Histone deacetylase 6 reduction promotes aortic valve calcification via an endoplasmic reticulum stress-mediated osteogenic pathway

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ABSTRACT

Objective: Aortic valve (AoV) calcification occurs via a pathophysiologic process that includes osteoblastic differentiation of valvular interstitial cells (VICs). Histone deacetylases (HDACs) have been shown to be involved in the pathogenesis of vascular diseases. Here, we investigated the role of HDAC6 in AoV calcification.

Methods: AoV cusps from patients with aortic stenosis (n = 7) and normal controls (n = 7) were subjected to determination of calcified nodules and HDAC6 expression. Human VICs were cultured in osteogenic media and treated with 10 μ M tubacin or HDAC6 small interfering RNA silencing to inhibit HDAC6. Treatment with 100 μ M tauroursodeoxycholic acid was used to suppress endoplasmic reticulum stress. Activating transcription factor 4 (ATF4) small interfering RNA was used to knock down ATF4. Alizarin red staining was used to evaluate calcified nodules formation of VICs cultured with osteogenic media for 14 days.

Results: HDAC6 expression was significantly reduced in AoV tissue of patients with aortic stenosis compared with controls. Tubacin treatment or HDAC6 silencing markedly promoted osteoblastic differentiation accompanied by endoplasmic reticulum stress activation in VICs. The HDAC6 inhibition-induced osteogenic pathway was mediated by endoplasmic reticulum stress/ATF4 pathway as indicated by tauroursodeoxycholic acid pretreatment or ATF4 silencing. Finally, alizarin red staining showed that HDAC6 inhibition promoted osteoblastic differentiation of VICs, which could be suppressed by tauroursodeoxycholic acid.

Conclusions: HDAC6 inhibition promotes AoV calcification via an endoplasmic reticulum stress/ATF4-mediated osteogenic pathway. HDAC6 may be a novel target for AoV calcification prevention and treatment. (J Thorac Cardiovasc Surg 2019;158:408-17)

HDAC6 suppression promotes aortic valve calcification via ER stress.

Central Message

HDAC6 reduction is linked with AoV calcification. Inhibition of HDAC6 promotes osteoblastic differentiation of VICs via ER stress.

Perspective

HDACs have been involved in the pathogenesis of cardiovascular diseases, but their roles in AoV calcification remain unknown. Here we show HDAC6 reduction in the AoV cusps of patients with CAVD. Mechanistically, HDAC6 suppression promotes osteoblastic differentiation of VICs via an ER stress/ATF4-mediated osteogenic pathway.

See Commentary page 418.

Calcified aortic valve disease (CAVD) that may finally develop into aortic valve (AoV) stenosis is the most common valvular disease in developed countries and is a

significant cause of morbidity and mortality.¹ CAVD is characterized by mineralized nodules formation on the valve cusps and is not a passive degenerative process but

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Abbreviations and Acronyms

AoV	= aortic valve
ATF	= activating transcription factor
CAVD	= calcified aortic valve disease
ER	= endoplasmic reticulum
GRP78	= glucose-regulated protein 78
HDAC	= histone deacetylase
IRE1 α	= inositol-requiring transmembrane kinase and endonuclease-1 α
PERK	= protein kinase-like endoplasmic reticulum kinase
shRNA	= short hairpin RNA
siRNA	= small interfering RNA
TUDCA	= tauroursodeoxycholic acid
VIC	= valvular interstitial cells

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rather a chronic active disease process, including lipoprotein deposition, valve fibrosis, and osteoblastic differentiation of valvular interstitial cells (VICs).² However, there is no effective pharmacologic therapy other than surgical or interventional AoV replacement.³⁻⁵

Histone deacetylases (HDACs) are a group of enzymes that lead to the deacetylation of the lysine residues on histone and nonhistone proteins.⁶ It has been reported that HDAC inhibitors promote osteoblast maturation.⁷ Azechi and colleagues⁸ found that inhibition of HDAC activity by nonspecific inhibitor trichostatin A can promote vascular calcification. Kwon and colleagues⁹ found that HDAC1 has a protective effect on vascular calcification. These studies indicate that HDAC may also be involved in the pathogenesis of AoV calcification.

