Directed differentiation of embryonic origin–specific vascular smooth muscle subtypes from human pluripotent stem cells

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Vascular smooth muscle cells (SMCs) arise from diverse developmental origins. Regional distribution of vascular diseases may, in part, be attributed to this inherent heterogeneity in SMC lineage. Therefore, systems for generating human SMC subtypes of distinct embryonic origins would represent useful platforms for studying the influence of SMC lineage on the spatial specificity of vascular disease. Here we describe how human pluripotent stem cells can be differentiated into distinct populations of SMC subtypes under chemically defined conditions. The initial stage (days 0–5 or 0–7) begins with the induction of three intermediate lineages: neuroectoderm, lateral plate mesoderm and paraxial mesoderm. Subsequently, these precursor lineages are differentiated into contractile SMCs (days 5–19+). At key stages, the emergence of lineage-specific markers confirms recapitulation of embryonic developmental pathways and generation of functionally distinct SMC subtypes. The ability to derive an unlimited supply of human SMCs will accelerate applications in regenerative medicine and disease modeling.

INTRODUCTION

Vascular SMCs make up the walls of blood vessels, and their contractile properties help sustain vessel tone to mediate blood flow[1]. Interestingly, fate-mapping studies have demonstrated that the vasculature is populated by a mosaic of SMCs arising from distinct developmental lineages with defined boundaries separating regions of origin-specific SMCs[2]. An intriguing hypothesis is that the well-documented spatial distribution of vascular diseases is due in part to disparities in SMC embryonic origins (in addition to differences in hemodynamics and vessel structure)[3,4]. Therefore, a model system of embryologically distinct SMCs that originate from the same genetic source may be valuable for understanding the intrinsic differences between various regions of the vasculature.

Limitations of primary vascular SMCs as a model system

Primary vascular SMCs are typically derived under serum-containing conditions in which the SMCs tend to undergo phenotypic switching from a nondividing contractile phenotype to a proliferative dedifferentiated phenotype[5]. This results in reduced protein and mRNA levels of SMC markers. Moreover, primary SMC culture can be contaminated by other cell types, for instance, endothelial cells and fibroblasts. Although endothelial cells may be separated out by cell sorting for endothelial surface markers, there is a lack of definitive SMC surface markers to facilitate purification. Even with a variety of commonly known intracellular SMC markers, e.g., smooth muscle α-actin (ACTA2), calponin (CNN1) and SM22α (TAGLN), these have variable expression and poor specificity, as they may also be expressed by fibroblasts and other myogenic cell types. This problem is exacerbated by the fact that markers to distinguish between SMC subtypes from different developmental lineages have yet to be established. Owing to the difficulty in obtaining human SMC progenitors from either fetal or adult human tissues in sufficient quantities, the relationship between heterogeneity of SMC embryonic origins and anatomic localization of vascular diseases has not been studied extensively. Although there are commercially available human SMCs from various vascular tissues, such as coronary artery and aorta, these are often collected from heterogeneous sources. Inter-individual variability due to diverse genetic backgrounds may confound analysis of lineage-dependent effects on the functional phenotypes of SMC subtypes. Recent findings also raise the possibility that some primary SMC cultures may actually be derived from resident vessel wall stem or progenitor cells[6]. Although these findings have been disputed by others[7], the multiple limitations of primary SMC cultures strongly indicate that novel sources of SMCs with vessel territory specificity are essential to the advancement of the field of vascular biology.

Generation of human vascular SMCs

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), collectively known as human pluripotent stem cells (hPSCs), can serve as an ideal source for deriving unlimited amounts of isogenic SMC subtypes. Previous studies have demonstrated SMC differentiation from hPSCs through either an intermediate progenitor stage or directed differentiation[8]. SMC precursors that have been identified by fate-mapping studies in embryos and by hPSC differentiation systems *in vitro* include cardiovascular progenitor[9–11], proepicardium[12,13], hemangioblast[14,15], somites[16–18], mesoangioblast[19] and neural crest[20,21]. Methods for inducing SMC differentiation bypassing a distinct progenitor stage have also been described[8]. However, there exists high variability across the many protocols in terms of efficiency and timescale, probably owing to the reliance on undefined serum-containing culture media and, in some cases, a heterogeneous differentiation method using embryoid bodies. Notably, the concept of obtaining SMC subtypes with different embryonic origins from a single hPSC source *in vitro* has only recently been introduced[22].

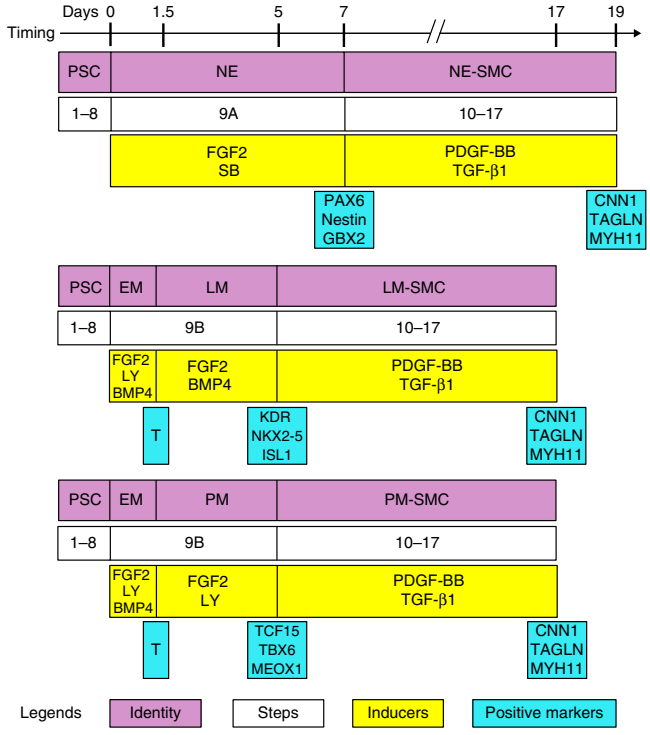

Figure 1 | Timeline outlining the generation of SMC subtypes (NE-SMC, LM-SMC and PM-SMC) from hPSCs. During the first phase, conditions (stated in yellow boxes) have been optimized to first induce the formation of precursor lineages (NE, LM and PM). Notably, before mesoderm specification into LM and PM, early mesoderm (EM) has to be obtained. Subsequently, SMC differentiation involves at least 12 d of treatment with PDGF-BB and TGF-β1. Quality control should be performed at the indicated time points (blue boxes) to check for positive expression of lineage-specific markers. LY, LY294002; SB, SB431542.

