Differentiation of Human Induced-Pluripotent Stem Cells into Smooth-Muscle Cells: Two Novel Protocols

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Abstract

Conventional protocols for differentiating human induced-pluripotent stem cells (hiPSCs) into smooth-muscle cells (SMCs) can be inefficient and generally fail to yield cells with a specific SMC phenotype (i.e., contractile or synthetic SMCs). Here, we present two novel hiPSC-SMC differentiation protocols that yield SMCs with predominantly contractile or synthetic phenotypes. Flow cytometry analyses of smooth-muscle actin (SMA) expression indicated that ~45% of the cells obtained with each protocol assumed an SMC phenotype, and that the populations could be purified to ~95% via metabolic selection. Assessments of cellular mRNA and/or protein levels indicated that SMA, myosin heavy chain II, collagen 1, calponin, transgelin, connexin 43, and vimentin expression in the SMCs obtained via the Contractile SMC protocol and in SMCs differentiated via a traditional protocol were similar, while SMCs produced via the Synthetic SMC protocol expressed less calponin, more collagen 1, and more connexin 43. Differences were also observed in functional assessments of the two SMC populations: the two-dimensional surface area of Contractile SMCs declined more extensively (to 12% versus 44% of original size) in response to carbachol treatment, while quantification of cell migration and proliferation were greater in Synthetic SMCs. Collectively, these data demonstrate that our novel differentiation protocols can efficiently generate SMCs from hiPSCs.

Introduction

Human induced-pluripotent stem cells (hiPSCs) can provide a theoretically unlimited number of terminally differentiated cells for use in tissue engineering, drug development, and autologous cell therapy; however, their utility will remain limited (particularly for clinical
applications) until efficient, standardized differentiation protocols are developed to satisfy the requirements of Good Manufacturing Practice. Protocols for differentiating hiPSCs into endothelial cells (hiPSC-ECs) [1] and cardiomyocytes (hiPSC-CMs) [2] have recently been improved, but conventional methods for generating hiPSC-derived smooth-muscle cells (hiPSC-SMCs) can take longer than four weeks [3] and may rely on co-culturing with feeder cells, which can lead to xenogenic contamination [4].

Because smooth muscle cells (SMCs) develop from a wide range of embryonic tissues, including the neural crest [5], the paraxial/somatic mesoderm [6], the lateral plate mesoderm [7], and the secondary heart field [8], many hiPSC-SMC differentiation protocols direct the cells toward an intermediate, origin-specific lineage [9, 10] before inducing the terminal SMC phenotype. Furthermore, somatic SMCs display a wide range of morphological and functional characteristics that are best described as a spectrum bounded by predominantly synthetic and contractile phenotypes [11]. Here, we present two hiPSC-SMC differentiation protocols. Both protocols begin by using a GSK inhibitor (CHIR99021) and bone morphogenic protein 4 (BMP-4) to direct the hiPSCs toward the mesodermal lineage; then, Synthetic hiPSC-SMCs are produced by culturing the cells with vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), or the Contractile hiPSC-SMC phenotype is induced with varying combinations of platelet-derived growth factor (PDGF), transforming growth factor (TGF), and FGF. Each protocol can be completed in two to three weeks and includes a 4- to 6-day selection period, which yields SMC populations that are ~95% pure and remain phenotypically stable for at least 20 generations.

**Methods**

**Cell lines**

The differentiation protocols were tested with hiPSCs that had been reprogrammed from human cardiac fibroblasts [12] or from human dermal fibroblasts [1] (GriPS, kindly provided by Dr. James Dutton, University of Minnesota, USA) and with H9 embryonic stem cells [13] (ESCs) (kindly provided by Dr James Thomson, University of Wisconsin, Madison, USA). Control assessments were performed with hiPSC-SMCs that had been differentiated via a conventional protocol [14] and in primary human aortic SMCs (HA-SMCs) (Life Technologies Corporation, Grand Island, NY, USA).

