GDF15/MIC-1 Functions As a Protective and Antihypertrophic Factor Released From the Myocardium in Association With SMAD Protein Activation

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Abstract—Here we identified growth-differentiation factor 15 (GDF15) (also known as MIC-1), a secreted member of the transforming growth factor (TGF)-β superfamily, as a novel antihypertrophic regulatory factor in the heart. GDF15 is not expressed in the normal adult heart but is induced in response to conditions that promote hypertrophy and dilated cardiomyopathy. To elucidate the function of GDF15 in the heart, we generated transgenic mice with cardiac-specific overexpression. GDF15 transgenic mice were normal but were partially resistant to pressure overload-induced hypertrophy. Expression of GDF15 in neonatal cardiomyocyte cultures by adenoviral-mediated gene transfer antagonized agonist-induced hypertrophy in vitro. Transient expression of GDF15 outside the heart by intravenous adenoviral delivery, or by direct injection of recombinant GDF15 protein, attenuated ventricular dilation and heart failure in muscle lim protein gene–targeted mice through an endocrine effect. Conversely, examination of *Gdf15* gene-targeted mice showed enhanced cardiac hypertrophic growth following pressure overload stimulation. *Gdf15* gene-targeted mice also demonstrated a pronounced loss in ventricular performance following only 2 weeks of pressure overload stimulation, whereas wild-type controls maintained function. Mechanistically, GDF15 stimulation promoted activation of SMAD2/3 in cultured neonatal cardiomyocytes. Overexpression of SMAD2 attenuated cardiomyocyte hypertrophy similar to GDF15 treatment, whereas overexpression of the inhibitory SMAD proteins, SMAD6/7, reversed the antihypertrophic effects of GDF15. These results identify GDF15 as a novel autocrine/endocrine factor that antagonizes the hypertrophic response and loss of ventricular performance, possibly through a mechanism involving SMAD proteins. **(*Circ Res*. 2006;98:342-350.)**

Key Words: cardiac ■ signaling ■ hypertrophy ■ growth factors ■ mouse genetics

Cardiac hypertrophy is typically characterized by an enlargement of the heart associated with an increase in cardiomyocyte cell volume that occurs during postnatal development, in response to physiologic stimuli such as exercise and in response to diverse pathophysiological stimuli such as hypertension, ischemic heart disease, valvular insufficiency, infectious agents, or mutations in sarcomeric genes.[1] Pathologic hypertrophic growth of the myocardium is a leading predictor for the development of arrhythmias and sudden death, as well as dilated cardiomyopathy and heart failure.[2,3] In general, the hypertrophic growth of the myocardium is regulated by endocrine, paracrine, and autocrine growth factors that activate membrane-bound receptors resulting in signal transduction that culminates in altered gene transcription and protein accumulation.[4] Both pro- and antihypertrophic growth factors have been characterized. For example, angiotensin II (Ang II) serves as an endocrine and autocrine growth factor underlying pathological cardiac hypertrophy, whereas insulin-like growth factor (IGF)-1 signaling is thought to function, in part, by mediating developmental and physiologic cardiac hypertrophy.[5] In contrast, the A- and B-type natriuretic factors/peptides (ANF and BNP) are thought to function as antihypertrophic neuroendocrine factors.[6]

A number of peptide growth factors/cytokines have been shown to affect cardiomyocyte biology, including Ang II, IGF-1, endothelin-1, ANF/BNP, cardiotrophin-1, tumor necrosis factor-α, interleukin-6, interleukin-1, epidermal growth factor, fibroblast growth factor, transforming growth factor (TGF)-β, and related members of the TGF-β superfamily.[7,8] A more recently characterized member of the TGF-β superfamily is growth-differentiation factor 15 (GDF15), first named macrophage inhibitory cytokine-1 (MIC-1) or PLAB

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(also later named PTGF, PDF, PL-74, and NAG-1). GDF15 is generated as a 40-kDa propeptide from which the N terminus is cleaved and a 30-kDa disulfide-linked dimeric protein is secreted as the active form.[9] GDF15 is only appreciably expressed in liver and placenta at baseline, but many tissues/cell-types show dramatic induction of expression following injury, hypoxia, or cytokine/growth factor stimulation.[9–14] Here we identified GDF15 as a novel cardiac-acting, protective autocrine/paracrine factor in vitro and in vivo.

Materials and Methods

Surgical Procedure, Animal Models, and mRNA Profiling

Gdf15 gene-targeted mice in the strain background C57BL/6SV129j were described previously and were generously provided by Dr Se-Jin Lee from The John Hopkins University.[10] GDF15 transgenic mice (FVBN strain) were generated by fusing a full-length GDF15 mouse cDNA (for the entire propeptide region) to the murine α-myosin heavy chain (MHC) promoter.[15] Transverse aortic constriction (TAC) was performed as described previously.[16] TAC mice were also subjected to measurement of pressure gradients across the aortic constriction. Briefly, under inactin anesthesia, the right carotid artery was cannulated with a Millar 1.4F catheter and the left carotid with a fluid filled PE catheter connected to a pressure transducer. The muscle lim protein (*mlp*) null mouse model of heart failure was previously described.[17] Transgenic mice expressing the activated calcineurin cDNA in the heart were also previously described.[18] All transgenic mice were maintained in the hemizygous state and were subjected to TAC analysis at 8 to 10 weeks of age. Experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee.

Echocardiography and Working Heart Analyses

Mice were anesthetized with 2% isoflurane and hearts were visualized using a Hewlett Packard Sonos 5500 instrument and a 15 MHZ transducer. Cardiac ventricular dimensions were measured on M-mode 3 times in a single session for the number of animals indicated. The isolated ejecting mouse heart preparation has been described in detail previously.[19]

Molecular Analyses

Western blotting and RT-PCR was performed as previously described.[20,21] Rabbit polyclonal antiserum (affinity purified) was generated against the mouse GDF15 epitope: NH_2-HRTDSGVSLQTYDD. The affinity-purified antiserum was used at a dilution of 1:500 for Western blotting. GDF15-specific RT-PCR was performed with the following primers: 5′-ATACTCAGTCCAGAGGTGAGAT-3′ and 5′-ATGCAGGCGTGCTTTGATCTG-3′, whereas L7 was used as a control.[21] The mouse cDNA encoding the entire GDF15 propeptide was obtained from the IMAGE consortium (GenBank accession no. AI528151). Generation of recombinant GDF15 protein was described previously.[22] Mice were injected SC for 14 days with 10 μg of GDF-15, twice a day, or BSA vehicle control.

