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Concise Review: Tissue-Specific Microvascular Endothelial Cells Derived From Human Pluripotent Stem Cells

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ABSTRACT

Accumulating evidence suggests that endothelial cells (ECs) display significant heterogeneity across tissue types, playing an important role in tissue regeneration and homeostasis. Recent work demonstrating the derivation of tissue-specific microvascular endothelial cells (TS-MVECs) from human pluripotent stem cells (hPSCs) has ignited the potential to generate tissue-specific models which may be applied to regenerative medicine and in vitro modeling applications. Here, we review techniques by which hPSC-derived TS-MVECs have been made to date and discuss how current hPSC-EC differentiation protocols may be directed toward tissue-specific fates. We begin by discussing the nature of EC tissue specificity in vivo and review general hPSC-EC differentiation protocols generated over the last decade. Finally, we describe how specificity can be integrated into hPSC-EC protocols to generate hPSC-derived TS-MVECs in vitro, including EC and parenchymal cell coculture, directed differentiation, and direct reprogramming strategies. STEM CELLS *2014;32:3037–3045*

INTRODUCTION

Endothelial cells (ECs), which line the blood vessel lumen, are not simply passive barriers but instead function as active gatekeepers that dynamically respond to changes in the microenvironment. Positioned at the interface between the circulating blood components and the surrounding tissue, ECs play a critical role in regulating physiological and pathological processes, including control of microvascular permeability, angiogenesis, coagulation, and inflammation. To perform such diverse functions, ECs exhibit a high degree of heterogeneity across developmental stages [1], vascular classes (i.e., capillary, arteriole, or venule), and tissue types.

The microvasculature of various tissues differs on both a structural and functional level in order to meet the specific needs of that tissue. For example, ECs residing in the brain form a continuous, highly impermeable barrier, the blood-brain barrier (BBB), in order to maintain the delicate biochemical balance necessary for proper brain function. Conversely, sinusoidal ECs in the liver are highly discontinuous to facilitate toxin clearance from the bloodstream, while glomerular ECs in the kidney help filter contents of the blood to remove waste products.

There exists much interest in generating tissue-specific microvascular endothelial cells (TS-MVECs) in vitro for use in both regenerative medicine and tissue modeling applications. Human pluripotent stem cells (hPSCs), specifically human embryonic and induced pluripotent stem cells (hESCs and hiPSCs, respectively), are an attractive source for generating TS-MVECs due to their capacity for extensive self-renewal and ability to differentiate into any somatic cell type. In particular, the ability to derive autologous cells and to study mechanisms of human tissue development in vitro makes hPSCs a particularly appealing source of TS-MVECs.

Over the last decade, the development and refinement of protocols to differentiate hPSCs to ECs have advanced the understanding of the role that human ECs play in both physiological and pathological tissue states. Recently, several exciting advances have demonstrated hPSC differentiation into ECs that exhibit tissue-specific characteristics. The objective of this review is to summarize these advances and suggest promising directions that may expand the applications of TS-MVECs.

CHARACTERIZING EC TISSUE SPECIFICITY IN VIVO

The ideal stem cell-derived TS-MVEC should match its in vivo counterpart as closely as

possible in terms of gene and protein expression, structure, and functional characteristics. In vivo studies have identified major structural (reviewed in [2]) and functional (reviewed in [3]) differences in capillaries across tissues, suggesting a high degree of heterogeneity of which we are now beginning to identify the molecular basis. The need for in vivo TS-MVEC characterization is supported by the observation that in the absence of microenvironmental context in vitro, primary cultured ECs dedifferentiate, losing expression of up to 50% of their tissue-specific genes [4].

A major function of ECs is the release of tissue-specific angiocrine factors that support tissue homeostasis and regeneration. For example, liver sinusoidal ECs (LSECs) influence liver regeneration via spatiotemporally regulated angiocrine signaling after partial hepatectomy [5, 6]). Ang-2 is initially downregulated [5], while Wnt2 and HGF are upregulated [6] in LSECs after hepatectomy, promoting hepatocyte growth and proliferation, which is followed by the gradual recovery of Ang-2 expression to promote angiogenesis [5]. In bone tissue, there are subtypes of capillary ECs, type H and type L ECs, which differ in their angiocrine responses [7, 8]. Type H ECs release niche signals that support the survival and proliferation of osteoprogenitor cells (including *Pdgfa* and *Pdgfb*), while type L ECs do not [7]. Interestingly, increasing the number of type H ECs by enhancing HIF1α levels increased the number of osteoprogenitors and enhanced bone formation [7].

