Human Menstrual Blood-Derived Mesenchymal Cells as New Human Feeder Layer System for Human Embryonic Stem Cells

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Human embryonic stem cells (hESCs) in general require coculture with feeder layers in order to remain undifferentiated. However, the use of animal-derived feeder layers is incompatible with the clinical setting. The objective of this work was to investigate whether human menstrual blood-derived mesenchymal cells (MBMCs) can substitute mouse embryonic fibroblasts (MEFs) as a feeder layer for H9-hESCs. Both feeder cell types were isolated and cultured in DMEM F-12 and high glucose DMEM, respectively. After three passages, they were inactivated with mitomycin C. To test MBMC feeder layer capacity, hESCs were grown over MBMCs and MEFs under standard conditions. hESC growth, proliferation, survival, and maintenance of the undifferentiated state were evaluated. hESCs grown over MBMCs preserved their undifferentiated state presenting standard morphology, expressing alkaline phosphatase, transcription factors *OCT3/4*, *SOX2*, and *NANOG* by RT-PCR and SSEA-4 and OCT3/4 by immunofluorescence assays. It is noteworthy that none of the feeder cells expressed these proteins. The average colony size of the hESCs on MBMCs was higher when compared to MEFs ($p < 0.05$; mean $\pm$ SD, $n = 3$). Growth factor analysis revealed amplification of the transcripts for *FGF-2*, *BMP4*, *TGF- β* , *VEGF*, and *PEDF* by RT-PCR in MBMCs and MEFs before and after inactivation. Furthermore, similar embryoid body formation, size, and morphology were observed in both feeder layers. In addition, EBs expressed marker genes for the three germ layers cultured on both feeder cells. In conclusion, MBMCs are able to maintain hESCs in an undifferentiated state with comparable efficiency to MEFs. Therefore, MBMCs are a suitable alternative to animal-derived feeder layers for growing hESCs.

Key words: Human embryonic stem cells (hESCs); Human feeder layer; Human menstrual blood-derived mesenchymal cells; Undifferentiated state

INTRODUCTION

Human embryonic stem cells (hESCs), derived from the blastocyst inner cell mass (ICM), were first established and cultured on mitotically inactivated primary mouse embryonic fibroblast (MEF) feeder layers by Thomson's group in 1998 (32). These cells present a continuous proliferation in an undifferentiated state resulting in an unlimited amount of cells, which have the potential to differentiate toward any type of cell in the human body. These characteristics make hESCs good candidates for cell-based therapies.

Although the use of MEFs as a feeder layer has been shown to be efficient and support the unlimited growth of hESCs, the use of animal-derived feeder cells is associated with xenocontamination, such as pathogen transmission and viral infection, and therefore is incompatible with the clinical use of the cells (2,9,14,17,24,29,31,36). To overcome these disadvantages, some alternatives have

been tested to efficiently culture hESCs. The use of matrices such as Matrigel™ (34), laminin (34), fibronectin (3), type I collagen (16), vitronectin (7), E-cadherin (26), and synthetic polymer (27) has been reported. However, feeder layer-free culture conditions can lead to genetic and epigenetic changes (10). In this scenario, feeder layer cells originating from human fetal and adult tissues including human fetal skin fibroblasts (29), fallopian tube epithelial cells (30), adult marrow cells (8), foreskin fibroblasts (2,13), adult uterine endometrial cells, adult breast parenchymal cells, embryonic fibroblasts (21), placenta cells (17,23), umbilical cord blood (35), umbilical cord stromal cells (9), amniotic mesenchymal cells (36), and bone marrow stromal cells (20) have been tested. However, obtaining these cells frequently is made difficult due to the invasiveness of collection procedures, ethical, and/or practical issues. Thus, the human menstrual blood has emerged as a useful source for feeder cells.

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Menstrual blood is derived from the uterine tissue. This tissue is prepared for embryo implantation, suggesting that the menstrual blood-derived cells might provide a natural environment for in vitro culture of hESCs (1,18). In addition, menstrual blood cells are widely available (12 times a year from a woman during child-bearing years), easily obtained by noninvasive procedure (24,28), possess capability of long-term proliferation (24,28), and are compatible with clinical application of hESC-derived cells.

In this study, we tested the use of human menstrual blood-derived mesenchymal cells (MBMCs) as a new feeder layer for supporting the unlimited growth and maintenance of pluripotency of undifferentiated hESC line H9 compared to MEF feeder layer standard conditions.

