Coronary Vessel Development

A Unique Form of Vasculogenesis

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Abstract—Development of the coronary vascular system is an interesting model in developmental biology with major implications for the clinical setting. Although coronary vessel development is a form of vasculogenesis followed by angiogenesis, this system uses several unique developmental processes not observed in the formation of other blood vessels. This review summarizes the literature that describes the development of the coronary system, highlighting the unique aspects of coronary vessel development. It should be noted that many of the basic mechanisms that govern vasculogenesis in other systems have not been analyzed in coronary vessel development. In addition, we present recent advances in the field that uncover the basic mechanisms regulating the generation of these blood vessels and identify areas in need of additional studies. (Arterioscler Thromb Vasc Biol. 2003;23:2138-2145.)

Key Words: coronary vessels n development n embryology n heart

A Brief Description of Coronary Vessel Development

Coronary vessel development is an example of vasculogenesis followed by angiogenesis with unique variations specific to heart development. Vasculogenesis has been described as the de novo generation of blood vessels, whereas angiogenesis can be thought of as the generation of capillaries, veins, and arteries from preexisting vessels. The process begins with the delivery of vasculogenic cell types to the surface of the heart after beating has begun. These cells must then disperse throughout the heart; differentiate into endothelial cells, smooth muscle cells, pericytes, and fibroblasts; subsequently form arteries, veins, and capillaries; and finally connect to the aorta and coronary sinus. Delivery of a population of cells to an existing organ requires dynamic cellular events, and coordination of cell movement with the precise timing of delivery, commitment, and differentiation is critical for proper vessel formation and organ development.

Originally, researchers thought that coronary vascular progenitors were derived from the cardiac mesoderm, like the other cell types in the myocardium and endocardium. These data were first challenged by Manasek, who demonstrated that the heart tube is initially composed of only endocardium and myocardium and that the epicardium arises from extracardiac regions. In more recent years, several studies have determined that progenitors of the epicardium and coronary vascular system are derived from extracardiac tissue, the proepicardial organ (PEO; Figure 1). Although the earliest location of these progenitors is still in debate, they appear to arise from splanchnic mesoderm. The PEO is associated with the septum transversum that grows from the dorsal body wall ventrally to divide the embryonic coelom into the pleuropericardial and peritoneal cavities and forms a portion of the diaphragm. However, the PEO is a transient structure that consists of a single epithelial layer folded into a shape resembling a grapelike cluster. In chicken (HH stage 17) and mouse (E9.5) embryos, the PEO migrates to and then over the surface of the heart to form the primitive epicardium (Figures 1 through 3). Experimental ablation of the PEO or genetic models that inhibit its migration result in absence of the epicardium.

Migration of the PEO to form the primitive epicardium occurs slightly differently in mouse and chicken embryos. In chickens, the PEO extends to the heart and attaches as a continuous epithelial sheet. On contact, the epithelium splits and advances in at least 2 directions to cover the heart and pericardial cavity (Figure 2). In contrast, murine PEO cells attach to the heart by way of cysts (vesicle-like structures) of PEO cells. These cysts float freely in the fluid of the pericardial cavity and then become attached to the surface of the heart. Attachment might occur near the original PEO location or at some distance away, but cells quickly contact each other and initiate formation of a continuous epithelium by E10.5 to 11. In both avian and murine embryos, once epithelial cells attach to the outer heart wall, the epithelium proliferates and forms a continuous epicardium that migrates over the myocardial heart tube.