The endoplasmic reticulum (ER) is a crucial luminal network for protein synthesis, folding, and transportation.¹⁰ ER homeostasis disruption leads to accumulation of unfolded and misfolded proteins in the ER lumen and creates a state defined as ER stress, which activates a complex signaling network termed the unfolded protein response. Unfolded protein response consists of 3 branches: protein kinase-like ER kinase (PERK), inositol-requiring transmembrane kinase and endonuclease-1 α (IRE1 α), and activating transcription factor (ATF) 6.¹¹ ER stress is associated with various pathophysiologic states,¹²⁻¹⁵ including AoV calcification.^{10,16,17} It has also been reported that ER stress, especially the

PERK/ATF4 pathway, is highly involved in the development of AoV calcification.^{10,18}

HDAC6 is a member of the HDAC IIb subfamily.¹⁹ Rao and colleagues¹¹ found that inhibition of HDAC6 in breast cancer cells induces acetylation of glucose-regulated protein 78 (GRP78) and triggers ER stress.¹¹ Because ER stress is involved in AoV calcification, we therefore hypothesized that HDAC6 reduction may promote AoV calcification by activating the ER stress-mediated osteogenic pathway.

MATERIALS AND METHODS

Human AoV Samples

The human study was approved by an ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Human specimens were from the Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. According to the Declaration of Helsinki, informed consents were signed. From October 2017 to April 2018, calcified and normal AoV were collected. We obtained calcified AoVs from patients who underwent valve replacement due to symptomatic, severe aortic stenosis without moderate or severe aortic regurgitation or mitral valve diseases ($n = 7$). Normal AoVs were from patients with acute Stanford type A aortic dissection accompanied by moderate aortic insufficiency (more than Grade II) who underwent emergency Bentall surgery ($n = 7$). Patients with bicuspid aortic valves and connective tissue disorders were excluded.

The tissues were put in phosphate buffered saline on ice after harvest and then were transported to the lab immediately. The tissues were divided into 3 parts. One part was used to extract protein for Western blot, 1 part was fixed in 4% paraformaldehyde for immunohistochemical staining and Von Kossa staining, and 1 part was digested by collagenase for cell culture and treatment.

Cell Culture and Treatment

VICs were isolated from normal AoV cusps by collagenase digestion.¹⁶ All experiments were performed with cells in passage 3 to 5. After 70% confluence, cells were incubated with indicated interventions in Dulbecco's modification of Eagle medium supplemented with 5% fetal bovine serum, 10 mM β -glycerophosphate (Sigma-Aldrich, St Louis, Mo), 10 nM dexamethasone (Sigma-Aldrich), 4 μ g/mL cholecalciferol (Sigma-Aldrich), and 8 mM calcium chloride (Sigma-Aldrich). If needed, pharmacologic reagents, including 10 μ M tubacin (Selleckchem, Houston, Tex), 100 μ M tauroursodeoxycholic acid (TUDCA) (Calbiochem, San Diego, Calif) were added.

Cell Transfection of Small Interfering RNAs

Small interfering RNAs (siRNAs) against indicated proteins were provided by Santa Cruz Biotechnology (Santa Cruz, Calif) and were designed against human HDAC6 or human ATF4. Scrambled siRNAs (Santa Cruz Biotechnology) were used as normal control. VICs were transfected with scrambled or indicated siRNAs using RNAiMAX (Invitrogen, Waltham, Mass) and Opti-MEM (Gibco, Carlsbad, Calif) according to manufacturer's recommendations.

Lentivirus Transfection

VICs were infected with recombinant lentiviruses expressing control short hairpin (shRNA) or HDAC6 shRNA (Jikai Genechem Co Ltd, Shanghai, China) according to manufacturer's instruction.

Western Blot

Tissue samples and cells were homogenized and subsequently performed for Western blotting as previously described.²⁰ The following

antibodies were used: phosphorylated IRE1 α (1:1000 dilution) (RB2150924) from Thermo Fisher Scientific (Waltham, Mass) phosphorylated PERK (1:500 dilution) (sc-32577) and β -actin (1:1000 dilution) (sc-47778) from Santa Cruz Biotechnology, *Runx2* (1:1000 dilution) (8486S), phosphorylated eukaryotic initiation factor 2 α (1:1000 dilution) (9721S) from Cell Signaling Technology (Danvers, Mass), and HDAC6 (1:1000 dilution) (12834-1-AP) from Proteintech (Rosemont, Ill). β -actin was used as normalization for total protein.

