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Abstract—Identifying the key factor mediating pathological cardiac hypertrophy is critically important for developing the strategy to protect against heart failure. Bone morphogenetic protein-4 (BMP4) is a mechanosensitive and proinflammatory gene. In this study, we investigated the role of BMP4 in cardiac hypertrophy, apoptosis, and fibrosis in experimentally pathological cardiac hypertrophy. The in vivo pathological cardiac hypertrophy models were induced by pressure-overload and angiotensin (Ang) II constant infusion in mice, and the in vitro model was induced by Ang II exposure to cultured cardiomyocytes. The expression of BMP4 increased in pressure overload, Ang II constant infusion-induced pathological cardiac hypertrophy, but not in swimming exercise-induced physiological cardiac hypertrophy in mice. BMP4 expression also increased in Ang II–induced cardiomyocyte hypertrophy in vitro. In turn, BMP4 induced cardiomyocyte hypertrophy, apoptosis, and cardiac fibrosis, and these pathological consequences were inhibited by the treatment with BMP4 inhibitors noggin and DMH1. Moreover, Ang II–induced cardiomyocyte hypertrophy was inhibited by BMP4 inhibitors. The underlying mechanism that BMP4-induced cardiomyocyte hypertrophy and apoptosis was through increasing NADPH oxidase 4 expression and reactive oxygen species-dependent pathways. Lentivirus-mediated overexpression of BMP4 recapitulated hypertrophy and apoptosis in cultured cardiomyocytes. BMP4 inhibitor DMH1 inhibited pressure overload–induced cardiac hypertrophy in mice in vivo. The plasma BMP4 level of heart failure patients was increased compared with that of subjects without heart failure. In summary, we conclude that BMP4 is a mediator and novel therapeutic target for pathological cardiac hypertrophy. **(*Hypertension*. 2013;61:352-360.)** ● **Online Data Supplement**

Key Words: ■ angiotensin II ■ apoptosis ■ bone morphogenetic protein-4 ■ fibrosis ■ hypertrophy

Cardiac hypertrophy is a compensatory mechanism in response to mechanical stimuli, exercise training, or pressure-overload. Cardiac hypertrophy is subdivided into physiological and pathological hypertrophy. Physiological cardiac hypertrophy does not progress to heart failure. However, pathological cardiac hypertrophy, characterized by cardiac hypertrophy, apoptosis, and fibrosis, progressively leads to heart failure.[1] Identifying the key factor mediating cardiac hypertrophy, apoptosis, and fibrosis is critically important for developing the strategy to protect against pathological cardiac hypertrophy.

Bone morphogenetic protein (BMP)-4, a member of the BMP family, has been extensively studied in embryo, bone, and cartilage development.[2] BMP4 is a mechanosensitive and proinflammatory gene,[3] and it induces endothelial cell apoptosis, and endothelium dysfunction[4,5] and promotes cardiomyocyte apoptosis after ischemia-reperfusion injury–induced myocardial infarction.[6] However, the role of BMP4 in pathological cardiac hypertrophy has never been identified. Here, we studied the changes of BMP4 expression in experimentally pathological cardiac hypertrophy in vivo and in vitro, the effects of BMP4 on cardiomyocyte hypertrophy, apoptosis, and cardiac fibrosis, and the underlying mechanism.

Methods

Pressure Overload–Induced Cardiac Hypertrophy In Vivo

The pressure-overload heart hypertrophy model was obtained by subjecting the animals to transverse aortic constriction (TAC) as described in our previous work.[7] Detailed information is described in the online-only Data Supplement.

Angiotensin (Ang) II–Induced Cardiac Hypertrophy In Vivo

Male mice (22–26 g body weight) were anesthetized with sodium pentobarbital (60 mg/kg, IP). A 1-cm incision was prepared in the

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midscapular region, and an osmotic mini-pump (Alzet model 2004; DURECT Corporation) was implanted. Pumps contained Ang II (Sigma-Aldrich, USA), which was dissolved in saline solution, and the infusion rate was 1.5 mg/kg per day for 4 weeks.

Swimming Exercise-Induced Physiological Cardiac Hypertrophy

Swimming exercise was performed as described.[8] In brief, the swimming-trained mice were subjected to a 90-minute swimming exercise session, twice a day, 5 days a week for 4 weeks. Water temperature was controlled at 30°C to 32°C.

Ang II–Induced Cardiomyocyte Hypertrophy In Vitro

Cardiomyocytes were isolated from 1- to 3-day-old neonatal rat hearts. Ang II treatment was used to induce cardiomyocyte hypertrophy. In the experiments of using Ang II, cardiomyocytes were incubated with 10 nmol/L and 100 nmol/L Ang II for 48 hours. The culture medium containing Ang II was changed every 24 hours. Cardiomyocytes were prepared for immunocytochemistry as described previously.[7] Detailed information is described in the online-only Data Supplement.

Results

BMP4 Expression Increases in Pathological Cardiac Hypertrophy In Vivo and In Vitro

Pressure-overload for 2 and 4 weeks induced significant cardiac hypertrophy (Figure S1A and S1B in the online-only Data Supplement). The mature and precursor of BMP4 protein and BMP4 mRNA expression were markedly increased in 2- and 4-week TAC-treated hearts (Figure 1A–1D). After 1-, 3-, and 7-day TAC treatment, no morphological cardiac hypertrophy was observed in TAC treated mice (Figure S2A and S2B). However, the BMP4 mature and precursor protein levels increased gradually during the time course (Figure 1E), in parallel with the gradual increase of β-myosin heavy chain protein level (Figure 1F). Constant release of Ang II by using Alzet pump induced cardiac hypertrophy (Figure S3A and S3B), in which BMP4 mRNA and protein expressions significantly increased (Figure 1G and 1H). Next, the changes of BMP4 expression in an in vitro pathological cardiomyocyte hypertrophy model were examined. Ang II

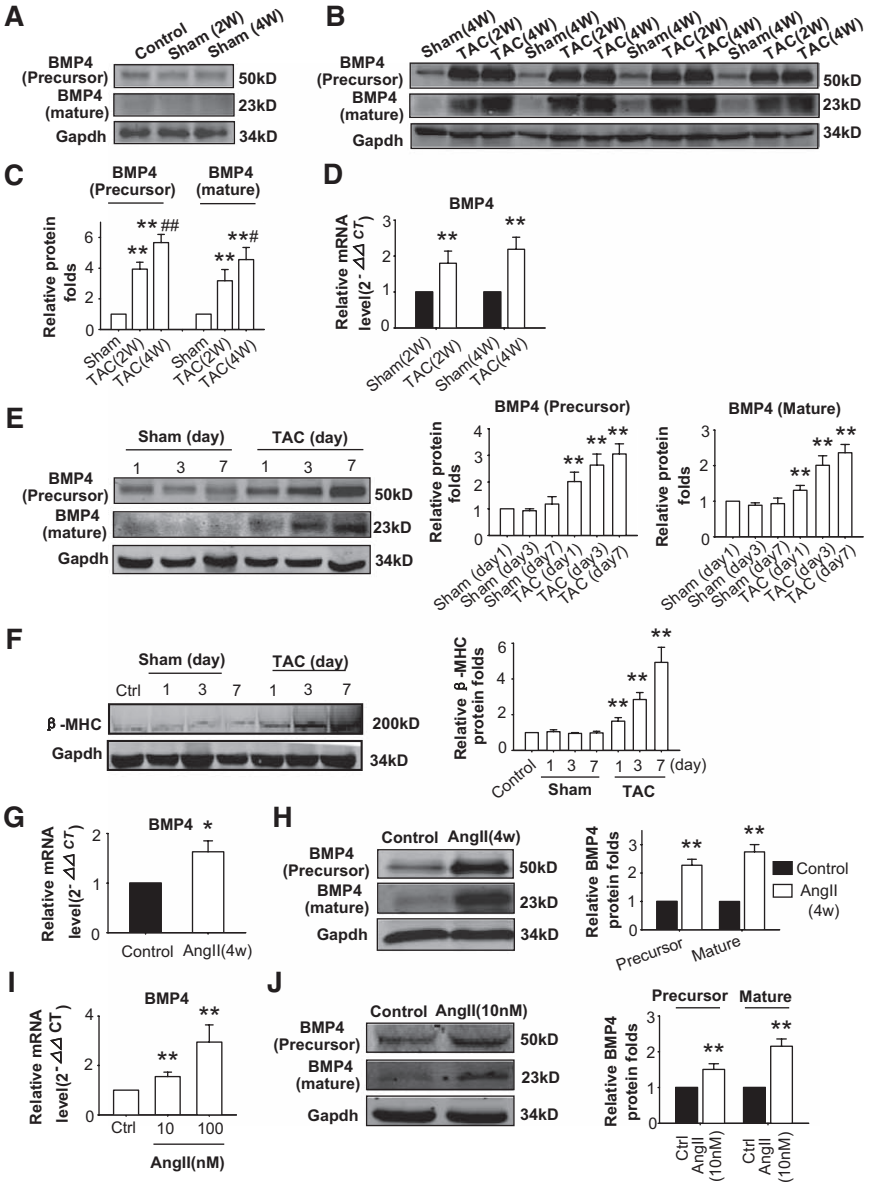

Figure 1. Cardiac bone morphogenetic protein-4 (BMP4) expression increases in pathological cardiac hypertrophy in vivo and in vitro. **A**, There was no difference of BMP4 protein level in control (ctrl) and sham (2 and 4 weeks) groups. The experiment was repeated 3 times individually. **B**, Representative Western blot for cardiac BMP4 using left ventricle tissue after 2 and 4 weeks of pressure-overload. **C**, Summarized data of cardiac BMP4 protein expression after 2 and 4 weeks of pressure-overload. n=6. ***P*<0.01 vs sham; #*P*<0.05, ##*P*<0.01 vs 2 weeks transverse aortic constriction (TAC). **D**, Summarized data of cardiac BMP4 mRNA expression after 2 and 4 weeks of pressure-overload. n=7. ***P*<0.01 vs sham. **E**, Representative Western blot and summarized data of cardiac BMP4 protein expression after 1, 3, and 7 days of TAC. n=6. ***P*<0.01 vs sham (day 1). **F**, β-myosin heavy chain (MHC) protein expression in left ventricle tissue after 1, 3, and 7 days of pressure-overload. n=6. ***P*<0.01 vs control. **G**, Angiotensin (Ang) II constant infusion for 4 weeks increased cardiac BMP4 mRNA expression. n=4, **P*<0.05, ***P*<0.01 vs control. **H**, Ang II constant infusion for 4 weeks increased cardiac BMP4 protein expression. n=5. ***P*<0.01 vs control. **I**, Ang II induced increase of BMP4 mRNA expression in cardiomyocytes in vitro. n=4. ***P*<0.01 vs control. **J**, BMP4 protein expression increased in cardiomyocytes treated with Ang II for 48 hours in vitro. ***P*<0.01 vs control. n=8.

(10 nmol/L) treatment for 48 hours induced significant cardiomyocyte hypertrophy (Figure S4); meanwhile, it also induced increases of BMP4 expression in mRNA and protein levels (Figure 1I and 1J). Taken together, BMP4 expression increased in pathological cardiac hypertrophy.

To indentify the specificity of BMP4 increase in pathological cardiac hypertrophy, we used the mice ramp swimming-exercise model.[8] After 4-week swimming exercise, mild cardiac hypertrophy was induced without alterations of pathological gene markers such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and β-myosin heavy chain (Figure S5A–S5C), and without detectable difference in fibrosis (Figure S12). The cardioprotective gene *Hsp70* was reported to be upregulated in exercise-induced (physiological) but not in pressure overload-induced (pathological) cardiac hypertrophy.[9] In the present work, Hsp70 protein expression increased in swimming exercise but not in TAC model (Figure S5D). Hence, we concluded that the present swimming exercise-induced cardiac hypertrophy was physiological. No changes of BMP4 mRNA and protein expressions occurred in this model (Figure S5E and S5F), indicating that BMP4 increase was specific in pathological cardiac hypertrophy.

BMP4 Induces Cardiomyocyte Hypertrophy and Apoptosis Through Increasing NADPH Oxidase 4 Expression and Reactive Oxygen Species-Dependent Pathways

We exposed cultured cardiomyocytes to BMP4 in the presence and absence of BMP4 inhibitor noggin. Cardiomyocytes demonstrated a dramatic increase in size and sarcomeric assembly after 48-hour exposure to 10 and 50 ng/mL BMP4. In the presence of 100 ng/mL noggin, the increases of cell area induced by 50 ng/mL BMP4 were inhibited (Figure 2A). BMP4 (50 ng/mL) induced significant increases of ANP, BNP, β-myosin heavy chain mRNA expressions, and protein/DNA ratio, and these increases were reduced by the treatment of BMP4 inhibitors, noggin (100 ng/mL) and DMH1(10 μmol/L; Figure 2B). We also proved that BMP4-induced cardiomyocyte death (Figure 2C) increased caspase-3 activity (Figure 2D), increased bax, and decreased bcl-2 protein expression (Figure 2E), and these changes were reversed by cotreatment with noggin or DMH1. BMP4 inhibitors alone showed no significant effect on cardiomyocyte hypertrophy, viability, and apoptosis (Figure S6). Taken together, BMP4 induced not only cardiomyocyte hypertrophy, but also cardiomyocyte apoptosis.

Ang II induced cardiomyocyte hypertrophy and increased expression of ANP and BNP in mRNA level (Figure 2 F and 2G). BMP4 inhibitors, noggin, and DMH1 completely suppressed Ang II–induced hypertrophy and Ang II–induced increase of BMP4 mRNA expression (Figure 2F and 2G), suggesting that BMP4 also mediated Ang II–induced consequences.

Excessive production of reactive oxygen species (ROS) plays an important role in pathological cardiac hypertrophy.[10] We found that BMP4 increased ROS production in cardiomyocytes and the increase was inhibited by DMH1 treatment (Figure 3A). NADPH oxidase (NOX) 2 and NOX4 are closely related to ROS production in cardiomyocytes. BMP4 treatment for 48 hours increased NOX4 but not NOX2 mRNA expression (Figure 3B), and the BMP4-induced increase of NOX4 mRNA expression was inhibited by noggin or DMH1 treatment (Figure 3C). BMP4 increased mitochondrial NOX4 protein level, which was inhibited by the treatment of noggin or DMH1, but BMP4 did not affect NOX2 protein level (Figure 3D). In in vivo pathological and physiological cardiac hypertrophy models, we found that NOX2 protein expression increased in swimming exercise-induced cardiac hypertrophy but not in TAC- or Ang II–induced cardiac hypertrophy (Figure S7A). NOX4 protein expression increased in all 3 hypertrophy models (Figure S7B). It was notable that the fold increase of NOX4 in TAC- and Ang II–induced cardiac hypertrophy was much higher than that in swimming exercise-induced cardiac hypertrophy (3.16±0.553 folds in TAC and 2.75±0.258 folds in Ang II treatment groups versus 1.23±0.023 folds in swimming-exercise group, $P<0.01$). Next, we further validated the role of NOX4 in BMP4-induced cardiomyocyte hypertrophy and apoptosis by using the NADPH oxidase inhibitor apocynin and the radical scavenger tempol (Figure S8).

