| | | | | |
|---|---|---|---|---|
| MYL 6 | Alkali, smooth, nonmuscle | Chr12q13.2 | 609931 | Chr 10 |

(Continued)

Table 4.1 (Continued)

| | Myosin light Chain type | Human | OMIM | Mouse |
|---|---|---|---|---|
| MYL 6B | Early and mature myotube, skeletal slow | Chr12q13.13 | 609930 | Chr 10 |
| MYL 7 | Regulatory, fetal heart, atrium | Chr 7p | 613993 | Chr 11 |
| MYL 9 | Vascular smooth muscle | Chr20q11.23 | 609905 | Chr 17 |
| | Actin type | Human | OMIM | Mouse |
| ACTA1 | ACTIN a, Skeletal | Chr 1q42.1 | 102610 | Chr 8 |
| ACTSA | ACTIN a, Smooth, Aortic | Chr 10q22–q24 | 102620 | Chr19 |
| ACTC | ACTIN a, Cardiac | Chr 15q14 | 102540 | Chr 2 |
| ACTB | ACTIN b, Cytoplasmic nonmuscle | Chr 7p22–p12 | 102630 | Chr 5 |
| ACTG1 | ACTIN g-1; Cytoplasmic nonmuscle | Chr 17q25 | 102560 | Chr11 |
| ACTG2 | ACTIN g-2, Smooth, Enteric | Chr 2p13.1 | 102545 | Chr 6 |

N/A: not available.

References: OMIM: Online Mendelian Inheritance in Man. http://omim.org/; Kendrick-Jones, J., Hodge, T. P., Lister, I. M. B., Roberts, R. C., Buss, I. F. Myosin Superfamily. http://www.mrc-lmb.cam.ac.uk/myosin/Review/Reviewframeset.html; Schiaffino and Reggiani (2011).

[a] Expression only in some mammalian species.

## 2.3. Head branchiomeric muscles

While some striated muscles in the head are derived from the somite, including the tongue and neck muscles, head muscles involved in mastication, facial expression, and pharyngeal and laryngeal function have a different origin. By grafting quail embryo parts into stage-matched chickens, head muscles were shown to be derived from the branchial arches, and were called branchiomeric muscles (Mootoosamy and Dietrich, 2002). Branchiometric muscles have a distinct lineage different from somite-derived muscle (Grifone et al., 2007; Noden and Francis-West, 2006; Tzahor, 2009), and two branchiomeric muscle lineages were shown in the chicken (TiroshFinkel et al., 2006) and the mouse (Lescroart et al., 2010). The first branchiomeric muscle lineage gives rise to the temporalis and masseter muscles (Larsen et al., 2009) and also contributes myocardial cells to the right ventricle. The second branchiomeric muscle lineage gives rise to the muscles of facial expression and also contributes to the myocardium at the arterial pole of the heart. Within this second lineage, a further subdivision is observed between the myocardium at the base of the pulmonary trunk and the aorta. These sublineages also contribute to the left or right muscles of facial expression, respectively. Head muscles cells and the cells of the SHF, therefore, are derived from a common lineage of cells from the branchial arches (Lescroart et al., 2010; Tirosh-Finkel et al., 2006).

## 2.4. Myogenesis of SMs

SM forms the greater part of the visceral musculature including the gastrointestinal tract, respiratory tract, urinary bladder, the uterus in females, and male and female reproductive tracts (Gabella, 2002). Contractile bundles propel contents in these organs by peristaltic motion. SM layers are also found within the walls of some arteries and veins, and the tension exerted by the muscle can regulate blood pressure by adjusting the radius of these vessels (Majesky, 2003). SM is an involuntary muscle not under conscious control. In contrast to cardiac or skeletal muscle, SM displays remarkable heterogeneity conducting diverse functions, and different SM types have different ontogeny. In the gastrointestinal tract, the first layer of SM differentiates from the undifferentiated mesenchyme to give rise to the circular inner layer, at day E11 in mice, followed by the longitudinal external layer and the muscularis mucosa, the thin layer of SM located outside of the lamina propria mucosae, one day later (McHugh, 1996; McLin et al., 2009). During blood vessel morphogenesis, endothelial cells assemble a tubular network, and vascular SM cells are recruited to nascent blood vessels (Herbert and Stainer, 2011). Progenitors

of vascular SMs are derived from multiple sources including the neural crest, SHF, somites, splanchnic mesoderm, mesothelial cells, and others (Majesky, 2003). In ureter myogenesis, the epithelial tube acquires a coat of SM cells after the basic shape of the ureter has been established (Lye et al., 2010).

The total amount of SM in the human body has been estimated to be 1 – 1.6 kg, or 1.5–2.2% of body weight, with the gut, uterus, and vessels having a significantly higher amount among the varieties of SM types (Gabella, 2002). Although being differentiated cell types with remarkable structural and functional diversity, SM cells retain a degree of morphological and functional plasticity that is triggered by environmental conditions (Owens et al., 2004). This capacity to modulate their phenotypes could result in pathological changes such as in airway SM in chronic asthma, resulting in increased mass and phenotype changes contributing to airway obstruction and hyperresponsiveness ( Jesudason, 2009). Because of this phenotypic plasticity added on top of diversity, it is difficult to establish definitive markers for SM cells. Nevertheless, SM isoforms of contractile apparatus proteins were selected to serve as markers for defining SM cells, which include SM a-actin, SM MyHC, calponin, caldesmon, smooth muscle protein 22-alpha (SM22a), aortic carboxypeptidase-like protein, desmin, telokin, and smoothelin (Owens, 1995; Owens et al., 2004). Actin and myosin are two contractile components, calponin and caldesmon are constituents of SM thin filaments (Duband et al., 1993; Makuch et al., 1991 ; Takahashi et al., 1988), desmin is an intermediate filament protein of both smooth and striated muscles, telokin is the C-terminal portion of SM myosin light chain kinase (Ito et al., 1989), and smoothelin is a SM-specific cytoskeletal protein (Van der Loop et al., 1996). The difficulty associated with these markers is that virtually all of them have been shown to be expressed in other cell types during development or in response to pathological

## 3. Signal Transduction in Cardiac Myogenesis

situations. For details of these markers, readers are referred to an excellent review by Owens et al. (2004).

