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Transcriptional Regulation of Heart Valve Progenitor Cells

Santanu Chakraborty, **Michelle D. Combs**, and **Katherine E. Yutzey**
Division of Molecular Cardiovascular Biology, Cincinnati, Children's Medical Center, ML 7020, 240 Albert Sabin Way, Cincinnati, OH 45229, USA

Katherine E. Yutzey: katherine.yutzey@cchmc.org

Abstract

The development and normal function of the heart valves requires complex interactions among signaling molecules, transcription factors and structural proteins that are tightly regulated in time and space. Here we review the roles of critical transcription factors that are required for specific aspects of normal valve development. The early progenitors of the heart valves are localized in endocardial cushions that express transcription factors characteristic of mesenchyme, including Twist1, Tbx20, Msx1 and Msx2. As the valve leaflets mature, they are composed of complex stratified extracellular matrix proteins that are regulated by the transcriptional functions of NFATc1, Sox9, and Scleraxis. Each of these factors has analogous functions in differentiation of related connective tissue lineages. Together, the precise timing and localized functions of specific transcription factors control cell proliferation, differentiation, elongation, and remodeling processes that are necessary for normal valve structure and function. In addition, there is increasing evidence that these same transcription factors contribute to congenital, as well as degenerative, valve disease.

Keywords

Transcription factor; Heart valve; Development

Introduction

The proper development and function of the heart valves are essential for unidirectional blood flow, and abnormal valve development can lead to significant cardiovascular disease. Cardiac valve defects represent approximately 20–30% of all congenital cardiovascular malformations with an incidence as high as 5% of live births [28,47]. In addition, approximately 100,000 inpatient valve procedures are performed in the USA per year [69]. In the mature heart, the mitral and tricuspid atrioventricular (AV) valves are localized in the left and right AV junctions, respectively, whereas semilunar valves (SL) are positioned at the roots of the aorta (aortic valve) and pulmonary artery (pulmonic valve) [56,63]. During pre-natal development, valve formation is controlled by complex interactions of signaling molecules and transcription factors that regulate cell proliferation, lineage diversification, differentiation, and leaflet remodeling [16]. Here, we review the transcription factors Twist1, Tbx20, Msx1, Msx2, NFATc1, Sox9, and Scleraxis and their roles in multiple aspects of valve progenitor cell specification and maturation.

Correspondence to: Katherine E. Yutzey, katherine.yutzey@cchmc.org.

Overview of Endocardial Cushion Formation and Valve Remodeling

The development of heart valves begins with the formation of endocardial cushions in the atrioventricular canal (AVC) and outflow tract (OFT) regions of the primitive heart tube [16, 46]. Endocardial cushion formation is highly conserved in vertebrates and is initiated at embryonic day (E) 3 in chick, E9.5 in mouse, and E31–E35 in human [23,43,44]. The endocardial cushions consist of highly proliferative undifferentiated mesenchymal cells localized between the endothelial endocardium and myocardial cell layers in the OFT and AVC. Endocardial cushion formation is initiated when signaling cues from the AVC and OFT myocardium result in epithelial to mesenchymal transformation (EMT) of adjacent endocardial cells. The transformed mesenchymal cells then migrate into the intervening cardiac jelly to form the endocardial cushions [42,46]. Cell lineage analyses with *Tie2Cre* × *Rosa26RLacZ* reporter mice demonstrate that the majority of cells present in the mature valves are of endothelial endocardial cushion origin [21,37]. The mesenchymal heart valve progenitor cells in the endocardial cushions are highly proliferative, migratory, and undifferentiated, within a loosely organized extracellular matrix (ECM) [3,21,37]. These cells express several transcription factors including Twist1, Tbx20, Msx1, and Msx2 that are associated with mesenchymal precursor populations in a variety of organ systems.

Both myocardial- and endocardial-derived signaling pathways affect endocardial cushion EMT and proliferation of valve progenitor cells. Mice lacking BMP2 in the myocardium or BMPR1a in the endocardium have no AV endocardial cushion mesenchymal cell formation and no expression of the mesenchymal transcription factors Twist1, Msx1, and Msx2 [41,53]. BMP signaling also has a likely role in regulation of endocardial cushion cell proliferation because mice lacking the BMP inhibitor Smad6 have increased proliferation of endocardial cushion cells [25]. In the endocardial endothelial cells, Notch1 signaling induces expression of the transcription factor Snail which suppresses the expression of *VE-cadherin* and promotes EMT [70]. Canonical Wnt/*β*-catenin signaling is also active in AV and OFT endocardial cushions and is required for EMT and mesenchymal cell proliferation [36,46]. Overall, multiple signaling pathways, including those activated by BMPs, Notch, and Wnts, control aspects of valve progenitor cell specification, proliferation, migration, and differentiation through their actions on specific transcriptional effectors (reviewed in more detail in [3,16]).

