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Twist1 promotes heart valve cell proliferation and extracellular matrix gene expression during development in vivo and is expressed in human diseased aortic valves

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ABSTRACT

During embryogenesis the heart valves develop from undifferentiated mesenchymal endocardial cushions (EC), and activated interstitial cells of adult diseased valves share characteristics of embryonic valve progenitors. Twist1, a class II basic-helix–loop–helix (bHLH) transcription factor, is expressed during early EC development and is down-regulated later during valve remodeling. The requirements for *Twist1* down-regulation in the remodeling valves and the consequences of prolonged Twist1 activity were examined in transgenic mice with persistent expression of Twist1 in developing and mature valves. Persistent Twist1 expression in the remodeling valves leads to increased valve cell proliferation, increased expression of Tbx20, and increased extracellular matrix (ECM) gene expression, characteristic of early valve progenitors. Among the ECM genes predominant in the EC, *Col2a1* was identified as a direct transcriptional target of Twist1. Increased Twist1 expression also leads to dysregulation of fibrillar collagen and periostin expression, as well as enlarged hypercellular valve leaflets prior to birth. In human diseased aortic valves, increased Twist1 expression and cell proliferation are observed adjacent to nodules of calcification. Overall, these data implicate Twist1 as a critical regulator of valve development and suggest that Twist1 influences ECM production and cell proliferation during disease.

Introduction

In the United States, 20–30% of all congenital cardiovascular malformations are associated with valve defects, and diseased valves requiring replacement often have congenital anomalies (Loffredo, 2000; Roberts and Ko, 2005). Heart valve development is characterized by the transition of highly proliferative undifferentiated progenitor cells of the endocardial cushions to quiescent valve interstitial cells (VIC) embedded in a complex stratified extracellular matrix (ECM) in the mature valves (Combs and Yutzey, 2009). Likewise, the initial stages of valve disease are characterized by activation of VICs, followed by increased production and disorganization of the valve ECM, leading to thickening of the valve leaflets (Hinton et al., 2006; Rabkin et al., 2001; Rabkin-Aikawa et al., 2004). Embryonic valve progenitor cells are characterized by cell proliferation, migration, and primitive ECM production, which are also characteristics of activated VICs in disease (Aikawa et al., 2006; Rabkin-Aikawa et al., 2004; Schoen, 2008). Several signaling pathways and transcription factors, including the bHLH factor Twist1, have been implicated in maintaining the valve progenitor phenotype during development based on cell culture studies (Shelton and Yutzey, 2008). However, the consequences of persistent expression of early endocardial cushion transcription factors during later stages of remodeling in vivo have not been previously demonstrated. Moreover, potential roles for regulators of valve progenitor cells in adult human valve pathogenesis or repair have not been reported.

The development of the heart valves initiates with the formation of endocardial cushions (EC) in the atrioventricular (AV) junction and outflow tract (OFT) of the primitive heart tube (Combs and Yutzey, 2009). Heart valve progenitor cells arise from an epithelial to mesenchymal transformation (EMT) and are a highly proliferative, migratory, and undifferentiated mesenchymal cell population within the EC. Heart valve remodeling during fetal and neonatal development is characterized by the reduced proliferation of progenitor cells with increased complexity and organization of stratified ECM in the

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semilunar (SL) and AV valve leaflets (Aikawa et al., 2006; Hinton et al., 2006). In addition, matrix metalloproteinases (MMPs) and ECM protein genes, including collagens 2 and 9 are preferentially expressed in EC, whereas fibrillar collagens 1 and 3 are predominant in the remodeling valves (Chakraborty et al., 2008). The mature AV and SL valve leaflets are stratified into elastin, proteoglycan, and collagen-rich ECM layers with VICs that exhibit little to no cell proliferation (Combs and Yutzey, 2009). The molecular and cellular mechanisms that govern the transition of proliferative, migratory, undifferentiated valve progenitor cells of the EC into the differentiated, relatively quiescent VICs of the mature valves are not well-characterized.

