Signaling Pathways Controlling Second Heart Field Development

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Abstract—Insight into the mechanisms underlying congenital heart defects and the use of stem cells for cardiac repair are major research goals in cardiovascular biology. In the early embryo, progenitor cells in pharyngeal mesoderm contribute to the rapid growth of the heart tube during looping morphogenesis. These progenitor cells constitute the second heart field (SHF) and were first identified in 2001. Direct or indirect perturbation of SHF addition to the heart results in congenital heart defects, including arterial pole alignment defects. Over the last 3 years, a number of studies have identified key intercellular signaling pathways that control the proliferation and deployment of SHF progenitor cells. Here, we review data concerning Wnt, fibroblast growth factor, bone morphogenetic protein, Hedgehog, and retinoic acid signaling that have begun to identify the ligand sources and responding cell types controlling SHF development. These studies have revealed the importance of signals from pharyngeal mesoderm itself, as well as critical inputs from adjacent pharyngeal epithelia and neural crest cells. Proliferation is emerging as a central checkpoint in the regulation of SHF development. Together, these studies contribute to defining the niche of cardiac progenitor cells in the early embryo, and we discuss the implications of these findings for the regulation of resident stem cell populations in the fetal and postnatal heart. Characterization of signals that maintain, expand, and regulate the differentiation of cardiac progenitor cells is essential for understanding both the etiology of congenital heart defects and the biomedical application of stem cell populations for cardiac repair. (Circ Res. 2009;104:933-942.)

Key Words: heart development ■ intercellular signaling ■ progenitor cells

Congenital heart defects occur in almost 1% of live births, reflecting the complex cellular processes underlying heart development.1 In the early embryo, the heart tube elongates by addition of myocardium from progenitor cells lying outside the heart itself. The source of the new myocardium is a recently identified progenitor cell population situated in pharyngeal mesoderm termed the second heart field (SHF).2–4 Direct or indirect perturbation of addition of SHF-derived cells to the heart tube results in congenital heart defects. Defining properties of the SHF include elevated proliferation and differentiation delay with respect to cells that contribute to the linear heart tube. In this review, we briefly introduce the SHF and then focus on recent data dissecting the intercellular signaling pathways that control SHF deployment. A central element in this process concerns the regulation of proliferation in pharyngeal mesoderm dorsal to the elongating heart tube. Detailed understanding of the signals that control SHF development in the early embryo will provide insights into the regulation of resident progenitor cells in the later heart and the directed differentiation of stem cells toward a cardiac fate.

Properties of the SHF

Three articles published in 2001 highlighted the previously known but poorly appreciated observation that the heart tube elongates by addition of cells to the arterial pole and furthermore identified the origin of these cells in mesoderm contiguous and dorsal to the heart tube.5–7 Gene expression, Cre lineage and retrospective clonal analysis studies revealed that these pharyngeal mesodermal progenitor cells, now termed the SHF, are situated medially to the primary or first heart field (FHF) that gives rise to the linear heart tube and are distinguished by the expression of transcription factors Isl1 and Tbx1 and the growth factors Fgf8 and Fgf10.5,8–11 The SHF contains multipotent cardiac progenitor cells that can give rise to myocardial, endocardial and smooth muscle cells12,13 and is characterized by elevated proliferation and differentiation delay with respect to the FHF.14 Whereas the FHF contributes to the left ventricle, right ventricle, and inflow region of the heart, the SHF contributes to the outflow tract (OFT), right ventricle, and inflow region; the left ventricle and OFT are therefore exclusive derivatives of the FHF and SHF, respectively.2,4
Despite these differences, the FHF and SHF are contiguous in the early embryo, and recent studies suggest that FHF cells transiently express genes that continue to be expressed in the SHF, including Isil and Fgfl8.15,16 The term SHF is thus a working definition of those cardiac progenitor cells in pharyngeal mesoderm that contribute progressively to the growth of the heart after the linear heart tube stage, reflecting both the position of progenitor cells in the early embryo and that of their descendants in the definitive heart.17 Here, we focus on the contribution of SHF cells to the cardiac OFT. Maximal extension of the OFT is essential for correct alignment of the base of the aorta with the left ventricle during cardiac septation.18 Direct or indirect perturbation of SHF deployment therefore leads to a spectrum of conotruncal congenital heart defects, ranging from failure of heart tube extension to arterial pole alignment defects observed in human patients, including double outlet right ventricle, overriding aorta, and tetralogy of Fallot. Anomalies in OFT development underlie 30% of human congenital heart defects.19 SHF progenitor cells may also contribute to later growth of the heart, because a small number of multipotent Isl1 progenitor cells are present in the fetal and early postnatal heart.12,20 Understanding the regulatory events that control SHF deployment therefore is a major goal of research into both the etiology of congenital heart defects and the application of stem cells for cardiac repair. This imperative is reflected in the rapid expansion in knowledge concerning gene regulatory networks and intercellular signaling pathways controlling SHF development.

