Generation of human vascular smooth muscle subtypes provides insight into embryological origin–dependent disease susceptibility

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Heterogeneity of embryological origins is a hallmark of vascular smooth muscle cells (SMCs) and may influence the development of vascular disease. Differentiation of human pluripotent stem cells (hPSCs) into developmental origin–specific SMC subtypes remains elusive. Here we describe a chemically defined protocol in which hPSCs were initially induced to form neuroectoderm, lateral plate mesoderm or paraxial mesoderm. These intermediate populations were further differentiated toward SMCs (>80% MYH11+ and ACTA2*), which displayed contractile ability in response to vasoconstrictors and invested perivascular regions in vivo. Derived SMC subtypes recapitulated the unique proliferative and secretory responses to cytokines previously documented in studies using aortic SMCs of distinct origins. Notably, this system predicted increased extracellular matrix degradation by SMCs derived from lateral plate mesoderm, which was confirmed using rat aortic SMCs from corresponding origins. This differentiation approach will have broad applications in modeling origin-dependent disease susceptibility and in developing bioengineered vascular grafts for regenerative medicine.

The walls of larger vessels and capillary beds are composed of vascular SMCs and pericytes, respectively1. Lineage tracking studies have shown that vascular SMCs in different vessels have distinct embryological origins2. For example, the basal aortic root is derived from the secondary heart field3, whereas the ascending aorta and the arch are neural crest derived4. Descending aortic SMCs originate from paraxial (somitic) mesoderm5. Coronary SMCs arise from the pro-epicardial organ, which is of lateral plate mesodermal origin6. Although information on the embryological origins of pericytes is relatively lacking, there is considerable phenotypic overlap between these cells and SMCs. Consistent with the phenotypic similarities, it appears that SMCs and pericytes in the same vascular region share common origins7–10.

The diversity of SMC origins may in part contribute to the site-specific localization of vascular diseases, including regional susceptibility to atherosclerosis11,12, vascular calcification13 and aortic aneurysm distribution14. Notably, aortic dissections occur preferentially at the interfaces where SMCs of distinct embryological origins meet. Together, these findings suggest that intrinsic lineage-derived differences between SMCs may play a role in the spatiotemporal pattern of vascular diseases. Therefore, a model system to investigate how SMC embryological origins influence development of vascular diseases would be valuable.

Currently, SMC derivation from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (iPSCs), collectively known as hPSCs, usually requires serum and does not encompass the concept of using a lineage-specific approach15. We report a chemically defined method for generating origin-specific SMCs from hPSCs through three intermediate lineages—neuroectoderm, lateral plate mesoderm and paraxial mesoderm. We used our in vitro system to predict the responses of different SMC subtypes to interleukin-1β (IL-1β), an inflammatory mediator, and validated the results using rat aortic SMCs of distinct origins. Our finding reveals that origin-specific SMCs exhibit differential activation of matrix metalloproteinase 9 (MMP9) and tissue inhibitor of metalloproteinase 1 (TIMP1). Hence, our SMC subtypes could potentially be used to study origin-dependent disease susceptibility and also serve as a source of therapeutic cells for vascular regenerative medicine.

RESULTS

Mesoderm subtypes specified by modifying BMP and PI3K

The H9 hESC line, which is chromosomally normal (Supplementary Fig. 1a), was used to study the specification of the various lineages from which SMCs arise. For neuroectoderm differentiation, we adopted our previously published protocol16, using fibroblast growth factor 2 (FGF2, 12 ng/ml) and the activin/nodal inhibitor SB431542 (10 µM). During the induction of neuroectoderm in hESCs, we observed a marked decrease in expression of the pluripotency marker NANOG within 2 d (Supplementary Fig. 1b). The expression of neuroectoderm markers (GBX2, OLIG3 and SOX1) increased over time and peaked between day 6 and day 8, as documented before16. Extensive immunostaining for neuroectoderm markers (PAX6 and NESTIN) was observed in both hESCs and human iPSCs after 7 d of FGF2 and SB431542 treatment. High efficiency of neuroectoderm
induction was observed, with >90% of the cells positive for both PAX6 and NESTIN (Supplementary Fig. 1c). For mesoderm subtype differentiation, we used our previously described 36-h early mesoderm differentiation protocol\textsuperscript{17}. A combination of FGF2 (20 ng/ml), phosphoinositide 3-kinase (PI3K) inhibitor (LY294002, 10 μM) and bone morphogenetic protein 4 (BMP4, 10 ng/ml), herein referred to as FLyB, was used (Fig. 1a, i). Subsequent mesoderm subtype specification was further investigated.

