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### Bone Morphogenetic Protein Signaling Pathways in Heart Development and Disease

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#### 1. Introduction

The heart is the first organ to develop and its proper formation is requisite for survival of the embryo. Heart development relies on exquisitely controlled signaling cascades that together weave the temporal and spatial cardiac gene expression patterns required for normal heart morphogenesis and function. Aberrations in cardiogenic signaling pathways or in cardiac gene expression patterns can result in congenital heart defects (CHDs), the most common type of birth defect worldwide and the leading noninfectious cause of infant death in the Western world (Hoffman 1995; Hoffman and Kaplan 2002). This review provides evidence from multiple experimental models that demonstrates the conserved, critical roles of Bone Morphogenetic Protein (BMP) signaling pathways throughout heart development, from induction of the cardiac mesoderm to the formation of the fourchambered heart. BMP signaling pathways have roles in developmental processes that can contribute to CHDs, including formation of the septa, valves, and outflow tract.

#### 2. Overview

#### 2.1 Heart development

During gastrulation, cardiac progenitors within the lateral plate mesoderm migrate in bilateral sheets of cells to the anterior of the embryo. Within the cardiac mesoderm, there are two populations of cells that contribute to the developing heart called the first and second heart fields (FHF and SHF, respectively). At embryonic (E) day 7.5 in mice, cells of the FHF form the cardiac crescent. At this stage, the second population of cardiac cells, the SHF, is medial and anterior to the FHF. As the embryo folds, at mouse E8.0, the cardiac crescent fuses along the midline and forms the heart tube while the SHF moves dorsally. The heart tube consists of an outer myocardial layer and an inner endocardial layer, separated by an extracellular matrix (ECM) called the cardiac jelly. SHF cells migrate through the pharyngeal mesoderm to populate the anterior and posterior regions of the heart tube. Starting from E8.5 in the mouse, the heart undergoes rightward looping. Regional proliferation along the myocardium of the outer curvature of the heart tube demarcates the future atrial and ventricular chambers. The myocardium of the inflow tract (IFT), outflow tract (OFT), atrioventricular canal (AVC) and inner curvature of the heart tube is characteristically nonproliferative. The FHF contributes primarily to the left ventricle as well as to part of the atrium, and the SHF contributes to the atria, right ventricle, and OFT. At about E9.5 in the

AVC, the endocardial cells respond to signals from the myocardium and undergo epithelial to mesenchymal transition (EMT) to form the cushions, the primordial valve structures. Cushions are also formed in the proximal region of the OFT at a slightly later stage. Around E10.0, another population of cells called the cardiac neural crest cells (CNCC) migrates from the dorsal neural tube and contributes to the developing OFT. By E11.5, the proepicardial cells have migrated around and enveloped the heart, forming the epicardium. Finally, septation and valve development result in a four-chambered heart with right, pulmonary, and left, systemic, halves by mouse E14.5. For a review, see (Evans et al. 2010).

#### 2.2 BMP signaling pathways

BMP ligands are conserved growth factors that belong in the Transforming Growth Factor-β (TGF $\beta$ ) superfamily. More than twenty BMPs have been identified and they have a myriad of functions during development. BMP precursor proteins are activated via endoproteolytic cleavage, glycosylated, and then secreted as homo- or hetero-dimers (Derynck et al. 1985; Derynck et al. 1986; Wozney et al. 1990). Once processed and secreted, BMP ligands relay their signal to the nucleus through signaling cascades that utilize unique combinations of serine threonine kinase receptors which respond to specific ligand combinations. There are three type I receptors (out of seven) and three type II receptors (out of five) that transduce the BMP signals. The type I receptors are ALK2 (ACVRI, ACTRI), ALK3 (BMPRIA/BRK-1), and ALK6 (BMPRIB, BRK-2) (Macías-Silva et al. 1998; Koenig et al. 1994; ten Dijke et al. 1994). The type II receptors are BMPR2 (BMPRII, BRK-3), ACVR2A (ACTRIIA), and ACVR2B (ACTRIIB) (Yamashita et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995; Kawabata, Chytil, and Moses 1995). The BMP dimer binds a type II receptor, which recruits and phosphorylates a type I receptor in its intracellular kinase domain (Yamashita et al. 1995). The type I receptor then phosphorylates an intracellular receptor-regulated SMAD protein (R-SMAD). SMAD1, SMAD5, and SMAD8 are activated specifically by BMP signals (Cárcamo, Zentella, and Massagué 1995; Wieser, Wrana, and Massagué 1995; Hoodless et al. 1996; Nishimura et al. 1998; Chen, Bhushan, and Vale 1997). After phosphorylation, activated R-SMADs form a complex with the common SMAD, SMAD4 (Zhang, Musci, and Derynck 1997). The R-SMAD-SMAD4 complex translocates to the nucleus, where it cooperates with other cofactors to regulate gene transcription; an example is illustrated elsewhere (Jiao, Zhou, and Hogan 2002). BMP signaling can occur independently of SMAD proteins in non-canonical pathways. For example, BMPs can activate MAP kinase pathways, resulting in the activation of p38 MAPK, PI3K, ERK, and JNK with downstream effects on cell proliferation and differentiation (Yamaguchi et al. 1995; Shibuya et al. 1998; Kimura et al. 2000; Lou et al. 2000; Lai and Cheng 2002; Yanagisawa et al. 2001; Xu et al. 1996).

