

Abbreviations

BMP	Bone morphogenic protein
EC	Endothelial cells
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal-transition
EPDCs	Epithelial progenitor derived cells
FGF	Fibroblast growth factor
HH	Hamburger–Hamilton stages (chick development)
PDGF	Platelet-derived growth factor
PE	Proepicardium
TGF- β	Transforming growth factor- β
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
Wt-1	Wilm’s tumor gene

Introduction

The coronary vasculature is formed by precursor cells that (1) originate outside the heart; (2) migrate to form the epicardium and subepicardium; (3) differentiate into endothelial cells, smooth muscle, and fibroblasts; and (4) migrate and assemble into vascular structures. This is an elaborate process involving vasculogenesis, angiogenesis, and arteriogenesis. Since the heart chambers are initially thin-walled, consisting primarily of a trabecular network of cardiomyocytes, O₂ diffusion from the chamber lumens is sufficient. However, the thickening of the compact regions makes this diffusion inadequate and sets into motion a cascade of events that facilitate the formation of a coronary vasculature. The initial set of vascular channels consists of endothelial-lined tubes that are not yet perfused because they lack connections to the aorta. Part of this network penetrates the root of the aorta just above the left and right cusps, establishing a functional coronary circulation. This event is followed by the development of larger coronary vessels. This chapter addresses these events and the factors that precipitate and regulate coronary vessel development.

Proepicardium

The generally accepted assumption that the progenitor cells of the coronary vasculature were derived from cardiac mesoderm was shown to be false nearly a half century ago by Manasek [1]. This assumption had already been challenged by His in 1885 [2], and later by Kurkiewicz [3], who noted that the pericardial mesothelium, known as pericardial villi, was the source of epicardial cells. A transient, grape-like cluster of cells called proepicardium (PE) develops from splanchnic mesoderm that is the source of the proepicardium, which in turn generates cells that form the epicardium and subepicardium, and subsequently, the coronary vasculature (Fig. 2.1). As described in mouse and rat [4–10], cells from the pericardial surface of the septum transversum form multicellular villi which reach the dorsal surface of the heart where they detach and adhere to the myocardium. Most of these studies, as well as one on the tree shrew [11], noted free floating vesicles that detach from the villi. The motion of the heart pulling on the vesicles when they attach has been proposed as the mechanism for their detachment [8]. In avian embryos, which have been extensively studied, the proepicardium forms at the ventral wall of the sinus venosum [1, 12, 13]. A simple cuboidal mesothelium with microvilli covers an extracellular matrix (ECM) core and forms villi that project as outgrowths [6, 14, 15]. The ECM core contains mesenchymal cells [16]. The villi project a bridge to the right atrioventricular myocardium, which contains an ECM of heparin sulfate and fibronectin between the proepicardial cells and the myocardium [16]. The tips of the villi adhere to the dorsal wall of the atrioventricular canal and proepicardial cells spread over the surface of the heart, forming a monolayer cover.

Origin of Proepicardium

The proepicardium is thought to arise from the lateral margins of the heart fields because it develops next to the sinus

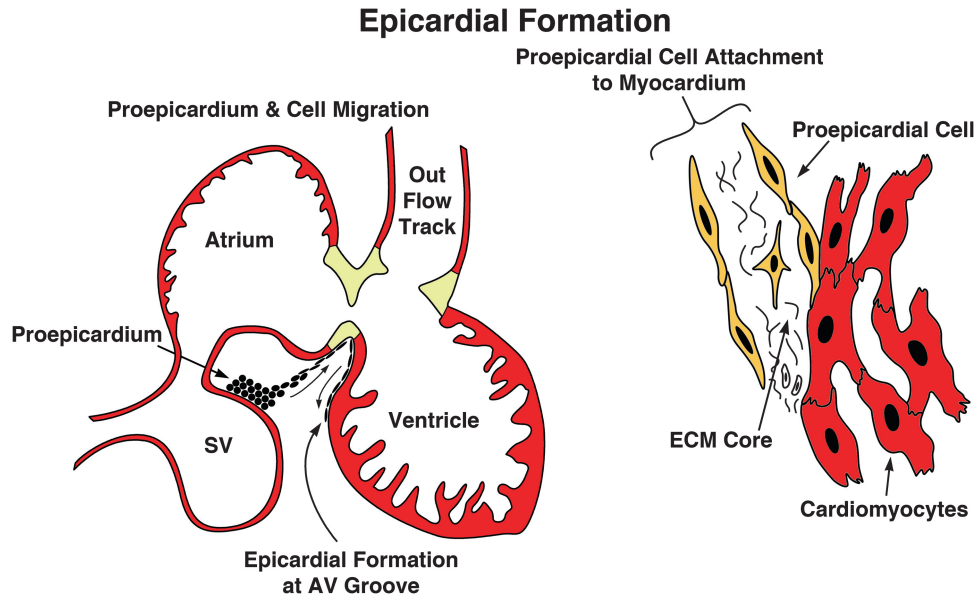


Fig. 2.1 Epicardial formation. In avians, proepicardial cells migrate from the ventral surface of the sinus venosus (SV) into the AV groove and then along the surface of the heart (*left illustration*). The proepicar-

dial cells form multiple villi with an extracellular matrix (ECM) core containing mesenchymal cells (*right illustration*). In mice, the proepicardium is inferior to the sinus venosus and adjacent to the liver bud.

venosus in the peripheral mesoderm [17]. Mesoderm overlying the liver bud endoderm has been documented as a source of proepicardial cells, suggesting a role for the liver as an inducer of the proepicardium [18]. The influence appears to be reciprocal in that the mesoderm acts as an inducer for hepatic cell differentiation and liver bud formation. In avians, the proepicardium develops on the wall of the sinus venosus, as seen in Fig. 2.1 [19]. In mouse embryos, the proepicardium is located on the pericardial surface of the septum transversum adjacent to the liver bud [20]. The role of proepicardium as a cell source for the epicardium and the coronary vasculature has been documented in a variety of vertebrates, a finding that suggests that this entity has been conserved through evolution. In addition to the studies on birds [13, 15, 21–23] and mammals [6–8, 24, 25], the proepicardium and epicardium have been studied in fish [10, 26, 27] and amphibians [28, 29]. The proepicardium consists of bilateral primordia. In avians, the right side persists while the left side regresses; whereas in mice, both primordia develop [30]. A key study documenting the proepicardium as the cell source of coronary vessels used replication-defective retrovirus expressing β -galactosidase injected into embryonic chick hearts [31]. Retroviral injections revealed that endothelial and smooth muscle cells first entered the heart at Stage 17, substantially after the myocardium has developed contractions. Subsequently, it was shown that the proepicardium includes a lineage of smooth muscle progenitor cells that migrate to the epicardium [32].

Specification of Proepicardial Cells

More recently, questions regarding the induction and specification of the proepicardium in the mesoderm have been addressed [33]. Induction of the proepicardial marker genes Wt-1 (Wilm's Tumor gene), capsulin, and Tbx18 in chick mesothelial cells was shown, both in vitro and in vivo, to be a function of the liver and did not occur until (Hamburger–Hamilton) HH stage 12 (about 15 somites). Thus, this induction involves paracrine signaling at a specific developmental stage. The Wt-1 positive epicardial progenitor cells appear to originate from precursors that express transcription factors Is11 and NKX2-5, which are required for the normal development of the proepicardium [34]. Capsulin, a transcription factor also called epicardin, is expressed in the septum transversum mesenchyme, then in the proepicardium, and subsequently, in the epicardium [35]. GATA-4 [36], α 4-integrin [37, 38], and BMP4 [39] are also expressed in proepicardial cells and are required for epicardial formation.

In the chick, bone morphogenic protein 4 (BMP4) appears in the right sinus horn and then in the proepicardium, whereas BMP2 occurs in the sinus venosus [39]. Ishii et al. [40] showed that proepicardial explants migrate toward the cocultured myocardium and that this preferential direction can be mimicked by BMP2/4 and inhibited by Noggin. Differentiation into epicardial and myocardial lineages is mediated by BMP-2 and fibroblast growth factor-2 (FGF-2) signaling [41]. FGF-2 stimulates differentiation into the

epicardial lineage, whereas coexpression of BMP-2 and FGF-2 inhibits differentiation into both myocardium and epicardium. FGF ligands maintain proliferation and prevent apoptosis of proepicardial cells [42].

Epicardium

Proepicardial Migration and Epicardial Formation

As illustrated in Fig. 2.1, proepicardial (PE) cells in both avians and mammals reach the dorsal surface of the heart, proliferate, and spread over the rest of the myocardial surface [4, 22, 43–45]. Their attachment to the myocardium, via an extracellular bridge in the multicellular villous projections of the proepicardium that reach the posterior myocardial surface at the atrioventricular junction, has been shown to be similar in both mammal and avian models [16, 25]. This conclusion was based on serial section reconstructions, electron microscopy, and immunofluorescence. Upon proepicardial attachment, the proteoglycan-rich extracellular bridge lies between the epicardium and myocardium. Based on experiments with chimeric chick embryos and quail PE

transplants, the inner curvature of the heart has been shown to be selectively permissive for the penetration of endothelial progenitor cells [46]. These mesothelial cells express cytokeratins, a distinguishing marker for epithelial cells [43]. The epicardial cells undergo a spatiotemporal migration over the surface of the heart in avian, amphibian, and mammalian species. They spread radially to the right and left from the AV sulcus, then cranially, caudally to the atria [5]. Epicardial formation is complete at embryonic (E) day 10.5 in mice [47], E13.5 in rats [25], and HH23 in chick [15]. Subsequently, the proepicardium disappears and the epicardium undergoes epithelial–mesenchymal transition (EMT). Thus, the fate of these epicardial (mesothelial) cells is to differentiate into coronary smooth muscle cells, fibroblasts, endothelial cells, and atrioventricular cushion mesenchyme, or remain in an undifferentiated state in the subepicardium [48].

