

Directed Differentiation of Skin-Derived Precursors Into Functional Vascular Smooth Muscle Cells

Sarah K. Steinbach, Omar El-Mounayri, Ralph S. DaCosta, Matthew J. Frontini, Zengxuan Nong, Azusa Maeda, J. Geoffrey Pickering, Freda D. Miller, Mansoor Husain

Objective—The goal of this study was to characterize the factors and conditions required for smooth muscle cell (SMC)-directed differentiation of Sox2⁺ multipotent rat and human skin-derived precursors (SKPs) and to define whether they represent a source of fully functional vascular SMCs for applications in vivo.

Methods and Results—We found that rat SKPs can differentiate almost exclusively into SMCs by reducing serum concentrations to 0.5% to 2% and plating them at low density. Human SKPs derived from foreskin required the addition of transforming growth factor- β 1 or - β 3 to differentiate into SMCs, but they did so even in the absence of serum. SMC formation was confirmed by quantitative reverse transcription-polymerase chain reaction, immunocytochemistry, and fluorescence-activated cell sorting, with increased expression of smoothelin-B and little to no expression of telokin or smooth muscle γ -actin, together indicating that SKPs differentiated into vascular rather than visceral SMCs. Rat and human SKP-derived SMCs were able to contract in vitro and also wrap around and support new capillary and larger blood vessel formation in angiogenesis assays in vivo.

Conclusion—SKPs are Sox2⁺ progenitors that represent an attainable autologous source of stem cells that can be easily differentiated into functional vascular SMCs in defined serum-free conditions without reprogramming. SKPs represent a clinically viable cell source for potential therapeutic applications in neovascularization. (*Arterioscler Thromb Vasc Biol.* 2011;31:2938-2948.)

Key Words: angiogenesis ■ vascular biology ■ vascular muscle ■ differentiation ■ skin-derived precursors

Skin-derived precursors (SKPs) are multipotent, Sox2⁺, nestin⁺ neural crest- and somite-derived adult stem cells present in the dermis of face and trunk, respectively, that can be expanded as spheres in suspension.¹⁻³ They derive from Sox2⁺ dermal precursors, which reside in the dermal papilla and sheath of hair and whisker follicles.⁴ SKPs can also be derived from glabrous skin, such as human foreskin, which expresses neural crest stem cell markers.⁵ However, the lack of definitive lineage tracing experiments in the human precludes us from unequivocally determining the origin of foreskin-derived SKPs. Despite differences in their embryonic origin, SKPs from face, trunk, and foreskin are transcriptionally and functionally similar and have analogous multilineage potential.^{2,6,7} Like neural crest stem cells, SKPs can differentiate into mesenchymal cell derivatives, such as adipocytes, bone and smooth muscle, and peripheral nervous system cell types, such as Schwann cells.^{1,4,8-10} Although SKPs have been shown to differentiate into smooth muscle cells (SMCs),^{1,8} the specific cues directing SKPs to the SMC lineage to high levels of enrichment have not been described.

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The SMC is a therapeutically relevant cell type shown to promote neovascularization and wound healing, and it has even been shown to improve the function of the heart after myocardial infarction.¹¹⁻¹⁵ Indeed, the SMC also represents a potential cell type for regenerative applications such as transplantation to promote angiogenesis and the generation of vascular grafts. Adult stem cells appear to be a more viable source for SMC acquisition than embryonic stem cells (ESCs) or induced pluripotent stem cells. For example, the Wobus method of differentiating SMCs from ESCs with retinoic acid and dibutyryl-cAMP resulted in only 25% of differentiated cells expressing α -smooth muscle actin (ASMA) based on fluorescence-activated cell sorting (FACS).^{16,17} Other directed methods of ESC differentiation result in a mixed population of cells, require extensive cell sorting, and may still induce teratoma formation.¹⁸ Selection-based methods of deriving SMCs from transgenic ESCs no longer form teratomas but require genetic modification, which can pose hazards, such as insertional mutagenesis.¹⁹

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From the McEwen Centre for Regenerative Medicine, Toronto, Ontario, Canada (S.K.S., O.E.-M., F.D.M., M.H.); Toronto General Research Institute (S.K.S., O.E.-M., M.H.) and Ontario Cancer Institute (R.S.D.), University Health Network, Toronto, Ontario, Canada; Robarts Research Institute, University of Western Ontario, London, Ontario, Canada (M.J.F., Z.N., J.G.P.); Department of Medical Biophysics (R.S.D., A.M.) and Heart and Stroke Richard Lewar Centre of Excellence, Department of Medicine (M.H.), University of Toronto, Toronto, Ontario, Canada; Hospital for Sick Children, Toronto, Ontario, Canada (F.D.M.).

Correspondence to Mansoor Husain, MD, 101 College St, TMDT 3-910, Toronto, Ontario M5G 1L7, Canada. E-mail mansoor.husain@utoronto.ca
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Other methods producing higher levels of SMC differentiation from ESCs use serum,²⁰ a hazardous option given that serum promotes nonspecific differentiation, contamination with animal products, and serum-derived infectious agents.²¹ Cells cultured in heterologous animal products can also develop xenoantigens, causing an immunogenic response after transplantation.^{22,23}

We hypothesized that growth factors used to differentiate SMCs from neural crest and mesenchymal stem cells would also differentiate SKPs into SMCs at high frequency. For example, adipose tissue-derived mesenchymal stem cells can be directed into forming SMCs using sphingosylphosphorylcholine (D-SPC) and transforming growth factor (TGF)- β 3 in combination, or sphingosine 1-phosphate (S1P).^{24,25} In addition, bone marrow-derived mesenchymal stem cells can be directed to form SMCs using TGF- β 1 and ascorbic acid.²⁶ Finally, cortical stem cells can differentiate almost exclusively into SMCs when cultured at low density.²⁷

In this report, we present findings that instructively promote the differentiation of neural crest-derived SKPs into vascular SMCs. We have systematically tested those factors and culture conditions that have been described as promoting SMC formation from other progenitors. We report on the temporal appearance of transcriptional, immunocytochemical, and FACS evidence of SMC-directed differentiation from both rat and human SKPs and demonstrate the functional capacity of the resulting vascular SMCs in contractile assays *in vitro* and angiogenesis assays *in vivo*.

Methods

Tissue Culture

Rat SKPs were generated from whisker pads of 3- to 5-day-old neonates from green fluorescent protein (GFP)⁺ or wild-type (Sprague Dawley) animals (SLC Japan) and cultured as described^{3,28} at 25×10^3 to 50×10^3 cells/mL. Human foreskins, ranging from 1 to 2 cm² in size, were acquired from voluntary circumcisions of children aged 4 weeks to 12 years of age (Hospital for Sick Children, Toronto, Ontario, Canada). SKP spheres were passaged at least twice for all experiments before differentiation *in vitro* at a cell density of 5×10^3 to 10×10^3 cells/cm² in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) (3:1) with 1% penicillin/streptomycin, supplemented with 0.5% to 2% fetal bovine serum (FBS). In DMEM/F12 (3:1) 10% to 20% FBS, TGF- β 1 (PeproTech), and TGF- β 3 (R&D Systems) were used at a final concentration of 2 ng/mL, ascorbic acid (Sigma) at a final concentration of 30 μ mol/L, D-SPC (Matreya) at a final concentration of 2 μ mol/L, and S1P (Sigma) at a final concentration of 1 μ mol/L. Cells were fed with fresh differentiation medium every 4 days and differentiated for 7 to 10 days. Primary coronary (Cascade Biologics) and bladder (Lonza) SMC lines were cultured in Medium 231 supplemented with Smooth Muscle Differentiation Supplement. The coronary SMC line was derived from an unknown source, and the bladder SMC line was derived from a 25-year-old Hispanic male.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA (2 μ g) extracted with Trizol (Invitrogen) was reverse transcribed into DNA with Superscript III (Invitrogen). RNA was treated with DNase I (Fermentas) to remove genomic DNA and spiked with enhanced green fluorescent protein RNA to monitor consistency between cDNA synthesis reactions. Random hexamers (Invitrogen), dNTPs (Fermentas), and finally RNase Inhibitor (New England Biolabs) were also added to the reaction mixture. Final primer concentrations were 0.2 μ mol/L, and an annealing temperature of 63°C was used during the amplification cycle for all primers.

Standard curves were made in triplicate using RNA isolated from tissues known to express genes of interest. Experimental samples from SKPs, SKP-derived SMCs, and control cell lines and tissues were run in duplicate. The relative quantification approach of quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to determine target concentrations between cell lines and tissues. With this approach, the absolute values of the target and housekeeping genes were calculated in the same sample. To obtain the absolute values of these 2 parameters, an external standard curve for each was used. The absolute value of the target was then divided by the absolute value of the housekeeping gene. The expression values plotted graphically represent the target/housekeeping gene expression ratio in that particular cell line or tissue. Light Cycler 480 software (version 1.5.0, Roche) was used to calculate the target and housekeeping gene expression levels, as well as target/housekeeping gene expression ratios.

Immunocytochemistry

Immunostaining was carried out in either 4-well chamber slides (Nunc) or 6-well plates/60-mm dishes with glass coverslips. Cells were fixed with paraformaldehyde solution (Cytifix, BD Biosciences) for 10 minutes and permeabilized with a saponin-based permeabilization buffer (Perm/Wash, BD) for 30 minutes. Antibodies against ASMA (Sigma), SM22 α (ProteinTech), and calponin (Abcam) were diluted 1:250 in Dako antibody diluent with background reducing agents. Fixed cells were incubated in antibody overnight at 4°C. Cells were washed 3 times in $1 \times$ PBS and incubated in secondary antibody (1:500) with 5 μ g/mL Hoechst 33258 for 1 hour at room temperature in the dark. After washing the cells three times with PBS, cells were mounted in PBS:glycerol (1:1).

