Serum-free differentiation of functional human coronary-like vascular smooth muscle cells from embryonic stem cells

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Aims

Despite the diverse developmental origins of vascular smooth muscle cells (VSMCs), recent attempts to generate VSMCs from human embryonic stem cells (hESCs) differentiated along various lineages did not yield distinct cell phenotypes. The aim of this study was to derive and characterize functional coronary-like VSMCs from hESCs using serum-free cardiac-directed differentiation.

Methods and results

Embryoid bodies (EBs) from three pluripotent stem cell lines subjected to cardiac-directed differentiation in defined media were characterized over 30 days for VSMC-specific gene expression by qRT–PCR, immunofluorescence microscopy and fluorescence-activated cell sorting (FACS). EBs composed of cardiomyocytes, endothelial cells (ECs), fibroblasts, and VSMCs underwent FACS on d28 to reveal that the VSMCs form a distinct subpopulation, which migrate with ECs in an in vitro angiogenesis assay. To enrich for VSMCs, d28 EBs were dissociated and cultured as monolayers. Over several passages, mRNA and protein levels of cardiomyocyte, endothelial, and fibroblast markers were abolished, whereas those of mature VSMCs were unchanged. Vascular endothelial growth factor and basic fibroblast growth factor were critical for the separation of the cardiac and VSMC lineages in EBs, and for the enrichment of functional VSMCs in monolayer cultures. Calcium cycling and cell shortening responses to vasoconstrictors in hESC-derived VSMCs in vitro were indistinguishable from primary human coronary artery SMCs, and distinct from bladder and aorta SMCs. VSMCs identically derived from green fluorescent protein -expressing hESCs integrated in and contributed to new vessel formation in vivo.

Conclusion

The ability to generate hESC-derived functional human coronary-like VSMCs in serum-free conditions has implications for disease modelling, drug screening, and regenerative therapies.

Keywords

Coronary smooth muscle cell • Human embryonic stem cell • Smooth muscle differentiation • Vascular endothelial growth factor

1. Introduction

Vascular smooth muscle cells (VSMCs) are derived from a complex and diverse population of precursor cells during embryonic development. Fate-mapping studies indicate that VSMCs of the coronary artery are derived from the proepicardium and the proepicardial mesothelium, which represent somatic and splanchnic tissues of lateral plate mesoderm. In contrast, VSMCs of aortic root originate from the secondary heart field, whereas those of aortic arch are derived from neural crest. Correspondingly, previous studies have
derived SMC-like cells from diverse sources such as embryonic stem cells (ESCs),17 cardiac-directed progenitors,17 neural crest-derived stem cells,17 skin-derived progenitors,17 and other human adult tissues.9–13 Having said this, current human ESC systems do not accurately reproduce mature SMCs based on precise lineage specification.14,15

Human ESCs represent a unique source of VSMCs given their unparalleled ability to yield specific lineages. Indeed, methods to derive specific VSMCs from hESCs might serve as a promising platform for regenerative medicine.16–18 Although many studies have reported the paralleled ability to yield specific lineages. Indeed, methods to derive specific VSMCs from hESCs might serve as a promising platform for regenerative medicine.16–18 Although many studies have reported striking differentiation capacity of hESCs towards SMC-like cells, the majority have included the serum, which represents an immunogenic and pleiotropic hazard.19,20 Ill-defined combinations of factors, together with inherent variability between lots, further limit the reproducibility of serum-based protocols. Moreover, these protocols have not been optimized for the generation of specific SMC lineages.

To date, studies have not characterized the stability of SMC-specific gene and protein expression and function over several passages. We believe that a mature and functional SMC population derived from hESCs had not been demonstrated as stable, or thoroughly compared with human SMCs from distinct lineages. It has also been suggested that SMCs of varying embryonic origins when tested under identical conditions in vivo respond in lineage-specific ways to factors that control development, growth, and tissue remodelling.21,22 In recent work by Cheung et al.23 a chemically defined method to derive lineage-specific VSMCs from human pluripotent stem cells yielded no clear phenotypic and functional differences between SMC subtypes, which were compared only with primary human aortic SMCs. However, these authors did not direct differentiation towards any specific organ.