Here we describe in detail the derivation of origin-specific SMC subtypes from hPSCs via three intermediate tissue lineages, neuroectoderm (NE), lateral plate mesoderm (LM) and paraxial mesoderm (PM), which together give rise to the majority of SMCs in the body. This chemically defined approach yields high purity of SMCs, with more than 80% of the cells expressing smooth muscle contractile proteins. These SMC subtypes display contraction and calcium influx upon treatment with vasoconstrictors, and they are capable of homing to peri-endothelial regions in an *in vivo* Matrigel angiogenesis assay. Notably, proper patterning of the intermediate tissue lineages is crucial for obtaining functionally distinct SMC subtypes, as demonstrated by their differential proliferative and secretory profiles in response to transforming growth factor (TGF)-β signaling[22]. Because of the absence of *in vivo* microenvironmental cues to guide differentiation, a potential issue is maturity of the resulting SMCs. Nevertheless, all hPSC-derived SMC subtypes express mature SMC markers at levels comparable to human adult aortic SMCs; further, they exhibit focal adhesion proteins, a characteristic of functional SMCs, and they display contractile ability[22]. This protocol is reproducible in iPSCs, thus allowing the generation of SMCs with monogenic disorders or other complex genotypes that show predisposition to vascular diseases.

Experimental design

The rationale behind this stepwise SMC differentiation procedure is based on insights from developmental studies. Previous fate-tracking experiments have revealed the diverse embryonic origins of vascular SMCs[2]. As discussed in the following section, the protocol can be used on hPSCs from a variety of origins. Once suitable starting cells have been selected, the protocol comprises three main stages: preparation of hPSCs for differentiation (Steps 1–8); specification of SMC precursor populations (Step 9); and finally the differentiation of the precursor populations into SMC subtypes (Steps 10–18). **Figure 1** gives a summary of the procedure.

Starting cell population considerations. The inherent differences among SMC subtypes may be established early during development by their respective embryonic origins. HPSC-derived SMCs demonstrate differential functional properties such as proliferation, migration and matrix degradation, similar to their *in vivo* SMC counterparts[22]. We have previously shown that this protocol is reproducible in two ESC lines (i.e., H1 and H9) and in a wild-type iPSC line[22]. Please note that discrepancies in the final yields and timing of differentiation can exist with other hPSC lines. Variability between hPSCs is likely to be influenced by genetic backgrounds, as well as by the method of reprogramming and cells of origin in the case of iPSCs. Given our experience with multiple hPSC lines that have different differentiation propensities, we point out that users of this protocol may need to carry out minor optimization of differentiation conditions.

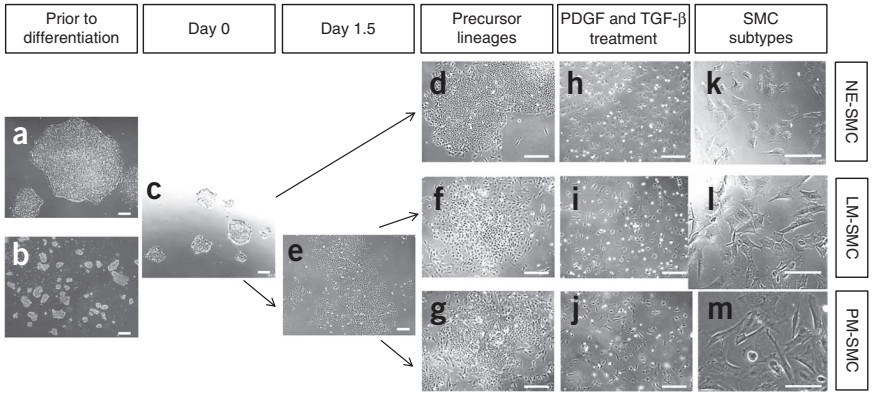

Figure 2 | Phase-contrast images of hPSCs during the course of differentiating into SMC subtypes. (**a**) HPSC colonies. (**b**) Fragmented hPSC colonies right after splitting. (**c**) HPSC colonies attach to plate 24 h after splitting. (**d**) NE induction after 7 d of treatment with FGF2 and SB431542 displays the neuroepithelial phenotype. (**e**) Early mesoderm formation is observed as dispersed colonies after 1.5 d of treatment with FGF2, LY294002 and BMP4. (**f**,**g**) Mesoderm specification into LM and PM by FGF2-BMP4 and FGF2-LY294002, respectively. (**h**–**j**) One day after splitting of precursor populations into medium containing PDGF-BB and TGF-β1. (**k**–**m**) Three SMC subtypes are obtained after 12 d of PDGF-BB and TGF-β1 treatment. Scale bars, 100 μm.