Calcineurin and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), the 2 Ca^{2+}-dependent signaling proteins, have been implicated in pathological cardiac hypertrophy.[11,12] BMP4 treatment did not change calcineurin, CaMKII, phosphorylated CaMKII (Figure S9A and S9B), as well as total p38, phosphorylated p38, total 1/2ERKs, and phosphorylated 1/2ERKs expressions in protein level (Figure S9D). However, BMP4 treatment increased oxidized CaMKII (ox-CaMKII) and phosphorylated JNK (P-JNK) in protein level and the increases were inhibited by noggin or DMH1 treatment (Figure S9C and S9E). Oxidation-dependent CaMKII activation plays critical role in cardiac hypertrophy and cardiac injury.[13,14] Both ox-CaMKII and JNK activation are dependent on ROS,[13–15] therefore we concluded that BMP-induced pathological cardiac hypertrophy was through ROS-dependent pathways.

BMP4-induced cardiomyocyte hypertrophy and apoptosis were further validated by lentivirus-mediated overexpression of BMP4 in cultured cardiomyocytes (Figure S10).

In cardiomyocytes, BMP4-induced increase of BMP4 mRNA was inhibited by noggin treatment (Figure S11A). BMP4-induced increase of BMP4 protein expression was suppressed by noggin or DMH1 treatment (Figure S11B). BMP2 and BMP4 share >80% amino acid homology and form a distinct subclass of BMPs. Several studies revealed that there was interaction between BMP2 and BMP4.[16,17] We found that BMP4 treatment did not affect BMP2 protein expression in cardiomyocytes (Figure S11C).

BMP4 Induces Cardiac Fibrosis

Pressure-overload and Ang II constant infusion but not swimming exercise induced significant cardiac fibrosis (Figure S12). So we further investigated the role of BMP4 in cardiac fibrosis. BMP4 at 10 and 50 ng/mL showed no effect on cell viability of cardiac fibroblasts (Figure 4A). Exposure to 10 and 50 ng/mL BMP4 induced significant increase of collagen in cardiac fibroblasts. In the presence of 100 ng/mL noggin, BMP4-induced increase of collagen was inhibited (Figure 4B). BMP4 increased BMP4 protein expression in cardiac fibroblasts (Figure 4C).

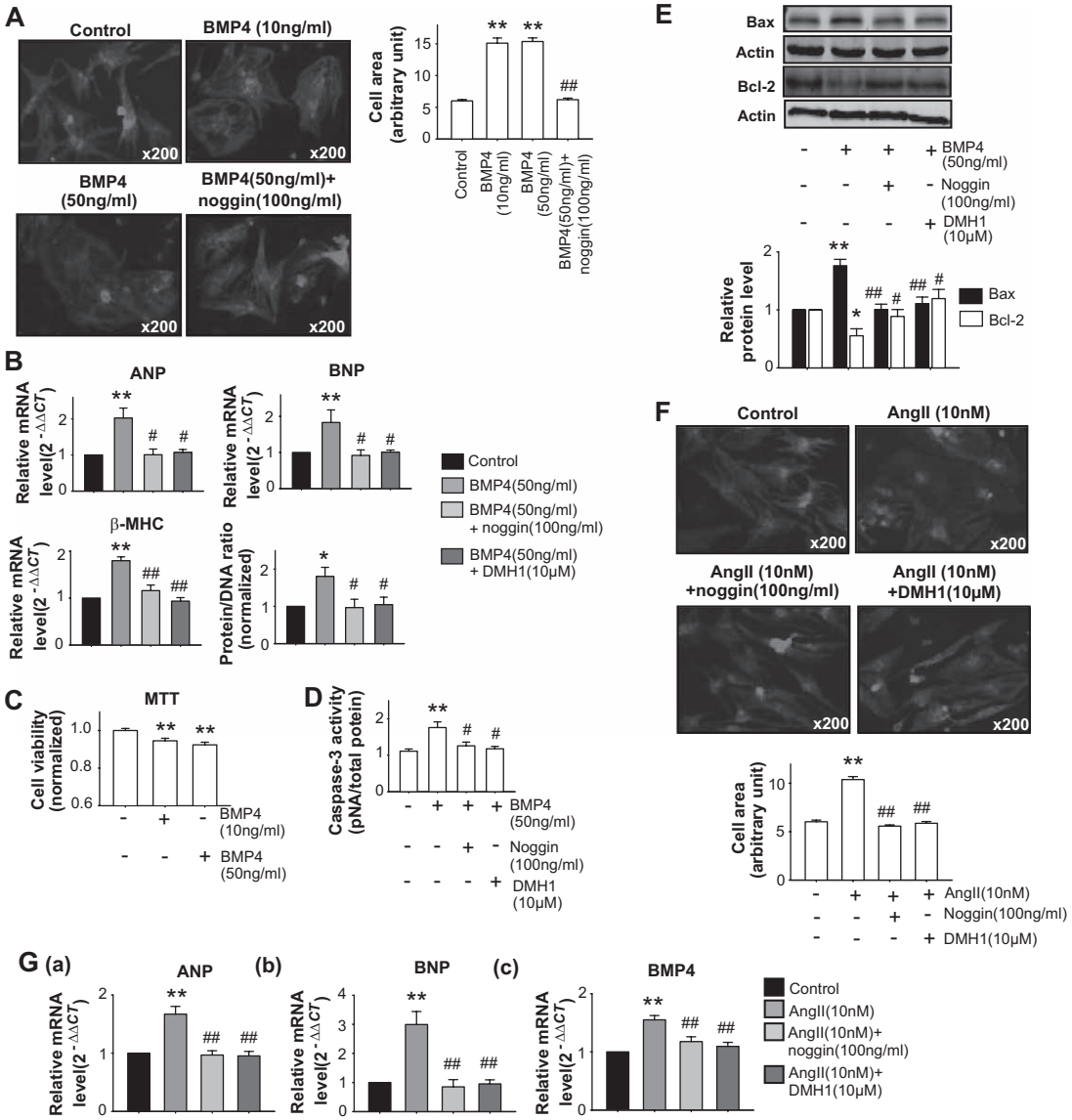

Figure 2. Bone morphogenetic protein-4 (BMP4) induces cardiomyocyte hypertrophy and apoptosis and BMP4 inhibitors suppress angiotensin (Ang) II-induced cardiomyocyte hypertrophy. **A**, BMP4 treatment induced increase of the cell area of cardiomyocytes which was inhibited by BMP4 inhibitor noggin. Cardiomyocytes were identified (×200) with sarcomeric α-actinin antibody (red signal), and nuclei were stained with bisbenzamide (blue). The BMP4-treated cells showed dramatically stretched state. The right panel showed the summarized data of cell area. Fifty cells were qualified in each group from 3 individual experiments. ***P*<0.01 vs control; ##*P*<0.01 vs 50 ng/mL BMP4. **B**, The increased atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (MHC) mRNA expression, and protein/DNA ratio induced by BMP4 were inhibited by BMP4 inhibitors noggin and DMH1. n=8 for ANP, BNP, β-MHC analysis, and n=6 for protein/DNA ratio analysis. **P*<0.05, ***P*<0.01 vs control; #*P*<0.05, ##*P*<0.01 vs 50 ng/mL BMP4. **C**, BMP4 reduced the cell viability of cardiomyocytes. n=5, ***P*<0.01 vs control. **D**, BMP4 treatment increased caspase-3 activity in cardiomyocytes and the increase was inhibited by BMP4 inhibitors noggin and DMH1. n=6,***P*<0.01 vs control; #*P*<0.05 vs BMP4. **E**, The increased bax and decreased bcl-2 protein expression induced by BMP4 were prevented by BMP4 inhibitors noggin and DMH1. n=6 for bax and bcl-2 analysis. **P*<0.05,***P*<0.01 vs control; #*P*<0.05, ##*P*<0.01 vs BMP4. **F**, The increased cell area of cardiomyocytes induced by Ang II was inhibited by BMP4 inhibitors noggin and DMH1. One hundred cells from 3 individual experiments were qualified in each group. ***P*<0.01 vs control; ##*P*<0.01 vs Ang II. **G**, The increased ANP, BNP, BMP4 mRNA expression induced by Ang II was inhibited by BMP4 inhibitors noggin and DMH1. n=9 for ANP, BNP, and BMP4 analysis,***P*<0.01 vs control; ##*P*<0.01 vs Ang II.

By immunostaining of α-SMA, the marker of myofibroblasts, we found that BMP4 induced fibroblasts to differentiate into myofibroblasts, which showed not only the clear fiber, but also the enlarged area, and the induced differentiation was inhibited by noggin or DMH1 treatment (Figure 4D). Differentiated myofibroblasts were further validated by measuring α-SMA protein expression (Figure 4E). However, BMP4 treatment showed no significant effect on matrix metalloproteinase-2 and matrix metalloproteinase-9 mRNA expression in cardiac fibroblasts (Figure S13).

Ang II stimulates fibroblasts to generate collagen and differentiate to myofibroblasts. Ang II treatment increased BMP4 protein expression (Figure 4F) and collagen production in cardiac fibroblasts, and the increased collagen was inhibited by noggin or DMH1 treatment (Figure 4G). Immunostaining of α-SMA showed that the induced differentiation of myofibroblasts

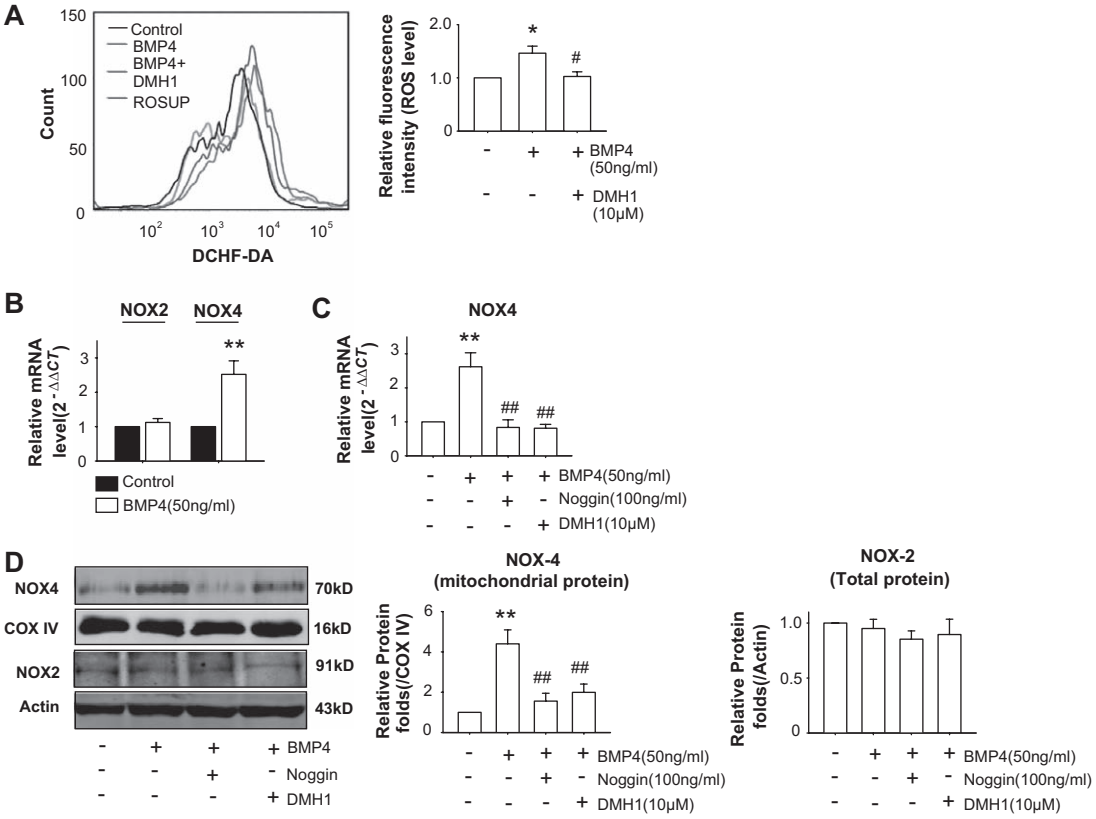

Figure 3. Bone morphogenetic protein-4 (BMP4) induces increase of reactive oxygen species (ROS) production and upregulation of NADPH oxidase (NOX) 4 expression in cardiomyocytes. **A**, Flow cytometry analysis showed that BMP4 induced increase of ROS production in cardiomyocytes, which was inhibited by DMH1. n=4 individual experiments, 10 000 cells in each experiment. ROSUP was positive control. **P*<0.05 vs control; #*P*<0.05 vs 50 ng/mL BMP4. **B,** BMP4 induced increase of NOX4 but not NOX2 mRNA expression in cardiomyocytes. n=4 individual experiments,***P*<0.01 vs control. **C**, BMP4 increased NOX4 mRNA expression, which was inhibited by noggin and DMH1. n=4 individual experiments,***P*<0.01 vs control. ##*P*<0.01 vs BMP4. **D**, BMP4-induced increase of mitochondrial NOX4 protein expression was inhibited by noggin and DMH1 in cultured cardiomyocytes. BMP4 did not affect NOX2 protein expression. n=9 for NOX4 analysis and n=6 for NOX2 analysis. ***P*<0.01 vs control; ##*P*<0.01 vs BMP4.

by Ang II was inhibited by noggin or DMH1 treatment (Figure 4H), and the detection of α-SMA protein expression showed similar results (Figure 4I). In cardiac fibroblasts, apocynin and tempol also inhibited BMP4-induced increase of α-SMA protein expression (Figure S14), indicating that NOX was involved in BMP4-induced fibroblast differentiation.

