

# Sestrin2 Is Increased in Calcific Aortic Disease and Inhibits Osteoblastic Differentiation in Valvular Interstitial Cells via the Nuclear Factor E2–related Factor 2 Pathway

Haixiong Wang, MD,\* Jicheng Xi, MD,\* Zhibiao Zhang, MD,\* Jun Li, MD,\* Liping Guo, MD,\* Na Li, MD,\* Yuehui Sun, MD,\* Xiaofang Li, MD,† and Xuebin Han, MD\*

**Abstract:** Sestrin2 (Sesn2) is involved in the progression of cardiovascular diseases, such as hypertension and myocardial infarction. This study aimed to examine Sesn2 expression in human calcific aortic valve disease (CAVD) and explore its possible mechanisms by which Sesn2 participates in this process. CAVD and normal aortic valves were collected. Sesn2 expression and sources were examined, and the results showed that Sesn2 expression was increased in aortic valves from patients with CAVD and was mainly secreted by macrophages. Additionally, U937 macrophages were pretreated with si-Sesn2 or cDNA-Sesn2 and further treated with oxidized low-density lipoprotein (ox-LDL); M1 macrophages and their markers were measured, and we found that pretreatment with si-Sesn2 increased ox-LDL–induced M1 macrophage polarization and marker mRNA levels, whereas pretreatment with cDNA-Sesn2 had the opposite effects. In ox-LDL–treated U937 macrophages, oxidative stress levels were increased in the si-Sesn2 pretreatment group and further increased by si-Nrf2 treatment, whereas oxidative stress levels were decreased in the cDNA-Sesn2 pretreatment group and significantly reversed by ML385, a specific Nrf2 inhibitor. The effects of Sesn2 on ox-LDL–induced oxidative stress and the osteogenic differentiation of ox-LDL–induced valvular interstitial cells (VICs) was examined by down-regulating Nrf2 pathway. When U937 macrophages were

co-cultured with VICs, downregulation of Sesn2 increased ox-LDL–induced osteogenic differentiation in VICs, whereas overexpression of Sesn2 exerted the opposite effects. Our study suggests that Sesn2 is increased in CAVD aortic valves and may participate in the development of CAVD by regulating oxidative stress via the Nrf2 pathway.

**Key Words:** calcific aortic disease, Sestrin2, M1 macrophage polarization, oxidative stress, valvular interstitial cell osteoblastic differentiation

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## INTRODUCTION

Calcific aortic valve disease (CAVD) is a common public health problem in elderly individuals in both developed and developing countries, and valve calcification is the main clinical feature.<sup>1</sup> Studies have shown that CAVD can result in more than 23,000 deaths per year in the United States, and although the number is lower in China, its incidence is still rising.<sup>2,3</sup> To date, no drugs have been found to delay the progression or improve the prognosis of CAVD. Aortic valve replacement may be the only effective treatment, but it is expensive and difficult for older patients to tolerate.<sup>4</sup>

Sestrin2 (Sesn2) is an antioxidant protein that has been reported to be widely expressed in many organs and tissues in mammals. Evidence has confirmed that although both immune cells and nonimmune cells can secrete Sesn2, immune cells are still the main sources of Sesn2, especially macrophages.<sup>5–8</sup> The expression of Sesn2 can be regulated by a variety of pathological factors, especially the oxidative stress.<sup>5,6</sup> Previous studies reported that Sesn2 expression is positively correlated with oxidative stress levels, suggesting that Sesn2 levels increase when oxidative stress levels increase.<sup>5,6</sup> Sesn2 can regulate many systemic diseases by regulating oxidative stress levels.<sup>9–11</sup>

Data from animal studies and clinical experiments have shown that Sesn2 can participate in the progression of a variety of cardiovascular diseases. Yang et al<sup>12</sup> reported that deletion of the dopamine D2 receptor could increase blood pressure in mice, and silencing of Sesn2 could further elevate blood pressure. In a mouse heart ischemia–reperfusion model, knockout or downregulation of Sesn2 expression significantly increased the myocardial infarction areas.<sup>13</sup> In a mouse model of cardiac fibrosis induced by ultraviolet radiation, Sesn2

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From the \*Department of Cardiology, Shanxi Cardiovascular Hospital, Taiyuan, Shanxi, China; and †Department of Digestive Oncology, Shanxi Bethune Hospital, Taiyuan, Shanxi, China.

