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Authors
Shen, Mengcheng
Quertermous, Thomas
Fischbein, Michael P
et al.

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REVIEW

Generation of Vascular Smooth Muscle Cells From Induced Pluripotent Stem Cells

Methods, Applications, and Considerations

Mengcheng Shen, Thomas Quertermous, Michael P. Fischbein, Joseph C. Wu

ABSTRACT: The developmental origin of vascular smooth muscle cells (VSMCs) has been increasingly recognized as a major determinant for regional susceptibility or resistance to vascular diseases. As a human material-based complement to animal models and human primary cultures, patient induced pluripotent stem cell iPSC-derived VSMCs have been leveraged to conduct basic research and develop therapeutic applications in vascular diseases. However, iPSC-VSMCs (induced pluripotent stem cell VSMCs) derived by most existing induction protocols are heterogeneous in developmental origins. In this review, we summarize signaling networks that govern in vivo cell fate decisions and in vitro derivation of distinct VSMC progenitors, as well as key regulators that terminally specify lineage-specific VSMCs. We then highlight the significance of leveraging patient-derived iPSC-VSMCs for vascular disease modeling, drug discovery, and vascular tissue engineering and discuss several obstacles that need to be circumvented to fully unleash the potential of induced pluripotent stem cells for precision vascular medicine.

Key Words: developmental biology ■ drug discovery ■ pluripotent stem cell ■ smooth muscle cell ■ tissue engineering ■ vascular diseases

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Vascular smooth muscle cells (VSMCs), the prominent cell type residing in the medial layer of major blood vessels, play a critical role in maintaining vascular wall integrity and blood pressure. Under physiological conditions, VSMCs are quiescent and express abundant contractile proteins, including α-SMA (alpha smooth muscle actin), SM22α, calponin, smoothelin, and SMMHC (smooth muscle myosin heavy chain).[1] However, VSMCs retain significant plasticity and, therefore, can undergo phenotypic switching and adopt a proinflammatory, proliferative, and synthetic phenotype in response to aging, vascular injury, or pathogenic cues.[1–3] Fate mapping studies revealed that VSMCs are derived from multiple developmental origins, such as the lateral plate mesoderm (LPM)-derived epicardium, LPM-derived second heart field (SHF), neural crest (NC), and paraxial mesoderm (PM)-derived sclerotome (Figure 1).[4] Notably, considerable evidence has shown that the developmental origin of VSMCs is a major determinant for regional propensity or resistance to vascular diseases, such as atherosclerosis,[5,6] vascular calcification,[7] and aortic aneurysm.[8,9] Thus, a better understanding of the intrinsic differences of signaling pathways that regulate the development, growth, and pathology between lineage-specific VSMC subtypes could advance the development of novel vascular therapeutics. As a complement to animal models and human primary cells, patient induced pluripotent stem cell (iPSC)-derived VSMCs are a virtually unlimited, immune-compatible cell source without ethical concerns, and have been increasingly used to study vascular diseases.[10–13] However, iPSC-VSMCs derived by most existing induction protocols are heterogeneous in developmental origins, which could hinder the translational efficacy of this model system.

In this review, we summarize the current knowledge of signaling networks that govern cell fate decisions of lineage-specific VSMC progenitors both in vivo and in vitro. We then briefly describe key regulators that terminally specify lineage-specific VSMCs. Finally, we discuss several promising applications and unmet challenges of leveraging patient-derived iPSC-VSMCs to achieve precision vascular medicine.

Correspondence to: Joseph C. Wu, MD, PhD, Stanford University School of Medicine, 265 Campus Dr, Rm G1120B, Stanford, CA 94305. Email joewu@stanford.edu

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Nonstandard Abbreviations and Acronyms

2/3D	2/3-dimensional
BMP	bone morphogenetic protein
CDM	chemically defined medium
CNC	cardiac neural crest
FGF	fibroblast growth factor
iPSC	induced pluripotent stem cell
LPM	lateral plate mesoderm
MRTF	myocardin-related transcription factor
NC	neural crest
PDGF	platelet-derived growth factor
PEO	proepicardium organ
PM	paraxial mesoderm
RA	retinoic acid
SHF	second heart field
SHH	sonic hedgehog
SMMHC	smooth muscle myosin heavy chain
TEVG	tissue-engineered vascular graft
TGF	transforming growth factor
VSMC	vascular smooth muscle cell
α-SMA	alpha smooth muscle actin

Developmental Ontogeny of VSMC Progenitor Types

The NC

The NC is a transient, multipotent, and migratory cell population from the dorsal neural plate border during gastrulation that gives rise to a plethora of neural and mesenchymal derivatives.[14] Molecular signals such as Wnt, FGF (fibroblast growth factor), Notch, and BMP (bone morphogenetic protein) are orchestrated in a highly sophisticated manner to regulate the induction, migration, specification, and differentiation of the NC.[15] Among these signals, an intermediate level of BMP signaling is crucial for NC induction and is fine-tuned by endogenous BMP antagonists (Figure 2).[16,17] The induction of the NC is initiated by the expression of neural plate border specifiers such as *Tfap2*, *Msx1/2*, *Dlx3/5*, *Pax3/7,* and *Zic1*.[14,18,19] These transcription factors act synergistically in a Wnt-dependent manner to activate NC specifiers such as *Snai2*, *Foxd3*, and *Sox9/10*.[18] Once premigratory NC cells reside within the dorsal neural tube, NC specifiers form an interconnected regulatory loop to promote epithelial-to-mesenchymal transition, delamination, migration, proliferation, and cell fate specification.[14,18,19]

The implementation of region-specific environmental cues along the rostral-to-caudal migratory route of delaminated NC cells synergistically patterns cranial, cardiac, vagal, and trunk NC subtypes, each of which contributes to a unique set of cell and tissue types.[18–20] The cardiac NC (CNC) is located at the most caudal region of the cranial NC. Fate mapping studies revealed that the CNC gives rise to VSMCs in the inner layer of the ascending aorta and arch, pulmonary trunk, ductus arteriosus, the brachiocephalic, right subclavian and carotid arteries, the septation of the cardiac outflow tract, and the face and forebrain (Figure 1).[21–24] However, the precise signaling networks that specifically imbue the CNC fate are not well understood.[25]

The Second Heart Field

The SHF is a subpopulation of the medial splanchnic mesoderm that can give rise to the myocardium, endothelial cells, and VSMCs.[26,27] During development, the SHF undergoes an anterior-to-posterior patterning in response to retinoic acid (RA) signaling. The anterior SHF, the progenitors that give rise to VSMCs in the aortic root and the outer layer of the ascending aorta (Figure 1),[28–30] expresses both general cardiac (eg, *Nkx2-5*, *Gata4*, and *Mef2c*) and region-specific (eg, *Isl1*, *Tbx1*, *Foxc1/2, Fgf8/10*, and *Foxh1*) markers.[31] Studies of diverse model organisms have revealed that FGF, Wnt, SHH (sonic hedgehog), Notch, and BMP signaling networks actively interact with SHF-selective markers to mediate the proliferation, maintenance, and differentiation of this progenitor type.[31]

The Epicardium

The epicardium is a sheet of mesothelium covering the outermost layer of the heart.[32] During development, a subset of epicardial cells undergo epithelial-to-mesenchymal transition in response to TGF (transforming growth factor)-β and PDGF (platelet-derived growth factor) signaling, and subsequently invade the underlying myocardium where they give rise to cardiac fibroblasts and coronary VSMCs (Figure 1).[33–40] However, discussions continue as to whether the epicardium contributes to the coronary endothelium.[37,38,41,42] Fate mapping studies have shown that the epicardium originates from the proepicardium organ (PEO), a transitory structure that arises from the posterior SHF-derived septum transversum.[43] Therefore, both the PEO and the epicardium are marked by SHF-selective markers *Nkx2-5* and *Isl1*.[38,44,45] Evidence shows that FGF signaling is required for the separation of the PEO lineage from the precardiac mesoderm, whereas BMP signaling is necessary for the specification of the PEO and the migration and attachment of its derivatives to the myocardium.[46] Using single-cell RNA sequencing technology, it has been shown that both the septum transversum and the PEO in the mouse embryonic heart are selectively marked by *Wt1*, *Tbx18*, *Tcf21*, *Sema3d*, and *Scx*, whereas the epicardium shares most of the epicardial genes with the PEO. However, *Raldh2*, a gene encoding RA synthesizing enzyme that regulates the expression of *Tcf21* and *Wt1*, marks the epicardium but not the PEO.[47–49]

The Sclerotome

The sclerotome, also known as ventral somitic mesoderm, originates from the PM[50] and gives rise to VSMCs in the

