

Review

The Epicardium and Coronary Artery Formation

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Abstract: The coronary system is the network of blood vessels that nourishes the heart muscle. After birth, proper coronary blood circulation is required to support heart homeostasis, and altered coronary function frequently leads to myocardial ischemia, infarction and heart failure. The epicardium plays a pivotal role during coronary blood vessel embryonic development, contributing cells to the coronary vasculature, but also secreting diffusible signals that regulate coronary morphogenesis and secondarily impact on ventricular compact myocardium growth. Accordingly, anomalous epicardium development gives rise to the multiple congenital defects of the coronary vascular system and the heart walls. In this review, we will summarize and discuss our current knowledge on the embryogenesis of coronary blood vessels, as related to epicardial development, and attempt to highlight the biomedical relevance of this tissue.

Keywords: coronary artery; embryo; heart vascularization

1. Introduction

The development of the embryonic epicardium and its role in heart development has attracted much attention during the last few decades. Our perception of the epicardium as a passive, protective epithelial structure has changed through time, and today, the embryonic epicardium is regarded as a complex tissue that does not only materially contribute cells to various cardiac tissues, but also acts as a paracrine signaling center providing instructive cues to adjacent tissues.

Most of the interest raised by epicardial studies focuses on the participation of this tissue and its derivatives in the morphogenesis of the coronary vascular system. Coronary blood vessels nourish the chamber myocardial walls in certain vertebrate species. In humans, coronary blood vessels are of extreme clinical relevance. Coronary arteriosclerosis is the prevalent condition underlying ‘coronary disease’ and associated heart failure (more than 80% of the ischemic episodes affecting the adult myocardium are due to anomalous coronary flow) [1]. In this biomedical context, research on the origin of coronary blood vessel cell progenitors, morphogenesis, patterning and coronary-myocardium molecular interactions will be instrumental to understanding congenital coronary anomalies and acquired disease, helping to develop new diagnostic and reparative strategies for these ailments. The main aim of this review is to summarize our current knowledge on epicardial involvement in coronary blood vessel normal and pathologic development, detailing the different cellular and molecular mechanisms driving the development of embryonic coronary vasculature.

2. The Cardiac Vascular System

The vertebrate circulatory network is a major complex organic system, which supplies the cells of the whole body with oxygen and nutrients and removes carbon dioxide and other metabolic waste from tissues. The blood exits the heart through the arteries, and it is pumped through the body, until it arrives at the capillary bed of the tissues, where the molecular exchanges take place. Afterwards, it returns to the heart through the venous system, completing a closed circuitry [2]. The adult vertebrate blood vascular system is mainly divided into veins, arteries and capillaries. Large arteries have the more complex tissue arrangement: the outer tunica adventitia, formed by fibrous cells, covers the surface of the vascular structure, and the tunica media, formed by smooth muscle and elastic fibers, forms the vessel wall. The innermost layer, the tunica intima, consists of a common internal endothelial lining of mesodermal origin. The intimal layer might also contain elastic fibers, though these might be absent in the earlier stages of development [3].

The heart, as the rest of the body organs, has its own vascular supply. The cardiac vascular network is known as the ‘coronary’ vascular system, because the distribution of its largest vessels over the heart surface reminds one of an inverted crown. From a historical point of view, only the arteries in the cardiac vascular system deserve the classical designation as ‘coronary’, since cardiac venous vessels have always been referred to as ‘cardiac veins’, suggesting that these two cardiac blood vessel types should be regarded as distinct anatomical entities, a point that was already clear in the descriptions of coronary anatomy by Galen [4]. However, in order to simplify the reading of the text, we will also refer to cardiac veins as ‘coronary veins’.

The coronary vascular network appears in the embryo when the luminal supply of oxygen is not enough to fulfill the metabolic requirements of the growing myocardial walls and, therefore, plays a critical role in sustaining the homeostasis of the embryonic and adult heart. Furthermore, coronary arteries are of major medical importance, as coronary artery disease is the source of seven million deaths per year [1], along with the consequent myocardial infarction and cardiac arrest. Therefore, the enlightening of coronary vessel morphogenesis, remodeling and maturation processes is essential for the understanding of the etiology of coronary disease and the development of effective diagnostics and/or therapeutics.

