

## Multipotent Menstrual Blood Stromal Stem Cells: Isolation, Characterization, and Differentiation

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The stromal stem cell fraction of many tissues and organs has demonstrated to exhibit stem cell properties such as the capability of self-renewal and multipotency, allowing for multilineage differentiation. In this study, we characterize a population of stromal stem cells derived from menstrual blood (MenSCs). We demonstrate that MenSCs are easily expandable to clinical relevance and express multipotent markers such as Oct-4, SSEA-4, and c-kit at the molecular and cellular level. Moreover, we demonstrate the multipotency of MenSCs by directionally differentiating MenSCs into chondrogenic, adipogenic, osteogenic, neurogenic, and cardiogenic cell lineages. These studies demonstrate the plasticity of MenSCs for potential research in regenerative medicine.

**Key words:** Menstrual blood; Stromal stem cells; Multipotent markers; Differentiation

### INTRODUCTION

Embryonic stem cells (ESCs) have the totipotent ability to differentiate into any cell type derived from all three germ layers (14,22). However, the potential for teratomas, along with limited availability of ESCs for large-scale clinical use, currently limit them strictly for scientific research. However, with the vast number of patients with a myriad of diseases, additional stem cell sources with practical clinical application are being sought. Many other sources of stem cells have been identified that are already being used in early phase clinical trials, including heart failure (18), spinal cord injury (17), and bone and cartilage repair (24). The stromal cell fraction of many tissues and organs has shown in vitro multipotency by differentiating into neurogenic, cardiogenic, osteogenic, adipogenic, and chondrogenic cell types (4,6,23,24,26). The fact that stromal cells can differentiate into ectoderm and mesoderm lineages further validates their multipotency. The question then becomes whether there is a source of multipotent stromal stem

cells that can be safely obtained, in a renewable fashion, and maintain potency to differentiate.

Recently, stromal cells have been identified in endometrial tissue (3,19). However, obtaining the cells directly would be a very invasive procedure. The endometrial lining of the uterus has a remarkable capacity for regeneration. During each menstrual cycle there is vast growth of tissue and blood vessels, which is shed at the end of the cycle. The shed blood and tissue contain a heterogeneous population of cells including some with regenerative capacity (5). The uterine stromal cells have similar multipotent markers commonly found in bone marrow mesenchymal stem cells and may actually originate in part from bone marrow (12,20,21). Important markers for determining multipotency are Oct-4 and SSEA-4, which are found to be expressed in many multipotent and pluripotent stem cells including ESCs, along with the cell surface marker c-kit (CD117) (8,10,25). In order to evaluate the practicality of obtaining multipotent stem cells from the uterus, in a safe and reproducible manner, we analyzed the shed menstrual

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blood and tissue to identify stromal cells (MenSCs). We present here a population of MenSCs that express the multipotent markers Oct-4, SSEA-4, and c-kit, along with their ability to be directionally differentiated in vitro into multiple cell lineages derived from mesoderm and ectoderm and the ability to be easily expanded.

## MATERIALS AND METHODS

### *Cell Procurement and Processing*

An endometrial/menstrual cell sample was procured by using a Divacup (Kitchener, ON) during the first few days of a menstrual cycle. The cells were harvested with the informed consent of the donor as approved by an institutional review board. The cells were transferred in phosphate-buffered saline (PBS) with penicillin/streptomycin and heparin. The sample was shipped at 4°C until it reached the processing laboratory within 24–48 h after procurement. The sample was centrifuged and supernatant was evaluated for bacteria. The cells were then cultured.

### *Cell Culture Method*

Cells were cultured with Chang Medium and seeded in a culture flask to obtain adherent cells (7). Chang Medium is composed of MEM alpha medium (Invitrogen, Carlsbad, CA), Chang B (basal) (18% v/v) (Irvine Scientific, Santa Ana, CA), Chang medium C from Supplement C106 (2% v/v) (Irvine Scientific), penicillin/streptomycin sulfate (Invitrogen), L-glutamine 2 mM (Invitrogen), and fetal bovine serum (FBS, 15% v/v) (Invitrogen). Cells were cultured for 7 days at which time the media was changed. On day 10 the cells were subcultured using TrypLE Express (Invitrogen) and 50,000 cells were obtained. The cells were subcultured until passage five, at which time the cells were processed by positive selection for c-kit+ (CD117) was performed using Magnetic Micro beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's directions using purified mouse anti-human CD117 monoclonal antibody (IgG1) with clone 104D2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Ten million cells were processed with 900,000 cells in the positive fraction that were cultured. The c-kit+ (CD117) cells were further subcultured an additional three passages in MSC-BM medium (Lonza, Basel, Switzerland). The cells were subcultured using 0.25% trypsin (Invitrogen) and cultured on flasks and plates seeded at 5000–7000 cells/cm<sup>2</sup>. Medium was changed every 2–3 days between passages after the first 7 days of culture.

### *Karyotype Analysis*

Karyotype analysis was performed at Cell line Genetics (Madison, WI) by standard cytogenetic protocol. In total, 50 metaphases were prepared by the air-drying

method and stained with trypsin Wright's stain protocol to produce G-banding.