Real-Time Cell Analysis

Rat and human SKPs were dissociated with collagenase XI, and 5000 cells were seeded onto 16-well E-plates (Roche). Rat SKPs were cultured in 1% to 2% FBS with or without 2 ng/mL TGF- β 1 and - β 3, and human SKPs were cultured in serum-free conditions with or without 2 ng/mL TGF- β 1 or - β 3 for 7 to 12 days at 37°C, 5% CO₂. Cells were monitored for proliferation and contraction in response to 10 μ mol/L phenylephrine using the xCELLigence real-time cell analyzer (Roche). This real-time cell system continuously monitors changes in impedance over time, represented arbitrarily as the cell index. Impedance was collected every 10 minutes for proliferation assays²⁹ and every 15 seconds for cell contraction assays.^{30,31} Each arbitrary measurement of cell index is defined as (Rtn-Rt0)/F1, where Rtn is the impedance of the well at time Tn, Rt0 is the impedance of the well at time T0 before cell attachment, and F1 is 15 ohm.

FACS

Rat and human SKPs were dissociated and then differentiated with TGF- β 1 or - β 3 and fixed with Cytifix (BD) for 30 minutes on ice. Cells were washed and permeabilized with Perm/Wash buffer (BD) and incubated in primary antibody or isotype control for 20 to 30 minutes on ice. After washing with Perm/Wash buffer (BD), cells were incubated with secondary antibody for 20 to 30 minutes on ice, light protected, washed again, and resuspended in staining medium ($1 \times$ Hanks' balanced salt solution, 2% FBS, 10 mmol/L Na₃N, 10 mmol/L HEPES, pH 7.2) and filtered through Nitex mesh before analysis on an LSRII flow cytometer with FACSDiva software (BD). FACS data were analyzed with FlowJo flow cytometry analysis software version 9-1.3.1.

In Vivo Angiogenesis Assays

Nondifferentiated and differentiated rat SKPs were resuspended at 1.5×10^6 cells/mL in 1.5 mL DMEM/F12 (3:1) with 2% FBS and 500 ng/mL basic fibroblast growth factor 2 (BD). A 1.5-mL cell suspension was diluted 1:1 with growth factor–reduced Matrigel (BD) and kept on ice. NOD-SCID mice were anesthetized with ketamine and xylazine (1:2). The cell/Matrigel suspensions (300 μ L) were injected subcutaneously in the mouse abdomen with 27-gauge

needles. After 2 to 3 weeks, the mice were anesthetized with isoflurane, and the Matrigel plug was exposed for intravital microscopy, as previously described.³² To observe blood flow within vessels, fluorescein isothiocyanate (FITC)-conjugated dextran (1 mg/mL) was injected via the tail vein.

Cell Tracker Red (Invitrogen)-labeled human SKP-derived SMCs were transplanted into 4- to 6-week-old athymic female nude mice and visualized in a dorsal window chamber model, as previously described.³³ SKP-derived SMCs from passage 2 in 50% Matrigel were injected directly into the retractor muscle of the mouse dorsal skin fold or subcutaneously. Fluorescence imaging was performed weekly over 4 weeks. Tail vein injections of FITC-conjugated dextran (1 mg/mL) and anti-CD31 (0.2 mg/mL) were performed to visualize blood flow and the endothelial layer of vessels, respectively.

Quantification of Human SKP-Derived SMC Integration

At 2 weeks after transplantation, the number of cells and vessels with or without human SKP-derived SMC (hSMC) integration were counted in 10 to 15 randomly selected fields of view (magnification) for each experiment. Vessel diameter was recorded for each vessel identified. The total number of hSMC per field of view and the numbers of hSMCs tightly integrated into vessels of 5 to 20, 21 to 50, and 51 to 100 μm diameter were recorded. The number of vessels with and without SMC integration was quantified and categorically grouped based on vessel size. Finally, the 2-dimensional area of a vessel and the percentage invested by hSMCs were computed using Image Browser version 4.2.0.121 (Zeiss).

Results

TGF- β 1 and - β 3 Promote Serum-Dependent Differentiation of Rat SKPs Into SMCs

SKPs were isolated from the whisker pads of Sprague Dawley neonatal rats, grown in SKP proliferation medium for 7 to 10 days, and passaged 2 to 4 times before differentiation. To assess whether SKPs expressed SMC-specific markers, they were probed with antibodies against SMC-specific markers. Immunofluorescent staining for ASMA, calponin, and SM22 α revealed little to no SMC-specific protein in undifferentiated SKP spheres (Supplemental Figure 1A, available online at <http://atvb.ahajournals.org>). When differentiated in 10% to 20% FBS, a time course experiment revealed that SMC formation occurred as early as 7 days and was stable for 21 days of culture (Supplemental Figure 1B to 1D). Although seeded at low density (5×10^3 cells/cm²), cells proliferated to confluence in the presence of 10% to 20% serum. Cells staining positive for SMC markers were large and flat in appearance, resembling neural crest stem cells isolated directly from embryonic day (E) 10.5 neural tubes.³⁴ After 7 days in 20% FBS alone, SMC formation was quantified to be \approx 4% to 29% on the basis of ASMA and SM22 α staining, respectively (Supplemental Figure 1D). When SKPs were differentiated under similar conditions but with TGF- β 1 or - β 3, the proportion of ASMA- or SM22 α -positive SMCs increased to 80% to 90% (Supplemental Figure 1D). Unlike other mesenchymal stem cells, such as bone marrow- or adipose tissue-derived stem cells, SKPs did not differentiate into SMCs in 10% to 20% FBS after the addition of ascorbic acid or D-SPC (Figure 1B and 1D and Supplemental Figure 1IA and 1IB). On the contrary, D-SPC suppressed SMC formation from rat SKPs (from 13–25% to 2–8%, $P < 0.05$) (Figure 1D).

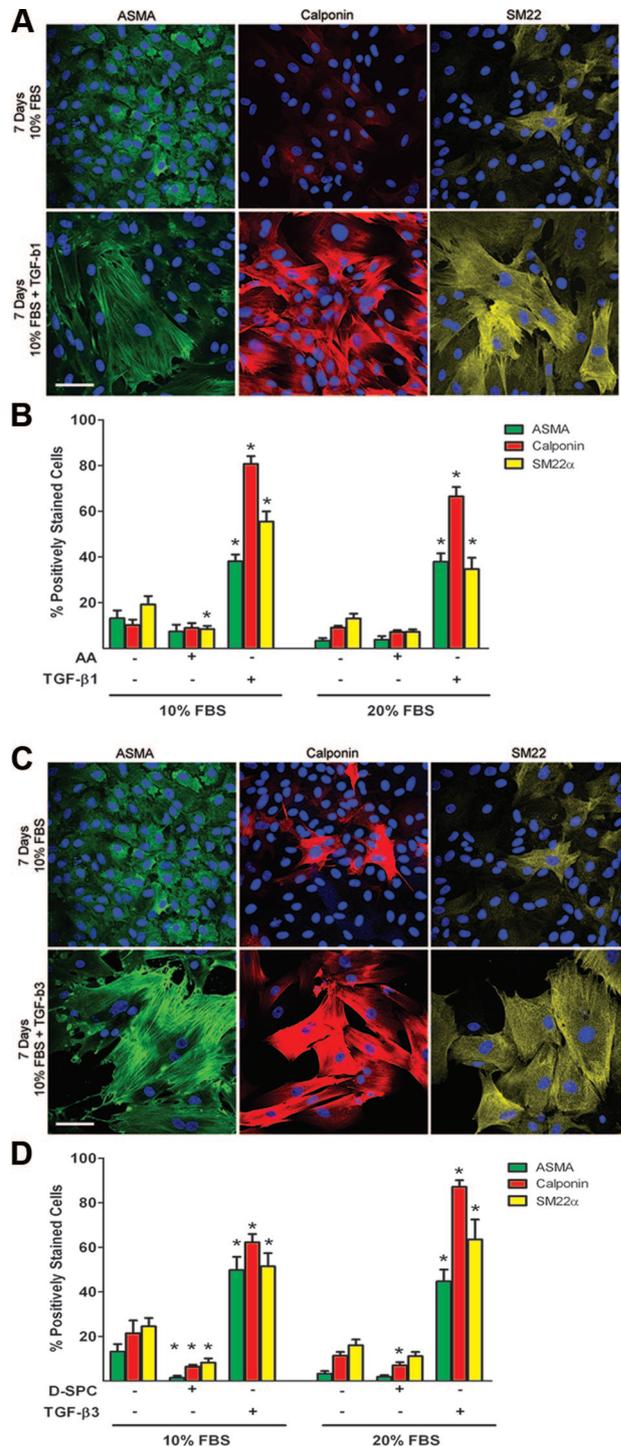


Figure 1. Transforming growth factor (TGF)- β 1 and - β 3 differentiate rat skin-derived precursors (SKPs) into smooth muscle cells (SMCs) in 10% fetal bovine serum (FBS). **A**, Rat SKPs were differentiated for 7 days in 10% FBS or 10% FBS+TGF- β 1 (2 ng/mL), formaldehyde fixed, and examined by confocal microscopy after staining for α -smooth muscle actin (ASMA) (green), calponin (red), SM22 α (gold), and nuclei (blue). **B**, Percentage of ASMA⁺, calponin⁺, and SM22 α ⁺ cells before and after ascorbic acid (AA) (30 $\mu\text{mol/L}$) vs TGF- β 1 (2 ng/mL) in 10% or 20% FBS was determined by manual counting of cells ($n=80$ –320 cells for each condition). **C**, SKPs were differentiated for 7 days in 10% FBS or 10% FBS+TGF- β 3 (2 ng/mL) and prepared as in **A**. **D**, Percentages of ASMA⁺, calponin⁺, and SM22 α ⁺ cells before and after sphingosylphosphorylcholine (D-SPC) vs TGF- β 3 in 10% or 20% FBS ($n=68$ –322 cells for each condition). Scale bar=60 μm .