3. The Embryonic Epicardium and the Coronary Vascular System Are a Developmental Unit

Coronary blood vessel and epicardium formation are inextricably related. During the early stages of heart development, the embryonic myocardium is the outermost tissue layer of the heart, remaining in direct contact with the pericardial cavity fluid. This condition does not allow for the development of vascular structures over the heart surface, as blood vessels require a highly specialized extracellular matrix (ECM) to form. Therefore, epicardial formation is required for coronary vessels morphogenesis.

3.1. The Proepicardium and the Formation of the Primitive Epicardium

In mammals, the monolayered embryonic epicardium derives from mesothelial cells (epithelial cells of mesodermal origin) of the septum transversum, most specifically from a transient accumulation of cluster of cells, the proepicardium [5,6]. The proepicardium can be easily identified from E9.5 onwards (in the human, Carnegie stage 12) as an outgrowth of the coelomic mesothelium at the ventrocaudal base of the developing heart and consists of a highly heterogeneous collection of different sub-populations of cells with distinct functions and downstream fates [7].

As indicated above, one of the main fates of proepicardial cells is to form the embryonic primitive epicardium (from E10 onwards in the mouse, human Carnegie stage 16). This requires the transfer of proepicardial cells to the heart surface, a process that may involve different cell mechanisms. A common one is the detachment of proepicardial cells to the pericardial cavity from where they will eventually attach to and spread over the heart surface. An alternate mechanism involves the direct attachment of the proepicardium to the myocardium, forming a permanent tissue bridge, although both mechanisms can be active together in the same animal model [8].

The spreading of the monolayered primitive epicardial epithelium over the bare myocardium marks the initiation of coronary vascularization. As the epicardium progressively covers different heart regions, a new extracellular matrix layer, the subepicardium, forms between the epicardium and the myocardium. This subepicardium constitutes a unique milieu that actively promotes the development of coronary blood vessels, an effect mediated by the scaffolding properties of many of the molecules secreted to the extracellular matrix by both the epicardium and the myocardium, as well as by the modulation of provascular cytokine activity [6,9] (Figure 1A–F).

3.2. Epicardial Epithelial-to-Mesenchymal Transition

The epicardium crucially participates in building an optimal environment for coronary blood vessel formation, but also provides cells that incorporate into this complex vascular network. This contribution is partially mediated by the full conversion of some epicardial epithelial cells into a subset of mesenchymal, epicardial-derived cells through an epithelial-to-mesenchymal transition. During epicardial epithelial-to-mesenchymal transition, epithelial epicardial cells undergo a complete phenotypical change, transforming into mesenchymal, invasive cells (epicardial-derived cells); that progressively migrate from the epicardium through the subepicardium; the compact and trabecular myocardium, contributing to coronary blood vessels, the ventricular myocardial and the atrioventricular valvular interstitium [10–15] (Figure 1D–F). Epicardial epithelial-to-mesenchymal transition is still

poorly characterized, as it remains unclear which molecules trigger the process. Some reports suggested TGF β as a positive regulator of epicardial epithelial-to-mesenchymal transition, whereas other studies regarded this same molecule as a negative regulator of the same process [16]. Recent data also indicate that the expression of the Wilms' tumor suppressor gene (Wt1)-dependent cadherin inhibitor, Snail, could be critical in the loss of cell adhesion required for epithelial-to-mesenchymal transition to proceed [17], results that are in conflict with two other pieces of work suggesting that epicardial epithelial-to-mesenchymal transition could be Snail-independent [18,19]. Retinoic acid has also been proposed to regulate epicardial epithelial-to-mesenchymal transition [20,21], as well as to participate in the differentiation of specific cell types from epicardial progenitor cells [20,22,23]. It also remains unclear when the embryonic epicardial epithelial-to-mesenchymal transition is over; multiple data indicate that epithelial-to-mesenchymal transition starts as soon as the primitive epicardium forms over the myocardium [10,11,13], but it is more difficult to define an endpoint for the process, although different pieces of evidence indicate that the transformation of the embryonic epicardium is not active after E14 in the mouse [24,25]. Remarkably, the ability of epicardial cells to undergo epithelial-to-mesenchymal transition is determined by their spindle orientation during cell division [24].

The extent of epicardial involvement in coronary development has focused a good part of modern research on epicardial embryonic development, but controversies remain on the quantitative and qualitative contribution of epicardial-derived cells and signals to coronary morphogenesis. A full discussion on this topic will be provided in the following sections.

