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Growth/differentiation factor 1 alleviates pressure overload-induced cardiac hypertrophy and dysfunction

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ABSTRACT

Pathological cardiac hypertrophy is a major risk factor for developing heart failure, the leading cause of death in the world. Growth/differentiation factor 1 (GDF1), a transforming growth factor-β family member, is a regulator of cell growth and differentiation in both embryonic and adult tissues. Evidence from human and animal studies suggests that GDF1 may play an important role in cardiac physiology and pathology. However, a critical role for GDF1 in cardiac remodelling has not been investigated. Here, we performed gain-of-function and loss-of-function studies using cardiac-specific GDF1 knockout mice and transgenic mice to determine the role of GDF1 in pathological cardiac hypertrophy, which was induced by aortic banding (AB). The extent of cardiac hypertrophy was evaluated by echocardiographic, hemodynamic, pathological, and molecular analyses. Our results demonstrated that cardiac specific GDF1 overexpression in the heart markedly attenuated cardiac hypertrophy, fibrosis, and cardiac dysfunction, whereas loss of GDF1 in cardiomyocytes exaggerated the pathological cardiac hypertrophy and dysfunction in response to pressure overload. Mechanistically, we revealed that the cardioprotective effect of GDF1 on cardiac remodeling was associated with the inhibition of the MEK–ERK1/2 and Smad signaling cascades. Collectively, our data suggest that GDF1 plays a protective role in cardiac remodeling via the negative regulation of the MEK–ERK1/2 and Smad signaling pathways.

1. Introduction

Heart failure is the leading cause of death globally. One of the major risk factors for developing heart failure is pre-existing cardiac remodeling, *i.e.*, cardiac hypertrophy, inflammation, fibrosis, and cardiomyocyte apoptosis [1,20]. Cardiac remodeling may occur with pressure overload (aortic valve stenosis and hypertension), with volume overload (valvular regurgitation), or following cardiac injury, including myocardial infarction, myocarditis, and idiopathic dilated cardiomyopathy [15]. Although the etiologies of these diseases are different, they share molecular, biochemical, and cellular events that collectively change the shape of the myocardium. Distinct remodeling events may initially be beneficial because they are initiated to compensate for cardiac dysfunction, but remodeling ultimately leads to a transition to heart failure [1]. Multiple signaling pathways mediating the development of pathological cardiac remodeling have been identified over the past several decades [5,9,12,15,21,23,31], but the molecular modulators that antagonize the development of cardiac remodeling and the transition to heart failure remain incompletely defined.

Growth/differentiation factor 1 (GDF1) is a transforming growth factor-β family member that was originally isolated from a mouse embryo cDNA library [28]. Two GDF1 transcripts [1.4 kilobases (kb) and 3.0 kb in length] displaying distinct temporal expression patterns were detected in a Northern blot analysis of embryonic mRNA [18]. Furthermore, primary GDF1 protein contains a polybasic proteolytic processing site where it is cleaved to produce a mature protein comprising seven conserved cysteine residues, indicating that GDF1 is post-translationally regulated. Concerning its functional role, GDF1 is a regulator of cell growth and differentiation in both embryonic and adult tissues [2,8]. Recent studies in rodents suggest that GDF1 is involved in establishing left-right asymmetry during early embryogenesis and in neural development during later embryogenesis [3,29]. Mice deficient in GDF1 exhibit a spectrum of defects related to left-right axis formation, including visceral situs inversus, right pulmonary isomerism, and a range of cardiac anomalies [28]. Kaasinen et al. [13] reported that mutations in GDF1 could cause inherited right atrial isomerism, and

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heterozygous loss-of-function mutations in the human GDF1 gene contribute to cardiac defects and vessel remodeling [14]. Furthermore, Wall et al. [30] demonstrated that mature GDF1 activates a Smad2-dependent signaling pathway and is sufficient to reverse the left-right axis. GDF1 overexpression significantly rescues developmental anomalies, such as pericardial edema, circulation failure, and heart malformation, as well as cardiac toxicity caused by morpholinos or arsenite [22]. Together, these findings suggest that GDF1 plays a critical role in cardiac physiology and pathology. However, the role of GDF1 in cardiac remodeling has not been investigated. The current study featured the following objectives: 1) to determine whether GDF1 is altered in dilated cardiomyopathy (DCM) patients and a model of pressure overload-induced cardiac hypertrophy; 2) to determine whether GDF1 expression affects cardiac hypertrophy; and 3) to identify the mechanisms that would be involved in any such effects that are observed.

To this end, we employed knockout (KO) mice with a cardiac-specific deletion of the GDF1 gene and transgenic (TG) mice with cardiac-specific overexpression of GDF1 to determine the role of GDF1 in pathological cardiac remodeling. Our results demonstrate that GDF1-TG mice are resistant to cardiac remodeling via inhibition of MEK–ERK1/2 and Smad signalings, whereas cardiac-specific GDF1-KO mice display the opposite phenotype in response to pressure overload. Our study shows a previously unrecognized therapeutic potential for GDF1 in the treatment of pathological cardiac remodeling and heart failure.

2. Methods

2.1. Antibodies

GDF1 expression in human and mouse samples was determined by Western blotting using a GDF1-specific antibody (R&D Systems, AF858, 1:1000 dilutions). Antibodies to ERK1/2 (#4695, 1:1000 dilution), phospho-ERK1/2$^{Thr202/Thr204}$ (#4370, 1:1000 dilution), MEK1/2 (#9122, 1:1000 dilution), phospho-MEK1/2$^{Ser217/221}$ (#9154, 1:1000 dilution), mTOR (#2983, 1:1000 dilution), phospho-mTOR^{Ser2448} (#2971, 1:1000 dilution), FOXO3A (#2497, 1:1000 dilution), phospho-FOXO3A$^{Ser318/321}$ (#9465, 1:1000 dilution), P38 (#9212, 1:1000 dilution), phospho-P38$^{Thr180/Thr182}$ (#4511, 1:1000 dilution), JNK1/2 (#9258, 1:1000 dilution), phospho-JNK1/2 (#4668, 1:1000 dilution), AKT (#4691, 1:1000 dilution), phospho-AKT^{Ser473} (#4060, 1:1000 dilution), GSK3β (#9315, 1:1000 dilution), phospho-GSK3β (#9322, 1:1000 dilution), FoxO1 (#2880, 1:1000 dilution), phospho-FoxO1^{ser256} (#9461, 1:1000 dilution), and α-actinin (#3134, 1:1000 dilution) were purchased from Cell Signaling Technology (Danvers, MA, USA). The GAPDH (MB001, 1:10,000 dilution) antibody was purchased from Bioworld Technology (Harrogate, UK). Antibodies against atrial natriuretic peptide (ANP, sc20158, 1:200 dilution), lamin B (sc6217, 1:200 dilution), and β-myosin heavy chain MHC (β-MHC, sc53090, 1:200 dilution) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Study animals

All experiments involving animals were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University. Experiments were performed using male mice that were 8–10 weeks of age with body weights of 23.5–27.5 g. These mice were anesthetized with 1.5–2% isoflurane by inhalation or with pentobarbital (30 mg/kg, Sigma) by intraperitoneal injection. The mice were housed with an alternating 12-h light and dark cycle in temperature-controlled rooms and had free access to food and water. The following animals were used.

2.2.1. Cardiac-specific GDF1 conditional knockout mice

Male GDF1-floxed conditional mutation mice (B6.129X1-Gdf1^{tm1Dmus}/Kctt, C57BL/6 background) were ordered from the European Mouse Mutant Archive (EMMA, EM: 02230). To obtain cardiac-specific GDF1 knockout mice, GDF1-floxed mice were crossed with mice that carried the α-MHC-MerCreMer transgene [MEM-Cre-Tg (Myh6-cre/Esr1, Jackson Laboratory, 005650)]. Six-week-old GDF1-Cre mice (with MEM-Cre and the GDF1 genes) were then injected with tamoxifen (80 mg/kg/day, Sigma, T-5648) on 5 consecutive days to induce Cre recombinase expression in these mice. GDF1-Cre mice were identified using a PCR analysis of cardiac genomic DNA with the following primers: primer 1: 5′-ATGCCTTCCTTCAGGTC ACTT-3′, primer 2: 5′-CTCCACATTCGACAGG TCAAA-3′, and primer 3: 5′-GTACTTGG ATCGGTTTGTCTC-3′.

2.2.2. Cardiac-specific GDF1 transgenic mice

Transgenic mice (C57BL/6 background) with cardiac-specific GDF1 expression were generated by subcloning the full-length mouse GDF1 cDNA (Origene, MC202978) downstream of the cardiac α-myosin heavy chain (α-MHC) promoter. The linearized α-MHC-GDF1 plasmid was microinjected into mouse oocytes, which were introduced into pseudopregnant females to obtain the desired transgenic mice. The transgenic mice were confirmed by PCR analyses of tail genomic DNA using the forward PCR primer 5′-ATCTCCCCCATAAGAGTTTGAGTC-3′ and the reverse PCR primer 5′-CCCTGTATCTTCACTCTCAGCC-3′. Four independent lines were obtained for GDF1-Tg mice, and each had the same phenotype.