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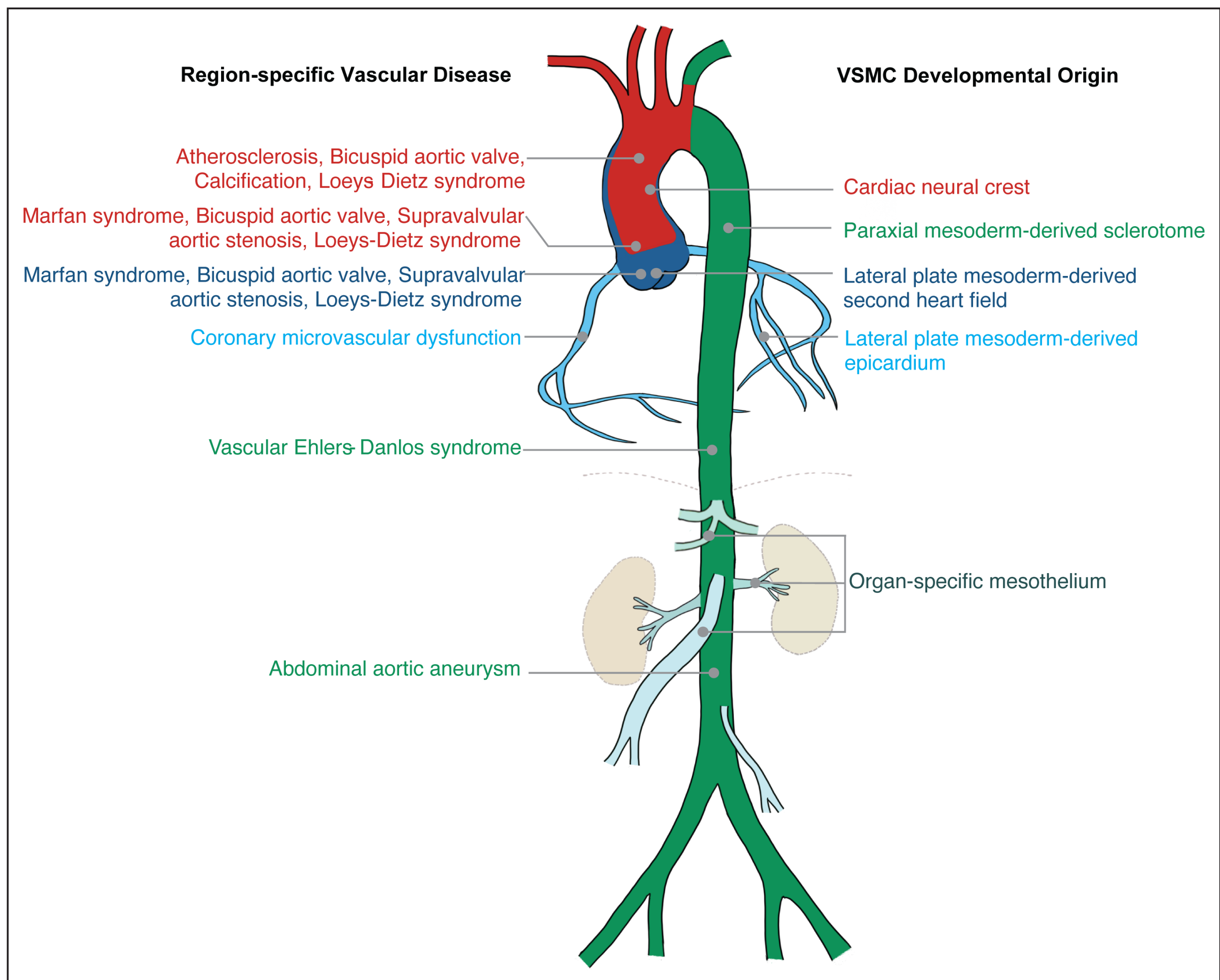

Figure 1. The developmental origin of vascular smooth muscle cells (VSMCs) is a major determinant for regional susceptibility to vascular diseases.
VSMCs residing in each color-coded (red, neural crest; dark blue, lateral plate mesoderm-derived second heart field; light blue, lateral plate mesoderm-derived epicardium; and green, paraxial mesoderm-derived sclerotome) region along the vascular tree originate from different developmental origins. Sharp boundaries with no intermixing of VSMCs of different lineages can be observed in the adjacent regions.

descending aorta and its main branches (Figure 1).[51–53] PM development is composed of 3 stages: presomitic mesoderm specification, somitogenesis, and somite specification.[50] While a low level of BMP signaling specifies the PM,[54] the canonical Wnt signaling is essential to PM development.[55] However, the precise roles of FGF and RA signaling in somitogenesis remain controversial.[56] The sclerotome, which is marked by *Pax1/9*, *Nkx3-2*, *Foxc2*, and *Sox9*, is derived from the ventromedial part of the somite by the interplay between Notch, FGF, Wnt, BMP, and SHH signaling pathways (Figure 2).[57,58]

Generation of Lineage-Specific iPSC-VSMC Progenitor Types

The first and only available induction protocol so far to systemically derive lineage-specific iPSC-VSMC subtypes was developed by the Sinha group.[59] In this protocol, the authors first generated 3 early-stage multipotent intermediate lineages, namely, neuroectoderm, LPM, and PM from iPSCs. They then treated each intermediate lineage with PDGF-BB and TGF-β1 to induce lineage-specific VSMC subtypes. However, the low specificity of the derived intermediate types raises concerns regarding the lineage authenticity of the terminally differentiated VSMC subtypes. Specifically, the PAX6$^+$ neuroectodermal cells are a population of ectodermal derivatives distinct from the SOX10$^+$ NC cells, and there is no evidence to support that PAX6$^+$ neuroectoderm has the potential to interconvert into the NC in vitro. Both the LPM and PM exhibit substantial multipotency and can give rise to diverse somatic cell types other than VSMCs. Thus, lineage-specific VSMC subtypes generated from their immediate progenitors (ie, CNC, SHF, epicardium, and sclerotome) represent better analogs of their in vivo counterparts. Interestingly, the investigators were able to further differentiate iPSC-LPM into the epicardium and

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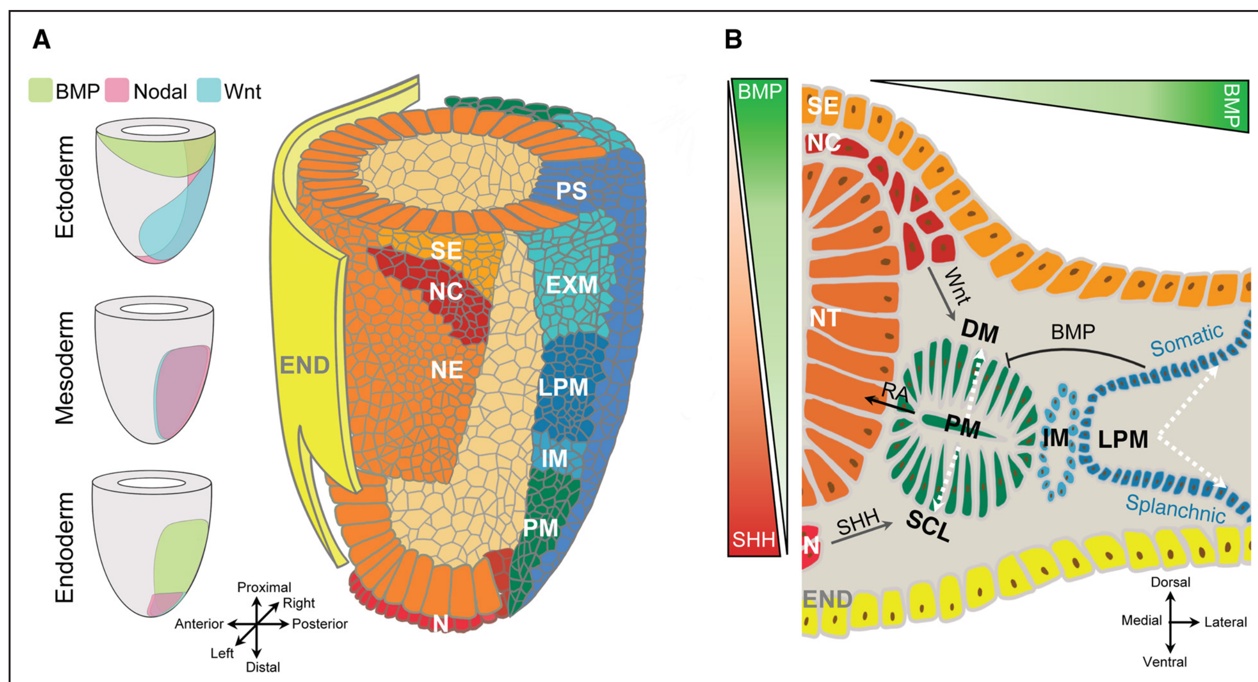

