Contractile smooth muscle cells derived from hair-follicle stem cells

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Aims We hypothesized that hair-follicle stem cells can differentiate toward smooth contractile muscle cells, providing an autologous cell source for cardiovascular tissue regeneration.

Methods and results Smooth muscle progenitor cells (SMPCs) were obtained from ovine hair follicles using a tissue-specific promoter and fluorescence-activated cell sorting. Hair-follicle smooth muscle progenitor cells (HF-SMPCs) expressed several markers of vascular smooth muscle including α-actin, calponin, myosin heavy chain (MHC), caldesmon, smoothelin, and SM22. HF-SMPCs were highly proliferative and showed high clonogenic potential without any signs of chromosomal abnormalities as evidenced by karyotype analysis. HF-SMPCs compacted fibrin hydrogels to a similar extent as vascular smooth muscle cells from ovine umbilical veins (V-SMCs), indicating the development of the force-generating machinery. In addition, cylindrical tissue equivalents prepared with HF-SMPCs displayed significant contractility in response to vasoactive agonists including KCl and the thromboxane A2 mimetic U46619, suggesting that these cells had developed receptor and non-receptor-mediated pathways of contractility. Finally, transforming growth factor-β1 promoted differentiation of HF-SMPCs toward a mature SMC phenotype as suggested by increased expression of MHC and enhanced matrix compaction.

Conclusion Our results suggest that hair follicles may be an easily accessible, autologous, and rich source of functional SMPC for cardiovascular tissue engineering and regenerative medicine.

KEYWORDS Stem cells; Hair follicle; Smooth muscle cells; Contractility; Cardiovascular tissue engineering; Cell therapy

1. Introduction

Smooth muscle cells (SMCs) comprise the muscle of several tissues and organs including the bladder, abdominal cavity, gastrointestinal tract, respiratory tract, and blood vasculature where they provide contractile function and play a critical role in major human diseases, including atherosclerosis, hypertension, and asthma.1 As a result, SMCs are very important for the development of cell therapies especially for the treatment of cardiovascular disease. Although SMCs can be isolated from existing blood vessels, the process is invasive, requires major surgery and injures the donor site. In addition, mature SMCs have limited proliferative capacity thus necessitating multiple tissue harvests for repeat grafting procedures.

The hair follicle is a very rich source of multipotent adult stem cells and as such it may be an easily accessible, alternative source of autologous SMC. Epidermal stem cells are located in the bulge area of the hair follicle as shown by label retaining assays2,3 as well as by transgenic mice designed to express EGFP in that region.4,5 In a pioneering study, Lako et al.6 first demonstrated that cells from the dermal papilla or dermal sheath but not from the epidermal compartment of hair follicles could reconstitute multiple lineages of the haematopoietic system in lethally irradiated mice. In the presence of appropriate induction medium, cells form the dermal papilla or dermal sheath were also shown to differentiate toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages similar to bone marrow mesenchymal stem cells.7,8 Additionally, nestin-positive cells from the skin and hair follicles were shown to express neuronal and glial markers and were able to reconstitute the vasculature upon transplantation.9–16 Taken together, these data suggest that hair follicles contain multipotent stem cells with the ability to regenerate the epidermis as well as mesenchymal and haematopoietic tissues.

Previous studies reported differentiation of stem cells from bone marrow17–19 or cord blood18 into SMC by supplementing the culture medium with one or more growth factors. Although these methods are straightforward, they cannot achieve isolation of a pure population of SMCs without contamination from fibroblasts or other cells. To address this issue, we proposed a novel method of SMC...
isolation that relies on expression of a fluorescent marker protein (EGFP) from the smooth muscle alpha-actin (\text{SM\alpha\alpha}) promoter.\textsuperscript{20} To this end, bone marrow mesenchymal stem cells were transfected with a plasmid encoding for EGFP under the \text{SM\alpha\alpha} promoter and were subsequently separated from other cells by fluorescence-activated cell sorting. Using biochemical and functional assays, we showed that the resulting cells—termed bone marrow smooth muscle progenitor cells (BM-SMPCs)—expressed SMC-specific proteins and developed contractile phenotype. Most notably, vascular grafts engineered with BM-SMPCs were implanted into the jugular veins of an ovine animal model and demonstrated successful matrix remodelling, including production of elastin fibres.

