GDF11 Does Not Rescue Aging-Related Pathological Hypertrophy

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**Rationale:** Growth differentiation factor 11 (GDF11) is a member of the transforming growth factor-β superfamily of secreted factors. A recent study showed that reduced GDF11 blood levels with aging was associated with pathological cardiac hypertrophy (PCH) and restoring GDF11 to normal levels in old mice rescued PCH.

**Objective:** To determine whether and by what mechanism GDF11 rescues aging dependent PCH.

**Methods and Results:** Twenty-four–month-old C57BL/6 mice were given a daily injection of either recombinant (r) GDF11 at 0.1 mg/kg or vehicle for 28 days. rGDF11 bioactivity was confirmed in vitro. After treatment, rGDF11 levels were significantly increased, but there was no significant effect on either heart weight or body weight. Heart weight/body weight ratios of old mice were not different from 8- or 12-week-old animals, and the PCH marker atrial natriuretic peptide was not different in young versus old mice. Ejection fraction, internal ventricular dimension, and septal wall thickness were not significantly different between rGDF11 and vehicle-treated animals at baseline and remained unchanged at 1, 2, and 4 weeks of treatment. There was no difference in myocyte cross-sectional area rGDF11 versus vehicle-treated old animals. In vitro studies using phenylephrine-treated neonatal rat ventricular myocytes, to explore the putative antihypertrophic effects of GDF11, showed that GDF11 did not reduce neonatal rat ventricular myocytes hypertrophy, but instead induced hypertrophy.

**Conclusions:** Our studies show that there is no age-related PCH in disease-free 24-month-old C57BL/6 mice and that restoring GDF11 in old mice has no effect on cardiac structure or function. (Circ Res. 2015;117:926-932. DOI: 10.1161/CIRCRESAHA.115.307527.)

**Key Words:** aging ■ body weight ■ cardiac function tests ■ growth differentiation factors ■ transforming growth factor beta

Cardiovascular function can decline in old age, and the disease independent factors that induce these changes are not well known. A recent study suggests that disease-free aging induces pathological cardiac hypertrophy (PCH) in 24-month-old mice and that this results in large part from an age-related reduction in the circulating blood levels of growth differentiation factor 11 (GDF11), a member of the transforming growth factor-β superfamily of cytokines. GDF11 and related family members generally reduce skeletal muscle protein synthesis and repair and enhance protein degradation, which leads to muscle atrophy in adults. Loss of these factors, particularly myostatin (also called GDF8), is primarily associated with skeletal muscle hypertrophy but with limited effects on the heart. Recent work suggests that the circulating levels of GDF11 decrease with aging, and restoring a youthful circulation containing normal levels of GDF11 to old mice via parabiosis reversed age-dependent PCH. A major finding of this study was that restoring youthful levels of GDF11 by injecting recombinant (r) GDF11 into old animals restored normal myocyte size and gene expression in the old mouse heart. Related studies suggest that restoring GDF11 can also have beneficial effects on skeletal muscle and brain function, suggesting that restoring GDF11 to levels seen in young animals could reverse critical aspects of age-related brain, skeletal muscle, and cardiac dysfunction. These studies suggest that restoring normal GDF11 levels in old age can reverse aging effects on critical organ systems.

In This Issue, see p 905
Editorial, see p 906
The idea that a member of the TGF super family of cytokines, GDF11, is singularly responsible for aging-related organ dysfunction is not supported by some recently published reports. A study to reexamine the idea that reduced GDF11 in aging is responsible for defective skeletal muscle repair could not confirm most aspects of the studies related to GDF11-induced rescue of skeletal muscle wasting with aging. This newest report showed that aging involves skeletal muscle wasting and that increased rather than decreased levels of myostatin and GDF11 are involved. These data suggest that inhibition rather than stimulation of myostatin/GDF11 signaling in aging could blunt the associated skeletal muscle dysfunction.

The goals of this study were to reexamine the idea that restoring youthful levels of GDF11 in old mice, by injection of rGDF11, reverses PCH and imparts a youthful phenotype to the old heart. If these findings could be confirmed, we then planned to explore what aspects of pathological myocyte function were rescued by rGDF11 treatment.