**Figure 1** Induction of mesoderm subtypes from hPSCs. (a) (i) HPSCs were initially differentiated to early mesoderm using FGF2, LY294002 and BMP4 (depicted as FLyB) for 36 h. Mesoderm subtype specification required another 3.5 d of FGF2+LY294002 (FLy) or FGF2+BMP4 (FB50) for paraxial or lateral plate induction. (ii) BMP concentration gradient exists along the primitive streak (PS). On the basis of fate map studies, mesoderm subtypes emerge along the gradient on the embryo cylinder. (b) *KDR* and *MEOX1* expression levels of hESCs differentiated for 36 h in FLyB, followed by 3.5 d in FGF2+LY294002 with Noggin (Nog), BMP4 (B) and/or activin (A) were determined by qRT-PCR. BMP4 concentrations ranged from 0 ng/ml (B0) to 100 ng/ml (B100). Data represent means ± s.e.m. (n = 3). Significant differences compared to day 0 population are indicated in black, whereas that between two groups are indicated in red (*, P < 0.05; **, P < 0.01). (c) Lateral plate and paraxial mesoderm marker expression levels. (d) Temporal qRT-PCR to analyze mesoderm specification. After a common 36-h treatment of FLyB, lateral and paraxial mesoderm were specified using FB50 and FLy, respectively. (e) Percentage of KDR\textsuperscript{+} and TCF15\textsuperscript{+} cells at days 3 and 5 determined by flow cytometry. (f) At day 5, FB50- and FLy-treated populations were immunostained for mesoderm subtype-specific markers. Scale bars, 100 μm.
During embryogenesis, specification of lateral plate mesoderm and paraxial mesoderm follows a posterior-anterior BMP gradient along the primitive streak18 (Fig. 1a, ii). We hypothesized that by varying the BMP4 concentration according to these developmental principles, we would obtain the relevant mesoderm subtypes from hPSCs. Studies have suggested that BMP signaling is a key driver of mesoderm19–23, and BMP4 also cooperates with activin A to induce differentiation of mesendoderm, the precursor of mesoderm and endoderm16. We examined the roles of BMP4 and activin A in mesoderm subtype specification from the early mesoderm stage with constant concentrations of FGF2 (20 ng/ml) and LY294002 (10 µM). In the presence of activin A (10 ng/ml), BMP4 promoted the expression of the lateral plate marker KDR in a dose-dependent way (from 0 ng/ml to 100 ng/ml, that is, B0 to B100, respectively) (Fig. 1b). Endogenous BMP signaling (B0 condition) was insufficient to promote KDR expression since Noggin, a BMP antagonist, produced the same effect as B0. The absence of activin A did not affect the development of lateral plate mesoderm as KDR was expressed as long as BMP4 was added. In contrast, absence of activin A promoted the expression of the paraxial marker MEOX1 in the absence of BMP (Noggin and B0 conditions). Activin A was therefore dispensable for mesoderm formation, whereas a high concentration of BMP4 enhanced lateral plate mesoderm specification but inhibited paraxial mesoderm specification.

We then evaluated the effect of 10 µM LY294002 (Ly) on mesoderm subtype specification. LY294002 has been shown to facilitate differentiation of hESCs25. Notably, we found that, in the presence of 20 ng/ml FGF2 (P) alone, Ly significantly promoted the expression of a panel of paraxial markers, MEOX1 (P = 0.0051), TBX6 (P = 0.0027), TCF15 (P = 0.0001) and PAX1 (P = 0.0087), versus no LY294002 (Fig. 1c). Again, the presence of 50 ng/ml BMP4 (B50) inhibited the expression of all paraxial markers and promoted the expression of lateral plate markers. On the other hand, Ly significantly downregulated the lateral plate markers, LMO2 (P = 0.00024 in P, P = 0.00002 in FB50); PECAM1 (P = 0.0037 in P, P = 0.00167 in FB50); NKX2-5 (P = 0.0170 in F); and ISL1 (P = 0.0132 in F) compared to the no LY294002 condition. Hence, we established the optimal conditions for lateral plate and paraxial mesoderm specification to be FB50 and FLy, respectively.

We next determined the timescale of mesoderm subtype differentiation. NANOG expression decreased markedly within 2 d of differentiation of hESCs (Fig. 1d). Brachyury (T), a primitive streak and early mesoderm marker, emerged transiently and peaked at 36 h after FLYb treatment. Further specification demonstrated that FB50 increased the expression of lateral plate genes (MESP1, KDR, NKX2-5 and ISL1) from as early as day 2 of differentiation, peaking at days 4 to 5. In contrast, FLY (blue lines) upregulated paraxial genes (TBX6, MEOX1, TCF15 and PAX1) only, with peak expression between days 3 and 5. Flow cytometry confirmed that FB50 treatment significantly promoted lateral plate mesoderm development, with 82.4 ± 5.1% KDR-expressing cells at day 3 compared to 2.12 ± 0.21% under the FLY treatment (P = 0.0004) (Supplementary Fig. 1d). Meanwhile, FLY treatment resulted in 67.5 ± 7.3% TCF15-expressing cells at day 5, versus 1.25 ± 0.05% using FB50 (P = 0.0008), indicative of paraxial mesoderm formation primarily. Representative flow cytometric plots are shown in Figure 1e. Day 5 populations generated by either FB50 or FLY treatment also stained positively for their respective mesoderm subtype markers by immunofluorescence (Fig. 1f). We therefore identified day 5 as the time point when the relevant mesoderm subtypes were optimally specified (Fig. 1d–f and Supplementary Fig. 1c).