Histopathology and Immunohistochemistry

Human AoV tissues were rapidly harvested, rinsed in phosphate buffered saline, fixed in 4% paraformaldehyde in phosphate buffered saline, and embedded in paraffin for histopathologic analyses. Immunohistochemical staining was performed using the following antibodies: HDAC6 (1:200 dilution) (12834-1-AP) from Proteintech. At least 2 of the 3 AoV cusps for each patient were analyzed.

Von Kossa Staining

Sections were stained with a von Kossa staining kit (Abcam, Cambridge, United Kingdom) according to manufacturer's instruction.¹⁷

Alizarin Red Staining

For analysis of osteoblastic differentiation in VICs, cells were incubated with indicated interventions in a conditioning media (growth medium supplemented with 10 mM β -glycerophosphate, 10 nM dexamethasone, 4 μ g/mL cholecalciferol, and 8 mM calcium chloride) for 14 days as reported previously.^{17,21} Media were changed every 3 days. Alizarin red staining for calcium deposits was performed as described.²² To quantitatively analyze alizarin red stain, wells were rinsed with distilled water, and alizarin red stains were bleached with 10% acetic acid at 85°C. Supernatant was spectrophotometrically analyzed at 450 nm.²²

Statistical Analysis

All values are presented as mean $\pm$ standard deviation. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test and visualized by Q-Q plot, statistical differences were evaluated by Student *t* test or analysis of variance followed by Bonferroni's multiple comparison test by SPSS version 18.0 (IBM-SPSS Inc, Armonk, NY).

Sample Size Estimation

Stata software version 12.0 (StataCorp, College Station, Tex) was used to estimate the sample size for 2-sample comparison of mean in human study. Sample size was set as identical in 2 groups. Alpha was set as 0.05, power was set as 0.8. Results of mean and standard deviation values in the human study were used.

RESULTS

HDAC6 Is Reduced in AoV Cusps of Patients With CAVD

To confirm whether HDAC6 is involved in AoV calcification, we first determined the levels of HDAC6 in AoV samples from patients with CAVD and normal controls. The clinical characteristics of patients are listed in Table 1. Von Kossa staining confirmed a clear calcium deposit in the AoV tissues from patients with CAVD (Figure 1, A). Immunohistochemical staining (Figure 1, B) and Western blot (Figure 1, C) revealed that HDAC6 was significantly reduced in calcified AoV samples compared with controls.

TABLE 1. Clinical characteristics in patients with calcified aortic valve disease (CAVD) versus control patients

Characteristic	Control (n = 7)	CAVD (n = 7)	P value
Age (y)	62.6 $\pm$ 5.2	64.8 $\pm$ 6.1	.523
Male gender	4 (57.1)	5 (71.4)	.577
Body mass index	22.9 $\pm$ 3.1	23.2 $\pm$ 5.1	.908
Smoking	5 (71.4)	6 (85.7)	.515
Hypertension	3 (42.9)	5 (71.4)	.28
Hypercholesterolemia	4 (57.1)	6 (85.7)	.237
Diabetes mellitus	2 (28.6)	3 (42.9)	.577
Coronary heart disease	2 (28.6)	3 (42.9)	.577
Peripheral vascular diseases	0 (0)	1 (14.3)	.299
Aortic insufficiency*	7 (100)	0 (0)	<.001
Antiplatelets	3 (42.9)	6 (85.7)	.094
ACEi/ARB	2 (28.6)	3 (42.9)	.577
Beta blockers	1 (14.3)	0 (0)	.299
Calcium channel blockers	1 (14.3)	0 (0)	.299
Diuretics	0 (0)	2 (28.6)	.127
Statins	3 (42.9)	6 (85.7)	.094
LVEF (%)	55.3 $\pm$ 3.5	52.1 $\pm$ 7.3	.363
Transvalvular gradient (mm Hg)	15.4 $\pm$ 5.5	79.7 $\pm$ 6.3	<.001
Aortic valve area (cm ²)	3.1 $\pm$ 0.4	0.7 $\pm$ 0.1	<.001

Values are presented as mean $\pm$ standard deviation or n (%). CAVD, Calcified aortic valve disease; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blockers; LVEF, left ventricular ejection fraction. *Defined as Grade I or greater.