It has recently been demonstrated that BMP signaling can regulate microRNA (miRNA) biosynthesis. miRNAs are short non-coding RNA that target messenger RNA (mRNA) in a sequence-specific manner for post-transcriptional degradation and translational inhibition. miRNAs are transcribed as primary miRNAs (pri-miRNAs), which are processed by the Drosha complex within the nucleus. pri-miRNA processing results in a shorter pre-miRNA, which is exported from the nucleus to the cytoplasm where it is cleaved into its mature miRNA structure by Dicer. For a review on miRNA during cardiovascular development and disease, the reader is referred to Liu and Olson (2010). Activated R-SMADs can directly interact with the microprocessor complex, Drosha, independent of the common SMAD, SMAD4, to promote the biosynthesis of miRNAs such as *miR-21* (Davis et al. 2008; Ji et al.

2007; Warner et al. 2004; Fukuda et al. 2007; Davis et al. 2010). *miR-21* is upregulated in damaged cardiovascular tissue (Ji et al. 2007).

The timing, duration, and gradient of BMP ligands affect the outcomes and add to the complexity of BMP signaling pathways. After BMP processing and secretion, access to the receptors and retention in the ECM are inhibited by extracellular factors such as noggin, chordin, follistatin, cerberus, and gremlin (McMahon et al. 1998; Streit et al. 1998; Sasai et al. 1995; Hemmati-Brivanlou, Kelly, and Melton 1994; Fainsod et al. 1997; Bouwmeester et al. 1996; Hsu et al. 1998). These inhibitors bind BMP ligands, interfering with ligand-receptor interaction (Zimmerman, De Jesús-Escobar, and Harland 1996; Hsu et al. 1998; Yamashita et al. 1995; Piccolo et al. 1996; Iemura et al. 1998). BMP signaling is also regulated at the membrane, for instance by the pseudoreceptor BAMBI (BMP and Activin membrane bound inhibitor). BAMBI lacks the intracellular domain needed to transduce the signal and, upon binding BMP receptors, it inhibits the formation of an active BMP receptor complex (Onichtchouk et al. 1999; Grotewold et al. 2001). Alternatively, BMP signaling can be enhanced at the membrane level by modulators such as DRAGON, which acts as a coreceptor and presents BMPs to the receptors (Samad et al. 2005; Babitt et al. 2005; Babitt et al. 2006). Another example is endoglin, a transmembrane protein that binds to BMP ligands and enhances BMP signaling (Barbara, Wrana, and Letarte 1999; Scherner et al. 2007). Intracellularly, BMP signaling can be downregulated by SMURF, an E3 ubiquitin ligase that promotes R-SMAD degradation, receptor turnover, and facilitates inhibition by the inhibitory SMADs, SMAD6 and SMAD7 (Murakami et al. 2003; Kavsak et al. 2000; Ebisawa et al. 2001). SMAD6 and SMAD7 inhibit BMP signaling cascades through binding active type I BMP receptors and preventing R-SMAD activation, and by competing with SMAD4 for R-SMADs (Imamura et al. 1997; Hata et al. 1998; Hanyu et al. 2001). Lastly, crosstalk with other signaling pathways affects R-SMAD phosphorylation, activity, turnover and nuclear accumulation (Pera et al. 2003; Sapkota et al. 2007; Fuentealba et al. 2007; Suzawa et al. 2002).

#### 3. BMP signaling in heart development

#### 3.1 Cardiac specification and heart tube formation

In this section, we will review the functions of different components of BMP signaling during the initial stages of heart development.