Another major function of ECs is to regulate the permeability of ions, small molecules, proteins, and cells. As one example, the highly impermeable brain ECs comprising the BBB are characterized by lack of fenestrae, diminished pinocytotic activity, expression of polarized efflux transporters, and high transendothelial electrical resistance (TEER) (reviewed in [9]). In fact, the TEER of brain capillaries has been measured up to $\sim$6,000 $\Omega \times cm^2$ depending on the age and species of the animal tested [10, 11], compared to below 20 $\Omega \times cm^2$ in peripheral vessels [12]. Paracellular permeability is mediated by tight junction complexes that are heterogeneously expressed and differentially organized across tissues [13]. For instance, highly impermeable brain ECs express tight junction proteins occludin and claudin-5, whereas highly permeable LSECs do not express occludin and only heterogeneously express claudin-5 [14].

Gene microarrays have proven useful in identifying the molecular determinants of EC tissue specificity [15, 16]. Daneman et al. compared the transcriptomes of brain-, lung-, and liver-derived ECs in a Tie2-GFP mouse [15]. By focusing on sets of genes that have functional significance in producing the barrier phenotype of brain ECs, Daneman et al. described a number of brain EC-specific genes compared with lung and liver ECs, including tight junction proteins (occludin, Marveld2, and Jam4) and transporters from the Slc, Slco, ATP, and ABC transporter families. Pathway analysis of BBB-enriched genes identified the canonical Wnt and retinoic X receptor signaling pathways as upregulated in the brain vasculature. Interestingly, both canonical Wnt signaling [17–19] and retinoic acid signaling [20] have been implicated in the induction of brain-specific endothelial properties during development.

More recently, Nolan et al. isolated ECs from nine different tissues in mice via intravital labeling and fluorescent-activated cell sorting (FACS) purification [16]. They found that ECs from distinct tissues display significant differences in their transcriptomes, with the most dissimilar ECs (kidney and testis) only exhibiting an R^2 correlation in gene expression of 0.796, while the most similar ECs (heart and muscle) exhibited an R^2 of 0.976. Furthermore, Nolan et al. identified sets of transcription factors, angiocrine factors, and surface markers that were differentially expressed between tissues. For example, the transcription factor SFPI1 was enriched in liver- and bone marrow-derived ECs, the angiocrine factor interleukin 33 was enriched in kidney ECs, and the surface marker CD133 was enriched in brain- and testes-derived ECs. However, clear examples of tissue-specific endothelial markers are rare. Collectively, these analyses indicate that tissue specificity should be defined by a unique combination of genes or proteins rather than a single factor.

Stem Cell-Derived ECs

Human Stem Cell Sources

There are several distinct stem cell sources for deriving human ECs, including both pluripotent and adult stem cells. Adult stem cell populations, including bone marrow mononuclear cells [21], peripheral blood mononuclear cells [22–26], adipose-derived stem cells [27], and cardiac progenitors [28], have been shown capable of differentiating into ECs. However, adult stem cells are limited in their differentiation capabilities, often consist of heterogeneous populations [29], and in some instances lose proliferative and differentiation capacity with aging [30]. The derivation of hESCs from the inner cell mass of the blastocyst [31] and, later, the generation of hiPSCs from terminally differentiated somatic cells [32] have overcome several of the limitations of adult stem cells. The ability to use hPSC-derived ECs that represent a patient-specific phenotype [33] makes hPSCs a very powerful resource to further understand ECs in both their physiologic and pathophysiologic states and may play a critical role in cellular regeneration.

Characterization of Stem Cell-Derived ECs

There is no single distinct marker of ECs; instead a combination of markers is beneficial in EC identification. The most definitive constitutively expressed endothelial markers include PECAM (CD31), vascular endothelial (VE)-cadherin (CD144), endothelial nitric oxide synthase (eNOS), von Willebrand Factor (vWF), vascular endothelial growth factor receptor-2 (VEGFR-2), and Tie-2 [34]. A number of functional assays can be used to characterize the phenotypes of hPSC-derived ECs. The release of nitric oxide by ECs is critical in the regulation of blood flow and blood pressure in vivo [35] and can be measured in vitro [36]. The uptake of acetylated low-density lipoprotein is another characteristic of healthy ECs [37]. ECs are activated by inflammatory cytokines, such as TNF-α, inducing an upregulation of adhesion molecules including ICAM-1, which captures circulating leukocytes and may be vital in paracrine signaling of endothelial progenitor-like cells to damaged vasculature [38]. In vitro angiogenic and vasculogenic assays that require EC proliferation and migration [39, 40] are conducted by seeding VEGF-treated ECs onto growth-factor reduced Matrigel and observing tube-like networks that contain lumens. Such assays can also be conducted in vivo by