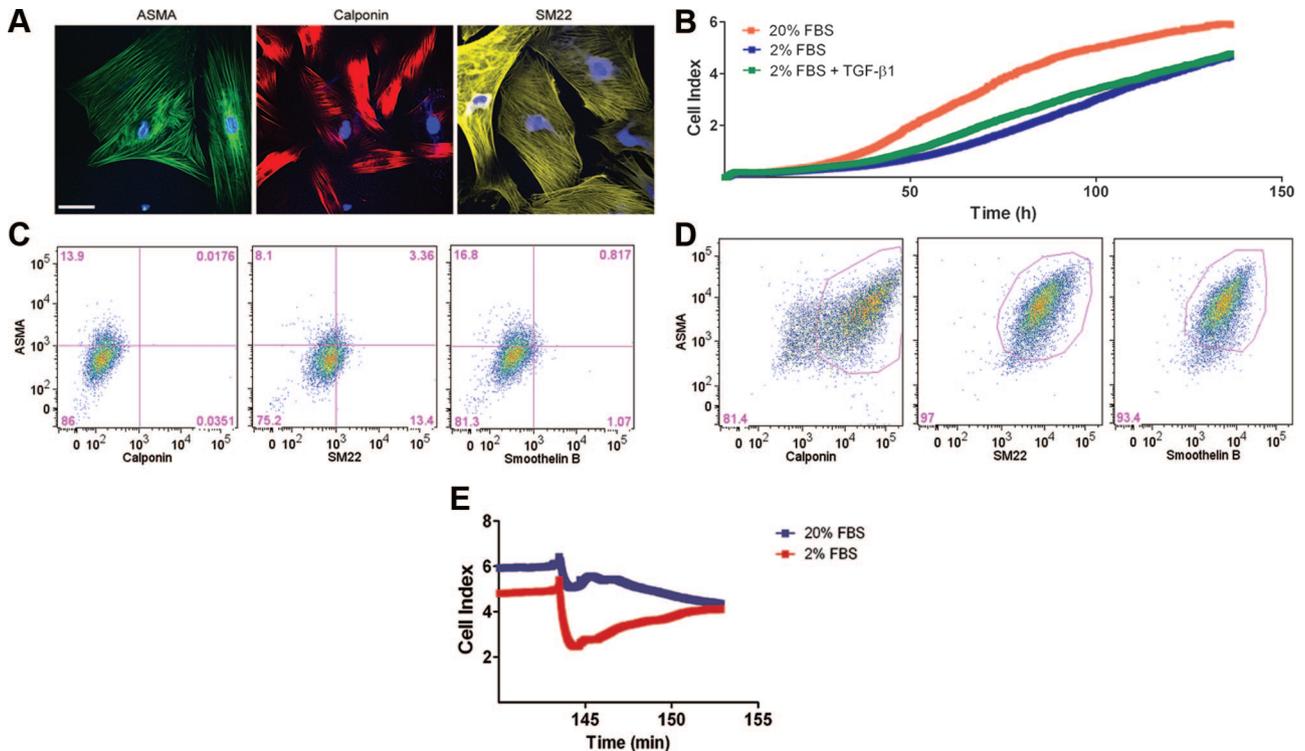


Figure 2. Reduction of fetal bovine serum (FBS) to 2% differentiates rat skin-derived precursors (SKPs) almost exclusively into smooth muscle cells (SMCs) at low density. **A**, Dissociated SKP spheres were plated at 5×10^3 cells/cm² and allowed to differentiate in Dulbecco's modified Eagle's medium/Ham's F-12 medium (3:1) with 2% FBS for 7 days. Cells were formaldehyde fixed and stained for α -smooth muscle actin (ASMA) (green), calponin (red), SM22 α (gold), and nuclei (blue). **B**, Cell proliferation was assessed using real-time cell analysis (xCELLigence). Dissociated rat SKPs (5000 cells/well) were seeded onto 16-well E-plates and allowed to differentiate in 20% FBS, 2% FBS, and 2% FBS+transforming growth factor (TGF)- β 1 (2 ng/mL). **C**, Fluorescence-activated cell sorting (FACS) analysis of formaldehyde-fixed dissociated rat SKPs double-stained for ASMA, calponin, SM22 α , and smoothelin-B. **D**, FACS analysis of rat SKP-derived SMCs differentiated for 7 days in 2% FBS using SMC markers described in C. **E**, Representative image of real-time cell analysis of differentiated rat SKP-derived SMCs treated with 10 μ mol/L phenylephrine. Each curve represents integrated impedance data from ≥ 5000 cells. Scale bar=60 μ m.

Low Cell Density and Reduced Serum Concentrations Direct Rat SKPs to Form Functional Vascular SMCs

Seeding cortical stem cells at low density induces the formation of SMCs.²⁷ We wondered whether limiting cell proliferation by decreasing serum would similarly enhance SMC differentiation from SKPs. Reducing serum to 2% in DMEM/F12 (3:1) and plating rat SKPs at low density (5×10^3 cells/cm²) induced $\approx 98\%$ of them to differentiate into SMCs, as determined by immunofluorescent staining (Figure 2). Similar effects were observed by further lowering serum to 1% and 0.5% (data not shown). Addition of TGF- β 1 and - β 3 had no discernable effect on SMC formation from rat SKPs under these conditions (data not shown), suggesting that low serum and limited cell-cell contact were sufficient to induce SMC-directed differentiation in rat SKPs. Indeed, TGF- β 1 had little effect on the proliferation of rat SKPs cultured in 2% FBS as measured by real-time analysis over 6 days (Figure 2B).

Formation of SMCs from SKPs was also quantified by FACS. Based on the expression of individual markers ASMA, calponin, SM22 α , and smoothelin-B, differentiation of SMCs from SKPs occurred with 70% to 98% efficiency (data not shown). Corroborating these results, FACS revealed that 81% to 97% of SKP-derived SMCs coexpressed ASMA

and calponin, SM22 α , and smoothelin-B, compared with 0% to 17% of undifferentiated SKPs (Figure 2C and 2D).

Real-time analysis with a continuous cell impedance system (xCELLigence) was also used to examine whether SKP-derived SMCs manifest functional responses over minutes (Figure 2E). Optimally differentiated SKP-derived SMCs (2% FBS; see Figure 2A and 2D) showed stronger and more stable contractions in response to the α -adrenergic receptor agonist phenylephrine (10 μ mol/L) than poorly differentiated controls (20% FBS; see Figure 1B and 1D) (Figure 2E).

ASMA, calponin, and SM22 α have been extensively used to identify SMCs.³⁵ However, these markers are also expressed in other cell types. To test whether the smooth muscle (SM)-like cells being generated from SKPs were bona fide SMCs and to further characterize their nature, we performed extensive qRT-PCR for additional lineage markers and transcription factors and compared the results to undifferentiated SKPs and SMC-poor and SMC-enriched tissues in vivo. Expression levels of nestin, a pan-neural filament marker of self-renewing SKPs, were as high as expected in SKPs but dramatically decreased in SKP-derived SMCs, supporting the loss of potential for self-renewal (Figure 3A).

Consistent with data from immunofluorescence and FACS, mRNA levels of ASMA, calponin, and SM22 α were in-

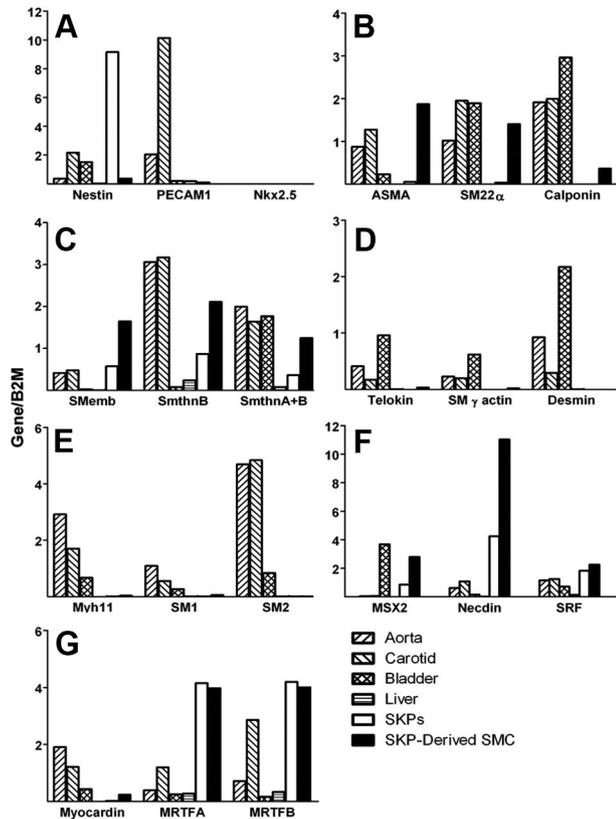


Figure 3. Expression levels of smooth muscle cell (SMC)-specific contractile genes and transcription factors in skin-derived precursors (SKPs), SKP-derived SMCs, and adult SMC-enriched tissues. RNA was isolated from adult rat aorta, carotid, bladder, and liver, as well as from rat SKP spheres and rat SKP-derived SMCs. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Green, with relative gene expression levels normalized to β 2-microglobulin. **A**, Relative gene expression levels of Nestin, PECAM1 and Nkx2.5. **B**, Relative gene expression levels of ASMA, SM22 α and Calponin. **C**, Relative gene expression levels of SMemb, SmthnB and SmthnA+B. **D**, Relative gene expression levels of Telokin, SM γ actin and Desmin. **E**, Relative gene expression levels of Myh11, SM1 and SM2. **F**, Relative gene expression levels of MSX2, Necdin and SRF. **G**, Relative gene expression levels of Myocardin, MRTFA and MRTFB. Data are representative of 3 separate experiments.