We performed a blinded study in which we treated 24-month-old C57BL/6 mice with rGDF11 for 28 days, following the protocol used previously. We measured cardiac structure and function before and after rGDF11 treatment and then measured heart and myocyte size and changes in molecular remodeling after treatment. Our studies suggest that while hearts of older mice are larger there is no pathological hypertrophy present. We also found that daily injection of rGDF11 significantly raised blood levels of rGDF11 in old mice. However, we did not observe any reduction in heart or myocyte size nor did we observe any changes in cardiac performance. We also showed that rGDF11-induced hypertrophy in neonatal myocytes and did not block phenylephrine-induced neonatal myocyte hypertrophy.

Methods

For detailed Methods refer to Online Data Supplement.

Briefly, all animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine and conducted in accordance with the Guide for the Use and Care of Laboratory Animals. Twenty-four–month-old C57BL/6 male mice were provided by Boehringer Ingelheim Pharmaceuticals. Functional characterization, analysis of antibody selectivity, and dosing of rGDF11 are described in detail in the Online Data Supplement. Cardiac function before and after rGDF11 treatment was measured by echocardiography using the Vevo2100 ultrasound system, and hemodynamic parameters were measured using ADInstruments Powerlab 16/30 as described in the Online Data Supplement. Tissue processing, histology, heart weight/body weight (HW/BW) ratio, myocardial fibrosis, and hypertrophy were measured in vivo and in vitro as described in the Online Data Supplement.

Results

Recombinant GDF11 Is Functional and Can Be Selectively and Reliably Detected

Using Western analysis, we first tested the specificity of the reagents used in the previous study (Abcam anti-GDF11) to document reduced GDF11 with aging and reductions in cardiac hypertrophy after rGDF11 injections. We found, as reported recently by others, that the Abcam GDF11 antibody readily detected both GDF11 and myostatin (Online Figure 1A), suggesting that this is not an appropriate reagent to define GDF11 changes with aging or after rGDF11 injection. The Abcam antibody also did not readily detect the nonreduced forms of either GDF11 or myostatin. We then tested the specificity of an R&D systems GDF11 antibody (Online Figure 1B). This antibody had high specificity for GDF11 versus myostatin and was able to detect both reduced and nonreduced forms of GDF11. This antibody was used to detect GDF11 in the present experiments.

We documented the bioactivity of recombinant proteins before injecting them into old mice. The rGDF11 protein was analyzed for its ability to induce Smad2/3 (its known signaling pathway) activity using HepG2 Smad2/3 luciferase reporter cells (Online Figure IC). rGDF11 induced Smad2/3 activity with an EC50 and EC90 of 1.9 and 8.6 nmol/L, respectively (Online Figure IC), documenting that rGDF11 binds to its native receptor with high affinity.

GDF11 Blood Levels Increase After Injection of rGDF11

We next performed studies to determine whether a daily intraperitoneal injection of rGDF11 (0.1 mg/kg for 28 days) into 24-month-old male mice reverses any existing PCH. We measured the circulating levels of rGDF11 in the plasma of old mice either 1 to 3 hours after rGDF11 injection (for peak levels) or 24 hours after injection (for trough levels; Online Table I). These studies showed that rGDF11 rises to a detectable peak within a few hours and then falls to low levels within 24 hours. Importantly, the native GDF11 levels in old mice were below the quantification level (0.1 ng/mL) of this assay. Therefore, we were unable to determine whether GDF11 levels in the blood decreased with age in C57BL/6 mice, similar to what has been reported recently.

GDF11 Has No Effect on Cardiac Hypertrophy

PCH was assessed using HW/tibia length and HW/BW ratios. Heart size was also determined with echocardiographic measures. In addition, immunohistochemistry and qRT-PCR (quantitative real-time polymerase chain reaction) were used to examine cardiac fibrosis and the presence of common markers of PCH. rGDF11-treated 24-month-old male animals were compared with vehicle-treated animals. We also studied 8- or 12-week-old young mice to define the magnitude of age-dependent PCH.

