Life Sciences 228 (2019) 72–84

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

CC chemokine receptor 2 functions in osteoblastic transformation of valvular interstitial cells

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ARTICLE INFO

Keywords:
CCR2
Calcified aortic valve disease
Aortic valvular interstitial cells
PI3K/Akt signaling

ABSTRACT

Aims: Calcific aortic valve disease (CAVD) emerges as a challenging clinical issue, which is associated with high cardiovascular mortality. It has been demonstrated that osteoblastic transformation of AVICs is a key mechanism of CAVD and C-C motif chemokine receptors (CCRs) may favor this process. Thus, we aimed to investigate whether CCRs were involved in osteoblastic transformation of AVICs during the development CAVD.

Main methods: We first analyzed microarray data (GSE51472 and GSE12644) to identify differentially expressed genes between CAVD aortic valve tissue and normal samples, followed by verification of immunohistochemistry, qPCR and western blotting. Primary aortic valvular interstitial cells (AVICs) were incubated with specific inhibitors and/or siRNA of CCR2 and CCL2 under pro-calcifying medium. The levels of CCL2 in the medium were measured by ELISA. In addition, we used recombinant CCL2 to activate CCR2 in calcifying AVICs. Alizarin red S staining and calcium deposition were used to evaluate the degree of calcification. Western blotting was used to determine osteoblastic transformation of AVIC and total Akt and phosphorylated Akt expression.

Key finding: CCR2 levels were remarkably up-regulated in calcified aortic valve and calcifying AVICs. Silencing CCR2 inhibited the osteoblastic transformation and calcification of AVICs. In addition, recombinant CCL2 activated CCR2 and accelerated AVICs calcification through PI3K/Akt pathway.

Significance: We characterize abnormal activation of CCL2/CCR2 axis as a promoter of AVICs osteoblastic transformation and calcification. Our findings implicate the CCL2/CCR2-PI3K/Akt pathway as a potential target for treatment of CAVD.

1. Introduction

Calcific aortic valve disease (CAVD) is considered to be a motivator of aortic stenosis which shows an increased incidence of development countries [1,2]. Despite the advance on valve replacement and intervention therapy for aortic stenosis, however, the molecular mechanisms of transformation of calcific aortic valve are still unclear. Thus, it is necessary to understand the progression of CAVD and search for the key molecules for better management of aortic stenosis.

The heart valve contains a heterogeneous group of cells including valvular endothelial cells (VECs) and valvular interstitial cells (VICs). The valve surfaces were covered by a monolayer of VECs which provides a physical barrier between blood and valve tissue. Within the valve tissue, VICs is the most abundant cell type, interacts with extracellular matrix (ECM) and serves as the valve biomechanical coaptation during cardiac cycle. CAVD is a chronic disorder characterized by a cascade of cellular phenotypic transition and angiogenesis which leads to the fibrotic thickening and extensive formation of calcific masses within the aortic valve leaflet. Recently, VICs were found to regulate VECs network organization and promote VECs angiogenesis sprouting

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https://doi.org/10.1016/j.lfs.2019.04.050
Received 30 January 2019; Received in revised form 11 April 2019; Accepted 19 April 2019
Available online 26 April 2019

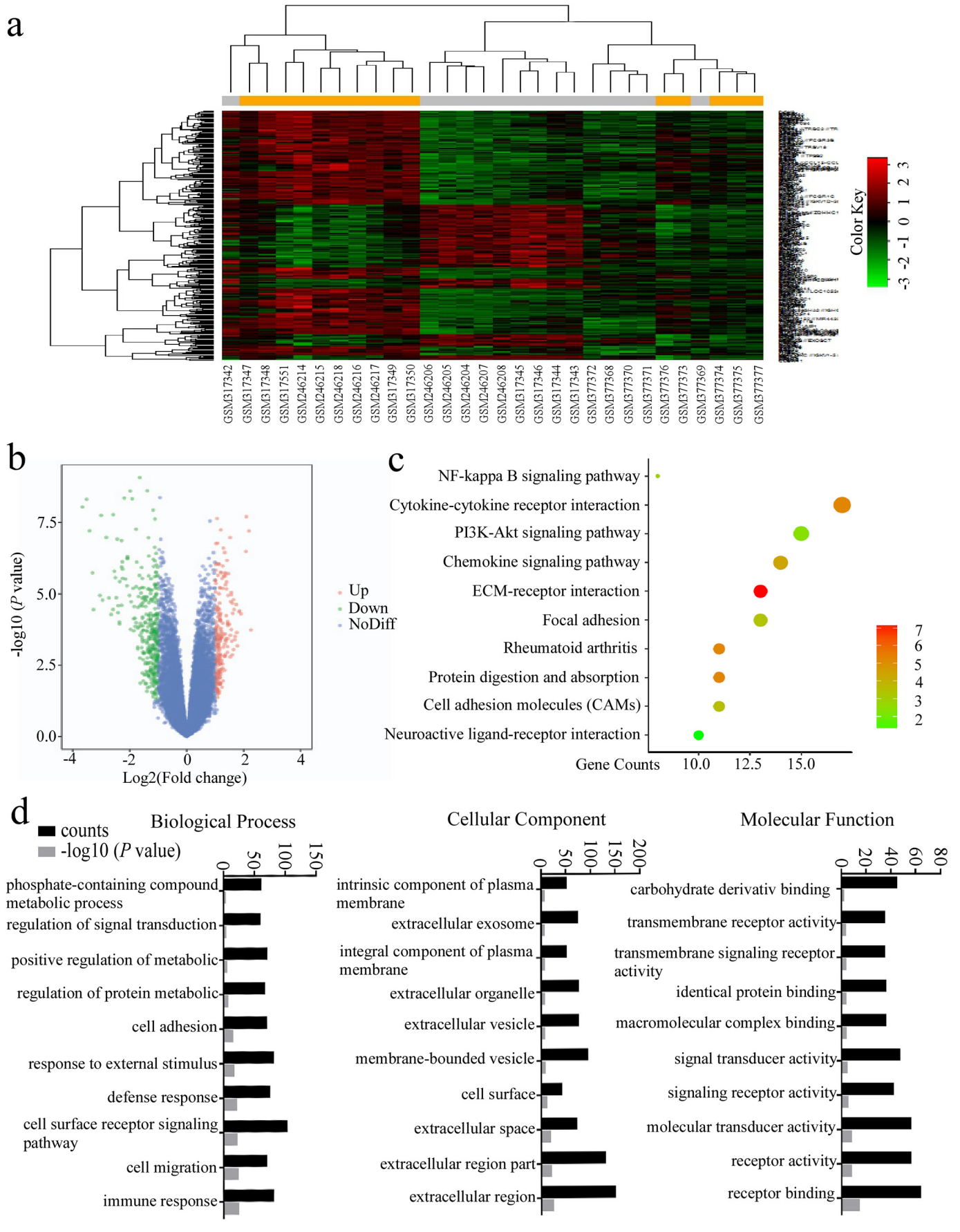

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Fig. 1. Identification of differentially expressed genes (DEGs) in calcific aortic valve and normal valve.
a. Heatmap of genes in calcific aortic valve and normal valve. The orange part represents the calcific aortic valve and the grey part represents the normal valve group. b. Volcano plot of genes in calcific aortic valve and normal valve. Red dot represents genes up-regulated with log2FC > 1 and $P < 0.05$, green dot represents genes down-regulated with log2FC < -1 and $P < 0.05$, blue dot represents no difference with $P > 0.05$. c. KEGG pathway analysis of DEGs. Gene counts refer as the number of genes enriched in cluster d. Gene ontology analysis of DEGs including biological process, cellular component and molecular function of DEGs. Counts refer as the number of genes enriched in the cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
KEGG pathway enrichment analysis of differentially expressed genes (DEGs) in calcific aortic valve disease.