**Synthetic and contractile hiPSC-SMC differentiation protocols**

hiPSCs and ESCs were cultured in mTeSR™ medium on Matrigel-coated plates, with daily medium changes, until confluent (~2 days); then, differentiation into mesodermal-lineage cells was initiated on Day 0 by culturing the cells with CHIR99021 (5 μM) and BMP-4 (10 ng/mL) in RPMI1640 medium and 2% B27. Differentiation into Synthetic SMCs or Contractile SMCs began on Day 3. Synthetic SMCs were produced by culturing the cells with 25 ng/mL VEGF-A and FGFβ in RPMI1640 and 2% B27 minus insulin from Day 3 to Day 7, with 25 ng/mL VEGF-A and FGFβ in RPMI1640 and 2% B27 from Day 7 to Day 9, and with 10 ng/mL PDGFβ and 3 ng/mL TGFβ in RPMI1640 and 2% B27 from Day 10 to Day 14. Contractile SMCs were produced by culturing the cells with 25 ng/mL VEGF-A and FGFβ in RPMI1640 and 2% B27 minus insulin from Day 3 to Day 7, and with 5 ng/mL PDGFβ and 2.5 ng/mL TGFβ in RPMI1640 and 2% B27 from Day 7 to Day 14. The differentiated cells were enriched for SMCs by maintaining them in 4 mM lactate RPMI1640 metabolic medium for 4 to 6 days (Fig 1).
**Quantitative real time polymerase chain reaction (QRT-PCR)**

Total RNA was extracted with RNeasy Mini as directed by the manufacturer’s instructions (QIAGEN). cDNA was prepared with a Maxima First Strand cDNA Synthesis Kit (Thermal Scientific Inc), and the QRTPCR mixtures were prepared with SYBR Green PCR Master Mix (Thermal Scientific Inc). QRTPCR reactions were performed on a 7500 Fast Real-time PCR System (Applied Biosystems) and by using the Quantitation-comparative CT setting. The QRTPCR thermal cycling program included 40 cycles, and each cycle consisted of enzyme activation for 2 min at 95°C, denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C, and extension for 30 sec at 70°C; primer sequences are listed in Table 1. Duplicate measurements were performed for each analysis and were normalized to the endogenous level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Immunofluorescent imaging and flow cytometry**

For immunofluorescent imaging, adherent cells were fixed, permeabilized, blocked with 5% donkey serum, and incubated with primary antibodies at 4°C overnight; then, the primary antibodies were labeled with fluorescent secondary antibodies (Jackson Lab, USA) (Table 2), cell nuclei were labeled with DAPI, and the cells were imaged under an Olympus 200M microscope. For flow-cytometry analyses, cells were fixed with 4% paraformaldehyde for 10 min and incubated with UltraV (Thermo Scientific, USA) block for 7 min at room temperature; then, the cells were labeled with primary phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated anti-SMA antibodies and with isotype-control antibodies (BD Pharmingen, USA).
for 30 min at 4°C in phosphate-buffered saline (PBS) and 2% fetal bovine serum (FBS), washed with 2% FBS/PBS, and re-suspended in 0.3 mL 2% FBS/PBS containing 5 μL of propidium iodide (10 μg/mL). Flow-cytometry analyses were performed with a FACS Aria instrument (BD Biosciences, USA), and control assessments were performed with undifferentiated hiPSCs.

### Cell migration

Cells were cultured in 6-well plates (4×10⁵ cells/well) overnight at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS under a water-saturated, 5% CO₂ atmosphere; then, the cultures were scratched with a 200-μL pipette tip and the medium was changed to DMEM containing 1% FBS. Images were taken 10 hours later, and cell migration was quantified as the number of cells present in the scratched region.

#### Table 1. QRT-PCR Primer Sequences.

| Gene                        | Primer Sequences       |
|-----------------------------|------------------------|
| Alpha SMA 2                 | Forward: GAT CTG GCA CCA CTC TTT CTA C |
|                             | Reverse: GAT CTG GCA CCA CTC TTT CTA C |
| Calponin                    | Forward: ATG GCC GAT TGC CAC TCC AAC |
|                             | Reverse: ATG GCC GAT TGC CAC TCC AAC |
| Myosin heavy chain 11       | Forward: AGG GCA ACC TAG ACA AGA ATA AG |
|                             | Reverse: CTG GAT GTG GAG AGT GGA GAT G |
| Transgelin                  | Forward: GAA ACA GAG CCC AGG TTA |
|                             | Reverse: GAA ACA GAG CCC AGG TTA |
| VE cadherin                 | Forward: GAT GGT GAG CCC AGG TTA |
|                             | Reverse: GAT GGT GAG CCC AGG TTA |