Twist1, a class II category basic-helix–loop–helix (bHLH) transcription factor, regulates morphogenesis in a variety of developing organ systems, including the heart, and also promotes EMT during development and in tumor metastasis (Barnes and Firulli, 2009; Yang et al., 2004). Twist1 haploinsufficiency in humans is associated with Saethre–Chotzen syndrome, characterized by craniofacial abnormalities, skeletal anomalies, and limb patterning defects (Reardon and Winter, 1994). During development, *Twist1* is highly expressed in EC, and its expression is down-regulated during heart valve remodeling (Chakraborty et al., 2008). In cultured avian embryonic EC, Twist1 promotes cell proliferation and migration, while inhibiting differentiation (Shelton and Yutzey, 2008). However, the requirements for *Twist1* down-regulation in the remodeling valves and the consequences of prolonged Twist1 activity in adult valves have not been determined previously.

In this study, transgenic mice with persistent Twist1 expression in remodeling and adult valves were generated in order to determine the effects of prolonged Twist1 expression on valvulogenesis. Increased Twist1 expression leads to increased cell proliferation, prolonged expression of endocardial cushion ECM proteins, and abnormal valve morphogenesis. In adult human diseased valves, Twist1 expression is induced in regions of increased cell proliferation and ECM disorganization. Together these studies provide in vivo evidence for Twist1 function in heart valve development and disease.

Materials and methods

Generation of mice

Female *CAG-CAT-Twist1* mice (Connerney et al., 2006) were bred with *Tie2Cre* males (Kisanuki et al., 2001) (Jackson Laboratories, stock number: 004128) to generate double transgenic embryos, neonates, and adult offspring. Timed matings were established, with the morning of an observed copulation plug set at E0.5. All studies were performed on cohorts of *CAG-CAT-Twist1;Tie2Cre* double transgenic (DTG) animals compared to *CAG-CAT-Twist1* single transgenic (STG) littermate controls. *CAG-CAT-Twist1;Tie2Cre* DTG animals were obtained at expected Mendelian ratios, and no embryonic or adult morbidity or mortality was observed. Genotyping for the *Cre* and *Twist1* transgenes was performed by PCR of DNA isolated from embryonic yolk sacs or 3 weeks postnatal tail clips. The following forward and reverse primer sequences were used: *Cre*: (5′-GCG GTC TGG CAG TAA AAA CTA TC-3′ and 5′-GTG AAA CAG CAT TGC TGT CAC TT-3′) and *Twist1*: (5′-GCA GTG GTG GAA TGC CTT TA-3′ and 5′-TGT GGT ATG GCT GAT TAT GAT CTC-3′). All experiments involving animals were carried out with experimental protocols and procedures reviewed and approved by the Cincinnati Children's Medical Center Biohazard Safety Committee and Institutional Animal Care and Use Committee.

In situ hybridization (ISH) and immunohistochemistry

E12.5 whole embryos, E17.5, P7, and adult mouse hearts were collected and washed in ice-cold 1× phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Tissues were subsequently processed for paraffin embedding as previously described (Lincoln et al., 2006) and sectioned at 14 μm for *in situ* hybridization, 5 μm for histological Masson's trichrome staining, or 7 μm for immunofluorescence. The generation of the *Twist1* antisense RNA probe and ISH were performed as previously described (Chakraborty et al., 2008). For histology, following deparaffinization and hydration through a graded ethanol series (100%, 95%, 75%, and 50%), tissues were stained with Masson's trichrome as previously described (Hinton et al., 2006; Levay et al., 2008).

