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Review

The role of secondary heart field in cardiac development

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ABSTRACT

Although de la Cruz and colleagues showed as early as 1977 that the outflow tract was added after the heart tube formed, the source of these secondarily added cells was not identified for nearly 25 years. In 2001, three pivotal publications described a secondary or anterior heart field that contributed to the developing outflow tract. This review details the history of the heart field, the discovery and continuing elucidation of the secondarily adding myocardial cells, and how the different populations identified in 2001 are related to the more recent lineage tracing studies that defined the first and second myocardial heart fields/lineages. Much recent work has focused on secondary heart field progenitors that give rise to the myocardium and smooth muscle at the definitive arterial pole. These progenitors are the last to be added to the arterial pole and are particularly susceptible to abnormal development, leading to conotruncal malformations in children. The major signaling pathways (Wnt, BMP, FGF8, Notch, and Shh) that control various aspects of secondary heart field progenitor behavior are discussed.

The history of the search for heart fields

The earliest cardiac fate mapping studies date back to the 1940s, when Rawles (1943) identified regions of myocardial developmental potential by grafting fragments of head-process stage chick embryos to determine which regions could generate beating tissue. This early study was the first to define a cardiogenic field, which was noteworthy in that it consisted of two broad fields that were bilateral with respect to the primitive node, and this region was defined as the area of the lateral plate mesoderm that has the potential to form myocardium.

Knowing where the cardiac potential boundaries were allowed more refined experiments that prospectively labeled and observed presumptive heart field cells. Time-lapse cinematography in chick embryos showed that the cardiogenic fields moved as a cohesive unit that retained its spatial relationship during migration between Hamburger Hamilton stage (HH) 6+(1951) to HH9–10 (Dehaan, 1963). Based on observations that no cell mixing occurred when tritium-labeled tissue fragments were grafted into unlabeled host embryos as young as HH5, Rosenquist and DeHaan (1966) hypothesized that the heart field is prepatterned. Using even smaller defined regions of the cardiac field, Stalsberg and DeHaan (1969) mapped subdivisions using radioactively labeled transplants (Fig. 1). These transplants were done in embryos in New culture at HH5, and the embryos could be followed only through HH13, after the heart tube had formed. This study confirmed both Rawles' previous observation of the bilateral heart fields and also the fact that these transplanted regions represented cohesive groups of cells that did not intermingle with unlabeled host cells.

Because all the early mapping studies were performed in New cultures, cells could only be followed for 24–36 h, ending when the heart has only recently closed dorsally to form a tube. de la Cruz and colleagues (1977) used an iron oxide marking technique *in ovo* to allow the embryos to develop longer (Fig. 1). This marking technique allowed the embryos to survive until HH35, when the four-chambered heart with two arterial trunks had formed. If the cranial-most aspect of the outflow pole was labeled at HH12, this region was incorporated into the right ventricular trabeculae by HH22. If the same marking was performed at HH22, the cranial-most outflow region was incorporated into the myocardium beneath the pulmonary semilunar valve cusps. de la Cruz postulated that these regions were formed by a secondary source of myocardium, but she did not look for this additional population.

The source of outflow tract myocardium

The question of where the outflow tract myocardium originated remained open for a number of years, mainly because mapping studies were primarily carried out in explanted embryos. As mentioned previously, developmental failure occurs in explanted embryos before the definitive outflow myocardium has been added to the heart tube. As late as 2001, avian mapping studies carefully analyzed embryos starting from HH4 to 8 (Redkar et al., 2001); however, each stage was mapped for only 20 h, thereby not addressing the source of the outflow tract myocardium (Fig. 1). In

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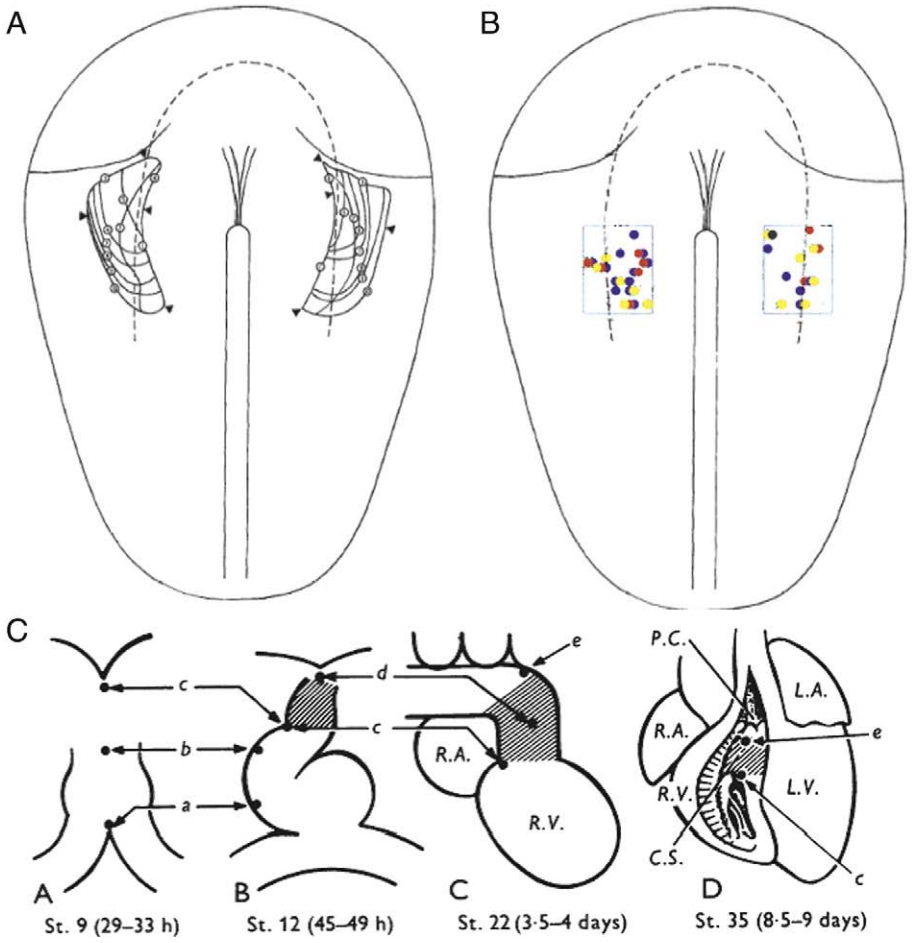

