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Connecting the coronaries: How the coronary plexus develops and is functionalized

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Abstract

The establishment of the coronary circulation is one of the final critical steps during heart development. Despite decades of research, our understanding of how the coronary vasculature develops and connects to the aorta remains limited. This review serves two specific purposes: it addresses recent advances in understanding the origin of the coronary endothelium, and it then focuses on the last crucial step of coronary vasculature development, the connection of the coronary plexus to the aorta. The chick and quail animal models have yielded most of the information for how these connections form, starting with a fine network of vessels that penetrate the aorta and coalesce to form two distinct ostia. Studies in mouse and rat confirm that at least some of these steps are conserved in mammals, but gaps still exist in our understanding of mammalian coronary ostia formation. The signaling cues necessary to guide the coronary plexus to the aorta are also incompletely understood. Hypoxia-inducible transcription factor-1 and its downstream targets are among the few identified genes that promote the formation of the coronary stems. Together, this review summarizes our current knowledge of coronary vascular formation and highlights the significant gaps that remain. In addition, it highlights some of the coronary artery anomalies known to affect human health, demonstrating that even seemingly subtle defects arising from incorrect coronary plexus formation can result in significant health crises.

Keywords

coronary artery; coronary ostium; development; arteriogenesis

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None.

As the heart begins forming, the myocardial and endocardial layers of the heart are each composed of a single layer, and oxygen and nutrients are passaged via simple diffusion. With the growth of the ventricles, endothelial-lined trabeculae in the ventricles maintain sufficient surface area such that a vasculature is not required to supply oxygen and nutrients until as late as embryonic day (E) 15.5 in the mouse (Olivey and Svensson, 2010). However, a functional vasculature is required for the heart to undergo successful compaction of the ventricular myocardium. The coronary vasculature develops in two stages: first, a vascular plexus forms and encompasses the heart, and then, this plexus remodels into a mature vasculature that connects to the aorta. In the mature heart, the connections that bridge the plexus and the aorta occur through two ostia or openings through which the left and right coronary arteries connect to the aorta (Figure 1). The specific segments of the coronary arteries that connect to the aorta are referred to as the coronary stems, and this term will be used to distinguish the specific segment of the main coronary arteries that penetrates the aorta from the general coronary vasculature. These main coronary arteries subsequently branch off the base of the aorta, with the left coronary artery further dividing into the circumflex artery, which curves around the base of the left ventricle, and the left anterior descending artery, which reaches toward the apex of the left ventricle. Together, these arteries supply the mature ventricles with oxygen and nutrients.

Origin of the coronary endothelium

The coronary plexus first forms as a series of discontinuous endothelial patches that spread from the sinus venosus around the ventricles to form a complete plexus. The origin of these endothelial cells is highly controversial, having been attributed to the proepicardium, the sinus venosus, and the endocardium. The original studies detailing the origins of the coronary endothelium relied on avian species due to their amenability to *in ovo* manipulation, have been thoroughly discussed by Riley and Smart (2011), and will be briefly summarized. These initial studies used retroviral labeling, either *in ovo* or in an *in vitro* proepicardial explant that was subsequently transplanted, and quail-chick chimeras in which a quail proepicardial explant was placed in a chick host (Mikawa and Fischman, 1992; Poelmann et al., 1993; Perez-Pomares et al., 1998) (see also (Greulich and Kispert, 2013) for a detailed description of these studies). In these experiments, labeled proepicardial cells contributed to the coronary vessels, and transplanted quail cells expressed the quail endothelial marker QH-1. The inclusion of liver primordium in some of the proepicardial explants has led to the hypothesis that the liver is also a source of coronary endothelial cells (Poelmann et al., 1993; Lie-Venema et al., 2005). However, subsequent studies have not supported this hypothesis.

More recent proepicardial lineage-tracing studies in mouse (summarized, along with additional mouse-based experiments, in Table 1) have challenged the long-held assumption that the proepicardium gives rise to coronary endothelium. Lineage-tracing studies using the standard proepicardial marker Tbx18 showed that Tbx18-lineage cells give rise not only to the epicardium but also to myocardium, cardiac fibroblasts, pericytes, and coronary smooth muscle (Cai et al., 2008). However, these experiments did not identify Tbx18 lineage-traced cells within the coronary endothelial population, even with the use of sensitive detection methods such as fluorescence activated cell sorting (Cai et al., 2008). Zhou et al. (2008)

simultaneously performed similar lineage analyses using the WT-1 promoter, with similar results. Zhou et al. also found that WT-1-derived proepicardial cells gave rise to myocardium and coronary smooth muscle. In rare cases, though, WT-1-derived cells also contributed to the coronary endothelial population, but this event was too infrequent to support WT-1-positive proepicardial cells as the major source of coronary endothelial cells (Zhou et al., 2008). However, just as the avian transplantation studies can be compromised by dissection integrity, Cre-mediated lineage tracing has its own unique pitfalls. In the case of the WT-1-Cre lines, these shortcomings include potential low levels of recombination beyond the targeted tissue (Zhou and Pu, 2012) and expression of WT-1 in the coronary endothelium and even some cardiomyocytes (Greulich and Kispert, 2013). In the Tbx18-lineage study, the myocardial “progeny” are more likely a second population of Tbx18-expressing cells within the myocardium rather than descendants of the earlier Tbx18-expression proepicardium (Christoffels et al., 2009). Although species-specific differences may explain, at least in part, the apparent inconsistencies between the chick and mouse data, one alternative hypothesis is that some coronary endothelial cells derive from a source other than the Tbx18-/WT-1-positive proepicardium.