Inhibition of HDAC6 Activates an Osteogenic Pathway in VICs

VICs are the main cellular components of AoVs, and the activation of osteogenic pathways is an important mechanism of AoV calcification.^{2,10} VICs were incubated with tubacin, a specific inhibitor of HDAC6. As indicated in Figure 2, A, and Figure E1, tubacin treatment markedly increased the expression of *Runx2* in VICs. The effect of HDAC6 on *Runx2* was further confirmed by HDAC6 silencing in VICs because knockdown of HDAC6 significantly induced *Runx2* expression (Figure 2, B).

HDAC6 Reduction Induces ER Stress in VICs

A previous report indicates that HDAC6 suppression can activate ER stress in breast cancer cells.¹¹ We determined ER stress under HDAC6 inhibition in VICs. As shown in Figure 3, A, and Figures E1 and E2, HDAC6 inhibition by tubacin significantly triggered the expressions of ER stress markers, except for ATF6, in VICs. Moreover, knockdown of HDAC6 by siRNA in VICs showed the same result as tubacin treatment; that is, HDAC6 silencing markedly induced ER stress in VICs (Figure 3, B).

FIGURE 1. Histone deacetylase 6 (*HDAC6*) is reduced in aortic valve cusps of patients with calcified aortic valve disease (CAVD). A, Representative von Kossa staining of the AoV cusps of normal controls and patients with CAVD. The AoV cusps of patients with CAVD showed significant calcified nodules formation. B, Immunohistochemical staining of HDAC6 in the AoV cusps of normal controls and patients with CAVD. HDAC6 staining was significantly reduced in AoV cusps of patients with CAVD. C, Western blot analysis of HDAC6 in AoV cusps from normal controls and patients with CAVD. HDAC6 expression was significantly reduced in AoV cusps of patients with CAVD. Scale bar: 50 μ m. N = 7 for each group. **P* < .05 versus normal.

FIGURE 2. Inhibition of histone deacetylase 6 (*HDAC6*) activates an osteogenic pathway in valvular interstitial cells (VIC). A, Tubacin (10 μ M) treatment significantly increased *Runx2* expression in VIC. B, Knockdown of HDAC6 significantly induced *Runx2* expression in VIC. **P* < .05 versus dimethyl sulfoxide (DMSO) or scrambled small interfering RNA (scr siRNA). *Runx2*, Runt-related transcription factor 2.

FIGURE 3. Histone deacetylase 6 (*HDAC6*) reduction induces endoplasmic reticulum (ER) stress in valvular interstitial cells (VIC). A, Tubacin (10 μ M) treatment significantly increased the expressions of ER stress markers phosphorylated protein kinase-like ER kinase (*p-PERK*), phosphorylated inositol-requiring transmembrane kinase and endonuclease-1 α (*p-IRE1 α*), phosphorylated eukaryotic initiation factor 2 α (*p-eif2 α*), and activating transcription factor 4 (*ATF4*) in VIC. B, *HDAC6* silencing markedly induced the expression of ER stress markers (*p-PERK*, *p-eif2 α* , and *ATF4*) in VIC. * $P < .05$ versus dimethyl sulfoxide (*DMSO*) or scrambled small interfering RNA (*scr siRNA*).

The Effect of *HDAC6* Inhibition on ER Stress and Osteogenic Pathway Activation Is More Profound in VICs From Normal Control Patients

We checked the effect of *HDAC6* inhibition in VICs from normal AoVs and from calcified AoVs. As shown in Figure E3, both ER stress markers phosphorylated PERK and ATF4, and osteogenic pathway markers *Runx2* and alkaline phosphatase were significantly induced in VICs from calcified AoVs. *HDAC6* inhibition by tubacin led to a more significant induction of ER stress and osteogenic pathways in VICs from the control AoVs (Figure E3).

HDAC6 Suppression-Induced Osteogenic Pathway Is Mediated by ER Stress in VICs

Our previous reports confirm that ER stress activation induces AoV calcification.^{10,16,17} Because our data show that *HDAC6* inhibition triggers ER stress and osteogenic pathways in VICs, the next question is whether alleviating ER stress rescues osteogenic pathway activation induced by *HDAC6* suppression. As depicted in Figure 4, A and B, preincubation of TUDCA, the ER stress suppressor,¹⁰ significantly relieved ER stress activation as well as *Runx2* expression after tubacin treatment. We also tested the effect of TUDCA in *HDAC6*-silenced cells. Inhibition of ER stress by TUDCA markedly reduced *Runx2*

expression that was induced by *HDAC6* silencing (Figure 4, C and D).