Fig. 1. The heart field at HH 5, as defined by (A) Stalsberg and DeHaan (1969) and (B) Redkar et al (2001; adapted to the Stalsberg and DeHaan schematic). While Stalsberg and DeHaan observed non-overlapping regions that gave rise to distinct components of the heart, this same organization is not observed at HH5 in the Redkar study. In (B), red dots indicated regions that gave rise to atria, yellow dots indicated regions that gave rise to the sinus venosus, blue dots correlated with ventricles, and black dots indicated regions that gave rise to bulbus arteriosus. (C) The existence of cells that contribute to the outflow tract later in development was suggested by the work of de la Cruz et al (1977). Lowercase letters indicate specific positions that were labeled and where these points end up as the heart continues to develop. Note the fact that cells labeled at the distal outflow tract as late as HH22 are below the pulmonary outflow valves at HH35. Abbreviations: R.A., right atrium; R.V., right ventricle; P.C., pulmonary semilunar valve cusps; C.S., crista supraventricularis; L.A., left atrium; L.V., left ventricle.

addition, this study concluded that there was no identifiable organization within the heart field, which remains somewhat puzzling in light of earlier and later studies.

The same year, three different groups described a population of cells that contributed to the heart after the initial heart tube had formed. Kelly et al (2001) created a fibroblast growth factor (FGF)10-nlacZ reporter mouse that showed expression in the myocardium of the right ventricle and outflow tract and in the pharyngeal mesoderm at E9.5 (Fig. 2A). DiI labeling in this reporter mouse determined that the right ventricle and the outflow tract myocardium are added from both the pharyngeal arch core and splanchnic mesoderm from E8.25 to E10.5 (Kelly et al., 2001). Both FGF10 and the nlacZ transcripts are down-regulated as these secondarily added myocardial cells are added to the heart tube, whereas β-galactosidase (β-gal) protein encoded by nlacZ is still present in these cells, supporting their origin as the FGF10-positive cells in the pharynx.

Two additional studies identified sources of the myocardial cells that contributed to the lengthening outflow tract, using chick as the model. Mjaatvedt et al (2001) labeled myocardial progenitor cells using either Mitotracker or a replication-deficient adenovirus that expressed β-gal. After labeling cells cranial to the heart tube and observing labeled cells in the outflow tract, Mjaatvedt et al defined this progenitor population as the anterior heart field. When Mjaatvedt et al ablated the bilateral heart fields as defined by Rosenquist and DeHaan (1966), the embryos only formed a rudimentary heart tube, leading to the assumption that the outflow tract progenitors were a separate population from the bilateral heart fields (Fig. 2B).

Waldo et al (2001) also used cell labeling to determine the origin of the outflow tract myocardial progenitors. After observing that heart field markers Nxk2.5 and Gata4 were expressed in the pharyngeal mesoderm caudal to the outflow tract at HH14, this region was labeled with Mitotracker. Embryos that developed to HH22 showed robust labeling in the proximal outflow tract. Interestingly, HNK1, an antibody commonly used to identify migrating cardiac neural crest cells in the chick, also labeled this population of splanchnic mesoderm, but only near the outflow tract. HNK1 was found to colocalize with the myocardial marker MF20 at the junction of the splanchnic mesoderm with the outflow tract. More discrete than either the Kelly or the Mjaatvedt study, this population was termed the secondary heart field (Fig. 2C).

Relationship of the second, anterior, and secondary heart fields

Since their initial description, the relationships between these three novel regions of myocardial progenitors and the bilateral heart fields have been extensively refined. One of the key steps in this refinement was the identification of Islet (Isl)1 as a heart field marker (Yuan and Schoenwolf, 2000). Isl1 expression begins as asymmetric