In summary, we devised a method to derive either paraxial or lateral plate mesoderm subtypes from hESCs.
Figure 2 Efficient differentiation of intermediate lineages into vascular SMCs. (a) A schematic which outlines the conditions for deriving origin-specific SMCs from hPSCs. FlyB+FB and FlyB+Fly, described in Figure 1, generate the lateral plate mesoderm (LM) and paraxial mesoderm (PM), respectively. HPSCs were treated with FGF2+SB431542 (FSb) for 7 d to induce neuroectoderm (NE) differentiation. For further differentiation into vascular SMCs, each intermediate population was subjected to PDGF-BB+TGF-β1 (PT) for 12 additional days. The SMC subtypes, namely the neuroectoderm-derived SMCs, lateral mesoderm-derived SMCs, paraxial mesoderm-derived SMCs are abbreviated as NE-SMC, LM-SMC and PM-SMC, respectively. (b) qRT-PCR demonstrated increasing expression of SMC markers during the 12 d of PT treatment on the three intermediate populations. Data represent means ± s.e.m. (n = 3). (c) Flow cytometric analysis demonstrated that the SMC differentiation protocols were highly efficient and reproducible in three hPSC lines with 83–92% of the resulting populations positive for both MYH11 and CNN1. Value in each quadrant is the percentage of positively expressing cells in the entire population (top left: CNN1+; top right: MYH11+/CNN1+; bottom right: MYH11+). (d) Microarray gene expression heat map of control hESCs versus SMC subtypes obtained after 12 d of PT treatment. Red (upregulation) and blue (downregulation) depict differential gene expression from the mean across all samples. (e) The majority of our SMC subtypes immunostained positively for CNN1 and TAGLN. HUVEC was used as a negative control, whereas human aortic SMCs were used as a positive control. Scale bars, 100 µm. (f) Western blot analysis confirmed the presence of the mature SMC proteins, MYH11 and SMTN, in the hPSC-derived SMCs and human aortic SMCs but not HUVECs.
Immunocytochemistry showed extensive staining for SMC markers in the derived SMCs and in the positive control, human aortic SMCs, but not in the negative control, human umbilical vein endothelial cells (HUVECs) (Fig. 2c). Western blot also demonstrated the presence of mature SMC proteins, MYH11 and SMTN, in our derived SMCs and human aortic SMCs, which were absent in HUVECs (Fig. 2f). Overall, these results suggest that by mimicking embryonic development, we developed a robust process to directly differentiate hPSCs into origin-specific SMCs using a series of growth factor manipulations in a chemically defined medium.

**Origin-specific vascular SMCs are functional**

Despite the divergent initial differentiation routes, microarray analysis revealed that the three types of derived SMCs shared many genes (3,604) differentially upregulated compared to hESCs (false-discovery rate, 0.1%) (Fig. 3a). Among the highly enriched functional classifications of the 3,604 genes, there were the classical SMC functionality categories, such as blood vessel morphogenesis, extracellular matrix (ECM) interaction and actin cytoskeleton organization (Fig. 3a; gene lists comprising the highlighted groups in yellow found in Supplementary Table 1). Alternatively, the significantly enriched functional classifications of the nonoverlapping gene subsets revealed inherent differences among SMC subtypes (Supplementary Fig. 3a). For example in neuroectoderm (NE)-SMC, there were categories related to myelination and synaptic transmission, both of which are neuronal characteristics, supporting the common neuroectoderm origin of NE-SMC and neurons. In particular, lateral plate mesoderm (LM)-SMC was highly enriched in genes promoting cell migration and consistent with this enrichment, these cells also displayed the greatest migration in a scratch assay (Supplementary Fig. 3b).

To assess SMC contractile potential, we initially performed immunostaining for vinculin and phalloidin staining for actin filaments. Well-developed focal adhesion complexes were detected in all three SMC subtypes (Supplementary Fig. 3c). The cells were then preloaded with a calcium-sensitive dye, Fluo-4. Carbachol (100 µM) stimulated an increase in Fluo-4 fluorescence intensity in the derived SMCs within 1 min of treatment (Fig. 3b, i), indicating increased intracellular calcium flux. After 2 min of treatment, Fluo-4 intensity decreased, approaching original basal levels by 5 min. The same trend was observed in the positive control, human aortic SMCs but not in the negative control HeLa cells. Freshly dissociated rat aortic SMCs served as the optimal physiological control and demonstrated prolonged increase in intracellular calcium over the first 3 min. The derived SMCs exhibited similar peak fluorescence responses to both the cultured and freshly dissociated SMC controls (Fig. 3b, ii). Time-lapse microscopy showed that our SMCs and human aortic SMCs contracted in a tonic fashion during the 10 min of carbachol treatment (Fig. 3c and Supplementary Videos 1–5), consistent with the sustained contraction usually manifested by vascular SMCs in controlling vessel tone. Contracting cells exhibited a 10–20% change of cell surface area (n = 20). Over 50% and 20% of all derived SMCs contracted upon carbachol and angiotensin II treatments, respectively (Supplementary Fig. 4a, b). Paraxial mesoderm (PM)-SMC demonstrated a significantly higher percentage of contractile cells than other SMC subtypes (P = 0.036 versus NE-SMC in carbachol; P = 0.027 versus NE-SMC and P = 0.013 versus LM-SMC in angiotensin II).

To determine whether the hPSC-derived SMCs could contribute to vessel formation in vivo, we implanted Matrigel plugs with our SMCs and HUVECs (ratio 1:2) subcutaneously into immunodeficient mice for 2 weeks. Sections were immunostained using human-specific SMTN and PECAM1 antibodies. Luminal structures composed of
HUVECs (PECAM1*) were observed (Fig. 3d). HPSC-derived SMCs (SMTN*) were recruited to peri-endothelial regions, reminiscent of their biological niche. There was 30–45% SMC coverage around the endothelial vessel structures with LM-SMCs displaying lower coverage levels (31.5 ± 5.1%) than the other two groups (43.1 ± 2.6% NE-SMC and 48.9 ± 3.1% PM-SMC). These results confirmed that the in vitro-derived SMCs were functional.

Origin-specific MKL2 requirement and responses to cytokines

To determine whether our system reliably generated origin-specific SMCs from hPSCs, we validated our model against reported findings from developmental studies. MKL2 is a transcriptional co-activator from developmental studies. MKL2 is a transcriptional co-activator that plays a role in early embryogenesis. Previous in vivo studies revealed the unique requirement for MKL2 during SMC differentiation from neural crest but not mesoderm. We postulated that MKL2 was essential only for NE-SMC specification and not for LM- or PM-SMC specification. To test this hypothesis, we silenced MKL2 using short interfering RNA (siRNA) in our intermediate populations (Fig. 4a,b). We then induced SMC differentiation and found that the MKL2 siRNA-treated cells showed a significant decrease in SMC gene expression in NE-SMCs (CNN1, P = 0.0006; MYH11, P = 0.015; SMTN, P = 0.0023) compared to scrambled siRNA control, whereas LM-SMCs and PM-SMCs were unaffected (Fig. 4c). Likewise, MKL2 knockdown resulted in a significant decrease in the percentage of MYH11*ACTA2* NE-SMCs (55.0 ± 4.8% MKL2 siRNA versus 86.7 ± 2.1% scrambled control, P = 0.0076, Fig. 4d), but no reduction in SMCs derived from mesoderm origins.