ATF4 Activation Mediates Osteogenic Pathway Activation Triggered by *HDAC6* Inhibition

ER stress promotes oxidized low-density lipoprotein-induced osteoblastic differentiation of VICs through the ATF4 pathway.¹⁰ We tested whether an *HDAC6* suppression-induced osteogenic pathway is mediated by ATF4. Knockdown of ATF4 significantly suppressed *Runx2* expression that was induced by tubacin treatment (Figure 5, A-C) or *HDAC6* silencing (Figure 5, D-F) in VICs.

Alleviating ER Stress Prevents Osteoblast Differentiation of VICs Induced by *HDAC6* Inhibition

The activation of the osteogenic pathway leads to the osteoblastic differentiation of VICs, resulting in intracellular and extracellular calcium deposition and the formation of calcified nodules.² The final question would be whether osteoblast differentiation induced by *HDAC6* suppression could be alleviated by ER stress inhibition. VICs were transfected with lentiviral *HDAC6* shRNA or scramble shRNA. The transfected cells were incubated

FIGURE 4. Histone deacetylase 6 (*HDAC6*) suppression-induced osteogenic pathway is mediated by endoplasmic reticulum (*ER*) stress in valvular interstitial cells (*VIC*). **A**, Tauroursodeoxycholic acid (*TUDCA*) (100 μ M) preincubation significantly relieved *ER* stress activation as indicated by expressions of phosphorylated protein kinase-like *ER* kinase (*p-PERK*), phosphorylated inositol-requiring transmembrane kinase and endonuclease-1 α (*p-IRE1 α*), phosphorylated eukaryotic initiation factor 2 α (*p-eif2 α*), and activating transcription factor 4 (*ATF4*) induced by tubacin (10 μ M). **B**, *TUDCA* pretreatment markedly reduced *Runx2* expression induced by tubacin. **C**, *TUDCA* incubation significantly attenuated *ER* stress activation (*p-PERK*, *p-IRE1 α* , *p-eif2 α* , and *ATF4*) caused by *HDAC6* silencing. **D**, *TUDCA* treatment markedly suppressed *Runx2* expression induced by *HDAC6* silencing. **P* < .05 versus dimethyl sulfoxide (*DMSO*) or scrambled small interfering RNA (*scr siRNA*). #*P* < .05 versus tubacin or *HDAC6* siRNA. *Runx2*, Runt-related transcription factor 2.

FIGURE 5. Activating transcription factor 4 (*ATF4*) mediates osteogenic pathway activation triggered by histone deacetylase 6 inhibition (*HDAC6*). A to C, Knockdown of *ATF4* significantly suppressed *Runx2* expression induced by tubacin treatment in valvular interstitial cells (VIC). D to F, Knockdown of *ATF4* significantly suppressed *Runx2* expression caused by *HDAC6* silencing in VIC. * $P < .05$ versus dimethyl sulfoxide (*DMSO*) or scrambled small interfering RNA (*scr siRNA*). # $P < .05$ versus tubacin or *HDAC6* siRNA. *Runx2*, Runt-related transcription factor 2.

with or without TUDCA and were cultured in osteogenic media for 14 days. As shown in Figure 6, A and B, lentiviral *HDAC6* shRNA transfection markedly suppressed *HDAC6* expression. Alizarin red staining showed that knockdown of *HDAC6* promoted calcified nodules formation, whereas inhibition of ER stress by TUDCA suppressed the effect (Figure 6, C).

DISCUSSION

Despite the clinical importance of heart disease and intense study in AoV calcification, the mechanisms underlying the development of AoV calcification remain unclear.³⁻⁵ The current study shows that *HDAC6* reduction is linked with CAVD in human beings. Our data indicate that *HDAC6* inhibition promotes AoV calcification by ER stress/*ATF4*-mediated osteogenic pathway.

The function of *HDACs* is transcriptional regulation and posttranslational modification of proteins.⁶ In the field of cardiovascular medicine, studies have found that class I and class II *HDAC* inhibitors can prevent myocardial hypertrophy

and myocardial injury after myocardial infarction, and may inhibit fibrosis.^{19,23} *HDAC2* and *HDAC3* can maintain endothelium integrity and reduce atherosclerosis.^{19,23,24}

However, *HDAC* inhibition seems to be harmful in vascular calcification. Studies have shown that *HDAC* inhibitors promote vascular calcification.⁸ *HDAC1* has a protective effect on vascular calcification, but the study shows that *HDAC6* is unchanged in vascular calcification.⁹ Our results show that *HDAC6* is reduced in calcified AoV, indicating the pathogenesis of AoV calcification is not identical to vascular calcification.