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H. Wang and J. Xi contributed equally to this work.

Correspondence: Xuebin Han, Department of Cardiology, Shanxi Cardiovascular Hospital, Taiyuan, Shanxi, China (e-mail: [hanxuebin669799@163.com](mailto:hanxuebin669799@163.com)) or Xiaofang Li, Department of Digestive Oncology, Shanxi Bethune Hospital, Taiyuan, Shanxi, China (e-mail: [1xf838428781@163.com](mailto:1xf838428781@163.com)).

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deficiency further increased cardiac fibrosis marker expression.<sup>14</sup> Liu et al reported that *Sesn2* was increased in both the plasma and the aortas of aortic dissection patients and may participate in aortic dissection by reducing vascular smooth muscle cell apoptosis via the Nrf2 pathway.<sup>6</sup> In our previous study, plasma *Sesn2* levels were increased in chronic heart failure patients and were closely related to prognosis.<sup>15</sup> Until now, the role of *Sesn2* in CAVD has not been reported. In this study, we examined *Sesn2* expression in CAVD and explored the possible mechanisms.

## MATERIALS AND METHODS

### Collection of Aortic Valve Tissue

Diseased and normal aortic valves were collected from patients with CAVD ( $n = 20$ ) and patients undergoing heart transplantation in the vascular surgery department ( $n = 10$ ) of the Shanxi Cardiovascular Hospital from 2016 to 2019, respectively. No donors had aortic valve abnormalities caused by other diseases, including congenital diseases, rheumatic heart disease, or endocarditis. All aortic valves were divided into 2 parts and stored in 4% paraformaldehyde and liquid nitrogen for further examination. This study complied with the Declaration of Helsinki. The families of donors signed informed consent forms, and this study was approved by the Ethics Committee of Shanxi Cardiovascular Hospital (approval no. DC.W.2015-11). The clinical characteristics are shown in **Supplemental Digital Content 1** (see **File**, <http://links.lww.com/JCVP/A839>).

### Cell Culture, Transfection, and Treatment

Valvular interstitial cells (VICs) and human U937 monocytes were used in this study. VICs were isolated as previously described.<sup>16</sup> In brief, fresh normal aortic valves were transferred to ultraclean benches and washed with 4°C PBS. Then, the aortic valves were cut into small pieces and digested repeatedly with 1.5% collagenase until completely digested. Then, after being neutralized with FBS, all cells were collected and centrifuged for 10 minutes at 1000g. Furthermore, the cells were cultured in DMEM with 10% FBS, and streptomycin and penicillin G were also added. Human U937 monocytes were purchased from the Cell Center of Wuhan University and cultured in DMEM containing 10% FBS. U937 monocytes were treated with 50 ng/mL macrophage colony-stimulating factor (M-CSF; PeproTech, Cat No.: 300-25-10ug) for 9 days to promote their differentiation into macrophages. In this study, VICs and U937 macrophages at passages 4–6 were used for subsequent experiments.

Small interfering RNA (siRNA) against *Sesn2* (si-Sesn2) was transfected into U937 macrophages to decrease *Sesn2* expression using Lipofectamine 2000 (RiboBio, Guangzhou) according to the manufacturer's instructions, and the sequences were described in a previous study.<sup>7</sup> Complementary DNA (cDNA) targeting *Sesn2* (cDNA-Sesn2; RiboBio, Guangzhou) was used to upregulate *Sesn2* expression in U937 macrophages, and the sequences were derived from a previous article.<sup>17</sup> In this study, si-Sesn2

reduced *Sesn2* mRNA expression by 71% in U937 macrophages, whereas cDNA-Sesn2 increased *Sesn2* mRNA levels by 1.2-fold. The results are shown in **Supplemental Digital Content 2** (see **Fig. 2**, <http://links.lww.com/JCVP/A839>).

