Tbx20 Regulation of Endocardial Cushion Cell Proliferation and Extracellular Matrix Gene Expression

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Abstract

While recent work has implicated Tbx20 in myocardial maturation and proliferation, the role of Tbx20 in heart valve development remains relatively unknown. Tbx20 expression was manipulated in primary avian endocardial cells in order to elucidate its function in developing endocardial cushions. Tbx20 gain of function was achieved with a Tbx20-adenovirus, and endogenous Tbx20 expression was inhibited with Tbx20-specific siRNA in cultured endocardial cushion cells. With Tbx20 gain of function, the expression of chondroitin sulfate proteoglycans (CSPG), including *aggrecan* and *versican*, was decreased, while the expression of the matrix metalloproteinases (MMP) *mmp9* and *mmp13* was increased. Consistent results were observed with Tbx20 loss of function where the expression of *CSPG* genes increased and *MMP* genes decreased. In addition, cushion mesenchyme proliferation increased with infection of a Tbx20-adenovirus and decreased with transfection of Tbx20-specfic siRNA. Furthermore, BMP2 treatment resulted in increased *Tbx20* expression in endocardial cushion cells, and loss of Tbx20 led to increased *Tbx2* and decreased *N-myc* gene expression. Taken together, these data support a role for Tbx20 in repressing extracellular matrix remodeling and promoting cell proliferation in mesenchymal valve precursor populations in endocardial cushions during embryonic development.

Keywords

Tbx20; endocardial cushion development; aggrecan; versican; mmp9; mmp13; cell proliferation; siRNA; chicken

Introduction

Heart valve development is a complex process that is essential to normal heart function. Congenital valve defects can result in valve dysfunction (Rabkin-Aikawa et al., 2005) and there is mounting evidence that early developmental defects in valvulogenesis can lead to valve disease later in life (Cripe et al., 2004; Garg et al., 2005). Much is known about the early events in valve formation, however relatively little is known about how primitive endocardial cushions remodel into mature valves. T-box transcription factors function in various aspects of cardiogenesis, including cardiac lineage determination, chamber specification, epicardial development, and specialization of the conduction system (Plageman and Yutzey, 2005; Stennard and Harvey, 2005). Tbx20 has been implicated in cardiac muscle maturation and is

expressed in developing heart valves, however its specific role in valves and their precursor cell populations has not been identified.

Recently, the role of Tbx20 in mammalian heart development was investigated using targeted mutagenesis and RNAi strategies in mice. In these studies, loss of Tbx20 function resulted in decreased or delayed cardiac gene expression, hypoplasia of the myocardium, and decreased chamber maturation (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). In addition, a specific role for Tbx20 in myocardial proliferation was proposed through increased *N-myc* gene expression (Cai et al., 2005). In each case, mutant mice lacking *Tbx20* gene expression were embryonic lethal due to failure of cardiac muscle maturation at approximately embryonic day (E) 9, prior to valve development (Cai et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). Further evidence for Tbx20's role in heart muscle development comes from loss of function studies in other animal model systems. In zebrafish and *Xenopus* embryos, the loss of Tbx20 results in heart looping and chamber maturation defects (Brown et al., 2003; Stennard et al., 2003; Szeto et al., 2002). Taken together, these studies have defined a role for Tbx20 in early cardiac muscle development where it promotes primitive, proliferative myocardium. Later in development, Tbx20 is expressed in the myocardium as well as in the atrioventricular (AV) canal and outflow tract (OFT) endocardial cushions and in the mitral and tricuspid valves (Plageman and Yutzey, 2004; Stennard et al., 2003; Yamagishi et al., 2004). While the loss of function studies were very informative regarding Tbx20's role in regulating myocardial proliferation and maturation, the role of Tbx20 in valve development is still unclear.

Heart valvulogenesis is initiated in the AV canal and OFT by signaling events originating in the myocardium that cause cells in the endocardium to undergo an epithelial to mesenchymal transformation (EMT) and migrate into the intervening cardiac jelly. The result of EMT is the formation of endocardial cushions composed of highly proliferative, undifferentiated, mesenchymal valve progenitor cells embedded in an unorganized extracellular matrix (ECM) (Armstrong and Bischoff, 2004; Hinton et al., 2006; Lincoln et al., 2006b; Person et al., 2005; Schroeder et al., 2003). These cushions will ultimately elongate and undergo ECM remodeling in order to form functionally mature valves (Hinton et al., 2006; Lincoln et al., 2004). One hallmark of the transition from endocardial cushion to remodeling valve is a decrease in endocardial cushion cell proliferation (Hinton et al., 2006; Lincoln et al., 2004). As previously reported, murine endocardial cushion cells are approximately 6 times more proliferative than cells in the remodeling valve leaflet (Hinton et al., 2006) and decreased proliferation is also a feature of valve remodeling in avian embryos (Lincoln et al., 2004). In addition, valve remodeling is characterized by increased organization and complexity of the ECM. During endocardial cushion formation, proteoglycans are diffusely and variably expressed throughout the cushion (Hinton et al., 2006). However, beginning in the remodeling stage of valve development and continuing into postnatal time points, the ECM becomes stratified into three distinct layers: the elastin rich atrialis, the proteoglycan rich spongiosa, and the collagen rich fibrosa (Flanagan and Pandit, 2003; Hinton et al., 2006; Lincoln et al., 2006b). While recent progress has been made to describe this process histologically, the molecular mechanisms governing this transition have not been elucidated.

The process by which endocardial cushions develop into mature valve leaflets is marked by the expression of specific ECM proteins and remodeling enzymes (Lincoln et al., 2006b). Aggrecan (agg) and versican (vers) are major proteoglycan constituents of the spongiosa layer in avian valves. Both are large chondroitin sulfate proteoglycans (CSPG) that aggregate with hyaluronan to form hydrated compressible ECM (Arciniegas et al., 2004; Luo et al., 2000). These CSPGs are not only found in developing valves, but also in articular cartilage, where they function to provide resistance against compressive forces (Arciniegas et al., 2004; Pirok et al., 1997). The proper distribution and organization of ECM in remodeling valves is

important for normal valve function (Hinton et al., 2006; Rabkin et al., 2001) and is dependent on a coordinated deposition and degradation of individual matrix proteins. As in other tissues, matrix metalloproteinases (MMP) can mediate the degradation and remodeling of ECM components in the heart (Coker et al., 1998; Rabkin et al., 2001; Sternlicht and Werb, 2001). Family members, including MMP9 (Gelatinase B) and MMP13 (Collagenase-3), are involved in the degradation and reorganization of collagens, elastin, and proteoglycans (Passi et al., 1999; Rabkin-Aikawa et al., 2005; Sternlicht and Werb, 2001). In addition, increased expression of MMP9 and MMP13 was observed in diseased human mitral and aortic valves with disorganized ECM (Rabkin et al., 2001; Soini et al., 2001). Taken together, normal valve maturation requires regulated ECM organization and remodeling, however the mechanisms controlling this process are still unclear.

To investigate the role of Tbx20 in endocardial cushion maturation and valve development, the expression of Tbx20, CSPGs, and MMPs in the endocardial cushions and remodeling valves of avian embryos was examined. *Tbx20* and *MMP* genes are expressed at higher levels in endocardial cushions relative to remodeling valves, while *CSPG* genes are expressed at higher levels in remodeling valves relative to endocardial cushions. In addition, a primary chicken endocardial cushion culture system was used for Tbx20 gain and loss of function studies. The effects of altered Tbx20 expression on *CSPG* and *MMP* gene expression as well as the ability of Tbx20 to affect endocardial cushion cell proliferation were examined. In these studies, increased Tbx20 expression led to repression of *CSPG* genes and increased expression of *MMP* genes, whereas the opposite was observed with loss of Tbx20 function. Furthermore, increased Tbx20 function led to increased proliferation in endocardial cushion cells, while loss of Tbx20 led to decreased proliferation. These findings coincide with high levels of Tbx20 promoting proliferation in endocardial cushions while lower levels of Tbx20 in remodeling valves corresponds to decreased rates of proliferation. Taken together, these studies are consistent with a role for Tbx20 in antagonizing the transition from endocardial cushion to remodeling valve.