Figure 2. Schematics of key morphogenetic signals that specify different vascular smooth muscle cell (VSMC) progenitor types. **A**, Lineage specification signals and patterns in the gastrulating mouse embryo at embryonic day 7.5. The specification of the neural crest (NC) takes place in the proximal anterior region of the ectoderm. The divergence of mesodermal subtypes occurs along the posterior-to-anterior axis of the primitive streak. These processes are mediated predominantly by dynamic BMP (bone morphogenetic protein), Wnt, and nodal signaling gradients in each germ layer through autocrine, paracrine, and juxtacrine effects. Intermidate levels of FGF (fibroblast growth factor) and Notch signaling are expressed throughout the ectoderm (not shown). **B**, A planar view (the medial-to-lateral part of a cross section) of the NC and mesodermal subtypes. An intermediate level of BMP signaling is crucial for NC specification. High levels of BMP give rise to the lateral plate mesoderm (LPM), whereas low levels of BMP generate the paraxial mesoderm (PM). A posterior-to-anterior decreasing Wnt/FGF signaling gradient and an anterior-to-posterior decreasing retinoic acid (RA) signaling gradient enforce the specification of mesodermal subtypes (not shown). White dotted arrow lines indicate divergent progeny cell fates of the PM and the LPM. The combination of SHH (sonic hedgehog) activation and Wnt inhibition specifies the sclerotome (SCL). The SCL and the splanchnic mesoderm (the precursor of the SHF and the EPI) are mesodermal progenitors of VSMCs. DM indicates dermomyotome; END, endoderm; EXM, extraembryonic mesoderm; IM, intermediate mesoderm; N, notochord; NT, neural tube; NE, neuroectoderm; PS, primitive streak; and SE, surface edctoderm. Adapted from Peng et al[87] with permission. Copyright ©2019, Springer Nature.

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then coronary VSMCs,[60] suggesting that it is feasible to improve the lineage specificity of VSMCs by refining the induction conditions for the progenitor types.

With advances of in vivo evidence regarding the developmental signaling networks that specify the NC, SHF, epicardium, and sclerotome discussed in the previous section, several induction protocols for each progenitor type have been developed since the last decade. In general, the combination, dose, and timing of different morphogens (eg, TGF-β, FGF, Wnt, BMP, RA, and SHH) used to specify VSMC progenitor types vary drastically across protocols, as variations such as basal induction media, cell densities at the initiation of differentiation, and differentiation routes can significantly affect the specificity and yield of desired cell types (Tables 1 and 2).

Generation of the NC

During the early attempts to induce neural cells from human embryonic stem cells using either stromal cell-based co-culture systems[61–63] or neural rosette/embryoid body-based strategies,[64–66] a small fraction of NC cells spontaneously emerged in the cultures. However, some major drawbacks of these approaches were the use of mouse stromal cell lines, in which the precise contributions of specific signaling pathways required to generate the NC were elusive and the time-consuming (>21 to 40 days) and low efficiency (<5% before manual enrichment) nature of obtaining the NC.

In this regard, efforts have been devoted to optimizing combinations of Wnt, activin/nodal, FGF, and BMP signals in a time- and dose-dependent manner to maximize the induction efficiency of the NC. Under adherent culture conditions, the dual inhibition of BMP and activin/nodal signaling (termed dual Smad inhibition) of human pluripotent stem cells (hPSCs) in a gradient knockout serum replacement and N2 basal medium gave rise to predominantly neuroectodermal cells marked by PAX6 and SOX1, as well as a small fraction (<20%) of $PAX6^-$ NC cells.[67,68] A modified dual Smad inhibition protocol by the inclusion of a neural priming step was shown to significantly improve the NC induction efficiency to over 80% ($p75^+$ cells).[69] Interestingly, lowering the density of hPSCs at the initiation of neural differentiation also improved NC induction efficiency.[67,69]

Table 1. Summary of Differentiation Protocols for the Neural Crest From HPSCs in Monolayer Cultures

Reference	Induction method	Time	Markers	Efficiency	Remarks
Chambers et al[67]	1.8×10^4 hPSCs/cm^2 were treated with DSi in a gradient KSR/N2 medium	11 d	p75, HNK1, PAX7, TFAP2A	20% PAX6$^-$	Most derivatives were PAX6$^+$ neuroectodermal cells; a low cell density at the initiation of differentiation improved NC induction efficiency
Kreitzer et al[69]*	10^4 hPSCs/cm^2 were neural primed in an N2/B27 medium and then cultured in the DSi medium	8 d	p75, HNK1, TFAP2A, SOX10	80% p75$^+$	NC cells showed mixed axial identities (ie, cranial, cardiac, and trunk NC)
Mica et al[68]	(1.8–2.5)×10^4 hPSCs/cm^2 were treated with DSi and 2 days of CHIR in a gradient KSR/N2 medium	11 d	p75, HNK1, TFAP2A/B, PAX3/7, SNAI2, SOX9/10	40%–67% SOX10$^+$	The first study showed the necessity of Wnt signaling activation in NC induction; a p75$^+$/HNK$^+$/SOX10$^-$ non-NC cell population was observed; the NC did not arise from PAX6$^+$ NE; late Wnt activation directed cells to a NE fate; RA and FGF2 induced a vagal NC identity
Fattahi et al[79]	10^5 hPSCs/cm^2 were treated with DSi for 10 days, CHIR for the first 3 days, and RA starting at day 6 in a gradient KSR/N2 medium	11 d	CD49d, PAX3, FOXD3, TFAP2B, SOX10	62%–75% CD49d$^+$	The first study used CD49d for NC purification
Menendez et al[70]*	10^5 hPSCs/cm^2 were treated with BIO and SB in the StemPro medium	12 d	p75, HNK1, TFAP2A, SOX9/10, PAX3, ZIC1	>90% p75$^+$/HNK$^+$	Cell passaging during differentiation significantly enriched the NC population
Fukuta et al[71]*	20 hPSCs/cm^2 were treated with CHIR and SB in a CDM	7 d	p75, TFAP2A, TWIST, SNAI2, SOX9/10	70%–80% p75$^+$	Two p75 populations were generated, and the p75^{low} population expressed neural markers; RA induced cardiac and trunk NC fates; both BMP activation and inhibition repressed NC induction
Leung et al[72] Gomez et al[73]	2×10^4 hPSCs/cm^2 were treated with CHIR in a B27 medium	5 d	ZIC1, MSX1/2, SNAI2, PAX3/7, TFAP2A, SOX10	53%–72% SOX10$^+$	A 2-day pulse activation of Wnt signaling was sufficient to induce NC; BMP or FGF inhibition promoted a neural fate; TGF inhibition repressed NC induction; no SOX10 and PAX6 co-expression was observed
Tchieu et al[75]	(2.5–3)×10^5 hPSCs/cm^2 were treated with SB, gradient CHIR, and 2 days of 1 ng/mL BMP4 in an E6 medium	12 d	TFAP2A/B, PAX3, SOX10	30%–58% SOX10$^+$	High BMP4 induced a non-neural fate; TFAP2A/B are not specific markers for the NC
Hackland et al[76]*	10^4 hPSCs/cm^2 were treated with CHIR, SB, BMP4, and DMH1 in an N2 medium	7 d	p75, TFAP2A, PAX3, SOX10	80% SOX10$^+$	The first study precisely controlled BMP signaling by saturation of BMP ligands and partial inhibition of BMP receptor activity
Halaidych et al[170]*	10^5 hPSCs/cm^2 were treated with SB, CHIR, and FGF2 in a CDM	12 d	p75, HNK1, TFAP2A, SOX9/10	40%–50% p75$^+$/HNK$^+$	This protocol was modified from Cheung et al.[59] Results showed that Wnt signaling was essential to give rise to an NC fate; serial passaging improved NC purity to >90%

All protocols except one[75] conducted neural crest (NC) terminal differentiation experiments. Cranial NC is the default axial identity generated by most protocols, unless otherwise stated. BIO/CHIR99021 (CHIR), Wnt signaling activators; SB431542 (SB), TGF-β signaling inhibitor; DMH1, BMP type I receptor inhibitor. BMP indicates bone morphogenetic protein; CDM, chemically defined medium; DSi, dual Smad inhibition; FGF, fibroblast growth factor; hPSC, human pluripotent stem cell; KSR, knockout serum replacement; NC, neural crest; NE, neuroectoderm; RA, retinoic acid; and TGF, transforming growth factor.

*The derived NC is expandable in vitro.

