

Endothelial ontogeny and the establishment of vascular heterogeneity

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Abstract

The establishment of distinct cellular identities was pivotal during the evolution of Metazoa, enabling the emergence of an array of specialized tissues with different functions. In most animals including vertebrates, cell specialization occurs in response to a combination of intrinsic (e.g., cellular ontogeny) and extrinsic (e.g., local environment) factors that drive the acquisition of unique characteristics at the single-cell level. The first functional organ system to form in vertebrates is the cardiovascular system, which is lined by a network of endothelial cells whose organ-specific characteristics have long been recognized. Recent genetic analyses at the single-cell level have revealed that heterogeneity exists not only at the organ level but also between neighboring endothelial cells. Thus, how endothelial heterogeneity is established has become a key question in vascular biology. Drawing upon evidence from multiple organ systems, here we will discuss the role that lineage history may play in establishing endothelial heterogeneity.

KEYWORDS

coronary endothelial cell, heterogeneity, lineage tracing, lymphatic, ontogeny, single cell

INTRODUCTION

In multicellular organisms, circulatory systems evolved to overcome the constraints of passive diffusion, and they exist in various forms across the animal kingdom.^[1] The endothelial lining of circulatory systems is a unique feature of vertebrates, and it creates a controlled barrier for the movement of fluid, macromolecules and cells in blood and lymphatic vessels. Across the vascular tree, endothelial cells (ECs) display heterogeneous characteristics that are essential for the function of individual organs. For example, arterial and venous

ECs form continuous monolayers surrounded by vascular smooth muscles cells that together control blood flow to individual tissues; capillary ECs display organ-specific traits that lead to the formation of continuous (myocardium), fenestrated (kidney glomerulus) or discontinuous (liver sinusoids) endothelium; lymphatic capillaries are supported by a discontinuous basement membrane and display specialized cell-cell junctions that facilitate the uptake of interstitial fluid and immune cells. The widespread implementation of single-cell sequencing technology has revealed that molecular heterogeneity exists not only between ECs in different systems (e.g., arteries, veins, capillaries,

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lymphatics) or organs (e.g., brain, heart, liver, lungs), but also between adjacent ECs in the same vascular bed. These findings have raised a number of fundamental questions for the field of vascular biology: How is EC heterogeneity established? Do different subtypes of ECs play distinct roles during organ development, homeostasis or repair? While the role that local environmental cues play in the acquisition of organotypic EC characteristics is being described in increasing detail, the influence of cellular ontogeny in this process has received less attention.

ENDOTHELIAL CELL SPECIFICATION AND DIVERSIFICATION

Development of the cardiovascular system begins with blood vessel formation, where angioblasts are specified from mesodermal progenitors in a process known as vasculogenesis.^[2] Different functions for distinct cell lineages can be observed beginning at these early steps of differentiation of the vascular system. For example, angioblast populations in anatomically distinct regions of the lateral plate mesoderm acquire arterial or venous fate prior to forming vessels.^[3,4] In addition to the lateral plate mesoderm, angioblasts have been shown to derive from paraxial mesoderm in fish,^[5,6] birds,^[7] and mammals.^[8]

Mechanistic differences in the transcriptional regulation of angioblast specification have arisen during vertebrate evolution, likely due to divergent environmental and physiological selection pressures as body plans became more complex. For example, through the induction of *etsrp*, *tal1*, and *lmo2*, the bHLH-PAS transcription factor Npas4l is essential for hematoendothelial progenitor specification in zebrafish.^[2,9] In contrast, this gene was lost in the mammalian lineage where another ETS transcription factor, ETV2, appears to play a more dominant role. *Etv2* knockout mice lack hematoendothelial progenitors,^[10] which is analogous to Npas4l-deficient zebrafish. *Etsrp* in fish and ETV2 in mammals induce the initial expression of the vascular endothelial growth factor (VEGF) receptor gene, *VEGFR2* (also known as *FLK1*),^[3,10] which is essential for angioblast differentiation, behavior and proliferation.^[3,11–14] Under the influence of VEGF and other factors, angioblasts differentiate into blood endothelial cells (BECs) and assemble into the major arterial and venous vessels of the body. These vessels then provide a template from which BECs sprout to form the tissue-specific networks that deliver oxygen and nutrients to all organs and tissues.^[15,16]

Lymphatic ECs (LECs) form slightly later in development, and are primarily derived from progenitors in the cardinal vein,^[17,18] although recent studies have uncovered additional non-venous sources. Regardless of their origin, the specification of LECs is usually dependent on the transcriptional master regulator PROX1,^[19–21] whose expression is initially induced by SOX18^[22] and NR2F2.^[23] Budding and migration of lymphatic progenitors from the cardinal vein relies on proteolytic cleavage and activation of VEGFC by CCBE1 and ADAMTS3 in neighboring tissues.^[24–26] Subsequently, a PROX1-VEGFR3 feedback loop is required to maintain LEC fate,^[27] through the repression of

