CHAPTER ONE

How to Make a Heart: The Origin and Regulation of Cardiac Progenitor Cells

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Contents

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Abstract

The formation of the heart is a complex morphogenetic process that depends on the spatiotemporally regulated contribution of cardiac progenitor cells. These mainly derive from the splanchnic mesoderm of the first and second heart field (SHF), with an additional contribution of neurectodermally derived neural crest cells that are critical for the maturation of the arterial pole of the heart. The origin and distinguishing characteristics of the two heart fields, as well as the relation of the SHF to the proepicardial organ and to a proposed third heart field are still subjects of debate. In the last ten years many genes that function in the SHF have been identified, leading to the establishment of a gene regulatory network in the mouse embryo. It is becoming increasingly evident that distinct gene networks control subdomains of the SHF that contribute to different parts of the heart. Although there is now extensive information about mutant phenotypes that reflect problems in the integration of progenitor cells into the developing heart, relatively little is known about the mechanisms that regulate SHF cell behavior. This important source of cardiac progenitor cells must be maintained as a proliferative, undifferentiated cell population. Selected subpopulations, at different development stages, are directed to myocardial, and also to smooth muscle and endothelial cell fates, as they integrate into the heart. Analysis of signaling pathways that impact the SHF, as well as regulatory factors, is beginning to reveal mechanisms that control cardiac progenitor cell behavior.

1. Introduction

The heart occupies an important place in the popular imagination. In medieval times, in Europe, it was regarded as the seat of courage. King Richard I of England, renowned for his bravery, was called Richard the Lion Heart and the great queen Elizabeth I proudly claimed, in terms now politically incorrect, "I know that I have the mind and body of a weak and feeble woman, but I have the heart and stomach of a king, and of a king of England too!". After this period of male bravure, the heart, also regarded as the site of the soul and hence a holy relic, is now mainly a symbol of love, much evident in the commercialization of romance on St Valentine's Day. Fixation on the heart reflects its obvious role as a vital organ. The beating of the heart as it pumps blood around the body is synonymous of life and until very recently the official definition of death was arrest of the heartbeat.

The heart is the first organ to form in the embryo where its early function is essential for the circulation of nutrients and removal of waste, as soon as the number of cells reaches a point where diffusion is no longer efficient. Early heart defects are a frequent source of lethality when genes are mutated in mouse models. In the human population almost 1% of

newborn children have some form of congenital heart defects and cardiac malformations probably account for as many as 30% of embryos/foetuses lost before birth (Bruneau, 2008). These figures indicate the vital requirement for a fully functional heart and also reflect the degree of precision required during cardiogenesis. The construction of the heart is a complex process, involving the integration of different cell populations at distinct sites as development proceeds. In this review, we discuss the origins of cardiac progenitor cells and the regulation of their contributions to the heart. The characterization of the second heart field (SHF) as a major contributor, with increasingly detailed genetic information about the regulatory factors and signaling pathways that affect the behavior of cells that transit through this field has considerably advanced our understanding of cardiogenesis. Knowledge about the progenitor cells that form the heart is also of importance for potential stem cell therapies in the context of the failing adult heart.

We shall discuss the formation of the heart from the stand point of mammalian cardiogenesis. In this context, the mouse is the best studied model. We shall particularly focus on the properties of the SHF because it constitutes a major source of cardiac progenitor cells as the primitive heart tube grows. However, the formation of the tube and the contribution of other sources of cells will also be considered in the context of cardiogenesis, briefly summarized as follows (see Fig. 1.1).

The first differentiated myocardial cells are detected in the cardiac crescent, in splanchnic mesoderm underlying the head folds. As the embryo grows, the crescent fuses at the midline to form the primitive cardiac tube which rapidly begins to pump blood. It is now established that cardiac progenitor cells mainly lie medially and posteriorly to the crescent and then are located behind the heart tube, extending posteriorly and also anteriorly into pharyngeal mesoderm, as a result of morphogenetic movements as the embryo develops. These progenitor cells constitute the SHF, in contrast to the region of the crescent referred to as the first heart field (FHF). In the mouse embryo, the early heart tube has a mainly left ventricular identity and its expansion depends on contributions from the SHF (Buckingham *et al.*, 2005). The early FHF-derived cardiac tube thus provides a scaffold for subsequent growth.

Other cell populations also contribute to the formation of the heart, in addition to the splanchnic mesoderm of the heart fields. The proepicardial organ (PEO) is a transitory mesenchymal structure that forms at the posterior end of the heart tube. Cells from the PEO grow over the myocardium of the tube to form the outer layer of the epicardium. Some of these epicardial cells undergo an epithelial/mesenchymal transition (EMT) and enter the heart where they contribute the smooth muscle of the coronary blood vessels and also constitute the population of cardiac fibroblasts/interstitial cells.

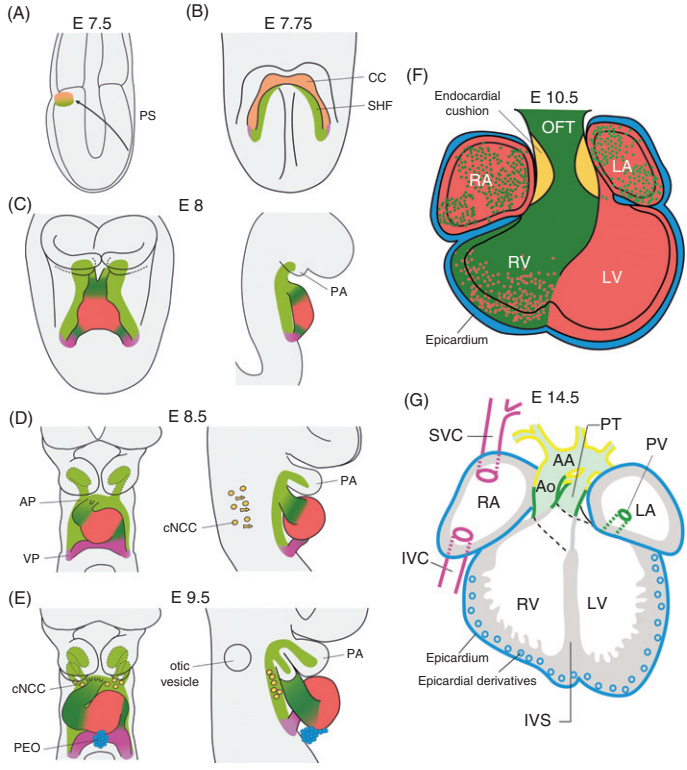

Figure 1.1 (A) Migration of cells anteriorly from the primitive streak (PS). (B) Formation of the cardiac crescent (CC), with the second heart field (SHF) lying medial to it. (C–E) Front (left) and lateral (right) views of the heart tube as it begins to loop with contributions of cardiac neural crest cells (cNCC), which migrate from the pharyngeal arches (PA) to the arterial pole (AP). The proepicardial organ (PEO) forms in the vicinity of the venous pole (VP). (F) The looped heart tube, with the cardiac compartments—OFT, outflow tract; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. (G) The mature heart which has undergone septation—IVS, interventricular septum; AA, aortic arch; Ao, aorta; PT, pulmonary trunk; PV, pulmonary vein; SVC, superior caval vein; IVC, inferior caval vein. The first heart field (FHF) and its myocardial contribution are shown in red, the SHF and its derivatives in dark green (myocardium) and pale green (vascular endothelial cells), cNCC in yellow (vascular smooth muscle of the AA, endocardial cushions), and PEO derivatives in blue. (See Color Insert.)

The functional form of the mature arterial pole of the heart depends on neural crest cells. These are of neurectodermal origin and migrate from the dorsal neural tube. Cardiac neural crest (Hutson and Kirby, 2007) transits through the posterior pharyngeal arches and invades the anterior domain of the SHF before entering the anterior part of the heart tube.

This constitutes the outflow tract of the heart and neural crest plays a major role in the remodeling of this region, contributing to septum and valve formation, which results in the separation of the myocardial base of the pulmonary trunk and aorta. These great arteries connect with right and left ventricles, respectively, and ensure blood flow to the lungs and body.

The venous pole of the heart, initially located at the posterior end of the heart tube, moves anteriorly as the tube undergoes looping. The inflow region of the tube develops with the addition of myocardium that will form the atria and then the base of the pulmonary and caval veins that recycle blood from the lungs and body to the left and right atria, respectively. The development of the cardiac chambers results from expansion of regions of the heart tube and subsequent septation and valve formation to give the mature heart (Fig. 1.1).

2. The Origin of the Heart Fields and Cardiac Progenitor Cell Behavior

Cardiac mesoderm derives from the anterior part of the primitive streak (PS), as shown previously by cell labeling and grafting experiments in the mouse (Lawson *et al.*, 1991; Tam *et al.*, 1997) as well as for the chick embryo (see Kirby, 2007). Since in both amniote models, the outflow tract of the heart tube has been shown to derive from the SHF, the question of whether there is pre-patterning of progenitor cells already in the streak can be addressed using the avian embryo, which is more amenable to experimental manipulations.

This question is intimately linked to the mode of migration of cells from the streak to the heart-forming region of splanchnic mesoderm (Fig. 1.1). Earlier experiments suggested that cells that give rise to outflow tract myocardium are situated more anteriorly in the cardiogenic region of the streak (Garcia-Martinez and Schoenwolf, 1993); however, these and other fate mapping experiments were limited by the technical methods of the time (see Abu-Issa and Kirby, 2007). This was also the case for studies at later developmental stages when it was difficult to ensure that only cardiac progenitors were labeled. Thus Stalsberg and DeHaan (1969) had concluded that migrating cells behaved as cohesive groups, whereas Redkar *et al.* (2001) suggested that there was considerable dispersion. In a recent fate mapping analysis, using sophisticated time lapse imaging microscopy after marker electroporation in the quail embryo, Cui *et al.* (2009) showed that cardiac progenitor cells, originating from similar anterior/posterior levels in the streak to those previously identified, change their relative positions as

they migrate. This results in medial/lateral repositioning as the region where cardiogenic mesoderm is located undergoes morphological changes driven by endodermal folding. This would be consistent with the medial/lateral location of first and SHFs, respectively. They concluded that cardiac progenitors can move as cohorts of cells that will contribute to specific regions of the heart tube.

The results of retrospective clonal analysis in the mouse embryo had shown that a period of dispersive progenitor cell growth precedes coherent growth that accompanies cardiogenesis (Meilhac *et al.*, 2003). This retrospective approach does not throw any light on the spatial location of progenitors; however, it provides important temporal insights. Notably it distinguishes two myocardial cell lineages that segregate early, around the onset of gastrulation (Meilhac *et al.*, 2004). These two lineages can be equated with the contribution of first and SHFs, in that the first is the exclusive source of the early left ventricle whereas the second is the exclusive source of outflow tract myocardium. Both lineages contribute to other parts of the heart. Prospective clonal analysis in the mouse embryo will be required to address spatial issues of myocardial lineage segregation, as well as the timing, at or before gastrulation.

The issue of cell behavior in the SHF is linked to that of proliferation. Two recent studies in the chick embryo use three-dimensional reconstructions to interpret BrdU data on cell proliferation (Soufan *et al.*, 2006; Van den Berg *et al.*, 2009). They conclude that the early heart tube has a low level of proliferation and that changes in cell size play a significant role in its expansion. Growth of the heart tube also depends on the addition of progenitor cells. This mainly occurs in a proliferative center which is located in a dorsal/medial position, in the posterior SHF. Cell tracing experiments suggest that cells move anteriorly from this proliferative zone to contribute to the arterial as well as the venous pole of the cardiac tube and indeed impairment of proliferation in this posterior zone affects both poles of the heart. This finding for the chick embryo has to be equated with observations on clonal growth and cell fate determination for the mouse embryo. Retrospective clonal analysis suggests that the mouse heart tube is more proliferative (Meilhac *et al.*, 2004) and the anterior as well as the posterior region of the SHF is clearly proliferating, as indicated by mutants that affect proliferation in the anterior SHF, with consequences for arterial pole formation (see Section 6.3).

Explant experiments and dye-tracing of cells show that the anterior part of the SHF is programmed to make outflow tract and right ventricular myocardium and contributes to this part of the heart (Zaffran *et al.*, 2004). In contrast, the posterior part of the SHF is programmed to make atrial myocardium and indeed cells in this domain contribute to the atria (Galli *et al.*, 2008). These observations do not preclude that there may be posterior/anterior movement of cardiac progenitor cells, but would suggest that such a phenomenon may be limited. However, more detailed spatiotemporal fate mapping in the mouse SHF is required to clarify this issue.

3. Markers of Cardiac Progenitors and the Distinction Between First and Second Heart Fields (FHF and SHF)

3.1. Cardiac progenitors and cell fate determination

One of the first markers of cardiac progenitor cells is Mesp1, which is required for the delamination of these cells from the primitive streak. A *Mesp1*$^{Cre/+}$ line crossed to the *Rosa-26* conditional reporter marks all cardiac cells in the heart of mesodermal origin (Saga *et al.*, 1999) and has proved to be very useful for genetic experiments in which mutations are targeted to cardiac mesoderm. Experiments with embryonic stem (ES) cells have led to the proposal that this transcription factor may act as a master regulator of cardiovascular cell fates (Bondue *et al.*, 2008), down-regulating pluripotency genes and early mesodermal genes and up-regulating genes for key cardiac transcription factors such as Gata4 or Nkx2-5. In keeping with this, injection of *Mesp1* RNA into *Xenopus* embryos leads to ectopic heart formation (David *et al.*, 2008); however, these authors emphasize an indirect role for Mesp1 in the induction of the Wnt inhibitor, Dkk1, and effects on endodermal induction of cardiogenesis. Another series of experiments in ES cells also points to the role of Mesp1 in promoting cardiovascular cell fate in the presence of Dkk1 (Lindsley *et al.*, 2008). These authors also demonstrate that Mesp1 triggers an epithelial/mesenchymal transition (EMT) in ES cell embroid bodies. In the mouse embryo, Mesp1 is implicated in EMT, required for exit of cells from the streak, but the gene is rapidly down-regulated thereafter and it is not clear whether it directly activates transcriptional regulators of the cardiac program, which are detected later.

Genes such as *Gata4* or *Nkx2-5* are expressed in the cardiac crescent where myocardial cell differentiation first takes place. T-box transcription factors, such as Tbx5 and Hand1/2 (basic helix–loop–helix), as well as Mef2c (MADS-box) factors, are also implicated in the differentiation of cells in the crescent, as well as in the heart. In the context of master regulators, it was shown recently that Gata4 and Tbx5, in the presence of the chromatin remodeling component, Baf60c/Smarcd3 specifically associated with cardiogenesis, can induce beating myocardial tissue when ectopically expressed in mesoderm (Takeuchi and Bruneau, 2009). Gata4 and Baf60c induce Nkx2-5 expression which acts with Gata4 to initiate the cardiac program; Tbx5 is required for full differentiation. The concept of a master regulator gene comes from the demonstration that transcription factors of the MyoD family can play this role for skeletal myogenesis (Weintraub *et al.*, 1991). In this case, these factors have intrinsic chromatin remodeling activity (Tapscott, 2005), which cardiac regulatory factors appear to lack; hence the requirement for Baf60c. Skeletal myogenesis is also exceptional in its dependence on a single determination factor, whereas

most tissue programs depend on combinations of factors. These may also be interchangeable, which would explain why no single mutation in a cardiac regulatory gene completely abolishes myocardial cell differentiation.