Regulation of Epicardial Formation (Fig. 2.2)

As already discussed, proepicardial cells require a number of genes to migrate and form an epicardium. Coupling of proepicardial cells on the surface of the myocardium requires the gap junction protein connexin 43 (Cx43), since its

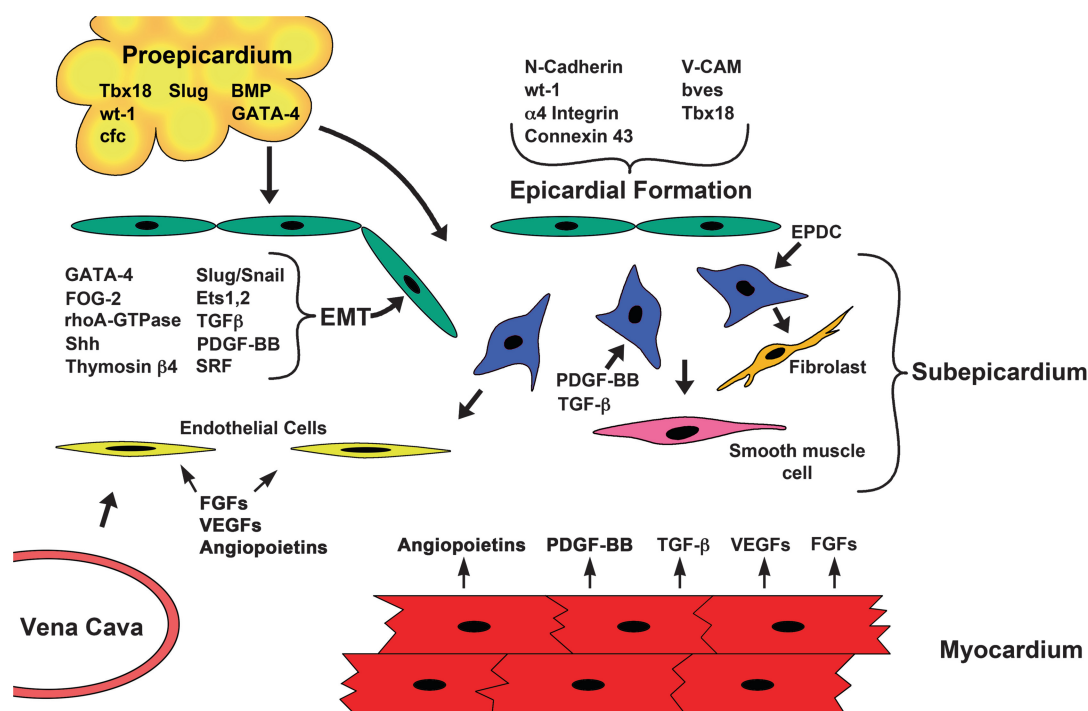


Fig. 2.2 A summary of epicardial formation, epicardial mesenchymal transition (EMT) and vascular cell differentiation. Proepicardial cell migration results in the formation of an epicardium and is influenced by a variety of transcription factors and adhesion molecules. EMT is also influenced by a wide range of molecules. The main source of vascular

cells is the epicardium/subepicardium; however, some endothelial cells may also originate in the vena cava. Subepicardial cells derived from the epicardium (EPDC) undergo differentiation to vascular phenotypes (endothelial, smooth muscle, fibroblasts) in response to growth factors. These events are influenced by signaling from the myocardium

absence results in coronary patterning defects, such as anomalous origins of coronary arteries [49]. These findings are not surprising since gap junctions are membrane channels enabling cell–cell communication. The major adhesion molecule, N-cadherin, is required for the formation of both gap and adherens junctions, as well as the Cx43 α 1 protein subunit expression [50]. Decreases in N-cadherin are associated with a loss of cell–cell contact in the epicardium, which occurs when angiotensin-1 is overexpressed [51]. Epicardial attachment and proliferation are dependent upon transforming growth factor β (TGF- β) signaling, as documented in epithelial-specific Alk5 (a TGF- β receptor) mutant mice [52]. Erythropoietin is essential for proliferation, differentiation, and survival of erythroid progenitor cells and is also required for adhesion of the epicardium to the heart and for capillary formation in the myocardium [53]. Mice homozygous for either erythropoietin or its receptor experience epicardial detachment and vascular abnormalities, as well as ventricular hypoplasia.

When the α 4-integrin ligand, vascular cell adhesion molecule (VECAM-1) gene is disrupted, epicardial formation is prevented [54]. Similarly, GATA-4 null embryos lack a proepicardium and therefore fail to develop an epicardium [36]. WT-1 is also expressed by proepicardial cells and is critical for epicardial development [47]. A role for retinoic acid is suggested by its presence in both intact epicardial cells and in those that migrate to the heart to form the epicardium [55]. The development of the proepicardium is influenced by FGF-2 as evidenced by its enhanced growth during *ex vivo* exposure to this growth factor [41]. Finally, a role for FGF signaling in proepicardial development was also demonstrated by its reduced growth after exposure to FGFR-1 inhibitors.

Epicardial–Myocardial Signaling

Epicardial cells not only serve as a source of coronary progenitor cells but also provide autocrine and paracrine signals (Fig. 2.2). Moreover, signals from cardiomyocytes contribute to coronary vascular development via the release of a number of growth factors, e.g., FGFs, vascular endothelial growth factor (VEGF), angiotensin-2 (Ang2), and platelet-derived growth factor (PDGF) (see review by [56]).

Signals from the epicardium are important for the induction of cardiomyocyte proliferation. Thus, the symbiotic relationship between the epicardium and myocardium facilitates the development of both vascular cells and cardiomyocytes.

Epithelial–Mesenchymal Transition and Cell Fate

Epicardial cells undergo epithelial–mesenchymal transition (EMT), specification, and differentiation into various cell lineages (Fig. 2.2). This process includes their delamination, that is, detachment of cell–cell junctions, a change in phenotype, and migration. EMT occurs during several developmental processes, as well as during tumor progression and metastases, and nonmalignant degenerative disorders. Three events characterize EMT [57]. First, epithelial cell release occurs when cell–cell contacts disintegrate, epithelial markers are repressed, and cell polarity is lost. Second, the change in phenotype, with loss of polarity, involves cytoskeletal remodeling—a phenomenon that includes a transformation of cortical actin into actin stress fibers. This transformation to actin stress fibers (which are composed of polymerized F-actin and myosin II filaments) provides the molecular basis for contraction, and thus motility. Finally, the invasive characteristic of these transformed cells requires their ability to degrade adjacent basal laminae and extracellular matrices, and subsequently to synthesize a new ECM.

Activation of EMT (Fig. 2.2)

Signaling pathways implicated for EMT include Wnt, Hedgehog, TGF- β , and Notch. Snail 1 transcription factors repress adhesion molecules, which allows delamination of epicardial cells; Wt-1, expressed by migrating mesenchymal cells, promotes EMT by binding to Snail 1 and E-cadherin and induces or inhibits, respectively, their cell activity [58]. Stable expression of Snail 1 in epithelial cells induces a loss of the adhesion molecule, E-cadherin, thus facilitating a change in phenotype [59]. The importance of Slug (a member of the Snail family) in EMT is supported by data that indicate its strong transient immunoreactivity in avian epicardial mesothelial cells [60] and its relationship to the dissolution of mesothelial desmosomes (strong cell–cell adhesion junctions), thus enabling cell migration. Epicardium-derived cells express the T-box gene, Tbx18 [61], that encodes transcription factors and is present in both proepicardium and epicardium [62]. A role for the Ets-1 transcription factor in EMT and EC formation was suggested by the observation that it is present in PE, increases with the establishment of the epicardium, and is highest during EMT [63]. Moreover, Ets-1 and Ets-2 are considered essential for normal coronary development [64]. That study revealed that coronary abnormalities (i.e., underdevelopment, irregular distribution, and often failure of one or both coronary arteries to form) were due to defective EMT. In embryonic avian hearts, EMT activation has been found to occur in response to hypoxia and HIF-1 α ; the latter was elevated by the active form of Notch 1 [65]. These authors suggested that this effect is most notable in the hypoxic sulcus regions of the heart which experience the first signs of vasculogenesis; they

proposed that the hypoxia-HIF1-VEGF-Notch pathway may play a role in EMT and coronary progenitor cell differentiation.

Proepicardium–Epicardium–Coronary Vessels: A Continuum

The transformation of cells from the proepicardium to epicardium to the coronary vasculature can be considered a continuum. For example, GATA-4 is essential for the formation of the proepicardium, epicardium and EMT, as well as for coronary vessel formation [66]. Communication between cells and the ECM constitutes another signaling process that activates EMT. For example, β -catenin, an intracellular anchor protein that links cadherins with actin is essential for epicardial function [67]. Mice homozygous for β -cadherin do not undergo expansion of the subepicardium and experience impaired of EPC differentiation into coronary smooth muscle cells. The gap junction protein Cx43 is required not only for epicardial formation but also for coronary vasculogenesis. Cell motility, and consequently migration of epithelial-derived cells in Cx43 knockout mice, is blunted due to cytoarchitectural perturbations; these mice exhibit defects in the vascular plexus [68].