creased by the differentiation conditions of low serum and low cell contact and comparable to levels observed in adult SMC-enriched tissue (Figure 3B). Smoothelin-B, a vascular specific SMC marker, was upregulated in SKP-derived SMCs and comparable to levels expressed in adult vascular SMC-enriched beds (aorta and carotid), suggesting that SKP-derived SMCs were vascular in nature (Figure 3C). Primers for smoothelin-A, a truncated isoform of smoothelin-B, do not distinguish between them.³⁶ Nevertheless, “combined” expression levels of smoothelin-A and-B were higher in bladder, consistent with smoothelin-A being expressed in visceral, not vascular, SMCs (Figure 3C). Expression of telokin and smooth muscle γ -actin, both visceral SMC markers,^{37,38} was notably absent in SKP-derived SMCs but present in the adult rat bladder, providing further evidence that SKP-derived SMCs were not visceral in nature (Figure 3D). Interestingly, nonmuscle myosin heavy chain B

(NMMHC-B) (SMemb) was highly expressed in SKP-derived SMCs and reached levels higher than in adult SMC-enriched tissues (Figure 3C). SMemb is highly expressed in embryonic SMCs, in SMCs grown in tissue culture, and in phenotypically modulated SMCs (ie, atherosclerotic lesions and the neointima).^{39,40} Thus, although expanded from adult dermis, SKP-derived SMCs appeared to be embryonic. This is further corroborated by their low levels of total SM-myosin heavy chain (Myh11) and the SM1 isoform (Figure 3E). Although expression of both markers was increased in SKP-derived SMCs as compared with undifferentiated SKPs, their levels do not reach those observed in adult SMC-enriched tissues. Importantly, SM2, which is typically only expressed in fully differentiated, contractile SMCs, was not detected in SKP-derived SMCs (Figure 3E). Finally, desmin, an SMC intermediate filament protein, was not expressed in SKPs or SKP-derived SMCs (Figure 3D). Desmin is expressed in perivascular pericytes, suggesting that the SKP-derived cells were not pericytes.⁴¹

We also assessed the expression levels of transcription factors involved in SMC differentiation. Serum response factor is a ubiquitously expressed transcription factor known to be involved in SMC differentiation.⁴² Expression levels of serum response factor were higher in SKPs and SKP-derived SMCs than in adult SMC-enriched tissues, such as carotid, aorta, and, to a lesser extent, bladder (Figure 3F). Myocardin, another transcriptional regulator involved in SMC differentiation, was also expressed at varying levels in the carotid, aorta, and bladder; at unexpectedly lower levels in SKP-derived SMCs; and not at all in liver or undifferentiated SKPs (Figure 3G). In contrast, the myocardin-related transcription factors (MRTFs) A and B were highly expressed in SKP spheres and SKP-derived SMCs as compared with adult SMC-enriched beds (Figure 3G).

Interestingly, expression levels of neccdin and MSX2, 2 proteins involved in the differentiation to SMCs from mesoangioblasts (a vessel-associated stem cell)⁴³ were increased in SKP spheres, as well as in SKP-derived SMCs, as compared with adult SMC-enriched beds, with particular prominence of neccdin (Figure 3F). Cardiac-specific markers Nkx2.5 (Figure 3A) and TnnI3 (data not shown), as well as the endothelial marker PECAM1 (CD31), were not expressed in undifferentiated SKPs or SKP-derived SMCs (Figure 3A).

TGF- β 1 or - β 3 Enables Serum-Free Differentiation of Human SKPs Into SMCs

Unlike rat SKPs, human SKPs could not be differentiated into SMCs by lowering serum concentration alone (Supplemental Figure III). Human foreskin-derived SKPs required TGF- β 1 or - β 3 for efficient differentiation into SMCs (Supplemental Figure III). However, as with rat SKPs, an inverse correlation between serum concentration and SMC differentiation was observed in human SKPs, where lower serum concentrations in addition to TGF- β 1 or - β 3 increased the extent of SMC differentiation (Supplemental Figure III).

We next determined whether we could culture and differentiate human foreskin-derived SKPs in defined media entirely without serum. This would enable SKP-derived SMCs

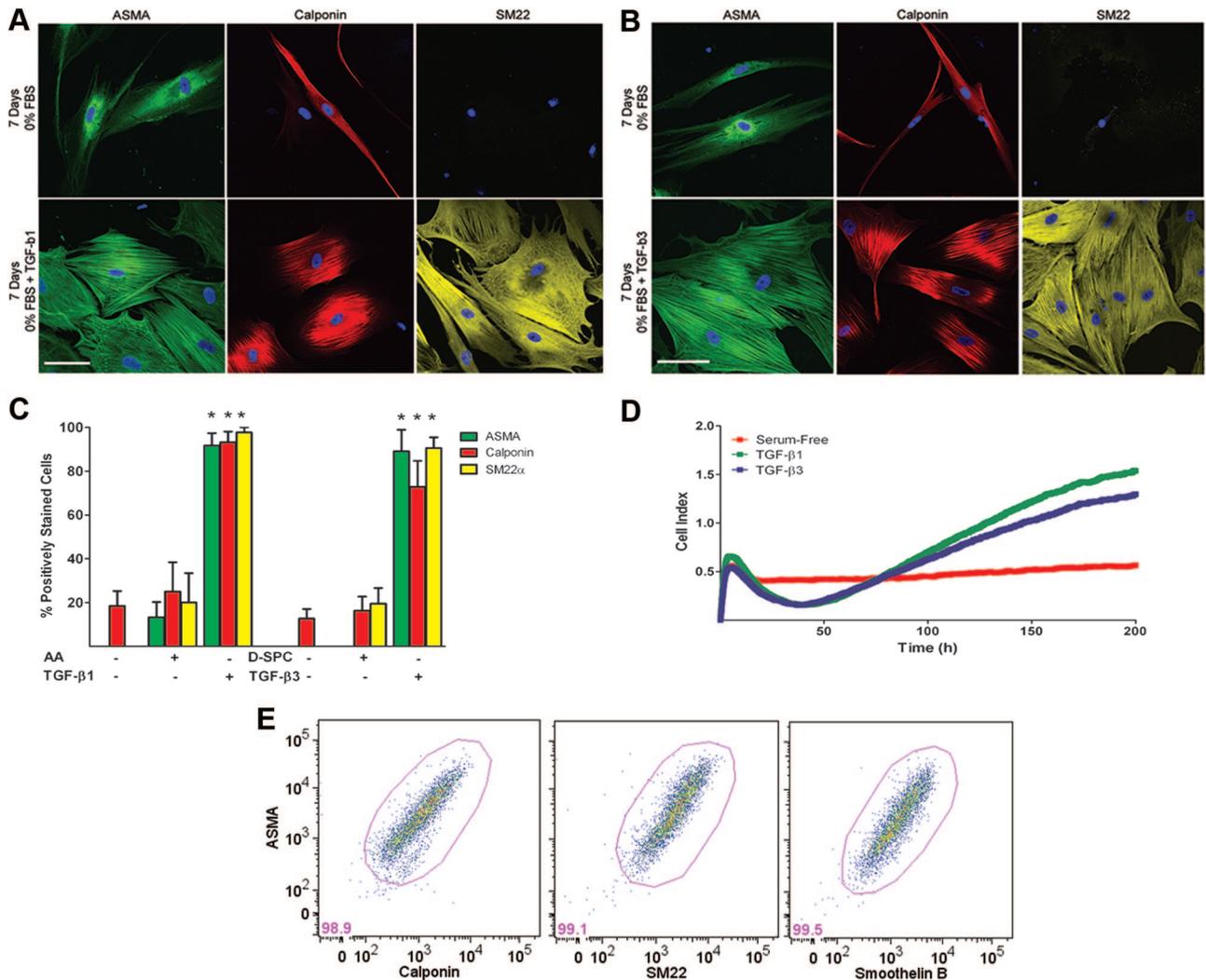


Figure 4. Serum-free differentiation of human skin-derived precursors (SKPs) into smooth muscle cells (SMCs) with transforming growth factor (TGF)- β 1 or TGF- β 3. Human foreskin-derived SKPs were differentiated serum-free with or without TGF- β 1 (2 ng/mL) (A) or TGF- β 3 (2 ng/mL) (B) for 7 days. Cells were formaldehyde fixed, and percentages of α -smooth muscle actin (ASMA)-, calponin-, and SM22 α -stained cells were quantified before and after TGF- β 1 and TGF- β 3 (C) ($n=32$ – 55 cells for each condition). D, Real-time analysis of proliferation of human SKP-derived SMCs in serum-free conditions with or without TGF- β 1 or TGF- β 3. E, Fluorescence-activated cell sorting analysis of human SKP-derived SMCs differentiated for 7 days serum-free. Cells were double-stained for ASMA, calponin, SM22 α , and smoothelin-B. Scale bar=60 μ m.

to be used in the clinical setting. In the absence of serum, human SKPs did not differentiate (Figure 4A to 4C) and died after 18 days (data not shown). However, in the presence of either TGF- β 1 or - β 3, human SKPs differentiated almost exclusively into SMCs and remained viable (Figure 4A to 4C). Real-time cell analysis with the xCELLigence system confirmed that TGF- β treatment induced the proliferation, survival, and differentiation of human SKPs in serum-free conditions (Figure 4D). Immunofluorescence for ASMA, calponin, and SM22 α revealed that SMC formation was as high as 97% in the presence of TGF- β 1 or - β 3 (Figure 4C). This was corroborated by FACS, which showed that \approx 99% of SKP-derived SMCs had simultaneous expression of ASMA and calponin, SM22 α , and smoothelin-B (Figure 4E). Addition of ascorbic acid or D-SPC did not induce SMC formation from human SKPs (Figure 4C). In fact, human SKPs appeared unhealthy or died after 18 days if cultured with only these agents (Supplemental Figure IVA and IVB).

