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Functional Recapitulation of Smooth Muscle Cells Via Induced Pluripotent Stem Cells From Human Aortic Smooth Muscle Cells

Tae-Hee Lee,* Sun-Hwa Song, Koung Li Kim, Ji-Yeun Yi, Ga-Hee Shin, Ji Yeon Kim, Jihoon Kim, Yong-Mahn Han, Sang Hun Lee, Suk-Ho Lee, Sung Han Shim, Wonhee Suh*

Rationale: Generation of induced pluripotent stem (iPS) cells has been intensively studied by a variety of reprogramming methods, but the molecular and functional properties of the cells differentiated from iPS cells have not been well characterized.

Objective: To address this issue, we generated iPS cells from human aortic vascular smooth muscle cells (HASMCs) using lentiviral transduction of defined transcription factors and differentiated these iPS cells back into smooth muscle cells (SMCs).

Methods and Results: Established iPS cells were shown to possess properties equivalent to human embryonic stem cells, in terms of the cell surface markers, global mRNA and microRNA expression patterns, epigenetic status of OCT4, REX1, and NANOG promoters, and in vitro/in vivo pluripotency. The cells were differentiated into SMCs to enable a direct, comparative analysis with HASMCs, from which the iPS cells originated. We observed that iPS cell–derived SMCs were very similar to parental HASMCs in gene expression patterns, epigenetic modifications of pluripotency-related genes, and in vitro functional properties. However, the iPS cells still expressed a significant amount of lentiviral transgenes (OCT4 and LIN28) because of partial gene silencing.

Conclusions: Our study reports, for the first time, the generation of iPS cells from HASMCs and their differentiation into SMCs. Moreover, a parallel comparative analysis of human iPS cell–derived SMCs and parental HASMCs revealed that iPS-derived cells possessed representative molecular and in vitro functional characteristics of parental HASMCs, suggesting that iPS cells hold great promise as an autologous cell source for patient-specific cell therapy. (*Circ Res*. 2010;106:120-128.)

Key Words: human smooth muscle cell ■ induced pluripotent stem cells ■ reprogramming

The therapeutic potential of embryonic stem (ES) cells is based on the ability of ES cells to recover or replace damaged tissues through self renewal and differentiation.[1] As a practical candidate for regenerative medicine, ES cells should be harmonized with the immune system of the patient. In the past, however, it was very difficult to make patient-specific ES cells for several reasons. For example, somatic cell nuclear transfer experiments with human oocytes have been extremely inefficient and present ethical problems, and methods to induce cell fusion between ES cells and somatic cells present difficulties in removing ES cell chromosomes from fused tetraploid pluripotent stem cells.[2–4] However, Yamanaka and colleagues recently developed a relatively simple and highly innovative method for the generation of ES cell–like pluripotent cells, so-called induced pluripotent stem (iPS) cells, from human and mouse fibroblasts.[5,6] Fibroblasts were reprogrammed into an embryonic-like state by retroviral transduction with 4 transcription factors (OCT4, SOX2, KLF4, and c-MYC), and these reprogrammed cells were shown to be remarkably similar to ES cells, in terms of morphology, gene expression, and differentiation potential in vitro and in vivo.[5,6] Simultaneously, Thomson and colleagues reported the generation of iPS cells from human fibroblasts by using a different gene combination (OCT4, SOX2, NANOG, and LIN28) than that used by Yamanaka and colleagues.[7] In addition, several other investigators have

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reported the generation of human iPS cells from adult somatic cells, mostly fibroblasts, indicating that iPS cell technology is likely to be highly reproducible.[8–10] Because it successfully overcomes serious limitations imposed by previous technologies, such as somatic cell nuclear transfer and cell fusion, iPS cell technology is expected to advance the development of patient-specific cell therapy by providing abundant, pluripotent patient-specific stem cells. In addition, iPS cell technology can be used to construct cell based-model systems for studying human inherited genetic disorders, an effort which has been limited because of the difficulty of isolating or maintaining symptomatic cells from patients.

Successful application of iPS cell technologies depends on the assumption that cells differentiated from patient-derived iPS cells have the same, or substantially similar, characteristics to those of the somatic cells of the patient. Therefore, it is necessary to perform a comparative analysis of the molecular and biological properties of cells differentiated from iPS cells and those of the same lineaged somatic cells from which the iPS cells were originated. To address this issue, we generated iPS cells from human aortic vascular smooth muscle cells (HASMCs) and differentiated these iPS cells back into smooth muscle cells (SMCs). We then characterized and compared the gene expression and cellular functionality of iPS cell–derived SMCs with the parental HASMCs from which the iPS cells were derived.

Non-standard Abbreviations and Acronyms

EB	embryoid body
ES	embryonic stem
iPS	induced pluripotent stem
MEF	mouse embryonic fibroblast
HASMC	human aortic vascular smooth muscle cell
miRNA	microRNA
SES	smooth muscle cell–derived ES-like
SMA	smooth muscle actin
SMC	smooth muscle cell

Methods

Reagents

Human recombinant basic fibroblast growth factor was obtained from R&D Systems (Minneapolis, Minn). Primary antibodies used in the study were SSEA-4, NANOG, OCT4, AP (R&D Systems), α-smooth muscle actin (α-SMA) (Dako Inc, Carpinteria, Calif), vimentin (Abcam, Cambridge, MA), α-fetoprotein (R&D Systems), and β-III tubulin (Chemicon International Inc, Temecula, Calif). Growth factor–reduced Matrigel was purchased from BD Biosciences Inc (Palo Alto, Calif).