MATERIALS AND METHODS

Isolation and Culture of MEFs

This study was approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol - 026) and was carried out in compliance with the National Institute of Health policies.

Mice were obtained from the Institute of Biophysics Carlos Chagas Filho (IBCCF, Rio de Janeiro, Brazil). Animals were housed at controlled temperature (23°C) with daily exposure to a 12:12 light–dark cycle.

MEFs were isolated from embryos obtained from pregnant C57Bl/6 mice at embryonic days 12 to 14. Head, visceral organs, and extremities were removed. The remaining tissue was cut into small pieces and incubated in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Gaithersburg, MD, USA) with 0.25% of the trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Sigma Chemical Co., St. Louis, MO, USA) for dissociation. After the enzymatic treatment, the fibroblasts were incubated in DMEM/F-12 supplemented with 20% (v/v) fetal bovine serum (FBS) (Cutilab, Campinas SP, Brazil), 2 mM L-glutamine (Sigma), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco) and cultured at 37°C, 5% CO₂, and 95% humidity. In the third passage, MEFs were plated onto 1.0% (v/v) gelatin-coated (Sigma) six-well plates (35 mm; Corning, São Paulo, SP, Brazil) at 1 × 10⁵ cells/well. These cells were mitotically inactivated using 10 µg/ml mitomycin C (Sigma) for 3 h, washed three times with phosphate-buffered saline (PBS; LGC Biotecnologia, Cotia, SP, Brazil), and then used as feeder cells.

Isolation and Culture of MBMCs

Menstrual blood was collected from young healthy women, aged from 18 to 26 years. The collection protocol was approved by the Ethics Committee of the Clementino Fraga Filho University Hospital (HUCFF) (Protocol - 056/09). The material was subjected to processing by Ficoll Histopaque® 1077 (Sigma) (25), and the cells were isolated and cultured in high (4.5 g/L) glucose

Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C, 5% CO₂, and 95% humidity. In the third passage, human MBMCs were plated onto 1.0% (v/v) gelatin-coated six-well plates at 1 × 10⁵ cells/well (35 mm). These cells were mitotically inactivated using 10 µg/ml mitomycin C (Sigma) for 3 h, washed three times with PBS, and then used as feeder cells.

hESC Culture on MEF and MBMC Feeder Layers

The female hESC line H9 (32) was kindly donated to Federal University of Rio de Janeiro by Dr. James Thomson (Morgridge Institute for Research, Madison, WI, USA). At 56 passages, all the colonies of H9-hESCs, plated in 35-mm dishes (Corning), were transferred onto the two above-mentioned inactivated feeder cell layers (MBMCs and MEFs) by mechanical harvesting with a needle under a microscope (Olympus, São Paulo-SP, Brazil) and were then cultured in DMEM/F-12 supplemented with 20% (v/v) knockout serum replacement (KSR; Gibco), 4 mM L-glutamine (Sigma), 55 µg/ml gentamicin (Schering-Plough, Kenilworth, NJ, USA), 0.1 mM β-mercaptoethanol (Gibco), 1% (v/v) nonessential amino acids (Gibco), and 10 ng/ml basic fibroblast growth factor (bFGF; Gibco) at 37°C, 5% CO₂, and 95% humidity. The cells were passaged every 5 days by mechanical dissociation under microscope. When the passages were carried out, all hESC colonies were transferred from one to two wells (35 mm) containing inactivated feeder cells. Culture media was changed every day.

Average Colony Size of hESCs

Ten random colonies cultured on feeder layers at the first (P1) and fourth (P4) passages were measured from the second to the fourth day after plating ($n=3$). A total of 180 colony images were obtained by phase-contrast optic microscopy (Olympus). Each colony was outlined, and the size was calculated using ImageJ (NIH, Bethesda, MD, USA). Calibration and conversion of the unit pixel pattern to µm² was carried out. The images were obtained at the same time throughout the experiment.

