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)

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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 500g 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 medium⁷ (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

reaction (PCR) performed using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies). The primers for each gene were as follows: β -ACTIN forward: 5'-ATTGCCACAGGATGCA GAA-3'; β -ACTIN reverse: 5'-ACATCTGCTGGAAGGTGGACAG-3'; ALP forward: 5'-CCCAAAGGCTTCTTCTTG-3'; ALP reverse: 5'-CTG GTAGTTGTTGTG AGCAT-3'; TWIST1 forward: 5'-GTCCGCAGTCT TACGAGGAG-3'; TWIST1 reverse: 5'-TGGAGGACCTGGTAGAG GAA-3'; COL1 forward: 5'-TCCAACGAGATCGAGATCC-3'; COL1 reverse: 5'-AAGCCGAATTCCTGGTCT-3'; CN forward: 5'-CTTTGTG TCCAAGCAGGAGG-3'; CN reverse: 5'-CTGAAAGCCGATGTGGT CAG-3'; RUNX2 forward: 5'-GGAGTGGACGAGGCAAGAGTTT-3'; RUNX2 reverse: 5'-AGCTTCTGTCTGTGCCTTCTGG-3'.

The amplification conditions were as follows: 1 cycle of 95°C for 30 seconds and 40 cycles of 95°C for 5 seconds followed by 60°C for 30 seconds. The results were analyzed using the comparative CT method. β -ACTIN served as the internal control.

Western Blot

For protein quantification, we used an Enhanced Bicinchoninic Acid Protein Assay Kit (P0010; Beyotime Institute of Biotechnology) using the manufacturer's instructions. In brief, bovine serum albumin solutions with known concentrations were used to achieve a standard curve. The concentrations of protein samples were determined using the standard curve. Next, 30 μ g of total protein of each sample was loaded and run on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, Mass). The blots were blocked and incubated overnight at 4°C with the primary antibody, followed by incubation with a horseradish peroxidase conjugated secondary antibody.

ALP Activity Assay

The VIC lysates were prepared as described previously.¹⁵ ALP activity was measured using an ALP assay kit (Genmed Scientifics, Wilmington, Del). The results were normalized to the total protein level.

Luciferase Assay

The plasmid pGL3-RUNX2-P2 containing -206 to -1671 bp of the RUNX2 P2 region was constructed by PCR cloning of human genomic DNA using the following primers: forward, 5'-GTAGGTAACCAAC CATCTTTCTTCCTTGATG-3', and reverse, 5'-GTAGCTAGCTAGGC TCCTTCCTTTCCCTTCTT-3'. The underlined sequences indicate the restriction enzyme sites for KpnI and NheI, respectively. The PCR products were subcloned into a pGL3-basic vector upstream of the luciferase gene. Two mutant plasmids of pGL3-RUNX2-P2 containing site-directed mutations (Site-Directed Gene Mutagenesis Kit; Beyotime Institute of Biotechnology) of E-box-820 (pGL3-P2-820M) and E-box-1333 (pGL3-P2-1333M) were generated using the following primers: pGL3-P2-820M: 5'-AAATGTCCAGCTATGGCAGGCAGTTCCTAAAACTA-3' and 5'-TAGTTTATAGGAAGTGCCTGCCATAGCTGGACATTT-5'; pGL3-P2-1333M: 5'-TGGTCGTAGACACCGACGAGCTCTGACTCT CATCA-3' and 5'-TGATGAGAGTCAGAGCTCGTCCGGTGTCTAC GACCA-3'. The underlined sequences indicate the mutated E-boxes.

The reporter constructs and an internal control plasmid pRL-TK were co-transfected into human aortic VICs using Lipofectamine 2000 (Invitrogen, Life Technologies). Luciferase activity was measured 48 hours after transfection, using the Dual-Luciferase Reporter Assay (Promega, Madison, Wis). The firefly luciferase activity was normalized by renilla luciferase activity to eliminate the influence of any transfection efficiency difference.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the EZ-ChIP Chromatin Immunoprecipitation Kit (EMD Millipore). The human aortic VICs were transfected with pTWIST and treated with