Cell Culture

Human ES cells (H9; Wicell Research Institute, Madison, Wis) and iPS cells were cultured on 60-mm dishes containing mitomycin C–treated mouse embryonic fibroblast (MEF) feeder cells in standard human ES cell medium (ReproCell Inc, Tokyo, Japan). For passaging, human iPS cells were incubated with ES dissociation solution (ReproCell Inc) at 37°C. When colonies were detached from the dishes, dissociation solution was aspirated. Cell clusters were washed with ES cell medium and collected in 1.5 mL tubes. After the spontaneous precipitation of pellets for 5 minutes, medium was washed out and cells were resuspended with fresh medium. Cells were then transferred to a new dish with MEF or STO feeder cells and maintained in ES cell medium containing 5 ng/mL basic fibroblast growth factor. STO cells were maintained in DMEM containing 10% FBS, 1% penicillin, and streptomycin. HASMCs were purchased from ScienCell Research Laboratories (Carlsbad, Calif) and maintained in SMC-specific medium, SMCM (ScienCell Research Laboratories). HUVECs were purchased from Cambrex Bio Science Inc (Walkersville, Md) and cultured with endothelial growth medium (EGM)-2.

Lentiviral Transduction and iPS Cell Generation

Human full length cDNAs for OCT4, SOX2, NANOG, LIN28, and KLF4 were subcloned into a modified pLenti6/R4R2/V5-DEST lentiviral vector (Invitrogen Inc, Carlsbad, Calif) with human elongation factor-1α promoter. Plasmids were transiently transfected with other packaging plasmids into 293FT cells, according to the protocol of the manufacturer (Invitrogen Inc). After the appropriate viral preparation, lentiviral supernatant was added to the HASMC dish at a 1:1 (vol/vol) ratio with SMCM in the presence of polybrene (8 μg/mL) for 12 hours. After 12 hours, cells were second-infected with the lentiviral supernatant for an additional 12-hour period. Cells were then maintained in fresh SMCM containing blastcidin (Invitrogen Inc) for subsequent 4 days. After trypsinization, 5×10^4 transduced cells were transferred to 100-mm dishes containing mitomycin C–treated MEF. The next day, the medium was replaced with human ES medium and changed every other day thereafter. After 3 weeks, colonies with ES cell morphology were manually picked and transferred to new dishes containing mitomycin C–treated MEF for the further expansion.

Immunocytochemistry

Immunocytochemistry was performed according to the previously described method.[11] Briefly, cells were grown on cover glass and fixed in 4% paraformaldehyde. They were permeabilized with PBST (0.1% Triton X-100 in PBS), incubated with blocking solution (Dako Inc), and subsequently incubated overnight with primary antibodies in blocking solution. Cells were then washed and incubated with Alexa 488– or Alexa 594–conjugated secondary antibodies (Invitrogen Inc) diluted at a ratio of 1:250 (vol/vol) in blocking solution for 1 hour at room temperature. Visualization was accomplished using a Zeiss LSM 500 confocal microscope.

Teratoma Formation

Approximately 1×10^6 iPS cells were suspended in a DMEM/Matrigel solution (BD Biosciences Inc) at a ratio of 1:1 (vol/vol), and the cell/Matrigel suspension was subcutaneously injected into the dorso-lateral area of NOD/SCID mice (Charles River Laboratories, Yokohama, Japan). Six weeks after injection, mice were anesthetized by intraperitoneal injection of ketamine-xylazine (50 mg/kg and 5 mg/kg, respectively). Xenografted masses were harvested, fixed, embedded in paraffin, and sectioned. Tissue sections were stained with hematoxylin/eosin, oil red O, or antibodies against nestin and cytokeratin 17 (R&D Systems). The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Pochon CHA University. All procedures were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996).

SMC Differentiation and Isolation

To differentiate iPS or H9 cells into SMCs, embryoid bodies (EBs) were transferred to gelatin-coated plate and maintained in SMCM medium for 10 days. Colonies with SMC-like cell morphology were manually picked and examined for the expression of SMC-specific

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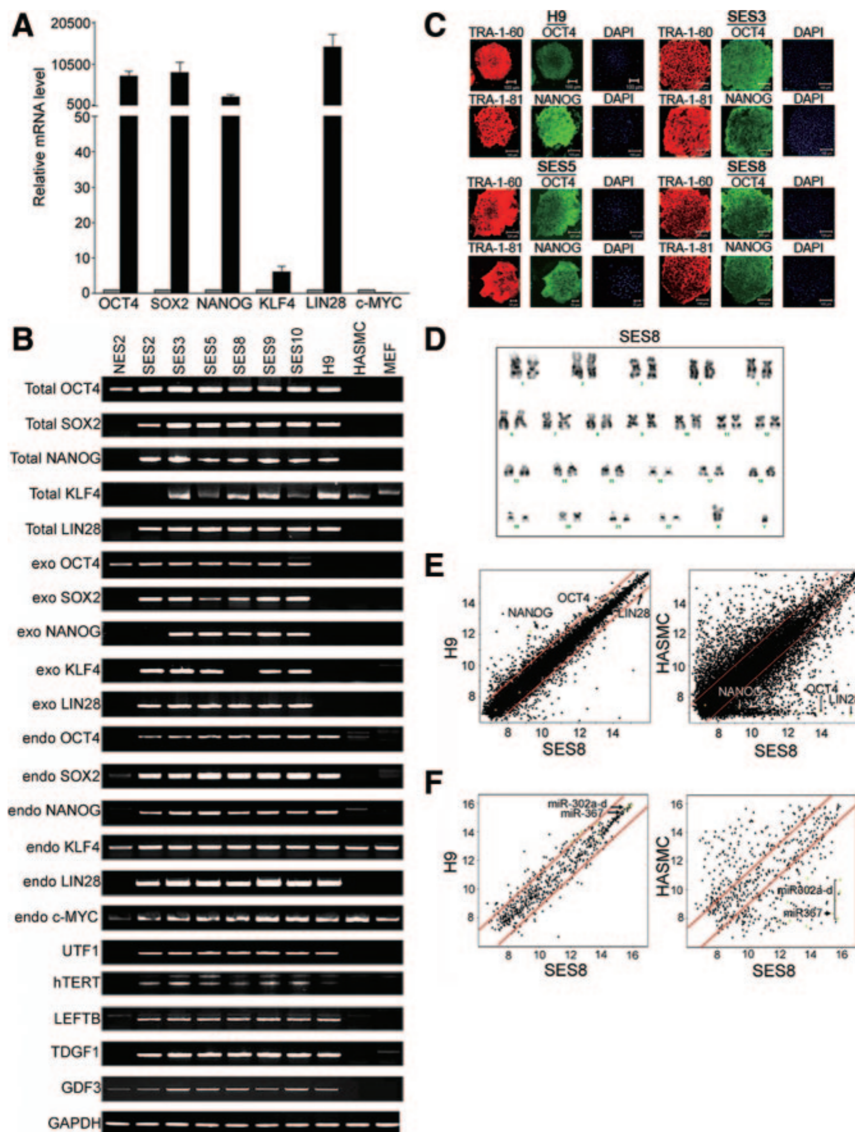