### *RNA Extraction and RT-PCR Analysis*

Total cellular RNA was isolated using RNeasy mini kit (Qiagen Inc., Valencia, CA). Total cellular RNA was collected from approximately 100,000 cells per experiment. To eliminate DNA contamination, the samples were treated with 2.0 U of DNase I (Invitrogen) at 37°C for 15 min followed by inactivation with the addition of EDTA 2 mM at 65°C for 10 min. The samples were concentrated by ethanol precipitation and resuspended in RNase-free water. Prior to cDNA synthesis, the samples were screened for genomic DNA contamination by PCR on RT controls. Total RNA was transcribed into cDNA using the Omniscript RT kit and later purified with the QIAquick PCR purification kit (Qiagen Inc.). For each PCR reaction, 20 ng of cDNA template was used in a 25-μL reaction volume with HotStar Taq Plus and Quantitect Primers (Qiagen Inc.). All targets were amplified at an annealing temperature of 55°C for 30 cycles. Amplification products were identified by size on a 2% agarose gel.

### *Flow Cytometry*

For cell surface antigen analysis after c-kit enrichment, the cells were harvested, washed in ice-cold blocking buffer (MEM/HEPES + 2% BSA), and incubated for 30 min on ice in blocking buffer containing the specific FITC-, APC-, phycoerythrin (PE)-, or PECy5-labeled antibodies. Antibodies for human cell surface antigens CD9, CD29, CD34, CD38, CD44, CD45, CD49f, CD90, CD105, CD117, CD166, LIN, MHC I, and MHC II were from Becton, Dickinson and Company (Franklin Lakes, NJ), while CD133 was from Miltenyi Biotec and SSEA-4 was from Chemicon. In all experiments, the corresponding isotype-matched antibodies (Becton, Dickinson and Company) were used as negative controls. Data (20,000 events) were collected using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA) and analyzed on FACS Diva software (BD Biosciences).

### *Adipogenic Differentiation*

Cells were plated onto 0.2% gelatin (Sigma) at 20,000 cells/cm<sup>2</sup> in hMSC Adipogenic Differentiation BulletKit (ADB) (Cambrex, East Rutherford, NJ); ADB medium consisted of 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 10 μg/ml recombinant human insulin, 100 mM indomethacin, and 5% FBS (Hyclone). Cells were in ADB for 7 days, in adipogenic maintenance media (AM) [DMEM-LG/GL (Invitrogen) + 1% penicillin/streptomycin (Invitrogen) + 15% FBS (Hyclone) + 10 μM insulin (Sigma)] for 3–4 days,

and then interchanged from AM to ADB every 3–4 days for up to 20 days.

#### Osteogenic Differentiation

Cells were plated onto 0.2% gelatin (Sigma) at 20,000 cells/cm<sup>2</sup> and treated with hMSC Osteogenic Differentiation BulletKit (ODB) (Cambrex), ODB medium consisted of 100 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate, 0.2 mM ascorbate, and 5% FBS and were in ODB with one-half medium changes every 3–4 days for up to 20 days.

#### Chondrogenic Differentiation

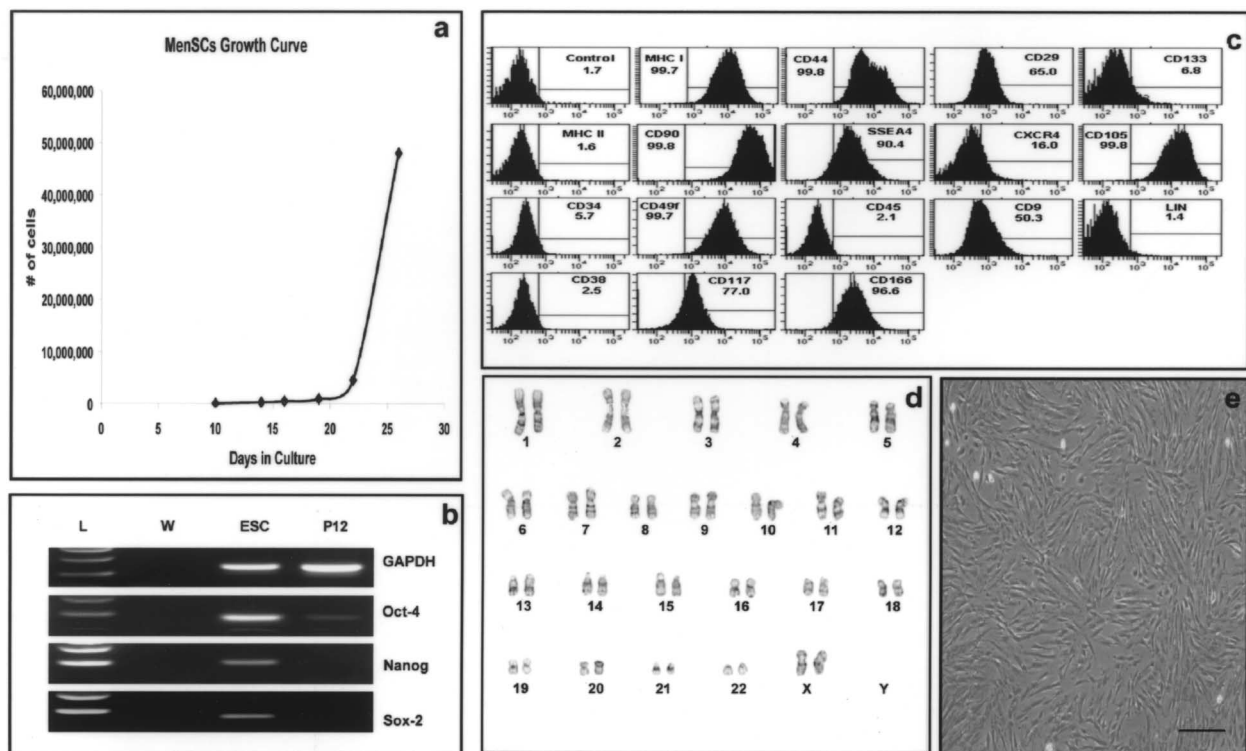
Cells were plated onto 0.2% gelatin at 6,000 cells/cm<sup>2</sup> in hMSC Chondrogenic Differentiation BulletKit (CDB) (Cambrex) + 1% FBS + 20 ng/ml TGF- $\beta$ 3 (R&D Systems, Inc., Minneapolis, MN) added fresh. Cells received full medium changes every 3–4 days for 14–20 days.