Table 1. Methods of differentiating human pluripotent stem cells into endothelial cells

Differentiation technique	Source	Differentiation factors	Purification	Expansion factors	Differentiation efficiency	Characterization	Reference
OP9 coculture	hESC	N/A	MACS $CD34^+VEGFR\text{-}2^+$ (~10% of total population)	ESFM media bFGF acidic FGF + heparin	~90% $CD144^+/vWF^+$	Ac-LDL uptake In vitro vascular tube formation Inflammatory cytokine activation $CD144^+$, $CD31^+$, vWF^+, $eNOS^+$, $VEGFR\text{-}2^+$	[41]
	hESC + hiPSC	N/A	MACS $CD34^+CD31^+$ (3%–6% of total population)	ESFM media bFGF acidic FGF + heparin	N/A	In vitro vascular tube formation $CD144^+$, $CD31^+$, vWF^+, $VEGFR\text{-}2^+$	[42]
Embryoid body formation	hESC	bFGF VEGF IGF EGF	FACs CD31 (12% of total population)	EGM-2 media	~93%–97% $CD31^+$/ $CD144^+/vWF^+$	Ac-LDL uptake In vitro and in vivo vascular tube formation $CD144^+$, $CD31^+$, vWF^+	[43]
	hiPSC	N/A	MACS $CD144^+$ (18% of total population)	EC medium	~95% $CD144^+$	Ac-LDL uptake In vitro vascular tube formation Inflammatory cytokine activation $CD144^+$, $CD31^+$, vWF^+, $eNOS^+$, $VEGFR\text{-}2^+$	[44]
	hESC	N/A	FACS $CD31^+$ (2% of total population)	EGM-2 media	~80% $CD31^+$	Ac-LDL uptake In vitro and in vivo vascular tube formation $CD144^+$, $CD31^+$, vWF^+, N-cadherin$^+$	[45]
	hESC	bFGF VEGF	FACs $CD31^+/CD144^+$ (10%–15% of total population)	EGM-2 media VEGF	~98% $CD31^+/CD144^+$	Ac-LDL uptake In vitro and in vivo vascular tube formation $CD144^+$, $CD31^+$, vWF^+	[46]
	hiPSC	BMP4 VEGF	FACs $CD31^+$ (5%–20% of total population)	EGM-2MV media	75%–90% $CD31^+$	Ac-LDL uptake In vitro and in vivo vascular tube formation $CD144^+$, $CD31^+$, vWF^+, $eNOS^+$	[39]
	hESC	BMP4 Activin A bFGF VEGF SB431542	MACS $CD31^+$ (2% of total population)	Advanced DMEM/F-12 SB431542	36-fold net expansion ($CD144^+/VEGFR\text{-}2^+$)	In vivo vascular tube formation $CD144^+$, $CD31^+$, vWF^+, $eNOS^+$	[47]
	hESC	BMP4 SCF Flt3 IL-3 IL-6 G-CSF VEGF	FACs $CD144^+$ (10% of total population)	N/A	97% ($CD31^+$)	In vitro vascular tube formation Inflammatory cytokine activation Endothelial Cell Migration Assay Wound-Healing Assay $CD144^+$, $CD31^+$, vWF^+	[48]
2D differentiation	hESC + hiPSC	bFGF Activin A BMP4 VEGF	MACS $VEGFR\text{-}2^+$ (6%–73% of total cell population)	ECM media EGF bFGF VEGF IGF-1 RA	93%–95% ($CD31^+/CD144^+$)	Ac-LDL uptake In vitro and in vivo vascular tube formation Endothelial cell migration assay Nitric oxide measurement VE-cadherin$^+$, $CD31^+$, vWF^+, $eNOS^+$	[40]
	hESC	bFGF VEGF BMP4	MACS $CD34^+$ (10% of total population)	EGM-2MV media bFGF VEGF	>85% (Adherent/ $CD31^+/CD144^+$)	Ac-LDL uptake In vitro vascular tube formation $CD144^+$, $CD31^+$, vWF^+, $VEGFR\text{-}2^+$, $Tie\text{-}2^+$	[49]
	hESC	BIO VEGF	MACS $CD144^+$ (20% of total population)	Stem Pro-SFM media VEGF	95% ($CD144^+$) 100-fold net expansion	Ac-LDL uptake In vitro vascular tube formation	[37]

