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MMP-12–Induced Pro-osteogenic Responses in Human Aortic Valve Interstitial Cells

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Abstract

Background: Calcific aortic valve disease (CAVD) is an age-related and slowly progressive valvular disorder. Overexpression of matrix metalloproteinase 12 (MMP-12) has been found in atherosclerosis, stiffed vascular tissue, and calcified aortic valves. We hypothesized that MMP-12 may induce the pro-osteogenic responses in human aortic valve interstitial cells (AVICs).

Methods: Human AVICs were isolated from normal and calcified aortic valves. Cells were treated with MMP-12. The pro-osteogenic marker Runt-related transcription factor 2 (RUNX-2), bone morphogenetic protein 2 (BMP-2), and alkaline phosphatase (ALP), as well as MMP-12–associated signaling molecules, were analyzed.

Results: Human calcified aortic valves expressed significantly higher MMP-12 than normal human aortic valves. MMP-12–induced the expression of RUNX-2, BMP-2, ALP, and calcium deposit formation. Suppression of MMP-12 by its inhibitor decreased the expression of RUNX-2, BMP-2, and ALP. MMP-12–induced osteogenic responses were associated with higher levels of phosphorylation of p38 mitogen-activated protein kinases (MAPK), low density lipoprotein-related protein 6 (LRP-6), and β-catenin signaling molecules. Calcified aortic valves exhibited markedly higher levels of LRP-6 and β-catenin levels. Inhibition of either p38 MAPK or LRP-6 attenuated MMP-12–induced expression of RUNX-2, BMP-2, and ALP. Suppression of p38 MAPK abrogated MMP-12–induced activation of LRP-6 and β-catenin signaling pathways.

Conclusions: MMP-12 induces pro-osteogenic responses in AVICs by activation of p38 MAPK-mediated LRP-6 and β-catenin signaling pathways. The study revealed that the potential role of MMP-12 in the pathogenesis of CAVD and therapeutically targeting MMP-12 may suppress the development of CAVD.

Keywords

Aortic valve; Pro-osteogenic proteins; MMP-12; Signal transduction

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

Introduction

Calcific aortic valve disease (CAVD) is a common heart disorder in older people. The initial form of CAVD, termed aortic sclerosis, is present in about one-third of individuals aged >65 y.[1] Owing to its association with age, the prevalence of CAVD is predicted to rise as life expectancy increases. CAVD is characterized by progressive valvar remodeling, with fibrosis and calcification of the leaflets.[2] The mechanisms underlying CAVD have not been fully identified. Many factors are involved in the progression of CAVD, including aging, atherosclerosis, and inflammatory diseases.[3]

The ability of aortic valves to function is mainly depended on the tissue extracellular matrix (ECM). Growing evidence indicates that the valve ECM may influence the cellular signaling processes to contribute to the pathobiology of CAVD.[4] Matrix metalloproteinases (MMPs) are a group of zinc endopeptidases functioning in the ECM and degrading both matrix and nonmatrix components.[5] Recently, MMP-12 (human macrophage elastase) has been found to be involved in the pathogenesis of atherosclerosis,[6,7] chronic obstructive pulmonary disease, and arterial stiffening.[8] Furthermore, gene expression studies have found that MMP-12 is markedly upregulated in calcific aortic valves as compared with normal valves.[9]

Several pathways are potentially involved in CAVD in terms of cellular mechanisms. wingless/integrated (Wnt)/β-catenin signaling pathway has been identified in experimental hypercholesterolemia model of CAVD and human calcific aortic valves.[10,11] The aim of this study was to compare the expression of MMP-12, low density lipoprotein-related protein 6 (LRP-6) (a coreceptor of WNT), and β-catenin in human aortic valve tissues from normal and diseased subjects and then to find if MMP-12 can induce the pro-osteogenic responses in human aortic valve interstitial cells (AVICs). Our results indicate that expression of MMP-12, LRP-6, and β-catenin is significantly higher in tissues from calcified aortic valves than that from normal aortic valves. MMP-12–induced pro-osteogenic responses in AVICs are regulated by p38 mitogen-activated protein kinases (MAPK)-mediated LRP-6 and β-catenin signaling pathways.