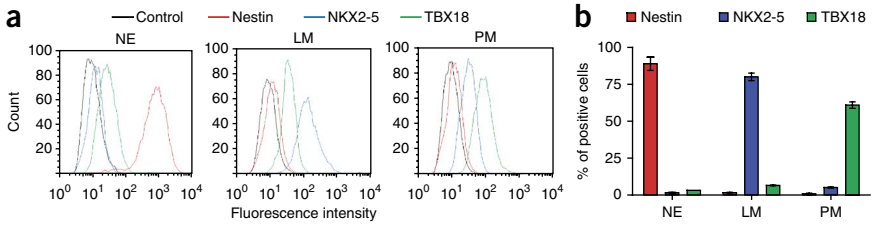

Figure 3 | Flow cytometry analysis of lineage-specific markers on the precursor populations. (**a**) Representative plots of flow cytometry for nestin, NKX2-5 and TBX18 markers on NE, LM and PM populations. (**b**) The percentage of positively stained cells in NE, LM and PM populations. Values represent mean ± s.e.m. (n = 3).

Both NE and PM protocols may benefit from a further addition of noggin or bone morphogenetic protein (BMP) inhibitor. As the LY294002 compound is effective in suppressing the expression of Nanog during mesoderm differentiation, users could consider testing LY294002 in hPSC lines that have difficulties in NE differentiation.

Although we normally maintain our hESCs in chemically defined medium (CDM), as specified in the Materials section, this protocol has also proven to work for human ESCs that have been routinely maintained and passaged using other serum-free commercially available media. These include NutriStem XF/FF culture medium (Stemgent), mTeSR1 (Stemcell Technologies) and Essential 8 medium (Life Technologies). The cells should be switched to CDM with polyvinyl alcohol (CDM-PVA) for differentiation, although the original matrix coating (in the case of vitronectin or Matrigel) can be maintained. Most human iPSCs and some human ESCs, in contrast, seem to be best maintained on feeder cells in KnockOut serum replacement–based media. We recommend the prior adaptation of these iPSCs to feeder-free conditions in one of the aforementioned serum-free media and gelatin-coated dishes for one passage before proceeding with differentiation. However, in the case of lines that adapt poorly to feeder-free conditions, hPSC clumps may be plated directly onto gelatin-coated plates after feeder depletion. CDM-PVA should be used as the basal medium for differentiation in all cases to keep culturing conditions as chemically defined as possible.

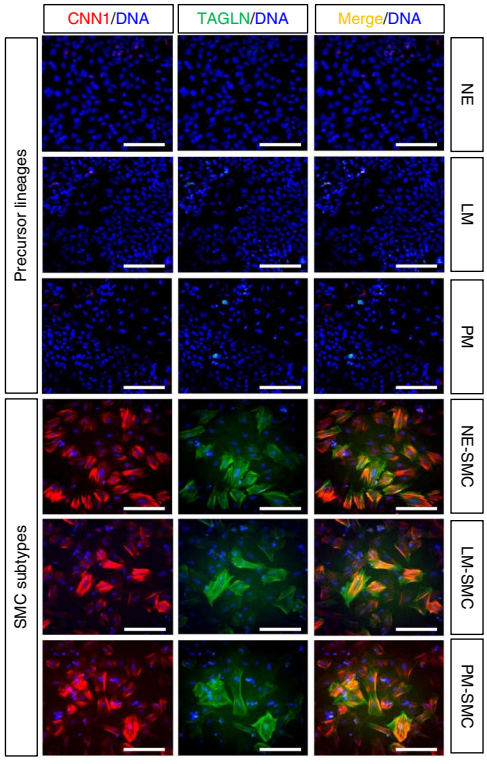

Figure 4 | Immunocytochemistry of SMC markers on precursor lineages and SMC subtypes. Only the hPSC-derived SMC subtypes are stained positively for the contractile proteins, CNN1 (red) and TAGLN (green), but not their respective precursor lineages. DNA is detected by DAPI staining (blue). Scale bars, 100 μm.

Preparation of hPSCs for differentiation. Healthy hPSC colonies should exhibit clear boundaries (**Fig. 2**). Before differentiation, hPSCs are first allowed to grow to confluence. HPSC colonies should then be broken up into relatively small clumps (**Fig. 2b**). We find that clump sizes are crucial to determining the efficiency of early mesoderm formation. Clumps that are too small may affect the viability of hPSC colonies after splitting. In contrast, big clumps may result in inefficient differentiation. Once the hPSC colonies have adhered to the culture plate after 24 h (**Fig. 2c**), differentiation can proceed.

Specification of SMC precursor populations. During this phase of differentiation, hPSCs are induced to form three different multipotent populations (NE, LM and PM) that have the ability to give rise to SMCs (**Fig. 1**). A chemically defined basal medium is used throughout the entire process. However, different combinations of growth factors and small molecules are added to the medium to induce NE, LM and PM separately. This is the definitive step that specifies the origins of SMC subtypes. Subsequent conversion of each of the three multipotent intermediate populations into SMC subtypes involves the use of a single SMC induction protocol for all lineages.

Over the period of NE induction, colonies grow bigger in size, giving rise to neuroepithelial morphology by day 7 (**Fig. 2d**). Approximately 90% of the cells should be double-positive for the NE markers PAX6 and nestin[22]. Proper formation of early mesoderm can be identified by the appearance of dispersed colonies after 1.5 d (**Fig. 2e**), as, characteristically, hPSCs undergo epithelial-to-mesenchymal transition when they are exposed to mesoderm differentiation conditions. At this point, the gene expression levels of the early mesoderm marker brachyury (encoded by *T*) peaks[22]. By mimicking embryonic ligand gradients[23], manipulated BMP4 concentration in CDM to obtain LM (**Fig. 2f**) and PM (**Fig. 2g**) distinctively by day 5. Emergence of KDR (80–90% of the cells) and TCF15 (60–75% of the cells) expression can be monitored to check for the efficiency of LM and PM specification, respectively[22]. Flow cytometric analysis of lineage-related markers demonstrated the specificity of differentiation conditions in deriving the distinctive precursor populations (**Fig. 3**).