Thus, in an attempt to identify other sources of coronary endothelium, recent studies in the mouse have employed clonal analyses to begin elucidating the contributions of the sinus venosus and the endocardium to the coronary plexus. Because the mouse embryo is less amenable to *in vivo* manipulation than the avian embryo, a key step for addressing the original of the coronary endothelium was identifying a coronary endothelium-specific gene. Red-Horse et al. (2010) took advantage of the apelin-lacZ knock-in mouse, which shows expression in adult coronary endothelium but not endocardium (Sheikh et al., 2008). An analysis of apelin expression during coronary plexus formation confirmed that apelin is expressed in the developing coronary endothelium and also showed that apelin expression is continuous with the sinus venosus (Red-Horse et al., 2010). This expression pattern, in combination with clonal analyses based on an inducible VE-cadherin Cre mouse line, suggested that VE-cadherin-positive endothelial cells from the sinus venosus migrate into the heart to generate the majority of the coronary plexus (Red-Horse et al., 2010). Further, subsequent clonal analyses using the Apelin promoter suggest that arterial and venous coronary endothelial cells share a common progenitor (Tian et al., 2013b).

As a potential resolution of the apelin expression pattern, the historical avian experiments, and the proepicardial lineage-tracing studies in mice,Katz et al. (2012) identified a population of WT1-negative, Tbx18-negative, semaphorin 3D-positive proepicardial cells. Lineage traces of these semaphorin 3D-expressing cells show that they migrate from the proepicardium into the heart and give rise to a portion of the coronary smooth muscle and endothelium, including both arterial and venous coronary endothelial cells (Katz et al., 2012). Further, lineage-traced cells were detected in the sinus venosus, though semaphorin 3D itself was not actively expressed by the sinus venosus endothelium. Thus, a population of semaphorin 3D-lineage proepicardial cells may migrate through the sinus venosus along their path to form the epicardium and subsequently generate coronary endothelium (Katz et al., 2012). Semaphorin 3D-lacZ knock-in mice that were crossed with an apelin lineage reporter mouse line showed that the apelin-derived endothelium does not actively express

semaphorin 3D (Tian et al., 2013b). This more recent study does not exclude the possibility that the semaphorin 3D-positive proepicardial lineage may downregulate semaphorin expression prior to migration to the sinus venosus and is consistent with the endogenous semaphorin 3D expression pattern (Katz et al., 2012). However, semaphorin 3D-derived coronary endothelial cells were detected in the ventricle prior to the timing suggested based on apelin expression (Red-Horse et al., 2010) and PECAM expression (Lavine et al., 2006), with the caveat that neither of these latter studies present results at E11.0. Further, this earlier time point is more consistent with the description by Viragh and Challice (1981) of capillaries beginning to form as early as E10.0 near the sinus venosus. Thus, the semaphorin 3D-derived coronary endothelial cells and the apelin-expressing coronary endothelial cells may represent distinct populations, or apelin may not be the first marker of coronary endothelium. The idea that two distinct populations specifically contribute to the coronary venous endothelium, predominantly through an epicardial-endocardial gradient, is plausible but not the most straight-forward solution. Thus, we hypothesize that the origin of the coronary endothelium may be more similar to the origin of the myocardium, which is described as deriving from the first and second heart fields, even though these two fields are part of the same cardiogenic mesoderm (Abu-Issa and Kirby, 2007). Further study is required to resolve these populations and will hopefully clarify the origin of the semaphorin 3D- and apelin-positive lineages.

Based on the position of the apelin-positive endothelial cells within the subepicardial myocardium and their early expression of venous markers (Red-Horse et al., 2010) as well as the apelin-based clonal analyses (Tian et al., 2013b), the coronary endothelial cells of the intramyocardial coronary arteries are hypothesized to derive from the subepicardial coronary veins. However, as an alternative to the proepicardium and sinus venosus, a subset of the endocardium has also been hypothesized to invade the ventricular myocardium to generate coronary endothelial cells within specific regions of the heart (Red-Horse et al., 2010; Wu et al., 2012). Based on the clonal analysis under the VE-cadherin promoter, most coronary endothelial cell clones derive from sinus venosus sprouts (Red-Horse et al., 2010). However, the VE-cadherin Cre is expressed in both the coronary endothelium and the endocardium (Alva et al., 2006), and additional clones, specifically those that contained blood islands near the interventricular groove, also contained endocardial cells (Red-Horse et al., 2010). Similarly, using lineage tracing of Nfatc1 descendants,Wu et al. (2012) identified Nfatc1-derived endothelial cells within the coronary plexus. Because Nfatc1 is actively expressed in the endocardium and is specifically excluded from the proepicardium, the epicardium, and coronary endothelial cells, the authors posit that the endocardium contributes specifically to the myocardial coronary arteries that are closest to the endocardium (Wu et al., 2012). Because Nfatc1 expression extends to the atrial endocardium and the sinus venosus (Misfeldt et al., 2009), Wu et al. evaluated whether Nfatc1-driven Cre was expressed in sinus venosus endothelium or proepicardially derived cells that have migrated through the sinus venosus. However, Cre expression was not detected at E9.5 in the sinus venosus, suggesting that the Nfatc1 lineage-traced cells derive strictly from the endocardium. Further, subsequent analyses support the hypothesis that blood islands bud from the endocardium (Tian et al., 2013b).

Though complicated and still controversial, the origin of the arterial and venous coronary endothelial cells has benefitted from over 30 years of study. However, significantly less is known about the origin of the lymphatic coronary endothelial cells. The lymphatic vessels develop following the blood vessels, both in the heart and in other organs (Ratajska et al., 2014). Quail-chick chimeras that replace the chick proepicardium with the quail epicardium suggest that proepicardium-derived quail coronary endothelial cells only contribute to a single lymphatic trunk at the base of the heart (Wilting et al., 2007). The remaining lymphatic endothelial cells are thought to sprout from existing veins (Srinivasan et al., 2007). The discovery of lymphatic-specific markers, such as Prox-1 and Pkd1, should help confirm the origin of these specific endothelial cells.