### 3.1. Signal transduction in cardiomyocyte precursors

#### 3.1.1. Developmental origins of cardiomyocyte progenitors

Decades of descriptive embryology and cell lineage tracings have improved our understanding of the development of the heart and developmental origins of cardiomyocyte progenitors or precursors of each

component of the heart (Fig. 4.1; Kodo and Yamagishi, 2011; Srivastava, 2006; Vincent and Buckingham, 2010). Formation of the precardiac mesoderm is considered to be the first step in cardiac development. In this process, a specific subset of mesodermal cells, generated during embryonic gastrulation, migrate anterolaterally to form a crescent shape on approximately embryonic day (E)7.5 in the mouse embryo, corresponding roughly to week 2 of

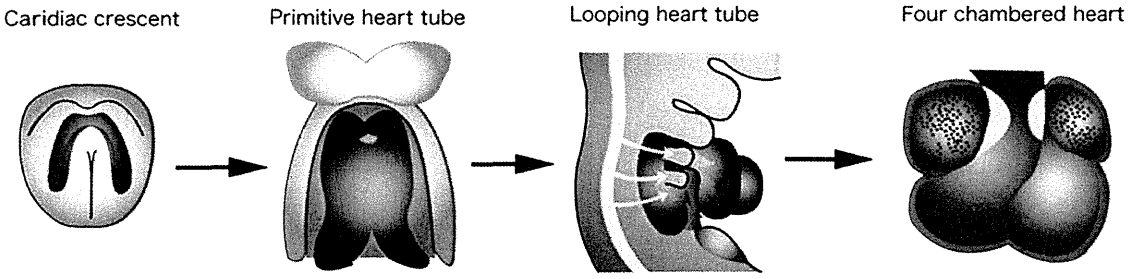



Figure 4.1 Developmental origins of the heart. Frontal views of embryos at cardiac crescent and primitive heart tube stage, and a lateral view of embryo at looping heart stage, and the four-chambered heart are shown. In the cardiac crescent, the first heart field (FHF) cells form a crescent shape in the anterior region of embryo with the second heart field (SHF) cells medial to it. In the primitive heart tube, the SHF cells lie dorsal to the heart tube in the splanchnic mesoderm of pharyngeal region. In the looping heart tube, the SHF cells migrate into the anterior and posterior ends of the tube ( blue allows). Subsequently, cardiac neural crest cells (cNCC) also migrate into the outflow tract from the dorsal neural tube (yellow arrows) to remodel the outflow tract and the aortic arch system. In the four-chambered heart, the FHF cells form the left ventricle and atria (red) whereas the SHF cells form the outflow tract, the right ventricle, and part of atria (blue). cNCC contribute to the outflow tract cushions (yellow) that eventually form the aortopulmonary septum. Epicardium is derived from proepicardial organ ( green ).

human gestation. By E8.0 in mice, or week 3 in humans, a pair of endocardial tubes appears in the cardiac crescent and fuses along the ventral midline to form a primitive heart tube, consisting of an interior layer of endocardial cells and an exterior layer of myocardial cells, separated by the extracellular matrix, or "cardiac jelly," that mediates reciprocal signaling between the two layers. The crescent-shaped pool of cardiogenic progenitor cells is referred to as the "first heart field".

In addition to the FHF, there is a second source of myocardial cells. In the mouse embryo, this source lies medially to the cardiac crescent and then lies behind the forming heart tube, extending into the mesodermal layer of the pharyngeal arches. This second source is referred to as the "second heart field" (Fig. 4.1; Kodo and Yamagishi, 2011; Srivastava, 2006; Vincent and Buckingham, 2010). The heart tube derived from the FHF may predominantly provide a scaffold upon which cells from the SHF migrate into both arterial and venous poles of the heart tube, where they subsequently construct the requisite cardiac components. As the heart tube

forms, SHF cells migrate into the midline and position themselves dorsal to the heart tube in the pharyngeal mesoderm. Upon rightward looping of the heart tube, SHF cells cross the pharyngeal mesoderm into the arterial and venous portions, populating a large portion of the outflow tract, future right ventricle, and atria. The addition of the SHF-derived myocardium to the outflow tract results in its elongation. This elongation is necessary to allow the outflow tract to rotate for building a correct alignment of the pulmonary and aortic trunks with their respective ventricles. In the arterial pole of the heart, it has been shown that the SHF contributes to both myocardial and SM lineages. Finally, progenitor cells derived from the FHF contribute exclusively to the left ventricle and all other parts of the heart except the outflow tract, whereas those from the SHF, which segregates rather later, contribute exclusively to the outflow tract and all other parts of the heart, except the embryonic left ventricle. In murine embryos, the entire outflow tract myocardium and most of right ventricular myocardium are formed from progenitor cells located in the anterior part of the SHF. In contrast, the myocardial contribution of the SHF cells appears to be limited to a part of the outflow tract in chick embryos (Waldo et al., 2001).

Neural crest cells (NCC) are multipotential cells that delaminate from the dorsal neural tube that is derived from the ectoderm and migrate widely throughout the body as mesenchymal cells. A subregion of the cranial NCC originating between the otocyst and somite 3 has been called the "cardiac NCC" that migrates into the third, fourth, and sixth pharyngeal arches and the cardiac outflow tract (Hutson and Kirby, 2007). They contribute to remodeling of the six pairs of bilaterally symmetric pharyngeal arch arteries connecting to the dorsal aorta, that eventually results in the formation of the thoracic aorta, and proximal subclavian, carotid, and pulmonary arteries. In addition, they also form the septum of the outflow tract that divides the embryonic single truncus arteriosus into the aortic and pulmonary trunk. Many signaling pathways are involved in the migration and condensation of cardiac NCC, including reciprocal signaling between the cardiac NCC and cells originated from the SHF, that are eventually essential for the development of the outflow tract and the aortic arch system (Hutson and Kirby, 2007; Kodo and Yamagishi, 2011; Srivastava, 2006; Vincent and Buckingham, 2010; Waldo et al., 2001).

### 3.1.2. Islet1 as a marker for cardiomyocyte progenitors

Insight into the molecular mechanism underlying the development of cardiac progenitor cells would provide new therapeutic approaches for cardiac repair. In the past 10 years, numerous transcription factors that function during cardiac development have been identified. LIMhomeodomain transcription factor, Islet1, has been defined as a

marker for a cardiac progenitor cell lineage that is capable of differentiating into all three major cell types of the heart, namely cardiomyocytes, SM cells, and endothelial cells (Laugwitz et al., 2005; Moretti et al., 2006). Islet1 knockout mice showed developmental defects of the right ventricle and outflow tract (Cai et al., 2003). During development, the expression of Islet1 is first detectable in the area comprising the dorsal-medial aspect of the cardiac crescent and then behind the forming heart tube, expanding the splanchnic mesoderm of the pharyngeal region. The field where Islet1 is expressed is now commonly defined as the SHF. Expression of Islet1 is extinguished as progenitor cells express markers of cardiac differentiation, although Islet1 is necessary for SHF-derived cells to populate the heart. Cardiac progenitor cells in the SHF appear to be regulated by complex positive and negative signaling networks (Rochais et al., 2009), including members of the Wnt, Fgf, bone morphogenetic protein (Bmp), hedgehog (Hh), and so on (Fig. 4.2). Such signals may be autocrine, but often arise from the adjacent endoderm or other cell lineages.