2.3. Aortic banding

The pressure overload-induced cardiac hypertrophy mouse model via aortic banding (AB) was established as previously described [12,20,23]. After anesthesia and once the absence of reflexes had been established, the left side of the chest of each mouse was opened to identify the thoracic aorta, which was tied against a 26G (for body weights of 25–27.5 g) or 27G (for body weights of 23.5–25 g) needle by a 7–0 silk suture; the needle was then removed, and the thoracic cavity was closed. Finally, adequate constriction of the aorta was determined by Doppler analysis. A similar procedure without aortic constriction was performed in the sham group.

2.4. Treatment of mice with U0126

U0126, an inhibitor of MAPK kinase (MEK) 1/2, was obtained from Cell Signaling Technology (Beverly, MA), dissolved in dimethyl sulfoxide, and administered at a constant volume of 1 ml per 100 g of body weight by intraperitoneal injection every 3 days (1 mg/kg/3 days) [20].

2.5. Echocardiography and hemodynamic measurements

Mice were anesthetized with 1.5–2% isoflurane by inhalation, as described previously [12,20,23,31], and then echocardiography was performed to evaluate left ventricle (LV) function and structure using a Mylab30CV (ESAOTE) machine with a 15-MHz probe. To measure LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), and LV fractional shortening, M-mode tracings derived from the short axis of the left ventricle at the level of the papillary muscles were recorded; parameters were obtained from at least three beats and averaged. A 1.4-French catheter-tip micromanometer catheter (SPR-839; Millar Instruments) was inserted into the left ventricle via the right carotid artery to obtain invasive hemodynamic measurements. An Aria pressure-volume conductance system coupled with a PowerLab/4SP A/D converter was used to record and store the pressure and dp/dt continuously, which were then displayed on a personal computer.

2.6. Histological analysis

Hearts were arrested with 1 M KCl and fixed in 10% formalin for >24 h. Hearts were paraffin embedded and cut into 5-μm sections.

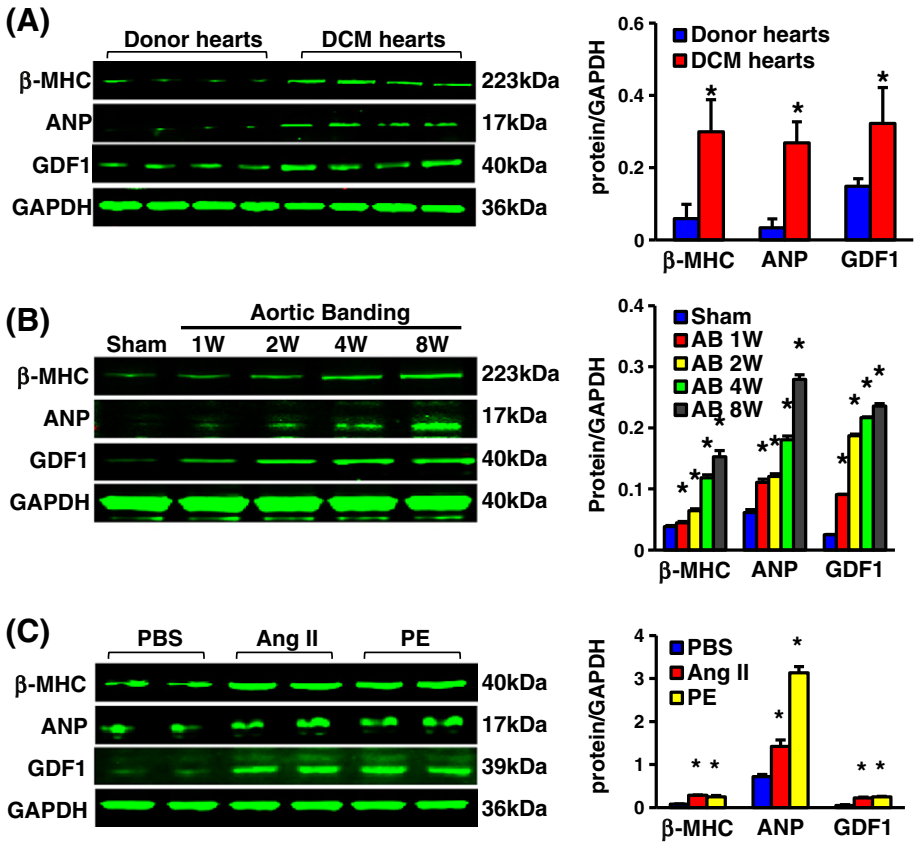

Fig. 1. GDF1 expression is up-regulated in DCM Human hearts and in hypertrophic murine hearts. (A–C) β-MHC, ANP, and GDF1 protein levels in left ventricular samples from (A) donor hearts and DCM hearts; (B) mice at the indicated times after sham or AB operation; and (C) NRCMs treated with Ang II or PE for 48 h (n = 3 independent experiments, $^{*}p < 0.05$ vs. donor or sham or PBS). Left: Representative blots; Right: Quantitative results.

Heart sections were stained with hematoxylin and eosin (H&E) to assess morphology, picrosirius red (PSR) to assess fibrosis content, or FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp.) to determine the myocyte cross-sectional area (CSA), which was measured using a quantitative digital image analysis system (Image-Pro Plus 6.0).

2.7. Cultured neonatal rat cardiac myocytes and recombinant adenoviral vectors

Primary neonatal rat cardiomyocytes (NRCMs) were cultured as previously described [20]. Briefly, neonatal hearts of 1- to 2-day-old Sprague–Dawley rats were removed from the thoracic cavities after euthanization. PBS containing 0.125% trypsin was used to digest finely minced heart tissue. NRCMs were enriched by differential pre-plating for 2 h; seeded at a density of 1×10^5 cells/well onto gelatin-coated, six-well culture dishes; and cultured in media consisting of DMEM/F12 medium, 10% FBS, BrdU (0.1 mM), and penicillin/streptomycin at 37 °C for 48 h. Subsequently, culture media were changed to serum-free DMEM/F12 for 12 h before adenoviral infection and/or Ang II (1 μM), phenylephrine (PE, 100 μM), or TGFβ1 (10 ng/ml) treatment. To overexpress GDF1, the full-length rat Gdf1 cDNA under the control of the cytomegalovirus (CMV) promoter was subcloned into a replication-defective adenoviral vector. AdGFP, a similar recombinant adenovirus expressing GFP, was used as a control. To knockdown GDF1, AdshGDF1 adenoviruses were generated based on three rat shGDF1 constructs obtained from SABiosciences (KR49323G). The adenovirus that showed the greatest decrease in GDF1 levels was selected for further experiments. AdshRNA was the non-targeting control. NRCMs were infected with adenovirus for 24 h at a multiplicity of infection of 100.

2.8. Immunofluorescence analysis

Immunofluorescence staining was performed to determine the cell surface area. Briefly, NRCMs were treated with the indicated adenoviruses for 24 h and then stimulated with Ang II (1 μM) or phenylephrine (PE, 100 μM) for 48 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature followed by three washes in PBS. Thereafter, the cells were permeabilized with 0.2% Triton X-100 for 5 min, blocked with 10% BSA for 1 h, and stained with α-actinin antibody at a dilution of 1:200 at 4 °C overnight. Subsequently, the cells were washed in PBS and incubated with secondary antibodies for 1 hour at room temperature. After washing, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were collected and analyzed using Image-Pro Plus 6.0 software.

2.9. Quantitative real-time PCR and western blotting

Total RNA was extracted from mouse hearts and NRCMs using TRIzol (Invitrogen), and cDNA was generated with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Transcripts were then amplified using SYBR Green (Roche), and GAPDH served as the endogenous reference gene. Western blotting was performed using the extracts from cardiac tissues and NRCMs. The protein concentration was measured using a Pierce® BCA Protein Assay Kit (Pierce). Protein (50 μg) was loaded into each lane for SDS-PAGE (Invitrogen) and then transferred to a

PVDF membrane (Millipore). Blots were incubated with the indicated primary antibodies overnight at 4 °C, followed by incubation with a secondary IRDye® 800CW-conjugated antibody (LI-COR Biosciences, at a 1:10,000 dilution). Signals were detected using an Odyssey Imaging System (LI-COR Biosciences). GAPDH was used as the loading control.

2.10. Human heart samples

All procedures involving human tissue samples were approved by the Renmin Hospital of Wuhan University Review Board, Wuhan, China. Informed consent was obtained from the families of prospective heart donors. Samples of failing human hearts were collected from the left ventricles of DCM patients during orthotopic heart transplantation. Control samples were obtained from the left ventricles of normal heart donors who died in accidents but whose hearts were not suitable for transplantation for noncardiac reasons.

2.11. Statistical Analysis

Data are expressed as the mean ± SD. Comparisons between two groups were evaluated by *Student's t-test*. Differences among multiple groups were assessed by one-way ANOVA followed by *Tukey's post-hoc* test. Differences were considered to be statistically significant at values of $p < 0.05$.