DEGs	Gene count	*P*-value	Gene symbols
Cytokine-cytokine receptor interaction	17	5.58E−06	LEPR, IL21R, LIFR, CXCL8, CCL19, CCL8, KIT, CCL5, IL7R, CXCL12, CCL4, TNFRSF11B, PPBP, PRLR, CXCL13, CXCL16, CCR2
PI3K-Akt signaling pathway	15	0.0052	IBSP, COL4A4, COL4A3, TNC, ITGA4, KIT, IL7R, COL4A5, PRLR, COL6A6, COL1A1, COL11A1, THBS2, SPP1, FN1
Chemokine signaling pathway	14	4.26E−05	CXCL5, HCK, CXCL8, CCL19, CCL8, CCL5, CXCL12, CCL4, CCL18, PPBP, CXCL13, CXCL16, CCR2, SHC4
ECM-receptor interaction	13	6.31E−08	IBSP, COL4A4, COL4A3, TNC, ITGA4, COL4A5, SDC1, COL6A6, COL1A1, COL11A1, THBS2, SPP1, FN1
Focal adhesion	13	4.69E−04	IBSP, COL4A4, COL4A3, TNC, ITGA4, COL4A5, COL6A6, COL1A1, COL11A1, THBS2, SPP1, SHC4, FN1
Protein digestion and absorption	11	5.11E−06	COL4A4, COL4A3, ATP1B1, COL6A6, SLC16A10, CPA3, COL1A1, ATP1A2, COL11A1, COL10A1, COL4A5
Rheumatoid arthritis	11	5.11E−06	HLA-DQB1, CD86, CXCL5, CXCL8, ACP5, ITGB2, HLA-DPA1, CCL5, CXCL12, HLA-DOB, MMP1
Cell adhesion molecules (CAMs)	11	3.19E−04	HLA-DQB1, CD86, SDC1, CADM1, CD2, CNTN1, ITGB2, HLA-DPA1, CLDN11, ITGA4, HLA-DOB
Neuroactive ligand-receptor interaction	10	0.077	GPR83, AGTR1, ADRB1, C5AR1, PRLR, GZMA, LEPR, ADRA2A, FPR1, CTSG
NF-kappaB signaling pathway	8	0.0012	LCK, BCL2A1, CXCL8, CCL19, LBP, CXCL12, CCL4, PLAU

phenotype which potentially contributing to neovascularization of CAVD [3]. When co-cultured with VICs, the pro-calcifying medium induced calcification of VECs was inhibited which was characterized by decreased level of osteocalcin and osteopontin [4]. In addition to VICs-VECs interactions, the phenotypic transformation of VICs into osteoblastic-like cells is responsible for increasing production of bone matrix, extracellular matrix and contributes to the active deposition of calcium within the valve leaflet [4,5]. These studies indicate that VICs play an important role in valve calcific processes.

C-C motif chemokine receptors (CCRs) superfamily belongs to G-protein coupled receptors. C-C motif chemokine binding and receptors activation are required to exert significant signaling transduction in progression of chronic diseases including atherosclerosis, obesity and type 2 diabetes [6]. Increasing evidence indicates that CCRs are not only involved in inflammation but also cardiovascular calcification. A clinical research indicated that CCR5 polymorphisms were associated with the degree of heart valve calcification [7]. Polymorphism of CCR2 Val64 alleles was significantly correlated with coronary artery calcification and was highly related to atherosclerosis [8]. In addition, CCR2-knockout mice which could be protected from aneurysm formation, exhibited preserved elastic lamellae, and showed reduced levels of matrix metalloprotein 2 (MMP-2) as well as MMP-9 in the aorta [9]. MMP2 and MMP9 are regarded as the biomarker of CAVD [10,11]. These evidence strongly suggested that CCRs predominantly function in calcification pathological process of cardiovascular system. However, the roles of CCRs in osteoblastic transformation of aortic valvular interstitial cells (AVICs) as well as their involvement in CAVD are unknown. In an attempt to identify pivotal CCR in CAVD, we screened for the differential expressed genes that were up-regulated in CAVD. Among CCRs family, only CCR2 was up-regulated in calcific aortic valve tissue. Blockade of CCR2 attenuated osteoblastic transformation and calcification of AVICs. Mechanism analysis revealed that CCR2 was activated by CCL2 and promoted the development of CAVD via PI3K/Akt pathway. Our findings uncover the significant role of CCR2 in osteoblastic transformation of AVICs and provide the CCL2/CCR2-PI3K/Akt pathway as a potential target for treatment of CAVD.

2. Material and method

2.1. Data extraction and processing

Two original microarray datasets GSE51472 and GSE12644 were downloaded from GEO database. Differentially expressed genes (DEGs) between calcified and normal valve samples were identified through R software (version 3.4.3) with criteria of log2FC > 1 or log2FC < -1 and $P < 0.05$. The functional annotations as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs were analyzed by DAVID (https://david.ncifcrf.gov/). The protein-protein interaction (PPI) were downloaded from STRING (https://string-db.org/) and constructed by Cytoscape (version3.5.1). The node modules among the PPI network were analyzed by Cytoscape plug-in Molecular Complex Detection (MCODE) and Centiscape2.2.

2.2. Patient samples

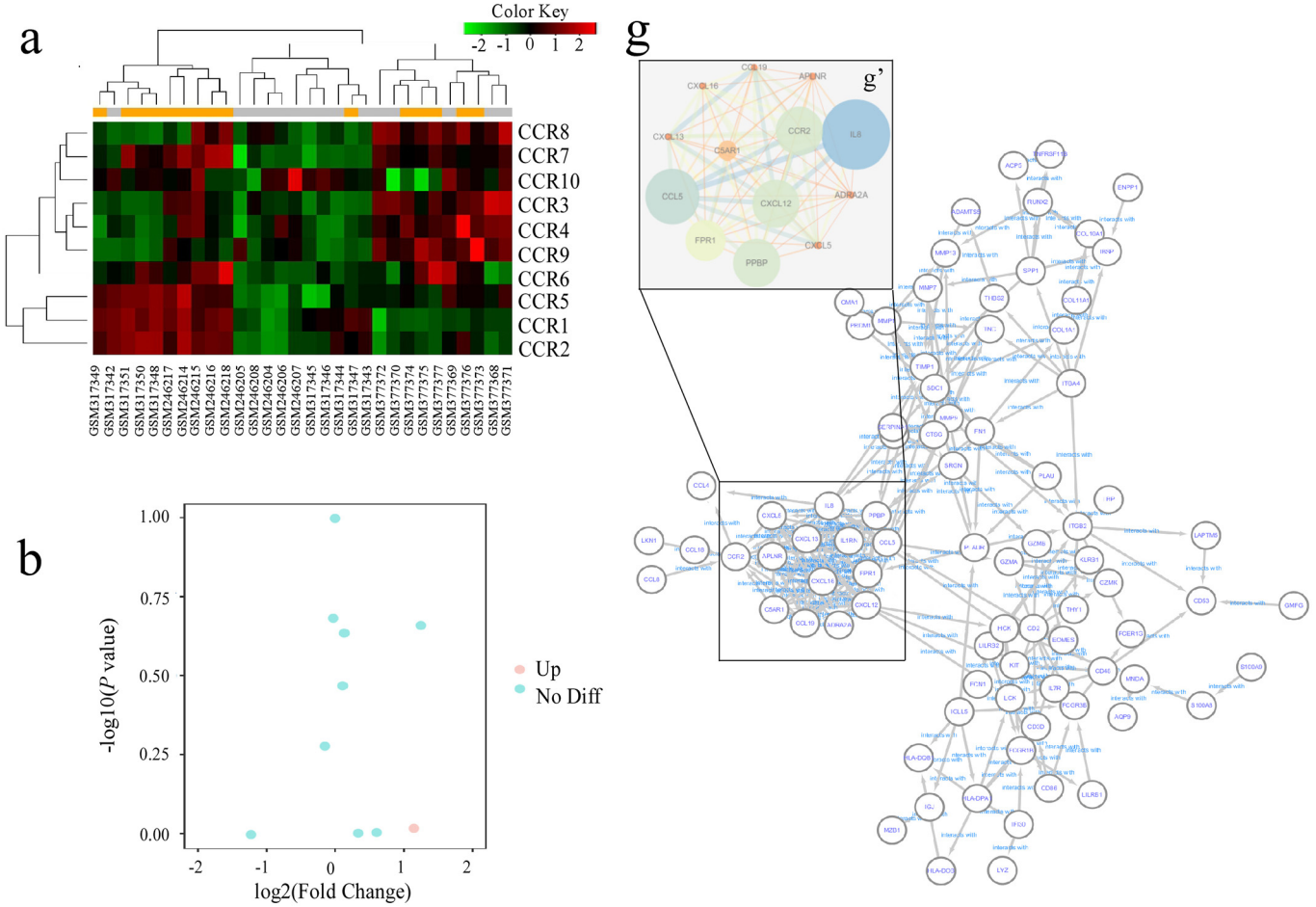
A total of 15 patients with CAVD and 8 patients who underwent heart transplantation regarded as normal controls were enrolled in Sun Yat-sen Memorial Hospital from 2015 to 2018 and this study was approved by the institutional Ethics Committee of Sun Yat-sen Memorial Hospital. All patients have signed the informed consent form of the use of clinical specimens for research uses. Patients with congenital aortic valve abnormality, rheumatic disease, and endocarditis were excluded. Their paraffin-embedded aortic valve tissue samples were collected for immunohistochemistry assay.

