Coronary Vessel Development
The Epicardium Delivers
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Coronary artery disease accounts for 54% of all cardiovascular disease in the United States. Understanding how coronary vessels develop is likely to uncover novel drug targets and therapeutic strategies that will be useful in directing the repair or remodeling of coronary vessels in adults. Recent insights have identified the importance of cells derived from the proepicardium and epicardium in the formation of coronary vessels. This article reviews the basic steps in coronary vessel development, the molecules implicated in these steps, and the pressing questions awaiting answers. (Trends Cardiovasc Med 2004;14:247–251) © 2004, Elsevier Inc.

The origin of the coronary vessels has been debated in the scientific literature for over a century. Development of coronary vessels coincides temporally and spatially with formation of the epicardium (EP), not only with respect to individual embryos during organogenesis, but also phylogenetically, with respect to the increasing oxygen demand of hearts during vertebrate evolution (Ostadal et al. 1975). Three distinct mechanisms have been proposed for coronary vascular formation: (1) migration of endocardial cells to the subepicardial space or trapping in sinioids formed by trabeculation of the ventricular muscle (Grant 1926, Viragh and Challice 1981), (2) formation by the process of angiogenesis as an outgrowth of the proximal aorta (Bennett 1936, Goldsmith and Butler 1937), and (3) derivation from the proepicardium (PE) and EP (Mikawa and Fischman 1992, Poelmann et al. 1993). Although each of these mechanisms has enjoyed varying degrees of acceptance by the scientific community, the importance of epicardially derived cells in coronary vessel formation has now been demonstrated in a number of experimental systems. Before detailing specific experiments and hypotheses, we begin with an overview of epicardial and coronary vessel formation.

- **EP and Origin of Coronary Vessels**

The EP originally was thought to be derived from the myocardium, but increasing evidence has identified the PE as the source for the majority of the mature EP that covers the myocardium (Manasek 1968, Manner 1993). In chick embryos, the PE arises from mesothelial cells along the caudal border of the pericardial cavity (Figure 1A, Figure 2A) (Ho and Shimada 1978). These mesothelial cells initially form villi, but soon develop into a small, bulbous mass adjacent to the sinus venosus. The PE enlarges, contacts the heart at the atrioventricular (AV) groove, and migrates to the heart, assisted by glycosylated microfibrils (Figure 1B) (Nahirney et al. 2003) that bridge the gap between the myocardium and the PE. Cells of the PE maintain polarity as they migrate over the heart as an intact epithelium with the formerly luminal surface in contact with the myocardium (Figures 2B–D). In mammals, clusters of PE cells detach as vesicles that are transferred to the heart via the pericardial fluid (Figure 1B) (Komiyama et al. 1987, Kuhn and Liebherr 1988). In both avians and mammals, subpopulations of cells of the PE undergo epithelial–mesenchymal transformation (EMT) soon after contacting the myocardium, and cells migrate into the subepicardial space (Figure 1C). A subset of these cells migrates further into the compact zone of the myocardium. The fate of these transformed cells is intimately linked to coronary vessel development (Mikawa and Fischman 1992, Poelmann et al. 1993). If the PE is prevented from interacting with the heart, coronary vessel development is absent (Gittenberger-de Groot et al. 2000, Kwee et al. 1995, Yang et al. 1995). Coronary vessel formation begins as angioblasts coalesce to form a primitive vascular plexus in the subepicardial space and myocardium (Figure 1D). These endothelial tubes join to form larger vessels that are recognizable as coronary arteries and veins. Once established, the coronary vessels link to the ascending aorta and the right atrium and recruit PE-derived cells to form the smooth muscle and fibroblast components of the vascular network. Therefore, coronary vessels form by a process of vasculogenesis after precursor cells are delivered to the heart by the PE (Munoz-Chapuli et al. 2002). However, whether the PE contributes precursor cells for all cell lineages in the coronary vessels—endothelial, smooth muscle, and fibroblast—or is required for subsequent delivery and support of these precursors has been hotly debated.

- **Proepicardial and Epicardial Contributions to the Vasculature: Are Endothelial, Smooth Muscle, and Fibroblast Precursors Derived from the PE?**

The origin of coronary vessel endothelial cells remains controversial. Quail-to-chick PE transplants have been interpreted to suggest that coronary vascular endothelial cells do not arise in the PE. When the quail PE is removed and transplanted isochronically into an
intact chick embryo adjacent to the endogenous chick PE and sinus venosus, PE-derived structures arise as chimeras containing both chick and quail cells. Poelmann et al. (1993) observed that grafted quail PE supplied smooth muscle cells and fibroblasts to the host heart, but did not result in quail-derived endothelial cells in the coronary vasculature. However, quail PE grafted with liver contributed quail-derived endothelial cells to the host embryo (Poelmann et al. 1993). Liver alone grafted into the pericardial space also contributed endothelial cells to the coronary vessels. These data suggested that the PE did not contribute endothelial cells to the developing coronary vessels.