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Wnt signaling has been implicated as a principal NC inducer in all vertebrates, with the canonical β-catenin-dependent signaling being required for the initial NC induction and the noncanonical signaling for the acquisition of a migratory NC phenotype.[14] Accordingly, the combination of an early pulsive Wnt activation and dual Smad inhibition has been shown to significantly divert the PAX6$^+$ neural fate toward the p75$^+$/HNK1$^+$ NC fate.[68] By contrast, using a knockout serum replacement-free chemically defined medium (CDM), activin/nodal inhibition and concurrent Wnt activation were sufficient to give rise to >90% p75$^+$/HNK1$^+$ NC cells.[70,71] In a B27-based basal medium, early activation of Wnt signaling alone was sufficient to give rise to the NC, whereas delayed activation of Wnt signaling skewed hPSCs to nonectodermal and placodal fates.[72,73] Notably, it has been shown that SOX10$^+$ and PAX6$^+$ cells in the same cultures did not exhibit a co-expression pattern or a spatial relationship. Moreover, knocking out *PAX6* promoted NC induction,[72] further supporting the claim that the PAX6$^+$ neuroectoderm-derived VSMCs are not of the NC lineage.[59]

It is evident that an intermediate level of BMP signaling is required for NC specification in vivo.[16,17] However, conflicting results were reported on the ways of modulating BMP signaling in vitro, with some studies showing that BMP inhibition was required for NC induction,[67–69,74] whereas others showed that BMP activation was essential for NC formation.[70,72,75] It has been suggested that variations of BMP ligand levels in different NC induction media may reconcile the contradictory BMP4 modulation strategies across these NC induction protocols: serum or knockout serum replacement-containing media may

Table 2. Summary of Differentiation Protocols for Mesodermal Subtypes From HPSCs in Chemically Defined Systems

Reference	Induction method	Cell type	Time	Markers	Efficiency	Remarks
Yang et al[91]	hESC-EBs were treated with activin A+BMP4+FGF2+DKK1+VEGF in the StemPro medium	CM	15 d	T, VEGFR2, ISL1, NKX2-5, TBX5/20	80% VEGFR2$^+$	Derived mixed lineages: cardiac (VEGFR2^{low}/c-Kit$^-$) and hematopoietic mesoderm (VEGFR2^{high}/c-Kit$^+$), and endoderm (VEGFR2$^-$/c-Kit$^+$)
Vallier et al[92]	hESCs were treated with activin A+FGF2+BMP4 for 2 days; SU5402+activin A for 3 days; and BMP4+FGF2+ SB for 4 days in a CDM	CM	9 d	T, GATA4/6, CXCR4, SOX17, EOMES, HAND1	21% PDGFRA$^+$	BMP4-dependent specification of mesendoderm; activin A signaling intensity specify mesoderm vs endoderm
Bernardo et al[93]; Cheung et al[59]	hPSCs were treated with FGF2+LY+BMP4 for 1.5 days, then either FGF2+BMP4 or FGF2+ LY for 3.5 days to generate LPM or PM, respectively, in a CDM	LPM and PM	5 d	T, EOMES; ISL, NXK2-5, MESP1, MIXL1, VEGFR2; TCF15, TBX6, MEOX1	70%–80% T$^+$; 77%–87% VEGFR2$^+$ for LPM; 60%–75% TCF15$^+$ for PM	Mesodermal subtypes were used to derive lineage-specific VSMCs
Umeda et al[171]	hPSCs were treated with BIO+Noggin+SB or activin A in a CDM	PM	8 d	MIXL1, MEOX1/2, TCF15, MESP2, PDGFRα	30%–60% VEGFR2$^-$/PDGFRA$^+$	Activation or inhibition of Wnt signaling in this protocol was determined by Wnt-induced endogenous nodal signaling across lines
Loh et al[97]	hPSCs were treated with day 1: activin A+CHIR+FGF2+PIK90 to induce APS; day 2: A83-01+CHIR+LDN+FGF2 to PM, day 3: A8301+LDN+C59+PD0325901, to ES days 4–6: SAG+C59 to SCL; day 1: activin A+ BMP4+CHIR+FGF2+PIK90 to MPS, day 2:A83-01+BMP4+C59 to LPM, days 3–4 A83-01+BMP4+C59+FGF2 to cardiac mesoderm in a CDM	CM, PM, SHF, ES, etc	4-6 d	T, EOMES; FOXF1, NKX2-5, ISL1, GATA4/6, HAND1, MEF2C, MIXL1, MESP1/2; MSGN1, CDX2, FOXC2, TBX6, MEOX1/2	>90% NKX2.5; >90% DLL1 for PM, >95% FOXC2 for ES	The first stepwise protocol that derived 12 highly pure mesodermal subtypes; identified surface markers for PM and CM
Xi et al[96]	hPSCs were treated with CHIR for 2 days, LDN+SB for 2 days, and SAG+FGF2 for 2 days in a CDM	PM, ES, and SCL	4–6 d	MEOX1, TCF15, PAX3, MSNG1, FOXC2; PAX1/9	90% PAX3$^+$/FOXC2$^+$ for ES	Identified BMP and TGF-β signaling are major regulators unique to human somitogenesis from human presomitic mesoderm RNA sequencing data
Nakajima et al[98]	hPSCs were treated with CHIR+SB+FGF2+DMH1 for 4 days to induce PM, CHIR+SB for 4 days to ES, and SAG+DMH1 for 3 days to SCL in a CDM	PM, ES, and SCL	4–11 d	MSGN1, TBX6, DLL1; MEOX1, TCF15, PAX3; PAX1/9, NKX3-2	86% DLL1$^+$/PAX3$^-$ for PM; 75% DLL1$^+$/PAX3$^+$ for ES; 45% PAX1/9$^+$ for SCL	The PM surface marker DLL1 identified by Koh et al.[172] was used for cell purification
Matsuda et al[100]	hiPSCs were treated with day 1: activin A+FGF2+CHIR, day 2: LDN+SB+CHIR+FGF2, day 3: PD173074+XAV939, day 4–6: SAG+LDN in a CDM	PM, ES, and SCL	5 d	T, MSGN1; MIXL1, TBX6, DLL1; MEOX1, FOXC2, TCF15, PAX3	Not specifically reported, but should be at least equivalent to the values from Koh et al.[172]	Modified from Koh et al,[172] and the ES was directly induced by FGF inhibition rather than MEK inhibition
Witty et al[94]	Day 4 hESC-EBs from Yang et al[91] were treated with BMP4+CHIR+SB+VEGF for 2 days in the StemPro medium	EPI	19 d	WT1, TBX18, ALDH1A2, ZO1	85% and 95% WT1$^+$ before and after replating	Preplated EPI expressed PDGFRA$^+$ cardiac progenitor genes
Iyer et al[60]	LPM from Cheung et al[59] were treated with BMP4+Wnt3a+RA for 10 days in a CDM	EPI	15 d	WT1, TBX18, TCF21	60% WT1$^+$	Generated three heterogenous EPI populations (WT1$^+$/TCF21$^+$, WT1$^+$/TCF21$^-$, and WT1$^-$/TCF21$^+$)
Bao et al[95]	hPSCs were sequentially treated with CHIR, IWP2, and CHIR in the RPMI medium	EPI	16 d	WT1, TBX18, TCF21, ALDH1A2,	95% WT1$^+$	Passage cells at a low density gave rise to homologous EPI; the EPI was renewable by inhibiting TGF-β signaling

A83-01/SB431542 (SB), TGF-β signaling inhibitors; BIO/CHIR99021(CHIR)/Wnt3a, Wnt signaling activators; C59/DKK1 (dickkopf homolog 1)/IWP2/XAV939, Wnt signaling inhibitors; DMH1/LDN193189 (LDN), BMP signaling inhibitors; LY294002 (LY)/PIK90, phosphoinositide 3-kinase inhibitors; PD173074/SU5402, FGF signaling inhibitors; PD0325901, MEK inhibitor; SAG, sonic hedgehog signaling agonist. APS/MPS indicates anterior/posterior primitive streak; BMP, bone morphogenetic protein; CDM, chemically defined medium; CM, cardiac mesoderm; EB, embryoid body; EPI, epicardium; ES, early somite; FGF, fibroblast growth factor; hiPSC/ESC, human induced pluripotent/embryonic stem cell; MEK, mitogen-activated protein kinase kinase; PDGFRA, platelet-derived growth factor receptor α; LPM, lateral plate mesoderm; PM, paraxial mesoderm; RA, retinoic acid; SCL, sclerotome; SHF, second heart filed; TGF, transforming growth factor; VSMC, vascular smooth muscle cell; and VEGF (vascular endothelial growth factor)/VEGFR, vascular endothelial growth factor/receptor.