Histology and Cell Surface Area Measurements

Serial 5-μm histological heart sections were cut and stained with hematoxylin/eosin, Masson's trichrome, or wheat germ agglutinin–tetramethylrhodamine B isothiocyanate conjugate (50 μg/mL) to identify sarcolemmal membranes for measuring myocyte cross-sectional area. All measurements of cellular areas from histological sections included 4 independent hearts for each cohort, from which at least 300 myocytes were counted for each heart from the same region of the left ventricle.

Cell Culture and Recombinant Adenovirus

All in vitro experiments were performed in neonatal ventricular cardiomyocytes isolated from 1- to 2-day-old rats under previously described conditions.[20,21] A GDF15 recombinant adenovirus was generated using the full-length mouse GDF15 cDNA subcloned into the shuttle vector pACCMVpLpA as previously described.[20] AdGDF15 or Adβgal (control) were used to infect cultured cardiomyocytes at an approximate multiplicity of infection of 50 for a period of 2 hours, followed by assessment of myocyte hypertrophy 24 or 48 hours afterward.[20,23] Assessment of cultured rat neonatal cardiomyocyte cell surface area (hypertrophy) and [^{3}H]-leucine incorporation was performed as previously described.[23] Tail vein infusion of Adβgal or AdGDF15 in *mlp* null mice used a dosage of 5×10^8 plaque-forming units (11 days later mice were analyzed). Recombinant human GDF15 and TGF-β were purchased from R&D Systems and used at a concentration of 3 pmol/L and 2 ng/mL, respectively. Transfections were performed with Fugene (Roche) in neonatal rat cardiomyocytes. Dr Jeff Wrana provided the luciferase reporters BRE and I-BRE, which were derived from the *Smad7* gene regulatory region and are regulated by SMAD1 (and 5/8).[24] The GDF8 expressing adenovirus was provided by Dr Anthony Rosenzweig. Adenoviruses encoding SMAD2, -6, and -7 were generously provided by Dr Aristidis Moustakas (Uppsala, Sweden).

Statistical Analysis

Statistical analyses between experimental groups were performed using Student's *t* test, 1-way ANOVA, or 2-way ANOVA, with post hoc Tukey's test or Dunett's test (InStat, Prism and SPSS software). Data were reported as mean±SEM unless otherwise noted. Probability values of <0.05 were considered significant.

Results

Identification of GDF15 As a Cardiac Inducible Factor

To further elucidate the molecular mechanisms that underlie cardiomyopathy we performed an Affymetrix-based GeneChip screen of altered mRNA transcripts in a mouse model of dilated cardiomyopathy caused by myocyte enhance factor 2C (MEF2C) overexpression in the heart (manuscript submitted). This analysis identified the TGF-β superfamily member GDF15 as a transcript that was specifically induced within the cardiomyopathic heart of adult mice. This result was confirmed by RT-PCR from 4 wild-type and 4 MEF2C transgenic hearts, with no change in L7 mRNA levels (Figure 1A). Rabbit polyclonal antiserum showed no detectable GDF15 propeptide in wild-type hearts, whereas both MEF2C and calcineurin transgenic models of dilated/hypertrophic cardiomyopathy showed expression of the propeptide in the heart (Figure 1B). Most nonspecific bands demonstrated equal loading between the samples (Figure 1B). A recombinant adenovirus expressing full-length GDF15 was used as a migration control (Figure 1B). These results were extended to a model of pathophysiological hypertrophy caused by pressure overload in wild-type mice or *Gdf15* null mice. The data demonstrate induction of GDF15 mRNA within 1 day of pressure overload that was maintained at least through 14 days in wild-type mouse hearts but not *Gdf15* null hearts (Figure 1C). Similar results were observed in 2 additional independent experiments, consistent with pressure overload-induced GDF15 mRNA induction reported in previous studies.[25,26]