We hypothesized that environmental cues of a specific organ are needed for mature phenotypic and functional differences to emerge in its developing VSMCs. In the current study, we identify developmental stage with lineage-specific populations of VSMCs derived from cardiac-directed hESC differentiation in serum-free conditions. Importantly, our chemically defined protocol promotes cardiac differentiation from lateral plate mesoderm without induction of somatic mesoderm markers.24 Embryoid bodies (EBs) differentiated under this protocol are composed of endothelial cells (ECs), cardiomyocytes (CMs), fibroblasts, and VSMCs, and yield a distinct subpopulation of VSMCs by d28. These VSMCs were subsequently characterized over multiple passages, and compared with mature primary human SMCs from three distinct developmental origins. Over several passages, hESC-derived VSMCs from our cardiac-directed protocol were highly enriched and expressed multiple mature SMC-specific genes and proteins, containing all the cellular components required for Ca2+ cycling and contraction in response to agonists. Importantly, these hESCs-derived VSMCs were functionally and phenotypically indistinguishable to human coronary artery SMCs (hCSMCs) and clearly different from human aorta and human bladder SMCs (hASMCs and hBSMCs). Finally, we demonstrate that our hESC-derived ‘coronary-like’ VSMCs are capable of integrating into and participating in vasculogenesis in vivo.

2. Methods

2.1 Maintenance and differentiation of hESCs

hESC line HES2 and H7 [NIH code ES02 from ES Cell International (ESI) and Wi Cell product number WA07, respectively] and the hiPS lines, MSC-iPS1 (also known as Y2-1 was maintained as described).24–27 The differentiation protocols used are detailed in the Supplementary material online.

2.2 FACS

EBs were harvested and dissociated to single cells with collagenase type I (Sigma C-0130) containing 10 μg/mL DNase (CALBIOCHEM# 260913) and trypsin (Sigma T-4799) as previously described.28 Fluorescence-activated cell sorting (FACS) and antibodies used for flow cytometry are detailed in the Supplementary material online.

2.3 Cell culture

To obtain monolayers of hESC-derived VSMCs, EBs were dissociated on d28 to single cells as described.27 Dissociated cells were counted and plated on 0.65% Matrigel as following: 5 × 105 cells/well for eight-well slides for immunofluorescence microscopy (IFM) and Ca2+ imaging; 104 cells/well for 24 well plates for mRNA extraction and qRT–PCR analysis; 105 cells/well for 12-well plates for FACS; and 0.5 × 106 cells/well for six-well plates for passaging. Cell culture is further described in the Supplementary material online.

2.4 IFM

Staining for IFM was carried out in eight-well chamber slides (Lab Tek II), six-well plates or 60 mm dishes (Greiner Bione) with glass coverslips, or frozen sections from Matrigel plugs. Method and antibodies used for IFM are described in the Supplementary material online.

2.5 qRT–PCR

RNA was extracted from EBs, monolayers of hESC-derived VSMCs, and three human primary SMCs (hCSMCs, hASMCs, and hBSMCs) using the PicoPure RNA Isolation Kit (Arcturus). The qRT–PCR method is detailed in the Supplementary material online, with primers used listed in Supplementary material online, Table S1.

2.6 Calcium imaging

Passage (P) one to three monolayers of hESC-derived VSMCs (105) were grown on eight-well glass-bottom chamber slides thinly coated with Matrigel in serum-free conditions. Other cell types tested under identical culture conditions included positive and negative controls consisting of primary hASMCs, hBSMCs, hCSMCs, mouse embryonic fibroblasts (MEFs), and hECs, respectively. Imaging methods, including contraction assays, are detailed in the Supplementary material online.

