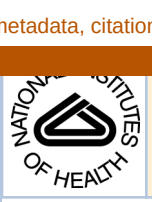

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The Opening Act: Vasculogenesis and the Origins of Circulation

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Abstract

Previous studies on cellular and molecular mechanisms that regulate vascular development identified key signaling pathways and transcription factors. These findings supported the notion that the formation of vasculature is predominantly regulated by genetic programs, which is generally accepted. However, recent progress in understanding nongenetic factors that can modify the preprogrammed genetic mechanisms added another layer of complexity to our current understanding of vascular development. Here, we briefly summarize historic viewpoints and evolutionary perspectives on vascular development. We also review the cellular and molecular mechanisms that govern the emergence of the endothelial lineage and the subsequent process of vasculogenesis during development, with an emphasis on vascular endothelial growth factor and angiopoietin signaling cascades. Finally, we discuss epigenetic factors such as hemodynamic forces and hypoxic responses that can modulate and override the predetermined genetic mechanisms of vascular development.

Keywords

vasculogenesis; VEGF; angiopoietin; endothelial progenitor; development

The cellular ontogeny and evolutionary origin of the endothelial lineage and the vascular system have been a center of investigation for centuries. Collective efforts so far have determined that the endothelial lineage has a surprisingly heterogeneous origin. Likewise, it is becoming increasingly clear that the molecular mechanisms that govern the initiation of the endothelial lineage are surprisingly intricate.[1,2] In recent years, the specification and differentiation of the endothelial lineage have been extensively studied in an effort to delineate genetic mechanisms that modulate this process. These studies have resulted in the identification of numerous diverse signaling pathways associated with the endothelial lineage, including vascular endothelial growth factor (VEGF) and angiopoietin.

Historical Perspective on the Circulatory System

The existence of the circulatory system has been known to many cultures from ancient times. For instance, Yellow Emperor's Manual of Corporeal Medicine, written in the second century B.C. in China, described the circulation pattern of the blood within the body.[3] In ancient Greece, a group of natural philosophers, including the famous Aristotle, recognized that higher animals contain blood.[4] The anatomic structure of the circulatory system,

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None.

however, was not identified until Erasistratus, who discovered membranous structures within the heart that connected to large blood vessels.[5] In the second century A.D., Claudius Galenus subsequently demonstrated that blood vessels are used as a conduit for the circulating blood. These observations were rediscovered later during the pinnacle of the Islamic Golden Age. Ibn al-Nafis of Damascus wrote a description about the circulation of blood which was later translated into Latin and served to influence many Renaissance scientists such as Giordano Bruno and Michael Servetus.[6,7] However, the actual importance of the circulatory system was not widely recognized until the seminal publication by William Harvey in the seventeenth century, the *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animali-bus*, (*An Anatomic Exercise on the Motion of the Heart and Blood in Living Beings*). William Harvey was the first person to suggest the presence of a complete circulatory loop within the body, including the pulmonary circulation, and delineated the function of the heart.[8,9] With the advent of the microscope, the Italian scientist Marcello Malpighi discovered that arteries and veins are connected via capillary vessels.[9]

Although the physiological aspects of the circulatory system were relatively well known by the end of the Renaissance era, its origin during development remained largely unexplored. In the late 1800s, Wilhelm His suggested that the cellular constituents of blood vessels were generated from extraembryonic tissue and that blood vessels themselves were formed by a series of morphogenetic events.[10] He used the term “angioblast” to describe the mesenchymal cells that give rise to the cellular constituents of blood vessels. Although this suggested origin of blood vessels by His was proven wrong, he did correctly point out that blood vessels do develop via distinct morphogenetic events. In the late 1910s, Florence Sabin observed that, during development, red blood cells and blood vessels originate concomitantly in adjacent regions of the avian embryo, and postulated that these two lineages might share the same progenitors. She adopted the term “angioblast” from His to describe this hypothetical progenitor,[11] which was later renamed “hemangioblast” by Murray to properly reflect that this progenitor is thought to generate both endothelial and hematopoietic lineages.[12] Recently, it has been shown that the endothelial lineage shares common progenitors with diverse cell types derived from the mesoderm, including cardiomyocytes, smooth muscle cells, as well as subtypes of blood cells.[13,14] Furthermore, in lower vertebrates, it has been reported that mesodermal cells programmed to generate kidney or other mesodermal cell types can differentiate into endothelial progenitors under certain circumstances, [15,16] suggesting that the emergence of the endothelial lineage might be far more diverse than previously thought.