To examine whether human SKP-derived SMCs were functional, their responses to a vasoconstrictor was also monitored using the xCELLigence system. Consistent with their characterization by qRT-PCR (see below), immunofluorescence, and FACS, human SKP-derived SMCs manifest stable contractile responses to phenylephrine (data not shown).

Gene Expression Analysis of Human Foreskin SKP-Derived SMCs Reveals Neural Crest-Derived Immature Vascular SMCs

Human foreskin-derived SKPs were differentiated in 0.5% serum supplemented with TGF- β 1 for 7 to 10 days. Quantitative real-time PCR was performed to examine relative expression levels of SMC markers in undifferentiated SKPs, SKP-derived SMCs, and primary human SMC lines from coronary arteries and bladder. Similar to rat SKPs, nestin expression in human foreskin-derived SKPs decreased on differentiation (Figure 5A). Expression of another SKP

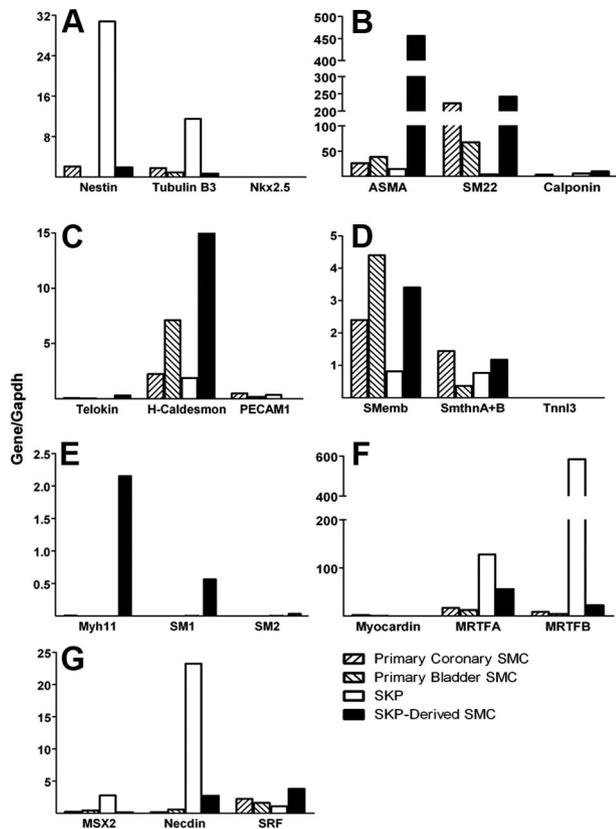


Figure 5. Expression levels of smooth muscle cell (SMC)-specific contractile genes and transcription factors in human skin-derived precursors (SKPs), SKP-derived SMCs, and primary SMC cell lines. RNA was isolated from human primary carotid and bladder SMC lines, as well as from human foreskin-derived SKPs and human SKP-derived SMCs. qRT-PCR was performed using SYBR Green, with relative gene expression levels normalized to GAPDH. **A**, Relative gene expression levels of Nestin, Tubulin β III and Nkx2.5. **B**, Relative gene expression levels of ASMA, SM22 α and Calponin. **C**, Relative gene expression levels of Telokin, H-Caldesmon and PECAM1. **D**, Relative gene expression levels of SMemb, SmthnA+B and TnnI3. **E**, Relative gene expression levels of Myh11, SM1 and SM2. **F**, Relative gene expression levels of Myocardin, MRTFA and MRTFB. **G**, Relative gene expression levels of MSX2, Necdin and SRF. Data are representative of 3 separate experiments.

marker, the neuronal precursor marker tubulin β III, also decreased on differentiation to SMCs (Figure 5A). Expression levels of the SMC markers ASMA, SM22 α , calponin, and h-caldesmon all increased in SKP-derived SMCs as compared with undifferentiated SKPs and were comparable to levels in primary SMC lines (Figure 5B and 5C). Similar to data in rat, expression levels of smoothelin-A and -B combined and SMemb increased on differentiation, indicating that the differentiated SMCs were vascular and embryonic in nature (Figure 5D). Although primary bladder SMCs also expressed high levels of SMemb and lower levels of visceral SMC markers telokin and SM- γ -actin (not shown) than expected, these visceral SMC markers were expressed at lower levels than smoothelin-A and -B in human SKP-derived SMCs (Figure 5C and 5D). In contrast to rat SKPs however, human SKP-derived SMCs expressed higher levels of SM-myosin heavy chain (total Myh11 and SM1) than cultured human primary SMC lines expected to express high

levels of these markers (Figure 5E). The SM2 isoform present in fully differentiated SMCs was not expressed in human SKP-derived SMCs, indicating that the latter were not fully differentiated or embryonic, consistent with their SMemb expression levels (Figure 5D and 5E). As previously observed in rat SKPs, human SKPs manifest low levels of myocardin as compared with MRTF-A and -B in both spheres and SKP-derived SMCs (Figure 5F). Indeed, MRTF-B was particularly abundant in foreskin-derived SKPs (Figure 5F), a finding consistent with their similarities to neural crest precursors.⁴⁴ Finally, like rat, human SKPs also showed increased expression levels of the mesoangioblast markers neccin and MSX2 (Figure 5G).

Transplanted SKP-Derived SMCs Functionally Integrate Into Blood Vessels

GFP⁺ rat SKPs were differentiated for 10 days in 2% FBS, trypsinized and resuspended in growth factor-reduced Matrigel supplemented with basic fibroblast growth factor 2 (500 ng/mL). This cell/Matrigel mixture was injected subcutaneously into 8- to 10-week-old male NOD-SCID mice. After 2 to 3 weeks, Matrigel plugs were exposed, and functional vessels penetrating the plugs were visualized with intravital microscopy, following a tail vein injection of FITC-conjugated dextran. In plugs containing undifferentiated GFP⁺ rat SKP spheres, no GFP⁺ vessels were ever observed (Figure 6A). In plugs containing differentiated GFP⁺ rat SKP-derived SMCs, functional GFP⁺ vessels were observed (Figure 6B). Higher magnification revealed that GFP⁺ SKP-derived SMCs wrapped around the vessels, just like endogenous vascular SMCs, confirming their vascular nature and supporting their ability to participate in neovascularization.

Human foreskin-derived SKPs were differentiated with 2 ng/mL TGF- β 1 \times 7 days, and labeled with Cell Tracker Red (CTR⁺, Invitrogen). After trypsinization, they were injected into the dorsal fold of nude mice at a concentration of 7.5×10^5 cells/mL in growth factor-reduced Matrigel supplemented with basic fibroblast growth factor 2. Vessel formation was visualized using a dorsal window chamber model,³³ and high-resolution images of FITC-dextran-perfused vessels and CTR⁺ hSMCs were acquired to assess integration. As observed with rat SKP-derived SMCs, human SKP-derived SMCs integrated into the vasculature (Figure 6C). z-Stacks showed intimate integration and wrapping of the hSMC around microvessels (Figure 6C). A representative cross-sectional view of a CTR⁺ hSMC wrapped around a FITC-dextran-perfused vessel showed that it was in immediate juxtaposition to the vessel (Figure 6D). Quantification of these data revealed that $\approx 30\%$ of all human SKP-derived SMCs present in randomly selected fields of view had similarly integrated into the vasculature, with the majority of cells integrating into vessels of 21 to 50 μ m diameter (Figure 6E). However, overall, there were more microvessels of 5 to 20 μ m diameter that showed integration with human SKP-derived SMCs (Figure 6E). The percentage area of vessels integrated with hSMC was also quantified and found to be similar for small (5 to 20 μ m) and medium (21 to 50 μ m) vessel diameters (Figure 6E). Together, these analyses revealed that human SKP-derived SMCs are capable of inte-