First, U937 macrophages were divided into 5 groups, cultured in calcification medium with 10% FBS, and then pretreated with PBS, siRNA (negative control for si-Sesn2), si-Sesn2, cDNA (negative control for cDNA-Sesn2), and cDNA-Sesn2. Then, U937 macrophages were treated with 100  $\mu$ g/mL of oxidized low-density lipoprotein (ox-LDL; AngYuBio, Cat No.: AY-1501). Twenty-four hours later, U937 macrophages were collected, and the expression of M1 macrophages and their related markers, as well as Nrf2 levels, were examined. In addition, some of U937 macrophages that were treated with si-Sesn2 and cDNA-Sesn2 were further administered ox-LDL and 2  $\mu$ M ML385 (MedChemExpress, Cat No.: HY-100523), a specific Nrf2 pathway inhibitor.<sup>18</sup> Then, oxidative stress was measured, including ROS levels, total SOD activity, GSH levels, and MDA levels. Finally, U937 macrophages were pretreated with si-Sesn2, cDNA-Sesn2, or ML385 and then cocultured with VICs for 24 hours. After treated with PBS or ox-LDL, the mineralization of VICs was examined.

### Western Blot Analysis

Aortic valve tissue and human U937 macrophages were lysed using RIPA lysis buffer, and total proteins were obtained and adjusted to the same concentration. Total proteins were added to a 10% SDS polyacrylamide gel to electrophoretically separate proteins by their different molecular weights, and all proteins were then transferred to Immobilon-FL PVDF membranes. After being blocked with 3% BSA for 90 minutes at room temperature, the membranes were incubated with primary antibodies for 2 hours at 37°C and with secondary antibodies for 1 hour at room temperature. Then, the expression of target proteins was examined by Odyssey. The target proteins in aortic valve tissue were *Sesn2* (GeneTex, Cat No.: GTX116925, 1:1500) and GAPDH (GeneTex, Cat No.: GTX100118, 1:5000), and the target proteins in human U937 monocytes were Nrf2 (GeneTex, Cat No.: GTX103322, 1:1500) and GAPDH.

### Immunofluorescence Staining

Fresh aortic valve tissue was fixed immediately in 4% paraformaldehyde, cut into 4- to 5- $\mu$ m-thick sections after paraffin embedding, and then transferred onto slides. To measure *Sesn2* levels in aortic valve tissue, slides were immunofluorescently stained with *Sesn2* antibodies (Abcam, Cat No.: ab236025, 1:150). In addition, double immunofluorescence staining with *Sesn2* and F4/80 antibodies (GeneTex, Cat No.: GTX26640, 1:100) was used to determine whether *Sesn2* was derived from macrophages. The same methods were used to examine CD86 expression in U937 monocytes and the osteoblastic differentiation of VICs.

### Flow Cytometry

U937 macrophages were washed with PBS 3 times and collected. Then, U937 macrophages were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human F4/

80 (Abcam, Cat No.: ab60343, 1:100) and phycoerythrin-labeled (PE)-conjugated anti-human CD86 antibodies (eBioscience, Cat No.: 12-0809-42, 0.5 μg/test) for 30 minutes at room temperature away from the light. In addition, for compensation and to confirm antibody specificity, some U937 cells were incubated with isotype controls. Therefore, macrophages were defined as F4/80<sup>+</sup>CD68<sup>+</sup>. Both the antibodies were purchased from BD Sciences.

### Examination of Oxidative Stress

The treated U937 macrophages were collected, washed, and then stained with 5 μmol/L of DCFH-DA (Sigma, Cat No.: D6883) in the dark at 37°C for 45 minutes. Furthermore, the fluorescence intensity, which represents reactive oxygen species (ROS) levels, was visualized using a fluorescence microscope. In addition, the supernatant was collected, and total SOD activity (Cat No.: S0109), GSH levels (Cat No.: S0073), and MDA levels (Cat No.: 0131S) (Beyotime Sciences, China) were measured according to the experimental procedures provided by the supplier.

### Quantitative Real-time PCR

Treated U937 macrophages were collected, aortic valve tissue was lysed using TRIzol reagent W, and total RNA was collected. A total of 2 μg of total RNA was synthesized into cDNA using a reverse transcription kit according to the manufacturer's instructions. Furthermore, the expression of target mRNAs was examined using polymerase chain reaction amplifications with LightCycler 480 SYBR Green Master Mix (Roche, Germany). The target mRNAs included CD38, CD80, CD86, inducible nitric oxide synthase (iNOS), osteopontin (OPN), alkaline phosphatase (ALP), and runt-related transcription factor 2 (Runx2), and the results were normalized to GAPDH levels. The primers used in this study are shown in **Supplemental Digital Content 2** (see **Table 2**, <http://links.lww.com/JCVP/A839>).