Formation of the heart valve leaflets occurs with elongation and ECM remodeling of the endocardial cushions. This process is characterized by decreased cell proliferation and increased deposition and complexity of ECM [27,37]. The mature valve leaflets are stratified into three layers, the elastin-rich atrialis (AV valves)/ventricularis (SL valves), the proteoglycan-rich spongiosa and highly organized collagen fiber-rich fibrosa [27,39,50]. These diversified matrix compartments share structural and biomechanical properties with other types of connective tissues [56]. There is increasing evidence for conserved regulatory hierarchies of signaling molecules and transcription factors that control both heart valve maturation and differentiation of cartilage, tendon, and bone precursors [10,26,34,39].

Transcriptional Regulation of Valve Development

Over the past several years, significant progress has been made in elucidating the transcriptional regulatory hierarchies that control valve development (Table 1). These studies demonstrate similarities in the regulatory interactions that control endocardial cushion mesenchyme proliferation and gene expression with other mesenchymal progenitor populations in the embryo. A recent microarray gene expression profile of murine E12.5 AV endocardial cushions versus E17.5 remodeled valves identified several transcription factors including *Twist1*, *Tbx20*, *Msx1* and *Msx2* that are expressed in mesenchymal valve progenitor cells [10]. Later stages of valve development share transcriptional regulatory mechanisms with development

of osteoclasts, cartilage, and tendons, related to ECM remodeling and compartmentalization [39]. NFATc1 is required in osteoclast differentiation, as well as in the transition from proliferation to remodeling of the valve primordia [17,20,51]. In addition, Sox9, which is required for cartilage precursor differentiation, and Scleraxis, important in the development of tendons, are critical for normal ECM compartmentalization in the developing valves [35,38, 40]. Here we review the individual functions and downstream targets of these transcription factors in endocardial cushion formation and valve leaflet maturation (Table 2).

Twist1

Twist, a class II basic-helix-loop-helix (bHLH) transcription factor, was first identified in *Drosophila* as a critical regulator of mesoderm formation [9]. In vertebrates, Twist1 promotes cell proliferation and migration in a variety of mesenchymal, embryonic, and transformed cells [4]. In humans, Twist1 haploinsufficiency causes Saethre-Chotzen syndrome (OMIM #10400), which is characterized by craniofacial abnormalities and also is associated with congenital heart defects [52]. *Twist1* null mouse embryos demonstrate a failure in neural crest migration, hypoplastic limb buds, and vascular defects with lethality by E11.5 [14,62,72]. While the initial stages of endocardial cushion formation are apparently normal in *Twist1* null embryos [72], embryonic lethality precludes the detailed assessment of Twist1 function in endocardial cushion maturation and differentiation.

During heart valve development, Twist1 is expressed throughout the endocardial cushions of the AVC and OFT, and expression is down-regulated in the remodeling valves [10,41]. Endocardial cushion expression of *Twist1* is induced by BMP2 in both chicken and mouse embryos [41,60]. Twist1 gain and loss of function studies in avian endocardial cushion cell cultures demonstrate that Twist1 promotes cell proliferation and migration, while increasing the expression of *Cadherin 11 (cdh11)*, *Periostin (POSTN)*, and *Matrix metalloproteinase (Mmp)2* [60]. Twist1 also promotes expression of *Tbx20*, and a Twist1-responsive regulatory element is located in chicken *Tbx20* gene flanking sequences [60], (Horn and Yutzey, unpublished). In transgenic mice, induced expression of Twist1 in the developing valves leads to increased cell proliferation, increased expression of Tbx20, prolonged expression of primitive ECM genes, and abnormal valve remodeling (Chakraborty and Yutzey, unpublished). Expression of *hyaluronan and proteoglycan link protein1 (Hapln1)*, *collagen type II, alpha1 (Col2a1)*, *Mmp2*, and *Mmp13*, characteristic of endocardial cushion ECM, is increased in the Twist1 transgenic mouse valves, and *Col2a1* is a direct downstream target of Twist1 [10], (Chakraborty and Yutzey, unpublished). Together, these studies demonstrate a role for Twist1 in promoting endocardial cushion cell proliferation, migration, and primitive ECM gene expression. In addition, the loss of Twist1 is required for the normal progression of valve leaflet stratification and remodeling.