Figure 1. Generation and characterization of iPS cells from HASMCs. A, Quantitative RT-PCR analyses of the endogenous expression of OCT4, NANOG, SOX2, LIN28, KLF4, and c-MYC in human ES cells (H9, black bar) and HASMCs (gray bar) (n=3; means±SEM). B, Expression profile of human ES cell marker genes, endogenous genes, and lentiviral transgenes in iPS cell clones. NES indicates non–ES-like cells; endo, endogenous genes; exo, lentiviral transgenes; total, endo+exo. RT-PCR analysis specific for lentiviral DNA showed the presence or absence of transgene expression in iPS cells. GAPDH was used as a loading control in RT-PCR. C, Representative immunofluorescence analysis of iPS cells. Clear expression of ES cell–specific surface antigens such as TRA-1 to 60, TRA-1 to -81, NANOG, and OCT4 was observed. Nuclei were stained with DAPI. Scale bar=100 μm. D, Karyotyping analysis of SES8 cells. E and F, Scatter plots comparing the (E) global mRNA and (F) miRNA expression patterns between iPS cells (SES8) and H9 cells and between SES8 cells and parental HASMCs. Red lines indicate 2-fold changes in mRNA or miRNA expression levels between the paired cells. Positions of selected mRNA and miRNA were indicated as arrows in scatter plots.

marker genes. Positive cells were transferred to new culture dishes for further expansion, and were checked for their expression of SMC-specific marker genes. During the course of the experiment, the cells were used until passages 3 to 10 and were checked routinely for the α-SMA expression.

Bisulfite Genomic Sequencing

Genomic DNA was subjected to sodium bisulfite treatment using the EZ DNA Methylation-Gold Kit according to the protocol of the manufacturer (Zymo Research, Orange, Calif). Aliquots of bisulfite-treated DNA (25 to 50 ng) were amplified by PCR.

The resulting amplified products were purified by using NucleoSpin Extract Kit (MACHEREY-NAGEL, Germany), and subcloned into pGEM-T EASY vector (Promega, Madison, Wis). For each cell type, 10 clones were sequenced using M13R primers. Sequencing results were further analyzed by using a web-based program (Blast-2) or software (BiQ Analyzer). A bisulfite conversion of at least 95% was accepted. Primer sequences for OCT4, REX1, and NANOG promoter are listed in Table V in the Online Data Supplement, available at http://circres.ahajournals.org.

Cytosolic Ca^{2+} Measurement

Cytosolic $[Ca^{2+}]$ in SMCs was measured according to the previously described method.[12] Briefly, cells were incubated with normal Tyrode solution (NaCl 148 mmol/L, KCl 5 mmol/L, $CaCl_2$ 2 mmol/L, $MgCl_2$ 1 mmol/L, glucose 10 mmol/L, Hepes 10 mmol/L, pH 7.4) containing 2 μmol/L Fura-2 acetoxymethyl ester/0.1% pluronic F-127 for 15 minutes at room temperature. Ca^{2+} transients were evoked by the treatment of 50 mmol/L of KCl or 5 μmol/L carbachol. The fluorescence imaging of SMCs was performed using a ×40 water immersion objective (LUMPlanFl, Olympus) and charge-coupled device camera (SensiCam, PCO, Kelheim, Germany). Images were taken with double wavelength excitation at 340 and 380 nm. A ratio of fluorescence intensity at 340 and 380 nm (F_{340}/F_{380}) was regarded as a parameter representing cytosolic $[Ca^{2+}]$.

Results

Generation of iPS Cells

For the generation of iPS cells from somatic HASMCs, we first examined expression of reprogramming transcription factors in HASMCs by using quantitative RT-PCR. As compared with expression levels in H9, human ES cells, the expression of OCT4, SOX2, NANOG, and LIN28 in HASMCs were markedly low, whereas the expression of KLF4 was moderately low (Figure 1A). Furthermore, the expression of c-MYC was very low in both cell lines (Figure

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1A). From these results, we speculated that c-MYC, a potent oncogene,[14,15] was not absolutely necessary for generating iPS cells from HASMCs. In this study, the 5 transcription factors that showed relatively low expression levels in the HASMCs were used to reprogram HASMCs into iPS cells. Therefore, lentiviruses containing human OCT4, SOX2, KLF4, NANOG, and LIN28 were introduced into HASMCs. After culturing transduced HASMCs onto MEF feeder cells for 3 weeks, 10 human ES cell–like colonies and 2 fibroblast-like cell clusters (non-ES (NES) cells; negative controls) were manually picked, cultured, and expanded. The efficiency of the generation of iPS cells from HASMCs was 0.002% and comparable to the previous reports.[6,7]

Of the 6 iPS cells initially established, 2 iPS cell lines (SES3 and SES8; SMC-derived ES-like cells are designated as SES cells in this study) have been maintained in continuous culture to date, a period which represents more than 30 passages. As shown in Online Figure I, the morphology of the SES8 cells was very similar to that of H9 cells.

