Co-ordinating Notch, BMP, and TGF β Signalling During Heart Valve Development

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Abstract

Congenital heart defects affect approximately 1–5% of human newborns each year and of these cardiac defects, 20–30% are due to heart valve abnormalities. Recent literature indicates that key factors and pathways that regulate valve development are also implicated in congenital heart defects and valve disease. Currently, there are limited options for treatment of valve disease and therefore, having a better understanding of valve development can contribute critical insight into congenital valve defects and disease. There are three major signalling pathways required for early specification and initiation of Endothelial-to-Mesenchymal Transformation (EMT) in the cardiac cushions: BMP, TGF β , and Notch signalling. BMPs secreted from the myocardium setup the environment for the overlying endocardium to become activated, Notch signalling initiates EMT, and both BMP and TGF β synergizes with Notch to promote the transition of endothelia to mesenchyme and to promote mesenchymal cell invasiveness. Together, these three essential signalling pathways help to form the cardiac cushions and populate them with mesenchyme and, consequently, set off the cascade of events required to develop mature heart valves. Furthermore, integration and cross-talk between these pathways generate highly stratified and delicate valve leaflets and septa of the heart. Here, we discuss BMP, TGF β , and Notch signalling pathways during mouse cardiac cushion formation and how they together produce a coordinated EMT response in the developing mouse valves.

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Keywords

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CONGENITAL HEART DEFECTS AND VALVE DISEASE

Congenital abnormalities of the heart affect up to 5% of human newborns and approximately a third of these cardiac defects are due to heart valve malformations [1,2]. The most prevalent heart valve malformations are bicuspid aortic valve (BAV), in which patients exhibit two aortic valve cusps instead of three; and mitral valve prolapse (MVP), in which patients suffer from floppy mitral valve leaflets that can slip past their normal position into the left atrium. Reports on the prevalence of congenital heart defects vary widely since BAV and MVP are not usually included. BAV affects 2% of the general population but the consequences are rarely seen until adulthood [1,2] whereas MVP affects up to 5% of the general population but is rarely detected in newborns as symptoms are frequently not severe [1].

According to the World Health Organization (WHO), cardiovascular diseases are the number one cause of death worldwide; in 2008 approximately 17.3 million people died from cardiovascular disease, which accounts for 30% of global deaths. In the US, approximately 3–5% of cardiovascular deaths are due to valve disease [3]. Adult valve disease can become evident as stenosis, a narrowing of the valve opening resulting in less blood flow; or as regurgitation, an incomplete closure of the valve causes backflow of blood in the heart. If valve disease goes undiagnosed it can lead to secondary effects, such as improper ventricular function and eventually heart failure. Initial stages of heart valve disease involve activation of valve interstitial cells (VICs), which leads to abnormal extracellular matrix (ECM) deposition and disorganization. There are two types of ECM changes that can occur in the heart valves during valve disease: myxomatous disease involves increased deposition of proteoglycans, loss of collagen, and destruction of elastin fibrils leading to “floppy” valves and regurgitation, while fibrotic disease involves degradation of proteoglycans along with increased levels of collagen and elastin fibre fragmentation resulting in stiffening of the valve leaflets known as valve stenosis [4–6]. Valve fibrosis can often progress further, leading to valve calcification [7]. To date, little is known about the progression of valve calcification.

Interestingly, many cases of valve disease in adults involve pre-existing defects in the heart valves and suggest that abnormalities occurring during embryonic valve development may lead to susceptibility to valve disease later in life [8–11,7]. Recent literature suggests that key pathways and factors regulating valve development are also implicated in valve disease and congenital heart defects [12,13]. Unfortunately, there are few options available for treatment of valve disease, the main option being valve replacement where additional surgeries are often required [14]. Having a better understanding of valve development may provide key insights into congenital valve defects and disease. Furthermore, the search for alternate treatments or biomarkers for earlier detection requires a more extensive

understanding of the molecular mechanisms involved in heart valve development and disease.

EMBRYONIC DEVELOPMENT OF THE HEART AND CARDIAC VALVES

The heart is the first organ to develop and function in the embryo. It delivers sufficient oxygen and nutrients to the growing embryo and establishes proper blood flow. The first step of heart development is specification of cardiac progenitor cells (CPCs), which occurs prior to their ingress through the primitive streak during gastrulation (embryonic day (E) 6–7.0 in mouse). Subsequently, CPCs undergo an epithelial-to-mesenchymal transition (EMT) and migrate out from the primitive streak to form the left and right heart fields (E7.5) that move laterally and fuse on the anterior side of the embryo, creating the cardiac crescent at E8.0 (reviewed in [15]). The differentiation of the cells within the cardiac crescent generates the endocardial and myocardial progenitor cells and together they make up the primary heart field (PHF). The PHF will go on to form the left ventricle, atrioventricular canal (AVC), portions of the right ventricle, and regions of the atria. An additional heart forming region anterior to the PHF, known as the secondary heart field (SHF), will contribute to parts of the right ventricle and atria and the outflow tract (OFT). The two arms of the cardiac crescent fuse along the embryonic midline generating the linear heart tube (E8.5) [15]. The heart tube consists of a monolayer of endothelium and several layers of myocardium at E9.0 (Figure 1). The linear heart tube loops rightward and forms the chambers of the heart and this looping brings the chambers into their final positions in the mature heart. Two constrictions appear in the looped heart, the AVC, between the atria and ventricle, and the OFT, between the ventricle and great arteries (Figure 1) and these regions will eventually form the septa and mature valves of the heart (additional information on early heart formation in [7,16,15]).

The septum divides the heart into four functional chambers and valves ensure uni-directional blood flow. During cardiac valve development, the AVC and the OFT will form four sets of heart valves: two sets of atrioventricular (AV) valves and two sets of semilunar (SL) valves, respectively. The AV valves are made up of the mitral valve, which regulates blood flow from left atrium to left ventricle; and the tricuspid valve, which prevents blood backflow between right atrium and right ventricle. The two SL valves are the aortic valve, which regulates blood flow from the left ventricle into the aorta; and the pulmonary valve, which regulates blood flow between the pulmonary artery and right ventricle [17].

Cardiac valve formation is initiated through increased production of cardiac jelly, ECM secreted by the myocardium into the interstitial space between the endocardium and myocardium, which is restricted to the AVC and OFT and creates swellings known as cardiac cushions. Although the cushions are initially acellular, endocardial cells overlying the cushion undergo EMT (also referred to as EndMT to indicate the endothelial origin of the cells) to form mesenchymal cells [18]. In the mouse, EMT begins at approximately E9.5 in the AVC and E10.5 in the OFT (Figure 1). During EMT, endocardial cells lose cell-cell junctions and cell polarity, transition into mesenchymal cells, and acquire a migratory phenotype [19]. The mesenchymal cells invade the ECM and populate the cardiac cushions [20,21]. The OFT, which will form the SL valves, develops similarly with the exception that neural crest cells migrate and contribute to the OFT cushions (reviewed in [22]).