#### Neural Differentiation

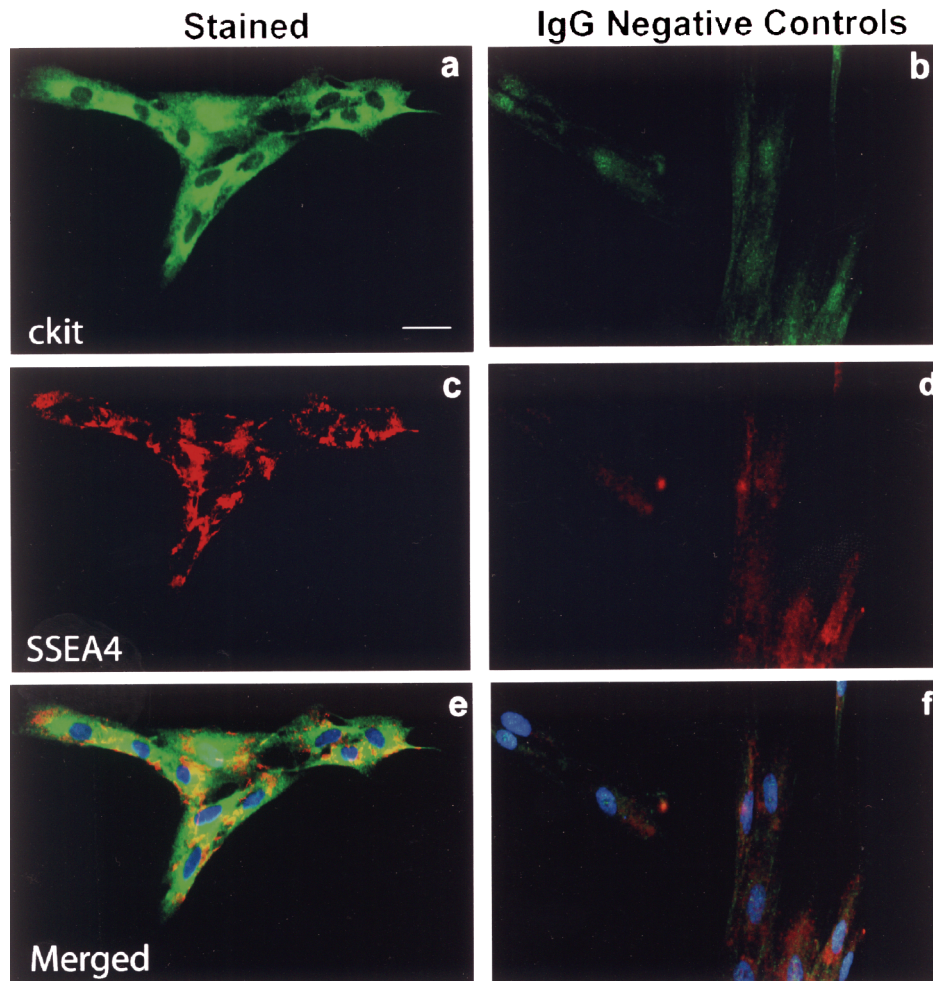
Cells were plated onto fibronectin-coated cover slips (BD Biosciences) at 10,000 cells/cm<sup>2</sup> in Neural Induction Media (NIM) consisting of DMEM-F/12 (Invitrogen) + 1% penicillin/streptomycin + 2 mM Glutamax (Gibco) + 1  $\times$  N-2 supplement (Invitrogen) plus the following condition: 1) 10 or 100 ng/ml fibroblast growth factor (bFGF) for 4 days, 2) passed 1:2 in 10 ng/ml bFGF + 10 ng/ml platelet-derived growth factor (PDGF) + 20 ng/ml epidermal growth factor (EGF) for 5 days, and 3) 10 ng/ml bFGF + 10 ng/ml PDGF excluding EGF for 7 days totaling 16 days. Cells were cultured on fibronectin-coated cover slips and full medium changes occurred every 2–3 days with fresh growth factors (all from R&D Systems, Inc.).