3.2. Markers of the heart fields

Expression of *Islet1* in the SHF first led to an appreciation of the full extent of this field and its contribution to the venous, as well as the arterial, pole of the heart (Cai *et al.*, 2003). Islet1 has been regarded as a marker of the SHF. However, more recently Islet1 protein has been detected in the cardiac crescent and, in the absence of Nkx2-5, Islet1 expression is maintained in differentiating myocardial cells of the crescent as well as the heart at later stages, suggesting that this regulation operates in both heart fields (Prall *et al.*, 2007). *Islet1-Cre* activation of a highly sensitive conditional *FLAP* reporter in the *Gata4* gene resulted in expression in the FHF as well as the SHF (Ma *et al.*, 2008). Furthermore, a conditional *Rosa26* reporter when activated by a new *Islet1-Cre* line is more broadly expressed in the heart than was seen previously, although part of the left ventricle remains negative (Sun *et al.*, 2007). This observation, also reported with other *SHF-Cre* driver lines, such as *Tbx1-Cre* (Brown *et al.*, 2004) or *Mef2c-Cre* (Verzi *et al.*, 2005), may also reflect subsequent expansion of initially right ventricular myocardium into the left ventricular compartment as the heart tube matures. Such expansion of genetically marked cells is also seen for *Tbx2*, a marker of the atrioventricular canal (AVC); cells that had expressed *Tbx2* subsequently expand into part of the left ventricular myocardium (Aanhaanen *et al.*, 2009). In this case, as AVC myocardium is derived from both first and second lineages, this does not necessarily imply that this is now a second lineage contribution (Harvey *et al.*, 2009). Despite detection of Islet1 in the FHF, it is notable that *Islet1* null mutants still form the primitive cardiac tube and the mutant phenotype primarily reflects a problem with the SHF contribution to the heart (Cai *et al.*, 2003). Islet1 positive cardiac progenitors have now been identified in *Xenopus*, where they co-localize initially with Nkx2-5 positive cells but subsequently appear to constitute a progenitor cell field; knock-down of Islet1 suggests that it is not essential for early heart formation (Brade *et al.*, 2007). Thus there is some indication of an equivalent to the SHF in *Xenopus*, and there is evidence for early segregation of two cardiogenic lineages, such that the second lineage, as in the chick, appears to mainly contribute to the outflow tract (Gessert and Kühl, 2009). In zebrafish, there is evidence for two phases of myocardial differentiation, a first Islet1-dependent contribution to the venous pole, and a second Fgf8-dependent addition of cardiomyocytes to the arterial pole (de Pater *et al.*, 2009).

In addition to *Islet1*, a number of genes expressed in the SHF have now been characterized (Fig. 1.2). In many cases, such as *Foxc1/Foxc2* (Seo and Kume, 2006), there is no evidence that they are also expressed in the FHF. Furthermore, as in the case of *Islet1*, when mutated they give rise to typical

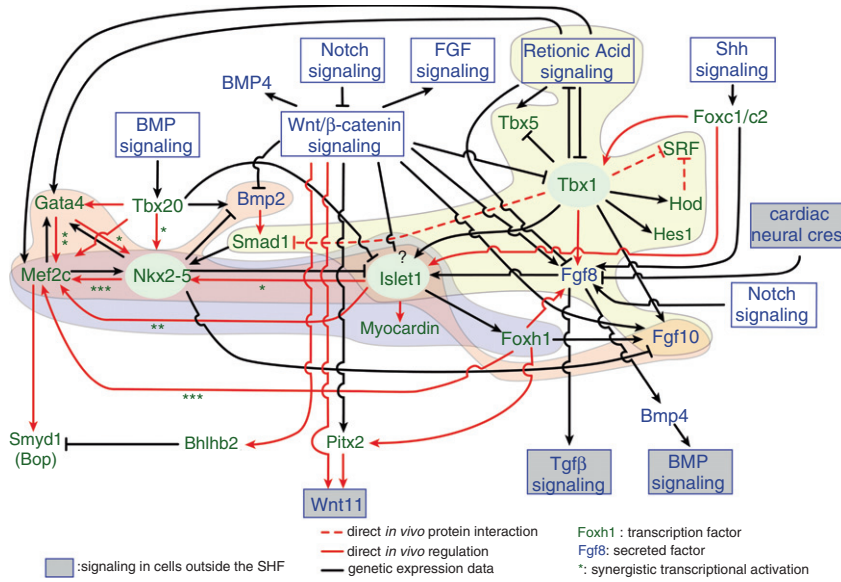

Figure 1.2 Regulatory network in the SHF. Three nodes of regulation are highlighted: Tbx1 (light grey), Islet1 (dark grey), and Nkx2-5 (mid-grey). Direct *in vivo* regulations are indicated by dark grey lines, direct protein–protein interactions are indicated by dashed lines, and genetic expression data are indicated by dark lines. Asterisks highlight synergistic transcriptional activation of enhancers. Grey boxes indicate effects outside of the SHF. The question mark above Islet1 is discussed in Section 6.3: several groups reported that *Islet1* is activated by the Wnt/β-catenin pathway, but a recent report suggested that down-regulation of *Islet1* by Wnt/β-catenin is required for SHF progenitor proliferation.

SHF phenotypes. The question of whether there is a continuum between FHF and SHF to the point where they are regarded as more or less differentiated regions of the same field is partly semantic. They are clearly juxtaposed in the mouse at E7.75 and contiguous in the chick at later stages (Abu-Issa and Kirby, 2008), where Islet1 is also characteristically expressed in undifferentiated cells (Yuan and Schoenwolf, 2000). Distinct first and second myocardial cell lineages exist in the mouse (Meilhac *et al.*, 2004) and mutant phenotypes indicate that many cardiac regulators are mainly functional in the progenitor cell population of the SHF, where a gene regulatory network, based on analysis of mutants and transcriptional regulatory elements, is observed (Fig. 1.2). A classic example of this is provided by two enhancers of the *Mef2c* gene, which are targets of Islet1 and Gata factors (Dodou *et al.*, 2004) and of Foxh1 and Nkx2-5 (von Both *et al.*, 2004), respectively, to drive SHF expression. Another example is provided by the recent identification of an *Islet1* enhancer active in the SHF that requires Foxc2 binding sites for activity, with an implication of Gata4 as well as Fox

factors (Kang *et al.*, 2009; Kappen and Salbaum, 2009). Furthermore, factors present in the SHF may interact with each other, as shown for Tbx1 and SRF (Chen *et al.*, 2009), adding a further level of complexity to a network in which Tbx1 activates *Hod* and Hod represses *SRF* (Liao *et al.*, 2008). Another level of factor interaction is illustrated by Tbx20 which synergizes with Islet1 and Gata4 to activate the *Mef2c* enhancer and an *Nkx2-5* cardiac enhancer (Takeuchi *et al.*, 2005).

4. Cardiac Progenitor Contributions to the Cell Types of the Heart

4.1. Cell types derived from the SHF

As stated in Section 1, the FHF is the major contributor to early left ventricular myocardium, whereas the SHF contributes to myocardium of other regions of the heart and most notably to that of the outflow tract. However, myocardium is not the only derivative of the SHF. The origin of the endocardium has been controversial. This endothelium forms the inner sheath of the cardiac tube and compartments of the heart as they develop. It plays a critical role in trabeculation of chamber myocardium and in valve formation, initiated by delamination of endocardial cells to form the cushions (Kirby, 2007). Retroviral tracing in the avian embryo (Wei and Mikawa, 2000) had suggested that myocardial and endocardial progenitors are already distinct at gastrulation. More recently, lineage studies in the zebrafish suggest that endocardial cells are derived from a hematopoietic/vascular lineage (Bussmann *et al.*, 2007). However, genetic tracing in the mouse embryo suggests that cells that had expressed *Islet1* (Cai *et al.*, 2003; Moretti *et al.*, 2006), *Nkx2-5* (Stanley *et al.*, 2002), or activated the *Mef2c SHF* enhancer (Verzi *et al.*, 2005) contribute to endocardium and myocardium. Furthermore *Flk1* expressing progenitors contribute to both tissues (Motoike *et al.*, 2003). *Nfatc1* now provides a marker for endocardium that distinguishes these cells from other endothelial cells. Using this marker in the ES cell model system, it has been shown that multipotent Flk1 positive cardiac progenitors give rise to both endocardial and myocardial derivatives (Misfeldt *et al.*, 2009). This is, therefore, in keeping with the prevailing view that the endocardium is an SHF derivative. Interestingly, a recent paper (Ferdous *et al.*, 2009) showed that the endothelial/endocardial fate in the developing embryo depends on an Ets-related protein, Etsrp17. This is a direct activator of the endothelial *Tie2* gene. Upstream of endocardial formation, Nkx2-5 transactivates the *Etsrp17* gene. Etsrp17 is first detected in the cardiac crescent, suggesting that FHF derivatives may also contribute to endocardium.

The mesoderm of the arches can be regarded as an extension of the SHF, which becomes incorporated into the pharyngeal arches as these transitory structures bulge out as pouches on either side of the pharyngeal region (Fig. 1.1). SHF marker genes such as *Fgf10* (Kelly *et al.*, 2001), *Islet1* (Cai *et al.*, 2003), and *Tbx1* (Xu *et al.*, 2004) are expressed in the mesodermal core of all the arches. In the chick embryo, dye-labeling experiments have demonstrated the contribution of the mesodermal core of arches 1 and 2 to outflow tract myocardium (Kirby, 2007) and this has also been shown in the mouse embryo for arch 2 (Kelly *et al.*, 2001). Endothelial cells of the derivatives of the pharyngeal arch arteries, at the arterial pole of the heart (Fig. 1.3) derive from SHF mesoderm of the posterior arches (3–6). In the mouse embryo, this is supported by genetic tracing experiments showing that these endothelial cells derive from progenitors that have expressed *Mesp1* and *Islet1* (Sun *et al.*, 2007) and activated the *Mef2c SHF* enhancer (Verzi *et al.*, 2005) and also by phenotypes such as that of the *Tbx1* mutant (Zhang *et al.*, 2005) or of *Fgf8/Fgf10* double mutants targeted to the mesoderm of the SHF (Watanabe *et al.*, 2010).

The ES cell system also shows that smooth muscle cells derive from the multipotent cardiac progenitors that give rise to myocardium and endothelial derivatives (Moretti *et al.*, 2006; Wu *et al.*, 2006). In the chick embryo, cell tracing and ablation experiments in the SHF have established that this is the source of smooth muscle cells in the outflow tract that will contribute to the sub-pulmonary and aortic smooth muscle at the base of these great arteries. This SHF contribution follows that of myocardium, which is contributed over a first 24-h period (HH14-18) (Waldo *et al.*, 2005b; Ward *et al.*, 2005). In the mouse embryo, cells that have expressed *Mesp1* (Saga *et al.*, 2000) and *Islet1* (Moretti *et al.*, 2006; Sun *et al.*, 2007) also contribute to smooth muscle at the arterial pole of the heart and *Tbx1* regulates this derivative, as well as myocardium (Chen *et al.*, 2009). It is not yet clear whether SHF cells are multipotent for smooth, myocardial, and endothelial lineages as shown by clonal analysis for ES cells. However, interestingly, the rare Islet1 positive cells detected in foetal hearts (Laugwitz *et al.*, 2005), which probably represent undifferentiated SHF progenitors, are multipotent in this respect (Bu *et al.*, 2009).

4.2. Neural crest

Cardiac neural crest, that invades the mesodermal core of the posterior pharyngeal arches, contributes the smooth muscle of the pharyngeal arch arteries and their derivatives at the arterial pole of the heart (Fig. 1.3). Neural crest cells from the arches also migrate through the anterior SHF into the outflow tract where they form the endocardial cushions (Fig. 1.1). In the absence of neural crest, outflow tract septation and arterial pole maturation are compromised (Hutson and Kirby, 2007).

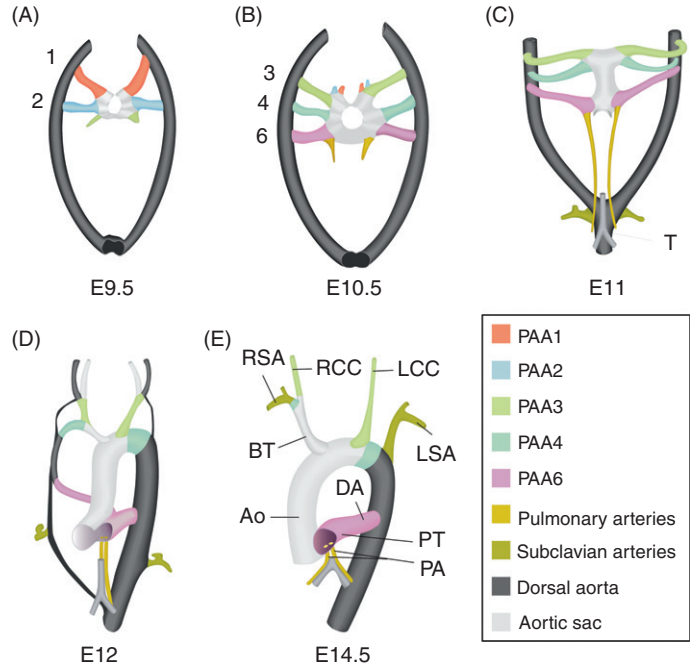

Figure 1.3 Pharyngeal arch artery remodeling. Ventral representation of the arterial network connected to the heart as development proceeds. The arterial pole of the heart (not represented here) feeds into the aortic sac (grey). The heart is connected to the paired dorsal aortas (black) via the artery of each of the five pharyngeal arches; the pharyngeal arch arteries (PAAs). During development, the pharyngeal arches and their arteries are symmetrically formed following a rostro-caudal, temporal gradient. (A) At E9.5, only the PAA1 (orange) and PAA2 (blue) are connected to the aortic sac. (B) At E10.5, the PAA1 and PAA2 are no longer connected directly to the heart but form the capillary beds of their respective pharyngeal arches. The heart is now connected via PAA3 (light green), PAA4 (dark green), and PAA6 (purple). (C) At E11, the network is still symmetric and the pulmonary arteries (dark yellow) are clearly visible. (E) From E11.5, remodeling of the PAAs is initiated via the increase of blood flow in the left PAA6. This results in the stabilization of the aortic arch on the left side at the expense of the right side. Segments of the dorsal aortas are degenerating, leading to the individualization of the future common carotid arteries (RCC, right common carotid and LCC, left common carotid). (D) At E14.5, the left PAA4 contributes to the segment of the aortic arch between the LCC and the left subclavian artery (in bronze), whereas the right PAA4 will form a segment that connects the RSA to the brachycephalic trunk (BT—remains of the right aortic arch). The BT is also connected to the derivative of the right PAA3, the right common carotid (RCC). The right PAA6 is not maintained, whereas the left PAA6 contributes to the *ductus arteriosus* (DA). The DA is an embryonic shunt that connects the (left) dorsal aorta to the pulmonary trunk. At birth, the DA closes, allowing the establishment of the pulmonary and systemic blood circulations (adapted from Kaufman and Bard (1999); Ao, aorta; PT, pulmonary trunk; T, trachea; PA, pulmonary arteries). (See Color Insert.)

Neural crest ablation not only affects septation, but also results in a reduction in outflow tract myocardium and consequent arterial pole defects (Waldo *et al.*, 2005a). The effect of neural crest on outflow tract development, demonstrated by experiments in the chick embryo, is also shown for the mouse, for example in the phenotype of mutants for *Tbx3*, expressed in neural crest (Mesbah *et al.*, 2008), as well as at other sites in the heart. Pax3 is a key regulator of neural crest and in the *Splotch*^{2H} mouse, where Pax3 function is affected, neural crest migration is reduced, again resulting in outflow tract defects, including ectopic myocardial differentiation with abnormal distribution of Islet1 positive cells of the SHF (Bradshaw *et al.*, 2009). Interactions between neural crest and the anterior SHF affect the behavior of both cell populations and their contributions to the heart (see Section 6.4).

