# ONLINE SUPPLEMENT

# Tumor Suppressor A20 Protects against Cardiac Hypertrophy and Fibrosis through Blocking TAK1-Dependent Signaling

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**Running Title:** A20 inhibits cardiac hypertrophy

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## Methods and Materials

### Materials

The antibodies against ERK1/2, P38, JNK, Caspase-3/8/9, phospho-Smad2, TAK1, phospho-p65, IKKα, IKKβ, phospho-IκBα, and IκBα were purchased from Cell Signaling Technology. [$^3$H]-leucine and [$^3$H]-proline were purchased from Amersham. The BCA protein assay kit was purchased from Pierce and the IKK activity kit was obtained from B&D Bioscience. All other antibodies were purchased from Santa Cruz Biotechnology. TGF-β1 was purchased from R&D Systems. Fetal calf serum (FCS) was obtained from Hyclone. Wild type rat A20 cDNA (AdA20) and siA20 adenoviral (AdsiA20) were made as described previously.[1] Cell culture reagents and all other reagents were obtained from Sigma.

**Animals, Aortic banding surgery, Blood pressure and Echocardiography**

All protocols were approved by institutional guidelines. All surgeries and subsequent analyses were performed in a fashion blinded for genotype. Transgenic mice were produced as described previously.[2] We used 8-10 week-old male mice with cardiac-specific expression of human A20 and their control littermates. Genotyping was performed by polymerase chain reaction (PCR) as described previously.[2] Aortic banding (AB) was performed as described previously.[3, 4] Age- and sex-matched WT and TG mice were anesthetized with isoflurane. A 7.0 nylon suture ligature was tied against a 27-gauge needle at the transverse aorta to produce a 65–70% constriction following removal of the needle. Doppler analysis was performed to ensure that physiologic constriction of the aorta was induced. Hearts and lungs of sacrificed mice were dissected and weighed to compare heart weight/body weight (HW/BW, mg/g) and lung weight/body weight (LW/BW, mg/g) in TG and control mice. A microtip catheter transducer (SPR-839, Millar Instruments, and Houston, Tex) was inserted into the right carotid artery and advanced into the left ventricle under pressure control. After stabilization for 15 minutes, the pressure signals and heart rate were recorded continuously with an ARIA pressure-volume conductance system coupled with a Powerlab/4SP A/D converter, stored, and displayed on a personal computer as described previously.[3, 4] Echocardiography was performed by SONOS 5500 ultrasound (Philips Electronics, Amsterdam) with a 15-MHz linear array ultrasound transducer. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. LVEDD and LVESD were measured from the M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

**Histological analysis and determination of apoptosis**

Hearts were excised, placed in 10% potassium chloride solution, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (4-5 μm thick) were prepared and stained with hematoxylyn and eosin (H&E) for histopathology or Picrosirius Red (PSR) for collagen deposition, then visualized by light microscopy. For myocyte cross-sectional area, a single myocyte was measured with an image quantitative digital analysis system (NIH Image 1.6). The outline of 100 to 200 myocytes was traced in each group. Cell death by apoptosis was evaluated by a TUNEL assay that was performed in sections with use of the CardiaoTACS in situ Apoptosis Detection Kit (R&D Systems, Minneapolis, USA) according to the manufacture's recommendations. Caspase-3/8/9 activities were also used to examine the effects of A20 on apoptosis.

**Western Blot Analysis and Northern blot**

All procedures were performed as previously described. Protein extracts from different groups of myocardium (50 μg) were fractionated on a 10% polyacrylamide gel under reducing conditions, transferred to nitrocellulose membranes, and probed with various antibodies. After incubation with a secondary peroxidase-conjugated antibody, signals were visualized by Chemiluminescence kit (Amersham, Sunnyvale, CA). We Northern blot to detect mRNA levels of ANP, BNP, β-MHC, α-MHC, α-skeletal actin, and sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA2a), as well as fibrosis markers including TGFβ1, TGFβ2, CTGF, Collagen I and Collagen III. Total RNA was extracted from frozen, pulverized mouse tissues using TRIzol (Invitrogen). The detailed information for Northern blot was described in previous work.[2] We normalized results against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression.

**Electrophoretic Mobility Shift Assay, IKK Assay and TAK1 kinase Assay**

Electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega, Madison, WI). Nuclear proteins were isolated using our previously method.[5, 6] Protein concentrations were measured by BCA Protein Assay Reagents (PIERCE, Rockford, IL) using bovine serum albumin (BSA) as a standard. To determine the effect of A20 on IKK activation, the IKK assay was performed as described previously.[5, 6] TAK1 immunoprecipitates were assayed using His-MKK6 as substrate as described previously.[7]