### Statistical Analysis

The data in this study are expressed as the mean ± SD. The differences in the mean values between 2 groups were compared with Student's *t* test, and differences in the mean values of 3 or more groups were compared with one-way analysis of variance, followed by Tukey's multiple

comparisons test. When the *P* value was less than 0.05, the difference was considered to be statistically significant.

## RESULTS

### Sesn2 Expression is Increased in CAVD Patients

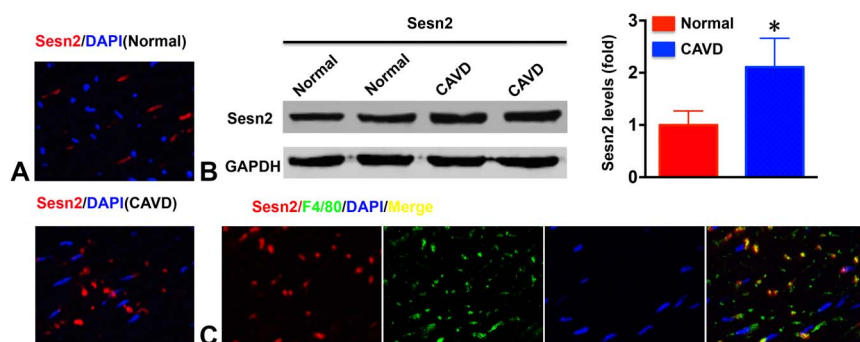
Sesn2 expression was first examined, and the western blot and immunofluorescence results showed that the Sesn2 levels increased approximately 1.1-fold in the CAVD group compared with the normal group (Figs. 1A, B). The double immunofluorescence staining results showed that Sesn2 was highly expressed in the aortic valve macrophages of patients with CAVD (Fig. 1C). The mRNA expression levels of iNOS, Sesn2, ALP, Runx2, and OPN were increased in patients with CAVD (Fig. 2A). In addition, the mRNA levels of Sesn2, ALP, Runx2, and OPN were positively correlated with iNOS mRNA expression in patients with CAVD (Fig. 2B).

### Sesn2 Attenuates ox-LDL-induced M1 Macrophage Polarization In Vitro

Flow cytometry showed that neither siRNA nor cDNA affected M1 polarization in ox-LDL-induced U937 macrophages. si-Sesn2 significantly increased the percentage of ox-LDL-induced U937 macrophages, whereas cDNA-Sesn2 exerted the opposite effect (Fig. 3A). Similar trends for CD86 intensity were observed in M1 macrophages (Fig. 3B). In addition, pretreatment with si-Sesn2 increased the mRNA expression levels of CD38, CD80, CD86, and iNOS, whereas pretreatment with cDNA-Sesn2 reduced the mRNA expression of these genes (Fig. 3C).

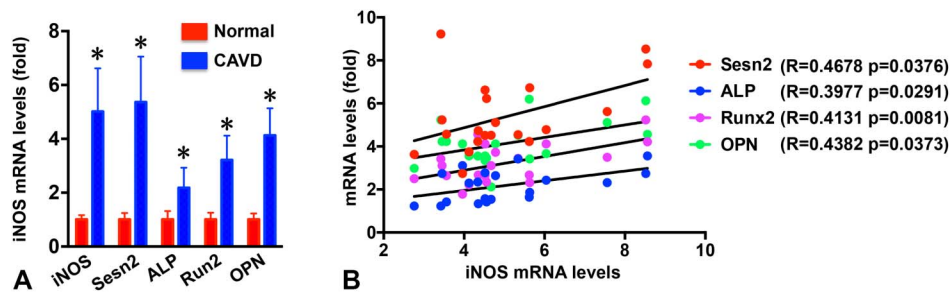
### Sesn2 Alleviates M1 Macrophage-related Oxidative Stress Induced by ox-LDL In Vitro

The effect of Sesn2 on Nrf2 expression was measured, and the results showed that pretreatment with si-Sesn2 decreased Nrf2 expression, whereas pretreatment with cDNA-Sesn2 increased Nrf2 levels in ox-LDL-induced U937 macrophages (Fig. 4A). In addition, treatment with si-Sesn2 increased ox-LDL-induced ROS and MDA levels and decreased SOD activity and GSH levels, whereas treatment with cDNA-Sesn2 decreased ROS and MDA levels and



**FIGURE 1.** Sesn2 levels in aortic valves from patients with CAVD. A and B, Sesn2 expression in normal donors and patients with CAVD was examined by immunofluorescence staining (×400) and western blotting. C, Double immunofluorescence staining with anti-Sesn2 and anti-F4/80 antibodies (×400). N = 10 for the normal group and 20 for the CAVD group. \**P* < 0.05 versus the normal group.