Materials and methods

Chicken embryo collection

Fertilized white leghorn chicken eggs (CBT Farms, MD) were incubated at 38°C under high humidity. Embryos were collected at Hamburger Hamilton (HH) stages 25, 26, 34, and 36, corresponding to embryonic days 4.5, 5, 8, and 10, respectively (Hamburger and Hamilton, 1951). For histology, hearts were dissected in $1\times$ phosphate-buffered saline (PBS) and fixed for 2 h in 4% paraformaldehyde/PBS. After fixation, embryonic tissue was dehydrated in a graded ethanol/PBS series (25%, 50%, 75%, 95%, 100%) and washed in xylene before being embedded in paraplast (Sigma) for further processing. All animal procedures were approved and performed in accordance with institutional guidelines.

In situ hybridizations

The chicken *Tbx20* sequence (Genbank accession number AB070544) was amplified from HH stage 20 heart cDNA using previously reported degenerate primers 5′-TGCTGRAAGTARTGRTG-3′ and 5′-GTGGAYAAYAAGAGATA-3′ where R represents purine and Y represents pyrimidine (Iio et al., 2001; Plageman and Yutzey, 2004). The chicken *aggrecan* sequence (Genbank accession number U78555) was amplified from HH stage 34 wing cDNA using the previously reported primers 5′-CTGCGTTCCCTGAGATTAC-3′ and 5′-TTGCCAGGTCGATCTCAC-3′ (Li et al., 1996; Lincoln et al., 2006a). The chicken *versican* sequence (Genbank accession number NM_204787) was amplified from HH stage 36 wing cDNA using the primers 5′-CAAGGCGCTGAGTGCTAAATG-3′ and 5′-AGGGGCTAATACTGCTCTGG-3′ (Shinomura et al., 1993). The chicken *mmp9* sequence

(Genbank accession number AF222690) was amplified from HH stage 30 heart cDNA using the primers 5′-GCTGCCACTTCCCCTTCATCTTTG-3′ and 5′-CGGGGGCCCACTGCGTTCTTG-3′ (Hahn-Dantona et al., 2000). The chicken *mmp13* sequence (Genbank accession number AF070478) was amplified from HH stage 30 cardiac outflow tract cDNA using the primers 5′-TGATGCCATAACAAAACTTCGTG-3′ and 5′-AGATGCTAGATTGCTGGGACTTA-3′ (Lei et al., 1999). All sequences were amplified by reverse transcriptase polymerase chain reaction (RT-PCR), subcloned into pGEM T-vector (Promega), and confirmed by sequencing. For each sequence, antisense RNA probes were generated as previously reported (Ehrman and Yutzey, 1999) with the following modifications. The *Tbx20* probe was synthesized with T3 polymerase from a plasmid linearized with Xho I. The *aggrecan* and *mmp13* probes were synthesized with Sp6 polymerase from plasmids linearized with Nco I. The *versican* and *mmp9* probes were synthesized using T7 polymerase from plasmids linearized with Not I.

In situ hybridization of tissue sections was performed as previously described (Somi et al., 2004) with the following modifications. Paraformaldehyde-fixed chicken hearts were embedded in paraffin, and 14μm sections were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific). Sections were then deparaffinized in xylene, rehydrated through an ethanol/distilled water series (100%, 95%, 75%, 50%), and then rinsed in 1×PBS. Sections were treated with 20μg/ml proteinase K/PBS for 6 min at 37°C. Hybridizations using 170μl of 0.5μg/ml DIG labeled riboprobe were carried out in Coverwell Incubation Chambers (Grace Biolabs). Color reactions using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche) were allowed to develop from 3-48 h. Slides were then rinsed in 1×PBS/0.1%Tween 20, dehydrated in a graded ethanol series, rinsed in xylene, and cover slipped using Cytoseal (Electron Microscopy Sciences).

Endocardial cushion cell culture

Embryonic chicken hearts were collected at HH stage 25, and pre-fused endocardial cushions were dissected using tungsten needles. Cushion cells free of myocardial contamination were cultured as previously reported (Lincoln et al., 2006a) with the following modifications. Dissected cushions from 12 embryos were plated onto a 0.01% collagen coated two-well chamber slide (Labtek) with 1ml media (10% fetal bovine serum, 1% penicillin/streptomycin, 1×M199 (Invitrogen)). Endocardial cushion cultures were incubated for a total of 4 days. In some cases, recombinant human BMP2 or recombinant human Noggin (R&D Systems) was added to the culture media, with a final concentration of 200ng/ml at the time of plating and replenishment after 48 h. Cells were collected after 4 days in culture for RNA isolation or fixed in 4% paraformaldehyde/PBS for immunohistochemistry.

Recombinant adenovirus

A recombinant adenovirus containing the full length coding region of murine Tbx20 (AdTbx20) was generated from a pAC-CMV-Tbx20 expression plasmid (Plageman and Yutzey, 2004) using previously described methods (Gomez-Foix et al., 1992). For Tbx20 gain of function studies, endocardial cushion cells cultured for 24 h were infected with 10^8 plaque forming units of the AdTbx20 virus or a β-galactosidase (Adβ-gal) control virus in serum free media (1 ×M199 (Invitrogen)). Cells were incubated with the infection media for 6 h, which was then replaced with new supplemented culture media. After 48 h, RNA was isolated or cells were fixed in 4% paraformaldehyde/PBS for immunohistochemistry. Infection efficiency was determined to be greater than 90% in Adβ-gal infected cultures as measured by staining with 1mg/ml X-gal (Amresco) using previously a reported method (Liberatore et al., 2002). High expression of the murine Tbx20 viral transcript was confirmed using RT-PCR and ectopic Tbx20 protein expression was confirmed by immunohistochemistry with a rabbit polyclonal antibody directed against Tbx20 (Orbigen).

Tbx20 siRNA

A 19 nucleotide RNA duplex corresponding to the chicken *Tbx20* sequence (Genbank accession number AB070544) was designed using BLOCK-iT™ RNAi Designer (Invitrogen). The Tbx20 siRNA with sequence 5′-GCAUCCAUUGCUACACCUAdTdT -3′ and 5′-UAGGUGUAGCAAUGGAUGCdTdT-3′ and a scrambled control siRNA with sequence 5′-CCGGUAAUGACACCCAAUUdTdT-3′ and 5′-AAUUGGGUGUCAUUACCGGdTdT-3′ were obtained from Invitrogen. A final siRNA concentration of 100nM was used with Lipofectamine 2000 (Invitrogen) to transfect cultured endocardial cushion cells as described by the manufacture's protocol. Transfected cells were incubated 48 h before RNA isolation or fixation for immunohistochemistry. The transfection efficiency was determined by co-transfection of the FITC-labeled (λex=494nm) BLOCK-iT™ Fluorescent Oligo (Invitrogen) along with siRNA oligos. Co-transfected cells were cultured and fixed in 4% paraformaldehyde/PBS for 15 min and then counterstained with a 1:1000 dilution of TO-PRO-3 iodide (λex=642) (Molecular Probes) in 1×PBS. Fluorescence was detected using a Nikon PCM 2000 confocal microscope and images were obtained using Simple PCI software. The percent of positively transfected cells was calculated by dividing the number of FITC/TO-PRO-3 iodide double-labeled nuclei by the number of total TO-PRO-3 iodide-labeled nuclei per microscopic field. In three independent experiments, a total of 10 fields containing at least 75 cells per field were counted for each treatment group. The level of *Tbx20* mRNA expression was determined using real time RT-PCR, and Tbx20 protein expression was detected by immunohistochemistry on Tbx20 siRNA and scrambled control transfected cultures using a rabbit polyclonal antibody directed against Tbx20 (Orbigen).

Immunohistochemistry

Endocardial cushion cell cultures were fixed with 4% paraformaldehyde/PBS for 30 min, washed three times in PBS/0.1%Tween 20, and treated with 3% hydrogen peroxide/PBS for 30 min. Immunohistochemistry was performed using an ABC peroxidase staining kit (Pierce) according to the manufacture's protocol. A rabbit polyclonal antibody directed against Tbx20 (Orbigen) was used at a 1:200 dilution in blocking solution. Mouse monoclonal antibodies directed against aggrecan (Abcam) and versican (Developmental Studies Hybridoma Bank) were used at a 1:200 dilution in blocking solution. All primary antibodies were incubated overnight at 4°C. Detection of antibody reactivity was visualized using DAB substrate (Pierce).