#### Cardiac Differentiation

Cells were plated onto 0.2% gelatin-coated cover slips (VWR) at 30,000 cells/cm<sup>2</sup> and in low-adhesion



**Figure 1.** MenSCs grow rapidly, have a normal karyotype, and express multipotent markers. (a) MenSCs doubling time of 24–36 hours allows for rapid expansion to 48 million cells in eight doublings starting with only 50,000 cells. (b) MenSCs express multipotent marker Oct-4 at the RNA level up to 12 passages as determined by RT-PCR: L, ladder; W, water; ESC, embryonic stem cells; P12, passage 12. (c) Flow cytometry: MenSCs are positive for stromal cell and/or mesenchymal stem cell markers such as CD44, CD105, CD166, CD90, CD49f, MHC I, CD29, and CD9 while negative for CD38, CD133, CD45, CD34, MHC II, and LIN, and mildly positive for CXCR4. In addition, flow cytometric analysis confirmed that MenSCs highly express the pluripotent marker SSEA-4 and c-kit+ (CD117). (d) No chromosomal aberrations are expressed by these cells as determined by karyotype analysis at passage 12. (e) Cultured MenSCs appear to have stromal cell morphology. Scale bar: 20  $\mu$ m.



**Figure 2.** MenSCs stain positive for (a) c-kit, (c)SSEA-4 and (e) c-kit+ and SSEA-4 colocalization. Negative controls did not stain for c-kit or SSEA-4 (b, d, f). Scale bar: 20  $\mu$ m.

dishes (Fischer) at 156,000 cells/cm<sup>2</sup> in Cardiac Media consisting of DMEM-LG + 1% penicillin/streptomycin + 2 mM Glutamax (Gibco) + 1% FBS (Hyclone). After 2 days, cell aggregates were plated onto the cell monolayer. After 2 days medium was changed to Cardiac Medium with either 8  $\mu$ M 5-aza-2'-deoxycytidine (Aza) or 400–800  $\mu$ M S-nitroso-N-acetylpenicillamine (SNAP) (both from Sigma). Full media changes occurred with fresh Aza and one half medium changes with fresh SNAP every 2–3 days for 12 days. Some MenSC cultures were induced to undergo cardiogenesis by allowing the cultures to become overcrowded; medium was changed every 2–3 days.

#### Immunocytochemistry

All cells were fixed in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for 10 min at

room temperature (RT). Adipogenic induced cells were stained for fat vacuoles using the oil red O staining kit (American Master Tech Scientific, Lodi, CA). Briefly, cells were washed with 70% ethanol (EMD Chemicals Inc., San Diego, CA), incubated for 10 min at RT with oil red O, and counterstained with Modified Mayer's Hematoxylin (MMH) (American Master Tech Scientific, Lodi, CA). Osteogenic-induced cells were stained for calcium deposits using alizarin red S (Fisher Scientific, Pittsburg, PA). Briefly, cells were washed two times with water, incubated 1 h at RT with 0.0075% alizarin red S (Fisher Scientific) diluted in dH<sub>2</sub>O, and counterstained with MMH. Chondrogenic-induced cells were stained for sulfated proteoglycans using alcian blue (Sigma). Briefly, cells were incubated with 1% alcian blue (Sigma) in 0.1 N HCl (Sigma) for 1 h RT, washed one time with 0.1 N HCl (Sigma) for 5 min at RT, and