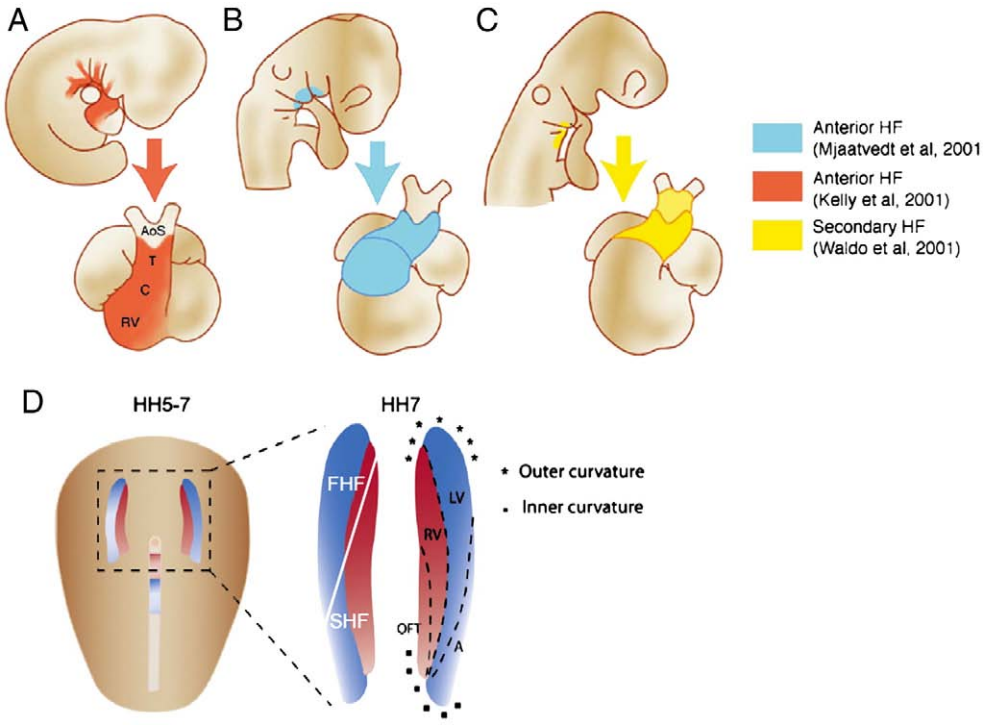

Fig. 2. The novel secondarily added cell populations identified by (A) Mjaadvedt et al., (B) Kelly et al., and (C) Waldo et al. (A) An FGF10-lacZ mouse showed expression in the right ventricle, conus, and truncus at E9.5. (B) Mitotracker-labeled cells from the pharyngeal arch cores, cranial to the heart tube, contributed to the conus and truncus. (C) DiI-labeled splanchnic mesoderm caudal to the heart tube contributed to the conus and truncus. (D) While the outflow tract (conus and truncus) appeared to form from a distinct population of cells, these progenitors are part of the bilateral heart fields. The position of the chamber progenitors is shown in the right heart field, and the approximate positions of the first and second heart fields (FHF and SHF, respectively) are shown in the left heart field. The most medial portion of the heart field at HH5–7 gives rise to the secondary heart field. Abbreviations: RV, right ventricle; LV, left ventricle; C, conus; T, truncus; AoS, aortic sac; OFT, outflow tract; A, atria. (A-C) from Abu-Issa et al (2004) with permission. (D) adapted from Abu-Issa and Kirby, 2008 with permission.

fields that encompass the rostrolateral mesoderm at HH4 (Yuan and Schoenwolf, 2000). As the initial heart tube begins to fuse at HH8–9, Isl1 expression is lost from the differentiating myocardium but remains in the specified but undifferentiated splanchnic mesoderm in chick (Yuan and Schoenwolf, 2000). Isl1 expression in the mouse also exhibits a complimentary pattern with differentiated myocardial marker MLC2a, indicating that it is only present in progenitors (Cai et al., 2003). However, lineage analysis of an Isl1-cre reporter in mouse showed Isl1-lineage-labeled myocardial cells in the right ventricle, the outflow tract, the atria, and parts of the left ventricle (Cai et al., 2003). This expression was similar to the pattern seen in the anterior heart field defined in the FGF10-nlacZ mouse for the right ventricle and outflow tract but with the addition of the atria, which were not included in the definition of the anterior heart field (Kelly et al., 2001). Knockout of Isl1 yielded a heart tube roughly lacking the regions where it was traced using the Isl1-cre (Cai et al., 2003). The expression pattern and phenotype of the Isl1-cre and Isl1-null mice suggested that there was both a "primary" heart field that formed the initial heart tube and a "second" heart field that produced myocardium contributing to both the arterial and venous poles of the heart tube.

Another key step in understanding the myocardial progenitor fields used clonal analysis of myocardial specific (cardiac actin promoter driven) nlaacZ expression. nlaacZ only infrequently undergoes intragenic recombination to form positive nlacZ clones (Meilhac et al., 2003). By retrospectively analyzing the nlacZ expression patterns, Meilhac et al showed that there were two distinct cardiac lineages separated by time (Meilhac et al., 2003, 2004). These lineages were referred to as the first and second lineages. Cells of the primitive left ventricle were completely derived from the first lineage, while cells of the distal outflow tract were derived from the second lineage. The two lineages were mixed in all the other chambers. Although these cells cannot be identified spatially in the heart field, the idea of an early differentiating population of myocardial cells fit well with the Isl1-cre mapping and expression studies, thus reinforcing the idea of a first and second heart field.

Using markers of the second heart field, Prall et al (2007) showed that one problem in the Nkx2.5-null mouse was that the second heart field, which should remain as progenitors as the first heart field differentiates, differentiated with the first heart field to form the initial cardiac tube, leaving no progenitors for subsequent addition. Nkx2.5 has multiple cis-regulatory elements; while one of the elements drives expression in the entire heart, two elements are specific for the right ventricle and outflow tract (Schwartz and Olson, 1999). These additional elements suggest that there are other regulatory networks that maintain Nkx2.5 expression in the regions of myocardial progenitors that are added after the linear heart tube has formed. In fact, Nkx2.5 inhibits bone morphogenic protein (BMP) signaling by inhibiting Smad1, which holds the second lineage myocardial cells in a proliferative state as the first heart field/lineage differentiates (Prall et al., 2007).