Table 1. *Continued*

Differentiation technique	Source	Differentiation factors	Purification	Expansion factors	Differentiation efficiency	Characterization	Reference
	hESC + hiPSC	BMP4, GSK3β Inhibition, VEGF,	FACs CD 144$^+$, CD34$^+$, CD31$^+$ (50% of total population)	EBM-2 Media FGF VEGF DAPT (Notch Inhibitor)	~80% (CD31$^+$/CD144$^+$)	CD144$^+$, CD31$^+$, VEGFR-2$^+$ In vitro and in vivo vascular tube formation CD144$^+$, CD31$^+$, vWF$^+$	[50]
Direct reprogramming	Human fibroblasts	N/A	N/A	EGM-2 media	23%–34% (CD144$^+$/CD31$^+$)	In vitro and in vivo vascular tube formation CD144$^+$, CD31$^+$, VEGFR-2$^+$, eNOS$^+$, vWF$^+$	[51]
	Human fibroblasts	EGM-2 Media bFGF VEGF BMP4	N/A	EGM-2 media SB431542	97% (CD31$^+$)	Ac-LDL uptake In vitro and in vivo vascular tube formation CD31$^+$, CD144$^+$, vWF$^+$	[52]
	Human amniotic cells	EGM SB431542	FACs CD144$^+$, VEGFR-2$^+$, CD31(~35% of total population)	EGM media	99% (CD144$^+$/CD31$^+$)	Ac-LDL uptake In vitro and in vivo vascular tube formation CD31$^+$, CD144$^+$, VEGFR-2$^+$	[53]

Abbreviations: bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein-4; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; FACS, fluorescent-activated cell sorting; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; MACS, magnetic activated cell sorting; vWF, von Willebrand Factor.

suspending cells into a Matrigel mixture with basic fibroblast growth factor (bFGF) and subcutaneously injecting into an immune-deficient animal. The Matrigel plug can later be removed and capillaries identified via expression of endothelial- and species-specific markers.

Deriving ECs from hPSCs

The differentiation of hPSCs to ECs has significantly progressed over the last decade, and select advances are summarized in Table 1. First, primary cell coculture has been used in directing hPSCs toward an EC fate. Both hPSCs [41] and hiPSCs [42] were induced to differentiate into ECs in the presence of the mouse bone marrow stromal cell line OP9. The strong hematopoiesis-promoting activity of OP9 cells may be attributed to differences in Notch ligand expression, protein expression, and unidentified paracrine factors, which may contribute to hESC differentiation to ECs. The authors of these studies hypothesized that secreted factors may contribute to directing hPSCs to an endothelial state, raising the possibility that paracrine signaling may regulate the derivation of tissue-specific ECs from hPSCs.

An additional method often used to obtain hPSC-derived ECs uses embryoid body (EB) formation. A number of EB protocols use growth factors to first induce a CD31+CD34+ progenitor population that, once isolated and exposed to the proper environmental cues, can generate ECs. Levenberg et al. demonstrated that after 10 days of culture of hESC-derived EBs, 2% of cells expressed CD31 [45]. The addition of growth factors IGF and EGF in combination with VEGF and FGF2 yielded ~12% CD31+ cells [43]. Rufaihah et al. formed EBs from hiPSCs in the presence of bone morphogenetic protein-4 (BMP4) and VEGF [39]. Following EB formation and 10 days of cell expansion in the absence of BMP4, approximately 15% of the population expressed CD31. In a recent study by James et al., hESCs were aggregated into EBs and exposed sequentially to BMP4, Activin A, and FGF2. On days 4–7, EBs were plated onto Matrigel and treated with VEGF-A. These conditions generated relatively sparse population of ECs as indicated by a VE-Cadherin-GFP reporter (~0.2%). However, inhibition of TGFβ at day 7 increased endothelial differentiation efficiency by 10-fold. Subsequent TGFβ inhibition of magnetic activated cell sorting (MACS)-purified CD31+ cells increased their expansion 36-fold [47]. These studies indicate that manipulation of specific signaling pathways following the initial differentiation to EC progenitors increases cell proliferation and conservation of the EC phenotype, approaches which could prove vital in the derivation of tissue-specific ECs. Pure populations of ECs can be readily obtained using EB differentiation protocols because of the ease of sorting and expanding cells expressing EC markers.