Characterization of iPS Cells

We first characterized the expression of human ES cell marker genes and lentiviral reprogramming transgenes in the established iPS cells. RT-PCR analysis showed that the 6 SES cell lines exhibited strong expression of stemness marker genes (UTF1, hTERT, LEFTB, TDGF1, and GDF3) and reactivation of pluripotency-related endogenous genes (OCT4, SOX2, and NANOG), whereas the parental HASMCs and NES cells did not (Figure 1B). These results were further confirmed by immunofluorescence analysis data which showed SES cells stained strongly positive for human ES cell–specific markers such as TRA-1 to -60, TRA-1 to -81, NANOG, and OCT4 (Figure 1C). When the expression of lentiviral transgenes was examined using specific primers, 4 SES cell lines (SES3, -5, -9, and -10) showed expression of all 5 transgenes, whereas 2 SES cell lines (SES2 and -8) were found to express only 4 transgenes (Figure 1B). In particular, the SES8 cells contained exogenous OCT4, SOX2, NANOG, and LIN28 genes, which constitutes the same combination of genes used previously by Thompson and his colleagues.[7] Genomic PCR analysis confirmed that the lack of NANOG or KLF4 gene expression in the SES2 and SES8 cells was attributable to the absence of the lentiviral transgene integration but not attributable to gene silencing (data not shown). Karyotyping and DNA fingerprinting analyses demonstrated that SES8 cells comprised normal chromosome (Figure 1D) and were derived from HASMCs, not from the contamination of female H9 cells which were the only human ES cells available in our laboratory (Online Table I).

Global Gene Expression Profile of Human iPS Cells

cDNA microarrays were used to compare the global gene expression patterns between SES8 cells and H9 cells, and between the SES8 cells and HASMCs. Scatter plots of these data showed an intensive correlation of SES8 cells with H9 cells, as compared with HASMCs from which the SES8 cells were derived (Figure 1E). Notably, SES8 cells exhibited strong induction of developmental pluripotency-associated 4 (DPPA4), DNA methyltransferase 3B (DNMT3B), sal-like 4 (SALL4), and reduced expression 1 (REX1) genes similar to levels observed in H9 cells, and substantial loss of SMC-specific genes including collagen type VI α_3 (COL6A3), COL1A2, SMA α2 (ACTA2), and fibulin 5 (FBLN5) (Online Table II). In addition, we investigated whether micro (mi)RNA expression was changed during reprogramming processes. micro (mi)RNA patterns showed a higher degree of similarity between SES8 cells and H9 cells, than between SES8 cells and HASMCs (Figure 1F). In particular, ES cell–specific miR302-367 clusters, recently reported to be regulated by OCT4, NANOG and SOX2,[16,17] were highly expressed in both SES8 cells and H9 cells, in contrast to a 50- to 200-fold lower expression level observed in HASMCs. These data demonstrated that SES8 cells reprogrammed from HASMCs became highly similar to human ES cells in terms of miRNA, as well as mRNA expression patterns.

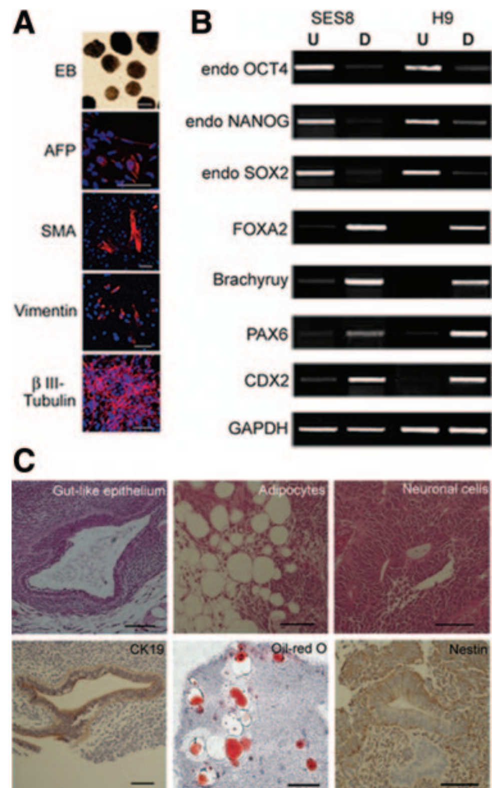

Figure 2. Pluripotency of HASMC-derived iPS cells. A, In vitro EB-mediated differentiation of SES8 cells into 3 germ layers. EBs were cultured in differentiation medium on gelatin-coated plate and stained with α-fetoprotein (endoderm), α-SMA (mesoderm), vimentin (mesoderm), or β_3-tubullin (ectoderm). Nuclei were stained with DAPI. Scale bar=100 μm. B, Changes in the expression levels of endogenous pluripotency-related genes and various differentiation markers were assessed in undifferentiated (U) and in vitro-differentiated (D) SES8 and H9 cells using RT-PCR. C, Teratoma derived from SES8 cells containing all 3 germ layers. Teratoma were harvested 6 weeks after subcutaneous injection of SES8 cells into NOD/SCID immunodeficient mice, and their sections were stained with hematoxylin/eosin, oil red O (adipocyte, mesoderm), and antibodies against nestin (neural rosette, ectoderm) and cytokeratin 17 (gut-like epithelium, endoderm). Scale bar=200 μm.