Tbx20

Tbx20 is a member of the *Tbx1* subfamily of *T-box* genes and is expressed in multiple organs, including the heart, of many species including *Drosophila*, *Xenopus*, avians, rodents, and humans [49,66]. Tbx20 has both activator and repressor functions that are dependent on regulatory element context and interactions with specific cofactors [48,64]. Human *TBX20* mutations are associated with a complex spectrum of developmental and functional cardiac abnormalities including valve defects [32]. Mice lacking Tbx20 have reduced myocardial proliferation and loss of heart chamber maturation, with embryonic lethality by E10.5 [7,61, 65,68]. The early embryonic lethality of these mice prevented analysis of the role of Tbx20 in valve formation, however, global knockdown of Tbx20 with RNA interference provided initial evidence for Tbx20 function in valve morphogenesis [68].

During heart valve development, *Tbx20* is strongly expressed in the endocardial cushion mesenchyme in both mouse and chicken embryos [48,64]. Tbx20 gain and loss of function studies performed in chicken AVC explants demonstrated that Tbx20, like Twist1, promotes cell proliferation and migration, while repressing ECM maturation [59,60]. Tbx20 promotes expression of the ECM remodeling enzymes, *Mmp9* and *Mmp13*, while repressing expression of the chondroitin sulfate proteoglycans, *aggrecan* and *versican* [59]. In addition, *Tbx20* expression is induced by BMP signaling, and *Tbx20* is a direct downstream target of Twist1 [59,60], (see above; Horn and Yutzey, unpublished). In cardiomyocytes, Tbx20 promotes cell proliferation through activation and binding to regulatory elements of *N-myc* and *Tbx2* genes [7]. Corresponding alterations in the expression of these genes also is observed in avian endocardial cushion cells with reduced expression of Tbx20 [59]. Together these studies demonstrate that Tbx20 promotes cell proliferation, while inhibiting maturation, of multiple cardiac cell lineages, including endocardial cushion cells. However, the consequences of altered Tbx20 function on heart valve development and morphogenesis in vivo are yet to be determined.

Msx1 *and* Msx2

Msx1 and *Msx2* belong to a subfamily of the *Nk*-like homeobox genes related to *Drosophila* muscle-segment homeobox (*msh*) [19]. During embryogenesis, Msx1 and Msx2 are often expressed together in mesenchymal structures, including the limb buds, pharyngeal arches, neural crest, and endocardial cushions of the heart [13,19]. In humans, mutations in *MSX1* cause orofacial clefting and tooth agenesis [5,71], and mutations in *MSX2* cause Boston-type craniosynostosis with premature fusion of skull bones and orofacial bone abnormalities [30]. However, no cardiac abnormalities have been reported with mutation of either human *MSX1* or *MSX2* genes. Similarly, mice lacking either Msx1 or Msx2 have no reported cardiac anomalies, but embryos lacking both Msx1 and Msx2 have severe endocardial cushion and conotruncal defects [12,13]. Interestingly, Msx2 expression is increased in adult human calcific valve disease, and ectopic expression of Msx2 in transgenic mice induces an osteogenic gene program in the valves through increased Wnt signaling [58].

In developing endocardial cushions, both *Msx1* and *Msx2* are expressed in endocardial and mesenchymal cells during EMT in overlapping patterns [13]. Loss of both Msx1 and Msx2 leads to a reduction of endocardial cushion formation, while no endocardial cushion or valve defects are associated with loss of either Msx1 or Msx2 [13,54,55]. These results support redundant functions for Msx1 and Msx2 during AV valve morphogenesis. In the Msx1/2 mutant embryos, endocardial expression of *Notch1*, *BMP2/4*, and *NFATc1* is reduced, and patterning of the AVC myocardium also is abnormal, leading to compromised EMT [13]. Secondary heart field and neural crest anomalies related to defects in cell proliferation and migration also occur with loss of both Msx1 and Msx2 [12]. Together, the loss of both Msx1 and Msx2 lead to a spectrum of cardiac malformations including double outlet right ventricle (DORV), pulmonary stenosis, atrial and ventricular septal defects, and hypoplastic ventricles [12,13]. Based on these studies, Msx1 and Msx2 have overlapping functions in endocardial cushion EMT and also potentially in cell proliferation and survival. Further studies are necessary to define the specific direct downstream targets of these proteins as well as their interacting factors in valve development.