**Cultured neonatal rat cardiac myocytes and fibroblasts**

Primary cultures of cardiac myocytes were prepared as described previously.[3, 4] Cells from the hearts of 1- to 2-day-old Sprague-Dawley rats (Charles River Laboratories) were seeded at a density of $1\times10^6$/well onto 6-well culture plates coated with fibronectin (Becton Dickinson) in plating medium consisting of F10 medium supplemented with 10% FCS and penicillin/streptomycin. After 48 hours, the culture medium was replaced with F10 medium containing 0.1% FCS and BrdU (0.1 mM), then infected with different adenoviruses followed by Ang II (1 μM) treatment. Viability was determined by cell number, frequency of contractions, cellular morphology, and trypan blue exclusion. Cultures of neonatal rat ventricular nonmyocytes, which have been shown to be predominantly fibroblasts, were prepared as described previously.[3] The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. For the cell infections, $1\times10^6$/well cardiac myocytes or cardiac fibroblasts were cultured in 6-well plates and exposed to $2\times10^8$ pfu of each virus in 1 ml of serum-free medium for 24 hours. The cells were then washed and incubated in serum-containing media for 24 hours. The viruses included AdA20 to overexpress A20, AdsiA20 to downregulate A20 expression, and their respective control viruses AdGFP and Adsi-control, as well as AddnTAK1 to block TAK1 activation, AdcaTAK1 to activate TAK1 and their control AdGFP.

**[$^3$H]-Leucine incorporation and surface area**

[$^3$H]-Leucine incorporation was measured as described previously.[4, 5] Briefly, cardiac myocytes were infected with different adenoviruses for 24 hours and subsequently stimulated with Ang II (1 μM) and coincubated with [$^3$H]-leucine (2 μCi/mL) for the indicated time. At the end of the experiment, cells were washed with Hanks' solution, scraped off the well, and then treated with 10% trichloroacetic acid at 4°C for 60 minutes. The precipitates were then dissolved in NaOH (1

N) and subsequently counted with a scintillation counter. For surface areas, the cells were fixed with 3.7% formaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS, and stained with α-actinin (Sigma) at a dilution of 1:100 by standard immunocytochemical techniques.

**Collagen synthesis assay**

Collagen synthesis was evaluated by measuring [$^3$H]-proline incorporation as described previously.[3] In brief, cardiac fibroblasts were infected with different adenoviruses, made quiescent by culturing in 0.1% FCS DMEM for 24 h, and subsequently incubated with TGF-β1 and 5 μCi/ml [$^3$H]-proline for the indicated time. Cells were washed with PBS twice, treated with ice-cold 5% trichloroacetic acid (TCA) for one hour and washed with distilled water twice. Cells were then lysed with 1 N NaOH solutions and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number.

**Reporter assays**

Cardiac myocytes or cardiac fibroblasts were seeded in triplicate in 6-well plates. Cells were infected with different adenoviruses for 24 hours and then transfected with 0.5 μg of ANF luciferase reporter construct, and internal control plasmid DNA using 10 μl of LipofectAMINE reagent (Invitrogen), according to the manufacturer's instructions. Cardiomyocytes were then treated with Ang II and fibroblasts with TGF-β1. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer's protocol. The luciferase activity was normalized by control plasmid. All experiments were done in triplicate and repeated at least three times.

**Statistical Analysis**

All values are expressed as mean±SEM. Differences between two groups were determined by a Student's t test. Comparison between groups on Western blotting data was assessed by One-Way ANOVA followed by a Bonferroni correction. A value of $P<0.05$ was considered statistically significant.

## References


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4. Li HL, She ZG, Li TB, Wang AB, Yang Q, Wei YS, Wang YG, Liu DP. Overexpression of myofibrillogenesis regulator-1 aggravates cardiac hypertrophy induced by angiotensin II in mice. *Hypertension*. 2007; 49: 1399-1408.
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7. Zhang D, Gaussin V, Taffet GE, Michael LH, Overbeek PA, Schneider MD. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. *Nat Med*. 2000; 6: 556-563.

# Online Supplement Tables

**Table S. Echocardiographic and anatomic data showed the effects of A20 on cardiac hypertrophy after at 8 weeks aortic banding.**

| Parameter | WT-sham mice | TG-sham mice | WT-AB mice | TG-AB mice |
|---|---|---|---|---|
| **Number** | n=14 | n=15 | n=13 | n=14 |
| **BW (g)** | 26.9±1.2 | 27.6±1.4 | 27.5±1.3 | 27.2±1.5 |
| **HW/BW (mg/g)** | 4.73±0.12 | 4.69±0.11 | 7.87±0.17* | 5.42±0.15†* |
| **LW/BW (mg/g)** | 4.56±0.13 | 4.55±0.16 | 7.14±0.22* | 5.31±0.11†* |
| **CSA ($\mu m^2$)** | 279±38 | 268±43 | 449±51* | 311±27†* |
| **SBP (mmHg)** | 114.5±2.7 | 109.7±1.8 | 150.7±4.8* | 145.1±5.4* |
| **HR (beats/min)** | 503±47 | 498±43 | 532±47 | 501±37 |
| **PWT(mm)** | 1.18±0.02 | 1.20±0.01 | 2.47±0.04* | 1.56±0.03†* |
| **LVEDD(mm)** | 3.47±0.04 | 3.52±0.03 | 5.12±0.04* | 4.02±0.05†* |
| **LVESD(mm)** | 1.68±0.03 | 1.72±0.03 | 3.46±0.04* | 2.22±0.02* |
| **LVSD (mm)** | 0.65±0.02 | 0.67±0.02 | 1.43±0.02* | 1.05±0.02†* |
| **LVPWD (mm)** | 0.63±0.04 | 0.62±0.03 | 1.27±0.02* | 0.79±0.02†* |
| **FS (%)** | 51.6±1.2 | 51.1±2.4 | 32.4±1.4* | 44.8±1.3†* |

*$P$<0.01 was obtained for the WT-sham values; † $P$<0.01 was obtained for the WT-AB values after AB.