Following invasion into the cardiac cushion, the mesenchymal cells proliferate, differentiate and remodel to form thin delicate valve leaflets and septal structures of the mature heart [4,23]. The cardiac cushions form the valves and septa through remodelling and maturation (E10.5-birth), and elongation (E14.5-birth). Valve remodelling can be divided into a number of overlapping steps: proliferation and expansion of mesenchyme cells (E10.5–E12.5), differentiation of mesenchyme cells (E12.5–E16.5), and valve maturation and condensation (E15.5-birth) [4,24–26]. To date, the majority of studies have concentrated on the initial EMT process as AVC explant cultures allows for robust measurement of EMT [19]. In contrast, our understanding of differentiation, maturation and condensation are lacking. This may be due to the absence of an established culture model system to examine the later stages of valve development.

The adult heart valve leaflets are highly organized structures composed of three stratified layers (atrialis in AV valves or ventricularis in SL valves, spongiosa and fibrosa) which are mainly composed of elastin, proteoglycans, and collagens, respectively [23]. In AV valves, the fibrosa layer is located on the ventricular side of the valve (in SL valves, it is located away from the ventricle) and maintains its strength and integrity [27]. The atrialis and ventricularis face toward blood flow and provide the flexibility of valve [28]. The middle layer of the valve, the spongiosa, acts as a sponge and allows for compression of the valve to absorb the pressure from blood flow. The valve leaflets are enclosed in a sheath of valvular endocardial cells (VECs) with valvular interstitial cells (VICs) dispersed throughout the valve leaflet. VICs are descendants of the mesenchymal cells found in the cardiac cushions during embryogenesis. Lineage tracing studies in mice using *Tie2*, which is expressed in endocardial cells prior to EMT, shows that the bulk of cells present in the valves after birth are derived from endocardium with the exception of the AV parietal leaflets [29–31]. A recent study indicates that epicardial-derived cells start to migrate into the minor AV cushions at E12.5 and selectively contribute to parietal leaflets of the mouse AV valves [31]. VICs play an important role in maintaining proper valve homeostasis but can be aberrantly activated during valve disease [32]. Studies focussing on how VICs are generated during development, how they maintain homeostasis in the adult, and how they become activated will all play an important role in understanding the mechanisms of valve defects and disease.

Recently, a number of studies have suggested that crucial developmental signalling pathways, involved in normal valve formation, such as transforming growth factor beta (TGF β), bone morphogenetic protein (BMP), and Notch, are activated during heart valve disease. Thus, increasing our understanding of how these signalling pathways function and interact during heart valve development provide key insights into mechanisms of adult heart valve disease.

NOTCH, BMP AND TGF β SIGNALLING PATHWAYS

Although numerous signalling pathways are involved in the formation of the cardiac valves, three major signalling pathways are required for early specification and initiation of EMT in the cardiac cushions. Initially, BMPs from the myocardium signal to the overlying endocardium to create an environment for EMT. Following this, Notch signalling is required for the initiation of EMT and together BMP and TGF β signalling pathways synergize with

Notch to promote transdifferentiation of the endothelial cells to mesenchyme and mesenchymal cell invasiveness. Together, these three crucial signalling pathways create the cardiac cushions and populate them with mesenchyme cells, setting off the cascade of events required to form mature heart valves and septa.

TGF β superfamily signal transduction

BMPs and TGF β s are part of the TGF β superfamily. This family comprises over 30 ligands that can be categorized into subgroups: activins/inhibins, nodals, BMPs, growth and differentiation factors (GDFs), Müllerian inhibiting substance (MIS) and TGF β s. To activate signalling, the ligands bind to a tetrameric, transmembrane receptor complex that contains two type I and two type II receptors. In mammals, there are five distinct type II receptors and seven type I receptors, which form specific combinations that dictate ligand binding specificity. These receptors phosphorylate and activate an intracellular canonical signalling pathway that is mediated by receptor-regulated Smad proteins (R-Smads). Following phosphorylation, R-Smads interact with the binding partner, Smad4, and move into the nucleus where they interact with DNA-binding proteins to regulate transcription of TGF β superfamily responsive genes [33,34] (Figure 2).

In BMP signalling, there are three possible type I receptors (ALK2 (Activin-Like Kinase), ALK3 and ALK6) and three possible type II receptors, (BMPRII, ActRII or ActRIIB). The binding of BMP ligands (BMP2, 4, 5, 6, or 7) to their receptors causes phosphorylation of the BMP R-Smads, Smads 1, 5 and 8, which interact with Smad4. The Smad complex moves to the nucleus to regulate BMP-responsive genes (Figure 2).

There are three TGF β ligands: TGF β 1, TGF β 2 and TGF β 3. To activate canonical TGF β signalling, a TGF β ligand binds to the tetrameric receptor complex that comprises of two TGF β type II receptors (TGFBR2, also known as T β RII) and two TGF β type I receptors (ALK5, also known as T β RI). The binding of the TGF β ligand to T β RII leads to activation of ALK5 and results in phosphorylation of the TGF β R-Smads, Smad2 and Smad3. As in BMP signalling, the phosphorylated R-Smads interact with Smad4 to regulate gene expression (Figure 2). In endothelial cells, there is another TGF β type I receptor known as ALK1 that can also mediate TGF β signalling. In contrast to ALK5, ALK1 phosphorylates and activates the BMP R-Smads, Smads 1/5/8. Thus, TGF β ligands can activate both arms of the intracellular pathway, depending on the receptor-ligand binding. An alternative pathway to the canonical TGF β signalling pathway functions in a Smad-independent fashion in response to TGF β ligand, activating pathways such as MAP kinase (MAPK), Rho-like GTPase, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways. Interestingly, MAPK, Rho-like GTPase, and PI3K/AKT pathways all have important roles in EMT although these pathways likely play a secondary, yet collaborative role during EMT in the developing cardiac cushions.

A number of additional factors are known to regulate TGF β signalling, such as the TGF β co-receptors, Betaglycan (also known as TGFBR3) and Endoglin. Betaglycan and Endoglin promote ligand binding specificity to the receptor complex at the surface of the cell. The TGF β signalling pathway can be further modulated by numerous intracellular and extracellular factors (see review [35]). For example, two inhibitory Smads (I-Smads), Smad6

and Smad7, can negatively regulate TGF β superfamily signalling [36,37]. I-Smads function as intracellular antagonists of TGF β signalling by inhibiting R-Smad phosphorylation, blocking Smad4 binding to R-Smads, and interacting with the receptors [38].

BMP signalling in cardiac valve development

BMP signalling is crucial for valve formation in two phases: first, it establishes an environment that allows endocardium to become activated, and together with Notch and TGF β signalling promotes EMT and invasion of the mesenchyme into the cardiac cushions. BMP ligands 2, 4, 5, 6, and 7 are all expressed in AVC and OFT myocardium during valve formation [39–44]. The BMP type I receptor, ALK2 is expressed in endocardium and a subset of mesenchymal cells in the heart at E10 [45]. During mouse embryonic development, ALK3 and BMPRII are ubiquitously expressed [46,47]. ALK6 has a more restricted expression pattern but is not expressed in the developing mouse heart [46].

