Article

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A Role for Bromodomain Containing Protein 4 in Aortic Valvular Interstitial Cells

Qinzheng Wang[1], Lijuan Zong[2], Zhanhui Du[2], Ping Yang[1], Linghan Meng[3], Xinmeng Yang[4], Quansheng Xing[3,*]

[1]Qingdao Medical College, Qingdao University, 266071 Qingdao, Shandong, China
[2]Birth Defect Prevention and Control Centre of Qingdao, Qingdao Women and Children's Hospital Affiliated to Qingdao University, 266034 Qingdao, Shandong, China
[3]Department of Cardiac and Great Vascular Surgery, Peking University People's Hospital, Qingdao, 266111 Qingdao, Shandong, China
[4]Heart Center, Qingdao Women and Children's Hospital Affiliated to Qingdao University, 266034 Qingdao, Shandong, China
*Correspondence: xingqs0214@163.com (Quansheng Xing)
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Background: Calcific aortic valve disease (CAVD) is a common valvular heart condition globally. Bromodomain-containing protein 4 (BRD4) is strongly associated with the development of various cardiovascular pathologies, most notably atherosclerosis (AS). Since CAVD and AS exhibit striking similarities in risk determinants, it is increasingly hypothesized that early-stage pathological changes in both diseases may converge on shared molecular mechanisms. To date, however, no functional or mechanistic data have been reported regarding BRD4's contribution to CAVD. This study aimed to elucidate the functional role of BRD4 in osteogenic differentiation of aortic valvular interstitial cells (AVICs).

Methods: We first detected BRD4 and associated protein levels in aortic valve tissues of patients with CAVD or aortic regurgitation (AR) who underwent aortic valve replacement using immunohistochemistry (IHC) and Western blot (WB) analyses. Then AVICs were isolated from AR patient valves and cultured with complete medium (CM) and osteogenic medium (OM) for *in vitro* validation. To determine whether BRD4 inhibition alleviates osteogenic differentiation and calcification of AVICs, we knocked down BRD4 expression or applied the BRD4 inhibitor JQ1. The impact of BRD4 on oxidative stress, inflammatory response, and apoptosis in AVICs was assessed using WB, reactive oxygen species (ROS) assays, and Enzyme-linked immunosorbent assay (ELISA). To elucidate the mechanism of BRD4 action, we employed inhibitors targeting relevant signaling pathways.

Results: BRD4 was significantly upregulated in calcific aortic valves using IHC and WB experiments ($p < 0.001$). BRD4 inhibition attenuated calcification of AVICs in osteogenic medium, accompanied by a decrease in runt-related transcription factor 2 (Runx2) ($p < 0.001$) and osteopontin (OPN) ($p < 0.001$). Further investigations demonstrated that BRD4 suppression reduced ROS generation ($p < 0.01$), lowered secretion of inflammatory cytokines (IL-6, IL-8, and MCP-1) ($p < 0.001$), and diminished expression of the pro-apoptotic protein Bax ($p < 0.05$) in AVICs. Mechanistically, in OM-treated AVICs, the down-regulation of BRD4 increased PI3K ($p < 0.05$) and p-AKT ($p < 0.01$) levels and reduced the expression of Runx2 ($p < 0.01$), OPN ($p < 0.01$), and Bax ($p < 0.001$), while the AKT inhibitor reversed this scenario.

Conclusions: BRD4 promotes the calcification of AVICs *in vitro* via the PI3K/AKT signaling pathway. This research represents the initial investigation into the function of BRD4 in valvular calcification, indicating its potential as a therapeutic target for CAVD.

Keywords: BRD4; calcific aortic valve disease; valve interstitial cells; calcification; PI3K/AKT pathway

Introduction

Calcific aortic valve disease (CAVD) is a common heart valve condition distinguished by gradual development of fibrosis and calcification within the leaflets of the aortic valve [1]. The incidence of CAVD rises significantly with aging [2]. In the context of the global population aging trend, epidemiological data suggest that an estimated over 9.4 million individuals worldwide are afflicted with CAVD [3]. Despite significant advancements in CAVD research over the last two decades, there remains a lack of efficacious pharmaceutical interventions capable of attenuating or reversing disease progression [4,5]. In the case of advanced-stage patients with critical aortic stenosis due to CAVD, surgical or transcatheter aortic valve replacement represents an effective therapeutic approach [6].

The aortic valve interstitial cells (AVICs), which serve as the core components of the aortic valve, are disseminated throughout the fibrous layer adjacent to the aorta, the middle spongy layer, and the ventricular layer in proximity to the left ventricle [7]. Within these three tissue layers, the fibrous layer harbors a substantial quantity of colla-

gen, the spongy layer consists of loose connective tissue abundant in glycosaminoglycans and proteoglycans, and the ventricular layer contains a relatively high number of elastic fibers [8]. AVICs, along with various elements of the extracellular matrix, are critically involved in the onset and development of CAVD. The transition of AVICs from quiescent fibroblast-like cells to myofibroblast-like cells and osteoblast-like cells represents the primary etiology of valve calcification [9,10]. Osteoblast-like AVICs can upregulate the expression of markers such as alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), and BMP2, which subsequently triggers calcium deposition and bone formation [11–14].

As a key member of the bromodomain and extraterminal domain (BET) protein family—which comprises BRD2, BRD3, and BRDT—Bromodomain-containing protein 4 (BRD4) is critically involved in decoding epigenetic signals by precisely and efficiently recognizing the acetylated lysine residues on histones H3 and H4 to mediate protein-protein interactions and regulate the activity of relevant transcription factors [15,16]. BRD4 ubiquitously exists in virtually all tissues, predominantly localized within the cell nucleus [17]. BRD4 can interact with CDK9 and CyclinT1, which leads to the liberation of the inhibitory section of positive transcription elongation factor b (P-TEFb), and recruits P-TEFb via the CTD to phosphorylate RNA polymerase II at the transcription start site, thus facilitating transcriptional elongation [18]. BRD4 has been intensively investigated in the context of cardiovascular diseases. In pulmonary arterial smooth muscle cells, BRD4 inhibition has been shown to downregulate tumor necrosis factor-α (TNF-α)-induced expression of both IL-6 and IL-8 at the protein and mRNA levels, thereby reducing the inflammatory response [19]. Inhibiting BRD4 was found to attenuate pathological cardiac hypertrophy by reducing reactive oxygen species (ROS) generation to suppress myocardial fibrosis and inflammation [20]. In the field of atherosclerosis (AS), BRD4 inhibitors can reduce the levels of proinflammatory markers, including SELE, VCAM-1, and IL-6, expressed by human umbilical vein endothelial cells stimulated by TNF-α and restore the permeability and integrity of endothelium [21]. This mechanism aids in preserving the functionality of vascular endothelial cells. Moreover, BRD4 has been shown to play a role in the migration and proliferation of vascular smooth muscle cells in AS [22,23]. Research has indicated that CAVD and AS share comparable risk factors, such as smoking, obesity, diabetes, hyperlipidemia, and hypertension [6,24]. The similarity suggests that at least common disease pathways exist in the initial phases of these two diseases [25]. Whether BRD4 is involved in the CAVD remains unclear.