4.3. The proepicardial organ

The PEO is a transitory structure, which forms as a group of cells close to the venous pole of the heart tube (Fig. 1.1). It has been thought to be derived from coelomic mesenchyme of the *septum transversum* (Männer *et al.*, 2001) and not from the SHF (Wessels and Pérez-Pomares, 2004); however, its relation to the SHF is not clear (see Section 5). In the early embryo this mesenchyme expresses *Islet1* (Ma *et al.*, 2008), so that the PEO and subsequent epicardium and coronary blood vessels are marked by *Islet1-Cre* genetic tracing (Moretti *et al.*, 2006; Sun *et al.*, 2007; Zhou *et al.*, 2008b). *Nkx2-5-Cre* tracing also marks these cells (Zhou *et al.*, 2008a, b) and, contrary to Islet1, Nkx2-5 is required for PEO development (Zhou *et al.*, 2008b). *Wt1* and *Tbx18* are expressed in the PEO and in the epicardium. *Wt1* mutant mice have coronary vascular defects and it has now been shown that this is due to a direct activation by Wt1 of the *Snail* gene, required for EMT of epicardial cells (Martínez-Estrada *et al.*, 2010). ES cells mutant for *Wt1*, fail to form cardiomyocytes, as well as other mesodermal derivatives, but this probably results from a failure of EMT required for embroid body "gastrulation."

The smooth muscle cells of the coronary vasculature arise from the epicardium and until recently it was thought that the endothelial cells also came from this source. However, genetic tracing experiments with fluorescent markers (Red-Horse *et al.*, 2010) now show that endothelial cells in the coronary vessels and capillaries derive from the venous plexus, at the *sinus venosus*, which invades the heart after formation of the epicardium. The question of whether the PEO can give rise to myocardium is a subject of debate. Myocardial differentiation of PEO-derived cells had been reported *in vitro* but only when levels of FGF versus BMP signaling were manipulated (Kruithof *et al.*, 2006; Van Wijk *et al.*, 2009). Previous fate mapping studies in the chick or mouse had not shown any

proepicardial contribution to myocardium (Winter and Gittenberger-de Groot, 2007). Using a *Wt1* $^{GFP\text{-}Cre/+}$ line, the fate of PEO cells has been followed and, surprisingly, in addition to the expected derivatives, cardiomyocytes were detected in the walls of the cardiac chambers and in the interventricular septum (Zhou *et al.*, 2008a). Furthermore a subset of GFP-positive non-myocardial cells, isolated from foetal hearts, differentiated into myocardium in culture. In a parallel experiment, a *Tbx18-Cre* line was used to trace cells that had expressed this gene, and also resulted in the labeling of cardiomyocytes (Cai *et al.*, 2008). Labeling of the smooth muscle of coronary blood vessels and of cardiac fibroblasts was consistent with the observation that a subset of PEO cells express *Tbx18*. A caveat for all such genetic tracing experiments is that the interpretation depends on the expression of the *Cre* driver—whether this precisely reflects expression of the endogenous gene and whether the expression of the gene in question is restricted to the postulated progenitor source (see Christoffels *et al.*, 2009). In the case of *Tbx18*, there is later expression in the myocardium and notably in the interventricular septum, where many labeled cells were present in the genetic tracing experiment, which complicates the interpretation. However, as in the case of *Wt1*, dye-labeled epicardium resulted in labeled cardiomyocytes after culture, consistent with some myocardial contribution (Cai *et al.*, 2008; Zhou *et al.*, 2008a). The suggestion that PEO-derived cells can contribute to myocardium is important in a therapeutic context also, since the fibroblasts of the heart, which come from the epicardium, are a potential endogenous source of cardiac stem cells. Unlike the rare Islet1 positive cells present in the foetal heart (Laugwitz *et al.*, 2005), which are no longer present in the adult, the fibroblast population is maintained. An association between the epicardium and regeneration is demonstrated in the zebrafish heart where up-regulation of *Raldh2* in the epicardium, triggered by injury, leads to a retinoic acid activated cascade which results in the extensive cardiac repair that characterizes this organism (Lepilina *et al.*, 2006), although this is probably not due to direct formation of myocardium from epicardially derived cells (Jopling *et al.*, 2010; Kikuchi *et al.*, 2010).

5. Subdomains of the SHF

Genes that function within the genetic network of the SHF (Fig. 1.2) are not all expressed throughout this heart field. Cellular level resolution is lacking and even *Islet1* may not be expressed in all progenitors. The results of *Cre* tracing experiments tend to be interpreted in terms of onset of expression of the *Cre* driver, but heterogeneity between cells in the SHF may also affect the results and this is, of course, also the case for mutant

phenotypes. If information on expression/co-expression of SHF genes at the cellular level is still lacking, there is increasing evidence for the existence of subdomains of the SHF, distinguishable by gene expression and by their contribution to the heart (Fig. 1.4).

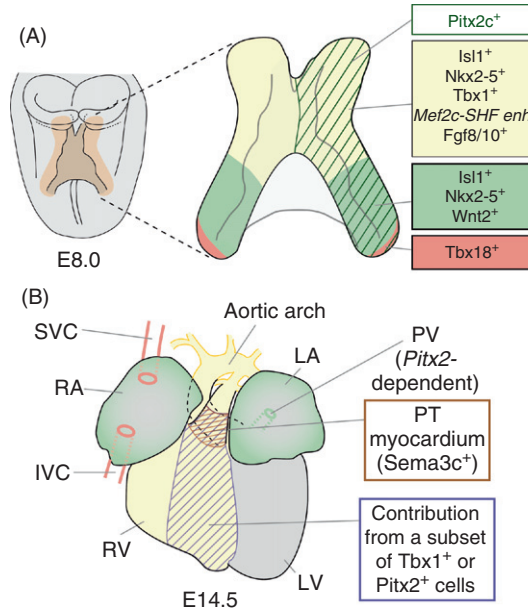

Figure 1.4 Subdomains within the SHF. (A) The SHF is characterized by the expression of Nkx2-5 and Islet1 shown at mouse embryonic day (E) 8.0. The anterior part of the SHF (light grey) is composed of cells that also express Tbx1, Fgf8, or Fgf10, and activate the *Mef2c*-SHF enhancer, whereas Wnt2 is expressed in addition to Nkx2-5 and Islet1 in the posterior SHF (mid-grey). At the most posterior and lateral side of the SHF resides a domain that is characterized by the expression of Tbx18 but not Nkx2-5 (dark grey). Pitx2c is expressed only on the left side of the SHF (diagonal stripes). (B) These different subdomains contribute to specific domains within the four-chambered heart shown at E14.5. Atrial myocardium contains cells that have expressed Islet1 and Nkx2-5 from the posterior SHF (mid-grey). The myocardium of the caval veins (IVC and SVC) is derived from cells that had expressed *Tbx18* (dark grey), whereas pulmonary vein (PV) myocardium is derived from cells of the posterior SHF (mid-grey) that also expressed Pitx2. Cells of the anterior part of the SHF contribute to the right ventricle (RV) and outflow tract myocardium. Interestingly, a subset of $Tbx1^+$ and $Pitx2^+$ cells contribute to a more centrally located region of the RV (stipled stripes), encompassing the interventricular septum domain, including the myocardium at the base of pulmonary trunk (PT) (darker diagonal stripes). This pulmonary trunk (PT) myocardium is characterized by the expression of *Sema3c* and is constituted from SHF progenitors that had responded to Shh. In *Tbx1* mutants, this domain is affected (see Section 6.3). RA, LA; right and left atria, respectively; RV, LV; right and left ventricles, respectively.

5.1. The anterior SHF and contributions to the arterial pole of the heart

The anterior part of the SHF is marked by expression of *Fgf8, Fgf10* (Kelly *et al.*, 2001), and *Tbx1* (Xu *et al.*, 2004) and cells that have transcribed these genes form the arterial pole of the heart (Fig. 1.4). The *Mef2c SHF* enhancer also functions here (Dodou *et al.*, 2004). Within this anterior region, subpopulations can be distinguished. Tbx1 controls the addition of cells that will constitute pulmonary trunk myocardium at the outlet of the right ventricle (Maeda *et al.*, 2006) and this is severely reduced in *Tbx1* mutant hearts (Théveniau-Ruissy *et al.*, 2008) where the SHF contribution to smooth muscle is also affected (Chen *et al.*, 2009). In human congenital heart disease, outflow tract alignment defects, such as tetralogy of Fallot, are frequent and probably result from a deficit in sub-pulmonary myocardium (Van Praagh, 2009). Further evidence that different progenitor cell populations form subpulmonary versus sub-aortic myocardium comes from retrospective clonal analysis and transgene expression profiles, which distinguish superior/inferior regions of the outflow tract and myocardium at the base of each artery (Bajolle *et al.*, 2008). The transgenes that mark different SHF domains and arterial pole derivatives reflect insertion site effects now shown to correspond to the *Sema3c* gene that marks sub-pulmonary myocardium (Théveniau-Ruissy *et al.*, 2008) and to the *Hes1* gene, also expressed in sub-aortic myocardium. *Hes1* mutants have outflow tract defects, leading to overriding aorta and ventricular septal defects (Rochais *et al.*, 2009; Van Bueren *et al.*, 2010). It has been suggested, as a result of experiments in the chick embryo, that the SHF contribution to smooth muscle, and probably also to myocardium at the base of the pulmonary trunk, spirals into the outflow tract from the right part of the SHF (Ward *et al.*, 2005). However, genetic tracing experiments in the mouse (see Section 6) suggest that cells that had expressed *Pitx2*, that marks the left side of the SHF, contribute to pulmonary trunk myocardium (Ai *et al.*, 2006). Interestingly cells that have expressed both *Pitx2* and *Tbx1* (Huynh *et al.*, 2007; Maeda *et al.*, 2006) also contribute to a central subdomain of the right ventricle extending into the interventricular septal region. The pulmonary trunk is further distinguished in fate mapping experiments which show it is derived from SHF cells that had received a sonic hedgehog (Shh) signal (Hoffmann *et al.*, 2009).

The anterior part of the SHF extends into the mesodermal core of the pharyngeal arches which gives rise to arterial pole myocardium or endothelial cells of the pharyngeal arch arteries (Section 4.1). In addition to myocardial versus endothelial cells derived from the mesodermal core of anterior compared to posterior pharyngeal arches, there are differences between progenitor populations that assume the same cell fate, but derive from different arches. This probably reflects differences in the pharyngeal environment which in turn reflects the progressive development of the

pharyngeal arches on the anterior/posterior axis. Thus, for example, the arterial derivatives of the fourth pharyngeal arch are particularly subject to interruption or hypoplasia. In mutants in which *Tbx1* is deleted in mesoderm, with consequent loss of pharyngeal arches 3–6 and failure of pharyngeal arch artery development, restoration of mesodermal *Tbx1* expression rescues most of these defects, but not the fourth pharyngeal arch phenotype (Zhang *et al.*, 2006), now shown to be due to *Gbx2* regulation by Tbx1 in the pharyngeal ectoderm (Calmont *et al.*, 2009). Gain of function *Tbx1* expression also specifically affects the fourth pharyngeal arch arteries as well as pulmonary trunk myocardium (Vitelli *et al.*, 2009). The mesodermal core of the anterior pharyngeal arches (1–3) not only contributes to arterial pole myocardium (1–2) or endothelial cells of pharyngeal arch arteries (3), but also to skeletal muscles of the head (Noden and Francis-West, 2006). These contributions have now been mapped for the first two arches in the chick embryo and an overlapping gradient of gene expression for skeletal myogenic versus SHF markers demonstrated, following a proximal/distal gradient within the core mesoderm (Nathan *et al.*, 2008; Tirosh-Finkel *et al.*, 2006). Genetic tracing experiments in the mouse indicate that cells that had expressed *Islet1*, for example, populate a subset of head muscles as well as giving rise to myocardium (Harel *et al.*, 2009; Nathan *et al.*, 2008). Again these results point to subdomains of the SHF, with different potential cell fates.

5.2. The posterior SHF and formation of the venous pole

Cells that contribute to the atria, at the venous pole of the heart, express Islet1, but not the anterior SHF markers (Cai *et al.*, 2003; Galli *et al.*, 2008) (Fig. 1.4). This posterior contribution of Islet1 positive cells to the atria and atrio-ventricular canal depends on Wnt2 signaling leading to *Gata6* activation in a feed-forward regulatory loop that is specific to this domain (Tian *et al.*, 2010). Furthermore, explant experiments suggest that this region of the SHF is programmed to assume an atrial fate (Galli *et al.*, 2008), whereas explants from the anterior region form right ventricular or outflow tract myocardium (Zaffran *et al.*, 2004). In the posterior domain, there is also evidence of regional heterogeneity, during the complex development of the venous pole (Anderson *et al.*, 2006). The dorsal mesenchymal protrusion (or *spina vestibuli*) is a morphologically distinct structure that contributes to the atrio-ventricular septum and undergoes a later myocardial transition (Mommersteeg *et al.*, 2006; Snarr *et al.*, 2007b). The mesodermal cells that form this structure are Islet1 positive and the dorsal mesenchymal protrusion, formed from the dorsal mesocardium is thought to be a SHF derivative (Meilhac *et al.*, 2004; Snarr *et al.*, 2007a). Its formation is particularly dependent on Shh signaling (Goddeeris *et al.*, 2008) (see Section 6.6).

As the venous pole develops, after formation of atrial myocardium (by E9.5), the *sinus venosus* develops with formation of myocardium around the caval veins. This is marked by the expression of *Tbx18*, and the absence of expression of *Nkx2-5*, both in the surrounding mesenchyme of the SHF, as well as the caval vein myocardium (Christoffels *et al.*, 2006). Tbx18 is required for the correct development of this part of the *sinus venosus* into caval vein myocardium. Explant experiments now confirm that these Tbx18 positive progenitor cells, that are Nkx2-5, and also Islet1, negative, form Nkx2-5 negative myocardium. In close proximity to these progenitors, Tbx18 negative, Islet1 positive cells contribute to pulmonary vein myocardium, with overlapping expression only at the lateral most border of these domains. Tbx18 positive cells are first detected caudal/lateral to the cardiac crescent (Fig. 1.1) and cell, as well as genetic, tracing experiments show that they subsequently contribute to the venous pole. Labeled cells were also seen in the PEO (Section 4.3). Interestingly, cells in the Tbx18 positive domain of cardiogenic mesoderm had initially expressed *Islet1* and *Nkx2-5*. The question, therefore, arises of whether this constitutes another heart field (Christoffels *et al.*, 2006; Mommersteeg *et al.*, 2010) and also of the relation with the PEO. Formal cell lineage studies will show whether there is an additional early lineage segregation, as seen for the first and second myocardial cell lineages (Meilhac *et al.*, 2004). However, the Tbx18 positive population clearly represents a distinct domain which contributes to a specific part of the venous pole and is characterized by the presence of a different transcriptional network from most of the posterior SHF. Recent results in the chick embryo suggest that *Tbx18* expressing cells of the PEO and subdomain of the second/third heart field derive from a common precursor pool and demonstrate how segregation to epicardial or myocardial lineages is promoted by FGF or BMP signaling, respectively, both in explants and *in vivo* (Van Wijk *et al.*, 2009).

6. Molecular Mechanisms that Govern SHF Cell Behavior–Transcriptional Regulators and Signaling Pathways

Signaling pathways and their interaction with transcriptional regulators underlie the patterning of the SHF, in terms of both its extent and its subdomains. The contribution and future cardiac identity of progenitor cells depends on the signals to which they are exposed. In the SHF, cells are maintained as proliferating, non-differentiated progenitors until they enter the heart tube. Indications of how regulatory networks (Fig. 1.2) and, particularly, signaling pathways affect SHF cell behavior are beginning to emerge (Fig. 1.5).