2.7 Angiogenesis assays

A sprouting assay was adapted for use with hESC-derived EBs.29 EBs on d28 were resuspended in neutralized acid-solubilized 2.5 mg/mL rat tail type-1 collagen (BD Biosciences #354236) and allowed to grow for 7 days in serum-free media. The method is further detailed in the Supplementary material online. Cells were then fixed and immunostained as described above.

2.8 Animal studies

All animal procedures were conducted in accordance with appropriate regulatory standards under protocol # 1566.7 approved by the University Health Network Institutional Animal Care and Use Committee (NIH Publication #85-23, revised 1996).

2.9 Analysis of vascular integration in vivo

Window chambers were surgically implanted as described previously.30 NOD-SCID 4–6-week-old mice (strain NCRNU-M, Taconic, Hudson, NY, USA) were placed under general anaesthesia (ketamine 80 mg/kg; xylazine 5 mg/kg injected ip) for both initial surgery and subsequent in vivo imaging. The adequacy of anaesthesia was monitored by the loss...
of reflexes, and the degree of muscle relaxation. At the time of surgery, and prior to placement of the transparent glass coverslip (12 mm), a concentrated suspension of green fluorescent protein (GFP⁺) hESCs-derived VSMCs from P2 in 50% Matrigel (150 000 cells/µL) was injected either directly into the retractor muscle of the mouse dorsal skin fold to examine the capacity of the hESCs-derived SMCs to integrate into native blood vessels, or subcutaneously to determine the ability of these cells to incorporate into new blood vessels. Further details regarding these models and the intravital confocal microscopy and 3D image visualization used for their analyses are provided in the Supplementary material online.

2.10 Statistical analysis

Data shown are mean ± SEM. Statistical analyses were performed by Student’s unpaired t-test or One-way ANOVA was followed by Tukey’s for multiple comparisons. Analyses were performed on Graphpad Prism v4.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1 Characterization of SMC gene expression in EBs directed towards cardiac differentiation

Using a staged cardiac-directed serum-free protocol described previously, hESCs were differentiated over 30 days (Figure 1A). Briefly, this involved the formation of a population of primitive streak-like hESCs, induction and specification of cardiovascular mesoderm, and subsequent expansion of resulting cardiovascular lineages. Directed differentiation was mediated by specific factors, namely activin A, bone morphogenetic protein 4, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and dickkopf homolog 1, which have been shown to support cardiomyocyte development.31–34 We posited that such a schema would provide the spatiotemporal cues necessary for the development of myocardium-associated coronary artery-like VSMC (Figure 1A) and Supplementary material online, Figure S1A).

Expression of cell type-specific mRNA and protein markers was quantified by qRT–PCR and FACS, respectively. Developing EBs contained cells that expressed VSMC-specific markers by d12, with the mRNA levels of these markers peaking between d14–22 when compared with undifferentiated hESCs (Figure 1B). qRT–PCR revealed the appearance of smooth muscle α-actin (ASMA), calponin-1 (CNN1), transgelin (SM22a), myosin heavy chain polypeptide-11 (MYH11), MYH11 isoforms SM1 and SM2, metavinculin (MVCL), smoothelin A/B (SMTN), and caldesmon-1 (HCAD). By d30, significant reductions in ASMA (27.1 ± 6.7 vs. 10.3 ± 0.8), SM22a (0.5 ± 0.06 vs. 0.17 ± 0.0016), MVCL (0.04 ± 0.01 vs. 0.01 ± 0.001), MYH11 (0.09 ± 0.01 vs. 0.04 ± 0.01), SM1 (0.1 ± 0.01 vs. 0.04 ± 0.002), SM2 (0.011 ± 0.0001 vs. 0.003 ± 0.002), and SMTN (0.07 ± 0.01 vs. 0.13 ± 0.02) were detected when compared with levels observed on d14 (n = 3, P < 0.05 for each). While the expression of SM1 and SM2 suggested a ‘mature’ VSMC phenotype, the embryonic SMC marker MYH10 remained abundant. Of note, increased levels of MYH10 have been described in proliferating VSMCs of experimental CALDES monolayers.35,36 However, by d30, the MYH10 gene expression level (0.6 ± 0.06) was significantly lower than on d14 (1.11 ± 0.16), d22 (1.2 ± 0.1) and d26 (1.45 ± 0.15) of developing EBs (n = 3, P < 0.04 for each). These key findings identified d28 as the developmental stage at which cardiac-directed EBs harbour a ‘mature’ VSMC population.