Monolayer-based two-dimensional (2D) directed differentiation techniques using small molecules, growth factors, and extracellular matrix proteins have also been designed to differentiate hPSCs to ECs. For example, Wang et al. cultured hESCs in a differentiation medium containing VEGF, bFGF, and BMP4 for 10 days, producing CD31$^+$CD34$^+$ angioblast-like cells that accounted for ~20% of the total cell population [49]. Following culture in endothelial growth medium supplemented with VEGF and bFGF, the majority of the cells formed adherens junctions, imported dil-acetylated low-density

Table 2. Methods of differentiating human pluripotent stem cells into tissue-specific microvascular endothelial cells

Source	Type of tissue specificity	Factors influencing tissue specificity	Differentiation efficiency	Reference
hESC + hiPSC	Brain	Neural cell coculture	>60%	[59]
hESC + hiPSC	Brain	Neural cell coculture, retinoic acid	>60%	[57]
hESC	Heart	VEGF-A	8%–10% of purified progenitor population	[56]

Abbreviations: hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

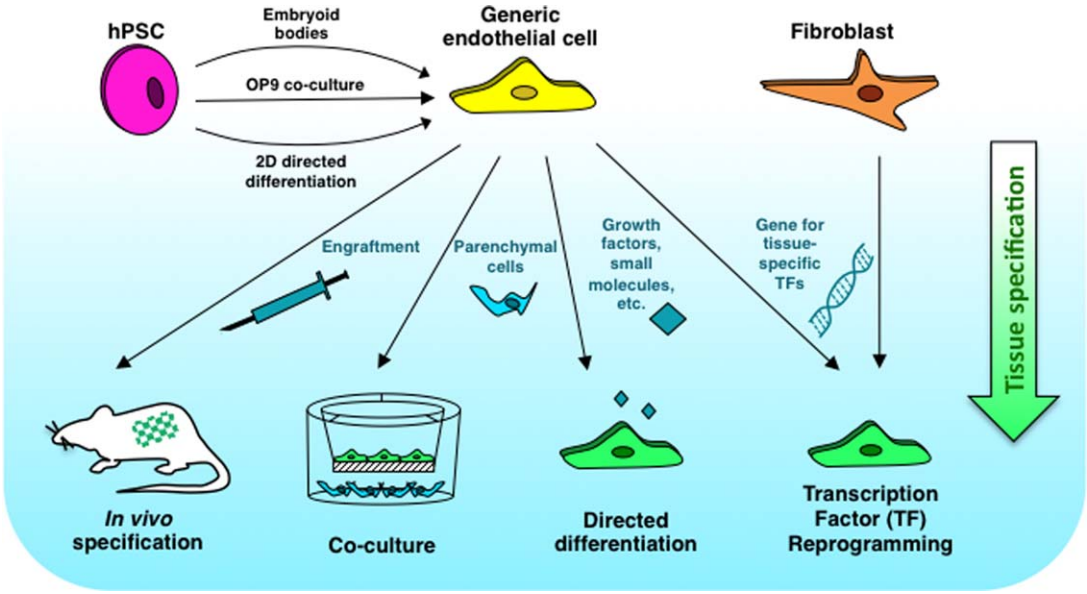

Figure 1. In vivo and in vitro approaches to generate hPSC-derived tissue-specific microvascular endothelial cells. Abbreviations: 2D, two-dimensional; hPSC, human pluripotent stem cell.

lipoprotein, and expressed VE-cadherin, CD31, vWF, Tie2, and VEGFR-2. In a similar study by Tatsumi et al., hESCs were differentiated in the presence of a glycogen synthase kinase-3β inhibitor and VEGF [37]. FACS analysis revealed that ~20% of the population expressed VE-cadherin, VEGFR-2, CD34, and CD31. Following VE-cadherin-based MACS purification, the ECs imported dil-acetylated low-density lipoprotein and formed capillary-like tubes in vitro. In another comprehensive study by White et al., hiPSCs and hESCs cultured in BMP4, Activin A, VEGF, and bFGF yielded 6%–70% VEGFR-2$^+$ cells, indicating high variability between cell lines [40]. However, following VEGFR-2$^+$ MACS isolation, the ECs could be expanded in Endothelial Cell Growth Medium (EGM) media containing EGF, IGF-1, bFGF, VEGF, and retinoic acid (RA), yielding 95% CD31$^+$ cells. The CD31$^+$ population imported dil-acetylated low-density lipoprotein, formed capillary-like tubes in vitro and in vivo, generated NO, and expressed a variety of endothelial markers. These 2D differentiation platforms permit finer chemical control over the microenvironment during differentiation than afforded by coculture and EB methods, and thus may be more amenable to identifying and optimizing the presentation of specific cues that promote EC differentiation. Although there has been extensive progress in the techniques and protocols used to develop stem cell-derived ECs, additional effort is necessary in understanding how to direct hPSCs to TS-MVECs.