#### 3.1.1 BMP ligands

Initial insight into the roles of BMP signaling pathways in cardiac specification came from studying the *BMP2/4* ortholog, *Dpp*, in *Drosophila melanogaster*. *Dpp*-deficient larva did not form the precursor cells for the heart organ, the dorsal vessel, while ectopic DPP caused ectopic formation of the dorsal vessel precursor cells (Xu et al. 1998; Frasch 1995; Yin and Frasch 1998). In the anterior region of chick embryos, the endoderm expresses BMP2 and 5, and the ectoderm expresses BMP4 and BMP7 (Schultheiss, Burch, and Lassar 1997; Somi et al. 2004). *In vivo* and *in vitro* experiments using chicken embryos revealed that both the FHF and the SHF pre-cardiac mesodermal cells differentiate in response to BMP signals (Waldo et al. 2001; Tirosh-Finkel et al. 2006). In mice, BMP2, BMP4, BMP5, and BMP7 are expressed in the anterior mesoderm (Zhang and Bradley 1996; Dudley and Robertson 1997; Solloway and Robertson 1999). Regardless of the differences in BMP expression patterns between species, it has been well-established that BMP signaling pathways induce precardiac

mesoderm to undergo cardiac differentiation (Alsan and Schultheiss 2002; Barron, Gao, and Lough 2000; Tirosh-Finkel et al. 2010). *Bmp2* deletion in mice causes embryonic lethality between E7.5-E9.0 (Zhang and Bradley 1996). Some mutant embryos lack hearts altogether and others develop ectopic heart tubes in the exocoelomic cavity, suggesting a critical role of BMP signaling for heart formation (Zhang and Bradley 1996).

BMP signaling pathways induce cardiac differentiation through upregulation of cardiogenic genes. Expression of the cardiac transcription factors *Nkx2.5* and *Gata4* is initiated by BMP signaling (Frasch 1995; Schultheiss, Burch, and Lassar 1997; Andrée et al. 1998; Schlange et al. 2000; Jamali et al. 2001; Liberatore et al. 2002; Shi et al. 2000; Lien et al. 2002; Reiter, Verkade, and Stainier 2001; Schultheiss, Xydas, and Lassar 1995). The *Nkx2.5* promoter region contains evolutionary conserved BMP-response elements that are necessary for its cardiac expression (Lien et al. 2002; Liberatore et al. 2002; Brown et al. 2004). BMP signaling also activates the expression of myocardin, a cardiac and smooth muscle-specific transcriptional cofactor for serum response factor, a regulator of cardiac differentiation (Arsenian et al. 1998; Wang et al. 2001; Callis, Cao, and Wang 2005). SMAD1 is also a transcriptional cofactor for myocardin to activate downstream gene expression (Callis, Cao, and Wang 2005).

#### 3.1.2 BMP receptors

The BMP type I receptor ALK3 is widely expressed in mouse embryos and Alk3 deletion causes embryonic lethality at E8.0 with no mesoderm formation (Mishina et al. 1995; Dewulf et al. 1995). ALK2, another type I receptor, is expressed in Hensen's node and in the primitive streak (Gu et al. 1999; Mishina et al. 1999). Deleting *Alk2* in mouse embryos results in gastrulation defects and embryonic lethality before E9.5 (Gu et al. 1999; Mishina et al. 1999). The third type I receptor, ALK6, is not expressed during early heart development and disrupting its function does not affect mouse cardiogenesis or viability (Dewulf et al. 1995; Yi et al. 2000). Knockout of the type II receptor, BMPR2, which is expressed widely throughout chicken embryos and during mouse cardiomyogenesis, causes embryonic lethality at gastrulation (Ehrman and Yutzey 1999; Stern et al. 1995; Feijen, Goumans, and van den Eijnden-van Raaij 1994; Beppu et al. 2000). In mice, ACVR2A is expressed after cardiomyocyte formation at E9.5 and ACVR2B is ubiquitously expressed during cardiomyogenesis (Feijen, Goumans, and van den Eijnden-van Raaij 1994; Beppu et al. 2000). Disruption of Acvr2a alone does not cause heart defects and disruption of Acvr2b causes heart defects later in development (Matzuk, Kumar, et al. 1995; Oh and Li 1997). However, deletion of both Acvr2a and Acvr2b results in embryonic death at gastrulation, suggesting functional redundancy of these type II receptors (Song et al. 1999).