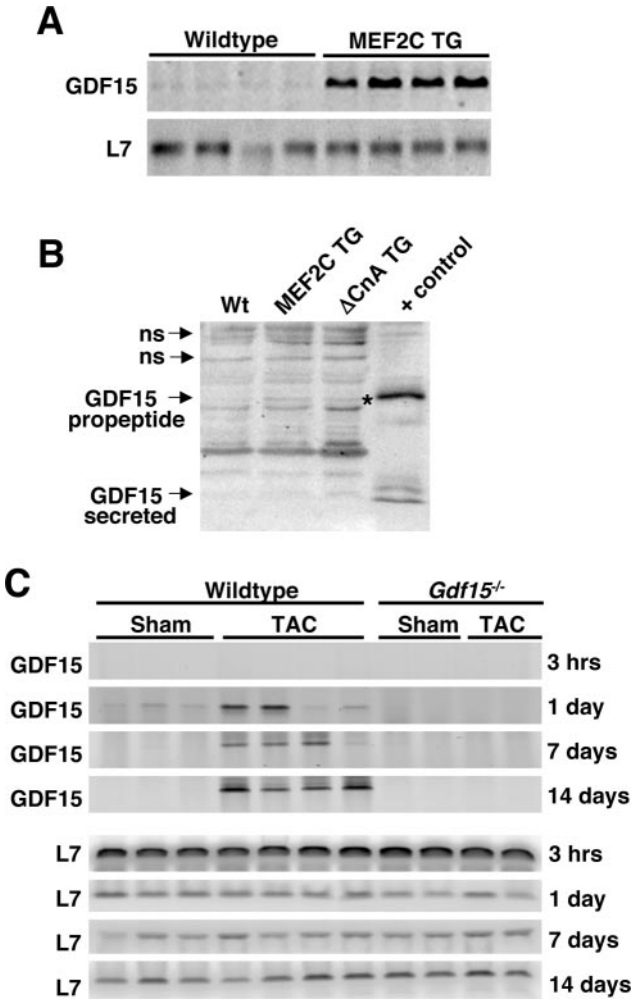

Figure 1. GDF15 is induced in the injured mouse heart. A, RT-PCR for GDF15 and L7 mRNA from 4 wild-type and 4 MEF2C cardiomyopathic transgenic hearts. B, Western blot with mouse-specific GDF15 affinity-purified antiserum from wild-type, MEF2C transgenic, and activated calcineurin transgenic hearts. ns indicates nonspecific bands; asterisk, the GDF15 propeptide. C, RT-PCR for GDF15 and L7 mRNA levels in the hearts of wild-type or *Gdf15*$^{-/-}$ mice subjected to sham procedure or TAC for the indicated times. Separate animals were used for each lane. Similar results were observed in 2 additional independent experiments.

Characterization of GDF15 Transgenic Mice

To examine the functional effects associated with GDF15 induction a cDNA encoding full-length GDF15 was subcloned into the α-MHC promoter vector[15] to permit generation of transgenic mice with cardiac-specific overexpression (Figure 2A). Five independent transgenic lines were initially generated and characterized by Western blotting. Three lines consisting of low, medium, and high expression were selected for in-depth analysis (Figure 2B) (lines 22.1 and 22.2 were not fully evaluated). A recombinant adenovirus expressing full-length GDF15 was used as a Western blotting migration control, and no changes in GAPDH protein levels were observed (Figure 2B). GDF15 protein was not detectable in wild-type mouse hearts, so that quantification of fold overexpression was made relative to the amount of induction observed in calcineurin transgenic hearts (Figure 2C). Given this reference point, the low expressing line showed approximately 3-fold more GDF15 propeptide expression. Dissociation and purification of cardiomyocytes from wild-type and calcineurin transgenic hearts showed that GDF15 protein is expressed in myocytes (Figure 2D).

GDF15 transgenic mice showed no pathological alterations in ventricular chamber dimensions, ventricular or septal wall thicknesses, fractional shortening, or ventricular-weight normalized to body-weight (Table I in the online data supplement available at http://circres.ahajournals.org). Extensive histological analysis also revealed no gross morphological alterations or fibrosis (data not shown). However, the high expressing GDF15 transgenic line did demonstrate slightly enlarged atria, which we believe is attributable to a developmental effect associated with high levels of atrial GDF15 expression during embryogenesis.

GDF15 Transgenic Mice Are Protected From Pressure Overload Hypertrophy

Medium expressing transgenic mice demonstrated a reduction in their ability to hypertrophy following TAC compared with age- and strain-matched littermates (Figure 3A). TAC was also repeated in high expressing GDF15 transgenic mice, which showed only a 17±5% increase in ventricle-weight normalized to body weight compared with a 38±8% increase in wild-type controls after TAC stimulation (data not shown). Males and females both appeared to have a similar hypertrophic profile (supplemental Table II). Cardiomyocyte cell size was also quantified from histological sections from wild-type and medium-expressing GDF15 transgenic mice at baseline and following TAC. Consistent with the gross ventricular weight measurements, GDF15 transgenic mice showed less cellular enlargement in the left ventricle after TAC compared with nontransgenic controls (Figure 3B). TAC-induced cardiac hypertrophy over 2 weeks did not alter ventricular performance as assessed by echocardiography in either wild-type or GDF15 transgenic mice (Figure 3C). Even though the GDF15 transgene attenuated the hypertrophic response, histological analysis of disease was not informative because control mice showed minimal remodeling and fibrosis given the relatively robust cardiac characteristics of the FVBN strain background (data not shown). Intriguingly, TAC induced similar increases in fetal marker gene expression between wild-type and GDF15 transgenic mice (Figure 3D) (see Discussion).

To better address the ability of GDF15 to alter heart failure and ventricular dilation, *mlp*$^{-/-}$ mice (N=9 to 10) were subjected to systemic delivery of either Adβgal (control) or AdGDF15 by intravenous injection of 5×10^8 plaque-forming units. AdGDF15 injected mice showed increased/induced GDF15 propeptide mostly in the kidney, but not the heart, suggesting that at this dosage of adenovirus GDF15 could have an endocrine effect on the heart (Figure 3E). Cardiac function was assessed in each animal before and after injection by echocardiography. Adβgal-injected *mlp*$^{-/-}$ mice maintained depressed fractional shortening and dilation of the left ventricle, whereas AdGDF15-injected *mlp*$^{-/-}$ mice showed an increase in fractional shortening from 20±3% to 32.5±2%, as well as a significant decrease in ventricular