DMH1 Inhibits Pressure Overload–Induced Pathological Cardiac Hypertrophy In Vivo

Alzet pump containing the small molecule chemical DMH1 was implanted subcutaneously in mice after TAC operation. The rate of constant release of DMH1 was 2 mg/kg per day for 4 weeks. Administration of DMH1 completely suppressed TAC-induced cardiac hypertrophy and fibrosis, as demonstrated by the decreased heart weight/body weight index, left ventricle weight/body weight index, ANP, BNP, β-myosin heavy chain mRNA expressions, myocyte cross section area, fibrosis ratio, collagen I, and NOX4 levels (Figure 5A–5E). DMH1 treatment inhibited TAC-induced increases of BMP4 and ox-CaMKII in protein level (Figure 5F and 5G).

Plasma BMP4 Level in Heart Failure Patients

Finally, we examined the plasma BMP4 levels in heart failure patients. The plasma BMP4 level of heart failure patients was increased compared with that of subjects without heart failure (Figure S15).

Discussion

Our data indicated that BMP4 was not only a pathological marker, but also an essential mediator for pathological cardiac hypertrophy. The underlying mechanism of BMP4-induced pathological cardiac hypertrophy was summarized in Figure 6. We suggest that BMP4 is a novel therapeutic target for pathological cardiac hypertrophy.

Several studies about BMP4 in cardiovascular system have implied that BMP4 might be involved in pathological cardiac hypertrophy, for example, BMP4 stimulates ROS production through NADPH oxidases in endothelium,[5,18] exaggerates cardiac ischemia-reperfusion injury by promoting cardiomyocytes apoptosis.[6] However, the role of BMP4 in pathological cardiac hypertrophy has never been identified. We found that BMP4 induced cardiomyocyte hypertrophy and apoptosis through increasing NOX4 expression and ROS-dependent pathways. There might be multiple ROS-dependent pathways involved in pathological cardiac hypertrophy. In the present work, we measured the p-JNK and ox-CaMKII expressions, but we did not

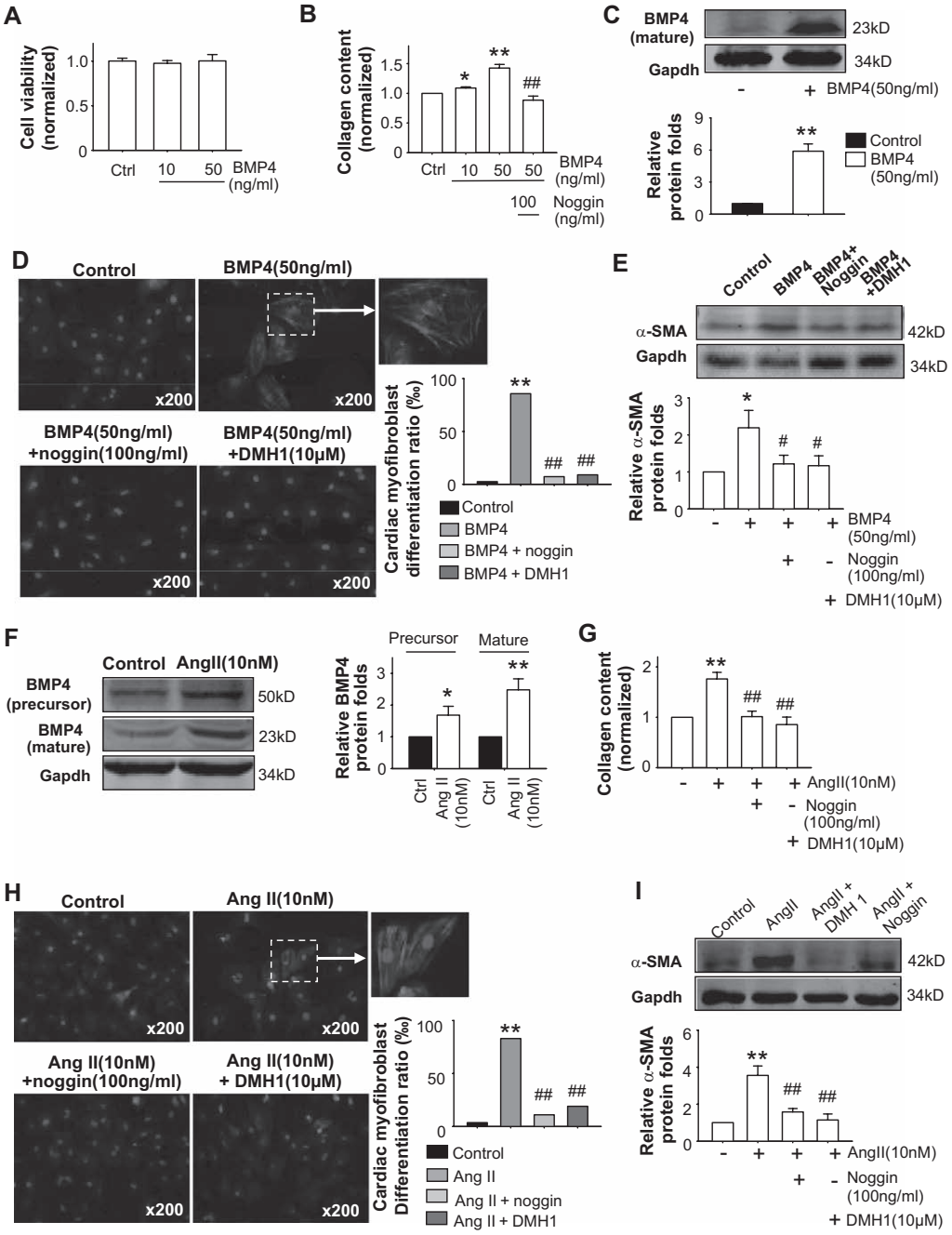

Figure 4. Bone morphogenetic protein-4 (BMP4) induces cardiac fibrosis. **A**, BMP4 (10, 50 ng/mL) had no effect on viability of cardiac fibroblasts. n=6. **B**, BMP4-induced increase of collagen production in cardiac fibroblasts and the increase was inhibited by noggin. n=5, **P*<0.05, ***P*<0.01 vs control; ##*P*<0.01 vs 50 ng/mL BMP4. **C**, BMP4 increased BMP4 protein expression in cardiac fibroblasts. n=8, ***P*<0.01 vs control. **D**, BMP4 induced fibroblasts to differentiate into myofibroblasts and the differentiation was inhibited by BMP4 blockers noggin and DMH1. Fibroblasts were immunostained for smooth muscle α-actin (SMA) (red) and nuclei (blue). BMP4-treated fibroblasts had clearly visible SMA positive stress fibers. The framed area was enlarged as shown. Five hundred cells were analyzed for myofibroblast differentiation ratio in each group. χ^2-test was used, ***P*<0.005, vs control; ##*P*<0.005 vs BMP4. **E**, BMP4 increased α-SMA protein expression in cardiac fibroblasts and the increase was inhibited by cotreatment of noggin or DMH1. n=7, **P*<0.05 vs control; #*P*<0.05 vs BMP4. **F**, Angiotensin (Ang) II treatment increased BMP4 protein expression in cultured cardiac fibroblasts. n=7, **P*<0.05, ***P*<0.01 vs control. **G**, BMP4 inhibitors noggin and DMH1 inhibited Ang II–induced collagen increase in cardiac fibroblasts. n=5, ***P*<0.01 vs control; ##*P*<0.01 vs Ang II. **H**, Ang II-induced differentiation from fibroblasts into myofibroblasts was inhibited by BMP4 blockers noggin and DMH1. The framed area was enlarged as shown. One thousand cells were analyzed for myofibroblast differentiation ratio in each group. χ^2 test was used, ***P*<0.005 vs control; ##*P*<0.005 vs Ang II. **I**, The increased α-SMA protein expression induced by Ang II was inhibited by BMP4 blockers noggin and DMH1. n=6, ***P*<0.01 vs control; ##*P*<0.01 vs Ang II.

exclude the possibility that there were other ROS-dependent pathways involved in BMP4-induced pathological cardiac hypertrophy.

Both NOX2 and NOX4 have been reported to be involved in pathological cardiac hypertrophy.[19] We found that BMP4 treatment increased NOX4 but not NOX2 expression in

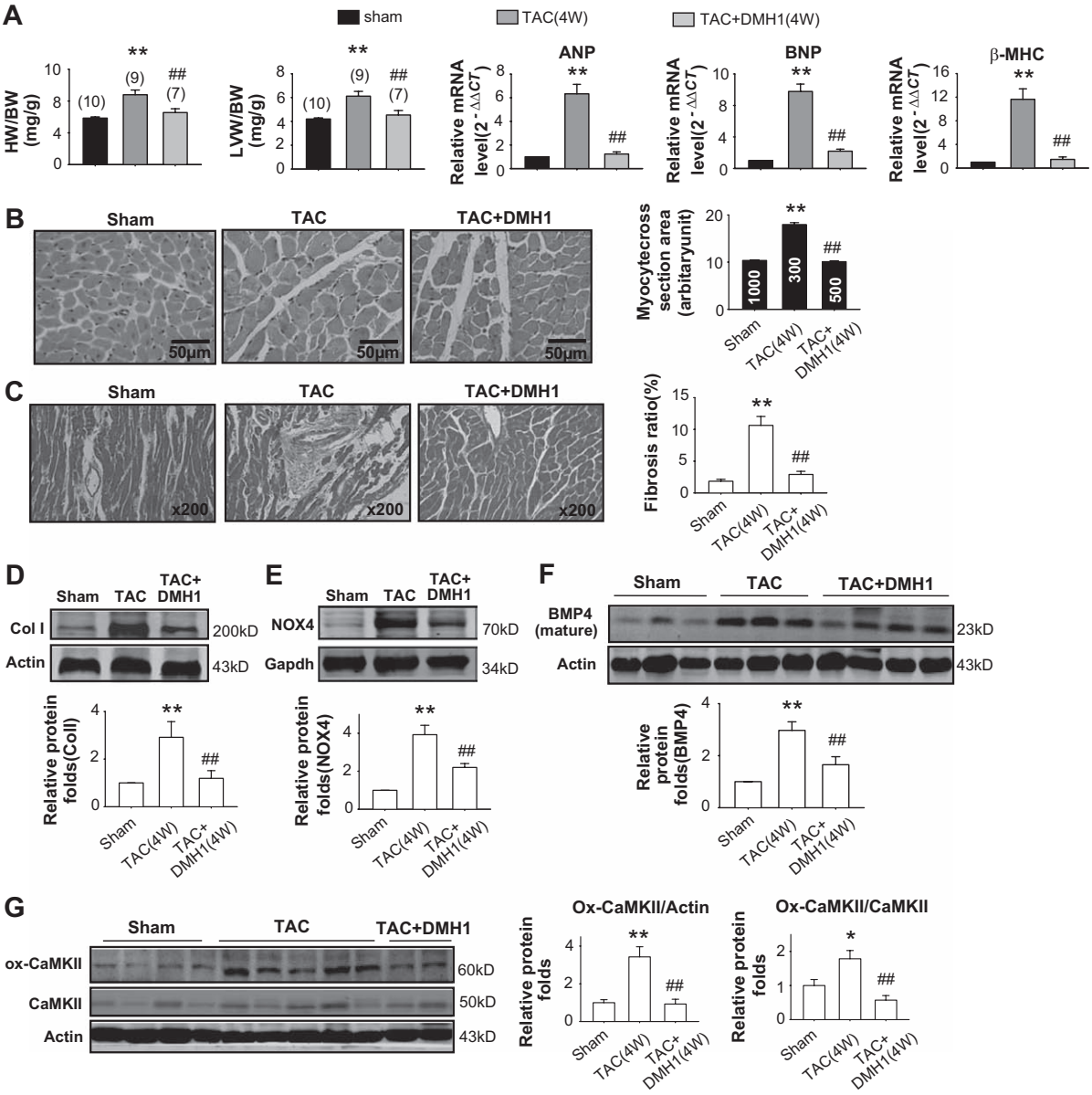

Figure 5. Bone morphogenetic protein-4 (BMP4) inhibitor DMH1 inhibits pressure overload-induced pathological cardiac hypertrophy in vivo. **A**, Administration of DMH1 suppressed transverse aortic constriction (TAC)-induced cardiac hypertrophy as demonstrated by the decreased heart weight (HW)/body weight (BW) index, left ventricle weight (LVW)/BW index, and atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (MHC) mRNA expressions. n=6 in each group, ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. **B**, Representative photographs of Hematoxylin and eosin stained sections and the summarized data of cardiomyocyte cross section area. ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. **C**, Representative photographs of Masson trichrome stained sections and the summarized data of cardiac fibrosis ratio. ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. n=3 hearts in each group. Average 10 stained sections for each heart. **D,** Representative Western blot for cardiac collagen I using left ventricle tissue and the summarized data. ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. n=6 in each group. Col I indicates collagen I. **E**, Representative westernblot for cardiac NADPH oxidase 4 (NOX4) using left ventricle tissue and the summarized data. ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. n=7. **F**, Representative Western blot for cardiac mature BMP4 using left ventricle tissue and the summarized data. ***P*<0.01 vs sham; ##*P*<0.01 vs TAC. n=7 hearts in each group. **G**, DMH1 treatment reduced TAC-induced increase of oxidized-calmodulin–dependent protein kinase II (CaMKII) protein expression. **P*<0.05, ***P*<0.01, vs sham; ##*P*<0.01 vs TAC. n=7 in each group.

cardiomyocytes. Therefore, we further compared NOX2 and NOX4 expressions in in vivo pathological and physiological cardiac hypertrophy models in mice. Results showed that the alterations of NOX4 and NOX2 expressions in in vivo pathological cardiac hypertrophy were consistent with the effects of BMP4 on NOX4 and NOX2 expressions in vitro, indicating that NOX4 played a more important role in pathological cardiac hypertrophy.

We observed the changes of BMP4 expression in TAC- and Ang II constant infusion-induced cardiac hypertrophy in mice in vivo. TAC treatment increases left ventricular end-systolic pressure, left ventricular end-systolic volume, and left ventricular end-diastolic volume.[20] In this model, the upregulated BMP4 expression in the heart might come from the local pressure and volume overload. Ang II shows multiple effects, such as increase of the systemic blood

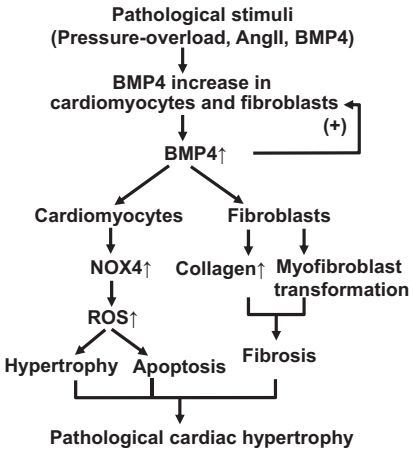

Figure 6. Proposed mechanism underlying bone morphogenetic protein-4 (BMP4)–induced pathological cardiac hypertrophy. Ang indicates angiotensin; NOX4, NADPH oxidase 4; and ROS, reactive oxygen species

pressure, increase of sympathetic activity, and increase of Ang II–aldosterone system. Therefore, in Ang II constant infusion-induced cardiac hypertrophy model, we extrapolated that the upregulated BMP4 expression might come from the pressure-overload and the direct stimulation of hormones.