In the present study, we found that BRD4 was highly expressed in the cardiac valves of individuals with CAVD. Inhibition or knockdown of BRD4 expression significantly attenuated the calcification process of AVICs under osteogenic conditions, involving the mediation of the NF-κB signaling pathway. These findings suggested that targeting BRD4 depletion might serve as a novel therapeutic strategy for CAVD.

Methods

Human Aortic Valves

Human aortic valve tissues were collected from patients undergoing surgical aortic valve replacement at Qingdao Women and Children's Hospital Affiliated to Qingdao University between December 2020 and June 2021. Aortic valve specimens exhibiting calcification were surgically excised from ten patients with a confirmed diagnosis of CAVD. For the control group, aortic valves were obtained from eleven individuals suffering from severe aortic regurgitation (AR). The absence of any valvular thickening or calcification in these control samples was confirmed through both computed tomography and transthoracic echocardiography. Patients suffering from rheumatic diseases, Marfan syndrome, or infective endocarditis were excluded from the study. Before enrolling in this study, all patients provided their informed consent by signing a formal document. The research protocol for the human subjects involved in this study adhered to the principles outlined in the Declaration of Helsinki. Approval for the protocol was granted by the ethics committee of the Qingdao Women and Children's Hospital (QFELL-YJ-2020-80). During the surgical procedure, the aortic valve tissue was immediately rinsed with sterile, cold saline (4 °C) to remove any remaining blood clots and other contaminants. The valve leaflets were then allocated for various analyses: some were used directly in cell culture experiments, others were processed for protein extraction for Western blot analysis, and the remaining samples were fixed in a 4% paraformaldehyde solution for subsequent immunohistochemical staining.

Immunohistochemistry (IHC)

For histological and immunohistochemical analysis, tissue samples were initially fixed in 4% paraformaldehyde for a period of 24 hours, followed by embedding in paraffin. To retrieve antigens, sections were incubated in Tris-EDTA buffer. Subsequently, the sections underwent wash with phosphate-buffered saline (PBS) and were then incubated with a peroxidase blocking solution to suppress activity of endogenous peroxidase. The primary antibodies used in the experiment included BRD4 (128874, Abcam, Cambridge, UK, 1:200), Runx2 (20700-1-AP, Proteintech, Chicago, IL, USA, 1:300), and OPN (22952-1-AP, Proteintech, 1:300). Horseradish peroxidase (HRP)-goat anti-rabbit IgG (PV-9001, ZSGB-Bio, Beijing, China) was applied as a secondary antibody. Color development was achieved using DAB chromogenic solution (PV-9000, ZSGB-Bio). All histopathological sections were digitized

using a 3DHISTECH panoramic scanner (MIDI II, 3DHISTECH, Budapest, Hungary).

Cell Culture and Treatment

To isolate AVICs, human aortic valves from patients with AR were subjected to enzymatic digestion. This procedure was carried out on the basis of the previously established protocols [26,27]. The leaflets were trimmed of the annulus and other tissues and rinsed with pre-cooled PBS. Initial enzymatic treatment was performed using collagenase II (C8150, Solarbio, Beijing, China, 2 mg/mL) at 37 °C for 10 min. To eliminate the native endothelium, both surfaces of the leaflets were abraded with sterile cotton-tipped applicators. Following a second wash with PBS, the tissue was directly minced into 2 mm^3 pieces and continuously digested in 2 mg/mL collagenase II solution at 37 °C for 6 h, with gentle mixing every 30 min via manual tube inversion. When the tissue appeared as a transparent flocculent, it indicated that the digestion was complete and the samples were centrifuged. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2, using a complete medium (CM) composed of Dulbecco's modified Eagle medium (DMEM) (11885084, Thermo Fisher, Waltham, MA, USA). This medium was supplemented with 10% fetal bovine serum (FBS) (A5256701, Thermo Fisher), along with 100 µg/mL streptomycin (P1400, Solarbio) and 100 U/mL penicillin (P1400, Solarbio). AVICs between passages 3 and 7 were utilized in our experiments. All cell cultures were screened to confirm the absence of mycoplasma contamination. To induce osteoblastic differentiation, AVICs were cultured until they reached a confluence of 70–80%. Subsequently, the culture medium was replaced with an osteogenic medium (OM). This specialized medium was formulated with DMEM supplemented with 10% FBS, 100 µg/mL streptomycin, 100 U/mL penicillin, 50 µM ascorbic acid (IA0530, Solarbio), 10 mM β-glycerophosphate (IG1340, Solarbio), and 10 nM dexamethasone (D8040, Solarbio). JQ1 (S7110, Selleckchem, Houston, TX, USA) was dissolved in DMSO and added to the OM at various concentrations. BRD4 expression was knocked down via short hairpin RNA (shRNA) in AVICs treated with OM. To evaluate the effect of BRD4 on the calcification process of AVICs, MK2206 (5 µM) (108232, MedChemExpress, NJ, USA), an inhibitor of AKT, was administered to the cells. Fresh culture medium was supplied at three-day intervals.

Western Blotting

Protein expression was detected in both aortic valve tissues and AVICs. A BCA protein assay kit (CW0014S, CWBio, Taizhou, China) was employed to determine protein concentrations. Proteins were separated using 8–12% SDS-PAGE (PG111-113, YamayBio, Shanghai, China), and then electrotransferred onto PVDF membranes (0000240177, Millipore, Billerica, MA, USA). The membranes were first blocked with 5% skim milk powder (PS112, YamayBio) for one hour and then incubated with primary antibodies overnight at 4 °C. The specific antibodies used included BRD4 (128874, Abcam, 1:2000), Runx2 (20700-1-AP, Proteintech, 1:1000), ALP (A0514, ABclonal, Wuhan, China, 1:1000), OPN (22952-1-AP, Proteintech, 1:1000), β-Tubulin (ABL1030, Abbkine, Wuhan, China, 1:1000), β-Actin (M3028, Immunoway, San Jose, CA, USA, 1:2000), Bcl-2 (26593-1-AP, Proteintech, 1:1000), Bax (60267-1-Ig, Proteintech, 1:2500), PI3K (4249, Cell Signaling Technology, Danvers, MA, USA, 1:1000), p-AKT (Ser473) (4060, Cell Signaling Technology, 1:1000), and AKT (4691, Cell Signaling Technology, 1:1000). Following washing with PBS-T, the membranes were subjected to a one-hour incubation at room temperature with HRP-conjugated secondary antibodies, including goat anti-mouse IgG (SA00001-1, Proteintech, 1:10,000) or goat anti-rabbit IgG (SA00001-2, Proteintech, 1:5000). After incubation, the membranes were washed again with PBS-T. For detection, an enhanced chemiluminescence (ECL) kit (GK10008, GlpBio, CA, USA) was used to visualize the specific antigen-antibody interaction bands. Quantification of the band intensities was subsequently performed using ImageJ software (V1.54, National Institutes of Health, Bethesda, MD, USA). To standardize the protein band signals, β-Tubulin or β-Actin was utilized as an internal loading control.