The EC marker platelet/EC adhesion molecule 1 (PECAM1, a.k.a. CD31) and the fibroblast-specific marker discoidin domain receptor tyrosine kinase 2 (DDR2) were also quantified. Compared with d3, there were significant increases in DDR2 (0.08 ± 0.003 vs. 15.5 ± 3) starting at d14, and in CD31 (0.001 ± 0.0001 vs. 0.3 ± 0.09) starting at d26 (Figure 1, n = 3, P < 0.05 for each). Similar patterns of expression were observed in the H7 hESC line (Supplementary material online, Figure S2).

To accurately define the proportion of differentiated VSMCs within EBs, FACS of cells dissociated from whole EBs (from both HE52 and H7 lines) were performed on d13, d21, and d28 (Figure 2 and Supplementary material online, Figure S2). At the earlier time points, cells co-expressing CM- and VSMC-specific markers were identified, a finding consistent with the notion that developing CMs transiently express VSMC genes that are turned off at later stages of development.36 Indeed, cells from EBs dissociated on d13 and d21 revealed ASMA⁺/TNN13⁺, ASMA⁺/TNN3⁺, and ASMA⁺/TNN13⁻ subpopulations. By d28, there was a complete loss of the double-positive ASMA⁺/TNN13⁺ population and clear separation of two putatively more lineage-specific populations of ASMA⁺/TNN13⁺ and ASMA⁺/TNN13⁻. Similar profiles were observed with CNN1 and SM22a for the HE52 line. Together, these data further suggest that the d28 EB represents a developmental stage with a specific and differentiated VSMC population.

3.2 VEGF and bFGF are required for cardiovascular lineage specification in EBs, and for VSMC enrichment in monolayers

Based on data suggesting a role for VEGF-R2 in differentiation of VSMC from mouse ESC,35 we examined the roles played by VEGF and bFGF in the separation of cardiovascular lineages in human EBs. In this experiment, EBs at d11 were maintained in serum-free medium supplemented with VEGF, bFGF, both or neither (Supplementary material online, Figure S1A), and examined 10 (d21) and 17 (d28) days later by qRT–PCR for SMC, fibroblast, CM, and EC markers. Unlike results obtained when EBs are exposed to both VEGF and bFGF (Figure 1), the absence of VEGF (VEGF⁻/bFGF⁺) resulted in a significant increase in CM gene expression (TNNI3) in d28 EBs (P < 0.05) (Supplementary material online, Figure S1B). However, FACS revealed that these EBs (VEGF⁻/bFGF⁺ and VEGF⁻/bFGF⁻) do not yield distinct populations of CMs and SMCs, with a high percentage of cells remaining ASMA⁺/TNN13⁻ (Supplementary material online, Figure S1C). In the absence of bFGF (VEGF⁺/bFGF⁻ and VEGF⁻/bFGF⁻), there were significant decreases in EC gene expression (CD31) in d28 EBs (P < 0.05) (Supplementary material online, Figure S1B), with FACS indicating that bFGF alone is not sufficient to promote separation of CM and VSMC lineages in d28 EBs (Supplementary material online, Figure S1C). Together, these data suggest that prolonged exposure to both VEGF and bFGF (d11–d28) is required for complete cardiovascular lineage specification.