Based on these studies, we hypothesized that we can isolate functional SMCs from hair follicles by employing \text{SM\alpha\alpha}-driven EGFP expression and fluorescence-activated cell sorting. Using this strategy, we showed that the hair follicle is a rich source of smooth muscle progenitor cells with very high proliferation potential and contractile function. In addition to \text{SM\alpha\alpha}, HF-SMPCs expressed several markers of SMC lineage including calponin, myosin heavy chain (MHC), smoothelin, SM22, and caldesmon. Clonogenic and long-term growth assays showed that HF-SMPCs had much higher proliferation potential than mature vascular smooth muscle cells (V-SMCs) from newborn animals. Karyotype analysis showed that HF-SMPCs were normal with no sign of transformation, suggesting that these cells can be expanded to the numbers necessary for development of cell therapies. Notably, HF-SMPCs exhibited ability to generate force as shown by compaction of fibrin hydrogels and contractility in response to vasoactive agonists. Our study suggests that hair follicle may be a readily accessible source of autologous, highly proliferative, and functional SMC that can be used for tissue engineering and regenerative medicine.

2. Materials and methods

2.1 Retroviral vector encoding EGFP under the control of \text{SM\alpha\alpha} promoter

The rat \text{SM\alpha\alpha} promoter was cloned into the promoterless EGFP reporter vector pEGFP-1 (Clontech, Mountain View, CA, USA) as described previously.\textsuperscript{20} The \text{SM\alpha\alpha}-EGFP sequence from this vector was amplified by high fidelity PCR with forward primer: CCTCTAGAC-CAGCGTCTTGCATGATA containing the XbaI site (underlined); and reverse primer: AAATTGGAGCCCTAAAGAGCTCGTCCATGCCG containing the XhoI site (underlined). The PCR reaction was carried out with denaturation for 30 s at 94°C; annealing for 30 s at 55°C, and extension for 90 s at 72°C. The PCR product was subsequently excised with XbaI and XhoI and subcloned into the same sites of the self-inactivating retroviral vector pQCXIX (Clontech), removing the CMV promoter and IRES sequence. The resulting retroviral vector, termed pQ-SM\alpha\alpha-EGFP, encodes EGFP under control of \text{SM\alpha\alpha} promoter.

2.2 Retrovirus production

VSV-G pseudotyped retrovirus was produced by transient transfection of Phoenix-gp packaging cells (kindly provided by Dr Gary Nolan, Stanford University) with the retroviral vector pQ-SM\alpha\alpha-EGFP and the vesicular stomatitis virus glycoprotein (VSV-G) encoding plasmid. Briefly, Phoenix-gp cells were plated in T-75 tissue culture flask (5 × 10\textsuperscript{5} per flask) and incubated overnight. The next day, plasmid DNA (10 \mu g of pQ-SM\alpha\alpha-EGFP and 10 \mu g of VSV-G) and transfection reagent Fugene 6 (Roche, Indianapolis, IN, USA) were mixed (1:3; \mu g: \mu L) in 800 \mu L of DMEM without serum or antibiotics. The mixture was incubated at room temperature for 45 min and then added to the culture medium overlaying the packaging cells. The next day, the medium was replaced with fresh culture medium and retrovirus was harvested 24 h later.

2.3 Isolation of smooth muscle progenitor cells from ovine hair follicles

Full-thickness skin was harvested from a newborn lamb under aseptic condition. All procedures and protocols in this study were approved by the Laboratory Animal Care Committee of the State University of New York at Buffalo. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Skin tissue was trimmed to remove underlying fat tissue, cut into 2 × 4 mm pieces, and subsequently digested with 1 mg/mL of Collagenase Type I (Invitrogen, Carlsbad, CA, USA) at 37°C with occasional agitation. After 4 h of digestion, single-hair follicles were released from the full-thickness skin, filtered through 40 \mu m cell strainer (BD Biosciences, San Jose, CA, USA), and washed extensively with PBS. Then, hair follicles were placed each in a well of a 96-well plate (BD Biosciences) and cultured in 100 \mu L of DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% PBS (Gibco) to allow for cell migration onto the tissue culture plastic. Cells that originated from the bulge region were visually identified as epidermal keratinocytes, while cells migrating from the dermal sheath or papilla had the morphological appearance of mesenchymal cells. The wells populated with cells originating from the dermal sheath or papilla were selected, pooled, and expanded.