BEC fate; loss of PROX1 in formed lymphatics leads to blood pooling and the acquisition of BEC-like characteristics.^[28] Although the transient presence of red blood cells is a physiological feature of developing mesenteric lymphatics,^[29] blood flow through lymphatics in pathological settings leads to the loss of LEC identity.^[30] Other transcription factors known to contribute to the induction and maintenance of LEC fate include GATA2,^[31,32] HHEX,^[33] and FOXC2.^[34,35]

In order to meet the divergent physiological requirements of their environment, ECs acquire distinct organotypic characteristics that are established through organ-specific signaling and underpinned by organ-specific enhancer usage.^[36,37] For example, selective expression of WNT ligands in the neuroepithelium stimulates the specification of central nervous system BECs, establishing the blood brain barrier.^[38–40] Similarly, a tissue-specific requirement for VEGFR3-PI3K signaling has been demonstrated in LECs, whereby loss of PIK3CA leads to defects in mesenteric but not dermal lymphatic development.^[41] Further levels of specialization have recently been shown among BECs within the same vessel bed. In an aortic injury model, BEC regeneration was shown to rely on a subpopulation of highly proliferative ECs.^[42] Furthermore, in the alveolar endothelium of the lung, two distinct subtypes of capillary BECs exist: *Car4*⁺ aerocytes and *Car4*[−] general capillaries. *Car4*⁺ aerocytes display an extended morphology and are uniquely dependent on constitutive VEGF signalling. Gene expression analysis suggests that the two cell types may differentially control gas exchange, leukocyte trafficking, and vasomotor tone.^[43,44]

As well as responding to organ-specific signalling inputs, BECs are known to secrete organ-specific angiocrine cues that play instructive roles during tissue development, homeostasis, and regeneration.^[45] During embryonic development, BECs promote pancreatic islet differentiation^[46] and cardiomyocyte proliferation.^[47] In adults, BECs coordinate metabolic zonation in the liver^[48] and maintenance of stem cell niches in tissues that include liver,^[45] bone,^[49] brain,^[50] and skin.^[51] Moreover, there is an increasing appreciation of the role that blood vessels play during organ regeneration; chemokine signaling mediated by CXCL12-CXCR4 is a key mediator of the rapid vascular invasion and remodeling that is essential for cardiac regeneration^[52,53] as well as angiocrine support during liver^[54] and lung^[55] regeneration. Other signaling cues, such as Angiopoietin-2, have been shown to play more tissue-restricted roles in organ repair.^[56] LECs also provide angiocrine support during heart regeneration in zebrafish^[57,58] and mouse,^[59] and in the latter at least in part through secretion of RELN. They also influence the regeneration of other organs through the regulation of immune cell clearance^[60] and modulation of stem cell function.^[51]

TRANSCRIPTIONAL HETEROGENEITY IN THE ENDOTHELIUM

While it has been appreciated for many years that BECs and LECs in different organs and tumors have diverse properties,^[16] the advent

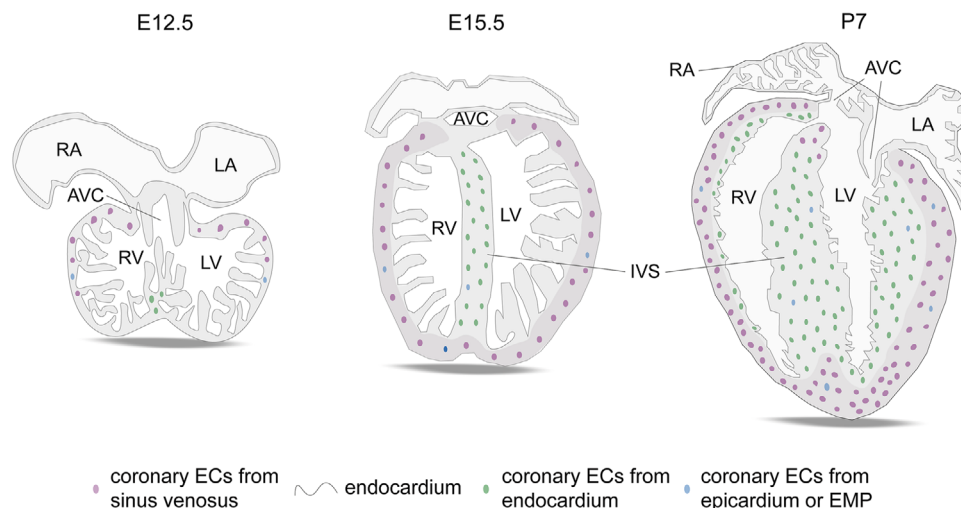


FIGURE 1 Schematic representation showing coronary BECs derived from the sinus venosus (magenta), endocardium (green) and epicardium (blue) in the developing mouse heart (RA – right atrium; LA – left atrium; RV – right ventricle; LV – left ventricle; AVC – atrioventricular canal; IVS – interventricular septum)

of single-cell RNA sequencing (scRNA-seq) has uncovered the existence of an unexpectedly high level of transcriptional heterogeneity, even amongst cells within the same vascular beds and vessels.^[61] BECs within capillary beds in the heart and brain are not one homogeneous cell subtype, but they rather exist along a continuum between arterial and venous transcriptional states.^[62,63] During development, the heart capillary plexus also contains a transcriptionally distinct population of “pre-artery” cells that are the precursors of coronary arteries.^[63]