BrdU incorporation and quantification

BrdU positive nuclei were identified via immunohistochemistry using a BrdU detection kit (Zymed). BrdU labeling reagent (Zymed) was diluted 1:100 in culture media and incubated with endocardial cushion cell cultures for 1.5 h prior to fixation with cold 70% ethanol for 15 min at 4°C. Cells were treated with 3% hydrogen peroxide/methanol for 10 min, washed three times with 1×PBS, and incubated in blocking solution for 10 min. A biotinylated mouse anti-BrdU primary antibody was used followed by a streptavadin-peroxidase conjugated secondary antibody. A colorimetric reaction was carried out using DAB followed by counterstaining with hematoxylin. The percent of proliferating cells was calculated by dividing the number of BrdU labeled nuclei by the number of total nuclei per microscopic field. In three independent experiments, a total of 10 fields containing at least 75 cells per field were counted for each treatment group. Statistical significance of observed differences was determined by Student's t-test.

RT-PCR analysis of gene expression

Total RNA was isolated from cultures of 12 endocardial cushions per experimental group using 200μl Trizol reagent (Invitrogen) and cDNA was generated from the entire RNA sample from each group using SuperScript II (Invitrogen), as described by the manufacture's protocol. Total

RNA was also isolated from 6 chicken AV canals at HH stages 25 and 36 using 800μl Trizol reagent and cDNA was generated from 5μg of each RNA sample using SuperScript II as described by the manufacture's protocol. 1μl cDNA was used for analysis by semi-quantitative RT-PCR or quantitative real time RT-PCR (MJ Research Opticon 2). RT-PCR reactions were performed at 35 cycles using 20 pmol of the following primers: *Tbx20* 5′-CAGGCAACGCAAAGCAGAG-3′ and 5′-TTGGCATGTGGAAAGAAGG-3′, *aggrecan* 5′-CCTGCCTGACCTCTTTGC-3′ and 5′-TGGGGAGGAGGGCAACAT-3′, *versican* 5′-CCTCACTGGTAAGCCCACAT-3′ and 5′-TGATTCTTCTTGGCCCATTC-3′, *mmp9* 5′-GCCACTTCCCCTTCATCT-3′ and 5′-GTTGCCACCATTGGTGTA-3′, *mmp13* 5′-TGATGCCATAACAAAACTTCGTG-3′ and 5′-AGATGCTAGATTGCTGGGACTTA-3′, *N-myc* 5′-ACCACTTTTCCATCGGTCAG-3′ and 5′-TTGGTTGGATCATGGGTTTT-3′, *Tbx2* 5′-AACACGGCTTTACCATCCTG-3′ and 5′-TTCAGCTGCGTGATCTTGTC-3′, *β-actin* 5′-ATCACAGGGGGTGTGGGTGTT-3′ and 5′-AATGAGAGGTTCAGGTGCCC-3′. Gene expression levels determined by quantitative real time RT-PCR were calculated as previously reported (Lincoln et al., 2006a) with the following modifications. A standard curve was generated for each primer set using HH stage 34 whole heart cDNA and all values were normalized to *GAPDH* expression. Consistent *GAPDH* expression in all experimental groups was confirmed by normalizing *GAPDH* values to *β-actin* expression. Real time RT-PCR results represent at least three independent experiments (n=3–6) with reactions performed in duplicate. For developmental studies, expression is represented as arbitrary units of fluorescence intensity for data generated with equivalent RNA input and normalized to *GAPDH*. For endocardial cushion cultures, expression was calculated as fold increase or percent decrease determined by dividing the experimental value by the control value. The control value was then set to 1 or 100%, respectively. Statistical significance of observed differences was determined by Student's t-test.

Results

CSPG and MMP genes are differentially expressed in endocardial cushions and remodeling valves

Tbx20 is expressed in chicken and mouse endocardial cushions and in the remodeling valves (Plageman and Yutzey, 2004; Stennard et al., 2003; Yamagishi et al., 2004). In addition, *CSPG* genes, including *aggrecan* and *versican*, and *MMP* genes, including *MMP9* and *MMP13*, are expressed in mature and diseased valves. In situ hybridizations were performed on sectioned embryonic chicken hearts in order to localize the expression of *aggrecan (agg)*, *versican (vers)*, *mmp9*, and *mmp13* in relation to *Tbx20* during the stages of endocardial cushion formation (HH stage 25) and valve remodeling (HH stage 34) in vivo. At HH stage 25, *Tbx20* is expressed at high levels throughout the entire AV endocardial cushion (Fig. 1A). In contrast, *agg* expression is restricted to the subatrial region (arrow) of the endocardial cushion and is excluded from the core (star) of the cushion (Fig. 1C). *vers* is also expressed in the subatrial region of the endocardial cushion and excluded from the core region (Fig.1E). In contrast, *mmp9* is expressed throughout the entire cushion including the subatrial and core regions, and expression was also observed in the subepicardial space (open arrowhead) at this stage (Fig.1G). Similarly, *mmp13* is expressed throughout the subatrial and core regions of the endocardial cushion at HH stage 25 (Fig.1I).

In HH stage 34 remodeling valves, *Tbx20* is expressed at more diffuse levels in the leaflets (arrowhead) and tips (asterisks) of the remodeling mitral valve (Fig.1B). In contrast, *agg* becomes highly expressed throughout the valve leaflet but is expressed at lower levels at the most distal tips (Fig.1D). *vers* is also expressed throughout the remodeling valve but is predominant at the distal tips of the valve leaflets (Fig.1F). By HH stage 34, *mmp9* is expressed at low levels in the remodeling mitral valve, with expression being concentrated at the atrial

aspect of the valve leaflet and the distal tips (Fig.1H), while *mmp13* is expressed at low levels in the ventricular aspect of the valve leaflet and at the distal tips (Fig.1J). Similar expression of *Tbx20*, *agg*, *vers*, *mmp9*, and *mmp13* was found in the remodeling tricuspid valve leaflets with the exception of the muscular portion of the mural leaflet (data not shown). Together these expression studies show that *Tbx20*, *mmp9*, and *mmp13* are expressed throughout the core of the endocardial cushion while the expression of *agg* and *vers* is restricted to the subatrial region. Later in remodeling valves, the expression of *agg* and *vers* is expanded throughout the valve leaflet while *Tbx20*, *mmp9*, and *mmp13* expression is more diffuse and compartmentalized compared to the expression in endocardial cushions.

Tbx20, CSPG and *MMP* expression was further quantified using RNA isolated from AV canals of HH stage 25 and HH stage 36 chicken embryos. The expression levels of *Tbx20*, *agg*, *vers*, *mmp9*, and *mmp13* were measured using quantitative real time RT-PCR (Fig.2). At HH stage 25 (endocardial cushion stage), the expression of *Tbx20* is approximately 1.5 fold higher than later at HH stage 36 (valve remodeling stage). Similarly, the expression of *mmp9* and *mmp13* is more than 2 fold higher at HH stage 25 than later at HH stage 36. In contrast, the expression of *agg* and *vers* is more than 4 fold higher in remodeling valves (HH stage 36) than in endocardial cushions (HH stage 25). These results indicate that the expression of *Tbx20*, *mmp9, and mmp13* is relatively higher in endocardial cushions than in remodeling valves. In contrast, expression of *agg* and *vers* is increased in remodeling valves relative to undifferentiated endocardial cushions.

Tbx20 gain of function results in decreased CSPG expression and increased MMP expression in cultured endocardial cushion cells

The ability of Tbx20 to affect CSPG and MMP expression in undifferentiated endocardial cushions was examined. Primary cultures derived from unfused endocardial cushions removed from the AV canals of HH stage 25 chicken embryos were infected with an adenovirus that expresses murine Tbx20 (AdTbx20) or a control adenovirus that expresses β-gal (Adβ-gal). Endocardial cushion cells were infected with greater than 90% efficiency, as determined by X-gal staining of Adβ-gal infected cultures (data not shown). High levels of expression of the Tbx20 viral transcript were determined by real time RT-PCR and increased Tbx20 protein was detectable by immunohistochemistry (data not shown). The effect of increased Tbx20 on the expression of *CSPG* genes was determined by real time RT-PCR. Endocardial cushion cultures infected with AdTbx20 had an approximately 60% reduction in *agg* (Fig. 3E) and *vers* (Fig. 3F) mRNA levels relative to Adβ-gal infected controls. This reduction was confirmed by immunohistochemistry using antibodies specifically directed against Agg and Vers. Expression of Agg and Vers protein was apparent in cells infected with Adβ-gal (Fig.3A,C), but not in cells infected with AdTbx20 (Fig.3B,D). In addition, the expression of *mmp9* and *mmp13* mRNA was measured by real time RT-PCR in cells infected with Adβ-gal or AdTbx20. In cells infected with AdTbx20, the expression of *mmp9* was increased 3 fold (Fig.3G), and the expression of *mmp13* was increased 5.5 fold (Fig.3H). These studies demonstrate that Tbx20 gain of function results in decreased *agg* and *vers* expression and increased *mmp9* and *mmp13* expression in endocardial cushion cell cultures.