A recent work reported that BMP4 was involved in valvular interstitial cell activation in human myxomatous mitral valve prolapse.[21] They found that in vitro hBMP4 treatment of isolated human mitral valvular interstitial cells mimicked the cellular activation and extracellular matrix remodeling as seen in mitral valve prolapse tissues. Mitral valve prolapse was associated with development to progressive mitral regurgitation, which in turn, predisposed to eccentric left ventricular hypertrophy. Our data together with this report indicated that BMP4 mediated not only the pathological cardiac hypertrophy, but also the mitral valve prolapse, suggesting that BMP4 might be a target with wide clinic potential.

Heart failure is generally accompanied with endothelial dysfunction.[22] Therefore, in addition to the heart, the increased plasma BMP4 in heart failure patients may also come from the injured endothelium, from which BMP4 can also be secreted.[23] Because BMP4 induced cardiac hypertrophy, apoptosis, and fibrosis, the increased plasma BMP4 might be an important factor to promote human heart failure progress.

Perspectives

BMP4 induces pathological cardiac hypertrophy. Furthermore, BMP4 upregulates itself, which magnifies the harmful effects of BMP4. Therefore, blockade of BMP4 and its related signaling is of therapeutic significance. It is exciting that the small molecule BMP4 inhibitor such as DMH1 has been developed, which make it promising to develop the kind of small molecule chemicals for clinic.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Bone morphogenetic protein-4 (BMP4) expression increases in pathological but not physiological cardiac hypertrophy.
- BMP4 induces cardiac hypertrophy, apoptosis, and fibrosis.
- BMP4 induces cardiac hypertrophy and apoptosis through increasing NADPH oxidase 4 expression and reactive oxygen species-dependent pathways.

What Is Relevant?

- Chronic hypertension induces pathological cardiac hypertrophy and heart failure.
- Pathological cardiac hypertrophy is characterized by hypertrophy, apoptosis, and fibrosis.

Summary

BMP4 mediates cardiac hypertrophy, apoptosis, and fibrosis in experimentally pathological cardiac hypertrophy. BMP4 is a novel therapeutic target for pathological cardiac hypertrophy.

ONLINE SUPPLEMENT

Bone Morphogenetic Protein-4 Mediates Cardiac Hypertrophy, Apoptosis and Fibrosis in Experimentally Pathological Cardiac Hypertrophy

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Short title: BMP4 mediates pathological cardiac hypertrophy

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Supplemental Methods

Agents

Angiotensin Ⅱ(Ang Ⅱ) and DMH1 were purchased from Sigma (sigma-Aldrich); Tempol was purchased from Santa Cruz Biotechnology and PhosSTOP from Roche; Anti-BMP-4, -BMP-2, -CaMKII, -NOX-4 and -NOX-2(gp-91) antibodies were from Santa Cruz; Anti-p-CAMK-Ⅱ was from Promega; Anti-calcineurin was from BD Biosciences; Anti-HSP-70,-Bax and -Bcl-2 were from Cell signaling Technology. Anti-GAPDH was from kangchen (China). Anti- sarcomeric α-actinin was from Abcam. Anti-β-MHC was form Sigma. Anti-p-P38, anti-P38, anti-p-ERK1/2, anti-ERK1/2, anti-p-JNK, anti-JNK were from cell signaling technology. Anti-ox-CaMKII was from Millipore. Recombinant human BMP-4, BMP-2 and recombinant human noggin were purchased from R&D Systems. The real-time PCR primers were shown in **Table-S1.**

Animals

Kunming mice (male) and Wistar rats (SLAC Laboratory Animal Co. Ltd, Shanghai, China) were used. The animals were kept under standard animal room conditions (temperature 21±1 °C; humidity 55–60%) with food and water continuously available for 1 week before the experiment. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, PR China.

Pressure Overload-induced Cardiac Hypertrophy *in vivo*

The pressure-overload heart hypertrophy models were obtained by subjecting the animals to transverse aortic constriction (TAC) as described in our previous work[1]. Adult male mice (22-26g body weight) were anesthetized and placed in the supine position and a midline cervical incision was made to expose the trachea. After successful endotracheal intubation, the cannula was connected to a volume cycled rodent ventilator (UGO BASILE S.R.L. Italy). The chest was opened and the thoracic aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle which was subsequently removed. This maneuvers increased systolic pressure about 1.5 folds and induced cardiac hypertrophy (1). The chest was closed and the animals were kept ventilated until recovery of autonomic breath. At the pre-determined time points (day 1,3,7 and week 2,4), surviving animals were sacrificed and the heart was quickly excised and weighted in cold (4°C) buffer. The left ventricle and right ventricle were separated and weighted, and left ventricle tissue was then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent western blot or real-time PCR analysis. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, PR China.

Angiotensin II (Ang II)-induced cardiac hypertrophy *in vivo*

Male mice (22-26g body weight) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A 1-cm incision was prepared in the mid-scapular region and an osmotic mini-pump (Alzet model 2004; DURECT Corporation) was implanted. Pumps contained Ang II (Sigma-Aldrich, USA) which was dissolved in saline solution and the infusion rate was 1.5 mg/kg per day. After 4 weeks, mice were sacrificed, hearts were weighted and left cardiac ventricles (LCV) were fixed in 10% formalin for histology or quickly frozen in liquid nitrogen for quantification of gene expression.

Swimming exercise-induced physiological cardiac hypertrophy

Swimming exercise was carried out as described [2]. In brief, the swimming-trained mice were subjected to a 90-min swimming exercise session, twice a day, 5 days a week for 4 weeks.

Water temperature was controlled at 30-32°C.

Angiotensin II-induced cardiomyocyte hypertrophy *in vitro*

Ang II treatment was used to induce cardiomyocyte hypertrophy. In the experiments of using Ang II, cardiomyocytes were incubated with 10nmol/L and 100nmol/L Ang Ⅱ for 48hrs. The culture media containing Ang Ⅱ was changed every 24 hrs. Cardiomyocytes were prepared for immunocytochemistry as described previously [1]. Monoclonal antibody against sarcomeric α-actinin (Sigma) was added at dilutions of 1:200. Nuclear staining was performed with 1.3μmol/L bisbenzimide (Sigma). The cardiomyocyte surface was depicted by using Image-Pro Plus Version (5.0.1) software and the relative surface area was read with the arbitrary units (the number of pixels) for evaluating hypertrophy [1].

Isolation and culture of cardiomyocyte and cardiac fibroblast

Cardiomyocytes and cardiac fibroblasts were isolated from 1-3-day-old neonatal rat hearts using a conventional method adopted by our and other studies [1,3]. Briefly, after hearts were digested by trypsin, cells were suspended in DMEM (Dulbecco's modified Eagle's medium/medium) with 10 %FBS, and pre-cultured in humidified incubator (95%air-5% CO_2) for 90 min to obtain cardiac fibroblasts for their selective adhesion. Then the suspended cardiomyocytes were plated in another dish. The purity of cardiomyocytes was increased by supplementing BrdU(5-Bromo-2'-deoxyuridine) to prevent non-cardiomyocytes from developing. Culture medium was renewed after 48hrs and cells were further cultured for 24hrs. Then, both cardiomyocytes and cardiac fibroblasts were cultured in non-serum DMEM for 12hrs before experiments.

Protein/ DNA Ratio Analysis

After standard culture procedures, the total protein and DNA ratio of cardiomyocytes was analyzed. In a brief, cells were rinsed in cold PBS twice and scraped with 100ul of lysis buffer (150mM Nacl ,15mM sodium citrate and 0.25%SDS PH=7.0).The collected cells were immediately frozen and stored at -20℃. Then samples were thawed and vortexed, 1ul sample was applied to determine the total protein content using BCA method. DNA concentrations were detected by using a DNA Quantitation Kit (Sigma-Aldrich).

BMP4 inhibitor DMH1 treatment *in vivo*

After TAC operation, 1-cm incision was prepared in the mid-scapular region of mice and an osmotic mini-pump (Alzet model 2004; Alza) containing DMH1(Sigma-Aldrich,USA)was implanted. The constant infusion rate of DMH1 was 2 mg/kg per day. After 4 weeks, mice were sacrificed and hearts were harvested for analysis.

Drug treatment

Ang Ⅱ(10nmol/L and 100nmol/L), BMP4 (50ng/ml), BMP2(50ng/ml), Noggin (100ng/ml) and DMH1(10μmol/L) were applied for 24hrs in cardiac fibroblasts and 48hrs in cardiomyocytes. The culture media containing different drugs was renewed every 24 hrs. In the experiments, BMP4, BMP2 and noggin were dissolved in PBS solution and diluted in the culture media as the ratio 1.25:5000. DMH1 was dissolved in DMSO and diluted in the culture media as the ratio 0.7:1000. Under this condition, these vehicles showed no effect.

Real-time PCR Analysis

One microgram of total RNA from each sample was used to generate cDNA by using M-MLV reverse transcriptase per manufacturer's specifications (Promega Corporation,USA).
Real-time PCR was cycled in 95°C/15s, 60°C/30s and 72°C/30s for 40 cycles, after an initial denaturation step at 95°C for 10 min using SYBR Green PCR Master Mix purchased from

Applied Biosystems (USA). Amplification was performed by using 7500 Fast Real-Time PCR Systems (Applied Biosystems,USA) and the products were routinely checked by using dissociation curve software. Transcript quantities were compared by using the relative quantitive method, where the amount of detected mRNA normalized to the amount of endogenous control (GAPDH). The relative value to the control sample is given by $2^{-\Delta\Delta CT}$. The real-time PCR primer sequences were shown in **Table-S1.**

Western Blot

Cellular and tissue proteins were extracted with lysis buffer which contained 1% protease inhibitor solution. After complete homogenization on ice rotator, samples were centrifuged at 13500g for 15min at 4℃ to precipitate cell debris. The supernatants were transferred into fresh tubes and protein concentrations were determined with BCA Protein Assay Kit(Bio-Rad). The proteins were electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 2 hours at room temprature, the membranes were incubated with the primary antibodies at 4℃ over night. After washing with PBS-0.1% Tween 20(PBST), membranes were incubated with fluorescence-conjugated goat anti-rabbit IgG , goat anti-mouse IgG or donkey anti-goat IgG (1:10000 dilution, Invitrogen) at room temperature for 1h. Western blot bands were quantified by using Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software.

MTT assay

Viability of cells cultured in the 96-well culture plates was assessed by measuring mitochondrial dehydrogenase activity, using the colorimetric MTT assay, based on the fact that viable cells (but not dead cells) can reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Collagen assay

Collagen assay was performed using the Sircol collagen assay method (biocolor, UK). Briefly, 100μl test samples of treated cardiac fibroblasts were mixed with 1ml Sirco dyes reagent and incubated at 25°C for 30 minutes. After centrifugation at 1,0000 g for 10 minutes, the supernatants were removed. Then 1ml alkali regent was added to pellet to release the bound dye into solutions. The absorbance of each sample was measured by a microplate Reader at 540nm.

TUNEL staining

After 3 times PBS washing, treated myocytes were fixed by 4% paraformaldehyde, permeabilized in 0.1% Triton x-100 sodium citrate buffer. Then an In Stiu cell death detection kits (Roche) were used to label apoptotic cells, and the nuclei were stained with DAPI. The numbers of total cells and Tunel positive cells were automatically counted by Image-Pro plus version. The apoptosis rate was defined as ratio of apoptotic cells to total cells.

Detection of reactive oxygen species (ROS) by flow cytometry

ROS level of cardiomyocytes was quantified by using ROS assay kit (Beyotime Institute of Biotechnology, China). Cells were harvested by trypsin and exposed for 20 min at 37 ℃ to 10 μmol/L DCFH-DA, which became fluorescent when oxidized to DCF within the cells. Labeled cells were washed twice in PBS and then subject to cytometric analysis (excitation: 488nm; emission:525nm). The model of flow cytometry was FACSAria (BD). ROS level was represented with fluorescence intensity. The number of analyzed cells was 10000 in each individual experiment.

Caspase-3 activity assay

Caspase-3 activity was determined by using Caspase-3 Activity Assay Kit (Beyotime Institute

of Biotechnology, China), which is based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product, p-nitroaniline (pNA). Briefly, the cells were harvested and washed with cool PBS twice,then the cells were lysed with lysis buffer (100μl per 2×106cells) for 15 min on ice. The lysate was centrifuged at 13500r/min for 15 min at 4 °C, then collected the supernatant and protein concentration was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). After incubating the mixture composed of 40μl of cell lysate, 50μl reaction buffer and 10μl of 2mmol caspase-3 substrate(Ac-DEVD-pNA)in 96-well plates at 37℃ overnight, the absorbance of p-nitroanilide at 405nm was determined by using a microtiter plate reader (Bio-TEK Epoch, BioTek Instrument, VT,USA). Caspase-3 activity was calculated as a ratio of p-nitroanilide content to total protein amount. The detail analysis procedure was described in the manufacturer's protocol (Beyotime Institute of Biotechnology, China).

Infection of cardiomyocytes with lentiviruses expressing rBMP4

Cardiomoyotes were plated in 6 wells plate(2×10^5cells /well) and cultured in a humidified incubator (95% air-5% CO_2) at 37°C. After 48hrs incubation, culture medium was renewed and cells were further cultured for 24hrs. Then, the cells were prepared for lentivirus infection with rBMP4 lentiviral packaging prepared by 3DBiopharm Co Ltd (Shanghai, China) which was shown in **Figure-S10A.** Infection was carried out in DMEM with 10% FBS at MOI (multiplicity of infection) of 20 for 48hrs to obtain the maximal efficiency and the minimal cell loss. Polybrene (6μg/ml) was added to ensure the infection efficiency. After 48hrs infection, culture medium was changed to the medium without lentiviruses and the cells were further cultured for 24hrs. BMP4 overexpression induced by rBMP4 lentiviral transfection was confirmed by western blot examination (**Figure-S10B**). The performance of lentiviral transfection was based on the company's instruction.

Plasma BMP4 measurement

The human plasma BMP4 concentration was measured with BMP4 human Elisa Kit (Abcam Inc, USA) according to the specifications.