Evolution of the Circulatory System

The essential function of the circulatory system is to provide essential nutrients and oxygen, as well as to remove cellular waste. Various model organisms that are commonly used to study biological sciences reflect distinct evolutionary stages of the circulatory system. In the animal kingdom, some phyla, notably Platyhelminthes, Nematoda, and Porifera, lack any circulatory system. Instead, their body structure allows for all cells to directly absorb nutrients and oxygen. For instance, a well known animal model, *Caenorhabditis elegans*, has a primitive internal cavity known as the pseudocoelom, which allows every cell in the body to have direct access to nutrients and oxygen.[17] In addition, this organism possesses a rudimentary excretory system consisting of two cells which extend a tubular structure along the body axis to regulate osmotic pressure and remove excessive ions and cellular metabolites.[17] However, the majority of Bilateria have a functional circulatory system. Depending on whether blood and interstitial fluid mix, the circulatory system in these animals is divided into two major groups: the open circulatory system and the closed circulatory system. In the phyla with the open circulatory system such as Arthropoda, the blood can freely blend with the interstitial fluid to generate hemolymph. The internal organs

in these animals are submerged within an internal cavity called the hemocoel and directly receive oxygen and nutrients, and deposit metabolic wastes.[18] The hemolymph is drawn back through the ostia (open end pore) when the heart dilates, a process that is often facilitated by muscular movement. Closed circulatory systems can be found in Chordata, Annelida, as well as some species of Mollusca (Cephalopods). In the closed circulatory system, the blood circulates only within the internal lumen of the blood vessels and does not mix with interstitial fluid.

Cellular Origin of Endothelial Cells During Development

During development, endothelial cells appear to emerge from mesodermal tissue residing within diverse regions of the embryo, ranging from blood islands in the yolk sac[19,20] and within the embryo proper[21,22] to atypical regions such as allantois[23,24] and placenta[25,26] (Figure). In mice, the first site the endothelial lineage appears is the extraembryonic visceral mesoderm in the yolk sac, where endothelial progenitors contribute to the blood islands.[27,28] Endothelial progenitors in the embryo proper subsequently appear in the rostral region of the embryo around embryonic day (E) 8, based on the expression pattern of various endothelial markers. In the avian model, formation of endothelial cells can be observed as early as the gastrulation stage. Similar to the endothelial progenitors in mice, endothelial progenitors in avian embryos are closely associated with the hematopoietic lineage.[29–31]

Based on the temporal and spatial proximity of endothelial and hematopoietic lineages during avian development, in the early 1900s Sabin postulated that these two lineages share a common progenitor, which was later named the “hemangioblast” by Murray.[11,12] Since then, much indirect evidence supporting this common progenitor notion has been reported, including the phenotype of VEGF receptor2 (Flk1/KDR) knock-out mice[27] and the zebrafish mutant, *clo*.[32] Recently, studies using embryonic stem (ES) cell–derived embryoid bodies provided more evidence that supports the existence of the hemangioblast. Using ES cells containing blast colony-forming cells (BL-CFC), Choi and colleagues reported that a single progenitor-derived embryoid body contains both endothelial, hematopoietic, and smooth muscle lineages, implying the existence of the hemangioblast.[33] Although these observations do not provide direct evidence of the hemangioblast, they provide invaluable insights into understanding the endothelial or hematopoietic lineage specification process. Despite these findings, providing in vivo evidence that directly supports the existence of the hemangioblast as a bipotential progenitor during development has been extremely difficult because of the technical limitations innate to commonly used model systems. Previously, based on their fate mapping and lineage tracing data, Kinder and colleagues suggested that endothelial and hematopoietic lineages might arise independently, alluding to the fact that the hemangioblast might not exist in vivo.[34] However, Huber and colleagues took this one step further and isolated potential hemangioblast cells (identified by the expression of both brachyury and Flk1) from E7.5 mouse embryos and showed that these cells could give rise to both hematopoietic and vascular cells.[35] Recently, Vogeli and colleagues demonstrated that developing zebrafish gastrula contain progenitors that generate both endothelial and hematopoietic lineages using single cell resolution fate mapping.[36] Surprisingly, they also found that the hemangioblast gives rise to only a small fraction of the endothelial lineage, whereas the majority of the endothelial lineage derives from endothelial-specific progenitors, or angioblasts.[36] Similar findings have also been reported in mammalian and avian models.[37,38] Although the precise molecular and cellular mechanisms modulating the specification of endothelial progenitors are largely unknown, recent identification of Islet1, Nkx2.5, and Flk1-positive multipotent cardiovascular progenitor cells within the nascent mesodermal cells provide an important insight into the developmental ontogeny of the endothelial lineage[13,14] (Figure). It is likely that the hemangioblast is an integral component of early vascular development as well as primitive hematopoiesis. However, whether the

hemangioblast exists beyond the early developmental stage to contribute to definitive hematopoiesis, and if so, also plays a critical role in postembryonic stage, still needs to be determined.

