Twist-related protein 1 negatively regulated osteoblastic transdifferentiation of human aortic valve interstitial cells by directly inhibiting runt-related transcription factor 2

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Objective: Valve calcification involves transdifferentiation of valve interstitial cells (VICs) into osteoblasts. Twist-related protein 1 (TWIST1) has been established as a negative regulator of osteoblast differentiation in both mouse and human mesenchymal stem cells, but its function in human aortic VICs is unknown. In our study, we determined the mechanism of TWIST1 action in regulating osteoblastic transdifferentiation of human aortic VICs.

Methods: Human calcified and noncalcified aortic valves were examined for TWIST1 expression. Human aortic VICs were isolated and cultured.

Results: The data showed that calcified aortic valves express lower levels of TWIST1. In vitro experiments showed that TWIST1 overexpression inhibited the transdifferentiation of VICs into osteoblasts by decreasing the expression of runt-related transcription factor 2 (RUNX2) and its downstream osteoblastic markers. Through chromatin immunoprecipitation and dual luciferase assays, we found that TWIST1 repressed the expression of RUNX2 by directly binding to an E-box located at −820 bp of the RUNX2 P2 promoter region and inhibiting its activity.

Conclusions: Our study results suggest that TWIST1 could play an important role in preventing human aortic valve calcification by negatively regulating osteoblastic transdifferentiation of human aortic VICs through direct inhibition of RUNX2. (J Thorac Cardiovasc Surg 2014;148:1700-8)

Supplemental material is available online.

Calcific aortic valve disease (CAVD) is the most common heart valve disease in the elderly. It is characterized by thickening of the valve leaflets and formation of calcium nodules.[1] The pathophysiologic mechanisms underlying CAVD have not been fully elucidated. For several years, CAVD was thought to be a passive process of valve degeneration with calcium accumulation. However, recent studies have shown that aortic valve calcification is a complicated, actively regulated process involving inflammation, extracellular matrix remodeling, and, most importantly, osteoblast differentiation that results in ectopic bone formation.[2,3] Runt-related transcription factor 2 (RUNX2) is a master transcription factor for osteoblasts that plays a critical role in bone formation and transcriptionally regulates several osteoblast markers and bone matrix protein genes, including osteopontin (OPN), osteocalcin (OCN), alkaline phosphatase (ALP), and type I collagen (COL1).[4,5] Once RUNX2 has been expressed, the cells are committed to an osteoblast phenotype and will undergo calcification.[3] The presence and upregulation of RUNX2 and these bone-specific proteins in calcified aortic valves strongly support the concept that aortic valve calcification is an actively regulated process associated with an osteoblast phenotype.[6]

Valve interstitial cells (VICs) are the main source of the osteoblasts in calcified aortic valves.[7] VICs are mainly derived from embryonic progenitor endothelial and mesenchymal cells that initiate the process of valve formation in the embryo.[8] Other sources of VICs include bone marrow-derived cells, circulating cells, and resident valvular progenitor cells, from which progenitor VICs are derived.[8] VICs comprise a diverse, dynamic, and highly plastic population of resident cells crucial to valve function by regulating the remodeling of collagen and other extracellular matrix components.[8] In normal aortic valves, the VICs will be quiescent. However, in disease states, the

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Abbreviations and Acronyms

ALP = alkaline phosphatase
CAVD = calcific aortic valve disease
ChIP = chromatin immunoprecipitation
COL1 = type I collagen
MSC = mesenchymal stem cell
OCN = osteocalcin
OIM = osteogenesis-inducing medium
OPN = osteopontin
pTWIST1 = TWIST1 expression plasmid
RUNX2 = runt-related transcription factor 2
siRNA = small interfering RNA
TWIST1 = twist-related protein 1
VEC = valve endothelial cell
VIC = valve interstitial cell

VICs will be activated and undergo transdifferentiation into a variety of other cell types, including osteoblasts,[7,8] which express bone markers and matrix proteins, such as RUNX2, ALP, OCN and OPN, and lead to valve calcification. Therefore, inhibition of osteoblastic transdifferentiation of VICs could slow or halt the process of valve calcification and CAVD.

Twist-related protein 1 (TWIST1), a basic helix-loop-helix transcription factor, plays a crucial role in the negative regulation of osteoblast differentiation and early osteogenesis.[9-11] In transgenic mice, TWIST1 overexpression can inhibit osteoblast differentiation during skeletogenesis, and TWIST1 deficiency will lead to premature osteoblast differentiation.[10] More importantly, only after TWIST1 expression has decreased can osteoblast-specific gene expression occur. During the process of human mesenchymal stem cell (MSC) differentiation into osteoblasts in vitro, TWIST1 also acts as a critical negative modulator and represses MSC osteogenesis.[11] TWIST1 has also been reported to negatively regulate osteoblastic differentiation in human periodontal ligament cells.[12] These findings all suggest strong inhibition of osteoblast differentiation by TWIST1.