Our results demonstrate that *HDAC6* inhibition activates ER stress in VICs. The result is in consistent with the studies in cancer cells.^{11,25} Li and colleagues¹¹ reported that *HDAC6* prevents acetylation of *GRP78*, the molecular chaperone that colonizes the ER,¹¹ at lysine residue 633.²⁵ In nonstressed cells, *GRP78* binds to ER transmembrane receptors and keeps them inactive. When acetylated, *GRP78* reduces binding to *PERK* and *IRE1α* on the ER membrane, and activates ER stress.¹¹

FIGURE 6. Alleviating endoplasmic reticulum stress prevents osteoblastic differentiation of valvular interstitial cells (VIC) induced by histone deacetylase 6 (*HDAC6*) inhibition. A and B, Lentiviral *HDAC6* short hairpin RNA (*shRNA*) transfection markedly suppressed *HDAC6* expression in VIC. C, Alizarin red staining showed that knockdown of *HDAC6* promoted calcified nodules formation, whereas tauroursodeoxycholic acid (*TUDCA*) suppressed the effect. * $P < .05$ versus scrambled (*scr*) *shRNA*. # $P < .05$ versus *TUDCA* + *scr shRNA* in panel B or *HDAC6 shRNA* in panel C.

ATF4 downstream of ER stress serves not only an ER stress sensor but a transcriptional factor of genes involved in osteogenic pathways as well.^{18,26,27} Our previous work^{10,17} indicates that ATF4 is essential in the activation of osteoblastic differentiation of VICs. Masuda and colleagues¹⁸ further confirmed that ATF4 knockout prevents AoV and vascular calcification in vivo.¹⁸ Because *HDAC6* suppression activates ER stress/ATF4 pathway, it is reasonable that ATF4 mediates the osteogenic effect of *HDAC6* inhibition in VICs. Indeed, our data show that ATF4 silencing attenuates osteogenic pathway activation induced by *HDAC6* suppression.

Runx2 is a transcription factor that is required for osteoblast differentiation. It has been confirmed that *Runx2* binds with osteoblast-specific cis-acting element, termed osteoblast-specific cis-acting element, to activate downstream osteoblast-related genes.²⁸ Previous studies

report *Runx2* plays a critical role in smooth muscle cell osteogenic phenotype change and mineral deposition.²⁹ Our previous report also shows that *Runx2* is essential in the development of AoV calcification.¹⁰

Our study shows that *HDAC6* inhibition promotes AoV calcification, suggesting that activation of *HDAC6* may prevent AoV calcification. However, inhibition of *HDAC6* inhibits cardiac fibrosis,³⁰ and reduces reactive oxygen species generation and confers a protective effect against myocardial ischemia/reperfusion or hypoxia/reoxygenation injury.³¹ Inhibition of *HDAC6* also suppresses endothelial cell migration and angiogenesis,³² and prevents endothelial apoptosis.³³ These data indicate the differential role of *HDAC6* in various cell types.

HDACs, including *HDAC6* inhibitors, have been applied in clinical trials for their anticancer effects.^{19,23} As our study implicates, *HDAC6* inhibition is associated with

FIGURE 7. Proposed mechanism of histone deacetylase 6 (*HDAC6*) suppression-triggered osteoblastic differentiation of valvular interstitial cells (*VIC*). *HDAC6* inhibition leads to acetylation of glucose-regulated protein 78 (*GRP78*), and subsequently induces endoplasmic reticulum (*ER*) stress. Activating transcription factor 4 (*ATF4*) activation downstream of *ER* stress further induces the expression of runt-related transcription factor 2 (*Runx2*), which promotes the osteoblastic differentiation of *VIC*. *siRNA*, Small interfering RNA; *ATF6*, activating transcription factor 6. *PERK*, protein kinase-like ER kinase; *p-PERK*, phosphorylated protein kinase-like ER kinase; *IRE1α*, inositol-requiring transmembrane kinase and endonuclease-1α; *p-IRE1α*, phosphorylated inositol-requiring transmembrane kinase and endonuclease-1α; *p-eif2α*, eukaryotic initiation factor 2α; *shRNA*, short hairpin RNA.