OIM for 48 hours before they were collected and lysed. DNA sonication was performed using a VCX400 ultrasonic processor (Sonics & Materials, Newtown, Conn) with the recommended shearing conditions. The immunoprecipitating antibody of TWIST1 was anti-TWIST antibody [Twist2C1a]-ChIP grade (ab50887). For a negative control, normal mouse IgG in the EZ-ChIP Kit was used. The primers of the E-boxes in the RUNX2 P2 region were as follows: E-box-100 forward: 5'-GGAG GAATCTGAGCGTGC-3', reverse: 5'-TGGTGAAGCGGTGTGGGTT-3'; E-box-820 forward: 5'-CACCTCATTCTTGTCTCTA-3', reverse: 5'-GCCTCTGTGCTGACTTCT-3'; E-box-1333 forward: 5'-AGTTTGAG GCTGGTCGTAGA-3', reverse: 5'-ATGCTTCCGTGGCTGTTTATT-3'; E-box-1556 and -1604 forward: 5'-ATAAGATGGGAGGGCGTGAG-3', reverse: 5'-GGGAAATCCGCTTGAGGCTA-3'; E-box-2056 forward: 5'-AGTGTCTGTGGAGGGATTG-3', reverse: 5'-GTGGTTGCGTGACT CGGGAT-3'; E-box-2333 and -2462 forward: 5'-TCCCTGTGACC GAAAAGATA-3', reverse: 5'-GGTGACTCCGAGAAGCGAT-3'; E-box-2651 forward: 5'-GCCCAAAGTAAACTAGCC-3', reverse: 5'-CATT GCTGTCTACCTTAC-3'.

Statistical Analysis

All data were evaluated using the Statistical Package for Social Sciences, version 19.0 (SPSS, Chicago, Ill). Student's *t* test was applied to compare the differences between groups.

RESULTS

Calcific Aortic Valves Express Lower TWIST1 Levels

First, we examined the expression of TWIST1 in calcified (CAVD, *n* = 28) and noncalcified (*n* = 12) aortic valves. Quantitative real-time PCR and Western blot showed significantly lower levels of TWIST1 mRNA and protein in the CAVD than in the noncalcified valve group (Figure 1, A and B), suggesting that TWIST1 might play a role in inhibiting aortic valve calcification. Next, using immunohistochemistry, we found that in normal valves, RUNX2 was rarely expressed (Figure 1, D), but TWIST1 was expressed in all 3 layers of the valve (Figure 1, C). In calcified valves, RUNX2 was expressed throughout the valve, especially in the thickened fibrosa layer (Figure 1, F). Only a small amount of TWIST1 expression was found near the border of the valve (Figure 1, E). These results suggest that TWIST1 might suppress RUNX2 expression in normal valves, and its decrease in calcified valves would weaken this suppression.

Identification of VICs Cultured In Vitro

VICs have no specific cell marker. In the present study, immunofluorescence for mesenchymal cell marker, vimentin detection, and flow cytometry for endothelial cell marker CD31 detection were applied for VIC identification. The cell vimentin-positive rate reached nearly 100%, and the CD31-positive rate was less than 2% (Figure E1). These results suggested that the VICs cultured in vitro were high in purity and had very little valve endothelial cell contamination.

TWIST1 Upregulated During VIC Osteoblastic Transdifferentiation In Vitro

When treated with OIM, VICs expressed greater levels of osteoblast-related genes, including RUNX2, OCN, and OPN

FIGURE 1. The expression of twist-related protein 1 (*TWIST1*) in human calcified and noncalcified aortic valves. A, Quantitative real-time polymerase chain reaction showed that *TWIST1* mRNA levels were lower in calcified valves (calcific aortic valve disease [CAVD], n = 28) than in noncalcified valves (control [CTL], n = 12). **P* < .05 versus CTL. B, Western blot showed that *TWIST1* protein expression was also downregulated in the calcified valves (CAVD). β -ACTIN was used as a control for protein loading. C-F, Immunohistochemical staining for *TWIST1* and runt-related transcription factor 2 (*RUNX2*) in calcified (CAVD) and normal (CTL) aortic valves ($\times 400$), with insets of greater magnification. Arrows point to positive staining. f, Fibrosa; s, spongiosa; v, ventricularis; mRNA, messenger RNA.

(Figure 2, A-D). The ALP activity also increased (Figure 2, E). These results suggest that the VICs undergo osteoblastic transdifferentiation in OIM. It was interesting to note that *TWIST1* expression was also upregulated in VICs treated with OIM (Figure 2, F and G). It is unknown whether this upregulation is a self-protective response against the pathologic factors or is a pathologic factor itself that promotes osteoblastic transdifferentiation of VICs. We performed additional experiments to clarify these 2 possibilities.