Altogether, these mouse studies provide additional insights into the classical work performed in chick and quail. A mosaic source of coronary endothelium, including a subset of the proepicardium and endocardial derivatives, is the easiest explanation for the divergent findings. Subsequent analyses that determine whether differences are present among different coronary arteries, veins, and lymphatics (e.g., those within the interventricular septum vs. the free ventricular walls) may help clarify this hypothesis. Additional improvements in microscopy that allow for more detailed clonal analyses, for example using all 90 fluorescent combinations produced by the Brainbow 3.1 reporter, will also help identifying the lineage relationships among the types of coronary endothelium. Until live fluorescent imaging can be used to follow the presumptive coronary endothelial lineage in the developing mouse embryo, lineage tracing combined with in-depth histological analyses may help determine the exact migratory pathway of the proepicardial derivatives to clarify the spatial relationships among the proepicardium, sinus venosus, epicardium, and subepicardial coronary endothelium.

Aside from the origin of the coronary endothelium, the subsequent formation of the coronary plexus has been well described and is straight-forward in avian and mouse species. The coronary plexus develops by wrapping around the heart using the same pattern of addition as the epicardium, starting from the sinus venosus and spreading to the ventral side of the ventricles (Viragh and Challice, 1981; Lavine et al., 2006; Red-Horse et al., 2010). The recently created wealth of transgenic mouse lines should help address other underappreciated aspects of coronary plexus development. Thus, we will now highlight one particularly understudied area: the final stage of development in which the coronary plexus connects to the aorta.

Coronary stem formation

Despite decades of study, the connection process between the coronary plexus and aorta is incompletely understood. In one of the seminal works describing this connection in mouse, Viragh and Challice (1981) suggested that vascular growth within the wall of the aorta connects to the vascular plexus within the ventricles. This theory was readily accepted, with similar patterns of outgrowth described in species as diverse as shark and pig (see references within (Waldo et al., 1990) for historical studies), and subsequent authors either accepted the proposed mechanism or attributed their own inability to find coronary arteries budding off the aorta to the rapidity with which the buds must connect with the coronary plexus (e.g.,

(Hutchins et al., 1988; De Andres et al., 1990). However, follow-up analyses in quail, rats, and humans found no evidence of outgrowth from the aorta towards the developing plexus (Bogers et al., 1988; Bogers et al., 1989). Instead, as Waldo et al. (1990) describe based on work in chick, the coronary plexus grows toward the aorta and mounts a "controlled invasion" to connect with the wall of the aorta and form distinct openings, known as ostia (Figure 1). Indeed, most of our knowledge concerning the connection between the coronary plexus and aorta is due to observations made in chick and quail. Based on these studies, coronary stem formation begins with a fine network of vessels connecting to the aorta (Bogers et al., 1988; Bogers et al., 1989; Waldo et al., 1990; Poelmann et al., 1993; Ando et al., 2004; Nanka et al., 2008; Tomanek et al., 2008). These vessels then coalesce to form two distinct ostia. In the quail, the remodeling from a fine network to distinct ostia takes approximately three days, spanning E6–9. Cardiac neural crest (CNC)-derived cells, which populate the outflow tract cushions and septate the outflow tract into an aorta and pulmonary artery, are not necessary for the fine coronary network to connect to the aorta (Waldo et al., 1994). However, the CNC derivatives do promote the addition of smooth muscle to the coronary stems (Hood and Rosenquist, 1992). This addition occurs after the fine network has connected to the aorta and is hypothesized to promote the stabilization of two specific stems.

Coronary stem formation has been analyzed albeit in a limited fashion in rat as well and follows a mostly similar pattern. By E15.5, a network of peritruncal, lumenized vascular clusters are present, and these clusters coalesce into channels (Ratajska and Fiejka, 1999). However, whether these channels connect to the aorta prior to their coalescence into two distinct ostia remains unclear. Even though the coronary plexus has fully encompassed the heart by E16.5 in the rat (Hanato et al., 2011), no coronary ostia are present at this stage. The first true ostia are observed between E17.5–18.5 (Tomanek et al., 1996; Ratajska and Fiejka, 1999). As observed in the chick, the coronary stems in the rat are initially devoid of smooth muscle, which is added only after the ostia have formed within the aorta (Tomanek et al., 1996).

In the mouse, the connection of the plexus to the aorta has only recently begun being addressed. The early description by Viragh and Challice indicates that the first capillaries are found in the heart by E10 and that blood-filled capillaries appear in the wall of the aorta by E13 (Viragh and Challice, 1981), and subsequent work supports these observations (Gonzalez-Iriarte et al., 2003). However, neither study reported that these capillaries directly connected with the aorta. Thus, whether the mouse (or rat) developed a fine network of vessels that connect with the aorta prior to the observation of two distinct stems, which are present by E14.0 and encompassed by smooth muscle by E15.0 (Compton et al., 2007), has remained unclear until very recently. Using the apelin^{CreERT2} mouse, coronary endothelial cells were labeled at E10.5 and formed the endothelium of the coronary stems, supporting the in-growth of the coronary vasculature as a common developmental mechanism (Tian et al., 2013a).

In the human, the general pattern of coronary plexus development appears to be conserved with other species. Blind-ended capillaries are first observed at the base of the interventricular sulcus at Carnegie stage 14, and these capillaries spread across the

ventricles, approaching the atrioventricular sulcus and covering the ventral side of the ventricles (Hutchins et al., 1988). These capillaries then remodel to form a rudimentary plexus that covers the heart. Coronary stems are not observed until Carnegie stage 18 and show the same pattern of coronary vessels invading the aorta rather than forming as outgrowths from the aorta (Hutchins et al., 1988). The placement of the coronary ostia after formation in humans is consistent with observations in all other species examined, with one ostium present in each of the sinuses of Valsalva (Hutchins et al., 1988).

