts albeit they are slightly difficult to handle compared to fibroblasts. First, MSCs can be cultured for more than 25 passages without any alteration in their characteristics, whereas the fibroblasts can be propagated steadily not more than 4–5 passages. Therefore, MSC as feeder layers is cost-effective and becomes economically sustainable in maintaining routine cultures of hESC. Second, MSCs secrete essential growth factors like bFGF and IGF [Gnecchi et al., 2008] which are essential to maintain self renewal and pluripotency of hESC. Third, hESC media has been shown to be effective in maintaining MSCs [Rajala et al., 2010] and therefore can be employed for regular culturing of both the cell types (MSC and hESC) which could make it easier than using two different media for maintenance of feeders and hESC cultures separately. Fourth, MSCs do not evoke an immune reaction post-transplantation since they do not generate alloreactive T lymphocytes in culture [Koç and Lazarus, 2001]; hence this property could certainly be exploited for therapeutic application of hESCs grown on MSC.

Based on our present data we envisage several advantages of hESC cocultured with HF-MSCs over other traditional feeders. (a) HF-MSCs offer potentially low bioburden because they are not automatically exposed to the external environment and hence to adventitious agents; (b) foreskin samples are easily accessible by a simple noninvasive procedure over complicated surgical interventions; (c) HF-MSC conditioned medium can be exploited for feeder-free growth of hESCs, thus avoiding potential viral transfer from the MEF-conditioned medium; (d) HF-MSC culture is simple and faster compared to other sources of MSCs (data not shown) and last but not the least (e) avoids the sacrifice of pregnant animals for MEF.

In conclusion, we have developed a robust protocol to selectively isolate MSCs of human origin from discarded foreskin and illustrated their utility as a specific and unique co-culture system for long term proliferation of hESCs. This co-culture system successfully prolongs growth of undifferentiated hESC with a stable karyotype, with intact in vitro differentiation potential. Currently we are studying the role of the growth factors and cytokines secreted by HF-MSCs which are potentially responsible for successful maintenance of the hESC.

ACKNOWLEDGMENTS

The work was supported by Institute for Medical Research (IMR), Ministry of Health, Malaysian research Grant (JPP-IMR-08-019) under Stempeutics Research Malaysia Sdn Bhd. and IMR joint research collaboration. The authors would also like to thank the Director General of Health Malaysia and the Director of the Institute for Medical Research for encouraging and facilitating the publication of this paper.

REFERENCES

Blanpain C, Fuchs E. 2006. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 22:339–373.

Cheng L, Hammond H, Ye Z, Zhan X, Dravid G. 2003. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells 21:131–142.

Cortes JL. 2009. Mesenchymal stem cells facilitate the derivation of human embryonic stem cells from cryopreserved poor-quality embryos. Hum Reprod 24:1844–1851.

Crigler L, Kazhanie A, Yoon TJ, Zakhari J, Anders J, Taylor B, Virador VM. 2007. Isolation of a mesenchymal cell population from murine dermis that contains progenitors of multiple cell lineages. FASEB J 21:2050–2063.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.

Durig J, Rosenthal C, Halfmeyer K, Wiemann M, Novotny J, Bingmann D, Duhrsen U, Schirrmacher K. 2000. Intercellular communication between bone marrow stromal cells and CD34+ haematopoietic progenitor cells is mediated by connexin 43-type gap junctions. Br J Haematol 111(2):416–425.

Fernandes KJ, McKenzie IA, Mill P. 2004. A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6:1082–1093.

Fu X, Li J, Sun X, Sun T, Sheng Z. 2005. Epidermal stem cells are the source of sweat glands in human fetal skin: Evidence of synergetic development of stem cells, sweat glands, growth factors, and matrix metalloproteinases. Wound Repair Regen 13:102.

Gnecchi M, Zhang Z, Ni A, Dzau VJ. 2008. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103(11):1204–1219.

Haniffa MA, Collin MP, Buckley CD, Dazzi F. 2009. Mesenchymal stem cells: The fibroblasts' new clothes. Hematologica 94:258–263.

Huang HI, Chen SK, Ling QD, Chien CC, Liu HT, Chan SH. 2010. Multilineage differentiation potential of fibroblast-like stromal cells derived from human skin. Tissue Eng Part A 16(5):1491–1501.

Ji L, Liu YX, Yang C, Yue W, Shi SS, Bai CX, Xi JF, Nan X, Pei XT. 2009. Self-renewal and pluripotency is maintained in human embryonic stem cells by co-culture with human fetal liver stromal cells expressing hypoxia inducible factor 1α. J Cell Physiol 22:54–66.

Jones DL, Wagers AJ. 2008. No place like home: Anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 9:11–21.