Cell Fate

From the foregoing, one can conclude that the fate of proepicardial cells is to (1) remain as epicardial cells or (2) invade the subepicardium and undergo assembly into blood vessels. Thus, there must be both intrinsic and extrinsic factors that determine which of the three fates a cell will experience. One important intrinsic factor is the ATP-dependent SWI/SNF chromatin remodeling complex, BAF180 [69]. Ablation of this regulator results in impaired EMT, arrested epicardial remodeling, limited migration of epicardial cells, and failure to develop a coronary plexus. Signaling by FGFs and TGF β s has been shown to stimulate EMT and cell migration and invasion [70–74]. In this regard, TGF β s weaken epithelial intercellular adhesion molecules [70, 75, 76]. Thus, the ability of cells to undergo EMT, and consequently invasion and migration, requires attenuation of V-CAM-1, β catenin, and E-cadherin [70]. It has been proposed that a transmembrane glycoprotein, podoplanin, stimulates EMT by downregulating E-cadherin and thereby facilitating the detachment of epicardial progenitor-derived cells (EPDCs) from the epicardium [77]. That study documented a reduced EMT and enhanced E-cadherin in podoplanin knockout mice. The repression of adhesion molecules is facilitated by Slug and Snail transcription factors which, as previously noted, are essential for EMT [59, 78]. A decrease in the adhesion molecule α 4-integrin, which normally restrains EMT, enhances the invasiveness of EPDCs [76]. That study concluded that α 4-integrin levels may determine the molecule's effect, i.e., high levels inhibit, while low levels stimulate invasiveness of EPDCs.

Progenitor Cell Migration and the Subepicardium

Thymosin β 4 (T β 4), a G-actin binding protein, has been found to induce mobilization of EPDCs and their inward migration and differentiation into endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) [79, 80]. These studies revealed that progenitor cells in T β 4 knockout mice persisted in or near the epicardium, indicating a loss of paracrine signaling from the myocardium where T β 4 is normally expressed. Embryonic and postnatal day 1 epicardial explants produced outgrowths comprising smooth muscle α -actin and Tie-2 (angiopoietin receptor) positive cells, characteristic of smooth muscle and endothelial cells, respectively. Addition of T β 4 to the cultured explants increased the numbers of these cells, with additional increases occurring when VEGF and FGF-7 were added. Establishment of cell polarity is requisite for the development of many tissue types. Cardiomyocytes in the developing heart express the cell polarity gene, Vang12, which is disrupted in loop tail (Lp) mutants. In these hearts, limited EPDC migration is due to actin cytoskeletal disruptions and a reduction in subepicardial fibronectin causes a decrease in VSMCs in coronary vessels, a consequence of RhoA/Rho kinase signaling defects [81].

These findings taken together illustrate that several molecules facilitate EPDC activation, migration, and differentiation, and they underscore the importance of myocardial signaling for coronary vessel formation.

The Subepicardium

This region, illustrated in Fig. 2.3, contains EPDCs, has a rich ECM containing collagens I, IV, V, and VI [7, 82], proteoglycans and laminin [7], vitronectin, fibrillin-2, elastin [83], and the extracellular glycoprotein, tenascin-X [84]. The migration of EPDCs is also associated with vitronectin and fibronectin deposition since migratory sites in both the subepicardium and myocardium are positive for these ECM components [83]. With the formation of a rich ECM and the infiltration of many cells, the subepicardium expands and accumulates types I and III collagens [82]. Following its early expression in the subepicardium, tenascin-X is then expressed in the myocardium in association with developing blood vessels [84]. Its pattern of expression suggests a role in the migration of connective tissue cells. Blood islands (endothelial and blood cells that form a vesicular-like structure) are seen during the early stages of coronary vessel development, primarily in the subepicardium, but also in the myocardium [15, 44, 85, 86].

Myocardial Influences

The myocardium, an important source of growth factors such as FGFs, VEGFs, and angiopoietins, provides para-

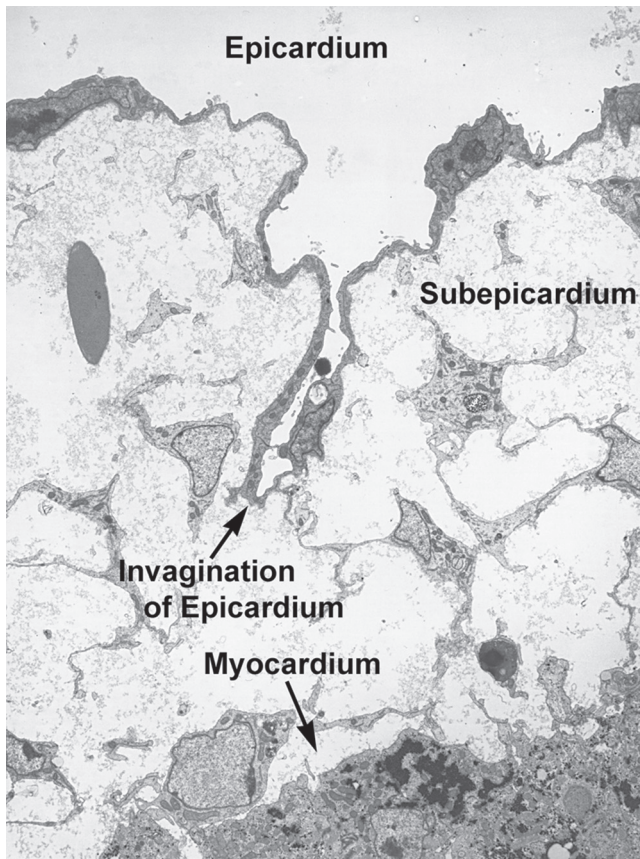


Fig. 2.3 Electron micrograph of cells of the epicardium and subepicardium of a quail heart. The epicardial invagination is a step in delamination of epicardial cells during their transformation into mesenchymal cells. Subepicardial cells often appear linked by long cytoplasmic processes (as seen here). Red blood cells are common in this region. The rich extracellular matrix contains many collagens, proteoglycans, laminin, vitronectin, fibrillin-2, tenascin, and elastin

crine signaling to the epicardium and EPDCs (Fig. 2.2). Not surprisingly, EMT is stimulated by FGFs [73, 79], VEGF, and epidermal growth factor [73]. Consistent with FGF's role in this process is an upregulation of FGFR-1, the major receptor for FGF signaling; its overexpression increases EMT [72]. FGFR-1 plays a key role in EPDCs invasiveness of the subepicardium, as well as their migration to the myocardium. This study also revealed that FGFR-1 is already expressed in a subset of proepicardial cells as well as epicardial cells and that FGFR-1 signaling activation increases delamination of epicardial cells and their invasiveness. Moreover, FGFR-1 overexpression increased the proportion of ECs in the FGFR-1 overexpressing progeny. Angiopoietin-1 is a stabilization factor for developing vessels by facilitating "tight" vessels that are resistant to leakage [87]. However, its overexpression in embryonic mice caused decreases in N-cadherin, a loss of cell-cell contact and defective development of the epicardium [51].

Coronary Cell Lineages and Differentiation

Knowing that subepicardial cell differentiation provides coronary ECs and VSMCs, as well as fibroblasts (summarized in Fig. 2.2), a number of investigators have addressed factors that predetermine or subsequently regulate cell fate. That smooth muscle cells and fibroblasts are derived from EPDCs has been consistently documented [31, 32, 88–95] and is based on data from several types of experimental approaches, including interspecies chimeras [88, 91, 96] and retroviral tagging of the proepicardium [31, 32]. Several reviews of this topic have been published [5, 10, 33]. Although the derivation of coronary endothelial cells has been more controversial, their derivation from the proepicardium is now documented [20, 97]. In the following sections, the origins of smooth muscle and endothelial cells are discussed in more detail.

Coronary Endothelial Cells

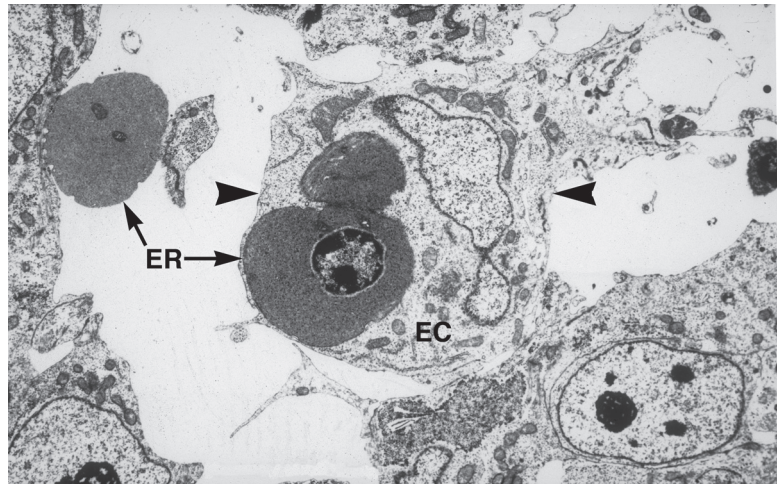
Data from Avians

In avians, the coronary EC precursor, the angioblast, is VEGFR-2 positive and is first recognized as such when the proepicardium attaches to the heart [98]. Epicardial markers cytokeratin, Wt1, RALDH2, and the retinoid X receptor are expressed in angioblasts found in either the epicardium or subepicardium. RALDH2 is the major retinoic acid dehydrogenase that catalyzes retinaldehyde conversion into retinoic acid during avian embryonic cardiac development and is expressed in epicardial derived cells [55]. Cells derived from the PE migrate from a rich retinoic acid environment into the myocardium to form endothelial tubes [90]. Epicardial retinoid receptor α mutant ventricles in E15 mice have tortuous vessels and altered branching patterns indicative of defective arteriogenesis [99]. The vasculogenic potential of proepicardial cells prior to epicardial attachment is indicated by experiments that transplanted the proepicardium onto collagen gels and found angioblastic differentiation, as revealed by the QH1 marker specific for quail ECs [98]. The differentiation process was markedly enhanced when the proepicardia were exposed to VEGF and FGF-2.