HR=heart rate; BW=body weight; HW=heart weight; CSA=cardiomyocyte cross-sectional area; PWT=posterior wall thickness; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; LVSD=left ventricular septum, diastolic; LVPWD=left ventricular posterior wall, diastolic. FS=fractional shortening. SBP=systolic blood pressure; All values are mean ± SEM.

# Online Supplement Figures

## Figure S1

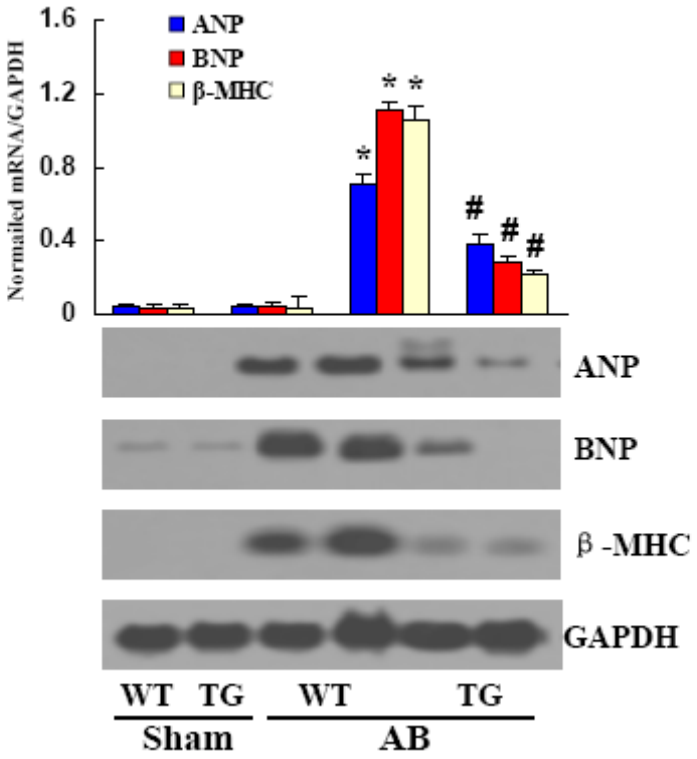


**Figure S1. Forced A20 expression attenuates the expression of cardiac hypertrophy markers in vivo.**
Total RNA was isolated from hearts of mice of the indicated groups, and expression of ANP, BNP and β-MHC induced by AB were determined by Northern blot analysis. Data represent typical results of 3-4 different experiments as mean±SEM (n=5 mice/per group). **P*<0.01 *vs* WT/sham. #*P*<0.01 *vs* WT/AB after AB.