Material and methods

Reagents

The antibody against MMP-12 was purchased from Thermo Fisher Scientific (PA5-13181, 1:1000 dilution; Rockford, IL). Antibody against bone morphogenetic protein 2 (BMP-2) was purchased from ProSci, Inc (XP-5111, 1:1000 dilution; Poway, CA). The antibody against alkaline phosphatase (ALP) was purchased from ABCAM (ab108337, 1:1000 dilution; Cambridge, MA). Antibodies against Runt-related transcription factor 2 (RUNX-2) (12,556, 1:1000 dilution), phosphorylated p38 MAPK (9211, 1:1000 dilution), total p38 MAPK (9212, 1:1000 dilution), phosphorylated LRP-6 (2568, 1:1000 dilution), total LRP-6 (2560,1:1000 dilution), phosphorylated β-catenin (s41/45) (9565, 1:1000), phosphorylated β-catenin (s33/37/41) (9561, 1:1000 dilution), total β-catenin (9562, 1:1000 dilution), and β-actin (4967, 1:1000 dilution) were purchased from Cell Signaling, Inc (Beverly, MA). Recombinant human MMP-12 was incubated with assay buffer (50-mM Tris, 10-mM $CaCl_2$,

150-mM NaCl, 0.05% Brij-35, pH 7.5) at 37°C for 30 h to activate enzyme, as per the manufacturer's protocol (R&D Systems). MMP408 (MMP-12 inhibitor) is from EMD Millipore corporation (Billerica, MA). DBM1285 dihydrochloride (p38 MAPK inhibitor) is from TOCRIS Bioscience (Bristol, UK). Recombinant human DKK1 (LRP-6 inhibitor) is from R&D Systems, Inc (Minneapolis, MN). Medium 199 was purchased from Lonza (Walkersville, MD). Laemmli sample buffer and nitrocellulose membranes for immunoblotting were purchased from Bio-Rad (Hercules,CA). Lipopolysaccharide (Escherichia coli 0111:B4) and all other chemicals were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO).

Cell isolation and culture

Normal aortic valve leaflets were collected from the explanted hearts of 6 patients with cardiomyopathy and undergoing heart transplantation at the University of Colorado Hospital. Calcific aortic valve leaflets were obtained at the time of aortic valve replacement from 6 separate donors. These valve leaflets were thin and did not exhibit histological abnormality. All patients gave informed consent for the use of their own valves for this study approved by the University of Colorado Denver Institutional Review Board.

AVICs were isolated from normal or calcific human aortic valves and cultured in a M199 medium.[12] Briefly, valve leaflets were subjected to sequential digestions with collagenase, and cells were collected by centrifugation. Cells were cultured in a M199 growth medium containing penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum. Cells from passages 2 to 6 were used for this study. Cells were treated when they reached 80% to 90% confluence.

Immunoblotting

Western blot was applied to analyze MMP-12, LRP-6, β-catenin, BMP-2, ALP, RUNX-2, phosphorylated and total β-catenin, phosphorylated, and total p38 MAPK with β-actin as a loading control. AVICs in culture were lysed in 1× Laemmli sample buffer with β-mercaptoethanol. Cell lysates were resolved on 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and the proteins were transferred onto polyvinylidene difluoride membranes. After being blocked with 5% skim milk solution, membranes were incubated with primary antibodies, followed by peroxidase-linked secondary antibodies specific to the primary antibodies. Protein bands were revealed using the enhanced chemiluminescence system. Band density was analyzed using the National Institutes of Health Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Alizarin red S staining

To examine the formation of calcium deposits, cells were treated with MMP-12 (80 ng/mL) for 21 d in a conditioning medium (growth medium supplemented with 10 mmol/L of β-glycerophosphate, 10 nmol/L of vitamin D3, 10 nmol/L of dexamethasone, and 8 mmol/L of $CaCl_2$). Alizarin red S staining for calcium deposits was performed. Briefly, cell monolayers were washed three times with Phosphate buffer saline and fixed for 10 min with 4% paraformaldehyde, followed by incubation with 0.2% Alizarin red S solution (pH 4.2) for 30

min. Excessive dye was removed by washing with distilled water. Alizarin red S staining was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan). To quantitatively analyze Alizarin red staining, stains were bleached with 10% acetic acid at 85°C. The supernatant was analyzed spectrophotometrically at 450 nm.

Casein zymography

Conditioned media were harvested from AVICs cultures (at 48 h). Twenty microliter aliquots of 10× concentrated conditioned media were electrophoresed on 10% SDS-polyacrylamide gel with 1 mg/mL of β-casein.[13] After electrophoresis, the gel was washed with Triton X-100 buffer and then incubated at 37° C for 24 h. After washing, the gel was stained in Colloidal Coomassie Brilliant Blue G-250 for 24 h. Relevant band intensities were quantified by scanning densitometric analysis.

Statistical analysis

Data are presented as mean ± standard error (SE). Statistical analysis was performed using analysis of variance; $P < 0.05$ was considered significant.