The following antibodies were used for immunofluorescence (IF): Myc (1:400; Cell Signaling, 2272), Phospho-Histone H3 (p-HH3) (1:100; Millipore, 06-570), Ki-67 (1:50; Santa Cruz Biotechnology, 7846), Tbx20 (1:100; Orbigen, PAB 11248), Collagen (Col)2a1 (1:50; Santa Cruz Biotechnology, 52657), Mmp2 (1:50; Abcam, 37150), Mmp13 (1:50; Millipore, MAB 13426), Col1 (1:40; Millipore, AB 765P), Col3 (1:50; Rockland, 600-401-105-0.5), and Periostin (Postn) (1:100; Abcam, 14041). Following deparaffinization and hydration, tissue sections for IF were subjected to antigen retrieval in a microwave oven with citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) for 4.5 min at 90% power, followed by 7 min at 80% power, and 7 min at 70% power and then 2 washes, separately in distilled water and in 1×PBS for 2 min each. Tissue sections were incubated with blocking reagent (1% BSA, 0.01% cold water fish skin gelatin, 0.1% Tween-20 and 0.05% sodium-azide in 1×PBS) for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies with appropriate dilutions in 1×PBS using Coverwell chamber slides (Grace Biolabs). Following incubations, sections were washed in 1×PBS for 10 min, incubated with either corresponding Alexa 488 or 568 fluorescent-conjugated secondary antibodies (Molecular Probes, 1:100) for 1 h, and washed in 1×PBS. Nuclei were stained with ToPro3 (Molecular Probes, 1:1000) for 20 min at room temperature. Tissue sections were washed thoroughly in 1×PBS for 10 min and mounted in Vectashield (Vectorlabs). Fluorescent images were captured with Zeiss LSM 510 confocal microscope and LSM version 3.2 SP2 software.

RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

AV valves were isolated from E17.5 embryos or P7 neonates generated from matings of *CAG-CAT-Twist1* females and *Tie2Cre* males. After genotyping, valve tissue from 4 to 6 embryos or 2 to 3 neonates was pooled for RNA isolation, and real-time qRT-PCR was performed as previously described (Chakraborty et al., 2008). There was minimal myocardial contamination of the valve RNA samples as indicated by qRT-PCR analysis of the myocardial specific gene, cardiac troponin I (*cTnI*). Forward and reverse primer sequences designed for qRT-PCR are *Tbx20*: (5′-GCA GCA GAG AAC ACC ATC AA-3′ and 5′-AAT GAC ACG CGG ATG GTG GGG AAC-3′), *Col2a1*: (5′-GGA AAG TCT GGG GAA AGA GG-3′ and 5′-GGA ACC ACT CTC ACC CTT CA-3′), *Mmp2*: (5′-GAT GTC GCC CCT AAA ACA GA-3′ and 5′-TGG TGT TCT GGT CAA GGT CA-3′), *Mmp13*: (5′ ATC CTG GCC ACC TTC TTC TT-3′ and 5′-TTT CTC GGA GCC TGT CAA CT-3′), *Col3a1*: (5′-GCA CAG CAG TCC AAC GTA GA-3′ and 5′-TCT CCA AAT GGG ATC TCT GG-3′), *Postn*: (5′-CGA AGG GGA CAG TAT CTC CA-3′ and 5′-AGG TCG GTG AAA GTG GTT TG-3′), and *cTnI*: (5′-GAA GCA GGA GAT GGA ACG AG-3′ and 5′-TTA AAC TTG CCA CGG AGG TC-3′). The primer sequences for *Col1a1* qRT-PCR were described previously (Chakraborty et al., 2008). All the amplification reactions were performed with 34 cycles of 94 °C for 30 s; 55 °C for 45 s; and 72 °C for 30 s. A standard curve was generated for each experimental primer set with either E17.5 limb or E13.5 whole embryo cDNA, and all values were normalized to ribosomal protein *L7* expression (Hemmerich et al., 1993). The average base-line levels of all the genes relative to *L7* expression in STG AV valves were calculated as described previously (Sengupta et al., 2009). For each gene, normalized expression in STG AV valves is set to 1 and then fold-change is calculated for corresponding DTG AV valves. qRT-PCR results represent three independent experiments (biological n = 3)