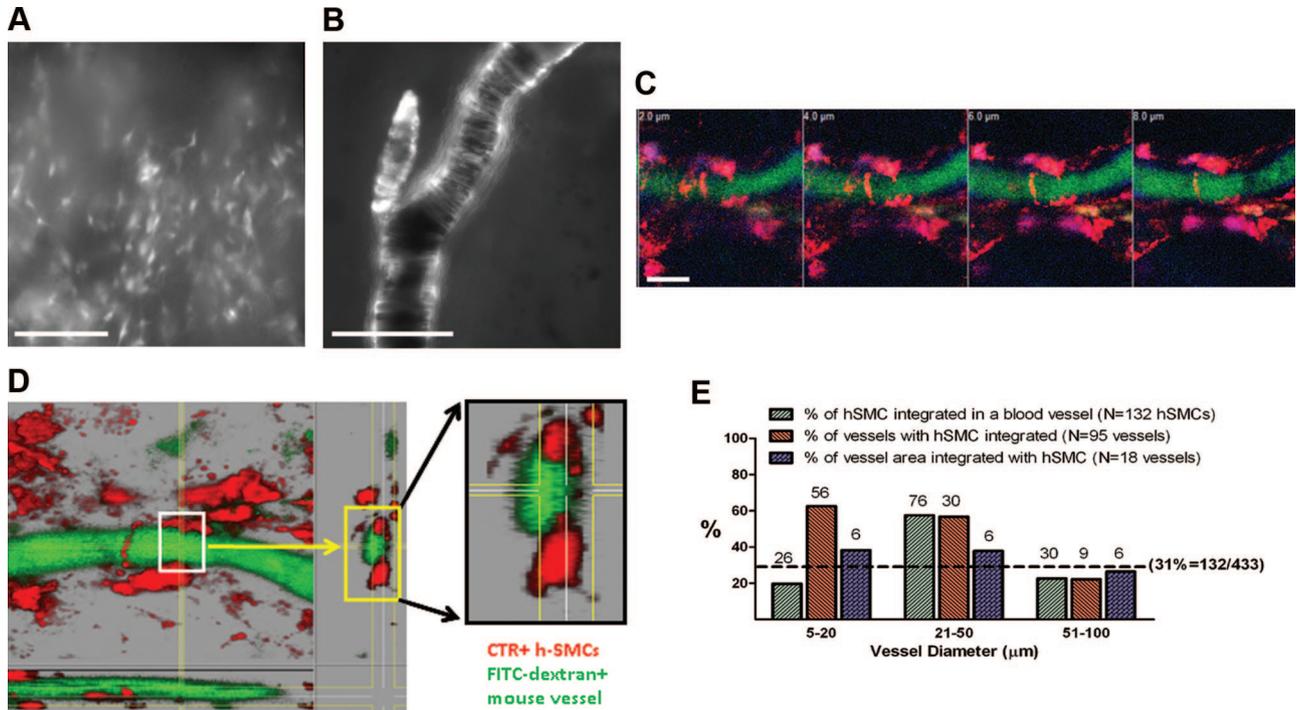


Figure 6. Rat green fluorescent protein (GFP)⁺ skin-derived precursor (SKP)-derived smooth muscle cells (SMCs) integrate into vasculature in vivo. Rat GFP⁺ SKPs were differentiated for 7 days in 2% fetal bovine serum (FBS). Dissociated GFP⁺ rat SKP (A) and SKP-derived SMCs (B) were combined with growth factor-reduced Matrigel and injected subcutaneously into 8- to 10-week-old NOD-SCID mice. After 2 weeks, the Matrigel plug was exposed, and fluorescein isothiocyanate-conjugated dextran was injected through the tail vein. Vessels were imaged with intravital confocal microscopy. Scale bar=50 μm. C, Human foreskin-derived SKPs were differentiated with transforming growth factor (TGF)-β1×7 days in serum-free conditions, labeled with Cell Tracker Red (CTR), combined with growth factor-reduced Matrigel and injected into the dorsal fold of 8- to 12-week-old nude mice. Multiple serial z-stacks of a representative vessel showing an integrated human SKP-derived SMCs are shown. D, A cross-section of an integrated SKP-derived SMCs from a representative image is shown. E, Quantification of human SKP-derived SMC integration. Shown are the percentages of integrated SKP-derived SMCs, vessels with integrated SKP-derived SMCs, and area of vessels integrated with SKP-derived SMCs. The dotted line denotes the overall percentage of human SKP-derived SMCs (h-SMCs) integrated into vessels.

grating into the neovasculature of mice, with a greater ability to invest smaller (<50 μm) rather than larger (51 to 100 μm) vessels.

Discussion

Transplantation of SMCs have been shown to promote neovascularization of ischemic tissues.^{11–15} Current methods for generating SMCs from ESCs or induced pluripotent stem cells are neither efficient nor free of serum. In addition, there remain risks of teratoma or tumor formation with the injection of pluripotent cells for regenerative therapies.⁴⁵ Although adult mesenchymal stem cells are another accessible progenitor, they also require serum for differentiation into SMCs.^{24–27} In this respect, adult stem cells derived from neural crest, such as skin, may represent a better source of this therapeutically important cell type. The current study establishes the SKP as a clinically viable source of vascular SMCs capable of supporting neovascularization.

In rat SKPs, the addition of either TGF-β1 or -β3, but neither ascorbic acid nor D-SPC in 10% to 20% FBS resulted in the efficient (40% to 80% by immunocytochemistry) formation of SMCs. Decreasing the serum concentration to 0.5% to 2% and reducing cell-cell contact resulted in near exclusive differentiation of rat SKPs into functional vascular SMCs (up to 97% by FACS). This effect was supported by

quantitative real-time PCR, revealing levels of marker expression comparable to those in adult vascular SMC-enriched tissues. Although human foreskin-derived SKPs required little to no FBS (1% to 0%), they did require instructive signals from TGF-β1 or -β3 to efficiently (up to 99% by FACS) differentiate into functional vascular SMCs. As with rat, qRT-PCR analyses indicated that SMCs differentiated from human SKPs expressed marker genes at levels comparable to human coronary SMCs. Finally, a Matrigel angiogenesis assay revealed that both rat and human SKP-derived SMCs were capable of functioning in vivo as SMCs for new blood vessel formation.

Evidence supporting directed differentiation to the SMC lineage included qRT-PCR and immunostaining, with distinct stress fiber formation (Figures 2 to 5). Although this ultrastructural morphology supports a mature contractile SMC phenotype, other data suggest the SMCs formed from SKPs remain immature. For example, rat SKP-derived SMCs expressed high levels of SMemb, levels higher than that observed for adult rat SMCs. SMemb is expressed in embryonic SMCs, as well as phenotypically modulated SMCs, such as those in the neointima.^{39,40} This suggests that SKP-derived SMCs are embryonic in nature, a finding corroborated by little to no SM-myosin heavy chain expression (little total Myh11 and little to no SM1 and SM2). Having said this, rat

SKP-derived SMCs expressed smoothelin-B at levels comparable to those observed in adult aortic and carotid SMCs, indicating that this cell type is vascular in nature. Smoothelin-B is also a marker of fully differentiated mature vascular SMCs,⁴⁶ indicating that these cells could be functional, although embryonic. Consolidating this finding, telokin and SM γ -actin (not shown), both visceral SMC markers, were not expressed in the rat SKP-derived SMCs or in negative controls such as the aorta, carotid and liver, whereas they were expressed in bladder. Importantly, similar observations were made in human foreskin-derived SKPs, where levels of SMemb were increased on SMC-directed differentiation with TGF- β 1 or - β 3 and comparable to those observed in primary SMC lines. Treatment of rat and human SKPs with phenylephrine elicited sustained contractions lasting minutes, indicating that the SKP-derived SMCs were functional despite high levels of SMemb and low levels of SM-myosin heavy chain.

It was interesting to note that SKPs and SKP-derived SMCs express only low levels of myocardin. MRTF-A and -B are structurally and functionally similar to myocardin and may be compensating for the function of myocardin in these cells.^{42,47–51} Indeed, evidence supporting this can be found in studies of the developing aorta. Myocardin was detected by in situ hybridization in the dorsal aorta at E11.5.⁵² This was in contrast to SM22 α and ASMA expression, which began at E9.5 in this region,⁵² and MRTF-B, which began to be expressed in the dorsal aorta at E8.5.⁵³ MRTF-B is also expressed in the cardiac neural crest, where myocardin is absent and which is known to contribute SMCs to various embryonic tissues.⁵³ Given that MRTF-B is structurally similar to myocardin and can also act as a transactivator of genes containing CArG box promoter elements,⁵⁴ we postulate that MRTF-B may act to initiate SMC differentiation in the dorsal aorta and in the neural crest–derived SKPs and SKP-derived SMCs we have examined.

In the mesenchymal adult stem cell population, sphingolipids have been shown to have instructive effects in generating SMCs. D-SPC is a naturally occurring sphingolipid derived from the degradation of sphingomyelin and is structurally similar to SIP.⁵⁵ However, unlike SIP, it is less abundant and has no known receptor.⁵⁵ In this study, neither D-SPC nor SIP induced the differentiation of neural crest–derived SKPs into SMCs (Supplemental Figures IV and V). Rather, D-SPC suppressed SKP-derived SMC differentiation. These findings are in direct contrast to the reported effects of these sphingolipids on other adult mesenchymal stem cells.^{24,25}

Members of the TGF- β superfamily (such as TGF- β 1, - β 2, and - β 3) have been shown to have instructive effects on neural crest stem cells taken from explants of the E10.5 neural tube, directing them into SMCs.³⁴ In both neural crest and mesenchymal systems, TGF- β acts instructively to influence cell fate decisions rather than selectively to support survival of lineage committed progenitors.³⁴ This widely expressed cytokine is produced by both inflammatory and vascular cells, as well as in human and mouse atherosclerotic plaques.^{56,57} Developmentally, TGF- β and its downstream signaling events are essential for vasculogenesis. Only 50%

of embryos with homozygous deletion of TGF- β 1 survive beyond E10.5 because of hematopoietic and vasculogenic defects in the yolk sac, whereas the other 50% of newborn animals die of a wasting syndrome attributed to a high inflammatory response.^{58–60} TGF- β 2 is also essential for normal hematopoiesis and vasculogenesis in the yolk sac, in addition to the development of several organs.⁶¹ In accord with this, histological analysis of the TGF- β 1 and - β 2 knockout embryos are nearly identical.⁶¹ Mutation of endoglin, a TGF- β and - β 1 binding protein, results in poor vascular SMC development and arrested endothelial remodeling.⁴⁴ By contrast, TGF- β 2 and - β 3 knockout animals differ from the TGF- β 1 deletion mutant and do not share overlapping phenotypes.⁶² It is thus curious that both TGF- β 1 and - β 3 elicited analogous differentiation responses in SKPs, possibly via shared receptors or signaling pathways in this progenitor.