Our studies showed that rGDF11 had no effect on the HW or BW of old mice (Figure 1A). HW/BW and HW/tibia length ratios were not significantly different between rGDF11 and vehicle-treated animals. In addition, the HW/BW ratio of 24-month-old animals was not significantly different from 8- or 12-week-old mice (Figure 1A). These results show that
although the HW (and BW) of 24-month-old animals is greater than that of young animals, there is no pathological hypertrophy, but rather normal growth associated with changes in body mass.\textsuperscript{10–12}

Myocyte cross-sectional area was measured in vehicle- and rGDF11-treated 24-month-old mouse hearts by performing morphometric analysis of cardiac histological sections. No differences in myocyte cross-sectional area between rGDF11- and vehicle-treated 24-month-old animals were observed (Figure 1B).

mRNA levels of the hypertrophic markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-myosin heavy chain (αMHC), and βMHC were also measured in vehicle- and rGDF11-treated 24-month-old mice. We also compared young and old mice to explore age-related changes in these parameters. There were no significant differences in ANP, BNP, αMHC, or βMHC mRNA expression between rGDF11- and vehicle-treated animals (Figure 1C). Although there were no differences in ANP mRNA levels between young and aged mice, BNP was greater in 8-week-old animals versus other ages. In addition, there seem to be age-related reductions in αMHC mRNA and increases in βMHC. These likely represent maturational changes in these molecules.\textsuperscript{13}

Cardiac fibrosis was measured in vehicle- and rGDF11-treated 24-month-old mice by measuring the percentage of collagen in cardiac histological sections using Masson trichrome staining. There was no significant difference in fibrosis between rGDF11- and vehicle-treated animals (Online Figure IIA). We also tested the ability of rGDF11 to stimulate fibroblast activation in vitro using primary cultures of normal human dermal fibroblasts. rGDF11 stimulated fibroblast activation with an EC50 of 176pM (Online Figure IIB). This was similar to the effects of myostatin, which had an EC50 of 83pM.

GDF11 Had No Effect on Cardiac Function

Cardiac structure and function remained unchanged at 1, 2, and 4 weeks of rGDF11 treatment as measured by echocardiography (Figure 2A and 2B.) In terminal hemodynamic studies, there was no difference in max pressure, max dP/dT, min dP/dt, end diastolic pressure, or τ between rGDF11- and vehicle-treated animals (Figure 3). Collectively, these experiments show that rGDF11 had no effects on cardiac structure or function.

GDF11 Did Not Prevent Phenylephrine-Induced Hypertrophy In Vitro

Finally, we tested the effects of rGDF11 treatment on phenylephrine-induced hypertrophy in cultured neonatal rat ventricular myocytes.\textsuperscript{1} Neonatal rat ventricular myocytes were treated with rGDF11 at 3 concentrations: 0.5, 5, and 50 nmol/L with and without simultaneous phenylephrine treatment. GDF11 treatment failed to inhibit phenylephrine-induced increases in myocyte surface area, but instead caused a dose-dependent increase in myocyte size (Figure 4A). rGDF11 failed to inhibit phenylephrine-induced increases in ANP and BNP mRNA expression, whereas by itself it induced a dose-related increase in ANP and BNP mRNA compared with controls (Figure 4B and 4C).

Discussion

Aging-related cardiomyopathy is often secondary to the accumulation of cardiovascular disease and disease-induced changes in cardiac structure and function lead to cardiomyopathy.\textsuperscript{14–16} True age-dependent changes in cardiac structure