Tbx20 loss of function results in increased CSPG expression and decreased MMP expression

In order to achieve endogenous Tbx20 loss of function, a Tbx20-specific siRNA was transfected into primary chicken endocardial cushion cells. Scrambled control siRNA was also transfected in parallel experiments. To determine the efficiency of transfection, a fluorescently labeled oligonucleotide was co-transfected with the siRNA, and cells were counterstained with TO-PRO-3 iodide to visualize the nuclei (Fig.4A,C,E). Primary endocardial cushion cells were transfected with greater than 70% efficiency in the presence or absence of co-transfected

siRNA (Fig.4G). Knockdown of Tbx20 protein expression was evident using immunohistochemistry with a Tbx20-specific antibody (Fig.4B,D,F). Cells transfected with Tbx20-specific siRNA had significantly reduced Tbx20 expression in the nucleus (Fig.4B) relative to scrambled siRNA(Fig.4D) or untransfected controls (Fig.4F). In addition, transfection with Tbx20-specific siRNA resulted in an approximately 85% reduction of *Tbx20* mRNA levels (Fig.4H) as determined by real time RT-PCR. There was no detectable change in *Tbx20* gene expression following transfection of the scrambled siRNA control (Fig. 4H). These data indicate that primary chicken endocardial cushion cells can be efficiently transfected with sequence-specific siRNA in order to produce a significant loss of Tbx20 function.

The regulatory effects of Tbx20 knockdown on *CSPG* and *MMP* expression were examined in primary endocardial cushion cells removed from HH stage 25 AV canals. In cells transfected with Tbx20-specific siRNA, *agg* expression increased approximately 4 fold (Fig.5A) and *vers* expression increased approximately 6 fold (Fig.5B). In contrast, *mmp9* expression decreased approximately 80% (Fig.5C) and *mmp13* expression decreased approximately 50% (Fig.5D) as measured by real time RT-PCR. Taken together, loss of Tbx20 function leads to increased expression of *CSPG* genes and decreased expression of *MMP* genes. These results from loss of Tbx20 function are consistent with the adenoviral gain of Tbx20 function data and support a role for Tbx20 in repressing CSPG expression and promoting MMP expression in primitive endocardial cushion cells.

Tbx20 promotes endocardial cushion cell proliferation

Heart valve development is characterized by high proliferation in endocardial cushions and decreased proliferation in remodeling valves (Hinton et al., 2006; Lincoln et al., 2004). Targeted mutagenesis of *Tbx20* in mice suggests Tbx20 promotes myocardial cell proliferation in the primitive heart tube (Cai et al., 2005). To determine if Tbx20 has a similar role in endocardial cushion cells, Tbx20 gain and loss of function studies were performed and cell proliferation was assessed. Endocardial cushion cultures were infected with AdTbx20 or Adβ-gal for gain of function studies or transfected with Tbx20-specific siRNA or scrambled control siRNA for loss of function studies. The number of cells in S-phase of the cell cycle was measured by BrdU incorporation and used as an indication of proliferation. A proliferation index was determined by dividing the number of BrdU labeled nuclei by the number of total nuclei per microscopic field. The normal proliferation index of endocardial cushion cells infected with Adβ-gal (Fig.6A) or transfected with scrambled siRNA (Fig.6C) was approximately 20% (Fig.6E). Cells that were infected with AdTbx20 (Fig.6B) were significantly more proliferative, with a proliferation index of over 40% (Fig.6E). In contrast, cells transfected with Tbx20-specific siRNA (Fig.6D) were significantly less proliferative, with a proliferation index of less than 10% (Fig.6E). These data are consistent with a role for Tbx20 in promoting proliferation in endocardial cushions.

BMP2 induces Tbx20 and altered Tbx20 function affects the expression of Tbx2 and N-myc in endocardial cushion cells

BMP2 induces Tbx20 expression in cardiac primordia prior to cardiomyogenic differentiation and its expression in AV canal myocardium is required for endocardial cushion formation (Ma et al., 2005; Plageman and Yutzey, 2004; Rivera-Feliciano and Tabin, 2006). The ability of BMP2 to induce Tbx20 expression in endocardial cushion cells was investigated. AV endocardial cushions were removed from the surrounding myocardium of HH stage 25 chicken embryos and cultured with or without the addition of recombinant human BMP2 or Noggin, a BMP inhibitor. RNA was isolated and *Tbx20* expression levels were measured using real time RT-PCR. Addition of BMP2 induced *Tbx20* expression while addition of Noggin repressed endogenous *Tbx20* expression below levels in untreated controls (Fig. 7A). These data

demonstrate that BMP2 can induce *Tbx20* in endocardial cushion cells and that inhibition of endogenous BMP signaling by Noggin decreases *Tbx20* gene expression levels. *Bmp2* is required for endocardial cushion formation (Ma et al., 2005; Rivera-Feliciano and Tabin, 2006; Sugi et al., 2004), and the ability of BMP2 to induce Tbx20 in endocardial cushion cells is consistent with Tbx20 regulation by BMP signaling in the early stages of endocardial cushion development.

It was previously reported that Tbx20 affects myocardial proliferation by directly repressing *Tbx2,* and thus relieving the direct repression of Tbx2 on *N-myc* expression (Cai et al., 2005). To determine if a similar mechanism occurs in endocardial cushion cells, cultured cells were transfected with Tbx20-specific siRNA or scrambled control siRNA. Changes in *Tbx2* and *N-myc* mRNA levels were measured by real time RT-PCR. In cells transfected with Tbx20-specific siRNA, *Tbx2* expression increased by approximately 2 fold (Fig.7B), while *N-myc* expression decreased by approximately 60% (Fig.7C). These studies provide evidence that Tbx20 similarly regulates endocardial cushion and myocardial cell proliferation through *Tbx2* and *N-myc* gene expression. Taken together, these results are consistent with a role for Tbx20 in maintaining the endocardial cushions as primitive, undifferentiated, and proliferative.

Discussion

Gain and loss of function strategies were used to examine the role of Tbx20 in developing avian endocardial cushions. During normal valve development *Tbx20*, *mmp9*, and *mmp13* are expressed at higher levels in endocardial cushions relative to remodeling valves, while *agg* and *vers* are expressed at higher levels in remodeling valves relative to endocardial cushions. Additionally, endocardial cushion cells are more proliferative than cells in remodeling valves (Hinton et al., 2006). In these experiments, increased Tbx20 leads to decreased CSPG expression and increased MMP expression whereas the opposite trend occurs with loss of Tbx20. Furthermore, increased Tbx20 induces cell proliferation in endocardial cushion cultures, while proliferation decreases with loss of Tbx20. These results are consistent with Tbx20 expression in highly proliferative, unremodeled valve precursors and suggest that Tbx20 functions to maintain valve precursors in an immature, highly proliferative state, as has also been noted for Tbx20 function in primitive myocardium. Taken together, a model for the functions of Tbx20 in valve development (Fig.8) can be generated in which high levels of Tbx20 early in cushion mesenchyme maintain primitive, proliferative, unremodeled, endocardial cushions, while lower levels of Tbx20 later in development occur in more highly differentiated, less proliferative, remodeling valves.