Passage 4 cells were transduced with \text{SM\alpha\alpha}-EGFP recombinant retrovirus in the presence of 8 \mu g/mL polybrene and EGFP\textsuperscript{+} cells were subsequently sorted using fluorescence-activated cell sorting. These cells showed high proliferation capacity, expressed several SMC markers, and exhibited contractility (see Methods and Results section). Therefore, they were termed hair-follicle smooth muscle progenitor cells (HF-SMPCs).

Ovine V-SMCs from umbilical veins of near-term foetal lambs were isolated as described previously\textsuperscript{21} and served as positive controls. Finally, an epidermal keratinocyte cell line (HaCaT; kind gift of Dr Satrajit Sinha, Department of Biochemistry; SUNY at Buffalo) served as negative control.

2.4 Proliferation and clonogenic assays

Passage 10 HF-SMPCs or V-SMCs were seeded at 10\textsuperscript{5} cells per well in 6-well plates and cultured in DMEM containing 10% FBS. Every three days, the cells were trypsinized, counted with haemocytometer, and subcultured at the same density for 6 weeks until V-SMCs stopped proliferating. The cumulative cell number was normalized by the seeding density (10\textsuperscript{5} cells/well) and plotted over time. The experiment was repeated three times.

For clonogenic assays, HF-SMPCs or V-SMCs were seeded (400 cells/dish) in a 100 mm culture dish (BD Biosciences, Franklin Lakes, NJ, USA) and cultured for 10–12 days in DMEM containing 10% FBS. The cells were then fixed with a solution containing acetic acid and methanol (1:3 v/v) for 5 min; washed twice with PBS; and stained with 10 mL trypan blue (Gibco) for 5 min. Cells were washed once with PBS and photographed at a fixed distance using a gel documentation imaging system (UVG, Upland, CA, USA). Images were analysed using the NIH software Image J (version 1.3; National Institutes of Health, USA) to determine the area and effective diameter of each clone. Only colonies with diameter larger than 2 mm were counted as those were considered to have originated from cells with high proliferation capacity, which were more likely to be stem or progenitor cells. The experiment was repeated at least three times with cells between passages 5 and 10.
2.5 Flow cytometry

Single-cell suspension of HF-SMPCs or V-SMCs was fixed in 2% ice cold formaldehyde for 10 min, washed twice with PBS, and permeabilized in permeabilization buffer [PBS containing 0.1% (w/v) saponin (MP Biomedical, Inc., Irvine, CA, USA) and 0.05% (w/v) NaN₃ (Fisher Scientific, Pittsburgh, PA, USA)] for 10 min. Cells were resuspended for 30 min in 100 μL of blocking buffer [permeabilization buffer containing 1% (w/v) BSA] and stained with mouse monoclonal anti-human SMαA (1:50 dilution in blocking buffer; 1 h at room temperature; SeroTec, Oxford, UK) or anti-human smooth muscle calponin (1:50 dilution in blocking buffer; 1 h at room temperature; DakoCytomation, Carpinteria, CA, USA). Thereafter, the cells were washed twice with PBS and stained with anti-mouse secondary antibody conjugated with Alexa Fluor® 647-R-phycocerythin (1:100 dilution; Invitrogen; 30 min at room temperature). After staining, cells were washed twice with PBS and processed for flow cytometry. Cells that were stained with secondary antibody only served as negative control.

2.6 Histology and immunostaining

Histology and immunostaining were performed as described previously.20,22 using the following antibodies in PBS containing 1% BSA and 0.01% triton X-100: mouse monoclonal anti-human SMαA (1:50 dilution; overnight at 4°C; SeroTec); mouse anti-human calponin (1:100 dilution; overnight at 4°C; DakoCytomation); mouse IgM isotype anti-bovine alpha actinin (1:100 dilution; overnight at 4°C; Sigma); Alexa Fluor488-conjugated secondary goat anti-mouse IgG or IgG (1:100 dilution; 1 hr at room temperature; Molecular Probes); Alexa Fluor594-conjugated secondary goat anti-mouse IgM (1:100 dilution; 1 hr at room temperature; Molecular Probes). No fluorescence was observed when cells or tissues were stained with secondary antibody only (no primary antibody; negative control).