As SHF cells contribute to the heart, they are exposed to a series of stage-dependent signals from surrounding cell types, including pharyngeal ectoderm and endoderm, as well as autocrine signals from pharyngeal mesoderm (Figure 1A and 1B). Neural crest cells further modulate signaling and impact on SHF development as they enter the pharyngeal region (Figure 1C through 1F). These signaling events have been shown by a series of genetic studies to play critical roles in SHF deployment. New data concerning the roles of fibroblast growth factor (FGF), canonical and noncanonical Wnt, bone morphogenetic protein (Bmp), Hedgehog (Hh), and retinoic acid signaling pathways during SHF development provide major insights into how SHF cardiac progenitor cells are regulated and, in particular, into how proliferation in the SHF is controlled during heart tube elongation. These studies, many of which use conditional mutagenesis in the mouse to circumvent early requirements for these signaling pathways, have begun to define the time and place of signal emission and reception during SHF development and thus contribute to defining the SHF progenitor cell niche. Detailed reviews of the SHF and gene regulatory networks involved in SHF development have been discussed elsewhere,2,4,21 as have details of the individual signaling pathways summarized in Figure 2.

Wnt/β-Catenin Signaling Regulates SHF Proliferation

Control of cardiac progenitor cell proliferation is emerging as a central feature of SHF development. A series of recent articles have demonstrated a critical upstream role for β-catenin, intracellular mediator of the canonical Wnt pathway, in this process. Signaling by secreted Wnt glycoproteins plays a pivotal role in the regulation of diverse stem cell populations22 and has been previously shown to have stage-dependent positive and negative effects on early mesoderm development and cardiac specification and differentiation (reviewed elsewhere23–25). Studies in embryonic stem cells have revealed a requirement for Wnt signals in early mesoderm development, yet, subsequently, Wnt signaling restricts cardiac differentiation to lateral splanchnic mesoderm.26–29 Active Wnt/β-catenin signaling is observed in the SHF and OFT.29–32 By deleting β-catenin specifically in cardiac mesoderm using a series of Cre lines it has now been shown that canonical Wnt signaling plays an important upstream role in positively regulating SHF proliferation, without affecting development of the linear heart tube.30–34 Right ventricular and OFT hypoplasia are observed in β-catenin conditional mutant embryos (Figure 3A and 3B). Transcriptional down-regulation of genes encoding Isl1, Bmp4, Fgf10, Shh and the noncanonical Wnt ligand Wnt11, suggests that canonical Wnt signaling acts upstream of other signaling pathways to control progenitor cell proliferation and differentiation. β-Catenin has been shown to directly activate transcription from the promoters of Isl1 and Fgf10.30,32 In addition to Fgf10, other Fgf ligand encoding genes, including Fgf3, Fgf16, and Fgf20 are downregulated in conditional β-catenin mutant embryos and reduced levels of phosphorylated extracellular signal-
regulated kinase (p-ERK), an intracellular target of FGF signaling, were observed in the SHF and OFT. Consistent with this upstream role in SHF development, activation of β-catenin by LiCl treatment or conditional expression of a gain-of-function mutation using the same Cre lines results in expansion of SHF progenitor cells and a greater number of Isl1-positive cells in the OFT, revealing that downregulation of Wnt/β-catenin signaling is required for differentiation (Figure 3C and 3D). Wnt3a has been shown to promote SHF proliferation in explants and Isl1-expressing cells in culture, yet the Wnt ligands required for normal SHF proliferation in the embryo remain unidentified. The expression profiles of Wnt2a, Wnt2b, and Wnt8 suggest that these genes are candidates for autocrine signaling during SHF and OFT development. It is important to note that β-catenin plays additional roles in cell-cell adhesion, complicating