The importance of BMP2 for valve formation was first indicated in experiments using chick AVC explant assays using antisense oligonucleotides against BMP2 to inhibit EMT [48]. In mouse, complete deletion of BMP2 causes embryonic lethality between E7 and E10.5 and mutant embryos have abnormal cardiac development: the heart develops in the exocoelomic cavity instead of the amniotic cavity [49]. At E9.5, a heart tube is identifiable in BMP2^{-/-} embryos but chambers are indistinguishable and thus precludes examination of BMP2 in cardiac cushion formation [49]. Given that BMP2 is highly expressed in the AVC myocardium prior to valve formation [50], Ma *et al.* deleted BMP2 specifically in the myocardium using *Nkx2.5-Cre* [39] and demonstrated that BMP2 is required for formation of cardiac jelly and for initiation and formation of the cardiac cushions [39]. Additionally, although explants normally require AVC myocardium for EMT to take place, BMP2 induces EMT in mouse AVC explants without AVC myocardium. Moreover, noggin, a BMP antagonist, blocks EMT [50] and addition of BMP2 to ventricular explants (normally unable to undergo EMT) induces EMT [51]. These together indicate that BMP2 is a critical factor required for EMT and initial formation of the cardiac cushions. Of interest, loss of BMP2 in myocardium did not affect OFT cardiac cushion development [39] and suggests that BMP2 is specifically required in AV valve development.

In contrast, BMP4 is highly expressed in OFT myocardium from E9 to E10.5 and loss of BMP4 in OFT myocardium causes impaired proliferation, decreased cushion growth, and septation defects [52]. This demonstrates that BMP4 is not required for EMT in OFT cushions but rather for growth. Interestingly, BMP2 and BMP4 double heterozygous mice (*Bmp2*^{+/-};*Bmp4*^{+/-}) have ventricular septal defects and abnormal valve structure after birth [53] and this suggests that the development of AVC cushions are sensitive to the dosage of BMP signalling. Complete knockouts of BMP5, BMP6, and BMP7 do not display defects in cardiac cushion formation [54–56]; however, BMP5/7 double knockout embryos lack cardiac cushions at E10.5 due to an overall hindrance in heart development [41]. The BMP6/7 double knockouts have defects in OFT cushions whereas the AVC cushions are not severely affected [42]. Thus, BMP ligands are required in specific and overlapping patterns during the development of AVC and OFT cardiac cushions.

Targeted loss of the BMP receptors, ALK2, ALK3, and BMPRII, result in embryonic lethality at gastrulation [57–59], and thus the role of BMP receptors in heart development was examined using tissue-specific deletion. Endocardial cells lacking ALK2 fail to transform into mesenchymal cells and populate the cardiac cushions and embryos that do survive to E14.5 have ventricular septal defects and valve abnormalities [45]. Endocardial loss of ALK2 leads to decreased phosphorylation of both BMP- and TGF β -specific Smads and reduction of *Snail*, a transcriptional repressor required for EMT [45]. The reduction in TGF β -Smad phosphorylation was surprising and suggest that BMP signalling allows endocardial cells to be responsive to TGF β signalling [45]. This endocardial responsiveness could be attributable to ALK2-mediated BMP signalling that leads to activation of Notch signalling resulting in TGF β 2 expression and subsequently induction of Smad2/3 signalling [51]. Similar to endocardial loss of ALK2, deletion of ALK3 in endocardium causes a failure in EMT with a dramatic reduction in mesenchymal cells in the cardiac cushions [60]. ALK3 was shown to be required for growth and survival of the cushion mesenchyme. Interestingly, loss of ALK3 did not affect TGF β -Smad phosphorylation [60]. Thus, endothelial ALK2 and ALK3 have specific and non-redundant roles during EMT and cushion formation. Of note, mice with myocardial specific loss of ALK3 have intraventricular septum defects, thinning of the myocardial walls, abnormal trabeculation, and cushion defects [61]. The cushion defects observed in the myocardial-specific ALK3 knockout suggest that BMP signalling in AV myocardium generates a secreted signal that is necessary for AVC cushion formation. Coinciding with this, mutant mice have lower levels of TGF β 2 in the AV myocardium, likely accounting for AVC cushion defects [61]. Interestingly, if ALK3 is specifically deleted from the AV myocardium only, mutant mice exhibit defects in AV valve leaflets and disruption of the annulus fibrosis [62], indicating that ALK3 is required for later stages of valve remodelling. Similar to the BMP ligands, no OFT cushion defects were seen in ALK2 and ALK3 knockout mice [45,60,61]. Thus, tissue-specific loss of BMP type I receptors in the developing heart indicates that they are essential for cardiac cushion formation and EMT in the AVC.

Given that complete loss of BMPRII causes early lethality, the function of BMPRII during heart development was examined using a mouse model with truncated BMPRII that reduces BMP signalling [63]. The truncation of BMPRII results in disrupted OFT septation, an absence of SL valves, and an interrupted aortic arch [63]. Initially, defects in the AVC and septa (AVC derived) were not described in mutant mice with truncated BMPRII, however, a recent review reported that these mice have atrial and ventricular septal defects and a common AVC [64]. Endocardial loss of BMPRII results in thickened valve leaflets and atrial and ventricular septal defects, and indicates that endocardial BMPRII expression is required for valve remodelling and septation [65]. Interestingly, truncated BMPRII mutant mice have a more severe phenotype than the endocardial loss of BMPRII. Thus, truncated BMPRII may act to sequester BMP ligands and receptors and prevent compensation through the use of other BMP receptors. Together, this data suggests that BMPRII is required for SL valve formation, valve remodelling and proper septation.

Overall, BMP ligands and receptors play critical roles in cardiac cushion development by patterning the AVC/OFT myocardium during initial cushion formation, providing a permissive environment for EMT, promoting initiation of EMT and mesenchymal cell

invasion, ensuring cushion mesenchyme growth and survival, and remodelling the valves. As the majority of the mouse models for BMP signalling result in defects during early cardiac cushion formation, the essential role of BMP signalling in the early stages of cardiac cushion formation is well documented, however, the roles of the BMP signalling molecules during valve remodelling and differentiation is less understood and indicates that additional research is required. For example in the adult heart, evidence indicates that BMP signalling may be abnormally activated during valve disease. BMP2 is increased in calcified regions in diseased valve leaflets [66,67] and there are higher levels of BMP signalling in fibrosa endothelium of human diseased aortic valves [68]. This suggests that BMP signalling may be involved in the process of calcification during valve disease.

TGF β signalling in cardiac valve development

TGF β 1, TGF β 2, and TGF β 3 signalling play critical roles in morphogenesis and development of numerous tissues including the cardiac valves [69]. During cushion formation, TGF β signalling promotes EMT and mesenchymal invasiveness [19,70]. TGF β 1 is initially expressed in the endocardium (E8.0) and becomes restricted to AVC endocardium at E9.5, when EMT takes place and is sustained in endocardial cells of cardiac valves until just after birth [71]. TGF β 2 is expressed in AVC endocardium, AVC myocardium, and cardiac cushion mesenchyme cells starting at E10 [19,72]. Interestingly, TGF β 3 is initially expressed after EMT at E11.0 in cushion mesenchyme and is not expressed in endocardium [19,72]. The expression patterns of TGF β 1 and TGF β 2 suggest that these ligands play an important role in EMT, whereas TGF β 3 may have a role in valve remodelling. Similar expression patterns for TGF β ligands are found in OFT cardiac cushions [72]. The TGF β receptors, ALK5 and T β RII are expressed in the developing heart tube, myocardium, endocardium and cushion mesenchyme cells [73–75]. ALK1 is expressed in endothelial cells and in embryonic heart [76]. The TGF β co-receptors have defined expression patterns during valve development: Betaglycan is found in myocardium and at low levels in AVC endocardial cells [77] while Endoglin is detected in endocardium and cushion mesenchyme with expression decreasing as the valves mature [78].