Results

The calcified aortic valves express higher MMP-12

To determine if expression of MMP-12 is increased in calcified aortic valves, we compared aortic valve leaflets from 6 valve-replacement patients to those from 6 explanted cardiomyopathy patients (patient demographic data are listed in Table). MMP-12 expression is seen in almost all tissues, but there is a significantly higher MMP-12 protein level in calcific aortic valve leaflets relative to normal valve leaflets (Fig. 1A). Similar to aortic valve leaflets, supernatants from the calcified AVICs show higher MMP-12 activity than those from normal AVICs as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis casein zymography (Fig. 1B). Owing to the SDS in the gel, the 54-kDa latent form of MMP-12 produced by AVICs was responsible for the major caseinolytic activity.[13]

MMP-12 protein induces the osteogenic responses in human cells

To determine if MMP-12 protein can induce the osteogenic responses in human cells, we isolated AVICs from aortic valve leaflets and then treated the cells with human MMP-12 recombinant protein. First, cells were cultured with different concentrations of MMP-12 for 48 h. As showed in Figure 2A, MMP-12 dose-dependently induced RUNX-2, BMP-2, and ALP expression in AVICs. Then, cells were treated with the same dose of MMP-12 protein at different time points. As showed in Figure 2B, MMP-12 time-dependently induced RUNX-2, BMP-2, and ALP in AVICs. On the other hand, MMP-12 inhibitor MMP408 significantly abrogated MMP-12–induced pro-osteogenic responses at 72 h. Then, cells were stimulated with MMP-12 in a conditioning medium for 21 d. Interestingly, MMP-12 induced substantially greater calcium deposition than control (Fig. 2C). These data indicated that MMP-12 contributes to the mechanism underlying osteogenic responses in human AVICs.

Active form of MMP-12 *induces the osteogenic responses in* AVICs

To determine which form of MMP-12 induces the osteogenic responses in AVICs, we treated AVICs with full-length MMP-12 and cleaved MMP-12. As showed in Figure 3, only cleaved MMP-12 (active form) induced RUNX-2, BMP-2, and ALP expression in AVICs.

Signaling pathway activated by MMP-12

To elucidate the mechanism by which MMP-12 induces the osteogenic responses, we firstly examined the expression of β-catenin and LRP-6 in calcified aortic valves *versus* control. Interestingly, both activated LRP-6 and total LRP-6, and total β-catenin was remarkably expressed in calcified aortic valves compared with normal (Fig. 4A).

Then, we examined the activation of p38 MAPK, LRP-6, and β-catenin, an important signaling pathway that regulate AVIC osteogenic responses. Human AVICs were treated with MMP-12 for 0-48 h. As showed in Figure 4B, MMP-12 markedly induced the phosphorylation of p38 MAPK, β-catenin (both s41/45 and s33/37/41), and LRP-6. To confirm the role of p38 MAPK and LRP-6 in mediating the osteogenic responses to MMP-12, we exposed AVICs to specific inhibitors before the exposure to MMP-12. Inhibition of p38 MAPK by DBM1285 or LRP-6 by DKK1 significantly reduced the osteogenic effects of MMP-12 (Fig. 4C). Furthermore, suppression of p38 MAPK abrogated MMP-12–induced activation of LRP-6 and β-catenin signaling pathway (Fig. 4D). Together, those observations revealed that p38 MAPK/LRP-6/β-catenin mediate the osteogenic responses to MMP-12 in AVICs (Fig. 5).

Comment

ECM pathological remodeling promotes CAVD progression. In this study, we found that MMP-12 is capable of inducing production of multiplepro-osteogenic factors inAVICs by activation of p38 MAPK-mediated LRP-6 and β-catenin signaling pathways.

MMP-12 is a canonical elastase secreted by macrophages and plays a major role in the development of aneurysm.[14] Produced as a 54-kDa zymogens, MMP-12 initially is inactive. With autolytic cleavage of the NH2-terminal domain, it becomes active (45 kDa). This form of enzyme can be readily processed to mature 22-kDA active enzyme with a loss of the COOH-terminal domain.[15] The major substrate for MMP-12 is elastin, which is richly present in arterial wall and other tissues.[16] The presence of macrophages is rare in normal human aortic valves, but excessive infiltration of macrophages was observed in calcified human aortic valves.[17] This implicates MMP-12 in the development of calcific aortic valve stenosis. Several forms of MMPs (MMP-1, 2, and 9) existed in calcified aortic leaflets.[18] A recent genomic study demonstrated that MMP-12 is the most upregulated gene in stenotic aortic cusps.[9] Furthermore, MMP-12 protein was directly recognized by immunohistochemical staining in the areas of prominent calcification.[16] In accordance with these observations, our study shows that diseased valves express greater levels of MMP-12 in vitro. Similarly, AVICs from diseased donors show higher MMP-12 activity than those from normal donors. We postulate that an inflammatory environment, with macrophage infiltration, promotes local production of MMP-12 and is a pathogenic mechanism for calcific aortic stenosis.