#### 3.1.3 SMAD proteins

In chicken embryos, the receptor-regulated SMAD proteins, SMAD1, SMAD5, and SMAD8, are enriched in the heart forming region (Faure et al. 2002). In mice, *Smad1* and *Smad5* mRNA are expressed in the mesoderm during cardiomyocyte formation (Tremblay, Dunn, and Robertson 2001). *Smad1* disruption in mice results in embryonic lethality at E10.5 from failure of umbilical-placental connections to form (Tremblay, Dunn, and Robertson 2001). Germline deletion of *Smad5* results in defective left-right symmetry with a heart looping abnormality and defective angiogenesis (Chang et al. 2000; Yang et al. 1999). Deletion of *Smad4*, the gene encoding the common SMAD, causes death before E7.5, with reduced size

100

and failure to gastrulate (Sirard et al. 1998). Conditional deletion of *Smad4* from the epiblast causes embryonic lethality by E8.5, but the heart tube forms and *Nkx2.5* is expressed (Chu et al. 2004). Heart tube formation and cardiac gene expression may occur in these mice because canonical BMP signaling occurs before *Smad4* deletion or because other R-SMAD transcriptional cofactors compensate for the loss of SMAD4.

#### 3.1.4 BMP inhibitors

Inhibition of BMP during gastrulation restricts the heart forming fields to discrete territories in the anterior of the embryo. Noggin, chordin, and follistatin are secreted from the notochord and bind BMP ligands, preventing receptor activation (McMahon et al. 1998; Streit et al. 1998; Sasai et al. 1995; Hemmati-Brivanlou, Kelly, and Melton 1994; Fainsod et al. 1997). The responsiveness of pre-cardiac mesoderm to inhibitory signals from the notochord is developmentally regulated. Ectopic application of noggin to stage 4 chick mesendoderm prevents the initiation of the cardiac gene expression and development of the contracting cardiomyocytes (Schultheiss, Burch, and Lassar 1997; Schlange et al. 2000). If noggin is applied to explants a stage later, the cardiac gene expression is initiated without spontaneous contraction of myocytes. If noggin is applied at stage 6, differentiation occurs normally (Nakajima et al. 2002). In mice, deletion of noggin or follistatin individually does not cause heart defects, but deletion of both reverses heart looping (Bachiller et al. 2000; McMahon et al. 1998; Matzuk, Lu, et al. 1995). Deleting chordin causes defects phenocopying those in DiGeorge syndrome (Bachiller et al. 2003).

#### 3.2 Cardiogenesis after heart tube formation

In this section, we will discuss BMP signaling during different cardiogenic processes after heart tube formation.

#### 3.2.1 Myocardial wall morphogenesis

During early heart development, myocardial walls expand through cardiomyocyte proliferation and differentiation. The ventricle chamber myocardium develops a latticework of muscular projections on the subendocardial surface called trabeculae. Trabecular myocardium generates contractile force, coordinates intraventricular conduction, and helps diffuse nutrients to the cardiomyocytes within the expanding heart wall prior to vascularization. Later in heart development, the trabecular myocardium undergoes remodeling and is incorporated into the compact myocardium, the interventricular septum, and the papillary muscles of the atrioventricular valves. For a review, see Dunwoodie (2007). Proper formation of myocardial walls is essential for embryo viability and postnatal cardiac function. Abnormal myocardial wall morphogenesis can result in left ventricular noncompaction, which may lead to cardiomyopathy (Pignatelli et al. 2003; Xing et al. 2006). BMP10 is initially expressed in the looping mouse heart within regions destined to be the atrial and ventricular chambers, and its expression is maintained in the chamber myocardium during heart development. (Neuhaus, Rosen, and Thies 1999; Somi et al. 2004;

myocardium during heart development. (Neuhaus, Rosen, and Thies 1999; Somi et al. 2004; Chen et al. 2004) Also, *Bmp10* is upregulated in mouse models of hypertrabeculation (Chen et al. 2004). Myocardial expression of BMP10 during chamber formation relies on endocardial expression of notch (Grego-Bessa et al. 2007). Deleting *Bmp10* in mice causes embryonic lethality at E9.0 with decreased cardiomyocyte proliferation, downregulation of cardiac genes *Nkx2.5* and *Mef2c*, and loss of trabecular myocardium (Chen et al. 2004). Removing both *Bmp6* and *Bmp7* in mice causes embryonic lethality at midgestation with hypoplastic ventricles and reduced trabeculations (Kim, Robertson, and Solloway 2001). Mice with conditional deletion of the BMP receptor *Alk3* from the myocardium die during embryogenesis and display underdeveloped myocardial walls and ventricle septal defects (VSD) (Gaussin et al. 2002). Specific inactivation of the common Smad, *Smad4*, from the myocardium likewise causes embryonic lethality at midgestation and disrupts myocardial wall formation and ventricle septation (Azhar et al. 2010; Song, Yan, et al. 2007; Qi et al. 2007; Wang, Xu, et al. 2005). Myocardial deletion of *Smad4* causes downregulation of genes encoding cell cycle regulators, cardiac structural proteins, and transcription factors. Together, these studies provide multiple lines of evidence that show BMP signaling is required for ventricular myocardial wall morphogenesis through regulation of cardiomyocyte proliferation, differentiation, and gene expression.