**FIGURE 2.** iNOS mRNA levels and expression of osteoblastic differentiation markers (VICs) in CAVD. A, The mRNA expression levels in the 2 groups were examined by RT-qPCR. B, Correlation between iNOS mRNA levels and *Sesn2*, ALP, Runx2, and OPN mRNA levels. N = 10 for the normal group and 20 for the CAVD group. \**P* < 0.05 versus the normal group.



increased SOD activity and GSH levels (Figs. 4B, C). When the Nrf2 pathway was inhibited by ML385, further ROS and MDA production and lower SOD activity and GSH levels were observed in both the si-Sesn2 and the cDNA-Sesn2 groups (Fig. 4B, C).

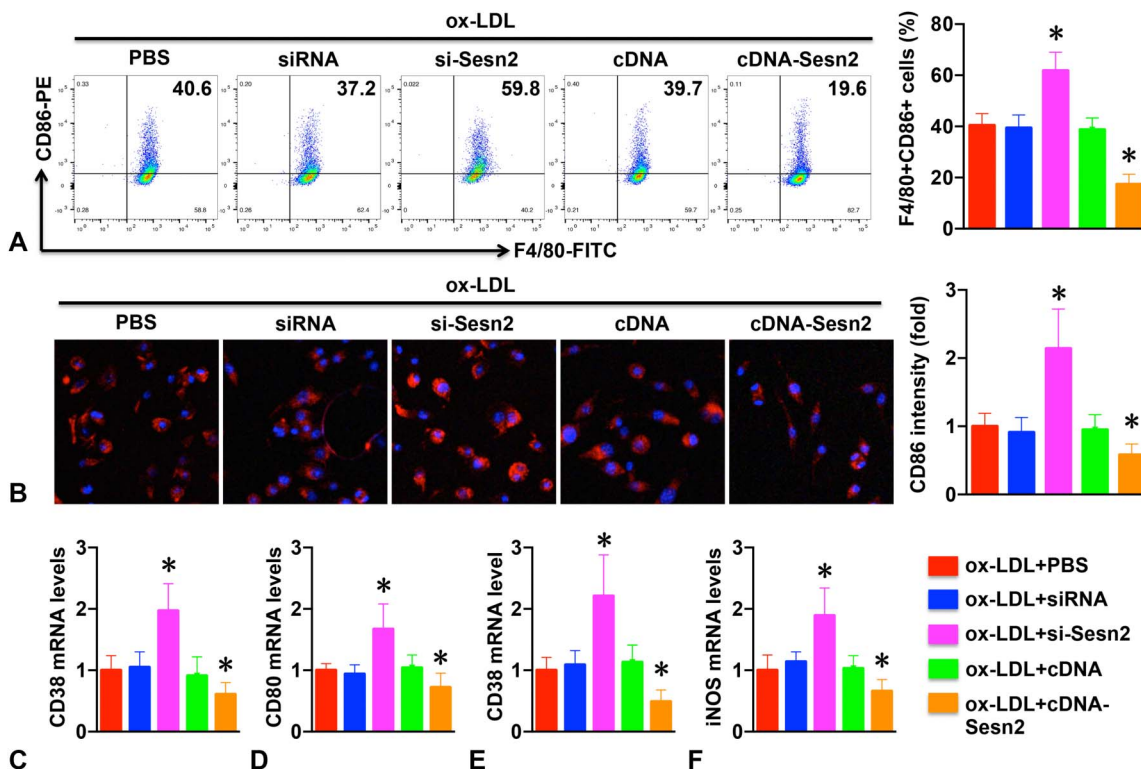
### Sesn2 Inhibits ox-LDL-induced Mineralization in VICs In Vitro