Soon after formation of the epicardium, the epithelial-mesenchymal transition (EMT) begins in specific regions of the heart, mainly in the atrioventricular and conoventricular grooves. EMT is the generation of mesenchyme or migratory cells from an epithelium. A variety of labeling techniques...
have been used to show that these cells become migratory mesenchyme and move into the subepicardial connective tissue space and subsequently into the myocardium itself (Figure 4).11,12,19,20 The work of several laboratories has shown that epicardially derived mesenchyme gives rise to most cells of the coronary system.5,7,11,20,21 This means that vasculogenic mesenchyme must travel extensively throughout the developing myocardium, because every cardiac myocyte of the avian and mammalian heart is in contact with at least 1 capillary. This extraordinary invasive activity can be best appreciated in the chick/quail chimera studies of Mann,12 who demonstrated that grafted PEO cells are later seen throughout the entire host heart, graphically demonstrating the pervasive nature of cardiac mesenchyme. Once these cells have migrated throughout the heart, they differentiate into vascular endothelium, smooth muscle, pericytes, and fibroblasts and form primary vascular plexi (Figure 5). Later, these structures are remodeled and reshaped by angiogenesis to form the major coronary vessels as well as all other smaller-caliber arteries, veins, and capillaries.22–24 Interestingly, most of the developmental events that lead to the differentiation and patterning of the coronary system proceed in the absence of blood flow, as the connection of the proximal coronary arteries to the aorta occurs relatively late in this process.

From these descriptive studies on coronary vessel development, we have learned that (1) a unique population of cells is delivered to the heart by the migration of PEO, (2) they subsequently undergo EMT from the epicardium, and (3) they migrate throughout the myocardium before differentiating into vasculogenic cells. Clearly, cell migration, cell lineage determination, and morphogenesis must be closely coordinated. In the following sections, we will review specific areas of emerging research on this process.

**Formation of the Epicardium**

The movement of the PEO to and over the heart is a particularly interesting event in embryology and heart development. Still, movement of PEO cells to the heart is poorly understood. Nahirney et al.16 have demonstrated a glycoprotein-rich bridge between the advancing PEO and the heart that spans the future pericardial cavity. The functional significance of this structure will be important to determine. Movement of the PEO to the heart is not random, usually making contact near the area of the atrioventricular junction. Identification of factors, either structural or chemotactant, that mediate the migration of cells to the heart is critical.

The migration of an epithelium or of groups of epithelial cells over a developing organ is a relatively rare event in
embryogenesis and is not well understood. Regulation of adhesion within cells of the migrating PEO and epicardium, as well as adhesion between the PEO/epicardium and the cellular and extracellular matrix (ECM) components of the myocardium, is essential to this process. Examination of the differential expression and distribution of extracellular matrix molecules and their cellular receptors suggests that these molecules are likely to regulate coronary vessel development.13,14,17,18,25

The importance of regulating cell adhesion during PEO migration to and over the heart is exemplified by the genetic analysis of vascular cell adhesion molecule-1 (VCAM-1)/α4-integrin interaction. VCAM-1 is a cytokine-inducible transmembrane protein of the immunoglobulin superfamily of cell adhesion molecules, and its only known ligand is α4β1-integrin. Integrins are a group of heterodimeric transmembrane receptors that mediate cell-cell and cell-matrix interactions.25,26 VCAM-1 is expressed in the developing myocardium from the initiation of heart development until E13.5,19 whereas α4-integrin is detected in the PEO and epicardium.17,18 These opposing expression patterns suggest a role for these molecules in the regulation of PEO and epicardial adhesion as well as movement on the myocardium.

Functional inactivation of VCAM-1 in knockout mice reveals that formation of the epicardium is severely inhibited. As a result, hearts of mutant mice leak blood into the pericardial space, have no subepicardial vascular development, and are not viable.14 Loss-of-function studies of α4-integrin in mice also showed that formation of the epicardium is severely inhibited.15 Further examination of this phenotype showed that cells are found in the PEO region but that cysts fail to bud out and attach to the heart.17 The few cysts that are able to reach the heart fail to migrate out as epithelium. Taken together, these studies indicate that this interactive adhesion system in both the epicardium and myocardium is essential for movement of PEO cysts, epicardial formation, and subsequent epicardial migration.