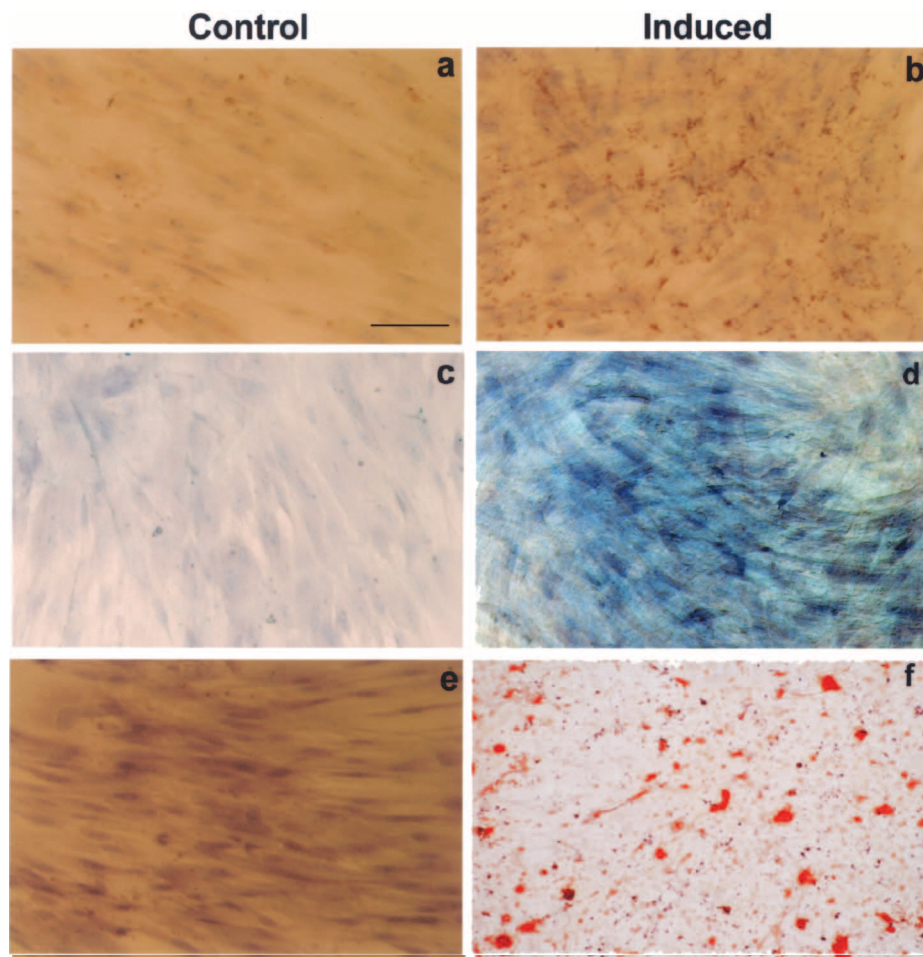
counterstained with MMH. For antibody staining, cells were washed in PBS and fixed in 4% paraformaldehyde for 10 min at room temperature (RT). Blocking solution, PBS/2%BSA/10% goat serum/Triton X-100 0.2%, was applied for 1 h at RT followed by incubation with primary antibody overnight at 4°C. After washing in PBS/Triton X-100 0.2%, cells were incubated with secondary antibody for 1 h at RT, washed in PBS, and mounted in Vectashield H-1000 mounting medium. Neural-induced cells were analyzed using the following primary antibodies: tub-III (1:100), Map2 (1:200), Vimentin (1:500), O4 (1:200) (Chemicon, Temecula, CA), GalC (1:200) (Sigma), and GFAP (1:200) (BD Biosciences). Cardiac-induced cells were analyzed using the following primary antibodies: troponin (1:200), connexin 43 (1:200) (Chemicon), and cardiac-actin (1:100) (RDI, Concord, MA).

Nuclei were stained using DAPI (Invitrogen). The specificity of antibodies was tested using human embryonic stem cell cultures and human neural and cardiac cells as positive controls. Negative controls for staining included corresponding IgG isotype control or omission of primary antibody. Fluorescence was analyzed using an Olympus BX-61 microscope with SlideBook image software while mesodermal staining was analyzed using a Leica DM IRB microscope with Microsuite Biological suite imaging software.

## RESULTS

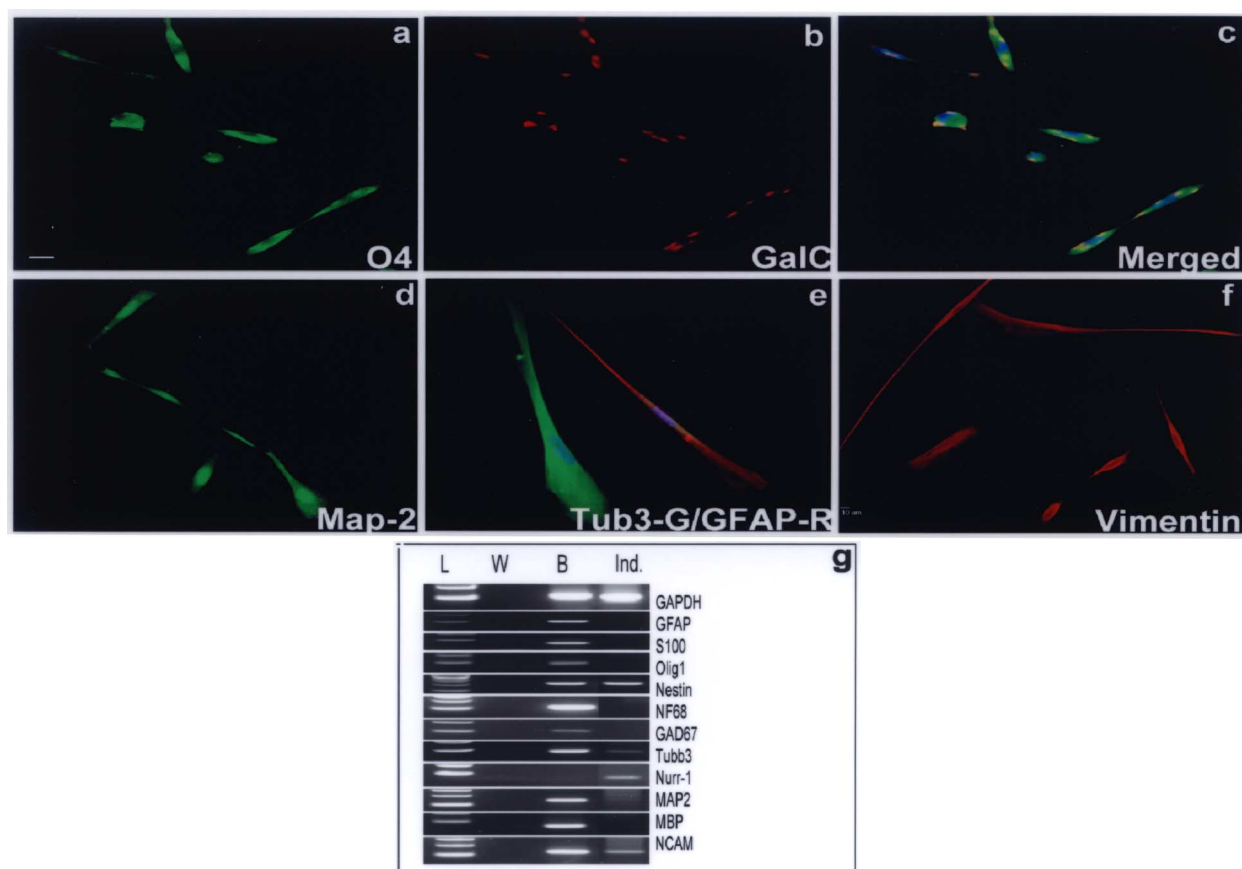
### *Growth, Multipotent Marker Expression, and Characterization of MenSCs*