Figure 2. Illustration showing the core features of the Wnt, Fgf, Bmp, Hh, and Notch signaling pathways. This simplified schema summarizes the key steps from ligand binding to target gene transcription for each pathway. Wnt/β-catenin signaling: binding of Wnt ligands to the Frizzled-LRP5/6 receptor complex allows the activation of the intracellular effector Disheveled (DSH), which results in the uncoupling of β-catenin from a multiprotein degradation complex composed of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase (GSK)β. β-Catenin then translocates to the nucleus and, in association with Lef/Tcf, activates target gene transcription. β-Catenin also exists in a cadherin bound form and regulates cell–cell adhesion. Wnt ligand binding can be blocked by binding to secreted frizzled-related molecules (FRZB). Noncanonical Wnt signaling: at least 2 divergent signaling pathways are involved in noncanonical Wnt signaling downstream of DSH. Activated DSH promotes small G protein (Rac and Rho) activation, leading to the activation of c-Jun N-terminal kinase (JNK) and Rho-associated kinase (ROCK). Ultimately, the ATF/CREB complex is activated and results in target gene transcription. The second pathway, independent of DSH, results in intracellular Ca²⁺ release and activation of the Ca²⁺/calmodulin-dependent kinase 2 (CamKII) and the protein kinase (PK)C pathways. Fgf signaling: binding of Fgf ligands leads to the auto-phosphorylation of the Fgf receptor tyrosine kinase, allowing interaction of the FRS2α docking protein and subsequent activation of the GRB2/SOS (growth factor receptor-bound protein 2/Son of sevenless) complex. Activated SOS, a guanine nucleotide exchange factor, in turn, activates the small G protein RAS, triggering a cascade of phosphorylation leading to the successive activation of RAF, MEK, and mitogen-activated protein kinase (ERK) kinases. p-ERK phosphorylates target transcription factors (including Ets family members) to activate gene expression. Sprouty blocks the phosphorylation cascade by interacting with the FRS2α/GRB2/SOS complex. Bmp signaling: Bmp ligands belong to the transforming growth factor (TGF)β family of signaling molecules and bind to a receptor complex composed of type 1 and type 2 Bmp receptors. On receptor activation, Smad proteins (Smad1/5/8) are phosphorylated and associate with a coactivator Smad (Smad 4). The resulting Smad complex enters the nucleus and activates target gene expression. Bmp signaling can be inhibited by the secreted proteins Noggin (NOG) and Chordin (CHORD) and intracellular Smad proteins (Smad 6/7). Ihh signaling: in the absence of Hh ligand, the Patched receptor inhibits activity of the transmembrane protein Smoothened (Smo). The COS2/Fu/Sufu (Costal-2/Fused Suppressor of fused) complex is thus able to convert the transcription factor Gli into a repressor form (GliR). In the presence of Hh ligand, Ihh binds to Patched and thereby releases the inhibition of SMO. SMO blocks the COS2/Fu/Sufu complex, leading to the generation of the activator form of Gli (GliA) that translocates to the nucleus to drive target gene transcription. Notch signaling: Interaction of the Notch receptor with transmembrane ligand proteins (Ser, Serrate; Del, Delta; Jag, Jagged) in a neighboring cell triggers the proteolytic cleavage of Notch by tumor necrosis factor (TNF)α-converting enzyme (TACE) and γ-secretase. This cleavage releases the Notch intracellular domain (NICD) and allows its translocation to the nucleus. NICD forms a transcriptional complex with recombinase signal binding protein (RBP)-Jk to activate target genes.
interpretation of the precise functions of β-catenin in SHF development.