To confirm that we had generated origin-specific SMC subtypes, we used cytokines known to induce unique responses in aortic SMCs of distinct origins. Angiotensin II (Ang II) promotes medial hyperplasia in the ascending aorta (neuroectoderm derivative) but not the descending aorta (mesoderm derivative). TGF-β1 also encourages greater cell proliferation in SMCs of neuroectoderm origin compared to those of mesodermal origin. Consistent with the previous studies, NE-SMC proliferated in response to Ang II (1 μM, red lines) and TGF-β1 (5 ng/ml, green lines) over 3 d, but LM-SMC and PM-SMC did not (Fig. 4e). Serum (10%, blue lines), a potent mitogen, induced proliferation in all the SMC subtypes although to a much lesser extent in PM-SMC. Cell cycle analysis confirmed that Ang II, TGF-β1 or serum each increased the proportion of NE-SMCs in S and G2-M phases compared to vehicle control after 24 h (Fig. 4f). LM-SMC and PM-SMC showed higher S and G2-M populations only upon serum treatment.

Figure 4 MKL2 knockdown and cytokine treatments validate the origin-specific characteristics of hPSC-derived SMC subtypes. (a) 60–65% knockdown of the MKL2 expression levels in the intermediate populations (NE, LM and PM) by MKL2 siRNA was verified by qRT-PCR. (b) Western blot analysis confirmed the effects of MKL2 siRNA knockdown on the protein levels compared to scrambled siRNA controls. (c) SMC gene expression levels after SMC differentiation of the siRNA-treated intermediate populations were determined by qRT-PCR. (d) Percentage of MYH11*ACTA2* SMCs obtained from the siRNA-treated intermediate populations was determined by flow cytometry. (e) Proliferation responses of the SMC subtypes were monitored by MTT assay every 24 h over 3 d of treatment with the cytokines indicated. (f) Cell cycle analysis of the SMC subtypes after 24 h of cytokine treatments. The percentage of cells in different phases of the cell cycle was quantified by the areas under the peaks (graph, far right). Black dashed lines divide the growth arrested cells in G0-G1 from the proliferating cells in S and G2-M of the control groups. Pi, propidium iodide. (g) Gene expression levels in control and TGF-β1–treated SMCs were determined by qRT-PCR after 10 h of treatment. (h) Western blot analysis was done to confirm the distinct secretory responses exhibited by TGF-β1–treated SMC subtypes. Data represent means ± s.e.m. (n = 3). Significant differences compared to the scrambled siRNA or vehicle controls are indicated (*, P < 0.05; **, P < 0.01, ***, P < 0.001).
Only NE-SMCs proliferated in response to TGF-β1 (Fig. 4e,f). TGF-β1 may have enhanced cell proliferation by autocrine production of TGF-β2 and PDGFA. We investigated whether TGF-β1 stimulated greater auto-induction of TGF-β2 and PDGFA in SMCs of a neuroectoderm origin. TGFβ2 (P = 0.002) and PDGFA (P = 0.0015) expression was significantly upregulated only in NE-SMCs after 10 h of TGF-β1 (5 ng/ml) treatment compared with vehicle control (Fig. 4g). Correspondingly, elevated TGFβ2 and PDGFA protein levels were found in NE-SMC lysates after TGF-β1 treatment (Fig. 4h). Taken together, the data on requirement for ML2 and their unique proliferative and secretory responses validate the hypothesis that our different SMC subtypes are analogous to the distinct lineage-dependent SMC populations documented in vivo.

**SMC subtypes predict origin-specific MMP and TIMP activity**

To investigate a link between SMC developmental origins and susceptibility to vascular diseases, we treated our SMC subtypes with IL-1β (10 ng/ml), an atherogenic cytokine. Rat SMCs isolated from various aortic regions (Supplementary Fig. 5) were tested in parallel. A panel of MMP and TIMP markers, known to be implicated in human atherosclerosis and aneurysm formation, was investigated. We discovered that, broadly, LM-SMCs, NE-SMCs and PM-SMCs modeled similar responses to IL-1β as the rat aortic SMCs (bottom panel) of corresponding origins—root, arch and thoracic descending (Fig. 5a). In particular, MMP9 and TIMP1 expression levels were differentially activated in our SMC subtypes, as were rat SMCs of distinct origins. The induction of MMP9 relative to control in the rat SMCs was ~100-fold greater than that in the hPSC-derived SMCs. This could be due to species-specific variations or different degrees of SMC maturity, either of which could reflect the differences in level of activation for certain genes. Western blot analysis confirmed that the hPSC-derived SMCs (top panel) could predict the differential levels of MMP9 and TIMP1 proteins in the rat aortic SMCs after exposure to the same stimulus (Fig. 5b). The human SMC subtypes also predicted the differential elastin (left panel) and collagen (right panel) degradation demonstrated by the rat aortic SMCs (Fig. 5c). In summary, these results suggest that adult SMCs display heterogenous matrix remodeling responses due, in part, to their different origins. Notably, our in vitro-derived SMC subtypes are able to effectively model and predict the properties of their in vivo counterparts and consequently may have relevance in predicting origin-dependent disease responses.