#### 3.2.2 Conduction system development

In vertebrates, regional differentiation of the myocardium allows for development of slowconducting, nonchamber myocardium (IFT, AVC, and OFT) and fast-conducting chamber myocardium (atria and ventricles). Proper formation of the AVC is important for establishment of the primary conduction system. The primary conduction system includes the atrioventricular node (AVN) and its associated structures. In mice, AVN precursor cells are observed in the AVC at E9.5. The AVN subsequently extends into the left ventricle and connects with the trabecular myocardium and the interventricular septum. (See reviews, (Christoffels et al. 2010; Moorman and Christoffels 2003)). The electrical impulse is carried from the atria, across the AVC to the ventricles (Valderrábano et al. 2006; Rentschler et al. 2002; de Jong et al. 1992). The AVC has a slower conduction rate than the atria and delays the atrial-ventricular electrical impulse (de Jong et al 1992).

BMP2 is necessary for AVC specification and expression of *Tbx2* (Yamada et al. 2000; Ma et al. 2005). TBX2 is a transcriptional repressor of chamber-specific genes and is specifically expressed in nonchamber myocardium of the IFT and the AVC (Aanhaanen et al. 2009; Habets et al. 2002; Yamada et al. 2000; Christoffels et al. 2004; Harrelson et al. 2004). In the AVC, BMP2 activates *Tbx2* transcription to suppress proliferation and inhibit the expression of chamber-specific genes *Nppa*, *Cx40*, *Cx43*, and *Chisel* (Ma et al. 2005; Shirai et al. 2009; Christoffels et al. 2004). BMP2 can directly regulate *Tbx2* through a SMAD-dependent enhancer upstream of its transcription start site (Singh et al. 2009). BMP signaling also promotes *Tbx2* transcription through SMAD1 inhibition of TBX20, a *Tbx2* repressor (Singh et al. 2009). The BMP2-TBX2 pathway is restricted to the AVC region by notch/HEY signaling in the developing heart chambers (Rutenberg et al. 2006; Kokubo et al. 2005).

Deletion of *Bmp2* from mouse myocardium decreases *Tbx2* expression and results in the expansion of chamber myocardium into the AVC region (Ma et al. 2005). Inactivation of the BMP receptor *Alk3* specifically in the AVC myocardium disrupts AV valve development and AVN morphogenesis, resulting in ventricular pre-excitation (Gaussin et al. 2005; Stroud et al. 2007). Lastly, removal of myocardial *Tbx2* results in abnormal AVC patterning and ventricular pre-excitation (Aanhaanen et al. 2011). Taken together, these data suggest that BMP2 regulation of *Tbx2* expression and AVC myocardial patterning is important for development of the AVN and proper atrial-ventricular conduction. Indeed, the phenotype resulting from AVC-depletion of *Alk3* resembles Wolff-Parkinson-White syndrome (WPWS, OMIM 224700), a pre-excitation syndrome that can present as tachycardia due to an

102

abnormal connection between the atria and ventricles (Gaussin et al. 2005). Recently, a heterozygous microdeletion was identified in a chromosomal region encompassing *BMP2* that is associated with predisposition to WPWS (Lalani et al. 2009).

#### 3.2.3 Valvulo-septal development of the atrioventricular canal and outflow tract

The AVC and OFT are septated by endocardial cushion maturation into valvulo-septal structures. Cushions develop in the AVC and OFT through the expansion of the ECM. Induction of cushion formation occurs within the looped heart, when the myocardium signals through the cardiac jelly to the endocardium. Endocardial cells then delaminate and invade the cardiac jelly to form the mesenchymal cells of the endocardial cushions. For reviews, see Person, Klewer, and Runyan (2005) and Butcher and Markwald (2007). The AVC cushions form earlier than the OFT cushions and develop into the mitral (left) and tricuspid (right) valves at the junction of the atria and ventricles. The OFT cushions, but not the AVC cushions, have a CNCC contribution (Kirby, Gale, and Stewart 1983; Waldo et al. 1998; Jiang et al. 2000). The cushions in the OFT develop into the semilunar valves in the aorta (left) and pulmonary artery (right). Congenital defects in valve formation and septation comprise the most common CHDs, while defects involving the OFT are found in 4 per 10,000 live births and are often lethal (Hoffman 1995; Edmonds and James 1993). Pathological mutations in the BMP receptor ALK2 have been found in patients with congenital defects in atrioventricular septum development, providing evidence for the importance of BMP signaling pathways in human heart development (Smith et al. 2009; Joziasse et al. 2011).