**Autocrine Fgf Signaling Drives SHF Proliferation and Deployment**

Fgf10, a target of Wnt signaling in the SHF, was the first identified endogenous marker of the SHF.\(^5\) However, embryos lacking Fgf10 do not have OFT alignment or septation defects despite abnormal ventricular morphogenesis and malpositioning of the heart in the thoracic cavity.\(^35\) Analysis of Fgf8 hypomorphic alleles and conditional ablation of Fgf8 using a series of Cre lines revealed that Fgf8 is likely to be the major Fgf ligand driving SHF development.\(^10,16,36,37\) Fgf8 is known to play an important role in specifying early cardiac mesoderm,\(^38\) and zebrafish fgf8 is expressed in ventricular myocardium and progenitor cells and is required for ventricle formation.\(^39\) Early loss of Fgf8 in cardiac mesoderm in the mouse results in survival and proliferative defects in the SHF and a failure to elongate the heart tube.\(^10,16\) The hearts of surviving mutant embryos display right ventricular and OFT hypoplasia and severe alignment defects. Ablation of Fgf8 in pharyngeal mesoderm at later stages results in failure of OFT alignment; decreased Fgf signaling in pharyngeal mesoderm and OFT myocardium was associated with reduced expression of Isl1, Bmp4, and Wnt11. These experiments suggested that mesodermal Fgf8 is required for SHF development and impacts on additional signaling pathways; Fgf8 in pharyngeal endoderm may also be required for OFT septation.\(^10,16\)

The major candidate gene for del22q11.2 (DiGeorge) syndrome, encoding the T-box transcriptional activator Tbx1, has been shown to regulate Fgf ligand expression in the caudal pharynx. del22q11.2 patients have craniofacial and cardiovascular defects including arterial pole alignment defects such as tetralogy of Fallot, and Tbx1-null mice have a short OFT at midgestation and a common ventricular outlet at later stages of development (Figure 3E through 3H; reviewed elsewhere\(^40\)). Conditional mutagenesis has shown that these defects are attributable to a requirement for Tbx1 in pharyngeal mesoderm during cardiac looping and that Tbx1 positively regulates proliferation in the SHF,\(^10,41\) likely through transcriptional regulation of Fgf10, Fgf8, and Fgfr1.\(^9,16,42,43\) Tbx1 is itself regulated positively by Shh signaling\(^44,45\) and negatively by retinoic acid signaling\(^46,47\) and thus plays an important role in integrating different signaling inputs during SHF development.

Two recent articles have now shown that the critical target cell type for Fgf signaling during OFT formation is the SHF itself.\(^48,49\) Conditional ablation of both Fgfr1 and Fgfr2 in the
SHF, but not in endothelial or neural crest cells, results in a reduction in proliferation within the SHF (Figure 3I through 3L). Similar observations were made using a gain-of-function Sprouty2 transgene, a known feedback inhibitor of Fgf signaling, and in mice lacking factor receptor substrate (FRS)2α, an adaptor protein mediating Fgf receptor function, in the SHF. This primary defect of the SHF results in a shorter OFT and is compounded by subsequent failure of normal myocardial differentiation and neural crest cell development, leading to defects in cushion morphogenesis in addition to alignment defects.58,40 Because loss of Fgf signaling in neural crest and endodermal cells does not affect OFT development, secondary loss of signals from the SHF and SHF-derived myocardium are likely to contribute to this progressive impairment of OFT development. Bmp4, Wnt11, and Sema3c were among the genes observed to be downregulated in conditional Fgfr1/Fgfr2 mutant embryos, and vascular endothelial growth factor A was found to be upregulated in the OFT of FRS2α conditional mutant embryos. Successive steps of SHF and OFT development are therefore controlled by a signaling cascade downstream of autocrine Fgf signaling. In Drosophila, the Fgf receptor Heartless is required for heart formation and controls directed migration of mesodermal cells inside the ectoderm to the dorsal position, where Bmp signaling activates the cardiomyogenic program.50 Whether Fgf signaling plays a role in progenitor cell migration during SHF development remains to be seen.