Immunofluorescence Staining

To characterize the phenotype of AVICs, we conducted immunofluorescence staining. AVICs were seeded into 12-well culture plates. After attaining 50% confluency, they were fixed with a 4% paraformaldehyde solution. For the detection of intracellular targets, cells were permeabilized with 0.5% Triton X-100 (T8200, Solarbio), followed by blocking with 5% skim milk. Primary antibody incubation was performed overnight at 4 °C, using the following dilutions: CD31 (A2104, ABclonal, 1:100), α-SMA (ab124964, Abcam, 1:300), and vimentin (10366-1-AP, Proteintech, 1:200). A subsequent incubation with suitable fluorescently-labeled secondary antibody—goat anti-rabbit IgG H&L (DyLight 488) (150077, Abcam, 1:200)—was conducted at 37 °C. Finally, the nuclei were stained with 4′,6-diamidino-2-phenylindole (104139, Abcam), and the samples were visualized using a fluorescence microscope (Axioscope 5, ZEISS, Oberkochen, Germany).

Alizarin Red Staining

To assess calcium deposition in AVICs, Alizarin red staining was performed. AVICs were cultured in 6-well plates and treated under different experimental conditions over a 14-day period. The cells were subsequently washed three times using PBS and fixed with 4% paraformaldehyde for 15 minutes. After three additional rinses with double-distilled water, Alizarin red staining solution (C0148S, Be-

yotime, Shanghai, China) was applied and incubated for 10 minutes. Finally, the stained samples were washed three times with double-distilled water and photographed for documentation.

Lentivirus Transfection

AVICs were transduced with recombinant lentiviruses expressing either control short hairpin RNA (shScramble) or BRD4-targeting short hairpin RNA (shBRD4) at a multiplicity of infection (MOI) of 50 in CM containing HiTransG P (Jikai Genechem, Shanghai, China). Sixteen hours after transduction, the CM was used to replace the viral medium. Transduction efficiency was assessed using fluorescence microscopy (Ti2-U, Nikon, Tokyo, Japan).

The nucleotide sequence of shBRD4:

Sence sequence: 5′-CCGGCCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGGTTTTTG-3′

Anti-sense sequence: 5′-AATTCAAAAACCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGG-3′

The nucleotide sequence of shScramble:

5′-TTCTCCGAACGTGTCCACGT-3′

Cell Viability Assay

To evaluate cell viability, the Cell Counting Kit-8 (CCK8) assay (PF00004, Proteintech) was employed. AVICs were seeded into 96-well plates and exposed to varying concentrations of JQ1 (1, 5, 10, 20, 30, 40, and 50 μM), which was diluted in CM. A control group was set up using a medium supplemented with an equivalent volume of DMSO. Following 24- or 48-hour incubation with JQ1, 10 μL of CCK8 solution was introduced into each well, and the plates were subsequently incubated for an additional hour. A blank control containing cell-free CM was included. Each experimental condition was performed in at least five replicate wells. The optical density (OD) at 450 nm was measured with a microplate reader (VICTOR Nivo, Revvity, Waltham, MA, USA).

Enzyme-linked Immunosorbent Assay (ELISA)

After a 3-day incubation under various treatment conditions, supernatants from AVICs were harvested. Using ELISA, the concentrations of IL-6 (PI330, Beyotime), IL-8 (PI640, Beyotime), and MCP-1 (PC130, Beyotime) in these supernatants were determined. A microplate reader (VICTOR Nivo, Revvity) was employed to measure the OD at 450 nm.

Reactive Oxygen Species (ROS) Assay

The intracellular ROS content was quantified with a commercial assay kit (50101ES01, Yeasen Biotechnology, Shanghai, China). After 3 days of incubation under respective treatment conditions, AVICs were treated with 10 μM DCFH-DA for 30 minutes. Following this incubation, the cells underwent two washes using a serum-free medium. A microplate reader was employed to detect fluorescence, with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical Analysis

All values are expressed as the mean ± standard deviation (SD). For comparisons involving two groups, the Student's *t*-test was applied. When comparing more than two groups, one-way analysis of variance (ANOVA) was performed, followed by post hoc testing using the Tukey-Kramer method. All statistical analyses were carried out using SPSS software (version 29.0, IBM, Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Elevated BRD4 Expression in the Aortic Valve of Patients With CAVD and AVICs Treated With OM

We obtained aortic valve tissue specimens from patients with CAVD and from those with isolated AR, who showed no evidence of significant calcification or stenosis. Patient clinical characteristics are summarized in Table 1. Baseline comparisons revealed no statistically significant differences between the two cohorts in age, sex, or other clinical parameters ($p > 0.05$). To elucidate the relationship between BRD4 and aortic valve calcification, BRD4 expression was first measured in human aortic valve specimens obtained from individuals with AR and those with CAVD. Immunohistochemical staining results showed the expression levels of BRD4 ($p < 0.001$), Runx2 ($p < 0.001$), and OPN ($p < 0.001$) were significantly higher in CAVD tissues compared to those in AR specimens (Fig. 1A). Similarly, Western blot analysis revealed a marked upregulation of BRD4 ($p < 0.001$), along with Runx2 ($p < 0.001$), OPN ($p < 0.001$), and ALP ($p < 0.001$), in the aortic valves of CAVD patients (Fig. 1B).

The third generation of AVICs after primary culture was characterized by immunofluorescence detection to determine cell phenotypes. AVICs demonstrated positive expression of vimentin and α-SMA proteins, while exhibiting negative CD31 expression (Fig. 1C). Following OM induction, alizarin red staining revealed substantial formation of calcium nodules ($p < 0.001$) (Fig. 1D). In addition, OM treatment led to a marked upregulation in the expression levels of BRD4 ($p < 0.001$), Runx2 ($p < 0.001$), OPN ($p < 0.01$), and ALP ($p < 0.001$) in AVICs (Fig. 1E). Together, increased BRD4 expression is associated with calcification of the aortic valve.

Suppressing BRD4 Function Reduces the Calcification Process in AVICs

We next investigated whether the inhibition of BRD4 could alleviate calcification of AVICs. Transfecting AVICs