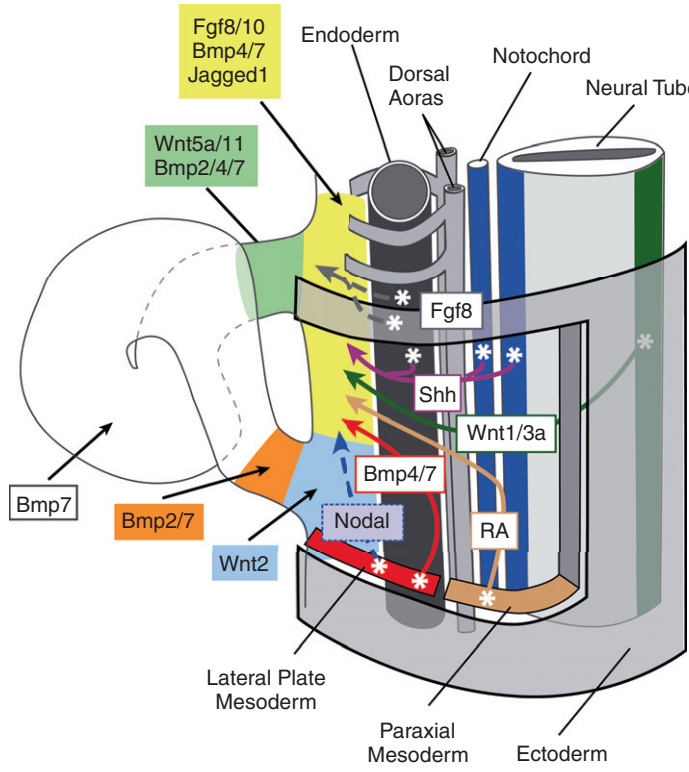

Figure 1.5 The SHF is a target of multiple signals. Schematic representation of a left-sided view of the heart region. The SHF itself is a source of signals: the Notch ligand Jagged1, is involved in the activation of *Fgf8*, but Notch signaling also interferes with canonical Wnt signaling, inhibiting progenitor proliferation. Fgf8 and Fgf10 are important for the promotion of SHF proliferation, whereas Bmp4/7 is required for the survival of the cNCCs. Bmp2, which is repressed by Nkx2-5 and also by canonical Wnt signaling, inhibits SHF proliferation. In the posterior SHF, Wnt2 controls the contribution of venous pole progenitors. Fgf8 also comes from the pharyngeal endoderm and ectoderm (grey arrow). The outflow tract is also a source of signals for the SHF: Bmp2 has been shown to be required for the deployment of SHF cells. Non-canonical Wnt11 and Wnt5a do not affect the SHF, but control outflow tract maturation. The midline is another signaling source. Shh (purple arrow), from the endoderm (in dark grey) (but also from the notochord and the floorplate, in blue), affects arterial pole formation, probably through an effect on SHF proliferation, and is required for the formation of the atrial septum at the venous pole. Also coming from the midline, canonical Wnts (Wnt1, Wnt3a, green arrow) from the dorsal neural tube (green domain) are important for the maintenance of proliferating progenitors within the SHF and inhibition of differentiation. On the contrary, lateral signals such as Bmp4 (red arrow) from the lateral plate mesoderm (red domain) promote cardiac differentiation. The paraxial mesoderm (posterior to the SHF, in brown) is a source of retinoic acid (RA, brown arrow) that is important in the early stages of cardiogenesis for anterior/posterior patterning and limiting the posterior boundary of the SHF. On the left side, and only between E8 and E8.5, Nodal (dashed blue arrow) activates *Pitx2c* asymmetrically on the left side of the SHF. Asymmetric *Pitx2c* expression is maintained independently of Nodal and affects both the arterial and venous poles of the heart (see Section 6.2). (See Color Insert.)

6.1. Anterior/posterior patterning of the SHF: retinoic acid signaling

The patterning of the SHF on the anterior/posterior axis depends on retinoic acid signaling, as indicated by the abnormal posterior expansion in expression of anterior SHF marker genes, such as *Tbx1, Fgf8*, and *Fgf10* in *Raldh2* mutant embryos (Ryckebusch *et al.*, 2008; Sirbu *et al.*, 2008). In avian embryos, vitamin A deficiency, or over-expression, of retinoic acid also up- or down-regulates *Tbx1* expression, respectively (Roberts *et al.*, 2006). Reduction in retinoic acid synthesis results in many of the phenotypic features of DiGeorge syndrome at the arterial pole of the heart, in which *TBX1* is implicated, and analysis of compound *Raldh2/Tbx1* mouse mutants indicates that decrease in levels of retinoic acid accelerates the recovery from arterial growth delay seen in *Tbx1*$^{-/-}$ embryos (Ryckebüsch *et al.*, 2010; Vermot *et al.*, 2003). This genetic interaction also operates in *Tbx1* mutant embryos in which the *Raldh2* expression domain is moved anteriorly and enzymes that degrade retinoic acid are down-regulated (Guris *et al.*, 2006; Ivins *et al.*, 2005; Liao *et al.*, 2008; Roberts *et al.*, 2006). In *Raldh2* mutants the posterior domain of *Islet1* expression in the SHF is also abnormally extended caudally (Ryckebusch *et al.*, 2008; Sirbu *et al.*, 2008), indicating that not only the patterning, but also the limits of the SHF are affected by retinoic acid.

In the zebrafish embryo, retinoic acid has been shown to control the size of the cardiac field (Keegan *et al.*, 2005). Retinoic acid levels regulate anterior/posterior expression of genes in the *Hox* clusters, which are potential effectors of retinoic acid signaling in the SHF. In zebrafish, this scenario is complex since indirect effects of Hox5b, acting in the forelimb field, downstream of retinoic acid signaling, may control signals that affect the number of atrial progenitors (Waxman *et al.*, 2008). In the mouse *Raldh2* mutant, despite the expansion of SHF marker expression, the heart tube fails to grow which may reflect the fact that although more cells express SHF genes, the level of expression is reduced (Ryckebusch *et al.*, 2008). A related phenomenon is seen in retinoic acid receptor mutants where the distal outflow tract fails to form. This late phenotype is associated with a marked reduction in *Mef2c* expression and genetic tracing with the *Mef2c SHF* enhancer (*Cre*) shows that this subpopulation of cells is strongly reduced and does not enter the heart, whereas *Islet1* and *Fgf8* expression in the SHF appears normal (Li *et al.*, 2010). This effect of interference in retinoic acid signaling on activation of the *Mef2c SHF* enhancer, probably mediated through Gata4, with failure to form the distal part of the arterial pole, provides another example of regulatory subdomains of the SHF (see Section 5).

6.2. Left/right patterning: Nodal signaling through Pitx2c

The SHF is also patterned on the left/right axis by the Nodal signaling pathway which leads to the activation of *Pitx2c* in the left side of the SHF. This transcription factor also has a dynamic expression pattern in the heart itself, where it is important for cardiac remodeling during development (Tessari *et al.*, 2008). Interference with this left/right signaling has a striking effect on cardiac morphogenesis, and asymmetry, exemplified by right atrial isomerism (see Franco and Campione, 2003; Tessari *et al.*, 2008), but interestingly *Pitx2* mutants still undergo cardiac looping and indeed signals that drive this striking aspect of asymmetric development remain poorly understood. In the posterior domain of the left SHF, where it is strongly expressed, Pitx2c functions to repress the acquisition of right atrial identity, followed with a transgenic marker in explant cultures, as well as *in vivo* (Galli *et al.*, 2008). Pitx2c also represses proliferation in the left *sinus venosus,* but not detectably in the left SHF. It is not clear whether these are direct effects, since the function of Pitx2 as a transcriptional activator or repressor, and its transcriptional targets, are poorly understood at present. At later stages in the posterior SHF, Pitx2 is required to initiate the formation of pulmonary vein myocardium, but the identity of this myocardium appears to depend on Nkx2-5, since in *Nkx2-5* mutants markers of caval vein myocardium begin to be expressed in pulmonary vein myocardium (Mommersteeg *et al.*, 2007). Here, too, the molecular role of Pitx2 and the nature of its interaction with Nkx2-5 are obscure.

In addition to its role at the venous pole, Pitx2c also plays a role in outflow tract development. By genetic lineage tracing and cell type specific conditional mutation, it is now clear that this is not due to a primary effect on neural crest cells, but due to the function of Pitx2c in the left anterior SHF and its derivatives (Ai *et al.*, 2006). Once the outflow tract is formed in the mouse embryo, it undergoes rotation, essential for the final juxtaposition of the great arteries, and this depends on Pitx2c (Bajolle *et al.*, 2006). Spiraling of the outflow tract structure and consequent rotation of the aortic sac, which affects the initially symmetrical blood flow, has been further investigated in *Pitx2* mutants, where Pitx2 indirectly induced asymmetric signaling, through PDGF and VEGF2 receptors, points to downstream mechanisms (Yashiro *et al.*, 2007). Genetic interaction between *Pitx2* and *Tbx1* has been demonstrated, with the suggestion that in the anterior SHF, *Pitx2* expression requires *Nkx2-5* and *Tbx1*, transitorily expressed on the left side (Nowotschin *et al.*, 2006). Foxh1, another transcription factor implicated in the development of the anterior SHF (von Both *et al.*, 2004), functions in the Nodal signaling pathway. It has now been shown that mutations in *FOXH1* and other mutants of this pathway are linked to arterial pole defects in the human population (Roessler *et al.*, 2008).

Sonic hedgehog signaling is involved in the establishment of left/right asymmetry and characterization of cardiac defects in the *Shh* mutant mouse

shows a phenotype at the arterial pole similar to tetralogy of Fallot, with complete pulmonary trunk atresia (Washington Smoak *et al.*, 2005), which may result from an asymmetric effect on the SHF. Furthermore, left atrial isomerism, with bilateral *Pitx2* expression, is seen in the *Shh* mutant embryo, in which the heart remains attached to the dorsal mesocardium (Hildreth *et al.*, 2009).

6.3. Maintenance of proliferation in the SHF: FGF, hedgehog, and canonical Wnt signaling

A number of signaling pathways that impact the SHF have been shown to promote proliferation. This is the case for FGF signaling. Fgf8 is produced by the mesodermal cells of the SHF, and also by endoderm, and ectoderm of the pharyngeal arches. *Fgf8* mutants do not gastrulate, but *Fgf8* hypomorphs have demonstrated its importance in arterial pole development, with defects that ressemble those in *Tbx1* mutants (Abu-Issa *et al.*, 2002; Frank *et al.*, 2002). Mutation of *Fgf8* in the *Tbx1* expressing domains of the SHF and endoderm/ectoderm of the arches results in phenotypes that suggest that *Fgf8* lies downstream of *Tbx1* and indeed a regulatory element on the *Fgf8* locus is directly activated by Tbx1 (Hu *et al.*, 2004). However, targeting of an *Fgf8* coding sequence to the *Tbx1* gene shows that, whereas Fgf8 can replace Tbx1 for many aspects of pharyngeal arch artery formation (Brown *et al.*, 2004), Fgf8 and Tbx1 play independent roles in outflow tract development. Notably Tbx1, but not Fgf8, is crucial in a *Hoxa3* expressing domain of the pharynx which includes the mesoderm of the anterior SHF (Vitelli *et al.*, 2006). Conditional mutants, in which *Fgf8* deletion has been targeted to specific expression domains, clarify its role in pharyngeal arch artery and in outflow tract development (Ilagan *et al.*, 2006; Macatee *et al.*, 2003; Park *et al.*, 2006). In addition to effects on myocardial derivatives, targeting *Fgf8* deletion to *Tbx1* expressing cells with a *Tbx1-Cre* shows its role in the SHF cells that will form smooth muscle (Brown *et al.*, 2004). Mesodermal *Fgf8* expression is clearly important for SHF development. *Fgf10* is also expressed in the SHF, but *Fgf10* mutants do not demonstrate an SHF phenotype (Marguerie *et al.*, 2006). However, mesodermal *Fgf8/Fgf10* compound mutants display increasingly severe pharyngeal artery and outflow tract defects, as alleles of *Fgf8* and *Fgf10* are removed. This shows that the SHF contribution to the arterial pole, which depends on maintenance of proliferation is very sensitive to FGF dosage (Watanabe *et al.*, 2010). Conditional deletion in cardiac mesoderm of *Fgfr1* or *Fgfr2*, which encode the principal receptors of Fgf8 and Fgf10, or of *Frs2α* which encodes an adaptor protein that links FGFR to MAPK and P13K signaling cascades within the cell, as well as conditional over-expression of Sprouty that interferes with this intracellular signaling, demonstrates an autocrine requirement for FGF signaling in the SHF (Park *et al.*, 2008; Zhang *et al.*,

2008). In these different genetic situations in the mouse embryo, and in chick, FGF signaling is required for SHF proliferation. In zebrafish embryos, a reduction in FGF signaling reduces the number of cardiomyocytes, particularly in the ventricle (where Fgf8 is expressed), and an ectopic increase in FGF increases the number of ventricular and atrial cardiomyocytes prior to cardiac tube differentiation (Marques *et al.*, 2008). In *Xenopus*, FGF from anterior neural ectoderm increases the extent of *Nkx2-5* expression in mesoderm, rendering it cardiogenic (Keren-Politansky *et al.*, 2009). In these models, FGF signaling, probably through its effects on proliferation, increases the extent of the heart field and consequent numbers of cardiomyocytes.

Hedgehog (Hh) signaling is also often associated with proliferation. A number of defects at the venous as well as at the arterial pole have been documented when Shh signaling from endoderm is abrogated (see Section 6.6; Goddeeris *et al.*, 2007; Hoffmann *et al.*, 2009). In the mouse model it is not clear that this is due to an effect on SHF proliferation; however, in the chick embryo, Shh signaling is clearly important for maintaining progenitor cell proliferation in the critical time frame which precedes addition of cells to the heart tube (Dyer and Kirby, 2009).

Canonical Wnt signaling, in addition to playing earlier roles in mesoderm development and negative modulation of cardiac specification, also promotes progenitor cell proliferation, as evidenced by work with ES cultures and embryo model systems (see Cohen *et al.*, 2008). Wnt/β-catenin signaling is active in the SHF and specific deletion of the gene encoding β-catenin in cardiac mesoderm leads to right ventricular and outflow tract hypoplasia, probably due to a reduction in SHF proliferation (Ai *et al.*, 2007; Cohen *et al.*, 2007; Klaus *et al.*, 2007; Kwon *et al.*, 2007; Lin *et al.*, 2007; Qyang *et al.*, 2007). SHF marker expression is reduced, including transcripts of *Islet1, Fgf10*, and *Shh*, suggesting an upstream role for canonical Wnt signaling, although this also reflects the reduction in cell number. β-Catenin can directly activate transcription from *Islet1* and *Fgf10* promoters (Cohen *et al.*, 2007; Lin *et al.*, 2007), but the significance of this is not demonstrated *in vivo*. Complementary gain of function experiments, by LiCl treatment and conditional expression of stabilized β-catenin, results in expansion of SHF progenitors. Transcriptome analysis of these Islet1 positive cells shows up-regulation of *Fgfs*, which will promote proliferation, when canonical Wnt signaling is increased (Kwon *et al.*, 2009).

A caveat for genetic experiments that depend on manipulation of β-catenin is that this has a second function in cell adhesion, which may also influence the SHF phenotypes. Of Wnt ligands potentially involved in autocrine signaling within the SHF, only Wnt2 expressed in the posterior SHF has been shown, in concert with β-catenin, to directly regulate proliferation of venous pole progenitors (Tian *et al.*, 2010).

