A Highly Efficient Method to Differentiate Smooth Muscle Cells From Human Embryonic Stem Cells

To the Editor:

The molecular mechanisms and the control of smooth muscle cell (SMC) differentiation have been extensively investigated because of its therapeutic potential. To date, different cell types have been used to study SMC differentiation, including a variety of mouse embryonic stem cells, adult stem cells, and others. Because several fundamental differences exist between mouse and human embryonic development, lack of a good model system to study SMC differentiation has hampered the progress of translating SMC knowledge to novel clinical therapies.

Human embryonic stem (hES) cells provide a valuable source of cells for studying human cell differentiation and developing therapeutic potentials in regenerative medicine. Since the initial report describing the derivation of hES cells, a variety of studies have established in vitro differentiation strategies to several lineages. Recently, it has been demonstrated that vascular progenitors derived from hES cells could be differentiated into endothelial cells and SMCs by endothelial growth medium with VEGF and PDGF, respectively. In the present study, we demonstrate a highly efficient and feasible cell culture–based methodology to differentiate hES cells to the SMC lineage by using a combination of cell culture medium and extracellular matrix environment.

Two well-studied, NIH-approved, human ES cell lines (H1 and H9) were induced to differentiate after the strategy shown in supplemental Figure I (available online at http://atvb.ahajournals.org). Throughout this process, undifferentiated hES cells underwent complex morphological changes (supplemental Figure II). Cells derived from the outgrowth of embryoid bodies (EBs) were subsequently plated in growth conditions (GC), consisting of smooth muscle growth medium (SMGM) and matrigel-coated plates. As a result, a morphologically homogeneous cell popu

Figure. Characterization of SMCs derived from hES cells. A, Immunohistochemical staining of SMC-like cells derived from hES cells with SMC-specific markers, SMMHC (left panel, green) and SM-α-actin (right panel, red). The nuclei (blue) were costained with DAPI. Bar=100 μm. B, Representative Western blots showing SMC-specific proteins including SMMHC, SM-α-actin, and caldesmon. β-tubulin was used as the loading control. C, Carbachol-induced contraction of SMC-like cells differentiated from hES cells. Photographs were taken before (left panel) and after (right panel) treatment with 1 mM carbachol for 10 minutes. Arrowheads show representative contracted cells grown in differentiation conditions. Bar=200 μm.
lation was achieved that remained so with culture time and passages (supplemental Figure III). On the other hand, these homogeneous cells underwent a dramatic morphological change when switching GC to differentiation conditions (DC), consisting of DMEM +5% FBS and a gelatin-coated surface. Cells growing in DC became larger and displayed an elongated spindle-shaped morphology, the characteristics of SMC-like cells (supplemental Figure IIF).

To characterize these cells as SMC-like cells, SMC markers were examined by immunohistochemical staining. As shown in Figure A, hES-derived cells growing in DC displayed smooth muscle (SM)-myosin heavy chain (SMMHC) and SM-α-actin expression. Fluorescence-activated cell sorter (FACS) analysis showed that both SMMHC- and SM-α-actin–positive cells significantly increased after the growth environment was switched to DC for 5 days (55.26±8.02% and 96.81±2.07%, respectively), compared with cells growing in GC (4.54±0.92% and 9.99±1.68, respectively, n=3, P<0.01) (supplemental Figure III).

To further demonstrate the effect of culture conditions on SMC differentiation, the expression of SMC-specific genes was determined by quantitative real-time reverse transcription polymerase chain reaction (PCR) (qRT-PCR). Myocardin, SM-α-actin, calponin, smoothelin, SMMHC, SM22α, and telokin were significantly up-regulated in hES cell–derived culture in DC for 5 days (supplemental Figure IV). Despite an increased expression of Flk1, a marker for cardiovascular progenitor cells, it was determined that endothelial cell markers (such as CD31 and Tie2) are not increased in this differentiation protocol. Consistent with the qRT-PCR data, Western blot analyses demonstrated that expression levels of SM-α-actin, h-caldesmon, and SMMHC were upregulated in a time-dependent manner in DC (Figure, B).

It is well-established that cultured SMCs are able to contract in response to external ligands. First, carbachol, a muscarinic agonist, was used to evaluate the contraction of derived SMC-like cells. Cells were treated for 1 minute with carbachol and observed microscopically up to 30 minutes. A large proportion of cells growing in DC changed shape in response to carbachol treatment (arrows in Figure, C and supplemental Movie 1). Also, a similar response to KCl was observed in these SMC-like cells (supplemental Movie II and arrows in supplemental Figure V).