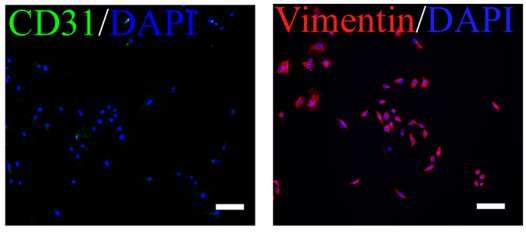
2.3. Animals

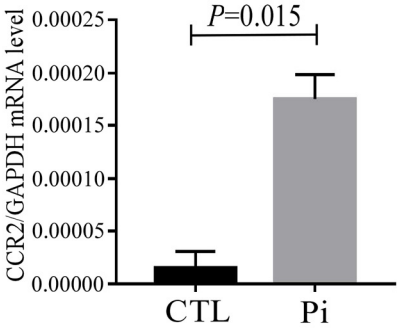
6 weeks male SD rat (n = 5) were purchased from Sun Yat-sen medical university and all experiments were approved by the institutional Ethics Committee of Sun Yat-sen University. All animal work was conformed to the Guidelines on Ethical Treatment of Experimental Animals from Ministry of Science and Technology of the People's Republic of China (MOST, [2006] 398) and guidelines from Chinese Association for Laboratory Animal Sciences.

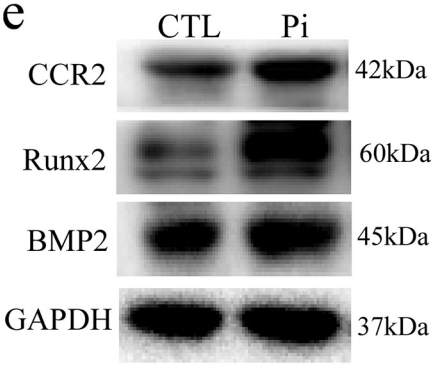
2.4. Cell isolation and culture

The primary rat AVICs were harvested and cultured as previously described [12]. The distal 1/3 part of the aortic valve leaflets was dissected under sterile conditions and endothelial cells were removed from leaflets by scalpel and seeded onto dishes with medium containing: M199 + 20% FBS, 100 U/mL penicillin, and 100 μg /mL streptomycin. In general, cells were used between passages 4–6. In order to provoke calcification, cells were incubated with pro-calcifying









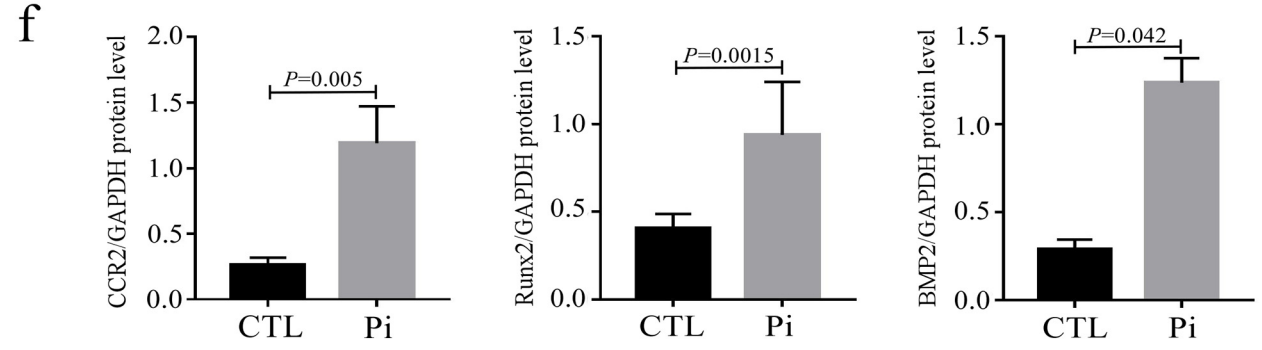


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Fig. 2. CCR2 potentially functioned in calcified aortic valve disease.
a. Heatmap of C-C motif chemokine receptor (CCRs) in calcific aortic valve and normal valve. The orange part represents the calcific aortic valve and the grey part represents the normal valve. b. Volcano plot of CCRs in calcific aortic valve and normal valve. Red dot represents genes up-regulated with log2FC $>$ 1 and $P < 0.05$, green dot represents genes down-regulated with log2FC $<$ −1 and $P < 0.05$, blue dot represents no difference with $P > 0.05$. c. Identification of aortic valvular interstitial cells (AVICs) with interstitial cells specific markers Vimentin (green) and endothelial cells marker CD31 (red). Cell nuclei were stained by DAPI (blue). Scale bar 50 μm. d. The mRNA expression levels of CCR2 were determined by qPCR in pro-calcifying medium induced AVICs compared to control group. All data is shown as the mean ± SD. e. The expression of CCR2 and osteoblastic markers Runx2 as well as BMP2 in pro-calcifying medium induced AVICs. f. Analysis of grey value of CCR2, Runx2 and BMP2 compared to control cells in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD. g. The constructed protein-protein interaction (PPI) network of DEGs and selected module with the highest scores which was screened out by MCODE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
The demographic and clinical characteristic of the patients.