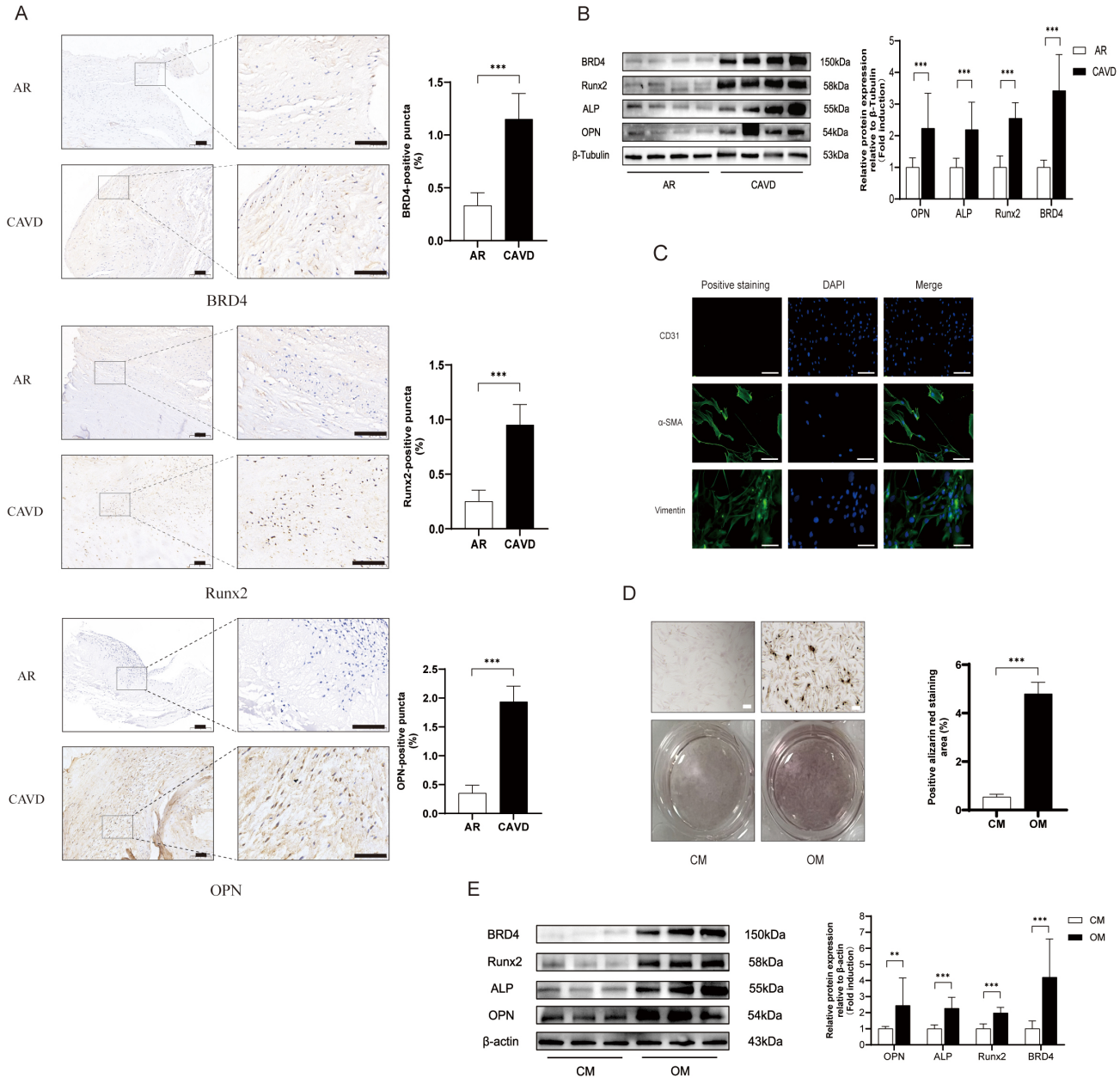

Fig. 1. BRD4 is activated in CAVD and AVICs treated with OM. (A) Immunohistochemical staining of BRD4, Runx2, and OPN in aortic valve tissues from patients with AR and CAVD. Scale bar: 100 µm. (B) Western blot analysis and quantification of BRD4, Runx2, ALP, and OPN protein expression levels in AR and CAVD valve tissues. (C) Immunofluorescence staining of CD31, α-SMA, and Vimentin in AVICs isolated from AR patients. Scale bar: 100 µm. (D) Representative images and quantitative analysis of alizarin red staining in AVICs cultured in CM or OM for 14 days. Scale bar: 100 µm. (E) Western blot and quantitative analysis of BRD4, Runx2, ALP, and OPN protein expression in AVICs treated with CM or OM for 14 days. n = 3 per group. The data are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$. BRD4, Bromodomain-containing protein 4; CAVD, calcific aortic valve disease; AVICs, aortic valvular interstitial cells; OM, osteogenic medium; OPN, osteopontin; AR, aortic regurgitation; CM, complete medium; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase.

with lentivirus carrying shRNA effectively knocked down the expression of BRD4, as evidenced by a significant decrease in BRD4 expression levels in shBRD4-mediated AVICs ($p < 0.001$) (Fig. 2A,B). Notably, shBRD4-mediated knockdown of BRD4 in AVICs with OM led to a substantial reduction in the protein levels of Runx2 (OM + shScramble vs OM + shBRD4, $p < 0.01$) and OPN (OM + shScramble vs OM + shBRD4, $p < 0.05$) (Fig. 2C). In addition, previous research has indicated that JQ1 is an effective BRD4 inhibitor. CCK-8 revealed no statistically sig-

Table 1. Basic information about patients.

	AR patients	CAVD patients	*p*-value
Number, n	11	10	-
Age (years)	65.7 ± 4.71	65.0 ± 6.36	0.768
Male, n (%)	7 (63.6)	7 (70.0)	0.562
BMI (kg/m^2)	24.0 ± 2.40	24.7 ± 3.81	0.614
Hypertension, n (%)	7 (63.6)	6 (60.0)	0.608
Diabetes, n (%)	3 (27.3)	2 (20.0)	0.550
Hyperlipemia, n (%)	2 (18.2)	3 (30.0)	0.450

Data are presented as mean ± SD or as n (%). BMI, body mass index.

nificant reduction in the viability of AVICs treated with 1 μM JQ1 for 24 or 48 hours relative to the CM group ($p > 0.05$) (Fig. 2D). Consequently, we opted for 1 μM JQ1 as the concentration for subsequent experiments. The result of alizarin red staining indicated that JQ1 inhibited the formation of calcified nodules induced by OM in AVICs ($p < 0.001$) (Fig. 2E). Consistent with this finding, JQ1 also lowered the protein levels of Runx2 (OM + DMSO vs OM + JQ1, $p < 0.001$) and OPN (OM + DMSO vs OM + JQ1, $p < 0.001$) in OM-induced AVICs (Fig. 2F). Overall, these results suggest that inhibition of BRD4 could alleviate calcification of AVICs.

Inhibition of BRD4 Reduces the Generation of ROS, Secretion of Inflammatory Factors, and Apoptosis in AVICs

Due to the involvement of oxidative stress, inflammatory response, and apoptotic cell death in the pathogenesis of CAVD, we investigated the relationship between BRD4 and these processes. According to ELISA data, culturing AVICs in OM for three days led to markedly higher concentrations of IL-6 ($p < 0.001$), IL-8 ($p < 0.001$), and MCP-1 ($p < 0.001$) compared to CM. This increase was attenuated when BRD4 was knocked down using shBRD4 over the same period (Fig. 3A). Compared to the AVICs cultured in CM, the production of ROS was elevated in the AVICs cultured in OM (CM vs OM, $p < 0.001$), whereas it was significantly lowered following the impact of shBRD4 (OM + shScramble vs OM + shBRD4, $p < 0.01$) (Fig. 3B). Furthermore, suppressing BRD4 resulted in downregulation of Bax (OM + shScramble vs OM + shBRD4, $p < 0.05$; OM + DMSO vs OM + JQ1, $p < 0.01$) and upregulation of Bcl-2 (OM + shScramble vs OM + shBRD4, $p < 0.01$; OM + DMSO vs OM + JQ1, $p < 0.05$) (Fig. 3C,D). In general, these findings reveal that in an osteogenic environment, AVICs with interference of BRD4 decrease the generation of ROS, secretion of inflammatory factors, and cell apoptosis.