As can be seen from the descriptions above, coronary stem placement is consistent across species. In avians, there is a distinct transition stage during which numerous endothelial strands penetrate the aorta before coalescing to form two distinction stems. A similar peritruncal ring of coronary vessels has been recently described in mouse (Tian et al., 2013a), but whether these small endothelial strands penetrate the aortic wall and remodel as in the chick has yet to have been definitively reported.

Coronary stem maturation

The coronary stems that connect to the aorta initially comprise an endothelial vessel only. After the coronary ostia are formed in chick and quail, smooth muscle cells and fibroblasts are recruited to the coronary stems (Hood and Rosenquist, 1992; Vrancken Peeters et al., 1997; Vrancken Peeters et al., 1999); vessels that lack a smooth muscle-based tunica regress in epicardial quail-chick chimeras (Poelmann et al., 1993). Based on experiments in chick, these additional smooth muscle cells and fibroblasts are derived from the proepicardium (Vrancken Peeters et al., 1999). The myocardium at the base of the chick aorta is enriched in vascular endothelial growth factor receptors (VEGFR) 2 and 3 (Tomanek et al., 2002), suggesting that VEGF signaling may be a positive migratory factor for the smooth muscle cells. Indeed, blocking VEGF signaling in chick reduces the recruitment of smooth muscle (Tomanek et al., 2008), and the VEGF120/120 mouse embryo, which produces a single highly soluble, non-heparin-binding VEGF isoform, shows disrupted smooth muscle surrounding the coronary stems (van den Akker et al., 2008). However, *in vitro* analyses of proepicardial quail explants show that both VEGF and retinoic acid inhibit smooth muscle differentiation. Based on the *in vivo* expression patterns of these molecules, the *in vitro* analyses suggest that these factors delay the smooth muscle differentiation of proepicardial derivatives until after the coronary ostia have formed within the aorta (Azambuja et al., 2010). The production of retinoic acid within the overlying epicardium in chick, quail, and mouse during ostia formation, though, supports the hypothesis that retinoic acid delays the differentiation of proepicardial derivatives until after the ostia are formed (Moss et al., 1998; Xavier-Neto et al., 2000). However, how VEGF specifically affects smooth muscle addition in the embryo is unclear.

Circulating platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)-2 also promote the investment of the coronary stems with smooth muscle cells and affect both the thickness of the smooth muscle tunica and the proportion of the stem that is encompassed in chick (Tomanek et al., 2008). PDGF-2 acts specifically as a smooth muscle cell chemoattractant (Dardik et al., 2005), and PDGF-2 homodimers also induce smooth muscle differentiation via the RhoA intracellular pathway (Lu et al., 2001). Although RhoA is also

part of the planar cell polarity pathway, the addition of smooth muscle to the coronary stems has not been addressed in mouse mutations for this pathway (e.g., *loop-tail*) despite disorganized smooth muscle addition to the coronary plexus within the ventricles in the *loop-tail* mouse (Phillips et al., 2008). Importantly, FGF-2 and PDGF-2 transcription is increased in bovine aortic endothelial cells in response to shear stress (Malek et al., 1993), suggesting that smooth muscle recruitment may be induced by the initiation of flow once the coronary ostia are patent.

Hypoxia-induced signaling during coronary stem formation

Hypoxia-induced vessel recruitment is a common theme in angiogenesis. The base of the aorta expresses the hypoxia-induced transcription factor Hif1α, whose expression in chick is induced in response to hypoxia and correlates with coronary ostium placement (Wikenheiser et al., 2009). Exposing quail embryos to hypoxia increases the capillary density throughout the myocardium, consistent with low oxygen levels promoting vascular growth (Nanka et al., 2008). When quail embryos are placed in a hypoxic environment at approximately Hamburger-Hamilton stage 12 (after 48 h of incubation), atretic coronary arteries are observed (Nanka et al., 2008). In contrast, exposing chick embryos to either hypoxic or hyperoxic conditions starting at Hamburger-Hamilton stage 25 (or approximately 4.5–5 days of incubation) does not cause coronary atresia but does lead to both anomalous origins of the coronary stems and additional stems (Wikenheiser et al., 2009). The results of these experiments appears to indicate that, whereas there is a specific window during which the oxygen levels can affect the formation of the coronary stems, the location at which these stems are placed can be affected over a much broader time frame.

Hif1α is the master switch downstream of hypoxia. Its transduction is instantaneous in response to hypoxia, and the protein is rapidly degraded in the presence of oxygen. Hif1α forms a heterodimer with ARNT and binds to hypoxia response elements within the promoters of its targets. Binding alone is insufficient to induce transcription, and co-transcription factors such as Smad3 are often required. Smad3 in particular is necessary to induce transcription of the VEGF receptors. Numerous pro-angiogenic factors are downstream of Hif1α; in addition to the VEGFs and their receptors, FGF-2 and PDGF are also induced by Hif1α (Liu and Simon, 2004). VEGF receptors are expressed at the base of the aorta during coronary stem formation (Tomanek et al., 2002), and PDGF-A and PDGF receptor α are expressed within the outflow tract during this time period (Van Den Akker et al., 2005). Despite Hif1α's necessity in mediating hypoxia, though, the endothelial-specific Hif1α conditional knockout mouse is viable (Tang et al., 2004), suggesting that hypoxia drives Hif1α-mediated angiogenesis by affecting the environment that needs vascularization as opposed to directly affecting the endothelial cells.