Methods that use scRNA-seq data to cluster similar cell types together, frequently identify multiple clusters within the arterial, venous, and capillary BEC populations, and the frequency and identity of these clusters varies amongst different organs.^[64,65] These data could indicate a previously unappreciated functional heterogeneity among BECs since gene expression profiles suggest a variety of functions, including being actively angiogenic or possessing differential metabolic capacities.^[66] This trend is also observed when restricting analyses to a large artery; scRNA-seq analysis of the adult aorta identified multiple BEC clusters, with expression patterns reflecting specific anatomical locations (i.e., branch points) and suggesting distinct roles in adhesion, signaling, and immune function.^[67] Although large-scale comparisons across different organs have failed to capture significant heterogeneity among LECs,^[64] targeted scRNA-seq of lymph node lymphatic vessels revealed multiple subpopulations that may play distinct roles in neutrophil homing.^[68]

More examples of EC transcriptional heterogeneity emerge on a frequent basis with new scRNA-seq datasets.^[69] For the most part, this heterogeneity remains at the descriptive stage, and it will be important to use the wealth of unique genetic markers identified for EC subtypes to make and utilize reagents to probe their function. In addition, future experiments incorporating lineage tracing with scRNA-seq will reveal whether EC heterogeneity is related to ontogeny.

DEVELOPMENTAL ORIGINS OF ENDOTHELIAL CELLS

The endothelium of different vascular beds has been shown to arise from multiple cellular sources in fish,^[2,5,6] birds,^[7,70–72] and mammals.^[8,73,74] For the purpose of this review, we will focus on the developmental origins of cardiac, liver, and lymphatic endothelial cells.

Blood vessel endothelium

Cardiac endothelial cells

The mature heart possesses three general types of endothelial cells that arise at different times in development from multiple sources – endocardial cells that line the lumen of the heart, coronary vessel BECs, and cardiac LECs (Figure 1). Extensive lineage analyses have established that all three EC subtypes arise from multiple sources in the developing embryo.

At the earliest stage of cardiac development, the cardiac crescent or heart tube consists of two layers: the endocardium and the outer muscle layer or myocardium. Endocardial cells are distinct and independent from other vascular endothelial cell populations in terms of morphology, function, and gene expression.^[75] Lineage analysis has indicated that they form a single layer of interspersed cells with heterogeneous origins. The endocardium consists of cells that migrate in from the surrounding embryonic vascular plexus, differentiate from $NKX2.5^+VEGFR2^+$ multipotent cardiac progenitors, or differentiate from $ISL1^+$ second heart field cardiac progenitors. Whether endocardial cells from different developmental origins are functionally distinct has not been tested, although deleting the second heart field-derived population was not detrimental and

stimulated cellular compensation.^[76] It will be interesting to probe for transcriptional heterogeneity amongst the endocardium using scRNA-seq and ascertain whether any heterogeneity can be traced to ontogeny.

During formation of the atrial and ventricular cardiac chambers, expansion of the myocardial layer requires a dedicated blood vessel network, known as the coronary vessels. The earliest coronary vascular endothelial cells emerge at embryonic day (E)10.5–E11.0 in the atrioventricular groove of the developing mouse heart.^[77] The coronary plexus originates on the dorsal surface of the heart, expands from the atrioventricular canal to reach the outflow tract, and subsequently populates the entire dorsal surface by E14.5.^[78,79] Subsequently, coronary BECs invade the myocardial wall of the ventricles, and gradually form arterial and venous networks to establish the coronary circulation.^[79] Given the complex mechanisms underlying its formation, understanding the developmental origins of the coronary endothelium may provide critical insight for the application of therapeutic revascularization in coronary artery disease.

At least four distinct developmental sources have been described for coronary BECs, including the proepicardium/epicardium, sinus venosus, ventricular endocardium, and yolk sac (Figure 1). The proepicardium is a transient structure that sits between the sinus venosus and the septum transversum between E9.0–E11.0 in mouse.^[80] Cells from the proepicardium attach to the atrioventricular groove of the heart and spread over the heart surface to form a single-cell layer called the epicardium.^[81] Ground breaking early studies using quail-chick chimeras proposed the proepicardium as a progenitor of both coronary BECs and smooth muscle.^[82] However, this interpretation largely relied on the specificity of the dissected tissue used for implantation, which in addition to the proepicardium, included the sinus venosus (discussed below as a second source).^[82] With the advent of Cre-loxP-mediated genetic lineage tracing in mouse, studies using epicardium-specific Cre drivers such as *Wt1*^{CreERT2}, *Tbx18*^{Cre}, and *Tcf21*^{MerCreMer} reported that epicardial cells were precursors of coronary smooth muscle cells but not coronary ECs in the developing mouse heart,^[83–85] refining the conclusions drawn from quail-chick chimera studies. Subsequent studies revealed that the mouse proepicardium itself is heterogeneous and identified additional markers of proepicardial subsets. Lineage labeling these newly identified subsets with *Scx*-Cre, *Sema3d*^{Cre}, and *Gata4-G2*-Cre led to the observation that as many as 20% of coronary ECs are derived from proepicardial/epicardial cells.^[86,87] However, recent spatiotemporal gene expression analyses revealed an overlap of the key proepicardial markers in the developing heart, supporting the notion that epicardial cells mainly form fibroblasts and mural cells, rather than coronary BECs.^[88]