Alpha SMA 2, alpha smooth-muscle actin 2; VE cadherin, vascular endothelial cadherin.

doi:10.1371/journal.pone.0147155.t001

#### Table 2. Antibodies.

| Antibody                  | Isotype/Source/Catalog Number/Clone       | Concentration |
|---------------------------|------------------------------------------|---------------|
| αSMA                      | Mouse IgG/Sigma/0837M4778V/Mab            | 1:150         |
| Connexin 43               | Rabbit IgG/Millipore/AB1728/Polyclonal    | 1:100         |
| Collagen I                | Mouse IgG/ Millipore /MAB3391/Mab        | 1:150         |
| Calponin                  | Mouse IgG/ Transduction Labs/8592591550/Mab | 1:100       |
| Vimentin                  | Goat IgG/R&D systems/AF2105               | 1:100         |
| Secondary antibody        | FITC Donkey Anti-mouse IgG/703-099-155   | 1:100         |
| Secondary antibody        | FITC Donkey Anti-rabbit IgG/711-095-152  | 1:100         |
| Secondary antibody        | FITC Donkey Anti-goat IgG/705-095-147    | 1:100         |
| Secondary antibody        | TRITC Donkey Anti-mouse IgG/Jackson Lab/712-025-150 | 1:100       |
| Secondary antibody        | TRITC Donkey Anti-rabbit IgG/ Jackson Lab/711-026-152 | 1:100       |
| Secondary antibody        | TRITC Donkey Anti-goat IgG/ Jackson Lab/705-025-147 | 1:100       |
| Secondary antibody        | Alexa-Fluor647 Donkey Anti-mouse IgG/ Jackson Lab/715-495-140 | 1:100       |
| Secondary antibody        | Alexa-Fluor647 Donkey Anti-rabbit IgG/ Jackson Lab/711-605-152 | 1:100       |
| Secondary antibody        | Alexa-Fluor647 Donkey Anti-goat IgG/ Jackson Lab/705-605-147 | 1:100       |
| Secondary antibody        | FITC Donkey Anti-mouse IgM/ Jackson Lab/715-096-020 | 1:100       |
| Secondary antibody        | TRITC Donkey Anti-mouse IgM/ Jackson Lab/715-096-020 | 1:100       |

doi:10.1371/journal.pone.0147155.t002
Contraction

**Cell contraction test.** Cells were seeded onto a gelatin-coated 6-well plate (2×10^5 cells/well), partially detached by incubating them with a non-enzymatic dissociation buffer (Versene, Invitrogen, USA), and then treated with 10 µM carbachol for 5 min. Images were obtained both 0 and 5 min after carbachol treatment, and contraction was evaluated by using CellC Software (Department of Signal Processing, Tampere University of Technology, [https://sites.google.com/site/cellcsoftware/download](https://sites.google.com/site/cellcsoftware/download)) to measure the cells’ cross-sectional surface areas.

**Gel contraction test.** SMCs (1×10^6) were suspended in 250 µL of an 8 mg/mL fibrinogen solution; then, the cell-containing fibrinogen solution was mixed with 250 µL of a 5 U/mL thrombin solution in one well of a 24-well plate and incubated at 37°C. Ten min later (after the mixture had formed a semi-solid gel) the gels were transferred to a 6-well plate and cultured with 300 U/mL aprotinin and 10 µM Rho kinase (ROCK) inhibitor (Y-27632; Millipore, USA) in 2 mL DMEM containing 5% PBS. Gel sizes were measured at 24-hour intervals over 3 days.