3. Results

3.1. GDF1 is up-regulated in human DCM hearts and murine hypertrophic hearts

To investigate the potential role of GDF1 in cardiac remodeling, we first examined whether GDF1 expression levels were altered in diseased hearts. Western blotting and real-time PCR showed that both GDF1

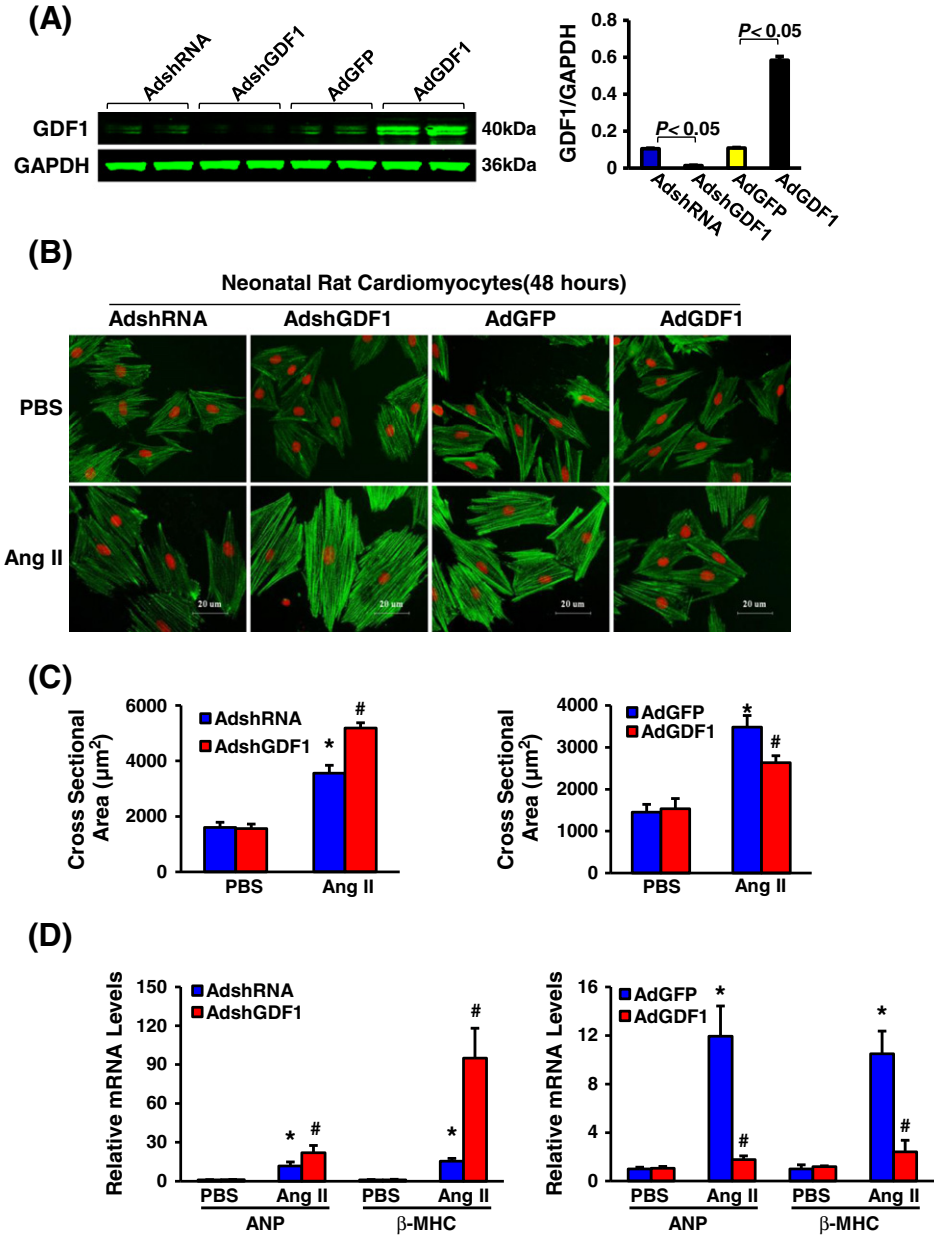

Fig. 2. GDF1 protects against Ang II-induced cardiomyocyte hypertrophy *ex vivo*. (A) GDF1 protein levels in NRCMs infected with AdshGDF1 or AdGDF1 (n = 3 independent experiments). Left: representative blots; right: quantitative results. (B) Representative images of NRCMs infected with AdshGDF1 or AdGDF1 and treated with 1 μM Ang II for 48 h. (C) CSA of NRCMs infected with AdshGDF1 (left) or AdGDF1 (right) and treated with 1 μM Ang II for 48 h (n = 100+ cells per experimental group). (D) The relative mRNA levels of ANP and β-MHC in NRCMs infected with AdshGDF1 (left) or AdGDF1 (right) and treated with 1 μM Ang II for 48 h. $^{*}p < 0.05$ vs. AdshRNA or AdGFP/PBS; $^{\#}p < 0.05$ vs. AdshRNA or AdGFP/Ang II.

protein and mRNA levels were dramatically increased in human DCM hearts compared with donor hearts (Fig. 1A and Supplemental Fig. S1A). Additionally, the β-MHC and ANP (two hypertrophic markers) expression levels were markedly elevated in these DCM hearts compared with donors (Fig. 1A and Supplemental Fig. S1B). Similarly, in a murine model of AB-induced cardiac hypertrophy, we found that GDF1 was progressively up-regulated from 1 week to 8 weeks after the AB operation (Fig. 1B and Supplemental Fig. S1C). Furthermore, using *ex vivo* cultured NRCMs treated with either Ang II or PE for 48 h to induce cell hypertrophy, we observed that GDF1 expression was also significantly elevated compared with control PBS-treated cells (Fig. 1C). Together, these data indicate that GDF1 may be involved in cardiac remodeling.

3.2. GDF1 protects against Ang II-induced cardiomyocyte hypertrophy ex vivo

The alterations in GDF1 expression in response to hypertrophic stimuli suggest that it has a potential role in the development of cardiac hypertrophy. We therefore performed gain- and loss-of-function experiments using cultured NRCMs. Cells were infected with either AdshGDF1 to knockdown GDF1 or AdGDF1 to overexpress GDF1 (Fig. 2A) and then treated with either 1 μM Ang II or PBS as a control for 48 h. Next, the cells were immunostained with α-actinin to measure cardiomyocyte size. GDF1 knockdown by shRNA (AdshGDF1) remarkably promoted Ang II-induced cell hypertrophy, whereas GDF1 overexpression (AdGDF1) significantly limited cell hypertrophy, as evaluated by cell surface area (CSA) (Fig. 2B/C). Supporting these observations, Ang II-induced expression of the hypertrophic markers ANP and β-MHC was markedly enhanced by GDF1 knockdown (Fig. 2D, left panel) but was suppressed by GDF1 overexpression (Fig. 2D, right panel). These *ex vivo* data indicate that GDF1 is a negative regulator of cardiac hypertrophy.

3.3. Cardiomyocyte-specific deletion of Gdf1 aggravates pressure overload-induced cardiac remodeling

We next generated conditional GDF1-KO mice (GDF1-Cre mice) by crossing GDF1-floxed mice with transgenic αMHC-MerCreMer (MEM-Cre) mice to further evaluate the anti-hypertrophic effects of GDF1 *in vivo*. Conditional knockout founders were identified by Western blot analysis (Fig. 3A). At baseline, GDF1-floxed mice showed no pathological alterations in cardiac structure or function (Supplemental Table S1). To induce Cre-dependent recombination, 6-week-old GDF1-