Data from Mice

In mouse embryos, angiogenic sprouts from the sinus venosus have been shown to be a source of ECs for capillaries, arteries, and veins [100]. The study used endothelial markers to establish the origin of the expanding vascular plexus at E11.5 on the dorsal cardiac surface near the sinus venosus. The plexus spread around the atrioventricular canal and outflow tract to the ventricular groove. Additional *in vitro* experiments revealed that if the sinus venosus and atrium

Fig. 2.4 Electron micrograph of blood island (between arrowheads) from an embryonic rat heart. *EC* endothelial cell; *ER* erythrocyte. The endothelial cells in blood islands are not fully differentiated



were removed, vascular sprouts did not develop. Recent work on the proepicardium indicates that it contains distinct cell compartments which give rise to coronary ECs, both in vitro and in vivo [97]. Cells that express scleraxis and semaphoring 3D also contribute to the early sinus venosus and cardiac endocardium, respectively. These findings reconcile earlier avian and mouse data that disputed the contribution of the PE to the coronary endothelium. The presence of ECs in the mouse PE prior to vasculogenesis in the heart had been previously documented [20].

Endothelial Cell Lineage Signaling

As noted in Chap. 1, the specification of endothelial cell lineage involves many signaling pathways such as Wnt/Frizzled, Delta/Notch, Ephrin/Eph, FGF, VEGF, and angiopoietin/Tie. Retroviral tagging experiments in quail proepicardium demonstrated for the first time that coronary ECs, as well as VSMCs and fibroblasts, originated from the structure that forms the epicardium [31, 32]. Support for this conclusion comes from subsequent studies using chick-quail chimeras [89–91, 101] or adenoviral tagging [88]. Since angioblasts are precursors of endothelial cells, the first sign of angioblasts in the formation of coronary vessels has been explored. A review of the subject suggests that all avian coronary cell lineages are segregated in the proepicardium prior to its contact with the heart [18].

Blood Islands and EPDCs

Blood islands (erythrocytes/erythroblasts, together with ECs that form a vesicular-like structure) are seen before and during the early stages of coronary vessel development (Fig. 2.4). Such structures have been described in embryonic hearts of humans [85, 102, 103], rats [104, 105], mice [106], and quail [107–109]. A review of these structures [110], also termed

“vascular vesicles,” indicates that they are present both prior and during coronary vasculogenesis and angiogenesis and suggests their role in vessel formation. A study on quail hearts documented the presence of hematopoietic, CD45⁺ precursors prior to blood island formation and concluded that hematopoiesis and vasculogenesis have a close temporal relationship [109]. Subsequently, my colleagues and I [108] used retroviral tagging to document that some erythrocytes in blood islands were progeny of the proepicardium. This finding is consistent with the presence of the CD45⁺ cells as early as HH23 (embryonic day 3.5–4.0) in the quail [109] and in the finding that red blood cells within blood islands of embryonic mouse hearts express the *Terr/119* antigen which specifies the later stages of erythroblast differentiation [110].

Coronary Smooth Muscle Cells

Marker Gene Expression

Differentiation of coronary VSMCs, which are primarily derived from EPDCs [88, 92, 96], requires activation of marker gene expression. Mikawa and Gourdie [32] were the first to document the migration of VSMCs from the proepicardium to the heart. They employed a PE culture system that is still used today. When proepicardial cells are cultured, they initially express smooth muscle α -actin, smooth muscle 22 α , and serum response factor (SRF), and then calponin, smooth muscle α -actin, and smooth muscle myosin heavy chain [92]. SRF is required for most VSMC markers; its role as a DNA-binding protein depends on coactivators and coexpressors [111]. Myocardin is one example of an SRF co-activator of smooth muscle differentiation and like other coactivators and corepressors, modifies CArG box-dependent transcription of SMC marker genes [111–113]. However, more recent data indicate that myocardin is essential for the development of cardiomyocytes and visceral smooth muscle cells, but not

for coronary VSMCs [114]. It is possible that two other myocardin-like transcription factors, i.e., MRTF-A and MRTF-B, could play the role of myocardin in coronary VSMCs. Smooth muscle differentiation requires SRF and its overexpression is sufficient for the induction of SMC markers [92]. By utilizing explanted PE cells, these investigators were able to provide the first evidence that SMC differentiation from the PE progenitor cells involves a multistep process, i.e., changes in gene expression patterns and cell morphology and cytoskeletal organization that is SRF dependent.

Signaling Molecules for Differentiation and Migration

Work from Majesky's laboratory showed that PDGF-BB stimulates a rhoA/p 160 RhoK pathway that mediates cytoskeletal actin reorganization in PE cells and their differentiation into VSMCs [93]. Thus, these differentiated cells are able to migrate and subsequently are recruited into the coronary vascular wall. Interaction between the notch ligand, jagged 1 (expressed by coronary ECs) and its receptor notch (expressed by coronary smooth muscle myoblasts), further stimulates smooth muscle differentiation and promotes expression of a mature VSMC phenotype [115, 116]. Epicardial cell migration and differentiation require PDGFR- β , as well as the PDGFR- β -driven phosphoinositide 3'-kinase signaling [117]. Epicardial cells in PDGFR- $\beta^{-/-}$ hearts have aberrant migration patterns into the myocardium that are associated with an irregular cytoskeleton. SRF expression in derivatives of the proepicardium is dependent on regulatory sequences that have been identified as an E-box/Ets containing cis-acting module in the SRF promoter that mediates expression in these cells [118]. That study revealed that this module is not expressed in the epicardium, a finding that suggests that these PE-derived cells either bypasses the epicardium or that the cis-acting module is operative only during migration from the epicardium. TGF- β , which is abundant in the developing heart [119], has been shown to induce EMT and VSMC differentiation in chick [75] and mice [120]. Immortalized epicardial cells were found to be dependent upon the TGF β type I receptor ALK5 kinase activity.

Fibroblasts and Pericytes

The major source of cardiac fibroblasts are EPDCs [121]. During embryonic heart development coronary fibroblasts express many genes important for the development of the ECM and its various components including integrins, fibronectin, transcription factors, cadherins, and growth factors. They, along with differentiating ECs, secrete molecules that comprise the ECM.

Pericytes also contribute to the formation of the myocardial capillary ECM and facilitate EC-pericyte adhesion [122]. It is likely that pericytes and ECs share a common progenitor

cell, since stem cell-derived Flk1⁺ (VEGFR-1⁺) cells can differentiate into either ECs or mural cells and assemble into the vasculature [123]. Differentiation into ECs was stimulated by VEGF₁₆₅ exposure. "Mural cells" can be VSMCs or pericytes and the distinction between the two can be problematic during differentiation. Indeed, pericytes may be precursors of VSMCs or they may develop from tissue resident stem cells, fibroblasts/myofibroblasts, or circulating progenitor cells [122]. The specific roles of pericytes in assembly of the vascular wall and role in coronary vascular function are discussed in subsequent sections of this chapter.

Establishing the Primary Coronary Plexus

The Growth Pattern

Vascular tubes form in the subepicardium and in the myocardium. Their formation occurs as ECs differentiate from progenitor cells and assemble to form a tubular network (vasculogenesis). In addition to the ECs in blood islands, some ECs form a large cytoplasmic vacuole (Fig. 2.5), i.e. an intercellular lumen [124, 125]. Vascular tubes fuse and branch (angiogenesis) to form a network that eventually extends throughout the myocardium. ECs in the capillary network mature during the last days of gestation. As observed in the rat, plasmalemmal vesicles increase in numbers and the basal lamina becomes continuous as the endothelial cells become more narrow and pericytes are recruited [126, 127].

The formation of vascular tubes (tubulogenesis) follows a VEGF gradient from the epicardium to the endocardium [128]. This study on rat embryos revealed that the highest VEGF concentration is initially in the epicardial region and then increases toward the epicardium between E13 and birth. Tubulogenesis follows the VEGF gradient. Moreover, the rate of myocardial growth, as evidenced in an avian embryo, influences the rate of myocardial vascularization [129]. Increased growth of the ventricular compact region was accomplished by banding the outflow tract of HH Stage 21 (3.5 days) chicken hearts, i.e., prior to the onset of coronary vasculogenesis and resulted in a 64% greater ventricular mass by HH stage 29 (6 days). However, vascular volume and numerical density were similar to the nonbanded hearts, indicating that vascular growth in the banded embryos increased in proportion to the increase in heart mass. Thus, vascularization in the embryonic heart is accelerated when myocardial growth is enhanced.