A link between Notch signaling and the canonical Wnt/β-catenin pathway has been demonstrated (Kwon *et al.*, 2009). Conditional deletion

of *Notch1*, with an *Islet1-Cre* line, promoted proliferation of Islet1 positive progenitors in the SHF at the expense of cardiogenesis, with absence of the arterial pole, including the right ventricle, as had been seen when β-catenin is over-expressed (Cohen *et al.*, 2007). In the absence of Notch1, higher levels of stabilized β-catenin and increased Wnt/β-catenin signaling were observed, suggesting that Notch signaling normally represses cardiac progenitor proliferation by negatively regulating the active form of β-catenin (Kwon *et al.*, 2009).

6.4. Interactions with neural crest in the SHF: FGF, Notch, and semaphorin signaling

FGF signaling in the SHF was thought to directly affect neural crest. However, targeted deletion, with a neural crest (*Pax3*)*Cre* line, of conditional mutants for the *Fgfr1* and *Fgfr2* genes or for *Frs2α* which encodes an FGFR adaptor protein, does not result in any arterial pole defects, in contrast to deletion in the SHF. This is also the case when Sprouty, the intracellular inhibitor of FGF signaling is up-regulated in neural crest cells (Park *et al.*, 2008; Zhang *et al.*, 2008). In mesodermal *Fgf8* mutants, BMP/TGFβ signaling is down-regulated in the SHF and it is proposed that this pathway normally provides a relay that then affects neural crest (Park *et al.*, 2008), which requires Smad4 mediated signaling for survival (Nie *et al.*, 2008). In addition to Fgf8 and Fgf10, Fgf15 is also implicated in outflow tract development. It is expressed, independently of Tbx1, in pharyngeal arch mesoderm (arch 3) and in the anterior SHF. In *Fgf15* mutants, outflow tract morphogenesis is abnormal, and this is probably at least partly due to effects on neural crest (Vincentz *et al.*, 2005). Neural crest also feeds back on FGF signaling in the SHF, since in chick embryos neural crest ablation results in an increase in Fgf8 which perturbs SHF development and this can be rescued when the level of Fgf8 is reduced (Hutson *et al.*, 2006). An increase in FGF signaling might be expected to increase the cardiac progenitor pool at the expense of differentiation; however, in *Splotch*^{2H} mutant embryos, where neural crest migration is reduced, ectopic myocardial differentiation is observed (Bradshaw *et al.*, 2009), suggesting perturbation of additional SHF regulators in this case.

Interference with Notch signaling in neural crest cells results in arterial pole and arch artery phenotypes (High *et al.*, 2008, 2007; Varadkar *et al.*, 2008). Manipulation, using *Islet1*- or *Mef2-Cre* lines of a dominant-negative form of Mastermind-like (MAML), a co-activator, of the Notch transcriptional complex, interferes with Notch signaling in the SHF and results in arterial pole defects (High *et al.*, 2009), probably also partly due to effects on neural crest. Similar effects are seen when the gene encoding for Jagged1 is conditionally deleted, indicating that this is the principal Notch ligand in

the SHF. Jagged1 is also produced by endothelial derivatives of the SHF, with important consequences for neural crest derived smooth muscle differentiation (High and Epstein, 2008). Interference with Notch signaling correlates with a decrease in BMP signaling, which in turn can be explained (Park *et al.*, 2008) by an observed down-regulation of *Fgf8* in the pharyngeal region (High *et al.*, 2009). Changes in the expression of other SHF marker genes, such as *Islet1, Fgf10*, or *Tbx1*, are not observed suggesting that this is a specific effect of Notch signaling acting upstream of *Fgf8*. Thus in addition to a direct effect of Notch signaling in the SHF on neural crest, there is also probably an indirect effect through Fgf8 which then affects BMP signaling.

Semaphorin signaling, functioning through Plexin receptors, is important for neural crest migration. Semaphorin3C which is expressed in a subdomain of the SHF and marks pulmonary trunk myocardium (Théveniau-Ruissy *et al.*, 2008), is required for normal neural crest migration into the arterial pole of the heart (Brown *et al.*, 2001; Feiner *et al.*, 2001). *Sema3C* is directly regulated by Gata6 in smooth muscle derived from neural crest, where it is also expressed (Lepore *et al.*, 2006); *GATA6* mutations in humans have been linked to arterial pole malformations (Kodo *et al.*, 2009). In the mouse embryo, PlexinD1, which interacts with Semaphorin3C, and PlexinA2, both expressed in cardiac neural crest, are required for correct outflow tract development (Toyofuku *et al.*, 2008). PlexinD1 is also expressed in endocardial and endothelial cells (Gitler *et al.*, 2004) and its expression in these Tie2 positive cells is essential for arterial pole development (Zhang *et al.*, 2009).

6.5. Prevention of differentiation in the SHF: canonical Wnt signaling and transcriptional repression

In addition to its role in promoting proliferation in the SHF there is evidence that canonical Wnt signaling also prevents the onset of differentiation. This was suggested by experiments in the *Xenopus* embryo where cardiac differentiation is negatively affected, with inhibition of Gata4/6 and Nkx2-5 expression (Lavery *et al.*, 2008). In experiments in which canonical Wnt signaling is increased by conditional expression of stabilized β-catenin (Kwon *et al.*, 2009), maintenance of Islet1 positive cells in the outflow tract would be in keeping with a delay in cardiac differentiation. Further analysis of genes expressed by Islet1 positive cells in which β-catenin is stabilized shows down-regulation of the gene encoding Myocardin which, together with SRF, promotes myocardial and smooth muscle differentiation, and also of the repressor Bhlhb2, shown to be a direct Wnt/β-catenin target (Fig. 1.2). However, surprisingly, down-regulation of the level of *Islet1* expression was also observed. Because of its presence in the SHF, Islet1 had been associated with proliferation; however, it may, at least when expressed at a high level, promote differentiation. Manipulation of Islet1 in the ES cell

system suggests that it can promote myocardial differentiation, and furthermore Islet1 directly activates a regulatory element of the *Myocardin* gene. This may explain why maintenance of Islet1 in cardiomyocytes in the *Nkx2-5* mutant (Prall *et al.*, 2007) is compatible with differentiation. The importance of differences in levels of Islet1 in determining its effects on differentiation or proliferation is further supported by manipulation of stabilized β-catenin versus Islet1 in ES-derived cardiac progenitors, leading to the conclusion that a low level of Islet1 is required for Wnt/β-catenin mediated proliferation of these cells (Kwon *et al.*, 2009).

It is possible that differences in function, according to the level of expression, may also hold for factors like Nkx2-5 or Mef2c, present both in the SHF and in the heart where they play a role in activating myocardial genes. In this context, modulation or prevention of expression of genes encoding factors that can promote differentiation will be critical for maintaining the progenitor cell pool. Tbx1 represses *SRF* and *Tbx5* expression in the SHF, since these genes, which are implicated in myocardial differentiation, are up-regulated in *Tbx1* mutants (Liao *et al.*, 2008).

6.6. Regulation of SHF differentiation potential, recruitment to the heart tube, and differentiation: Shh, Notch, BMP, and non-canonical Wnt signaling

A number of signaling pathways affect subdomains of the SHF (Section 5). Whereas they may primarily exert an effect on proliferation, as in the case of FGF signaling in the anterior SHF, in other cases it is beginning to be evident that they exert effects on myocardial differentiation potential, in terms of the SHF contribution to a region of the heart. At the arterial pole, Shh from the endoderm affects pharyngeal arch mesoderm and *Tbx1* expression (Yamagishi *et al.*, 2003), with additional effects on the maintenance and deployment of neural crest (Goddeeris *et al.*, 2007; Washington Smoak *et al.*, 2005). In the absence of Shh, pharyngeal vasculature as well as outflow tract development is affected (Kolesová *et al.*, 2008). The effect of Shh on *Tbx1* expression has particular implications for the formation of pulmnary trunk myocardium (Théveniau-Ruissy *et al.*, 2008). In the chick embryo, manipulation of Shh signaling affects migration of SHF cells into the outflow tract (Dyer and Kirby, 2009) suggesting that it is also important for SHF recruitment. In the absence of Shh signaling in the posterior SHF, there are specific venous pole defects. The source of the signal is the underlying pulmonary endoderm, where Islet1 is also present and is required for Shh expression (Lin *et al.*, 2006). Development of the dorsal mesocardial protrusion and subsequent formation of the primary atrial septum depend on this signaling pathway (Goddeeris *et al.*, 2008; Hoffmann *et al.*, 2009). Observations on the dorsal myocardium suggest that Shh plays

a role in the specification of atrial septal precursors and in the recruitment of cells from this part of the SHF. A role for Shh in cardiac cell specification is in keeping with observations in P19 embryonic carcinoma cells. In the absence of primary cilia, which are intimately related to Hh signaling, myocardial derivatives are compromised in these cells. Furthermore mouse mutants that lack cilia have cardiac abnormalities (Clement *et al.*, 2009; Slough *et al.*, 2008). During cardiogenesis in the zebrafish embryo, Hh signaling promotes cardiomyocyte formation as well as regulating the number of cardiac progenitor cells (Thomas *et al.*, 2008), pointing to a dual role.

Notch signaling is an important regulator of many developmental processes, with a spectrum of effects on cell behavior. Modification of this pathway in the heart leads to cardiac phenotypes which reflect the variety of its roles (High and Epstein, 2008). Components of the pathway are present in the SHF. Among Notch functions are regulation of cell fate choices and of the decision to differentiate rather than proliferate. In cells of the anterior SHF, a role in myocardial versus smooth muscle fates, for example, has not yet been demonstrated. However, effects on myocardialization of the outflow tract have been reported after interference with Notch signaling, by manipulation in the SHF of a dominant-negative form of MAML (see Section 6.4) (High *et al.*, 2009). MAML can interact with Mef2c (Shen *et al.*, 2006), which may complicate the interpretation. However, similar AP defects are seen when the gene for *Jagged1* is conditionally deleted, supporting the role of Notch in promoting differentiation.

BMP signaling promotes cardiac specification and myocardial differentiation, as established in classic experiments on the chick embryo (see Schultheiss *et al.*, 1997), and also more recently for mesoderm in the core of the anterior pharyngeal arches which can form cardiac or skeletal muscle (Tirosh-Finkel *et al.*, 2006). The overall picture (Fig. 1.5) in which SHF progenitors lie medially to the differentiating cardiac crescent, at a distance from sources of BMP emanating from lateral mesoderm, is consistent with their maintenance in an undifferentiated state. Medially produced signals, such as canonical Wnts from the neural tube, will promote proliferation (Section 6.1). Mouse mutant phenotypes demonstrate the importance of BMP signaling for heart development. Deletion of *Bmpr1a*, encoding the BMP type 1 receptor, in early cardiac mesoderm results in failure to form a differentiating cardiac tube, although Islet1 positive cardiac progenitors are present (Klaus *et al.*, 2007). Later conditional deletion in *Islet1* expressing cells results in an abnormal right ventricle and outflow tract where increased numbers of Islet1 positive cells suggest a differentiation defect, since *Islet1* is normally down-regulated in cardiomyocytes. This is accompanied by a reduction in Tbx20, required to repress *Islet1* in the outflow tract (Yang *et al.*, 2006). Bmp4 is required for outflow tract development (McCulley *et al.*, 2008), where it probably affects myocardium formation, as well as the survival of neural crest (Nie *et al.*, 2008). Outflow tract elongation is

reduced in embryos lacking both Bmp4 and Bmp7 (Liu *et al.*, 2004) and Bmp2, acting with Bmp4, is also implicated in cardiac development (Uchimura *et al.*, 2009). Bmp2 and Bmp4, expressed by the outflow tract myocardium, are candidate molecules for inducing differentiation of SHF progenitors at the arterial pole of the heart tube and it has also been proposed, from experiments in the chick embryo, that they play an additional role in the recruitment of these cells (Somi *et al.*, 2004; Waldo *et al.*, 2001).

Bmp4, together with other genes for BMP/TGFβ family members, are targets of FGF signaling in the SHF (Park *et al.*, 2008), where Wnt/β-catenin signaling also up-regulates *Bmp4* (Ai *et al.*, 2007) (Fig. 1.2). This might, at first sight, seem contradictory, since these signaling pathways promote proliferation in the SHF where maintenance of an undifferentiated state is important. However, the levels of expression are probably critical and different signaling thresholds may be required for an effect on proliferation or myocardial differentiation. A balance between FGF/BMP had been shown to be important in this context (Barron *et al.*, 2000). The importance of regulating the level of TGFβ/BMP signaling within the SHF is demonstrated by the role of Nkx2-5 which represses *Bmp2* expression. In the absence of Nkx2-5, there is cardiac over-specification and a reduction in proliferating SHF progenitors leading to outflow tract truncation. The SHF effect of Bmp2 is mediated by Smad1. In mesodermal *Smad1* mutants SHF proliferation and outflow tract length are increased; furthermore the *Nkx2-5* phenotype is alleviated on loss of *Smad1* alleles, demonstrating the presence of an Nkx2-5/Bmp2/Smad1 negative feedback loop that controls progenitor specification and proliferation in the SHF (Prall *et al.*, 2007). Tbx1 is another SHF transcriptional regulator that can affect BMP/TGFβ signaling, in this case by direct interaction with Smad1 (Fulcoli *et al.*, 2009).

Non-canonical Wnt signaling is associated with cardiac differentiation, as shown when Wnt5a, in combination with the canonical Wnt inhibitor Dkk-1, is added to stromal vascular cells (Palpant *et al.*, 2007). *In vivo* in lower vertebrates, non-canonical Wnt signaling induces cardiac specification as shown for Wnt11 in *Xenopus* embryos (Pandur *et al.*, 2002). In the mouse embryo, *Wnt5a* mutants have arterial pole defects, but these may be due to effects on neural crest, through PlexinA2 down-regulation (Schleiffarth *et al.*, 2007). *Wnt11* null mice have arch artery patterning and outflow tract defects (Zhou *et al.*, 2007) also seen with *Dishevelled* mutants (Etheridge *et al.*, 2008). However, expression of Islet1 and other markers is normal and this is probably not a SHF phenotype. Wnt11, expressed at a high level in the mouse outflow tract, is implicated in polarized cell behavior required for correct arterial pole development (Phillips *et al.*, 2005, 2007). Wnt11 mediated effects on cardiomyocyte organization are also important for ventricular myocardium development (Nagy *et al.*, 2010). *Wnt11* participates in a gene regulatory network in

which it is a direct target of Pitx2 and of canonical Wnt/β-catenin signaling, whereas *Tgfβ2* is a target of Wnt11 acting through ATF/CREB. *Wnt11, Vangl2, Scribble*, and *TGFβ2* mutants have a similar outflow tract phenotype suggesting that TGFβ2 is implicated in the cellular polarization required for outflow tract development (Zhou *et al.*, 2007).

7. Conclusion

Studies on the mouse embryo have provided extensive information about mutant phenotypes which affect different parts of the heart. The existence of subdomains within the SHF is now emerging, with potentially important consequences for the diagnosis and prognosis of human congenital heart disease as well as for the fundamental understanding of cardiogenesis. The developmental and regulatory history of these cell populations raises many of the same questions as do the first and second heart fields, in terms of lineage, cell behavior and gene networks. Complex genetic networks for transcriptional regulators and signaling pathways are documented for the SHF, but their spatiotemporal importance is poorly understood. Apparently confusing observations on the interactions between signaling pathways and effects on proliferation or differentiation probably partly reflect the lack of definition in terms of the location of subpopulations and developmental timing. Indications of the importance of the latter come from observations on SHF cell fate choices, illustrated by the addition of smooth muscle to the outflow tract after the myocardial contribution.