Taken together, the described results clearly indicate that this differentiation approach results in a highly efficient achievement of human SMCs as determined by the acquisition of SMC characteristics including expression of specific markers and functional reactivity in response to contractile agents. This simple and efficient cell model system will provide a useful tool to study human SMC differentiation and vascular development as well as potential therapeutic targets for treating vascular diseases.

Disclosures

None.

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Supplemental Text

Cell Culture and In Vitro Differentiation Procedure

Human ES cells (H1 and H9 cells from WiCell at Madison, WI, passages 32-45) were expanded routinely on irradiated mouse embryonic fibroblast (mEF) feeder layer. The hES cells were grown on inactivated mEFs in growth medium consisting of Knock-out Dulbecco’s modified Eagle’s medium (KO-DMEM), Knock-out serum replacement (KO-SR), L-glutamine, β-mercaptoethanol, non-essential amino acids, and human basic fibroblast growth factor (hbFGF), as previously described (Invitrogen, Carlsbad, CA). Cells were split once a week by incubation in 1mg/ml collagenase IV (Invitrogen) for 10 min at 37 °C and seeded on freshly prepared inactivated mEFs.

For formation of embryoid bodies (EBs), hES colonies were digested by using 1mg/ml collagenase type IV and transferred to an ultra-low attachment 6-well plate (Corning Incorp., Corning, NY) to allow their aggregation in suspension. Human EBs were grown in DMEM (high glucose) supplemented with 10% fetal bovine serum (FBS), L-glutamine, β-mercaptoethanol, and non-essential amino acids. Medium was changed every day.

Six-day old EBs were transferred to 6-well plates coated with 0.1% gelatin for 5-6 days according to the strategy shown in online supplement Fig. S1, followed by digestion with TrypLE™ Express (Invitrogen) and cultured on matrigel-coated 6-well plates with 5x10^5 cells/cm^2 in smooth muscle growth medium (SMGM) from Cambrex (Walkersville, MD) (growth conditions, GC). Medium was changed every day and further re-plated when 80-90% confluency is observed. To induce cell differentiation to SMC, cells were re-plated in DMEM + 5%FBS on gelatin-coated plates (differentiation conditions, DC).
**Immunocytochemistry and Fluorescence Activated Cell Sorting Analysis**

Derived cells were digested with TrypLE™ Express for 10-20 minutes. After cells were resuspended in DMEM containing 5% FBS, they were seeded onto 0.1% gelatin- or matrigel-coated chamber polystyrene vessels (BD Bioscience, Bedford, MA) and incubated in DMEM containing 5% FBS. Cells were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized using 0.5% Triton X-100 in PBS/1% serum, and incubated overnight with the primary antibodies, mouse anti-smooth muscle (SM)-α-actin 1:1000 (Chemicon Inter. Temecula, CA) and rabbit anti-SM myosin heavy chain (MHC) 1:300 (BTI, Stoughton, MA). After rinsing, secondary goat anti-mouse or rabbit immunoglobulin G (IgG) alexa fluor 594 (red) or 488 (green) 1:500 (Molecular Probe, Eugene, OR) were added to the samples, which were then incubated for an additional hour. The cell nuclei were stained with DAPI (Molecular Probe). Finally, the cells were rinsed once more and mounted with mounting media. The slides were analyzed using a fluorescence microscope (Olympus, Japan). Normal mouse IgG1 (DAKO Corporation, Carpinteria, CA) served as negative control.

For FACS analysis, after cells were resuspended in DMEM supplemented with 5% FBS and centrifuged, the pellets were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized using 0.1% Triton X-100 in PBS/2% serum, and incubated overnight with the primary antibody. Mouse IgG1 or IgG2a served as an isoform control, while 293 cells and human vascular SMC (Cambrex Bio Science, Walkersville, MD) served as a negative and positive control, respectively. Secondary goat anti-mouse IgG alexa fluor 488 (Molecular Probe, Eugene, OR) was added to the samples, which were then incubated for an additional hour. Finally, the cells
were rinsed once more and fluorescence was analyzed using FACSCalibur™ system (BD Biosciences, San Jose, CA) following the user’s guide.

**Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

Total RNA from undifferentiated ES cells, human EBs and differentiated cells was extracted by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and treated with DNase I (Qiagen, Valencia, CA). cDNA was synthesized from 5μg of total RNA with a superscript III first-strand synthesis system (Invitrogen). cDNA samples were subjected to PCR amplification with primers selective for human smooth muscle cell genes. PCR primers and reaction conditions are described in supplemental Table 1. All RNA samples were adjusted to yield equal amplification of 18S RNA as an internal standard.