4. Vascularizing the Vertebrate Heart

Only animals belonging to the subphylum Vertebrata have a specific coronary blood vessel supply to their functioning heart. Among them, only vertebrates with a compact ventricular layer possess a fully developed coronary vascular system. These include some elasmobranchs (sharks and rays), reptilians (crocodiles) and all avians and mammals. Vertebrates with a predominantly trabecular (spongy) ventricular myocardium can lack coronary blood vessels, as it happens to be the case in many teleosts (bony fishes), which have a fully avascular ventricle (classified into type Ia class fish ventricles (see [26]), implying that most of the oxygen needs of these animals are fulfilled from luminal blood [27]. Other teleosts have a partial coronary vascularization, which can range from a low number of superficial vessels located on the external epicardial heart surface with a poor capillary vascularization to a complex transmural vascular bed providing an extensive vascularization that can reach the ventricular trabeculae [28]. Amphibians have a spongy myocardium without evident coronary vascularization, with the oxygenation of the heart guaranteed by the pulmonary venous return and cutaneous respiration. On the other hand, reptiles, whose heart comprises a well-formed compact myocardium, have a coronary blood supply often formed by a common coronary stem connected to the aortic root, which soon subdivides into dorsal and ventral branches [27,29].

As can be inferred from the previous paragraph, the development of a complex and extensive coronary blood vessel network is linked to the presence of a compact myocardial layer. From a developmental perspective, it is well known that disruption of epicardial/coronary development in avians and mammals impacts myocardial growth [7], but also that interfering with myocardial gene expression may lead to anomalous coronary development [30,31]. It has been suggested that in lower

vertebrates with a spongy heart, coronary-like blood supply is a mere supplement to the basic cardiac luminal supply [27]. Accordingly, various reports have suggested a transmural gradient of oxygen concentration as the main driving force for the expansion of coronary vascularization from the outer (epicardial) to the inner (endocardial) sides of the heart [32].

5. The Elements of the Coronary System

5.1. Making the Coronary Tree Grow: Vasculogenesis versus Angiogenesis

For a long period of time, coronary blood vessels, and, most especially, coronary arteries, were thought to form from the aortic root and to progressively grow, eventually vascularizing the heart walls [33]. Such vascularization was supposed to happen by angiogenesis, *i.e.*, the formation of new blood vessels from pre-existing ones [34,35]. This latter interpretation was in conflict with studies that described the early embryonic coronary vessel network as a closed endothelial plexus isolated from the aortic endothelium [36]; all these works, indeed, suggested that the embryonic scaffold of the coronary system formed from non-aortic sources of endothelium, a point that was confirmed by classical embryological approaches using the avian embryo as an experimental model [37]. Moreover, additional studies characterized the earliest coronary blood vessels as structures formed by vasculogenesis [11,38], *i.e.*, the coalescence and fusion of vascular cell progenitors (called ‘angioblasts’) to form a vascular structure *de novo* [39] (Figure 1E–J).

Different hypotheses on the origin of coronary endothelium and the cellular mechanisms that build it have been coexisting through time (Figure 1D), and the controversy on the specific origin of coronary endothelium has persisted up to today. We believe that this situation has been strongly influenced by the extrapolation of results derived from different animal models, a certain research bias that favored a single origin for all coronary endothelium and our poor understanding of the intrinsic differences found between the arterial and venous components of the cardiac vasculature.

5.2. Chicken or Mice?

Two animal models have been preferentially used in the study of epicardial development. The avian system, mostly due to the possibility of using interspecific transplantations (e.g., quail-to-chick epicardial chimeras), has proven to be a useful tool to trace cell migration and differentiation of epicardial derivatives after proepicardial transplantations [11,14,40] (Figure 1H). This experimental approach is not biased by the dilution of the tracer (quail donor cells maintain the specific antigens used to identify them on a chick tissue background) and allows for a high number of replications, but is limited by the accuracy in the dissection and grafting of donor proepicardial cells in the host, as this step of the procedure is highly dependent on the microsurgical skills of the person performing the experiments.

Transgenic technologies in the mouse allow for the tracing of cells as based on the activation of gene transcription, a method that also avoids the dilution of the tag (as happens with classic vital dyes, like 1,1'-Diocadecyl-3,3',3' tetramethylindocarbocyanine perchlorate/DiI or 3,3'-Diocadecyloxacarbocyanine perchlorate/DiO). Such labeling can show real-time gene activity or irreversibly label groups of cells as based on their original expression of a given gene (e.g., Cre/Lox

technologies). The former approach is limited by the gene expression threshold allowing for the activation of the reporter construct, whereas the latter does not directly allow for the identifying of the exact time point for the activation of the reporter. Conceptually, the most significant caveat of this technology is the unequivocal association of the expression of a certain molecule to a single cell type, population or 'lineage'. In this context, several mouse Cre transgenic lines have been constructed using transcription factor promoters to drive Cre expression and further trace epicardial derivatives, including Wilms' tumor suppressor (Wt1) [41], Tbx18 (Tbx18) [42] or Scleraxis (Scx) [12].