Because Hif1α is at the top of the hypoxia-responsive cascade, numerous factors modulate its response (Figure 2). Cited2, which is expressed at the base of the aorta in mouse at E13.5, competitively binds Hif1α to downregulate signaling (Yin et al., 2002; Weninger et al., 2005). The Cited2 knockout mouse, which would thus have upregulated Hif1α signaling, is embryonic lethal starting at E13.0 and phenocopies the VEGF-A-overexpressing mouse in that it displays numerous cardiac phenotypes, including outflow tract alignment defects,

valve defects, and septal defects (Yin et al., 2002). Although no coronary artery anomalies have been specifically reported in the Cited2 knockout embryo, these embryos exhibit dysregulated plexus formation within the myocardium (Xu et al., 2007). In addition, the Cited2 knockout embryo displays intense hypoxia at the base of the aorta after this region should have blood flow through the coronary ostia (Xu et al., 2007), suggesting that this embryo has coronary artery anomalies. The Cited2 knockout mouse can be partially rescued by inactivating a single copy of Hif1α, thus titrating the dose of Hif1α (Xu et al., 2007). Specifically, the level of hypoxia at the base of the aorta decreases after the coronary stems have formed in both wild-type and the Cited2 knockout/Hif1α haploinsufficient mutant, suggesting that any impairment of the coronary vasculature can be rescued by regulating the dose of Hif1α (Xu et al., 2007).

Hif1α-induced signaling during coronary stem formation

Downstream of Hif1α is the VEGF signaling pathway. Of the VEGF family members, VEGF-B can affect the number of coronary stems that form. Injecting quail embryos with an antibody that specifically blocks VEGF-B leads to a reduction in the number of coronary ostia, with one or both ostia missing, without affecting overall capillary density in the myocardium (Tomanek et al., 2006); blocking VEGF-A has no effect. Similarly, injecting quail embryos with a VEGF trap that inhibits both VEGFR1 and VEGF2 also inhibits coronary stem formation. However, blood islands are observed within the peritruncal ring, indicating that only the last step of connecting the coronary plexus to the aorta is interrupted (Tomanek et al., 2006). Despite these findings in quail, the VEGF-B-null mouse is viable (Bellomo et al., 2000). Although the formation of the coronary stem specifically has not been addressed, no changes in the density of the coronary plexus are apparent in these mice (Bellomo et al., 2000). These findings suggest that there are species-specific differences among the coronary response to VEGF.

However, other VEGFs also affect endothelial cell behavior. Upon binding to VEGFR2, VEGF-A can interact with neuropilin-1, which induces the migration of HUVECs towards a VEGF-A gradient in a FAK-dependent manner (Herzog et al., 2011). The neuropilin-1 knockout embryo shows numerous vascular patterning defects but is embryonic lethal by E13.5, thus precluding an analysis of the coronary stems in this mouse (Kawasaki et al., 1999). VEGF-A also acts upstream of the PI3K/Akt pathway to phosphorylate the Forkhead transcription factors in cultured endothelial cells (Abid et al., 2004). This phosphorylation leads to increased cell survival and proliferation (Abid et al., 2004). Together, these studies suggest that endothelial cells respond to VEGF-A by migrating towards it, which then promotes their survival and subsequent proliferation. However, whether these mechanisms hold true in the *in vivo* context remains unknown.

Additional growth factors that are downstream of Hif1α and are involved in coronary stem formation include FGF-2 and PDGF. In the quail embryo, blocking either of these growth factors with circulating antibodies prior to coronary stem formation results in one or two missing ostia (Tomanek et al., 2006; Tomanek et al., 2008). PDGF-A is initially expressed in the chick outflow tract cushion mesenchyme, with expression decreasing during the connection and remodeling of the coronary arteries (spanning Hamburger-Hamilton stages

28–35), and receptor PDGFRα shows a similar temporal expression within the outflow tract myocardium (Van Den Akker et al., 2005).

In addition to inducing Hif1α, hypoxia also induces the transcription factor KLF-2 (Kawanami et al., 2009). This factor promotes angiogenesis and inhibits Hif1α and other hypoxia-related factors, thereby turning off this angiogenic switch (Kawanami et al., 2009). KLF-2 is also induced in endothelial cells by the shear stress induced by blood flow, which would further halt the formation of additional coronary stems once patent ostia were in place; however, the expression of KLF-2 specifically in the coronary endothelium is unknown (Lee et al., 2006).

Hypoxia may induce apoptosis around the coronary ostia

Whether hypoxia induces apoptosis and whether apoptosis is related or necessary for coronary stem insertion remain controversial. In chick, high levels of apoptosis are observed near the coronary ostia formation points under normoxic conditions (Velkey and Bernanke, 2001). However, studies in quail embryos raised under normoxic and hypoxic conditions show that coronary stem formation defects are highly prevalent in the hypoxic embryos, despite a dense vascular plexus in the peritruncal ring, even though no significant changes in apoptosis are present throughout the ventricles (Nanka et al., 2008). However, because apoptosis at the coronary stem formation points has not yet been specifically addressed in this model, the issue of whether apoptosis levels change with hypoxia near the coronary ostia remains unknown.

Cardiac neural crest contributions to the coronary arteries

Beyond hypoxia, CNC-derived cells also affect coronary artery development. Quail-chick chimera studies have shown that CNC-derived cells do not directly contribute to the coronary stems (Waldo et al., 1994). However, CNC-derived cells do give rise to the cardiac ganglia, which contact the tunica media of the coronary stems (Waldo et al., 1994). Despite the lack of direct contribution to the coronary stems, in the chick CNC ablation model, only one coronary ostium forms normally, whereas the other ostium is misplaced (Hood and Rosenquist, 1992). This original analysis did not clarify whether the ostium placement was a direct effect of the CNC derivatives or an indirect effect caused by the arterial pole defects observed in the CNC ablation model. A more recent study using bis-diamine in rats to disrupt CNC migration showed that the rats exhibit a poorly developed coronary plexus and coronary atresia (Hanato et al., 2011), thus indicating that the requirement for CNC derivatives is species-independent. However, because CNC derivatives do not migrate to the outflow tract in this model, this study leaves open the question of whether ostium placement is directly or indirectly affected by CNC-derived cells.