We then examined whether an enrichment of the ASMA⁺/TNN13⁻, CNN1⁺/TNN13⁻, and SM22a⁺/TNN13⁻ populations is possible at early developmental stages. Monolayers from d13 EBs revealed predominant TNN13⁺/ASMA⁺ and TNN13⁻/CNN1⁺ cells in culture with TNN13⁻ cells showing ectopic (nuclear) ASMA.
Figure 1 Developmental changes in marker genes in EBs during serum-free cardiovascular-directed differentiation of hESCs. (A) A schematic representation of the protocol used for hESCs differentiation toward cardiovascular lineages. (B) Quantitative RT–PCR was used to monitor putative lineage-specific gene expression levels in differentiating EBs over 30 days. Data shown are mean ± SD (n = 3 separate experiments) for mRNA levels normalized to the housekeeping gene TATA box binding protein. Significant findings are detailed in the Results.
CNN1, and SM22α staining (Supplementary material online, Figure S3B). In addition, beating CMs caused both retraction and detachment of monolayers from this stage (Supplementary material online, Movie S1). These data show that d13 EBs do not harbour bona fide SMCs and are difficult to maintain in monolayers.

Having established d28 as a developmental stage that harbours authentic VSMCs, we next dissociated d28 EBs into single-cell suspensions, plated them at low density, and continued to culture the constituent cell populations in serum-free medium supplemented with VEGF, bFGF, both, or neither (Supplementary material online, Figure S1A). Over three passages, both VEGF and bFGF were found to be critical for the maintenance and expansion of functional hESC-derived VSMCs (Supplementary material online, Figure S1E). Interestingly, culturing d28 EBs in bFGF alone (VEGF+bFGF−) promoted the expansion of ECs as shown by increased CD31 expression over three passages. Both VEGF and bFGF were required to eliminate CMs from monolayers, as evidenced by residual TNNI3 expression in all other conditions (Supplementary material online, Figure S1D).

Comparing cells freshly disassociated from d28 EBs with cells grown in monolayers with VEGF and bFGF for three passages, FACS revealed over 3- and 14-fold enrichment of ASMA-expressing cells from HES2 (6.5 ± 0.4 vs. 20 ± 2%; *P < 0.005) and H7 (5.4 ± 1.8 vs. 77.6 ± 8%; *P < 0.001) derived VSMCs, respectively (Figure 3A and Supplementary material online, Figure S2D). Additionally, there was near complete loss of TNNI3-expressing CMs (HES2: 24 ± 5 vs. 0.16 ± 0.1%, *P < 0.0001; H7: 16.6 ± 3 vs. 0.34 ± 0.3%; *P < 0.006). Of note, FACS analysis of Y2-1-derived VSMC monolayers also revealed a high percentage of ASMA+ cells at P3 with a complete loss of TNNI3-expressing CMs (Supplementary material online, Figure S3B). Consistent with this, expression levels of TNNI3, CD31, and DDR2 were significantly decreased in P3 vs. P1 monolayers (⁎*P < 0.001, ⁎*P < 0.05, and *P < 0.05, respectively; Figure 3B). To further assess the nature of enriched hESC-derived VSMCs in monolayer, we compared their gene expression profile to that of cultured primary coronary (hCSMCs), aorta (hASMCs), and bladder (hBSMCs) SMCs. Over three passages, hESC-derived VSMCs express many markers (ASMA, CNN1, SM22α, SMTN, HCAD, γ-actin, and myocardin) at levels mostly comparable with those obtained in hCSMC cultures (Figure 3B and Supplementary material online, Figure S5B). Of particular interest, P3 hESC-derived VSMCs and hCSMCs shared comparable levels of γ-actin, ASMA, and CNN1, a pattern that clearly distinguished them from hASMCs and hBSMCs. We observed similar findings in VSMCs derived from the H7 hESC and Y2-1 iPS lines (Supplementary material online, Figures S2B and S4A).
In hESC-derived VSMCs from EBs d28, a highly homogeneous and stable pattern of the expression of multiple VSMC-specific proteins was also observed over three passages (Figure 3C and Supplementary material online, Figure S6A). Furthermore, their pattern of VSMC-specific protein expression was comparable with that of primary hCSMCs, and not detected in primary human ECs or epithelial cells (Supplementary material online, Figure S6). IFM also confirmed the loss of ECs and fibroblasts from monolayers (Figure 3C).