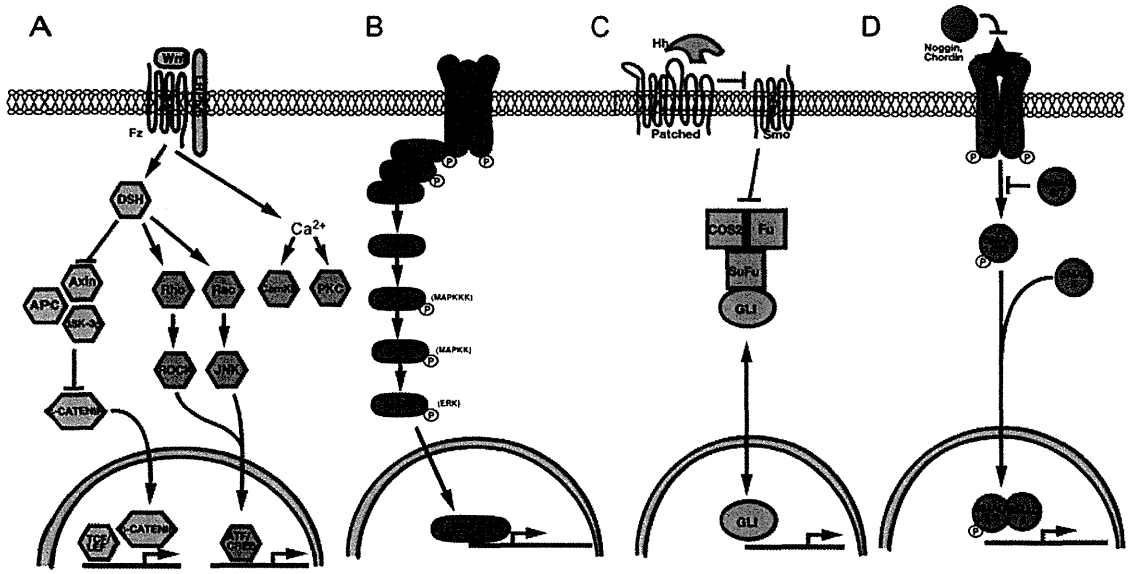
### 3.1.3. Wnt signaling in cardiac precursors

Canonical Wnt signaling promotes progenitor cell proliferation, in addition to playing earlier roles in mesodermal development and negative modulation of cardiac specification (Cohen et al., 2007). After the heart tube has formed, canonical Wnt signaling is maintained in the SHF. SHF cells may remain in an undifferentiated progenitor state until they are incorporated into the heart because they are close to inhibitory Wnt signals that emanate from the midline. Canonical Wnts (Wnt1, Wnt3a) from the dorsal neural tube are important for the maintenance of proliferating progenitors within the SHF and for the inhibition of differentiation. b-Catenin is required for SHF progenitors that are positive for Islet1. The genetic ablation of b-catenin results in embryonic lethality with pharyngeal arch remodeling defects and a single outflow tract, probably as a result of a reduction in SHF proliferation (Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007). The expression of Islet1, Fgf10, and Sonic hedgehog (Shh) are reduced, suggesting an upstream role for canonical Wnt signaling in the SHF, and in fact, b-catenin can directly activate transcription from Islet1 and Fgf10 promoters. Loss of Wnt signaling reduces the number of Islet1-positive cells, leading to defects of the outflow tract and right ventricle, whereas excess Wnt signaling expands the Islet1-positive population. Conditional expression of stabilized b-catenin results in the expansion of SHF progenitors. Transcriptome analysis of these Islet1-positive cells shows an upregulation of Fgfs, which promote proliferation, when canonical Wnt signaling is increased (Kwon et al., 2009). Also, higher levels of stabilized b-catenin and increased

Wnt/b-catenin signaling were observed in the absence of Notch1, suggesting a link between the canonical Wnt/b- catenin pathway and Notch signaling. Notch signaling normally represses cardiac progenitor proliferation by negatively regulating the active form of b-catenin. Of Wnt ligands potentially involved in autocrine signaling within the SHF, Wnt2 is expressed in the posterior region and directly regulates proliferation of venous pole progenitors in concert with b- catenin (Tian et al., 2010).

In addition to its role in promoting proliferation in the SHF, there is evidence to suggest that canonical Wnt signaling also prevents the onset of differentiation (Kwon et al., 2009). Conditional expression of stabilized

Figure 4.2 The core features of the Wnt, Fgf, Hh, and Bmp signaling pathways. ( A ) Wnt/b-catenin signaling: Binding of Wnt ligands to the Frizzled (Fz)–LRP5/6 receptor complex activates the intracellular effector Disheveled (DSH), resulting in the uncoupling of b-catenin from a multiprotein degradation complex composed of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase (GSK) 3b, which allows b-catenin translocates to the nucleus. b-Catenin activates target gene transcription in association with TCF/LEF. Noncanonical Wnt signaling: (1) Activated-DSH promotes small G protein (Rac and Rho), leading to the activation of c-Jun N-terminal kinase (JNK) and Rho-associated kinase (ROCK). The ATF/CREB complex is eventually activated, leading to target gene transcription. (2) Intracellular $Ca^{2b}$ release leads to activation of the $Ca^{2b}$/calmodulin-dependent kinase II (CaMKII) and the protein kinase (PK) C independent of DSH. (B) Fgf signaling: Binding of Fgf ligands leads to the autophosphorylation of the Fgf receptor tyrosine kinase, allowing interaction of the FRS2-docking protein and subsequent activation of the GRB2 (growth factor receptorbound protein 2) and SOS (son of sevenless) complex. Activated SOS, in turn, activates the small G protein RAS, triggering a cascade of phosphorylation leading to the activation of RAF, MEK, and mitogen-activated protein kinase (MAPK/ERK). pERK phosphorylates target transcription factors. (C) Hh signaling: In the absence of Hh ligand, the Patched receptor inhibits activity of the transmembrane protein Smoothened (Smo). The COS2/Fu/SuFu (Costal-2/Fused/Suppressor of fused) complex maintains the transcription factor Gli inactive. In the presence of Hh ligand, Hh binds to Patched and thereby releases the inhibition of SMO. SMO blocks the COS2/FU/SuFu complex, resulting in translocation of active Gli to the nucleus to drive target gene transcription. (D) Bmp signaling: Bmp ligands (Tgfb family of signaling molecules) bind to a receptor complex composed of Bmp receptors type 1 and type 2. On receptor activation, Smad proteins (Smad1/5/8) are phosphorylated and associate with a coactivator Smad (Smad4). The resulting Smad complex enters the nucleus to activate target gene expression.