Further lineage tracing analyses in mouse revealed that the sinus venosus and ventricular endocardium are the major sources of coronary BECs. Clonal analyses where the pan-EC *Cdh5*-CreERT2 line was used to sparsely label random BECs after sinus venosus formation, but prior to coronary development, showed a clonal relationship between coronary and sinus venosus endothelium, suggesting that the sinus venosus is a source of coronary BECs.^[79] This analysis also revealed

clones containing coronary BECs and endocardial cells, suggesting the endocardium as an additional source.^[79] These two sources were confirmed using direct lineage tracing in concurrent and follow up studies. Experiments using an *Nfatc1*^{Cre} line confirmed the endocardial origin of coronary BECs, and demonstrated that angiogenic ventricular endocardial cells invade the myocardium to form a large proportion of coronary vessels in the developing heart.^[89] Using the *Apln*^{CreERT2} line, lineage traced subepicardial BECs were found to invade the myocardium and contribute to the majority of ventricular wall coronary vessels,^[90] while studies that labelled sinus venosus derivatives using the *Aplnr*-CreERT2 identified this structure as the initial source of coronary BECs in the ventricular wall.^[91] Together, these studies firmly established that the sinus venosus and endocardium form the majority of BECs in the developing heart.

Several important questions remained due to the caveats of Cre-based lineage tracing experiments and variations in techniques for quantifying lineage-labeling experiments. Specifically, what was the precise contribution from the sinus venosus and endocardium?^[92] Using the more specific *Npr3*^{CreERT2} line, Zhang et al. reported that ventricular endocardial cells contribute minimally to coronary vessels in the ventricular free walls while instead giving rise to most BECs in the interventricular septum and many of those on the ventral side of the heart.^[93] This finding supported the concept of a compartmentalization of coronary development that was revealed by analyzing lineage labeling in whole heart preparations.^[91] Mechanistically, the contribution of sinus venosus endothelium to the coronary vasculature is controlled by Angiopoietin-1, Elabela/Apj, and VEGFC signaling,^[91,94–96] while the endocardial contribution is primarily VEGFA-stimulated.^[89] In addition to BECs formed during embryogenesis, a substantial number of new coronary vessels form after birth in the inner myocardium, where embryonic trabeculae coalesce to form the compact wall.^[97] These newly formed vessels were lineage-traced from *Nfatc1*^{CreERT2} positive cells labeled at E8, showing that they derive from the endocardium.^[97] It is not known whether endocardial- and sinus venosus-derived cells differ functionally once they join coronary vessels. The observation that endocardial ECs can compensate for the loss of sinus venosus-derived ECs to restore heart function is evidence that they become highly similar upon differentiation.^[96] However, whether they retain different cell states/transcriptomes or display different responses to injury or disease has not been directly tested.

A recent study reported a complementary source of intraembryonic ECs, derived from erythro-myeloid progenitors (EMPs) that originate in the yolk sac. Using a *Csf1r*-iCre transgene, Plein et al. reported that a wave of labeled EMPs contribute to ECs in the embryo, in addition to hematopoietic cells.^[98] Notably, as high as 20% of ECs in the E18.5 heart were genetically labeled, indicating that they derive from the yolk sac.^[98] However, a more recent study using a knock-in *Csf1r*^{JRES-iCre} driver line did not find any evidence that yolk sac EMPs contribute to blood vessels in any organs, including the heart.^[99] One key difference is that the *Csf1r*-iCre tool used by Plein et al. is a transgene, which may not always faithfully reflect endogenous *Csf1r* gene expression. Ectopic expression of Cre may transiently target a few coronary ECs, thereby complicating the interpretation of the fate mapping results. Assessing

the yolk sac contribution to coronary BECs would benefit from additional orthogonal approaches – retrospective reconstruction of lineage relationships between known EMP derivatives and embryonic ECs following unbiased genetic barcoding of yolk sac ECs, prior to the emergence of EMPs, may resolve this issue.