TWIST1 Inhibited Transdifferentiation of VICs Into Osteoblasts

To assess the effects of *TWIST1* on VIC osteoblastic transdifferentiation, we applied gain- and loss-of-function

strategies in the VIC osteogenic model. First, the VICs were transfected with p*TWIST1* and cultured in OIM for 4 days. An empty vector served as negative control. Cell phase contrast microphotographs showed that smaller cell clusters formed in the p*TWIST1* group than in the negative control empty vector plasmid group (Figure 3, A-C). Overexpression of *TWIST1* (Figure 3, D and E) suppressed the expression of *RUNX2* and its downstream osteogenic markers, including OCN, COL1, OPN, and ALP. The relative mRNA level of *RUNX2*, OCN, COL1, and ALP in the p*TWIST1* group compared with control was 0.72, 0.64, 0.74, and 0.59, respectively (Figure 3, F). The suppression of *RUNX2*, OCN, and OPN expression was also confirmed by Western blot

FIGURE 2. Establishment of human aortic valve interstitial cell (VIC) osteogenic models with osteogenesis-inducing medium (OIM). A-C, Quantitative real-time polymerase chain reaction showed that mRNA levels of the osteogenic markers were significantly upregulated in VICs treated with OIM for 2 (OIM2) or 4 (OIM4) days. Data represented as the fold change. D, The protein levels of runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and osteopontin (OPN) increased in VICs treated with OIM. E, Alkaline phosphatase (ALP) activity also increased. The data were normalized to the total protein level. F and G, TWIST1 expression was also upregulated during VIC osteoblastic transdifferentiation in vitro ($n = 3$ experiments for all tests; error bars represent $\pm$ standard deviation). * $P < .05$ versus control (CTL). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TWIST1, twist-related protein 1.

(Figure 3, G). The ALP activity decreased by 42% (Figure 3, H).

Next, the VICs were transfected with TWIST1 siRNA and cultured in OIM for 24 hours. Scrambled siRNA served as the negative control. The efficiency of siRNA transfection could reach $\leq 90\%$ (Figure 4, A and B). The expression level of TWIST1 mRNA was decreased by 68% (Figure 4, C), and the expression level of RUNX2, OCN, COL1, and ALP mRNA in the transfected group was upregulated by 49%, 53%, 93%, and 49%, respectively (Figure 4, D-F).

Taken together, overexpression of TWIST1 was decreased, and TWIST1 knockdown increased the expression of RUNX2 and its downstream osteogenic markers. These results suggest that TWIST1 suppresses

transdifferentiation of VICs into osteoblasts in vitro; thus, its upregulation during this process might be a self-protective response.

TWIST1 Directly Inhibited RUNX2 P2 Promoter Activity

Because the levels of both RUNX2 mRNA and protein were decreased by TWIST1, we speculated that TWIST1 might have an inhibitory effect on RUNX2 promoter activity, just as it did in human MSCs.¹¹ RUNX2 has 2 promoters, P1 and P2. Type I RUNX2, which is more widely expressed and sufficient for early osteoblastogenesis, is derived from the P2 promoter.¹⁶ We found 9 E-boxes in the P2 promoter region that extends to

FIGURE 3. Overexpression of twist-related protein 1 (*TWIST1*) inhibited the expression of runt-related transcription factor 2 (*RUNX2*) and its downstream osteogenic markers in valve interstitial cell (VIC) osteogenic models. VICs were transfected with *TWIST1* expression plasmid (*pTWIST1*) and treated with osteogenesis-inducing medium (OIM) for 4 days. The cells transfected with an empty vector (negative control empty vector plasmid [*pNC*]) served as the negative control. A, The VICs cultured in vitro showed elongated or spindle-shaped morphology. Smaller cell clusters formed in the (C) *pTWIST1* group than in the (B) *pNC* group. The overexpression of *TWIST1* was verified through (D) quantitative real-time polymerase chain reaction and (E) Western blot. F, The expression of runt-related transcription factor 2 (*RUNX2*), osteocalcin (*OCN*), type I collagen (*COL1*), and alkaline phosphatase (*ALP*) mRNA was downregulated in the *pTWIST1* group. G, The expression of *RUNX2*, *OCN*, and osteopontin (*OPN*) was inhibited by *TWIST1* in VIC osteogenic models. H, ALP activity also decreased in the VIC osteogenic models transfected with *pTWIST1* ($n = 3$ experiments for all tests; error bars represent $\pm$ standard deviation). * $P < .05$ versus *pNC*; # $P < .05$ versus *pNC* plus OIM.