BRD4 Induces AVICs Calcification Through the PI3K/AKT Pathway

The pathogenesis of CAVD is closely linked to the PI3K/AKT pathway, which regulates key cellular processes including proliferation, apoptosis, inflammation, and metabolism. Research has demonstrated downregulation of PI3K and p-AKT in the calcified aortic valve tissue [28]. Consistent with our findings, expression levels of PI3K ($p < 0.01$) and p-AKT (Ser473) ($p < 0.05$) were decreased in the CAVD group (Fig. 4A). Similarly, when AVICs were exposed to OM, the levels of PI3K ($p < 0.01$) and p-AKT (Ser473) ($p < 0.05$) declined, while an increase in the calcium-related protein expression of Runx2 ($p < 0.05$) and OPN ($p < 0.05$) was observed (Fig. 4B). Interestingly, suppression of BRD4 expression enhanced the activity of the PI3K/AKT pathway in OM-induced AVICs, resulting in elevated protein levels of PI3K ($p < 0.05$) and p-AKT (Ser473) ($p < 0.01$) (Fig. 4C). Moreover, we utilized the AKT high-selective inhibitor MK2206 to suppress the PI3K/AKT pathway. Result showed that the protein levels of OPN (OM vs OM + shBRD4, $p < 0.01$) and Runx2 (OM vs OM + shBRD4, $p < 0.01$) decreased with BRD4 knockdown was reversed by MK2206 (OPN, OM + shBRD4 vs OM + shBRD4 + MK2206, $p < 0.01$; Runx2, OM + shBRD4 vs OM + shBRD4 + MK2206, $p < 0.05$), as well as lowering the anti-apoptotic effect (Bax, OM + shBRD4 vs OM + shBRD4 + MK2206, $p < 0.01$; Bcl-2, OM + shBRD4 vs OM + shBRD4 + MK2206, $p < 0.05$) (Fig. 4D). The alizarin red staining revealed that MK2206 efficiently suppressed the anti-calcification effect of BRD4 knockdown, consequently facilitating the formation of calcium nodules (OM + shBRD4 vs OM + shBRD4 + MK2206, $p < 0.05$) (Fig. 4E). The above findings suggest that the role of BRD4 in promoting calcification may be closely associated with the PI3K/AKT pathway.

Discussion

This investigation revealed that BRD4 contributes to the calcification process of AVICs in CAVD, participating in both apoptotic and inflammatory pathways. Furthermore, the mechanistic analysis demonstrated that BRD4 promotes calcification in AVICs via activation of the PI3K/AKT pathway (Fig. 5). These results suggest that targeting BRD4 may represent a promising therapeutic strategy for CAVD.

Our research has revealed that an upregulation of BRD4 in the aortic valves of individuals suffering from CAVD. It has been well-established in multiple studies that BRD4 is critically involved in the development of diverse diseases, particularly within the realm of oncology. Nucleophosmin 1 facilitates the progression of prostate cancer through a c-Myc-mediated pathway, with BRD4 serving as an intermediary [29]. BRD4 is involved in the epigenetic regulation of enhancers through binding to the cis-

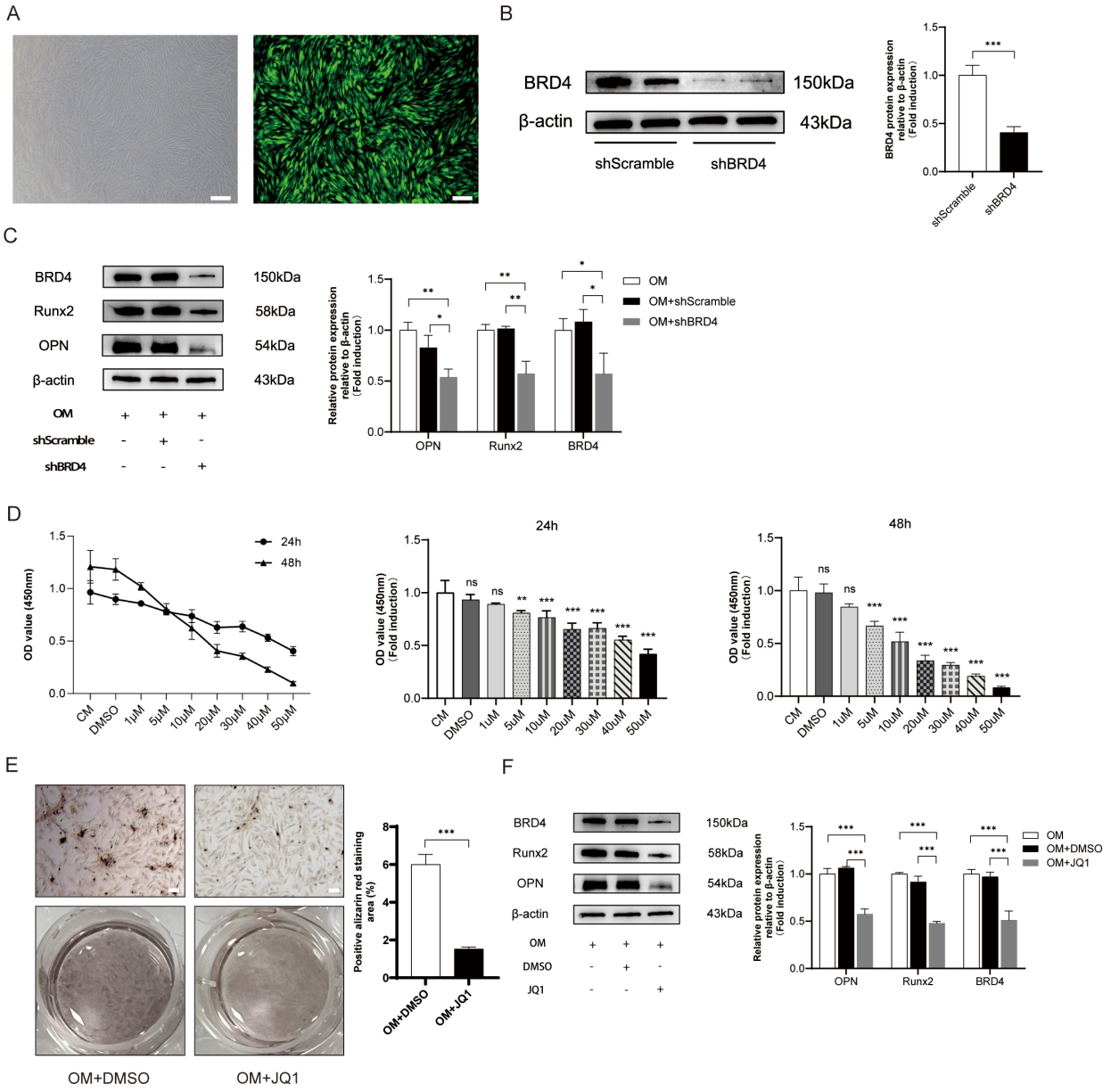

Fig. 2. Inhibition or genetic knockdown of BRD4 expression attenuates calcification in AVICs. (A) Fluorescence imaging of lentiviral transfection efficiency in AVICs. Scale bar: 100 µm. (B) Western blotting was employed to evaluate the knockdown efficiency of the BRD4 protein in AVICs. (C) Western blotting was performed to assess BRD4, Runx2, and OPN protein expression in AVICs from the OM, OM + shScramble, and OM + shBRD4 groups for 14 days. (D) The viability of AVICs was assessed using the CCK-8 following 24 h or 48 h of treatment under different conditions. CM versus other groups. (E) Alizarin red staining and quantitative analysis of AVICs cultured in OM for 14 days following treatment with 1 µM JQ1 or DMSO. Scale bar: 100 µm. (F) Western blotting was performed to assess BRD4, Runx2, and OPN protein expression in AVICs from the OM, OM + DMSO, and OM + JQ1 groups for 14 days. n = 3 per group. The data are expressed as mean ± SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, no significance. shBRD4, BRD4-targeting short hairpin RNA.