<table>
<thead>
<tr>
<th>Heart Weight (g)</th>
<th>Body Weight (g)</th>
<th>Tibia Length (mm)</th>
<th>HW/BW</th>
<th>HW/TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF11 (n=21)</td>
<td>150.60 ± 14.87*</td>
<td>33.65 ± 3.53*</td>
<td>18.07 ± 0.44*</td>
<td>4.74 ± 0.48</td>
</tr>
<tr>
<td>Vehicle (n=22)</td>
<td>159.67 ± 12.85*</td>
<td>34.19 ± 3.73*</td>
<td>19.02 ± 0.46*</td>
<td>4.70 ± 0.38</td>
</tr>
<tr>
<td>8 wk old (n=10)</td>
<td>108.38 ± 8.15</td>
<td>22.83 ± 1.28</td>
<td>16.39 ± 0.24</td>
<td>4.75 ± 0.21</td>
</tr>
<tr>
<td>12 wk old (n=4)</td>
<td>110.05 ± 4.56</td>
<td>24.15 ± 0.48</td>
<td>16.59 ± 0.00</td>
<td>4.56 ± 0.21</td>
</tr>
</tbody>
</table>

Figure 1. Assessment of hypertrophy in vivo. A, Heart weight (HW), body weight (BW), and tibia length (TL) were measured at time of euthanasia 28 days post initial injection of growth differentiation factor 11 (GDF11). No significant differences were found between GDF11- and vehicle-treated animals. *P<0.05 compared with 8-week-old mice. B, There was no difference in myocyte cross-sectional area between recombinant GDF11- and vehicle-treated animals. Hundred to 175 myocytes with nuclei from 6 animals were analyzed per group. Scale bar=50 μm. C, There was no difference in atrial natriuretic peptide (ANP) levels between young and aged mice. There was no significant difference between GDF11 and vehicle-treated animals. *P<0.05 vs 8-week-old mice. #P<0.05 vs 12-week-old mice. BNP indicates brain natriuretic peptide; and MHC, myosin heavy chain.
and function in cardiovascular disease–free individuals are not well understood and if defined could provide novel clues for protection from aging-specific cardiac functional decline.

Others have searched for aging-specific changes in factors that circulate in the blood that if corrected could prevent or reverse age-dependent decline in the function of critical organ systems. Indeed, a recent study, which has generated significant attention and collateral reporting (http://hsci.harvard.edu/aging-and-gdf11-what-we-know, http://www.nature.com/cr/journal/v24/n12/full/cr2014107a.html), suggests that the blood levels of the myostatin-related protein, GDF11, decrease with age. These myokine proteins are members of the transforming growth factor-β family of proteins. Myostatin can potently inhibit skeletal muscle growth and differentiation, with smaller effects on cardiac muscle. Reductions or loss of function of myostatin cause increases in skeletal muscle mass, with little or no effect on the heart.

A recent study reported that the circulating level of GDF11 decreases with aging, and this reduction correlated with the development of PCH in old mice. PCH was rescued in these old mice by either restoring a youthful circulation with parabiosis or by injection of rGDF11 to restore youthful GDF11 levels. Related studies suggest that restoring youthful GDF11 levels rejuvenates skeletal muscle and brain structure and function. These findings suggest that restoring youthful levels of GDF11 can reverse aging dependent decline of critical organ systems, and they were highly touted as the discovery of the long sought after tissue rejuvenation factor (http://hsci.harvard.edu/aging-and-gdf11-what-we-know). However, the skeletal muscle findings have recently been challenged and, considering our results in the old heart, it does not appear that GDF11 is an antiaging factor. A recent editorial has also highlighted that GDF-11 is not the long-sought rejuvenation factor.

The objectives of our experiments were to reexamine the idea that rGDF11 is a critical cardiac antiaging factor in the mouse. Our goal was to define the mechanisms underlying the ability of rGDF11 to reverse aging dependent cardiac pathological structural and functional remodeling.

We first set out to develop assays with appropriate sensitivity and specificity for GDF11 detection. We first examined the antibody used in previous work and found, similar to results

Figure 2. Cardiac structure and function measured by echocardiography. Mice received echocardiography at baseline, 1, 2, and 4 weeks after the start of injections. Recombinant growth differentiation factor 11 (GDF11) did not affect any (A) structural or (B) functional parameters measured. rGDF11 (n=21) or vehicle (n=22). EF indicates ejection fraction.
in another recent report, that this antibody also identified the highly homologous family member, myostatin (Online Figure IA). We then characterized an antibody (R&D systems) that detected GDF11 and not myostatin (Online Figure IB), and we used this antibody to determine that treatment increases the blood levels of rGDF11 in old mice. We found that endogenous levels of GDF11 were below our detection limits in both young and old mice. Therefore, we could not determine whether GDF11 levels fell with age similarly to what has been reported recently in a study of skeletal muscle. Our results show that the reagents used in the previous work are inadequate to determine whether GDF11 levels fall in old mice or increase after rGDF11 treatment.