The majority of the endothelial lineage does appear to originate from conventional progenitors such as angioblast and hemangioblast, which are predominantly derived from the posterior and lateral mesoderm. However, it is also critical to note that the entire mesoderm (excluding notochord and prechordal mesoderm) can be reprogrammed to generate endothelial cells during development,[39] suggesting that the ability to produce the endothelial lineage might be one of the intrinsic properties of the mesoderm. However, this plasticity of mesodermal cells diminishes as embryogenesis progresses, and only a small group of endothelial progenitors retain the ability to produce the endothelial lineage in later development.

Vasculogenesis: De Novo Formation of Vascular Structure

Vascular structures in vertebrates are formed by two distinct mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is a process by which new vessels are generated from endothelial progenitors via differentiation. The subsequent assembly of these differentiated cells leads to the formation of primitive blood vessels. Conversely, angiogenesis describes the process of new vessel formation from the sprouting or splitting of preexisting vessels. Although environmental factors such as hemodynamics[40,41] and hypoxic response[42,43] appear to modulate the vasculogenesis process, initial development of the vasculature seems to be genetically programmed.[44–46] In mice and chick, even before the onset of circulation, endothelial cells that are differentiated from the progenitor population assemble into a capillary network. After the onset of circulation, the capillary network remodels into arteries and veins to generate a functional circulatory loop.

For the functional vertebrate circulatory system, subsequent morphogenesis of a vascular lumen is essential. Previous studies on lumen formation using Mardin-Darby Canine Kidney (MDCK) cell culture provide critical insight on how vascular lumens might be generated.[47] Nascent MDCK cells initially establish polarity by actively sorting the apical membrane to the luminal side and subsequently stabilize by deposition of tight junctions. However, because MDCK cells are epithelial in origin, they do not necessarily reflect behaviors of endothelial cells during lumen formation. Furthermore, given that complex interactions between different cell types which might be essential to generate the complicated three-dimensional architecture of a vascular lumen in developing embryos, the precise cellular and molecular mechanisms that modulate vascular lumen formation in vivo still remain elusive. Recently, Kamei and colleagues demonstrated that intracellular vacuoles within endothelial cells, which were first described by Folkman and Haudenschild,[48] coalesce to create a functional lumen within the sprouting intersegmental vessels in developing zebrafish embryos.[49] This result suggests that vascular lumen formation might recapitulate the similar cellular and molecular processes that have been reported in various cell culture models.[49]

Many of the signaling pathways responsible for regulating vascular development elicit their function in a noncell autonomous manner,[2] suggesting that specific tissue interactions are critical during this process. In mice, it has been proposed that the endoderm is necessary and sufficient for the specification of the endothelial lineage.[50,51] Evidence supporting this claim include the fact that the endoderm is juxtaposed to the region of the mesoderm that produces endothelial progenitors and that some of the key signaling molecules that regulate endothelial specification are expressed in the endoderm.[51] In particular, Hedgehog has been implicated as a potential signaling molecule secreted by the endoderm because it is expressed in the visceral endoderm starting at E6.5, and loss of Hedgehog signaling causes

defects in vasculogenesis as well as hematopoiesis in embryoid bodies.[52] However, Vokes and Krieg analyzed this hypothesis and found that Hedgehog signal secreted from the endoderm is dispensable for the specification of the endothelial lineage, although essential for the subsequent morphogenesis of the vascular network in mammalian and avian model systems.[51] Likewise, Lawson and colleagues elegantly demonstrated that cell nonautonomous signals, namely Hedgehog from the hypochord and VEGF from the ventral somites, induce arterial differentiation.[53] Although the interaction between nascent endothelial progenitors and their surrounding tissue appear to be indispensable for the subsequent differentiation of either arterial or venous endothelial cells, there are always some exceptions. In zebrafish, the function of the endoderm is negligible for vascular development because endoderm-less mutant embryos form a fully lumenized and highly organized vascular network.[54]