Although they exhibit a few major differences with mammals in terms of cardiac development, zebrafish are an excellent model system to study coronary development and function. Mature mammalian heart ventricles are mostly a compact muscular layer, but zebrafish ventricular muscle is primarily trabeculated with a thin compact layer that expands during juvenile stages through expansion of cortical cardiomyocytes.^[100] Blood can access trabecular recesses so that coronary vessels are not initially required. Coronary vessels in zebrafish first develop in juveniles and closely follow cortical cardiomyocytes.^[53,101] Lineage tracing and clonal analysis revealed the endocardium as the origin of zebrafish coronary vessel endothelium,^[101] while the epicardium is the progenitor for perivascular cells.^[102] Although other lineage tracing techniques could reveal additional sources, it is tempting to speculate that sinus venosus-derived ECs provide an additional coronary source to support the mammalian-specific growth of the compact layer. Consistent with this hypothesis, inhibiting sinus venosus or endocardial angiogenesis in mouse delayed compact myocardium expansion.^[47] Interestingly, ectopic *Vegfaa* expression abnormally thickens the zebrafish compact layer, making the ventricle more structurally similar to that in mouse.^[103] It would be interesting to investigate whether this effect is due to angiogenesis from an additional progenitor source.

Liver endothelial cells

BECs are essential for liver development, homeostasis, and regeneration.^[54] Additionally, BECs also constitute the hematopoietic niche of the embryonic liver, supporting the expansion of hematopoietic stem cells before their migration to the bone marrow.^[104] Liver BECs have been described to arise from at least four distinct (but potentially overlapping) developmental sources. During early development, endodermal cells differentiate and proliferate within an epithelium, subsequently migrating into the surrounding connective tissue where they become vascularized. The closest mesoderm-derived mesenchyme surrounding the embryonic midgut endoderm and nascent liver bud is the septum transversum mesenchyme. A study using a liver bud culture system reported that BECs from the septum transversum interact with hepatic progenitor cells prior to liver bud emergence and promote the morphogenetic phase of liver organogenesis, when hepatic progenitors proliferate and migrate into the septum transversum mesenchyme to develop the liver bud.^[105] As such, the septum transversum mesenchyme is the major source of liver BECs, and may be critical for the earliest stages of liver organogenesis.^[106]

In addition to the septum transversum, differentiating endocardial cells are a second source of hepatic BECs, forming a primitive vascular plexus that surrounds the liver bud and contributes to the liver vasculature.^[107] A study using intersectional genetics showed that the

endocardium of the sinus venosus is also a source of hepatic BECs. Mechanistically, VEGF signaling regulates endocardial angiogenesis, which is required for the formation of the primitive vascular plexus surrounding the liver bud and subsequent liver organogenesis.^[107] A third source of hepatic BECs was identified through lineage tracing of paraxial mesoderm-derived cells using a *Pax3^{Cre}* line, showing that the somitic paraxial mesoderm is a source of liver BECs in mouse.^[108]

Hepatic BECs have also been described to arise from *FOXA2⁺VEGFR2⁺* endoderm-derived hepatic progenitors using human ES cell differentiation and mouse lineage tracing models.^[109] Using two distinct genetic lineage tracing strategies, Goldman et al. reported that *Foxa2* expressing cells contribute to the fetal liver endothelium,^[109] suggesting that ECs originate not only from mesoderm but also from endoderm. However, subsequent lineage tracing studies have shown that *Foxa2⁺* cells are found not only in the endoderm, but also in mesodermal cells of the anterior primitive streak.^[110] Given this labelling of some mesodermal progenitors, it is possible that the *Foxa2* lineage contribution to the liver vasculature is through a mesodermal rather than endodermal route.

Lymphatic vessel endothelium

The developmental source of lymphatic ECs has been the subject of interest for over a century. A venous origin for lymphatic vessels was first proposed by Louis-Antoine Ranvier,^[111] inspiring the experimental work of Florence Sabin who in 1902 provided evidence that lymphatics bud from the venous endothelium in pigs.^[111] Alternative origins were also proposed at the time, including the aggregation of mesenchymal cell-derived vesicles to form lymph sacs.^[112] It was not until more than 100 years later that studies in zebrafish^[113] and mouse^[17] showed that lymphatic endothelium is primarily derived from the sprouting, proliferation and migration of venous progenitors. Live imaging of transgenic zebrafish revealed parachordal ECs as the source of thoracic duct LECs,^[113] which are initially specified through asymmetric division of bipotent progenitors in the cardinal vein.^[19,114] In mouse embryos, *Tie2-Cre* based lineage tracing and loss-of-function analyses showed that ECs in the dorsal half of the cardinal vein transdifferentiate under the control of *SOX18*, *NR2F2* and *PROX1* to form LECs.^[17,22] Detailed morphogenetic analyses in mouse subsequently revealed that venous intersegmental vessels provide an additional source of LEC progenitors.^[115]