Recent analysis using both Nkx2.5 and Isl1 cre mice have shown that these genes have similar expression domains throughout the heart field in the lateral plate mesoderm (Ma et al., 2008) and that the difference between the first and second heart field progenitors or lineages is in the timing of differentiation because both express both transcription factors. Isl1 expression is generally limited to the progenitor population, and an Isl1-lacZ knock-in has shown that nearly all heart cells are Isl1 derivatives (Sun et al., 2007). In fact, this study highlights problems with the commonly used cre recombination technique: not all recombination occurs with the same efficiency (Ma et al., 2008; Vooijs et al., 2001). Isl1 expression is extinguished as the cells begin myocardial differentiation (with some exceptions; see, for example, Sun et al., 2007), while Nkx2.5 is expressed in progenitors, and expression is maintained at a somewhat lower

level as the cells begin to differentiate (Komuro and Izumo, 1993; Lints et al., 1993; Prall et al., 2007).

A new fate map of the cardiogenic fields in the chick finally revealed that the first and second heart fields are contiguous in the lateral plate mesoderm (Abu-Issa and Kirby, 2008). Parts of the cardiogenic fields were marked with fluorescent vital dyes, DiI or DiO, and embryos were allowed to develop to HH22. This new map elaborates on and refines the Stalsberg and DeHaan map (Stalsberg and DeHaan, 1969), suggesting why the Mjaavedt study found rudimentary heart tubes after their ablations. This new map clearly shows that the myocardial progenitors in the second heart field/lineage are located in the medial portion of the Nkx2.5/Isl1-positive field, supporting a single heart field that is spatiotemporally defined (Fig. 2D). Furthermore, the secondary heart field gives rise to the last myocardium to be added to the outflow tract and is located most caudally and medially in the heart field. Furthermore, the model shows how myocardial cells from the outflow and inflow portions of the heart can arise from contiguous regions of the heart field (Abu-Issa and Kirby, 2008).

While this unified heart field model clearly explains how a single (bilateral) cardiogenic field forms the heart, the terms *first heart field*, *second heart field*, *anterior heart field*, and *secondary heart field* are still particularly useful for describing distinct, well-defined populations of cells that are added at various times to the heart tube. The *first heart field* refers specifically to the first wave of mesodermal cells that differentiate to form the initial heart tube and express muscle-specific proteins such as MLC3F (Kelly et al., 1997); these cells are the first to down-regulate Isl1 expression (Prall et al., 2007) and express differentiation markers (Saga et al., 2000; Saga et al., 1999). The *second heart field* consists of the progenitors that are added to the formed heart tube at either the arterial or venous pole. The *anterior heart field* refers to the region defined by FGF10-lacZ expression that is restricted to the right ventricle and outflow tract myocardium progenitors (Kelly et al., 2001). A Mef2C promoter cre was initially reported to be an anterior heart field cre. This distinction is somewhat confused by recent reports that it is also expressed in some atrial progenitors, specifically the dorsal mesocardium, dorsal mesocardial protrusion, and the muscular atrial septa (Goddeeris et al., 2008; Verzi et al., 2005), suggesting that it is not specifically an anterior heart field marker. The *secondary heart field* refers specifically to the splanchnic mesoderm caudal to the outflow tract that gives rise to the most distal outflow tract myocardium and the most proximal smooth muscle that forms the tunica media of the arterial trunks as they leave the heart (Waldo et al., 2005b; Waldo et al., 2001); this specific region where the myocardium of the ventricular outflow meets the smooth muscle of the great arteries at the semilunar valves is the definitive arterial pole.

Using 3D reconstructions coupled with BrdU labeling to assess proliferation in the cardiogenic progenitor pool, van den Berg et al (2009) generated heat maps of proliferation in cardiogenic progenitors and myocardium in the chick. These maps showed that newly differentiated myocardial cells cease proliferation and that the cardiogenic progenitors at HH9–14 proliferate at very high rates. At HH9, the highly proliferative region is lateral to the inflow pole, and cells that are labeled in this region move either into the inflow pole or into the splanchnic mesoderm. At HH14, the splanchnic mesoderm between the inflow and outflow pole attachments to the body, corresponding to the *secondary heart field,* represents a particularly "hot spot" of proliferation (van den Berg et al., 2009). Tracing experiments showed that cells from this field are added only to the arterial pole (Ward et al., 2005), and injection of this region with a cell cycle-blocking drug causes malformations limited to the arterial pole (Hutson and Kirby, unpublished).