Approaches to Generate Stem Cell-Derived Tissue-Specific ECs

It is well accepted that tissue-specific characteristics of ECs are a consequence of the local microenvironment. The first studies demonstrating the notion of "organ imprinting" used chick-quail transplantations, which showed that the surrounding tissue was the source of tissue-specific signals, rather than the invading vessels being driven by intrinsic tissue-specific programs [54]. Since then, a number of studies have demonstrated that microenvironmental cues such as soluble signaling factors [55–57] and extracellular matrix composition [58] contribute to tissue specificity of microvascular ECs. Most recently, several studies have shown that TS-MVECs can be differentiated from hPSCs [56, 57, 59]. Here, we review the successful strategies that have emerged to date (summarized in Table 2) and suggest approaches to drive derivation of hPSC-derived TS-MVECs in the future (summarized in Fig. 1).

In Vivo Specification of Stem Cell-Derived ECs

As researchers continue to understand and improve the differentiation of hPSCs to ECs, the question remains whether these ECs can be directed to acquire tissue specificity by providing proper inductive cues. Nolan et al. [16] demonstrated this was possible by stimulating mouse ESCs (mESCs)

with BMP4, Activin-A, VEGF-A, and FGF2 as previously described by James et al. [47] followed by transduction with myrAkt1 [60]. 99.3% of cells in the purified population maintained expression of VE-cadherin and CD31 for 4 weeks and lacked markers predicted to be upregulated in certain tissues, such as CD133 (upregulated in brain ECs) or Vascular cell adhesion molecule (VCAM) (upregulated in liver ECs) [16].

Next, ECs generated from mESCs in this manner were injected into mice that had undergone a 70% hepatectomy. Surprisingly, mESC-ECs engrafted in the kidney as well as the regenerating liver, suggesting that the surgical procedure created a permissive environment for engraftment in the kidney. Notably, the two mESC-EC populations showed divergent expression patterns, with high VCAM expression present in the liver mESC-ECs only, and elevated Tie2 and endoglin expression in the kidney mESC-ECs only, consistent with global gene expression patterns in primary liver and kidney ECs [16]. Importantly, this suggests that stem cell-derived ECs are responsive to tissue-specific microenvironmental cues. However, expression of only a few tissue-specific markers was assessed in the engrafted cells, and in the absence of further phenotypic and functional analyses these cells would not be considered bona fide liver or kidney ECs. Moving forward, perhaps TS-MVECs can be generated in vitro provided that the relevant microenvironmental cues are presented to populations of generic hPSC-ECs in the proper spatiotemporal fashion. While the identities of many of the microenvironment cues that can drive formation of TS-MVECs remain unknown, it is conceivable that once such factors are identified, they can be introduced in vitro during or after EC differentiation to induce tissue specificity.

Generating hPSC-Derived TS-MVECs In Vitro

Coculture of ECs with Parenchymal Cells. Coculture of primary ECs with neural cells, including neural progenitor cells [55] and astrocytes [61], has been widely adopted as a means to enhance BBB-specific properties of ECs in vitro. For example, Boyer-Di Ponio et al. found that astrocyte coculture induced human cord blood-derived circulating endothelial progenitors to acquire BBB-specific markers and phenotypes [62]. In one of the few examples of functional hPSC-derived TS-MVECs to date, our group developed a strategy whereby hPSCs differentiate into a mixture of neural cells and ECs, resulting in a population of $>$60% brain-specific microvascular ECs (BMECs) [59] (reviewed in [63]). These hPSC-derived BMECs recapitulate many key characteristics of the in vivo BBB including expression of tight junction proteins, elevated TEER, and polarized efflux transporter activity. We hypothesized that the codifferentiating neural cell population provides physiologically relevant developmental cues, including Wnt signaling [17–19], that instruct the nascent ECs to become brain-specific.

Although the neural and EC progenitor populations arise concurrently during hPSC-derived BMEC differentiation [59], BMEC barrier and transporter phenotypes are enhanced when hPSC-derived BMECs are cocultured with cells from the neurovascular unit. Coculture of hPSC-derived BMECs with primary rat astrocytes increased TEER from $221 \pm 51\ \Omega \times cm^2$ in monoculture to $860 \pm 260\ \Omega \times cm^2$ in coculture, indicating tightening of the barrier [59]. In addition, a combination of retinoic acid treatment (as discussed later in *Directed Differentiation of TS-MVECs Via Microenvironmental Cues*) and sequential coculture of hPSC-derived BMECs with primary human pericytes followed by astrocytes and neurons differentiated from human neural progenitor cells achieved a TEER of approximately 5,000 $\Omega \times cm^2$ [57], approaching in vivo values [11].