TGF- β has varying effects on different cell types, depending on dosage and cell type, including proliferation, migration, or differentiation. For example, TGF- β has been shown to induce mesoderm from ectodermal progenitors (neural crest).³⁴ Also, effects of TGF- β on SMCs are density dependent, with an antiproliferative effect on cells cultured at low density and a proproliferative effect at high cell density, such that TGF- β has been implicated in the “hill-and-valley” appearance of SMC cultures in vitro.⁶³ These effects are also dose dependent. Neural crest stem cell-derived SMCs from the thoracic aorta respond to TGF- β 1 by increasing their proliferation in low concentrations (\approx 0.1 ng/mL) and decreasing their proliferation in high concentrations of TGF- β 1 (\approx 10 ng/mL).⁶⁴ It is thus tempting to speculate that the inductive differentiation of sparsely plated human neural crest–derived SKPs observed in response to the relatively low concentrations of TGF- β 1 (2 ng/mL) we used may be related to the ability of this agent (and particular dose) to inhibit cell cycle progression. Indeed, TGF- β 1 has been shown to cause G₁/G₀ cell cycle arrest in various cell types.⁶⁵

SMCs display remarkable structural and functional diversity. In the developing embryo, SMCs derived from the arch of the aorta, thoracic aorta, aortic root, innominate arteries, and common carotid, which are neural crest derived, are functionally distinct from SMCs derived from the abdominal aorta, which are splanchnic mesoderm in origin.⁶⁶ Both of these embryonic SMC populations display differential responses to TGF- β . For example, on the addition of TGF- β 1, cell proliferation was increased in neural crest–derived SMCs, whereas mesodermal-derived SMCs were either unresponsive or growth inhibited.⁶⁴ Also, c-Myb expression was induced on the addition of TGF- β 1 in neural crest–derived SMCs, whereas it was not in mesodermal-derived SMCs.⁶⁴

These known effects of TGF- β were mirrored or recapitulated in the neural crest–derived stem cells we studied. In the rat system, low serum and low cell density differentiated SKPs into SMCs almost exclusively, suggesting that “quiescence” may initiate the SMC program. In humans, more instructive and directive signals were required. The addition of TGF- β 1 or - β 3 was required to direct human foreskin and cleft palate SKPs (data not shown) into SMCs. Again, low cell densities and reduced serum concentrations (as well as

TGF- β 1 or - β 3) were required for optimal differentiation to SMCs whenever serum was used (Supplemental Figure III).

Our observation that human neural crest-like foreskin-derived SKPs can survive in serum-free conditions is of particular interest. It had been observed that ectoderm-sourced neural crest-derived SMCs display a greater capacity for growth in serum-free conditions.⁶⁷ In our current study, TGF- β 1 or - β 3 alone was enough to provide the survival signals required to maintain these cells in culture for extended periods of time.

Finally, the differentiated SKPs appear to be functional vascular SMCs in vivo, by virtue of their intimate integration around functional blood vessels. This provides evidence that these cells, when transplanted, can support angiogenesis and may be useful for the treatment of ischemic diseases or the repair of wounds. That these cells can be directed to differentiate and expanded in serum-free cultures over extended periods of time makes them attractive therapeutic candidates for further preclinical and clinical trials.

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Disclosures

None.

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Directed Differentiation of Skin-Derived Precursors Into Functional Vascular Smooth Muscle Cells

Sarah K. Steinbach, Omar El-Mounayri, Ralph S. DaCosta, Matthew J. Frontini, Zengxuan Nong, Azusa Maeda, J. Geoffrey Pickering, Freda D. Miller and Mansoor Husain