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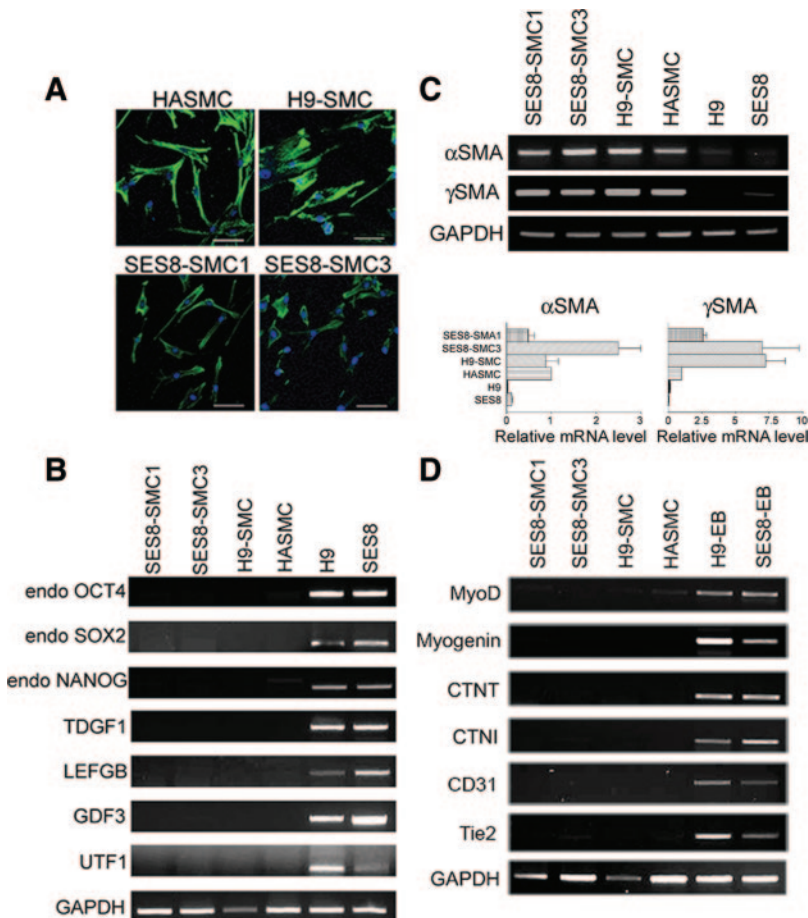

Figure 3. Generation of SMCs from iPS cells. A, α-SMA expression in HASMCs, iPS cell–, and H9 cell–derived SMCs (SES8-SMC1, SES8-SMC3, H9-SMC). Scale bar=100 μm. RT-PCR analyses showing the markedly reduced expression of endogenous pluripotency-related genes and human ES cell marker genes (B) and the expression of SMC marker genes (α- and γ-SMA) (C) but not skeletal myocyte-specific (MyoD, myogenin), cardiomyocyte-specific (CTNT, CTNI), or endothelial cell–specific (CD31, Tie2) genes (D) in SES8-SMCs and H9-SMCs. GAPDH was used as a loading control.

In Vitro Differentiation and Teratoma Formation of the iPS Cells

An EB culture system was used to determine whether human iPS cells exhibited an ability to differentiate into 3 germ layers in vitro. Cells differentiated from SES8 cells stained positively for endoderm (α-fetoprotein), mesoderm (α-SMA, vimentin), and ectoderm (βIII-tubulin) markers (Figure 2A). In accordance with these immunofluorescence data, RT-PCR analysis revealed that EB-mediated differentiation significantly upregulated forkhead box A2 (FOXA2, endoderm), BRACHYURY (mesoderm), paired box 6 (PAX6, ectoderm), and caudal-related protein (CDX2, trophectoderm) but markedly reduced the endogenous expression of OCT4, SOX2, and NANOG in both SES8 cells and H9 cells (Figure 2B). Furthermore, the pluripotency of SES8 cells in vivo was confirmed by subcutaneous transplantation into the lateral dorsal region of immunodeficient mice. Six weeks after cell injection, teratoma that contained derivatives of all 3 germ layers, including gut-like epithelium (cytokeratin 17, endoderm), adipose tissues (oil-red O, mesoderm), and neural tissues (nestin, ectoderm), were readily observed (Figure 2C). Taken together, these data demonstrated that SES8 cells had the capacity to differentiate to 3 germ layers both in vitro and in vivo.

Differentiation of iPS Cells Into SMCs

To differentiate SES8 cells into SMCs, EBs were seeded on gelatin-coated plates and maintained in SMC differentiation medium for 10 days. Colonies having SMC-like morphology were manually picked and transferred onto new culture dishes for further expansion and characterization. From these experiments, 2 SES8-derived SMC-like cell lines (SES8-SMC1 and SES8-SMC3) were established, in which all cells strongly expressed SMC-specific marker, α-SMA (Figure 3A), and they were confirmed to be derived from SES8 cells by DNA fingerprinting analysis (Online Table I).