Although MMP-12 is accumulated in calcific, stenotic areas of aortic valves, the role of MMP-12 in the pathogenesis of CAVD and the effect of MMP-12 on aortic valve cells had been unclear. It has been proposed that inflammatory cell invasion is involved in the early pathogenesis of CAVD.[19] The present study demonstrated that MMP-12 induces expression of RUNX-2, BMP-2, ALP, and calcium deposit formation. Inhibition of MMP-12 by its specific inhibitor MMP408 completely repressed the RUNX-2 but partially for BMP-2 or ALP. A higher dose of inhibitor might completely inhibit BMP-2 or ALP. RUNX-2 is a transcription factor that is involved in aortic valve stenosis.[20] BMP-2 is a potent osteogenic element and has been identified in calcified tissue.[21] ALP plays a role in the initiation process of calcification.[22] Because MMP-12 effectively upregulates these pro-osteogenic factors in human AVICs, it may contribute to the mechanism of CAVD.

In the present study, we observe the presence of higher levels of LRP-6 and β-catenin in diseased aortic valve tissues, which indicates that there might be a correlation between MMP-12 and WNT/β-catenin signaling pathway. Transcription of MMP-12 is responsive to WNT/β-catenin signaling pathway.[23] LRP-6 is an important coreceptor for WNT/β-catenin pathway.[24] It is involved in bone development, lipid metabolism, and cardiac valve disease.[25] Furthermore, canonical WNT signaling plays a crucial role in osteogenesis.[26] Indeed, stimulation of AVICs with recombinant MMP-12 induces the phosphorylation of LRP-6 and β-catenin. Moreover, MMP-12 also induces the phosphorylation of p38 MAPK in AVICs. Both pathways appear to be involved in MMP-12–mediated upregulation of RUNX-2, BMP-2, and ALP because the specific inhibitor for each of these signaling pathways attenuates MMP-12–induced expression of RUNX-2, BMP-2, and ALP. However, it is noteworthy that inhibition of LRP-6 only blocks MMP-12–induced β-catenin activation, but does not affect MMP-12–induced p38 MAPK activation. On the other hand, suppression of p38 MAPK abrogates both MMP-12–induced LRP-6 and β-catenin activations, which is consistent with a previous finding that p38 MAPK can phosphorylate LRP-6 to regulate WNT/β-catenin signaling.[27] Thus, p38 MAPK/LRP-6/β-catenin pathway plays a major role in MMP-12–mediated upregulation of RUNX-2, BMP-2, and ALP expression.

In conclusion, human calcified aortic valves express greater levels of MMP-12, LRP-6, and β-catenin. Recombinant MMP-12 induces the expression of RUNX-2, BMP-2, and ALP in human AVICs mainly through interaction with p38 MAPK/LRP-6/β-catenin pathway. These findings suggest that MMP-12 contributes to the mechanism underlying the initiation and progression of CAVD.unclear. It has been proposed that inflammatory cell invasion is involved in the early pathogenesis of CAVD.[19]

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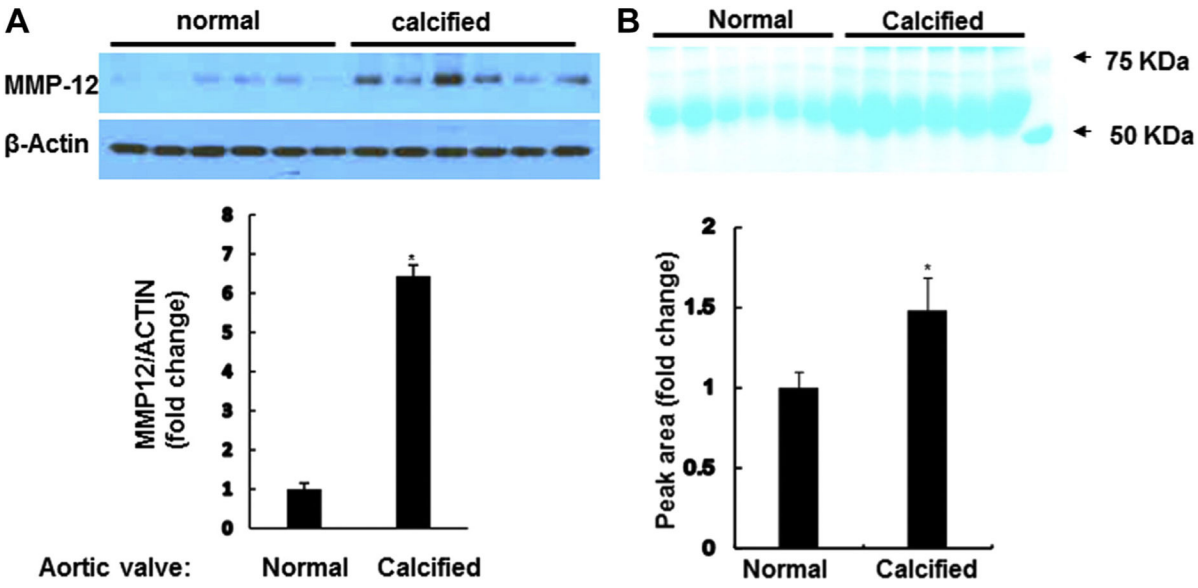

Fig. 1 –.