Human coronary stem defects

Aside from the obvious requirement of at least one functional coronary ostium, a wide variety of human coronary artery anomalies exist, and their presence is associated with additional health burdens. In particular, coronary anomalies are frequently associated with sudden death. In the 1970s, a correlation between a single ostium and sudden death was

established (Lipton et al., 1979). Although uncommon, subsequent case reports support this finding (Debich et al., 1989; Turkmen et al., 2007). An additional link has been established between left coronary artery atresia and myocardial infarction (Vidne et al., 1979). Although less dramatic than complete atresia, high take-off coronaries, in which the ostium connects distal to the aortic valve, are associated with sudden death in athletes (Maron, 2003). In an adolescent patient in whom both ostia opened into a single sinus, a thrombus that formed during surgery led to severe myocardial infarction and sudden death (Slavin and Rudoff, 1997). These cases touch only on missing ostia or misplaced ostia within the context of the aorta. However, further complications can arise when the coronary arteries connect with the pulmonary artery. Depending on the developmental of collateral coronary vessels that bridge the systemic and pulmonic circulatory systems, this anomaly can inhibit perfusion of the myocardium to varying degrees, leading to severe left-sided heart failure or to mild symptoms such as exercise intolerance (Aykan et al., 2012). Thus, even when two coronary ostia are present, their location is still critically important.

On the other side of the spectrum, some patients with narrow, or stenotic, coronary arteries adapt to the insufficient blood supply to parts of the heart through the development of a network of collateral coronary vessels that connect to the other coronary artery (Sahinarslan et al., 2010). This adaptive growth helps the non-stenotic coronary artery preserve the myocardium and can compensate for coronary artery disease, but great variability exists in the extent of this collateral growth (Sahinarslan et al., 2010). Patients with collateral coronary circulation have significantly lower one-year mortality rates after myocardial infarction compared with patients who lack this additional circulation (Wang et al., 2011). New subendocardial plexus formation has also been observed in patients with heart failure (Fulton, 1956; van den Wijngaard et al., 2010). This new growth is correlated with the abundance of $CD34^{+}/Flk1^{+}$ circulating endothelial progenitor cells (Kocaman et al., 2011; Tokgozoglu et al., 2011), suggesting that this remodeling may be predictable. These studies underscore the potential of coronary revascularization and highlight the importance of understanding how the coronary ostia are formed within the aorta. However, developing an effective collateral circulation requires at least one patent ostium.

Closing remarks

The establishment of the coronary circulation is critical for life. The early events in coronary vascular development have been well established, but despite decades of study, there are fundamental gaps in our understanding of how this vasculature connects to the aorta. In avians, the coronary plexus sends a fine network of vessels to invade the aorta, and these vessels coalesce to form two distinct stems. Whether mammalian species exhibit a similar transition period or immediately form two distinct coronary stems remains unknown. Because the avian models are so amenable to hypoxia and hyperoxia studies and are easily manipulated *in ovo*, knowing whether mammalian and avian coronary stem formation occurs similarly is critically important if we are to apply the results of avian studies to humans. Hif1α and its downstream targets, such as VEGF and FGF-2, promote coronary stem formation in avians and mouse, but whether Hif1α is the specific initiating factor that recruits the coronary plexus to the aorta is unknown. The placement of the two coronary ostia is consistent among species, but why the ostia form in those two specific aortic sinuses

remains unknown. Studies that elucidate the factors that recruit the coronary plexus to the aorta will be highly beneficial for understanding how the human coronary artery anomalies arise. With so many questions remaining unanswered, understanding the development of the coronary stems promises plenty of new growth.

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Hihglights

Key aspects of coronary vasculature formation remain incompletely understood.

Genetic lineage analyses have yielded novel coronary endothelium origin hypotheses.

How the coronaries form their stereotypical pattern in the aorta remains unknown.