**Western Blot Analysis**

Protein samples were extracted using the M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Antibodies against SM-α-actin (1:2000 dilution, Chemicon Inter. Temecula, CA) with, caldesmon (1:1000 dilution, Sigma-Aldrich, St.Louis, MO), SMMHC (1:3000 dilution, BTI, Stoughton, MA) and β-tubulin (1:10000 dilution, Upstate, Charlottesville, VA) would be used for testing SMC-specific protein expression. Immunoactivity was visualized by the enhanced chemiluminescence’s detection system (ECL, Amersham Biosciences) according to the manufacturer’s instructions.
Contractility assays

Agonist-induced contractile activity of the differentiated cells was assayed as described\textsuperscript{3}. Differentiated SMC-like cells were washed with PBS, stimulated with 1 mM carbachol or 40 mM KCl (Sigma-Aldrich, St. Louis, MO) in the DC and monitored under the microscope up to 30 min. Images of the same field before and after carbachol or KCl treatment were snapped and compared.

Statistical Analysis

All data, calculated as a percentage from three independent experiments, were expressed as the mean ± SD and were evaluated with chi-square test. A value of \( P<0.05 \) was considered statistically significant.

References


### Supplemental Table 1: Primers used for qRT-PCR

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Supplemental Figure Legends

**Figure S1. Schematic representation of the strategy for differentiation of hES cells to SMC.**

Human embryonic stem (hES) cells were cultured in suspension for 6 days to form embryoid bodies (EBs). The resulting EBs were transferred to a culture plate coated with 0.1% gelatin for an additional 6 days. The EBs outgrowth was dissociated and cultured in smooth muscle growth medium (SMGM) on matrigel-coated plates (growth conditions, GC) with a density of $5 \times 10^5$ cells/cm$^2$. Subsequent passages were done when cells reached 80-90% confluency. To induce cell differentiation to SMC, cells were cultured in DMEM + 5% FBS on gelatin-coated plates (differentiation conditions, DC).

**Figure S2. Representative phase-contrast photographs of the differentiation process of hES cells to SMC.** (A) Undifferentiated hES clone growing on mouse feeders. (B) Suspended EBs. (C) Outgrowth of EBs for 5 days. (D) Derived cells after dissociation from outgrowth of EBs grown in GC for additional 10 days. (E) Expanded cells cultured in the growth conditions (passage 4, additional 30 days). (F) Derived cells grown for 3 days in the DC.

**Figure S3. Expression of SMC-specific markers in SMC-like cells derived from hES cells by FACS analysis.** Cells switched from GC to DC for 5 days were analyzed for expression levels of SMMHC and SM-α-actin. Values in histogram plots indicate mean±SD from 3 independent experiments.
Figure S4. SMC and endothelial-specific gene expression measured by qRT-PCR analyses.

Total RNA was isolated from derived cells growing in GC and DC for 5 days. Total RNA isolated from human aortic smooth muscle cells (HASMC) was served as the positive control. Values for mRNA levels normalized by 18S rRNA levels were expressed as mean ± SD (n=3, *p <0.01 and #p<0.05 compared to the GC).

Figure S5. KCl-induced contraction of SMC-like cells differentiated from hES cells.

Photographs were taken before (left panel) and after (right panel) treatment with 40 mM KCl for 30 minutes. Arrowheads show representative contracted cells grown in differentiation conditions. Bar=250 μm.

Movies 1 and 2: SMC-like cells derived from hES cells in vitro acquire the ability to contract in response to carbachol and KCl, respectively. SMC-like cells cultured in DC were washed with PBS and refreshed with medium. Contraction was induced by additional 1 mM carbachol (Movie-1) or 40 mM KCl (Movie-2). After the stimuli were added into culture medium for 30 min, pictures were taken every 10 seconds for 30 min to document the morphological changes. Then, cell contraction movie was generated using Metaphor software from Molecular Devices (Downingtown, PA).
Induced SMC Differentiation Protocol

1. hES cells
2. EBs suspension in DMEM + 10% FBS for 6 days
3. Outgrowth of hEBs on gelatin-coated plates in DMEM + 10% FBS for 6 days
4. Cultured in GC
5. Switch to DC
GC
4.54% ± 0.92

DC
55.26% ± 8.02

SMMHC
9.99% ± 1.68

SM-α-actin
96.81% ± 2.07

Fluorescence Intensity

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