regulatory elements of TCF7L2, a key factor in the canonical Wnt signaling pathway, and this interaction modulates the expression of TCF7L2 to facilitate the progression of colorectal cancer [30]. In non-Hodgkin's lymphoma, BRD4 regulates the transcription of ferroptosis suppressor protein 1, which plays a protective role by inhibiting ferroptosis [31]. The significance of BRD4 in cardiovascular diseases cannot be overlooked. Inhibition of BRD4 has the potential to attenuate the inflammatory response of pulmonary artery smooth muscle cells triggered by TNF-α, which can alleviate vascular proliferation [19]. Interfering with BRD4 can restore the transactivational activ-

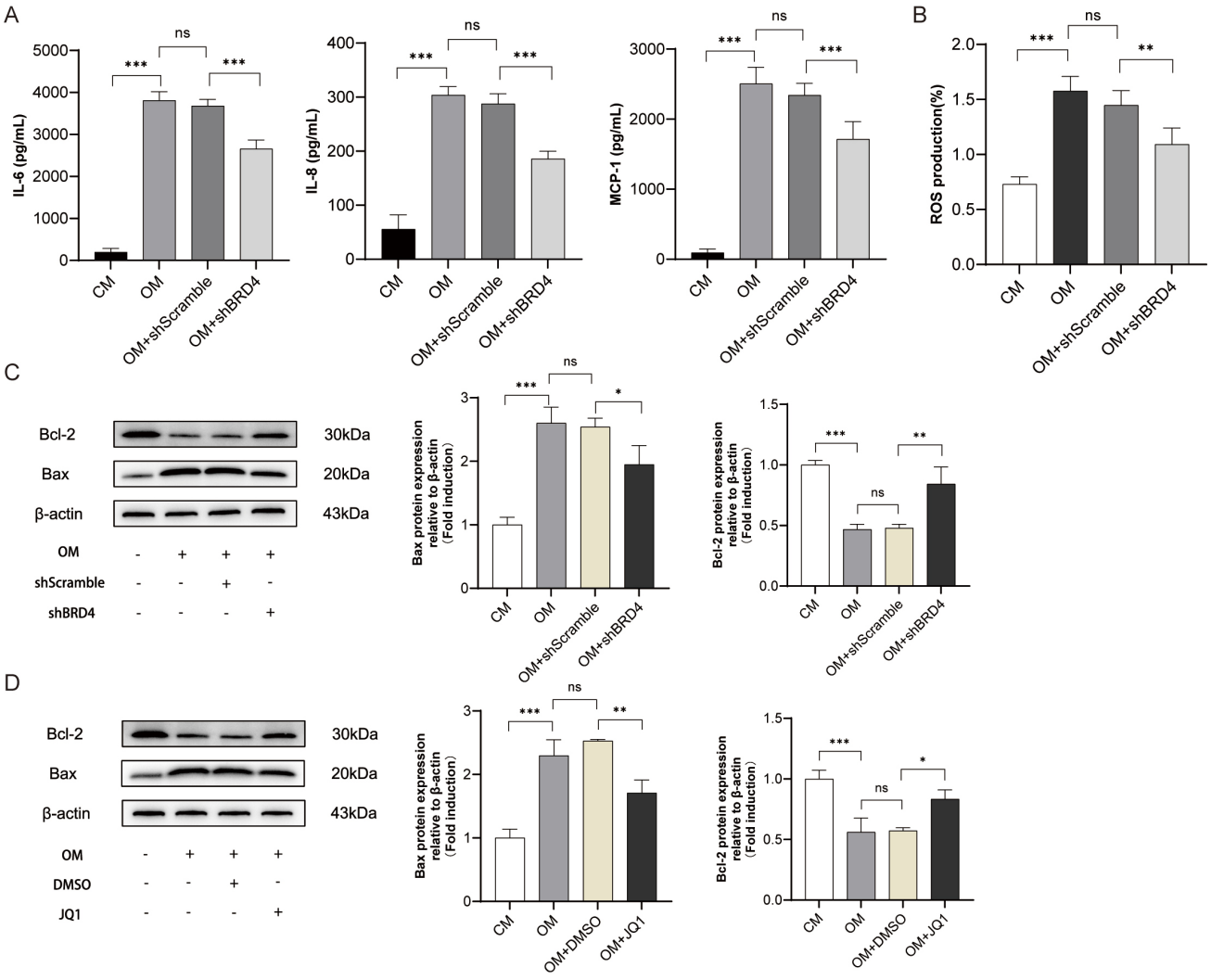

Fig. 3. BRD4 suppression in AVICs concurrently attenuates ROS production, inflammatory cytokine secretion, and apoptotic activity. (A) ELISA was used to measure the levels of L6, IL-8, and MCP-1 in the supernatant of AVICs from the CM, OM, OM + shScramble, and OM + shBRD4 groups after 3 days of culture. Compared across groups. (B) After 3 days of culture, ROS levels were measured in AVICs from the CM, OM, OM + shScramble, and OM + shBRD4 groups. Compared across groups. (C) Western blotting was performed to assess Bcl-2 and Bax protein expression in AVICs from the CM, OM, OM + shScramble, and OM + shBRD4 groups for 14 days. Compared across groups. (D) Western blotting was performed to assess Bcl-2 and Bax protein expression in AVICs from the CM, OM, OM + DMSO, and OM + JQ1 groups for 14 days. Compared across groups. n = 3 per group. The data are expressed as mean ± SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, no significance. ROS, reactive oxygen species; ELISA, Enzyme-linked immunosorbent assay.

ity of Forkhead box O1 (FOXO1), resulting in cell cycle arrest and thereby preventing the neointima formation in atherosclerosis [23]. This study is the first to identify abnormal expression of BRD4 in CAVD. Given that valve interstitial cells make up the majority of the aortic valve, we isolated AVICs from the regurgitant aortic valve and induced them using osteogenic medium. Protein studies revealed increased expression of BRD4 and osteogenic markers (Runx2, OPN, ALP) in AVICs when they were cultured under osteogenic conditions. This is analogous to the protein and immunohistochemistry findings of valve tissue. To elucidate the relationship between BRD4 and calcification, we intervened in BRD4 expression. JQ1, a pan-BET inhibitor, is a diazepine featuring a triazole structure [32]. It exhibits comparable inhibitory effects on the bromodomains BD1 and BD2. JQ1 not only directly binds to the acetylated lysine binding site of the BET protein, occupying the entire deep hydrophobic pocket formed by the ZA and BC loops to exert its inhibitory effect, but also interacts with the acetylated lysine binding site of BRD4 to displace BRD4 from nuclear chromatin [33]. The optimal concentration of JQ1 was ascertained through the CCK-8 assay. Regardless of whether the duration was 24 or 48 hours, JQ1 at concentrations higher than 5 µM influenced the cellular