Differentiation of precursor populations into SMC subtypes. In the next phase of differentiation, the intermediate populations are dissociated into single cells and then plated down in medium

supplemented with platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)-β1, inducers that are commonly used for SMC differentiation[24]. The dissociation step and optimal cell seeding density are important to promote a mesenchymal cell fate. We found that if cells were plated too dense, this encouraged massive proliferation, which hindered differentiation. By contrast, when cells were plated too sparsely, there was abundant cell death during PDGF-BB and TGF-β1 treatment. We also noticed that dissociated PM cells displayed less substrate adherence and proliferative capacity compared with NE and LM. This may be due to the use of LY294002, an inhibitor of phosphatidylinositol 3 (PI3)-kinase, during the induction of PM for the first 5 d. PI3-kinase belongs to a family of enzymes that regulate cell growth and proliferation. To maintain consistent cell density for all the three intermediate populations, NE and LM are seeded at a lower density of 20,000 cells per cm^2, whereas PM cells were seeded at a density of 30,000 cells per cm^2. Owing to the switch of culture conditions, it is expected that between 50 and 70% of the dissociated cells will eventually be attached to the plate 1 d after splitting (**Fig. 2h–j**).

PDGF-BB and TGF-β1 treatment takes place for at least 12 d to achieve maximal expression of SMC contractile genes[22]. The resulting SMC subtypes look spindle shaped or stellate (**Fig. 2k–m**), and they are highly enriched for calponin (CNN1) and SM22α (TAGLN) expression (**Fig. 4**). By flow cytometric analysis, more than 80% of the SMCs should be double-positive for CNN1 and smooth muscle myosin heavy chain 11 (MYH11)[22]. Characterization of SMC contractility could be performed by exposing the cells to depolarization agents and vasoactive agonists, as previously described[25,26]. The smooth muscle subtypes may be indistinguishable by morphology and by contractile gene and protein expression. However, functional attributes such as varied growth response toward angiotensin II and TGF-β1, as well as differential migratory capability, enable the SMC subtypes to be distinguished from one another[22]. In addition, this *in vitro* system of SMC subtypes was able to predict the different proteolytic abilities of SMCs that were isolated from various rat aortic regions of corresponding embryonic origins[22].

MATERIALS

REAGENTS

- Collagenase IV (Life Technologies, cat. no. 17104019)
- Advanced DMEM F12 (Life Technologies, cat. no. 12634028)
- KnockOut serum replacement (KOSR; Life Technologies, cat. no. 10828028)
- L-Glutamine (Life Technologies, cat. no. 25030024)
- β-Mercaptoethanol (Sigma, M6250) ! **CAUTION** β-Mercaptoethanol is toxic if it is ingested and inhaled. It can be harmful on extensive or prolonged contact with skin. Overexposure can result in death. Wear protective clothing, avoid contact and use it within a fume hood or in a well-ventilated area.
- Penicillin-streptomycin (Life Technologies, cat. no. 15140122)
- FBS (Biosera, cat. no. S1818) ▲ **CRITICAL** There can be batch-to-batch variability in serum. Identify the batch of serum that works best and consider stocking up with this lot number in bulk. Usually one in three batches of FBS works well, and this can be tested by plating cells in wells coated with different batches of FBS side by side for at least two passages to assess their sustainability of pluripotency.
- Gelatin (Sigma, cat. no. G1890)
- Water for embryo transfer (Sigma, cat. no. W1503)
- Ham's F-12 nutrient mix (Life Technologies, cat. no. 11765054)
- IMDM (Life Technologies, cat. no. 12440053)
- Advanced DMEM F12 (Life Technologies, cat. no. 12634028)
- Chemically defined lipid concentrate (Life Technologies, cat. no. 11905031)
- Monothioglycerol (Sigma, cat. no. M6145)
- Transferrin (30 mg ml^{-1}, Roche, cat. no. 652202)
- Insulin, 10 mg ml^{-1} (Roche, cat. no. 1376497)
- BSA (Europa Bioproducts) ▲ **CRITICAL** There can be batch-to-batch variability in serum. Identify the batch of serum that works best and consider stocking up with this lot number in bulk. Usually one in five or six batches of BSA works well, and this can be tested by plating cells in medium prepared with the different batches of BSA side by side for at least two passages to assess their sustainability of pluripotency.
- Poly(vinyl alcohol) (PVA; Sigma, cat. no. P8136)
- DMSO (Sigma, cat. no. D2650)
- Recombinant human activin A (R&D Systems, cat. no. 338-AC)
- Recombinant human BMP4 (R&D Systems, cat. no. 314-BP)
- Recombinant human FGF basic, 146 aa (R&D Systems, cat. no. 233-FB)
- LY294002 (Sigma, cat. no. L9908)
- SB431542 (Sigma, cat. no. S4317)
- Recombinant human PDGF-BB (PeproTech, cat. no. 100-14B)
- Recombinant human TGF-β1 (PeproTech, cat. no. 100-21C)
- Hydrochloric acid (HCl; Sigma) ! **CAUTION** HCl is hazardous, and it can cause severe skin burns and eye damage upon contact. Wear protective clothing and avoid ingestion and inhalation. Use it within a fume hood or in a well-ventilated area.
- TrypLE Express (Life Technologies, cat. no. 12604-013)
- H9 and H1 hPSC cell lines (WiCell) ▲ **CRITICAL** Alternative sources of hPSCs can be used, as discussed in the INTRODUCTION.
- Dulbecco's PBS (DPBS; Life Technologies, cat. no. 14190)
- CO_2 incubator (Sanyo, cat. no. MCO-18AC)
- Centrifuge (Eppendorf, cat. no. 5804)
- Counting chamber (Superior Marienfeld, cat. no. 0640410)
- Disposable serological pipettes, 5 and 10 ml (Corning, cat. nos. 4487 and 4488)
- Graduated filter tips, 1,000 μl, 200 μl, 20 μl, 10 μl (Starlab, cat. nos. S1122-1830, S1120-8810, S1120-1810, S1120-3810)
- Disposable borosilicate glass Pasteur pipettes (Fisher Scientific, cat. no. FB50253)
- Centrifuge tubes, 15 ml (Corning, cat. no. 430791)
- Syringe filter units (Millipore, cat. no. SLGP033RS)
- Vacuum filter, 500 ml (Corning, cat. no. 431097)