Increased Tbx20 expression promotes proliferation in endocardial cushion cells. In this report and in a previously published study, altered Tbx20 function affects the expression of *N-myc* and *Tbx2* (Cai et al., 2005). In studies of primitive mouse myocardium lacking Tbx20 it was suggested that Tbx20 acts to increase *N-myc* expression by repressing *Tbx2*, a transcriptional repressor (Cai et al., 2005). However it is also possible that Tbx20 directly activates *N-myc* gene expression since Tbx20 can have activator or repressor function depending on transcriptional partners or cellular context (Plageman and Yutzey, 2004; Stennard et al., 2003). The relevance of N-myc to normal heart development was demonstrated directly by targeted mutagenesis in mice. Mutant mice lacking N-myc have complex cardiac abnormalities including a lack of endocardial cushions, atrial and ventricular septal defects, and hypoplasia of the compact myocardium (Charron et al., 1992; Hurlin, 2005; Moens et al., 1993). Extensive cell culture studies have demonstrated that N-myc promotes cell cycle progression at the G1/S-phase, and studies of Tbx5 in Xenopus embryos support a role for this T-box family member in the G1 to S-phase transition in cardiac myocytes (Goetz et al., 2006; Hooker and Hurlin, 2006). In addition, *TBX2* and *TBX3* expression is increased and promotes proliferation in a subset of mammary carcinomas and breast cancer cell lines (Carlson et al., 2002; Fan et al.,

2004; Prince et al., 2004; Vance et al., 2005). The ability of Tbx20 to promote *N-myc* expression in the developing heart may be indicative of an important role in regulation of cell cycle progression at the G1/S-phase in both myocardial and endocardial cushion cell populations.

Bmp2 is expressed in the myocardium of the AV canal and is required for the initial stages of endocardial cushion formation (Ma et al., 2005; Rivera-Feliciano and Tabin, 2006; Yamada et al., 2000). In this study, we report BMP2 induces *Tbx20* in endocardial cushion cells and that loss of Tbx20 function in cushion cells results in increased *Tbx2* expression. In murine atrial and ventricular chamber myocardium, Tbx20 represses *Tbx2* expression, thereby allowing chamber-specific differentiation and myocardial proliferation to occur (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). A similar regulatory relationship may occur during endocardial cushion maturation. BMP2 can induce expression of both *Tbx20* and *Tbx2* in the primary heart field and AV canal (Plageman and Yutzey, 2004; Rivera-Feliciano and Tabin, 2006; Yamada et al., 2000). In addition, Tbx20 may exert positive feedback on *Bmp2* expression (Cai et al., 2005) although, decreased Tbx20 expression has also been observed to result in ectopic *Bmp2* expression, suggesting that Tbx20 can negatively feedback on *Bmp2* (Singh et al., 2005). In light of all of these studies, the mechanism that regulates the precise coordination of *Bmp2*, *Tbx20*, and *Tbx2* expression still remains unclear. In addition, future experiments are needed to assess independent functions of Tbx20 and Tbx2 in chamber myocardium and endocardial cushions.

Increased Tbx20 function leads to decreased expression of CSPGs and increased expression of MMPs in endocardial cushion cells. This is consistent with a role for Tbx20 in ECM remodeling during valve development. While altered Tbx20 function affects the expression of ECM remodeling genes, it is not known whether these interactions are direct or indirect. In previous studies a limited number of direct downstream targets of Tbx20 have been identified, including *Nppa* and *Tbx2* (Cai et al., 2005; Stennard et al., 2003; Takeuchi et al., 2005). To determine if Tbx20 interacts directly or indirectly with CSPG and MMP regulatory elements, promoter regions of murine Agg and Mmp13 were analyzed for T-box binding sites (TBE). While several conserved TBE sites were found in addition to binding sites for common T-box cofactors, Tbx20 alone was not sufficient to induce *Agg* or *Mmp13* reporter constructs and induction was not observed in co-transfection experiments (data not shown). However, these studies do not rule out the possibility that Tbx20 directly regulates *CSPG* and *MMP* gene expression through other regulatory elements or with additional co-factors. Direct transcriptional regulators of *Agg* and *Mmp13* have been identified in other cell types. Sox9 and Scleraxis can directly activate *Agg* expression in chondrocytic and osteoblastic cell lines respectively (Liu et al., 1997; Sekiya et al., 2000), while Tgf-β and core-binding factor 1 (CBFA1) can directly activate *Mmp13* expression in human fibroblasts and chondrocytic and osteoblastic cell lines (Jimenez et al., 1999; Sternlicht and Werb, 2001; Uria et al., 1998). It is possible that the effect of Tbx20 on *CSPG* and *MMP* expression is indirect through the expression of genes such as *Sox9*, *scleraxis*, *Tgf-β*, or *CBFA1*, which are also present in endocardial cushions and valve primordia (Lincoln et al.,2006a; Yutzey, unpublished). With the data currently available, the effect of Tbx20 as a direct or indirect transcriptional regulator of *CSPG* or *MMP* gene expression cannot be determined.

In general, the ECM is important for many cellular processes including cell shape, movement, growth, differentiation, and survival. Similarly, MMPs that degrade and remodel ECM components can also affect all of these processes by altering the structure and composition of the ECM (Sternlicht and Werb, 2001). During angiogenesis and bone development, MMPs function to create a permissive environment for cell migration (Sternlicht and Werb, 2001). Therefore, the expression of MMPs in endocardial cushions composed of a loosely arranged ECM is consistent with migratory, proliferative, mesenchymal cells. The observed decrease in *mmp9* and *mmp13* expression in remodeling valves may be required for organization of the

ECM and compartmentalization of interstitial cells (Hinton et al., 2006). Correct expression and assembly of CSPGs is also necessary for normal valve development. Transgenic mice containing an insertional mutation in the *versican* gene fail to form endocardial cushions (Kern et al., 2006; Mjaatvedt et al., 1998) and proteoglycans are integral components of the mature valve leaflet (Hinton et al., 2006). In this report, the observed differential expression of CSPGs and MMPs during endocardial cushion development and valve remodeling stages highlights the importance of establishing a balance between deposition and degradation of ECM components in order for normal valve development to occur.

Human heart valve disease is associated with abnormal organization and increased deposition of ECM constituents. In a recent study of pediatric aortic valve disease, ECM layers were unorganized and disproportionate and interstitial cell compartmentalization was abnormal (Hinton et al., 2006). Similarly, patients with congenital polyvalvular disease had AV and semilunar valves composed of abnormal ECM and disrupted valve architecture (Bartram et al., 2001). Furthermore, in cases of myxomatous and calcified adult aortic valves, disorganized ECM was accompanied by elevated levels of MMPs including MMP9 and MMP13 (Akhtar et al., 1999; Bailey et al., 2004; Rabkin et al., 2001; Soini et al., 2001). These features of valve disease, including unorganized ECM and interstitial cells with elevated MMP levels, are all characteristic of endocardial cushions. While features characteristic of valve disease have been described, relatively little is known about the molecular causes of valve pathogenesis. As shown in this study, altered Tbx20 function affects valve progenitor cell proliferation as well as expression of ECM components and remodeling enzymes in AV valve precursor cells. Therefore, it is possible that that misregulation of Tbx20 could lead to some of the characteristic features of valve disease. Further experiments examining the expression of Tbx20 in diseased valves may give insights into molecular mechanisms of valve disease.