At E9.5 in mice, BMP2 has weak expression in OFT myocardium which disappears by E10.5 (Lyons, Pelton, and Hogan 1990). BMP2 is strongly and persistently expressed in AVC and atrial myocardium at E10.5 (Lyons, Pelton, and Hogan 1990; Abdelwahid et al. 2001). It is also expressed in the cushion mesenchyme during valve remodeling and in adult mouse valves (Sugi et al. 2004). In mice, BMP2 enhances cardiac jelly formation, endocardial EMT, and AVC myocardial patterning (Ma et al. 2005; Rivera-Feliciano and Tabin 2006; Sugi et al. 2004; Camenisch et al. 2002). BMP2 upregulates *Twist1*, an inducer of EMT, and *Has2*, a component of the cardiac jelly necessary for EMT (Camenisch et al. 2000; Ma et al. 2005; Yang et al. 2004). Myocardial deletion of *Bmp2* decreases ECM in the AVC cushions, however the OFT cushions develop normally (Ma et al. 2005; Rivera-Feliciano and Tabin 2006). This suggests a compensatory mechanism in the OFT such as BMP4 signaling. Data suggests that BMP2 signaling interacts with notch1 and TGF $\beta$  signaling pathways to coordinate EMT (Luna-Zurita et al. 2010; Boyer et al. 1999; Yamagishi et al. 1999).

BMP4 is expressed in AVC myocardium in mice at E9.5, but at E10.5 its expression is largely restricted to the myocardium of the OFT (Jones, Lyons, and Hogan 1991; Abdelwahid et al. 2001). It is also expressed in the chicken OFT (Somi et al. 2004). BMP4 is 92% identical in the C-terminus to BMP2, and they have overlapping functions (Goldman, Donley, and Christian 2009; Uchimura et al. 2009). Conditional deletion of *Bmp4* from mouse myocardium causes atrioventricular septation defects, double outlet right ventricle (DORV, both arteries are connected to the right ventricle), and aortic arch artery malformations (Jiao et al. 2003; Liu et al. 2004). Mouse models with myocardial-specific deletion of *Bmp4* or with hypomorphic *Bmp4* alleles have impaired AVC cushion mesenchymal cell proliferation (Jiao et al. 2003; Kulessa and Hogan 2002). Mice compound heterozygous for *Bmp2*-null and *Bmp4*-null or *Bmp4*-hypomorphic alleles have VSD (Goldman, Donley, and Christian 2009). Decreased

expression of myocardial BMP4 does not affect OFT development, but it increases BMP7 expression (Liu et al. 2004). On a *Bmp7*-null background, BMP4 reduction causes a shortened OFT with hypoplastic OFT cushions, revealing dose-dependence and functional redundancy of BMP signaling in the OFT morphogenesis (Liu et al. 2004).

Despite being expressed during early heart development, single gene deletions of Bmp5, Bmp6, or Bmp7 do not cause heart defects, likely due to redundancy of the BMP signaling family members (Kingsley et al. 1992; Jena et al. 1997; Dudley and Robertson 1997; Luo et al. 1995; Solloway et al. 1998; Kim, Robertson, and Solloway 2001). BMP5 is expressed throughout the heart tube myocardium and later becomes restricted to the myocardium of the AVC and OFT in mouse and chicken embryos (Yamagishi et al. 2001; Solloway and Robertson 1999; Somi et al. 2004). In mice, BMP6 is expressed in OFT endocardium and myocardium, and within the OFT and AVC mesenchyme (Kim, Robertson, and Solloway 2001; Jones, Lyons, and Hogan 1991; Solloway and Robertson 1999; Yamagishi et al. 2001). BMP6 is not expressed in the developing chicken heart (Somi et al. 2004). BMP7 is robustly expressed throughout the myocardium of the developing hearts of mice and chickens (Solloway and Robertson 1999; Lyons, Hogan, and Robertson 1995; Somi et al. 2004). Combinations of gene deletions in mouse models reveal their essential roles in chamber formation and septal-valvulogenesis. Bmp5 and Bmp7 double deletion causes embryonic lethality at E10.5, with delayed heart development, no endocardial cushion formation or chamber septation, and abnormal pericardium (Solloway and Robertson 1999). Removal of Bmp6 and Bmp7 results in defects in OFT cushion development, chamber septation, and myocardial wall formation (Kim, Robertson, and Solloway 2001). Deletion of Bmp5 and *Bmp6* does not cause heart defects (Solloway et al. 1998).