The calcified aortic valves show higher MMP-12 protein level and activity than normal aortic valves. (A) Normal and calcified aortic valves are homogenized, and the expression of MMP-12 is analyzed by immunoblotting ($n = 6$). (B) Scanned image of a casein zymography gel. Supernatants from the calcified AVICs show higher MMP-12 activity than those from normal AVICs ($n = 6$); $^*P < 0.05$ versus normal. (Color version of figure is available online.)

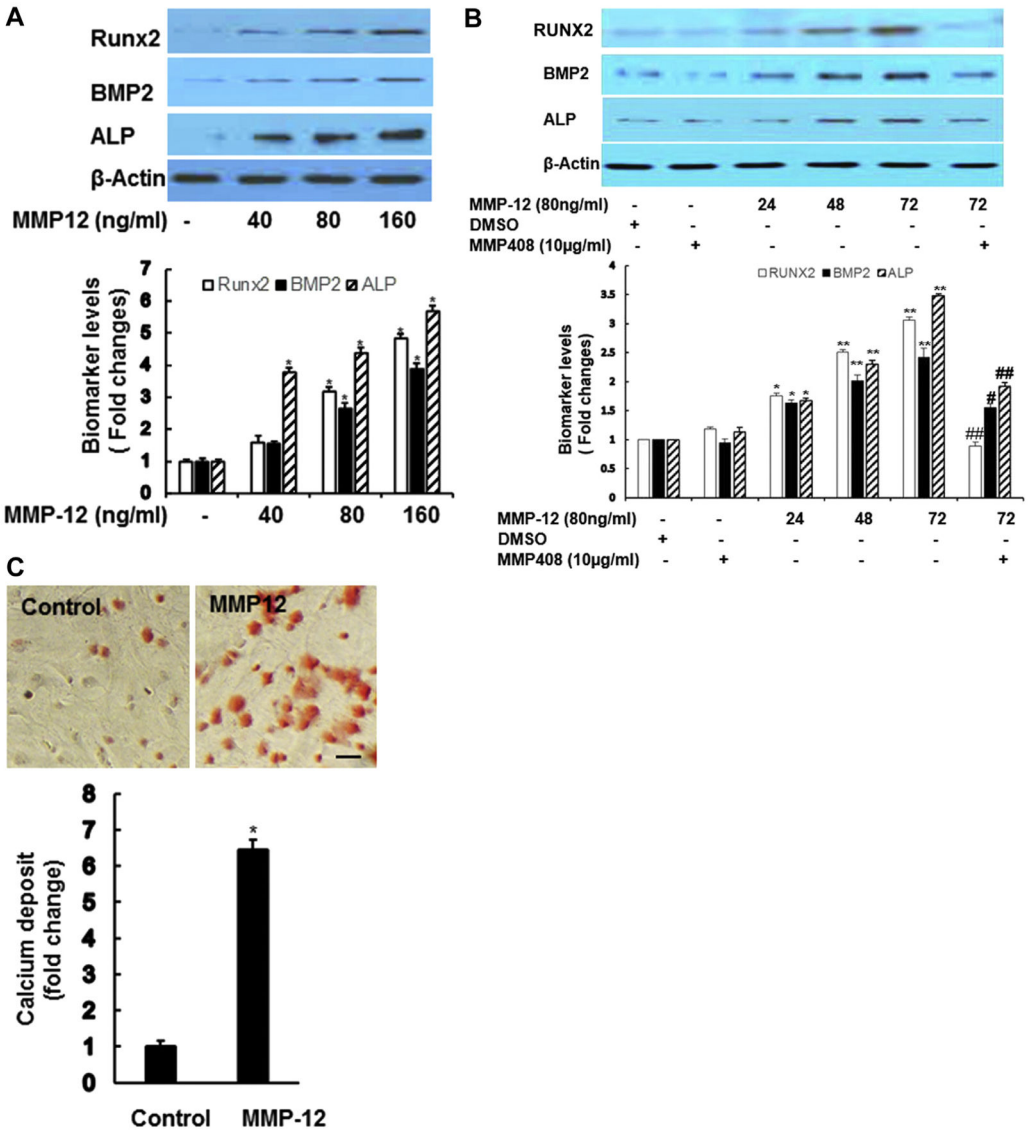

Fig. 2 –.