2.7 Transmission electron microscopy

For transmission electron microscopy (TEM) fibrin-based TEVs were prepared from V-SMCs or HF-SMPCs. After for two weeks culture, the tissues were cut into ~1 mm² pieces and fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2 for 2 h; then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h and stored in buffer. Dehydration was accomplished via graded ethanol and acetone prior to embedding in Epon-Araldite resin. Sixty to 100 nm thin sections were cut with a Sorvall MT2 ultramicrotome, mounted on copper mesh grids, and stained with uranyl acetate and lead citrate. The samples were observed and photographed with a Hitachi H500 electron microscope operating at 75 kV. Negatives were scanned at 1200 dpi with an Agfa T1200 scanner using IPLab and Adobe Photoshop software, unsharp masked, and resampled to required resolution for publication.

2.8 Western blots

Western blots for SMαA, calponin, and MHC were performed as described previously.20

2.9 Compaction of fibrin hydrogels

Three-dimensional fibrin gels were prepared with HF-SMPCs, V-SMCs, or HaCaT cells. For each gel, cells (10⁶ cells/mL) were resuspended in 200 μL of 12.5 U/mL thrombin and mixed with 800 μL of 3.125 mg/mL fibrinogen (BD Biosciences, Franklin Lakes, NJ, USA). The mixture was allowed to polymerize in a well of a 24-well plate (polymerization time: ~15 s) to form gel with final concentration of fibrinogen and thrombin at 2.5 mg/mL and 2.5 U/mL, respectively. After 1 h, the gels were released from the wells and incubated in DMEM ± 10% FBS in the presence or absence of TGF-beta1 (2 ng/mL) as indicated. Cell culture medium was replenished daily. At the indicated times, the gels were photographed at a fixed distance using a gel documentation imaging system (UVG). The area of each gel was measured using Image J and normalized to the initial area (t = 0 h).

2.10 Cylindrical smooth muscle tissue equivalents

Tissue equivalents containing HF-SMPCs or V-SMCs were prepared as described previously.20,21,23 Briefly, HF-SMPCs or V-SMCs were suspended in thrombin and mixed with fibrinogen at a ratio of 1:4 yielding fibrin gels with final concentration of fibrinogen and thrombin at 2.5 mg/mL and 2.5 U/mL, respectively. For each tissue, 1 mL of fibrin containing 1 × 10⁶ cells was polymerized around a 6.0 mm mandrel of poly(di-methyl siloxane) placed in the middle of each well. After 1 h, the gels were detached from the walls and incubated in 2 mL of DMEM containing 10% FBS. The next day, the medium was supplemented with 2 μg/mL insulin, 2 ng/mL TGF-β1, 300 μM ascorbic acid phosphate, and 2 mg/mL e-aminocaproic acid. Thereafter, cell culture medium was replenished every three days.

2.11 Contractility and mechanical properties of tissue equivalents

After two weeks in culture, the length and diameter of cylindrical tissue equivalents were measured using an electronic digital calliper. Then the tissues were released from the mandrel and mounted on two stainless hooks in an isolated tissue bath and incubated in Krebs-Ringer solution. The tissues were continuously bubbled with 94% O₂, 6% CO₂ to obtain a pH of 7.4, a PCO₂ of 38 mmHg, and a P O₂ > 500 mm Hg at 37°C. Each construct was mounted on stainless steel hooks through the lumen, one was fixed, and the other one was connected to a force transducer. Tissues were equilibrated at a basal tension of 1.0 g and constant length for 30-60 min. After equilibration, potassium chloride (KCl, 118 mM) or the thromboxane A2 mimetic U46619 (10⁻⁶ M) was added to the tissue bath and isometric contraction was recorded using a PowerLab data acquisition unit and analysed with Chart5 software.

For measuring mechanical properties, tissue equivalents were mounted on the force transducer and stretched incrementally until they broke, yielding the ultimate tensile strength (UTS) and break length of each tissue. The initial tissue length corresponds to the length under a passive tension of 1.0 g. Broken constructs were dehydrated with series of ethanol washes, air dried, and weighted. UTS was normalized by the dry weight of each construct and expressed in units of Newton per gram of dry tissue weight (N/g dry weight). The elastic modulus was calculated as the slope of the linear part of the length–tension curve.

2.12 Statistical analysis

Data were expressed as mean± standard deviation and statistical significance (defined as P < 0.05) was determined using Student’s t-test.