Although the venous endothelium is the primary source of LECs, other cellular sources have been reported to contribute to zebrafish,^[116] frog,^[117] avian,^[118] and mouse^[41,119,120] lymphatics. In mouse embryos, lineage tracing with *Tie2-Cre* revealed incomplete labelling of dermal lymphatics.^[120] Lymphatic vessels at the dorsal midline and lumbar regions of the dermis form through assembly of lymphangioblast clusters, which were reported to derive from a *Tie2* lineage negative source using the *Rosa26^{mTmG}* reporter.^[120] Subsequent analyses using *Cdh5-CreERT²*, *Sox18^{CreERT²}*, and *Tie2-Cre* lines in combination with a *Rosa26^{tdTomato}* reporter revealed that lymphangioblast clusters at the cervicothoracic midline are derived

from the dermal capillary plexus.^[121] These findings were supported by the identification of LEC progenitors within the dermal capillary plexus that were labelled with *Prox1*^{CreERT2}. However, the direct cellular precursor of lymphangioblast clusters in the lumbar dermis is unclear.^[120,121] In addition, the identification of a tissue-specific requirement for VEGFR3-PI3K signaling during the formation of mesenteric lymphatics led to the discovery that *Tie2* expressing hemogenic endothelium is a source of mesenteric LECs.^[41,122] Lineage tracing with *cKit*^{CreERT2} showed that LEC clusters in the embryonic mesentery are derived from non-venous hemogenic endothelium.^[41]

LECs in the developing heart also arise from multiple sources in both mouse and zebrafish.^[57,119] In mouse, a subset of lymphatic vessels is not lineage-labeled by the *Tie2-Cre* transgene, suggesting that they arise from a *Tie2* negative endothelial or non-endothelial source.^[119] Lineage tracing with *Wt1*^{CreERT2}, *Mesp1*^{Cre}, *Nkx2.5*^{Cre}, and *Wnt1-Cre* ruled out the epicardium, cardiac mesoderm and neural crest as possible sources. Another study also ruled out the coronary vessels and endocardium using *Aplnr-CreERT2* and *Bmx-CreERT2*, respectively.^[57] Instead, the yolk sac hemogenic endothelium was proposed as a source based on lineage tracing with *Vav1-Cre*, *Csf1r-MeriCreMer* and *Pdgfrb-Cre*. The precise identity and functional properties of the *Tie2* lineage negative yolk sac hemogenic endothelial source of cardiac LECs is unclear; the *Tie2-Cre* transgene labels the majority of yolk sac derived myeloid cells, which would be expected to share the same cellular source as non-venous cardiac LECs.^[123] Two independent studies provided substantial evidence that one non-endothelial source for cardiac LECs in mouse is the second heart field.^[124,125] Clonal relationships between second heart field progenitors and LECs, in addition to lineage labeling with *Isl1*^{Cre}, demonstrated their contribution to LECs located on the pulmonary artery and ventral aspect of the cardiac ventricles. In mouse and zebrafish, lymphatics can be observed forming through both sprouting and coalescence of LEC clusters, suggesting dual origins or developmental mechanisms.^[57] Supporting this notion, in the zebrafish heart, lymphatic sprouting follows and is dependent on coronary arteries, while LEC cluster formation is completely independent of coronary development.^[57,58]

Chimeric transplantation in avian embryos^[118,126,127] and lineage tracing analyses in mouse^[108] showed that the initial mesodermal progenitor source of most LECs is the paraxial mesoderm (PXM), which is also known to give rise to skeletal muscle. Transplantation of PXM from quail embryos into the jugular region of chick embryos showed this tissue to be a source of venous and lymphatic ECs.^[127] Subsequent analyses of quail-chick chimeras showed that the lateral plate mesoderm does not contribute to the jugular lymph sac, and that while deeper regions of the avian lymph sacs are derived from venous endothelium, superficial lymphatics are derived directly from the dermomyotome.^[127] Lymphatic ECs have also been shown to derive from the PXM in mouse.^[108] Lineage tracing with the *Pax3*^{Cre} line showed that somitic PXM-derived cells make a preferential contribution to lymphatic rather than blood vessel endothelium, contributing to the dorsal half of the forming cardinal vein, where they begin to express PROX1. *Myf5*^{Cre} and *Pax7*^{Cre} were used to understand the spatiotemporal differentiation of ECs from the somitic PXM (Figure 2),

revealing that lymphangioblast specification from bipotent musculoendothelial progenitors begins in early somitogenesis and becomes restricted to the hypaxial domain of the dermomyotome during development. Following specification, *Pax3* lineage-derived ECs were shown to form the cardiopulmonary, liver, subcutaneous, and dermal lymphatics, but were not identified in the gut. In the dermis, *Pax3* and *Myf5* lineage cells are the source of lumbar LEC clusters, which were shown to arise from a *Tie2* lineage negative population.^[120] Following conditional deletion of *Prox1* in the *Tie2* lineage, blood-filled lymphatics and isolated clusters of dermal LECs remained in the lumbar region.^[120] In contrast, conditional deletion of *Prox1* in the *Pax3* lineage led to an absence of lumbar lymphatics,^[108] indicating that these cells are PXM-derived. Taken together, these findings raise the possibility that in addition to differentiating from PXM-derived venous endothelium, LECs may arise directly from *Pax3* and *Myf5* positive dermomyotome, a tissue that gives rise to superficial LECs in birds.^[118] A technical limitation of using *Pax3*^{Cre} for lineage tracing of PXM-derivatives is the restricted labelling of somitic PXM,^[128] meaning that ECs in the head and neck are not labelled.^[108] Using *Myf5*^{Cre}, which labels muscle precursors throughout the embryo, it was shown that cranial musculoendothelial progenitors are a source of LECs in the jaw, meninges, and ear skin.^[108] The second heart field, which gives rise to clonally related cardiac and neck muscle,^[129] is a third musculoendothelial source that has been shown to give rise to LECs on the ventral side of the heart and in the anterior jugular lymph sacs.^[108,124,125]