Our H7 and Y2-1-derived SMCs showed similarly homogenous and stable patterns of the expression of multiple SMC-specific proteins (Supplementary material online, Figures S2C and S4B).

3.3 Both VEGF and bFGF are required for hESC-derived VSMCs to cycle intracellular Ca\(^{2+}\) and contract in response to agonists

An important characteristic of functional VSMCs is their ability to contract in response to agonists, which is mediated by increased intracellular Ca\(^{2+}\) levels triggering the contractile apparatus. To examine whether hESC-derived VSMCs maintained in serum-free medium supplemented with VEGF, bFGF, both, or neither exhibited functional Ca\(^{2+}\) responses, cells were loaded with the Ca\(^{2+}\)-sensitive dye Fluo-4 AM, with their responses to phenylephrine (PE) and norepinephrine (NE) quantified by confocal microscopy. Frame-by-frame image analysis of regions of interest (ROIs) was performed. ROIs corresponded to single Ca\(^{2+}\)-cycling cells, and their average fluorescence intensities normalized to baseline were plotted over time. Both VEGF and bFGF were essential for hESC-derived VSMC responses to PE and NE, with phasic Ca\(^{2+}\) cycling activity (Figure 4A and Supplementary material online, Figure S1E). On the other hand, ‘SM-like’ cells from d13 EBs had sparse response to PE, no response to NE, with monolayers from this stage showing spontaneous rapid Ca\(^{2+}\) cycling typical of CMs (Supplementary material online, Figure S3C). Primary hASMC, hBSMC, and hCSMC were also tested in this manner. hCSMCs responded to PE stimulation, and were equally responsive to NE, a finding also observed in hESC-derived VSMCs. On the other hand, hASMCs responded more vigorously to PE than NE. Overall, a greater proportion of cells in each field were found to cycle Ca\(^{2+}\) in the hESC-derived VSMCs after agonist stimulation vs. hASMCs and hCSMCs. hBSMSCs spontaneously cycled Ca\(^{2+}\) under basal conditions, without enhanced responses to PE or NE (see Supplementary material online, Movie S2). As negative controls, MEFs and primary...
human coronary artery ECs (hCAECs) did not spontaneously cycle Ca\textsuperscript{2+} and did not respond to either PE or NE (Supplementary material, Figure S7B). Responses to agonists were also plotted as maximal fluorescence intensities normalized to baseline for each cell type (Figure 4B). The response of hESC-derived VSMCs to NE is more similar to hCSMCs than hASMCs. Overall, hBSMCs had the weakest response to PE and NE. Frequency of Ca\textsuperscript{2+} cycling was also quantified for each group (Figure 4C). hBSMCs exhibited the greatest Ca\textsuperscript{2+} cycling frequency of all groups tested. Interestingly, as hESC-derived VSMCs were passaged to P6, their frequency response approximated hCSMCs, which also show impaired Ca\textsuperscript{2+} cycling over repeated passaging. Ca\textsuperscript{2+}-cycling frequencies at baseline and after stimulation with PE and NE were also plotted. Although hASMCs maintain some level of spontaneous Ca\textsuperscript{2+} cycling at baseline, this was not observed in hCSMCs or P2, P3, or P6 hESC-derived VSMCs. Both H7- and Y2-1-derived VSMCs responded to both PE and NE with phasic Ca\textsuperscript{2+} cycling activity (Supplementary material online, Figures S2F, S4D and Movie S3).