Studies on the embryonic development of coronary blood vessels using these two animal models (avian and mice) have yielded different results. While it is widely accepted that coronary smooth muscle cells and an important part of interstitial fibroblasts derive from the epicardium [43,44], results on the origin of coronary endothelium obtained from these two animal models bear a significant discrepancy. In short, the avian proepicardium and epicardium show a high vasculogenic potential and a major contribution to coronary endothelium (Figure 1H), whereas the mouse transgenic lines used up to date identify a variable contribution of proepicardial/epicardial cells to the coronary endothelium. This interesting aspect of coronary development will be studied in detail below.

5.3. Epicardially-Derived Cell (EPDC) Adult Fates

An important part of epicardial-derived cells is still forming part of coronary blood vessels postnatally, but it is not clear which number of embryonic epicardial-derived cells remain in the adult coronary vascular system, as the normal cellular turnover of the adult tissue is expected to progressively substitute at least part of these cells. At the same time, some epicardial-derived cells incorporate into coronary blood vessels during embryonic development, while other epicardial-derived cells take residence in the interstitial space between cardiomyocytes. Furthermore, although they are generally referred to as fibroblasts, most of them do not differentiate into characteristically secretory fibroblasts and retain the genetic profile of undifferentiated epicardial cells still expressing genes such as Wt1 and GATA-4. It has been hypothesized that epicardial-derived fibroblasts play a crucial role in the fibrotic response that characterizes ventricular remodeling after myocardial infarction [45], but further research is needed to understand how the specific responses of these cells to cardiac damage are articulated.

6. Epicardial Cell Contribution to Coronary Blood Vessel

6.1. Sources of Coronary Endothelial Cells

Despite the biomedical relevance of coronary endothelium, the origin of this tissue and the developmental mechanisms that participate in its differentiation, organization and maturation are not completely understood. As pointed out above, earlier reports proposed that coronary endothelium rose from endothelial sprouts at the base of the aorta, but this idea was quickly refuted by the evolution of cell tracing techniques.

Seminal studies in the avian embryo show that the proepicardium gives rise to an important proportion of coronary endothelial cells, *in vivo* and *in vitro* [46] (Figure 1H); in this latter setting, endothelial differentiation depends on the addition of growth factors, like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2) [46], which are normally produced by the myocardium *in vivo*.

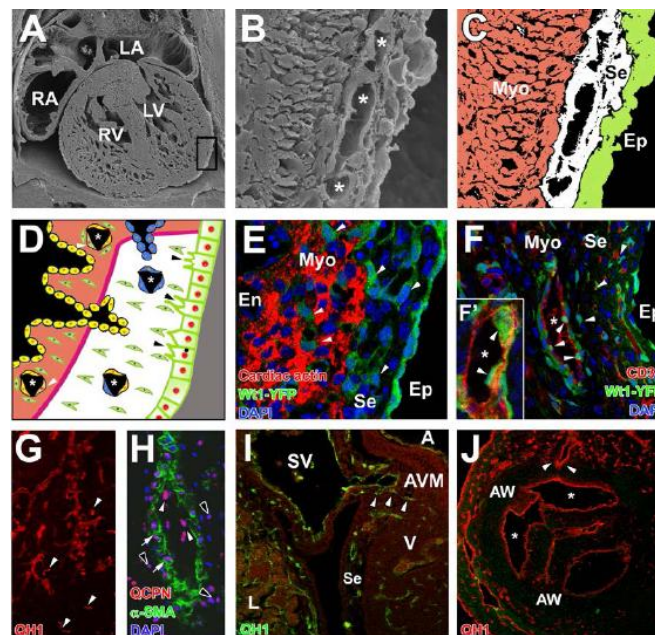
On the contrary, the contribution of epicardial-derived cells to the coronary system in the mouse continues to be debated. In order to demonstrate the embryonic origin and fate of mouse tissues, different mapping techniques have been used to study the progeny of individual and groups of cells, those being based on Cre-Lox technology, a gene targeting approach using site-specific recombinases, the one that is most frequently used (Figure 1E–F). This allows for the irreversible expression of a reporter under the promoter elements of specific genes. This method has been also modified to allow for a time-dependent activation of the recombination (and, hence, reporter activity) by an inducing agent (such as doxycycline, tetracycline, RU486 or tamoxifen). For the study of the origin of coronary blood vessels, Tbx18-, GATA5-, Sema3D-, Scx- and Wt1-Cre drivers have been used. Results from these studies identify a very different contribution of proepicardial-derived cells to coronary endothelium, ranging from virtually none to an average of 20%.