### Bmp Signaling and the Switch From Progenitor Expansion to Differentiation

In 2001, Mjaatvedt et al showed that myocardial differentiation in SHF explants could be induced by coculture with right but not left ventricular myocardial explants, suggesting a recruitment model for SHF deployment.6 Bmp signaling was known to be required for cardiac mesoderm specification and differentiation,51–53 and Waldo et al demonstrated that blocking Bmp signaling inhibited SHF differentiation, identifying Bmp2 as a candidate molecule for recruiting SHF cells to the arterial pole of the heart tube.7 These experiments led to the early conclusion that SHF development involves Bmp driven differentiation at the distal end of the heart tube and Fgf driven proliferation in pharyngeal mesoderm. Consistent with such a model, Bmp4 overexpression promotes a cardiac cell fate in cranial mesoderm.54 Mouse genetics has shown that Bmp4, a target of Wnt/β-catenin and Fgf signaling,33,48 is required for OFT septation, including smooth muscle and endocardial cushion development.55 OFT elongation is reduced in embryos lacking both Bmp4 and Bmp7,55 suggesting complexity of Bmp ligand involvement in SHF development.

Bmp signal reception is required in the SHF and OFT, as shown by conditional loss of the Bmp type 1 receptor Bmpr1a in Isl1-expressing cells, resulting in abnormal right ventricular and OFT morphology and septation defects.56 Proliferative defects in ventricular myocardium were associated with a decrease in expression of the T-box gene Tbx20 and an increase in Isl1 expression in the OFT.56 These results are consistent with a model by which Bmp signaling downregulates Isl1 expression as SHF-derived cells enter the OFT. Earlier deletion of Bmpr1a in cardiac mesoderm reveals that Bmp signaling is essential for differentiation of the linear heart tube, but is not required for SHF specification.31 In addition to promoting differentiation, Bmp ligands negatively impact progenitor cell proliferation. Increase in Bmp2 expression in embryos lacking the homeobox gene Nkx2.5 leads to cardiac overspecification and decreased proliferation of SHF cells.55 The intracellular signaling molecule Smad1 mediates the effect of Bmp2 on proliferation because elevated proliferation in the SHF and increased OFT length are observed in embryos lacking Smad1 in cardiac mesoderm; furthermore, loss of Smad1 alleles alleviates the Nkx2.5 phenotype.15 By negatively regulating Bmp2, Nkx2.5 may therefore act to brake cardiac specification in the SHF during normal heart tube elongation. Importantly, Bmp signaling is required for Nkx2.5 expression, revealing a negative feedback loop controlling the balance between proliferative modes during progenitor expansion and differentiation.

### Hh Signaling From Pharyngeal Endoderm Controls SHF Survival and Deployment

In addition to Wnt, Fgf, and Bmp signaling, Hh signaling also impacts on SHF development. Loss of the Hh receptor Smoothened results in severe defects in heart tube formation that appear to reflect compound roles of the ligands Shh and Ihh during early heart development, including the regulation of Nkx2.5 expression.58 Shh mutant embryos display defects in SHF deployment and survival; defective right ventricle and OFT development lead to alignment and septation defects, resulting in a common ventricular outlet.59 Conditional deletion of Shh has revealed that pharyngeal endoderm is the source of ligand required for SHF and OFT development; extensive cell death is observed in the SHF of conditional mutant embryos, resulting in defective OFT elongation.60 Intriguingly, Shh expression is regulated by Isl1 in pharyngeal endoderm.45 Conditional deletion of the Smoothened receptor in different pharyngeal cell types has revealed complex roles of Shh in controlling arterial pole development.45,60 Hh signal reception is required in neural crest cells for survival in the pharyngeal region, in the SHF for later OFT septation, and in pharyngeal endoderm itself to regulate a secondary signal that controls SHF survival and deployment.60 What this signal is and how Hh impacts on later OFT septation through an early role in the SHF remain unknown.