The role of the ECM in the attachment, formation, and movement of the epicardium is now becoming clear. Several studies have revealed a complex pattern of expression and localization of ECM components. For example, fibronectin is expressed throughout the developing epicardium but is most concentrated on the epicardial-myocardial interface.27,29 In addition, fibronectin is detected at the external surface of the myocardium where the advancing epicardium has not yet migrated. Other ECM molecules such as vitronectin, laminin, JB3, and fibrillin exhibit dynamic patterns of expression at or near the epicardial-myocardial interface and might provide cues to the epicardium for its differentiation during and after attachment and migration over the myocardium. Although a comprehensive understanding of the ECM in epicardial movement and adhesion during PEO and epicardial migration is not yet complete, these data suggest that an intricate modulation of the ECM facilitates a scaffolding for cell migration to and over the heart.

Obviously, expression of ECM and cell adhesion molecules is regulated by transcription factors, and thus, elucidation of their roles during coronary vasculogenesis is essential. A limited number of studies have reported the role of such transcription factors in the establishment and migration of epicardial cells. Studies on Wilms tumor 1 (WT1), a zinc-finger transcription factor whose function is necessary for urogenital development, exemplify the important role of transcription factors in the regulation of epicardial development.30–32 WT1 is expressed in the PEO and migrating epicardium and later, in the subepicardial and migrating
Regulation of Epicardial EMT

The EMT is a key morphogenetic mechanism that is observed in many different processes of embryogenesis, such as gastrulation, delamination of neural crest cells, and differentiation of somites. It remains to be determined how many aspects of epicardial EMT are shared with the generalized concept of EMT and the extent to which specific variation in epicardial EMT exists.

An initial step in the generation of mesenchyme from epithelium is the transmission of a stimulating signal from adjacent tissue. At present, a limited number of reports exist that describe myocardial signaling of epicardial EMT. In one such study, Morabito et al reported that there are both positive and negative regulators of epicardial EMT from the developing myocardium. In these analyses, epithelial epicardium was isolated and cultured in the presence of serum or heart-conditioned serum. EMT from the epicardium was induced in both situations. In addition, fibroblast growth factor (FGF)-1, -2, or -7 induced maximal stimulation of EMT, whereas transforming growth factor-β (TGF-β) family members did not stimulate epicardial EMT and actually inhibited the stimulatory effects of FGF-2. This finding contrasts with other examples of EMT, in which TGF-β induced EMT. Interestingly, high levels of FGF-2 are detected in the myocardium close to the epicardium, and application of FGF-2 but not of TGF-β to the heart stimulated epicardial EMT. Although the apparently adversarial roles of TGFs and FGFs in the epicardium require further examination, these studies clearly exemplify the potentially unique nature of epicardial EMT. At present, there are few data to suggest that the signaling mechanisms that govern vasculogenesis are different in coronary vessel development. Still, given the unique nature of this process, careful analysis of vasculogenic signaling is necessary for a comprehensive understanding of coronary vasculogenesis. It will be important to determine the degree to which these mechanisms are conserved or varied in the development of this unique vascular system.

Analyses of the transcription factors GATA and friend of GATA (FOG) in cardiac myocytes can lead to important insights into epicardial EMT. The GATA-4/5/6 subfamily is expressed in the heart and gastrointestinal tract in an overlapping and dynamic manner. FOG-2 physically interacts with GATA-4/5/6 and, like GATA-4/5/6, is expressed in developing cardiac myocytes. The importance of GATA-FOG interaction in coronary vessel development is exemplified by loss-of-function experiments. FOG-null mice develop an intact epicardium and express several epicardial markers, such as retinoic acid synthetic enzyme, endoglin, epicardin, and WT1 but do not produce mesenchyme. Mutation of the GATA-FOG interaction domain generally produces the same phenotype. These data suggest that although a GATA-4/FOG-2–dependent signaling system from the myocardium is not essential for the formation of the epicardium, it is essential for the subsequent production of mesenchyme. Taken together, these data demonstrate that epicardial adhesion, migration, and EMT are continuous yet separable events that are regulated by multiple, interacting factors.