While primary cell coculture with hPSC-derived ECs has the potential to induce EC tissue specificity, the development of hPSC differentiation protocols for various parenchymal cell types, such as cardiomyocytes [64], hepatocytes [65], or renal cells [66], provides the opportunity to construct cocultures of hPSC-derived ECs and hPSC-derived parenchymal cells. This strategy would permit the development of syngeneic models of ECs and parenchymal cells and represents an avenue to study mechanisms and dynamics of tissue-specification of EC progenitors in vitro.

Coculture may also provide a critical first step in understanding the mechanisms of EC specification by parenchymal cells. For example, proteomic analysis has been used to quantify changes in protein expression in primary bovine brain ECs stimulated by astrocyte coculture, highlighting astrocyte-induced changes in actin cytoskeleton [67] and the asymmetric dimethylarginine pathway [68]. In addition, genomic analysis of Human umbilical vein endothelial cells (HUVECs) cocultured with fibroblasts identified 323 genes differentially expressed upon coculture [69]; analysis of the promoters of these genes implicated a number of conserved C promoter binding factor 1/CBF1, Suppressor of Hairless, Lag-1 elements, suggesting regulation of fibroblast/EC interactions via Notch signaling. Ideally, as these signals are identified they can be incorporated into to the EC differentiation platform at the appropriate developmental stage to drive tissue specificity.

Directed Differentiation of TS-MVECs via Microenvironmental Cues. Directed differentiation strategies typically leverage existing endothelial differentiation protocols (as described in the previous section *Stem Cell-Derived ECs*) and incorporate elements of the in vivo microenvironment (i.e., growth factors, signaling proteins, tissue-specific matrices, and/or small molecules) to induce tissue specificity in the ECs. The role of extracellular matrices has been shown to be vital in regulating EC phenotype [70], and could play a critical role in deriving TS-MVECs. Small molecules and growth factors have already been successfully used in deriving both brain-specific ECs [57] and cardiac-specific ECs [56] from hPSCs.

The first step in adopting a directed differentiation strategy to produce TS-MVECs is the identification of candidate specification cues. For example, RA was hypothesized to enhance the BBB phenotype in the hPSC-derived BMECs for several reasons. First, retinol binding protein receptor *STRA6* has been detected in brain ECs but not peripheral ECs in adult mice, suggesting tissue specificity [71]. Moreover, addition of RA has been shown to upregulate certain BBB characteristics in a human immortalized BMEC cell line and has been suggested to be involved in BBB development in vivo [20]. Indeed, addition of RA during the later stages of hPSC-derived BMEC differentiation induced an earlier onset of VE-cadherin expression, enhanced tight junction fidelity, and increased TEER from an average of $228 \pm 57\ \Omega \times cm^2$ to

$2{,}940 \pm 800\ \Omega \times cm^2$ [57]. RA-treatment was synergistic with coculture of human pericytes and human astrocytes and neurons differentiated from Neural Progenitor Cells (NPCs), increasing TEER to $\sim 5{,}000\ \Omega \times cm^2$ [57].

In another example, Lui et al. [56] developed a method to direct hPSCs to a cardiac-specific EC fate. The authors identified VEGF-A as the most highly expressed growth factor in cardiac ECs and determined that its expression was specific to cardiac ECs. VEGF-A, either exogenously added or transfected into the progenitor population via chemically modified mRNA, drove hPSC-derived multipotent cardiovascular progenitors toward an endothelial fate (comprising 8%–10% of the purified progenitor population), and further, these ECs expressed cardiac-specific markers.

Interestingly, while VEGF-A was identified to be highly expressed by human cardiac ECs by Lui et al. [56], it was not identified as highly expressed by mouse cardiac ECs compared to other tissues in the microarray database presented by Nolan et al. This discrepancy might be attributed to species differences. Additionally, the presence of VEGF-A in the differentiation medium could explain how the hPSC-derived ECs differentiated by Nolan et al. acquired expression of select cardiac-specific markers [16]. Also, VEGF-A was highly expressed in cardiac-specific ECs themselves, rather than cardiac parenchymal tissue, suggesting that autocrine signaling may contribute to EC tissue specification.

Tissue-Specific Endothelial Reprogramming. During directed differentiation, microenvironmental cues stimulate a cascade of intracellular signaling that ultimately triggers a developmental program resulting in the expression of tissue-specific genes and acquisition of tissue-specific phenotypes. A more direct approach to generating TS-MVECs may be the forced expression of transcription factors (TFs) that regulate expression of tissue-specific endothelial genes.