Bmp4 and Fgf8 expression are not affected, although loss of Smoothened in the Isl1 expression domain perturbs expression of Neuruplin2, suggesting a potential role for Semaphorin signaling, known to be required for neural crest influx and OFT septation.45,61 These experiments demonstrate that the Shh phenotype is a composite of multiple roles of Hh signaling in different cell types, each leading to failure to secrete the OFT, making the important point that different upstream etiologies can converge on common end phenotypes. Gain- and loss-of-function experiments in zebrafish embryos have revealed an early requirement for Hh signaling in controlling myocardial progenitor cell numbers through a cell autonomous role in promoting progenitor cell specification.62 This role appears to be distinct from that in the SHF. A recent study has demonstrated that Shh signaling in pharyngeal mesoderm is required not only for OFT development but
also for atrioventricular septation.63 Isl1 expression analysis and Cre lineage experiments have shown that the dorsal mesenchymal protrusion, giving rise to part of the atrial septum extending toward the atrioventricular cushions, derives from the SHF.63,64 Furthermore, Smoothened activity is required in pharyngeal mesoderm but not differentiated endocardium or myocardium for normal atrioventricular septation.63 By implicating the SHF in development of this region of the heart, this study provides a new paradigm for understanding the etiology of atrioventricular septal defects.

**Neural Crest Cells Control SHF Development by Modulating Intercellular Signaling**

SHF development is intricately linked with that of the cardiac neural crest. Cardiac neural crest cells have long been known to be critical for OFT morphogenesis and septation.65,66 These cells, derived from the dorsal neural tube, migrate through the caudal pharyngeal arches into the distal OFT, where they drive OFT septation and contribute to valve and smooth muscle tissue. As neural crest cells move through the pharyngeal region, they are closely apposed to cells of the SHF (Figure 1D through 1F). Neural crest cells do not give rise to the myocardial walls of the OFT in birds and mammals, although they have been reported to give rise to myocardium in the developing zebrafish heart.67,68

The conditional mutagenesis experiments described above have shown that Shh signaling impacts independently on SHF and neural crest development during OFT morphogenesis and may thus coordinate the contribution of these 2 cell types to the heart tube.69,70 However, deployment of these 2 cell types is also interdependent. An elegant series of studies in the chick have shown that, before arriving in the heart tube, cardiac neural crest cells play a critical role in controlling addition of SHF cells to the heart tube.69-72 Consequent reduction in OFT neural crest cells play a critical role in controlling addition of SHF cells to the heart tube.69–72 Consequent reduction in OFT neural crest ablation.72,73

Precise levels of Fgf signaling are therefore critical for normal OFT development. The phenotypes observed after Fgf inhibition in the chick are similar to those observed after laser ablation of the SHF and include alignment defects such as overriding aorta and pulmonary atresia.74,75

The neural crest–mediated brake in SHF proliferation appears essential for distal OFT morphogenesis. Dialogue between these 2 cell types, derived from different regions of the embryo, yet moving together through the pharyngeal region to contribute to the heart, is thus critical in coordinating arterial pole development. However, how neural crest cells buffer the effect of Fgf signaling on the SHF is currently unclear, and the identification of signals exchanged between these 2 cell types is a major research goal. A complex and dynamic series of signal exchanges is likely. As noted above, Fgf signal reception by Fgfr1 and Fgfr2 is not required in neural crest cells for OFT morphogenesis, although elevated neural crest cell death in Fgf8 hypomorphic mutant embryos suggests an indirect role for Fgf signaling in neural crest survival in the pharyngeal region.36,37 Recent data have shown that cardiac neural crest development is critically dependent on neural crest autonomous ERK signaling.76 Conditional deletion of the Bmpr1a receptor in neural crest cells also leads to a shortened OFT, suggesting that a Bmp-regulated gene participates in the dialogue with SHF cells.77 In support of this hypothesis, deletion of Smad4 in neural crest cells leads to abnormal SHF development and a shorter OFT.78 Loss of Tbx3, expressed in neural crest cells and pharyngeal endoderm, results in elevated proliferation in the SHF and defective OFT elongation and alignment; Tbx3 target genes in neural crest cells may thus participate in signaling to the SHF.79