In response to stimulating signals, the epicardium must select specific cells for mesenchyme production and alter their adhesive characteristics for them to leave the epithelium. This characteristic is not uniform throughout the heart, because the production of mesenchyme from the epicardium varies in different regions of the heart. Much more mesenchyme is produced in the atrioventricular region than over the atria or ventricles, suggesting that (1) the epicardium is more sensitive to myocardial signals in different regions, (2) myocardial signals vary in nature and/or intensity throughout the heart, or (3) both situations exist.

The Epicardium Is Essential for Myocardial Differentiation

Myocardial signals are important for epicardial EMT, but emerging data show that the epicardium is also essential for myocardial differentiation. Careful inspection of genetic models that affect epicardial formation and subsequent production of mesenchyme reveal that although both WT1- and
FOG-2–null mice have a thinning of the myocardium, only the FOG-2–null genotype has a complete epicardium. However, it is important to note that formation of the epicardium alone is not sufficient to rescue the thin-wall phenotype seen in the FOG-2–null mouse. Therefore, it is possible that the presence of epicardially derived mesenchyme and/or its products in the developing myocardium might be essential to stimulate myocardial proliferation, growth, and/or differentiation before the completion of coronary vessel development. The terminal stages of cardiac organogenesis are obviously dependent on an intact coronary circulation to sustain myocardial growth, but the developing coronary system also provides critical morphogenetic signals to developing myocytes. A recent study from Sucov and colleagues (Chen et al) demonstrates that the epicardium is critical for sustaining the proliferative nature of embryonic myocytes. In addition, Mikawa and colleagues (Hyer et al) have demonstrated that coronary vascular development is essential for induction of conduction-system Purkinje cells in the avian heart. Elucidating the complex molecular mechanisms that regulate potential interactions between the epicardium, vasculogenic mesenchyme, and the developing myocardial wall will be an exciting area of future research.

**Migration of Vasculogenic Cells Within the Myocardium**

Mesenchymal cells that exit the epicardial epithelium are nonadherent or only weakly adherent and are capable of massive migration throughout the developing heart. Many factors play into this step in the migration of vasculogenic cells to their final point of differentiation.

First, vasculogenic cells must migrate throughout the developing myocardium. This is a more complex issue than at first glance, and a basic understanding of the development of the vertebrate myocardium is needed to appreciate this developmental process. The myocardium of the vertebrate heart begins as a simple polarized epithelium that is lined on the interior by the endocardium. On arrival of the epicardium, the 3 basic cell layers of the heart are established. Expansion or thickening of the myocardial wall is initiated by proliferation of myocytes in this myogenic epithelium. Mikawa and coworkers (Ong et al) have shown that the progeny of proliferating myocytes form transmural clones that expand into the lumen of the heart and trabeculate with their endocardial lining from the outer wall. An intriguing aspect of this process is understanding how mesenchymal cells are dispersed throughout the myocardium during the period of myocardial proliferation and morphogenesis without ever penetrating the endocardial lining.

Examination of sections of the trabeculating heart reveal that spaces appear in the developing heart wall. The importance of these spaces is that they provide a thoroughfare through which epicardially derived mesenchyme can move within the developing heart. The relation of myocardium and migrating mesenchyme can be best observed with antibodies directed against mesenchymal and myocyte antigens or in chick/quail chimaera. These spaces in the myocardium might arise by 1 or more developmental events. First, myogenic progeny expanding into the trabeculae are only loosely adherent to 1 another. Radice and coworkers (Ferreira-Cornwell et al) have shown that N-cadherin, a cell–cell adhesion molecule expressed in cardiac myocytes, is down-regulated in myocytes as they enter the trabeculae. Furthermore, Mikawa’s group has shown that overexpression of N-cadherin leads to improper migration and alignment of myocytes. Thus, the less-adherent nature of myocytes during trabeculation might lead to spaces in the heart wall through which cells can pass. In addition, there is abundant opportunity for remodeling of the ECM in the developing wall, because numerous matrix proteases are expressed at this stage of heart development. Again, these spaces are continuous with the subepicardial space but do not connect with the lumen of the heart, and the endocardial lining is not compromised. In summary, remodeling of the myocardial ECM and production of signaling molecules are likely to play important roles in mesenchymal migration and vasculogenesis.