Our studies show that treatment of old mice with rGDF11 had no effect on heart or myocyte size, overall cardiac structure, and cardiac pump function (Figures 1–3). Indeed, hearts and their myocytes are larger in old versus young mice. However, old mice have greater body weights than young mice, and 24-month-old mice have the same HW/BW ratio as 8- or 12-week-old mice, which is inconsistent with the presence of pathological hypertrophy. In addition, we could not find evidence for activation of pathological hypertrophy signaling in old animals. Collectively, our results suggest that the increase in heart size with aging is what is expected in healthy animals that have increases in their body mass. There are numerous reports showing that heart size changes with increases or decreases in body size in mature adult animals. Our results suggest that HW/tibia length cannot discriminate between pathological hypertrophy and physiological growth in mature animals, where tibia length does not change.

The suggestion of an antihypertrophic effect of a youthful circulation in old mice came from parabiosis experiments in which old and young mice had a shared circulation. In these studies, heart size was clearly reduced in old mice sharing a circulation with young mice. However, the old mice lost 25% to 30% of their BW during the month of parabiosis with the young mice, and the HW/BW ratios in these old mice seem to be unchanged. The unexplained data in this previous report, on which the conclusions in the parabiosis experiments rest, are that there was no change in the HW of old mice sharing a circulation with other old mice, even though these old mice also lost 25% of their BW. The observation that mice can reduce their BW by 25% with no corresponding change in HW does not fit with a large body of existing work.

We also studied the effects of GDF11 on phenylephrine-induced hypertrophy of neonatal rat ventricular myocytes. We found that rGDF11, by itself, activated pathological hypertrophy signaling and increased myocyte size, but it did not...
Figure 4. Growth differentiation factor 11 (GDF11) induces hypertrophy in neonatal rat ventricular myocytes. A, Hundred and fifty to 300 myocytes were analyzed from at least 20 fields of view per condition. B and C, GDF11 treatment increased expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression (n=3). *P<0.05 vs control. #P<0.05 vs phenylephrine (PE) 50 μmol/L.

not exacerbate or block the effects of phenylephrine reported previously.1

In summary, our studies show that daily injections of biologically active rGDF11 raised the blood levels of rGDF11 in old mice, but had no effect on heart and myocyte size, overall cardiac structure, and cardiac pump function. We also did not find evidence for the existence of pathological hypertrophy in 24-month-old disease-free C57BL/6 mice. These results do not support the idea that GDF11 should be part of an antiaging elixir.

Sources of Funding

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Disclosures

None.

References


15. Waller BF. The old-age heart: normal aging changes which can produce or mimic cardiac disease. Clin Cardiol. 1988;11:513–517.


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**Novelty and Significance**

**What Is Known?**

- Cardiovascular structure and function can become abnormal with aging, and these changes are often accompanied by the accumulation of cardiovascular disease risk factors, such as hypertension.
- Aging could also lead to cardiac structural and functional defects that are largely independent of cardiovascular disease, but the basis of these changes is not well understood.
- A recent study showed that restoring youthful levels of growth differentiation factor 11 (GDF11) by sharing the circulation of a young animal (parabiosis) or by injecting recombinant GDF11 (rGDF11) into old mice restored youthful levels of GDF11 and reversed pathological hypertrophy.

**What New Information Does This Article Contribute?**

- Daily injection of GDF11 into old mice increased the blood levels into a range of biological activity, but the rGDF11 injections did not affect the heart or myocyte size, cardiac fibrosis, or cardiac function.
- rGDF11 did not reduce phenylephrine-induced neonatal myocyte hypertrophy, but had prohypertrophic effects when tested alone.