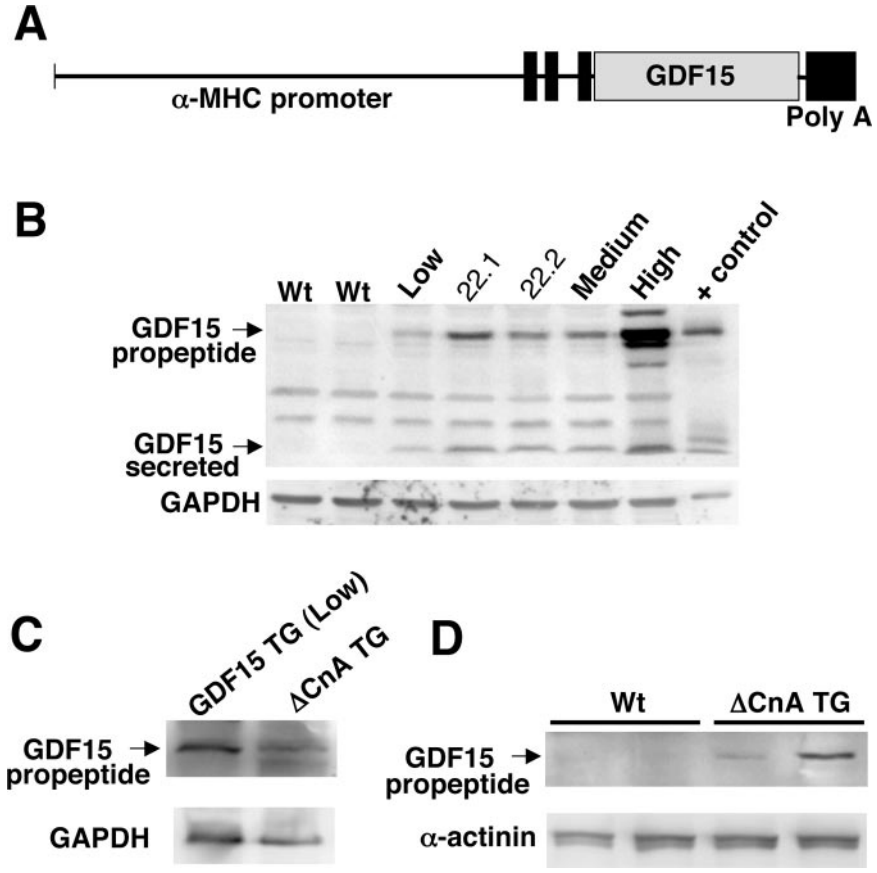

Figure 2. Generation of GDF15 transgenic mice. A, Schematic of the GDF15 transgene that was constructed with the 5.5-kb α-MHC mouse promoter. B, Western blot for GDF15 and GAPDH (control) protein from hearts of the indicated mice. C, Western blot for GDF15 protein from the hearts of a low expressing GDF15 or an activated calcineurin transgenic mouse. D, Western blot for GDF15 propeptide from purified adult cardiomyocytes from calcineurin transgenic or wild-type mice.

dilation, although a nonmyocyte effect cannot be excluded (Figure 3F through 3H). More astonishingly, twice daily injections of recombinant GDF15 protein (10 μg each) for 14 days also partially reversed heart failure in $mlp^{-/-}$ mice (N=6), whereas control BSA injections (N=6) had no significant effect (Figure 3I through 3K).

Analysis of Cardiac Hypertrophy and Function in *Gdf15* Gene-Targeted Mice

Because the gain-of-function phenotype suggested an antihypertrophic role for GDF15, it was of interest to corroborate this conclusion by also investigating *Gdf15* gene-targeted mice.[10] Western blotting of liver protein extracts demonstrated the complete lack of the propeptide in *Gdf15*-targeted mice (Figure 4A). *Gdf15* null mice had normal appearing hearts on histological examination, normal heart-weight to body-weight ratios, normal values of mean arterial blood pressure, normal baseline contractility, and normal heart rates compared with wild-type controls (supplemental Figure I). However, *Gdf15* null mice demonstrated significantly greater cardiac hypertrophy following 2 weeks of TAC stimulation compared with control mice (Figure 4B). No differences in pressure gradients across the aortic constriction were observed in either group (Figure 4C). Males and females both appeared to have a similar hypertrophic profile (supplemental Table III). Cardiomyocyte cell size was also quantified from histological sections, and although there was a trend toward a greater increase in the *Gdf15* null mice, it was not significant (Figure 4D). Echocardiographic assessment of ventricular performance showed reduced fractional shortening in *Gdf15* gene-targeted mice over 2 weeks of TAC, whereas wild-type controls were not compromised over this same, relatively short, time period (Figure 4E and Table 1). Whereas heart-weights and cellular areas were greater in *Gdf15* null mice subjected to TAC, echocardiographic assessment of septal and left ventricular wall thicknesses showed no increase, likely as a result of the onset of ventricular dilation and failure (see Discussion). Echocardiographic measurements normalized to body-weight showed identical results (supplemental Table IV).

To more definitively evaluate function in the experimental groups discussed above, an isolated working heart preparation was performed. After 2 weeks of TAC stimulation wild-type mice demonstrated a 25% reduction in +dP/dt, whereas *Gdf15* null mice demonstrated an 84% reduction in heart function (Figure 4F, Table 2). Assessment of −dP/dt and left ventricular pressure developed also revealed a similar profile of substantially greater decompensation in *Gdf15* null mice compared with wild-type mice following only 2 weeks of TAC stimulation (Table 2). TAC-induced upregulation of fetal markers genes was similar between wild-type and *Gdf15* null mice, although one might have expected to observe a greater increase in the *Gdf15* null mice (Figure 4G) (see Discussion).

GDF15 Attenuates Hypertrophy in Cultured Cardiomyocytes

The results observed in response to TAC stimulation were extended using an in vitro model of cellular growth in