Coculture with macrophages had no effect on ALP, Runx2, or OPN mRNA expression in VICs with or without si-Sesn2 or cDNA-Sesn2 in the absence of ox-LDL (Fig. 5). The mRNA levels of ALP, Runx2, and OPN were increased by ox-LDL and further elevated when si-Sesn2 was also present but were decreased by cDNA-Sesn2 (Fig. 5). The effects

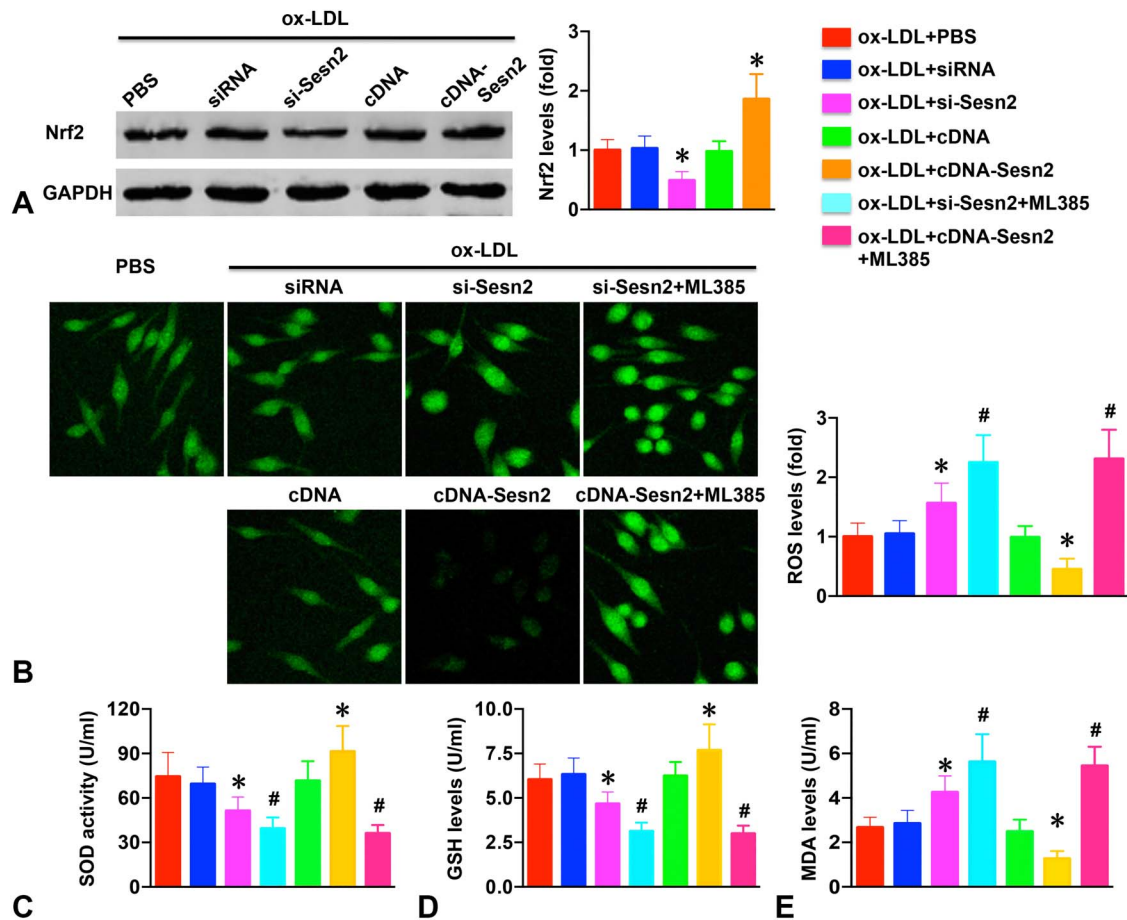
of si-Sesn2 and cDNA-Sesn2 on ALP, Runx2, and OPN were reversed by ML385 (Fig. 5).