Bmp signaling can be inhibited by the secreted proteins, Noggin and Chordin, and intracellular Smad proteins ( Smad 6/7).

b-catenin also shows, in Islet1-positive cells, downregulation of the gene encoding myocardin which, together with SRF, promotes myocardial and SM differentiation. Meanwhile, b-catenin induces both Bmp4 and noncanonical Wnt11 expression in the SHF, suggesting that early canonical Wnt signaling also sets the stage for differentiation. Although Islet1 had been mainly associated with proliferation, it may, at least when expressed at a high level, promote differentiation. It is capable for Islet1 to directly activate a regulatory element of the myocardin promoter. Differences in the levels of Islet1 in determining its effects on differentiation or proliferation are likely to be associated with the level of stabilized b-catenin in cardiac progenitor cells.

In contrast to that, canonical Wnt signaling is mainly responsible for promoting proliferation and maintaining cells in a progenitor state, while noncanonical Wnt signaling promotes cardiac differentiation and is specifically required for outflow tract development (Pandur et al., 2002). Wnt11 mutant embryos have a short outflow tract and develop alignment and septation defects likely mediated by the loss of transforming growth factor (Tgf)b2 signaling, known to be a critical regulator of the neural crest and endocardial cushion development during outflow tract septation (Zhou et al., 2007). Wnt5a is expressed in the SHF, and mutant embryos have a common ventricular outlet and abnormal neural crest invasion, although the expression domains of SHF genes Fgf8, Fgf10, and Tbx1 are unaltered, suggesting a role for noncanonical Wnt signaling from the SHF to neural crest cells (Schleiffarth et al., 2007).

### 3.1.4. Fgf signaling in cardiac precursors

Fgf signaling promotes SHF proliferation. Fgf8 is known to play an important role in specifying the early cardiac mesoderm and is likely to be the major Fgf ligand driving SHF development (Alsan and Schultheiss, 2002). Mice with Fgf8 hypomorphic alleles develop outflow tract defects reminiscent of those in the mouse mutant for T-box (Tbx) transcription factor, Tbx1 (Abu-Issa et al., 2002). Regulatory elements on the Fgf8 locus are directly activated by Tbx1, suggesting that Tbx1 may positively regulate proliferation in the SHF through transcriptional regulation of the Fgf8– Fgfr1 signaling pathway (Hu et al., 2004). Tbx1 itself is regulated positively by Shh signaling and negatively by retinoic acid signaling, and thus, plays an important role in integrating different signaling inputs during SHF development (Yamagishi et al., 2003).

Fgf8 is produced by the mesoderm, endoderm, and ectoderm of pharyngeal arches. Among those, mesodermal Fgf8 expression is clearly important for the development of the SHF (Abu-Issa and Kirby, 2007). Fgf10 is also expressed in the SHF. Although mice with mutation of Fgf10 do not show defects in SHF development, mesodermal Fgf8/Fgf10 compound mutants display increasingly severe defects in the outflow tract and pharyngeal arch arteries, suggesting that proliferation of progenitor cells in the SHF that contribute to the outflow tract myocardium is very sensitive to the dosage of Fgf signaling (Watanabe et al., 2010).

Conditional ablation of both Fgfr1 and Fgfr2 specifically in the SHF, but not in endothelial or neural crest cells, shows a reduction in proliferation of progenitor cells within the SHF, suggesting that autocrine Fgf signaling from the SHF-derived myocardium are likely to drive proliferation and deployment of SHF cells (Park et al., 2008). The results that Bmp4 and Wnt11, as well as Sema3c, were downregulated in these conditional Fgfr1/ Fgfr2 mutants further demonstrate an autocrine requirement for Fgf signaling in the SHF. Fgf signaling, probably through its effects on proliferation, increases the extent of the heart field and the consequent numbers of cardiomyocytes that eventually form the outflow tract.

In addition to Fgf8 and Fgf10, Fgf15 is also implicated in the development of the outflow tract. It is expressed, independently of Tbx1, in the pharyngeal arch mesoderm (arch 3) and in the SHF. Fgf15 mutants show outflow tract defects, probably at least partly as a result of abnormal development of the neural crest (Vincentz et al., 2005).

Fgf signaling is also thought to be involved in the interaction between progenitor cells derived from the SHF and neural crest. In mesodermal Fgf8 mutant mice, Bmp/Tgfb signaling, that normally provides a relay that affects the neural crest, is downregulated in the SHF, which requires Smad4-mediated signaling for survival (Nie et al., 2008; Park et al., 2008). In chick embryos, neural crest ablation results in an increase in Fgf8 that

perturbs SHF development, and the defect 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.

### 3.1.5. Hh signaling in cardiac precursors

Hh signaling is also often associated with proliferation. Shh, from the endoderm, the notochord, and the floorplate 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. Shh mutant embryos display defects in SHF deployment and survival, resulting in a common ventricular outlet, and the conditional deletion of Shh in mice has revealed that the pharyngeal endoderm is the source of a ligand required for SHF and outflow tract development (Goddeeris et al., 2007). Extensive cell death is observed in the SHF of these conditional mutant embryos, resulting in defective outflow tract elongation.

Shh expression is regulated by Islet1 in the pharyngeal endoderm (Lin et al., 2006). At the arterial pole, Shh from the endoderm affects the pharyngeal arch mesoderm and Tbx1 expression, with additional effects on the maintenance and deployment of the neural crest (Dyer and Kirby, 2009; Yamagishi et al., 2003). In the absence of Shh, the pharyngeal vasculature, as well as outflow tract development, is affected. The effect of Shh on Tbx1 expression has particular implications for the formation of the pulmonary trunk myocardium (The´veniau-Ruissy et al., 2008). In chick embryos, 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). Manipulation of Shh signaling in chicks affects migration of SHF cells into the outflow tract, suggesting that it is also important for SHF recruitment. Moreover, the absence of Shh signaling in the posterior SHF of mouse embryos results in 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 (The´veniau-Ruissy et al., 2008). Taken together, Hh signal reception is necessary for the survival of neural crest cells in the pharyngeal region, in the SHF, and in the pharyngeal endoderm itself to regulate a secondary signal that controls the survival and deployment of cells derived from the SHF.

Shh signaling in the pharyngeal mesoderm is required not only for outflow tract development but also for atrioventricular septation (Goddeeris et al., 2008). Development of the dorsal mesocardial protrusion, giving rise to part of the atrial septum extending toward the atrioventricular cushions, derived from the SHF depend on this signaling pathway. Shh may play a role in the specification of atrial septal precursors

and in the recruitment of cells from this part of the SHF. Hh signaling also promotes cardiomyocyte formation, as well as regulating the number of cardiac progenitor cells, pointing to a dual role, during cardiogenesis in zebrafish embryos (Thomas et al., 2008). Gain- and loss-of-function experiments in zebrafish embryos have revealed an early requirement for Hh signaling in controlling myocardial progenitor cell numbers through a cell autonomous role in promoting progenitor cell specification. This role appears to be distinct from that in the SHF.