The difference between species is bewildering, and this could either reflect an actual interspecific difference between coronary vessel development or could be a limitation of the use of only one molecular marker to define the (pro)epicardial population, as this approach might well exclude some key smaller populations, as suggested by Katz *et al.*, 2012 [47]. In this paper, a definite contribution of the epicardium to the coronary endothelium is shown; the authors characterized two new cellular compartments of the proepicardial organ, the Scleraxis and Semaphorin 3D positive cells, which seem to have relevance for coronary endothelium development as early as at E11.5. These two populations seem to be largely non-overlapping with the well-characterized Tbx18 and Wt1 marked epicardial cells and contribute to endothelial lineages in the heart and coronary vessels. In this study, the disparities between the mice and chick studies are also reconciled by a cross-species transplantation (mouse to chick proepicardial chimeric transplantation), which results in the expression of mouse epicardial markers, as indicated by the reporter activity in the chick coronary endothelium. The two endothelial cell populations traced seem to account for 30% of the endothelial cells in the coronary vessels by E16.5.

6.2. Sources of Coronary Smooth Muscle Cells

The first lineage tracing experiments used to tag (pro)epicardial derivatives unequivocally identified the differentiation of large numbers of these cells into the medial smooth muscle wall of coronary arteries [43,44]. As seminal studies on the fate of cardiac neural crest had soon discarded a significant contribution of this cell population to coronary arteries [48], an epicardial origin of coronary smooth muscle was rapidly accepted. *In vitro* evaluation of smooth muscle proepicardial differentiation potential, as well as genetic tracing of (pro)epicardial cells in the mouse confirmed that one of the main developmental fates of the (pro)epicardial lineage is to form the smooth muscle wall of coronary arteries. However, some authors have suggested an alternative origin for the smooth muscle of the proximal left and right coronary stems as based in the absence of epicardial-derived smooth muscle in these areas [49], but the origin of these cells still needs to be elucidated.

Figure 1. Coronary development in mouse and chick embryos. **A–C.** The heart of a 15.5-day-old mouse embryo (E15.5) is shown. The boxed area in **A** is magnified in **B**, allowing for the identification of developing coronary vessels (asterisks). Tissue arrangement in that area is shown in **C**. **D** summarizes current hypotheses on the origin of coronary endothelium (CoE, asterisks). Sources for coronary endothelium include the ventricular (yellow) and sinus venosus (blue) endocardium, as well as epicardial-derived cells (green cells in coronary vessels, white arrowheads). Note that some epicardial epithelial cells (green) display morphological features of epithelial-to-mesenchymal transition (black arrowheads). **E–F.** Epicardial-derived cell contribution to the developing heart can be traced in Wt1-Cre x ROSA26-eYFP (Wt1-YFP⁺) embryos (E12.5, **E**; E18, **F**) by the expression of the eYFP reporter (green). Wt1-YFP⁺ cells (arrowheads) progressively invade the subepicardial and myocardial layers and incorporate into developing coronary vessels, preferentially to prospective coronary arteries (asterisks). **G–J.** Coronary blood vessel development in chick embryos. **G.** Part of the primitive coronary capillary plexus (QH1⁺, red) forms by coalescence of isolated vascular progenitors ('angioblasts', arrowheads). **H.** Chimeric transplantation of embryonic quail proepicardium into recipient chick embryos allows for the following and studying of the developmental fate of cells from the epicardial lineage. Epicardial-derived cells (QCPN⁺, purple) are found in the endothelium (white arrowheads), the medial smooth muscle wall (α -SMA⁺, green) and the fibrous adventitia (black arrowheads). **I.** Sinus venosus endocardium also contributes to the development of the coronary vascular system in chick embryos. SV endocardial sprouts (QH1⁺, arrowheads) use the subepicardial space to immigrate into the ventricle. **J.** The preformed arterial component of the coronary vascular plexus joins the systemic blood flow at the aortic root (QH1⁺, red). Some of these capillaries (arrowheads) cross the forming aortic wall and open to coronary Valsalva sinuses (right and left, asterisks). Abbreviations: AVM, atrioventricular myocardium; AW, aortic wall; En, endocardium; Ep, epicardium; Myo, myocardium; Se, subepicardium; SV, sinus venosus. Panel J is reprinted with the permission of Academic Press [50].