Relationship of Coronary Tubulogenesis and Extracellular Matrix

Extracellular matrix (ECM) molecules serve as an anchoring scaffold and provide signals for growth and differentiation.

Fig. 2.5 This electron micrograph of an endothelial cell (between *arrows*) illustrates a large vacuole (Vac) containing debris. This vacuole appears to constitute a lumen as endothelial cells assemble to form a vascular plexus. The micrograph is from a rat embryonic heart. *Myo* myoblast



My colleagues and I used immunohistochemistry to elucidate the relationship of specific ECM components to vessel formation in embryonic rat hearts [125]. We found that fibronectin precedes vasculogenesis and appears to provide the extracellular milieu for EC precursor migration. Laminin deposition coincided with tube formation and was closely followed by type IV collagen. Collagens I and III first appeared in the adventitia of forming arterioles and venules.

Hypoxia Is an Initiator of Tubulogenesis

My colleagues and I have used a heart explant model in numerous studies on coronary vasculogenesis and angiogenesis first described by Bolander et al. [130]. When a portion of the heart, e.g., ventricular apex, is explanted onto a collagen gel, the epicardium delaminates to form tubes in the gel consisting entirely of endothelial cells. When embryonic quail hearts were cultured in a hypoxic environment, VEGF mRNA was upregulated and tubulogenesis was increased [131]. In contrast, when O_2 was increased to hyperoxic levels, VEGF mRNA was downregulated and coincided with a limited tubulogenesis. Several splice variants of VEGF-A are upregulated by hypoxia and VEGF₁₆₅ induced tubulogenesis [132]. The role of hypoxia in a quail in vivo model was also documented [133]. That study demonstrated that hypoxic regions of the embryonic heart were characterized by hypoxia inducible factor-1 α and 1 β (HIF-1 α , HIF-1 β) and VEGF expression. Capillary growth was notable in the areas of high VEGF expression. Subsequently, this group showed that experimental hypoxia in this model resulted in a higher capillary density, but also failure of the compact region of the myocardium to develop, coronary abnormalities, and embryonic death [134].

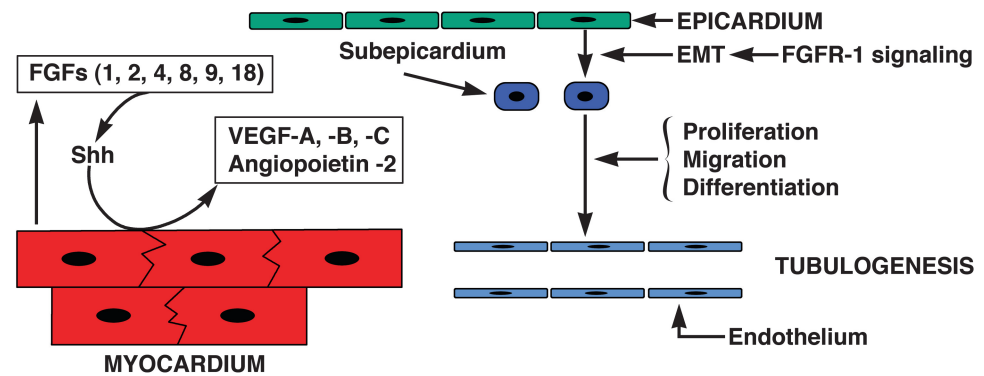
Further evidence that hypoxia plays a significant role in the developing heart comes from work on chick eggs subjected to a hypoxic environment [135]. Using EF5 (a fluorinated derivative of etanidazole, a marker for hypoxia), the pattern of greatest levels of hypoxia was found in or near regions which later contained major coronary vessels. This included the region where the peritruncal network surrounded the aortic root. In human embryos, EPAS1, a transcription factor that responds to hypoxia, overlaps to some extent with VEGFR-1 and Tie-2 in the myocardium and other tissues, and is often colocalized with HIF-1 α [136]. The link between hypoxia and VEGF in coronary vessels was documented in adult mice by exposing them to 6% ambient O_2 for 6 h [137]. This exposure caused an upregulation of VEGF and VEGFR-1 mRNA. Hypoxia has also been shown to increase the number of hemangioblasts [138] and ECs [139] in stem cell cultures.

Multiple Growth Factors Regulate Tubulogenesis (Fig. 2.6)

The importance of three major vasculogenic/angiogenic growth factors in coronary morphogenesis was established by in vitro experiments in my lab over a decade ago [140–142]. We documented a role for both VEGF and FGF-2 in enhancing tube formation in vitro [140] and in vivo [124, 141] models. Explanted embryonic ventricles were cultured on collagen gels and the vascular tubes outgrowing from the epicardium were quantified. Tubulogenic inhibition occurred when antibodies to VEGF-A, FGF-2, or soluble Tie-2 receptors (a receptor for angiopoietins) were added to the culture media. The greatest inhibition occurred with FGF-2 antibodies, which was, in part, attributed to a reduction of ECs.

Fig. 2.6 A summary of growth factor regulation of tubulogenesis in the embryonic heart. Sonic hedgehog, FGFs, VEGFs, and angiopoietin-2 provide important signals that are required for tubulogenesis. FGFR-1 signaling plays a key role in epithelial mesenchymal transition (EMT). As also shown in Fig. 2.2, the myocardium is a source of growth factors involved in tubulogenesis

GROWTH FACTOR SIGNALING IN CORONARY TUBULOGENESIS



That multiple growth factors are indeed effective in stimulating tubulogenesis was further supported by our data that indicated a greater (80–90%) inhibition of tube formation when two of the three growth factors were inhibited. The interdependency of angiopoietins, FGF-2, and VEGF-A was shown by experiments that included a protein for one growth factor in combination with inhibition of another growth factor. Under this condition, there was a threefold to fivefold reduction in tube formation.

VEGF Family Members Play Key Roles

VEGF ligands comprise a large family and include splice variants and three receptors. Although deletion of the VEGF-A gene is embryonic lethal in mice [143], its overexpression results in aberrant coronary development [144], a finding that supports the concept of a narrow window of its expression. However, in addition to VEGF-A, the developing heart also expresses an abundance of VEGF-B [145, 146], as well as detectable levels of VEGF-C [145] and VEGF-D [147]. Moreover, 3 VEGF receptors, 1, 2, and 3, are expressed in the developing heart along with neuropilin-1, a receptor that overlaps with VEGFR-1 (flt-1) [148]. VEGF-C synergizes with VEGF-A and enhances plasminogen activator activity in endothelial cell lines [149].

To specify the effects of VEGF family members on tubulogenesis in the embryonic heart, we inhibited various VEGFs by adding neutralizing antibodies to quail heart explants [124]. Surprisingly, inhibition of VEGF-B attenuated tubulogenesis the most, followed by anti-VEGF-C and then anti-VEGF-A. Consistent with a major role for VEGF-B in tubulogenesis was the finding that addition of soluble VEGFR-1, which binds VEGFs A and B and placental growth factor (PIGF), inhibited tube formation by 87%.

Addition of soluble receptor VEGFR-2, which does not bind VEGF-B and PIGF, but binds VEGFs A, C, D, and E, gave only half the effect of soluble VEGFR-1.

These data indicate that VEGF-B is an effective inducer of tubulogenesis in the embryonic heart. Indeed, work by Lavine and Ornitz [150] suggests that both VEGF-B and VEGF-A, along with angiopoietin 2, affect coronary tube formation and are activated by hedgehog signaling. The interactions involving multiple growth factors are discussed in the next section.

FGFs and Hedgehog Signaling (Fig. 2.6)

FGFs play many roles during development, including myocardial proliferation and coronary morphogenesis. In this regard, the epicardium regulates myocardial growth in response to erythropoietin signals [151]. By using both genetically engineered mice and organ cultures, Lavine et al. [152] were able to show how FGFs and hedgehog signaling drive coronary morphogenesis. This finding is consistent with the evidence that loss of hedgehog signaling decreases or eliminates vascular assembly in mice and avians, respectively [153]. The wave of hedgehog signaling required for VEGFs A, B, and C, and angiopoietin-2 expression occurs in response to FGF signaling. Thus, FGF signaling to the cardiomyocyte regulates hedgehog signaling, which then dictates the level of VEGF expression [154]. This work also documented FGF-9 as an essential growth factor for coronary development by noting that FGF-9^{-/-} embryonic hearts failed to develop a complete vascular plexus. Among the impairments in these mice were a poorly developed subepicardial

mesenchyme and a delay in subepicardial and myocardial blood vessels. These impairments were not associated with any notable abnormalities of the epicardium.

FGFs and VEGFs Cooperate in Tubulogenesis

Most FGF proteins activate FGFR-1, which is required for blood vessel development [155]. The role of this receptor in epicardial cell transition and differentiation of coronary lineages has been documented [72]. During EMT, FGFR-1 is upregulated and its overexpression increases EMT and epicardial cell delamination [72]. These data fit with the finding that FGF-2 is abundant in developing avian and rodent hearts [156–158] and with the data indicating a peak in FGF-2 transcripts during the early stage of myocardial vascularization [141]. Since FGFR-1 is a receptor for most FGFs, it is not surprising that other FGFs, in addition to 1, 2, and 9, are angiogenic. For example, FGF-1 or FGF-4 retroviral infection of the ventricular wall of quail embryos affected upregulation of FGFR-1 and VEGFR-2 in epicardial and subepicardial cells [159]. Experiments on embryonic quail and mouse heart explants in my lab revealed that FGFs 1, 2, 4, 8, 9, and 18 contribute to tubulogenesis primarily via FGFR-1 signaling and that the optimal tubulogenic response occurs when multiple FGF proteins are available [103]. FGF proteins enhanced three key elements of capillary plexus formation, i.e., EC proliferation, migration, and assembly into tubes. This work also documented the interdependence of the various FGFs on VEGF-A for their vasculogenic/angiogenic efficacy. Further documentation of the FGF–VEGF relationship comes from the finding that VEGF inhibition in mice results in decreased expression of FGF-9 and decreased myocardial vascularization [160]. Another finding demonstrating the importance of FGFR-1 signaling is that addition of mouse embryonic stem cells to embryonic heart explants, which enhances tubulogenesis, is negated when FGFR-1-dominant negative inhibitor is added to the culture medium [103].