Pathological hypertrophy and myocyte dysfunction can occur in response to cardiovascular diseases. GDF11 has been shown to reverse pathological cardiac hypertrophy; however, we were unable to confirm these findings. In addition, the reagents used in previous studies could not reliably detect GDF11, so the idea that GDF11 falls with aging and was increased in old animals after parabiosis with young animals or after injection of rGDF11 is not based on trustworthy data. Collectively, our results do not support the idea that GDF11 has any significant effect on the size, structure, or function of the old mouse heart.
GDF11 Does Not Rescue Aging-Related Pathological Hypertrophy
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Supplemental Material

Detailed Methods

Animals

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine and conducted in accordance with the Guide for the Use and Care of Laboratory Animals. Aged (24 months) C57BL/6 male mice were provided by Boehringer Ingelheim Pharmaceuticals.

Functional Characterization of GDF11

Functional activity of recombinant GDF11 (R&D Systems) was verified before use, by defining its ability to activate Smad2/3 signaling in HepG2 reporter cells. Briefly, HepG2 cells were transfected with Cignal Lentiviral (Qiagen) particles expressing an inducible firefly luciferase reporter under control of Smad2/3-specific TRE (AGCCAGACA). A puromycin-selected stable reporter cell line was used in the experiment as follows. The HepG2 Smad2/3 luciferase reporter cells were harvested, washed and resuspended at a concentration of 1X 10^6 cells per ml in Opti-MEM assay medium. Reporter cells were incubated in a 96-well plate at 50,000 cells per well with serial dilutions of rGDF11. After 24-hour incubation, samples were treated with 100ul STEADY-Glo reagent (Promega), and assayed for luciferase expression. Relative Luminescence Units (RLU) were plotted versus Log10 nano molar concentrations of the test rGDF11, where EC50 & EC90 values were calculated using a 4 Parameter Logistic Model, supported by Excel add-in Xlfit (ID Business Solutions Limited).

GDF11 Dosing and Injections

We followed the protocol used in the previous report. Investigators were blinded to treatment. Animals were given a daily single intraperitoneal injection of either rGDF11 (R&D Systems) at 0.1mg/kg or vehicle (60mM NaAcetate Buffer, pH 5.0 and 10% Trehelose) daily for 28 days. rGDF11 stock solution was dissolved in NaAcetate Buffer, pH 4.5 at a concentration of 1mg/ml. Stock solutions were diluted with the dosing solution (60mM NaAcetate Buffer, pH 5.0 and 10% Trehelose) to reach the final concentration of 0.1mg/kg. Boehringer Ingelheim Pharmaceuticals provided all solutions in a blinded fashion. Animals were weighed every day before dosing.

Circulating Levels of GDF11

rGDF11 and vehicle-treated animals were divided into two groups to determine peak and trough circulating levels of GDF11 in vivo and after injection. Preliminary studies showed that peak GDF11 blood levels were found within 2 hours of injection. Therefore, 1-3 hours before sacrifice, animals were given a final injection of rGDF11 or vehicle to determine the peak plasma levels of GDF11 post injection. Animals in the trough group were sacrificed 24 hours after their final injection. Plasma was collected from blood removed from the left ventricle via cardiac puncture.

Plasma levels of GDF11 were measured using the 2-Step Homebrew Assay Protocol for the Simoa Assay (Quanterix). Assay conditions were as follows: Capture was performed using R&D Systems anti-GDF11 antibody conjugated to paramagnetic beads (0.7mg/ml, 5.0E+06 final bead concentration), cat #1958-GD/CF. Detection was performed using R&D Systems anti-GDF11 antibody labeled with Biotin (60X,
final stock 1.8ug/ml), cat #1958-GD/CF. A standard curve was created using R&D Systems rGDF11 in 3% BSA with 0.05% Tween, cat#MAB19581/CGIM021408A. The Quanterix SBG enzyme was used at a final concentration of 100pM.