MenSCs rapidly expand at a doubling rate of 24–36 h; starting with 50,000 cells we obtained 48,000,000 by



**Figure 3.** MenSCs are easily differentiated into mesoderm tissue types. Staining with oil red O for fat vacuoles demonstrates differentiation into adipogenic tissue when induced (b) compared to noninduced controls (a). Staining with alcian blue for sulfated proteoglycans demonstrates differentiation into chondrogenic tissue types when induced (d) compared to noninduced MenSCs controls (c). Staining with alizarin red S (f) in induced cells shows calcium deposits when compared to the noninduced controls (e). Scale bar: 20  $\mu$ m.





**Figure 4.** MenSCs differentiate into neural tissue. (a–c) Neural-induced MenSCs differentiate into oligodendroglial cells expressing markers O4 and GalC. Moreover, they can be differentiated into neuronal cells and neural progenitors shown by the expression of (d) Map-2, (e) Tub-3, and (f) Vimentin (F), respectively. (g) RT-PCR data support and extend our immunocytochemistry staining demonstrating RNA expression of neural cells. Immunocytochemistry. RT-PCR: L, ladder; W, water; B, brain control; neural induced MenSCs. Scale bar: 50  $\mu$ m.

day 26 (Fig. 1a) and they maintained diploid cells without chromosomal aberrations as determined by karyotype analysis at passage 12 (Fig. 1d). Moreover, RT-PCR data demonstrated that MenSCs expressed the multipotent marker Oct-4 at passage 12, but not SOX-2 or Nanog (Fig. 1b). Flow cytometric analysis illustrated that MenSCs were positive for stromal cell and/or mesenchymal stem cell markers such as CD44, CD105, CD166, CD90, CD49f, MHC I, CD29, and CD9 while negative for CD38, CD133, CD45, CD34, MHC II, and LIN, and mildly positive for CXCR4 related to stem cell homing. In addition, flow cytometric analysis confirmed that MenSCs highly expressed the pluripotent marker SSEA-4 and c-kit<sup>+</sup> (CD117) (Fig. 1c). Also, SSEA-4 and c-kit<sup>+</sup> were colocalized on isolated clones from MenSCs (Fig. 2). Cultured MenSCs appeared to have stromal cell morphology (Fig. 1e). These data demonstrate that MenSCs are expandable and express multipotent stem cell markers.

#### *MenSCs Differentiate Into Mesodermal Lineage*

Stromal stem cells have the ability to differentiate into mesoderm tissues such as cartilage, adipose, and bone. MenSCs were induced to the adipogenic lineage (Fig. 3b), chondrogenic lineage (Fig. 3d), and osteogenic lineage differentiation (Fig. 3f). All displayed specific histological characteristics such as fat vacuoles found in adipocytes, sulfated proteoglycans staining for cartilage, and calcium deposits for bone. Figure 3a–d are negative controls. These data demonstrate that MenSCs differentiate into mesodermal tissues at varying degrees; for example, 40–50% of MenSCs differentiated to chondrogenic lineage, 60–70% for adipogenic, and 45% for osteogenic lineages, all similar to or slightly better than bone marrow-derived MSCs.

#### *MenSCs Differentiate Into Neural Lineage*

In order to demonstrate the plasticity of MenSCs, we differentiated cells toward ectodermal lineage and as-

sessed their cellular and molecular marker expression. When MenSCs were placed into medium containing FGF for 4 days, followed by the addition of FGF, PDGF, and EGF for 7 days, then cultured in FGF and PDGF excluding EGF for 5 days the cells expressed oligodendroglial markers O4 and GalC (Fig. 4a–c), the mature neuronal marker Map-2 (Fig. 4d), and Vimentin (Fig. 4f). Cells also expressed the astrocyte marker GFAP (Fig. 4e). RT-PCR data supports the immunocytochemistry data by confirming that MenSCs express several neural markers at the RNA level (Fig. 4g), including Nestin, NCAM, and Nurr-1. These data demonstrate the plasticity of MenSCs and the potential for differentiation into multiple neural phenotypes at a rate of 45–50%, which is similar to bone marrow-derived MSCs.