# Figure S2

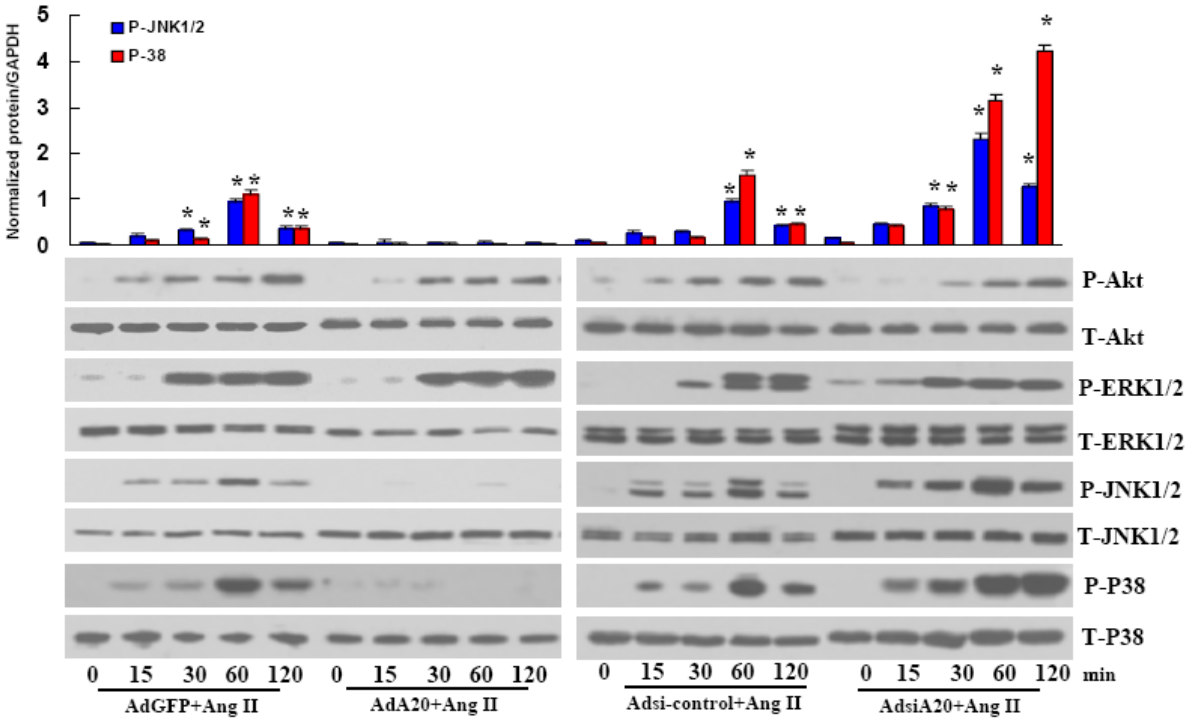


**Figure S2. Forced A20 expression attenuated mechanical stress-mediated activation of stress kinase activation *in vitro*.**
ERK1/2, P38, JNK1/2 and AKT activation in cardiomyocytes after infection with different adenovirus. Upper, quantitative results of phosphorylation of P38 and JNK1/2. Bottom, Representative blots of total and phosphorylated ERK1/2, P38, JNK1/2 and AKT. Cardiomyocytes were infected with AdA20, AdGFP, AdsiA20 or Adsi-control for 24 and then incubated with 1 μM Ang II for indicated time. Values are mean±SEM **P*<0.01 for difference from AdGFP+Ang II group at zero time point.

# Figure S3

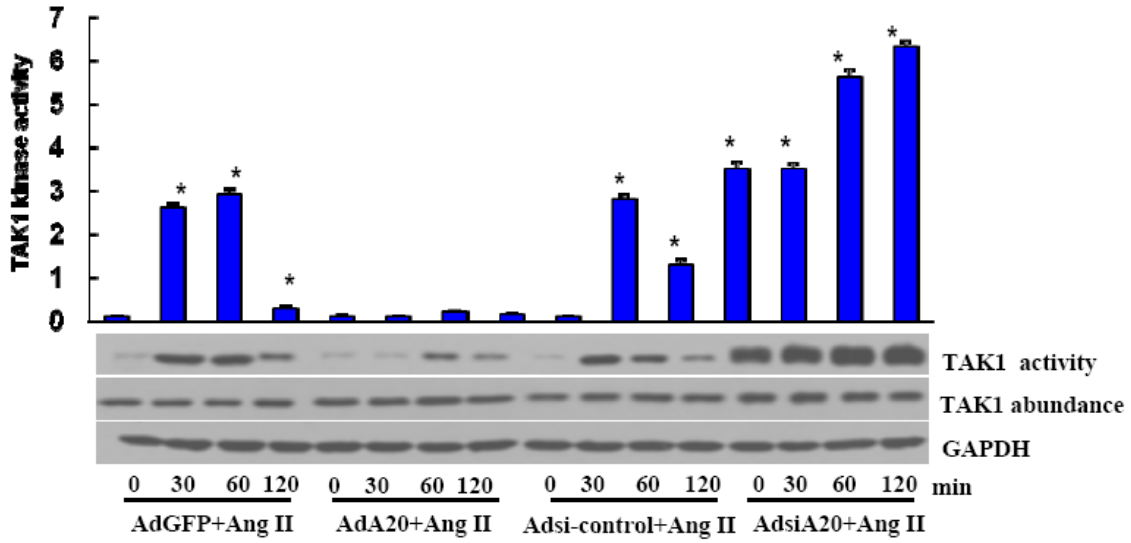


**Figure S3. Forced A20 expression impairs TAK1 signaling involved in hypertrophy *in vitro*.** The TAK1 activity and TAK1 protein expression in cardiomyocytes after infection with different adenovirus. Upper, quantitative results. Bottom, Representative blots. Cardiomyocytes were infected with AdA20, AdGFP, and AdsiA20 or Adsi-control for 24 hours and then incubated with 1 μM Ang II for indicated time. Values are mean±SEM *P<0.01 for difference from AdGFP+Ang II group at zero time point.