Western Blot Analysis
Recombinant human GDF8 (myostatin) and GDF11 were purchased from Peprotech (cat. # 120-00 and 120-11, respectively) or R&D Systems (cat. # 788-G8/CF and 1958-GD). 100ng of protein was resolved on 4-12% Bis-Tris mini gels (life technologies # NP0321) either under non-reducing or reducing conditions with 100mM DTT. Transfer to nitrocellulose membranes was done on an Invitrogen iBlot transfer system. Membranes were blocked for 1hr in 2% BSA (Promega # W3841) + 0.05% Tween-20 in Tris Buffered Saline, pH7.5. Primary anti-GDF11 antibodies (R&D Systems #MAB-19581 or abcam 124721) were used at 1ug/ml diluted in 2%BSA + 0.05% Tween-20 in Tris Buffered Saline, pH7.5, 1hr at room temperature with gentle rocking. Membranes were washed 3 times in 0.05% Tween-20 in Tris Buffered Saline, pH7.5. Secondary antibodies were (for R&D antibody), goat anti-rabbit IgG-HRP (life technologies #626520) or (for abcam antibody), goat anti-rabbit-HRP (life technologies #656120) used at 1:3000 dilution for 1hr at room temperature with gentle rocking. After 3 additional washes, membranes were developed using HRP Chemiluminescent Substrate Reagent Kit (Invitrogen #WP20005). Images were captured on a Bio-Rad Image Analyzer.

Echocardiography and Strain Analysis
Anesthetized mice underwent transthoracic echocardiography using a Vevo2100 ultrasound system (VisualSonics; Toronto, Canada). Repeated measurements were performed as previously described2-4 at baseline and at 1, 2 and 4 weeks post initial injection. Images were acquired in the short-axis B-mode and M-mode for analysis of cardiac function and dimensions.

In-Vivo LV Pressure Measurements
LV pressures were measured with a 1.4-Fr Millar pressure catheter (SPR-671, Millar Instruments, Houston, TX) connected to an ADInstruments PowerLab 16/30 (ADInstruments, Colorado Springs, CO) with LabChart Pro 6.0 software. Mice were anesthetized with 2.5% isoflurane to maintain HRs in the 450–470 beats/min range, and then a midline neck incision was made and the right carotid artery was exposed and the catheter introduced. The pressure catheter was then advanced through the aortic valves into the LV. The catheter was carefully adjusted to avoid direct contact with the ventricular wall so that smooth intra-LV pressure traces were recorded. Five minutes of baseline pressure were recorded. Intra-LV blood pressure was continuously measured. Pressure data were analyzed offline with the blood pressure module in the LabChart6.0 software.

Tissue Processing, Histology, Heart Weight to Body Weight Ratio (HW/BW), Myocardial Fibrosis and Myocyte Cross Sectional Area
Prior to sacrifice rGDF11 and vehicle treated animals were randomly divided to be used for molecular analysis or histology. Animals were sacrificed 24 hours after their 28th injection. All hearts were rinsed with PBS and weighed. Tibias were removed and measured to the nearest 0.5mm. The hearts from 50% of animals per group were immediately frozen for molecular analysis. The remaining hearts were perfusion-fixed with 10% formalin and paraffin embedded for histology following previously published protocols3-5.
Tissue blocks were sent to AML Laboratories (Baltimore, MD) for sectioning and staining for Hemotoxylin and Eosin. Myocyte cross sectional area was measured from 6 animals per group using H and E stained slides. 6 samples from each group were stained with Masson’s trichrome (Sigma-Aldrich; St. Louis, MO) for collagen deposition. Myocyte cross-sectional area and Fibrotic area were quantitated with NIH ImageJ software (http://rsbweb.nih.gov/ij/). At least 100 myocytes from 4 sections of the heart were analyzed per animal to assess myocyte cross sectional area. 12 fields of view were analyzed per animal for collagen deposition. Fibrotic area was measured by visualizing all blue-stained areas. Color based- thresholding was used to differentiate between the total area of collagen deposition, stained in blue, and myocyte areas in each section. Fibrosis is presented as the sum of the blue-stained areas divided by total ventricular area.