6.3. Sources of Coronary Fibroblasts

Not much has been published on the differentiation of epicardial-derived cells into cardiac fibroblasts, but it is widely accepted as being one of the main cell kinds deriving from the (pro)epicardial cell lineage [7] (Figure 1H). Recent studies have identified the transcription factor, Tcf21, as a bona fide marker for epicardial-derived fibroblasts in the mouse heart, sharply discriminating between fibroblasts and smooth muscle cells [25]. These findings argue against the idea of a late specification of epicardial-derived cardiac fibroblasts and smooth muscle cells from a common progenitor pool [49].

Many important questions related to the fibroblastic derivatives of epicardial cells remain without answer. It is unknown what is the percentage of cardiac fibroblasts contributed to the heart by epicardial cells (or any other cell source), nor is it known in detail how important these cells are for normal cardiac performance and pathological responses to stress or damage. Our research team has characterized the migratory and proteolytic properties of epicardial-derived cells and their ability to differentiate into fibroblasts and has identified epicardial-derived cardiac fibroblasts as an heterogeneous population, proposing the existence of various lineages of epicardial-derived fibroblasts [45].

7. Building an Arteriovenous Coronary Vascular Tree

Coronary Arteries and Veins

Arteries and veins are very distinct vessels, structurally and functionally, and play crucial roles in circulating blood to and from sources of oxygen, designed to regulate the pressure underlying blood flow. Arteries are the pressure recipients of the circulatory system, as they carry blood from the heart under high pressure and are surrounded by multiple layers of smooth muscle cells and extracellular matrix components, which provide strength and elasticity to their vascular walls. On the other hand, veins have thinner and less elastic vascular walls and, thereby, have to rely on specialized valves to return blood to the heart, under low pressure [3].

The identity of arteries and veins is specified during embryonic development and is influenced by hemodynamic forces [51]. Molecular markers that distinguish arteries from veins are expressed before the beginning of blood circulation, during embryonic development [52–54]. The ultimate vessel identity and function are plastic properties, as shown experimentally by the manipulation of blood flow and the observation that it can alter the fate of arteries and veins [51,55].

Although vein and artery endothelial origins are still controversial, the molecular pathways leading to different fates are better characterized. In zebrafish and mammals, Notch family members mediate the choice of fate between arterial or venous endothelium, as Notch activity promotes the formation of arteries. VEGF also plays an important role upstream of Notch in specifying arterial fate, along with sonic hedgehog protein (Shh). Other markers are quite specific to the vessel's fate, such as the transmembrane ligand, ephrinB2, expressed in arteries, smooth muscle cells, pericytes and mesenchyme surrounding the sites of vascular remodeling and its cognate, tyrosine kinase receptor EphB4, expressed in the endothelium of veins and lymphatic vessels [25]. These markers are respectively expressed on the arterial and venous endothelial cells of the developing murine yolk sac prior to blood flow, and the knockout of either of these genes results in embryonic lethality at E11.0, due to failed vascular remodeling [23,52]. In the same study, however, it became clear that ephrinB2