Embryoid Body Formation

Embryoid bodies (EBs) were generated from cultured hESCs at the fourth passage. The hESCs were dissociated by enzymatic digestion (TrypLE™, Gibco), further broken into small clumps using 1-ml pipette tips, and cultured in suspension in 60-mm plates (Falcon, São Paulo-SP, Brazil) coated with poly 2-hydroxyethyl methacrylate (Sigma). The EBs were grown in DMEM/F-12 supplemented with 20% (v/v) KSR, 4 mM L-glutamine, 55 µg/ml gentamicin, 0.1 mM β-mercaptoethanol, and 1% (v/v) nonessential amino acids. After 3 days, EBs were collected

Table 1. Primers Used for RT-PCR Assays to Analyze Undifferentiated H9-hESCs

Gene Product	Sense Primer Sequence Orientation: 5'–3'	Antisense Primer Sequence Orientation: 3'–5'	Size (Base Pairs)
OCT3/4	AGCCTGAGGGCGAAGCAGGA	CCCCAGGGTGAGCCCCACAT	236
SOX2	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA	126
NANOG	CAGCCCTGATTCTTCCACCAGTCCC	TGGAAGGTTCCCAGTCGGGTTTACC	391
GAPDH	ACCATGGGGAAGGTGAAGGT	CATGGGTGGAATCATATTGG	163

OCT3/4, octamer-binding transcription factor 3/4; SOX2, sex-determining region Y box 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and transferred to 2% (v/v) gelatin-coated dishes for two more weeks and then analyzed by RT-PCR.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the cells using RNeasy® Mini Kit (Qiagen, São Paulo-SP, Brazil) following the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA using random primers and High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. In addition, a sample free of reverse transcriptase (RT-) was included for detecting contamination of genomic DNA. Specific sense and antisense primers were designed to remove the introns and to eliminate possible contamination of genomic DNA. The mRNAs of β -actin or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were used as internal controls of amplification. The sequences of primers and size of expected products are shown in Tables 1 and 2. Aliquots (1 μ l) of each cDNA sample were amplified in a Peltier Thermal Cycler PTC-200 (MJ Research, Waltham, MA, USA) in a 20- μ l reaction mixture containing 1 $\times$ PCR Buffer (Promega, São Paulo SP, Brazil), 2.5 mM of each $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 mM each of sense and antisense primers, and 1.25 units of Go Taq® DNA Polymerase (Promega). The PCR program consisted of denaturation at 95°C for 5 min,

30 cycles of denaturation at 95°C for 1 min, annealing at 56–62°C (Table 3) for 1 min, and extension at 72°C for 1 min, followed by a final extension of 72°C for 10 min. The PCR products were analyzed on a 2% agarose gel (Sigma) and revealed using ethidium bromide (Sigma).

Real-Time Polymerase Chain Reaction

Real-time RT-PCR analysis was performed on an ABI Prism 7500 Real-time PCR system (Applied Biosystems) using the Maxima SYBR Green qPCR Master Mix (2 $\times$) (Thermo Scientific, Waltham, MA, USA). The expression value of each gene was normalized against the amount of *GAPDH* and calculated by the $2^{-\Delta\Delta Ct}$ method. The expression level of each gene in MEFs was used as control. The normalized expression values for all samples were averaged, and average fold change was determined. Details of the primers used for quantitative PCR are in Table 4.

Alkaline Phosphatase Activity

To stain undifferentiated cells, a kit for alkaline phosphatase detection (Millipore, São Paulo-SP, Brazil) was used. hESCs at fourth passage were fixed with 4% (v/v) paraformaldehyde (Vetec Química Fina Ltda., Duque de Caxias, Rio de Janeiro, Brazil) for 2 min at room temperature, permeabilized with 0.5% (v/v) Triton X-100 (Sigma), and incubated with a solution containing Fast Red Violet (0.8 g/L; Millipore Corporation, Billerica, MA, USA),

Table 2. Primers Used for RT-PCR Assays to Analyze Growth Factor Expression

Gene Product	Sense Primer Sequence Orientation: 5'–3'	Antisense Primer Sequence Orientation: 3'–5'	Size (Base Pairs)
qmBMP4	ATTGCAGCTTTCTAGAGGTCC	GGGAGCCAATCTTGAACAAAC	149
qhBMP4	GCACTGGTCTTGAGTATCCTG	TGCTGAGGTTAAAGAGGAAACG	135
qmFGF-2	GTCTATCAAGGGAGTGTGTGC	CTGGAGTATTTCCGTGACCG	150
qhFGF-2	ACCCCTCACATCAAGCTACAAC	AAAAGAAACACTCATCCGTAACAC	141
qmTGF- β	CCTGAGTGGCTGTCTTTTGA	CGTGGAGTTTGTATCTTTGCTG	124
qhTGF- β	GCCTTTCCTGCTTCTCATGG	TCCTTGCGGAAGTCAATGTAC	150
qmPEDF	ACATCCACAGCACCTACAAG	TCCCATAGGACTTCTCCAGAG	141
qhPEDF	AGCTGAGTTATGAAGGCGAAG	ATCCTCGTTCCACTCAAAGC	149
qmVEGF	GAACTTTCTGCTCTCTTGGG	CTTCGCTGGTAGACATCCATG	144
qhVEGF	AGGGCAGAATCATCACGAAG	GGATGGCTTGAAGATGTACTCG	130
GAPDH	ACCATGGGGAAGGTGAAGGT	CATGGGTGGAATCATATTGG	163
β -actin	CATCACTATTGGCAACGAGCG	ATGGATGCCACAGGATTCCA	85