#### *MenSCs Differentiate Into Cardiogenic Lineage*

MenSCs can be differentiated into cells of the cardiac lineage using two different techniques. Immunocytochemistry demonstrated positive staining for the cardiac markers actin, troponin, and connexin 43 when the cells were differentiated using either 8  $\mu$ M Aza or 800  $\mu$ M

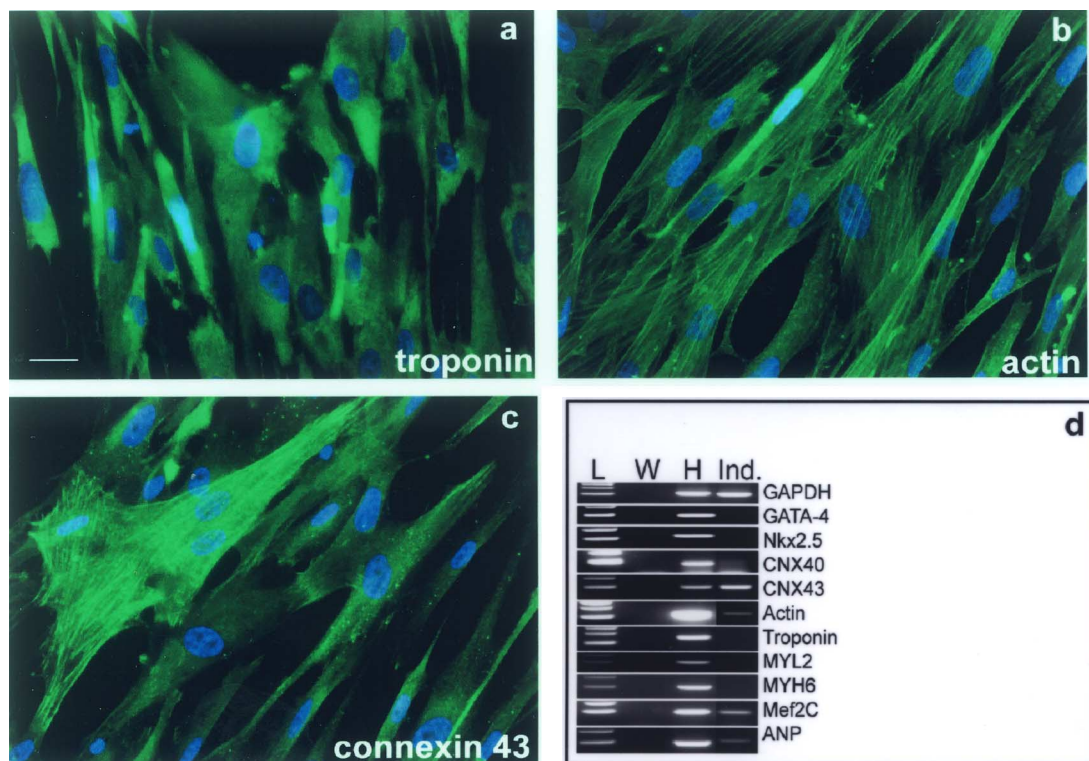
SNAP (Fig. 5a–c). RT-PCR data support immunocytochemistry findings by demonstrating that differentiated MenSCs express cardiac markers at the RNA level (Fig. 5d), when cells are overgrown with MenSCs cell aggregates. These data confirm that MenSCs express cardiogenic markers at the cellular and molecular level at a rate of 50–60%, which is similar to bone marrow-derived MSCs.