In conclusion, multiple FGFs may play a role in coronary tubulogenesis. FGFR-1 plays an early role in the vascularization process by enhancing EMT and epicardial delamination. Finally, FGFs depend on VEGF-A for their angiogenic effects in the myocardium.

Other Key Molecules for Tubulogenesis

FOG-2, a cofactor for GATA transcription factors, is required for heart morphogenesis and formation of the coronary vasculature as documented in FOG-2^{-/-} embryos [161]. Coronary

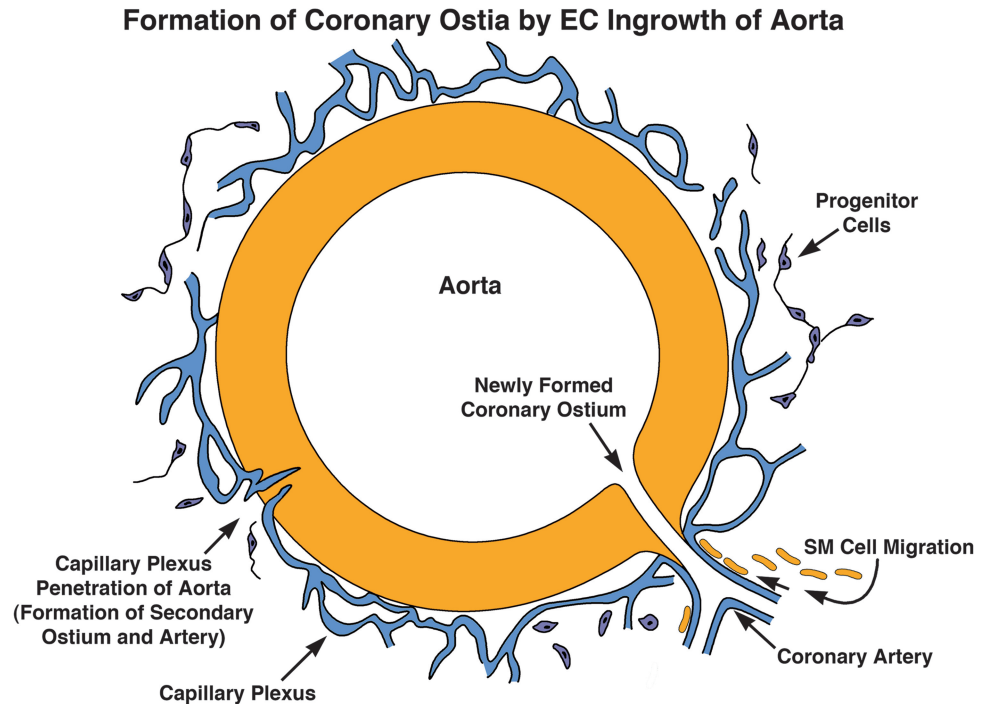
vessels are absent in these embryos despite the formation of an epicardial layer and markers of cardiac vessel development (ICAM-2 and VEGFR-2). Transgenic reexpression of FOG-2 rescues the vascular phenotype. Thus, failure of coronary vessel formation is linked to the inability to activate EMT. FOG-2 and its interaction with GATA-4 were found to be essential in mouse hearts prior to E12.5 and for maintenance of the coronary vasculature in the adult heart [162]. The importance of both spatial and temporal expression in coronary morphogenesis is also recognized in calcineurin-NFAT signaling [163]. Calcineurin is activated by Ca²⁺ signals that are outcomes of tyrosine kinase activity. Coronary morphogenesis (tube formation) was induced by calcineurin-NFAT signaling in ECs between E10.5 and E11.5, while deletion of this signaling at later time points had no effect [163]. Moreover, deletion of calcineurin in either epicardial or myocardial cells had no effect. These data indicate specific temporal and spatial requirements of NFAT signaling for coronary angiogenesis, and the importance of the transcription factor NFAT in this process.

Formation of the Coronary Ostia and Onset of Coronary Circulation

Vascular Tube Ingrowth into the Aorta

As the primary capillary plexus expands throughout the heart, a capillary ring encircles the root of the aorta (Fig. 2.7). The ECs that form this ring penetrate the aorta just above the left and right coronary cusps and form channels through the aortic wall. Formation of coronary ostia via ingrowth, rather than outgrowth, was not established until 1989 [164]. This work in chick hearts was verified in both avian and rodent models [23, 156, 165], as well as in human hearts (unpublished data from my lab). This process is characterized by aortic penetration of multiple capillary strands which fuse to form the left and right coronary ostia [166]. Redundant channels are eliminated by apoptosis at the time of ostial formation [167]. The fusion of the capillary strands occurs at E7 in quail and this event is associated with VSMC recruitment at the sites of penetration followed by the formation of coronary trunks at E8–E9 [166]. Similar observations on rat heart serial sections document the formation of a plexus of epithelial cords or capillary-like channels formed at the aorta root, followed by recruitment of mesenchymal cells that became smooth muscle α -actin positive after ostial formation [168]. Remodeling of the coronary stems involves a fourfold diameter increase between E18 and E21 [169]. The formation of coronary ostia is dependent on epicardial cell-derived Fas ligand, which induces apoptosis at the sites in the aorta where the two ostia are formed [170]. Thus, apoptosis forms a channel that becomes lined by ECs.

Fig. 2.7 The coronary ostia are formed by the ingrowth of a capillary plexus. The endothelial-lined ostium is connected to the tubular network. The coronary artery stems recruit smooth muscle cells (SM). In this cartoon, one coronary artery (seen on the right of the illustration) has already formed (usually the left) and the second is in the early stages of formation, as indicated by penetration of endothelial strands into the aortic wall. The penetration of the endothelial strands requires apoptosis in the aortic wall to form the coronary ostium



Signals for Coronary Ostial and Stem Formation

The mechanisms that limit aortic ingrowth of the capillary plexus to only two sites, i.e., above the left and right coronary cusps, are not well understood. This event requires not only spatial but also temporal regulation, as noted by formation of the left ostium prior to the right in humans [102], chickens [171], and rats [168].

Onset of Coronary Blood Flow Triggers Arteriogenesis

Arteriogenesis (formation of arterioles) has long been recognized as a response to mechanical stimuli and was discussed in Chap. 1. Experiments in fetal lambs documented a relationship between coronary development and blood flow [172]. After 4 days of intermittent adenosine into the circumflex artery, maximal coronary flow nearly doubled in the absence of any change in oxygen content, tension, or arterial pressure. The authors noted that blood flow regulates vessel growth via endothelial signaling in response to shear stress, stretch, and pulsatile strain. These factors can induce vascular growth by several mechanisms, for example NO stimulation of FGF-2 [173] or VEGF expression [174]. Fluid shear stress induces the differentiation of Flk-1 positive (VEGF-R2⁺) embryonic stem cells in ECs [175]. Flow affects cells via a mechanical (shear) force and by enhancing rate-dependent mass transport. In these experiments, the former was found to be the mechanism inducing stem cell differentiation.

The addition of ECs into the developing artery or arteriole is but one component of the process. Differentiation and recruitment of mural cells (VSMCs and pericytes) are the second component, which requires their interaction with ECs. Smooth muscle cells are recruited in response to PDGF-BB secreted by ECs, a topic discussed later in this chapter. This recruitment is regulated by the extracellular glycoprotein, tenascin C [176]. In avian embryonic hearts, tenascin immunohistochemistry revealed that tenascin was deposited around the developing coronary stem and that it colocalized with VSMC α -actin. Tenascin C has been shown to enhance PDGFR- β and promote PDGF-induced proliferation and migration of VSMCs [177]. This was a result of enhanced crosstalk signaling of the integrin α V β 3-PDGFR- β complex. The muscularization process of endothelial channels destined to become coronary arteries and arterioles begins at the aortic root (Figs. 2.7 and 2.8) and spreads distally. Thus, the tunica media is developed last in the smallest branches. This pattern was quantified in our studies on quail coronary arteries/arterioles [178].

These data fit with the observation that arteries and arterioles develop only after the establishment of coronary flow and that the developmental pattern proceeds in a proximal to distal sequence.

Parasympathetic Nerves

The sites of coronary ostia are associated with parasympathetic ganglia, as described in chicks [165], quail [107], and humans (Tomanek, unpublished data). The idea that parasympathetic nerves promote growth of the coronary vessels at their origins was suggested by Bogers et al. [179] and is supported by the finding that neural crest ablation limits the number of coronary artery stems or alters their position [180].