**Noncanonical Wnt Signaling in OFT Development**

An alternative Wnt signaling pathway operates not through β-catenin but JNK and protein kinase C to effect intracellular cytoskeletal changes associated with the establishment of planar cell polarity.23 Wnt11 is required for cardiac development in Xenopus through a noncanonical Wnt pathway.80 The phenotypes of mouse mutants lacking the noncanonical Wnt ligands Wnt5a and Wnt11 have recently been described and reveal a role for this pathway in OFT development.81,82 Previous analysis of Vangl2 mutant mice had implicated planar cell polarity in later stages of OFT development, in particular in myocardialization of OFT cushions during septation.83 Wnt5a is expressed in the SHF and mutant embryos have a common ventricular outflow and abnormal neural crest invasion, although the expression domains of SHF genes Fgf8, Fgf10 and Tbx1 are unaltered, suggesting a role for noncanonical Wnt signaling from the SHF to neural crest cells.81 In agreement with such a hypothesis, the Fz2 receptor is transiently expressed in cardiac neural crest cells.84 Wnt11 expression in the OFT is regulated by Wnt/β-catenin and Fgf signaling pathways, consistent with hierarchies of signaling pathways controlling OFT development.16,32 Wnt11 is also a direct transcriptional target of the transcription factor Pitx2, a gene that transmits embryonic laterality signals to the developing heart tube. Failure to correctly establish laterality through
Nodal signaling in the early embryo results in complex arterial pole defects, and loss of Pitx2 in the SHF and OFT leads to arterial pole alignment defects and transposition of the great arteries. Wnt11 mutant embryos have a short OFT and develop alignment and septation defects likely mediated by loss of transforming growth factor β2 signaling, known to be a critical regulator of neural crest and endocardial cushion development during OFT septation. It remains to be seen whether noncanonical Wnt signaling plays earlier roles in SHF development.

**Retinoic Acid Signaling Defines the Posterior Limit of the SHF**

The above pathways predominantly affect survival, proliferation, and deployment of SHF progenitor cells, in addition to subsequent differentiation of OFT myocardium and OFT remodeling. Less is known about the signals that define the limits of the SHF in pharyngeal mesoderm. Recent evidence suggests that retinoic acid signaling may participate in this process. Excess or reduction of retinoic acid is known to result in cardiac defects; for example, altered retinoic acid levels in avian embryos support a role for retinoic acid signaling in development of the venous pole of the heart. Analysis of mouse embryos lacking the retinaldehyde dehydrogenase gene Raldh2 reveals that a number of SHF genes are abnormally expanded in a posterior direction, including Isl1, Tbx1, Fgf10, and Fgf8 (Figure 3Q through 3T). Similar effects on Tbx1 expression are observed in vitamin A–deficient avian embryos and addition of retinoic acid soaked beads locally downregulates Tbx1 expression. Early derepression of Fgf signaling in the posterior domain of Raldh2 mutant embryos may contribute to the upregulation of other SHF markers. Explant analysis has shown that despite expanded expression of SHF genes, cardiac differentiation is blocked and the heart tube of Raldh2 mutant embryos fails to extend. Retinoic acid signaling is in turn controlled by signals from the SHF: in Tbx1 mutant embryos, the Raldh2 expression domain is shifted anteriorly and retinoic acid catabolizing enzymes are downregulated. In the zebrafish, retinoic acid has been shown to regulate the size of the cardiac field, intriguingly, recent evidence suggests that this effect is mediated indirectly by adjacent cell types and that Hox gene expression in the forelimb field may control signals restricting atrial progenitor cell numbers.