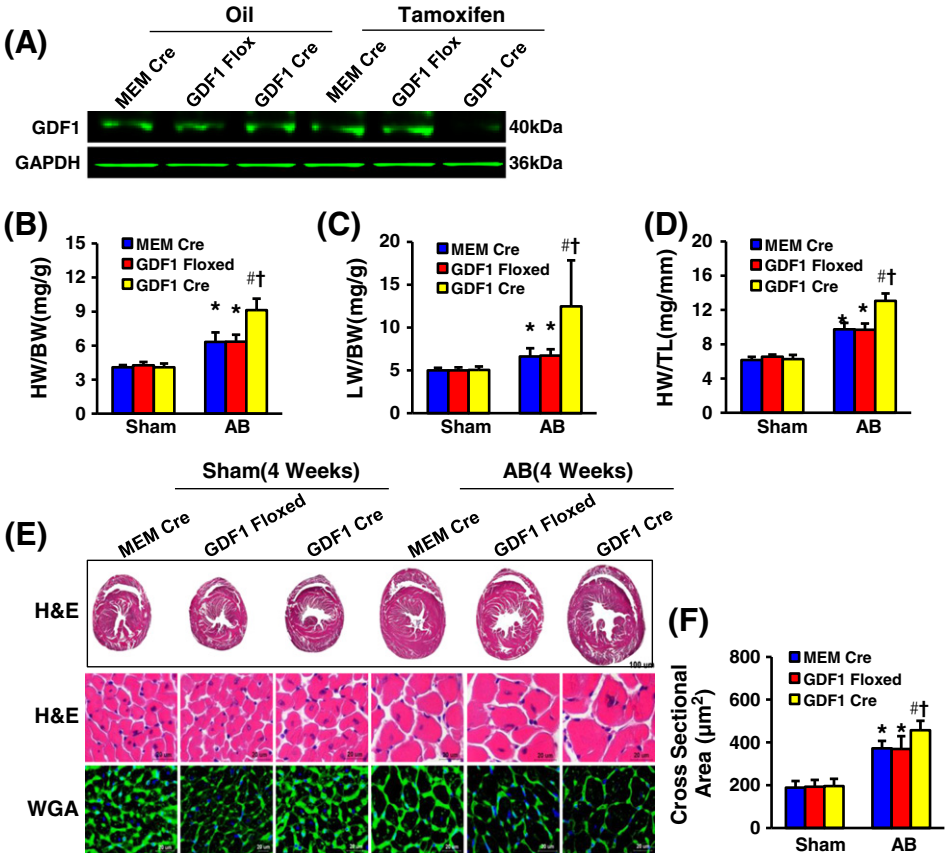

Fig. 3. GDF1 deficiency aggravates pressure overload-induced cardiac remodeling. (A) Representative Western blots for GDF1 expression levels in hearts from the indicated groups. (B–D) Statistical results for the following ratios in the indicated groups (n = 11–15 mice per experimental group): (B) HW/BW, (C) LW/BW, and (D) HW/TL. (E) Images of heart sections from MEM Cre, GDF1 Floxed and GDF1 Cre mice at 4 weeks after a sham or AB operation (n = 5–7 mice per experimental group). Sections were stained with hematoxylin and eosin (H&E) or FITC-conjugated wheat germ agglutinin (WGA). (F) Statistical results for CSA (n = 120+ cells per experimental group). (G) Upper panel: Representative echocardiographic images at 4 weeks after AB in MEM Cre, GDF1 Floxed and GDF1 Cre mice. Lower panel: Statistical results for the echocardiographic parameters in MEM Cre, GDF1 Floxed and GDF1 Cre mice (n = 6–11 mice per experimental group). (H) Hemodynamic parameters for MEM Cre, GDF1 Floxed and GDF1 Cre mice (n = 6–8 mice per experimental group). (I) The relative mRNA levels of ANP, BNP, and β-MHC in samples from MEM Cre, GDF1 Floxed and GDF1 Cre mice (n = 4 mice per experimental group). (J) Images of picrosirius red (PSR)-stained heart sections from MEM Cre, GDF1 Floxed and GDF1 Cre mice at 4 weeks after a sham or AB surgery (n = 5–7 mice per experimental group). (K) Statistical results for left ventricular collagen volume (%) (n = 25+ fields per experimental group). (L) The relative mRNA levels of CTGF, collagen I, and collagen III in left ventricular samples from MEM Cre, GDF1 Floxed and GDF1 Cre mice (n = 4 mice per experimental group). $^{*}p < 0.05$ vs. MEM Cre/sham or GDF1 Floxed/sham; $^{\#}p < 0.01$ vs. GDF1 Cre/sham; $^{\dagger}p < 0.05$ vs. MEM Cre/AB or GDF1 Floxed/AB.

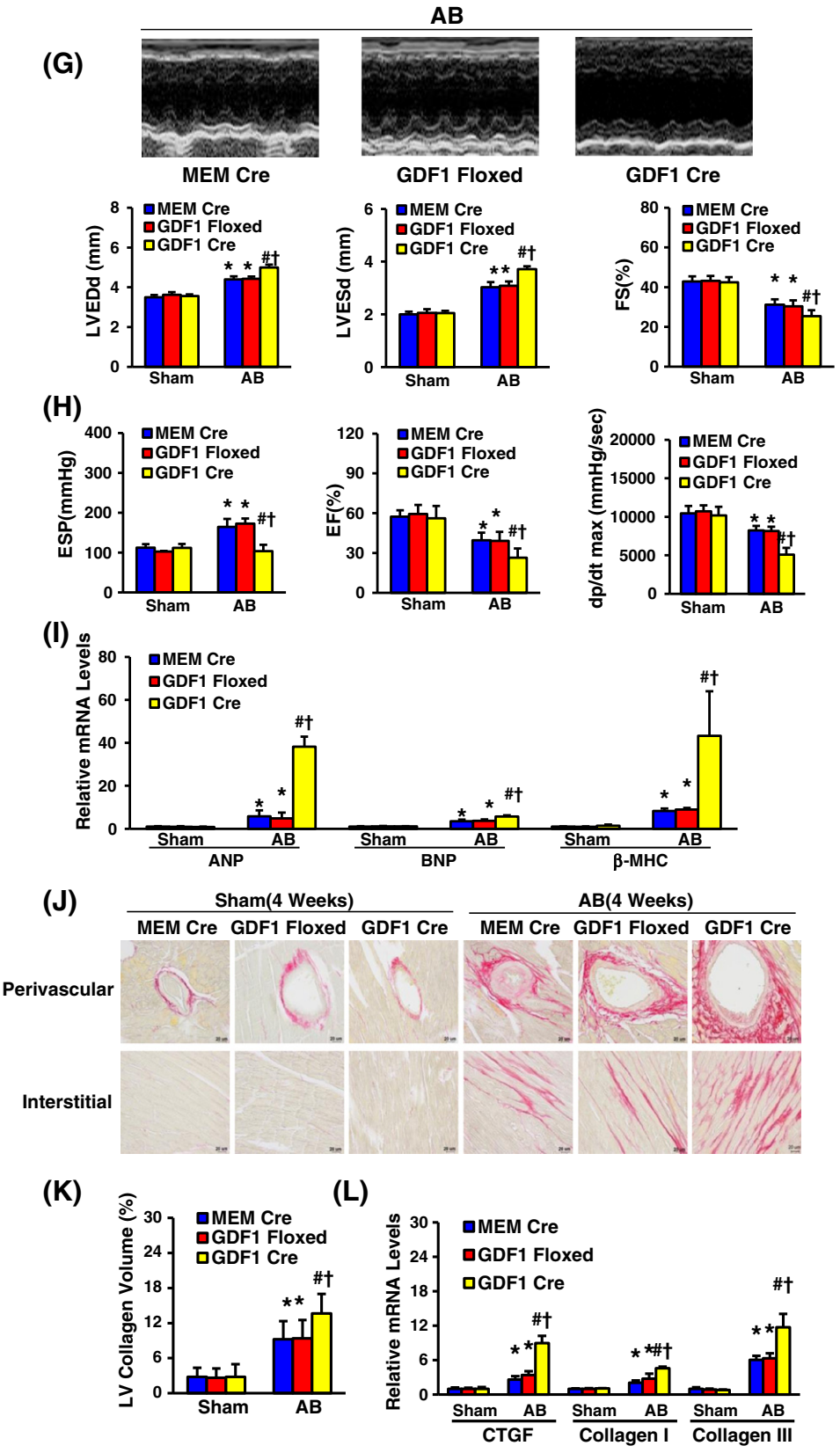

Fig. 3 (*continued*).

Cre mice were treated with tamoxifen (80 mg/kg/day, i.p.) for 5 consecutive days prior to the experiments. Four weeks after AB, GDF1-Cre mice exhibited a remarkable deterioration of cardiac hypertrophy compared with the control groups (GDF1-floxed mice and MEM-Cre mice), as evidenced by increased ratios of heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) (Fig. 3B–D). Histological examination of the hearts revealed an increased cardiomyocyte CSA in GDF1-Cre mice 4 weeks after AB (Fig. 3E/F).

Echocardiography and hemodynamic measurements showed that GDF1 deficiency significantly aggravated AB-induced cardiac dilation and dysfunction compared to control mice (Fig. 3 G/H). Additionally, 4 weeks after AB, the mRNA levels of hypertrophic markers (ANP, BNP, and β-MHC) were much higher in GDF1-Cre hearts compared to the control hearts (Fig. 3I).

To further determine the effects of GDF1 deficiency on maladaptive cardiac remodeling, we measured cardiac fibrosis, an important feature of developing pathological cardiac hypertrophy [20]. Paraffin-embedded slides were stained with PSR to assess the extent of cardiac fibrosis. We observed that both perivascular and interstitial fibrosis were much more prominent in AB-treated GDF1-Cre hearts compared to control hearts (Fig. 3J). Consistent with these results, the content of LV collagen and the mRNA levels of fibrotic markers (CTGF, collagen I, and collagen III) were greater in GDF1-null hearts compared with controls at 4 weeks after AB (Fig. 3 K/L). Thus, GDF1 deficiency aggravates pathological cardiac hypertrophy in response to pressure overload.