NFATc1

Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATc1/NFAT2/NFATc) is a transcription factor belonging to the NFAT family [29]. NFAT factors contain a Rel homology region DNA binding domain and are regulated via dephosphorylation by calcineurin, which promotes nuclear localization. NFATs regulate proliferation, differentiation, and homeostasis in numerous cell types during embryogenesis and throughout life [18]. Surprisingly, NFAT

mutations have not been linked to human disease. Mice lacking *NFATc1* expression experience embryonic lethality by E14.5 due to lack of endocardial cushion growth and remodeling [20, 51]. In addition, NFATc1 regulates immune cell function and osteoclast differentiation [67]. In osteoclasts, NFATc1 is activated by RANKL signaling and promotes expression of the ECM remodeling enzyme *Cathepsin K (CtsK)* [67]. This same regulatory interaction occurs during heart valve development [17,34].

NFATc1 is expressed specifically in endocardial endothelial cells of the primitive heart tube and is restricted to the AVC and OFT endothelial cells at the early stages of endocardial cushion formation [20]. Endothelial-specific expression of *NFATc1* rescues the heart defects in *NFATc1*−/− mutants, demonstrating the specific requirement for endothelial expression of NFATc1 in normal valve morphogenesis [11]. EMT is apparently normal in NFATc1 null endocardial cushions. Further studies in NFATc1 null mice and avian endocardial cushion cell cultures demonstrate that NFATc1 is required both for endocardial endothelial cell proliferation and induction of *CtsK* gene expression [17,34]. NFATc1-dependent endocardial cushion endothelial cell proliferation is induced by VEGF in conjunction with MEK1-ERK1/2 activation. At later stages, RANKL treatment promotes NFATc1 nuclear localization and *CtsK* expression, while repressing cell proliferation, in conjunction with JNK activation. In vivo, *VEGF*, NFATc1, and activated ERK1/2 are localized to proliferating endocardial cushion endothelial cells, whereas *RANKL*, activated JNK1/2, and CtsK are expressed in the valve primordia during later remodeling stages [17]. Additional NFATc1 transcriptional targets in the developing valves include its own promoter in an autoregulatory interaction, as well as the calcineurin modulatory enzyme *DCSR1/MCIP1/RCAN1* [33,74]. NFATc1 also is expressed in adult human pulmonary valve endothelial cells in which VEGF can induce cell proliferation via an NFATc1-dependent mechanism, supporting a role for NFATc1 in adult valve homeostasis [31]. Together, these studies demonstrate a complex critical role for NFATc1 in the transition from endocardial cushion growth to remodeling during valve development, as well as in maintenance of the mature valves.

Sox9

Sox9 (SRY-box containing gene 9) is a high mobility group transcription factor expressed in several embryonic tissues including cartilage, where it is required for cell lineage expansion and differentiation [1]. Conditional mutagenesis of *Sox9* in mice demonstrates a requirement for Sox9 in cartilage precursor cell proliferation and differentiation [1]. There is increasing evidence for shared regulatory pathways in heart valve development with development of structurally related connective tissues including cartilage [39]. Therefore, a similar strategy was used in the developing heart valves to demonstrate that Sox9 is required for endocardial cushion cell lineage expansion as well as later differentiation evident in expression of genes associated with cartilage [40]. In humans, mutations in *SOX9* are associated with campomelic dysplasia, a disease characterized by generalized hypoplasia of endochondral bones and sex reversal with occasional defects in pancreas and/or kidney [24,73]. While *SOX9* mutations have not been associated with human valve malformations, increased SOX9 expression has been reported with mitral valve calcification in human patients [8].

In mouse and chicken embryos, Sox9 is expressed in the endocardial cushions and remodeling valve leaflets, but not in the supporting chordae tendineae [38,40]. Loss of *Sox9* in mice results in embryonic lethality between E11.5 and E12.5 with hypoplastic endocardial cushions [2]. In addition, NFATc1 is misexpressed in the cushion mesenchymal cells, which may indicate disruption of the endothelial delamination process during EMT [2]. *Tie2Cre* mediated loss of Sox9 in the endocardial cushions demonstrates that Sox9 also is required for cell proliferation and expansion of the valve progenitor pool [40]. In differentiating cartilage progenitor cells, Bmp2 activates expression of *Sox9* and the cartilage differentiation marker *aggrecan* [15]. This

same regulatory interaction is observed in cultured avian endocardial cushion cells [38]. Likewise targeted loss of Sox9 with *Col2a1Cre* in the remodeling valve leaflets in mice results in decreased expression of cartilage-associated proteins, Col2a1 and Hapln1, further supporting the parallels between valve and cartilage development [40]. In adult mice, heterozygous loss of *Sox9* in *Col2a1Cre* expressing cells results in thickened valve leaflets and calcification characteristic of valve disease [40]. Together these studies demonstrate that Sox9 has critical functions in endocardial cushion formation and valve remodeling and may also serve as a protective factor in calcific valve disease.

