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Twist1 function in endocardial cushion cell proliferation, migration, and differentiation during heart valve development

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Abstract

Twist1 is a bHLH transcription factor that regulates cell proliferation, migration, and differentiation in embryonic progenitor cell populations and transformed tumor cells. While much is known about Twist1's function in a variety of mesenchymal cell types, the role of Twist1 in endocardial cushion cells is unknown. Twist1 gain and loss of function experiments were performed in primary chicken endocardial cushion cells in order to elucidate its role in endocardial cushion development. These studies indicate that Twist1 can induce endocardial cushion cell proliferation as well as promote endocardial cushion cell migration. Furthermore, Twist1 is subject to BMP regulation and can induce expression of cell migration marker genes including *Periostin, Cadherin 11*, and *Mmp2* while repressing markers of valve cell differentiation including *Aggrecan*. Previously, Tbx20 has been implicated in endocardial cushion cell proliferation and differentiation, and in the current study, Tbx20 also promotes cushion cell migration. Twist1 can induce *Tbx20* expression, while Tbx20 does not affect *Twist1* expression. Taken together, these data indicate a role for Twist1 upstream of Tbx20 in promoting cell proliferation and migration and repressing differentiation in endocardial cushion cells during embryonic development.

Keywords: Twist1; Tbx20; Endocardial cushion development; Cadherin 11; Periostin; Mmp2; Aggrecan; Versican; Cell proliferation; Cell migration; siRNA; Chicken

Introduction

Heart valve development is characterized by the activity of complex regulatory pathways, several of which have also been associated with adult valve disease (Bartram et al., 2001; Cripe et al., 2004; Garg et al., 2005). The onset of heart valve development is marked by the appearance of endocardial cushions in the atrioventricular (AV) canal and outflow tract of the looped heart tube (Armstrong and Bischoff, 2004). Cushion development is initiated by signaling events originating in the myocardium that cause endocardial cells to undergo an epithelial to mesenchymal transformation (EMT) and migrate into the intervening cardiac jelly (Barnett and Desgrosellier, 2003). The resulting endocardial cushions are made up of highly proliferative, migratory, undifferentiated mesenchymal cells embedded in a loose extracellular matrix (Armstrong and Bischoff, 2004; Hinton et al., 2006; Lincoln et al., 2006; Person et al., 2005; Schroeder et al., 2003; Shelton and Yutzey, 2007). As endocardial cushions remodel into mature valve leaflets, the valvular interstitial cells become less proliferative, more compartmentalized into distinct regions of the valve, and more differentiated (Hinton et al., 2006). In addition, the extracellular matrix of the valves becomes highly organized and stratified into three distinct layers (Hinton et al., 2006; Lincoln et al., 2006; Rabkin-Aikawa et al., 2005). While much is known about the events that initiate endocardial cushion development, relatively little is known about the molecular mechanisms that govern the transition from primitive endocardial cushions to mature valve leaflets.

Twist1 is a basic helix–loop–helix (bHLH) transcription factor that was first identified in *Drosophila* as a critical regulator of mesoderm formation (Thisse et al., 1988). Previous studies have identified roles for Twist1 in migration, differentiation, and proliferation of mesenchymal cell populations. In Twist deficient mouse models, loss of mTwist results in

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abnormal limb development, failure of the neural tube to close, hypoplastic branchial arches, somite abnormalities, and lethality by embryonic day 11.5 (Chen and Behringer, 1995; O'Rourke et al., 2002; Soo et al., 2002; Zuniga et al., 2002). Heterozygous hTWIST mutations in human patients have been linked to Saethre–Chotzen syndrome, a disease characterized by craniofacial abnormalities, skeletal anomalies, and limb defects (Bourgeois et al., 1998; Reardon and Winter, 1994; Zackai and Stolle, 1998). In the heart, *Twist1* is expressed in the mesenchyme of developing endocardial cushions (Ma et al., 2005). However, the function of Twist1 in heart valve development has not been reported.

Tbx20, a T-box transcription factor, is highly expressed in developing endocardial cushions and continues to be expressed at lower levels in the mature mitral and tricuspid valves (Plageman and Yutzey, 2004; Shelton and Yutzey, 2007; Stennard et al., 2003; Yamagishi et al., 2004). Mutant mice with loss of Tbx20 function have hypoplastic myocardium, chamber maturation defects, and are embryonic lethal prior to the onset of endocardial cushion development (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). Mutations in human TBX20 are associated with defects in septation, chamber growth, and valvulogenesis (Kirk et al., 2007). In avian endocardial cushion cells, Tbx20 can promote cell proliferation and *N-myc* gene expression, as has also been demonstrated in maturing myocardium (Cai et al., 2005; Shelton and Yutzey, 2007). In addition, Tbx20 can promote an immature extracellular matrix in developing cushions by inducing the expression of matrix remodeling enzymes like *Mmp9* and *Mmp13* and repressing the expression of *Aggrecan* (Agg) and *Versican* (Vers), chondroitin sulfate proteoglycans that mark mature stratified extracellular matrix (Shelton and Yutzey, 2007). Overall, Tbx20 functions to maintain proliferative, undifferentiated, mesenchymal cushions during valve development.