### 3.1.6. Bmp signaling in cardiac precursors

Bmp signaling is known to be required for cardiac mesoderm specification and differentiation (Schultheiss et al., 1997). SHF progenitors lie medially to the differentiating cardiac crescent, at a distance from sources of Bmp emanating from the lateral mesoderm, consistent with their maintenance in an undifferentiated state. Mouse mutants with Bmpr1a, that encodes the Bmp receptor type 1 in the early cardiac mesoderm, results in a failure to form a differentiating heart tube, although Islet1 positive cardiac progenitor cells are present (Klaus et al., 2007). Later conditional deletion of Bmpr1a in Islet1-expressing cells results in an abnormal right ventricle and outflow tract with increased numbers of Islet1-positive cells, suggesting a differentiation defect as Islet1 is normally downregulated in cardiomyocytes. This is accompanied by a reduction in Tbx20 that is required to repress Islet1 in the outflow tract (Yang et al., 2006).

Bmp4 is required for outflow tract development where it probably affects myocardium formation as well as the survival of the neural crest (Nie et al., 2008). Outflow tract elongation is reduced in embryos lacking both Bmp4 and Bmp7 (Liu et al., 2004). Bmp2, acting with Bmp4, is also implicated in cardiac development (Uchimura et al., 2009). Bmp2 and Bmp4 are expressed in the outflow tract myocardium. Blocking of Bmp signaling inhibits SHF differentiation. Bmp2, and also Bmp4, are proposed to be candidate molecules for inducing differentiation of SHF progenitors at the arterial pole of the heart tube, and may play an additional role in the recruitment of these cells (Waldo et al., 2001). Bmp signaling downregulates Islet1 expression as SHF-derived cells enter the outflow tract.

Bmp4, together with other genes for the Bmp/Tgfb family members, are targets of Fgf signaling in the SHF (Park et al., 2008). Wnt/b- catenin signaling, which plays a role in proliferation and maintenance of an undifferentiated state in the SHF, also upregulates Bmp4 (Ai et al., 2007). The expression levels of these signal transductions are probably critical and different signaling thresholds may be required for an effect on proliferation or myocardial differentiation. In this context, a balance

between Fgfs and Bmps should be important. The importance of regulating the level of Bmp/ Tgfb signaling within the SHF is demonstrated by the role of Nkx2–5 that represses Bmp2 expression (Prall et al., 2007). Over-specification of cardiomyocytes and a reduction in proliferating SHF progenitors, leading to outflow tract truncation, are evident in the absence of Nkx2–5. Smad1 is supposed to mediate the effect of Bmp2 on the SHF. In mesodermal Smad1 mutant mice, SHF proliferation and the outflow tract length are increased. The Nkx2–5 phenotype is alleviated on loss of Smad1 alleles. These findings demonstrate a negative feedback loop involving Nkx2–5, Bmp2, and Smad1 that controls the balance between progenitor proliferation and differentiation in the SHF. Bmp/Tgfb signaling can also be regulated by Tbx1 through direct interaction with Smad1 (Fulcoli et al., 2009). Development of the SHF involves Bmp-driven differentiation at the distal end of the heart tube and Fgf-driven proliferation in the pharyngeal mesoderm.

### 3.1.7. Signaling transduction in the proepicardium

The proepicardium (PE) is another lineage of cardiac precursor cells developing from the coelomic mesothelium which overlays the liver bud, and the expression of PE-specific genes is induced in naï¨ve mesothelial cells in response to a localized liver-derived signal (Kodo and Yamagishi, 2011 ; Srivastava, 2006; Vincent and Buckingham, 2010). Cells of the epicardium and the coronary vessels arise from the PE, which develops as multiple epithelial villi protruding from the pericardial mesothelium immediately posterior to the sinoatrium of the looping stage embryonic heart. The PE extends toward the primitive heart, attaches, and spreads over the myocardial surface, forming the epicardium (Fig. 4.1). During PE growth and epicardial formation, some PE/epicardial cells undergo an epithelial– mesenchymal transformation (EMT) and are localized to the subepicardial space, giving rise to the precursors of coronary vessels and connective tissue cells (Olivey and Svensson, 2010). As for the development of the coronary vasculature, SM cells arise from the epicardium, and endothelial cells and capillaries are derived from the venous plexus, at the sinus venosus, which invades the heart after formation of the epicardium.

Although the PE has been thought to be derived from the coelomic mesenchyme of the septum transversum and not from the SHF (Wessels and Pe´rez-Pomares, 2004), its relationship to the SHF is still unclear since it forms as a group of cells close to the venous pole of the heart tube. Islet1 is expressed in PE in the early embryo and Islet1-Cre genetic tracing marks PE and subsequent epicardium and coronary blood vessels (Zhou et al., 2008a,b). It is also ambiguous whether the PE can give rise to the myocardium of the heart. Although fate-mapping studies showed no

contribution of PE-derived cells to the myocardium (Winter and Gittenberger-de Groot, 2007), manipulation of Fgf versus Bmp signaling levels resulted in myocardial differentiation of PE-derived cells in vitro (Van Wijk et al., 2009). Transcription factors Wt1 and Tbx18 are expressed in PE/epicardial cells and are thought to play a critical role in signal transduction essential for development of the myocardium and coronary SM. Mice with a Wt1 mutation show coronary vascular defects as a result of a failure of direct activation of the downstream target genes required for EMT of epicardial cells (Martı´nezEstrada et al., 2010). ES cells with a WT1 mutation fail to form cardiomyocytes, as well as other mesodermal derivatives, and probably results from a failure of EMT. When the fate of PE-derived cells were traced using a Wt1-GFP-Cre line, GFP-positive cardiomyocytes were detected in the walls of the cardiac chambers and in the interventricular septum, suggesting that a subset of PE-derived cells may differentiate into the myocardium (Zhou et al., 2008a,b). Fate mapping of Tbx18-expressing cells using a Tbx18-Cre line also showed the labeling of cardiomyocytes, as well as the SM of coronary blood vessels and of cardiac fibroblasts (see the below section) (Cai et al., 2008).

## 3.2. Transcriptional regulation during cardiac myogenesis

### 3.2.1. A role of GATA genes in cardiac development

Members of zinc-finger transcription factors, termed GATA factors, have essential roles in the differentiation and survival of many cell types, including cardiomyocytes (Brewer and Pizzey, 2006; Molkentin, 2000). Structures of GATA factors are well conserved and widespread between species. Six GATA transcription factors have been identified in vertebrates, each of which contains a highly conserved domain, consisting of two zinc fingers of the motif Cys-X2-Cys-X17-Cys-X2-Cys (Evans and Felsenfeld, 1989; Tsai et al., 1989), that directs binding to the nucleotide sequence element ( A/T ) GATA(A/G) (Ko and Engel, 1993; Merika and Orkin, 1993). Based on their expression patterns, GATA proteins have been divided into two subfamilies, Gata1, 2, and 3; and Gata4, 5, and 6. Gata1, 2, and 3 are mainly associated with haematopoietic lineages and some ectodermal derivatives (Shimizu and Yamamoto, 2005), whereas Gata4, 5, and 6 are involved in cardiogenesis and the formation of a subset of endodermal tissues (Charron and Nemer, 1999).