BMP4, bone morphogenetic protein 4; FGF-2, fibroblast growth factor 2; TGF- β , transforming growth factor- β ; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor.

Table 3. Annealing Temperatures for Primers Used in RT-PCR Assays

Gene Product	Annealing Temperature	Gene Product	Annealing Temperature
OCT3/4	56°C	qhTGF- β	61°C
SOX2	56°C	qmIGF1	61°C
KLF4	56°C	qhIGF1	60°C
NANOG	56°C	qmPEDF	57°C
qmBMP4	59°C	qhPEDF	59°C
qhBMP4	59°C	qmVEGF	60°C
qmFGF-2	59°C	qhVEGF	60°C
qhFGF-2	58°C	GAPDH	62°C
qmTGF- β	60°C	β -actin	58°C

naphthol phosphate (4 mg/ml; Millipore Corporation), and water in a 2:1:1 ratio at room temperature away from light for 15 min. Red color reaction in cells was observed by phase-contrast optic microscopy (Olympus).

Immunocytochemistry

The hESCs were fixed in 4% (v/v) paraformaldehyde for 15 min at room temperature and permeabilized with 0.3% (v/v) Triton X-100. Fixed cells were first incubated for 30 min at room temperature with 2% bovine serum albumin (BSA; Sigma) to reduce nonspecific binding. Then, cells were incubated overnight at 4°C with primary antibodies, such as octamer-binding transcription factor 3/4 (OCT3/4; Abcam, Cambridge, MA, USA; ab-19857, diluted 1:200) and stage-specific embryonic antigen-4 (SSEA-4; Chemicon International, Temecula, CA, USA; MAB4304, diluted 1:100). After that, the cells were incubated for 1 h at room temperature with secondary antibodies, such as Cy3-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA, USA; 111-165-144, diluted 1:1,000) and Alexa 488 (Invitrogen A-11001, diluted 1:400). The coverslips were then washed three times for 10 min each with PBS, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma), and coverslips were mounted in an antifading solution VECTASHIELD® H-1000 (Vector Laboratories, Inc., Burlingame, CA, USA). Fluorescence was observed on

inverted fluorescence microscope (Zeiss, São Paulo-SP, Brazil; Axiovert 130), coupled to a system for capturing digital photomicrographs. The specificity of the immunofluorescent staining was assessed for each experimental condition by performing the reaction in the absence of primary antibodies.

Statistical Analysis

GraphPad Prism 5.0 (San Diego, CA, USA) was used for colony size and cell growth analysis. All quantitative data were presented as mean $\pm$ SEM, and the statistical significance was determined using a two-way analysis of variance (ANOVA) test. GraphPad Prism 5.0 was used for relative mRNA levels by quantitative RT-PCR analysis. All quantitative data were presented as mean $\pm$ SD, and the statistical significance was determined using a one-way analysis of variance (ANOVA) test. Results were considered significant when the value of $p < 0.05$. The data were subjected to Bonferroni post hoc test to determine where the significance lies.

RESULTS

Feeder Layer Cell Culture

To evaluate the morphology of the cells used as a feeder layer for hESC expansion, MBMCs and MEFs were plated and maintained under the same culture conditions. Figure 1 shows that MBMCs grow as monolayers on culture plates and present fibroblast-like morphology, with thin and elongated adherent cells, and elliptical nuclei similar to MEFs. Incubation with mitomycin C for inactivation did not alter cell morphology. MEFs and MBMCs were continuously grown for about 10 passages, and cryopreserved cells, at early passages, maintained similar characteristics after freeze–thawing process (data not shown).

hESC Morphology

hESC colonies grown over MBMC feeder layers exhibited typical morphology of undifferentiated hESCs, such as rounded shape, delineated colony borders, high nucleus-to-cytoplasm ratio, one to three prominent nucleoli, and close spacing between the cells without any difference in comparison to cells grown over MEFs (Fig. 2).