Cell proliferation

SMCs (1×10^6 cells/mL in RPMI1640) were serially diluted in a 1:2 ratio, and 100 µL of solution for each cell concentration was added to the wells of a 96-well plate. The cells were cultured with PDGFβ and TGFβ in DMEM containing F12 and 5% FBS for 2 hours under 5% CO₂, then, 20 µL of One Solution Cell Proliferation Assay reagent (Promega, USA) was added to the well, and the cells were cultured under the same conditions for another 90 min. Proliferation was quantified by measuring optical density at 490 nm with an ELISA reader (Energy H2, BioTek instrument, USA) both before (i.e., at 0 min) and after the 90-min culture period; measurements were blanked with the One Solution Cell Proliferation Assay reagent, and the measurement at 0 min was used to define the baseline.

Statistics

Data are presented as mean±standard deviation (SD) and were evaluated for significance via the student T test (unpaired, 2 tailed); the Bonferroni correction was used when more than two groups were being compared. Statistical analyses were performed with SPSS software (version 20.0; IBM, Armonk, New York, USA) and a p-value of less than 0.05 was considered significant.

Results

**Differentiation of hiPSCs into hiPSC-SMCs**

The efficiency of our differentiation protocols was evaluated in cardiac-lineage hiPSCs (chiPSCs), dermal-lineage hiPSCs (dhiPSCs), and embryonic stem cells (ESCs). The cells were incubated on Matrigel-coated plates for two days until they reached near-complete confluence. Differentiation into the mesodermal lineage was initiated on Day 0 and produced cultures of uniform morphology with a small amount of cell death (10%~15%) that can likely be attributed to CHIR99021 toxicity. Induction of the SMC phenotypes began on Day 3, and then the differentiated cells were purified by maintaining them in metabolic medium for 4 to 6 days beginning on Day 14 (Fig 1). Before purification, flow cytometry analyses indicated that SMA was expressed by ~45% of chiPSC-derived SMCs (Synthetic: 46%, Contractile: 44.5%; n = 6) (Fig 2), by 35% (Contractile; n = 3) to 75% (Synthetic; n = 3) of dhiPSC-SMCs, and by ~80% (Synthetic: 83%, Contractile: 78%; n = 3) of hESC-derived SMCs. The proportion of SMA⁺ cells increased to ~95% after purification (chiPSC-SMCs, Synthetic: 95.1%, Contractile: 94.5%; n = 6. dhiPSC-SMCs, Synthetic: 97%, Contractile: 96.4%; n = 3. hESC-SMCs, Synthetic: 98%, Contractile: 98.6%; n = 3).
Characterization of Synthetic and Contractile hiPSC-SMCs

The specificity of the two hiPSC-SMC differentiation protocols was evaluated by comparing the expression of cell-specific markers in chiPSC-derived Synthetic and Contractile SMCs, in
hiPSC-SMCs that were generated via conventional differentiation methods, and in primary human aortic SMCs (HA-SMCs). Myosin heavy chain 11, calponin, and transgelin mRNA levels in conventional hiPSC-SMCs and Contractile chiPSC-SMCs were similar and significantly higher than in Synthetic chiPSC-SMCs (Fig 3A), but Synthetic chiPSC-SMCs were more likely
to express collagen 1, connexin 43, or vimentin (Fig 3B). Assessments of cell migration (Fig 4A) and proliferation (Fig 4B) were also significantly greater in Synthetic chiPSC-SMCs than

![Graph showing cell migration and proliferation](image)

**Fig 4. hiPSC-SMC functional assessments.** (A) $4 \times 10^5$ Synthetic or Contractile chiPSC-SMCs were cultured on gelatin-coated plates for 24 hours; then, the plate was scratched with a 200-μL pipette tip, and images of the scratched area were obtained 0 and 10 hours later. Migration was quantified by counting the number of cells that had migrated into the scratched area ($p<0.01$). (B) $1 \times 10^6$/mL Synthetic or Contractile chiPSC-SMCs were suspended in 100 μL of RPMI1640 and cultured in the presence of PDGFβ or TGFβ for 90 min; then, the solutions were serially diluted in half six times, and cell concentrations were evaluated via optical density measurements at 490 nm ($p<0.01$). (C) $2 \times 10^5$ Synthetic or Contractile chiPSC-SMCs were cultured on gelatin-coated plates for 24 hours; then, the cells were treated with carbachol to induce contraction, and images were obtained 0 and 5 min later. (D) Contraction was evaluated by calculating the mean cell surface area at each of the two time points ($p<0.01$). (E) $1 \times 10^6$ Synthetic or Contractile chiPSC-SMCs were suspended in a fibrinogen gel; then, the gels were cultured with aprotinin and Rho kinase inhibitor, and the surface area of the gels was measured 0 and 3 days later ($p<0.01$).