Plasma samples

The plasma collection was approved by the Ethics Committee of Harbin Medical University. The plasma samples of heart failure patients were provided by the cardiology department, first affiliated hospital of Harbin Medical University. The control plasma samples were provided by the clinical laboratory, the second affiliated hospital, Harbin Medical University. The control plasma samples were from subjects of department of ENT (ear-nose-throat) when the routine blood index checks were finished. The residual plasma was used for the present study. The subjects as control were without heart diseases. The age of subjects as control (57.0±2.0 years) matched with the heart failure patients (63.2±2.5 years) as possible.

The plasma fraction was prepared immediately by centrifugation at 3,000g for 5 min at 4 ℃. Samples were then immediately divided into aliquots (0.5 ml) in cryovials and frozen at -80℃until the analysis procedures were carried out. The information of subjects as control was shown in **Table-S2**, and the clinical parameters of heart failure patients were shown in **Table-S3.**

Data analysis

Data were presented as mean±SEM. Significance was determined by using Student t test or one-way ANOVA, followed by Tukey post test. $P<0.05$ was considered significant.

Supplemental Results

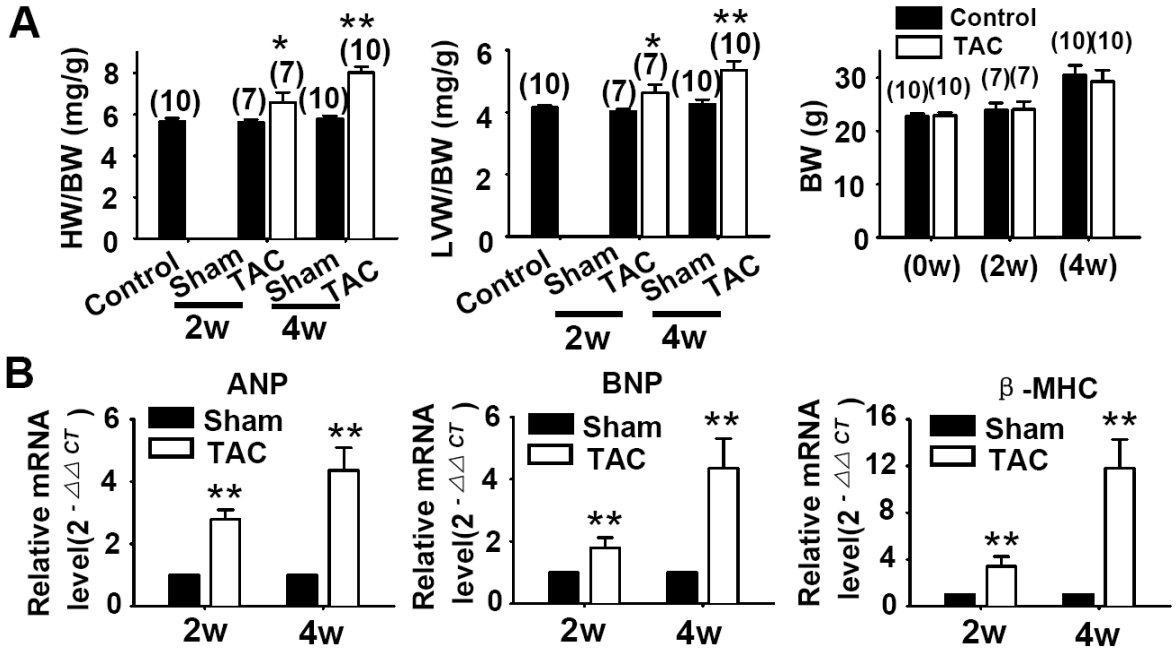

Figure S1. Pressure-overload induced cardiac hypertrophy in mice. A. Heart weight/body weight (HW/BW),left ventricle weight/body weight (LVW/BW) and body weight (BW) after 2 and 4weeks of pressure overload in mice. The number was shown in brackets. **P*<0.05,***P*<0.01, vs.sham. **B.** Cardiac mRNA levels of ANP, BNP, and β-MHC after 2 and 4weeks of pressure overload in mice. n=5 in each group. ***P*<0.01, vs.sham. TAC, transverse aortic constriction.

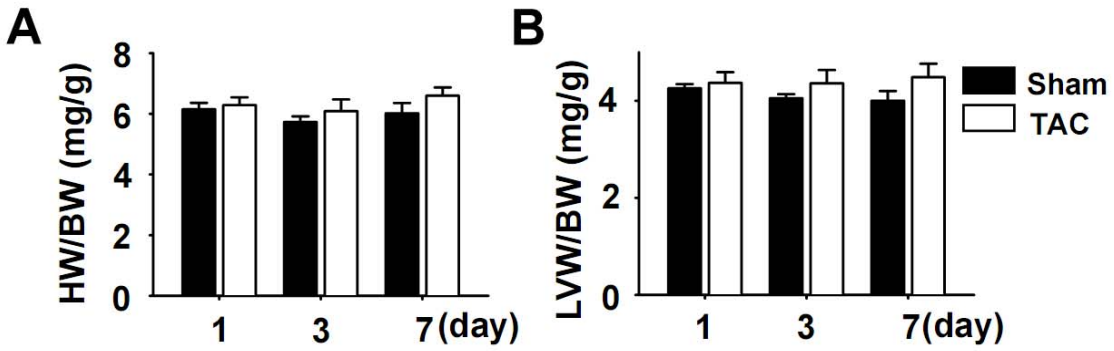

Figure S2. No cardiac hypertrophy was observed in mice treated with TAC for 1, 3, and 7 days. There was no difference of HW/BW and LVW/BW among the groups. n=6 in each group. TAC, transverse aortic constriction.

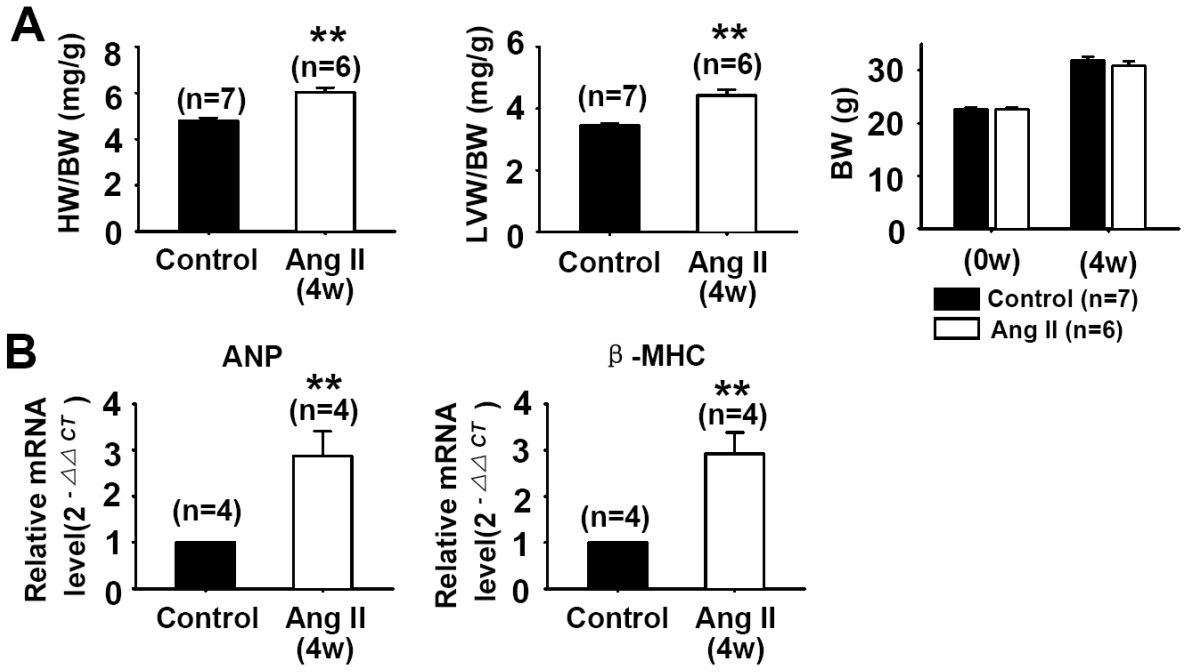

Figure S3. Ang II constant infusion (4 weeks) induced cardiac hypertrophy in mice. A. Ang II constant infusion for 4 weeks increased HW/BW and LVW/BW index. The number was shown in brackets. ***P*<0.01, vs. control. **B.** Ang II constant infusion for 4 weeks increased cardiac ANP, β-MHC mRNA expression. n=4, ***P*<0.01, vs. control.

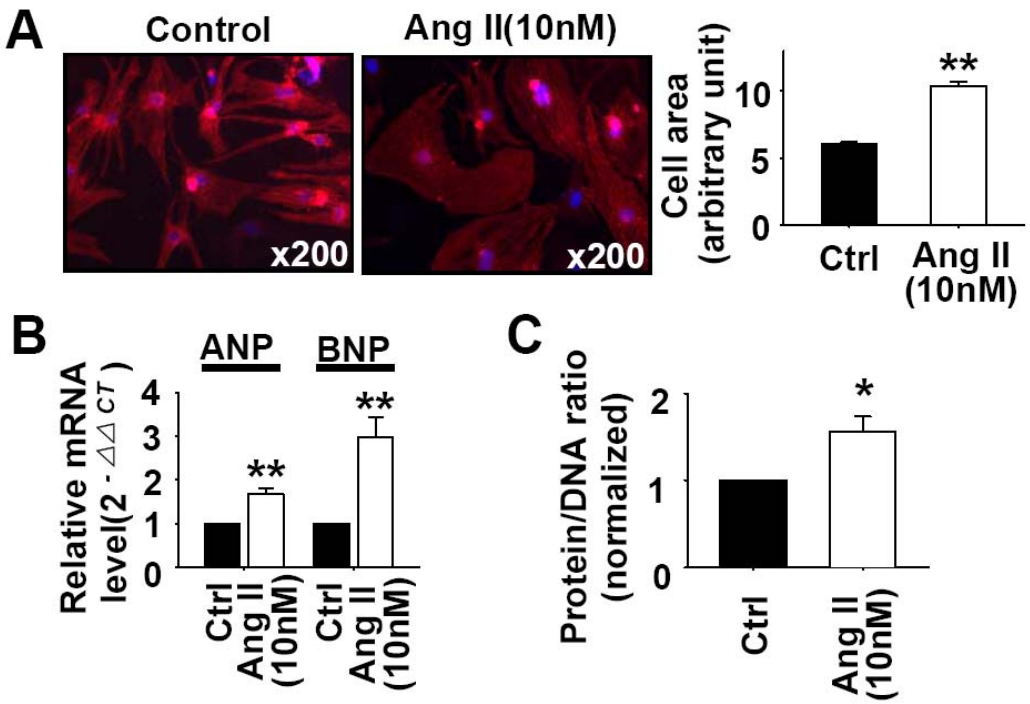

Figure S4. Ang II induced cardiomyocyte hypertrophy *in vitro*. A. Ang II induced increase of cell area of primary rat cardiomyocytes. The left panel showed the representative photographs of cardiomyocytes and the right panel showed the summarized data of cell area. Cells were stimulated with Ang II. Cardiomyocytes were identified (x200) with sarcomeric α-actinin antibody (red signal), and nuclei were stained with bisbenzamide (blue). One hundred cells were qualified in each group. ***P*< 0.01 vs. control. **B.** Ang II induced increases of ANP and BNP mRNA expression in cardiomyocytes. n=5 individual experiments. **C.** Ang II induced increases of protein/DNA ratio in cardiomyocytes. n=3 individual experiments. **P*<0.05 vs. control. Ctrl, control.

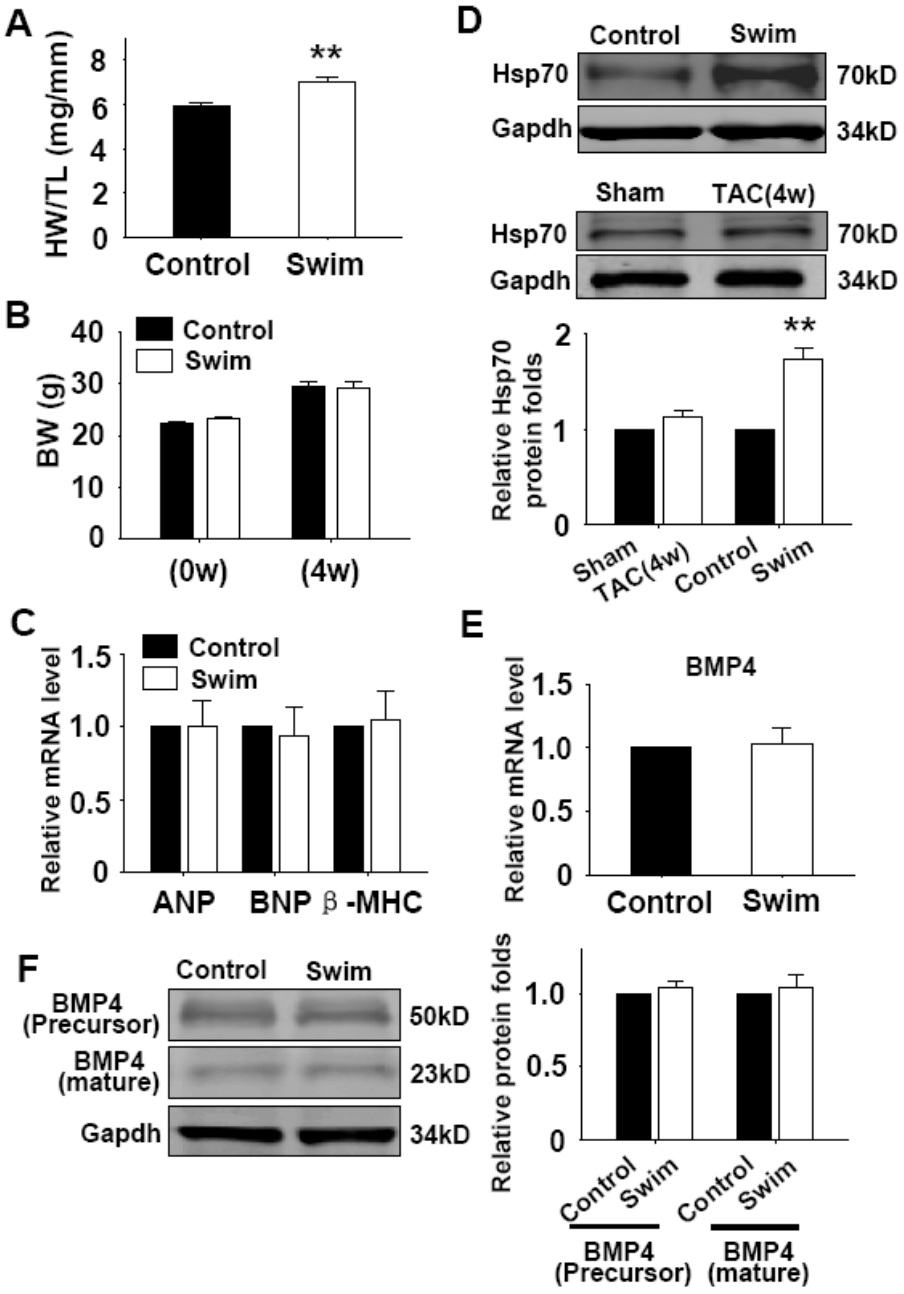

Figure S5. No change of cardiac BMP4 expression in mice after 4 weeks of swimming exercise. A. Four-week swimming exercise increased heart weight/tibial length (HW/TL) in mice. n=7 in each group. ***P*<0.01, vs. control. **B.** Body weight during swimming exercise. n=7 in each group. **C**. Four-week swimming exercise did not affect cardiac ANP,BNP,β-MHC mRNA expression in mice. n=5. **D.** Four-week swimming exercise but not TAC increased cardiac Hsp70 protein expression. n=5 in each group. ***P*<0.01, vs. control. **E.** Four-week swimming exercise did not affect cardiac BMP4 mRNA expression in mice. n=5 in each group. **F.** Four-week swimming exercise did not affect cardiac BMP4 protein expression in mice. n=5 in each group.