However, it is unknown whether TWIST1 inhibits osteoblastic transdifferentiation in human aortic VICs. In the present study, we aimed to determine whether TWIST1 negatively regulates VIC osteoblastic transdifferentiation and the mechanism of TWIST1 function.

METHODS

Tissue Specimens

Human calcified aortic valves were obtained from patients undergoing aortic valve replacement for calcific aortic stenosis in accordance with protocols approved by the Medical Ethical Committee of Changhai Hospital and according to the Declaration of Helsinki. The major concomitant procedures included mitral valve replacement (n = 2), mitral valve plasty (n = 7), tricuspid valve plasty (n = 6), ascending aorta plasty (n = 3), and coronary artery bypass grafting (n = 2). The general clinical data of the patients are listed in Table E1. Noncalcified aortic valves were obtained from patients undergoing heart transplantation or from autopsy samples of those who had died of noncardiac reasons and had no history of valvular heart disease. The noncalcified valve donors were aged 44.8 ± 11.4 years (range, 24-63), and 8 of the 12 were women. All patients or their direct relatives gave written informed consent. The normal and diseased valves used in the present study were all tricuspid aortic valves. Bicuspid and monocuspid aortic valves were excluded. The normal control aortic valves were thin and smooth, with a thickness of about 1 mm. The diseased valves were thick and stiff, with obvious calcific nodules formed in the cusp belly.

Isolation and Culture of VICs

VICs were isolated from the noncalcific region of the mildly calcified valves using a modification of a previously described method.[13] The valve leaflets were digested in collagenase type II solution (2.0 mg/mL) for 30 minutes at 37°C. After vigorous vortexing to remove the endothelial cells, the leaflets were further digested in fresh collagenase type II solution (2.0 mg/mL) for 2 hours at 37°C. We then spun the tube vigorously and removed the remaining undigested leaflets. The suspension was centrifuged at 500*g* for 5 minutes to collect the isolated cells. The cells were resuspended in Dulbecco's modified Eagle medium with penicillin G, streptomycin, and 10% fetal bovine serum, plated onto a 6-well dish, and cultured with 5% carbon dioxide at 37°C. Immunofluorescence for mesenchymal cell marker vimentin detection and flow cytometry for endothelial cell marker CD31 detection were applied for VIC identification. The cells were subcultured when they had reached 70% to 90% confluence. The cells from passages 3 to 7 were used for the experiments. Each experiment was repeated 3 times using cells from different valves.

VIC Osteogenic Model

The VICs were treated with osteogenesis-inducing medium[7] (OIM) for 4 days to induce osteoblast differentiation when they had reached 70% to 80% confluence. This OIM consisted of complete growth medium supplemented with 50 ng/mL bone morphogenic protein-2, 50 μg/mL ascorbate-2-phosphate, 10 nmol/L dexamethasone, and 10 mmol/L β-glycerol phosphate. In our study, the OIM was supplemented with bone morphogenic protein-2 because of its physiologic importance in valve calcification.[14]

TWIST1 Overexpression and Silencing in VICs

TWIST1 expression plasmid (pTWIST1) and a negative control empty vector plasmid were purchased from Science Peptide Biological Technology (Shanghai, China). The TWIST1-specific small interfering RNA (siRNA) was purchased from GenePharma (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, Calif) according to the manufacturer's instructions. Carboxyfluorescein-labeled siRNA was used to test the transfection efficiency.

Immunohistochemical Staining

In brief, the specimens were embedded in paraffin and sectioned at 5 μm thickness. After dewaxing, rehydration, and antigen retrieval, the sections were incubated with anti-TWIST1 antibody (1:50, ab50887; Abcam, Cambridge, UK) and anti-RUNX2 antibody (1:100, BS2831; Bioworld Technology, Minneapolis, Minn) overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. A DAB Horseradish Peroxidase Color Development Kit (P0202; Beyotime Institute of Biotechnology, Shanghai, China) was used for color development.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted and cDNA synthesized using the PrimeScript RT Reagent Kit (TaKaRa Bio, Ōtsu, Shiga, Japan). SYBR Premix EX Taq (TaKaRa Bio) was used for quantitative real-time polymerase chain