One potential strategy toward reprogramming cells to TS-MVECs uses cell reprogramming via TFs that are common among all ECs, followed by exposure of these reprogrammed ECs to microenvironments that impart tissue specificity. Indeed, ECs have been generated via transdifferentiation of human fibroblasts [51, 52]. Both of these studies used expression of pluripotency factors—either *OCT4*, *SOX2*, *KLF4*, and *C-MYC* [51] or *OCT4* and *KLF1* alone [52]—in combination with permissive culture conditions to obtain transdifferentiated ECs. In contrast, reprogramming of human amniotic cells to vascular ECs was accomplished via expression of 3 ETS family TFs (*ETV2*, *FLI1*, and *ERG1*), which were shown to induce vascular-specific genes while silencing nonvascular-specific genes [53]. In addition, reprogramming of mouse fibroblasts using four TFs implicated in the endothelial-to-hematopoietic transition and hematopoietic stem cell specification (*GATA2*, *GFI1b*, *C-FOS*, and *ETV6*) induced an endothelial precursor cell phenotype [72]. While these studies have successfully produced reprogrammed ECs, it has not yet been shown that these cells can acquire tissue specificity.

A second potential strategy toward reprogramming TS-MVECs uses expression of endothelial tissue-specific TFs, which may be identified by mining genomics data for transcription factors that are heterogeneously expressed across vascular beds [16]. For example, the transcription factor *SFPI1* is highly enriched in bone marrow and liver ECs [16], and may potentially regulate expression of bone marrow endothelial- and liver endothelial-specific genes. Ultimately, there may be different approaches to reprogram cells into tissue-specific ECs depending on both input cell type (i.e., EC vs. non-EC) and combinations of TFs (i.e., EC identity factors vs. tissue-specific TFs).

Conclusions and Future Challenges

The generation of TS-MVECs derived from hPSCs has the potential to reshape our understanding of endothelial function in health, disease, and development, create opportunities for therapeutic discovery, and offer an EC source that is well-suited for regenerative medicine. Defining the genes, proteins, and phenotypes that constitute endothelial tissue specificity is an active area of research, and the mechanisms directing this specificity are largely unknown. Given the substantial overlap in gene expression patterns between ECs from various tissues [16], researchers should be careful to avoid reducing complex EC heterogeneity to a few markers. Moving forward, the expansion of genomics and proteomics databases in conjunction with the investigation of tissue-specific EC phenotypes in vivo will be crucial for defining what constitutes hPSC-derived TS-MVEC behavior in vitro.

In this review, we have described various methods to generate TS-MVECs via hPSC differentiation and somatic cell reprogramming, including in vivo- and in vitro-guided approaches. Ultimately, the optimal approach depends on the desired application. In vivo-guided hPSC differentiation or EC maturation does not require a priori knowledge of specification mechanisms, which is of particular advantage as in vitro-guided processes aim to recapitulate in vivo signaling processes. However, ECs differentiated and matured in vivo face engraftment, viability, and isolation challenges [73] and are likely subject to the same dedifferentiation processes as primary TS-MVECs should they be removed and expanded ex vivo. In vitro specification methods, conversely, could potentially generate the large quantities of purified TS-MVECs required for screening or therapeutic applications. Additionally, in vitro specification methods may be more controlled and better defined. However, the ability to derive fully mature, phenotypically relevant TS-MVECs has proven challenging and may be difficult to accomplish in an in vitro setting without a deeper understanding of specification mechanisms.

To date, researchers have generated hPSC-derived ECs populations displaying aspects of brain specificity [57, 59] and heart specificity [56]. These exciting breakthroughs provide evidence that hPSCs are indeed capable of differentiating into TS-MVECs that could be useful for scientific and pharmaceutical applications. While hPSC-derived TS-MVECs are also a promising source of cells in regenerative medicine applications where vascularization is important, several key criteria must first be met. First, molecular and phenotypic hallmarks of tissue specificity in ECs must be identified. Also, epigenetic memory may influence hiPSC differentiation [74] and somatic cell reprogramming [75], thus cell sources for generating TS-MVECs must be carefully

considered. TS-MVEC differentiation conditions would ideally be completely defined, and reprogramming methods should use nonintegrating methods to minimize off-target effects. In addition, hPSC-derived TS-MVECs should be mature and fully differentiated. Finally, protocols to successfully transplant and engraft hPSC-derived TS-MVECs must be developed.

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Author Contributions

H.K.W, S.G.C, E.V.S., and S.P.P.: conception and design, manuscript writing, and editing. H.K.W. and S.G.C. contributed equally to this article.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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