VEGF Signaling

One clue that the sites of coronary ostial and arterial stem formation are influenced by VEGF signaling came from a study in my lab that discovered intense VEGFR-2 and VEGFR-3 transcripts at the sites of the arterial stems [124]. This region is also characterized by intense VEGF staining of precursor cells. A subsequent study documented the dependency of coronary ostia and arterial stem formation on VEGF signaling [108]. When a soluble VEGFR-1/VEGFR-2 chimera was administered to embryonic chicks, the treatment either prevented coronary artery stem formation or limited formation to one stem. Moreover, antibodies to VEGF-B, but not VEGF-A, limited coronary artery stem formation. These data demonstrate that 1) VEGF signaling is not only important for tubulogenesis but also for coronary ostial and artery stem formation, and 2) VEGF-B is important for both tubulogenesis and coronary artery stem formation.

Based on these *in vivo* and on the *in vitro* studies detailed earlier in this chapter and above, VEGF-B appears to be the most significant VEGF ligand in the avian heart since its inhibition attenuates the formation of vascular tubes and prevents the formation of coronary ostia and arterial stems.

FGF-2 and PDGF Signaling

Coronary artery stem formation and arterial growth are also influenced by FGF-2 and PDGF signaling [178]. Neutralizing antibodies to FGF-2 and/or PDGF-BB were injected into the vitelline vein of quail embryos at various stages of development. If administered prior to ostial formation, the embryos usually developed only one or lacked both coronary artery stems. When coronary arteries were formed, as occurred more frequently when the neutralizing antibodies were administered at the onset of coronary ostial formation, arterial segments were characterized by a thinner tunica media and VSMC investment did not extend as far distally as in the controls. Moreover, administration of VEGF-trap (the VEGFR-1/VEGFR-2 chimera that binds ligands for either of these receptors) administered after ostial formation also limited medial development of coronary arteries.

In conclusion, these data indicate that VEGFs, FGF-2, and PDGF-BB facilitate the development of coronary arteries.

VSMC Recruitment and Assembly

Coronary VSMCs in avians have been shown to be derived entirely from the epicardium as demonstrated by labeling proepicardial cells [32, 88, 90, 91, 101]. In mice, however, VSMCs in a short segment of the proximal coronary stem are derived from the neural crest while the remaining coronary VSMCs are derived from the epicardium [181]. VSMCs are recruited either *de novo* from mesenchymal progenitors adjacent to the vessel or from a pool of preexisting VSMCs. The initial phase involves induction of VSMC and pericyte progenitors followed by their migration and proliferation [182]. An absence of PDGF-B signaling attenuates the number of coronary VSMCs and pericytes. These data fit with the finding that PDGF-B inhibition limits coronary artery stems and the development of the tunica media of coronary arteries as well as the assembly of VSMCs distal to the origins of the coronary arteries [178].

As stated earlier, the earliest appearance of coronary VSMC markers in the artery stems occurs only after the establishment of a coronary circulation. In the rat, smooth muscle α -actin (SM α -actin) was observed on E16 and smooth muscle-myosin heavy chain on E17 [183]. In quail embryonic hearts, SM α -actin becomes detectable around E8 [180], corresponding to the first signs of coronary ostial and stem formation. The visualization of smooth muscle cell markers also occurs in a proximal to distal sequence as noted in chick hearts [180] and fits with the sequence of tunica media formation. This study also documented the importance of the presence of the neural crest for an orderly development of coronary VSMCs.

VSMC recruitment is also influenced by signaling via the TGF- β type I receptor, Alk5 [52]. Alk5/Gata5-Cre mutant mouse hearts experience defective development of the VSMC layer surrounding coronary arteries, but contain a higher number of small vessels lacking VSMCs. Since the main coronary arteries were similar in the mutants and controls, the authors concluded that the role of Ak5-mediated signaling is to recruit VSMCs during remodeling of vascular tubes downstream of the major coronary artery stems.

The processes of VSMC recruitment into coronary arteries via PDGF and Angiopoietin/Tie-2 signaling pathways require the transcription factor CHF1/Heg 2 [184]. Knockout of this transcription factor in mice resulted in sparse VSMCs in coronary arteries and abnormally remodeled venous networks. Fibronectin and laminin may also affect VSMCs

migration and coronary artery formation. Data from quail embryos indicate VSMC association with fibronectin strands and the presence of fibronectin in the developing tunica media. These glycoproteins are generously expressed in embryonic human coronary arteries [185].

Inhibition of VSMC Differentiation

A delay in epicardial VSMC differentiation and migration is necessary because the EC vascular tube network must be in place prior to VSMC recruitment. It has been established that early vasculogenesis in the mammalian heart occurs in a rich retinoic acid environment [186]. More recent work has shown that VSMC differentiation is prevented by the high levels of both retinoic acid and VEGF, which are characteristic of the early stage of vascular tube formation [187]. The study revealed that in quail proepicardial cultures, VEGF decreases the number of SM α -actin expressing cells and concomitantly increases the EC cell phenotype. Similarly, retinoic acid abrogated smooth muscle α -actin expression. My colleagues and I have shown that VEGFR-2 and VEGFR-3 receptor density in quail hearts is highest at E6 corresponding to the onset of tubulogenesis and falls by E8–E9, when coronary ostia and stems are formed [124]. However, some VEGF level is required for the formation of the coronary ostia [108] and also the subsequent optimal formation of the tunica media of coronary arteries [178].

Assembly of the Coronary Arterial Tree

Recruitment of VSMCs in the arterial tree, as already noted, occurs in a base-apex direction. Work from my lab [178] showed that VSMC incorporation in embryonic day 9 quail hearts is limited to a distance of 500–900 μ m from the origin of the main coronary artery. By day 10 all hearts had VSMCs 800 μ m to more than 1 mm from the origin of the major coronary artery. The study also documented the importance of FGF-2, PDGF-BB, and VEGF in facilitating the muscularization of arteries. For example, administration of anti-FGF-2 or anti-PDGF reduced the distance of muscularization by more than 60% when administered at the time the main coronary artery stems were being formed. The extent of muscularization was also impaired. VEGF-Trap (the VEGFR-1/VEGFR-2 chimera) also reduced the extent of arterial development during this time period. The assembly of the coronary arterial tree follows a similar course in mice (unpublished data). Arterioles are formed during late gestation and in the early postnatal period.

Development of the Arterial Wall

Because of the rapid growth of the heart, remodeling of the larger vessels is an early event. This allows the growth of the

vasculature to parallel the growth of the ventricular wall, as documented in chick embryos [129]. Innervation of the arterial wall is also progressive, i.e., larger vessels are innervated first, as documented in the quail [178]. Nerve fibers appear initially on the tunica adventitia (E10) and later (E12–E15) in contact with the tunica media; the number of nerve fibers associated with blood vessels increased significantly between E10 and E15. We also found a close association between VSMCs and both fibronectin and laminin; the former is generously distributed at sites of vessel formation and aligned with VSMCs. Fibronectin forms a fine network in the tunica adventitia and extends into the media. Laminin forms a ring around the artery or arteriole in addition to its fine network within the media.

Formation of Coronary Veins and Lymphatics

Coronary Veins

Compared to coronary arteriogenesis, the formation and development of the coronary venous system have received much less attention. A study utilizing chicken quail chimeras reported that precursor cells formed small vessels that grew into the sinus venosus and contradicted the assumption that veins developed as outgrowths of the sinus venosus [23]. Thus, this observation suggests that like capillary ingrowth into the aorta to form the coronary arteries, venous formation also constitutes a process of ingrowth. As demonstrated in the dog fish, progenitor cells from the subepicardial mesenchyme migrate, form capillary-like structures, and coalesce to form veins [9]. A system of venules at the atrioventricular sulcus is evident at E16 in rat heart and the larger veins are already positioned posteriorly during this development period [188].

As discussed previously, hedgehog signaling plays a key role in coronary vascularization. Moreover, hedgehog signaling regulates the formation of specific cell types as revealed by a study that used conditioned gene targeting to document that (1) cardiomyoblasts and perivascular cells are targets of hedgehog signaling and (2) these two cell types control development of distinct vascular subtypes [154].

Most significantly, these experiments showed that hedgehog signaling to the cardiomyoblast is essential for the development of coronary veins, while its signaling to perivascular cells is essential for arterial development. As discussed in Chap. 1, venous and arterial fates are established at the time of, or prior to, vascular plexus formation.

Lymphogenesis in the Heart

Lymphatic vessels in the human heart have been found to often accompany subendocardial branches of the coronary arteries along their adventitia [189]. These vessels are present in the subepicardium, myocardium, and subendocardium [190]. Although the development of the chick cardiac lymphatic vessels was described more than 4 decades ago [191, 192], only more recently has the topic received much attention. The availability of antibodies for lymphatic ECs, e.g., Prox1 (a homeobox transcription factor) and LYVE-1 (the hyaluronan receptor), has provided the opportunity to explore the derivation and assembly of lymphatic cells in the heart. Wilting and colleagues [193] demonstrated the presence of lymphatic vessels in the subepicardial mesenchyme in E9 chicks and noted their attachment to lymphatic trunks. Using quail-chick chimeras, these authors showed that the lymphatic ECs are not derived from the proepicardium. Additionally, they noted a lymphatic collecting vessel that appeared to have venous anastomoses. Lymphatic vessel formation in mice involves a phenotype change in ECs; their precursors stem from leukocytes and monocytes (A. Ratajska, personal communication). Cardiac lymphatic vessel formation begins simultaneously at the arterial and venous poles.