**DISCUSSION**

We have developed a chemically defined monolayer system to generate origin-specific vascular SMCs from hPSCs with high efficiency. The initial induction to the intermediate lineages—neuroectoderm, lateral plate mesoderm and paraxial mesoderm—is instrumental in defining our subsequent SMC subtypes. Using developmental insights into the BMP gradient along the primitive streak, we have established conditions for mesoderm specification. Directed differentiation toward the paraxial mesoderm has not been previously reported. Moreover, our unpatterned lateral plate mesoderm is nonspecified tissue, in contrast to the specific lateral plate mesoderm derivatives, such as blood or cardiac lineages, generated by other published protocols that also use BMP4. The hPSC-derived SMCs possess contractile function and participate in blood vessel formation in vivo. Although the SMC subtypes appear phenotypically and functionally indistinguishable, we provide evidence that they represent truly origin-specific subtypes. More specifically, we show that they recapitulate differential requirements for SMC fate commitment and unique biological responses to cytokines consistent with previous in vivo and ex vivo studies. Our in vitro–derived SMC subtypes could be used to predict differential ECM remodeling responses of regionally distinct rat aortic SMC populations to IL-1β. Currently, there are no published data that functionally distinguish between SMCs from the lateral plate mesoderm and paraxial mesoderm. Nevertheless, we find that our PM-SMCs are less prone to serum-stimulated proliferation and ECM degradation compared to LM-SMCs and NE-SMCs. Moreover, a greater proportion of PM-SMCs become contracting...
cells in response to vasoconstrictors, suggesting that PM-SMCs may be relatively resistant to phenotypic modulation and retain a more differentiated phenotype than other SMC subtypes. Gene subsets uniquely upregulated in each of the SMC subtypes (Supplementary Fig. 3a) reveal previously unknown differences, which may guide further studies on the functional relevance of the PM-SMC population to vascular pathology.

The diverse embryological origins of vascular SMCs are well recognized. However, it remains unclear whether the site-specific presentation of some acquired vascular diseases is attributable to SMCs' intrinsic developmental origins. We have shown that origin-specific SMCs exhibit differential MMP and TIMP activation in response to IL-1β. MMP activation is associated with SMC phenotypic modulation, which precedes aneurysm formation in experimental models. Degradation of the vessel wall connective tissues by MMPs is a major pathological remodeling process in an array of human vascular diseases. We hypothesized that the differential proteolytic ability of the medial SMC subtypes could influence the weakening of the aortic wall to different extents. Consequently, regional discrepancies in vessel mechanical properties may lead to aortic dissection at the boundaries where SMCs switch origins (Fig. 6). MMPs also induce SMC migration during atheroma formation in atherosclerosis. The most deleterious atherosclerotic lesions occur frequently in the coronary and carotid arteries, which arise from the lateral plate mesoderm and neuroectoderm, respectively. Accordingly, our results demonstrate that LM-SMCs and NE-SMCs are more predisposed to MMP activation than are PM-SMCs. We propose that hard-wired embryonic programs contribute to disease susceptibility, although other factors such as hemodynamic forces and physical structure of the vessels also play important roles.

The described differentiation system broadly covers most of the origins of vascular SMCs. Further patterning of the intermediate lineages, for example, the lateral plate mesoderm into secondary heart field or pro-epicardial lineage, may mimic more closely the precise origins of SMCs in the aortic root and coronary artery, respectively. Our protocols could potentially be extended to encompass these intermediate subpopulations in future studies. Moreover, a small number of SMC populations may be derived from embryonic tissues other than neuroectoderm, lateral plate mesoderm and paraxial mesoderm. For example, renal SMCs are thought to originate from the metanephric mesenchyme, an intermediate mesoderm derivative that is not modeled here. Additionally, it is likely that the hPSC-derived SMCs represent a fetal phenotype, possibly due to lack of heterotypic cell-cell interactions and in vitro microenvironment cues. In spite of this, our in vitro SMC differentiation system defines conditions for generating SMCs from different embryological origins. Elucidating the molecular mechanisms underlying origin-dependent differences in SMC behavior may provide clues to understanding how SMC origins influence a variety of vascular disease patterns.

Furthermore, our ability to produce large amounts of SMC subtypes from hPSCs should be beneficial in far-reaching applications in vascular disease modeling and regenerative medicine. Genetic syndromes and hereditary influences seem to be closely linked to SMC dysfunction in the thoracic aorta. Congenital vascular diseases, such as cono–truncal defects or CADASIL, feature mutations that predominantly affect neural crest–derived SMCs. Disease modeling with the appropriate origin-specific SMCs generated from patient-derived iPSCs is therefore vital for accurate assessment and therapeutic discovery. Patient-matched SMCs may also be used to construct bioengineered blood vessels for coronary and peripheral artery bypass or hemodialysis grafts. In tissue or whole-organ regeneration, optimal outcomes require adequate vascularization. Therefore, to maximize success in both disease modeling and regenerative medicine, it may be useful to focus on SMCs that are derived from the same embryonic lineage as in the affected tissue or organ.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Accession codes. Raw and processed data are available from the ArrayExpress microarray data repository under accession numbers E-MTAB-781 (SMC) and E-MTAB-464 (hESC).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

C.C. and S.S. developed the concept of generating origin-specific SMCs and designed the experiments. A.S.B. and R.A.P. developed the mesoderm specification protocols. C.C. performed experiments, analyzed data, and wrote and prepared