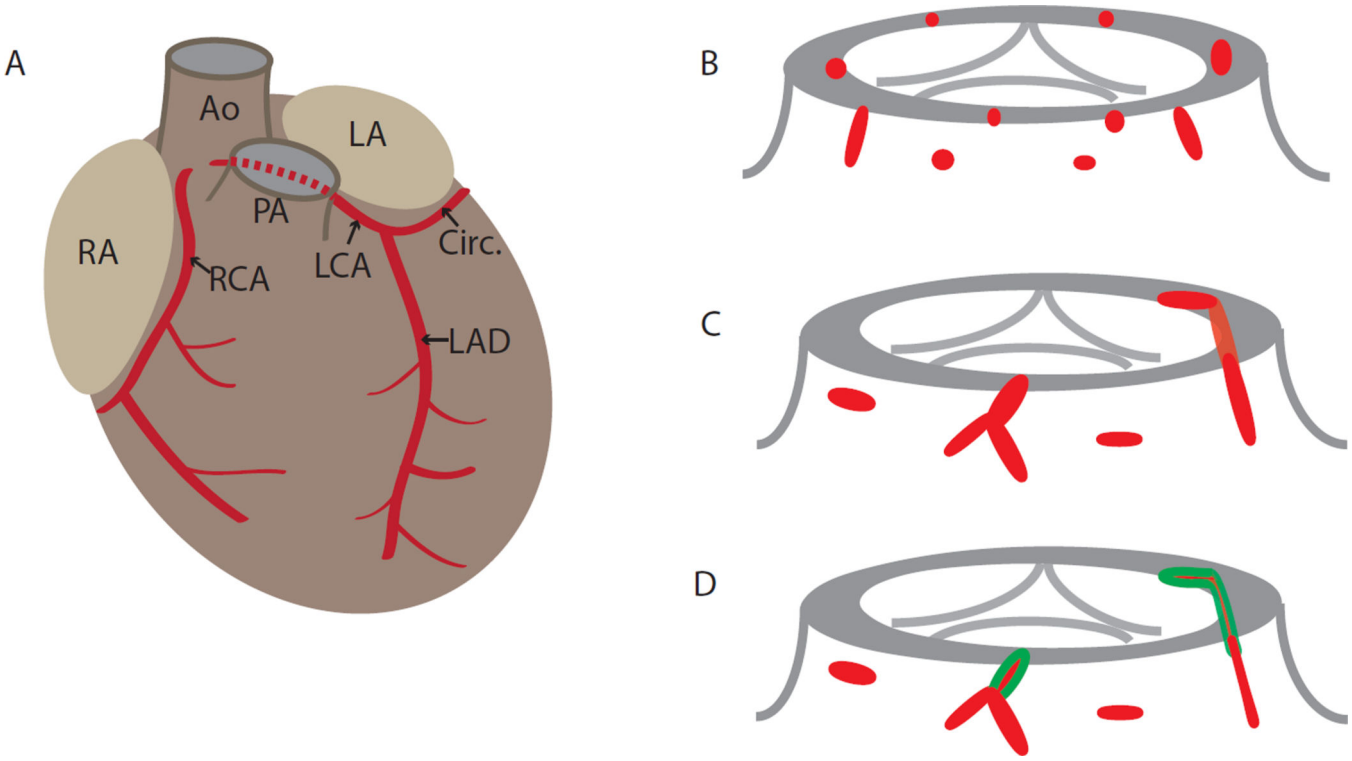

Figure 1. Coronary artery anatomy and stem formation

(A) In the mature heart, two main coronary arteries are present. The left coronary artery (LCA) runs between the pulmonary artery (PA) and the left atrium (LA) and branches into the circumflex (Circ.) artery and the left anterior descending (LAD) artery. The right coronary artery (RCA) branches off the right side of the aorta (Ao) and follows the right atrium (RA) as it travels to the apex of the heart. (B–D) A schematic overview of coronary ostia formation in avians. B) Thin blood vessels (denoted in red) from the coronary plexus invade the peritruncal region and penetrate the aortic wall. C) These small vessels coalesce to form two distinct ostia. D) Smooth muscle cells (denoted in green) encompass the coronary stems, beginning at the ostia themselves and over time encompassing the rest of the coronary arteries. All three of these steps have been documented in chick and quail; whether the thin vessels (B) are initially present before coalescing into two distinct ostia in mammals is unknown.