**Commitment of Vasculogenic Cells**

A central dogma in vasculogenesis is that angioblasts present throughout the embryo induce local mesenchyme to differentiate into smooth muscle or pericytes. Coronary vessel development is a form of vasculogenesis followed by angiogenesis. As such, commitment of angioblasts, interaction between angioblasts and mesenchyme, and diversification into arterial and venous structures are hallmarks of this process. Still, there are interesting peculiarities about coronary vasculogenesis that appear to vary from the current theories on de novo blood vessel formation during embryogenesis.

First, it is intriguing that both angiogenic cells and smooth muscle progenitors are present within the same epithelium, the PEO/epicardium. At present, we do not know of another example of this kind of vasculogenesis. Also, it is unknown whether cells commit to the angiogenic, smooth muscle/pericyte, and fibroblast lineages while in this epithelium, during EMT, or later in development. It is apparent that only delaminated mesenchyme differentiates into endothelial cells, but whether these cells are designated for this lineage while they reside in the epithelium is unknown. It is interesting to note that in vitro analysis of the PEO suggests that most cells have the potential to differentiate into smooth muscle. Our own work suggests that the EMC cell line derived from rat epicardium retains the potential to produce smooth muscle cells. Although it is quite likely that coronary vasculogenesis uses the same molecular signals as in other systems, a comprehensive analysis of angiogenic markers during the formation and movement of the PEO is needed.

Yet another variation in coronary vasculogenesis is that the early heart is devoid of angioblasts and “local mesenchyme,” and it is not until the PEO delivers these progenitors to the surface of the heart that they even arrive at the organ. Experimental and genetic ablation of these progenitors leads to an absence of coronary blood vessels. Furthermore, these cells must travel long distances before they differentiate into components of the blood vessel. This situation leads to a
particularly interesting question, namely, “How do cells determine when to interact, cease migration, and differentiate into components of the blood vessel?” There is the potential for cell signaling and interaction during this entire period of time as cells travel through the same tissue spaces. Obviously, the composition of the ECM is critical to the movement of progenitors and the construction of nascent blood vessels. Characterization of matrix molecules such as collagen IV, fibronectin, laminin-5, and vitronectin illustrates the differential distribution of connective-tissue elements within the heart wall during coronary vessel development.23,29,65,82–84 Furthermore, the deposition of signaling molecules within the connective-tissue space is also a potential regulator of mesenchymal migration. The role of I signaling molecule, platelet-derived growth factor and vascular endothelial growth factor, in the differentiation of smooth muscle from the PEO in vitro has been demonstrated.85,86 Thus, although the molecular interactions between angioblasts and mesenchyme leading to the production of nascent blood vessels have been well documented,72,74,84 how they are regulated in the case of coronary development is unknown. The ECM is likely to provide a scaffold for the deposition of growth factors that stimulate the proliferation of endothelial and smooth muscle progenitors as well as to concentrate or dilute chemotactic agents. Clearly, the differential expression and deposition of matrix molecules play critical roles in the differentiation of vasculogenic cells within the heart wall.