Antibodies

- KDR (R&D Systems, cat. no. FAB357C) ▲ **CRITICAL** We have also used PerCP/Cy5.5 anti-human CD309 (VEGFR2, Flk-1) (BioLegend, cat. no. 338915). The recommended concentration for both is 10–100 μl of 10^6 cells.
- PAX6 (Covance, cat. no. PRB-278P). Recommended dilution is 1:200
- Nestin (BD Biosciences, cat. no. 611659). Recommended dilution is 1:500
- TCF15 (Santa Cruz Biotechnology, cat. no. sc-98796). Recommended dilution is 1:400
- CNN1 (Sigma, cat. no. C2687). Recommended dilution is 1:20,000
- TAGLN (Abcam, cat. no. ab14106). Recommended dilution is 1:500
- NKX2-5 (Santa Cruz Biotechnology, cat. no. sc-14033). Recommended dilution is 1:200
- TBX18 (Santa Cruz Biotechnology, cat. no. sc-17869). Recommended dilution is 1:200

EQUIPMENT

- Phase-contrast inverted microscope (Nikon, Eclipse TS100)
- Fluorescence microscope (Zeiss, Axiovert 200M)
- Flow cytometer (Beckman Coulter, CyAn ADP Analyzer)
- Plates, 6 and 12 wells (Corning, cat. nos. 3516 and 3513)

REAGENT SETUP

Human pluripotent stem cells Before commencing the procedure, maintain hPSCs in CDM-BSA supplemented with FGF2 (12 ng ml^{-1}) and

activin A (10 ng ml^{-1}). The maintenance of hPSCs under these conditions is described in more detail in ref. 27.

Chemically defined medium, CDM (500 ml) Basal CDM consists of 250 ml of IMDM, 250 ml of F12 nutrient mix, 5 ml of chemically defined lipid concentrate, 250 µl of transferrin, 350 µl of insulin and 20 µl of monothioglycerol. An additional 5 ml of penicillin-streptomycin is optional. We recommend adding insulin after filtration because of the risk of adsorption to some filters, which can lead to an inappropriate concentration of insulin in the medium and variable results. For hPSC maintenance, make up CDM-BSA by dissolving 2.5 g of BSA in CDM. For differentiation, make up CDM-PVA by dissolving 0.5 g of PVA in CDM. Sterilize the medium with a 500-ml vacuum filter unit. Store the medium at 4 °C for up to 4 weeks.
▲ CRITICAL Add PVA into a small volume of CDM and bring it to boil in order to dissolve the PVA.

Gelatin, 500 ml Add 0.5 g of gelatin into 500 ml of water for embryo transfer. Heat the mixture at 56 °C for about 30 min until gelatin is dissolved. Sterilize it with a 500-ml vacuum filter unit. Store the gelatin at room temperature (typically 19– 24 °C in our laboratory) for up to 2 months.

Collagenase, 500 ml Combine 400 ml of advanced DMEM F12, 100 ml of KOSR, 5 ml of L-glutamine and 3.5 µl of β-mercaptoethanol. Add 500 mg of collagenase IV to the medium. Pipette up and down (gently, to avoid frothing) to dissolve collagenase IV completely. Sterilize collagenase by using a 500-ml vacuum filter unit. Store it at 4 °C for up to 4 weeks or divide it into aliquots and store them in a −20 °C freezer for up to 12 months.

MEF medium, 500 ml Combine 450 ml of advanced DMEM F12, 50 ml of FBS, 5 ml of L-glutamine and 3.5 µl of β-mercaptoethanol. An additional 5 ml of penicillin-streptomycin is optional. Sterilize the medium with a 500-ml vacuum filter unit. Store the medium at 4 °C for up to 4 weeks.

Activin A, 10 µg ml^{-1}, (1,000× stock) Dissolve 50 µg of activin A in 5 ml of sterile DPBS containing at least 0.1% (wt/vol) BSA. Divide it into aliquots and store them at −80 °C for up to 3 months. It is stable at 4 °C for 1 month after reconstitution.

BMP4, 10 µg ml^{-1}, (1,000× stock) Dissolve 50 µg of BMP4 in 5 ml of sterile 4 mM HCl containing at least 0.1% (wt/vol) BSA. Divide it into aliquots and store them at −80 °C for up to 3 months. It is stable at 4 °C for 1 month after reconstitution.

FGF basic, 4 µg ml^{-1}, (1,000× stock) Dissolve 25 µg into 6.25 ml of sterile DPBS containing at least 0.1% (wt/vol) BSA. Divide it into aliquots and store them at −80 °C for up to 3 months. It is stable at 4 °C for 1 month after reconstitution.

PDGF-BB, 10 µg ml^{-1}, (1,000× stock) Dissolve 10 µg into 1 ml of sterile 4 mM HCl containing at least 0.1% (wt/vol) BSA. Divide it into aliquots and store them at −80 °C for up to 3 months. It is stable at 4 °C for 1 month after reconstitution.