**A Potential Role for Notch Signaling in Controlling Differentiation Delay in the SHF**

Proliferation and differentiation delay are the defining properties of the SHF yet little is known about how this second attribute is controlled. Notch signaling regulates diverse progenitor cell populations in the embryo and plays multiple roles in heart development (reviewed elsewhere) yet, to date, has not been implicated directly in SHF development. However, mutant embryos in which the canonical Notch pathway is inactive develop up to midgestation suggesting that this signaling pathway is not required for initial heart tube formation. In Xenopus embryos, Notch signaling represses cardiomyocyte differentiation in the dorsal pericardial wall, functioning downstream of Nkx2.5 activation.

The Notch ligand Serrate is expressed in this region, whereas the Notch1 receptor is expressed throughout the heart; active Notch signaling is thus restricted to the dorsal pericardial wall. Recent evidence that Xenopus Isl1 is expressed in the dorsal pericardial wall supports the possibility that this is an analogous structure to the SHF and may contribute to the frog heart after metamorphosis. Whether Notch signaling plays a role in SHF development is an area of active research.

**Biomedical Perspectives: Isl1 and Resident Cardiac Progenitor Cells**

Understanding the signals that control development of the SHF in the early embryo can provide insight into the pathways that maintain and direct the differentiation of cardiac progenitor cells in the later heart. A small number of Isl1-positive cells are present in the fetal and early postnatal heart and have been proposed to represent residual SHF cells that identify a resident progenitor cell population potentially contributing to growth of the heart. Isl1 plays a central role in the SHF transcriptional hierarchy, regulating multiple signaling pathways required for SHF survival, proliferation, and differentiation, including Fgf, Bmp, and Shh ligand expression. Isl1 expression is also a target of different signaling pathways revealing feedback regulation in the control of SHF progenitor cells. Lineage tracing studies and purification of Isl1-positive progenitor cells from the early embryo or later heart have shown their self-renewal capacities and ability to differentiate into fully mature cardiac myocytes, endothelial cells and smooth muscle cells. In addition to Isl1-positive cells, other resident progenitor cells, extracardiac stem cells, and pluripotent embryonic stem cells are the focus of intense research efforts to harness cardiac progenitor cells for myocardial repair. The signals regulating progenitor cell development in the early embryo may also regulate Isl1 and other progenitor cell populations in the later heart and can potentially be used to direct embryonic stem and induced pluripotent stem cells toward a cardiac fate. For example, Wnt/β-catenin signaling stimulates proliferation of purified Isl1-positive cells, and a recent report in zebrafish shows that Wnt signaling is upregulated during cardiac regeneration, highlighting the potential importance of this signaling pathway for cardiac repair. Similarly, Notch signaling appears to block differentiation and allow expansion of activated cardiac precursor cells in failing hearts. These and other studies will contribute to optimizing techniques to maintain, expand, and trigger differentiation of cardiac progenitor cells for therapeutic applications.

A number of new questions and perspectives concerning the etiology of congenital heart defects emerge from these studies. We have seen that SHF development depends on a complex cascade of upstream signals (Figure 4). A current challenge is to understand how these multiple signal inputs are integrated as the topography of progenitor cells changes during OFT extension. Cross-regulation between different pathways, as seen above, is a recurrent theme, and common intracellular signaling intermediates suggest that activation of one pathway may enhance or block responsiveness to another pathway. Intracellular signaling pathways provide a potential link between cardiac function and form during heart devel-
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Conclusions

Multiple signaling pathways converge to regulate addition of SHF cells to the poles of the heart tube. The signaling molecules identified to date constitute part of the signaling environment or niche that maintains SHF cells in a progenitor cell state and controls their progressive differentiation. Many of the signaling pathways converge on the regulation of SHF proliferation and myocardial differentiation; the control of differentiation delay and SHF movement into the OFT remain more obscure. Continued elucidation of the gene regulatory networks and signaling pathways that control SHF development will undoubtedly identify mechanisms underlying these processes and define the extent to which they differ from the control of differentiation of the linear heart tube. Insights gained into the etiology of congenital heart defects and the control of progenitor cell fate highlight the biomedicai importance of the SHF. As such, further exploration of the mechanisms underlying SHF progenitor cell development in the early embryo will contribute to realizing the potential of progenitor and pluripotent stem cells for cardiac repair.

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Disclosures

None.

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