Collectively, these studies highlight the heterogeneous developmental origins of ECs, even within individual organs, suggesting that their functions may be related to their ontogeny during development.

ONTOGENY AS A REGULATOR OF ENDOTHELIAL HETEROGENEITY

Morphologically and functionally distinct cell types arise through the hierarchical diversification of developmental lineages. Conversely, phenotypic convergence of anatomically distinct cell lineages towards morphologically and functionally similar cell types can also occur.^[130] For example, in *C. elegans*, where the fate of every cell can be followed from fertilization to adulthood, morphometric^[131] and molecular^[132] analyses have revealed phenotypic and transcriptional convergence of cells derived from distinct lineages during specification of neuroblasts and muscle cells. Furthermore, in the *Drosophila* optic lobe, neurons with distinct lineage histories converge towards transcriptionally similar states that may play distinct roles.^[133,134] Moreover, lineage tracing^[96,107,135] and single-cell transcriptomic^[136–140] analyses have revealed that convergence of anatomically distinct cell lineages towards analogous cellular states is prevalent in vertebrates, including during the specification of monocytes, vascular smooth muscle cells and ECs. However, the extent to which lineage history may impact the function of individual cells of a certain type remains largely unexplored.

Recently, single-cell transcriptomic^[137] and lineage barcoding^[141] analyses in mouse showed that while extra-embryonic and intra-embryonic endoderm-derived cells of the gut tube are molecularly