Similar to BMP2, TGF β signalling in valve development was initially suggested as critically important when EMT was inhibited in chick AVC explants treated with antisense oligonucleotides to TGF β 3 [79]. In chick explants, addition of TGF β 1 and TGF β 3 induces EMT in the absence of the normally required AV myocardium [70,80]. Addition of neutralizing antibodies for TGF β 2 or TGF β 3 to chick AVC explants inhibits activation of endocardial cells (TGF β 2), and formation and migration of cushion mesenchymal cells (TGF β 3) [81]. This data indicates that TGF β signalling plays a significant role in chick cardiac cushion development and therefore may play a similar role in mouse valve development.

To examine the functions of TGF β signalling during mouse valve formation, complete and tissue-specific knockout mice were generated for TGF β ligands and receptors. Despite the specific expression of TGF β 1 in AVC and OFT endocardium, *Tgfb1*^{-/-} mice generated by two separate groups have no reported cardiac abnormalities. In one study, loss of TGF β 1 leads to embryonic lethality at E10.5 due to defects in haematopoiesis and vasculature [82],

whereas the second study observes postnatal lethality as a result of major immune system complications [83]. Cardiac defects such as disorganized valves are only seen in TGF β 1 null mice when a null mother (given immunosuppressive injections to survive to breeding age) gives birth to a null embryo [82,84], indicating that TGF β 1 is involved in cardiac valve formation and that maternal TGF β 1 is sufficient to rescue valve formation. Complete absence of TGF β 2 leads to multiple organ defects resulting in perinatal lethality [85]. *Tgfb2* null mice exhibit numerous defects in the OFT, AVC, septa and aortic arch [85,86], which indicates that TGF β 2 is critical for cardiac valve and septal development. Not only does TGF β 2 promote EMT in chick explants, TGF β 2 plays a key role in termination of EMT as *Tgfb2*^{-/-} mice have hypercellular cardiac cushions and valves [87]. Moreover, *Tgfb2*^{-/-} cardiac valves have impaired mesenchymal cell differentiation and abnormal ECM composition, indicating that TGF β 2 has a second essential role in valve remodelling and differentiation [88]. TGF β 3 null mice do not have cardiac valve defects but TGF β 3 has been implicated in epithelial-mesenchymal interactions in the developing lung and palate [89]. Collectively, this data indicates that TGF β 1 and TGF β 2 are essential for valve development whereas TGF β 3 likely do not play critical roles.

Complete loss of ALK5 or T β RII causes abnormal vascular development of the yolk sac and defects in hematopoiesis leading to lethality by E10.5 [90,91]. To circumvent the early lethality, tissue-specific knockout mice have been used to assess TGF β receptor functions in heart valve development. Interestingly, loss of ALK5 in endothelium using Tyrosine kinase 1 (*Tie1*)- or 2- (*Tie2*) *Cre* strains results in embryonic lethality at E10.5 and E13, respectively [92,93]. More specifically, embryos lacking ALK5 in *Tie1* expressing cells die at E10.5 due to vascular defects in the yolk sac, similar to the complete knockout mouse [92], while loss of ALK5 in *Tie2* expressing cells leads to embryonic lethality at E13 as a result of hypoplastic cardiac cushions and abnormal myocardial trabeculation [93]. Both *in vitro* and *in vivo* data show that no mesenchymal cells migrate into the cushions in *Tie2* endothelial-specific ALK5 knockout mice [93]. This data suggests that ALK5 is required in endothelial cells for normal vascular development and is essential for EMT during valve development. Similar to ALK5 mutant mice, complete deletion of ALK1 causes major defects in the vasculature, resulting in embryonic lethality at midgestation [94]. Interestingly, blood vessel abnormalities in ALK1 mutants are similar to those identified in TGF β 1, T β RII, and Endoglin mutant mice, further supporting that ALK1 mediates TGF β 1 signalling in the endothelium [94]. Additionally, in endothelial cells, ALK1 was shown to bind to both TGF β 1 and T β RII, and ALK1 signalling can inhibit ALK5-mediated TGF β 1 signalling, which suggests that a balance between ALK1 and ALK5-mediated TGF β 1 signalling is required in endothelial cells for vascular development [94].

Deletion of T β RII in endothelial cells using *Tie1-Cre* generates vascular defects in the yolk sac comparable to the complete loss of T β RII [92]. Loss of T β RII *Tie2-Cre* results in embryonic lethality by E11.5 or E12.5 [95]. Approximately 65% of the mutant embryos have severe yolk sac defects and growth arrest at E9.5 and do not survive past E11.5. The remaining T β RII mutant embryos do not display overall growth retardation and survive until E12.5 but die as a result of haemorrhaging and cardiac defects. Of note, mutants have decreased mesenchymal cell proliferation specifically in the inferior cardiac cushion but do not have EMT defects as mesenchymal cells are still evident in the cushions [95]. This data

suggests that T β RII is not involved in initial cushion formation and EMT but rather proliferation of cushion mesenchyme. If deletion of T β RII is induced using tamoxifen at E11.5 using a CreERT system (tamoxifen-activated *Cdh5*(PAC)-CreERT2) in the endothelium, embryos die at E15.5 due to abnormal ventricular septation, failure of cushion fusion and haemorrhaging in cerebral blood vessels [96].

Deletion of either of the TGF β co-receptors, Endoglin and Betaglycan, results in embryonic lethality due to cardiac defects [97–100]. Endoglin^{-/-} embryos die at E11 with enlarged ventricles, dilated OFT, cardiac cushions that lack mesenchyme cells, and angiogenic defects in yolk sac; whereas, Betaglycan null mice are embryonic lethal at E14.5 due to coronary vascular developmental defects such as hypercellular epicardium, dysmorphic vessels at the AV groove and subepicardial haemorrhage [97–100]. Betaglycan null mice also have defects in septation, OFT alignment, and myocardial thinning, although these defects are likely not the cause of lethality [100].

Overall, TGF β signalling plays an essential role in the initial promotion and cessation of EMT, and in cushion mesenchyme proliferation and differentiation during heart valve development. Tissue-specific knockout mouse models suggest that the TGF β receptors have very diverse and specific roles dependent on the tissue and time point in which they are expressed. This makes it difficult to determine their roles in developing heart valves. Moreover, early lethality of many of the mouse models precludes our understanding of the potential roles of these signalling components in later stages of valve remodelling and differentiation. The use of inducible knockout systems will be highly valuable in teasing out the exact roles of TGF β signalling components during heart development. Furthermore, TGF β signalling plays a significant role in maintaining adult heart health by regulating cardiac fibrosis and hypertrophy after injury and hypertension [101,33]. In a normal adult valve, VICs, the main cellular component, are quiescent and maintain the integrity of the valve. When VICs are injured, TGF β signalling is involved in activating VICs [102], sustaining VIC activation and regulating *in vitro* valve repair via activated VICs [103]. Persistent activation of VICs can lead to abnormal ECM composition and can alter the mechanical properties of the valve, thus making the valve more susceptible to disease. TGF β 1 has been found in calcified aortic valve cusps and promotes calcification of aortic VICs [104]. TGF β signalling has been associated with a number of valve diseases [105–107] suggesting that aberrant activation/inhibition of this pathway during embryonic development may lead to valve disease later in life. Additional studies on the role of TGF β signalling in later stages of heart valve development and adult VIC activation are required to aid in the discovery and design of new potential therapeutics for congenital heart defects and valve disease.