AoV calcification, so these agents might be cautiously introduced in patients with CAVD and conditions that may lead to CAVD such as uremia.³⁴ However, the side effects of *HDAC6* inhibitors on AoV calcification need to be further explored in clinical trials.

Our study has some limitations. First, the sample size is small in the human study. We used our data, set alpha as 0.05 and power as 0.8, to calculate the estimated sample size. The results show that the sample size in the study was enough (immunohistochemical study: control group mean, 28.71 ± 6.29 ; CAVD group mean, 14.07 ± 2.39 [lowest $n = 2$ for each group]; Western blot analysis: control group mean, 1.000 ± 0.404 ; CAVD group mean, 0.393 ± 0.096 [lowest $n = 4$ for each group]). However, future studies with a large sample size are needed to confirm the association between *HDAC6* levels and the degree of AoV calcification. In addition, the ideal control AoVs should be AoVs from patients who died as a result of head trauma or stroke. The AoVs in our control group may not be the true control because they had moderate aortic insufficiency. Another limitation is that we only used human VICs to explore the mechanisms, although

VIDEO 1. The description of the conclusion and significance of the study. Video available at: [https://www.jtcvs.org/article/S0022-5223\(18\)32930-1/fulltext](https://www.jtcvs.org/article/S0022-5223(18)32930-1/fulltext).

viability and stability of the cells may influence the results. Further in vivo study is required.

CONCLUSIONS

The present study provides evidence that *HDAC6* is associated with AoV calcification. *HDAC6* inhibition leads to osteoblast differentiation of VICs via *ER* stress/*ATF4*-mediated osteogenic pathway. The proposed mechanism is outlined in Figure 7 and Video 1. Our study results indicate that *HDAC6* may be a novel target against AoV calcification.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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FIGURE E1. Different concentration of tubacin treatment in valvular interstitial cells (VIC). Treatment with 10 μ M tubacin treatment significantly increased expressions of endoplasmic reticulum (ER) stress markers phosphorylated protein kinase-like ER kinase (*p-PERK*), phosphorylated inositol-requiring transmembrane kinase and endonuclease-1 α (*p-IRE1 α*), phosphorylated eukaryotic initiation factor 2 α (*p-eif2 α*), activating transcription factor 4 (*ATF4*), and *Runx2* expression in VIC. Treatment with 20 μ M tubacin significantly induced *p-IRE1 α* , *p-eif2 α* , and *ATF4* expression, but had no significant effect on *p-PERK* and *Runx2* in VIC. A, Western blot of ER stress markers (*p-IRE1 α* , *p-PERK*, *p-eif2 α* , and *ATF4*) and *Runx2* after different concentration of tubacin treatment in VIC. B, Statistical analysis of *Runx2* protein expression after different concentration of tubacin treatment in VIC. C, Statistical analysis of ER stress markers (*p-IRE1 α* , *p-PERK*, *p-eif2 α* , and *ATF4*) after different concentration of tubacin treatment in VIC. ns, No significance. * $P < .05$ versus dimethyl sulfoxide (DMSO). *Runx2*, Runt-related transcription factor 2.

FIGURE E2. Inhibition of histone deacetylase 6 (*HDAC6*) has no effect on inducing activating transcription factor 6 (*ATF6*) expression in valvular interstitial cells (VIC). A, Tubacin treatment (10 μ M) did not induce *ATF6* expression in VIC. B, Silencing of *HDAC6* did not induce *ATF6* expression in VIC. DMSO, Dimethyl sulfoxide; scr siRNA, scrambled small interfering RNA; ns, no significance.

FIGURE E3. The effect of tubacin in valvular interstitial cells (VIC) from patients with calcified aortic valve disease (CAVD) and normal controls. Tubacin treatment (10 μ M) significantly induced endoplasmic reticulum (ER) stress markers phosphorylated protein kinase-like ER kinase (*p-PERK*) and activating transcription factor 4 (*ATF4*), as well as *Runx2* and alkaline phosphatase (*ALP*) expression in VIC from calcified aortic valves and in normal controls. *ATF4*, *p-PERK*, *Runx2*, and *ALP* expressions were significantly increased in VIC from patients with CAVD compared with controls. The effect was more profound in treated VIC from the normal controls. **P* < .05 versus normal VIC + dimethyl sulfoxide. #*P* < .05 versus CAVD VIC + dimethyl sulfoxide. *Runx2*, Runt-related transcription factor 2; *DMSO*, dimethyl sulfoxide.