# Figure S4

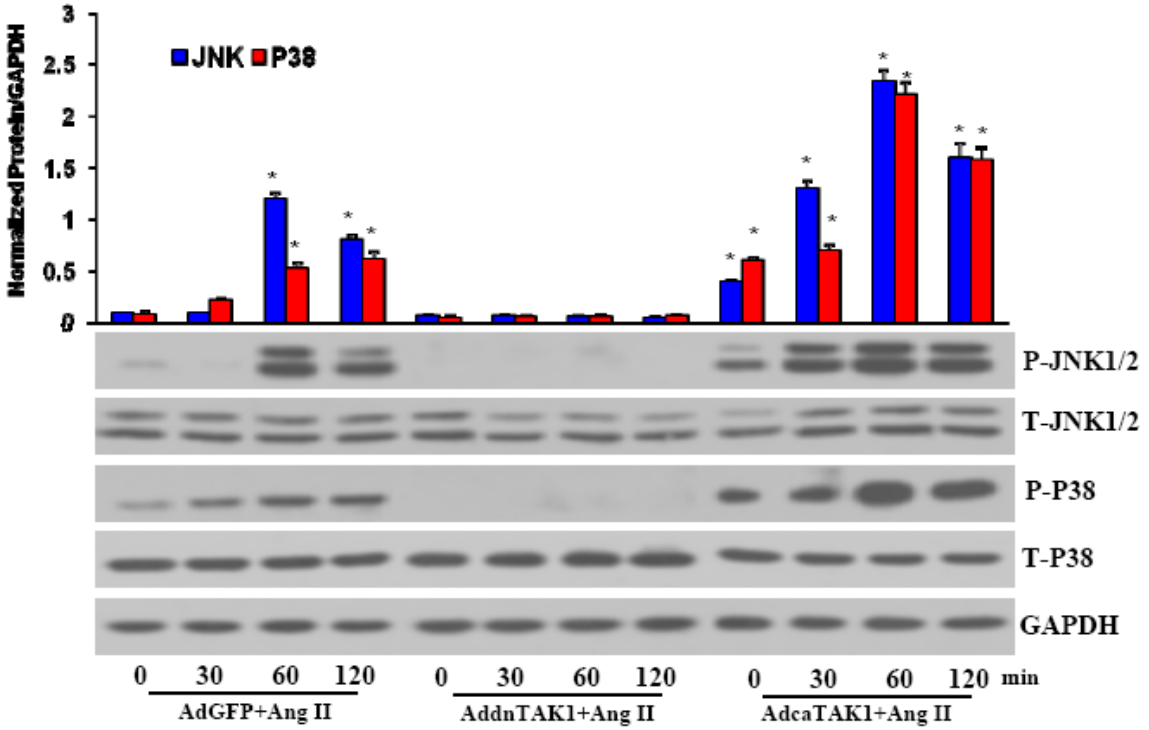


**Figure S4. The effects of TAK1 on the phosphorylation of P38 and JNK1/2 induced by Ang II treatment at time points indicated.**

Cardiomyocytes were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 1 μM Ang II for indicated time. Values are mean±SEM. **P*<0.01 for difference from AdGFP+Ang II group at zero time point.

# Figure S5

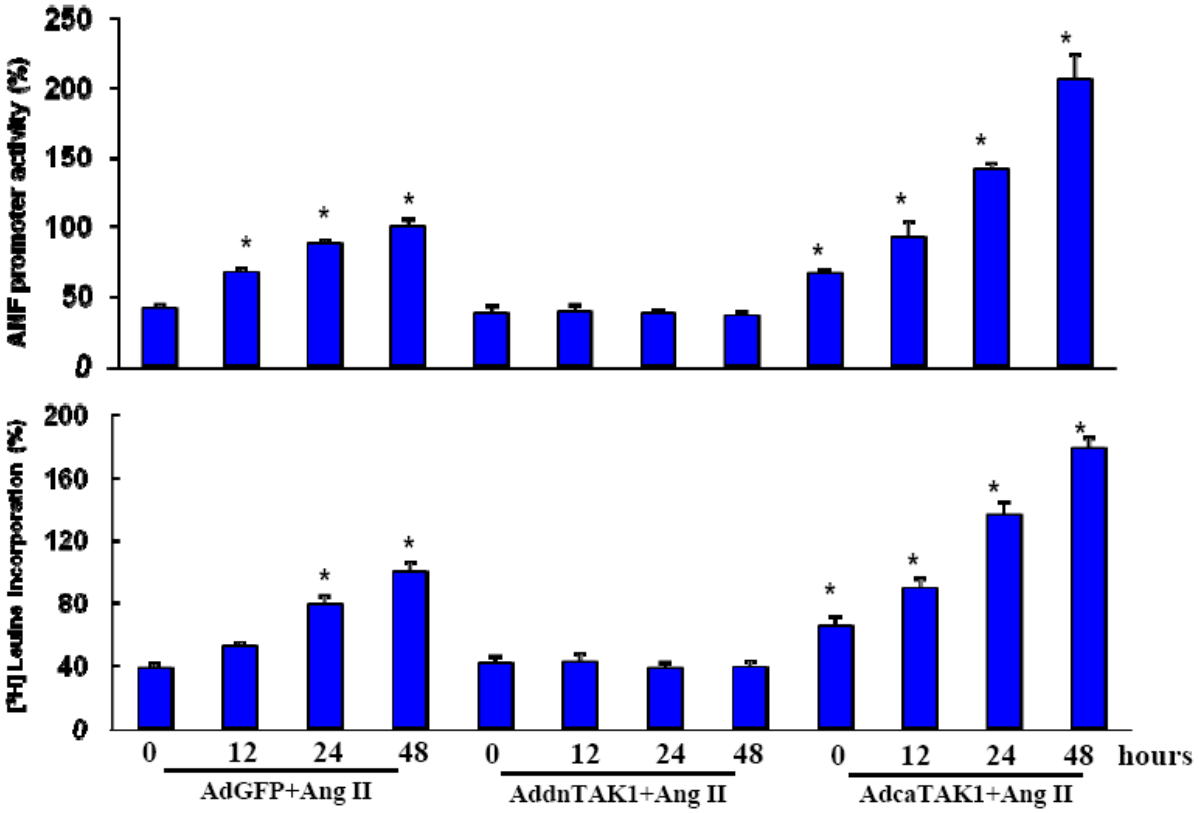


**Figure S5. The effects of TAK1 on the [$^3$H]-Leucine incorporation and ANP promoter activity induced by Ang II treatment at time points indicated.** Cardiomyocytes were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 1 μM Ang II for indicated time. Values are mean±SEM **P*<0.01 for difference from AdGFP+Ang II group at zero time point.