We next examined primary hCSMCs and hESC-derived VSMCs undergoing physical contractions as manifest by cell shortening in response to agonists. Representative and grouped P3 hESC-derived VSMC and hCSMC data revealed no differences in PE-induced cell shortening (Supplementary material online, Figure S7A; \( n = 4–6 \) cells/group). Together, these results showed that hESC-derived VSMCs obtained from our cardiac-directed protocol have Ca\textsuperscript{2+} and contractile responses similar to those of primary hCSMCs, and clearly distinct from those of hASMCs and hBSMCs.

3.4 VSMCs from hESC-derived cardiac-directed EBs participate in angiogenesis

To assess the functional capacity of the vascular components of hESC-derived cardiac-directed EBs, we first employed a sprouting angiogenesis assay. This in vitro assay recapitulates angiogenesis in a natural sequence that includes EC sprouting, development of a branched network of angiogenic sprouts, with co-migration of VSMCs and ECs. Upon incubating d13, d21, and d28 EBs in serum-free media supplemented with VEGF and bFGF, we observed sprouting after 4 days in EBs from d21 to d28, and a complex network of interconnected vascular structures after 7 days. Importantly, there was no sprouting observed in EBs from d13
IFM showed that vascular cells migrated away from d21 to d28 EBs and assembled in highly organized cord-like structures (Figure 5A and Supplementary material online, Figure S2E). Multiple mature VSMC-specific markers were expressed by cells (VSMCs) aligned alongside cells expressing CD31 (i.e., ECs), including ASMA, CNN1, SM22α, and HCAD in d28 EBs (Figure 5C). Of interest, quantitative analysis indicated that 72 ± 6% (n = 27) of cells that sprouted out from d21 EBs stained positive for both a SMC-specific marker and CD31 (Figure 5B). Conversely, 83 ± 6% (n = 17) of cells migrating out from d28 EBs were positive for either a SMC-specific marker (ASMA, CNN1, or SMTN) or CD31. Moreover, outgrowths from d28 EBs contained both CD31- and ASMA-expressing cells that were in direct contact with one another (Figure 5A). Having said this, there was no observable difference in sprouting kinetics, branching points or total sprouts between d21 and d28 EBs after 7 days of culture. To support these observations, we also seeded cells dissociated from d28 EBs on Matrigel. As before, hESC-derived SMCs from d28 EBs aligned themselves into networks forming a primitive vascular plexus (Supplementary material online, Figure S8). These results suggest that while both d21 and
d28 EBs contain vascular cells, the vascular component of hESC-derived cardiovascular EBs is more mature at d28, with EC and VSMCs manifesting lineage specification (and presumed greater functional capacity) not seen at d21 (or d13) of EB development.

3.5 GFP-labelled hESC-derived VSMCs integrate into native vessels in vivo

To examine the functional capacity of hESC-derived VSMCs in vivo, we next assessed the ability of these cells to incorporate into new vasculature. hESC-derived VSMCs at P2 were generated from a GFP\(^+\) expressing HES2 cell line. Cells were injected either directly into the retractor muscle fascia of the dorsal skin fold below a transparent glass coverslip in a window chamber model (to assess integration with existing blood vessels), or subcutaneously (to assess new blood vessel formation) in NOD-SCID mice. Intravital multispectral fluorescence confocal microscopy was used to track the initial migration and subsequent clustering of GFP\(^+\) hESC-derived VSMCs around native mouse vessels during the first 2 weeks after their delivery. By Week 3 post-injection, there was obvious homing and integration of GFP\(^+\) hESC-derived VSMCs around host vasculature (Figure 6A and Supplementary material online, Figure S9). Histological analysis of
dissected Matrigel revealed the presence of GFP+ donor cells within the smooth muscle-layer of vessels invading the Matrigel, which were detected by staining with anti-GFP antibody. GFP+ cell populations strongly expressed ASMA. Finally, GFP+/ASMA+ and GFP+/CNN1+ cells were also present in mouse vessels invading Matrigel, suggesting that hESC-derived VSMCs were able to contribute and integrate into newly formed mouse vasculature (Figure 6B). These results demonstrate that hESC-derived VSMCs behave analogously to native mature VSMCs in their ability to integrate and contribute to new vessel formation in vivo.