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carry exogenous BMP ligands, which could exceed the threshold of BMP required for NC induction and would, therefore, require BMP inhibition. On the contrary, CDM does not contain enough BMP ligands at the initiation of NC differentiation and thereby requires BMP activation to specify an NC fate. A high cell density at the initiation of NC induction can also increase endogenous BMP levels. To resolve this BMP modulation strategy paradox, a protocol termed top-down inhibition was developed.[76] In particular, the authors saturated all the BMP receptors in the culture by adding an excessive amount of BMP4 ligand and fine-tuned BMP signaling activity by titrating the concentration of a BMP receptor type 1 inhibitor to achieve a partial inhibitory effect. The inclusion of a Wnt agonist and a TGF-β inhibitor in the same culture medium in conjunction with a low cell seeding density strategy synergistically improved the intra- and inter-line NC induction efficiency. Once the NC was generated, low levels of FGF2 were used for cell renewal.[62,64,71,77]

In most NC induction protocols, 2 surface markers, p75 and HNK1 were most commonly used to enrich the NC population.[62,64,67,69–71,74] However, both HNK1 and p75 are also expressed in non-NC populations[78] and even in hPSCs.[76] Indeed, some protocols were shown to give rise to 2 p75 populations. While bona fide NC markers such as SOX10 were found to be enriched predominantly in the p75^{high} population, neural markers such as PAX6 and SOX1/2 were exclusively expressed in the p75^{low} fraction.[70,71,76] TFAP2A, a commonly used NC marker, was also highly expressed in non-neural ectodermal cells.[72,75] Interestingly, CD49d/α4 integrin was found to be a cell surface marker that reliably marked SOX10^{+} NC, and therefore, may be used to purify or enrich bona fide NC.[79,80] The NC generated by most protocols exhibited a cranial identity.[67,70–76] However, FGF signaling has been implicated in caudalizing the cranial NC to acquire a cardiac or vagal identity in addition to supporting NC survival and proliferation.[68,71] Similarly, RA can promote an anterior-to-posterior identity transition of the NC,[71,81,82] although early RA treatment derailed NC specification by promoting a neural fate.[82] However, a protocol to precisely derive a pure culture of CNC is not yet available. Since *MAFB* has been recently identified as a marker specific to the CNC,[83] it may be used to generate a reporter system to refine the differentiation conditions for this particular NC subtype. A brief summary of monolayer-based NC induction protocols is shown in Table 1.

Generation of Mesodermal Subtypes

It is now well accepted that the induction and patterning of mesoderm subtypes are regulated by extensive crosstalk among Wnt, FGF, BMP, activin/nodal, and RA signaling.[84–87] A gradient of BMP activities along the mediolateral axis is conserved across species to drive a posterior-to-anterior patterning of the mesoderm and gives rise to several subtypes, namely, axial, paraxial, intermediate, and LPM.[88] As the organism matures, the LPM segregates into the precardiac mesoderm, which further diverges based on the levels of BMP signaling and gives rise to the SHF and the PEO/epicardium.[89] Meanwhile, the PM undergoes somitogenesis to generate blocks of somitic cells that further specify into the sclerotome or the dermomyotome in response to SHH or Wnt signaling, respectively (Figure 2).[90]

Although embryoid bodies were the first widely used strategy to generate different early embryonic tissues, including the mesoderm, the undefined culture conditions and heterogenous derivatives make it challenging to identify specific factors required to induce mesodermal subtypes and their derivatives. Moreover, the paucity of lineage-specific surface markers prevents manual purification of a desired mesodermal subtype. To circumvent these challenges, several chemically defined differentiation protocols have been developed in the last decade (Table 2). The induction protocols of the precardiac mesoderm, the precursor for both cardiomyocytes and lineage-specific VSMC subtypes (ie, SHF and epicardium), have been extensively developed by researchers in the cardiac field. The pioneering work by Yang et al[91] showed that treating human embryonic stem cell-embryoid bodies with activin A, BMP4, and FGF2 can induce a primitive streak-like population marked by *T* and VEGFR2. These progenitors were further propagated and specified by a Wnt signaling inhibitor DKK1 (dickkopf homolog 1), VEGF (vascular endothelial growth factor), and FGF2 to generate heterogenous mesodermal subtypes that can give rise to cardiac, endothelial, and VSMC populations. By contrast, a later study using the same set of growth factors in a chemically defined monolayer culture system showed that BMP4 is a major inducer of mesendoderm, the precursor of the mesoderm and the endoderm. Activation or inhibition of activin/nodal signaling at a later stage of differentiation increased the proportion of definitive endoderm or mesoderm, respectively.[92] Accordingly, treating human embryonic stem cells with BMP4 and FGF2 in the absence of activin A was shown to direct mesodermal derivatives marked by *T* and *CDX2*. Although the induction efficiency of mesoderm was shown to be greatly enhanced by a phosphoinositide 3-kinase inhibitor (LY294002), several mesodermal subtypes were observed in the cultures.[93] Subsequently, Cheung et al[59] optimized the BMP levels in the original protocol[93] and treated hPSCs with BMP4, FGF2, and LY294002 to derive early mesoderm, which was further treated with either BMP4 and FGF2 to generate the LPM or LY294002 and FGF2 to generate the PM. Activating BMP, Wnt, and RA signaling in the LPM was shown to give rise to the epicardium.[60] Consistently, embryoid body-derived PDGFRA^{+} mesodermal cells[91] were also shown to generate the epicardium after being exposed to BMP and Wnt signals.[94] However, stage-restricted activation and inhibition of Wnt signaling alone have been shown to be sufficient to derive a homogenous population of the epicardium (>90% WT1^{+}),[95] raising the

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question of the necessity of BMP signaling for a robust epicardium differentiation. Intriguingly, the same study also showed that the inhibition of activin/nodal signaling enabled long-term renewal of the epicardium in a xeno-free, chemically defined condition, which could open up an avenue for large-scale production of this cell type for cardiac regenerative medicine.[95]

The induction of the PM, an early precursor that can specify into the dermomyotome and the sclerotome, has been shown to require activation of Wnt signaling and concomitant inhibition of BMP and activin/nodal signaling.[96–100] In vivo evidence suggests that oscillation of Notch signaling and the antagonizing effects of Wnt/FGF signaling are essential for somitogenesis.[90] Although several studies tried to recapitulate this signal transition pattern in vitro by the manipulation of Wnt and FGF/ERK signaling,[97,100] activation of Wnt signaling alone seems to be sufficient to elicit the same effect.[98,101] Inhibition of BMP signaling not only promotes a medial mesodermal fate (eg, PM), but also directs somite specification.[97,98,100] SHH signaling has been shown to be indispensable for sclerotome induction,[58,102] and the combination of an SHH agonist and a Wnt inhibitor recapitulates the antagonizing relationship of the 2 signaling pathways at this developmental stage (Figure 2).[97,102]

Using stepwise strategies, several induction protocols have generated highly homogenous populations of the PM and its derivatives. For example, a careful modulation of the minimal inductive and inhibitory signals concomitantly at each fate-decision bifurcation during mesoderm specification led to the generation of 12 highly pure mesodermal subtypes, including early mesoderm (>98% $MIXL1^+$), the precardiac mesoderm (>90% $NKX2\text{-}5^+$), and the PM (>92% $TBX6^+/CDX2^+$).[90] Notably, the transcriptional profile of the precardiac mesoderm generated by this protocol mirrors that of the SHF. As such, it can be used to specify SHF-VSMCs. Two recent stepwise induction protocols, which generated homogeneous somitic mesoderm[100] and septum transversum,[103] can be readily refined to derive the sclerotome and the epicardium by manipulating Wnt/SHH[97,100] and RA signaling,[60] respectively.

The field is advancing as a fast pace. Collectively, it is now feasible to derive definitive VSMC progenitor types from iPSCs by recapitulating the dynamic interplay of diverse morphogenetic signals along their developmental trajectories in vitro. The derived definitive progenitor types can be further specified into physiologically relevant, lineage-specific VSMC subtypes via a complex signaling network (Figure 3).

Signaling Pathways in Lineage-Specific VSMC Specifications and Functions

VSMC specification has been shown to be tightly regulated by serum response factor-myocardin coactivator