TGF-β1, 2 µg ml^{-1}, (1,000× stock) Dissolve 10 µg into 5 ml of sterile 4 mM HCl containing at least 0.1% (wt/vol) BSA. Divide it into aliquots and store them at −80 °C for up to 3 months. It is stable at 4 °C for 1 month after reconstitution.

LY294002, 10 mM, (1,000× stock) Dissolve 1 mg into 290.9 µl of DMSO. Divide it into aliquots and store them at −80 °C for up to 12 months.

SB431542, 10 mM, (1,000× stock) Dissolve 5 mg into 1.3 ml of DMSO. Divide it into aliquots and store them at −80 °C for up to 12 months.

EQUIPMENT SETUP

Cell culture incubation Adjust the incubator to 37 °C and connect it to a CO_2 source for a final concentration of 5%. Replenish the water to maintain humidity.

Gelatin-coated plate Coat the culture plates with 0.1% (wt/vol) gelatin solution for at least 15 min at room temperature. Aspirate the gelatin and add a bare minimum amount of MEF medium to cover the well. Then place the culture plate in a CO_2 incubator for at least 24 h before seeding cells.

PROCEDURE

Preparation of hPSCs for differentiation ● TIMING 1 d (day −1)

1| Check that the hPSCs are growing healthily in CDM-BSA supplemented with FGF2 (12 ng ml^{-1}) and activin A (10 ng ml^{-1}) (**Fig. 2a**). Proceed to the next step when the colonies reach ~70% confluency.
? TROUBLESHOOTING

2| Aspirate the CDM and add enough collagenase to cover the bottom of the culture plate. Collagenase should have been warmed in a 37 °C water bath before addition to the hPSCs.

3| Leave the cells in prewarmed collagenase at room temperature for 5–10 min until the edges of colonies begin to show signs of rolling up. Aspirate and discard the collagenase and add fresh CDM to wash away the remnant collagenase. An alternative to this step for more sensitive cell lines is to split without collagenase by incubating the cells with PBS (without calcium or magnesium). The use of PBS also circumvents the need to test every new batch of collagenase.
▲ CRITICAL STEP Treatment of hPSCs with collagenase should not exceed 10 min, so as to avoid colony fragments from lifting off the plate.

4| Aspirate and discard the CDM wash. Replenish the CDM again and scrape the cells from the plate with a 5-ml serological pipette in a left-right movement. Then scrape perpendicularly to break the colonies up into fragments. Repeat this process until all colonies are completely scraped off the plates.

5| Collect the colony fragments in a 15-ml tube and centrifuge the cells for 3 min at 200*g* at room temperature.
▲ CRITICAL STEP If the culture of cells is good but some differentiation is seen at the periphery of the colonies, then a gradient split should be done instead. For that, instead of centrifuging the cells, place them in a 15-ml tube with plenty of wash medium and leave them to sink by gravity toward the bottom of the tube. As soon as the clumps are at the bottom of the tube, carefully aspirate the medium and all the floating cells within it. This will remove single cells that are differentiated and that will not sink to the bottom of the tube.

6| Aspirate and discard the supernatant and then gently triturate the cell pellet in CDM-PVA (warmed at 37 °C) supplemented with FGF2 (12 ng ml^{-1}) and activin A (10 ng ml^{-1}). Slowly pipette the mixture up and down three or four times with a 5-ml serological pipette to further break the colonies up into smaller clumps (**Fig. 2b**).
▲ **CRITICAL STEP** As clump sizes can affect the differentiation efficiency, gentle trituration here is essential to ensure that small clumps of 50–100 cells are plated for differentiation.

7| Plate the cells at a 1:10 ratio onto gelatin-coated plates (preparation of plates is described in Equipment Setup). If necessary, add extra CDM-PVA supplemented with FGF2 (12 ng ml^{-1}) and activin A (10 ng ml^{-1}).

8| Maintain the cells at 37 °C in a 5% CO_2 incubator for 24 h.

Specification of SMC precursor populations
9| Check that the hPSC colonies are adhered to the plate (**Fig. 2c**). If so, proceed with induction of NE (option A) and mesoderm differentiation (option B). If you ultimately wish to have approximately equal-sized populations of NE, LM and PM cells, perform option B (PM) with double the starting amount of cells as option A (NE) or option B (LM).

(A) NE induction ● TIMING 7 d (days 0–7)

(i) *Day 0*. Aspirate and discard the medium and wash the cells with CDM-PVA to remove the remaining FGF2 and activin A.

(ii) Add CDM-PVA supplemented with FGF2 (12 ng ml^{-1}) and SB431542 (10 μM). Continue to incubate the cells, by monitoring the NE differentiation culture daily under a phase-contrast microscope. If dark 'lumps' appear in the middle of colonies owing to proliferating cells piling up, the lumps should be mechanically removed by marking the spot where each lump is with a microscope object marker, and then removing it under the hood with the aspirator.

(iii) *Days 2, 4, 6*. Replace half of the medium every 2 d until day 7. No passaging is required.

(iv) *Day 7*. NE cells with neuroepithelial morphology should be observed by day 7 (**Fig. 2d**). A small proportion of cells can be fixed for immunocytochemistry to confirm positive staining for PAX6 and nestin. The expected outcome is that over 90% of the cells are $PAX6^+/nestin^+$ (ref. 22). Alternatively, flow cytometric analysis of nestin expression can be performed (**Fig. 3** and **Supplementary Methods**).
▲ **CRITICAL STEP** We advise proceeding with SMC differentiation immediately, as maintenance conditions for NE cells have not yet been optimized.
? TROUBLESHOOTING

(B) Mesoderm induction ● TIMING 5 d (days 0–5)

(i) *Day 0*. Aspirate and discard the medium and wash the cells with CDM-PVA to remove the remaining FGF2 and activin A.