3.2.1.1. Expression of GATA transcription factors in the heart Focusing on the heart, transcripts of Gata4, 5, and 6 are all present in the precardiac mesoderm at embryonic day (E)7–7.5 (Charron and Nemer, 1999) and are thought to be some of the earliest genes reported to be expressed in cardiomyocyte precursors in murine embryos. The expression of all three

factors is subsequently maintained as the cells within the cardiac crescent migrate from the anterolateral mesoderm to form the primary heart tube. As for Gata4, early cardiac expression at E8.0 is confined to the posterior end of the heart tube. During the looping stage at E8.5–9.0, its expression extends throughout the heart tube (Nemer and Nemer, 2003). The expression pattern of Gata6 has been reported to be almost identical to that of Gata4 within the developing heart up to E9.5 (Nemer and Nemer, 2003). By E11.5, Gata4 and Gata6 are detected in the myocardium and endocardium of the atria and ventricles and within the proximal outflow tract (Nemer and Nemer, 2003). Both proteins are detected in the endocardial cushions of the atrioventricular canal and the outflow tract at E10.5–11.5 (Nemer and Nemer, 2003). Between E12.5 and E13.5, Gata6 is detectable in the vascular SM in the caval veins and dorsal aorta. Cardiac expression of Gata4 and 6 persists in the adult in the endocardium and myocardium (Charron and Nemer, 1999). The expression of Gata5 in murine cardiogenesis initially overlaps substantially with that of Gata4 and 6. However, by E12.5, Gata5 has become restricted to the atria. By E16.5, Gata5 can no longer be detected within the mouse heart (Morrisey et al., 1997).

3.2.1.2. GATA factors in cardiac differentiation The process of early embryonic development involved in cardiogenesis is well conserved between species. In flies, cardiac precursor cells are specified from paired progenitor fields and move into a linear tubular structure like that in vertebrates. In Drosophila, GATA-binding factor Pannier is expressed within the dorsal, heart-forming mesoderm of embryos and is required for cardiogenesis (Sorrentino et al., 2005).

In vertebrate embryos, cardiac GATA factors are coexpressed within the cardiac mesoderm from the very early stages in its specification (Nemer and Nemer, 2003). Targeted disruption of Gata4, 5 and 6 in the mouse has revealed phenotypes consistent with their individual expression patterns.

Mice null for Gata4 die between E8.0–9.0 as a result of defects in heart morphogenesis and ventral closure of the foregut (Molkentin et al., 1997). Gata4 null mice show cardia bifida resulting from the failure of ventral fusion of the lateral aspects of the embryo. This defect of heart formation in Gata4 null mice is likely a secondary effect associated with an intrinsic defect in the endoderm that underlies the splanchnic mesoderm containing the cardiac field.

On the other hand, targeted disruption of the Gata5 gene in the mouse did not result in a developmental lethality (Morrisey et al., 1997). Interestingly, a Gata5 null mutation in zebrafish resulted in an embryonic lethality with an almost identical phenotype to that observed in Gata4 null

mice, suggesting a reversal in the roles of Gata4 and Gata5 between fish and mouse (Reiter et al., 1999).

Gata6 null mice die during early embryogenesis (E5.5–7.5) as a result of defects in visceral endoderm function and subsequent extraembryonic development that are consistent with the expression pattern of Gata6 in the embryonic primitive endoderm (Morrisey et al., 1998). Expression of Gata4 and 6 is dependent on each other given the observation that Gata6 is upregulated in Gata4 null mice and that Gata6 null embryos show downregulation of Gata4 (Morrisey et al., 1998). Although the mechanism underlying this regulation has not been elucidated, it is suggested that Gata4 negatively regulates Gata6 expression, whereas Gata6 positively regulates Gata4 expression. Conditional inactivation of Gata6, specifically in the cardiac NCC, causes persistent truncus arteriosus, a severe congenital heart defect characterized by an embryonic single outflow tract without septation, suggesting an essential role of Gata6 during development of the outflow tract (Lepore et al., 2006). Mice compound heterozygous for Gata4 and Gata6 null alleles die in utero and exhibit a spectrum of congenital heart defects, involving the atrioventricular septum and outflow tract (Xin et al., 2006). Gata6 may function in concert with Gata4 to direct tissuespecific gene expression essential for formation of the mammalian heart.

3.2.1.3. Target genes of GATA transcription factors during cardiac development Gata4 regulates expression of a number of cardiac structural genes (Table 4.2). GATA factors also regulate developmental expression of the cardiac transcription factor Nkx2–5, suggesting the existence of a reinforcing transcriptional regulatory circuit between Nkx2–5 and GATA factors in the heart (Lien et al., 1999). In Drosophila, pannier regulates expression of the myocyte enhancer factor-2 (MEF2) gene in cardioblasts, extending the role of GATA factors as regulators of heart gene expression to include invertebrates (Sorrentino et al., 2005). Taken together, GATA factors play roles as regulators of structural genes in the heart, as well as the gene network essential for development of the heart.

Table 4.2 Downstream target cardiac genes for GATA transcription factors

| Target geneReference | |
|---|---|
| a-Myosin heavy chain, | Molkentin et al. (1994), Charron et al. (1999) |
| Cardiac troponin-C | Ip et al. (1994), Morrisey et al. (1996) |
| atrial natriuretic factor | Gre′pin et al. (1994) brain natriuretic peptide Thuerauf et al. (1994) |

| | |
|---|---|
| Myosin light chain 1/3 | McGrew et al. (1996) |
| Sodium/calcium exchanger | Nicholas and Philipson (1999), Cheng et al. (1999) |
| A1 adenosine receptor | Rivkees et al. (1999) |
| Cardiac-restricted ankyrin repeat protein | Kuo et al. (1999) |
| Cardiac troponin-I | Murphy et al. (1997), Di Lisi et al. (1998) , and Bhavsar et al. (2000) |
| m2 muscarinic receptor | Rosoff and Nathanson (1998) a- |
| Cardiac actin | Sepulveda et al. (2002) b-Myosin heavy chain Charron et al. (1999), Hasegawa et al. (1997), and Wright et al. (2001) |
| Slow myosin heavy chain 3 | Wang et al. (1998) |
| Myosin light chain 2 | Latinkic et al. (2004) |

3.2.1.4. Interactions between GATA and other transcription factors Interactions between transcription factors are essential for correct temporal and spatial gene expressions. Synergistic transcriptional activation is observed between Gata4 and Nkx2–5, acting on a subset of cardiac promoters that contain both GATA- and Nkx-binding element, NKE sites(Brewer and Pizzey, 2006; Molkentin, 2000). This is also observed when Gata4 is substituted by Gata5, but not by Gata6 (Brewer and Pizzey, 2006; Molkentin, 2000). In contrast, both Gata4 and 6 can act synergistically with MEF2 proteins, while Gata5 cannot (Brewer and Pizzey, 2006). Synergistic interactions with coactivators have also in some cases been reported for all three heart-expressed GATA family members (Table 4.3).