3000 bp upstream of the transcription start site (Figure 5, A). To identify which E-box *TWIST1* was bound to, we applied a ChIP assay. Two potential target E-boxes were identified through ChIP, located at 820 bp (E-box-820) and 1333 bp (E-box-1333) upstream of the transcription start site (Figure 5, B and C). Next, we constructed a plasmid pGL3-RUNX2-P2 containing -206 to -1671 bp of the

P2 region. The inhibition of P2 promoter activity by *TWIST1* overexpression was further confirmed in human aortic VICs using a dual luciferase reporter system (Figure 5, D). To find the exact target site of *TWIST1*, we constructed 2 mutant plasmids of pGL3-RUNX2-P2 using site-directed mutagenesis: E-box-820 (pGL3-P2-820M) and E-box-1333 (pGL3-P2-1333M). Overexpression of

FIGURE 4. Downregulation of twist-related protein 1 (*TWIST1*) increased the mRNA expression of runt-related transcription factor 2 (*RUNX2*) and its downstream osteogenic markers. Valve interstitial cell (VIC) osteogenic models were transfected with *TWIST1*-specific small interfering RNA (*si-TWIST1*) or scrambled negative control siRNA (*si-NC*). A and B, A carboxyfluorescein-siRNA was used to test the transfection efficiency, which could reach $\leq 90\%$. C, *TWIST1* mRNA expression was decreased by 68%. D-G, The mRNA expression level of *RUNX2*, osteocalcin (*OCN*), type I collagen (*COL1*), and alkaline phosphatase (*ALP*) in the *si-TWIST1* group was upregulated by 49%, 53%, 93%, and 49%, respectively ($n = 3$ experiments for all tests; error bars represent $\pm$ standard deviation). * $P < .05$ versus *si-NC*.

TWIST1 inhibited the promoter activity of pGL3-P2-1333M but not pGL3-P2-820M (Figure 5, D). These results suggest that *TWIST1* inhibits *RUNX2* P2 promoter activity possibly by directly binding to E-box-820.

DISCUSSION

In the present study, we have demonstrated that the expression of *TWIST1* in calcified human aortic valves is downregulated. CAVD involves multiple pathogenetic factors that promote VIC osteoblastic transdifferentiation by directly or indirectly upregulating *RUNX2* expression. Our results have shown that *TWIST1* suppressed human mature aortic VIC osteoblastic transdifferentiation by directly binding to and inhibiting the P2 promoter of *RUNX2*, a core transcription factor in osteoblastogenesis. Thus, *TWIST1* could play an important role in preventing aortic valve calcification by directly suppressing *RUNX2* expression. In contrast, its decreased expression might contribute to valve calcification.

In a previous study that included bicuspid aortic valves, which were excluded in our study, Wirrig and colleagues¹⁷ found *TWIST1* expression was increased in adult human calcified aortic valves through immunohistochemistry.

However, the difference did not reach statistical significance.¹⁷ In our study, we found a large variation in *TWIST1* expression among the specimens. We increased the sample size and applied multiple detective methods and found that *TWIST1* expression was relatively lower in the calcified valves. Cheek and colleagues¹⁸ reported that the characteristics of myxomatous valve disease and increased *TWIST1* expression were found in osteogenesis imperfecta murine mice, with a mutation in *Colla2*. Because overexpression of *TWIST1* could not lead to valve thickening in postnatal mice,¹⁹ it remains uncertain whether the increased *TWIST1* expression in osteogenesis imperfecta murine mice is inseparable from myxomatous valve disease or is just parallel to the disease.

CAVD has also been linked to valve endothelial cell (VEC) dysfunction. VECs could play a complicated role in valve calcification. On the one hand, VECs have the potential and can be induced to transdifferentiate into osteoblastic VICs, which will lead to valve calcification.²⁰ On the other hand, VECs can attenuate VIC activation and osteoblastic transdifferentiation through the secretion of nitric oxide.²¹ In the present study, we cultured VICs of high purity, to exclude any VEC regulation on the VIC phenotype.

FIGURE 5. Twist-related protein 1 (*Twist1*) suppressed runt-related transcription factor 2 (*RUNX2*) expression by directly binding to the RUNX2 P2 promoter. A, Nine E-boxes (CANNTG) were in the RUNX2 P2 promoter region. B and C, Two putative target E-boxes of TWIST1 were identified using chromatin immunoprecipitation (ChIP) assays, E-box-1333 and E-box-820. We cloned the 1466-bp region that included these 2 E-boxes into a pGL3-basic vector (A) and constructed 2 plasmids that were mutated in E-box-1333 or E-box-820 using site-directed mutagenesis. D, The dual luciferase reporter system showed that TWIST1 could inhibit the promoter activity of pGL3-RUNX2-P2 and pGL3-P2-1333M but had little repression effect on pGL3-P2-820M ($n = 3$ experiments for all tests; error bars represent $\pm$ standard deviation). * $P < .05$; $\Delta P > .05$. IgG, Immunoglobulin G.