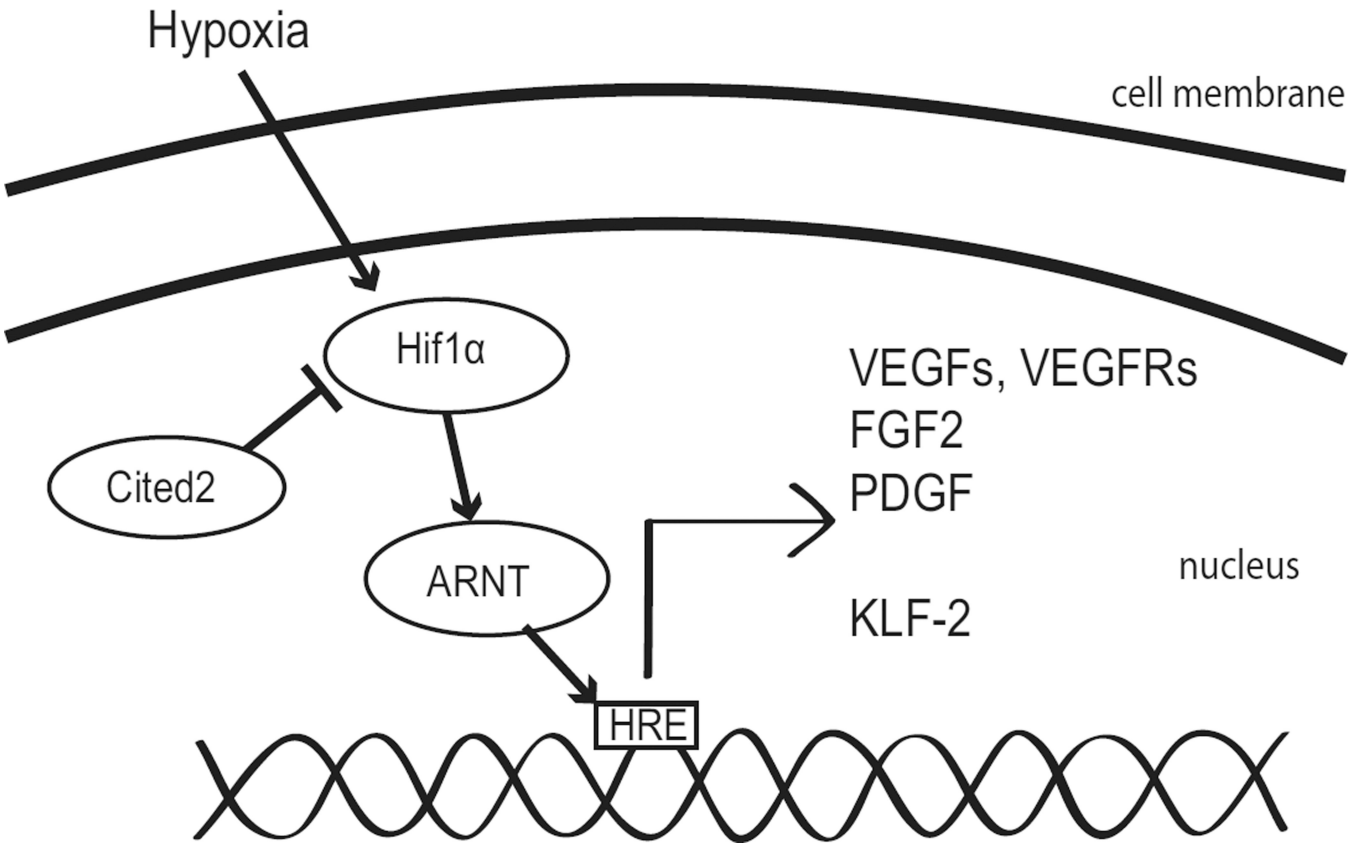

Figure 2. Signaling during coronary stem formation

Prior to formation of the coronary stems, the peritruncal region is hypoxic. Hypoxia stabilizes Hif1α, which is observed where the ostia will form. The stabilized Hif1α can then bind to ARNT, which allows this dimer to bind to hypoxia response elements (HREs) to induce the transcription of downstream targets. Downstream targets include ligands and receptors that further promote coronary stem formation, including VEGF and its receptors, FGF-2, and PDGF. Additional targets include the shear stress-responsive gene KLF-2. The Hif1α repressor Cited2 is also present around the aorta of the mouse and can inhibit the Hif1α-ARNT interaction.

Table 1

A summary of the genetic approaches used for lineage analysis of the coronary endothelium.

Gene	Study type	Active expression	Lineage-traced progeny	Ref.
Apelin (*Apln*)	LacZ knock-in	CE, SV		(Red-Horse et al., 2010; Tian et al., 2013b)
	$Apln^{CreER}$, tamoxifen-inducible	Cells expressing Apelin do not co-express Sema3D, Tbx18, or WT-1		(Tian et al., 2013b)
	Co-culture of SV/atria and ventricles using $Apln^{CreER}$ and WT embryos		Labeled coronary endothelial cells only formed in ventricles when $Apln^{CreER}$ SV/atria were paired with WT ventricles	
	Inducible Cre×$R26R^{mTmG}$		Induction at E10.5 only labels intramyocardial coronary endothelial vessels; induction at E12.5 only labels IVS vessels	
	Clonal analysis, inducible Cre×$Rosa26^{Rainbow}$		Clones contained subepicardial venous and intramyocardial arterial endothelial cells	
Mef-2c	Cre×$R26^{RFP}$, lineage trace		Coronary vessels in IVS	(Tian et al., 2013b)
Nfat-c1	ISH, IHC, *Nfat-c1*-LacZ	Endocardium, potentially faint protein expression in epicardium		(Wu et al., 2012)
	Cre×$R26^{fstz}$		Endocardium, cushion mesenchyme, subset of SV endothelium, arterial coronary endothelial cells	
	Clonal analysis, TetO-inducible Nfatc1-BAC-Cre		Majority of clones were in intramyocardial vessels; some were also in subepicardial vessels	
Scleraxis (*Scx*)	*Scx*-GFP transgenic	Subdomain of PEO at E9.5; epicardium at E10.5-E12.5; developing valves at E12.5		(Katz et al., 2012)
	GFP-Cre×$R26R^{LacZ}$		Coronary endothelial cells, endocardium, and others; begin penetrating RV wall at E10.25 and appear first in sub-epicardial myocardium; contributes to both arterial and venous coronary endothelial cells; some *Scx*-lineage cells express Nfat-c1 at E11.0	
	Mouse-chick chimera		Epicardial and coronary endothelial cells; coronary vessels showed mosaic chick/mouse origin	

Gene	Study type	Active expression	Lineage-traced progeny	Ref.
	In vitro clonal analysis		*Scx*$^{+}$ proepicardial cells give rise to a greater percentage of endothelial cells than *Scx*- proepicardial cells	
Semaphorin3D (*Sema3d*)	GFP-Cre fusion knock-in	Subdomain of PEO at E9.0; epicardium E10.5-E14.5; developing valves at E12.5		(Katz et al., 2012)
	GFP-Cre×*R26R*^{LacZ}		Coronary endothelial cells, SV, coronary smooth muscle, and others, first appearing in sub-epicardial myocardium; begin penetrating LV and IVS at E12.5	
	Mouse-chick chimera		Endothelium and coronary endothelial cells; coronary vessels showed mosaic chick/ mouse origin	
Tbx18	ISH, IHC, nLacZ knock-in	PEO, epicardium, myocardium		(Cai et al., 2008) (Christoffels et al., 2009)
	Cre×*R26R*^{lacZ}		Coronary smooth muscle, myocardium	
VE-cadherin	Clonal analysis, tamoxifen-inducible		Single clones marked both SV and coronary endothelial cells; coronary arterial, venous, and capillary endothelial cells were labelled; some coronary endothelial cell clones did not co-label with SV	(Red-Horse et al., 2010)
WT-1	Mouse-chick chimera, WT1$^{GFP-Cre}$, and WT1^{CreERT2}	Subset of PEO, endocardium, CE, myocardium	Coronary smooth muscle	(Katz et al., 2012; Rudat and Kispert, 2012; Zhou and Pu, 2012)
	Nkx2.5-Cre and Isl1-Cre×*R26R*^{fsLz} and IHC		WT-1-positive proepicardial cells derived from Nkx2.5/ Isl1-expressing progenitors	(Zhou et al., 2008)

Abbreviations: CE, coronary endothelium; IHC, immunohistochemistry; ISH, *in situ* hybridization; IVS, interventricular septum; LV, left ventricle; PEO, proepicardial organ; RV, right ventricle; SV, sinus venosus