Endocardial cushions are characterized by highly proliferative mesenchymal cells in a loosely organized extracellular matrix. Studies performed in chicken and mice show that endocardial cushion cells are approximately 6 times more proliferative than cells in remodeling valve leaflets (Hinton et al., 2006; Lincoln et al., 2004). Another hallmark of developing endocardial cushions is the migratory nature of the mesenchymal cells that make up the cushions. At the onset of cushion development, endothelial cells transform into migratory mesenchymal cells by becoming activated and losing cell–cell contact (Eisenberg and Markwald, 1995; Markwald et al., 1977). A role for Twist1 and Tbx20 in this aspect of endocardial cushion development has not been previously demonstrated. However, Twist1 promotes cell migration in tumor metastasis and cranial neural crest cells (Chen and Behringer, 1995; Soo et al., 2002; Yang et al., 2004), and Tbx20 promotes cell migration in cranial motor neuron cell bodies (Song et al., 2006). Cell migration is facilitated by cell adhesion proteins, matricellular factors, and remodeling enzymes including Periostin (Postn), Cadherin11 (Cad11), and Matrix Metalloproteinase 2 (Mmp2). The roles of Twist1 in regulating these migration markers or proliferation and differentiation of endocardial cushion cells have not been reported.

To investigate the role of Twist1 in the transition from endocardial cushion to remodeling valve, its expression was compared to known markers of cell proliferation, migration, and maturation in avian endocardial cushions and remodeling valves. *Twist1*, *Tbx20*, *Cad11*, *Postn*, and *Mmp2* are all expressed at higher levels in endocardial cushions relative to remodeling valve leaflets. Additionally, a primary chicken endocardial cushion culture system was used to determine the role of Twist1 in cushion cell proliferation and migration. Like Tbx20, Twist1 can induce cell proliferation in endocardial cushion cells. Moreover, both Twist1 and Tbx20 can promote endocardial cushion cell migration. Furthermore, Twist1 can affect the expression of cell migration and differentiation marker genes including *Cad11*, *Postn*, *Mmp2*, and *Agg*. Finally, it was determined that Twist1 can induce the expression of *Tbx20*, but Tbx20 does not affect the expression of *Twist1*. Taken together, these studies are consistent with Twist1 acting upstream of Tbx20 to regulate aspects of endocardial cushion cell proliferation, migration, and differentiation.

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 and 36 corresponding to embryonic days 5 and 10, respectively (Hamburger and Hamilton, 1951). For histology, hearts were dissected in 1× phosphate-buffered saline (PBS) and fixed for 2 h in 4% paraformaldehyde/PBS. After fixation, embryonic tissue was dehydrated in a graded ethanol/water series (25%, 50%, 75%, 95%, 100%) and washed in xylene before being embedded in paraplast (Sigma-Aldrich) for further processing. All animal procedures were approved and performed in accordance with institutional guidelines.

In situ hybridizations

Chicken *Twist1* sequence (753 bp; Genbank accession number NM20204739.1) was amplified from HH stage 30 wing cDNA using the primers 5′-GCAAGATCCAGACCCTCAAG-3′ and 5′-CTCCTCAGTGGCTCATAGGC-3′. Chicken *Tbx20* sequence (820 bp; Genbank accession number AB070544) was amplified from HH stage 20 heart cDNA as previously reported (Iio et al., 2001; Plageman and Yutzey, 2004; Shelton and Yutzey, 2007). Chicken *Cadherin 11* sequence (685 bp; Genbank accession number AF055342) was amplified from HH stage 34 heart cDNA using the primers 5′-AGAGCTGAAGCACGGGATAA-3′ and 5′-GCTTGTGCCGTGAGAGTGTA-3′. Chicken *Periostin* sequence (1001 bp; Genbank accession number NM001030541) was amplified from HH stage 37 heart cDNA using the primers 5′-TAATGCTCTCCACCACCACA-3′ and 5′-TCTGCTGGCTTGATGATTTG-3′. Chicken *Mmp2* sequence (604 bp; Genbank accession number NM204420) was amplified from HH stage 34 heart cDNA using the primers 5′-TGGAGGAGACTCCCATTTTG-3′ and 5′-GGCAGCAACCAAGAAGAGAC-3′.

To ensure identity and specificity, 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, digoxigenin (DIG)-labeled antisense RNA probes were generated as previously reported (Ehrman and Yutzey, 1999; Shelton and Yutzey, 2007) with the following modifications. The *Twist1*, *Cadherin 11*, and *Mmp2* probes were synthesized with SP6 polymerase from plasmids linearized with *Nco*I. The *Periostin* probe was synthesized with SP6 polymerase from a plasmid linearized with *Sac*II. In situ hybridization of tissue sections was performed as previously described (Shelton and Yutzey, 2007; Somi et al., 2004). Briefly, 14 μm paraffin