Deletion of Alk3 from the myocardium or the endocardium disrupts endocardial cushion formation (Gaussin et al. 2002; Song, Fässler, et al. 2007; Ma et al. 2005). Myocardial deletion of *Alk3* causes VSD and hypoplastic AVC cushions, with decreased TGFβ signaling in the AVC myocardium (Gaussin et al. 2002). Deleting Alk3 specifically from the AVC myocardium disrupts AV valve maturation (Gaussin et al. 2005). Endocardial deletion of Alk3 causes hypoplastic cushions with reduced cushion mesenchyme to about 20% of normal (Ma et al. 2005; Song, Fässler, et al. 2007; Park et al. 2006; Rivera-Feliciano and Tabin 2006). Endocardial deletion of Alk2 causes failure of EMT in AVC cushions along with decreased expression of EMT proteins MSX1 and SMAD2, an intracellular modulator of TGFβ signaling (Wang, Sridurongrit, et al. 2005). The role of ALK2 in cushion formation appears to be specific to the endocardium, as conditional deletion of Alk2 from the myocardium has no effect on cushion development (Wang, Sridurongrit, et al. 2005). Ectopic expression of active ALK2 in the chicken ventricle endocardium induces EMT (Desgrosellier et al. 2005). CNCC-depletion of Alk3 or Alk2 disrupts CNCC invasion, resulting in a shortened OFT with defective proximal septation (Stottmann et al. 2004; Kaartinen et al. 2004). Hypomorphic Bmpr2 alleles cause defects in proximal OFT septation and loss of semilunar valve formation, while AVC cushions form normally (Délot et al. 2003). However, completely abrogating *Bmpr2* in mouse hearts causes an array of CHDs, such as DORV, VSD, and AVC cushion defects (Beppu et al. 2009). Disruption of Acvr2b causes postnatal death with abnormal cardiac septation (Oh and Li 1997). Deletion of Smad4 from the myocardium affects OFT positioning, with a DORV phenotype in one mouse model (Azhar et al. 2010). Conditional deletion of Smad4 in CNCC reduced the contribution of CNCC to OFT, causing defects in OFT cushion formation, septation, elongation, and positioning (Jia et

al. 2007; Ko et al. 2007; Nie et al. 2008). Deletion of *Smad8* does not affect viability or heart development, but mice display defects in pulmonary vascular remodeling (Huang et al. 2009).

BMP signaling regulates SHF myocardialization and OFT morphogenesis in part by promoting *miR*-17-92 cluster transcription (Wang et al. 2010). The *miR*-17-92 cluster has roles in lung and heart development (Lu et al. 2007; Ventura et al. 2008). It is expressed as a primary transcript that encodes six miRNAs (*miR*-17, -18a, -19a, -20a, -19b-1, and -92a-1). BMP regulates the transcription of *miR*-17-92 through SMAD binding sites in the 5' region (Wang et al. 2010). In turn, *miR*-17-92 negatively regulates *Isl1* and *Tbx1* mRNA stability and translation (Wang et al. 2010). Deleting BMP reduces *miR*-17-92, causes misexpression of *Isl1* and *Tbx1*, and leads to defects in proximal OFT septation (Wang et al. 2010).

Inhibition of BMP signaling is also critical for normal valvulo-septal formation. For example, *Nkx2.5* is required for OFT development, in part by repressing BMP signaling (Prall et al. 2007). Deleting *Nkx2.5* results in expansion of SHF specification due to increased BMP expression, decreased proliferation, and failed OFT truncation. Disrupting the misregulated BMP signaling in the *Nkx2.5* mutants by deleting *Smad1* effectively rescues the proliferation and the OFT defects. Mutations in the BMP-inhibitor *Smad6* cause hyperplasia of cardiac valves and OFT septation defects, due to unregulated BMP signaling (Galvin et al. 2000). Noggin blocks EMT in mouse explants and overexpression of noggin in chicken embryos causes OFT septation defects (Sugi et al. 2004; Allen et al. 2001). Mutations in chordin cause abnormal OFT septation, resembling syndromes associated with loss of CNCC (Bachiller et al. 2003).