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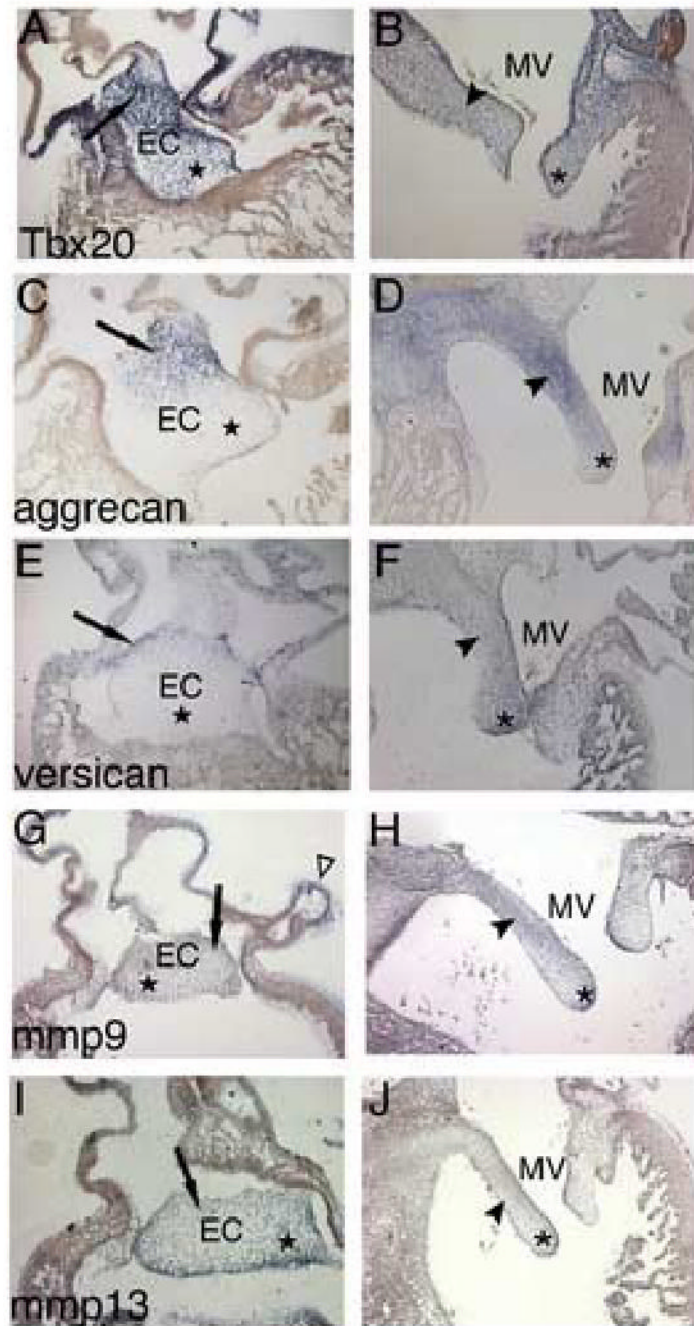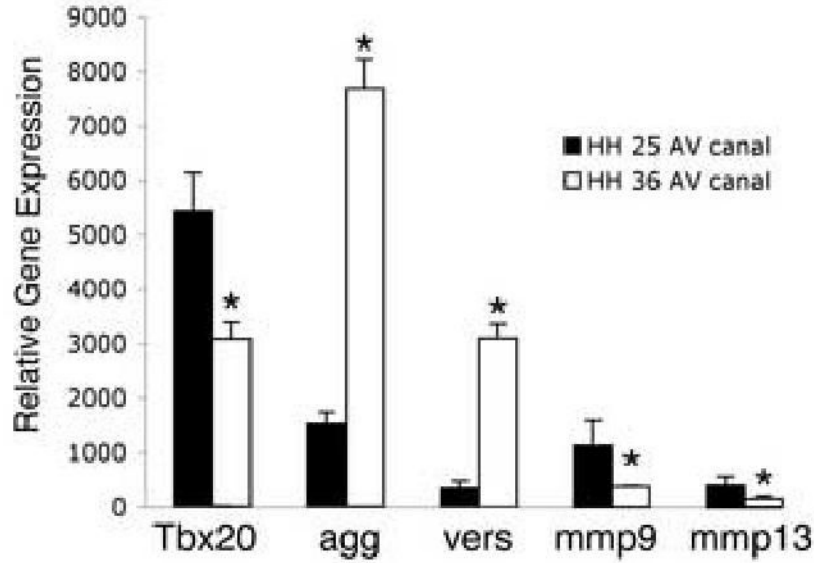
Figure 1. CSPG and MMP genes are differentially expressed in endocardial cushions and remodeling valves

Expression of *Tbx20*, *aggrecan*, *versican*, *mmp9*, and *mmp13* was examined in sectioned HH stage 25 and HH stage 34 chicken hearts. In situ hybridizations show *Tbx20* (A), *mmp9* (G), and *mmp13* (I) are expressed throughout the entire AV endocardial cushion including the subatrial region (arrow) and cushion core (star). In addition, expression of *mmp9* was also detected in the subepicardial space (open arrowhead in G). In contrast, *aggrecan* (C) and *versican* (E) are expressed only in the subatrial region of the cushion (arrows). In remodeling mitral valves, *Tbx20* (B) is expressed in the valve leaflet (arrowhead) and distal tips (asterisk). At HH stage 34, *aggrecan* (D) is expressed at high levels in the valve leaflet, but is excluded

from the distal tips, while *versican* (F) is expressed throughout the leaflet, with increased expression at the distal tips. In remodeling valves, *mmp9* (H) is expressed throughout the leaflet with concentrated expression in the atrial aspect of the leaflet and in the distal tips, while *mmp13* (J) is expressed in the ventricular aspect of the leaflet and in the distal tips. EC, endocardial cushion; MV, mitral valve.

Figure 2. Differential expression of *Tbx20*, *CSPG*, and *MMP* genes during endocardial cushion and remodeling valve stages

Expression of *Tbx20*, *aggrecan*, *versican*, *mmp9*, and *mmp13* was examined in isolated AV canals from HH stage 25 or HH stage 36 chicken embryos using real time RT-PCR. The expression of *Tbx20*, *mmp9*, and *mmp13* was decreased at HH stage 36 compared to the expression at HH stage 25. In contrast the expression of *aggrecan* and *versican* at HH stage 36 was increased compared to the expression at HH stage 25. Values on the y-axis represent arbitrary units of fluorescence intensity for data obtained with equivalent RNA input normalized to *GAPDH*. These data are representative of 3 independent real time RT-PCR experiments performed in duplicate (n=3). Statistical significance of observed differences between gene expression levels at HH stage 25 and HH stage 36 is indicated by an asterisk (P<0.05), and error bars represent standard error of the mean.
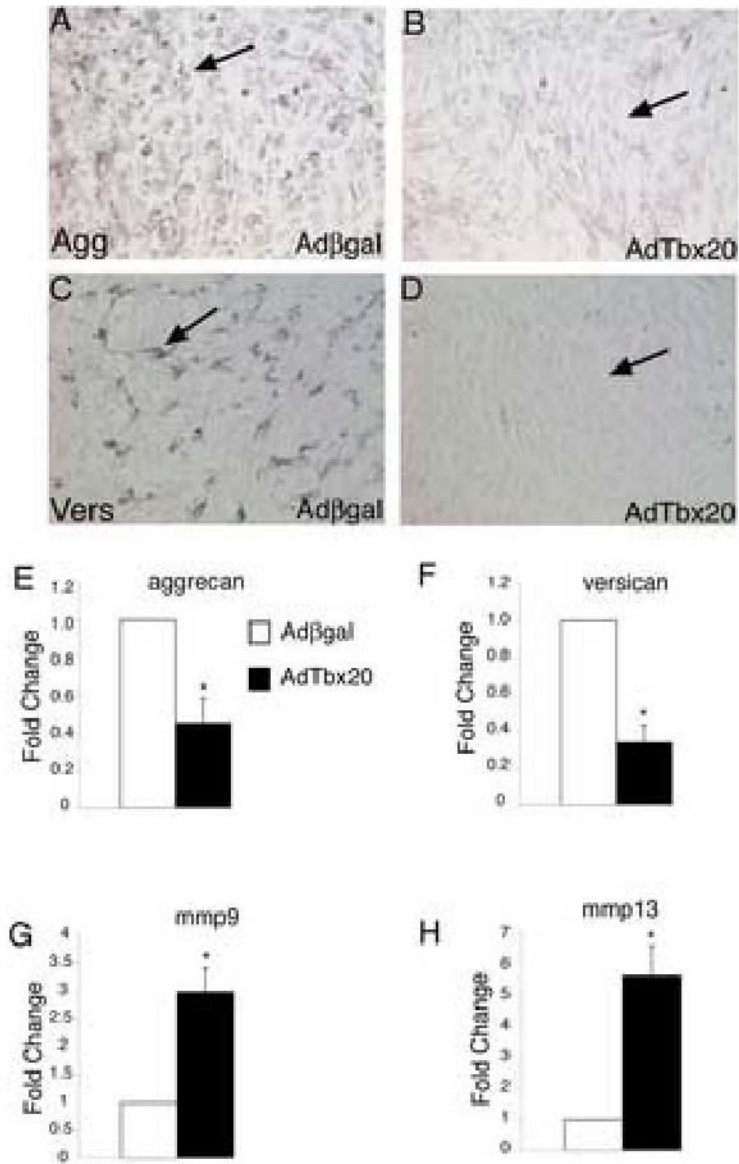
Figure 3. Tbx20 gain of function results in decreased expression of CSPGs and increased expression of MMPs