Labeling cells of the PE before migration to the heart has generally supported the view that endothelial cells derive from the PE itself. Mikawa and Fischman (1992) used both vital dye and a replication-incompetent retrovirus expressing β-galactosidase (β-gal) to label PE cells. Viral labeling allowed for infected cells and their progeny to be identified from a time shortly after infection until hatching. Discrete β-gal-positive colonies of either smooth muscle cells or endothelial cells along short segments of the coronary arteries were noted in hatched chicks. Injections using low-titer virus resulted in labeling of either smooth muscle cells or endothelial cells, but never both, demonstrating that endothelial cells and smooth muscle cells originated from precursor cells committed before the PE contacted the heart. Endothelial cell labeling was most common in embryos in which virus was targeted near the dorsal mesocardium, which is continuous with the liver. These data could be interpreted to support the hypothesis that endothelial cells in the coronary vasculature
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In a subsequent study, Mikawa and Gourdie (1996) injected very low titers of β-gal virus (10 or fewer infectious particles) into the PE. In this instance, virus labeled only coronary vascular smooth muscle cells, whereas higher-titer virus labeled coronary vascular endothelium and fibroblasts as well. These data strongly support the hypothesis that smooth muscle cell precursors exist in the PE and argue against a common progenitor of smooth muscle cells and fibroblasts. In total, these studies demonstrate that smooth muscle cell and fibroblast progenitors are found within the PE. Further, they suggest that angio blasts reside at the proximal border of the PE in close association with the liver and arrive at the myocardium after other progenitor cells have begun to seed the myocardium.

**Molecular Signals that Direct Coronary Vessel Formation**

Studies using experimental embryology, genetic manipulation, and in vitro assays have been useful tools in revealing the roles of specific molecules during coronary vessel development. This section discusses a subset of molecules whose functions have been investigated. Although several molecules have been found to be expressed in the PE, functional studies have failed to identify those required for PE formation. In contrast, several molecules have been shown to be required for EP formation and maintenance. Vascular cell adhesion molecule 1 (VCAM-1) is expressed throughout the myocardium (Kwee et al. 1995) and becomes localized to the outer compact layer that abuts the newly formed EP by embryonic day (ED) 11.5. VCAM-1-deficient mice lack an EP at ED 11.5. The VCAM-1 counterreceptors, α4β1-integrin heterodimers, are expressed in the PE and EP (Sengbusch et al. 2002, Yang et al. 1995). Mice deficient in α4 form an EP by ED 10.5 that degenerates (Yang et al. 1995), suggesting that VCAM-1 and α4 integrin are required for maintenance of the EP. A second line of α4 null mice has fewer vesicles released from the PE that are less likely to attach to the myocardium and fail to form an EP (Sengbusch et al. 2002). Together, these data suggest multiple roles for α4 integrin during PE and epicardial development, including budding of the PE, PE cell attachment, cell migration, and maintenance of PE-derived cells.

Two zinc finger transcription factors have been implicated in epicardial EMT. Wilms’ tumor 1 (WT-1) is expressed in the PE, EP, and mesenchyme (Moore et al. 1999, Perez-Pomares et al. 2002b). Mice homozygous null for WT-1 form a partial EP at the AV groove and the caudal aspect of the heart by ED 12.5, with fewer subepicardial cells present (Moore et al. 1999), suggesting that WT-1 is required for epicardial formation, maintenance, and EMT. Friend of GATA 2 (FOG-2) is a cofactor for GATA 4 (Lu et al. 1999, Svensson et al. 1999) required for epicardial EMT. FOG-2 null mice form a complete EP, but lack coronary vessels, because the EP fails to undergo EMT (Tevosian et al. 2000). EMT is rescued by overexpression of FOG-2 in the myocardium, demonstrating the importance of a myocardially derived signal in the regulation of epicardial EMT. Mice homozygous for a GATA-4 allele with an inactivated FOG-binding domain phenocopy the FOG-2 null mice (Crispino et al. 2001), suggesting that a FOG-2/GATA-4 complex is required for epicardial EMT.