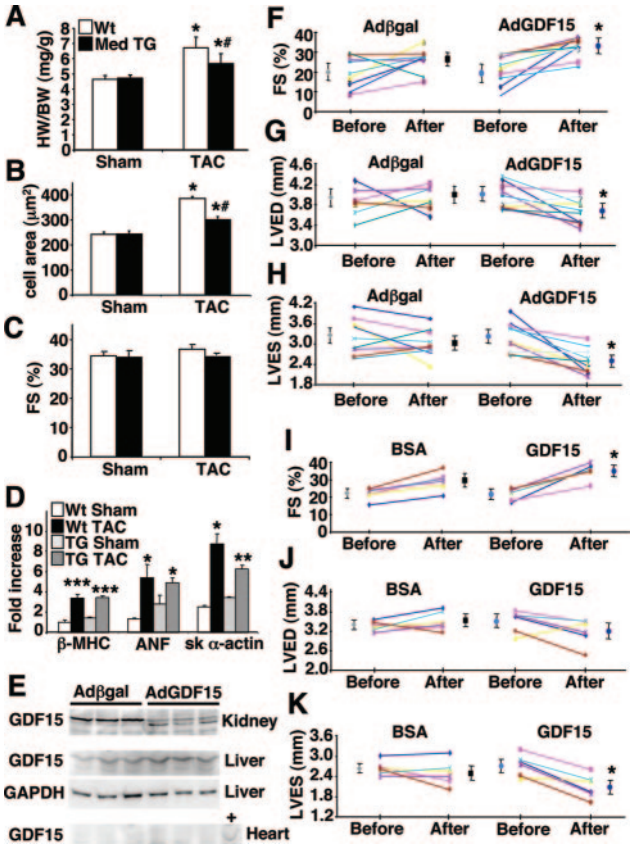

Figure 3. Assessment of induced cardiac hypertrophy and functional performance in GDF15 transgenic mice. A, Heart-weight (HW) normalized to body-weight (BW) in adult wild-type and GDF15 medium expressing transgenic mice subjected to sham procedure or 2 weeks of TAC (N=8 mice in each group). *$P<0.0001$, sham vs TAC; #$P<0.001$, wild type vs medium transgenic, with a significant interaction ($P=0.0027$). B, Assessment of cellular surface area within the left ventricles of adult wild-type and GDF15 medium–expressing transgenic mice subjected to sham procedure or 2 weeks of TAC. *$P<0.0001$, sham vs TAC; #$P=0.0001$, wild type vs medium transgenic, with a significant interaction ($P=0.0001$). C, Assessment of left ventricular fractional shortening as measured by echocardiography in shams or mice subjected to TAC (N=8 mice in each group). D, Assessment of mRNA levels for β-MHC, ANF, and skeletal α-actin (N=2 hearts in each group). ***$P<0.001$, **$P<0.01$, *$P<0.05$ vs corresponding sham. E, Western blot analysis of GDF-15 propeptide from kidney, liver, and heart. F through H, Fractional shortening or left ventricular end diastolic/systolic dimensions (LVED/LVES) in individual *mlp*$^{-/-}$ mice before and after Adβgal or AdGDF15, 1-time systemic injection (11 days later mice were examined). *$P<0.01$ after injection vs before injection. I through K, Echocardiographic parameters in individual *mlp*$^{-/-}$ mice before and after BSA (control) or recombinant GDF15 injections (14 days). *$P<0.01$ after injection vs before injection.

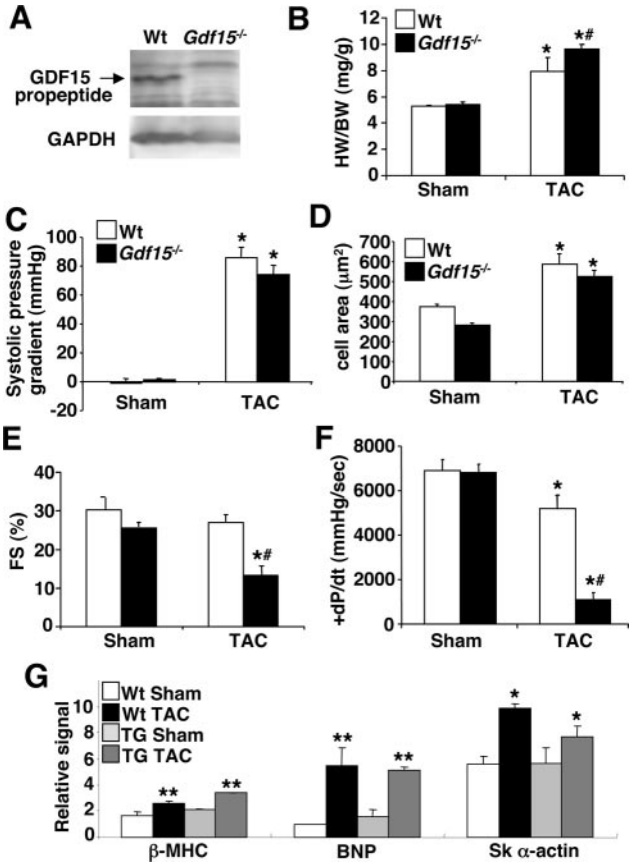

Figure 4. Assessment of induced cardiac hypertrophy and functional performance in *Gdf15* null mice. A, Western blot for GDF15 and GAPDH (control) from 4 day-old neonatal mouse livers from wild-type or *Gdf15* null mice. B, Heart-weight (HW) normalized to body-weight (BW) in adult wild-type and *Gdf15* null mice subjected to sham procedure or 2 weeks of TAC (N=6 to 7 mice in each group). *$P<0.0001$, sham vs TAC; #$P<0.001$, wild-type vs *Gdf15* null, with a significant interaction ($P=0.0017$). C, Measurement of the average systolic pressure gradient across the aortic constriction using a 1.4F Millar catheter for all the mice shown in B (N=6 to 7 mice per group). *$P<0.0001$, sham vs TAC. D, Assessment of cellular surface area within the left ventricles of adult wild-type and *Gdf15* null mice subjected to sham procedure or 2 weeks of TAC (N=4 hearts per group). *$P<0.0001$, sham vs TAC. E, Assessment of left ventricular fractional shortening as measured by echocardiography in shams or mice subjected to TAC (N=5 to 6 mice in each group). *$P<0.002$, sham vs TAC; #$P<0.001$, wild type vs *Gdf15* null, with a significant interaction ($P=0.04$). F, Assessment of cardiac ventricular performance using an ex vivo isolated working heart preparation measured as +dP/dt (mm Hg/sec). *$P<0.0001$, sham vs TAC; #$P<0.0001$, wild-type vs *Gdf15* null, with a significant interaction ($P=0.0001$). G, Assessment of fetal gene mRNA levels by RT-PCR analysis (N=2 hearts in each group). **$P<0.01$, *$P<0.05$ vs corresponding sham.

isolated rat neonatal cardiomyocytes cultured under serum-free conditions. Neonatal myocyte cultures were infected with AdGDF15, or Adβgal as a control, and allowed to incubate for 24 hours before stimulation with the combination of PE (50 μmol/L)/Ang II (1 μmol/L), or 1% FBS. GDF15 overexpression in culture significantly reduced the increase in cell surface area induced by the agonists used compared with Adβgal infection (Figure 5A). [^{3}H]-Leucine incorporation was also reduced by AdGDF15 infection (Figure 5B). Similarly, purified recombinant GDF15 was transiently applied to cardiomyocyte cultures treated with PE+Ang II, also demonstrating a significant attenuation of cardiac hypertrophy (Figure 5C).