Quantitative data are also mainly lacking and are probably very important in determining the impact of a signaling pathway or a transcriptional regulator. The level of BMP/TGFβ signaling in the SHF required for neural crest survival, for example, is probably not the same as the level that promotes myocardial differentiation. The importance of gene dosage can be approached by study of an allelic series, as in the case of *Fgf8/Fgf10*, where progressively more severe phenotypes point to the underlying importance of FGF signaling on SHF proliferation. However, in this example, quantitative effects on interacting factors and signaling pathways in the FGF network have not yet been evaluated. The suggestion that higher levels of Islet1 promote differentiation rather than SHF proliferation illustrates the importance of quantitative considerations. Moving from a qualitative to a quantitative level of description and extending this to the cellular level is a major challenge for developmental biology in general. The cellular and molecular mechanisms that underlie the behavior of cardiac progenitor cells are also poorly understood at present. Here the challenge is to distinguish regulatory circuits, within the cells, that promote proliferation, prevent or promote differentiation, direct cell fate choices and affect cell interactions and cell movement. Dissection of regulatory

outcomes, through a pathway like that of canonical Wnt signaling, is beginning to provide insight at this level. In addition to analysis of mutant phenotypes in the embryo, model cell systems, such as cardiogenic progenitors derived from ES cells, facilitate regulatory analysis, into the role of Wnt signaling for example. Of course, such cell culture systems present an artificial situation, with the risk that selection of a progenitor cell type or conjunction of regulatory inputs is a product of the *in vitro* system rather than a major component of the *in vivo* situation. Verification of a hypothesis, in the *in vivo* embryonic context, is often difficult, but essential. Explants, of regions of the SHF, for example, can provide a useful intermediate state for regulatory studies.

Analysis of outcomes at a single cell level in the SHF will depend on resolutive imaging techniques and genetically manipulable fluorescent probes. This technology is developing rapidly and provides a complementary approach to high-throughput analyses of gene expression or regulation which is also becoming practicable at the level of isolated single cells. Integrating this information for cardiac progenitor cell behavior in normal and mutant, or experimentally perturbed, contexts will depend on sophisticated modeling. In the long term, the aim is to transform the current crude level at which cardiogenesis is perceived into a precise spatiotemporal map of cardiac progenitor cell regulation that predicts behavior.

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REFERENCES

Aanhaanen, W. T. J., Brons, J. F., Domínguez, J. N., Rana, M. S., Norden, J., Airik, R., Wakker, V., de Gier-de Vries, C., Brown, N. A., Kispert, A., Moorman, A. F. M. and Christoffels, V. M. (2009). The tbx2+ primary myocardium of the atrioventricular canal forms the atrioventricular node and the base of the left ventricle. *Circ. Res.* **104**, 1267–1274.

Abu-Issa, R. and Kirby, M. L. (2007). Heart field: from mesoderm to heart tube. *Annu. Rev. Cell. Dev. Biol.* **23**, 45–68.

Abu-Issa, R. and Kirby, M. L. (2008). Patterning of the heart field in the chick. *Dev. Biol.* **319**, 223–233.

Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K. and Meyers, E. N. (2002). Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development* **129**, 4613–4625.

Ai, D., Fu, X., Wang, J., Lu, M. F., Chen, L., Baldini, A., Klein, W. H. and Martin, J. F. (2007). Canonical wnt signaling functions in second heart field to promote right ventricular growth. *Proc. Natl. Acad. Sci. USA* **104**, 9319–9324.

Ai, D., Liu, W., Ma, L., Dong, F., Lu, M.-F., Wang, D., Verzi, M. P., Cai, C., Gage, P. J., Evans, S., Black, B. L., Brown, N. A. and Martin, J. F. (2006). Pitx2 regulates cardiac left-right asymmetry by patterning second cardiac lineage-derived myocardium. *Dev. Biol.* **296**, 437–449.

Anderson, R. H., Brown, N. A. and Moorman, A. F. M. (2006). Development and structures of the venous pole of the heart. *Dev. Dyn.* **235**, 2–9.

Bajolle, F., Zaffran, S., Kelly, R. G., Hadchouel, J., Bonnet, D., Brown, N. A. and Buckingham, M. E. (2006). Rotation of the myocardial wall of the outflow tract is implicated in the normal positioning of the great arteries. *Circ. Res.* **98**, 421–428.

Bajolle, F., Zaffran, S., Meilhac, S. M., Dandonneau, M., Chang, T., Kelly, R. G. and Buckingham, M. E. (2008). Myocardium at the base of the aorta and pulmonary trunk is prefigured in the outflow tract of the heart and in subdomains of the second heart field. *Dev. Biol.* **313**, 25–34.

Barron, M., Gao, M. and Lough, J. (2000). Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. *Dev. Dyn.* **218**, 383–393.

Bondue, A., Lapouge, G., Paulissen, C., Semeraro, C., Iacovino, M., Kyba, M. and Blanpain, C. (2008). Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* **3**, 69–84.

Brade, T., Gessert, S., Kühl, M. and Pandur, P. (2007). The amphibian second heart field: xenopus islet-1 is required for cardiovascular development. *Dev. Biol.* **311**, 297–310.

Bradshaw, L., Chaudhry, B., Hildreth, V., Webb, S. and Henderson, D. J. (2009). Dual role for neural crest cells during outflow tract septation in the neural crest-deficient mutant Splotch(2H). *J. Anat.* **214**, 245–257.

Brown, C. B., Feiner, L., Lu, M. M., Li, J., Ma, X., Webber, A. L., Jia, L., Raper, J. A. and Epstein, J. A. (2001). PlexinA2 and semaphorin signaling during cardiac neural crest development. *Development* **128**, 3071–3080.

Brown, C. B., Wenning, J. M., Lu, M. M., Epstein, D. J., Meyers, E. N. and Epstein, J. A. (2004). Cre-mediated excision of fgf8 in the tbx1 expression domain reveals a critical role for fgf8 in cardiovascular development in the mouse. *Dev. Biol.* **267**, 190–202.

Bruneau, B. G. (2008). The developmental genetics of congenital heart disease. *Nature* **451**, 943–948.

Bu, L., Jiang, X., Martin-Puig, S., Caron, L., Zhu, S., Shao, Y., Roberts, D. J., Huang, P. L., Domian, I. J. and Chien, K. R. (2009). Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature* **460**, 113–117.

Buckingham, M., Meilhac, S. and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat. Rev. Genet.* **6**, 826–835.

Bussmann, J., Bakkers, J. and Schulte-Merker, S. (2007). Early endocardial morphogenesis requires scl/tal1. *PLoS Genet.* **3**, e140.

Cai, C.-L., Liang, X., Shi, Y., Chu, P.-H., Pfaff, S. L., Chen, J. and Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev. Cell* **5**, 877–889.

Cai, C., Martin, J., Sun, Y., Cui, L., Wang, L., Ouyang, K., Yang, L., Bu, L., Liang, X., Zhang, X., Stallcup, W., Denton, C., McCulloch, A., Chen, J. and Evans, S. (2008). A myocardial lineage derives from tbx18 epicardial cells. *Nature* **454**, 104–108.

Calmont, A., Ivins, S., Van Bueren, K. L., Papangeli, I., Kyriakopoulou, V., Andrews, W. D., Martin, J. F., Moon, A. M., Illingworth, E. A., Basson, M. A. and Scambler, P. J. (2009). Tbx1 controls cardiac neural crest cell migration during arch artery development by regulating gbx2 expression in the pharyngeal ectoderm. *Development* **136**, 3173–3183.

Chen, L., Fulcoli, F., Tang, S. and Baldini, A. (2009). Tbx1 regulates proliferation and differentiation of multipotent heart progenitors. *Circ. Res.* **105**, 842–851.

Christoffels, V. M., Grieskamp, T., Norden, J., Mommersteeg, M. T. M., Rudat, C. and Kispert, A. (2009). Tbx18 and the fate of epicardial progenitors. *Nature* **458**, E8–9.

Christoffels, V. M., Mommersteeg, M. T. M., Trowe, M.-O., Prall, O. W. J., de Gier-de Vries, C., Soufan, A. T., Bussen, M., Schuster-Gossler, K., Harvey, R. P., Moorman, A. F. M. and Kispert, A. (2006). Formation of the venous pole of the heart from an Nkx2-5-negative precursor population requires tbx18. *Circ. Res.* **98**, 1555–1563.

Clement, C. A., Kristensen, S. G., Møllgård, K., Pazour, G. J., Yoder, B. K., Larsen, L. A. and Christensen, S. T. (2009). The primary cilium coordinates early cardiogenesis and hedgehog signaling in cardiomyocyte differentiation. *J. Cell. Sci.* **122**, 3070–3082.

Cohen, E. D., Tian, Y. and Morrisey, E. E. (2008). Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. *Development* **135**, 789–798.

Cohen, E. D., Wang, Z., Lepore, J. J., Lu, M. M., Taketo, M. M., Epstein, D. J. and Morrisey, E. E. (2007). Wnt/beta-catenin signaling promotes expansion of isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J. Clin. Invest.* **117**, 1794–1804.

Cui, C., Cheuvront, T. J., Lansford, R. D., Moreno-Rodriguez, R. A., Schultheiss, T. M. and Rongish, B. J. (2009). Dynamic positional fate map of the primary heart-forming region. *Dev. Biol.* **332**, 212–222.

David, R., Brenner, C., Stieber, J., Schwarz, F., Brunner, S., Vollmer, M., Mentele, E., Müller-Höcker, J., Kitajima, S., Lickert, H., Rupp, R. and Franz, W.-M. (2008). MesP1 drives vertebrate cardiovascular differentiation through dkk-1-mediated blockade of wnt-signalling. *Nat. Cell Biol.* **10**, 338–345.

de Pater, E., Clijsters, L., Marques, S. R., Lin, Y. F., Garavito-Aguilar, Z. V., Yelon, D. and Bakkers, J. (2009). Distinct phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. *Development* **136**, 1633–1641.

Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S.-M. and Black, B. L. (2004). Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development* **131**, 3931–3942.

Dyer, L. and Kirby, M. (2009). Sonic hedgehog maintains proliferation in secondary heart field progenitors and is required for normal arterial pole formation. *Dev. Biol.* **330**, 305–317.

Etheridge, S. L., Ray, S., Li, S., Hamblet, N. S., Lijam, N., Tsang, M., Greer, J., Kardos, N., Wang, J., Sussman, D. J., Chen, P. and Wynshaw-Boris, A. (2008). Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. *PLoS Genet.* **4**, e1000259.

Feiner, L., Webber, A. L., Brown, C. B., Lu, M. M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J. A. and Raper, J. A. (2001). Targeted disruption of semaphorin 3c leads to persistent truncus arteriosus and aortic arch interruption. *Development* **128**, 3061–3070.

Ferdous, A., Caprioli, A., Iacovino, M., Martin, C. M., Morris, J., Richardson, J. A., Latif, S., Hammer, R. E., Harvey, R. P., Olson, E. N., Kyba, M. and Garry, D. J. (2009). Nkx2-5 transactivates the ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. *Proc. Natl. Acad. Sci. USA* **106**, 814–819.

Franco, D. and Campione, M. (2003). The role of pitx2 during cardiac development: Linking left–right signaling and congenital heart diseases. *Trends Cardiovasc. Med.* **13**, 157–163.

Frank, D. U., Fotheringham, L. K., Brewer, J. A., Muglia, L. J., Tristani-Firouzi, M., Capecchi, M. R. and Moon, A. M. (2002). An fgf8 mouse mutant phenocopies human 22q11 deletion syndrome. *Development* **129**, 4591–4603.

Fulcoli, F. G., Huynh, T., Scambler, P. J. and Baldini, A. (2009). Tbx1 regulates the BMP-smad1 pathway in a transcription independent manner. *PLoS ONE* **4**, e6049.

Galli, D., Domínguez, J. N., Zaffran, S., Munk, A., Brown, N. A. and Buckingham, M. E. (2008). Atrial myocardium derives from the posterior region of the second heart field, which acquires left–right identity as pitx2c is expressed. *Development* **135**, 1157–1167.

Garcia-Martinez, V. and Schoenwolf, G. C. (1993). Primitive-streak origin of the cardiovascular system in avian embryos. *Dev. Biol.* **159**, 706–719.

Gessert, S. and Kühl, M. (2009). Comparative gene expression analysis and fate mapping studies suggest an early segregation of cardiogenic lineages in xenopus laevis. *Dev. Biol.* **334**, 395–408.

Gitler, A. D., Lu, M. M. and Epstein, J. A. (2004). PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. *Dev. Cell* **7**, 107–116.

Goddeeris, M. M., Rho, S., Petiet, A., Davenport, C. L., Johnson, G. A., Meyers, E. N. and Klingensmith, J. (2008). Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart. *Development* **135**, 1887–1895.

Goddeeris, M. M., Schwartz, R., Klingensmith, J. and Meyers, E. N. (2007). Independent requirements for hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development. *Development* **134**, 1593–1604.

Guris, D. L., Duester, G., Papaioannou, V. E. and Imamoto, A. (2006). Dose-dependent interaction of tbx1 and crkl and locally aberrant RA signaling in a model of del22q11 syndrome. *Dev. Cell* **10**, 81–92.

Harel, I., Nathan, E., Tirosh-Finkel, L., Zigdon, H., Guimarães-Camboa, N., Evans, S. M. and Tzahor, E. (2009). Distinct origins and genetic programs of head muscle satellite cells. *Dev. Cell* **16**, 822–832.

Harvey, R. P., Meilhac, S. M. and Buckingham, M. E. (2009). Landmarks and lineages in the developing heart. *Circ. Res.* **104**, 1235–1237.

High, F. A. and Epstein, J. A. (2008). The multifaceted role of notch in cardiac development and disease. *Nat. Rev. Genet.* **9**, 49–61.

High, F. A., Jain, R., Stoller, J. Z., Antonucci, N. B., Lu, M. M., Loomes, K. M., Kaestner, K. H., Pear, W. S. and Epstein, J. A. (2009). Murine jagged1/notch signaling in the second heart field orchestrates fgf8 expression and tissue–tissue interactions during outflow tract development. *J. Clin. Invest.* **119**, 1986–1996.

High, F. A., Lu, M. M., Pear, W. S., Loomes, K. M., Kaestner, K. H. and Epstein, J. A. (2008). Endothelial expression of the notch ligand jagged1 is required for vascular smooth muscle development. *Proc. Natl. Acad. Sci. USA* **105**, 1955–1959.

High, F. A., Zhang, M., Proweller, A., Tu, L., Parmacek, M. S., Pear, W. S. and Epstein, J. A. (2007). An essential role for notch in neural crest during cardiovascular development and smooth muscle differentiation. *J. Clin. Invest.* **117**, 353–363.

Hildreth, V., Webb, S., Chaudhry, B., Peat, J. D., Phillips, H. M., Brown, N., Anderson, R. H. and Henderson, D. J. (2009). Left cardiac isomerism in the sonic hedgehog null mouse. *J. Anat.* **214**, 894–904.

Hoffmann, A. D., Peterson, M. A., Friedland-Little, J. M., Anderson, S. A. and Moskowitz, I. P. (2009). Sonic hedgehog is required in pulmonary endoderm for atrial septation. *Development* **136**, 1761–1770.

Hu, T., Yamagishi, H., Maeda, J., McAnally, J., Yamagishi, C. and Srivastava, D. (2004). Tbx1 regulates fibroblast growth factors in the anterior heart field through a reinforcing autoregulatory loop involving forkhead transcription factors. *Development* **131**, 5491–5502.

Hutson, M. R. and Kirby, M. L. (2007). Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. *Semin. Cell Dev. Biol.* **18**, 101–110.

Hutson, M. R., Zhang, P., Stadt, H. A., Sato, A. K., Li, Y.-X., Burch, J., Creazzo, T. L. and Kirby, M. L. (2006). Cardiac arterial pole alignment is sensitive to FGF8 signaling in the pharynx. *Dev. Biol.* **295**, 486–497.

Huynh, T., Chen, L., Terrell, P. and Baldini, A. (2007). A fate map of tbx1 expressing cells reveals heterogeneity in the second cardiac field. *Genesis* **45**, 470–475.