3.2.1.5. GATA transcription factors implicated in congenital heart diseases Most congenital heart diseases involve specific developmental processes and usually result from a failure of specific morphogenetic events. Such processes in cardiogenesis frequently involve transcriptional regulatory circuits and thus it is unsurprising to find transcriptional regulators as genetic causes in the etiology of several congenital heart diseases. Abnormal expression of GATA4, 5, or 6 might be expected to be associated with specific human congenital heart diseases, based on their essential roles in cardiac development. Garg et al. reported that GATA4 mutations cause familial

Table 4.3 Cofactors for GATA transcription factors

| Gene | Reference |
|---|---|
| NF-AT3 | Molkentin et al. (1998) |
| SRF | Belaguli et al. (2000) |

| | |
|---|---|
| dHAND | Dai et al. (2002) |
| SMAD1/4 | Brown et al. (2004) |
| Tbx5 | Garg et al. (2003) |
| c-Fos | McBride et al. (2003) |
| Kruppel family protein Zfp260 | Debrus et al. (2005) |
| YY1 | Bhalla et al. (2001) |
| STAT proteins | Wang et al. (2005) |
| MEF2 | Morin et al. (2000) |
| FOG-2 | Lu et al. (1999), Svensson et al. (1999, 2000), and Tevosian et al. (1999) |
| p300 | Dai and Markham (2001), Kakita et al. (1999), and Wada et al. (2000) |
| Hairy-related transcription factor family | Kathiriya et al. (2004) |
| Hey basic helix–loop–helix transcription factors | Fischer et al. (2005) |
| Jumonji | Kim et al. (2004) |
| RXRa | Clabby et al. (2003) |

cardiac septal defects (Garg et al., 2003). A wide range of mutations has now been identified in patients with various congenital heart diseases and, in some cases, specific genotype–phenotype correlations were observed (Fig. 4.3).

Recently, we screened for mutations in cardiac transcription factors in patients with selected nonsyndromic persistent truncus arteriosus, and identified two different GATA6 mutations in two probands that were not found in 182 unrelated controls with no congenital heart disease (Kodo et al., 2009). Our subsequent biological analyses revealed that SEMA3C and PLXNA2 were directly regulated by GATA6, and both GATA6 mutant proteins failed to transactivate these genes. SEMA3C and PLXNA2 mediate a signal transduction essential for neurovascular guidance as a ligand and a receptor, respectively, during development. Transgenic analysis further suggested that the expression of Sema3c and Plxna2 in the outflow tract was dependent on GATA transcription factors during heart development. Together, our data implicate mutations in GATA6 as novel genetic causes of a type of congenital heart disease involving outflow tract development, as a result of the disruption of the direct regulation of semaphorin–plexin signaling.

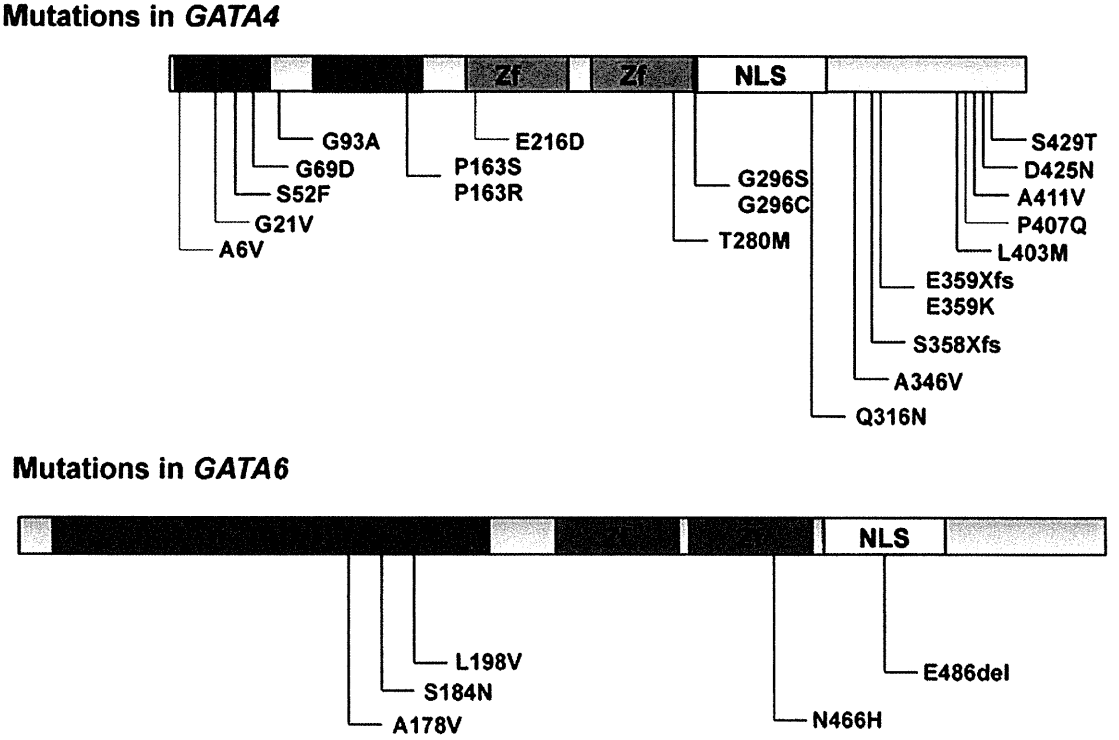



Figure 4.3 Schematic of GATA4 and GATA6 proteins, indicating the location of mutations reported to date. TAD, transcription activation domain; Zf, zinc finger domain; and NLS, nuclear localization signal.

Two other reports (Lin et al., 2010; Maitra et al., 2010) came out on mutations in GATA6 after our study that are associated with congenital heart diseases including atrioventricular septal defects, tetralogy of Fallot, and atrial septal defects (Fig. 4.3). It is of note that the GATA6 mutations identified in humans are predominantly associated with outflow tract defects, whereas GATA4 mutations are commonly associated with septal defects, although there are some phenotypic overlaps (Kodo, K., and Yamagishi, H., unpublished observations) likely to result from a redundant role of both genes. Although the clinical phenotype of individuals with GATA6 mutations commonly involves the outflow tract, it is distinct from that of the 22q11.2 deletion syndrome, the most common genetic cause of outflow tract defects, rather manifesting as nonsyndromic congenital heart diseases. TBX1 has been proposed as a major genetic determinant of the 22q11.2 deletion syndrome. Although no direct molecular link has been demonstrated between GATA6 and TBX1 until recently, a report (The´veniauRuissy et al., 2008) and our preliminary data suggest that the expression of Sema3c in the outflow tract and pharyngeal region is altered in mouse embryos deficient for Tbx1 (Kodo, K., and Yamagishi, H., unpublished observations), suggesting that GATA6 may share, at least in part, a common molecular pathway with TBX1 during outflow tract development.