	CAVD valves (m = 15)	Non-CAVD valves (n = 8)	*P*-value
Age	66 ± 2.289	45.86 ± 7.33	0.0029*
Male (%)	80%	50%	0.1819
Smoking (%)	60%	12.50%	0.0743*
Hypertension (%)	66.70%	12.50%	0.0272*
Diabetes (%)	26.70%	37.50%	0.6570
BMI (kg/m^2)	26.19 ± 0.9123	20.31 ± 2.216	0.0244*
Aortic peak gradient (mmHg)	_	61.5 ± 5.519	
Triglycerides (mmol/L)	1.142 ± 0.1085	1.548 ± 0.3197	0.1348
LDL-c (mmol/L)	2.771 ± 0.2256	3.316 ± 0.4142	0.2491
HDL-c (mmol/L)	1.036 ± 0.07872	0.918 ± 0.1206	0.4449
Creatinine (μmol/L)	95.13 ± 5.633	102.6 ± 5.834	0.4287
Creatinine clearance (ml/min)	66.2 ± 3.95	66.96 ± 9.865	0.9366

Values are shown as mean ± SD or %.
BMI: body mass index; LDL-c: low-density lipoprotein; HDL-c: high-density lipoprotein.
* When significant difference was found between CAVD valves and Non-CAVD valves group. ($P < 0.1$).

medium containing: M199 + 5%FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and NaH_2PO_4 at 2.0 mM for 7 days. CCR2 pharmaceutical inhibitor RS102895 [13] was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Recombinant Rat CCL2 was purchased from PeproTech (Rocky Hill, NJ, USA). Predesigned rat CCR2 and CCL2 small interfering siRNAs were purchased from IGEbio (IGEbio, Guangzhou, China). The sequence of CCR2 siRNA-1 is 5′-AUAAUGAC CAACAUGUUGC-3′ and siRNA-2 is 5′-UAAAUGAUAGGAUUAACGC-3′. The sequence of CCL2 siRNA-1 is 5′-CUGUAGUAUUUGUCACCAA-3′ and siRNA-2 is 5′-GCUGGAGAACUACAAGAGA-3′. SiRNAs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.5. Enzyme-linked immunosorbent assay (ELISA)

CCL2 protein level was detected in cell culture supernatant of AVICs after exposing to control or pro-calcifying medium after 3 days. CCL2 was measured using ELISA kits (Cusabio, Wuhan, China) according to manufacturer's instructions.

2.6. Immunofluorescence assay

Passage 2 of AVICs were seeded on 12 well plates and fixed with 4% paraformaldehyde. Then they were washed with PBS for three times and were blocked in 5% BSA for 1 h. Cells were incubated with CD31 (Abcam, ab24590, diluted 1:100, RRID:AB_448167), Vimentin (Abcam, ab45939, diluted 1:100, RRID:AB_2257290) antibody overnight and washed for three times. Subsequently, they were incubated with fluorescent-labeled anti-mouse (Cell Signaling Technology, 4408S, diluted 1:1000, RRID:AB_10694704) and anti-rabbit F(ab′) fragment secondary antibody (Cell Signaling Technology, 4412, diluted 1:1000, RRID:AB_1904025) individually. For immunofluorescence staining of human normal and calcified aortic valve tissue, slices were incubated with CCR2 (Abcam, ab21667, diluted 1:100, RRID:AB_446468) and CD68 (Abcam, ab955, diluted 1:100, RRID:AB_307338) overnight, subsequently, fluorescent-labeled anti-mouse (Cell Signaling Technology, 8890, diluted 1:1000, RRID:AB_2714182) and anti-rabbit secondary antibody (Cell Signaling Technology, 4412, diluted 1:1000, RRID:AB_1904025) were used individually. Nuclei were stained with DAPI (Life Technologies, diluted 1:1000). Cells and tissue samples were viewed by a fluorescence microscope (Olympus).

2.7. Von Kossa staining and Immunohistochemistry

Human calcified aortic valve tissue samples and normal control samples were fixed with 4% paraformaldehyde, and embedded in paraffin. 4 μm slices were subsequently stained with Von Kossa assay for detection of calcium deposition according to the manufacturer's instructions. Immunohistochemical staining was applied to detect Runt-related Transcription Factor 2 (Runx2) (Abcam, ab23981, diluted 1:1000, RRID:AB_777785), Bone Morphogenetic Protein (BMP2) (Abcam, ab14933, diluted 1:1000, RRID:AB_2243574) and CCR2 (Bioss, bs-10964R, diluted 1:500, RRID:AB_10856929) for normal and calcified sections. Image-Pro Plus (MediaCybernetics) was using to semi-quantitative the expression value between these 2 groups.

2.8. RNA extraction and quantitative real-time PCR assay

The RNA extraction protocol was performed according to the manufacturer's instructions. RNA was reverse transcribed using the Quantitec Reverse Transcription Kit from Takara. Quantitative real-time PCR (qPCR) was performed with a SYBR Green PCR kit from Takara and using the Roche LightCycler 96 System. GAPDH and CCR2 were quantified by qPCR at the following conditions: an initial 15 min run at 95 °C before starting, then 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s for a total of 40 cycles. GAPDH was used as the reference control gene for other genes. Primer sequences were as follows: GAPDH, 5′-TCCTACCCCCAATGTATCCG-3′ and 5′-CCTTTAGTGGGCCC TCGG-3′, CCR2, 5′-GTTCTCTTCCTGACCACCTTC-3′ and 5′-CTTCGGA ACTTCTCACCAACA-3′, CCL2, 5′-TGCTGCTACTCATTC ACTGGC-3′ and 5′-CCTTATTGGGGTCAGCACAG-3′.

2.9. Western blotting

AVICs were lysed in 1 × cell lysis buffer (Beyotime) with halt protease inhibitor (Cell Signaling Technology). The protein content was detected by BCA method. Denatured Proteins were loaded onto 10% SDS polyacrylamide gels followed by electrophoresis and blotting onto nitrocellulose membranes. Membranes were blocked with 5% BSA, and incubated with primary antibodies directed against Runx2 (Abcam, ab23981, diluted 1:1000, RRID:AB_777785), BMP2 (Abcam, ab14933, diluted 1:1000, RRID:AB_2243574), CCR2 (Bioss, bs-10964R, diluted 1:1000, RRID:AB_10856929), total Akt (Cell Signaling Technology, 4691, diluted 1:1000, RRID:AB_915783), phosphorylation Akt ser473 (Cell Signaling Technology, 4060, diluted 1:1000, RRID:AB_2315049), GAPDH (Cell Signaling Technology, 5174, diluted 1:1000, RRID:AB_