Studies of EMT in explanted PE and EP have identified candidate factors that regulate EMT. Both vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) stimulate EMT of epicardial cells in vitro (Morabito et al. 2001). Whereas transforming growth factor β (TGF-β) has been noted to inhibit EMT in epicardial explants (Morabito et al. 2001), TGF-β stimulates EMT in PE explants (H.E. Olivey, N.A. Mundell, and J.V. Barnett, unpublished observations). Recent evidence identifying functionally antagonistic TGF-β signaling pathways involving the activation of different activin receptor-like kinases in mediating endothelial cell transformation, migration, and proliferation (Goumans et al. 2003, Lai et al. 2000) and cardiac myocyte gene expression (Ward et al. 2002) may be one mechanism to explain these apparently contradictory effects of TGF-β. FGF, VEGF, and TGF-β ligand expression patterns support roles during epicardial transformation (Molin et al. 2003, Morabito et al. 2001, 2002).
How these and other factors interact to regulate transformation, and if they act on specific populations of precommitted cell lineages, remain to be determined.

Final steps in coronary vessel development include vessel patterning, attachment of the vascular network to the systemic circulation, and recruitment of smooth muscle. Connexin 43 (Cx43) mRNA is expressed abundantly in PE cells, and Cx43−/− mice display defects in coronary vessel patterning (Li et al. 2002). Although neural crest cell ablation results in similar defects in coronary vessel patterning, these are not seen in neural crest specific loss of Cx43 (Li et al. 2002, Sullivan et al. 1998). These data suggest a primary role for Cx43 and PE cells in coronary vessel patterning. Little is known about the molecular regulation of the attachment of the coronary vessels to the systemic circulation. Formation of the coronary orifice has been demonstrated to be dependent on the proper formation of the parasympathetic ganglia (Waldo et al. 1994). Expression of VEGFR-2 and -3 in the truncus arteriosus prior to coronary artery ingrowth suggests a role for VEGF in this process (Tomanek et al. 2002). Penetration of the coronaries into the aorta is accompanied by apoptosis of cells at the site of attachment (Velkey and Bernanke 2001).

Smooth muscle recruitment and differentiation occurs in a proximal to distal fashion after attachment of the coronary arteries to the aorta. Molecules known to direct smooth muscle cell differentiation outside of the heart are likely to play similar roles during coronary vessel development. Serum response factor (SRF), a MADS box transcription factor, is expressed in vivo in the PE and subepicardial mesenchyme but is absent from the EP, although expression has been noted in EP-derived cells in vitro (Landerholm et al. 1999, Nelson et al. 2004). Misexpression of dominant negative SRF in PE explants reduced the expression of smooth muscle markers without affecting EMT, demonstrating that SRF is required for smooth muscle cell differentiation in vitro. Platelet-derived growth factor (PDGF-BB)-stimulated smooth muscle differentiation was found to require the activity of rhoA and p160rho kinase (Lu et al. 2001). Inhibition of p160rho kinase decreases SRF transcription and, in vivo, the EP and subepicardial form apparently normally, but mesenchyme is lacking from the myocardium (Lu et al. 2001). These data suggest that p160rho kinase is required for the migration or survival of mesenchyme in the myocardium. Mice homozygous null for PDGF-B, or the cognate receptor PDGFR-β, have generalized vascular smooth muscle defects, including lack of smooth muscle in intramyocardial vessels, whereas subepicardial vessels are only partially ensheathed by smooth muscle (Galvin et al. 2000), suggesting that bone morphogenetic protein and TGF-β signaling, as well as PDGF, play a role in smooth muscle differentiation or recruitment.

• **Pressing Questions**

The determination of the lineage of PE-derived endothelial cells, smooth muscle cells, and fibroblasts is of major importance. How are these cells specified and fated to the PE? Of particular interest is the origin of endothelial cells. Are coronary endothelial cells specified prior to PE cells entering into the heart? Why are these cells delivered late relative to mesenchyme seeding the heart? When does commitment of smooth muscle progenitor cells in the PE occur? Given that most studies have focused on arterial development, how closely does development of the venous system follow that of the arterial system? Finally, is the developmental program that generates precursor cells and vessels retained in the EP or mesenchyme of the adult? Can this program be reactivated in adults? The answers to these questions promise general insight into organogenesis and may suggest novel therapeutic approaches to coronary vessel repair in humans.

• **Acknowledgments**

The authors have attempted to distill the major ideas and discoveries that have shaped this area. Inevitably, owing to limited space, some insights have not been discussed and some references have been omitted. They apologize for this and encourage the reader to explore the area fully. J.V.B. wishes to acknowledge the support of HL67105, HL52922, the March of Dimes, and the American Heart Association. The authors thank Drs. Jorg Manner and Patrick Nahirney for supplying figures and Drs. David Bader and Christopher Brown for critically reading the manuscript.