Significance of the secondary heart field

Conotruncal malformations in children generally involve the outlet myocardium and not the right ventricle. Hence, the progenitors that form the definitive arterial pole seem to be the most susceptible to insult during development. These progenitors are the last to be added from the secondary heart field. *In ovo* DiI-labeling showed that the secondary heart field contributes both myocardium and smooth muscle to the arterial pole (Waldo et al., 2005b). The myocardium spirals into the developing outflow tract, such that the right side of the secondary heart field ends up as the subpulmonary myocardium. Because the chick lies with its right side up, only the right side of the secondary heart field is accessible with any degree of confidence, and it was speculated that the left side provides the subaortic myocardium, although this result has never been shown experimentally (Ward et al., 2005) (Fig. 3). Myocardium is contributed from the secondary heart field progenitors over a 24-h period in chick, from HH14 to HH18. At HH18, secondary heart field progenitors continue to be incorporated into the arterial pole but as smooth muscle instead of myocardium. Interestingly, the spiral pattern that is observed during the addition of myocardium is not observed when the smooth muscle is added. As a result, the right side of the secondary heart field generates smooth muscle that incorporates into the aortic wall and surrounds the coronary stems (Sun et al., 2007; Waldo et al., 2005b), while the left side contributes smooth muscle to the pulmonary trunk. These cell tracings were confirmed by ablation of the right side of the secondary heart field (Ward et al., 2005). If the ablation was done at HH14, pulmonary stenosis or atresia and coronary artery defects were observed, indicating that both the subpulmonary myocardium and aortic smooth muscle were affected. If the ablation was performed at HH18, only coronary artery defects were observed because, at this stage, the right side of the secondary heart field provided only the smooth muscle to the base of the aorta.

As the secondary heart field-derived myocardium is added to the outflow tract, the outflow tract lengthens. This added length is necessary to allow the outflow tract to rotate sufficiently for correct alignment of the pulmonary and aortic arterial trunks with their respective ventricles (Yelbuz et al., 2002). Ablating the right secondary heart field, thereby reducing the secondary heart field population, resulted in misalignment of the aorta as well as pulmonary stenosis or atresia (Ward et al., 2005). The type of atresia produced was unlike commonly reported pulmonary atresias, which are diagnosed in humans by the presence of a strand of the remaining pulmonary trunk or an atretic pulmonary semilunar valve. In the case of right secondary heart field ablation, the pulmonary atresia was produced by failure of addition of the subpulmonary myocardium, leading to a defect that appeared similar to common trunk. However, further studies have shown that the outlet septum, which is formed by

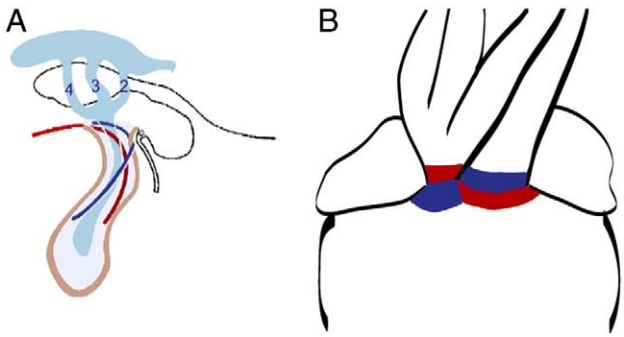

Fig. 3. The secondary heart field spirals as it begins migration. (A) During the addition of myocardium, progenitors from the right side of the secondary heart field spirals caudal and to the left of the outflow tract (depicted in red). The left side of the secondary heart field is speculated to spiral cranial and to the right of the outflow tract (depicted in blue) (from Ward et al., 2005, with permission). This pattern is only maintained during addition of myocardium; smooth muscle is added without spiraling. (B) Because of the initial spiral, progenitors from the right side of the secondary heart field become the subpulmonary myocardium and the aortic smooth muscle (shown in red). The left side of the secondary heart field thus is assumed to provides subaortic myocardium and smooth muscle to the base of the pulmonary artery (shown in blue).

cardiac neural crest cells, still forms even though the subpulmonary myocardium is reduced (Dyer and Kirby, 2009).

While an important role for cardiac neural crest is septation of the outflow tract (Kirby et al., 1983), neural crest cells also modulate FGF8 signaling in the pharynx during the time that the secondary heart field contributes myocardium to the outflow tract (Waldo et al., 2005a). Thus, ablating the cardiac neural crest leads to elevated FGF8 signaling associated with excessive proliferation of secondary heart field progenitors and failure of the myocardial cells to be added to the heart. These defects result in malalignment of the arterial trunk, which is a common trunk in this case because the cardiac neural crest cells do not reach and septate the outflow tract (Kirby et al., 1983).

The subpulmonary and subaortic myocardium can be identified molecularly. Recent work has shown that the subpulmonic myocardium expresses the transgene 96–16, which was identified as semaphorin 3C (Bajolle et al., 2006; Theveniau-Ruissy et al., 2008). In a complementary pattern, the sub-aortic myocardium expresses the transgene T55, recently identified as Hes1, a transcriptional repressor in the Notch signaling family (Bajolle et al., 2008; Rochais et al., 2009). Both of these genes are asymmetrically expressed as early as E9.5 (Bajolle et al., 2008). These expression patterns suggest that intrinsic differences in the myocardium may determine the placement of the outflow septum and, thus, may direct cardiac crest cells into the outflow cushions.

Signaling and the secondary heart field

In order to proceed from unspecified mesoderm to cardiogenic mesoderm to myocardium and smooth muscle, the second heart field receives signals that specify it and then orchestrate differentiation. Initially, a portion of the lateral plate mesoderm is induced, establishing its fate as cardiogenic mesoderm. However, the second heart field is held as specified but undifferentiated cardiogenic mesoderm as the first heart field forms the initial heart tube and differentiates into contractile myocardium and most of the other cells from the second heart field are added to the elongating tube. Throughout the early stages of its development, the secondary heart field is adjacent to the pharyngeal endoderm, a rich source of signals. As the secondary heart field is added to the outflow tract, it is exposed to a new set of signals that result in its differentiation into either myocardium or smooth muscle.