Analysis of Signaling Pathways Downstream of GDF15 in Cardiomyocytes

Members of the TGF-β superfamily traditionally signal through membrane bound receptors that result in the phosphorylation and activation of SMAD transcription factors, as well as through direct and indirect effects on other signaling

TABLE 1. Echocardiographic Analysis of *Gdf15* Gene-Targeted and Wild-Type Mice After Two Weeks of TAC or Sham Operation

	Wild-Type (Sham) (N=6)	Wild-Type (TAC) (N=6)	*Gdf15*$^{-/-}$ (Sham) (N=5)	*Gdf15*$^{-/-}$ (TAC) (N=6)
LVED, mm	4.14±0.43	4.13±0.46	3.85±0.36	4.25±0.15
LVES, mm	2.90±0.57	3.02±0.45	2.87±0.32	3.69±0.20*
Septum, mm	0.78±0.10	0.96±0.15*	0.69±0.17	0.79±0.07*
LV wall, mm	0.78±0.15	0.98±0.09*	0.58±0.06	0.78±0.10*
FS, %	30.3±6.9	27.0±3.5	25.5±3.1	13.1±5.1*†

Mice were 8 to 10 weeks of age. All measurements are means±SD, and significance was assigned by 2-way ANOVA. Septal and left ventricular (LV) wall thicknesses were assessed in diastole and shown as millimeters (mm). LVED indicates left ventricular end-diastolic dimension; LVES, left ventricular end-systolic dimension; FS, fractional shortening. *P<0.05 vs sham, †P<0.05 vs wild type.

kinase pathways. Acute treatment with recombinant GDF15 or TGF-β showed a prominent induction of SMAD2/3 phosphorylation and a detectable, albeit mild, induction of SMAD1/5/8 phosphorylation (Figure 6A). GDF15 also transiently activated Akt and extracellular signal-regulated kinases (ERK1/2), but not p38 or c-Jun N-terminal kinase (JNK) (Figure 6B). Transcriptional reporter plasmids specific to TGF-β/activin (typically SMAD2/3 response) or bone morphogenic proteins (typically SMAD1/5/8 response) were also used. Conditioned media from Adβgal-, AdGDF15-, and AdGDF8 (myostatin)-infected cells were used to stimulate cardiomyocytes previously transfected with the SMAD2/3-luciferase reporter (3TP-luc), or the SMAD1/5/8 reporter (BRE), or a slightly different SMAD1/5/8 reporter (I-BRE) (Figure 6C). The data show a greater than 2-fold activation of the 3TP-luciferase reporter (SMAD2/3) by GDF15 conditioned media (6 hours of stimulation), but not by Adβgal or AdGDF8 conditioned media (Figure 6C). By comparison, the bone morphogenic protein (SMAD1/5/8 activating) sensing reporters did not respond to either GDF15 or GDF8 over the time course used here (Figure 6C).

From a mechanistic perspective, SMAD2 overexpression with a recombinant adenovirus showed similar inhibition of cardiomyocyte hypertrophy compared with GDF15 in response to both PE+Ang II and serum stimulation, although coinfection of AdSMAD2 with AdGDF15 did not result in greater inhibition suggesting saturation of the inhibitory effect (Figure 6D). More importantly, coinfection of the inhibitory SMADs, AdSMAD6 or AdSMAD7, reversed the antihypertrophic effects of GDF15 following agonist stimulation (Figure 6E). AdGDF15 or AdSMAD2 also attenuated protein synthesis/accumulation following agonist stimulation (Figure 6F). Collectively, the observations described above are consistent with the hypothesis that GDF15 negatively regulates the hypertrophic response, in part, through SMAD proteins.

TABLE 2. Isolated Working Heart Preparation Assessment of Cardiac Function in Hearts From Wild-Type and *Gdf15*$^{-/-}$ Mice As Sham or TAC Operated for Two Weeks

	Wild-Type (Sham)	Wild-Type (TAC)	*Gdf15*$^{-/-}$ (Sham)	*Gdf15*$^{-/-}$ (TAC)
Working Heart	N=4	N=4	N=4	N=4
Heart Rate, bpm	424±2	397±12*	440±4	467±15*
+dP/dt, mm Hg/sec	6918±246	5180±476*	6801±173	1106±144*†
−dP/dt, mm Hg/sec	4797±163	3466±346*	4499±139	841±92*†
LVP, mm Hg	122±3	96±9*	124±3	48±2*†

Mice were 10 weeks of age. All measurements are means±SEM, and statistical significance was assessed by 2-way ANOVA. +/−dP/dt represents a measure of contractility based on the derivative of pressure change (mm Hg) vs time (sec). LVP indicates left ventricular pressure developed. All hearts were paced, although heart rate varied slightly. *P<0.01 vs sham, †P<0.01 vs wild type.