Smads, the mediators of TGF β and BMP signalling

The targeted loss of Smad1, Smad2, Smad4, or Smad5 results in embryonic lethality [108], while deletion of Smad3 or Smad8 are viable without any reported cardiac defects [109–111]. Smad1 knockout embryos have major defects in chorioallantoic fusion and do not survive past E9.5 [112,113]. Loss of Smad2 or Smad4 causes major abnormalities in mesoderm formation and the embryos fail to undergo normal gastrulation [114–117].

Deletion of Smad5 causes defects in vasculature, craniofacial abnormalities and mutants share a number of similarities with BMP2 knockout mice [118]. Interestingly, mice that are deficient in I-Smads have cardiovascular malformations. For example, the complete loss of Smad6 causes OFT septation defects, hyperplasia of the valves and OFT ossification at 6 weeks of age [119]. Thus, Smad6 has an important role in the development of the cardiac cushions via inhibition of BMP and TGF β signalling, and plays an essential role in valve homeostasis in the adult [119]. Deletion of Smad7 also results in valve malformations, including ventricular septal defects, OFT defects, and failure of compaction in the myocardium resulting in lethality at late gestation [120]. Some Smad7 mutants survive but display severe arrhythmias and a decline in cardiac function [120]. The loss of Smad7 in cardiac cushions and endocardium leads to increased Smad2/3 phosphorylation and elevated levels of apoptosis, suggesting that Smad7 regulates TGF β signalling in these cells to prevent apoptosis [120]. Endocardial loss of Smad4 results in severe AVC and OFT cushion defects such as an absence of EMT, acellular cushions, and reduced endocardial proliferation, and illustrates that Smad4 is essential for cardiac valve development [121]. Given that Smad4 is common to both TGF β and BMP signalling, Smad4 deletion in the endocardium supports that both TGF β and BMP signalling play an important role in the formation of the cardiac valves.

Notch signal transduction

Activation of Notch requires the binding of a transmembrane Notch ligand on a signalling cell to a transmembrane Notch receptor on a signal-receiving cell. In the mammalian system, there are four Notch receptors, Notch 1–4, and five Notch ligands, Delta-like (Dll) 1, 3, 4 and Jagged (Jag) 1, 2. Activation of Notch signalling has three major steps: ligand binding, release of the Notch intracellular domain (NICD) via two proteolytic cleavages of the Notch receptor, and finally translocation of NICD into the nucleus to function as transcription factor. In brief, when a Notch ligand binds the receptor, the Notch receptor undergoes a conformational change that exposes the extracellular cleavage site S2. S2 cleavage is mediated by a disintegrin and metalloproteinase 10 and/or 17 (ADAM10 and/or ADAM17) [122,123]. The extracellular portion of the Notch receptor released by this cleavage is endocytosed into the signalling cell [124]. As a consequence of loss of the extracellular portion of the Notch receptor, the remaining fragment becomes susceptible to a second proteolytic cleavage by the γ -secretase complex at the S3 site [125]. This releases NICD and allows it to translocate into the nucleus [125,126] where it binds to Recombination signal-Binding Protein 1 for J-Kappa (RBPJ also known as CSL: CBF1 (C Promoter-Binding Factor 1), Suppressor of Hairless, and Lag-1) through the RBPJ-interacting domain [127–129]. Binding of NICD to RBPJ displaces the repressor complex that is bound to RBPJ in the absence of Notch signalling and recruits coactivators such as Master-mind-Like (MAML) [130,131]. As a complex, NICD, RBPJ, and MAML lead to direct activation of Notch target genes (Figure 2B), such as the hairy enhancer of split (HES) and hairy/enhancer of split-related with YRPW motif (HEY, also called HESR, CHF, HRT) family proteins [132,133]. Previous work from our group has identified *Acta2* (also known as smooth muscle actin, SMA), *Snai2*, *Smad3*, and *Runx3* as direct Notch target genes in the developing heart [134–137]. Additional Notch target genes such as *c-Myc* and cyclin D1 and

D3 are reviewed in [138], however, these targets genes have been identified in other systems and not in developing heart and heart valves.

Notch signalling in cardiac valve development

Notch signalling is involved in numerous developmental events and processes such as heart valve development, angiogenesis, hematopoietic expansion and differentiation, and somitogenesis [139–142]. Notch signalling components are widely expressed during development of the mouse heart and during valve formation Notch signalling is an essential driver of EMT. *Notch1* is initially expressed in the cardiac crescent, throughout the endocardium with elevated levels in the AVC and OFT regions of the heart tube, and in cardiac cushion mesenchyme at E9.5 [143,144]. *Notch2* is highly expressed in AVC (E12.5) and OFT (E11.5 and E14.5) endocardium and cushion mesenchyme and is expressed in atrial and ventricular myocardium at later time points [145,146]. *Notch3* is expressed in the cardiac crescent only during its formation [143] whereas *Notch4* is expressed in E10.5 endocardium during cushion formation [147]. Of the Notch ligands, *Jag1* and *Jag2* are expressed during heart valve formation. *Jag1* is expressed in AVC and OFT endocardium and atrial myocardium from E10.5–E12.5 [148]. *Jag2* is expressed in OFT myocardium from E11.5–E15.5 [149]. Of the Dll ligands, only *Dll4* is expressed in the developing heart within the cardiac crescent, endocardium (after E8.5) and ventricular endocardium after E11.5 [150,151]. The Notch targets, *Hey1* and *Hey2* are expressed in the heart tube, endocardium (*Hey2* specifically in the AVC and OFT endocardium at E11) and atrial (*Hey1*) and ventricular (*Hey2*) myocardium at E10.5 [152,144].

To examine the functions of Notch signalling during valve development, transgenic mouse models have been generated for downstream, intracellular effectors, such as NICD, RBPJ and MAML. The loss of Notch intracellular effectors can lead to a complete block in Notch signalling, while overexpression of NICD leads to constitutive activation of Notch signalling. This allows examination of effects in the presence or absence of Notch signalling. Gain of function experiments with constitutive endocardial Notch activation using NICD leads to the up-regulation of a mesenchymal gene program in the ventricular endocardium and ventricular explants have the ability to undergo a non-invasive EMT and interestingly upon addition of BMP2 ventricular explants can undergo a full invasive EMT [51]. This data indicates that Notch signalling plays an important role in endocardial patterning of the AVC and chambers of the heart and that BMP2 has a key role in inducing invasive EMT. Conversely, absence of RBPJ causes a loss of cushion mesenchyme in valve regions, EMT defects, and collapsed endocardium in the developing heart [144]. *Rbpj* null mice have additional embryonic abnormalities such as growth retardation, incomplete turning, and placenta, neural tube and somite defects, which result in embryonic lethality at E10.5 [153]. Endothelial-specific *Rbpj* null embryos have a comparable phenotype to the complete loss of *Rbpj* with severe growth retardation and vascular remodelling abnormalities [153,154]. Interestingly, in *Rbpj* null embryos the endocardium overlying AVC cardiac cushions appears to be activated but the cells fail to invade the cushions due to preservation of their adherens junctions and maintenance of close associations [144,51]. This data suggests that Notch activation and signalling via RBPJ is essential for the endothelial cell lineage and EMT in the cushions. Over-expressing dominant-negative MAML (dnMAML), a pan Notch

inhibitor, in the endothelium results in lethality before E10.5 with abnormalities similar to endothelial-specific Notch mutant mice [155]. Endothelial-specific dnMAML mutants have a severe delay in development, pericardial effusions, and major defects in the vasculature [155]. Additionally, the induction of dnMAML in the endothelium at E8.5 and E9.5 causes a decrease in the number of mesenchymal cells in the AVC cushions [136] and demonstrates that loss of Notch signalling severely impairs cardiac cushion development by blocking EMT.