Molecular Pathways That Regulate the Specification of the Endothelial Lineage

The molecular mechanisms that regulate vascular development appear to be highly conserved among vertebrates. Given that endothelial progenitors emerge from the mesoderm, it is expected that several signaling molecules that function during the patterning of the mesoderm would be critical for the specification of the endothelial lineage (Figure). To date, many signaling pathways including the Wnt/Frizzled, Delta/Notch, bone morphogenic protein (BMP), Ephrin/Eph receptor, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) signaling pathways, as well as several transcription factors such as Scl, Runx-1, and Ets have been shown to be essential for the specification of the endothelial lineage and subsequent vasculogenesis. The list of factors whose function is essential for vascular development is continuously increasing with the recent addition of microRNA,[55,56] BMP antagonist BMPER/Crossveinless,[57,58] as well as lysocardiolipin acyltrans-ferase.[59,60] However, many of these factors do not function exclusively within the endothelial lineage. For instance, arguably the most critical signaling pathways for the endothelial lineage, the VEGF and the angiopoietin/Tie signaling pathways, appear to be critical for the development of nonendothelial lineages such as hematopoietic stem cells as well as neural stem cells.[61–64]

Vascular Endothelial Growth Factor and Related Molecules

Although a number of signaling pathways and their effectors have been implicated in vascular development in vertebrates, VEGF is regarded as the most critical factor for the emergence of the endothelial lineage and is also the most extensively studied signaling pathway within the context of vascular development. The function of VEGF is required for all aspects of vascular development—from the earliest event of the endothelial lineage specification to vessel maintenance. During development, activation of the VEGF signaling pathways is the earliest known landmark that defines the endothelial lineage commitment within the nascent mesoderm. The biochemical and molecular properties of VEGF have been extensively studied because of its essential role during vascular development. Biochemically, VEGF is a member of the platelet-derived growth factor (PDGF) family and its function is transduced via an array of tyrosine kinase receptors. In mammals, 5 VEGF molecules, 3 major receptors, VEGFR1/Flt1, VEGFR2/KDR, and VEGFR3/Flt4, and 2 coreceptors, Neuropilin 1 and 2, have been identified. Attenuation of any of these VEGFs or their receptors results in embryonic lethality. Similar phenotypes have been reported in *Xenopus* and chick embryos with compromised VEGF signaling. In zebrafish, VEGF also appears to be indispensable for the formation of the endothelial lineage, and subsequent differentiation into arterial endothelial cells by inducing arterial specific Notch activation.

It has been proposed that VEGF signaling is transduced via the VEGFR2/KDR receptor whereas VEGFR1/Flt1 modulates this process, functioning as a reservoir for VEGF ligands.[65–67] In agreement with its proposed function, the main receptor for VEGF signaling, VEGFR2/KDR, is widely used as a molecular marker that defines the endothelial lineage during development alongside PECAM and the ETS-related transcription factor, Fli. The expression of VEGFR2/KDR can be readily observed in early stages of development in mice,[27] chick,[22] and zebrafish.[67,68] In mammalian and avian model systems, *flk1/vegfr2* is the earliest known endothelial-specific marker, being expressed as early as the 5 somite stage (12 hpf) within the presumptive endothelial progenitors alongside progenitors for other mesodermal lineages in the lateral plate mesoderm.[22,27] Mice deficient in *VEGFR2/KDR* die between E8.5 and E9.5 in utero because of defects in endothelial and hematopoietic lineages.[27] At E7.5, homozygous null *VEGFR2/KDR* mice are completely devoid of blood islands, and no organized vasculature can be detected in the embryo proper. In contrast, mice that lack functional *Flt1* form both embryonic and extraembryonic endothelial cells, but display grossly dysmorphic vasculature, indicating that the function of Flt1 is dispensable for the specification of the endothelial lineage. VEGF signaling appears to function in a more complex manner in zebrafish partly because of genome duplication.[68] There are at least 4 functional VEGF receptors that have been identified in zebrafish to date: VEGFR2/KDR, KDR-related (KDRL), VEGFR1/Flt1, and VEGFR3/Flt4, with KDRL being the main transducer of VEGF signaling.[68] Interestingly, unlike in mammalian model systems, mutation in KDRL does not cause any obvious early vascular defects.[67,68] Early stages of vascular development, including the specification of the endothelial lineage, occur normally in homozygous *KDRL* mutant zebrafish embryos that completely lack a functional KDRL receptor. Later vascular development, such as intersegmental vessel formation, is however compromised. Morpholino-mediated knockdown of all VEGF receptors causes significant reduction of the endothelial lineage, suggesting that different VEGF receptors synergistically interact to mediate VEGF signaling in zebrafish.[67,68]