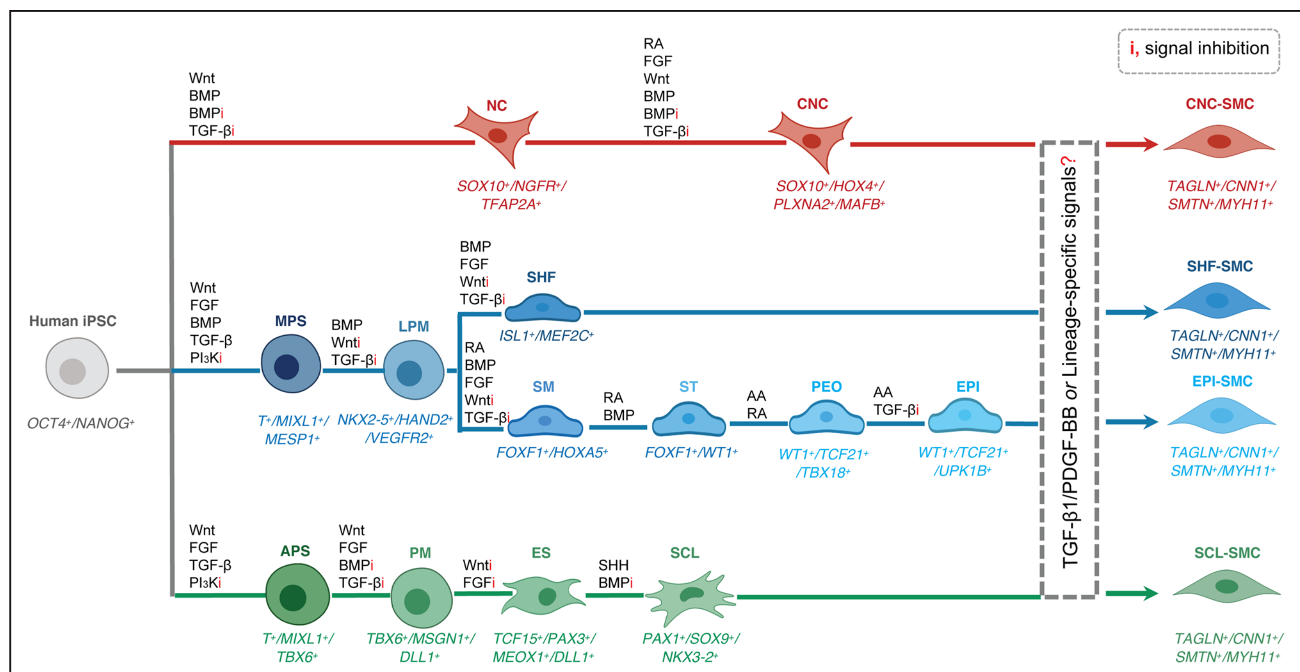

Figure 3. A proposed stepwise induction protocol to derive lineage-specific vascular smooth muscle cell (VSMC) subtypes from human induced pluripotent stem cells (iPSCs).
The proposed strategy to derive lineage-specific VSMC subtypes from human iPSCs can be achieved by integrating recently developed stepwise induction protocols for definitive progenitor types with VSMC-specifiying growth factors. Key morphogenetic signals and selective markers that specify each cell type are listed. AA indicates ascorbic acid; APS/MPS, anterior/posterior primitive streak; BMP, bone morphogenetic protein; CNC, cardiac neural crest; EPI, epicardium; ES, early somite; FGF, fibroblast growth factor; LPM, lateral plate mesoderm; NC, neural crest; PDGF, platelet-derived growth factor; PEO, proepicardial organ; PI_3K, phosphoinositide 3-kinase; PM, paraxial mesoderm; RA, retinoic acid; SCL, sclerotome; SHF, second heart field; SHH, sonic hedgehog; SM, splanchnic mesoderm; ST, septum transversum; and TGF, transforming growth factor.

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complex, TGF-β, PDGF, Notch, RA, reactive oxygen species, RhoA, ECM, and noncoding RNAs.[104–106] Several of them have been identified to show lineage-specific effects. Myocardin and MRTF (myocardin-related transcription factors)-A/B, transcriptional coactivators of serum response factor, have been shown to play unique roles in the differentiation of lineage-specific VSMCs.[104] While knockout of myocardin in the mouse resulted in embryonic lethality partially due to failure of VSMC differentiation of the somitic origin,[107,108] deletion of *Mrtfb* specifically led to defective VSMC differentiation of the CNC origin.[109,110] By contrast, VSMCs in *Mrtfa*-null mice were phenotypically normal.[111,112] TGF-β1-mediated specification of NC-VSMCs and PM-SMCs is regulated by Smad2/MRTF-B and Smad3/myocardin, respectively.[113–115]

In addition to directing early specification of lineage-specific VSMCs, several signaling regulators (eg, Notch, TGF-β, and PDGF) have been shown to lead to variations in growth, migration, metabolism, matrix production, and functional properties of VSMC subtypes at maturity.[1,9,104–106,116] Accumulating evidence also suggests that *KLF4*, a pluripotency factor, is repressed during VSMC differentiation.[117–120] By contrast, the induction of *KLF4* is essential for reprogramming differentiated VSMCs to multipotent progenitors, which can generate diverse mesenchymal cell types in response to pathogenic stressors and contribute to vascular diseases.[121–125] Notably, it has been shown that *KLF4*-dependent activation of the noncanonical p38 pathway in Marfan patient iPSC-derived neuroectoderm-VSMCs, but not in mesoderm-VSMCs. This partially accounted for the diseased phenotype via enhanced cell apoptosis.[126] Therefore, modulation of *KLF4* activity may represent an effective route (1) to improve the yield and purity of iPSC-VSMCs during differentiation and maintenance and (2) to preserve the cell identity and functional properties of differentiated iPSC-VSMCs. Since it has been shown that *KLF4* is a major target of various microRNAs during VSMC development,[117–120] it would be interesting to determine whether *KLF4* is regulated by distinct microRNAs in lineage-specific VSMCs and their progenitors.

Given that TGF-β1 and PDGF-BB have been unequivocally used to specify diverse progenitor types into VSMCs in vitro,[127,128] it would be meaningful to identify whether manipulation of other above-mentioned signaling pathways can direct lineage-specific progenitors to form VSMC subtypes that better recapitulate their in vivo counterparts.

Strengths and Challenges of Using Lineage-Specific iPSC-VSMCs for Basic Research and Clinical Applications

As most therapeutic discoveries obtained from animal models are ineffective in human clinical trials,[129] patient-specific iPSCs which carry both the causal genetic defects and permissive genetic background have been increasingly used as a powerful tool for disease modeling, drug discovery, and regenerative medicine.[130–132] To date, patient-specific iPSC-VSMCs (induced pluripotent stem cell VSMCs) have been used to model a handful of vascular diseases such as Hutchinson-Gilford progeria syndrome,[133–139] supravalvular aortic stenosis,[140–143] hypertension,[144] aortic aneurysm,[126,145,146] and atherosclerosis.[147] In general, these studies demonstrated that patient-derived iPSC-VSMCs could recapitulate several pathological features of vascular diseases. Thereby, this technology may provide insight into pathogenic mechanisms and therapeutic targets (Table 3). However, the fidelity of vascular disease modeling can be significantly affected by iPSC-VSMC quality (eg, maturity, homogeneity, and lineage specificity) and culture modality (eg, culture medium compositions, culture system complexity, and mechanical forces), neither of which was adequately specified in most studies.

To faithfully recapitulate vascular disease phenotypes in vitro, iPSC-VSMCs should closely resemble their in vivo counterparts at both molecular and functional levels. Therefore, before performing downstream experiments, VSMC identity should be verified by evaluating the expression levels of VSMC-specific contractile markers and the contraction responses to vasoconstrictors such as carbachol. It should be noted that since most VSMC markers such as α-SMA, SM22α, and calponin are also detectable in other cell types under certain conditions, it is important to tease out the expression levels of mature VSMC markers smoothelin and SMMHC in differentiated cells.[2] However, the 2 markers are not always readily detectable in iPSC-VSMCs, as immaturity is an inherent limitation of virtually all stem cell-derived cell types in vitro. Thus, to promote VSMC maturation, several strategies with low-to-moderate efficacy such as small molecules or growth factors,[148–152] mechanical stretching,[153] and endothelial cell co-culture[154] have been used. More effective VSMC maturation-promoting small molecules are expected to be discovered. It should also be noted that some hereditary thoracic aortic aneurysms are associated with mutated genes encoding contractile proteins such as *ACTA2* and *MYH11*, which would inevitably lead to suppressed expression of these markers in VSMCs. In such cases, it is crucial to include isogenic controls to help distinguish poor quality differentiations from genuine disease phenotypes of the examined VSMCs. Isogenic controls are also important in conditions where diseases are caused by multi-variants or exhibit mild clinical manifestations. Thus far, isogenic controls were rarely used in vascular disease modeling studies (Table 3). Finally, the inherent phenotypic plasticity of VSMCs can suppress the expression of all contractile markers. Therefore, VSMC identity should be verified before cells are exposed to synthetic phenotype-promoting cues, such as serum or inflammatory cytokines.

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Table 3. Summary of Studies Using iPSC-Derived VSMCs for Vascular Disease Modeling