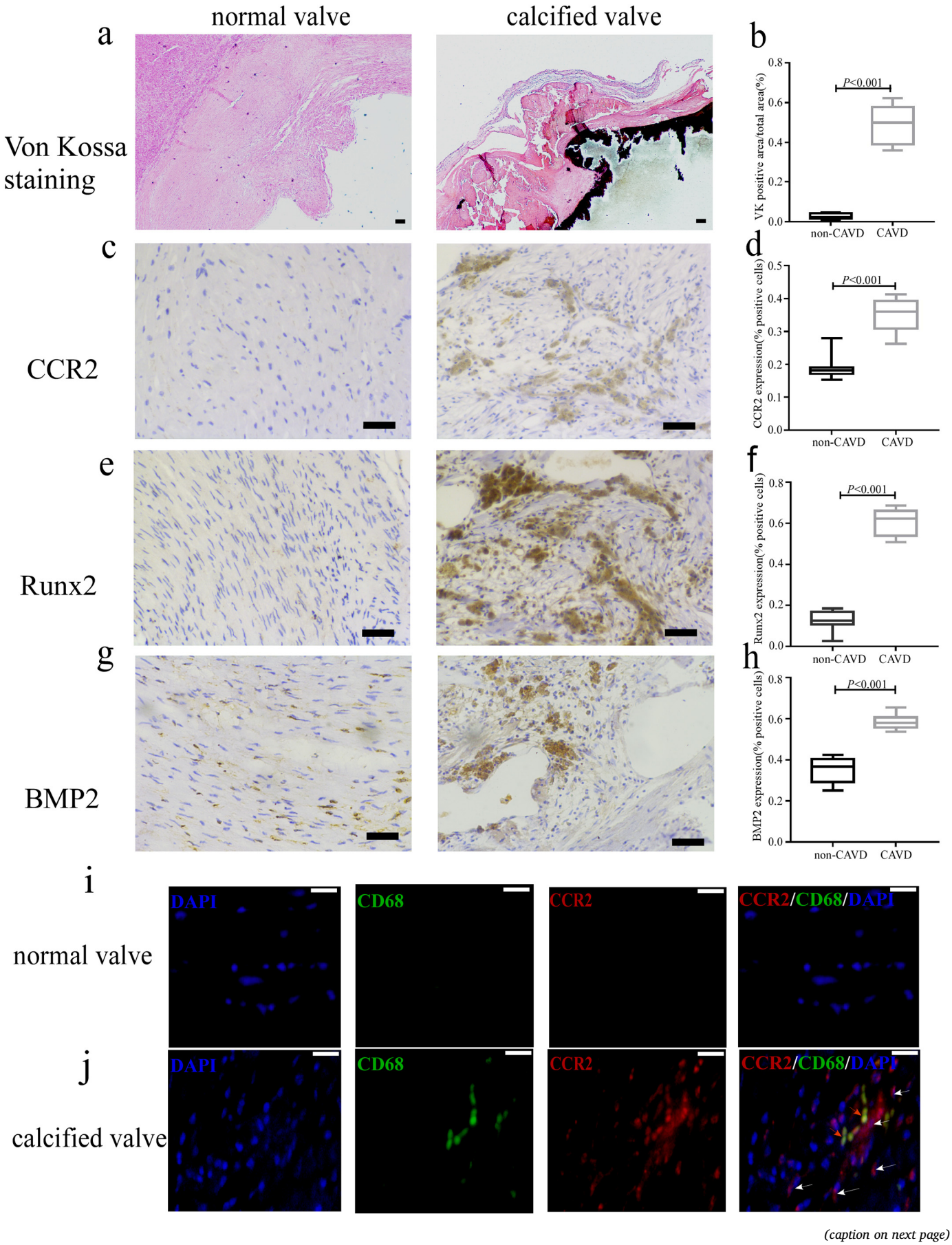

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Fig. 3. Calcific aortic valve of CAVD patients showed high level of CCR2 and calcification markers.
a. The representative images of Von Kossa S staining of aortic valve sample from CAVD patients and control patients. Scale bar: 50 μm. b. The degree of calcific aortic valve of CAVD (n = 15) and normal (n = 8) determined by Von Kossa S staining. c. The representative images of CCR2 immunohistochemistry staining of CAVD and normal samples which have been used for Von Kossa S staining. Scale bar: 50 μm. d. The levels of CCR2 in CAVD (n = 15) and normal samples (n = 8) were determined by immunohistochemistry. e. The representative images of Runx2 immunohistochemistry staining of CAVD and normal samples which have been used for Von Kossa S staining. Scale bar: 50 μm. f. The levels of Runx2 in CAVD (n = 15) and normal samples (n = 8) were determined by immunohistochemistry. g. The representative images of BMP2 immunohistochemistry staining of CAVD and normal samples which have been used for Von Kossa S staining. Scale bar: 50 μm. h. The levels of BMP2 in CAVD (n = 15) and normal samples (n = 8) were determined by immunohistochemistry. All data is shown as the mean ± SD. i. The representative images of immunofluorescence staining of normal valve and calcified valve which have been used for Von Kossa S staining. CD68 (green), CCR2 (red) and cell nuclei (DAPI, blue).Co-expression of CCR2 and CD68 were indicated by orange arrow, while CCR2-postive cells without colocalizing with CD68 were indicated by white arrow. Scale bar 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

10622025) and followed by an HRP-labeled secondary antibody (Cell Signaling Technology, 7074, diluted 1:5000, RRID:AB_2099233). Protein detection was done by Western blot chemiluminescence detection (Millipore). The expression of target protein was normalized to GADPH.

2.10. Alizarin red S staining

AVICs were seeded on 6 well plates and cultured with growth medium or pro-calcifying medium for 7 days. After washing with PBS, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.2% Alizarin red solution for 15 min, excessive dye was removed by PBS.

2.11. Quantification of calcium deposition

AVICs were collected and decalcified with 0.6 M HCL for 24 h. The calcium deposition was determined using calcium quantification kits (NanJing JianCheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions. The calcium deposition was measured by atomic absorption spectroscopy at 630 nm. The calcium content was normalized by total protein concentration.

2.12. Statistics analysis

All data are presented as mean ± standard deviation of 3 independent experiments. Comparisons between values of different groups were analyzed by one-way ANOVA or Student *t*-test for multigroups or two groups individually. All data statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Result

3.1. Identification of differentially expressed genes in calcific aortic valve disease

A total of 327 differentially expressed genes were identified between calcified aortic valve and normal aortic valve, including 201 up-regulated genes and 126 down-regulated genes (Supplementary Table 1, Fig. 1a–b). By using the biological process of GO term analysis, we found most of the candidate genes were enriched in cell surface receptor signaling pathway, immune response as well as response to external stimulus. As for cellular component of GO term analysis, differentially expressed genes were enriched in extracellular region, extracellular region part and membrane-bounded vesicle. Molecular function analysis indicated that DEGs were mainly related to receptor binding, receptor activity and molecular transducer activity (Fig. 1d). Besides, through KEGG pathway analysis, we found that these DEGs in CAVD were highly correlated with cytokine-cytokine receptor interaction, PI3K/Akt signaling pathway and chemokine signaling pathway (Fig. 1c and Table 1).

3.2. CCR2 potentially functioned in CAVD

Since several studies indicated that CCRs superfamily potentially regulated the progression of CAVD [9–11], thus, we tended to explore the functional roles of CCRs in CAVD. From data mining of two GEO datasets (GSE51472 and GSE12644) analyzed above, we found that among all CCRs family, only CCR2 was significantly up-regulated in calcified valve when compared with normal valve (Fig. 2a–b). We first separated AVICs successfully by identifying AVICs with high expression of the interstitial cells specific markers Vimentin but negative for CD31 (Fig. 2c). We showed that the mRNA and protein levels of CCR2 were significantly increased in AVICs treated with pro-calcifying medium (Fig. 2d–e). Meanwhile, the calcification markers (Runx2 and BMP2) presented the same tendency as CCR2 (Fig. 2e–f). These suggested that high levels of CCR2 were correlated with osteoblastic transformation of AVICs. Of great interest, by constructing a protein-protein interaction (PPI) which contained 160 nodes and 613 edges, we found that CCR2 exhibited a higher degree in the highest-scored module which contained 13 nodes and 78 edges in the PPI network (Fig. 2g), these evidence indicated that CCR2 potentially functions in CAVD.

3.3. Calcified aortic valve of CAVD patients showed high level of CCR2 and calcification markers

To support this opinion, 8 normal and 15 CAVD patients' aortic valve samples were collected. The demographic and clinical data of the patients enrolled in the experiment are presented in Table 2. From the data, the aortic peak gradient of CAVD group reached to 61.5 ± 5.519 mmHg and tended to have significantly higher age (66 ± 2.289 versus 45.86 ± 7.33, $P = 0.0029$), smoking percentage (60% versus 12.5%, $P = 0.0743$), hypertension percentage (66.70% versus 12.5%, $P = 0.0272$) as well as body mass index (BMI, 26.19 ± 0.9123 kg/m^2 versus 20.31 ± 2.216 kg/m^2, $P = 0.0244$) than normal group. However, the data showed no difference among gender, diabetes percentage, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), Creatinine and Creatinine clearance between normal and CAVD groups. Prominent calcified regions were observed by Von Kossa staining in calcified valve. On the contrary, the normal valve barely formed calcified masses (Fig. 3a–b). Of great interest, in immunohistochemistry assay, the expression of CCR2 (18.83% ± 1.11% versus 35% ± 1.59%, $P < 0.0001$) and Runx2 (12.66% ± 1.77% versus 60.87% ± 2.0%, $P < 0.0001$) and BMP2 (35.21% ± 2.67% versus 58.45% ± 1.07%, $P < 0.0001$) was both significantly increased in human calcified aortic valve tissue compared to normal aortic valve tissue (Fig. 3c–h). Regions of CCR2 protein expression of diseased aortic valve samples were dominantly in the area of surrounding angiogenesis and calcification (Fig. 3c). Several studies also highlighted the osteoblastic markers including BMP2 and Runx2 were actively expressed in calcified models [14].