#### 4. BMP induction of stem cells and progenitor cells to a cardiac fate

Controlled differentiation of stem cells has relevance in translational research for pre-clinical cell grafting and for establishing cardiomyocyte cultures for drug discovery and toxicology. As primary inducers of cardiac differentiation, BMP cytokines have important roles in growth factor-based stem cell therapies for cardiac tissue repair. Use of growth factor peptides to induce cardiac muscle formation from embryonic stem (ES) cells has been researched for over a decade. BMP ligands have been proven to be important inductors of cardiac fate in multiple ES cell types. In mouse ES cells (mESC), BMP2 or BMP4 can activate cardiac differentiation, in combination with other factors such as Activin A or fibroblast growth factor 2 (FGF2) (Johansson and Wiles 1995; Kawai et al. 2004; Behfar et al. 2002). In the pluripotent mouse embryonal carcinoma cell line, P19C16, treatment with BMP4 promotes cardiomyocyte formation and expression of a-MHC (Monzen et al. 1999; Monzen et al. 2001). Addition of noggin, a BMP inhibitor, prevents differentiation and this can be abolished by overexpressing BMP2, or SMAD1 and SMAD4 (Monzen et al. 1999; Monzen et al. 2001). In human ES cells (hESC), BMP4 promotes a cardiac fate (Takei et al. 2009; Kattman et al. 2011). BMP stimulation induces Sox17 expression, which is important for directing mesoderm toward a cardiac fate (Stefanovic et al. 2009). Addition of BMP2 or BMP4 in hESC, along with Activin A and other factors, can reliably induce multipotent cardiovascular progenitors that can generate multiple cell lineages such as cardiomyocytes, smooth muscle cells, and endothelial cells in vitro and in vivo (Laflamme et al. 2007; Tomescot et al. 2007; Yang et al. 2008; Liu et al. 2007). Human induced pluripotent stem cells (iPSC) can also be induced to undergo cardiomyogenesis using similar multistep additions of factors, including BMP4 (Takahashi et al. 2007; Carvajal-Vergara et al. 2010; Kattman et al.

2011). BMP2 stimulation with FGF inhibition also induces multipotent cardiovascular progenitor cells in hESC, human iPSC, and primate ESC (Blin et al. 2010). BMP4 enhances simian ESC differentiation into cardiomyocytes as well (Hosseinkhani et al. 2007). Future studies of stem cells and progenitor cells may help develop peptide therapies to stimulate the proliferation and differentiation of self-renewing stem cells within the postnatal heart.

#### 5. Conclusion

CHDs occur in nearly 1% of newborns and in over 5% of fetuses that do not survive to term in the Western world (Hoffman 1995; Hoffman and Kaplan 2002). Due to advances in medicine, there is a growing number of children and adults living with CHDs who require lifelong healthcare (Hoffman and Kaplan 2002). Therefore, understanding the molecular mechanisms of heart development and the underlying causes of CHDs has immediate translational significance. BMP signaling pathways are critical regulators of heart development in species as varied as fruit flies, chickens, mice, and humans. Mutations in the BMP pathway have been identified in humans with CHDs. This review discussed the critical roles of BMP signaling pathways in cardiac specification from the mesoderm, myocardial wall formation, valve development, chamber septation, and outflow tract morphogenesis. Because cardiac morphogenesis and BMP signaling pathways are evolutionarily conserved, information gleaned from a variety of model systems provides valuable insight into human heart development and CHDs. In the future this insight may help develop diagnostic tests and therapeutic options for people with CHDs.

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Bone Morphogenetic Protein Signaling Pathways in Heart Development and Disease

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Bone Morphogenetic Protein Signaling Pathways in Heart Development and Disease

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#### Congenital Heart Disease - Selected Aspects Edited by Prof. P. Syamasundar Rao

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There are significant advances in the understanding of the molecular mechanisms of cardiac development and the etiology of congenital heart disease (CHD). However, these have not yet evolved to such a degree so as to be useful in preventing CHD at this time. Developments such as early detection of the neonates with serious heart disease and their rapid transport to tertiary care centers, availability of highly sensitive noninvasive diagnostic tools, advances in neonatal care and anesthesia, progress in transcatheter interventional procedures and extension of complicated surgical procedures to the neonate and infant have advanced to such a degree that almost all congenital cardiac defects can be diagnosed and "corrected". Treatment of the majority of acyanotic and simpler cyanotic heart defects with currently available transcatheter and surgical techniques is feasible, effective and safe. The application of staged total cavo-pulmonary connection (Fontan) has markedly improved the long-term outlook of children who have one functioning ventricle. This book, I hope, will serve as a rich source of information to the physician caring for infants, children and adults with CHD which may help them provide optimal care for their patients.

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