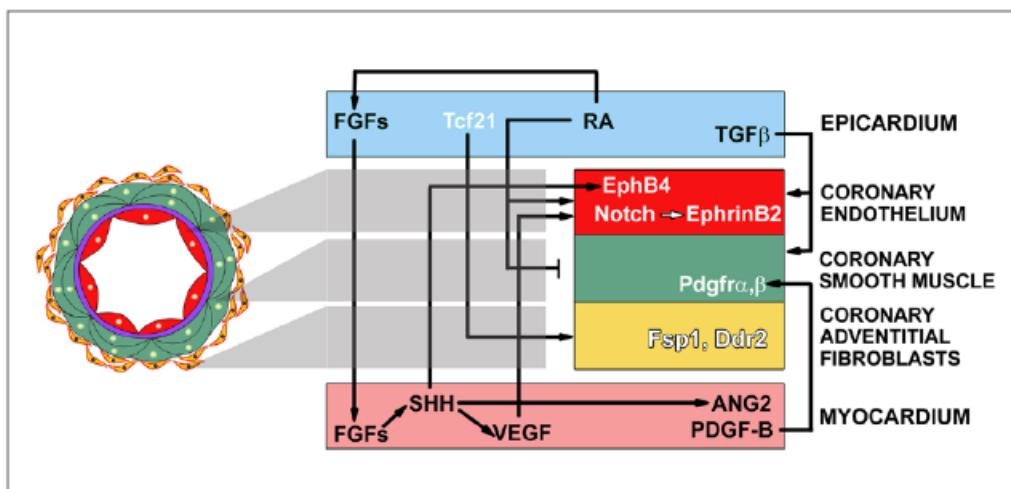
and EphB4 are not determinants of arterial-venous specification, as their deletion *per se* does not switch vessel identity [28]. It has also been shown that, as the zebrafish model suggested, Notch signaling determines arterial specification [24]. Specific knock-out of Notch pathway components results in downregulated ephrinB2 expression in arteries, whereas knock-in expression of Notch signaling components causes veins to ectopically express it.

Research on coronary blood vessel arteriovenous differentiation has not been very extensive. Remarkably, two recent papers have proposed different scenarios for this process. Through the analysis of coronary plexus development in Apelin-, EphB4- and EphrinB2-LacZ mice and fate mapping studies using a tamoxifen-inducible Vascular endothelial(VE)-cadherin-Cre driver, Red-Horse *et al.*, 2010 [56], suggested that coronary vessels are formed before E9.5, by the angiogenic sprouting venous endothelial cells coming from the sinus venosus; those venous sprouts are thought to be ‘reprogrammed’ as they expand to the ventricles to form coronary arteries. Unfortunately, VE-cadherin is not specifically expressed in endocardial cells, but also in all the vascular endothelium and many hemopoietic progenitors and is, therefore, a poor tool to trace endocardial derivatives.

Such findings have been directly challenged by Wu and colleagues [32], who propose a major ventricular endocardial origin for coronary arteries at E11.5. Using an NFATc1-Cre mouse line (NFATc1 being only expressed in endocardial and not vascular endothelial cells), the authors trace ventricular endocardial cells to the forming vascular outline of the arterial portion of the coronary system with a few marked cells found in coronary veins.

Together, these results seem to point to a diverse origin of the coronary endothelium, in which the arterial and venous systems have distinct origins at different morphological sites and in different stages of embryonic development. Due to its medical importance, this is a mystery that will require further effort to clarify the origins and the pathways involved in the assembly of this tissue.

Figure 2. Signaling during coronary blood vessel formation. The cartoon summarizes the molecular signaling active during coronary development. A color code (blue, epicardium; red, coronary endothelium; green, coronary smooth muscle; yellow, coronary fibroblasts; pink, myocardium) is used to identify the sources of secreted factors (capital letters, black). Known targets (receptors, transcription factors, signal transducers) characterizing coronary cell types are also shown (lower case letters, white).



8. Signaling Coordination during Epicardial Development

In parallel with its direct cellular contribution to the formation of coronary blood vessels, the embryonic epicardium has been characterized as a ‘signaling center’, meaning that this tissue produces itself multiple diffusible molecules that are secreted in a paracrine fashion; these molecules impact coronary vascular morphogenesis, but also the proliferation of the compact ventricular myocardium, thus guaranteeing the growth of the ventricular wall. We will detail the epicardium signaling role in a specific section.

How the myriad of signaling pathways involved in epicardial/coronary development are coordinated in space and time is not well understood. This is in part due to the frequent perception of the different transcription factors, signaling pathways and associated cellular mechanisms as isolated developmental circuitries and not as elements of a complex, integrated network. In this section, we will attempt to present molecular cross-talks as determinant factors in the triggering and coordination of cellular phenomena, whereas the essentials of this information are summarized in Figure 2.