Figure 6  The different embryological origins of aortic SMCs may contribute to the site of aortic dissection. Aortic SMCs originate from three distinct developmental lineages. The aortic root is derived from secondary heart field, a lateral plate mesoderm derivative (blue solid arrow), whereas the ascending aorta and arch are neural crest derived (red solid arrow). The descending aortic SMCs originate from paraxial/somitic mesoderm (green solid arrow). In this study, our in vitro hPSC–derived SMC subtypes predicted the differential MMP and TIMP activation in aortic SMCs of corresponding origins (dashed arrows) in response to an inflammatory mediator, IL-1β. We propose that the origin-specific SMCs display differential proteolytic ability in disease settings, which may result in differential loss of the structural integrity in different regions along the aortic wall. Such a difference in mechanical properties may predispose the sites of aortic dissection to occur preferentially at the boundaries between different SMC lineages (indicated by black jagged bolts).
the manuscript. A.S.B. performed part of the mesoderm validation experiments. M.W.B.T. gave advice regarding design of the microarray experiment, processed the resulting data and contributed to further analysis. S.S. supervised the project. All authors edited the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6, 389–395 (2000).
2. Majesky, M.W. Developmental basis of vascular smooth muscle diversity. Arterioscler. Thromb. Vasc. Biol. 27, 1248–1258 (2007).
3. Wasteson, P. et al. in dorsal secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. Dev. Biol. 281, 78–90 (2005).
4. Jiang, X., Rowitch, D.H., Soriano, P., McMahon, A.P. & Sucov, H.M. Fate of the mammalian cardiac neural crest. Development 127, 1607–1616 (2000).
5. Wasteson, P. et al. Developmental origin of smooth muscle cells in the descending aorta in mice. Development 135, 1823–1832 (2008).
6. Mikawa, T. & Gourdie, R.G. Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. Dev. Biol. 174, 221–232 (1996).
7. Etchevers, H.C., Vincent, C., Le Douarin, N.M. & Couly, G.F. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128, 1059–1068 (2001).
8. Vanrecken Peeters, M.P., Gittenberger-de Groot, A.C., Mentink, M.M. & Poelmann, R.E. Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium. Anat. Embryol. (Berl.) 199, 367–378 (1999).
9. Pouget, C., Pottin, K. & Jaffredo, T. Sclerotomal origin of vascular smooth muscle cells and pericytes in the embryo. Dev. Biol. 315, 437–448 (2008).
10. Armulik, A., Genove, G. & Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev. Cell 21, 193–215 (2011).
11. Haimovici, H. & Maier, N. Fate of aortic homografts in canine atherosclerosis. 3. Study of fresh abdominal and thoracic aortic implants into thoracic aorta: role of tissue susceptibility in atherogenesis. Arch. Surg. 89, 961–969 (1964).
12. DeBakey, M.E., Lawrie, G.M. & Glaeser, D.H. Patterns of atherosclerosis and their surgical significance. Ann. Surg. 201, 115–131 (1985).
13. Lenoux-Berger, M. et al. Pathologic calcification of adult vascular smooth muscle cells differs on their crest or mesodermal embryonic origin. J. Bone Miner. Res. 26, 1543–1553 (2011).
14. Ruddy, J.M., Jones, J.A., Spinale, F.G. & Ikonomidis, J.S. Regional heterogeneity of the resulting data and contributed to further analysis. S.S. supervised the project. All authors edited the manuscript.

21. Zhang, P. et al. Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. Blood 111, 1933–1941 (2008).
22. Yang, L. et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453, 524–528 (2008).
23. McLean, A.B. et al. Activin A efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25, 29–38 (2007).
24. Jain, R.K. Molecular regulation of vessel maturation. Nat. Med. 9, 685–693 (2003).
25. Kramer, J., Quenel, C., Meding, J., Cardoso, M.C. & Leonhardt, H. Identification and characterization of novel smoothelin isoforms in vascular smooth muscle. J. Vasc. Res. 38, 120–132 (2001).
26. Huang, X. & Saint-Jeanett, J.P. Induction of the neural crest and the opportunities of life on the edge. Dev. Biol. 275, 1–11 (2004).
27. Wang, D.Z. et al. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc. Natl. Acad. Sci. USA 99, 14855–14860 (2002).
28. Li, J. et al. Myocardin-related transcription factor B is required in cardiac neural crest-derived smooth muscle cells. J. Biol. Chem. 284, 15122–15127 (2009).
29. Owens, A.P. et al. Angiopoietin II induces a region-specific hyperplasia of the ascending aorta through regulation of inhibitor of differentiation 3. Circ. Res. 106, 611–619 (2010).
30. Topouzis, S. & Majesky, M.W. Smooth muscle lineage diversity in the chick embryo. Two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-beta. Dev. Biol. 178, 430–445 (1996).
31. Gasdon, P.F. Jr. et al. Differential response of mesoderm- and neural crest-derived smooth muscle to TGF-beta1: regulation of c-myb and alpha1 (I) procollagen genes. J. Vasc. Res. 38, 169–190 (1997).
32. Isoda, K. et al. Deficiency of interleukin-1 receptor antagonist promotes neointimal formation after injury. Circulation 108, 516–518 (2003).
33. Galis, Z.S. & Khati, J.J. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ. Res. 90, 251–262 (2002).
34. Owens, G.K., Kumar, M.S. & Warnhoff, B.R. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol. Rev. 84, 767–801 (2004).
35. Allawadi, G. et al. Smooth muscle phenotypic modulation is an early event in aortic aneurysms. J. Thorac. Cardiovasc. Surg. 138, 1392–1399 (2009).
36. Libby, P., Ridker, P.M. & Hansson, G.K. Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325 (2011).
37. Milewicz, D.M., Dietz, H.C. & Miller, D.C. Treatment of aortic disease in patients with Marfan syndrome. Circulation 111, e150–e157 (2005).
38. Loesy, B.L. et al. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. Nat. Genet. 37, 275–281 (2005).
39. Zhu, L. et al. Mutations in myosin heavy chain 11 cause a syndrome associating thoracic aortic aneurysm/aortic dissection and patent ductus arteriosus. Nat. Genet. 38, 343–349 (2006).
40. Guo, D.C. et al. Mutations in smooth muscle alpha-actin (ACTA2) lead to thoracic aortic aneurysms and dissections. Nat. Genet. 39, 1488–1493 (2007).
41. van de Laar, I.M. et al. Mutations in SMAD3 cause a syndromic form of aortic aneurysms and dissections with early-onset osteoarthritis. Nat. Genet. 43, 121–126 (2011).
42. Lindsay, M.E. & Dietz, H.C. Lessons on the pathogenesis of aneurysm from heritable conditions. Nature 473, 308–316 (2011).
43. Gittenberger-De Groot, A.C., Bartelings, M.M., Deruiter, M.C. & Poelmann, R.E. Basics of cardiac development for the understanding of congenital heart malformations. Pediatr. Res. 57, 169–176 (2005).
44. Kalimo, H., Ruchoux, M.M., Viitanen, M. & Kalaria, R.N. CADASIL: a common form of hereditary arteriopathy causing brain infarcts and dementia. Brain Pathol. 12, 371–384 (2002).
ONLINE METHODS