Reference	Vascular disease	Methods	SMC induction medium and time	SMC markers	Induction efficiency	Lineage specificity	Remarks
Zhang et al[133]*	HGPS	EB-MSCs	EGM-2 medium+5 mmol/L SPC+2 ng/mL TGF-β1, 21 days	ACTA2, CNN1, TAGLN, CALD1, SMTNB, MYH11	NR	Mesoderm	Checked phenotypes of other vascular cell types.
Liu et al[134]	HGPS	CD34+	SmGM-2 medium, not stated	CNN1	NR	Mesoderm	Identified DNA-dependent protein kinase catalytic subunit as a pathogenic driver
Zhang et al[135]*	HGPS	EB	SmGM-2 medium +5% FBS, 31 days	ACTA2, CNN1	NR	NR	Identified PARP1-dependent cell death accounted for diseased phenotypes
Atchison et al[136]*	HGPS	EB/TEBV	SmGM-2 medium +1%-5% FBS, 31 days	ACTA2, CNN1	NR	NR	Generated a 3D structure and applied physiological shear stress to the TEBVs
Ribas et al[137]*	HGPS	EB- CD34+/VOC	SmGM-2 medium+RA or PDGF-BB, not stated	ACTA2, CNN1, TAGLN, MYH11	70% ACTA2	Mesoderm	Employed a 3D structure with ECs, observed lovastatin and lonafarnib rescued strain-induced proinflammatory phenotypes
Bersini et al[138]*	HGPS	Direct reprogramming	DMEM medium +10% FBS, 7 days	ACTA2, CNN1	80% CNN1	NR	Diseased VSMCs showed BMP4 overexpression and damaged EC barrier
Atchison et al[139]*	HGPS	Monolayer/TEBV	Activin A (2 ng/mL)+ PDGF-BB (10 ng/mL)+ heparin (2 µg/mL) in a B27/N2 medium, 6 days	ACTA2, CNN1, MYH11	90% CNN1	Mesoderm	Identified everolimus increased vasoreactivity and improved HGPS-VSMC differentiation using the TEBV model
Ge et al[140]	SVAS	EB	SmGM-2 medium +5% FBS, 24 days	ACTA2, CNN1	96% CNN1	NR	Identified enhanced ERK1/2 responsible for VSMC hyperproliferation, recombinant elastin and RhoA rescued the diseased phenotype
Kinnear et al[141]*	SVAS	EB	Medium 231+5% FBS, 17 days	ACTA2, CNN1	92% CNN1	NR	Identified elastin-binding protein ligand 2 and rapamycin rescued diseased phenotype
Dash et al[142]*	SVAS	EB/TEVR	SmGM-2 medium, 21 days	ACTA2, CNN1, TAGLN, MYH11	92% CNN1	Putative LPM	TEVR retained high levels of SMC markers
Kinnear et al[143]*	SVAS	EB/biowires	Medium 231+5% FBS, 18 days	TAGLN	90% TAGLN	NR	Identified everolimus restored SVAS-VSMC functions; verapamil increased SMC differentiation and reduced proliferation
Biel et al[144]*	HNT	EB-CD40b+/CD91+	Medium 231+5% FBS, 14 days	TAGLN, MYH11, MLC, MLCK, CALM1	50% TAGLN	NR	Proposed that iPSC-VSMCs (induced pluripotent stem cell VSMCs) from hypertension patients are an ideal platform to identify SNPs in hypertension pharmacogenomics
Toyohara et al[147]	ATH	Monolayer	Activin A (12.5 ng/mL)+PDGF-BB (12.5 ng/mL) in a B27/N2 medium, 6 days	ACTA2, CNN1	NR	Mesoderm	Identified lower AADAC level in type 2 diabetes patients was responsible for higher risks of atherosclerosis.
Jiao et al[145]*	BAV-TAA	Monolayer	15% KSR+TGF-β1 (2 ng/mL) in a DMEM/N2 medium, 18-19 days	ACTA2, CNN1, TAGLN, MYH11	NC-SMC: 70% MYH11; PM-SMC: 90% CNN1	NC and PM	Identified rapamycin rescued aberrant differentiation of BAV-NC-SMCs, and BAV-PM-SMC phenotype was normal.
Granata et al[126]*	MFS-TAA	Monolayer	TGF-β1 (2 ng/mL) + PDGF-BB (10 ng/mL) in a CDM, 17–19 days	ACTA2, CNN1	NR	NE, LPM, and PM	Used isogenic controls; losartan partially rescued diseased phenotypes in NE-SMCs; KLF4-mediated noncanonical p38 pathway regulated NE-SMC apoptosis
Gong et al[146]*	LDS-TAA	Monolayer/TEVR	SmGM-2 medium +5% FBS, 31 days	ACTA2, CNN1, TAGLN, MYH11	NR	NC, CPC	Used a 3D model to better recapitulate diseased SMC phenotype, de novo generated isogenic mutation lines, LDS-CPC-SMCs showed disrupted TGF-β signaling

3D indicates 3-dimensional; ACTA2, alpha smooth muscle actin; ATH, atherosclerosis; BAV, bicuspid aortic valve; BMP, bone morphogenetic protein; CALD1, caldesmon; CDM chemically defined medium; CNN1, calponin; CPC, cardiac progenitor cell; EB, embryoid body; EC, endothelial cell; FBS, fetal bovine serum; HGPS, Hutchinson-Gilford progeria syndrome; HNT, hypertension; KSR, knockout serum replacement; iPSC, induced pluripotent stem cell; LDS, Loeys-Dietz syndrome; LPM, lateral plate mesoderm; MFS, Marfan syndrome; MSC, mesenchymal stem cell; MYH11, smooth muscle myosin heavy chain; NE, neuroectoderm; NR, not reported; PARP-1, poly(ADP-ribose) polymerase-1; PDGF, platelet-derived growth factor; PM, paraxial mesoderm; RA, retinoic acid; SMTNB, smoothelin-B; SPC, sphingosylphosphorylcholine; SVAS, supravalvular aortic stenosis; TAA, thoracic aortic aneurysm; TAGLN, SM22α; TEBV, tissue-engineered blood vessel; TEVR, tissue-engineered vascular ring; TGF, transforming growth factor; VOC, vasculature-on-chips; and VSMC, vascular smooth muscle cell.

*Contraction assays were performed to validate VSMC identity.

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Cell heterogeneity, a net outcome of variable proportions of developmentally and phenotypically distinct VSMC subtypes as well as non-VSMCs in the same differentiating cultures, can result in false genotype-phenotype correlations. However, as indicated in Table 3, most studies failed to report the purity, phenotypic status, or lineage specificity of iPSC-VSMCs. One common strategy to overcome cell heterogeneity is through immunophenotyping-based cell sorting, which relies heavily on the availability of cell type-specific cell surface markers and reliable antibodies. However, cell surface markers for VSMCs have yet to be identified. In this regard, the combination of multi-omics, cell surface capture technology, high-resolution mass spectrometry, and bioinformatics has been proposed to be a promising strategy to discover lineage- and phenotype-specific VSMC surface markers in the near future.[124]

Another challenge for iPSC-based disease modeling is to recapitulate in vivo environmental conditions that are typically not present or difficult to model under 2-dimensional (2D) conditions, which only captures a reductive snapshot of the disease. In this regard, several recent studies have adopted 3-dimensional (3D) cultures such as self-assembled vascular organoids,[155,156] tissue-engineered vascular rings,[142,146] vasculature-on-chips,[137,157,158] and tissue-engineered blood vessels[136,139] to model vascular diseases. Among these 3D models, vascular organoids share several inherent limitations with embryoid body-derived VSMCs, such as cell heterogeneity, lineage nonspecificity, and necrotic core formation. Therefore, this model may lead to significant intra- and inter-line variabilities.

Tissue-engineered vascular rings, a homologous 3D structure composed of only VSMCs, have been shown to be able to change their circumference or force generation in response to vasoconstrictors.[142] Since in vivo evidence shows that some vascular pathological phenotypes were only detectable after mechanical stretching,[159] it would be essential to evaluate the effect of mechanical contraction on VSMC dysfunction in vitro. Hypertension and supravalvular aortic stenosis are associated with changes in VSMC-mediated vascular contraction, both of which were initially modeled in monolayer conditions.[140,141,144] Therefore, it would be logical to re-examine whether novel phenotypes and pathogenic mechanisms can be observed in both diseases using this particular 3D model. Vasculature-on-chips are constructed in a native vascular architecture using purified iPSC-derived vascular cells. This permits the inclusion of different coating matrix, mechanical forces, as well as cell-cell and cell-matrix interactions to better recapitulate the in vivo environment.[158] However, the scalability of this system is largely restricted by the availability of equipped auxiliary devices. Alternatively, tissue-engineered blood vessels, a simplified version of vasculature-on-chips initially developed by Fernandez et al,[160] can be used in large scale. Interestingly, a recent study from the same group reported that this system could recapitulate the disease phenotypes of Hutchinson-Gilford Progeria syndrome, thereby dissecting differential pathogenic roles between VSMCs and endothelial cells.[139] Moreover, the investigators identified a rapamycin analog everolimus that can improve VSMC differentiation and vasoreactivity.[139] Collectively, 3D vascular models are shown to be superior to 2D models in vascular disease modeling. However, with the inclusion of other vascular types and extracellular matrix, it is crucial to take into account relative ratios and natural spatial arrangement between different cell types.[157] An inappropriate integration of these cell types could obscure phenotypic differences that may otherwise be distinguishable between control and diseased groups.