Scleraxis

Scleraxis (Scx) is a basic-helix-loop-helix (bHLH) transcription factor expressed in tendons and ligaments as well as in the developing heart valves [35,38,57]. In tendons, *Scx* is induced at the earliest stages of cell lineage specification, and it also regulates tendon differentiation through activation of structural protein genes including *Col14a1* and *tenascin* [6,57]. In mice, global deletion of *Scx*, results in severe tendon defects associated with limited use of all paws and back muscles with complete immobility of the tail [45]. These mice are viable, but they also have developmental defects in valve formation and progressive valve disease [35]. Interestingly, *Scx* mutations have not been reported in the human population, and there are no reports of Scx expression associated with human valve disease.

The chordae tendineae and other supporting structures of the valves share similarities in ECM composition and gene expression with tendon cell lineages [38]. In both mouse and chicken embryos, *Scx* expression is induced in the remodeling valves and is predominant in the chordae tendineae of the AV valves [35,38]. In developing tendons, FGF4 promotes expression of *Scx* and its downstream target tenascin, while inhibiting cartilage cell lineage development [22]. Likewise, in avian endocardial cushion cell cultures, FGF4 treatment promotes *Scx* and tenascin expression, while inhibiting expression of *Sox9* and *aggrecan* [38]. In mice, genetic deletion of *Scx* leads to thickening of the heart valves, decreased expression of *Col14a1*, and increased expression of *Sox9* [35]. In addition, mesenchymal cell markers *Msx1* and *Snail1* persist during the later stages of valve development, which may be indicative of a failure of these valve progenitors to differentiate [35]. Together these studies support a critical role for Scx in the specification and differentiation of heart valve supporting structures with molecular properties similar to tendons.

Overall Conclusions and Future Perspectives

Heart valve development is characterized by complex regulation of EMT, cell proliferation, cell lineage determination, ECM gene expression, and morphogenesis. Each of these processes is regulated by multiple signaling pathways and transcription factor interactions. Here we discuss the roles of seven transcription factors in specific aspects of heart valve development and maturation. Although significant progress has been made in this area, we are far from understanding all of the molecular regulatory interactions that control heart valve development. Certainly the complete regulatory networks are more complex than those presented here. Strikingly, several of the critical regulatory and structural genes expressed during valve development are associated with genetic lesions that cause congenital valve malformations. These genes and others expressed during valve development are strong candidates in future genetic studies of familial cardiovascular disease. In addition there is increasing evidence that critical regulators of valve development also are expressed during valve pathogenesis in adults. Therefore, the signaling pathways and transcriptional regulators active during valve development represent attractive therapeutic targets in the management and treatment of valve malformations and degenerative disease in a clinical setting.

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Table 1

Gene expression and associated human disease of transcription factors involved in heart valve progenitor cell development (see text for details and references)

Gene	Expression pattern	Associated human disease
Twist1	Endocardial cushion, mesoderm, cranial suture, neural crest, distal limb bud	Saethre-Chotzen syndrome
Tbx20	Primitive heart, endocardial cushion, heart valves, eye, ear, allantois, motor neurons	Defects in septum, chamber growth and valvulogenesis
Msx1	Endocardial cushion, pericardium, amnion, allantois, umbilical vein, neural plate, brain, cranial neural crest, tooth, limb bud	Orofacial clefting and tooth agenesis
Msx2	Endocardial cushion, pericardium, primitive streak, somatopleural lateral mesoderm, dorsal ectoderm, choroid plexus, dorsal region of the neural tube, limb bud	Boston-type craniosynostosis
NFATc1	Endocardial cushion/valve endothelial cells, coronary endothelium, immune cells, osteoclasts	Not reported
Sox9	Endocardial cushion, heart valves, cartilage progenitor cells	Campomelic dysplasia
Scx	Heart valves, ligaments, tendons, pericardium	Not reported

Table 2

Transcription factors and their downstream targets involved in heart valve development (see text for details and references)

Gene	Known downstream targets expressed in valves	Function in valve progenitor/mesenchymal cell development
Twist1	*Periostin, Tbx20, Col2a1, cdh11*	Proliferation/migration/ECM organization/collagen production
Tbx20	*N-myc, Tbx2, Isl1*	Proliferation/differentiation
Msx1	None identified	EMT/cushion formation
Msx2	None identified	EMT/cushion formation
NFATc1	*NFATc1, DSCR1, CtsK*	Cushion endothelial cell proliferation/valve ECM remodeling
Sox9	*Aggrecan, Col2a1, Col11a2, Hapln1*	Proliferation/differentiation
Scx	*Col14a1, tenomodulin*	Differentiation/ECM organization