HPSC culture. hESCs (lines H9 and H1 (WiCell), passages 65–85) were cultured in a chemically defined medium (CDM) as previously described 36. Human iPSCs were obtained from the Cambridge Biomedical Research Centre iPSC Core Facility. Briefly, human dermal fibroblasts were isolated from excess skin of patients undergoing plastic surgery and reprogrammed as previously described 37. Human iPSCs were grown on irradiated mouse feeders and cultured in the typical DMEM/F12 medium, containing 20% knockout serum replacement (Gibco) and 4 ng/ml FGF-2 (R&D Systems).

HPSC differentiation. For neuroectoderm differentiation, cells were grown in CDM + SB431542 (10 µM, Tocris) + FGF2 (20 ng/ml, R&D Systems) for 7 d. For early mesoderm formation, cells were initially grown in CDM+FGF2 (20 ng/ml, R&D Systems) + LY294002 (10 µM, Sigma) + BMP4 (10 ng/ml, R&D Systems) for 36 h. Further specification into lateral plate mesoderm required CDM+FGF2 (20 ng/ml, R&D Systems) + BMP4 (50 ng/ml, R&D Systems) for another 3.5 d; whereas paraxial mesoderm required CDM+FGF2 (20 ng/ml, R&D Systems) + LY294002 (10 µM, Sigma). Upon obtaining the intermediate populations, cells were trypsinized and cultured in SMC differentiation medium CDM+PDGF-BB (10 ng/ml, PeproTech) + TGF-B1 (2 ng/ml, PeproTech) for at least 12 d. Derived SMCs were maintained in CDM deprived of growth factors for at least 24 h before subsequent experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted with RNeasy Mini kit according to the manufacturer’s instructions (Qiagen). cDNA was prepared using Maxima First Strand cDNA Synthesis Kit (Fermentas). qRT-PCR mixtures were prepared with SYBR Green PCR Master Mix (Applied Biosystems). qRT-PCR reactions were done in technical duplicates with the 7500 Fast Real-time PCR System (Applied Biosystems), using the Quantification–comparative Ct settings. Obtained values were normalized to porphobilinogen deaminase. Primer sequences are listed in Supplementary Table 2.

Immunofluorescence and flow cytometry. For immunofluorescence, adherent cells were fixed and permeabilized. After blocking with 3% BSA, primary antibody was incubated at 4 °C overnight. Secondary goat anti-mouse Alexa Fluor 568 and goat anti-rabbit Alexa Fluor 488 antibodies (Invitrogen Molecular Probes) were used. Finally, nuclei were stained with DAPI. Images were acquired using Zeiss Axiosvert 200M microscope. For flow cytometry, harvested cells were fixed using the Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and stained according to the kit manual. Mouse IgG isotype controls (BD PharMingen, 555749, R&D Systems, IC002C) and nontargeting cells (H9 hESCs and HUVECs) were used. Primary antibodies used for this study are listed in Supplementary Table 3.

Western blot analysis. A portion of total protein (30 µg) was resolved with SDS-PAGE and transferred to nitrocellulose membranes. The membrane was probed with primary antibodies (Supplementary Table 3) and secondary anti-rabbit–HRP (Sigma) and anti-mouse–HRP (Sigma) antibodies. Detection was done using the ECL system (GE Healthcare). (For full-length blots, see Supplementary Fig. 6.)

Microarray analysis. Data import. Sample RNA from hESC and SCMC samples was hybridized to Illumina Human HT-12 v4 BeadArrays. Raw Illumina bead-level output was imported to the R statistical programming environment using functions of the beadarray package for the Bioconductor software suite.

Data processing. Signal intensities were background corrected using the RMA model 38, summarized and converted to log2 expression units using functionality of the beadarray package. Probe-sets without signal deemed significant above background level in all profiles of at least one sample group (Illumina signal detection statistic p < 0.01) were removed. Quantile normalization, implemented in the limma package for Bioconductor, was employed to equalize summarized expression intensity distributions across all sample profiles. Probe sets were annotated to gene targets using information available from the manufacturer. SCMC profiles (Illumina Human HT-12 v4) and published hESC profiles (Illumina Human WG-6 v3) were combined for comparison by quantile normalization on a subset of Illumina probe sets present on both arrays.