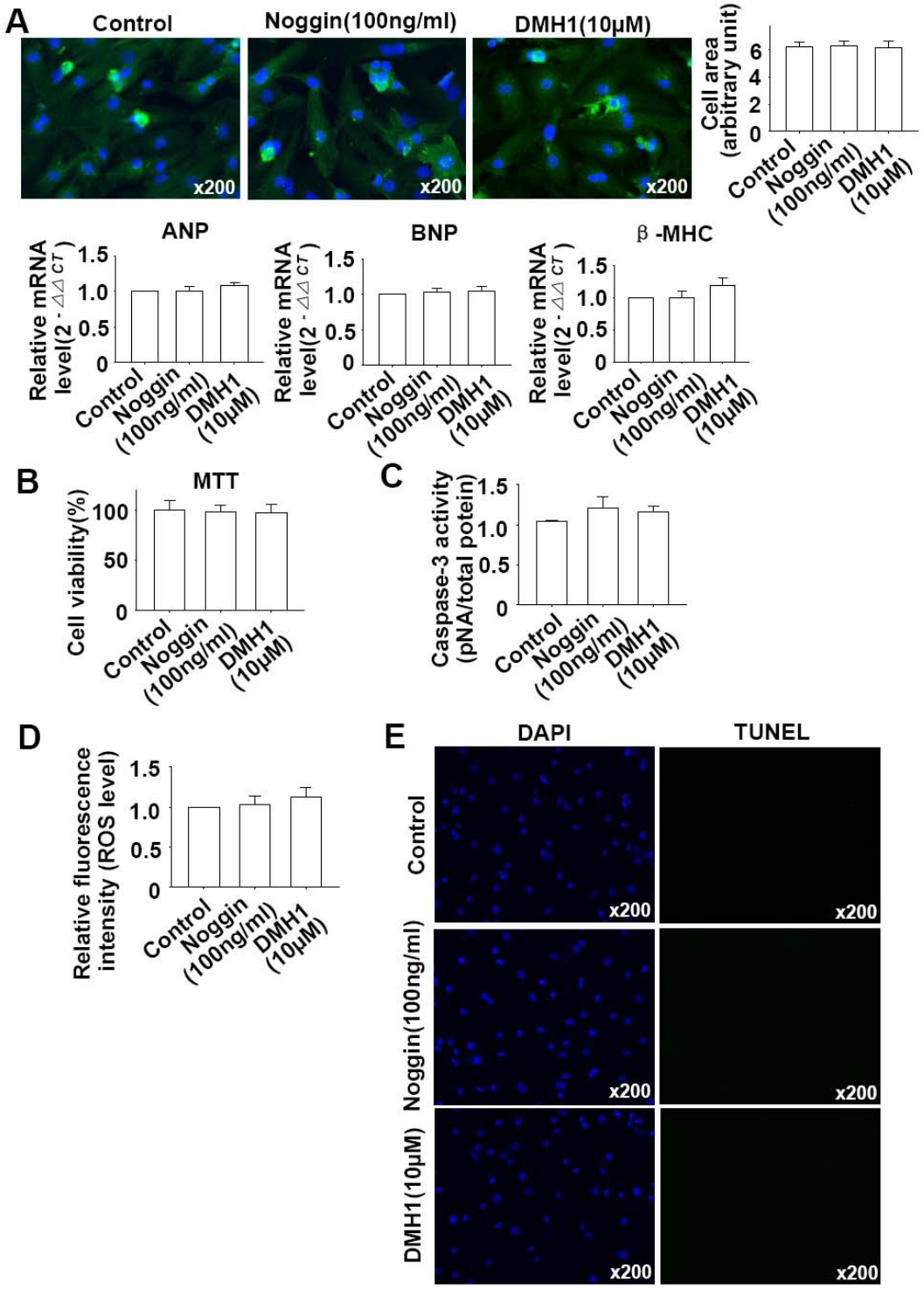

Figure S6. Noggin and DMH1 alone showed no effect on cardiomyocyte hypertrophy, viability and apoptosis. A. Noggin and DMH1 alone showed no effect on cardiomyocyte hypertrophy. For cell area evaluation, fifty cells were qualified in each group from three individual experiments. n=8 for ANP, BNP, β-MHC mRNA expression analysis. **B.** Cell viability was examined by MTT method. n=3 individual experiments. **C.** Noggin and DMH1 showed no effect on caspase-3 activity in cardiomyocytes. n=3 individual experiments. **D.** Noggin and DMH1 showed no effect on ROS production in cardiomyocytes. n=4 individual experiments. **E.** Noggin and DMH1 showed no effect on cardiomyocyte apoptosis evaluated by TUNEL staining.

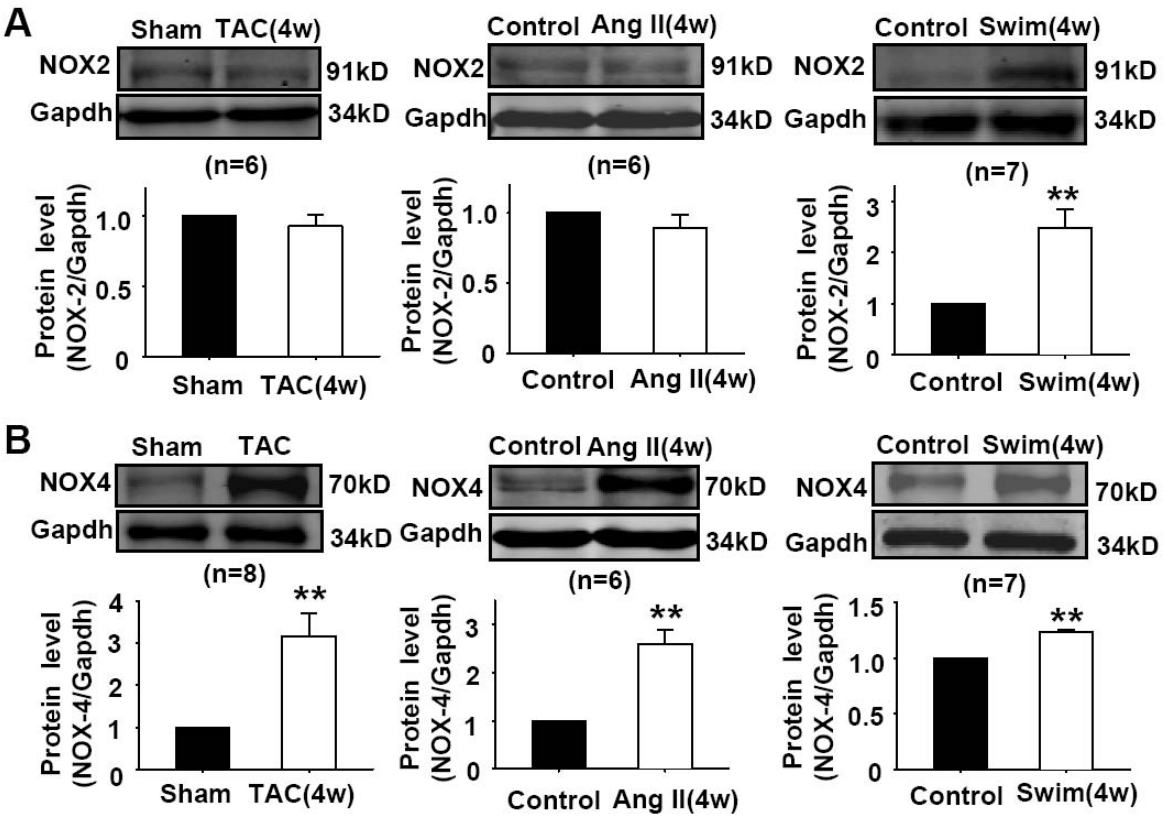

Figure S7.Changes of NOX2, 4 in pathological and physiological cardiac hypertrophy in mice. A. NOX2 protein expression increased in swimming-exercise but not TAC or Ang II induced cardiac hypertrophy in mice. The number was shown in brackets. ***P*<0.01, vs.control. **B.** NOX4 protein expression increased in swimming-exercise, TAC and Ang II induced cardiac hypertrophy in mice. Note: the fold increase of NOX4 in TAC and Ang II treatment groups is much higher than that in swimming-exercise group. The number was shown in brackets. ***P*<0.01, vs.sham or control.

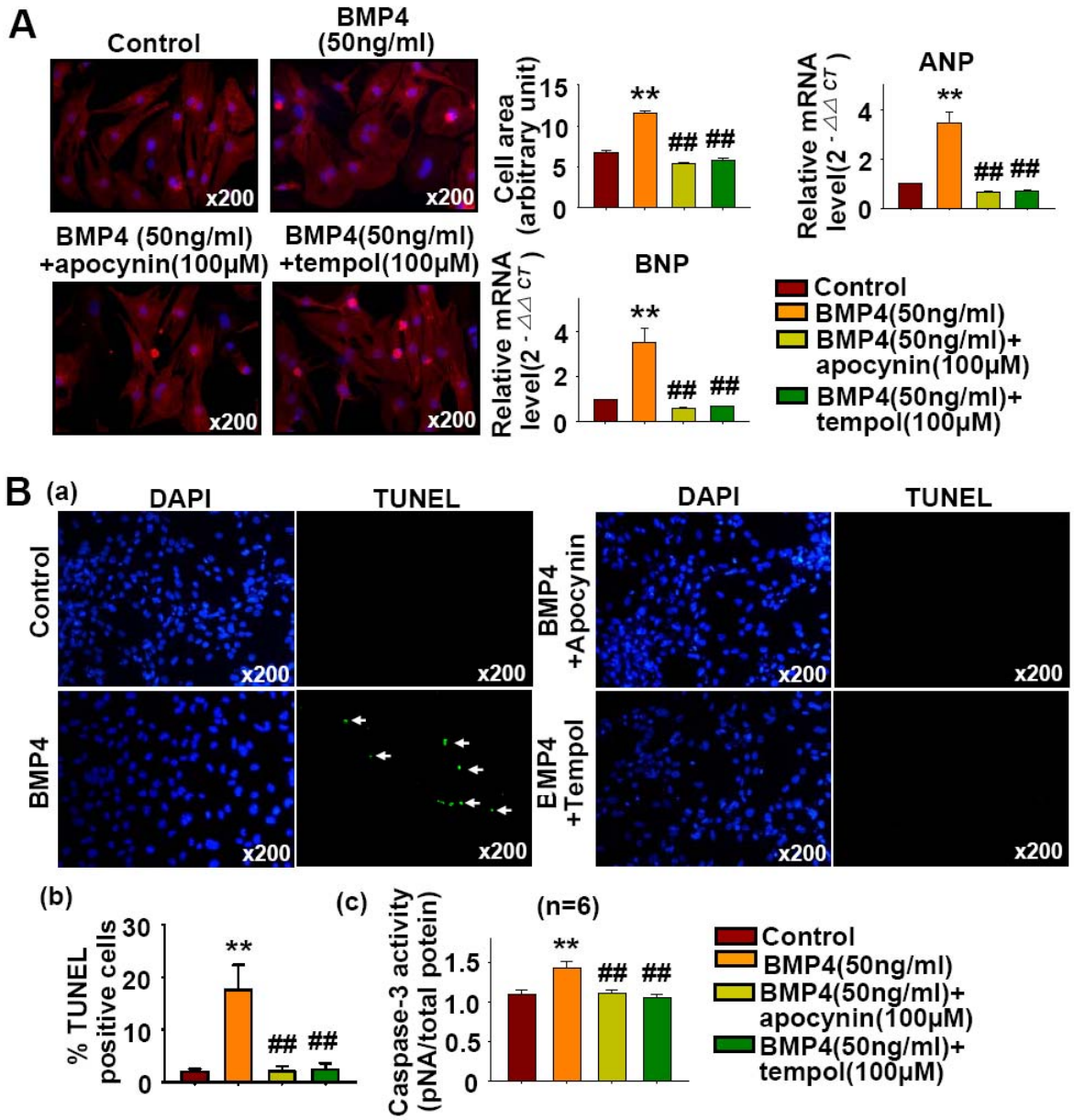

Figure S8. Apocynin and tempol inhibited BMP4-induced cardiomyocyte hypertrophy and apoptosis.

A. Apocynin and tempol inhibited BMP4-induced cardiomyocyte hypertrophy. For evaluation of cell area of cultured cardiomyocytes, one hundred cells from 3 individual experiments were qualified in each group. n=6 for ANP and BNP analysis. ***P*<0.01, vs. control. ##*P*<0.01 vs.BMP4. **B.** Apocynin and tempol inhibited BMP4-induced cardiomyocyte apoptosis. **(a)** TUNEL-positive nuclei were green, and DAPI was blue. BMP4 was 50ng/ml; Tempol was 100μmol/l; Apocynin was100μmol/l. **(b)** Bar graph showing the quantification of the apoptotic cells. The 2,000 cells from 3 individual experiments were analyzed in each group. ***P*< 0.01 vs. control; ##*P*<0.01 vs. BMP4. **(c)** Apocynin and tempol inhibited BMP4-induced increase of caspase-3 activity in cultured cardiomyocytes. n=6,***P*< 0.01 vs. control; ##*P*<0.01 vs. BMP4.