3. Results

3.1 Isolation of functional SMPCs from ovine hair follicles

Single-hair follicles were released from the dermis by digestion of newborn lamb skin with collagenase. After extensive washes, individual hair follicles were placed each in a well of a 96-well plate to allow for cell migration onto the tissue culture plastic (Figure 1A). Cells that originated from the bulge region were identified as epidermal keratinocytes, while cells migrating from the dermal sheath and papilla had the morphological appearance of mesenchymal cells. The wells populated with cells originating from the dermal sheath and papilla were selected and the cells pooled and expanded.
To obtain SMCs from the total population of hair-follicle cells, passage four cells were transduced with recombinant retrovirus encoding for green fluorescence protein (EGFP) under the control of the SMαA promoter and EGFP+ cells (~2% of total cells) were subsequently sorted using fluorescence-activated cell sorting. These HF-SMPCs were cultured for two to three more passages to obtain enough cells and then examined for expression of SMC markers. Specifically, RT–PCR showed that HF-SMPCs expressed several SMC markers, including SMαA, calponin, smoothelin, SM22, caldesmon, and MHC to similar extent as mature V-SMCs from the umbilical vein of newborn lambs (Figure 1B). Western blots (Figure 1C) confirmed strong expression of SMαA, calponin, and MHC in HF-SMPCs and V-SMCs. Human epidermal keratinocytes (HaCaT cells) did not express any of these proteins and were used as negative control. Flow cytometry provided quantitative data showing that similar to V-SMCs, nearly all HF-SMPCs expressed SMαA (99 ± 0.12%; n = 2) and calponin (99 ± 0.09%; n = 2). Finally, immunostaining showed similar fibrillar organization of SMαA, calponin, and alpha-actinin in HF-SMPCs and V-SMCs (Figure 1D). Interestingly, alpha-actinin was organized in Z-bodies as previously described for SMC and immature cardiomyocytes.24–26 MHC could not be detected due to lack of availability of anti-ovine antibody that was appropriate for flow cytometry or immunocytochemistry.

3.2 HF-SMPCs exhibited high clonogenic potential

Next we examined the proliferation ability of HF-SMPCs using both clonogenic and long-term proliferation assays. For clonal analysis, HF-SMPCs or V-SMCs were seeded at 5 cells per cm² and cultured for 10–12 days until large cell colonies were visible. HF-SMPCs formed significantly higher number of colonies than V-SMCs (Figure 2A). The fraction of HF-SMPCs that formed colonies larger than 2 mm in

Figure 1  HF-SMPCs express biochemical markers of V-SMCs. (A) Cells migrating out of the outer root sheath and dermal papilla. (B) RT–PCR showed that HF-SMPCs expressed smooth muscle cell markers as indicated. (C) Western blot for SMαA, calponin, and myosin heavy chain (MHC). Lysates from an epidermal keratinocyte cell line (HaCaT) served as negative control. Membranes were stripped and re-probed for beta-actin that served as loading control. (D) Immunocytochemistry for SMαA, calponin, and alpha-actinin. Insets show individual cells at higher magnification (60 x). (E) Cells stained with secondary antibody only served as negative control (Ctrl). Representative results from three independent experiments are shown.
diameter was 26.7 ± 5.9%. In contrast, only 3.7 ± 0.6% of V-SMCs were able of forming 2 mm or larger colonies. The distribution of colony size for both cell types is shown in Figure 2B. Proliferation assays showed that HF-SMPCs could be expanded a million fold over a period of 6 weeks in culture, while V-SMCs expanded only by a 1000-fold (Figure 2C). Despite the high proliferation rate, karyotype based on DAPI banding showed that HF-SMPCs were normal with no sign of transformation (Figure 2D).

3.3 HF-SMPCs generated force and compacted fibrin hydrogels

Next we examined the ability of HF-SMPCs to generate force and compact three-dimensional fibrin matrix. To this end, HF-SMPCs or V-SMCs (10^6 cells/mL) were embedded in fibrin hydrogels prepared with 2.5 mg/mL fibrinogen (FBG) and 2.5 U/mL thrombin. Each cell-containing gel was polymerized into a well of a 24-well plate. An hour after gelation, the hydrogels were released from the walls and compaction was monitored by measuring the area of each disk at the indicated times. As shown in Figure 3A, the rate and maximum extent of compaction were similar for both cell types, suggesting that HF-SMPCs possessed the contractile machinery to generate mechanical force and compact fibrin hydrogels. In contrast, HaCaT cells failed to compact the fibrin matrix (Figure 3B).