Despite the extensive studies on VEGF function in vascular development, upstream signaling cascades that regulate the initiation of VEGF and VEGFR2/KDR expression are still largely unknown. However, recent studies show that HoxB5 and FoxH1 can directly bind to the cis-regulatory elements of VEGFR2/KDR in mice and zebrafish, respectively.[69,70] Wu and colleagues reported that HoxB5 can bind the regulatory element within the first intron of the *Vegfr2/Kdr* locus to induce the expression of VEGFR2/KDR.[69] Interestingly, overexpression of HoxB5 causes a significant increase in the number of endothelial progenitors, suggesting that HoxB5 might be necessary and sufficient to initiate the specification of endothelial progenitors. In contrast, FoxH1 appears to function as a repressor for *vegf2/kdr* expression.[70] Choi and colleagues demonstrated that FoxH1 can bind the upstream enhancer of *vegf2/kdr* locus in zebrafish and attenuate its expression.[69] Taken together, these data suggest that the expression of VEGF signaling components is highly regulated by complex mechanisms.

Angiopoietins and Related Molecules

The second signaling pathway that predominantly functions within the endothelial lineage is the Angiopoietin (Ang)/Tie-2 pathway. Structural signatures of Angiopoietins are a fibrinogen-like domain which is essential for the binding to the Tie-2 receptor, and a coiled-coil domain which is used for oligomerization.[71–73] There are at least 3 members of the Ang family, Ang-1, Ang-2, and Ang-3 (mice)/Ang-4 (human), all of which bind to the receptor tyrosine kinase Tie-2. During development, Ang-1 can be detected as early as E9 in the myocardium and becomes more broadly expressed in later stages extending to the surrounding mesenchyme of developing blood vessels.[74] In contrast, the second Angiopoietin gene, Ang-2, is expressed in the smooth muscle cells associated with the major

vessels during development.[75,76] The expression of Tie-2 can be detected as early as E8 in the endocardium and endothelial cells within the dorsal aorta.[77–79] Mice deficient in Ang-1 or its receptor Tie-2 die at E9.5 and 12.5 because of multiple cardiovascular defects. However, vasculogenesis appears to occur normally in these embryos, and the primary reason for their early lethality seems to be the failure of endothelial cells to adhere to the surrounding extracellular matrix.[80] This phenotype clearly indicates that Ang-1/Tie-2 signaling is not essential for the specification of the endothelial lineage, but is required for the later aspects of vascular development such as patterning, migration, and maturation of the vascular network. Recent reports suggesting that Ang-1 promotes tight association of adjacent endothelial cells and between endothelial cells and vascular smooth muscle cells further support this conclusion. Interestingly, overexpression of another ligand of Tie-2, Ang-2, causes a phenotype similar to Tie-2–deficient mice,[81–83] suggesting that Ang-2 might function as an antagonist of Ang-1 to modulate the activity of Tie-2. Indeed, Scharpfenecker and colleagues found that Ang-2 is expressed before vessel remodeling, possibly functioning as a destabilizing signal, thereby antagonizing the function of Tie-2 in vascular maintenance.[84] Taken together, these findings indicate that both Ang-1 and Ang-2 signal through Tie-2 in a distinct manner, and complement VEGF signaling to modulate vascular development. The function of 2 additional Angiopoietins, Ang-3 and Ang-4, during development is largely unknown.[85,86]

In addition to the Angiopoietins, several molecules that possess a coiled-coil domain and a fibrinogen-like domain have been recently identified. Given their structural resemblance to the Angiopoietins, these molecules were named as angiopoietin-like proteins (Angptls).[87,88] Despite their structural similarities, Angptls do not bind to Tie-2 or Tie-1 receptors.[89] In humans, Angptl-1 and Angptl-2 have largely overlapping expression patterns, suggesting that these molecules might function redundantly. Recent studies in zebrafish demonstrated that Angptl-1 and Angptl-2 synergistically regulate the patterning of the vascular network. Embryos with attenuated Angptl-1 and Angptl-2 activity display defective intersegmental sprouting and increased level of apoptosis within the dorsal aorta.[90] Unlike Angptl-1 and Angptl-2, other Angptls do not seem to have any obvious function related to vascular development.[89,91]