### DISCUSSION

CAVD mostly occurs in elderly individuals older than 65 years, and the incidence rate increases with age. CAVD is the third major cause of chronic heart failure in elderly individuals after hypertension and coronary heart disease.<sup>19,20</sup> CAVD is one of the most serious public health problems worldwide, greatly reducing the quality of life of patients and even threatening their lives. At present, there is no effective drug to delay the progression of CAVD, and the only effective treatment is aortic valve replacement, which has very strict indications.<sup>19,20</sup>



**FIGURE 3.** Effect of *Sesn2* on ox-LDL-induced M1 macrophage polarization in vitro. A, F4/80+CD86+ cells were examined in the 5 groups by flow cytometry. B, CD86 intensity was measured using immunofluorescence staining. C, The mRNA expression levels of CD38, CD80, CD86, and iNOS were investigated by RT-qPCR. N = 6 in each group. \**P* < 0.05 versus the ox-LDL + siRNA or cDNA group.



**FIGURE 4.** Effect of Sesn2 on M1 macrophage-related oxidative stress in vitro. A, Nrf2 levels in each group were measured by western blotting. B, ROS levels were examined using DCFH-DA. C, SOD activity, MDA levels, and GSH levels were analyzed. N = 6 in each group. \* $P < 0.05$  versus the ox-LDL + siRNA or cDNA-Sesn2 group. # $P < 0.05$  versus the ox-LDL + si-Sesn2 or cDNA-Sesn2 group.

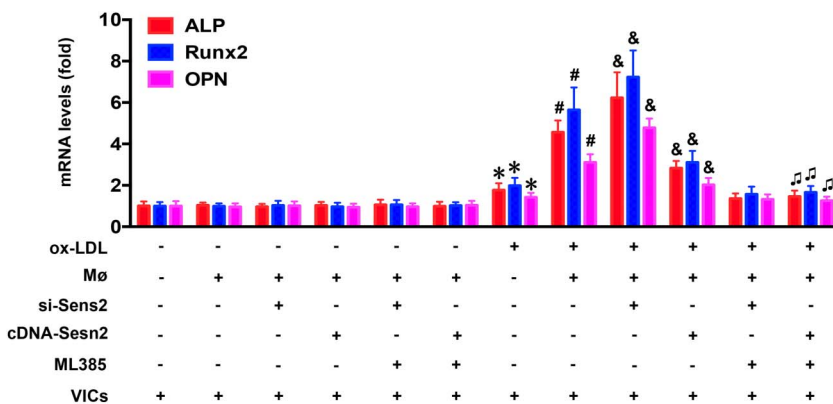
Therefore, it is important to clarify the pathogenesis of CAVD and find intervention targets to delay its progression. In this study, we found that oxidative stress may be involved in the progression of CAVD, and Sesn2 may delay its progression by reducing oxidative stress, suggesting that Sesn2 may have some value in delaying CAVD progression.

In previous studies on the pathogenesis of CAVD, researchers mostly focused on the osteogenic response of VICs caused by the inflammatory response.<sup>20</sup> It was reported that the infiltration of inflammatory factors, including IL-6, IL-21, IL-32, and IL-37, was significantly increased in the aortic valves of human patients with CAVD.<sup>16,21–23</sup> Data from clinical experiments have shown that the proinflammatory factors IL-6 and IL-21 can promote the osteogenic response in VICs in vitro, which may promote the progression of CAVD, whereas the anti-inflammatory factor IL-37 has the opposite effect.<sup>16,21–23</sup> Data from animal studies showed that the anti-inflammatory factor IL-37 alleviated the inflammatory response, prevented high-fat diet-induced aortic valve osteogenesis, and delayed the progression of CAVD in mice.<sup>24</sup> However, little attention has been given to the role of oxidative stress in CAVD. Our study showed for

the first time that the expression of Sesn2, an antioxidant protein, was significantly increased in the aortic valves of patients with CAVD and was mainly produced by aortic valve macrophages, which is consistent with previous reports. We also found that the mRNA levels of iNOS, an M1 macrophage marker, were positively correlated with both Sesn2 levels and VIC osteoblastic differentiation markers. This result suggests that Sesn2 may be involved in regulating CAVD by regulating M1 macrophage polarization.