Wnt signaling

The Wnt family is the earliest signaling family that affects cardiac induction, and there are three Wnt signaling pathways: canonical, non-canonical (also known as the planar cell polarity pathway), and Wnt/calcium (Kuhl et al., 2000; Wodarz and Nusse, 1998). Of these pathways, both the canonical and non-canonical pathways have clear roles in early heart development.

In the canonical pathway, extracellular Wnt binds the transmembrane receptor Frizzled and LRP5/6 to stabilize cytoplasmic β-catenin through Disheveled; stabilized β-catenin can then translocate to the nucleus, bind TCF/LEF, and induce transcription of downstream targets (Habas and Dawid, 2005). In *Xenopus*, canonical Wnts 3A and 8 inhibit cardiac induction in ventral marginal zone mesoderm explants, which are capable of forming beating heart tubes in the presence of the Spemann organizer and the cranial endoderm (Schneider and Mercola, 2001). Antagonizing the canonical Wnt signal by Dkk-1 or Crescent is necessary for the expression of cardiogenic transcription factors Nkx2.5 and Tbx5 as well as myocardial-specific proteins troponin-I and myosin heavy chain-α. Overexpression of either Wnt3A or Wnt8 inhibits myocardiogenesis (Schneider and Mercola, 2001). Interestingly, canonical Wnt repression is not an isolated requirement. Additional *Xenopus* experiments have shown that not only does Wnt signaling need to be downregulated to allow cardiac induction to occur, the signaling must be downregulated specifically in regions of high BMP activity (Marvin et al., 2001).

After the heart tube has formed, canonical Wnt signaling is maintained in the secondary heart field. β-catenin is needed in Isl1-expressing secondary heart field progenitors; genetic ablation results in pharyngeal arch remodeling defects, a single outflow vessel, and embryonic lethality (Lin et al., 2007). These defects indicate that the myocardial contribution from the secondary heart field has been disrupted; however, whether these mice also have smooth muscle defects is unclear. Loss of Wnt signaling reduces the number of Isl1-positive cells, leading to outflow tract and right ventricular defects, whereas excess Wnt signaling expands the Isl1-positive population (Cohen et al., 2007). Interestingly, when β-catenin is knocked out under the control of the Mesp1 promoter, proliferation in the splanchnic mesoderm is decreased at E8.0, followed by a decrease in Isl1 expression at E8.5 (Klaus et al., 2007). If it is knocked out under control of the Mef2c promoter, cell cycle gene cyclin D1 expression is lost in the right ventricle and outflow tract; not surprisingly, both of these regions are reduced in size compared to wild-type mice (Ai et al., 2007). Overexpression under the Mesp1 promoter inhibits heart tube formation and increases both Isl1 expression and first heart field proliferation (Klaus et al., 2007). Together, these data suggest that canonical Wnt signaling is responsible for promoting proliferation and maintaining cells in a progenitor state. However, β-catenin induces both BMP4 (Klaus et al., 2007) and non-canonical Wnt11 (Lin et al., 2007) expression in the secondary heart field, which also suggests that the early canonical Wnt signaling sets the stage for differentiation.

While the canonical Wnts are important for their ability to inhibit the initial cardiac induction, the non-canonical Wnts promote cardiac differentiation. Like the canonical pathway, non-canonical Wnts also bind to Frizzled but in the absence of LRP5/6; signaling proceeds through Disheveled, which modifies actin via the Rho/ROCK and Rac/JNK pathways (Habas and Dawid, 2005). Non-canonical Wnt5A, in combination with canonical Wnt inhibitor Dkk-1, induces cardiac differentiation in stromal vascular cells (Palpant et al., 2007). In addition, Wnt11 also induces cardiac differentiation in *Xenopus* (Pandur et al., 2002), and Wnt11-null mice have both arch artery patterning and outflow tract defects (Zhou et al., 2007). However, Isl1 expression appears normal in these mice (Zhou et al., 2007), suggesting that the initial specification of the secondary heart field occurs correctly. This expression pattern supports the idea that non-canonical Wnt signaling is important not for the early steps of induction or specification but for later differentiation.

In summary, canonical Wnt signaling inhibits cardiac induction and maintains the secondary heart field in an undifferentiated state. Its inhibition is essential for myocardial differentiation. Non-canonical Wnt signaling, on the other hand, promotes cardiac differentiation and is specifically needed for outflow tract development.

TGFβ superfamily

In heart progenitors, TGFβ superfamily signaling occurs primarily through BMPs. BMPs can bind both type I receptors, such as BMP receptor (BMPR) 1A (also known as ALK-3), and type II receptors, such as BMPR2 (van Wijk et al., 2007). The type I receptors phosphorylate SMAD1, 5, and 8 to induce intracellular signaling, and these activated SMADs bind the common binding partner SMAD4 (Chen et al., 2009). Surprisingly, mice lacking SMAD4 in the epiblast form rudimentary hearts but die by E8.5, suggesting that SMAD4-mediated BMP signaling is not required for differentiation of the initial heart tube myocardium (Chu et al., 2004) or that SMAD4-mediated BMP signaling happens earlier than the gene knockout occurred in these embryos. SMAD4-independent TGFβ signaling has been observed in other cell types, such as T cell activation and cancer cell migration (reviewed in Bommireddy and Doetschman, 2007; Giehl et

al., 2007), supporting the feasibility of the first hypothesis. Two additional SMADs–SMAD6 and SMAD7–are inhibitory; knocking out either of these two genes results in outflow tract defects (Chen et al., 2009; Galvin et al., 2000).