# Figure S6

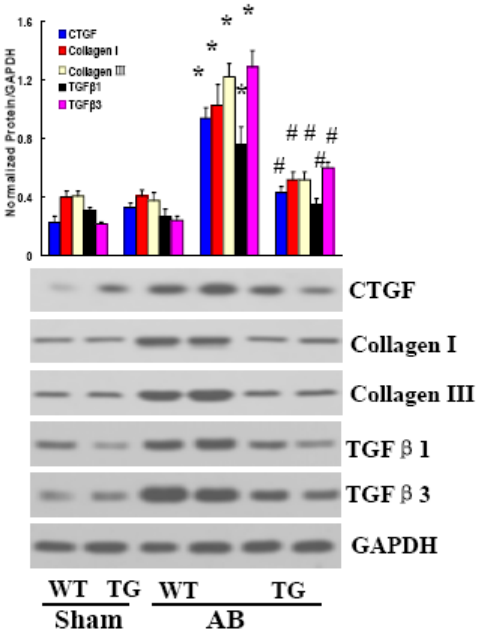


**Figure S6. The effects of A20 on the expression of fibrosis markers *in vivo*.**
Western blot analyses of CTGF, collagen I, collagen III, TGF-β1 and TGF-β3 were performed to determine protein expression levels in indicated groups. GAPDH was used as the normalization control. Data represent typical results of 3 different experiments as mean±SEM (n=4 to 5 mice/per group). **P*<0.01 *vs* WT/sham. $^{\#}$*P*<0.01 *vs* WT/AB after AB.

# Figure S7

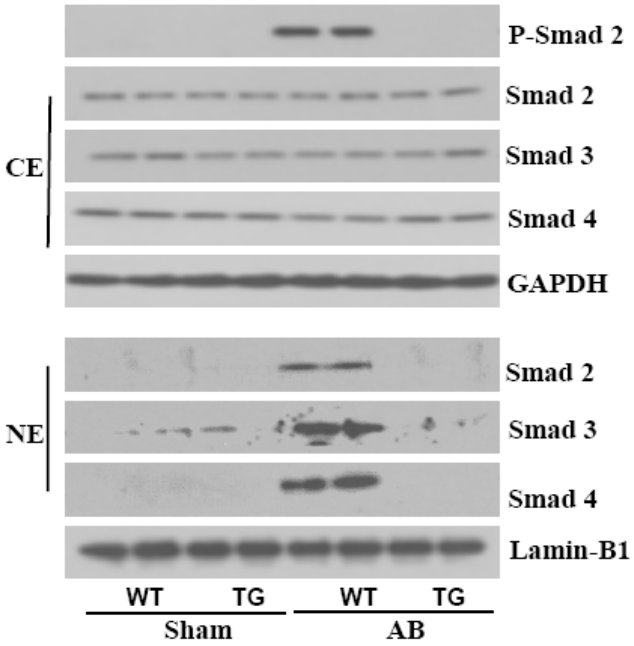


**Figure S7. The effects of A20 on Smad signaling *in vivo*.**
The effects of A20 on Smad 2 phosphorylation and Smad 2/3/4 protein expression as well as Smad 2/3/4 nuclear translocation in heart tissues in indicated groups. CE: Cytoplasmic extracts; NE: Nuclear extracts.