3.4. GDF1 overexpression attenuates pressure overload-induced cardiac remodeling

We then sought to address whether elevated GDF1 levels in the heart would attenuate pressure overload-triggered cardiac remodeling. To test this hypothesis, transgenic mice with the cardiac-specific overexpression of mouse GDF1 (TG mice) were generated using the α-myosin heavy chain promoter (α-MHC) (Fig. 4A). Four germ lines of GDF1 transgenic mice were established and verified by Western blotting analysis (Fig. 4B/C). We then selected transgenic line #4 (Tg4), which expressed the highest GDF1 levels, for the following experiments.

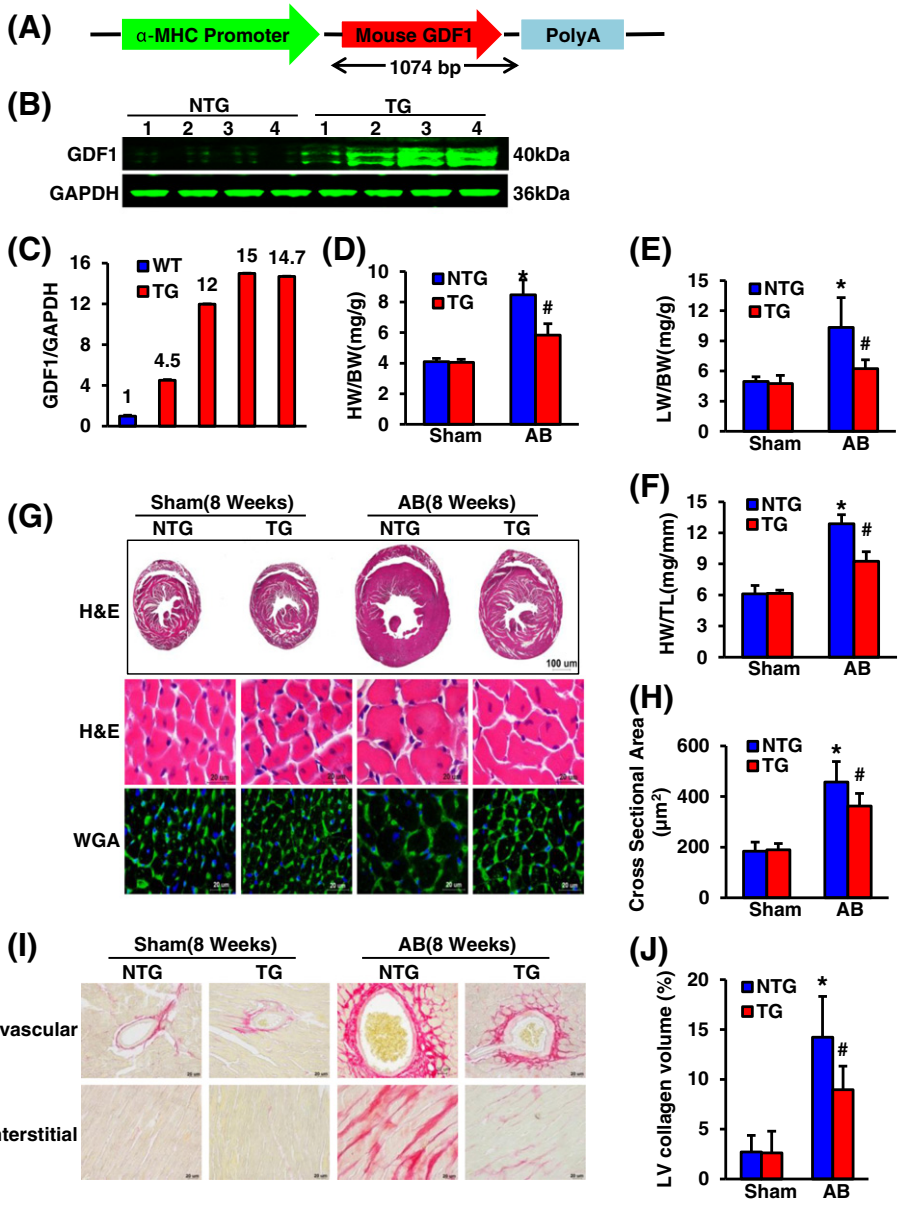

Fig. 4. GDF1 overexpression attenuates pressure overload-induced cardiac remodeling. (A) A schematic diagram of the construction of transgenic mice with full-length mouse GDF1 cDNA under the control of the α-myosin heavy chain promoter. (B) The GDF1 protein levels in samples from mice of four independent transgenic lines and their wild-type littermates. (C) The relative GDF1 protein levels in the heart tissue from four TG lines and their control WT mice. (D–F) Statistical results for the following ratios in the indicated groups(n = 13–15 mice per experimental group): (D) HW/BW, (E) LW/BW, and (F) HW/TL. (G) Images of heart sections from NTG and TG mice at 8 weeks after a sham or AB surgery(n = 5–6 mice per experimental group). Sections were stained with H&E or WGA. (H) Statistical results for CSA (n = 120+ cells per experimental group). (I) Images of PSR-stained heart sections from NTG and TG mice at 8 weeks after a sham or AB surgery(n = 5–6 mice per experimental group). (J) Statistical results for left ventricular collagen volume (%) (n = 25+ fields per experimental group). *$p < 0.05$ vs. NTG/sham; #$p < 0.05$ vs. NTG/AB.

Under basal conditions, GDF1 TG mice showed no apparent cardiac morphological or pathological abnormalities (Supplemental Table S1). GDF1-TG mice and their WT littermates (NTG mice) were subjected to either AB surgery or a sham operation. As expected, after 8 weeks, the AB-induced myocardial hypertrophic response was dramatically attenuated in GDF1-TG mice, as evidenced by lower ratios of HW/BW, HW/TL, and LW/BW in TG mice compared to NTG mice (Fig. 4D–F). Consistent with these results, cardiomyocytes in AB-treated GDF1-TG hearts were smaller than those of NTG mice (Fig. 4G–H). GDF1 overexpression also significantly alleviated AB-triggered cardiac dilation and dysfunction compared with controls, as determined by echocardiographic and hemodynamic analysis (Supplemental Fig. S2A–C). Furthermore, the AB-induced expression of hypertrophic markers (ANP, BNP, and β-MHC) was greatly reduced in GDF1-TG mice compared with NTG mice (Supplemental Fig. S2D). The effect of GDF1 overexpression on AB-induced cardiac fibrosis was determined by PSR staining of tissue sections and PCR analysis of fibrotic markers (collagen I, collagen III, and CTGF) in the left ventricle. In contrast to GDF1 deficiency, GDF1 overexpression remarkably attenuated AB-induced cardiac fibrosis compared with NTG controls (Fig. 4I/J and Supplemental Fig. S2E). Collectively, these gain-of-function data indicate that constitutive GDF1 expression in the heart protects against pressure overload-induced cardiac remodeling.

3.5. GDF1 modulates the MEK–ERK1/2 signaling pathway

The results above suggest that GDF1 may play a protective role against cardiac hypertrophy. However, the underlying mechanism by which GDF1 exerts its anti-hypertrophic response remains unknown. Therefore, we first examined whether the MAPK signaling cascade, a pathway associated with cardiac hypertrophy, might be altered in GDF1-floxed (GDF1-F, control) and GDF1-Cre (GDF1-C, KO) hearts in