As plenty of studies noted that monocyte/macrophage was involved in the development of CAVD and CCR2 was also highly expressed by monocyte/macrophage under inflammation [15]. Thus, we tended to investigate the source of CCR2 in calcified aortic valve. We performed a co-localization immunofluorescence of CCR2 and CD68 which is a

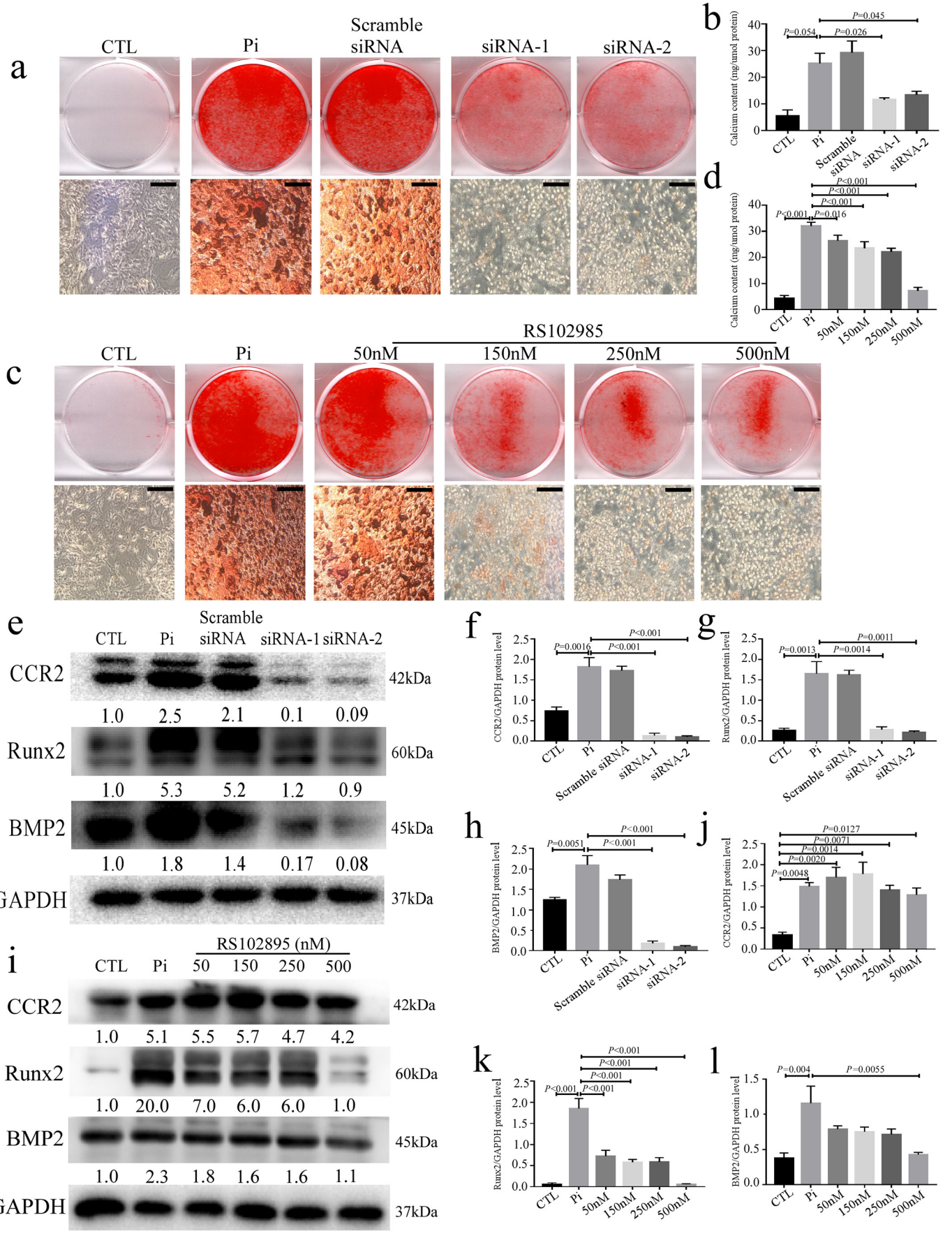

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Fig. 4. CCR2 blockade alleviated osteoblastic transformation of AVICs.
Alizarin red staining of AVICs treated with CCR2 siRNAs after 7 days pro-calcifying medium culture. Scale bar: 50 μm. b. Alizarin red staining of AVICs treated with CCR2 selective inhibitor (RS102895) after 7 days pro-calcifying medium culture. Scale bar: 50 μm. c. The levels of CCR2, Runx2 and BMP2 in pro-calcifying medium cultured AVIC after CCR2 knockdown. g. The levels of CCR2, Runx2 and BMP2 expression in pro-calcifying medium cultured AVICs after CCR2 inhibition. d. Analysis of grey value of CCR2, Runx2 (e) and BMP2 (f) compared to control cells after CCR2 knockdown in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD of 3 independent experiments. h. Analysis of grey value of CCR2, Runx2 (i) and BMP2 (j) compared to control cells after CCR2 inhibition by RS102895 in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD of 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

novel marker of monocyte/macrophage [16,17]. We found, in calcified aortic valve tissue, CCR2 was expressed in the areas where CD68 was absent, but could also be easily observed in the regions of overlapping with CD68, suggesting that monocyte/macrophage was not the main source of CCR2 in calcified aortic tissue. As expected, high expression of CCR2 was concentrated in calcified valve, while normal aortic valve tissue barely expressed CCR2 or CD68 (Fig. 3i). Thus, CCR2 expression was significantly increased in CAVD calcified aortic valve tissue and partially expressed in regions with the absent to monocyte/macrophage.

3.4. CCR2 blockade alleviated osteoblastic transformation of AVICs

In order to demonstrate the role of CCR2 in osteoblastic transformation of AVICs, we first showed that the expression of CCR2 was significantly decreased in CCR2 siRNAs-transfected AVICs (Fig. 4e–f). Alizarin red staining and Calcium deposition results showed that CCR2 siRNAs attenuated AVICs calcification in vitro (Fig. 4a–b). Moreover, CCR2 selective pharmaceutical inhibitor RS102895 inhibited AVICs calcification in a dose-dependent manner (Fig. 4c–d). To determine whether CCR2 inhibition was involved in alleviation of osteoblastic transformed AVICs, the expression of osteoblastic markers, Runx2 and BMP2, was also tested. We found that knockdown of CCR2 significantly decreased the expression of Runx2 and BMP2 in the pro-calcifying medium-induced AVICs (Fig. 4e–h). Consistently, both Runx2 and BMP2 expression was significantly down-regulated by RS102895, especially at the concentration of 500 nM (Fig. 4i–l). These suggested that CCR2 functions in the osteoblastic transformation of AVICs.

3.5. The CCL2/CCR2 axis promoted osteoblastic transformation of AVICs

CCL2 belongs to C-C chemokine subfamily and serves as a high-affinity ligand for CCR2, appearing to specifically bind to CCR2 only [6]. The CCL2/CCR2 axis is significantly involved in the progression of atherosclerosis [18]. Several studies demonstrated that the extent of aortic valve calcification was positively correlated with serum CCL2 levels [19]. Thus, we hypothesized that pro-calcifying medium could stimulate AVICs to secret more CCL2 and promote osteoblastic transformation of AVICs via binding to CCR2. To illuminate this point, we first measured the expression of CCL2 between control and pro-calcifying medium-induced AVICs. We found that AVICs produced CCL2 and showed a small increase in mRNA expression exposed to pro-calcifying medium (Fig. 5a). Furthermore, pro-calcifying medium-induced AVICs increased the protein production of CCL2 in the supernatant (Fig. 5b). Next, we treated AVICs with increasing dose of recombinant CCL2 (0, 20 and 200 ng/mL) in pro-calcifying medium for 3 days. CCL2 significantly increased the protein expression of osteoblastic markers Runx2 and BMP2 when compared to the group cultured without CCL2 (Fig. 5c–e). Moreover, CCL2 siRNA-AVICs exhibited significant suppression of osteoblastic markers Runx2 and BMP2 expression in pro-calcifying medium. These findings indicated that CCR2 was activated by CCL2 and the CCL2/CCR2 axis activation participated in the process of promoting osteoblastic transformation of AVICs.