With the same differentiation method, H9 cells were differentiated into α-SMA–positive cells (H9-SMCs), which were used as a positive control for the characterization of SES8-derived SMCs (Figure 3A). RT-PCR analysis revealed that differentiated SMCs (SES8-SMC1, SES8-SMC3, and H9-SMCs) barely expressed endogenous pluripotency-related transcriptional factors (OCT4, NANOG, and SOX2) or stemness marker genes (TDGF1, LEFGB, GDF3, and UTF1) (Figure 3B). To examine quantitative expression of known SMC-specific genes including α-SMA, smooth myosin heavy chain, calponin, myocardin, and caldesmon in SMCs derived from SES8 and H9 cells, we performed real-time RT-PCR. We found that SES8 and H9 cells constitutively expressed smooth myosin heavy chain, calponin, myocardin, and caldesmon, as did SMCs, implying that these genes may be not good indicators for differentiation of ES or iPS cells into SMCs (data not shown). However, we found that α- and γ-SMA were barely expressed in SES8 and H9 cells, whereas these genes were highly expressed in HASMCs, suggesting

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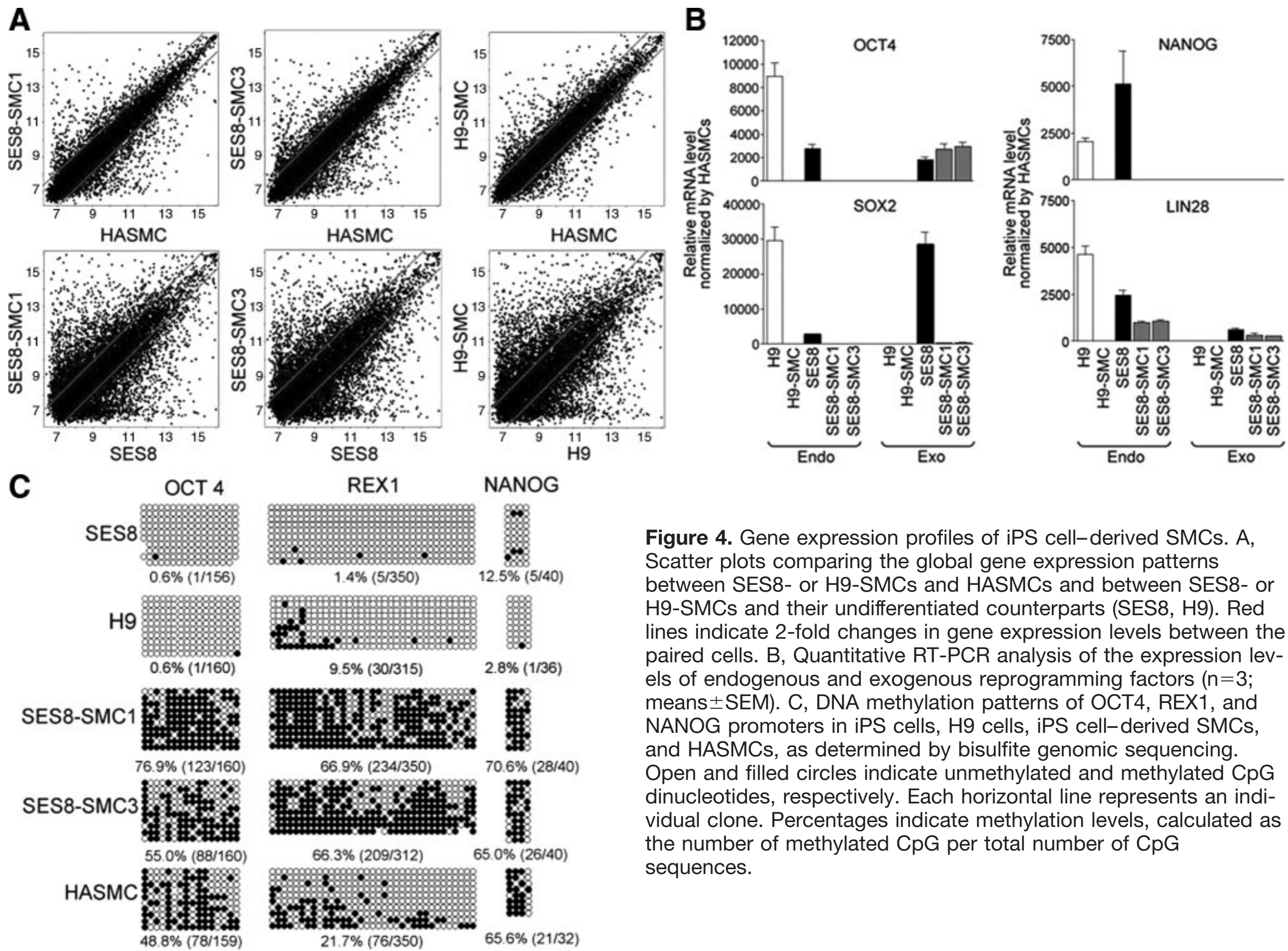

Figure 4. Gene expression profiles of iPS cell–derived SMCs. A, Scatter plots comparing the global gene expression patterns between SES8- or H9-SMCs and HASMCs and between SES8- or H9-SMCs and their undifferentiated counterparts (SES8, H9). Red lines indicate 2-fold changes in gene expression levels between the paired cells. B, Quantitative RT-PCR analysis of the expression levels of endogenous and exogenous reprogramming factors (n=3; means±SEM). C, DNA methylation patterns of OCT4, REX1, and NANOG promoters in iPS cells, H9 cells, iPS cell–derived SMCs, and HASMCs, as determined by bisulfite genomic sequencing. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively. Each horizontal line represents an individual clone. Percentages indicate methylation levels, calculated as the number of methylated CpG per total number of CpG sequences.

that α- and γ-SMA may be suitable markers for SMCs differentiated from human ES and iPS cells. When α- and γ-SMA expression was assessed with real time RT-PCR, they were found to be strongly induced in both H9-SMCs and SES8-SMCs (Figure 3C).

When the expression of skeletal myocyte–specific (Myo D and Myogenin), cardiomyocyte-specific (CTNI and CTNT) or endothelial cell–specific (CD31 and Tie2) genes were examined using RT-PCR, these genes were expressed at very low levels in SMCs, compared with H9 or SES8-derived EB (Figure 3D).