# Figure S8

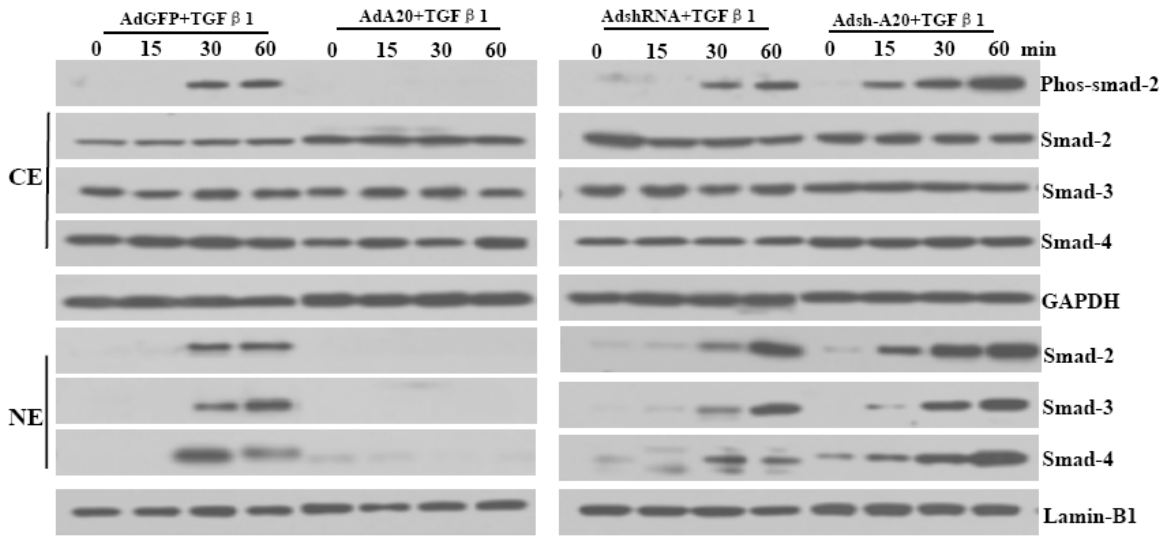


**Figure S8. The effects of A20 on Smad signaling *in vitro.***
The effects of A20 on Smad 2 phosphorylation and Smad 2/3/4 protein expression as well as Smad 2/3/4 nuclear translocation in cardiac fibroblasts. Cardiac fibroblasts were infected with AdA20, Adsi-control, AdGFP or AdsiA20 for 24 hours, and then incubated with 10 ng/mL TGFβ1 for indicated time.

# Figure S9

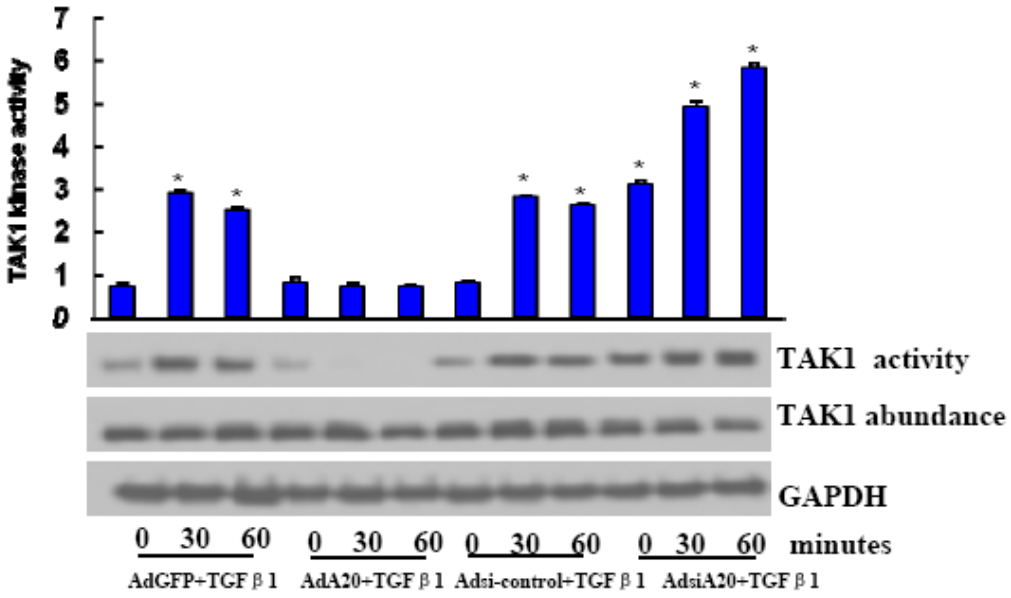


**Figure S9. The effects of A20 on TGF-β1-induced TAK1 activity *in vitro*.**
The TAK1 activity and TAK1 protein expression in cardiac fibroblasts after infection with different adenovirus. Upper, quantitative results. Bottom, Representative blots. Cardiac fibroblasts were infected with AdA20 or AdGFP, and AdsiA20 or Adsi-control for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM **P*<0.01 for difference from AdGFP+ TGFβ1 group at zero time point.

# Figure S10

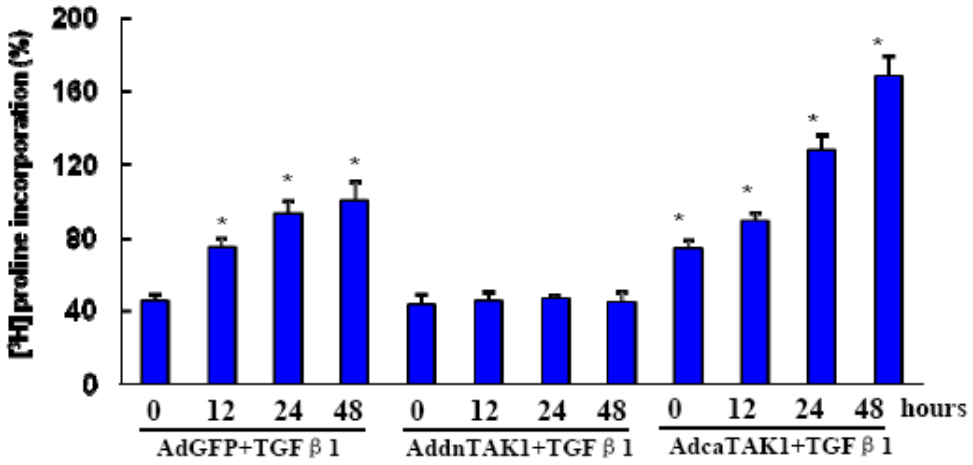


**Figure S10. The effects of TAK1 on [$^3$H]-proline incorporation.**
Cardiac fibroblasts were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM. **P*<0.01 for difference from AdGFP+ TGFβ1 group at zero time point.