MMP-12 induces pro-osteogenic protein expression. (A) MMP-12 dose-dependently induces RUNX-2, BMP-2, and ALP expression in AVICS ($n = 6$);*$P < 0.05$ versus normal. (B) MMP-12 time-dependently induces RUNX-2, BMP-2, and ALP in AVICs; MMP-12 inhibitor significantly abrogated MMP-12–induced pro-osteogenic responses ($n = 6$). *$P <$ 0.05 versus normal; **$P < 0.01$ versus normal; #$P < 0.05$ versus MMP-12 treatment (72 h); ##$P < 0.01$ versus MMP-12 treatment (72 h). (C) MMP-12 induces calcium deposit formation in AVICs ($n = 6$), *$P < 0.05$ versus normal. DMSO = dimethyl sulfoxide. (Color version of figure is available online.)

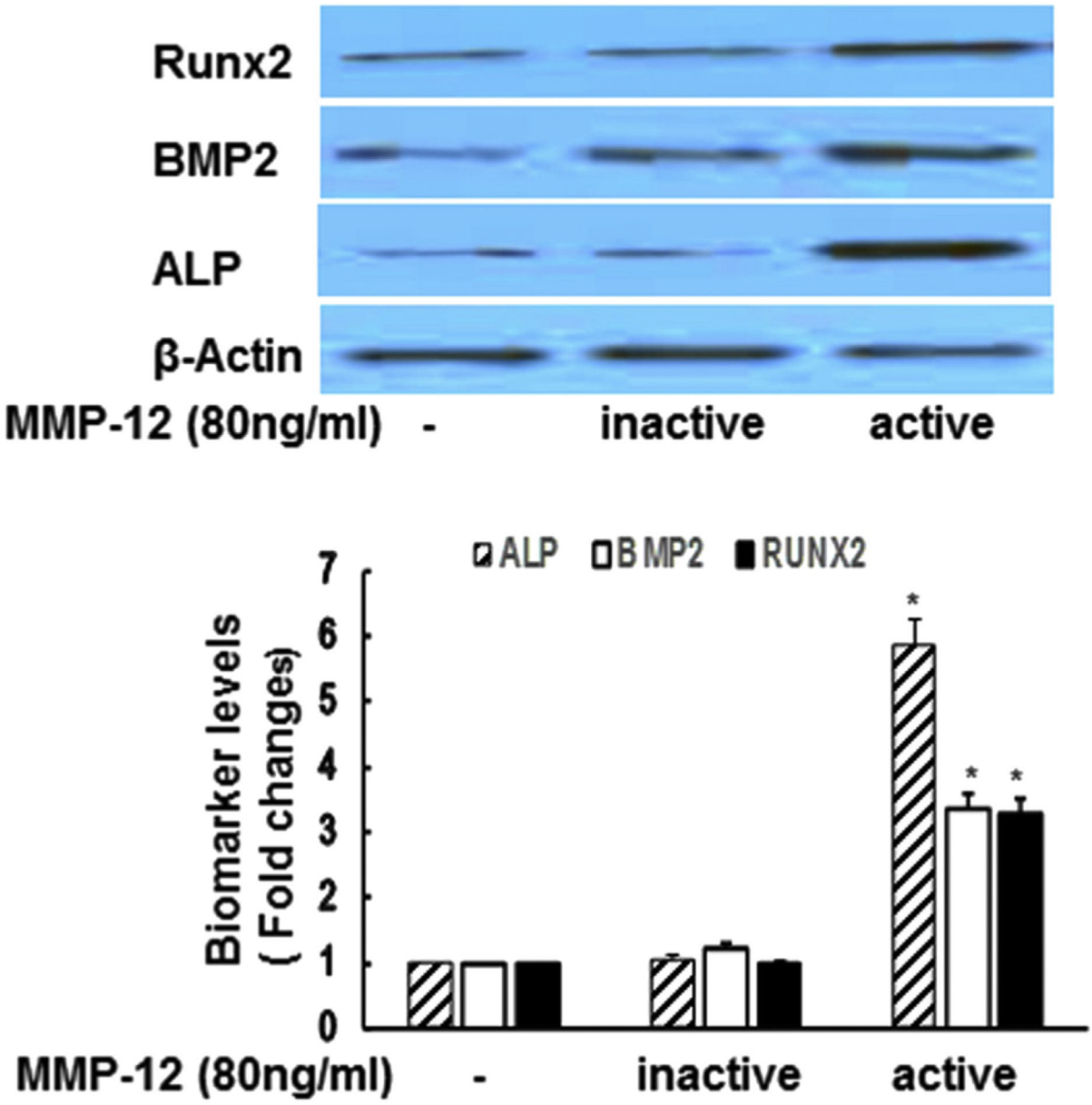

Fig. 3 –.