doi:10.1371/journal.pone.0147155.g004
in Contractile chiPSC-SMCs, but the two-dimensional surface area of Contractile
chiPSC-SMCs declined more extensively in response to carbachol treatment (Fig 4C and 4D).
Gels containing Contractile chiPSC-SMCs also contracted to 27% of their original size, com-
pared to 46% for gels that contained Synthetic chiPSC-SMCs (Fig 4E).

**Discussion**

Although the contractile activity of SMCs may be their most prominent characteristic, SMCs also
contribute to a variety of other physiological activities, including the growth and remodeling of
vessels in response to vascular injury, exercise, or pregnancy [15]. The functional diversity of
SMCs is accompanied by considerable phenotypic diversity, ranging from contractile cells at one
extreme to predominantly synthetic cells at the other. These two archetypal SMC phenotypes dif-
fer substantially in morphology, marker expression, and activity, including their rates of prolif-
eration and migration. Thus, the utility of hiPSC-derived SMCs for a particular application may
depend on the specific phenotype generated. For example, pharmacological studies of vasocon-
striction may be best performed with populations of primarily contractile SMCs, while tissue
engineering [9, 10, 16–19] and cell therapy could benefit from the inclusion of synthetic SMCs,
which are more proliferative and produce larger amounts of extracellular matrix material [20].

A number of highly efficient hiPSC-SMC differentiation protocols have been developed [14,
21, 22], but methods for specifying the contractile or synthetic SMC phenotype have been unavail-
able until recently [23, 24]. The results presented here indicate that our novel differentiation proto-
colks effectively direct hiPSCs toward either a contractile or synthetic SMC phenotype, and that the
SMC populations can be purified to ~95% via metabolic selection. Furthermore, the risk of xeno-
genic contamination is minimized, because the differentiation and purification procedures do not
require exposure to feeder cells, and the entire protocol (both differentiation and purification) can
be completed in just 2–3 weeks. The cells can also be quickly expanded in DMEM with 5 ng/mL
FGF and 5% serum or Medium 231 (Life Technologies, USA), and the SMC phenotype remains
stable for at least four months or 20 generations when the cells are maintained in 5% FBS or
RPMI-1640 medium with B27. Proliferation can be halted by limiting the serum concentration or
by reducing the cell density to ~10% or less (e.g., 5×10^4 cells/well in a 6-well plate).

The predominant phenotype of an hiPSC-SMC population can be characterized by evaluat-
ing the expression of a panel of cell-surface markers [23]. Marker expression in SMCs gener-
ated via our Contractile SMC differentiation protocol was similar to the expression in
conventionally produced hiPSC-SMCs, while the synthetic SMC differentiation protocol
yielded cells that produced more of the extracellular matrix proteins collagen 1 and connexin
43. These observations are consistent with the results from studies with primary SMCs that
have been isolated from swine coronary arteries [25, 26], although the pattern of gene expres-
sion appears to vary depending on whether the cells are studied in vivo or in vitro [11, 27]. Fur-
thermore, some evidence suggests that marker expression in SMCs derived from hiPSCs and
hESCs, or from hiPSCs that were generated from different somatic tissues, can also differ [23,
24]. Thus, the efficiency of our hiPSC-SMC differentiation protocols and subsequent function
of the differentiated cells may depend on which hiPSC (or hESC) line is used.

In conclusion, we have developed two novel, highly efficient differentiation protocols that
can be used to generate contractile or synthetic SMCs from hiPSCs in just 2–3 weeks while
minimizing the risk for xenogenic contamination.

**Acknowledgments**

The authors would like to thank W. Kevin Cukier-Meisner, PhD, ELS, for his editorial assis-
tance with this manuscript.
**Author Contributions**
Conceived and designed the experiments: LY JZ. Performed the experiments: LY ZG TN CJ LG JD CH. Analyzed the data: LY CH. Contributed reagents/materials/analysis tools: LY JZ. Wrote the paper: LY JZ.

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