### 3.2.2. A role of HAND genes in cardiac development

3.2.2.1. Normal development of the cardiac ventricles The formation of developing ventricles is first observed around embryonic day (E)8.5 in mouse embryos, approximately day 20 in human embryos (Srivastava et al., 1995). The developmental steps to form mature cardiac ventricles include looping of the primitive heart tube, ballooning of the cardiac ventricles, septation of the interventricular septum, and maturation of the ventricular myocardium. The looping of the heart tube leads the future right ventricle and the outflow tract to the right and ventral side, and the future left ventricle and the inflow to the left and dorsal side of the developing heart. The ballooning step progresses along with the cardiac looping. In this step, the primitive myocardium of the ventral side of the primitive straight heart tube gives rise to the outer curvature of the looping heart and rapidly proliferates to develop cardiac chambers toward the outside as the balloon fills out, resulting in the primitive right and left ventricles. Upon the ballooning process, the interventricular groove appears between the right and left ventricle, and the muscular interventricular septum protrudes inside the groove (Fig. 4.4). As the muscular

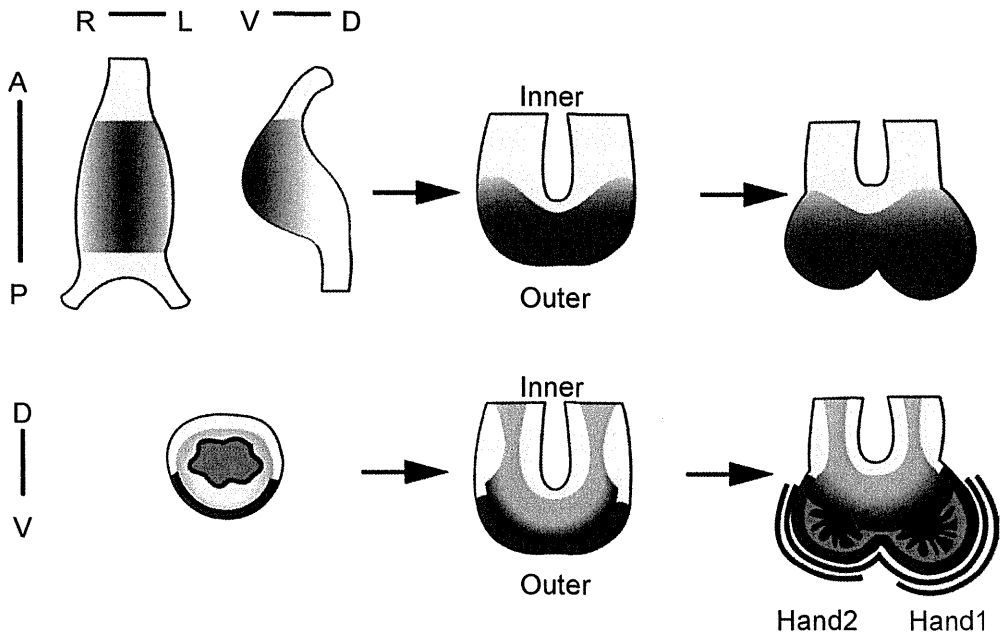



Figure 4.4 The ballooning process of developing cardiac ventricles. Upper panel shows the anterior view of the developing heart including the lateral view of the cardiac tube. Lower panel shows the axial view of the heart. As cardiomyocytes proliferate, the ventricular chambers grow and protrude outside like ballooning and interventricular groove between two ventricles passively appears prominent. Hand2 is expressed in the developing right ventricle while Hand1 is in the developing left ventricle. A–P, anterior–posterior; R–L, right-left; V–D; ventral–dorsal; D–V, dorsal–ventral; RV, right ventricle; and LV, left ventricle.

interventricular septum grows, the outflow tract septum dividing the aorta and pulmonary trunk and the endocardial cushion dividing the atrioventricular canal are fused to the muscular interventricular septum to form the membranous interventricular septum, leading to completion of the septation step. In the maturation step, cardiomyocytes in the ventricles give rise to the trabecular and the compact myocardial layer with fine gap junctions. The left ventricle hasa morecompact layerthan the right ventricle, andthese ventricles convey systemic and pulmonary circulation, respectively, after birth.

3.2.2.2. Expression and regulation of Hand genes during ventricular development Hand1/eHand and Hand2/dHand belong to a family of basic helix–loop–helix (bHLH) transcription factors and are indispensable for cardiogenesis, especially in ventricular development. Hand genes share common bHLH regions for DNA binding which are almost identical between Hand1 and Hand2. The Hand2 proteins share approximately 95% homology of the amino acid sequences among human, mouse, chicken, frog, and zebrafish, whereas the Hand1 proteins are less conserved across species. In zebrafish, an organism having only one cardiac ventricle, there is only one Hand gene that is closely related to Hand2 in the genomic sequence. This suggests that Hand2 may be evolutionally ancestral to Hand1 (Srivastava et al., 1995).

Hand genes direct a unique expression pattern during cardiac ventricular development. In chicks, Hand1 and Hand2 are expressed in both developing ventricles. In contrast, in mouse embryos, Hand1 is predominantly expressed in the left ventricle whereas Hand2 is expressed in the right ventricle in a complementary fashion (Srivastava et al., 1995). Intriguingly, genetic analysis of the developing heart in situs inversus mice showed that Hand1 and Hand2 are not expressed in the left-sided ventricle and rightsided ventricle, but in the morphological left ventricle and right ventricle, respectively (Thomas et al., 1998). Hand1 and Hand2 are also expressed in the NCC in partially overlapping patterns. Hand2 is more broadly expressed in the cardiac outflow tract than Hand1. Hand genes are downregulated after E13.5 during murine cardiac development and also after birth (Srivastava et al., 1995). In humans, HAND genes are expressed in both ventricles of adult hearts and Hand1 is reported to be downregulated in cardiomyopathies (Natarajan et al., 2001). Hence, the molecular mechanism that restricts the expression of Hand genes in the developing heart may be downregulated in the adult heart (Firulli, 2003).

Among the transcriptional factors related to developing cardiac ventricles other than Hand genes, Nkx2–5 and Gata4 are expressed throughout the ventricles during early development. Mef2c is