Table 4. Primers Used for Real-Time RT-PCR to Analyze H9-hESC Differentiation

Gene Product	Sense Primer Sequence Orientation: 5'–3'	Antisense Primer Sequence Orientation: 3'–5'	Size (Base Pairs)
AFP	GAATGCTGCAAAGTACACGCTGGAAC	TGGCATTCAAGAGGGTTTTCAGTCTGGA	281
Gata6	GCCAACTGTCACACCACAAC	TGGGGGAAGTATTTTGCTG	265
Brachyury	GCCCTCTCCCTCCCTCCACGCACAG	CGGCGCCGTTGCTCAGACACACAGG	274
MSX1	CGAGAGGACCCCGTGGATGCAGAG	GGCGGCCATCTTCAGCTTCTCCAG	305
Nestin	CACCTCAAGATGTCCCTCAG	AGCAAAGATCCAAGACGCC	176
GAPDH	ACCATGGGGAAGGTGAAGGT	CATGGGTGGAATCATATTGG	163

AFP, α fetoprotein; Gata6, guanine–adenine–thymine–adenine-binding protein 6; MSX1, muscle segment homeobox (Msh) homeobox 1.

Figure 1. Morphology of feeder layers. Menstrual blood-derived mesenchymal cells (MBMCs) (A) and mouse embryonic fibroblasts (MEFs) (B) at third passage. No morphological changes were observed in MBMCs (C) or MEFs (D) after inactivation with mitomycin C.

The Undifferentiated State of hESCs During In Vitro Culture

hESC colonies cultured on MBMCs expressed, by RT-PCR, several established undifferentiated state markers [transcription factors *OCT3/4*, *NANOG*, and sex-determining region Y box 2 (*SOX-2*)] similar to those when hESCs were cultured on MEFs. It is worth mentioning that inactivated MBMCs were used as a negative control (Fig. 2). Also, the positive staining for alkaline phosphatase in hESC colonies cultured on MBMCs indicates the maintenance of pluripotent cells in the colony as seen in MEF condition (Fig. 3). In addition, the hESC colonies grown over MBMCs were positively stained for transcription factor *OCT3/4* and *SSEA-4*, a glycolipid antigen expressed on hESCs (Fig. 3).

Comparison of the Average Colony Size of hESCs

The hESCs transferred onto either MBMC or MEF feeder layers maintained continuous growth and proliferation in passage 1 (P1) and passage 4 (P4) (Fig. 4). The average colony size of hESCs on MBMCs was higher when compared to MEFs ($p < 0.05$). The value of the

average colony size of hESCs grown over MBMCs and MEFs from the second to fourth day after plating is indicated in Table 5.

Growth Factors Analysis of Feeder Layers

Amplification of the transcript bone morphogenic protein 4 (*BMP4*), fibroblast growth factor 2 (*FGF2*), transforming growth factor- β (*TGF- β*), vascular endothelial growth factor (*VEGF*), and pigment epithelium-derived factor (*PEDF*) was similar in MBMCs and MEFs before and after inactivation. When hESCs were cultured on MBMCs and MEFs, all growth factor transcripts were also amplified (Fig. 4).

EB Formation

To determine the differentiation capacity of hESCs in vitro, we used suspension culture to generate EBs. The morphology of EBs derived from hESCs cultured on MBMCs was similar when compared to those cultured in MEFs (Fig. 5). Moreover, nestin (ectoderm), muscle segment homeobox (*Msh*) homeobox 1 (*Msx1*), and brachyury (mesoderm), α -fetoprotein (*AFP*), and

Figure 2. Morphology of cells and colonies of hESCs and expression of undifferentiated transcription factors. Human embryonic stem cells (hESCs) (H9) in coculture grown over MBMCs (A) and MEFs (B). RT-PCR of hESCs (H9) in passage 4 grown over MBMCs (C) and MEFs (D). Both feeder layers maintained expression of the core pluripotency transcription factors. Inactivated MBMCs were used as negative control (E). OCT3/4, octamer-binding transcription factor 3/4; SOX2, sex-determining region Y box 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

guanine–adenine–thymine–adenine-binding protein 6 (*GATA6*; endoderm) transcripts were detected by real-time PCR in EBs cultured for 7 and 14 days on both MBMC and MEF feeder layers. No significant difference was observed in the expression of these transcripts either on EBs in MBMC group (7 and 14 days) or in the MEF group (14 days) when compared to EBs in MEF group (7 days) (Fig. 5).