Early in development, BMPs induce the myocardial gene program after canonical Wnt signaling is repressed (Schultheiss et al., 1997). In the chick, BMP4 and 7 are expressed in the ectoderm, while BMP2 is expressed in the endoderm (Schultheiss et al., 1997); in mouse, however, BMPs 2, 4, 5, and 7 are present in the cardiogenic mesoderm (Dudley and Robertson, 1997; Solloway and Robertson, 1999; Zhang and Evans, 1996). Regardless of location, though, BMPs are responsible for inducing cardiac differentiation. BMP2 and BMP4 induce Nkx2.5, Gata4, and ventricular myosin heavy chain expression (Monzen et al., 1999; Schultheiss et al., 1997; Shi et al., 2000), and BMP7 can induce Nxk2.5 in the mesoderm cranial to the cardiogenic mesoderm (Schultheiss et al., 1997). These effects can be inhibited by applying the BMP inhibitor noggin (Schneider and Mercola, 2001; Schultheiss et al., 1997). While BMP2 induces ectopic cardiogenesis in cranial mesoderm, which has been exposed to Wnt signaling, it has no effect on caudal mesoderm (Schultheiss et al., 1997). These effects are likely via binding to type II receptors ALK3 and BMPR2, which are required to maintain Nkx2.5 expression in *Xenopus* (Shi et al., 2000). Because BMP has such a clear role in inducing myocardial differentiation and its expression is in the most lateral mesendoderm in the chick, Brand (2003) accurately predicted that the secondary heart field would lie medial to the primary heart field at these early stages, where it would be protected from being exposed to too much BMP ligand.

After specification, BMP signaling is still necessary for secondary heart field progenitors to differentiate as myocardium. In HH16 chick, BMP2 and BMP4 are both expressed where the outflow tract joins the body wall; in addition, BMP7 is expressed throughout the entire heart (Somi et al., 2004). In culture, chick secondary heart field explants differentiate into myocardium upon exposure to BMP2 (Waldo et al., 2001). Thus, to progress from lateral plate mesoderm to myocardium, both the first and second heart fields must down-regulate canonical Wnt signaling, receive high levels of BMP signaling, and up-regulate non-canonical Wnt signaling. In the secondary heart field, this process is delayed over an extended period to allow the progenitor cell proliferation.

Sonic hedgehog

Sonic hedgehog (Shh) is a secreted, cholesterol-modified protein that binds to transmembrane receptor Patched. Upon binding, Shh and Patched are internalized, which allows the transmembrane protein Smoothened to move into the cilium, where it activates the Gli proteins, allowing them to translocate into the nucleus and induce transcription of downstream targets (Rohatgi et al., 2007).

In the original Shh-null mouse, heart defects were noted. However, these defects were limited in description to “abnormalities” (Chiang et al., 1996). Ectopically expressed Shh could induce heart looping defects in the zebrafish (Schilling et al., 1999). Knocking out Shh, however, has not been shown to cause heart looping problems. The Shh-null mouse was later characterized as having a phenotype comparable to tetralogy of Fallot with complete pulmonary atresia (Washington Smoak et al., 2005), indicating that there may be a sided effect on the secondary heart field. A conditional knockout removing Shh from Nxk2.5-expressing cells also produced a single outflow tract, and cardiac neural crest-derived cells died in the pharyngeal arches (Goddeeris et al., 2007). While this study determined that Shh in the Nkx2.5-expressing domain, specifically, was required for arterial pole development, it did not elucidate the mechanism through which Shh causes arterial pole defects. Both of these studies indicate a crucial role for Shh in arterial pole development and suggest that it is of particular importance to the secondary heart field.

Recent work has shown that Sonic hedgehog is one of the factors that maintains proliferation in secondary heart field progenitors (Dyer and Kirby, 2009). This proliferation is essential to generate adequate myocardium and smooth muscle to form the arterial pole. Interestingly, the time frame in which proliferation is essential is quite narrow: in the chick, peak proliferation in the secondary heart field occurs between HH15 and 17. By the time the myocardium has been added to the outflow tract, proliferation has decreased in this region (Dyer and Kirby, 2009). Another critical point is that pulmonary atresia is the predominant defect that occurs in the absence of hedgehog signaling, and this defect occurs despite the presence of a neural crest-derived outflow tract septum. Because Shh has earlier roles in right-left asymmetry, it is unsurprising that Shh appears to affect one side of the secondary heart field (the right side) more than the other.

Fibroblast growth factor

A member of the receptor tyrosine kinase signaling pathway, FGF8 has a number of roles throughout heart development. In the early chick, it is expressed in the endoderm adjacent to precardiac mesoderm, and ectopic FGF8 expression induces myocardial gene expression in regions that have been exposed to BMP2 (Alsan and Schultheiss, 2002). If the endoderm is removed, FGF8 alone is necessary to induce Nkx2.5 and Mef2c expression in the cardiogenic mesoderm (Alsan and Schultheiss, 2002). The window of time in which the endoderm can induce differentiation is narrow; it actively signals between HH4 and 6, and by HH7, it can be removed without consequence (Gannon and Bader, 1995). Based on this early role in differentiation, it is not surprising that FGF8-null mice have early heart looping abnormalities (Meyers and Martin, 1999).