Adenoviruses expressing β-gal (Ad β-gal) or Tbx20 (AdTbx20) were used to infect primary avian HH stage 25 endocardial cushion cells. Immunohistochemistry with antibodies specific for Aggrecan (A,B) or Versican (C,D) was used to measure aggrecan and versican protein levels following infection. Cells infected with AdTbx20 had less immunoreactivity (B,D arrows indicate lack of staining) than cells infected with Adβ-gal (A,C arrows indicate staining). These results were confirmed by real time RT-PCR. Cells infected with AdTbx20 had reduced expression of *aggrecan* (E) and *versican* (F) (n=6, P<0.01). The expression of *mmp9* and *mmp13* was also measured in infected cells using real time PT-PCR. In cells infected with AdTbx20, *mmp9* (G) and *mmp13* (H) expression was increased relative to expression in cells infected with Adβ-gal (n=6, P<0.05). Fold changes were determined by dividing experimental values by control values with control values set to 1. Asterisks indicate

statistically significant differences from control values and error bars represent standard error of the mean.
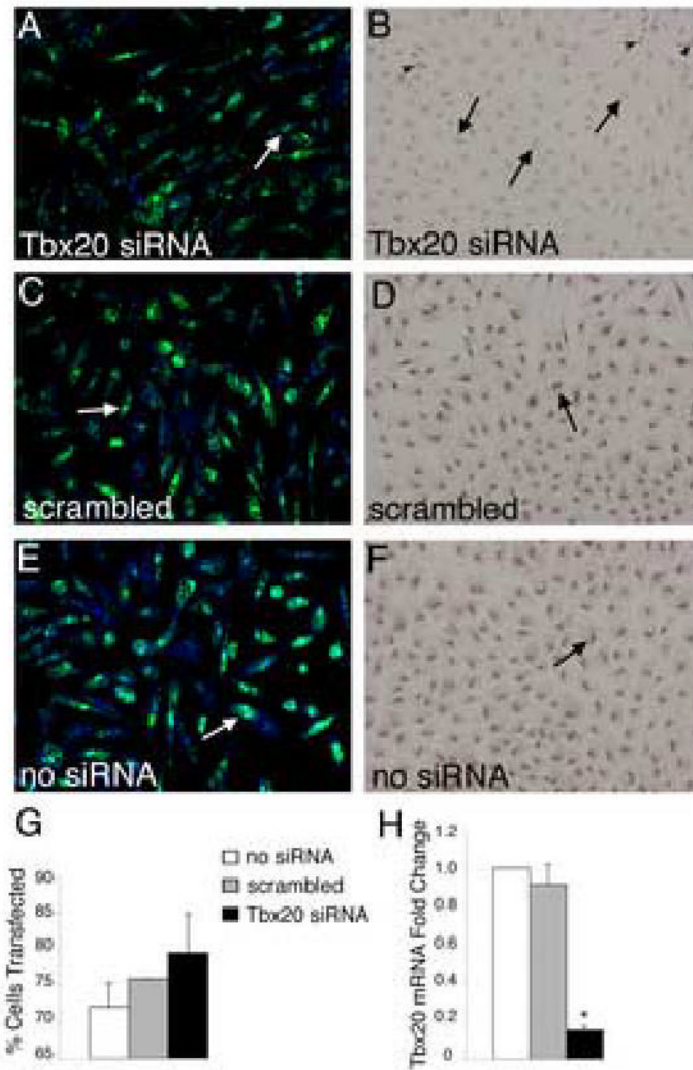
Figure 4. Tbx20-specific siRNA transfected into primary chicken endocardial cushion cells results in decreased Tbx20 mRNA and protein expression

(A,C,E) Primary chicken endocardial cushion cells were co-transfected with siRNA and FITC-labeled fluorescent oligos (green) to visualize transfection efficiency. Cells were counterstained with TO-PRO-3 iodide (blue) to visualize the nuclei. Examples of positively transfected cells are indicated by white arrows. Transfection efficiency was determined as the number of cells with incorporation of FITC-labeled oligonucleotide/total TO-PRO-3 positive nuclei (G). These analyses demonstrated that cultures were consistently transfected at 70–80% efficiency. (B,D,F) Tbx20 protein expression was examined by immunohistochemistry with an antibody specific for Tbx20 in parallel cultures of siRNA-transfected cells. Cells transfected with Tbx20-specific siRNA (B) had significantly reduced nuclear staining (arrows indicate cells with reduced Tbx20 immunoreactivity) compared to controls (D,F, arrows indicate Tbx20 immunopositive cells). In Tbx20-specific siRNA transfected cultures (B), a subset of cells remained Tbx20 immunopositive (arrowheads). *Tbx20* mRNA expression was quantified using real time RT-PCR (H). Cells transfected with Tbx20-specific siRNA had an 80% reduction in Tbx20 mRNA relative to untransfected and scrambled siRNA controls. Asterisks indicate statistically significant differences from control values and error bars represent standard error of the mean (n=3, P<0.01).
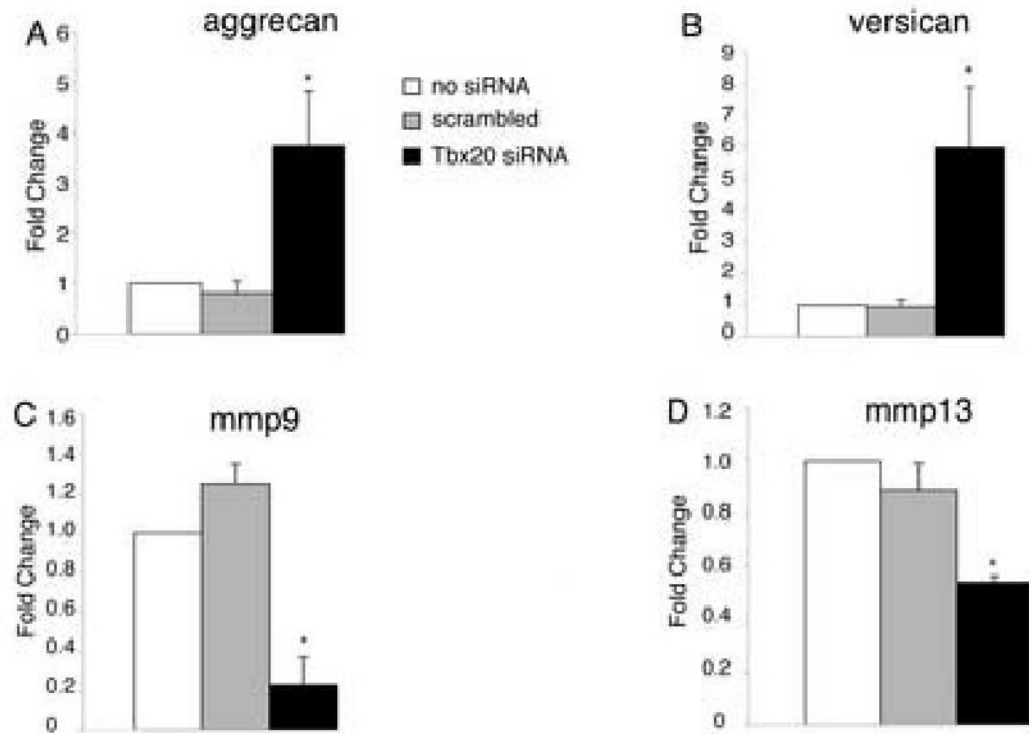
Figure 5. Tbx20 loss of function results in increased CSPG expression and decreased MMP expression