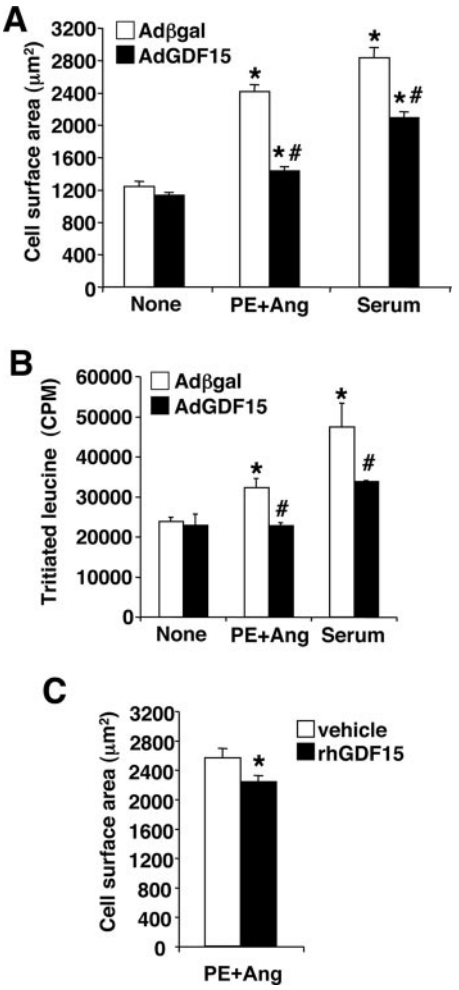

Figure 5. Assessment of hypertrophy in cultured neonatal cardiomyocytes. A, Cardiomyocytes were infected with Adβgal or AdGDF15 and stimulated with PE+Ang II or 1% FBS for 48 hours, and cell surface areas were measured. Three independent experiments and 500 total cells were analyzed. *P<0.05 vs none, #P<0.001 vs Adβgal PE+Ang II or 1% serum. B, The same as in A, except that incorporation rates of [^{3}H]-leucine were measured in triplicate experiments. *P<0.05 vs none, #P<0.05 vs Adβgal PE+Ang II or 1% serum. C, Assessment of cardiomyocyte cell surface area following 48 hours of recombinant human (rh) GDF15 or vehicle treatment in the presence of PE+Ang II. *P<0.05 vs vehicle in the presence of PE+Ang II.

Discussion

GDF15 is not normally expressed in the adult myocardium, although it is prominently induced following "injury" or in

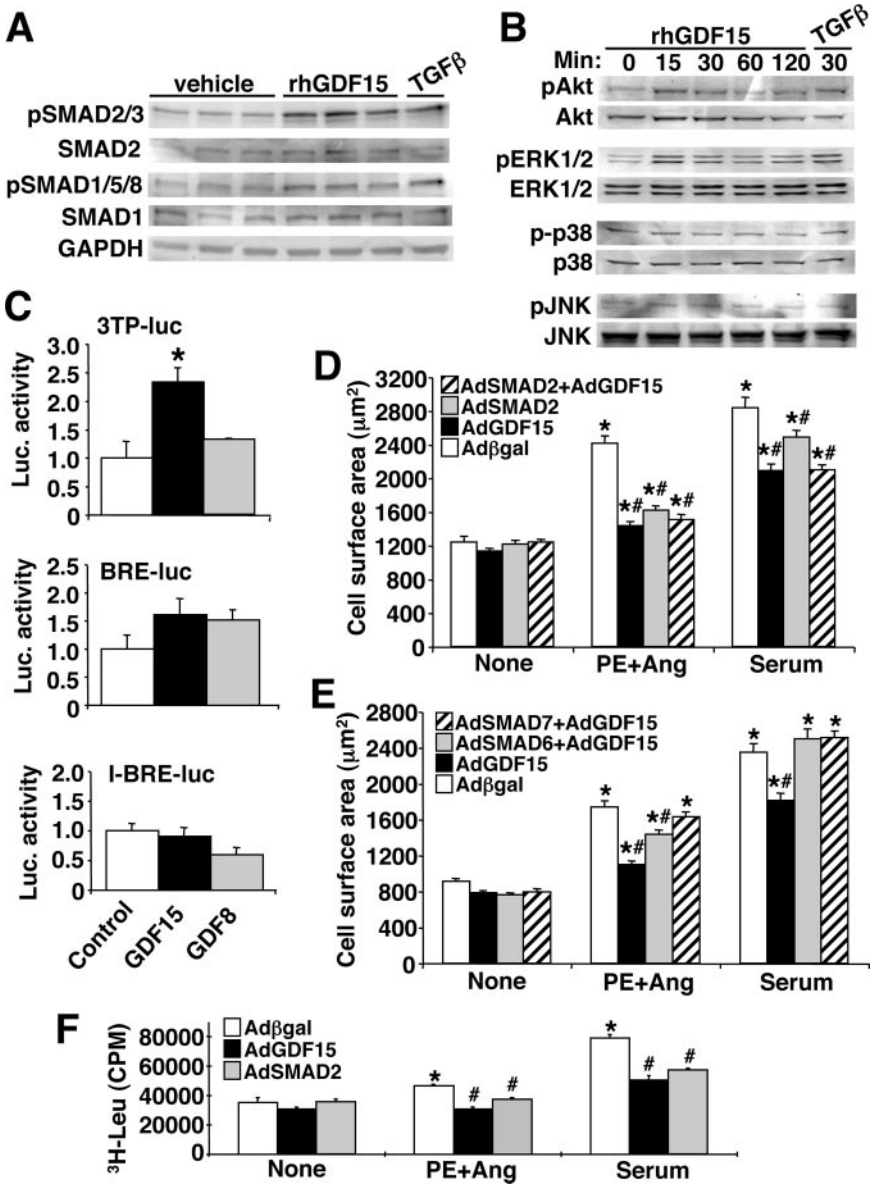

Figure 6. GDF15 induces SMAD2/3 transcriptional activity in neonatal cardiomyocytes. A, Western blot for SMAD2/3 and SMAD1/5/8 phosphorylation in cardiomyocytes stimulated with rhGDF15 or TGF-β for 30 minutes. Control Western blots for total SMAD1, SMAD2, and GAPDH are also shown. B, Western blot assessment for phosphorylation of Akt, ERK1/2, p38, and JNK from cells treated as in A. C, Cardiomyocyte cultures were transfected with 3TP-luc, BRE-luc, or I-BRE-luc and then, 24 hours later, incubated for 6 hours with conditioned media from Adβgal-, AdGDF15-, or AdGDF8–infected myocytes, *$P<0.01$ vs Adβgal. D, Cardiomyocytes were infected with Adβgal, AdGDF15, AdSMAD2, or AdSMAD2+AdGDF15 and stimulated with PE+Ang II or 1% FBS for 48 hours and cell surface areas were measured. *$P<0.05$ vs none, #$P<0.05$ vs Adβgal PE+Ang II or 1% serum. E, Cardiomyocytes were infected with Adβgal, AdGDF15, AdSMAD6+AdGDF15, or AdSMAD7+AdGDF15 and stimulated with PE+Ang II, or 1% FBS for 48 hours, and cell surface areas were measured. *$P<0.05$ vs none, #$P<0.05$ vs Adβgal PE+Ang II or 1% serum. F, Cardiomyocytes were infected with Adβgal, AdGDF15, or AdSMAD2 and stimulated with PE+Ang II, or 1% serum for 48 hours and incorporation rates of [^{3}H]-leucine were measured in triplicate experiments. *$P<0.05$ vs none, #$P<0.01$ vs Adβgal PE+Ang II, or 1% serum.