For the first time, we have demonstrated that TWIST1 suppresses human mature aortic VIC osteoblastic transdifferentiation. Previous studies have established that TWIST1 negatively regulates osteoblast differentiation in both mouse and human MSCs.⁹⁻¹¹ However, TWIST1 might have different functions in other cell lineages by targeting distinct transcription factors.²² To examine the role of TWIST1 in the process of human aortic VIC osteoblastic transdifferentiation, we established a VIC osteogenic model, which expressed greater levels of osteoblast-related genes, including RUNX2 and ALP.¹⁴ In this model, the overexpression of TWIST1 decreased, and the downregulation of TWIST1 increased the expression of RUNX2 and its downstream osteogenic markers. These results have indicated that TWIST1 negatively regulates VIC osteoblastic transdifferentiation and is an important negative regulator of valve calcification and ectopic osteogenesis. The upregulation of TWIST1 during VIC osteoblastic transdifferentiation in vitro might be a self-protective response of VICs against the pathologic environment to weaken its harmful effects. Although the upregulation of TWIST1 could not completely inhibit VIC osteoblastic transdifferentiation, it could, to some extent, suppress the process.

We also studied the mechanism by which TWIST1 negatively regulates VIC osteoblastic transdifferentiation. As a negative regulator of osteoblast differentiation, TWIST1 might function through different mechanisms in different cell lineages. TWIST1 can regulate osteoblast function through direct interaction with activating transcription factor 4.²³ TWIST1 silencing can upregulate osteoblast differentiation of murine mesenchymal cells by increasing fibroblast growth factor receptor 2 expression and its downstream signaling pathways.²⁴ Bialek and colleagues¹⁰ demonstrated that TWIST1 interacted with the Runt domain of RUNX2, decreasing its DNA binding and transactivation abilities. Yang and colleagues¹¹ found that the basic helix-loop-helix domain of TWIST1 could bind to the E1-box in the RUNX2 P2 promoter and downregulate its activity in human MSCs. In the present study, our data have shown that TWIST1 directly suppresses RUNX2 P2 promoter activity in human aortic VICs similarly to its activity in human MSCs. In conclusion, calcified aortic valves from patients with CAVD showed a lower expression of TWIST1 compared with noncalcified valves. TWIST1 suppressed osteoblastic transdifferentiation of VICs in vitro. Additional

investigation showed that TWIST1 directly bound to the RUNX2 P2 promoter and suppressed its activity in human aortic VICs. Our findings suggest that TWIST1 might inhibit aortic valve calcification by suppressing VIC osteoblastic transdifferentiation. The present study has provided new insights into the role of TWIST1 in valve calcification and a new potential target for clinical treatment of CAVD.

Study Limitations

First, the VIC osteogenic model might not fully simulate the in vivo valve calcification process. The development of an in vitro system that allows for independent modulation of the VIC phenotype is a challenging problem in CAVD research.²⁵ Second, the cause of the TWIST1 decrease during valve calcification remains unknown, and more work is needed to fully illuminate the mechanisms underlying the regulation of TWIST1 expression. Third, we only focused on the role of TWIST1 in VIC osteoblastic transdifferentiation. The overall role of TWIST1 in valve calcification remains to be explored. Finally, the role of VECs in valve calcification was not included in the present study and should be investigated in future studies.

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FIGURE E1. Vimentin and CD31 detection in valve interstitial cells (VICs). A, Vimentin (a mesenchymal cell marker) was expressed in all the cells (green fluorescence). B, Flow cytometry showed that the CD31-positive rate was <2%. *FSC-A*, Forward scatter area; *APC-A*, allophycocyanin area; *FL6-A*, fluorescence 6 (CD31 herein) area.

TABLE E1. General clinical data of patients undergoing aortic valve replacement (n = 28)

Variable	Value
Age (y)	
Mean ± standard deviation	62.9 ± 7.3
Range	52-77
Female gender (n)	10 (35.7)
Body mass index (kg/m ²)	23.0 ± 3.6
Diabetes mellitus (n)	3 (10.7)
Hypertension (n)	9 (32.1)
Aortic valve disease (n)	
Stenosis	16 (57.1)
Stenosis and insufficiency	12 (42.9)
Peak pressure gradient (mm Hg)	75.1 ± 29.1
Aortic annulus diameter (mm)	23.0 ± 1.9
Outcomes	
Early death (n)	0
Hospital stay (d)	
Mean ± standard deviation	15.3 ± 1.8
Range	12-19

Data in parentheses are percentages.