Gene Expression Profile and Epigenetic Modification of iPS Cell–Derived SMCs

The global gene expression profiles of SES8-SMCs, HASMCs, and SES8 cells were compared by using cDNA microarrays. Scatter plot analyses showed that the gene expression patterns of 2 SES8-derived SMCs and H9-derived SMCs were more closely correlated with expression patterns of HASMCs than with those of SES8 and H9 cells (Figure 4A). Online Table III provides a detailed comparison of SES8-, H9-derived SMCs, and HASMCs with regard to transcript levels of key genes determining SMC differentiation (MRTF-A/B [myocardin-related transcription factor], myocardin, serum response factor) and function (contractile proteins; SM22-α, α-SMA, and ion channels; Cav1.2, Slo).[18] These data highlighted that SMCs differentiated from iPS or H9 cells displayed mature SMC characteristics at the transcription level. However, closer examination of differentially expressed genes between SES8-SMCs and HASMCs showed that SES8-SMCs exhibited relatively high, though much lower than SES8 cells, expression of ES cell–specific genes, such as OCT4, LIN28, SALL4, and DPPA4, but not REX1 and AP, whereas H9-SMCs barely expressed these genes (Online Table IV). Quantitative RT-PCR results revealed that the expression of endogenous OCT4, SOX2, and NANOG genes were dramatically silenced during the differentiation of SES cells into SMCs, as observed in the differentiation of H9 cells into SMCs, whereas some lentiviral transgenes (OCT4 and LIN28) remained expressed in SES8-SMCs (Figure 4B). This result showed that residual expression of OCT4 and LIN28 in SES8-SMCs was derived from the lentiviral transgenes incorporated in SES8 cells but not from endogenous counterparts. Bisulfite genomic sequencing analyses revealed that promoter or enhancer regions of ES-specific marker genes such as OCT4, REX1 and NANOG were substantially demethylated in SES8 cells, indicating the epigenetic reprogramming into pluripotent ES-like state (Figure 4C). Moreover, the results showed that SES8-SMCs exhibited prominent promoter methylation patterns of OCT4, REX1, and NANOG similar to those of HASMCs, implying that the in

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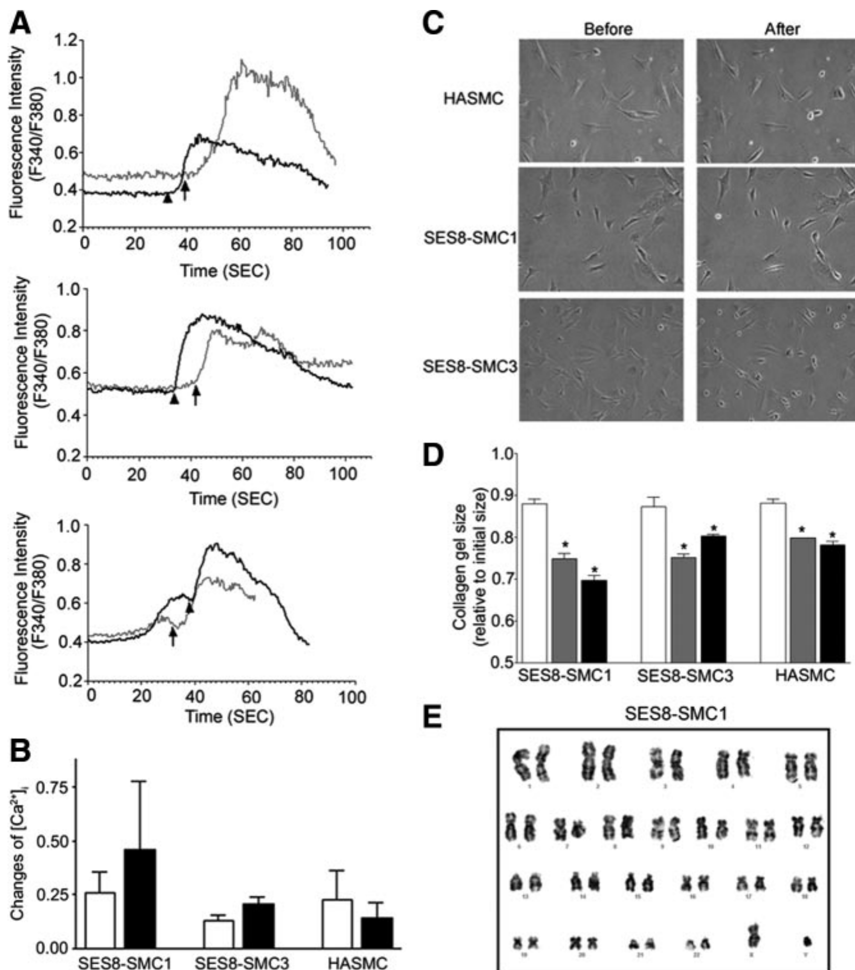

Figure 5. Functional characterization of iPS cell–derived SMCs. A, Representative graphs showing relative change in intracellular [Ca^{2+}] in response to membrane depolarization (50 mmol/L KCl, black line) and vasoconstrictor (5 μmol/L carbachol, gray line) in SES8-SMCs (SES8-SMC1 ⊤; SES8-SMC3 [middle]) and HASMCs (bottom). Concentration of intracellular calcium, $[Ca^{2+}]_i$, was measured with calcium indicator Fura-2 acetoxymethyl ester and expressed as a ratio of fluorescence intensity at 340 and 380 nm (F340/F380). The arrowheads and arrows show the time point for application of KCl and carbachol, respectively. B, Bar graphs showing the net changes in $[Ca^{2+}]_i$ on the treatment of KCl (white) or carbachol (black). (Changes of $[Ca^{2+}]_i$=peak $[Ca^{2+}]_i$−resting $[Ca^{2+}]_i$; n=13; means±SEM.) C, Representative images showing the contraction of HASMCs, SES8-SMC1, and SES8-SMC3 in response to carbachol. Bright-field images were taken before and 30 minutes after administration of carbachol (5 μmol/L). D, Collagen gel contraction assay of SMCs in response to vasoconstrictors. Collagen gels embedded with SES8-SMC1, SES8-SMC3, and HASMCs were incubated in basal α-MEM media without (white) or with carbachol (gray) or angiotensin II (black). The extent of SMC collagen gel contraction was estimated by dividing the gel size at 20 minutes by the initial gel size (n=3; means±SEM). *P<0.05 vs each α-MEM media control. E, Karyotyping analysis of iPS cell–derived SMCs.

vitro differentiation of SES8 cells into SMCs epigenetically knocked down the expression of endogenous pluripotency-related genes.