3.6. CCR2 alleviated osteoblastic transformation of AVICs through activating PI3K/Akt pathways

Previous studies demonstrated that phosphorylation Akt (p-Akt) was decreased in calcified AVICs [20,21] and CCR2 showed its potential regulation on PI3K/Akt signaling pathway. We tended to explore whether PI3K/Akt signaling pathway was involved in CCR2 dependent osteoblastic transformation of AVICs. We first employed KEGG analysis and found PI3K/Akt pathway was associated with CCR2 dependent chemokine signaling pathway (Fig. 6a). Therefore, we examined the activation of CCR2-PI3K/Akt signaling pathway in osteoblastic transformed AVICs. Consistently, p-Akt/t-Akt ratio was significant reduced in pro-calcifying medium-cultured AVICs (Fig. 6b–e). And we found that CCR2 siRNAs (Fig. 6b–c) and RS102895 (Fig. 6d–e) could reverse the reduction of p-Akt/t-Akt ratio in the osteoblastic transformed AVICs. These results demonstrated that CCR2 contributed to AVICs osteoblastic transformation through PI3K/Akt pathway.

4. Discussion

Growing evidence indicates the pathological processes of CAVD share similar process of the ectopic ossification in cardiovascular system which includes deposition of lipids, inflammatory, angiogenesis and ECM remodeling [22,23]. In the end-stage of CAVD, active forming of calcified mass and mineralized nodules on local valve cusps caused dysfunction of aortic valve [24]. Thus, it is of great importance to understand the pathological process as well as molecular mechanism of CAVD. In this study, we showed that CCR2 was upregulated in human calcified valve and calcifying AVICs. Besides, we found that CCL2/CCR2 axis activation participated in osteoblastic transformation and calcification of AVICs through PI3K/Akt signaling pathway. These suggested that targeting CCR2 might be promising for CAVD therapy.

It is widely acknowledged that the initiation and progression of CAVD involves several pathophysiological processes and abnormal function of AVICs contributes to CAVD. AVICs are the most abundant cell types in the valve. During the development of CAVD, AVICs undergoes osteoblastic transformation, produces collagen, remodeling of extracellular matrix and actives pro-calcifying signaling pathway [25]. In this regard, osteoblastic transformation of AVICs is critical for calcific changes in aortic valve under pathological circumstance. Recent studies showed that multiple cell groups such as AVECs, macrophages, dentric cells and T lymphocytes also functioned in the progression of CAVD [26,27]. More attention should be paid to these cell types and their involvement in CAVD in the future study.

CCRs superfamily is a member of G-protein coupled receptors which is an important regulator of inflammatory activity or immunology response [28]. Interestingly, some studies noted that CCRs were not only regulators of immunity but also associated with cardiovascular calcification. A cohort study unveiled that Ile64 variant of CCR2 was significantly related to lower degree of coronary artery calcification in first-degree relatives of subjects with premature coronary artery disease [8]. In addition, the Ile64 variant of CCR2 polymorphism has been reported to be associated with function reduction of CCR2 [29]. These strongly hint that CCR2 potentially regulate pathological cardiovascular calcification. However, no such direct evidence revealed that the functional roles of CCRs in CAVD. We, for the first time, revealed that

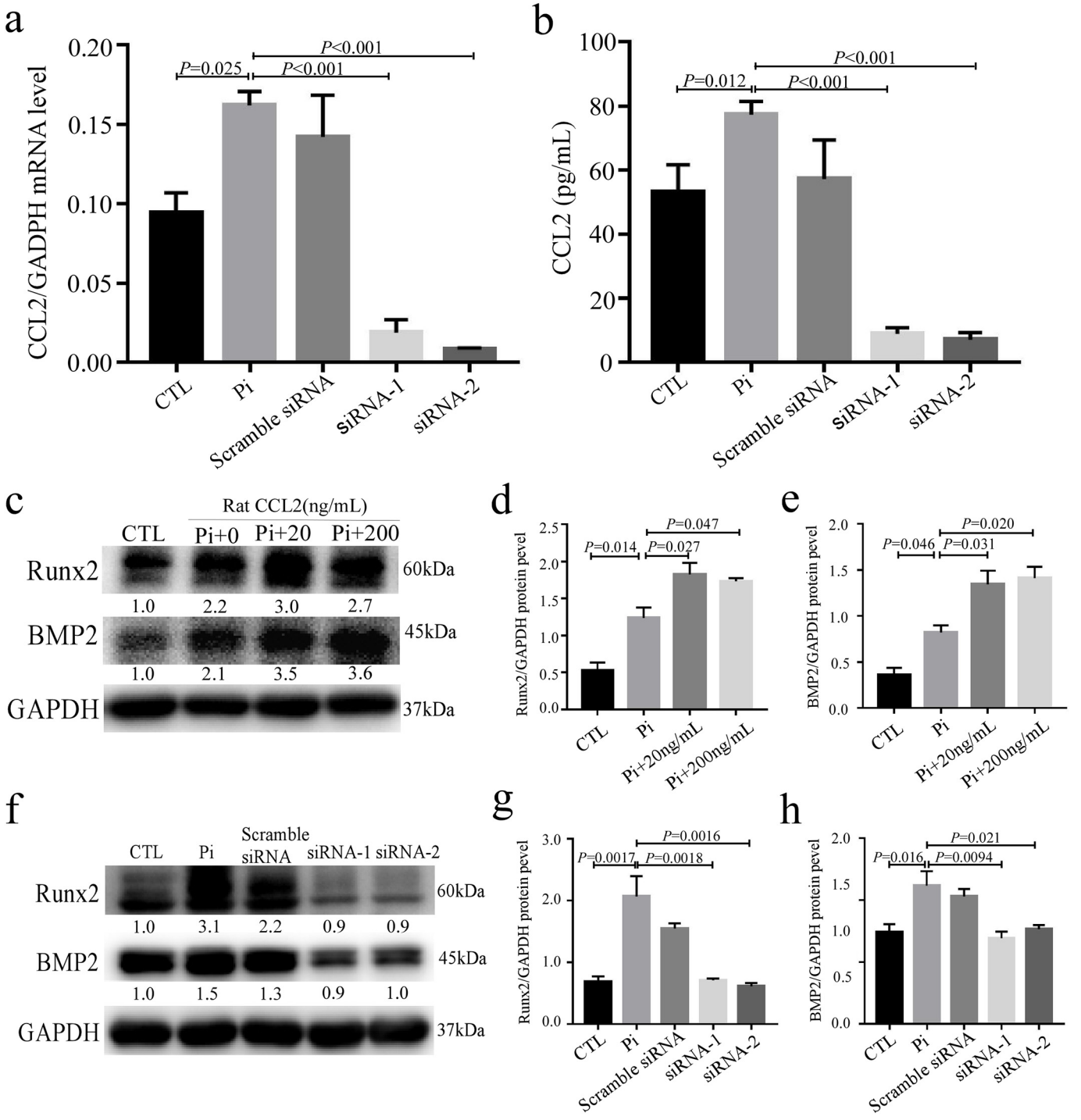

Fig. 5. The CCL2/CCR2 axis promotes osteoblastic transformation of AVICs.
a. The mRNA expression levels of CCL2 were determined by qPCR in pro-calcifying medium induced AVICs after CCL2 knock-down. All data is shown as the mean ± SD of 3 independent experiments. b. The protein expression levels of CCL2 were determined by ELISA in pro-calcifying medium cultured AVICs after CCL2 knock-down. All data is shown as the mean ± SD of 3 independent experiments. c. The levels of Runx2 and BMP2 in pro-calcifying medium cultured AVIC after treatment with CCL2. d. Analysis of grey value of Runx2 and BMP2 (e) compared to pro-calcifying medium cultured AVIC without CCL2. All data is shown as the mean ± SD of 3 independent experiments. f. The level of Runx2 and BMP2 in pro-calcifying medium cultured AVIC after CCL2 knockdown. g. Analysis of grey value of Runx2 and BMP2 (h) compared to control cells after CCL2 knockdown in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD of 3 independent experiments.