Kang JH, Wei YM, Zheng RL. 2001. Effects of diethyldithiocarbamate on proliferation, redifferention, and apoptosis in human hepatoma cells. Acta Pharmacol Sin 22(9):785–792.

Koç ON, Lazarus HM. 2001. Mesenchymal stem cells: Heading into the clinic. Bone Marrow Transplant 27(3):235–239.

Lan CC, Liu IH, Fang AH, Wen CH, Wu CS. 2008. Hyperglycaemic conditions decrease cultured keratinocyte mobility: Implications for impaired wound healing in patients with diabetes. Br J Dermatol 159(5):1103–1115.

Lavoie JF, Biernaskie JA, Chen Y, Bagli D, Alman B, Kaplan DR, Miller FD. 2009. Skin-derived precursors differentiate into skeletogenic cell types and contribute to bone repair. Stem Cells Dev 18:893–906.

Lee JB, Song JM, Lee JE, Park JH, Kim SJ, Kang SM, Kwon JN, Kim MK, Roh SI, Yoon HS. 2004. Available human feeder cells for the maintenance of human embryonic stem cells. Reproduction 128(6):727–735.

Montes R, Ligero G, Sanchez L, Catalina P, de la Cueva T, Nieto A, Melen GJ, Rubio R, García-Castro J, Bueno C, Menendez P. 2009. Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: Distinct requirements for TGF-beta and IGF-II. Cell Res 19(6):698–709.

Nieto A, Cabrera CM, Catalina P, Cobo F, Barnie A, Cortés JL, Barroso del Jesus A, Montes R, Concha A. 2007. Effect of mitomycin-C on human foreskin fibroblasts used as feeders in human embryonic stem cells: Immunocytochemistry MIB1 score and DNA ploidy and apoptosis evaluated by flow cytometry. Cell Biol Int 31:269–278.

Odorico JS, Kaufman DS, Thomson JA. 2001. Concise review: Multilineage differentiation from human embryonic stem cell lines. Stem Cells 19:193–204.

Ohyama M, Terunuma A, Tock CL. 2006. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 116:249–260.

Pal R, Totey S, Mamidi MK, Bhat VS, Totey S. 2009. Propensity of human embryonic stem cell lines during early stage of lineage specification controls their terminal differentiation into mature cell types. Exp Biol Med 234:1230–1243.

Raimondi C, Cortesi E, Gianni W, Gazzaniga P. 2010. Cancer stem cells and epithelial-mesenchymal transition: Revisiting minimal residual disease. Curr Cancer Drug Targets 10(5):496–508.

Rajala K, Lindroos B, Hussein SM, Lappalainen RS, Pekkanen-Mattila M, Inzunza J, Rozell B, Miettinen S, Narkilahti S, Kerkela E, Aalto-Setala K, Otonkoski T, Suuronen R, Hovatta O, Skottman H. 2010. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS ONE 5(4): e10246.

Richards M, Fong CY, Chan WK, Wong PC, Bongso A. 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 20:933–936.

Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A. 2003. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells 21:546–556.

Riekstina U, Muceniece R, Cakstina I, Muiznieks I, Ancans J. 2008. Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. Cytotechnology 58:153–162.

Saxena S, Hanwate M, Deb K, Sharma V, Totey S. 2008. FGF2 secreting human fibroblast feeder cells: A novel culture system for human embryonic stem cells. Mol Reprod Dev 75:1523–1532.

Shih DT, Lee DC, Chen SC, Tsai RY, Huang CT, Tsai CC, Shen EY, Chiu WT. 2005. Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. Stem Cells 23:1012–1020.

Stewart MH, Bendall SC, Bhatia M. 2008. Deconstructing human embryonic stem cell cultures: Niche regulation of self-renewal and pluripotency. J Mol Med 86:875–886.

Tiede S, Kloepper JE, Bodo E, Tiwari S, Kruse Paus R. 2007. Hair follicle stem cells: Walking the maze. Eur J Cell Biol 86:355–376.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784.

Toma JG, McKenzie IA, Bagli SD. 2005. Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23:727–737.

Weil BR, Abarbanell AM, Herrmann JL, Wang Y, Meldrum DR. 2009. High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. Am J Physiol Regul Integr Comp Physiol 296:1735–1743.

Xiao S, Zhu S, Ma B, Xia ZF, Yang J, Wang G. 2008. A new system for cultivation of human keratinocytes on acellular dermal matrix substitute with the use of human fibroblast feeder layer. Cells Tissues Organs 187(2):123–130.

Zhu WW, Li N, Wang F, Fu LL, Xu YL, Guan YQ, Zhang Y. 2009. Different types of feeder cells for maintenance of human embryonic stem cells. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 31(4):468–472.