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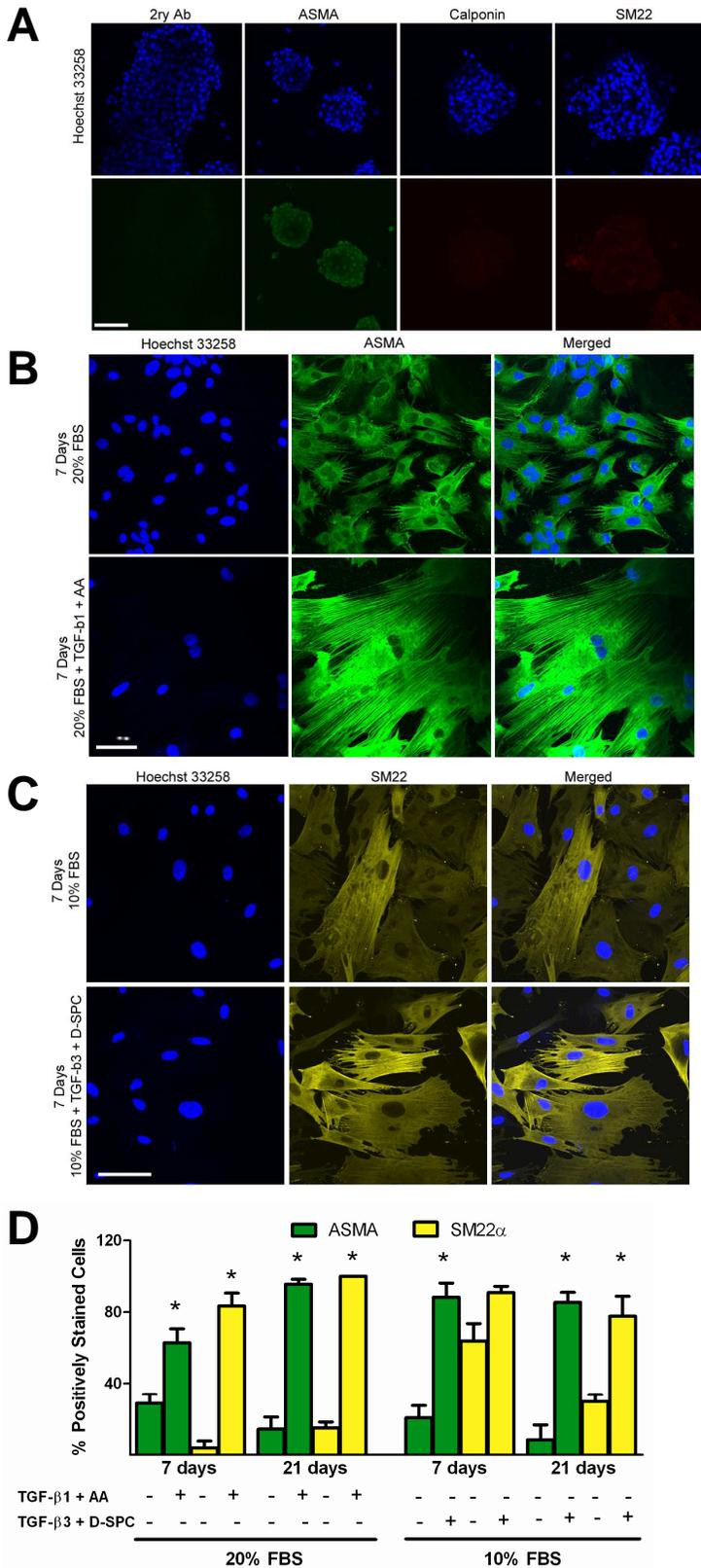
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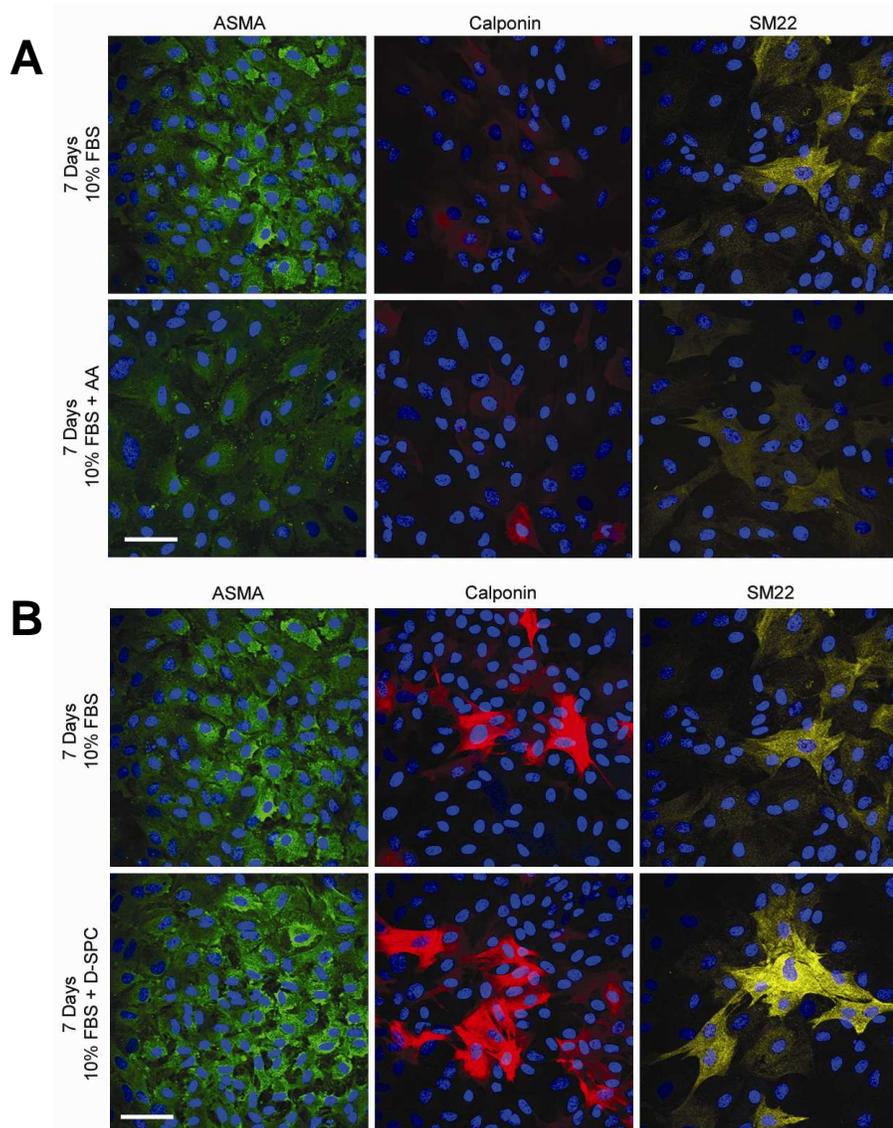
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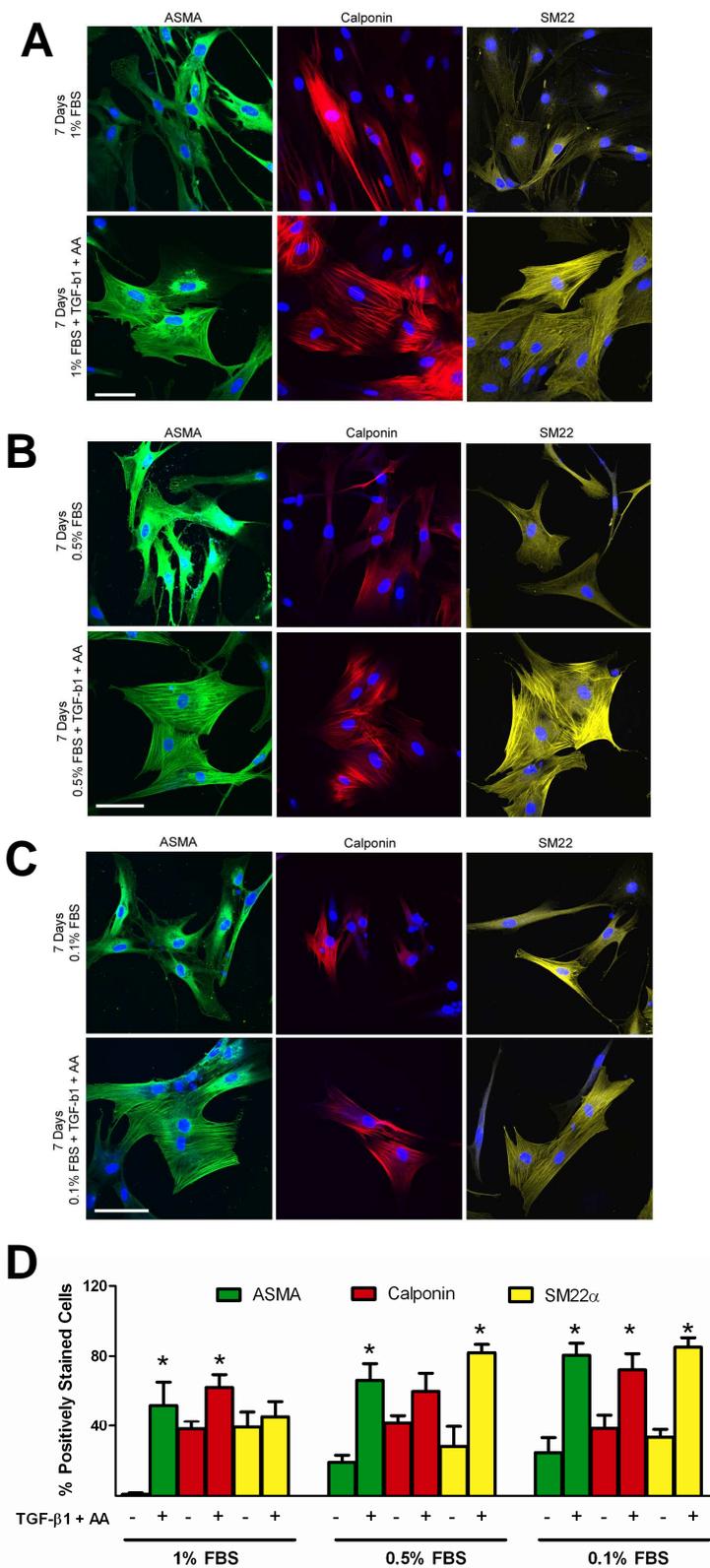
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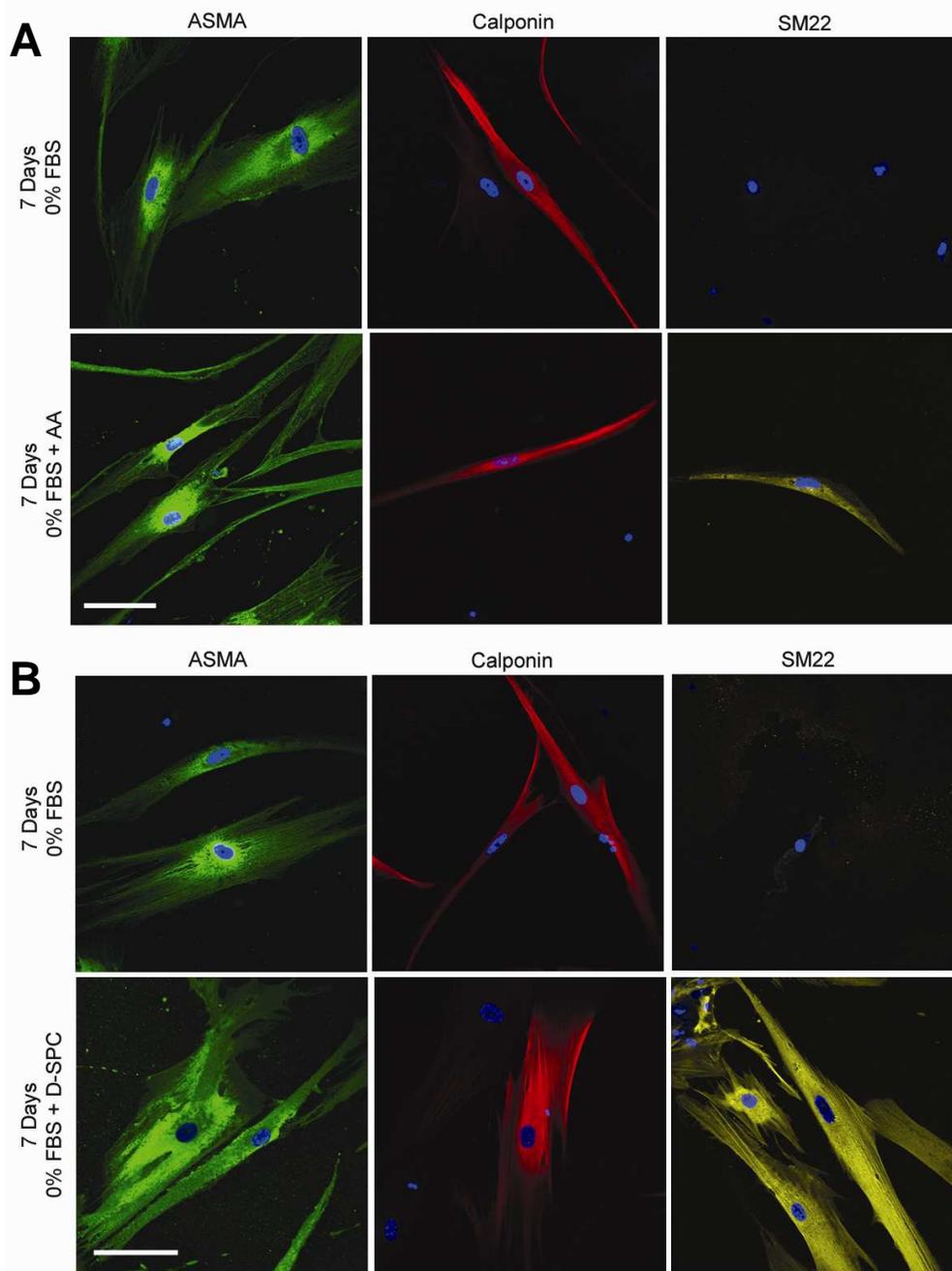
Supplementary Figure I. (A) Formaldehyde-fixed SKPs were examined by confocal microscopy after staining for secondary alone (2^o Ab, no colour), ASMA (green), calponin (red), SM22 α (gold), and nuclei (blue). Undifferentiated SKPs express little to no ASMA, calponin or SM22 α . Magnification bar= 60 μ m. Neonatal rat SKPs were differentiated x 7 d in: (B) 10% FBS, 10% FBS + TGF- β 1 (2 ng/ml) + AA (30 μ M), or (C) 10% FBS, 10% FBS + TGF- β 3 (2 ng/mL) + D-SPC (2 μ M). (D) SKPs were differentiated in the above conditions x 7 d and 21 d, and numbers of SMCs were quantified based on ASMA and SM22 α expression. N=75-315 cells counted per condition. * P <0.05 vs. untreated (FBS only) controls.



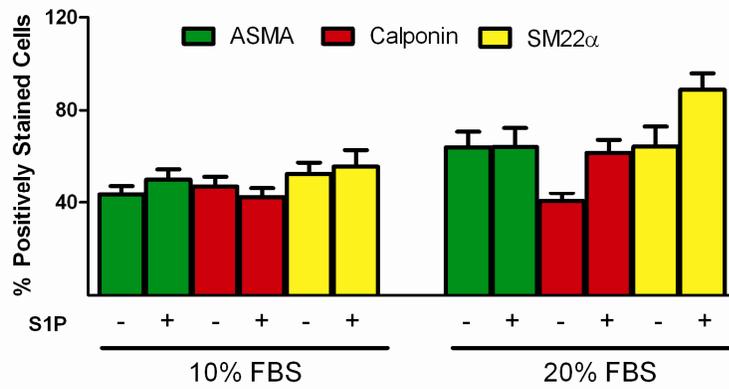
Supplementary Figure II. Neonatal rat SKPs were differentiated x 7 d in: (A) 10% FBS or 10% FBS + Ascorbic Acid (AA, 30 μ M), or (B) 10% FBS or 10% FBS + D-SPC (2 μ M).



Supplementary Figure III. Human foreskin-derived SKPs were differentiated in (A) 1%, (B) 0.5%, and (C) 0.1% FBS in the presence and absence of TGF- β 1 (2 ng/mL) + AA (30 μ M). (D) Numbers (%) of SMCs were quantified based on confocal micrographs showing ASMA, calponin and SM22 α staining. N=35-269 cells counted per condition. * P <0.05 vs. untreated (FBS only) controls. Magnification bar = 60 μ m.



Supplementary Figure IV. Neonatal human foreskin-derived SKPs were differentiated x 7 d (A) serum-free or serum-free + AA (30 μ M) and (B) serum-free or serum-free + D-SPC (2 μ M).



Supplementary Figure V. Neonatal rat SKPs were differentiated in 10% and 20% FBS in the presence and absence of S1P (1 μ M). Numbers of SMCs were quantified based on confocal micrographs with positive ASMA, calponin and SM22 α expression. N=62-265 cells counted per condition.