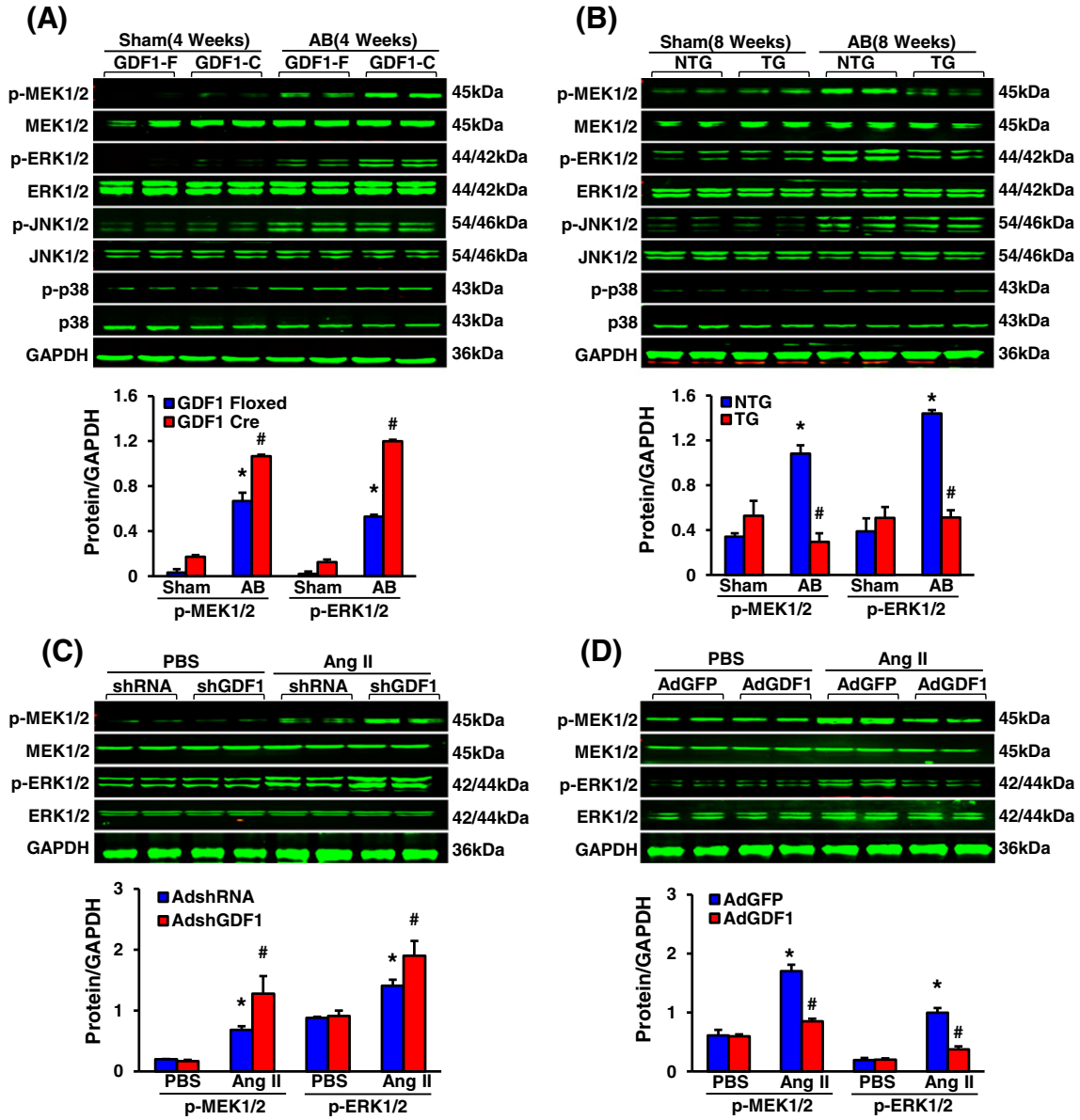

Fig. 5. GDF1 inhibits the MEK–ERK1/2 signaling pathway. (A–B) The levels of phosphorylated and total MEK1/2, ERK1/2, JNK1/2, and P38 proteins in samples from (A) GDF-F and GDF1-Cre mice and (B) NTG and TG mice at the indicated times after a sham or AB operation (n = 4,*$p < 0.05$ vs. GDF1-F or NTG/sham; #$p < 0.05$ vs. GDF1-F or NTG/AB). Upper: Representative blots; lower: quantitative results. (C–D) Levels of phosphorylated and total MEK1/2 and ERK1/2 proteins in samples of NRCMs infected with (C) AdshGDF1 or (D) AdGDF1 and then treated with Ang II (n = 4,*$p < 0.05$ vs. AdshRNA or AdGFP/PBS; #$p < 0.05$ vs. AdshRNA or AdGFP/Ang II). Upper: Representative blots; lower: quantitative results.

response to pressure overload. The immuno-blotting results revealed that although the phosphorylated levels of MEK1/2, ERK1/2, JNK1/2, and p38 were significantly increased in AB-treated hearts, the activation of MEK–ERK1/2 signaling was more pronounced in GDF1-Cs (KO) than in GDF1-Fs (WT) (Fig. 5A). Furthermore, both p38 and JNK1/2 were similarly activated in the two groups (Fig. 5A). PI3K–AKT is another important signaling pathway involved in cardiac remodeling. To further determine whether GDF1 blocks the AB-induced PI3K–AKT signaling response, we examined the activation of PI3K–AKT and its downstream targets, including GSK3β, mTOR, forkhead box O3A (FOXO3A), and forkhead box O1 (FOXO1). No differences were observed in the activation of AKT, GSK3β, FOXO1, or mTOR between GDF1-F and GDF1-C hearts after AB, except for FOXO3A (Supplemental Fig. S3). Based on the results obtained from GDF1-KO hearts (Fig. 5A), we next

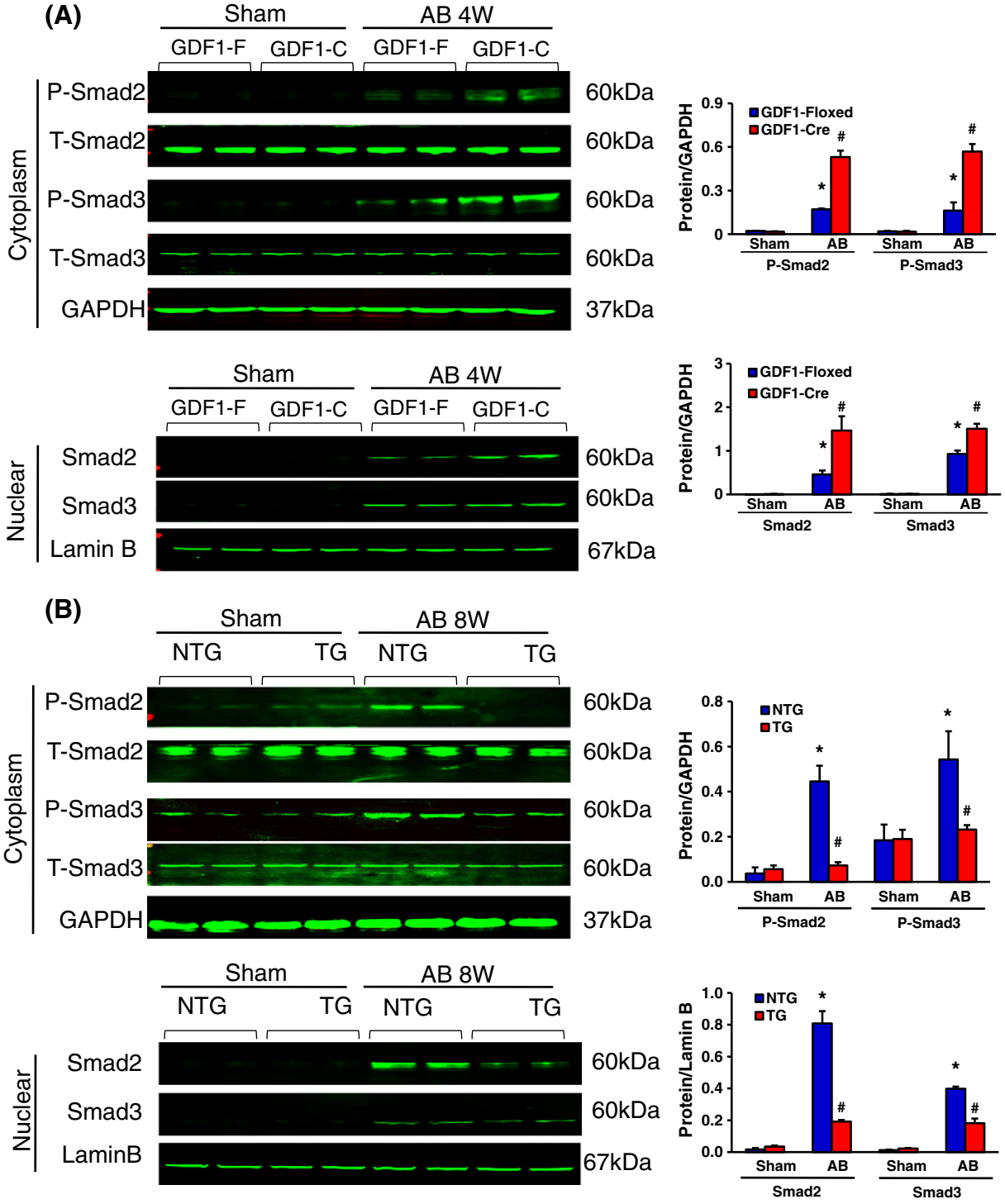

Fig. 6. GDF1 regulates Smad signaling. (A) Left: Representative western blots; right: quantitative results of cytoplasmic phosphorylated and total Smad2 and Smad3 protein levels and nuclear Smad2 and Smad3 protein levels from GDF1-F and GDF1-C mice after a sham or AB operation ($^{*}p < 0.05$ vs. GDF1-F/sham; $^{\#}p < 0.05$ vs. GDF1-F/AB). (B) Left: Representative western blots; right: quantitative results of cytoplasmic phosphorylated and total Smad2 and Smad3 protein levels and nuclear Smad2 and Smad3 protein levels from NTG and TG mice after a sham or AB operation ($^{*}p < 0.05$ vs. NTG/sham; $^{\#}p < 0.05$ vs. NTG/AB). (C) Left: Representative western blots; right: quantitative results of cytoplasmic phosphorylated and total Smad2 and Smad3 protein levels and nuclear Smad2 and Smad3 protein levels in cultured neonatal rat cardiomyocytes treated with 1 μM Ang II in the presence or absence of recombinant GDF1 ($^{*}p < 0.05$ vs. vehicle/PBS; $^{\#}p < 0.05$ vs. vehicle/Ang II).