(ii) Add CDM-PVA supplemented with FGF2 (20 ng ml^{-1}), LY294002 (10 μM) and BMP4 (10 ng ml^{-1}), and incubate the cells. Dispersed colonies should be observed by day 1.5 (**Fig. 2e**).
? TROUBLESHOOTING

(iii) *Day 1.5*. Aspirate and discard the medium. Wash cells with CDM-PVA. For LM specification, incubate the cells in CDM-PVA supplemented with FGF2 (20 ng ml^{-1}) and BMP4 (50 ng ml^{-1}). For PM specification, replenish with CDM-PVA supplemented with FGF2 (20 ng ml^{-1}) and LY294002 (10 μM).

(iv) *Day 3.5*. Replace half of the medium.

(v) *Day 5*. Analyze a small proportion of the resulting LM (**Fig. 2f**) and PM (**Fig. 2g**) populations by flow cytometry. The expected outcome is 77–87% KDR^+ for LM cells and 60–75% $TCF15^+$ for PM cells[22]. Alternatively, flow cytometric analysis of NKX2-5 and TBX18 can be performed (**Fig. 3** and **Supplementary Methods**).
▲ **CRITICAL STEP** We advise proceeding with SMC differentiation immediately, as maintenance conditions for LM and PM cells have not yet been optimized.
▲ **CRITICAL STEP** Visual morphology cannot be relied on to distinguish between mesoderm subtypes at this stage.
? TROUBLESHOOTING

Differentiation of precursor populations into SMC subtypes ● TIMING 12+ d (days 5–19+)
▲ **CRITICAL** Patterning for the distinct embryonic origins of SMCs occurs during Step 9. Subsequent SMC differentiation from the three precursor populations (Steps 10–18) proceeds under very similar chemically defined culture conditions.
10| Aspirate and discard the supernatant from all the intermediate populations and wash with CDM-PVA.

11| Treat the cells with TrypLE Express for 3–5 min at 37 °C in a 5% CO_2 incubator. Gently tap the plates and check them periodically under a phase-contrast microscope to ensure that all cells have been dissociated into single cells.

12| To each plate, add at least 10 volumes of CDM-PVA to dilute one volume of TrypLE Express used, and then collect the cell suspensions in separate 15-ml tubes. Centrifuge the tubes for 3 min at 200*g* at room temperature.

13| Aspirate and discard the supernatant and gently resuspend the cell pellets in CDM-PVA supplemented with PDGF-BB (10 ng ml^{-1}) and TGF-β1 (2ng ml^{-1}).

14| Plate NE and LM cells onto gelatin-coated plates at a seeding density of 20,000 cells per cm^2. Plate PM cells at a seeding density of 30,000 cells per cm^2. If necessary, add extra CDM-PVA supplemented with PDGF-BB (10 ng ml^{-1}) and TGF-β1 (2 ng ml^{-1}).
▲ **CRITICAL STEP** Different splitting ratios for the intermediate populations are essential owing to their different cell-substrate adhesion and proliferative capabilities during SMC differentiation.

15| Maintain the cells at 37 °C in a 5% CO_2 incubator. After 1 d of PDGF-BB and TGF-β1 treatment, 50–70% of the cells should have attached to the plate (**Fig. 2h–j**). Discard the medium to remove floating cells and replace it with fresh medium.
? TROUBLESHOOTING

16| Maintain the cells in this medium, differentiating for at least another 11 d. Replace half of the medium every 2–3 d. If cells become confluent, passage them at any point by repeating Steps 10–13 and then plating them onto gelatin-coated plated at the ratio of 1:2.
? TROUBLESHOOTING

17| After 12 d of PDGF-BB and TGF-β1 treatment, all the three SMC subtypes should appear spindle or stellate shaped (**Fig. 2k–m**). If desired, a small proportion of cells can be fixed for immunocytochemistry to confirm positive staining for CNN1 and TAGLN (**Fig. 4**).
? TROUBLESHOOTING

18| Differentiated SMCs remain quiescent when continued in culture under PDGF-BB and TGF-β1 treatment for another week. Thereafter, we observe progressive cell death. We thus recommend that differentiated SMCs be adapted to DMEM with 10% (vol/vol) FBS immediately after 12 d of PDGF-BB and TGF-β1 treatment. The SMCs will typically reach 80–90% confluence after 5–10 d of conditioning by DMEM with 10% (vol/vol) FBS. At this point, SMCs may either be cryopreserved or split for expansion. Cryopreservation using 90% (vol/vol) FBS + 10% (vol/vol) DMSO will suffice. Frozen SMCs can subsequently be revived on gelatin-coated plates with DMEM and 10% (vol/vol) FBS. For expansion, a splitting ratio of 1:3–1:6 is usually used during passaging. SMCs can be expanded about 10 passages, equivalent to 6–7 weeks in culture.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
1	The edges of hPSC colonies do not appear well defined	Use gelatin-MEF medium coated plates within 1 week of preparation. Do not use coated dishes that have dried out
		The FBS used to make the MEF medium should be hPSC compatible and the amount of MEF medium used to coat the plates should be just enough to cover the dish
		During passaging for hPSC maintenance, if some differentiation is seen at the periphery of the colonies, then a gradient split should be done. Instead of centrifuging the cells, place them on a 15-ml tube with plenty of wash medium and leave them to sink by gravity toward the bottom of the tube. As soon as the clumps are at the bottom of the tube, carefully aspirate the medium and all the floating cells within it. This will remove single cells that are differentiated and these will not sink to the bottom of the tube. A suboptimal hPSC culture with too many differentiated cells will negatively influence the subsequent SMC differentiation experiment