In addition, the expression of the transcript *OCT3/4* was analyzed in EBs by real-time PCR. *OCT3/4* was detected in H9 cells cultured in both feeder layers. However, the level of the expression of *OCT3/4* significantly decreased on EBs derived from H9 grown on MBMCs (7 and 14 days) when compared to MEFs (7 days) (Fig. 5).

DISCUSSION

In this study, we have demonstrated that MBMCs can replace MEFs as feeder cells in hESC culture systems and support their growth in an undifferentiated stage.

Despite some good results (2,8,9,13,17,20,21,23,35,36), the search for an ideal feeder layer that could maintain undifferentiated growth, the pluripotent state of hESCs in vitro, provide sufficient amount of starting material, be

easily accessible, and present high proliferation capacity and compatibility with clinical applications (35) is still mandatory. In this scenario, MBMCs emerge as an ideal feeder presenting additional advantages over other feeder layers such as noninvasive methods for cell acquisition, no ethical issues, and the possibility of creating a patient-specific banking.

The MBMCs presented mesenchymal characteristics (adherence to plastic dishes and fibroblast-like morphology) as described in the literature (24,28). Prior to coculture with H9-hESCs, MBMCs were inactivated with mitomycin C, cell cycle was arrested, and no morphological changes were observed.

H9-hESC colonies grown over MBMCs exhibited the morphological pattern of undifferentiated hESCs, similar to hESCs maintained on human adult uterine endometrial cells (21). MBMCs present an important advantage to be used as a feeder layer since they are obtained by a noninvasive procedure when compared to human adult uterine endometrial cells.

Spontaneous differentiation was not observed by our group when H9-hESCs were grown on MBMCs and MEFs. However, slight morphological changes and low

Figure 3. Expression of alkaline phosphatase (AP) and pluripotency markers in hESCs (H9). Expression of alkaline phosphatase in hESCs grown over MBMCs (A) and MEFs (B) after four passages. hESCs on MBMCs and MEFs were positive for stage-specific embryonic antigen 4 (SSEA-4) (C and I, respectively) and OCT3/4 (F and L, respectively). The nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI) (D, G, J, and M). Overlay image (E, H, K, and N). Scale bar: 10 µm.

differentiation rates were observed when hESCs were grown on human feeder cells derived from different sources including foreskin fibroblast, adult skin fibroblast, marrow stromal cells, and umbilical cord blood-derived fibroblast-like cells (2,8,30,35).

The use of enzymes to perform cell dissociation could explain, at least in part, the morphological alterations and cell differentiation observed by the above-mentioned authors. In consonance with those studies, we also detected that the colonies were not as round as they used to be, and there was an increase in spontaneous differentiation rates (data not shown) when enzymatic digestion was used to passage the H9-hESCs grown on MBMCs and MEFs. This limitation was bypassed using mechanical dissociation (9). Furthermore, mechanical disaggregation as a method to passage cells avoids culture contamination by animal-based ingredients present in enzymatic solutions that are therefore incompatible with the clinical setting.

The undifferentiated state of H9-hESCs cultured on MBMCs was confirmed by expression of transcription factors and proteins. Maintenance of pluripotency was confirmed by analysis of alkaline phosphatase, which was similar to H9-hESCs cultured on MEFs. Taken together, these results provide a complete characterization of undifferentiated hESCs and corroborate with data reported, which used human umbilical cord blood-derived stromal cells as feeder layer (9). Although the expression of OCT3/4 and SSEA-4 had been previously observed, no analysis of alkaline phosphatase was performed by the other groups that used foreskin cells and umbilical cord blood-derived fibroblast like as human feeder layer (9,13,35).

Throughout 25 days of culture, the growth rate was maintained in both feeder layers (MBMCs and MEFs) similar to what was observed using foreskin cells as a feeder layer (2). Related to hESC colony area, the studies described so far did not show whether feeder

Figure 4. Assessment of hESC growth and expression of growth factors in human and mouse-derived feeder cells. Comparison of hESC colony areas between MBMCs and MEFs in passage 1 (A) and passage 4 (B) (* $p < 0.05$) (mean $\pm$ SEM) ($n = 3$). Growth factor analysis revealed amplification of transcripts by RT-PCR in MBMCs (C), MEFs (D), inactivated MBMCs (E), inactivated MEFs (F), H9 grown over MBMCs (G), and H9 grown over MEFs (H). BMP4, bone morphogenetic protein 4; FGF2, fibroblast growth factor 2; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor.