Functional Characterization of SES8-SMCs

Next, we investigated whether iPS cell–derived SMCs have functional properties similar to somatic SMCs. Here, the calcium influx and subsequent contraction in response to vasoconstrictors, one of requisite characteristics of SMCs, was evaluated in SES8-SMCs and HASMCs. Fura-2 acetoxymethyl ester, a Ca^{2+}-sensitive dye, was used to monitor the intracellular calcium influx on addition of KCl or carbachol. SES8-SMCs exhibited increased intracellular calcium influx in response to vasoconstrictive carbachol and KCl-induced membrane depolarization similar to that of parental HASMCs (Figure 5A and 5B). Moreover, treatment with carbachol or angiotensin II induced a significant contraction of SES8-SMCs as observed in HASMCs (Figure 5C and 5D; Online Movies). Karyotyping analysis showed that SES8-SMC1 cells contained normal chromosomes (Figure 5E). Taken together, these results demonstrated that in in vitro conditions, the SMCs differentiated from SES8 human iPS cells possessed SMC-specific functionalities comparable to parental HASMCs.

Discussion

Since the recent discovery of iPS cell technology, many researchers have reported that iPS cells can be derived from mouse and human somatic cells by the viral transduction of defined reprogramming transcription factors. In mouse, iPS cells have been produced from a variety of lineage-committed somatic cells, such as fibroblasts,[6] hepatocytes,[19] B lymphocytes,[20] and neural stem cells.[8] However, most human iPS cells have been generated from fibroblasts or fibroblast-like synoviocytes,[6,7,9,10] with the exception of one recent report about iPS cell generation from human keratinocytes.[21] Our present study showing iPS cell generation from human aortic SMCs implies that human iPS cells, like murine iPS cells, can be generated from diverse somatic cell origins. Here, several iPS cell clones were generated by introducing 4 or 5 known reprogramming factors (OCT4, SOX2, NANOG, LIN28, and/or KLF4) into HASMCs. Among them, the SES8 cell clone with the same 4 lentiviral transgenes (OCT4, SOX2, NANOG, and LIN28) as those used by the Thomson laboratory was intensively analyzed and determined to have ES cell–specific properties.

It is thought that iPS cell technology may enable the generation of patient-specific, immunocompatible, pluripotent stem cells, which could potentially differentiate into any cell type for the treatment of incurable human diseases. In

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fact, several recent studies have shown that human iPS cells can be successfully differentiated into a variety of somatic cells including pancreatic β cells,[22] cardiomyocytes,[23] and neuronal cells.[24] Although these reports have described the great therapeutic potentials of cells differentiated from iPS cells, clinical applications will likely require more extensive data regarding how closely these cells represent the patient's somatic cells. With this rationale, we differentiated SES8 iPS cells back into SMCs and compared the genetic, epigenetic, and functional properties of SES8-derived SMCs with those of the parental HASMCs. SMCs differentiated from iPS cells were isolated homogeneously and maintained in in vitro long-term culture, without development of chromosomal abnormality. We found that 2 SMC clones (SES8-SMCs) differentiated from SES8 cells possessed not only global gene expression patterns similar to that of the parental HASMCs but also possessed SMC-specific functionalities such as the calcium influx and subsequent contraction in response to vasoconstrictors. However, SES8-SMCs expressed substantial levels of OCT4, LIN28, and several ES-specific genes that were, however, barely expressed in H9-SMCs. This residual expression of OCT4 and LIN28 resulted from the incomplete silencing of the corresponding lentiviral transgenes but not endogenous genes. In H9-SMCs, endogenous OCT4 and LIN28 were completely repressed under the same differentiation conditions, indicating that the differentiation method used in this study was sufficient to induce the complete differentiation of human ES cells into SMCs. When expression of the OCT4 transgene in SES8-SMCs was reduced by OCT4 small interfering RNA, these cells were not only elongated and spreading similar to HASMCs or H9-derived SMCs but also upregulated the SMC specific genes (α-SMA and myocardin) and downregulated the stemness genes (SALL4 and DPPA4) (see Online Figure II). These results indicate that the complete silencing of lentiviral transgenes in SES8 cells is necessary to induce complete differentiation into SMCs. Thus, our present study demonstrates that reprogramming of somatic cells without genome integration of transgenes should be strongly recommended to achieve complete differentiation of iPS cells.

Indeed, the Hochedlinger and Yamanaka groups have reported the generation of murine iPS cells by using a nonintegrating adenovirus delivery system or by using transient transfection with plasmids.[25,26] Recently, Jaenisch and colleagues have reported the generation of human iPS cells without genome integration of transgenes by using a Cre-lox system.[27] These reports introduce the possibility of practical application of iPS cell technology for regenerative medicine and genetic disorder research.

The present study is the first, to our knowledge, to report the generation of iPS cells from HASMCs and their differentiation back into SMCs. Moreover, the parallel comparative analysis of human iPS cell–derived SMCs and parental HASMCs showed that iPS cell–derived SMCs exhibited representative molecular and in vitro functional characteristics of parental HASMCs, suggesting that iPS cells hold great promise as an autologous cell source for patient-specific cell therapy.

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Disclosures

None.

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