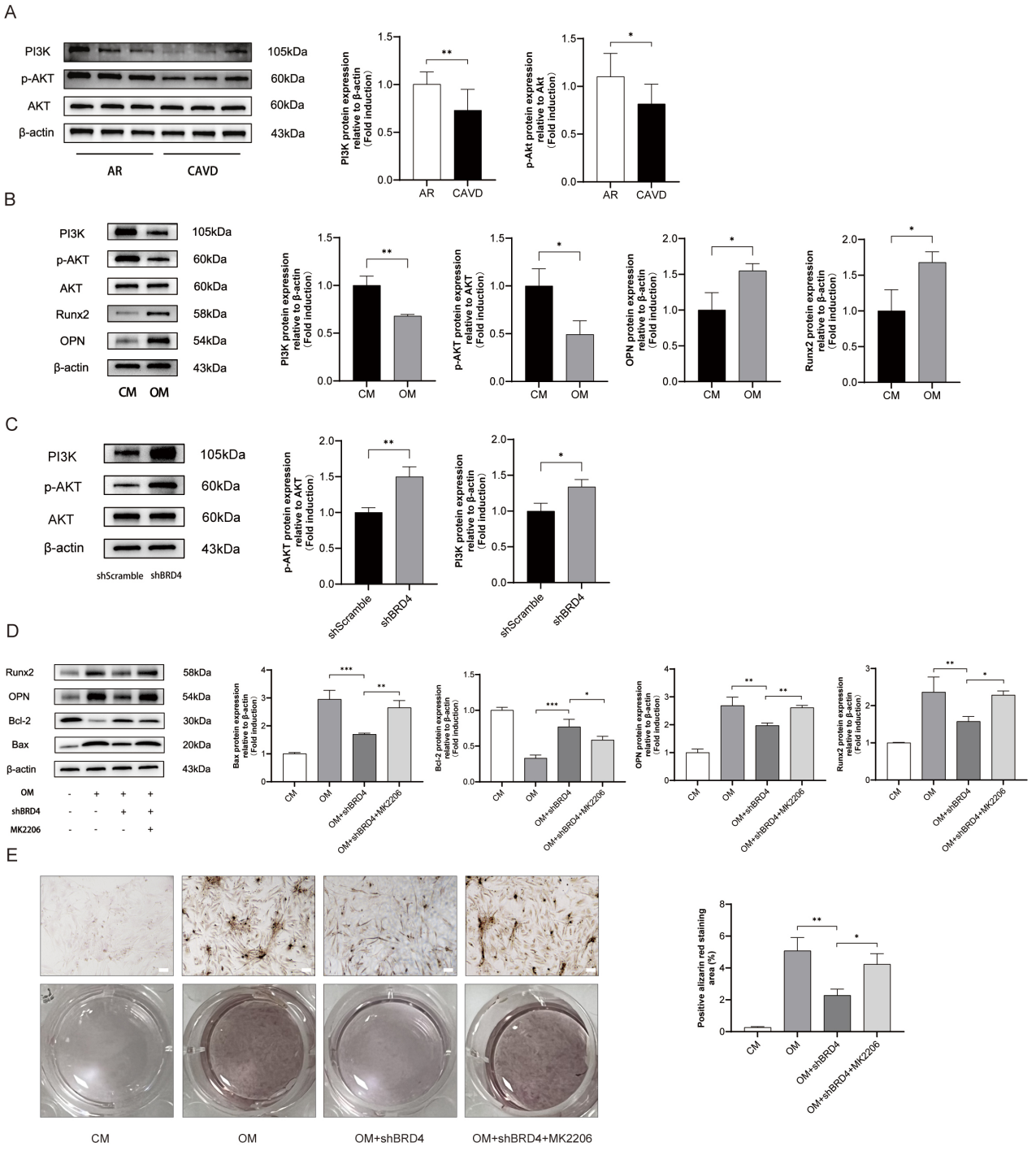

Fig. 4. BRD4 drives pathological calcification in AVICs via inhibition of the PI3K/AKT pathway. (A) Western blot analysis and quantification of PI3K, p-AKT (Ser473), and AKT protein expression levels in AR and CAVD valve tissues. (B) Western blot and quantitative analyses of PI3K, p-AKT (Ser473), AKT, Runx2, and OPN protein expression in AVICs treated with CM or OM for 14 days. (C) Western blotting was performed to assess PI3K, p-AKT (Ser473), and AKT protein expression in AVICs cultured in OM for 14 days following treatment with shScramble or shBRD4. (D) Western blot analysis and quantitative assessment of Runx2, OPN, Bcl-2, and Bax protein expression in AVICs after 14 days of treatment under four different conditions. Compared across groups. (E) Alizarin red staining and quantitative analysis of AVICs following 14 days of treatment under four different conditions. Scale bar: 100 μm. n = 3 per group. The data are expressed as mean ± SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

activity of AVICs. To guarantee safety, we chose 1 μM JQ1 for the subsequent experiments. Protein and alizarin red findings demonstrated that JQ1 decreased the levels of Runx2 and OPN and formation of calcific nodules in OM-