Juszynski et al. [194] followed the progression of Lyve-1 positive lymphatic ECs throughout embryonic/fetal and early postnatal development in mice. This detailed study showed that isolated Lyve-1 positive cells occur at E11 in the venous pole and in systemic veins, a finding that suggests their venous origin in the heart as in other organs (reviewed in Chap. 1). These cells are most numerous at the base of the heart and form tubules and collecting vessels by E14.5. The development of a lymphatic capillary plexus at E16.5 is followed by coalescence of vascular structures to form larger structures and the development of 2 collecting ducts from the right and left ventricles. A subsequent study that examined lymphatic markers during development in chick, quail, and mouse hearts documented Prox-1 positive cells on the developing aorta and pulmonary artery, which contribute to a branching lymphatic network that spreads over the heart [195]. The cells that form lymphatic vessels then express LYVE-1, VEGFR-3, and podoplanin. The data indicate that three cell types express lymphatic markers in the heart (1) Prox-1-positive cells, derived from an extracardiac source; (2) epicardial LYVE-1-positive cells that may incorporate into the lymphatic vasculature; and (3) LYVE-1-positive cells in the myocardium that do not become Prox-1-positive. VEGF-C and VEGF-D play key roles in lymphogenesis by activation of VEGFR-3 and its co-receptor neuropilin (reviewed by [196]).

Coronary Development in Humans

As noted in the previous sections, most of our knowledge regarding coronary vessel development is based on experimental animals. This raises the question: how similar is the process in humans to that of other mammalian and avian models? The following section summarizes what is known regarding the formation of coronary vessels in humans.

Early Development

Stages (S) 14–18 (32–44 days) are a period during which the premature vascular plexus forms and precedes the establishment of the coronary arteries (S18–19). Septation of the 4 heart chambers is completed by S18 [197]. The loose epicardial cells, blood islands, and subepicardial capillaries, characteristic of mammalian and avian coronary development, appear in humans during these developmental stages [85, 102, 198]; and Tomanek (unpublished data). Figure 2.8a, b is a micrograph of subepicardial blood islands and an expanded subepicardium with a network of loose cells at the A–V junction. The loose network of cells surrounding the aorta and pulmonary artery and parasympathetic ganglia is observed at the roots of these vessels [103]. That the subepicardial tubular plexus contributes to the formation of the coronary arteries was noted by Conte and Pelligrini [198]. They also described subepicardial vessels connected to the sinus venosus at S15 and noted that like in mammalian and avian models, these are developing veins. Hirakow [102] described the development of the cardiac vein at the sinus venosus from serially sectioned hearts. He found that the vascular structures were present in the wall of the sinus venosus at S16 (E37) and formed at least one channel by S17 (E41).

The formation of the coronary ostia and main coronaries occurs between S18 (E44) and S20 (E57) [85, 102, 103, 198]. Again, as in the case of venous formation at the sinus venosus, an invagination is seen at the aorta. The capillary-like network that penetrates the aorta in all other species studied is similar in humans (Tomanek, unpublished data). The left coronary artery develops first and was found to be present in 67% of S18 embryos, whereas both left and right were present by S19 [199] or S20 [102] and Tomanek (unpublished data). The coronary arterial tree develops rapidly. By the ninth week, the right coronary artery has sprouted branches to the sinoatrial node and the left coronary has divided into the left anterior descending and circumflex arteries [200]. VEGF receptors are present in coronary vessels as early as 5–6 weeks [201]. Their localization in the

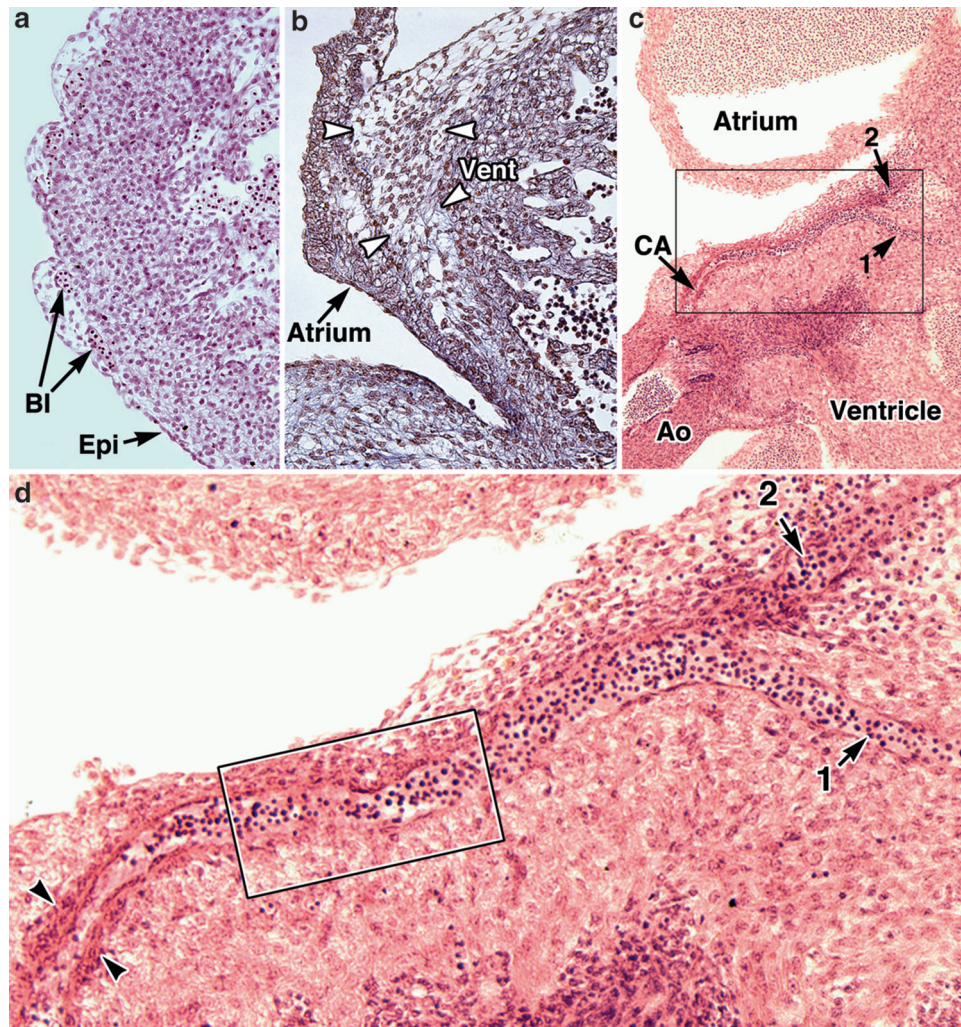


Fig. 2.8 Human embryonic hearts. (a) Blood islands (*arrows*) precede and accompany formation of the primary vascular plexus as seen here in a 41-day embryonic heart. (b) An expanded subepicardium between the atrium and ventricle is seen between the *arrowheads*. It contains a network of cells, many of which are progenitors of the coronary vasculature. This image is from a 44-day embryo. (c) Formation of a main coronary artery in a 48-day embryo. Near its origin (CA) at the aorta (Ao), smooth muscle has enveloped the endothelial tube. More distally,

smooth muscle has not yet completely covered the endothelial tube. Two branches (1 & 2) of the main coronary artery are seen. (d) This is an enlargement of the area in the *rectangle* in (c). The smooth muscle layer is complete at the root of the coronary artery (between *arrowheads*). Just distal to this region (indicated by the *rectangle*), the media of the artery is incomplete. In the branches (1 & 2), some smooth muscle is present in 2, but not in 1

hearts of different gestational ages is similar. VEGFR-1 and VEGFR-2 were documented in capillaries, myocardial arteries and veins, and epicardial veins. Epicardial arteries lacked VEGFR-2. Neuropilin, Tie-1, and Tie-2 were noted in all vessel types. Vascularization of the human heart occurs earlier in the left than in the right ventricle [202].

Later Development

The formation of the two main coronary arteries, late in the second month, establishes a coronary circulation and the

onset of an arterial hierarchy (Tomanek, unpublished data). Figure 2.8c is an image that illustrates the base to apex progression of medial development in a main coronary artery. During the subsequent 2 months, numerous endothelial-lined tubes become muscularized as branches of the arterial tree. Transmural arteries are evident by the fourth month. The remainder of in utero development is characterized by remodeling of the main coronary vessels into larger vessels to accommodate the increasing ventricular mass.

A collateral coronary circulation in human embryonic hearts has been described based on angiographic findings [203]. Collaterals were noted in 19–39 week embryos and

Synopsis of Prenatal Coronary Morphogenesis

The coronary vasculature is formed by proepicardial cells that originate outside the heart and migrate to form the epicardium and subepicardium. Epicardial cells undergo epithelial–mesenchymal transition, a process that involves many signaling molecules that determine cell fate. Formation of a coronary endothelial plexus precedes the development of coronary arteries and veins and is evoked by a relative hypoxia as the myocardium expands. The capillary plexus penetrates the aorta, a process that is VEGF dependent, and forms two coronary ostia. The onset of coronary blood flow evokes recruitment of VSMCs to the coronary stems; the media of the arteries then forms in a base to apex progression. This process is regulated by FGFs and PDGF. Veins develop as ingrowths of the sinus venosus. Venous and arterial fates are influenced by hedgehog signaling. The prenatal development of the coronary vasculature is strikingly similar in avians, mammals, and humans.

they were found to be more abundant and larger in diameter in the septum and subepicardium. The anastomoses were 3–50 µm in diameter and were observed in all regions of the heart.

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