Primary endocardial cushion cells were transfected with Tbx20-specific or scrambled control siRNA. Cells transfected with Tbx20-specific siRNA had increased expression of *aggrecan* (A) and *versican* (B) and decreased expression of *mmp9* (C) and *mmp13* (D), relative to untransfected and scrambled siRNA controls as measured by real time RT-PCR. Asterisks indicate statistically significant differences from control values and error bars represent standard error of the mean (n=4, P<0.05).
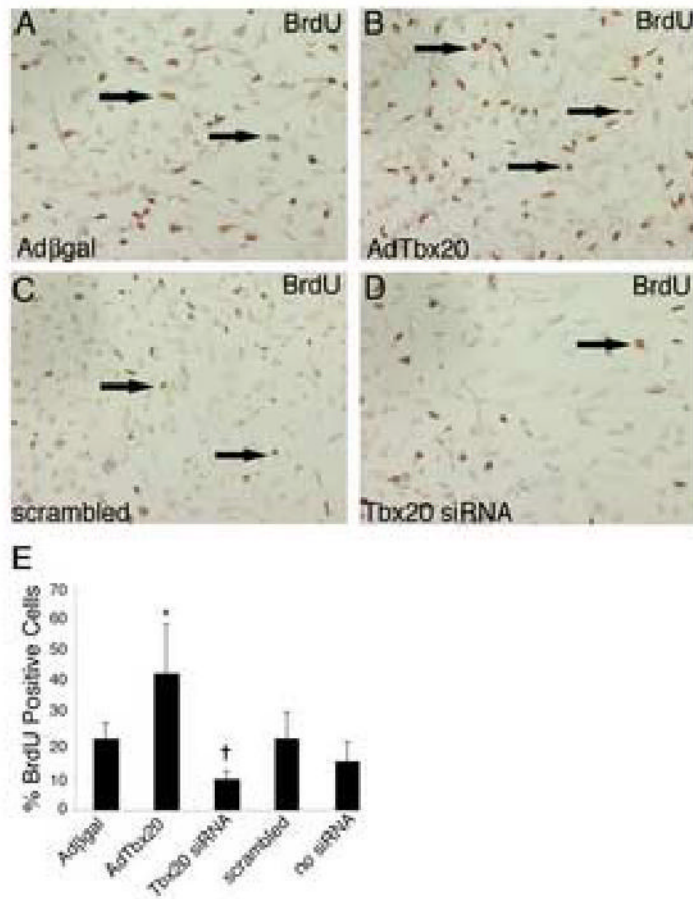
Figure 6. Tbx20 promotes endocardial cushion cell proliferation
Primary endocardial cushion cells were infected with adenoviruses expressing β-gal (Ad β-gal) or Tbx20 (AdTbx20) for gain of function or transfected with Tbx20-specific or scrambled control siRNA for loss of function. Proliferation was measured using immunohistochemistry for BrdU incorporation. Cells infected with AdTbx20 (B) exhibit increased BrdU incorporation (arrows indicate positively stained cells) relative to cells infected with Adβ-gal (A). Cells transfected with Tbx20-specific siRNA (D) are less proliferative than cells transfected with scrambled siRNA (C). The percent of BrdU positive cells relative to total nuclei in each group is quantified (E). The asterisk represents a statistically significant difference compared to the Adβ-gal control group while the cross represents a statistically significant difference compared to the no siRNA control group (n=3, P<0.01). Error bars represent standard error of the mean.
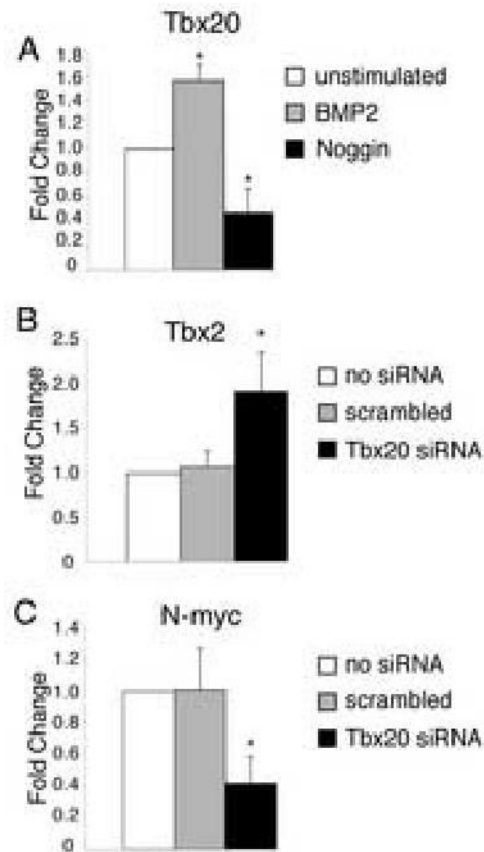
Figure 7. BMP2 induces Tbx20 and Tbx20 regulates the expression of Tbx2 and N-myc in endocardial cushion cells

Endocardial cushion cells were cultured with or without the addition of soluble recombinant BMP2 or Noggin, a BMP inhibitor. Addition of BMP2 resulted in increased *Tbx20* expression relative to untreated controls, while addition of Noggin attenuated that response (A), as measured by real time RT-PCR. The expression of *Tbx2* and *N-myc,* genes associated with Tbx20 regulated proliferation, was measured in siRNA transfected endocardial cushion cells using real time RT-PCR. In cells transfected with Tbx20-specific siRNA, *Tbx2* expression is increased (B) while *N-myc* expression is decreased relative to untransfected or scrambled siRNA controls (C). Asterisks indicate statistically significant differences from control values and error bars represent standard error of the mean (n=5, P<0.01).
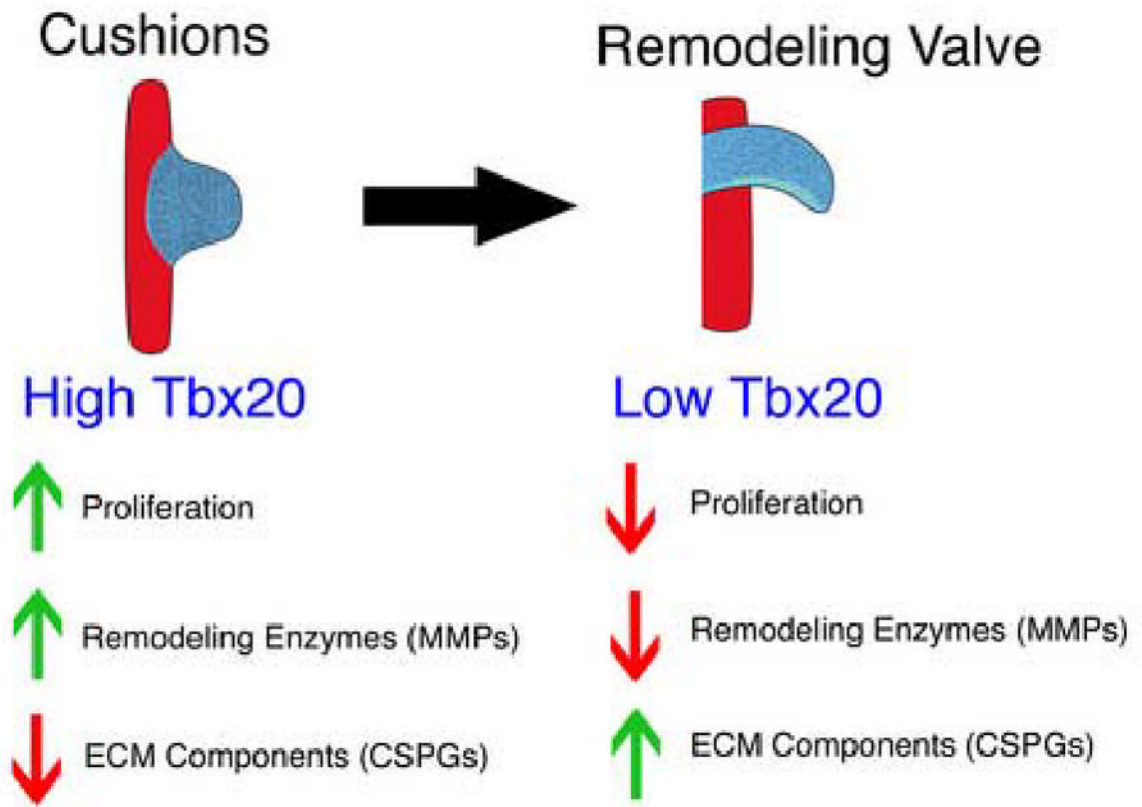
Figure 8. Model for Tbx20 function in endocardial cushions and remodeling valves
Endocardial cushions are characterized by highly proliferative mesenchymal cells and
unorganized extracellular matrix, while remodeling valves are characterized by less
proliferative interstitial cells and organized extracellular matrix. These molecular and cellular
characteristics are consistent with a model in which high levels of Tbx20 in endocardial
cushions induce proliferation while maintaining an unremodeled, unorganized extracellular
matrix. In contrast, lower levels of Tbx20 in remodeling valves allow for a less proliferative
cell population and a more remodeled and organized extracellular matrix.