failed hearts. Careful analysis of GDF15 mRNA levels showed detectable expression within 1 day of acute pressure overload stimulation within the mouse heart, which was maintained up to 8 weeks of stimulation, suggesting it might regulate aspects of both immediate and long-term cardiac "injury" (Figure 1C and elsewhere[25,26]). GDF15 expression is also maintained long-term in MEF2C or activated calcineurin cardiomyopathic transgenic hearts, further suggesting that it might regulate long-term adaptation or maladaptation of the heart. Using both gain- and loss-of-function genetic strategies, GDF15 was determined to function as a necessary protective factor to the heart in association with an alteration in the hypertrophic response, although other mechanisms underling the protective effect are likely (see below). Indeed, *Gdf15* gene-targeted mice not only showed greater hypertrophic enlargement following pressure overload stimulation, but they quickly developed a profile of compromised ventricular performance, further suggesting that GDF15 might antagonize the onset or severity of heart failure. Overexpression of GDF15 by systemic adenoviral delivery also attenuated heart failure in $mlp^{-/-}$ mice. More provocatively, injection of a "physiological" dosage of recombinant GDF15 protein for 14 days also attenuated heart failure in $mlp^{-/-}$ mice.

GDF15 transgenic and $Gdf15^{-/-}$ mice showed alterations in some experimental measures of hypertrophy but not others. More specifically, analysis of hypertrophic marker gene expression showed no differences when compared with controls, and echocardiographic assessment of septal and left ventricular wall thicknesses was not increased in $Gdf15^{-/-}$ mice to the same extent as wild-type controls following TAC. The reduced profile of septum and left ventricular wall thickening after TAC in $Gdf15^{-/-}$ mice is likely related to rapid decompensation and chamber dilation, resulting from either myocyte lengthening or, more likely, remodeling of the chambers and slippage of myocytes. With respect to hypertrophic marker gene expression, there is ample precedence in the literature showing discordance between expression of

ANF, BNP, skeletal α-actin, and β-MHC mRNA levels and hypertrophy, and atrophy even shows upregulated expression of many of these markers.[27–30] Thus, quantitation of hypertrophic marker gene expression does not always correlate with the magnitude and/or presentation of the hypertrophic response.

The observation that GDF15 expression is induced in the heart by disease causing stimuli is reminiscent of natriuretic peptide signaling. Both ANF and BNP are induced and secreted from the heart following acute and chronic stimulation associated with cardiac injury and long-standing disease, where they signal a protective and antihypertrophic response through their receptors.[6,31–34] Our results suggest that GDF15 might function in an analogous manner and, as such, may offer a new therapeutic strategy for the treatment of hypertrophic and dilated cardiomyopathy. Indeed, circulating GDF15 levels were shown to correlate with cardiac risk factors and propensity toward cardiovascular events.[35]

Here we have proposed a cardioprotective role for GDF15, although the exact mechanism of protection could be through antagonism of hypertrophy or reductions in cell death. The fact that GDF15 antagonizes cell death is supported in the accompanying article by Kempf et al,[36] in which GDF15 regulated cardiomyocyte viability in association with Akt activation. Consistent with these observations, GDF15 treatment in vitro protected cultured cerebellar granule neurons from low K^+-induced cell death, suggesting a protective mechanism of action in neurons.[37] However, other reports have suggested a proapoptotic role for GDF15 induction in prostate and colorectal cancer cells, although such induction could still be protective because it would enhance tumor dissemination.[38,39] These various studies highlight the fact that GDF15 function has yet to be fully elucidated, although its role in either protecting from or enhancing cell death may be cell type specific. The potential heterogeneity in the mechanism of GDF15 action in different tissues or cell types may be attributable to differential expression of the type I and type II TGF-β/activin receptors that have affinity of GDF15 (see below).

The data presented here suggest that GDF15 may protect the heart through an association with SMAD proteins. Indeed, Wang et al[40] recently published the phenotype of heart-specific *smad4*$^{-/-}$ mice, which showed greater cardiac hypertrophy and heart failure, consistent with our hypothesis. TGF-β family members function by binding to a heterodimeric cell surface receptor, which consists of 1 member of the type I serine/threonine receptor kinases (7 isoforms: ALK1 to -7) and 1 member of the type II receptors (5 isoforms: BMPRII, ActRII, ActRIIB, TβRII, AMHR).[41] Given the heterogeneity associated with each of the different receptor subtypes and their differential specificities for ligands, it is often difficult to conclusively determine the exact signaling mechanism whereby a given TGF-β superfamily member mediates its effects. Here we determined that GDF15 elicited a TGF-β/activin-like response through SMAD2/3. Furthermore, SMAD2 overexpression phenocopied the effects of GDF15, whereas overexpression of inhibitory SMAD6 or SMAD7 reversed the antihypertrophic effects of GDF15. It is interesting to note that TGF-β itself is likely a prohypertrophic regulatory factor, in contrast to GDF15, even though they both elicit a SMAD transcriptional response, although there is likely divergence in other signaling pathways that accounts for the different biologic responses. GDF15 treatment also transiently activated Akt and ERK1/2 signaling, both of which are thought to be cardioprotective pathways. Thus, although GDF15 might influence cardioprotection through multiple downstream signaling effectors, our results suggest that SMAD2/3 might at least function as part of the protective mechanism.

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