Data visualisation. Heat maps of relative gene expression were generated with the Heatmap Builder software 39 (downloaded courtesy of the Ashley Lab, Stanford). Venn diagrams representing subsets of genes significantly differentially regulated between SMC sample groups and the hESC profiles were created using functionality of the limma package for Bioconductor.

Overrepresentation analysis. The statistical overrepresentation of functional gene categories among genes deemed differentially expressed between sample group profiles was assessed using the DAVID bioinformatics resource 40. Category overrepresentation within a list of differentially expressed genes, obtained as described above, was determined.

Marker identification. Differential probe-set (or gene) expression between two sample groups was assessed through the output of a moderated t-test. To reduce errors associated with multiple hypothesis testing on such a scale, the significance P-values obtained were converted to corrected q-values using the FDR method previously published 41. Probe-sets with associated q < 0.001 (FDR 0.1%) were deemed to exhibit significant differential expression between sample groups.

Contraction study. SMCs were preloaded with the calcium-sensitive fluorophore, Fluo-4 a.m. (2.5 µM; Molecular Probes) at 37 °C for 1 h. Contraction was induced by treating the cells with carbachol (100 µM, Sigma). Intracellular calcium flux was measured using the FL1 channel of CyAn ADP flow cytometer (Beckman Coulter). Contraction videos of SMCs were acquired by time-lapse microscope (Olympus IX700) during 10 min of carbachol treatment. Change of cell surface area was assessed by ImageJ software.

Matrigel plug implantation. In vivo studies were compliant with the UK Home Office: Animals In Scientific Procedures guidelines and approved by the University of Cambridge. Derived SMCs and HUVECs (ratio 1:2) were resuspended in ice cold Matrigel basement membrane matrix (BD Biosciences) at a final cell concentration of 10⁶ cells/ml. Cell suspension (750 µl) was injected subcutaneously into Rag2-null immunodeficient male mice. After 2 weeks, the animals were euthanized and the Matrigel plugs were excised to be paraffin embedded. The sections were then immunostained and imaged with a confocal microscope (Zeiss, LSM700).

siRNA gene silencing. Transient knockdown of MKL2 was carried out with ON-TARGETPlus SMARTpool siRNA by DharmaFECT transfection reagent (Thermo Scientific Dharmacon). siRNA (10 nM) was used for transfection according to the manufacturer’s instructions. mRNA and protein analyses were done at 24 h and 48 h after transfection, respectively.

Proliferation assays. For the cell proliferation assay, hESC-derived SMCs were seeded 6,000 cells/well into 96-well plates. After incubation overnight, TGF-B1 (5 ng/ml, PeproTech), angiotensin II (10 µM, Sigma) or 10% FBS (Sigma) was added. Cell viability was measured as a function of the metabolic activity using tetrazolium salt, MTT (Sigma). At every time point, MTT (0.5 mg/ml) was incubated with the cells at 37 °C for 2 h and then solubilized using DMSO (Sigma) at 37 °C for 15 min. Absorbance was measured at 550 nm using the Wallac Envision 2104 multilabel reader (PerkinElmer). For cell cycle analysis, hESC-derived SMCs were harvested and the cell suspension was fixed by ice cold 70% ethanol for 30 min at 4 °C. To ensure that only DNA is stained, cells were treated with 100 µg/ml of RNase. Finally, cells were stained with 50 µg/ml of propidium iodide in the dark at 25 °C for 30 min. Cell fluorescence was measured by a flow cytometer (Beckman Coulter CyAn ADP) and cell cycle analysis was done using FlowJo software.

Rat primary SMC culture. Rats were euthanized using carbon dioxide and different aortic regions were surgically dissected (Supplementary Fig. 5). Aortic segments were incubated with 12.5 µl of enzyme solution comprising of 1 mg/ml collagenase II (Worthington), 1 mg/ml soybean trypsin inhibitor (Worthington), 9.3 units activity elastase (Worthington), 125 µl penicillin-streptomycin and 12.5 ml HBSS (Gibco) for 20 min at 37 °C. Adventitia was removed and aortic segments were rinsed to remove intimal cells. Remaining medial segments were snipped into 0.5- to 1-mm pieces and enzyme treated again for 1 h with occasional agitation. Dissociated SMCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin–streptomycin.
penicillin and 100 mg/ml of streptomycin. Rat SMCs were serum-starved for at least 24 h before experimentation. All experiments were done with cells during passages 2–4.

**Proteolytic assays.** 96-well plates were coated with either 0.1 mg/ml of elastin or collagen type I, of which both were FITC conjugated (AnaSpec). HESC-derived SMCs or rat SMCs were then seeded 6,000 cells/well. After incubation overnight, 10 ng/ml of IL-1β (PeproTech) was added in serum-free media. Activity of secreted proteases was assessed by the increase of fluorescence measured at excitation/emission = 490 nm/520 nm using the Wallac Envision 2104 multilabel reader (PerkinElmer). To take into account any differential cell proliferation due to IL-1β, the fluorescence readout was normalized against the corresponding cell number/well.

**Statistics.** Results are presented as mean ± s.e.m. of three independent experiments unless otherwise stated. Statistical P values were calculated by student’s t-test unless otherwise stated.

46. Brons, I.G. et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature **448**, 191–195 (2007).
47. Vallier, L. et al. Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. Stem Cells **27**, 2655–2666 (2009).
48. Irizarry, R.A. et al. Nucleic Acids Res. **31**, e15 (2003).
49. King, J.Y. et al. Pathway analysis of coronary atherosclerosis. Physiol. Genomics **23**, 103–118 (2005).
50. Dennis, G. Jr. et al. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. **4**, 3 (2003).
51. Storey, J.D. & Tibshirani, R. Proc. Natl. Acad. Sci. USA **100**, 9440–9445 (2003).