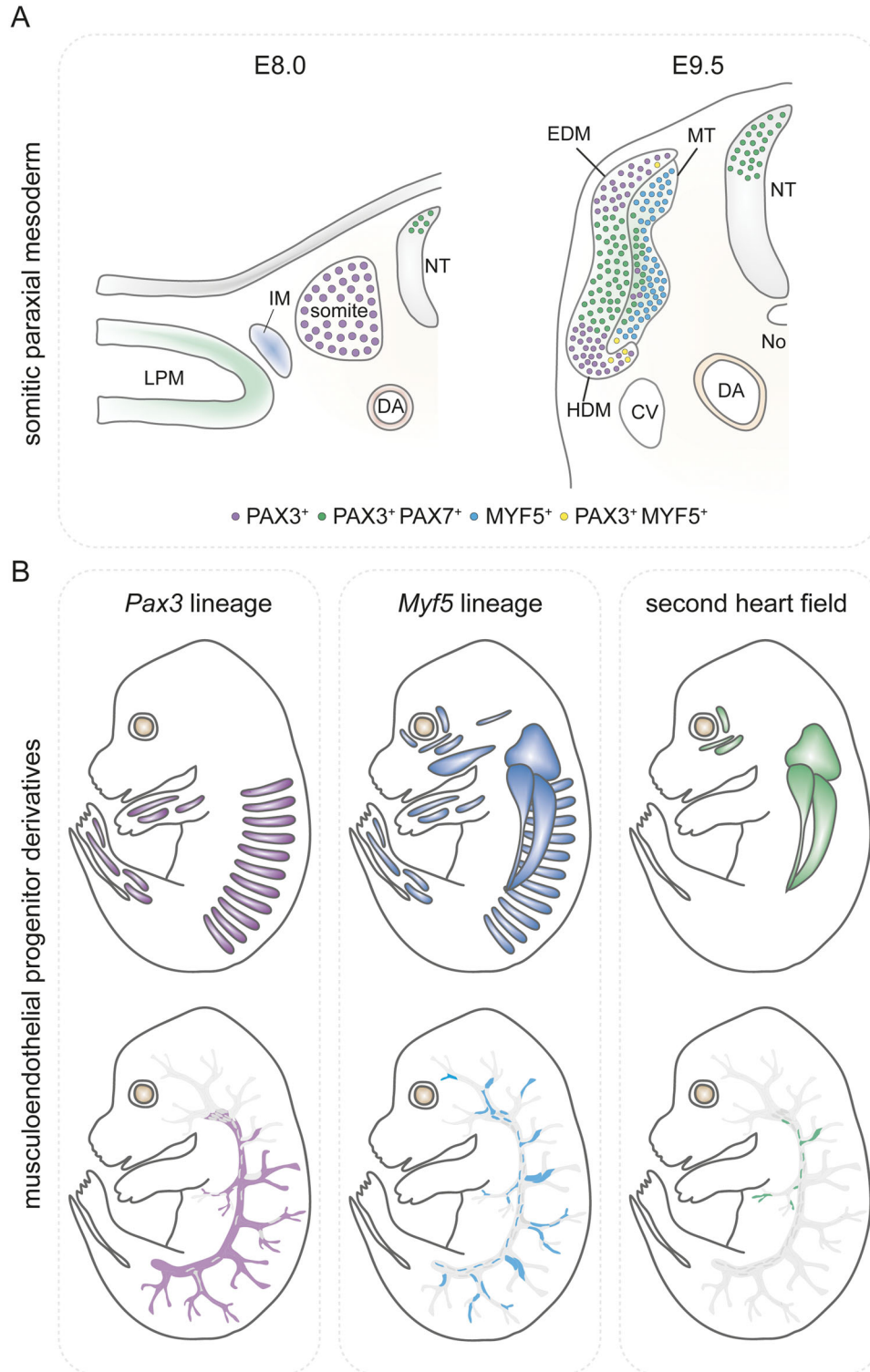


FIGURE 2 (A) Schematic representations of somitic musculoendothelial progenitor cell populations at E8.0 and E9.5. Lineage tracing has shown that ECs arise from *Pax3*⁺ cells in the somitic mesoderm and *Pax3*⁺/*Myf5*⁺ cells in the hypaxial dermomyotome (HDM) (LPM – lateral plate mesoderm; IM – intermediate mesoderm; DA – dorsal aorta; NT – neural tube; CV – cardinal vein; EDM – epaxial dermomyotome; MT – myotome; No – notochord). (B) Schematic representations of the contribution of musculoendothelial progenitor cell populations to skeletal muscle (above) and lymphatic endothelium (below). Lineage tracing of molecularly distinct progenitor populations (*Pax3*-lineage, *Myf5*-lineage, second heart field) leads to labelling of skeletal muscle and LECs in anatomically overlapping regions

similar, they retain lineage-dependent molecular characteristics. Vascular smooth muscle cells, which surround the endothelial layer of arteries and veins, are reported to derive from a number of distinct sources, including the neural crest,^[142] second heart field,^[143] pro-epicardial organ,^[144] and somites^[145]; and the anatomical distribution of vascular smooth muscle cells derived from different lineages correlates with the localized susceptibility of vessels to develop atherosclerotic plaques and aneurysms.^[146] There is also emerging evidence of morphological, molecular, and functional differences between ECs derived from distinct lineages. Coronary vessel ECs in mouse that are derived from different lineages are molecularly and phenotypically distinct, at least through developmental stages: ECs derived from the sinus venosus retain significantly higher expression of *Aplnr* and display a more elongated morphology.^[96] Future studies should investigate whether these two populations of coronary ECs remain distinct, either molecularly or functionally, throughout the life of the animal.

CONCLUSIONS AND OUTLOOK

The development and evolution of distinct cell types are underpinned by the activity of cell-type-specific transcription factor modules, which bind regulatory elements composed of transcription factor binding sites that reflect their tissue-specific function.^[130] During embryonic development, the rapid diversification of regulatory element accessibility and usage underpins the establishment of distinct cellular identities,^[147] and can preconfigure cells for allocation to specific fates.^[148,149] In the vasculature, regulatory elements that drive arterial,^[150] venous,^[151] lymphatic,^[152] and angiogenic^[153] gene expression have provided insight into the transcriptional control of endothelial diversification. However, it is unclear if ECs derived from distinct sources are endowed with a lineage-dependent transcriptional memory as a result of perduring differences in regulatory element configuration and usage. To address this gap in our knowledge, future experiments should use genome-wide methods to compare enhancer activity in ECs derived from distinct lineages. Furthermore, the rapid evolution of single-cell genomics has made it possible to assess chromatin accessibility and gene expression in individual cells, which may allow the identification of lineage-dependent characteristics in mixed populations of closely related ECs from different sources, such as in the heart or liver. Furthermore, analysis of somatic mitochondrial DNA mutations coupled to scRNA-seq may allow the identification of similar cell populations in human tissues.

It is tempting to speculate that while largely similar to their neighbors, ECs remain under the control of lineage-restricted gene regulatory networks that may impact various aspects of endothelial function beyond basal molecular and functional homeostasis. A recent study that used scRNA-seq and scATAC-seq to assess the molecular response of BECs to carotid artery ligation revealed reprogramming of enhancer accessibility that was linked to changes in gene expression and cell function.^[154] It will be interesting to determine if lineage-dependent differences in local chromatin composition impact endothelial function during development and in disease. This functional specialization may

include differential control of metabolism, vasorelaxation, angiocrine secretion, disease progression, and organ regeneration. It is also possible that lineage-specific characteristics are lost during aging or in disease settings where endothelial function is impaired. Further investigations should open a path to study the mechanisms and importance of EC lineage diversification in human development and disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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