3.4 HF-SMPCs displayed significant contractility

The defining property of mature V-SMCs is their ability to contract and generate force in response to vasoactive agonists. To examine whether HF-SMPCs exhibited functional properties of mature V-SMCs, we measured the isometric tension generated by cylindrical rings of fibrin-based tissue constructs containing HF-SMPCs or V-SMCs, as we reported previously.20,21,23

To this end, cylindrical tissue equivalents were cultured around 6 mm diameter mandrels for 2 weeks. At that time, HF-SMPCs and V-SMCs tissues compacted significantly
reaching wall thickness of 0.61 ± 0.06 mm (n = 4) and 0.65 ± 0.03 mm (n = 4), respectively. Both cell types appeared to be uniformly distributed in the hydrogel (H&E) and stained positive for SmαA and calponin (Figure 4A–C). In addition, TEM showed that both V-SMCs and HF-SMPCs in fibrin hydrogels appeared elongated and contained numerous microfilaments (7–10 nm in diameter) that were detected underneath the plasma membrane and organized parallel to the long axis of the cell (Figure 4D).

Tissue equivalents prepared with HF-SMPCs and V-SMCs exhibited similar UTS (HF-SMPCs: 252.7 ± 63.4 kPa, n = 4; V-SMCs: 251.1 ± 89 kPa, n = 4) and elasticity (HF-SMPCs: 40 ± 5.2 kPa, n = 4; V-SMCs: 46.3 ± 8.9 kPa, n = 4), suggesting that HF-SMPCs could remodel the fibrin matrix to a similar extent as V-SMCs. We also measured the isometric tension of tissue equivalents in response to KCl (118 mM) or U46619 (10⁻⁶ M). Notably, HF-SMPCs containing tissue equivalents (n = 23) showed similar vasoconstriction to both agonists as V-SMCs containing tissues (n = 23) (Figure 5A and B). These results strongly suggested that HF-SMPCs had developed active pathways of receptor and non-receptor mediated contractility.

3.5 TGF-beta1 increased expression of MHC and compaction of fibrin hydrogels

TGF-beta1 is known to affect V-SMCs differentiation and contractility. Therefore, we examined whether TGF-beta1 had a similar effect on differentiation of HF-SMPCs. Western blotting showed that similar to V-SMCs, HF-SMPCs expressed the late differentiation marker MHC, which was further increased by treatment with TGF-beta1 (Figure 6A).

We also examined whether TGF-beta1 affected the functional properties of HF-SMPCs by measuring its effect on compaction of fibrin hydrogels. Indeed, TGF-beta1 increased compaction of fibrin hydrogels containing V-SMCs or HF-SMPCs (Figure 6B), suggesting that similar to V-SMCs, TGF-beta1 increased the ability of HF-SMPCs to generate force.

4. Discussion

Despite significant progress toward development of biomaterials and methods to cultivate 3D vascular grafts, cell sourcing remains a major problem. Recent studies showed that function of V-SMCs from different species may behave differently and that vascular constructs made from human V-SMCs exhibited significantly lower mechanical properties when compared with those from rat cells. In addition, adult somatic cells were shown to exhibit limited replicative capacity especially when they originated from older donors, the population most likely to suffer from cardiovascular disease. Therefore, an easily accessible, autologous source of progenitor vascular cells is necessary to enable development of cell therapies for cardiovascular disease.

Interestingly, HF-SMPCs appeared in culture of HF cells that were grown in the presence of serum but with no additional differentiation promoting factors. HF-SMPCs cells appeared as early as passage four as evidenced by the activity of the alpha-actin promoter. Using the same medium, previous studies showed that rat hair-follicle cells were able to differentiate toward bone, cartilage, and fat cells, similar to bone marrow-derived mesenchymal stem cells. In the presence of embryonic stem-cell medium containing knock-out serum and basic fibroblast growth factor, human hair-follicle-derived cells remained undifferentiated for several passages while maintaining the ability to differentiate towards melanocytic, myogenic, and neuronal lineages. It would be interesting to examine the effect of embryonic stem-cell medium in preventing differentiation and maintaining the pluripotency of ovine HF cells that were used in this study.