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Solution
9A(iv)	Low percentage of PAX6$^+$/nestin$^+$ NE cells	Remove the dark lumps observed during neuroectoderm differentiation Check the dilution of antibodies Use FGF2 that is within five freeze-thaw cycles Check the levels of BMP expression in the hPSCs being used if you are using a line other than the ones tested for this protocol. If the levels of BMP in a different line are high, then add noggin or a BMP inhibitor to block endogenous BMP activity to counteract mesoderm differentiation
9B(ii)	Colonies do not appear dispersed	Gently triturate the hPSCs a bit more to break the colony fragments into smaller clumps before plating down for differentiation Little or no epithelial-to-mesenchymal transition at this step suggests the protocol was not efficient. Check the activity of LY294002 FGF and BMP If the cell line being used expresses very high levels of activin, you might need to block activin activity at this early step
9B(v)	Low percentage of KDR$^+$ LM cells and TCF15$^+$ PM cells	Check that the early mesoderm population appears as dispersed colonies before proceeding with LM and PM differentiation Check the levels of endogenous Wnt expression in the hPSCs being used if you are using a line other than the ones tested for this protocol. If the levels of Wnt in a different line are very low, then supplement Wnt exogenously to promote mesoderm induction Check the dilution of antibodies Use FGF2 and BMP4 that are within five freeze-thaw cycles Low levels of KDR: check BMP and FGF activity Low levels of TCF15: check the activity of LY294002 and FGF
15	Less than 50% of the cells from precursor populations attach to the plate	Use gelatin-coated plates within 1 week of preparation. Do not use coated dishes that have dried The FBS used to make the MEF medium should be hPSC compatible and the amount of MEF medium used to coat the plates should be just enough to cover the dish Ensure that the precursor populations are properly differentiated
16	Cells do not proliferate and gradually die off during PDGF-BB and TGF-β1 treatment	Increase seeding density in Step 14 and ensure that there is at least 50% cell attachment in Step 15 Ensure that the precursor populations are properly differentiated Use PDGF-BB and TGF-β1 that are within five freeze-thaw cycles
17	Cells do not appear spindle or stellate shaped after 12 d of PDGF-BB and TGF-β1 treatment	It is possible that some cells might take longer to differentiate into SMCs. Check CNN1 and TAGLN expression periodically. Lengthen the period of PDGF-BB and TGF-β1 treatment if necessary to achieve maximal SMC induction
18	SMCs show signs of senescence before passage 10	Split SMCs at a ratio of not more than 1:3 as cell-cell contacts seems to be important for stimulating proliferation

● TIMING

Steps 1–8, preparation of hPSCs for differentiation: 1 d
Step 9A, specification of SMC precursor populations—NE induction: 7 d
Step 9B, specification of SMC precursor populations—mesoderm induction: 5 d
Steps 10–18, differentiation of precursor populations into SMC subtypes: 12+ d

ANTICIPATED RESULTS

Knowledge from developmental studies has enabled us to devise this stepwise protocol using unique combinations of morphogens to mimic stages of normal development. First, three precursor lineages are derived that are known to give rise to vascular SMCs. Successful induction of these precursor populations is instrumental in their efficient differentiation into diverse subtypes of SMCs subsequently. Quality control by lineage-specific markers should be performed at the intermediate stages. Approximately 90% of NE cells coexpress PAX6 and nestin, whereas 77–87% of LM cells and 60–75% of PM cells stain

positively for KDR and TCF15, respectively[22]. Common SMC induction conditions are then used to differentiate the three intermediate lineages into SMCs. The majority of the resulting SMC subtypes coexpress CNN1 and TAGLN (**Fig. 4**), and over 80% become MYH11-expressing cells[22]. Given a multitude of microenvironment signals that guide differentiation of SMCs in embryos, some aspects of SMC development may be difficult to realize *in vitro*. Nonetheless, by using this approach, hPSC-derived SMCs that are obtained express SMC genes and proteins at comparable levels to primary cultures of human adult aortic SMCs, and they display equivalent contractile ability in terms of calcium signaling[22]. Over 50% of the SMC subtypes should display contraction upon treatment with vasoactive compounds such as carbachol[22]. The approximate yields of SMCs compared with the starting amount of hPSCs are summarized in **Supplementary Table 1**. Further scaling up of SMC cultures proceeds with ease, once the freshly differentiated SMC subtypes are adapted to serum-containing medium. Although the cells retain their SMC phenotype during this expansion, it is up to the end-user to confirm that the lineage-specific properties under study are retained. It should be noted that, although the cells that do not express SMC markers have not yet been fully characterized, we have transplanted the differentiated cell populations into immunodeficient NOD-SCID gamma mice and not seen any evidence of tumor formation over a 3-week follow-up period, thus excluding the presence of multipotent stem cells.

Advances in patient-derived iPSCs offer an avenue to capture human genetic diversity and complex genotypes of human diseases. For example, disease modeling of Hutchinson-Gilford progeria syndrome has been achieved with iPSC-derived SMCs[28]. With the availability of SMC subtypes using the approach described here, interrogation of lineage-dependent mechanisms may help unravel the molecular-genetic link between embryonic origins and disease susceptibility. For example, our recent study revealed that the homeobox positional identity in adult mice aortas is already established in hPSC-derived SMC subtypes, suggesting that embryonic origin-dependent differences may be responsible for regulating proinflammatory NF-κB activity[29]. Together, there could be a better understanding of how VSMC origins affect a variety of vascular disease patterns, and hence inform potential therapeutic strategies.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS C.C. and S.S. designed the protocol. C.C. performed the differentiation experiments, analyzed the data and wrote and prepared the manuscript. A.S.B. and R.A.P. established the mesoderm induction conditions and coedited the manuscript. S.S. supervised the project and edited the manuscript.

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