#### *Telomerase Activity*

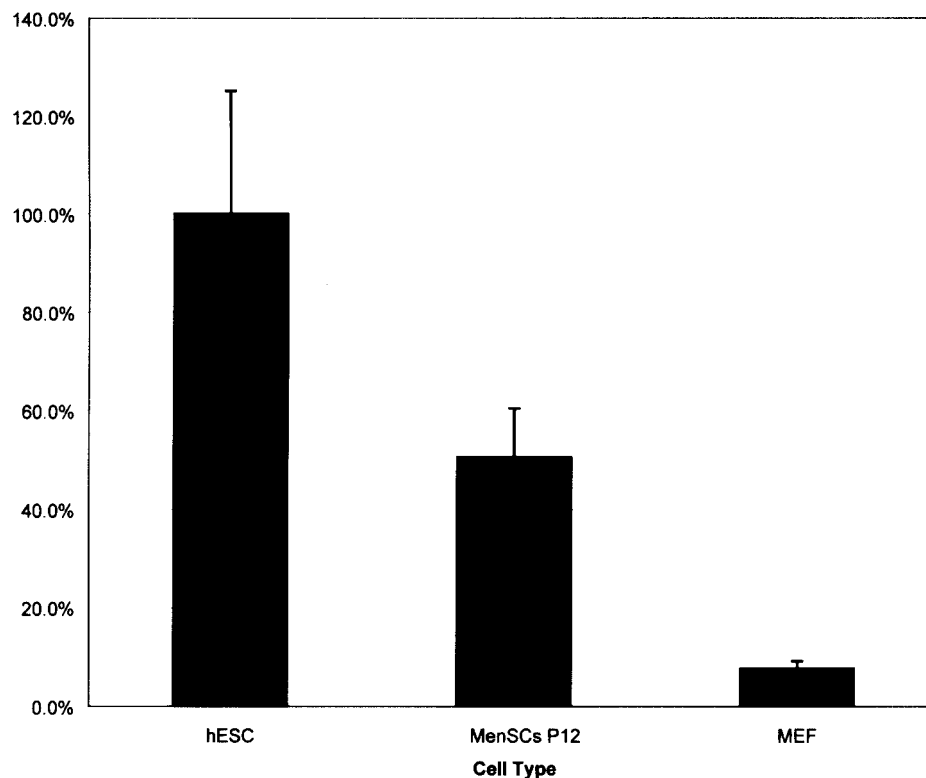
The MenSCs maintain greater than 50% of their telomerase activity even at passage 12 when compared to human embryonic stem cells, which is much better than bone marrow-derived MSCs (Fig. 6).

### DISCUSSION

Stromal stem cells have been shown to have great potential for future use in clinical translation of regenerative therapies (23,24,26). We have presented a population of stromal cells isolated from human menstrual blood (MenSCs). The MenSCs are characterized at both the cellular and molecular level, along with the ability to easily expand and differentiate. This study demon-



**Figure 5.** MenSCs differentiate into cardiac tissue. The addition of 8  $\mu$ M Aza produces cardiac cells with maximal expression of (a) troponin and (b) actin, demonstrated by immunocytochemistry. (c) Connexin 43 expression was accomplished by addition of 800  $\mu$ M SNAP, demonstrated by immunocytochemistry and RT-PCR. (d) RT-PCR: L, ladder; W, water; H, heart control; cardiac-induced (overcrowding) MenSCs. Scale bar: 50  $\mu$ m.



**Figure 6.** MenSCs express telomerase activity at passage 12. hESC, human embryonic stem cells; MEF, mouse embryonic fibroblasts.

strates that MenSCs are a unique cell population that can be safely isolated and provide an expandable source of stem cells from child-bearing aged women. The expression of multipotent markers Oct-4, SSEA-4, and c-kit (CD117) in the MenSCs is not common in most other adult stem cells. We have isolated clones with positive c-kit and SSEA-4 colocalization (Fig. 2). This unique population of MenSCs is different than one recently described by Cui et al. (5), which demonstrated skeletal muscle differentiation where they found menstrual blood cells expressing the following flow profile: positive CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, CD105, MHC-I and negative CD14, CD31, CD34, CD45, CD50, c-kit, CD133, MHC-II. Our cells (Fig. 1) have the multipotent markers mentioned above, which are absent in the cells identified and used by Cui et al. (5). Also, the MenSCs appear to have similar characteristics as the human endometrial stem cells identified by Cho et al. (3) with c-kit (CD117), Matthai et al. (15) with Oct-4, clonally expanded by Gargett et al. (9), and the mouse endometrial stem cells identified by Cervello et al. (1) with both c-kit+ (CD117) and Oct-4. Thus, it could be interpreted that MenSCs are the shed version of endometrial stem cells that can be easily harvested in a noninvasive manner. The expression of multipotent markers is indicative of cells that have the

capacity to differentiate into cell types derived from multiple germ layers. The transcription factor Oct-4 and SSEA-4 both are markers expressed by human embryonic stem cells (11), which are also highly expressed in our MenSCs, and may explain the rapid cell expansion (Fig. 1). It may also explain the ability to be directionally differentiated into several cell types (Fig. 3–5). The differentiated cell types demonstrate plasticity of the MenSCs by the fact that cells have not only phenotypic cell surface markers by flow cytometry and immunocytochemistry but also mRNA expression.

The need for regenerative therapies incorporating cells that have the ability to engraft and differentiate is vast. However, the ideal cell would also have the ability to be used in an allogeneic manner. Mesenchymal stem cells derived from bone marrow are currently in clinical trials after demonstrating safety and efficacy in animal models for allogeneic use due to their immunosuppressive properties (13,16). Due to their ease of collection and isolation, MenSCs would be a great potential source of multipotent cells if they also exhibited these properties.

Currently, we are evaluating the use of human MenSCs in vivo for neurodegenerative and cardiovascular regenerative therapies in animal models. We are also in the process of identifying the heterogeneous mixture of cell types found in menstrual blood and evaluating better



means to isolate and culture pure populations of MenSCs. In addition, we are also performing further studies from different donors to determine the reproducibility and efficiency of the multipotent differentiation potential of these cells. In summary, our study demonstrates that a unique and novel population of stromal stem cells can be collected, isolated, characterized, expanded, and differentiated from human menstrual blood.

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