Table 5. Values for the Average Colony Size of hESCs Over Time

Passage	Feeder Layer	Day 2	Day 3	Day 4
P1	MEF	73.016 $\pm$ 0.69 μm^2	165.018 $\pm$ 13.237 μm^2	376.262 $\pm$ 75.53 μm^2
	MBMC	640.142 $\pm$ 40.37 μm^2 *	863.965 $\pm$ 100.132 μm^2 *	1,090.943 $\pm$ 161.950 μm^2 *
P4	MEF	321.456 $\pm$ 21.4 μm^2	704.745 $\pm$ 10.8 μm^2	1,224.67 $\pm$ 222.8 μm^2
	MBMC	632.09 $\pm$ 79.06 μm^2	1,439.110 $\pm$ 28.72 μm^2 *	2021.058 $\pm$ 21.93 μm^2 *

Values are mean $\pm$ SEM; * $p < 0.05$. MEF, mouse embryonic fibroblast; MBMC, menstrual blood-derived mesenchymal cell.

Figure 5. Embryoid body (EB) formation and quantitative analysis of germ layer markers and OCT3/4. Morphology of EBs using hESCs (H9) plated without MBMC (A) and MEF (B) feeders. Positive expression of muscle segment homeobox (Msh) homeobox 1 (Msx1), brachyury, nestin, α fetoprotein (Afp), guanine-adenine-thymine-adenine-binding protein 6 (Gata6), and OCT3/4 using EBs plated without MBMCs (C) and MEFs (D) cultured for 7 and 14 days after adhesion. Quantitative RT-PCR analysis of Msx1 (E), brachyury (F), Nestin (G), Afp (H), Gata6 (I), and OCT3/4 (J). EBs grown in MEFs 7 days after adhesion were used as controls for relative quantification (** $p < 0.01$) (mean $\pm$ SD), ($n = 3$).

layers impact the size of the colony. Our data suggest that H9-hESC colonies grown on MBMCs were larger than those grown on MEFs. This result might be explained by provision of a natural environment to hESCs, similar to early in vivo development, enhancing growth in an undifferentiated state. Also, the presence of mesenchymal cells in MBMCs may help maintain self-renewal of hESCs by growth factor production (22).

As previously described, several groups reported that growth factors such as FGF, TGF- β , BMP-4, and PEDF are involved with growth of hESCs (4,5,12,19,33). More specifically, VEGF has been described as a growth factor secreted by menstrual blood-derived stem cells (6). In this context, we investigated whether growth factors might be involved with the increase in colony size. Our data showed no qualitative difference in growth factor expression produced by MBMCs compared to MEFs before and after inactivation and when H9-hESCs were grown on MBMCs. We have shown that H9-hESCs on MBMCs present a higher proliferation in comparison to MEFs, but further evaluation should be performed to find out why the colonies are larger when grown on MBMCs.

The morphology of EBs derived from hESCs cultured over MBMCs was similar to those cultured over MEFs. These results corroborate with data reported by several groups (9,15). It should be noted that for EB formation, hESCs were dissociated using Tryple for enzymatic digestion. The EBs from hESCs grown on MBMCs had a significant reduction of *OCT3/4* expression (100–1,000 times) when compared to MEFs. Although the reduction of *OCT3/4* expression at the fourth and eighth week of differentiation has been reported (9), no quantitative analysis was performed. Our data suggest that EBs formed after culturing hESCs over MBMCs differentiate faster, but further evaluation should be performed to find out why differentiation on MBMCs is faster than MEFs.

It is noteworthy to highlight that our group reported the rapid and efficient generation of induced pluripotent stem cells (iPSCs) from MBMCs, indicating that these cells can be used as a model to study patient-specific diseases, and in the future, it might be used in clinical settings (11).

CONCLUSION

In this work, we have identified a new human feeder layer presenting properties and advantages over other human feeder cells for hESC culture. Our culture system using MBMCs as feeder layer for hESCs is the closest and more suitable alternative to animal-free conditions for growing hESCs and, therefore, a good candidate for large expansion of cells for clinical application.

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