Ilagan, R., Abu-Issa, R., Brown, D., Yang, Y.-P., Jiao, K., Schwartz, R. J., Klingensmith, J. and Meyers, E. N. (2006). Fgf8 is required for anterior heart field development. *Development* **133**, 2435–2445.

Ivins, S., Lammerts van Beuren, K., Roberts, C., James, C., Lindsay, E., Baldini, A., Ataliotis, P. and Scambler, P. J. (2005). Microarray analysis detects differentially expressed genes in the pharyngeal region of mice lacking tbx1. *Dev. Biol.* **285**, 554–569.

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A. and Belmonte, J. C. I. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606–609.

Kang, J., Nathan, E., Xu, S., Tzahor, E. and Black, B. (2009). Isl1 is a direct transcriptional target of forkhead transcription factors in second heart field-derived mesoderm. *Dev. Biol.* **334**, 513–522.

Kappen, C. and Salbaum, J. M. (2009). Identification of regulatory elements in the isl1 gene locus. *Int. J. Dev. Biol.* **53**, 935–946.

Kaufman, M. H. and Bard, J. B. (1999). *The Anatomical Basis of Mouse Development*. Academic Press, London.

Keegan, B. R., Feldman, J. L., Begemann, G., Ingham, P. W. and Yelon, D. (2005). Retinoic acid signaling restricts the cardiac progenitor pool. *Science* **307**, 247–249.

Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* **1**, 435–440.

Keren-Politansky, A., Keren, A. and Bengal, E. (2009). Neural ectoderm-secreted FGF initiates the expression of Nkx2.5 in cardiac progenitors via a p38 MAPK/CREB pathway. *Dev. Biol.* **335**, 374–384.

Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. R. and Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by gata4+ cardiomyocytes. *Nature* **464**, 601.

Kirby, M. (2007). *Cardiac Development*. Oxford University Press, Oxford.

Klaus, A., Saga, Y., Taketo, M. M., Tzahor, E. and Birchmeier, W. (2007). Distinct roles of wnt/beta-catenin and bmp signaling during early cardiogenesis. *Proc. Natl. Acad. Sci. USA* **104**, 18531–18536.

Kodo, K., Nishizawa, T., Furutani, M., Arai, S., Yamamura, E., Joo, K., Takahashi, T., Matsuoka, R. and Yamagishi, H. (2009). GATA6 mutations cause human cardiac outflow tract defects by disrupting semaphorin–plexin signaling. *Proc. Natl. Acad. Sci. USA* **106**, 13933–13938.

Kolesová, H., Roelink, H. and Grim, M. (2008). Sonic hedgehog is required for the assembly and remodeling of branchial arch blood vessels. *Dev. Dyn.* **237**, 1923–1934.

Kruithof, B. P. T., Van Wijk, B., Somi, S., Kruithof-de Julio, M., Pérez Pomares, J. M., Weesie, F., Wessels, A., Moorman, A. F. M. and van den Hoff, M. J. B. (2006). BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. *Dev. Biol.* **295**, 507–522.

Kwon, C., Arnold, J., Hsiao, E. C., Taketo, M. M., Conklin, B. R. and Srivastava, D. (2007). Canonical wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proc. Natl. Acad. Sci. USA* **104**, 10894–10899.

Kwon, C., Qian, L., Cheng, P., Nigam, V., Arnold, J. and Srivastava, D. (2009). A regulatory pathway involving notch1/beta-catenin/isl1 determines cardiac progenitor cell fate. *Nat. Cell Biol.* **11**, 951–957.

Laugwitz, K.-L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L.-Z., Cai, C.-L., Lu, M. M., Reth, M., Platoshyn, O., Yuan, J.X.-J., Evans, S. and Chien, K. R. (2005). Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647–653.

Lavery, D., Martin, J., Turnbull, Y. and Hoppler, S. (2008). Wnt6 signaling regulates heart muscle development during organogenesis. *Dev. Biol.* **323**, 177–188.

Lawson, K. A., Meneses, J. J. and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113**, 891–911.

Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. and Poss, K. D. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607–619.

Lepore, J. J., Mericko, P. A., Cheng, L., Lu, M. M., Morrisey, E. E. and Parmacek, M. S. (2006). GATA-6 regulates semaphorin 3c and is required in cardiac neural crest for cardiovascular morphogenesis. *J. Clin. Invest.* **116**, 929–939.

Li, P., Pashmforoush, M. and Sucov, H. M. (2010). Retinoic acid regulates differentiation of the secondary heart field and TGFbeta-mediated outflow tract septation. *Dev. Cell* **18**, 480–485.

Liao, J., Aggarwal, V. S., Nowotschin, S., Bondarev, A., Lipner, S. and Morrow, B. E. (2008). Identification of downstream genetic pathways of tbx1 in the second heart field. *Dev. Biol.* **316**, 524–537.

Lin, L., Bu, L., Cai, C.-L., Zhang, X. and Evans, S. (2006). Isl1 is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. *Dev. Biol.* **295**, 756–763.

Lin, L., Cui, L., Zhou, W., Dufort, D., Zhang, X., Cai, C.-L., Bu, L., Yang, L., Martin, J., Kemler, R., Rosenfeld, M. G., Chen, J. and Evans, S. M. (2007). Beta-catenin directly regulates islet1 expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis. *Proc. Natl. Acad. Sci. USA* **104**, 9313–9318.

Lindsley, R. C., Gill, J. G., Murphy, T. L., Langer, E. M., Cai, M., Mashayekhi, M., Wang, W., Niwa, N., Nerbonne, J. M., Kyba, M. and Murphy, K. M. (2008). Mesp1 coordinately regulates cardiovascular fate restriction and epithelial–mesenchymal transition in differentiating ESCs. *Cell Stem Cell* **3**, 55–68.

Liu, W., Selever, J., Wang, D., Lu, M.-F., Moses, K. A., Schwartz, R. J. and Martin, J. F. (2004). Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. *Proc. Natl. Acad. Sci. USA* **101**, 4489–4494.

Ma, Q., Zhou, B. and Pu, W. T. (2008). Reassessment of isl1 and Nkx2-5 cardiac fate maps using a gata4-based reporter of cre activity. *Dev. Biol.* **323**, 98–104.

Macatee, T. L., Hammond, B. P., Arenkiel, B. R., Francis, L., Frank, D. U. and Moon, A. M. (2003). Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. *Development* **130**, 6361–6374.

Maeda, J., Yamagishi, H., McAnally, J., Yamagishi, C. and Srivastava, D. (2006). Tbx1 is regulated by forkhead proteins in the secondary heart field. *Dev. Dyn.* **235**, 701–710.

Männer, J., Pérez-Pomares, J. M., Macías, D. and Muñoz-Chápuli, R. (2001). The origin, formation and developmental significance of the epicardium: a review. *Cells Tissues Organs (Print)* **169**, 89–103.

Marguerie, A., Bajolle, F., Zaffran, S., Brown, N. A., Dickson, C., Buckingham, M. E. and Kelly, R. G. (2006). Congenital heart defects in fgfr2-IIIb and fgf10 mutant mice. *Cardiovasc. Res.* **71**, 50–60.

Marques, S. R., Lee, Y., Poss, K. D. and Yelon, D. (2008). Reiterative roles for FGF signaling in the establishment of size and proportion of the zebrafish heart. *Dev. Biol.* **321**, 397–406.

Martínez-Estrada, O. M., Lettice, L. A., Essafi, A., Guadix, J. A., Slight, J., Velecela, V., Hall, E., Reichmann, J., Devenney, P. S., Hohenstein, P., Hosen, N., Hill, R. E., Muñoz-Chapuli, R. and Hastie, N. D. (2010). Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of snail and E-cadherin. *Nat. Genet.* **42**, 89–93.

McCulley, D., Kang, J., Martin, J. and Black, B. (2008). BMP4 is required in the anterior heart field and its derivatives for endocardial cushion remodeling, outflow tract septation, and semilunar valve development. *Dev. Dyn.* **237**, 3200–3209.

Meilhac, S. M., Esner, M., Kelly, R. G., Nicolas, J.-F. and Buckingham, M. E. (2004). The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev. Cell* **6**, 685–698.

Meilhac, S. M., Kelly, R. G., Rocancourt, D., Eloy-Trinquet, S., Nicolas, J.-F. and Buckingham, M. E. (2003). A retrospective clonal analysis of the myocardium reveals two phases of clonal growth in the developing mouse heart. *Development* **130**, 3877–3889.

Mesbah, K., Harrelson, Z., Théveniau-Ruissy, M., Papaioannou, V. E. and Kelly, R. G. (2008). Tbx3 is required for outflow tract development. *Circ. Res.* **103**, 743–750.

Misfeldt, A., Boyle, S., Tompkins, K., Bautch, V., Labosky, P. and Baldwin, H. (2009). Endocardial cells are a distinct endothelial lineage derived from flk1+ multipotent cardiovascular progenitors. *Dev. Biol.* **333**, 78–89.

Mommersteeg, M. T. M., Brown, N. A., Prall, O. W. J., de Gier-de Vries, C., Harvey, R. P., Moorman, A. F. M. and Christoffels, V. M. (2007). Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. *Circ. Res.* **101**, 902–909.

Mommersteeg, M.T.M., Domínguez, J. N., Wiese, C., Norden, J., de Gier-de Vries, C., Burch, J. B. E., Kispert, A., Brown, N. A., Moorman, A. F. M. and Christoffels, V. M. (2010). The sinus venosus progenitors separate and diversify from the first and second heart fields early in development. *Cardiovasc. Res.* **87**, 92–101.

Mommersteeg, M. T. M., Soufan, A. T., de Lange, F. J., van den Hoff, M. J. B., Anderson, R. H., Christoffels, V. M. and Moorman, A. F. M. (2006). Two distinct pools of mesenchyme contribute to the development of the atrial septum. *Circ. Res.* **99**, 351–353.

Moretti, A., Caron, L., Nakano, A., Lam, J. T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S., Sun, Y., Evans, S. M., Laugwitz, K.-L. and Chien, K. R. (2006). Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151–1165.

Motoike, T., Markham, D. W., Rossant, J. and Sato, T. N. (2003). Evidence for novel fate of flk1+ progenitor: contribution to muscle lineage. *Genesis* **35**, 153–159.

Nagy, I. I., Railo, A., Rapila, R., Hast, T., Sormunen, R., Tavi, P., Räsänen, J. and Vainio, S. J. (2010). Wnt-11 signalling controls ventricular myocardium development by patterning N-cadherin and beta-catenin expression. *Cardiovasc. Res.* **85**, 100–109.

Nathan, E., Monovich, A., Tirosh-Finkel, L., Harrelson, Z., Rousso, T., Rinon, A., Harel, I., Evans, S. M. and Tzahor, E. (2008). The contribution of islet1-expressing splanchnic mesoderm cells to distinct branchiomeric muscles reveals significant heterogeneity in head muscle development. *Development* **135**, 647–657.

Nie, X., Deng, C.-X., Wang, Q. and Jiao, K. (2008). Disruption of smad4 in neural crest cells leads to mid-gestation death with pharyngeal arch, craniofacial and cardiac defects. *Dev. Biol.* **316**, 417–430.

Noden, D. M. and Francis-West, P. (2006). The differentiation and morphogenesis of craniofacial muscles. *Dev. Dyn.* **235**, 1194–1218.

Nowotschin, S., Liao, J., Gage, P. J., Epstein, J. A., Campione, M. and Morrow, B. E. (2006). Tbx1 affects asymmetric cardiac morphogenesis by regulating pitx2 in the secondary heart field. *Development* **133**, 1565–1573.

Palpant, N. J., Yasuda, S., MacDougald, O. and Metzger, J. M. (2007). Non-canonical wnt signaling enhances differentiation of sca1+/c-kit+ adipose-derived murine stromal vascular cells into spontaneously beating cardiac myocytes. *J. Mol. Cell. Cardiol.* **43**, 362–370.

Pandur, P., Läsche, M., Eisenberg, L. M. and Kühl, M. (2002). Wnt-11 activation of a non-canonical wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636–641.

Park, E. J., Ogden, L. A., Talbot, A., Evans, S., Cai, C.-L., Black, B. L., Frank, D. U. and Moon, A. M. (2006). Required, tissue-specific roles for fgf8 in outflow tract formation and remodeling. *Development* **133**, 2419–2433.

Park, E., Watanabe, Y., Smyth, G., Miyagawa-Tomita, S., Meyers, E., Klingensmith, J., Camenisch, T., Buckingham, M. and Moon, A. (2008). An FGF autocrine loop initiated in second heart field mesoderm regulates morphogenesis at the arterial pole of the heart. *Development* **135**, 3599–3610.

Phillips, H. M., Murdoch, J. N., Chaudhry, B., Copp, A. J. and Henderson, D. J. (2005). Vangl2 acts via RhoA signaling to regulate polarized cell movements during development of the proximal outflow tract. *Circ. Res.* **96**, 292–299.

Phillips, H. M., Rhee, H. J., Murdoch, J. N., Hildreth, V., Peat, J. D., Anderson, R. H., Copp, A. J., Chaudhry, B. and Henderson, D. J. (2007). Disruption of planar cell polarity signaling results in congenital heart defects and cardiomyopathy attributable to early cardiomyocyte disorganization. *Circ. Res.* **101**, 137–145.

Prall, O. W. J., Menon, M. K., Solloway, M. J., Watanabe, Y., Zaffran, S., Bajolle, F., Biben, C., McBride, J. J., Robertson, B. R., Chaulet, H., Stennard, F. A., Wise, N., Schaft, D., Wolstein, O., Furtado, M. B., Shiratori, H., Chien, K. R., Hamada, H., Black, B. L., Saga, Y., Robertson, E. J., Buckingham, M. E. and Harvey, R. P. (2007). An Nkx2-5/bmp2/smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* **128**, 947–959.

Qyang, Y., Martin-Puig, S., Chiravuri, M., Chen, S., Xu, H., Bu, L., Jiang, X., Lin, L., Granger, A., Moretti, A., Caron, L., Wu, X., Clarke, J., Taketo, M. M., Laugwitz, K.-L., Moon, R. T., Gruber, P., Evans, S. M., Ding, S. and Chien, K. R. (2007). The renewal and differentiation of isl1+ cardiovascular progenitors are controlled by a wnt/beta-catenin pathway. *Cell Stem Cell* **1**, 165–179.

Red-Horse, K., Ueno, H., Weissman, I. L. and Krasnow, M. A. (2010). Coronary arteries form by developmental reprogramming of venous cells. *Nature* **464**, 549–553.

Redkar, A., Montgomery, M. and Litvin, J. (2001). Fate map of early avian cardiac progenitor cells. *Development* **128**, 2269–2279.

Roberts, C., Ivins, S., Cook, A. C., Baldini, A. and Scambler, P. J. (2006). Cyp26 genes a1, b1 and c1 are down-regulated in tbx1 null mice and inhibition of cyp26 enzyme function produces a phenocopy of DiGeorge syndrome in the chick. *Hum. Mol. Genet.* **15**, 3394–3410.

Rochais, F., Dandonneau, M., Mesbah, K., Jarry, T., Mattei, M.-G. and Kelly, R. G. (2009). Hes1 is expressed in the second heart field and is required for outflow tract development. *PLoS ONE* **4**, e6267.

Roessler, E., Ouspenskaia, M. V., Karkera, J. D., Vélez, J. I., Kantipong, A., Lacbawan, F., Bowers, P., Belmont, J. W., Towbin, J. A., Goldmuntz, E., Feldman, B. and Muenke, M. (2008). Reduced NODAL signaling strength via mutation of several pathway members including FOXH1 is linked to human heart defects and holoprosencephaly. *Am. J. Hum. Genet.* **83**, 18–29.