To examine the effects of loss of specific components of Notch signalling during mouse development, ligand- and receptor-specific transgenic mice have been generated. Loss of *Notch1* leads to defects in angiogenesis, loss of cellularization in the AVC, blocked ventricular trabeculation, and disrupted vasculature resulting in embryonic lethality at E10.5 similar to *Rbpj* null mice [144,156,157]. *Notch1*^{-/-} mice have collapsed endocardium and lack cushion mesenchyme at the onset of valve formation, indicating that Notch1 is required for EMT [144]. Loss of *Notch1* results in a decrease of *Snail*, a known driver of EMT, which reduces cadherin-mediated cell adhesion. Additionally, AVC explants derived from *Notch1* mutant hearts contain very few cells capable of EMT, further supporting that Notch1 is required for EMT in cardiac cushions [144]. *Notch2* hypomorphic mice suffer from kidney abnormalities, eye defects, myocardial hypoplasia, and reduced ventricular trabeculation [158]. Tissue-specific deletion of *Notch2* in neural crest cells results in a constricted outflow tract due to decreased proliferation of the vascular smooth muscle derived from cardiac neural crest [146]. Double null *Notch1/Notch2* mice die during early development from abnormal left-right asymmetry caused by the lack of induction of *Nodal* [159], indicating redundant roles in Notch receptor signalling at this stage. This early embryonic lethality prevents examination of *Notch1/Notch2* double mutation in the heart valves. No valve defects are detected in *Notch2* mutant mice [158,146], suggesting that Notch1 may be sufficient for cardiac cushion development.

Mice with deletion of *Notch3* are viable and fertile and do not have any obvious defects [160]. Moreover, *Notch3* deletion does not enhance the *Notch1* mutant phenotype, suggesting that Notch3 does not play a redundant role with Notch1 during valve development [160]. *Notch4* null animals are viable and fertile [156] but combined *Notch1/Notch4* null animals have enhanced vascular defects when compared with *Notch1* null mice, including disrupted vasculature, growth retardation and pericardial effusions [156]. This data suggests that these Notch receptors have overlapping roles in endothelial signalling during development. To specifically study valve development, tissue-specific knockouts have been used to reveal additional roles of Notch signalling. Endothelial-specific deletion of *Notch1*, in which *Tie2-Cre* was used to delete *Notch1*, causes embryonic lethality at E10.5 due to vascular defects in the placenta, yolk sac, and embryo, and cardiovascular failure similar to complete loss of *Notch1* [161]. Together with Notch receptor knockout studies, Notch1 is essential for cardiac valve formation and Notch signalling is required in the endothelium during vascular and cardiac development.

Interestingly, Notch ligand mutant mice do not show abnormalities in cardiac valves, suggesting that redundancy plays a role during valve formation. For instance, *Jag1* null animals die at E10.5 from collapsed vasculature, haemorrhaging, and failure to remodel the

vascular plexus [162]. Endothelial-targeted *Jag1* null animals recapitulate the *Jag1* null mouse phenotype [155]. In contrast to *Jag1*, *Jag2* null animals have limb, thymic, and craniofacial defects that result in perinatal lethality but, similar to *Jag1* null, do not exhibit obvious defects in heart valve formation [163]. Loss of the Delta-like ligands (Dll) results in a variety of defects but also do not include cardiac valve defects. Of the Dll ligands, only *Dll4* is expressed in the embryonic heart in mice. *Dll4* heterozygous mice display pericardial effusions, growth retardation, vascular remodelling defects and are haploinsufficient lethal [154]. *Dll4* heterozygous mice have a similar vascular phenotype to Notch receptor and *Rbpj* knockout mice, suggesting that Dll4 is the predominant Notch ligand required for vascular development. Taken together, gene targeting of the Notch ligands in mice suggest that Notch ligand-specific functions are not required for cardiac valve development and redundancy plays a role in the lack of cardiac valve defects.

Loss of Notch target genes has further revealed the significance of Notch signalling during cardiac development. *Hey2* null mice have a similar phenotype to patients with Tetralogy of Fallot, a condition with a number of cardiac defects, including ventricular septal defects, pulmonary stenosis, overriding aorta, and right ventricular hypertrophy [164]. *Hey1/Hey2* double knockouts have cardiac cushion defects and lack mesenchymal cells in the cushions and *Hey1/HeyL* double knockouts fail to close the ventricular septum and have thickened valve leaflets [165,166].

Since the majority of mouse knockout studies result in embryonic lethality at E10.5, it is evident that Notch signalling has a critical role in early phases of cardiac valve formation following EMT. However, the roles in later valve development and in the adult valve disease are not fully explored. Interestingly, Notch has recently been implicated in valve calcification, a common form of heart valve disease [167], and mutations in *NOTCH1* have been linked with familial, nonsyndromic, autosomal-dominant calcific aortic valve disease (CAVD) and associated with BAV [168]. To further support a role for Notch signalling during valve disease, heterozygous *Notch* or *Rbpj* mice have a higher risk of developing calcification of the aortic valve [169,170]. Therefore, understanding the role of Notch signalling during late valve formation may provide key insights into the pathway's involvement during valve disease.

CROSS-TALK BETWEEN BMP, TGF β , AND NOTCH SIGNALLING

Synergy of Notch, BMP and TGF β signalling at the initiation of valve formation through induction of EMT is suggested through phenotypic similarities observed in transgenic mouse models and explant cultures. During formation of valves, it is possible that these signalling pathways act in parallel with one another and have their own separate roles. However, it is more likely that these pathways integrate and cross-talk to one another to generate the highly stratified and delicate valve leaflets and septa of the heart. Yet, how these signalling pathways are integrated in the heart valves to produce a coordinated EMT response remains a major question. Currently, our understanding of the molecular mechanisms involved in this cross-talk is limited and integration of the pathways primarily operates at the level of transcriptional regulation. First, Notch, BMPs and TGF β can cross-regulate each other's signalling pathway components in the developing heart. Second, the pathways can function

synergistically through direct DNA-binding of NICD and Smads to co-regulate expression of target genes.