- (1) Regulation of epicardial epithelial-to-mesenchymal transition: The *Wt1* gene encodes for a transcription factor reported to control the transcriptional repressor, Snail [17], epicardial retinoic acid signaling [20] and Wnt/beta-catenin-dependent signaling [18]. *Wt1* can be thus regarded as a coordinating gene in the regulation of epithelial-to-mesenchymal transition, as it promotes the loss of cell adhesion (via the cadherin repressor Snail), sustains pro-epithelial-to-mesenchymal transition epicardial retinoid synthesis and the associated activation of the canonical Wnt pathway.
- (2) Differentiation of coronary cell progenitors: As previously indicated, *Wt1* is involved in the regulation of epicardial retinoid signaling, and disruption of retinoic acid signaling in the epicardium downregulates PDGFR α,β expression in epicardial-derived cells, therefore altering coronary progenitor cell differentiation [20]. The Notch/Delta pathway, a key molecule in the activation of endothelial and endocardial signaling [57], is required for the promotion of arterial fate during coronary blood vessel development [58,59]. It is not known how Notch signaling interacts with the crucial function played by growth factors secreted by the embryonic myocardium, like FGFs and VEGF, in the regulation of coronary endothelial vasculogenesis [60]. These two growth factors are closely related, as VEGF has been reported to be dependent on myocardial FGF-induced Hedgehog activity [61]. On the other hand, Notch is also likely to be involved in the control of arteriovenous coronary endothelium differentiation. In this context, the downregulation of the nuclear transcription factor, COUP-TFII (a Notch repressor, [62]), and the upregulation of ephrinB2 tyrosine kinase expression in CA progenitor cells has been suggested to mark the beginning of coronary artery endothelium re-specification [56]. However, this point has not yet been proven, and the genetic regulation of this process remains unknown. Tcf21, another transcription factor, is required for the specification of fibroblast (but not smooth muscle cell) lineages from the epicardium prior to epicardial epithelial-to-mesenchymal transition [25].
- (3) Endocardium contribution to embryonic coronary vessels: It is still unknown which signals trigger the outgrowth of the endocardium to participate in coronary blood vessel formation. It has been suggested that the key mechanism is provided by a transmural (endocardial-to-

epicardial) increasing gradient of hypoxia that activates myocardial VEGF synthesis, promoting endocardial sprouting [32]. Spatio-temporal patterning of the endocardial sprouts have been proposed to be partially regulated by cell membrane-bound ephrins [56] and semaphorins [47].

- (4) Maturation of coronary blood vessels: The stabilization of the embryonic coronary endothelial outline requires perivascular/coronary smooth muscle differentiation and the initiation of endothelial-smooth muscle cell-to-cell interaction. Synergistic retinoic acid and VEGF signaling seems to regulate the physiological delay of coronary smooth muscle differentiation until an extensive coronary capillary network has formed [22]. FGFs seem to have a key developmental function in the regulation of transmural organization of coronary arteries [63,64]. Furthermore, cardiomyocytes also play an important role in the patterning and mural location of coronary arteries, as shown by experiments disrupting myocardial cell polarity [31]. On a final note, we want to emphasize that the definitive cues needed to stabilize the spatial patterning of major coronary vessels seems to be triggered by the activation of an effective blood flow in coronary arteries through unknown mechanisms.
- (5) Connection of primitive coronary arteries to the aortic root: Presumptive embryonic coronary arteries grow from the ventricle towards the aortic root, where they eventually connect to the systemic blood flow via the coronary ostia. It is well known that coronary ostia open to the left and right Valsalva sinuses (coronary sinuses) of the aortic valve [36,37,65], but the mechanisms that control the patterning of the two coronary stems remains unknown. Moreover, not many explanations are available on the developmental mechanisms that prevent coronary arteries from connecting to the posterior aortic sinus or to the pulmonary artery, and fewer are the explanations of the anomalous origin of coronary ostia from the so-called “wrong sinus” or from the pulmonary root. A plausible explanation would combine both repulsive signals emanating from the subpulmonary myocardium [66] and some pro-vascular signals especially active at the dorsolateral cardiac outflow tract [67].

9. Conclusions

Many aspects of the morphogenesis of this vascular bed remain a mystery. Coronary artery formation has, however, proven to be a unique model to study developmental phenomena related to the differentiation of vascular progenitors, vascular growth (vasculogenesis and angiogenesis) and arteriovenous specification and patterning. We believe that further research in the field will be instrumental in identifying the origin of coronary congenital defects, allowing for early diagnosis of these anomalies. Furthermore, developmental signals and gene regulatory networks involved in coronary blood vessel development could underlie the onset of adult coronary disease, partially accounting for the high impact of arteriosclerosis on coronary arteries as compared to other arterial vessels. The relevance of coronary developmental mechanisms in coronary neovascularization after vascular occlusion, ischemia and myocardial death needs more research and is still an open question.

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Conflicts of Interest

The authors declare no conflict of interest.

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