Although several studies used growth factor cocktails to differentiate cells from various sources into mature SMC, in no case has a purified population of contractile SMC been derived. In contrast, our work demonstrated that use of the SMαA promoter to drive expression of EGFP could successfully purify contractile SMC from bone marrow and in the present study from hair-follicle cells. A previous study employed the SM22 promoter to isolate SMC from bone marrow mononuclear cells. Selected clones expressed SMC markers only after long-term drug selection, suggesting that only a small fraction of cells with active SM22 promoter expressed SMC markers. In addition, functional properties of these cells such as force generation or contractility were not investigated. More recently, MHC and SMαA promoters were employed to drive expression of puromycin-N-acetyltransferase as a way to purify SMC from human embryonic stem cells (hESCs). In agreement with our study, the SMαA promoter resulted in contractile SMC that expressed multiple SMC differentiation specific markers. Surprisingly, the MHC promoter resulted in mixed population of cells with regards to expression of some SMC markers including SMαA. Collectively, these studies suggest that the SMαA promoter may provide a convenient and efficient way to isolate functional SMC from embryonic or adult stem-cell sources.

Our results are in agreement with a recent study from our laboratory that used the SMαA promoter and fluorescence-activated cell sorting to isolate BM-SMPCs.
Similar to HF-SMPCs, BM-SMPCs were highly proliferative; expressed several SMC markers at the mRNA and protein levels; and displayed receptor and non-receptor mediated contractility. Notably, vascular grafts from BM-SMPCs could withstand interpositional implantation into the jugular veins of lambs. Within 5 weeks post-implantation, BM-SMPCs remodelled the fibrin matrix and synthesized collagen and elastin. In contrast to V-SMCs from umbilical veins, BM-SMPCs organized elastin into fibres, an important characteristic of native vessels that has been difficult to recapitulate in engineered tissues.30 To establish the therapeutic potential of HF-SMPCs, we plan to employ the same animal model to examine implantability and extracellular matrix remodelling of HF-SMPCs-based vascular grafts.

The quintessential property of SMC is their ability to generate force in response to vasoactive agonists. Interestingly, when HF-SMPCs were embedded in fibrin hydrogels, they compacted the matrix to similar extent as V-SMCs, suggesting that these cells had developed the ability to generate force. In addition, rings of fibrin-based vascular constructs from HF-SMPCs exhibited vascular reactivity to vasoagonists such as KCl and U46619. KCl causes contraction by opening the L-type, slow calcium potential-dependent channels; and U46619 activates thromboxane A2 receptors. Consequently,
our data suggest that HF-SMPCs developed receptor and non-receptor-mediated pathways of contractility.

TGF-beta1 was shown to regulate proliferation and differentiation of SMC; upregulate SMC differentiation markers including SMαA, calponin, and MHC; stabilize the elastin mRNA; and increase the mRNA level and enzymatic activity of lysyl oxidase. TGF-beta1 also enhanced the mechanical strength and vascular reactivity of fibrin-based V-SMCs tissue equivalents. In addition, TGF-beta1 promoted differentiation of embryonic stem cells, bone marrow mesenchymal stem cells, and bone marrow multipotent adult progenitor cells into mature contractile SMC. In agreement with these studies, we found that treatment of HF-SMPCs with TGF-beta1 enhanced expression of the late SMC differentiation marker, MHC, and increased the extent of hydrogel compaction. These results suggest that similar to embryonic and mesenchymal stem cells, HF-SMPCs responded to TGF-beta1 by upregulating SMC-specific differentiation markers and improving their ability to generate force.

The ability to purify and culture autologous adult epithelial, mesenchymal, or neuronal cells from hair-follicles offers unique opportunities for regenerative medicine. In addition to engineering skin for burn victims or treatment of chronic wounds, our work shows that hair-follicle stem cells can be used for cardiovascular tissue regeneration. This is a fascinating prospect as HF-SMPCs may be used for engineering vascular grafts, heart valves, or cardiac patches to treat life-threatening cardiovascular disease using autologous cells from a readily accessible source. In addition to SMPC, HF cells may be able to differentiate toward other lineages such as cardiomyocytes or endothelial cells providing cell populations with great potential for the development of cardiovascular therapies. The therapeutic potential of hair-follicle stem cells will be greatly facilitated by the immune privilege that these cells
demonstrated in trans-species and human trans-gender transplantation studies. The pluripotency, potential immune privilege, and ease of accessibility make follicular stem cells an ideal cell source for cardiovascular tissue engineering and regenerative medicine.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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