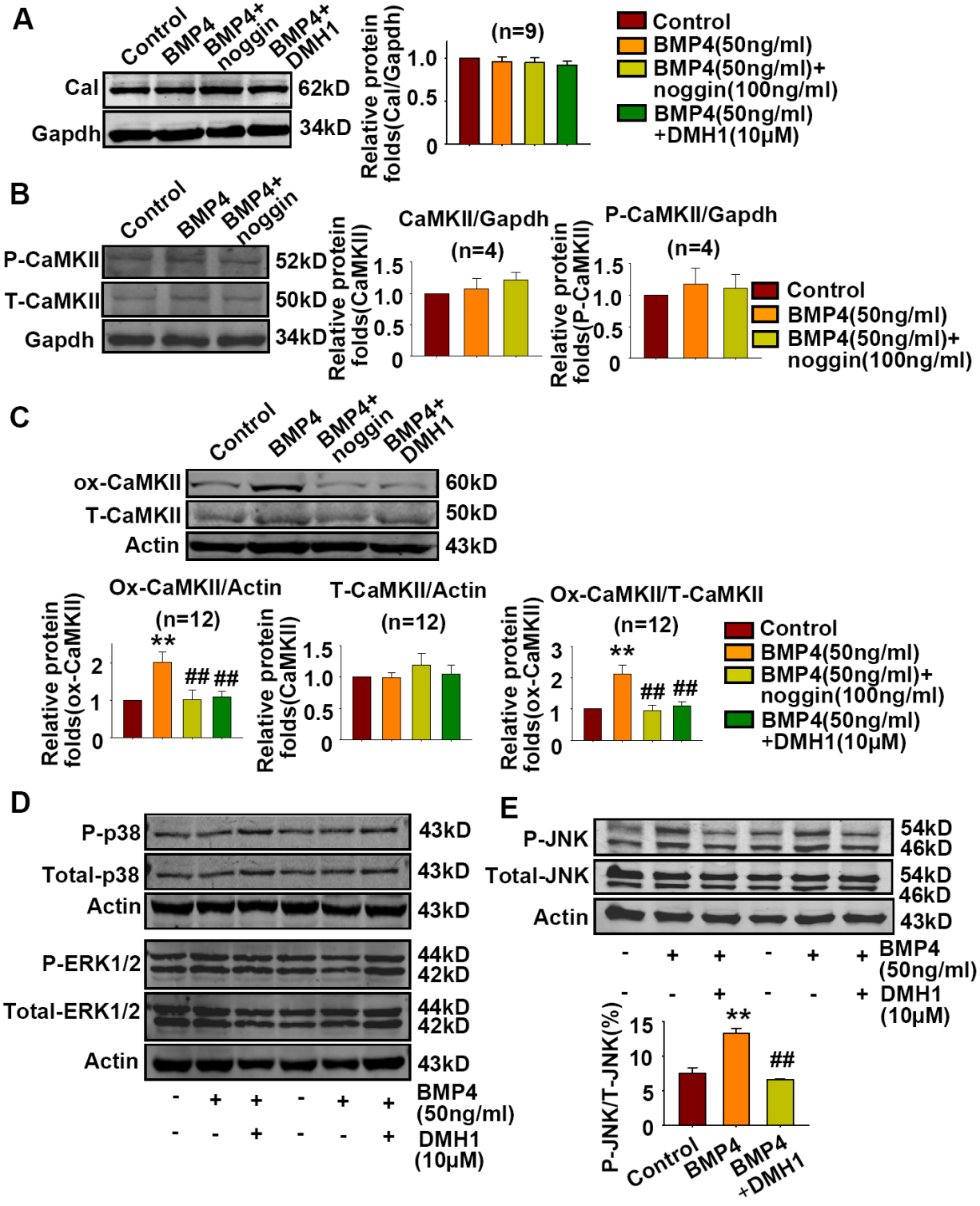

Figure S9. BMP4 treatment increased ox-CaMKII, P-JNK but did not alter calcineurin, CaMKII, P-CaMKII, P-p38,total-p38, P-ERK1/2 and total ERK1/2 in protein level in cultured cardiomyocytes. A. Representative westernblots and analyzed data for calcineurin in cultured cardiomyocytes.Cal, calcineurin. **B.** Representative westernblots and analyzed data for total CaMKII, phosphorylated CaMKII (P-CaMKII) in cultured cardiomyocytes. **C.** Representative westernblots and analyzed data for ox-CaMKII in cultured cardiomyocytes.

Noggin and DMH1 inhibited BMP4-induced increase of ox-CaMKII in protein level. **P<0.01 vs control; ##P<0.01 vs BMP4. **D.** Representative westernblots for P-p38, total p38, P-ERK1/2 and total ERK1/2 in cultured cardiomyocytes. **E.** DMH1 inhibited BMP4-induced increase of P-JNK in protein level. n=4, **P<0.01 vs control; ##P<0.01 vs BMP4.

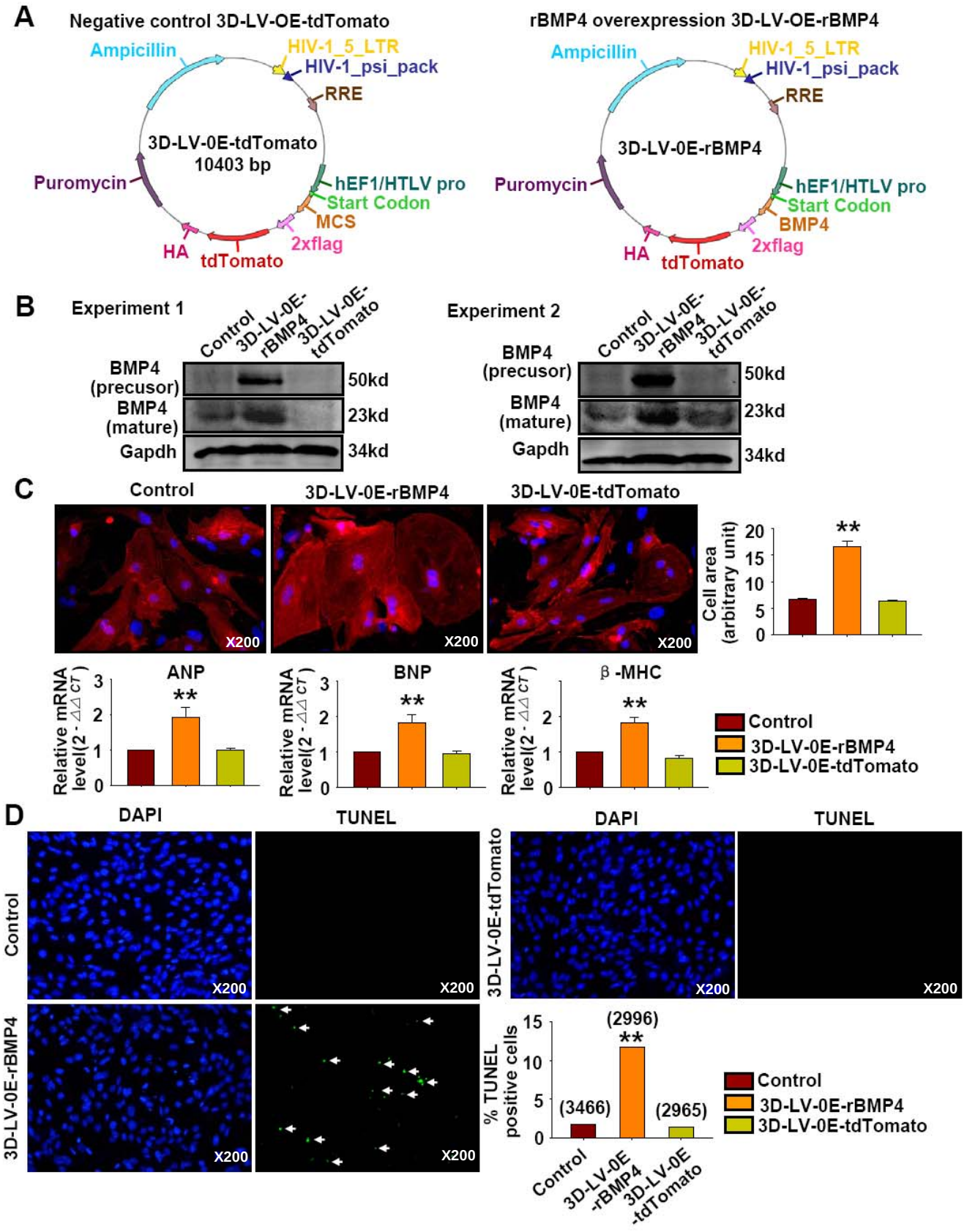

Figure S10. BMP4 overexpression induced by rBMP4 lentiviral transfection recapitulated cardiomyocyte hypertrophy and apoptosis *in vitro*. A. Establishment of rBMP4 lentiviral packaging. **B.** BMP4 overexpression induced by rBMP4 lentiviral

transfection was confirmed in two independent experiments. **C.** BMP4 overexpression by rBMP4 lentiviral transfection induced cardiomyocyte hypertrophy. The cell area and hypertrophy markers were evaluated. For evaluation of cardiomyocyte area, 250 cells from two independent experiments in each group were analyzed. n=8 for analysis of ANP, BNP, β-MHC mRNA expression. $^{**}P<0.01$, vs. control. **D.** TUNEL staining assay showed that BMP4 overexpression by rBMP4 lentiviral transfection induced cardiomyocyte apoptosis. TUNEL-positive nuclei are green, and DAPI is blue. The number of cells from two independent experiments was shown in the brackets. X^2-test was used, $^{**}P<0.005$, vs. control.

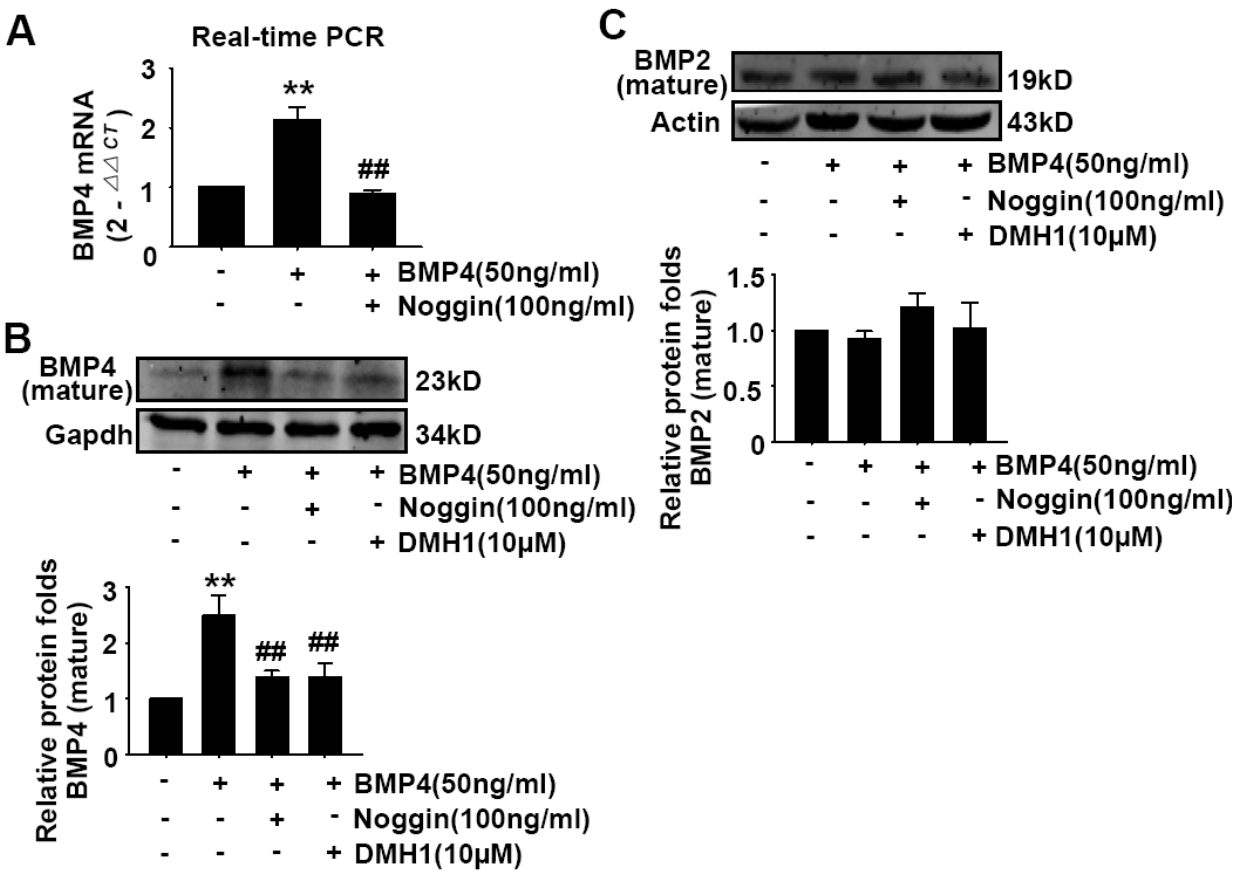

Figure S11. BMP4 induced increase of BMP4 expression in cultured cardiomyocytes. A. BMP4 induced increase of BMP4 mRNA expression was inhibited by noggin treatment. n=6,***P*<0.01, vs. control; ## *P*< 0.01 vs.50ng/ml BMP4. **B.** BMP4 induced increase of BMP4 protein expression was inhibited by noggin and DMH1 treatment. n=7, ***P*<0.01, vs. control; ## *P*< 0.01 vs.50ng/ml BMP4. **C.** BMP4 did not affect cardiac BMP2 protein expression in cardiomyocytes. n=4.