The inter-relationships between BMP, TGF β , and Notch signalling in heart valve development

A relationship between BMP and TGF β signalling was initially demonstrated in chick, where a synergistic association between TGF β 3 and BMP2 promote EMT in AV endocardial cells [48,171]. Transgenic mouse models for BMP signalling components support this model of cooperation since BMP signalling induces the expression of TGF β pathway components. For example levels of TGF β 2 in the AVC are reduced in myocardial-specific ALK3 mutant mice, indicating that myocardial BMP signalling is required to maintain TGF β 2 expression during cushion formation [61]. Moreover, addition of BMP2 to AVC explants increases TGF β 2 [50] and myocardial deletion of *Bmp2* leads to loss of TGF β 2 in AV cushions [39]. Additionally, ectopic activation of TBX2 (a target of BMP signalling) in ventricular myocardium results in an increase in TGF β 2 levels, which leads to formation of ectopic cardiac cushions in ventricular myocardium [172]. In addition to TGF β , myocardial deletion of *Bmp2* reduces *Notch1* and *Snail* expression in AVC endocardium, indicating that BMP2 signalling plays a critical role in maintaining Notch signalling in the AVC [39,51]. In summary, during formation of cardiac cushions, BMP2 signalling within myocardium acts to induce and maintain the expression of Notch1 and TGF β 2 to promote the initiation of EMT in the endocardium.

Notch has been shown to regulate BMP expression to pattern the developing heart. In the endocardium, Notch can repress expression of BMP2 via HEY proteins. Moreover, HEY proteins in chamber myocardium restrict myocardial BMP2 expression to the AVC myocardium [51]. Additionally, ectopic expression of NICD in myocardium expands *Hey1* and chamber-specific markers and reduces BMP2 in the AVC resulting in a loss of AVC identity; whereas ectopic Notch activation via NICD in the endocardium leads to expansion of the AVC phenotype into ventricular endocardium but does not affect the myocardium [51]. Therefore, Notch plays a key role in repressing a chamber-specific program in AVC endocardium and alterations to Notch signalling causes abnormalities in endocardial patterning. In the AVC, BMP2 and Notch signalling act in concert to promote EMT via induction of *Snail* expression and BMP2-driven *Snail* nuclear accumulation that induces a mesenchymal expression program [51]. Overall, endocardial Notch signalling restricts BMP2 signalling to valve forming regions and works together with BMP to promote EMT through induction of *Snail* in cardiac cushion mesenchyme.

Notch signalling can also influence TGF β signalling components in the developing heart. The loss of Notch signalling (via deletion of *Rbpj* or *Notch1*) in mouse embryos causes defects in EMT, decreased expression of *Snail*, and loss of TGF β 2 in AVC myocardium [144]. In addition, *Rbpj* mutants have reduced levels of TGF β receptors, ALK5, T β RII, and Betaglycan in endocardium, however, there was no change in Endoglin and the BMP receptors [144]. This data suggests that Notch signalling is required for TGF β 2 and TGF β receptor expression in the AVC. Therefore, Notch functions by lateral induction in AVC endocardium to promote TGF β -induced EMT.

In a recent study, it was shown that Notch signalling can activate the nitric oxide pathway by upregulating the nitric oxide receptors, soluble guanylyl cyclases (sGC), while inducing expression and secretion of Activin A, a TGF superfamily member, to stimulate nitric oxide biosynthesis in non-transforming endothelium [173]. Disruption of either Notch-sGC or Notch-Activin A axes resulted in blocked EMT. Although this is not a direct interaction between Notch and TGF β signalling, it highlights that there is indirect cross-talk between the nitric oxide, Notch and Activin pathways.

BMPs and TGF β signalling pathway interactions via ligand-receptor and Smad complexes

For simplicity, BMP and TGF β signalling pathways are often discussed as separate pathways although there is evidence to suggest that they cross-talk to one another by forming mixed receptor-ligand and Smad complexes. For example, BMP2 and TGF β 2 can bind to Betaglycan to induce EMT in chick ventricular and AVC explant assays [174,175], which suggests this interaction plays an important role in AVC cushion formation. Loss of ALK2 in endothelium leads to reduced phosphorylation of TGF β and BMP Smads [45] and indicates either that loss of BMP signalling reduces TGF β s expression (as mentioned earlier) or that BMP signalling can induce TGF β Smads specifically via different combinations of receptor-ligand complexes or vice versa. In support of this, TGF β s have been shown to induce both TGF β and BMP Smads in keratinocytes and authors suggest that TGF β activation of BMP Smads likely occurs via ALK2 (not ALK1, although this is also seen in other cell types) and is dependent on ALK5 [176]. To add to the complexity of these interactions, simultaneous activation of Smad2/3 and Smad1/5 causes the formation of mixed R-Smad complexes, such as a Smad1/2 complex, which can induce different sets of signalling pathways [177]. For instance, TGF β activation of the BMP Smads, Smad1/5, is required for anchorage independent growth and not for the growth inhibition that is characteristic of TGF β Smads [177]. Finally, R-Smads can compete for binding to Smad4 and thereby modulate both BMP and TGF β signalling [178]. Based on the above, BMP and TGF β signalling interact and create diverse signalling mechanisms via cross-talk to one another at multiple levels, including generation of different combinations of ligand-receptor complexes or mixed R-Smad complexes.

Synergistic activation of target genes via Notch and Smads

Notch can influence the TGF β pathway in endothelial cells via the expression of R-Smads and can synergize with Smad3 to regulate a subset of Smad3 target genes. Notch activation (via over-expression of NICD or ligand-induced activation) in endothelial cells leads to inhibition of TGF β -induced Smad1 and Smad2 and subsequently a decrease in expression of their target genes [136]. Interestingly, Notch activation in endothelial cells increases mRNA expression of Smad3, extends the protein half-life of Smad3, and has a role in regulation of specific Smad3 target genes [136]. To further support the effect of Notch on Smad3, a mouse that expresses the pan Notch inhibitor, dnMAML specifically in endothelial cells has reduced total Smad3 protein levels and Smad3 nuclear localization in cardiac cushion cells [136]. In addition, when Notch activation is coupled with TGF β stimulation, there is a synergistic effect on Smad3 target genes, *Ankrd1* and *Hey1*. This synergy between Notch and TGF β occurs by recruitment of Smad3 to both Smad and RBPJ binding sites followed by an induction of acetylation of histone H4 [136]. This demonstrates that Notch signalling

has a direct effect (positive and/or negative) on TGF β signalling via the R-Smads and that Notch and TGF β have a collaborative relationship to synergistically activate the expression of specific Smad3 target genes. Although it has not been demonstrated in the heart, NICD has been shown to directly interact with Smad1 and Smad3 to regulate target genes [179].

Common targets of Notch, TGF β and BMP signalling

The *Snail* family transcriptional repressors play an important role in EMT during development and metastasis [180]. Of interest to this review, the *Snail* family members illustrate how BMP, Notch and TGF β signalling pathways converge during heart valve formation. The first *Snail* family member described in heart valve development was *Slug* (*Snai2* in mouse), which was identified as a target of TGF β 2 signalling during valve formation and is required for initial stages of EMT in chick [181]. In mouse, a homologue of *Slug*, *Snail* is expressed in the AVC at the onset of EMT and has been shown to play a key role in the promotion of EMT via repression of E-cadherin [182]. The loss of *Snail* results in embryonic lethality due to EMT defects during gastrulation [183].