Active MMP-12 induces the osteogenic responses in AVICs ($n = 6$), *$P < 0.05$ versus normal. (Color version of figure is available online.)

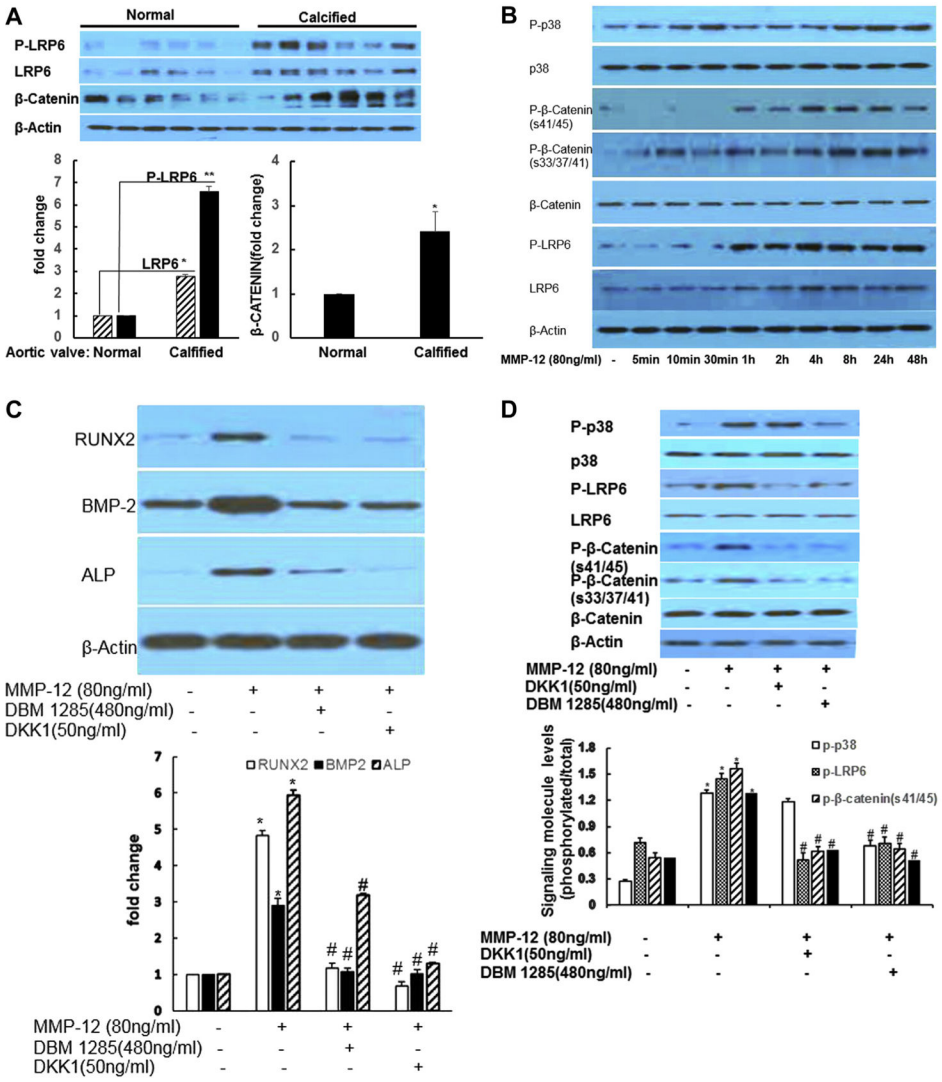

Fig. 4 –.

MMP-12 induces pro-osteogenic responses in AVICs by activation of p38 MAPK-mediated LRP-6 and β-catenin signaling pathways. (A) The calcified aortic valves express higher activated, total LRP-6, and β-catenin than normal aortic valves ($n = 6$). *$P < 0.05$ versus normal; **$P < 0.01$ versus normal. (B) MMP-12 activates p38 MAPK, β-catenin, and LRP-6 in AVICs. (C) Inhibition of p38 MAPK or LRP-6 abolishes MMP-12–induced osteogenic responses in AVICs ($n = 6$). *$P < 0.05$ versus normal, #$P < 0.05$ versus MMP-12 treatment. (D) Suppression of p38 MAPK abrogated MMP-12–induced activation of LRP-6 and β-catenin signaling pathway. *$P < 0.05$ versus normal, #$P < 0.05$ versus MMP-12 treatment. (Color version of figure is available online.)