Since patient-derived iPSC-VSMCs have been shown to effectively recapitulate vascular pathophysiology in vitro (Table 3), this powerful tool may be used to predict the severity of vascular diseases on a case-by-case basis via establishing genotype-phenotype correlations. In time, clinical interventions can be implemented to achieve personalized medicine. Patient-specific VSMCs have also been used to conduct drug screening in both 2D and 3D formats.[136,139,142,144] Due to the readily scalable nature of 2D cultures, it is feasible to test the responses of patient-specific VSMCs to various drugs and analyze a set of readouts such as contraction, proliferation, and apoptosis in an automated and high throughput manner. Once several of the most promising drug targets are identified from hundreds of thousands of candidates, they can be further tested in a more complex and physiological setup such as the vasculature-on-chips platform to narrow down the choices. Incorporating a microfluidic device and other vascular cell types in an appropriate ratio and architecture may make the responses of VSMCs to tested drugs more proximate to the in vivo conditions. In addition to screening novel vascular therapeutics, this platform can also be adapted to test drug-induced cytotoxicity, which has proven to be effective on iPSC-derived cardiomyocytes.[161,162] Recently, researchers ponder whether administering quinolone antibiotics could increase the risk of aortic dissection and rupture.[163,164] In this regard, it would be interesting to test the responses of VSMCs derived from patients with hereditary thoracic aortic aneurysms to quinolones. We expect these outcomes could provide some mechanistic insights to parse out what has been observed clinically (Figure 4).

With the advances in bioengineering tools, iPSC-VSMCs have also been increasingly used to develop tissue-engineered vascular grafts (TEVGs).[165–167] In general, iPSC-VSMCs are seeded alone or in combination with endothelial cells on natural or synthetic nanofibrous scaffolds to form TEVGs, which have been shown to form vascular structures when implanted in SCID mice. Thus, in cases where autologous vessels are not a viable therapeutic option for patients, iPSC-derived TEVGs represent

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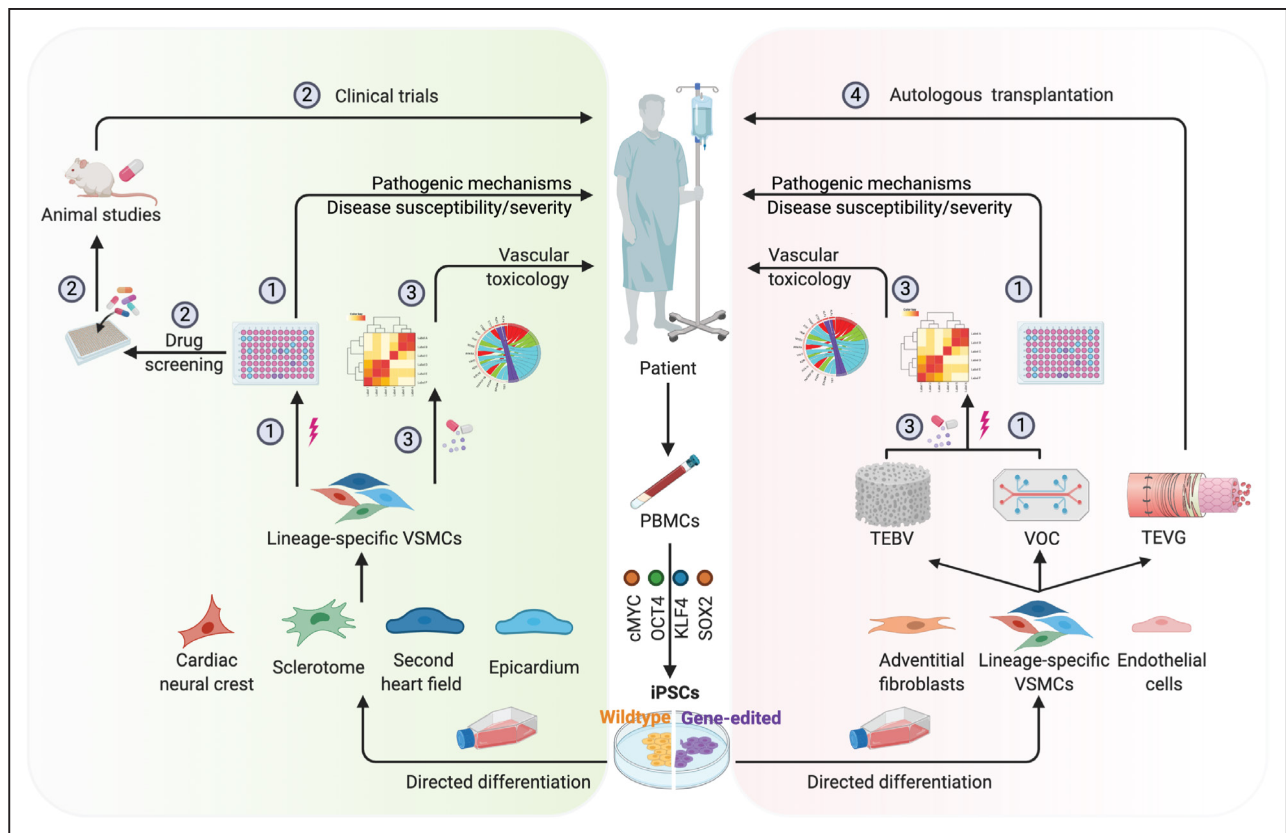

Figure 4. An overview of basic and translational applications of patient induced pluripotent stem cell (iPSC)-derived lineage-specific vascular smooth muscle cell (VSMC) subtypes.

With advances in iPSC biology and genome editing (to generate isogenic controls or correct pathogenic mutations), patient-derived lineage-specific iPSC-VSMCs (induced pluripotent stem cell VSMCs) can be used for (1) vascular disease modeling and susceptibility/severity prediction, (2) high throughput drug screening, and (3) candidate drug validation or drug-induced vascular toxicity evaluation in both 2D and 3D platforms. With the development of tissue engineering, (4) vascular grafts constructed from patients' iPSC-derived vascular cells are expected to be used for autologous transplantation in the future. PBMC indicates peripheral blood mononuclear cell; TEBV, tissue-engineered blood vessel; TEVG, tissue-engineered vascular graft; and VOC, vasculature-on-chips.

a promising alternative. Importantly, with more progress being made on refining protocols for precision genome editing and lineage-specific VSMC subtypes, mutation corrected and disease-resistant VSMC subtypes can be generated from patients' iPSCs. Once generated, VSMC subtypes can be constructed into immune-compatible TEVGs to stimulate in situ tissue repair (Figure 4). However, it takes several months to establish, characterize, and differentiate a new iPSC line to lineage-specific VSMCs and construct them into patient-specific TEVGs. The timeline can be even longer when gene editing is required. Therefore, this time-consuming and costly approach is more suitable for treating chronic vascular diseases rather than acute conditions. As an alternative, off-the-shelf, haplotype-matched, and immunocompatible iPSC-TEVGs could be used for allogenic implantation on any patient, which would greatly broaden the application flexibility and reduce the cost for future clinical practice.[167,168]

The applications of iPSC-VSMCs in regenerative medicine are appealing. Yet, numerous obstacles remain to be overcome before this tool is used in clinical practice. The primary concern of using TEVGs is the risk of tumorigenicity, as it has been reported that 25% of the grafts in SCID mice formed teratomas.[169] Therefore, one solution is to meticulously clean up undifferentiated cells before clinical use. More efforts should also be devoted to improving the survivability and integration efficiency of implanted TEVGs into the host vessel wall while maintaining the phenotypic and functional properties of iPSC-derived VSMCs within the grafts to minimize the risk of thrombosis, dilation, or rupture of the host vessel.[166]

CONCLUDING REMARKS

It is now evident that the development and pathophysiological functions of distinct regions of the vasculature are partially attributed to the developmental origin of VSMCs. Signaling pathways governing the development and phenotypic modulation of VSMCs are largely overlapped in vivo. Since patient iPSC-derived VSMCs share similar developmental trajectories and responses to pathological stimuli with their in vivo counterparts, they have been increasingly used to model diverse

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vascular diseases with success. However, the heterogeneity, immaturity, and lineage nonspecificity of iPSC-VSMCs and the oversimplified monoculture conditions could significantly confound the interpretation of observed phenotypes and translational values. To overcome these hurdles, stepwise, chemically defined protocols are being developed to derive lineage-specific iPSC-VSMC subtypes. Importantly, definitive progenitor types that are derived during iPSC-VSMC differentiation can serve as checkpoints to verify both cell population purity and lineage specificity.

With the advent of iPSC biology and multi-omics technologies, our knowledge in the understanding of the dynamic signaling networks that govern VSMC specification has considerably advanced over the years. This will expedite the refinement of existing protocols to generate more homologous and physiologically relevant iPSC-VSMC subtypes. The use of epigenetic mapping, chromatin accessibility, and other genomic features support this effort. Moreover, small molecule screening can be used to promote VSMC maturation and to enrich desired lineage-specific cell populations. Finally, developing a complex system that closely approximates the in vivo environment by including microenvironmental factors such as 3D structures and mechanical forces can further harness the power of iPSC-VSMCs in vascular disease modeling, drug screening, and personalized medicine.

ARTICLE INFORMATION

Affiliations

Stanford Cardiovascular Institute (M.S., T.Q., M.P.F., J.C.W.). Division of Cardiovascular Medicine, Department of Medicine (M.S., T.Q., J.C.W.), Department of Cardiothoracic Surgery (M.P.F.), and Department of Radiology (J.C.W.), Stanford University School of Medicine, CA.

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