CCR2 was up-regulated in human CAVD samples and the CCL2/CCR2 axis activation contributed to osteoblastic transformation of AVICs through PI3K/Akt signaling pathway. Interestingly, it was reported that other CCRs were related to ectopic ossification in cardiovascular system. For example, in the investigation of relationship between gene polymorphism and CAVD, patients who had a higher degree of calcification in aortic valve carried certain interleukin-10, connective tissue growth factor as well as CCR5 polymorphisms. More studies are needed to demonstrate the associations between CAVD and CCRs and the potential molecular mechanisms.

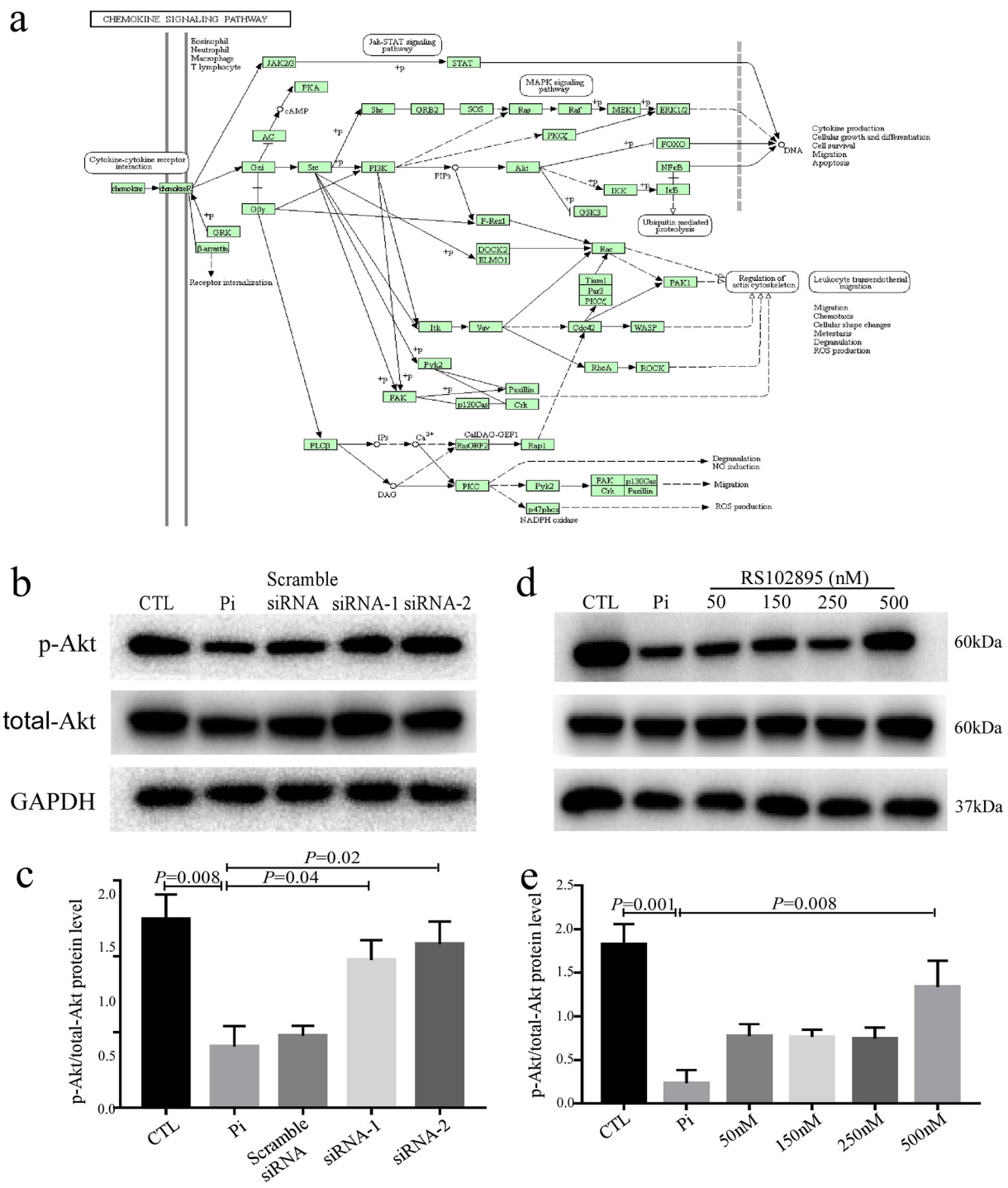

Fig. 6. CCR2 alleviated osteoblastic transformation of AVICs through PI3K/Akt pathways.
a. KEGG pathway analysis of relationship between CCR2 and PI3-K/Akt signaling pathway. b. P-Akt protein levels of pro-calcifying medium cultured AVICs after CCR2 knockdown by siRNAs. c. P-Akt protein levels of pro-calcifying medium cultured AVICs after CCR2 inhibition by RS102895. d Analysis of grey value of p-Akt/t-Akt ratio compared to control cells after CCR2 knockdown in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD of 3 independent experiments. e. Analysis of grey value of p-Akt/t-Akt ratio compares to control cells after CCR2 inhibition by RS102895 in pro-calcifying medium cultured AVIC. All data is shown as the mean ± SD of 3 independent experiments.

There are several limitations in our study. First, our experiments were performed under static conditions. Previous studies showed that mechanical force played an important role in CAVD. Porcine VICs in three-dimensional culture cannot mineralize even treated with pro-calcifying medium unless stimulated with mechanical stress [30]. The osteogenic deposit of VICs was more serious when exposed to a stiffness of 25 kPa [31]. Although calcification of VICs was successfully induced in our study, further studies should be carried out to reveal whether CCR2 can function in mechanical forces induced calcification. Second, we only performed the study in vitro, and CCR2 knockout mice should be used in the further exploration.

To our knowledge, this work demonstrated that CCR2 and CCL2 was an important regulator of AVICs osteoblastic transformation. Hence, this study illustrated that targeting the CCR2 may provide benefits in

reducing aortic valve osteoblastic transformation and calcification. CCR2 inhibition has the potential in the treatment of CAVD in the future.

5. Conclusions

CCL2/CCR2 axis activation was both in human calcific aortic valve tissues and calcifying AVICs. Furthermore, inhibition of CCR2 reduced osteoblastic transformation and calcification of AVICs through the activation of PI3K/Akt signaling pathway. Hence, further therapy targeting CCR2 may have potential in improving CAVD treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.050.

Acknowledgement

We specially acknowledged Professor Ximing Shen for performing technical assistance on the management of human aortic valve samples and Jingwei Gao for providing writing assistance.

Funding sources

This study was supported by grants from the National Natural Science Foundation of China (81370309 and 81700263), the Science and Technology Project of Shenzhen City of China (JCYJ20170307161535847) and Research Project (Doctoral Innovation Program) of Health Planning System of Shenzhen City (SZBC2017007). The funder had no roles in study design, data collection, data analysis and interpretation, or writing the manuscript.

Declaration of interests

The authors declare that they have no competing interests.

Author's contributions

E.Z., W.H. and Z.L. were responsible for most of the experiments. B.D. and X.S. recruited the aortic valve samples and collected clinical data. Z.H., Z.L. and X.W. were responsible for statistical analyzed and interpreted the data. X.K. and R.N. designed the study and they reviewed the final version of the manuscript and approve it for publication.

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