Ryckebüsch, L., Bertrand, N., Mesbah, K., Bajolle, F., Niederreither, K., Kelly, R. G. and Zaffran, S. (2010). Decreased levels of embryonic retinoic acid synthesis accelerate recovery from arterial growth delay in a mouse model of DiGeorge syndrome. *Circ. Res.* **106**, 686–694.

Ryckebusch, L., Wang, Z., Bertrand, N., Lin, S.-C., Chi, X., Schwartz, R., Zaffran, S. and Niederreither, K. (2008). Retinoic acid deficiency alters second heart field formation. *Proc. Natl. Acad. Sci. USA* **105**, 2913–2918.

Saga, Y., Kitajima, S. and Miyagawa-Tomita, S. (2000). Mesp1 expression is the earliest sign of cardiovascular development. *Trends Cardiovasc. Med.* **10**, 345–352.

Saga, Y., Miyagawa-Tomita, S., Takagi, A., Kitajima, S., Miyazaki, J. and Inoue, T. (1999). MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* **126**, 3437–3447.

Schleiffarth, J. R., Person, A. D., Martinsen, B. J., Sukovich, D. J., Neumann, A., Baker, C. V. H., Lohr, J. L., Cornfield, D. N., Ekker, S. C. and Petryk, A. (2007). Wnt5a is required for cardiac outflow tract septation in mice. *Pediatr. Res.* **61**, 386–391.

Schultheiss, T. M., Burch, J. B. and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11**, 451–462.

Seo, S. and Kume, T. (2006). Forkhead transcription factors, foxc1 and foxc2, are required for the morphogenesis of the cardiac outflow tract. *Dev. Biol.* **296**, 421–436.

Shen, H., McElhinny, A. S., Cao, Y., Gao, P., Liu, J., Bronson, R., Griffin, J. D. and Wu, L. (2006). The notch coactivator, MAML1, functions as a novel coactivator for MEF2C-mediated transcription and is required for normal myogenesis. *Genes Dev.* **20**, 675–688.

Sirbu, I. O., Zhao, X. and Duester, G. (2008). Retinoic acid controls heart anteroposterior patterning by down-regulating isl1 through the fgf8 pathway. *Dev. Dyn.* **237**, 1627–1635.
Slough, J., Cooney, L. and Brueckner, M. (2008). Monocilia in the embryonic mouse heart suggest a direct role for cilia in cardiac morphogenesis. *Dev. Dyn.* **237**, 2304–2314.
Snarr, B. S., O'Neal, J. L., Chintalapudi, M. R., Wirrig, E. E., Phelps, A. L., Kubalak, S. W. and Wessels, A. (2007a). Isl1 expression at the venous pole identifies a novel role for the second heart field in cardiac development. *Circ. Res.* **101**, 971–974.
Snarr, B. S., Wirrig, E. E., Phelps, A. L., Trusk, T. C. and Wessels, A. (2007b). A spatiotemporal evaluation of the contribution of the dorsal mesenchymal protrusion to cardiac development. *Dev. Dyn.* **236**, 1287–1294.
Somi, S., Buffing, A. A. M., Moorman, A. F. M. and van den Hoff, M. J. B. (2004). Dynamic patterns of expression of BMP isoforms 2, 4, 5, 6, and 7 during chicken heart development. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **279**, 636–651.
Soufan, A. T., van den Berg, G., Ruijter, J. M., de Boer, P. A. J., van den Hoff, M. J. B. and Moorman, A. F. M. (2006). Regionalized sequence of myocardial cell growth and proliferation characterizes early chamber formation. *Circ. Res.* **99**, 545–552.
Stalsberg, H. and DeHaan, R. L. (1969). The precardiac areas and formation of the tubular heart in the chick embryo. *Dev. Biol.* **19**, 128–159.
Stanley, E. G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L. and Harvey, R. P. (2002). Efficient cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-cre allele of the homeobox gene Nkx2-5. *Int. J. Dev. Biol.* **46**, 431–439.
Sun, Y., Liang, X., Najafi, N., Cass, M., Lin, L., Cai, C.-L., Chen, J. and Evans, S. M. (2007). Islet 1 is expressed in distinct cardiovascular lineages, including pacemaker and coronary vascular cells. *Dev. Biol.* **304**, 286–296.
Takeuchi, J. and Bruneau, B. (2009). Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* **459**, 708–711.
Takeuchi, J. K., Mileikovskaia, M., Koshiba-Takeuchi, K., Heidt, A. B., Mori, A. D., Arruda, E. P., Gertsenstein, M., Georges, R., Davidson, L., Mo, R., Hui, C.-C., Henkelman, R. M., Nemer, M., Black, B. L., Nagy, A. and Bruneau, B. G. (2005). Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development. *Development* **132**, 2463–2474.
Tam, P. P., Parameswaran, M., Kinder, S. J. and Weinberger, R. P. (1997). The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. *Development* **124**, 1631–1642.
Tapscott, S. J. (2005). The circuitry of a master switch: myod and the regulation of skeletal muscle gene transcription. *Development* **132**, 2685–2695.
Tessari, A., Pietrobon, M., Notte, A., Cifelli, G., Gage, P. J., Schneider, M. D., Lembo, G. and Campione, M. (2008). Myocardial pitx2 differentially regulates the left atrial identity and ventricular asymmetric remodeling programs. *Circ. Res.* **102**, 813–822.
Théveniau-Ruissy, M., Dandonneau, M., Mesbah, K., Ghez, O., Mattei, M.-G., Miquerol, L. and Kelly, R. G. (2008). The del22q11.2 candidate gene tbx1 controls regional outflow tract identity and coronary artery patterning. *Circ. Res.* **103**, 142–148.
Thomas, N. A., Koudijs, M., van Eeden, F. J. M., Joyner, A. L. and Yelon, D. (2008). Hedgehog signaling plays a cell-autonomous role in maximizing cardiac developmental potential. *Development* **135**, 3789–3799.
Tian, Y., Yuan, L., Goss, A. M., Wang, T., Yang, J., Lepore, J. J., Zhou, D., Schwartz, R. J., Patel, V., Cohen, E. D. and Morrisey, E. E. (2010). Characterization and in vivo pharmacological rescue of a wnt2-gata6 pathway required for cardiac inflow tract development. *Dev. Cell* **18**, 275–287.
Tirosh-Finkel, L., Elhanany, H., Rinon, A. and Tzahor, E. (2006). Mesoderm progenitor cells of common origin contribute to the head musculature and the cardiac outflow tract. *Development* **133**, 1943–1953.

Toyofuku, T., Yoshida, J., Sugimoto, T., Yamamoto, M., Makino, N., Takamatsu, H., Takegahara, N., Suto, F., Hori, M., Fujisawa, H., Kumanogoh, A. and Kikutani, H. (2008). Repulsive and attractive semaphorins cooperate to direct the navigation of cardiac neural crest cells. *Dev. Biol.* **321**, 251–262.

Uchimura, T., Komatsu, Y., Tanaka, M., McCann, K. and Mishina, Y. (2009). Bmp2 and bmp4 genetically interact to support multiple aspects of mouse development including functional heart development. *Genesis* **47**, 374–384.

Van Bueren, K. L., Papangeli, I., Rochais, F., Pearce, K., Roberts, C., Calmont, A., Szumska, D., Kelly, R. G., Bhattacharya, S. and Scambler, P. J. (2010). Hes1 expression is reduced in tbx1 null cells and is required for the development of structures affected in 22q11 deletion syndrome. *Dev. Biol.* **340**, 369–380.

Van den Berg, G., Abu-Issa, R., de Boer, B. A., Hutson, M. R., de Boer, P. A. J., Soufan, A. T., Ruijter, J. M., Kirby, M. L., van den Hoff, M. J. B. and Moorman, A. F. M. (2009). A caudal proliferating growth center contributes to both poles of the forming heart tube. *Circ. Res.* **104**, 179–188.

Van Praagh, R. (2009). The first Stella Van Praagh memorial lecture: the history and anatomy of tetralogy of fallot. *Semin. Thorac. Cardiovasc. Surg. Pediatr. Card. Surg. Annu.* **12**, 19–38.

Van Wijk, B., van den Berg, G., Abu-Issa, R., Barnett, P., Van Der Velden, S., Schmidt, M., Ruijter, J. M., Kirby, M. L., Moorman, A. F. M. and van den Hoff, M. J. B. (2009). Epicardium and myocardium separate from a common precursor pool by crosstalk between bone morphogenetic protein- and fibroblast growth factor-signaling pathways. *Circ. Res.* **105**, 431–441.

Varadkar, P., Kraman, M., Despres, D., Ma, G., Lozier, J. and McCright, B. (2008). Notch2 is required for the proliferation of cardiac neural crest-derived smooth muscle cells. *Dev. Dyn.* **237**, 1144–1152.

Vermot, J., Niederreither, K., Garnier, J.-M., Chambon, P. and Dollé, P. (2003). Decreased embryonic retinoic acid synthesis results in a DiGeorge syndrome phenotype in newborn mice. *Proc. Natl. Acad. Sci. USA* **100**, 1763–1768.

Verzi, M. P., McCulley, D. J., De Val, S., Dodou, E. and Black, B. L. (2005). The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev. Biol.* **287**, 134–145.

Vincentz, J. W., McWhirter, J. R., Murre, C., Baldini, A. and Furuta, Y. (2005). Fgf15 is required for proper morphogenesis of the mouse cardiac outflow tract. *Genesis* **41**, 192–201.

Vitelli, F., Huynh, T. and Baldini, A. (2009). Gain of function of tbx1 affects pharyngeal and heart development in the mouse. *Genesis* **47**, 188–195.

Vitelli, F., Zhang, Z., Huynh, T., Sobotka, A., Mupo, A. and Baldini, A. (2006). Fgf8 expression in the tbx1 domain causes skeletal abnormalities and modifies the aortic arch but not the outflow tract phenotype of tbx1 mutants. *Dev. Biol.* **295**, 559–570.

von Both, I., Silvestri, C., Erdemir, T., Lickert, H., Walls, J. R., Henkelman, R. M., Rossant, J., Harvey, R. P., Attisano, L. and Wrana, J. L. (2004). Foxh1 is essential for development of the anterior heart field. *Dev. Cell* **7**, 331–345.

Waldo, K. L., Hutson, M. R., Stadt, H. A., Zdanowicz, M., Zdanowicz, J. and Kirby, M. L. (2005a). Cardiac neural crest is necessary for normal addition of the myocardium to the arterial pole from the secondary heart field. *Dev. Biol.* **281**, 66–77.

Waldo, K. L., Hutson, M. R., Ward, C. C., Zdanowicz, M., Stadt, H. A., Kumiski, D., Abu-Issa, R. and Kirby, M. L. (2005b). Secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. *Dev. Biol.* **281**, 78–90.

Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H. and Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. *Development* **128**, 3179–3188.

Ward, C., Stadt, H., Hutson, M. and Kirby, M. L. (2005). Ablation of the secondary heart field leads to tetralogy of fallot and pulmonary atresia. *Dev. Biol.* **284**, 72–83.

Washington Smoak, I., Byrd, N. A., Abu-Issa, R., Goddeeris, M. M., Anderson, R., Morris, J., Yamamura, K., Klingensmith, J. and Meyers, E. N. (2005). Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. *Dev. Biol.* **283**, 357–372.

Watanabe, Y., Miyagawa-Tomita, S., Vincent, S. D., Kelly, R. G., Moon, A. M. and Buckingham, M. E. (2010). Role of mesodermal FGF8 and FGF10 overlaps in the development of the arterial pole of the heart and pharyngeal arch arteries. *Circ. Res.* **106**, 495–503.

Waxman, J. S., Keegan, B. R., Roberts, R. W., Poss, K. D. and Yelon, D. (2008). Hoxb5b acts downstream of retinoic acid signaling in the forelimb field to restrict heart field potential in zebrafish. *Dev. Cell* **15**, 923–934.

Wei, Y. and Mikawa, T. (2000). Fate diversity of primitive streak cells during heart field formation in ovo. *Dev. Dyn.* **219**, 505–513.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R. and Hollenberg, S. (1991). The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761–766.

Wessels, A. and Pérez-Pomares, J. M. (2004). The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **276**, 43–57.

Winter, E. M. and Gittenberger-de Groot, A. C. (2007). Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell Mol. Life Sci.* **64**, 692–703.

Wu, S. M., Fujiwara, Y., Cibulsky, S. M., Clapham, D. E., Lien, C. L., Schultheiss, T. M. and Orkin, S. H. (2006). Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137–1150.

Xu, H., Morishima, M., Wylie, J. N., Schwartz, R. J., Bruneau, B. G., Lindsay, E. A. and Baldini, A. (2004). Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development* **131**, 3217–3227.

Yamagishi, H., Maeda, J., Hu, T., McAnally, J., Conway, S. J., Kume, T., Meyers, E. N., Yamagishi, C. and Srivastava, D. (2003). Tbx1 is regulated by tissue-specific forkhead proteins through a common sonic hedgehog-responsive enhancer. *Genes Dev.* **17**, 269–281.

Yang, L., Cai, C.-L., Lin, L., Qyang, Y., Chung, C., Monteiro, R. M., Mummery, C. L., Fishman, G. I., Cogen, A. and Evans, S. (2006). Isl1cre reveals a common bmp pathway in heart and limb development. *Development* **133**, 1575–1585.

Yashiro, K., Shiratori, H. and Hamada, H. (2007). Haemodynamics determined by a genetic programme govern asymmetric development of the aortic arch. *Nature* **450**, 285–288.

Yuan, S. and Schoenwolf, G. C. (2000). Islet-1 marks the early heart rudiments and is asymmetrically expressed during early rotation of the foregut in the chick embryo. *Anat. Rec.* **260**, 204–207.

Zaffran, S., Kelly, R. G., Meilhac, S. M., Buckingham, M. E. and Brown, N. A. (2004). Right ventricular myocardium derives from the anterior heart field. *Circ. Res.* **95**, 261–268.

Zhang, Z., Cerrato, F., Xu, H., Vitelli, F., Morishima, M., Vincentz, J., Furuta, Y., Ma, L., Martin, J. F., Baldini, A. and Lindsay, E. (2005). Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development. *Development* **132**, 5307–5315.

Zhang, Z., Huynh, T. and Baldini, A. (2006). Mesodermal expression of tbx1 is necessary and sufficient for pharyngeal arch and cardiac outflow tract development. *Development* **133**, 3587–3595.

Zhang, J., Lin, Y., Zhang, Y., Lan, Y., Lin, C., Moon, A., Schwartz, R., Martin, J. and Wang, F. (2008). Frs2{alpha}-deficiency in cardiac progenitors disrupts a subset of FGF signals required for outflow tract morphogenesis. *Development* **135**, 3611–3622.

Zhang, Y., Singh, M., Degenhardt, K., Lu, M., Bennett, J., Yoshida, Y. and Epstein, J. (2009). Tie2cre-mediated inactivation of plexinD1 results in congenital heart, vascular and skeletal defects. *Dev. Biol.* **325**, 82–93.

Zhou, W., Lin, L., Majumdar, A., Li, X., Zhang, X., Liu, W., Etheridge, L., Shi, Y., Martin, J., Van de Ven, W., Kaartinen, V., Wynshaw-Boris, A., McMahon, A. P., Rosenfeld,

M. G. and Evans, S. M. (2007). Modulation of morphogenesis by noncanonical wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFbeta2. *Nat. Genet.* **39**, 1225–1234.

Zhou, B., Ma, Q., Rajagopal, S., Wu, S., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K. and Pu, W. (2008a). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* **454**, 109–113.

Zhou, B., von Gise, A., Ma, Q., Rivera-Feliciano, J. and Pu, W. T. (2008b). Nkx2-5- and isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem. Biophys. Res. Commun.* **375**, 450–453.