# Figure S11

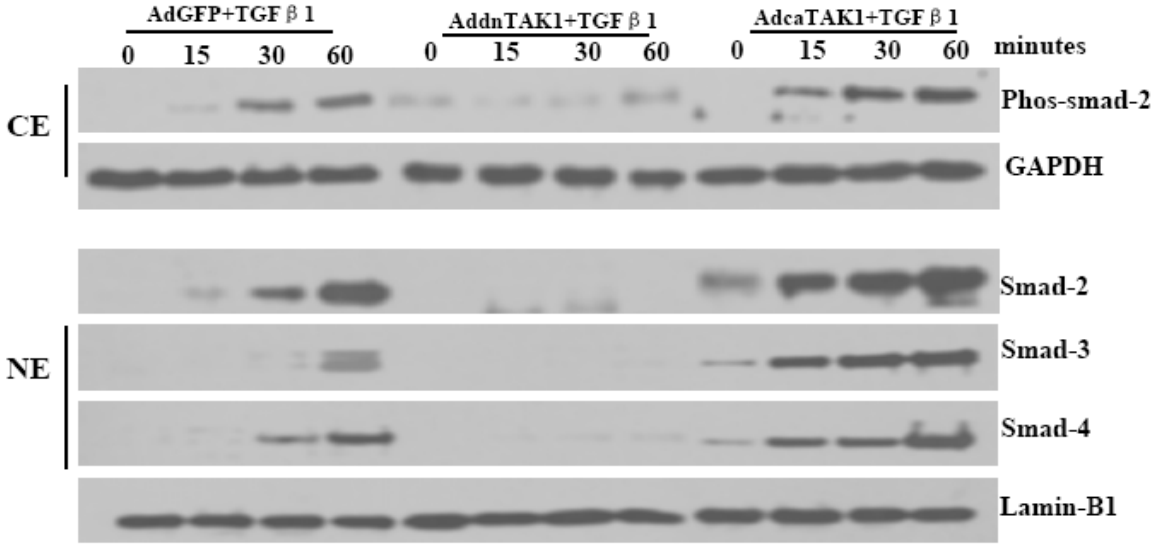


**Figure S11. The effects of TAK1 on Smad signaling *in vitro*.**
The effects of TAK1 on Smad 2 phosphorylation as well as Smad 2/3/4 nuclear translocation. Cardiac fibroblasts were infected with AdGFP as a control, or AddnTAK1, AdcaTAK1 for 24 hours and then incubated with 10 ng/mL TGFβ1 for indicated time. Values are mean±SEM. **P*<0.01 for difference from AdGFP+ TGFβ1 group at zero time point.

# Figure S12

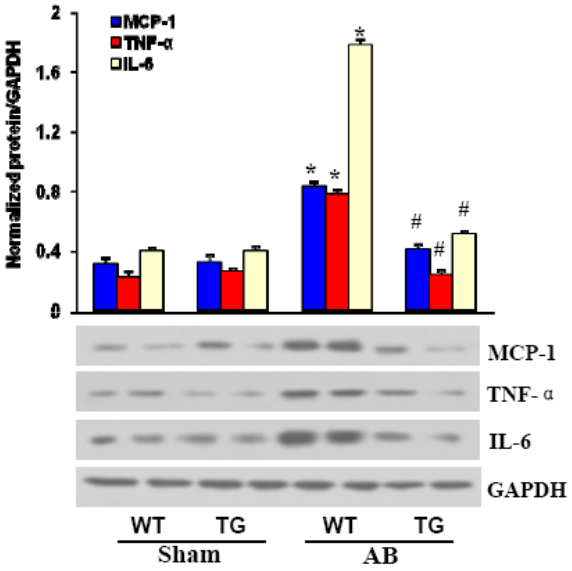


**Figure S12. The effect of A20 on proinflammatory cytokine induction.**
Western blot analysis of MCP-1, TNF-α and IL-6 protein expression in the myocardium obtained from indicated groups at 8 weeks AB (n=5). Values are mean±SEM. The results were reproducible in three separate experiments. Each assay was performed in triplicate. **P*<0.01 *vs* WT/sham. #*P*<0.01 *vs* WT/AB after AB.

# Figure S13

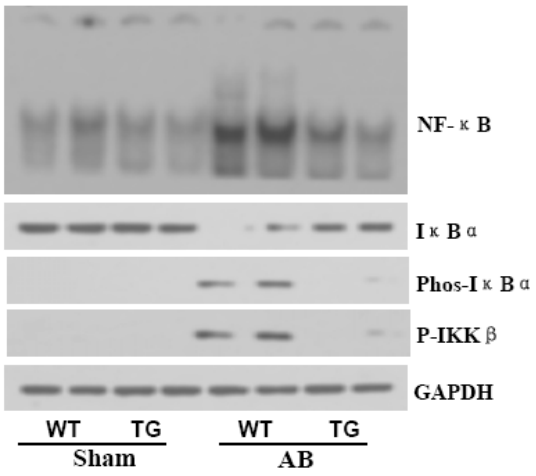


**Figure S13. The effect of A20 on NF-κB signaling pathways.**
The DNA binding activity of NF-κB and level of IκBα phosphorylation and degradation induced by AB were determined by Western blot. Upper, EMSA results, Bottom, Western blot representative blots.