Connecting the Coronary System to the Aorta
One of the most intriguing aspects of coronary development is that much of the initial differentiation and patterning of this system occurs in the absence of blood flow. Endothelial plexi are seen throughout the developing heart on its surface and throughout the trabeculating myocardial wall before connection to the aorta. Mikawa et al58 have shown that coronary vessels form in a segmental manner from a local population of cells that link or fuse. Some of the first vessels to form are those in the subepicardial space in the atrioventricular sulcus that will differentiate into the circumflex and right coronary arteries. In turn, these vessels give rise to smaller subepicardial arteries that invade the myocardial layer and interventricular septum, extending toward the apex of the heart.22,24 Intramyocardial vessels are arranged as a network with regular intervals, maintaining this pattern as the myocardial layer thickens. Although there is tremendous variation in the positioning of these vessels in the embryonic and adult heart, there is also constancy in the intervals between the vessels. The first steps in the diversification of coronary vessels into arteries, veins, and capillaries also occur before initiation of blood flow. Examination of the subepicardium reveals arteries and veins of different caliber even as mesenchyme is recruited to developing vessels.90 Epicardial vessels tend to be larger than the deeper and more peripheral arteries of the heart wall. The presence of arteries and veins of varying sizes in the heart before the flow of blood suggests a program of differentiation intrinsic to the developing coronary vessels. Thus, at the end of the vasculogenic period without blood flow, the general pattern of the coronary system is set, but significant remodeling of the major vessels and capillary system will take place after connection to the aorta.

Joining the coronary system to the general circulatory system is a complex and understudied developmental process, and whether this movement is directed by a chemotactic event or simply represents the “path of least resistance” is unclear at present. Initially, the proximal ends of the coronary arteries migrate toward the proximal aorta. The tips of the advancing coronary vessels must penetrate the tunica media of the aorta, pierce the endothelial lining, and establish continuity in the lumen.22,87 Initially, several coronary vessels approach the left and right aortic sinuses, but only 1 of these arteries will establish firm contact with each sinus and become the right and left coronary arteries. The mesenchyme of the approaching epicardial vessels meshes with that of the great vessels.22,87 When connecting coronary vessels approach the endothelium of the aorta, apoptotic cells are found along the aspects of these vessels and their attachment to the aorta.22 At the same time, connections between the developing venous plexus and the right atrium are observed, and these vessels become the coronary sinus and veins. It is fascinating to realize the precision with which the coronary arteries connect to the aorta, because they are located at the center of the aortic valve leaflets. Still, it is interesting to note that when anomalies do occur, they are invariably circumferential and not longitudinal. Elucidating the cellular and molecular regulation of this process is imperative for an understanding of coronary vessel formation.

Extensive remodeling of the coronary system continues after establishment of blood flow. With the corrosion casting technique, rat coronary vascular development of arteries, veins, and capillaries can be examined in 3 dimensions with beautiful results.23 As visualized with this methodology, the coronary system covers the heart as a discontinuous and randomly oriented vascular plexus of arteries, veins, and capillaries just after connection to the aorta. As the myocardial wall matures and myocytes orient along particular axes, capillaries also align along the same axis as the myocytes. This realignment might be driven by the contraction of cardiac myocytes as the heart wall undergoes morphogenesis and/or by the initiation of blood flow. Concurrently, the diameter of epicardial arteries and veins increases, and maturation of these vessels is visualized by changes in surface morphology. The degree to which changes continue after this time is unknown, but plasticity of the coronary system in response to injury or disease is widely known.

Therapies?
It should be noted that none of the authors are physicians, but we can speculate on how our understanding of coronary vasculogenesis might shed light on future therapeutic interventions. Obviously, coronary artery disease is a huge clinical problem. Is there anything that we have learned from the development of these vessels that could be applied to the repair or treatment of a diseased or damaged vessel? Maybe. One of the more interesting aspects of coronary vessel development is the realization that the epicardium is the source of stem cells for the generation of coronary vessels. We have recently reported that cell lines derived from cancers
of the adult epicardium retain the ability to produce smooth muscle cells.\(^7\) It might be possible to use this potential to generate vasculogenic cells to replace damaged cells or even to assist in the construction of new vessels.

**Brief Note**

Development of the coronary vascular system is a fascinating model for developmental biologists. It is also a process that has far-reaching consequences for the human condition. The basic road map describing the generation of coronary vessels is nearly complete, but a comprehensive understanding of any one of the potential mechanisms governing this process is lacking. Uncovering the mechanisms that direct coronary differentiation will provide the scientist and the clinician with insights into the generation and function of the heart. What could be better?

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**References**

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