In mouse, *Slug* was identified as a direct target of Notch and is required for the proper cellularization of the cardiac cushions [184]. *Slug* is expressed in cardiac cushion mesenchyme and a subset of AVC endocardial cells at E9.5 and loss of *Slug* results in a failure to populate the cardiac cushions with mesenchyme cells at E9.5 [184]. This effect is compensated for by *Snail* at E10.5 and cardiac cushion EMT is re-established [184]. Additionally, *Slug*, but not *Snail*, is directly upregulated by Notch whereas *Snail*, and not *Slug*, is induced by TGF β in endothelial cells [184]. Interestingly, when activation of Notch is combined with TGF β stimulation, there is a synergistic effect on the expression of *Snail* [184]. Recently, a link between BMP2 and *Snail* was established: the addition of BMP2 to endothelial cells leads to an increase in *Snail* mRNA and deletion of BMP2 in the myocardium results in a loss of *Snail* expression [51]. Since BMP, Notch, and TGF β signalling all target *Snail* expression, it is probable that *Snail* represents a critical point of convergence for Notch1, TGF β 2 and BMP2 induced signalling and suggest that they are dependent on *Snail* expression for complete activation of invasive EMT in the AVC. Therefore, BMP, Notch and TGF β play a key role in the promotion of cardiac EMT through regulation of *Snail* family members during heart valve formation. Taken as a whole, this data and all previously mentioned work supports that Notch, TGF β , and BMP signalling pathways cross-talk during the development of the cardiac valves (Figure 3).

PERSPECTIVES

As described above, TGF β , BMP, and Notch signalling play critical roles during early formation of cardiac cushions, particularly in the process of EMT. Prior to EMT, AV myocardium secretes BMPs to set up the appropriate environment for endocardial cells to become activated and following this, BMP signalling also plays an important role in EMT and promotes invasion of the mesenchyme. Loss of BMP signalling prevents initial formation of the cardiac cushions and tissue-specific deletion reveals additional roles in mesenchyme growth and survival, and valve remodelling. Following BMP signalling in the cardiac cushion, Notch signalling, a major driver of EMT, becomes activated in endocardial

cells and promotes EMT by activating *Snail*, which decreases cadherin-mediated adhesion (via inhibition of VE-cadherin, for example). This allows newly transformed mesenchymal cells to loosen their cell associations and migrate into the cardiac jelly. Together with Notch signalling, TGF β and BMP signalling are active in the cardiac cushions and play crucial roles in the promotion of EMT and mesenchymal cell invasiveness. At later time points, TGF β signalling has a role in the termination of EMT and in mesenchyme proliferation and differentiation. Interestingly, each of these signalling pathways plays a key role in EMT during valve formation, however, expression of BMP, TGF β , and Notch signalling components persist after cushion formation and, therefore, these pathways likely have additional roles in valve differentiation and remodelling.

There are a number of gaps in the field of cardiac valve development. For instance, there is a lack of information on events in cardiac cushions post-EMT. Many questions come to mind, such as: How are the cardiac cushions maintained? How is differentiation initiated in the cushion mesenchyme? What initiates cushion fusion and subsequent remodelling of the valves? Are there different subpopulations of valve precursor cells in the developing cardiac cushion? How do the valves become stratified into defined layers? Additional studies will need to address these types of questions to provide insights on descendants of the cardiac cushion mesenchyme (VICs) in adult valve during normal homeostasis, remodelling after injury, and induction of disease.

Although we only focused on a few, numerous signalling pathways and transcription factors are involved in cardiac valve development and developing regulatory networks that integrate signalling pathways and transcription factors is required to obtain a defined picture of how valves develop. In this review, we focussed on BMP, TGF β and Notch signalling as they are required to initiate valve formation and in their absence the cardiac cushions fail to form properly. Of note, there are a number of additional signalling pathways that are important for valve formation such as Wnt, vascular endothelial growth factor (VEGF), and Ephrin B (EphB). Furthermore, transcription factors have a crucial role in directing how valves develop by regulating downstream target genes. Transcription factors, such as Smads, Snails, Sox9, Runx, Twist1, and Tbx5 have central roles in valve formation. Strangely, there are very few known transcriptional targets for these transcription factors during heart valve development. By exploiting new technologies like chromatin immunoprecipitation coupled with next generation sequencing (ChIP-Seq), we can identify the collection of genes regulated by these transcription factors in the embryonic heart valves and explore the regulatory networks.

ECM creates a dynamic environment that is involved in the regulation of many cellular events in adult heart valves and alterations in composition can lead to valve defects and disease. The ECM can sequester ligands away from the receptors and thus, modulates signalling pathways and represents another level of regulation during heart valve development. Integration of signalling pathways, transcription factors and the ECM niche with *in vivo* disease mouse models will identify genes crucial for valve development and linked to congenital valve defects or involved in the initiation and progression of heart valve disease.

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Figure 1. Early development of the mouse embryonic heart

Initially, the heart forms a linear tube that generates swellings that create the future chambers of the heart, atria and ventricles at E8.5. At E9.0, the linear heart tube will begin looping to bring the chambers into their final positions. Endocardial cushion formation begins at E9.5 as AVC endocardial cells undergo endothelial-to-mesenchymal transformation (EMT) to create the AVC mesenchyme cells that fill the cardiac cushions and form the primitive heart valves. Following EMT (E10.5-birth), the primitive heart valves will remodel to generate the thin, delicate heart valve leaflets in the adult heart. In the first two panels, red depicts ventricles, and blue represents atria. The third panel is an inset from the E10.5 heart illustrating the AVC where red is the myocardium, green cells are endocardial cells, and orange cells are mesenchyme. The last panel depicts a cross-section through the adult heart where red/pink represents oxygen-rich blood and blue represents oxygen-depleted blood. AVC - atrioventricular canal, RA – right atria, LA – left atria, RV – right ventricle, LV – left ventricle, PA – pulmonary artery.

Figure 2. TGF β , BMP, and Notch signalling pathways

A: TGF β and BMP signalling pathways. To activate signalling, a TGF β or a BMP ligand binds their respective receptor complexes that contain two type I receptors and two type II receptors, which causes a phosphorylation event that activates the receptor Smads (R-Smads). Following this, R-Smads bind with their Co-Smad, Smad4, and move into the nucleus to activate TGF β or BMP-responsive genes, respectively. TGF β Smads = Smad2, Smad3. BMP Smads = Smad1, Smad5, Smad8 (S1/5/8). The yellow star with a P represents a phosphorylation event. B: Notch signalling pathway. Notch signalling is activated when a signalling cell with the Notch ligands, Jagged (Jag) or Delta-like (Dll) bind with a Notch receptor on a signal receiving cell. The binding of the ligand to the Notch receptor causes a conformational change and exposes two cleavage sites S2 and S3 in the Notch receptor. The first cleavage of Notch occurs at the S2 site via a disintegrin and metalloproteinase (ADAM) protein that releases the extracellular portion of Notch. The second cleavage of Notch occurs at the S3 site by γ -secretase complex and this releases the intracellular domain of Notch (NICD). NICD translocates into the nucleus and binds with RBPJ (Recombination signal-Binding Protein 1 for J-Kappa), MAML (Master-mind Like), p300, and co-activators (CoA) to activate Notch responsive genes. In the absence of NICD, RBPJ is bound by co-repressors (CoR) and cannot activate Notch responsive genes.

Figure 3. Cross-talk between BMP, TGFβ, and Notch signalling pathways
 Red represents Notch pathway induced interactions. Blue represents BMP induced interactions, and green represents TGFβ induced interactions.