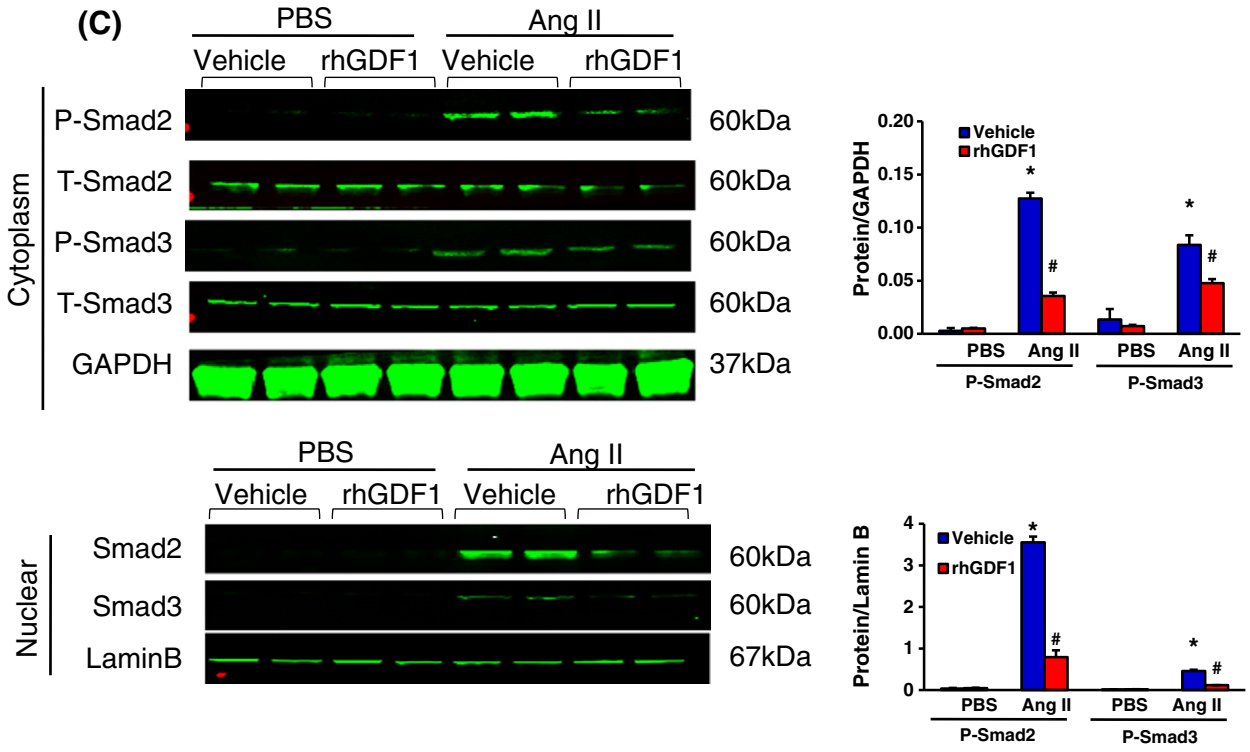

Fig. 6 (*continued*).

investigated whether GDF1 overexpression negatively affects the levels of phosphorylated MEK1/2 and phosphorylated ERK1/2 in response to pressure overload. As shown in Fig. 5B, the AB-triggered activation of MEK1/2 and ERK1/2 was almost completely blocked in GDF1-TG hearts. To exclude potential *in vivo* compensatory mechanisms, we further utilized cultured NRCMs to examine the effect of GDF1 on MEK–ERK1/2 signaling. We infected myocytes with either Ad-shGDF1 to knockdown GDF1 or Ad-GDF1 to overexpress GDF1 and then subjected these infected cells to 1 μM Ang II for 48 h. The Western blotting results showed that the Ang II-induced activation of MEK1/2 and ERK1/2 was promoted in GDF1-knockdown cells, whereas this activation was attenuated in GDF1-overexpressing cells (Fig. 5C/D). Our results demonstrate that GDF1-elicited anti-hypertrophic effect is largely associated with the inhibition of MEK–ERK1/2 signaling in hearts upon pressure overload.

3.6. GDF1 regulates Smad signaling pathway

GDF1 is a transforming growth factor-β (TGF-β) family member. TGF-β/Smad is an important signaling pathway involved in the development of cardiac remodeling. To further elucidate the cellular mechanisms underlying the cardio-protective effects of GDF1, we assessed the regulatory role of GDF1 on Smad cascade activation. Our results showed that GDF1-Cre mice induced higher levels of Smad 2/3 phosphorylation and nuclear translocation compared with that of MEM-Cre mice (Fig. 6A). Importantly, AB-induced the increased levels of Smad 2/3 phosphorylation and nuclear translocation were significantly attenuated in TG mice, compared with NTG controls (Fig. 6B). To confirm our *in vivo* data, we then treated neonatal rat cardiomyocytes with recombinant human GDF1, followed by the addition of Ang II for 48 h. Western blot analyses revealed that Ang II stimulation induced significant phosphorylation and nuclear translocation of Smad 2/3, which were almost completely suppressed by the administration of recombinant human GDF1 (Fig. 6C). These findings suggest that GDF1 attenuates cardiac remodeling partly by inhibiting Smad signaling.

3.7. Inhibition of MEK–ERK1/2 signaling rescued abnormalities in GDF1-deficient mice

The aforementioned experimental results suggested that ERK activation or inactivation would affect the role of GDF1 in the heart. To address this issue, we co-infected neonatal rat cardiomyocytes with Ad-GDF1 plus Ad-ERK, respectively, followed by the addition of Ang II for 48h. Our results of cell size analysis show that suppression of Ang II-induced cell hypertrophy by overexpression of GDF1 (Supplemental Fig. S4) was released by increased ERK expression (AdERK-cells). To further determine whether the abnormalities displayed in AB-treated GDF1-deficient mice could be reversed by pre-inhibition of MEK–ERK1/2 signaling, we treated MEM-Cre mice and GDF1-Cre mice with a specific MEK inhibitor, U0126, or PBS followed by AB. Western blotting analysis showed that the phosphorylated MEK and phosphorylated ERK1/2 levels were dramatically suppressed in U0126-treated samples compared with PBS-treated controls (Fig. 7A). Importantly, both the gravimetric data and histological examination demonstrated that U0126 treatment significantly reversed the deteriorative effects of GDF1 deficiency on the hypertrophic and fibrotic response 4 weeks after AB compared with PBS-treated controls (Fig. 7B–H). U0126 treatment also markedly limited the increases in the mRNA levels of hypertrophic (ANP, BNP, and β-MHC) and fibrotic markers (CTGF, collagen I, and collagen III) compared with expression in PBS-treated groups (Fig. S5A/B). Altogether, these data suggest that pre-inhibition of MEK–ERK1/2 signaling could resist the GDF1-null-induced pro-hypertrophic effects in the hearts upon pressure overload.

4. Discussion

The aim of our study was to examine the role of GDF1 in pressure overload-induced cardiac remodeling. Using both gain-of-function and loss-of-function approaches, we observed that GDF1 functions as a protective factor in the process of pathological cardiac hypertrophy. To our knowledge, this report is the first showing that GDF1 overexpression in