Despite the early abnormalities seen in FGF8-null mice, though, an FGF8 hypomorph does not have early lethal heart defects. Instead, the hypomorph exhibits double outlet right ventricle, persistent truncus arteriosus, and disrupted pharyngeal arch artery repatterning (Abu-Issa et al., 2002). These two FGF8-mutant mice suggest that the heart is sensitive to FGF8 dosage. Cardiac neural crest-derived cells, which septate the outflow tract, die as they leave the neural tube and in the pharyngeal arches, explaining both the repatterning defect and persistent truncus arteriosus (Abu-Issa et al., 2002). If FGF8 is conditionally ablated from Nxk2.5-expressing regions, atrial natriuretic factor (ANF) and myosin light chain 2v (Mlc2v) expression are expanded into the abnormal, shortened outflow tract, and BMP4 expression is lost from the outflow tract (Ilagan et al., 2006). Conditional ablation of FGF receptors or FRS2α (FRS2), an adaptor protein that links FGF receptor kinases to multiple signaling pathways, resulted in truncated outflow development that may have restricted the addition of the more proximal outflow myocardium because the outflow cushions fail to form in these mutants (Park et al., 2008; Zhang et al., 2008). Together, these data suggest that FGF signaling is important for addition of outflow tract myocardium from the secondary heart field and possibly pharyngeal arch core mesoderm.

The FGF8 that is present in the endoderm is regulated by cardiac neural crest-derived cells (Hutson et al., 2006). If the cardiac neural crest is ablated prior to migration into the pharynx, FGF8 signaling is up-regulated in the pharynx (Hutson et al., 2006). This up-regulation coincides with a failure of the secondary heart field to add myocardium to the outflow tract (Waldo et al., 2005a). These experiments indicate that the cardiac neural crest, in addition to forming the outflow tract septum, also has an indirect effect on the secondary heart field and thus outflow myocardium development, and that the FGF8 pathway is a common thread between the two populations.

Notch signaling

The Notch pathway is unique in that both the ligand and the receptor are membrane-bound necessitating proximity of signaling

and signal-receiving cells. This pathway is often involved in cell fate decisions. When Notch receptor binds one of its ligands, which include Delta, Serrate, and Jagged (depending on species), the receptor undergoes a series of proteolytic cleavages that release the Notch intracellular domain (NICD). The NICD can translocate into the nucleus and act as a transcriptional activator (reviewed in Mumm and Kopan, 2000).

Notch1 (known earlier as Motch or the mouse analog of Notch), is not observed in the mouse until E7.0 or mid-streak stage (Reaume et al., 1992), whereas mesodermal markers MESP1 and MESP2 are already expressed by primitive-streak stage (between E6.5 and E7.0) (reviewed in Buckingham et al., 2005). The Notch1-null mouse forms a heart tube but suffers severe pericardial effusion, indicating heart failure (Swiatek et al., 1994). These mice are indistinguishable from wild-type littermates through E8.5 but are severely growth arrested beyond this point and die between E9.5 and E10.5 (Swiatek et al., 1994). Embryonic death in Notch1-null embryos is not attributable to apoptosis, as no increase or change in apoptotic patterns are observed (Conlon et al., 1995). Recent work has shown that Notch1 negatively regulates β-catenin and that the Notch1-null mouse has an expanded progenitor pool that fails to differentiate (Kwon et al., 2009). The Notch2-null mouse is also embryonic lethal, with reduced myocardium and pericardial edema (McCright et al., 2001). Interestingly, Notch2 mRNA is observed in only the outflow tract between E11 and E14 (McCright et al., 2001), suggesting that it is maintained in the last cells that are added to the arterial pole. Cardiac precursors can differentiate in *Xenopus* without Notch signaling (Rones et al., 2000). In addition, this effect is downstream of Nkx2.5 and Gata4, suggesting that Notch's role is to keep these specified cardiac precursors in an undifferentiated state (Rones et al., 2000).

Recent work has reported conditional Notch knockouts using both the Isl1 and Mef2c cres (High et al., 2009). This restricted deletion allows the animals to survive as late as postnatal day 1. Unsurprisingly, knocking out Notch signaling earlier, using the Isl1 cre, results in a higher prevalence of more severe outflow tract and right ventricular defects. However, many of these mice exhibited arch artery remodeling defects and subsequent aortic arch defects, consistent with cardiac neural crest defects and again highlighting the relationship between the secondary heart field and the cardiac neural crest.

Conclusions

Understanding the secondary heart field and how it contributes to arterial pole development involves a set of complex, interconnected signaling pathways. In addition to being specified as cardiogenic but also being able to differentiate into at least two cell types, it must also be restricted from differentiating too early. Failure to maintain an undifferentiated secondary heart field progenitor population causes these cells to be added prematurely to the heart tube. In this case, proliferation is not maintained long enough to generate adequate progenitors to build a normal arterial pole. Reduced migration or abnormal proliferation in the secondary heart field leads to outflow tract defects. The cell signaling coordination required to balance proliferation, migration, and subsequent differentiation is complicated and leaves room for many errors. Over time, we will come to appreciate even more how these pathways are woven together and how the heart can compensate for imbalances in a single pathway.

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