4. Discussion

Although VSMCs have been previously derived and characterized from hESCs, our results advance this field in several important ways. By employing a developmental approach that recapitulates key events regulating cardiac lineage commitment in the embryo,27 we have (i) generated hESC-derived VSMCs that are functionally indistinguishable from primary hSMCs. To our knowledge, we are the first to generate such a specific and functionally distinct VSM population from hESCs. We have also demonstrated this population to have (ii) a remarkably stable functional phenotype (over at least six passages, Figure 4C), and (iii) be capable of integrating into both new and existing vasculature in vivo. Although the hESC-derived VSMCs generated by Cheung et al.23 also appear capable of forming new blood vessels in vivo, these authors added HUVEC to their Matrigel plugs and did not provide evidence of integration with existing host blood vessels. We have also (iv) directly compared and contrasted our hESC-derived ‘coronary-like’ VSMCs with three different primary human SMC cell types, each of which has a different developmental origin (coronary, aorta, and bladder). Despite directing their hESCs to differentiate along three different lineage paths (neuroectoderm, lateral mesoderm, and paraxial mesoderm), Cheung et al.23 were unable to functionally distinguish the resulting SMC from hASMCs. We believe that this failure to functionally specify their VSMCs may be the result of protocols that do not provide critical organ-specific cues. Additionally, we note that Cheung et al. analysed their lineage-specific SMCs at time points earlier (17d) than our study (28d+), including six passages over an additional 6 weeks. We can only speculate whether longer durations of culture in their chemically defined differentiation protocols would have mimicked the environmental cues inherent in our cardiac-differentiation system.

We also demonstrated (v) the importance of both VEGF and bFGF in cardiovascular lineage specification in EBs, and in the enrichment of ‘coronary-like’ VSMCs in monolayers. The importance of VEGF in our system is not unexpected, as both mouse VSMC progenitors and adult canine VSMCs29 have been shown to express VEGF-R2. Indeed, we also found abundant VEGF-R2 mRNA in our SMC system (Supplementary material online, Figure S10A).

Smooth muscle γ-actin is an early marker of smooth muscle differentiation. Previous studies indicated that mature SMCs express both α- and γ-actin isoforms with a ratio that varies depending on the type of muscle. For instance, VSMCs predominantly express the α-isoforms, whereas the γ-isoform predominates in visceral smooth muscle.42 Our results showed a γ/α-actin ratio of 0.25 ± 0.02 in HES2, 0.24 ± 0.02 in H7, and 0.33 ± 0.19 in Y2-1 IPS-derived VSMCs (P3), 0.3 ± 0.017 in hCSMSCs, 0.0007 ± 0.00008 in hASMCs, and 24.7 ± 3.6 in hBSCMs. Importantly, we observed no change in this γ/α-actin ratio over several passages in hESCs and IPS-derived VSMCs (Figure 3B, Supplementary material online, Figures S2B and S4A), further supporting the stability of this unique cell phenotype. In conclusion, we have described a chemically defined (serum-free) organ-specific differentiation system allowing for the induction and enrichment of functionally ‘mature’ coronary-like VSMCs. Comprehensive analyses of gene expression and cell physiology reveal enrichment for a very specific and highly stable SMC lineage derived from lateral plate mesoderm directed towards cardiac tissue. Functional characterizations including contractile responses and integration into new vessel formation in vivo support the opportunity to employ these cells for disease modelling, drug screening, and applications in cell-based therapies for regenerative medicine.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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References

HESCs-derived coronary-like vascular SMCs