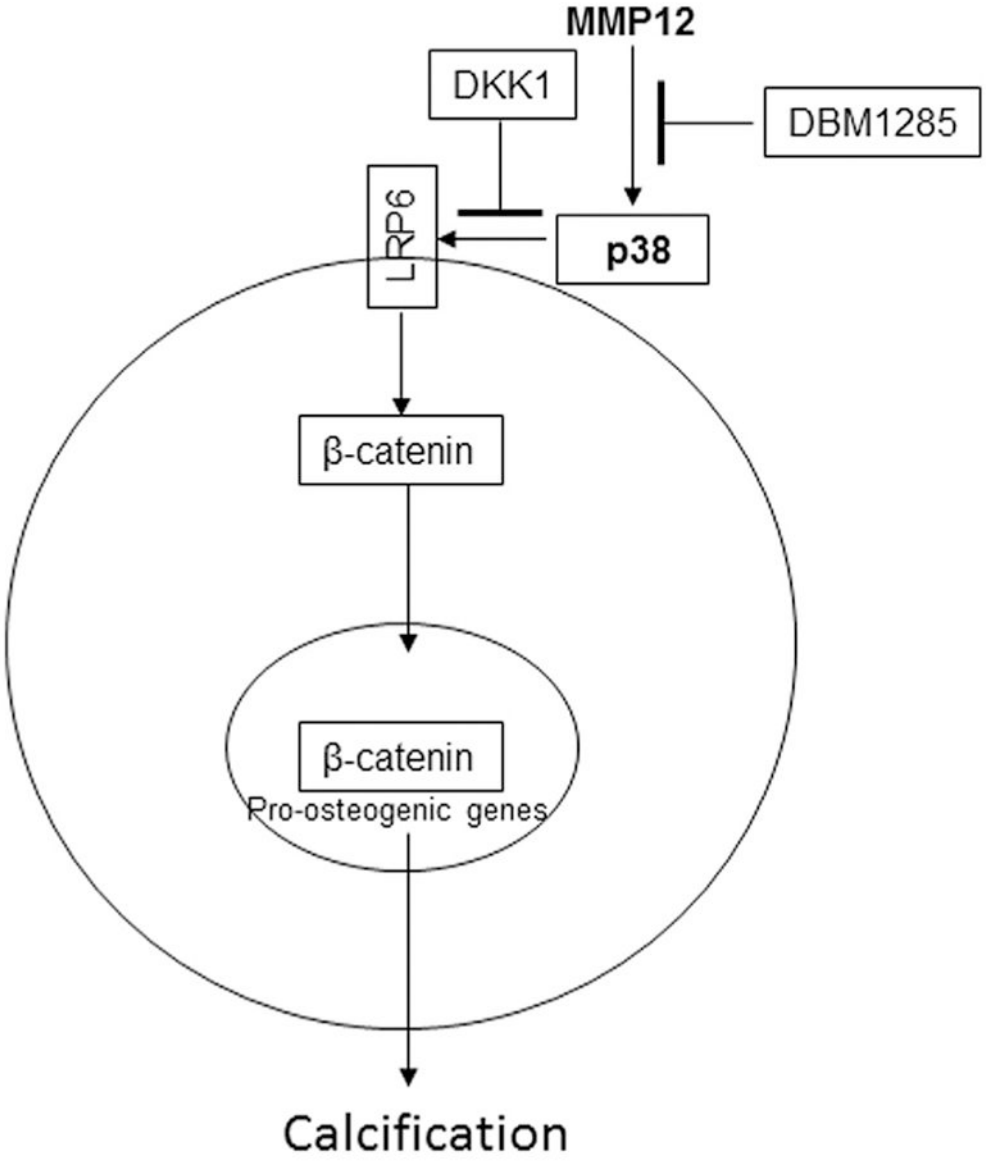

Fig. 5 –.
Suggested Mechanism of MMP-12–induced pro-osteogenic responses in human AVICs.

Table –

Patient demographic data: arotic value.

Patients	Diagnosis	Age (y)	Gender	Group
081806	Cardiomyopathy	58	F	Normal
052115	Cardiomyopathy	52	F	Normal
082410	Cardiomyopathy	57	F	Normal
011310	Cardiomyopathy	51	M	Normal
051310	Cardiomyopathy	60	M	Normal
010709	Cardiomyopathy	50	M	Normal
101510	CAVD	59	M	Calcified
120211	CAVD	62	M	Calcified
052110	CAVD	62	F	Calcified
092509	CAVD	67	F	Calcified
110306	CAVD	51	M	Calcified
073009	CAVD	68	M	Calcified