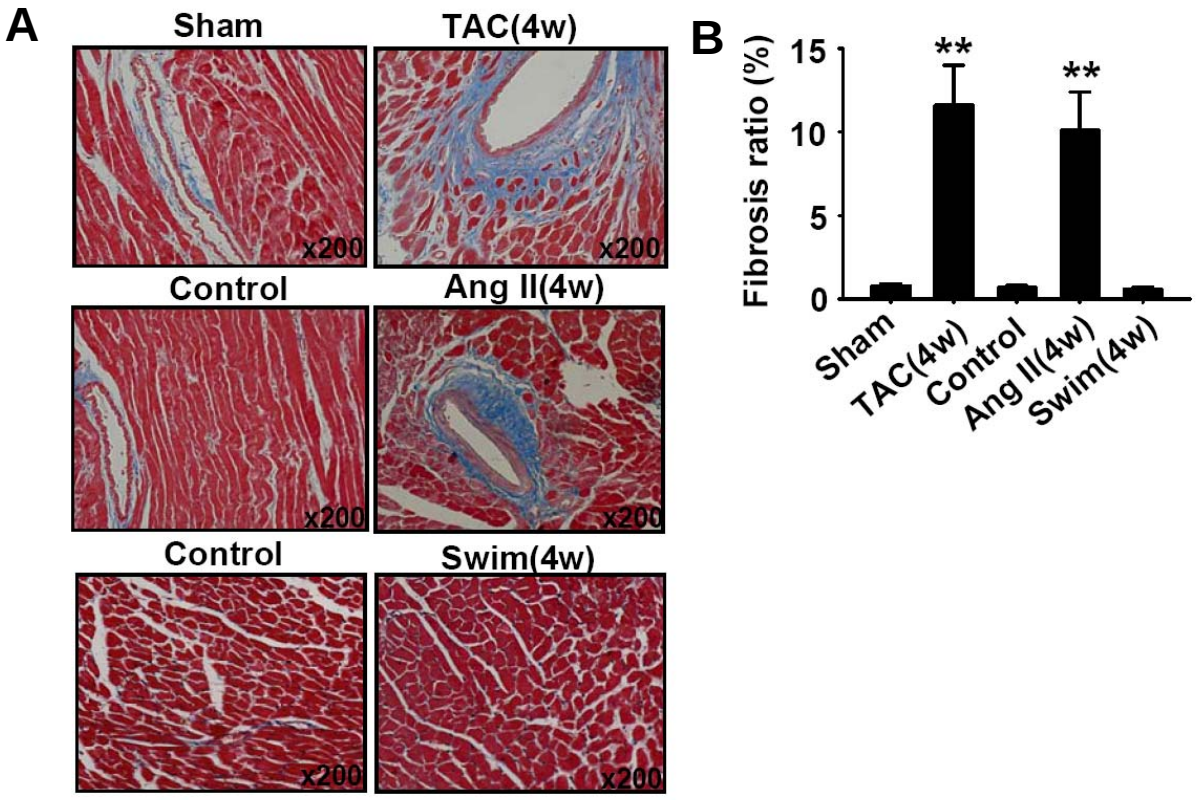

Figure S12. TAC or Ang II constant infusion but not swimming-exercise induced cardiac fibrosis in mice. Cardiac fibrosis was analyzed and quantified by using masson's trichrome method. The interstitial fibrosis was in blue staining. The degree of fibrosis was evaluated by the ratio of the area of blue staining to the total area. Image-Pro Plus Version (5.0.1) was used for analysis. **A.** Representative fields of Masson's trichrome stained heart sections in each groups. TAC and Ang II-treated animals showed more perivascular fibrosis in the hearts. **B.** The summarized data showed that TAC and Ang II, but not swimming exercise induced significant cardiac fibrosis. n=10. $^{**}P<0.01$ vs Sham or control.

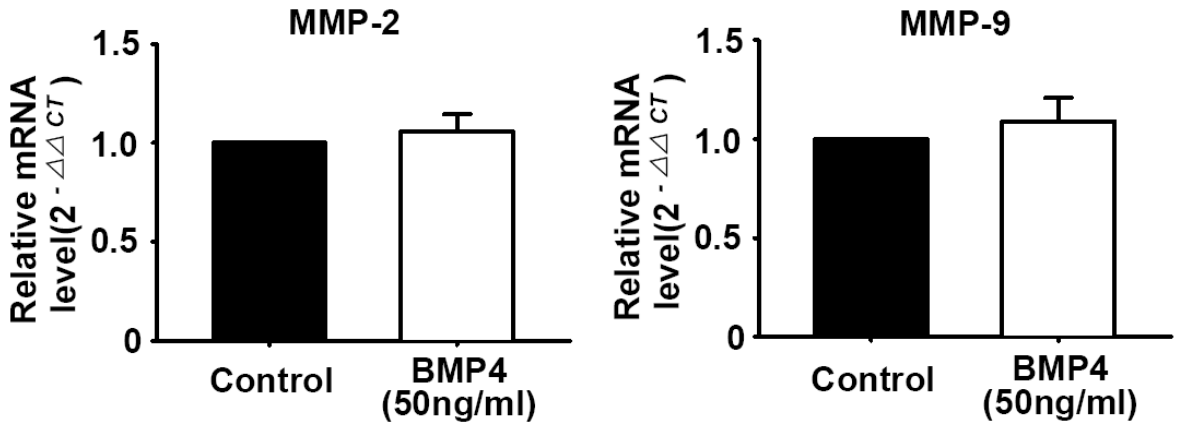

Figure S13. BMP4 treatment had no significant effect on matrix metalloproteinase-2 (MMP-2) and MMP-9 mRNA expressions in cardiac fibroblasts. n=6 in each group.

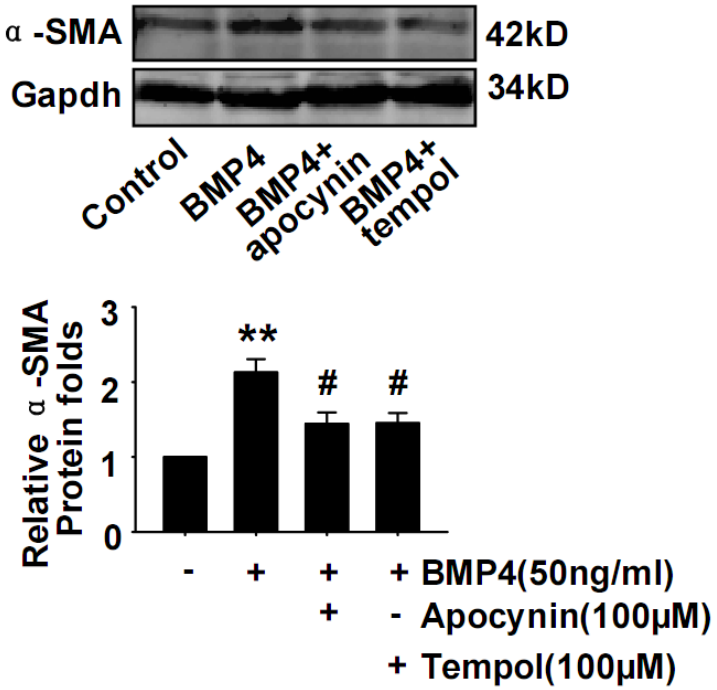

Figure S14. The increased α-SMA protein expression induced by BMP4 was inhibited by apocynin and tempol. n=6, ***P*<0.01, vs. control; #*P*< 0.05 vs. BMP4.

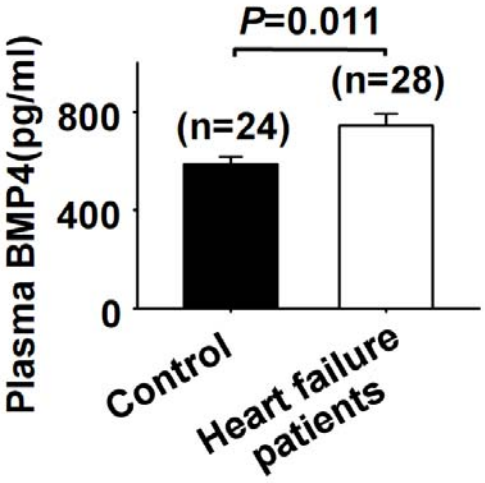

Figure S15. Plasma BMP4 level increased in heart failure patients. Compared with control, the plasma BMP4 levels were increased in heart failure patients.

References

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3. Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell*. 2009;16:233-244.

Table-S1. Sequences of qRT-PCR primers

Gene name	Accession No.	Species	Forward primer(5'-3')	Reverse primer (5'-3')
ANP	NM_008725.2 NM_012612.2	mouse rat	CTCCGATAGATCTGCCCTCTTGAA	GGTACCGGAAGCTGTTGCAGCCTA
BNP	NM_008726.4 NM_031545.1	mouse rat	TGATTCTGCTCCTGCTTTTC	GTGGATTGTTCTGGAGACTG
β-MHC	NM_080728.2 NM_017240.1	mouse rat	CCAGAAGCCTCGAAATGTC	CTTTCTTTGCCTTGCCTTTGC
BMP1	NM_009755.3	mouse	GACTCACGGCGGACTCTAAGC	GCGGCACTGACACTCGTAGC
BMP2	NM_007553.2	mouse	GACTCTGGTGAACTCTGTG	CTAACGACACCCGCAGCCCT
BMP3	NM_173404.3	mouse	GCACAGGGACAGAGACCAAACT	CTGCTGCCGCTGTACCTGTCAT
BMP4	NM_007554.2	mouse	CACCAGGGCCAGCACGTCAGAATC	AGTGAATGGCGACGGCAGTTCT
BMP4	NM_012827.2	rat	GGGAGGAGGAGGAAGAAGAG	TGGGATGCTGCTGAGGTT
NOX2	NM_023965.1	rat	CCAGTGAAGATGTGTTCAGCT	GCACAGCCAGTAGAAGTAGAT
NOX4	NM_053524.1	rat	AGTCAAACAGATGGGATA	TGTCCCATATGAGTTGTT
GADPH	NM_008084.2 NM_017008.3	mouse rat	AAGAAGGTGGTGAAGCAGGC	TCCACCACCCAGTTGCTGTA
MMP-2	NM_031054.2	rat	GGGTCTCAGAACGCCGTGGAG	ACAGGACGCAGAGAACCCGC
MMP-9	NM_031055.1	rat	GGGCATCTGGGGATTGAACTCAGC	AGCGCCCGACGCACAGTAAG

Table-S2. Information of subjects as control.

Sex	Number	Age (year)
Male	13	57.0±2.0
Female	11	

Table-S3. The clinical parameters of heart failure patients.

Patients	Sex	Age (y)	Clinical diagnosis	Heart function classification	Function of left ventricle		Left ventricle			Left atrium	Right atrium		Right ventricle
					EF %	FS %	IVST (mm)	LVPW (mm)	LVDd (mm)	Ad (mm)	Vd (mm)	Hd (mm)	Ad (mm)
			Normal values		**50-70**	**30-45**	**6-12**	**6-12**	**35-55**	**<30**	**34-49**	**32-45**	**7-23**
1	female	73	Dilated cardiomyopathy , CLBBB HF, AF	IV	33	16	10	10	61	41	52	43	20
2	female	75	Dilated cardiomyopathy , Hypertensive disease (I), HF,AF	IV	32	16	10	10	57	51	66	50	28
3	male	45	Dilated cardiomyopathy , CLBBB, HF	IV	36	18	8	8.3	72	42	48	32	23
4	female	59	Hypertensive disease (III), HF, AP, CAD	IV	47	24	10.4	10	70	43	46	31	22
5	female	58	RHD, MS, Arrhythmia, AF, HF	IV	55	29	9	9	45	50	55	42	22
6	male	69	CAD, AP, MI, HF	IV	48	25	11	11	75	55	58	45	23

7	female	44	RHD, VD, Arrhythmia, AF, HF	IV	50	26	7.7	8.5	54	51	58	44	21
8	male	76	CAD, Ischemic cardiomyopathy, HF	IV	26	12	10	9	75	52	57	41	24
9	female	52	Dilated cardiomyopathy, VPC, HF	IV	35	17	8	9.3	61.7	40	57	42	21
10	female	72	CAD, HF, Hypertensive disease (II), AF	IV	44	22	10.9	10.9	61	43	48	38	23
11	female	55	Dilated cardiomyopathy , HF, PVT	IV	27	13	10	9	72	45	57	42	20
12	female	59	CAD, Hypertensive disease (III) , HF	III	33	16	9	9	56	38	45	33	20
13	male	60	CAD, MI, Arrhythmia, AFL	IV	35	17	9	9	56	44	56	43	20
14	male	50	Dilated cardiomyopathy, HF, Hypertensive disease (III), AF	III	41	20	11.4	10.4	60	45	55	46	26
15	female	55	CAD, AP, HF, Hypertensive disease (III)	III	32	16	10.1	10.1	62	45	46	36	20
16	male	81	CAD, HF, MVP, AF	IV	42	21	11.4	10.8	67	49	69	50	22
17	male	66	Dilated cardiomyopathy , CAD, HF, AVB, LBBB	IV	43	22	12	11	71	45	56	42	22
18	male	67	CAD, MI, Hypertensive disease (III), Ischemic cardiomyopathy, HF	IV	39	20	10.4	10.4	60	43	45	35	20
19	female	70	CAD, HF, MI, AF, Ischemic cardiomyopathy,	IV	30	15	10	10	55	50	54	43	22

20	female	78	CAD, Hypertensive disease (III), MI, HF	IV	29	15	5.3	10.6	62	44	45	37	21
21	male	63	CAD, Ischemic cardiomyopathy, MI, HF	IV	32	16	9.5	9.5	62	42	46	38	22
22	female	41	Peripartum cardiomyopathy, HF	III	63	34	10	9	50	38	48	34	20
23	male	79	CAD, AP, HF	IV	31	15	8	7	55	40	46	37	20
24	female	75	CAD, Hypertensive disease (III) , HF	III	30	15	10	10	68	44	55	44	25
25	male	69	CAD, MI, HF	IV	39	19	9	10	58	36	46	36	20
26	female	63	Dilated cardiomyopathy, RBBB, HF	III	35	17	9.4	8.4	59	36	45	35	23
27	female	48	CAD, MI, VPC, Ischemic cardiomyopathy, HF	IV	28	14	7	9	64	43	53	40	24
28	male	78	Dilated cardiomyopathy, AF, VPC, HF	III	39	20	9	9	64	47	58	48	26

IVST: Interventricular Septal Thickness; LVPW: Left Ventricular Posterior Wall; LVDd: Left Ventricular Diastolic dimension; Ad: Anteroposterior diameter; Vd: Vertical diameter; Hd: Horizontal diameter; EF: Ejection Fraction; FS: Fractional Shortening; HF: Heart Failure; LBBB: Left Bundle Branch Block; RBBB: Right Bundle Branch Block; CAD: Coronary Artery Disease; MI: Myocardial Infarction; AF: Atrial Fibrillation; MVP: Mitral Valve Prolapse; VPC: Ventricular Premature Contraction; AP: Angor Pectoris; CLBBB: Complete Left Bundle Branch Block; RHD: rheumatic heart disease; MS: mitral stenosis; VD: valvular disease; PVT: paroxysmal ventricular tachycardia; AFL: atrial flutter; AVB: Atrioventricular block