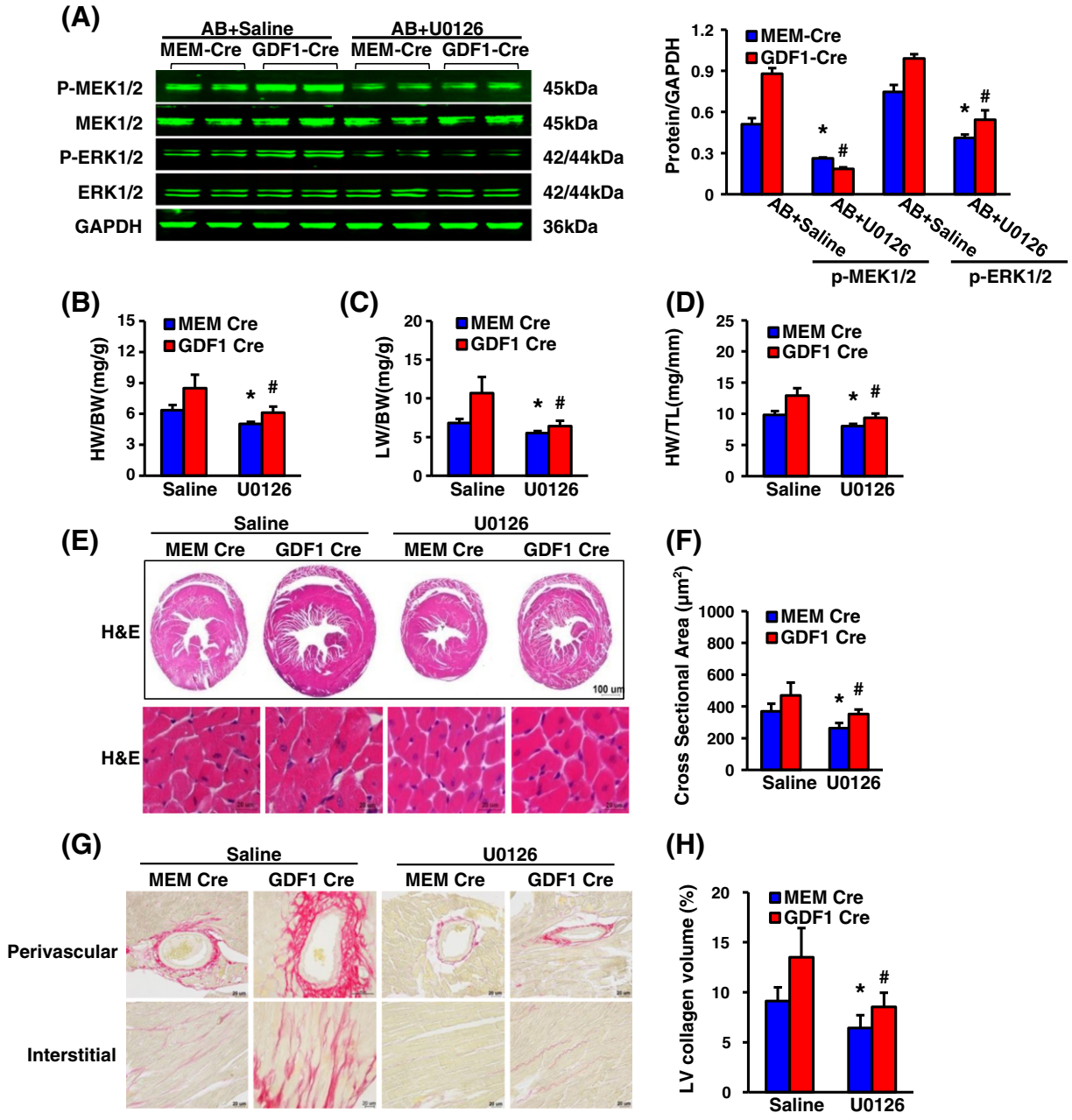

Fig. 7. Inhibition of MEK–ERK1/2 signaling rescued abnormalities in GDF1-deficient mice. (A) Left: Representative Western blots; right: quantitative results of the levels of phosphorylated and total MEK1/2 and ERK1/2 from MEM-Cre and GDF1-Cre mice treated with U0126 or PBS 4 weeks after AB. (B–D) Statistical results for the following ratios in the indicated groups (n = 10–15 mice per experimental group): (B) HW/BW, (C) LW/BW, and (D) HW/TL. (E) Images of heart sections of mice from the indicated groups. These sections were stained with H&E (n = 4–8 mice per experimental group). (F) Statistical results for CSA (n = 120+ cells per experimental group). (G) Images of heart sections of mice from the indicated groups. These sections were stained with PSR (n = 4–8 mice per experimental group). (H) Statistical results for left ventricular collagen volume (%) (n = 25+ fields per experimental group). $^{*}p < 0.05$ vs. MEM-Cre/saline; $^{\#}p < 0.05$ vs. GDF1-Cre/saline.

the heart profoundly blunts cardiac remodeling and dysfunction following chronic pressure overload. Conversely, disruption of GDF1 resulted in an exaggerated pathological cardiac remodeling response. These data demonstrate a previously unrecognized and important role for GDF1 in the regulation of cardiac remodeling and heart failure.

We found that GDF1 transgenic mice were resistant to pressure overload-induced cardiac remodeling, whereas GDF1-knockout mice displayed the opposite phenotype. These results indicate that GDF1 plays a critical role in protecting the heart against maladaptive responses to stress, and this finding is consistent with previous studies on other GDF proteins. For example, GDF5 regulates cardiac repair after myocardial infarction, and GDF15 antagonizes the pressure overload-induced hypertrophic response [32,33]. Additionally, GDF1 expression is up-regulated in human DCM hearts and murine hypertrophic hearts. However, the mechanism of GDF1 expression changes during cardiac remodeling is not known. A recent study has suggested that Smad2/3-dependent signaling pathway is involved in regulating GDF1 in myelomonocytic cells [7]. Therefore, determining whether this pathway also regulates GDF1 expression during the development of cardiac remodeling is important.

Mechanistically, the cardio-protective effect of GDF1 on pathological cardiac remodeling could be largely exerted by inhibiting MEK–ERK1/2 signaling. It is generally accepted that biomechanical stress induced by pressure overload triggers a variety of signal transduction molecules and pathways, which regulate the hypertrophic growth of cardiac myocytes [9]. Numerous studies have demonstrated that both the MAPK and AKT signaling pathways are often activated in response to extracellular stresses, and both have been shown to contribute to cardiac remodeling and heart failure [4,6,9]. The MAPK cascade comprises a sequence of successive kinases, including p38, JNKs, and ERKs [9]. The downstream targets of AKT signaling include GSK3β, mTOR, and FOXO transcription factors [9], which reprogram cardiac fetal gene expression and induce cardiac hypertrophy. In the present study, we found that activation of both MEK1/2 and ERK1/2 was blocked by cardiac GDF1 overexpression but enhanced by the loss of GDF1 in response to chronic pressure overload. However, GDF1 did not affect the phosphorylation of p38, JNK1/2, AKT, GSK3β, FOXO1, and mTOR, except for FOXO3a. Previous studies reported that ERK1/2 can modulate FOXO3a activation and nuclear translocation [34,35]. We speculate the alteration in FOXO3a may be due to the effect of GDF1 on MEK–ERK1/2 signaling pathway, which needs to be further investigated. Importantly, the aggravated effects of GDF1 deficiency on cardiac remodeling were mitigated by the MEK–ERK1/2 inhibitor, indicating that the MEK–ERK1/2 signaling pathway is critically involved in the anti-hypertrophic effects of GDF1. Thus, GDF1 may exert cardio-protective effects largely by inhibiting the MEK–ERK1/2 axis.

The TGF-β superfamily is a large family of structurally related cell regulatory proteins that function by binding to distinct complexes of type I and type II receptor serine-threonine kinases; each of these complexes bind to a different class of TGF-β ligands [24]. An unexpected finding in this study was that GDF1 exerts its cardio-protective effect through the inhibition, not the activation, of TGF-β canonical signaling. We observed that increased levels of Smad 2/3 phosphorylation and nuclear translocation were attenuated in GDF1 TG mice and promoted in GDF1 deficient mice in response to AB. This finding is in contrast to another TGF-β superfamily member, GDF15, which protects the heart from ischemia/reperfusion injury by activating PI3K–AKT-dependent signaling pathways and protects the heart against pressure overload-induced cardiac hypertrophy by activating Smad2/3 proteins [16,32]. In this context, defining the exact molecular mechanism through which a given TGF-β superfamily member exerts its effects is difficult due to the heterogeneity associated with each of the different receptor subtypes and their differential specificities for ligands. Interestingly, a previous study found that another GDF member, GDF3, interacts physically with BMPs and regulates cell fate in stem cells and early embryos by inhibiting its own subfamily, the BMP–GDF subfamily of TGF-β ligands [19]. Additionally, Derer et al. [7] reported that TGF-β down-regulates GDF1 expression through the Smad2/3 pathway in myelomonocytic cells. Therefore, the interactions between TGF-β superfamily members are complex. GDF1 may exert inhibitory effects on the MEK–ERK1/2 and Smad signaling pathways via interaction with its own subfamily members, a hypothesis that should be tested in future studies. Indeed, GDF1 has been shown to cooperate with GDF3 during early embryonic development [2].

The hypertrophic growth of cardiac myocytes in response to hypertrophic stimuli is regulated by endocrine, paracrine, and autocrine growth factors that activate membrane-bound receptor-mediated signal transduction pathways that, in turn, activate various transcriptional regulators [26]. Several antihypertrophic growth factors have been characterized in recent years. For example, A- and B-type natriuretic factors/peptides (ANP and BNP) are secreted from the heart following acute and chronic cardiac injury, and they signal a cardio-protective response through their receptors [10,11,17,25,27]. The observations that GDF1 expression is increased in human failing hearts and is induced in the heart in response to hypertrophic stimuli and that it functions as an antihypertrophic factor indicate that GDF1 might exert cardio-protective effects in an analogous manner and that it may serve as a novel remodeling biomarker in heart failure. However, it should be noted that human DCM may not be the best representative for AB. The alteration of GDF1 in human hearts in response to hypertrophic stimuli needs further study.

In conclusion, the present study defines the role of GDF1 in cardiac remodeling in response to pressure overload. The molecular mechanism for the protective role of GDF1 in the development of cardiac remodeling is largely dependent on the inhibition of the MEK–ERK1/2 and Smad signaling pathways. Our observations may help to develop novel therapeutic strategies for the treatment of cardiac remodeling and heart failure.

Sources of funding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.11.018.

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