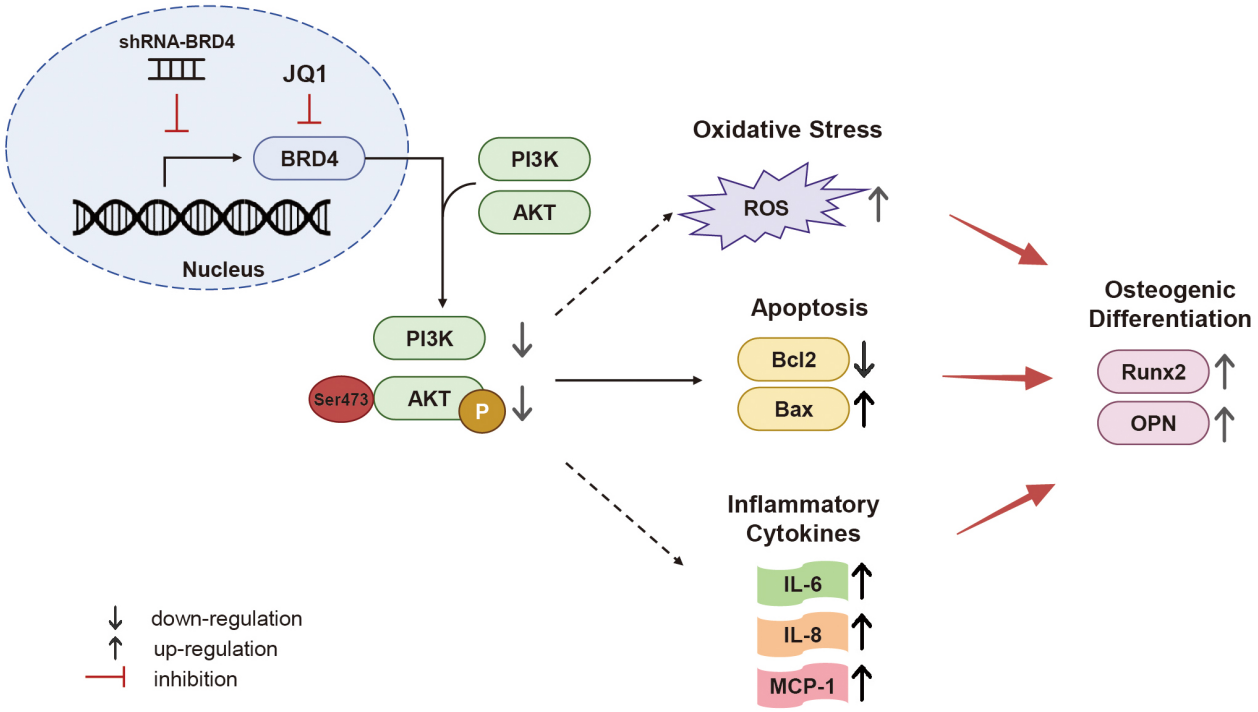

Fig. 5. Schematic model for the regulation of osteogenic differentiation by BRD4 in OM-treated AVICs. Elevated BRD4 expression suppresses PI3K and p-AKT protein levels, thereby inhibiting the PI3K/AKT pathway. This inhibition triggers apoptosis in AVICs, which is associated with upregulated expression of the osteogenic transcription factor Runx2 and the pro-calcific matrix protein OPN. Conversely, genetic or pharmacological inhibition of BRD4 restores PI3K/AKT activity and attenuates these pro-apoptotic and pro-osteogenic effects. Notably, interfering with the expression of BRD4 suppresses the production of ROS and the expression of inflammatory cytokines (IL-6, IL-8, and MCP-1). Given that dysregulation of the PI3K/AKT pathway has been consistently implicated in CAVD pathogenesis—particularly through its modulation of oxidative stress, apoptosis, and inflammatory responses—we propose that BRD4 upregulation contributes to ROS generation and sustained inflammation in AVICs by suppressing PI3K/AKT signaling, thereby promoting osteogenic differentiation. This figure was created by the authors using Microsoft Office PowerPoint (Version: Microsoft 365, Version 2405).

treated AVICs. Similarly, the knockdown of BRD4 expression via lentivirus transfection yielded the same results. Gilham *et al*. [34] demonstrated that disrupting BRD4 binding to chromatin can inhibit extracellular calcium deposition and prevent the induction of trans-differentiation markers in vascular smooth muscle cells under osteogenic conditions.

Currently, CAVD is considered to be an active, multistep disease process that involves lipid deposition, oxidative stress, inflammation, apoptosis, and mitochondrial dysfunction [35]. ROS have been implicated in human CAVD. ROS could directly increase BMP2 expression, which in turn regulates osteogenic differentiation through Runx2 and SMAD1 [36]. The oxidative stress and osteogenic differentiation of OM-induced AVICs were demonstrated to be ROS-dependent [37]. Moreover, inflammatory mediators such as TNF-α, IL-6, IL-8, and MCP-1 can facilitate the calcification process of AVICs [38–41]. Importantly, apoptotic bodies released from AVICs served as the core of calcium deposition and facilitated the formation of hydroxyapatite crystals [42]. Zinc supplementation was discovered to alleviate the degree of calcification in AVICs, reducing apoptotic and osteogenesis-related protein expression [43]. We also discovered that ROS, inflammatory cytokines, and pro-apoptotic protein Bax were all upregulated in AVICs induced by osteogenic medium. Conversely, knocking down BRD4 in AVICs led to a reduction in the above indicators, indicating that BRD4 is implicated in the calcification of AVICs via the disease process described above.

Previous studies have documented the involvement of the PI3K/AKT pathway in the pathogenesis of CAVD. Studies have indicated that a reduction in the proportion of phosphorylated AKT facilitates the formation of calcification within AVICs [44,45]. In macrophages, reduced expression of Triggering receptor expressed on myeloid cells 2 (TREM2) leads to lower phosphorylation in the Syk/PI3K/AKT cascade. This suppression elevates ROS, facilitates apoptosis and secretion of inflammatory cytokines such as IL-1β, IL-6, and TNF-α, and ultimately promotes the osteogenic differentiation of AVICs [46]. Previous research has revealed that the PI3K/AKT pathway is inhibited in calcified aortic valves [28,44]. Similarly, we

detected the down-regulation of PI3K and p-AKT (Ser473) in calcified aortic valves and *in vitro* AVICs. Notably, the knockdown of BRD4 elevated the levels of both PI3K and p-AKT. Nevertheless, the AKT inhibitor attenuated the anti-calcification and anti-apoptotic effects induced by the knockdown of BRD4. According to these results, the PI3K/AKT pathway contributes, at least partially, to the pro-calcific impact induced by BRD4 overexpression in AVICs. Taken together, this study found that BRD4 levels are significantly elevated in calcified aortic valves and in AVICs when cultured in OM. To the best of our knowledge, this research is the first to identify a link between BRD4 and the calcification process in AVICs. The osteogenic-like mechanism driven by BRD4, which is closely associated with the PI3K/AKT pathway, suggests its potential as a therapeutic target for CAVD.

Study Limitations and Future Perspectives

This study establishes a novel link between BRD4 and the PI3K/AKT pathway in CAVD pathogenesis, yet several aspects warrant further investigation to fully elucidate the mechanism and its therapeutic potential. Firstly, while we demonstrate that BRD4 suppression reactivates the PI3K/AKT pathway, the precise transcriptional mechanism by which BRD4 regulates this axis remains to be defined. Future studies employing techniques such as ChIP-seq in AVICs under calcifying conditions will be instrumental in identifying the direct genomic targets of BRD4 and clarifying whether it represses genes that activate PI3K/AKT signaling or activates specific phosphatases. Secondly, our observation that BRD4 inhibition reduces total intracellular ROS prompts the question of the subcellular source. The specific role of mitochondrial dysfunction was not assessed. Future work will apply MitoSOX staining and electron microscopy to determine if BRD4 drives mitochondrial ROS production and compromises mitochondrial health in calcifying AVICs. Third, the interplay between the inflammatory and apoptotic pathways observed herein represents a complex network. Although we propose a model where BRD4 coordinates these processes, the upstream regulatory crosstalk requires experimental validation. Future experiments involving simultaneous pathway inhibition and transcriptomic analysis will be essential to dissect their relative contributions and hierarchical relationships. Finally, the translational potential of targeting BRD4, highlighted by our *in vitro* findings, necessitates *in vivo* validation. Accordingly, future research will focus on evaluating the efficacy of pharmacological or genetic inhibition of BRD4 in established CAVD animal models. Successfully attenuating valve calcification *in vivo* will be a critical step toward validating BRD4 as a promising therapeutic target for CAVD.

Conclusions

In summary, our study demonstrates that BRD4 plays a critical role in promoting calcification of AVICs in CAVD, through modulation of ROS generation, apoptosis, and inflammatory responses. Mechanistically, BRD4 drives an osteoblast-like phenotypic transition in AVICs, a process tightly regulated by the PI3K/AKT pathway. These findings collectively suggest that BRD4 represents a promising therapeutic target for the intervention of CAVD.

Availability of Data and Materials

The authors declare that all relevant data of this study are available from the corresponding author upon reasonable request.

Author Contributions

Conceptualization, QX; methodology, ZD; validation, LM; formal analysis, QW and XY; investigation, QW and LZ; resources, XY and ML; data curation, PY; writing—original draft preparation, QW; writing—critically review and editing, LZ and PY; visualization, QW and LM; supervision, LZ and ZD; project administration, ZD and QX; funding acquisition, QX. All authors made critical revisions to the drafting of the manuscript or the important knowledge content, have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Before enrolling in this study, all patients provided their informed consent by signing a formal document. The research protocol for the human subjects involved in this study adhered to the principles outlined in the Declaration of Helsinki. Approval for the protocol was granted by the ethics committee of the Qingdao Women and Children's Hospital Affiliated to Qingdao University (QFELL-YJ-2020-80).

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Conflict of Interest

The authors declare no conflict of interest.

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