Clinically, CAVD is considered to be a biological process that is distinct from atherosclerosis. However, during the progression of CAVD, aortic valve endothelial injury, lipid infiltration and deposition, and the recruitment, infiltration, and polarization of macrophages and T lymphocytes in aortic valves were observed successively, which eventually led to aortic valve fibrosis and calcification. When these injuries are serious, they can cause hemodynamic changes.<sup>20,24,25</sup> In addition, CAVD is considered to have the same clinical risk factors as arteriosclerosis, such as dyslipidemia, smoking, and old age.<sup>20</sup> Therefore, ox-LDL can be used to simulate the pathological process of CAVD and has been used in animal experiments.<sup>24</sup> ox-

**FIGURE 5.** Effects of the Nrf2 pathway on osteoblastic differentiation of VICs. The mRNA expression levels of ALP, Runx2, and OPN in VICs were measured. N = 6 in each group. \*P < 0.05 versus the VICs group. #P < 0.05 versus the ox-LDL + VICs group. &P < 0.05 versus the ox-LDL + Mø group. ♪P < 0.05 versus the ox-LDL + Mø + si-Sesn2 + ML385 group.



LDL was used to induce the polarization of macrophages, and the effect of Sesn2 on macrophage polarization was examined. The results showed that Sesn2 could significantly reduce the expression of a variety of M1 macrophage markers and the percentage of F4/80+CD86+ cells. These results suggest that Sesn2 can inhibit the polarization of M1 macrophages.

Modern medicine divides macrophages activated by pathological factors into 2 categories: M1 macrophages, which have pro-inflammatory effects, and M2 macrophages, which have anti-inflammatory effects. An increasing number of studies have confirmed that macrophages can also play a strong role in the regulation of oxidative stress, and these cells are one of the main sources of oxidative stress.<sup>26,27</sup> Compared with M2 macrophages, M1 macrophages play a leading role in a variety of cardiovascular diseases, including hypertension and cardiac injury.<sup>26,27</sup> Data from clinical studies confirmed that an imbalance in M1 and M2 macrophages was present in the aortic valves of patients with CAVD, suggesting that M1 macrophage-mediated oxidative stress may be involved in the progression of CAVD.<sup>28</sup> Therefore, the effects of Sesn2 on M1 macrophage-mediated oxidative stress were examined, and the results showed that the upregulation of Sesn2 significantly reduced ox-LDL-induced M1 macrophage polarization-induced oxidative stress, suggesting that Sesn2 participates in the progression of CAVD by reducing M1 macrophage-related oxidative stress.

Nrf2 is an important signaling pathway that regulates oxidative stress. Nrf2 activation can induce the downstream HO-1 signaling pathway, inhibit the expression of Nox2 and Nox4, inhibit the expression of oxidants, and promote the expression of antioxidants. Therefore, Nrf2 is thought to be an antioxidant signaling pathway. In a previous study, Xiao et al<sup>6</sup> reported that the effect of upregulated Sesn2 expression on angiotensin II-induced oxidative stress was significantly reversed by silencing the Nrf2 pathway. In another study, Wang et al found that the overexpression of Sesn2 significantly improved neuronal injury induced by cerebral ischemia-reperfusion, and the protective effect of Sesn2 on neural cells was reversed by silencing Nrf2.<sup>29</sup> This evidence suggests that Sesn2 may regulate downstream signals by activating the Nrf2 pathway. In our study, we found that

treatment with si-Sesn2 and cDNA-Sesn2 decreased Nrf2 levels and Nrf2 expression, respectively, suggesting that Sesn2 may regulate ox-LDL-induced oxidative stress via the Nrf2 pathway. Next, the Nrf2 pathway was inhibited by ML385, and oxidative stress levels were increased significantly. These results suggest that Sesn2 regulates ox-LDL-mediated oxidative stress through the Nrf2 pathway.

VICs are the most important component of the aortic valve, and their normal function is very important for maintaining the morphology and function of the aortic valve. Osteoblastic differentiation can occur in response to pathological factors such as inflammation and hypoxia, and a large amount of ALP, Runx2, and OPN can be produced, which is a key step in the occurrence of CAVD.<sup>16,22,23</sup> Treatment with ox-LDL reduced the mRNA expression of ALP, Runx2, and OPN, which suggests that enhanced oxidative stress may be an important factor in the occurrence of CAVD. Sesn2 also reduces the mineralization of VICs, and this effect was reversed by ML385. These results demonstrate that Sesn2 can alleviate CAVD progression through the Nrf2 pathway by reducing M1 macrophage-related oxidative stress.

In conclusion, our study showed that Sesn2 inhibited the polarization of M1 macrophages and alleviated oxidative stress and VICs osteoblastic differentiation through the Nrf2 pathway to delay the progression of CAVD. Sesn2 may be a target for CAVD prevention.

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