Local Environmental Factors That Influence Early Vascular Development

As previously mentioned, early vascular development is largely governed by a genetic program. However, increasing evidence suggest that nongenetic components also modulate vascular development. In particular, these nongenetic factors seem to be the major driving force during vascular remodeling when the capillary plexus undergoes morphogenetic changes to create more defined arteries and veins. For instance, hypoxic conditions stimulate vasculogenesis in several model systems. As embryonic development proceeds, cells distantly located from a blood vessel undergo a hypoxic response, which triggers the stabilization of Hypoxia Inducible Factor-1 α (HIF-1 α), which in turn forms a heterodimeric transcription factor complex with constitutively expressed HIF-1β. The activation of HIF-1 induces an array of genes involved in vascular development including the VEGFs and their receptors, and the Angiopoietin receptor Tie-2.[92] Mice that lack a functional HIF-1α locus die in utero because of defects in vascular remodeling,[93] suggesting an essential role for the HIF pathway in vascular development. However, mouse embryos cultured under hypoxic conditions before the onset of circulation initiate normal vascular remodeling.[40] Similar results have been observed in other model systems,[94,95] alluding to the possibility that HIF-1α–induced regulation of vascular development might be dispensable for earlier stages of development. Although it is apparent that the HIF pathway does function during early vascular development, analyzing the exclusive function of HIF

during this process is problematic because of the fact that the HIF pathway interacts with other signaling pathways involved in vascular development, such as TGF-β and VEGF.

In many vertebrate model systems, the onset of circulation occurs while the vascular network is still forming.[96] The blood flow within the developing vessel generates mechanical forces, collectively known as hemodynamic forces, which can be recognized by endothelial cells.[97] As a result, endothelial cells within the vessels that are actively experiencing hemodynamic forces turn on the expression of eNOS.[98] A series of reports suggest that hemodynamic forces are essential for modulating vascular development. For instance, the secondary defects of the extraembryonic vasculature observed in mice embryos with impaired cardiac contractility support this notion.[40] Recent studies by Lucitti and colleagues analyzed this phenotype in detail and successfully demonstrated that the shear stress generated by circulating red blood cells is critical for inducing the expression of eNOS in endothelial cells, which can serve as a driving force for vascular remodeling during embryogenesis.[40] Although the exact mechanism is not known yet, it is very likely that mechanical forces created by the circulation can regulate subsequent development of the vascular network during development.

Concluding Remarks

In this review we have discussed how early vascular development proceeds, and we have explored some of the cellular and molecular mechanisms that regulate this process, emphasizing the specification of the endothelial lineage. Although the function of the circulatory system has been conserved throughout different phyla, the endothelial lineage in each species appears to possess diversity in terms of their origin and properties. The endothelial lineage emerges from heterogeneous progenitors residing in different regions within the developing embryo, and diverse signaling pathways and environmental cues regulate the induction of the endothelial lineage. With the help of novel technologies and various animal model systems, the complexity of the endothelial lineage has only just begun to be unveiled.

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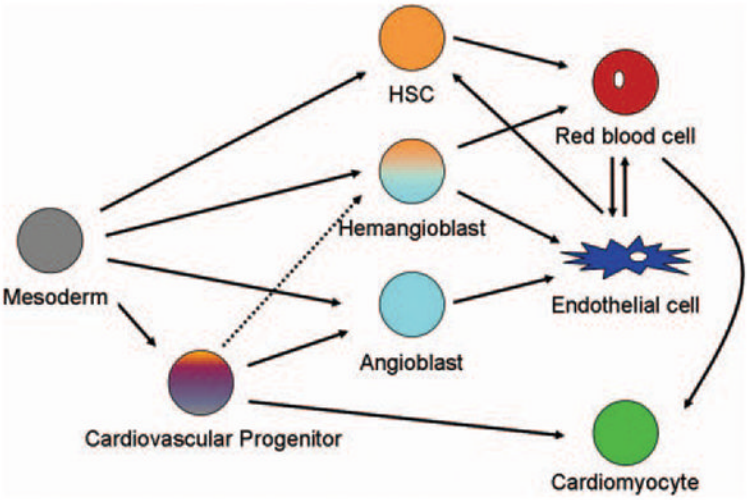

Figure.
Origin of the endothelial lineage. The endothelial lineage appears to emerge from diverse progenitors and has a close association with other lineages found in the cardiovascular system.