**In-Vitro Fibrosis Assay**

Normal human dermal fibroblasts (Lonza) were cultured in a 96-well plate at passage 3 to 90% confluence. Cells were serum starved for 24 hours. Cells were treated with a titration of TGF-β1, GDF-11, or GDF-8 at 1:3 dilutions, or medium control (all proteins purchased from R&D systems) for 48 hours. Cells were fixed in methanol for 30 min at -20°C. Fibronectin was labeled with 1ug/ml anti fibronectin goat IgG (Santa Cruz), at ambient temperature for 1hr. Alexa Fluor 555 conjugated anti goat IgG (Life Technologies) was used for secondary labeling, at ambient temperature for 1 hr. Fluorescence intensity at Ex: 555nm and Em: 580nm was determined using a Safire² microplate reader from Tecan. Fluorescence intensity values are plotted as a percent change from medium control.

**Real-Time Polymerase Chain Reaction (PCR)**

RNA was extracted from mouse hearts and from rat neonatal cardiomyocytes with TRIzol Reagent. The RNA was cleaned using the Quick-RNA™ MiniPrep (Zymo Research) clean-up protocol. Reverse transcription (RT) reaction was performed using the SuperScript III first strand synthesis system for RT-PCR (Invitrogen) and oligo-dt primers according to the manufacturer’s instructions. Real-time PCR was performed using the Quantifast Sybrgreen PCR kit (Qiagen). Data generated from mouse heart samples were normalized to 18SRNA expression, and data generated from rat neonatal cardiomyocytes were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following primer sets were used for mouse samples (forward, reverse): 18s 5’-GTAACCCGTGGACCCCATTT, 5’-CATCCAATCGGTAGTGGCG; atrial natriuretic factor (ANF) 5’-GCCCTGAGTGACGACTG, 5’-GGAAGCTGTTGACGCTTA; brain natriuretic factor (BNP) 5’-CTGCTGGAGCTGATAAGAGA, 5’-AGTCAGAAACTGGAGTCTCC; alpha myosin heavy chain (αMHC) 5’-ACCTACCAGACAGGGAGGAAG, 5’-ATTGTGTATTTGGCCACAGCG; beta myosin heavy chain (βMHC) 5’-ACCTCCAGACAGGGAGGAAG, 5’-TTGCAAAGAGTCCAGGTCAGGAG. The following primer sets were used for rat samples (forward, reverse): GAPDH 5’-GACATGCCCGCGCTGGAGAAAC, 5’-AGCCGAGATGCCCTTGTAGT; ANF 5’-ATCTGATGGATTCAAGACCC, 5’-CTCTGAGACGGTTGACTCC; BNP 5’-ACAATCCACGATGCAGAAGCT, 5’-GGGCTCCTGGTCTTCAGAG.

**In-Vitro Cardiac Myocyte Hypertrophy Assay**

Neonatal rat cardiac myocytes (NRCMs) were isolated from 1 day old rat pups. NRCMs were plated on coverslips and incubated overnight in DEM+10% FCS (12 well plates and 2x12 plates coverslips). After 24 hours, NRCMs were switched to serum free media (DMEM F12+1xITS). After 2 hours, cells were pretreated with rGDF11 at the following concentrations: 0.5nM, 5nM, and 50nM. Cells were incubated
with rGDF11 for 24 hours, before phenylephrine was added at 50 µM. Cells were incubated for an additional 24 hours for RNA preparation or 48 hours for analysis of cell size and myofibril organization. Cells were washed with cold 1x PBS before being processed for RNA isolation or fixed with 4% paraformaldehyde.

For analysis of myocyte surface area, cells were stained with rabbit anti-troponin I (Cell Signaling) and goat anti-ANP (Santa Cruz). Myocyte surface area was measured for at least 200 cells per condition using NIH ImageJ software.

Statistics

Data are reported as mean ± standard error of the mean. Unpaired t-test, two-way analysis of variance (ANOVA), or two-way ANOVAs for repeated measures were used to detect statistical significance with GraphPad Prism 6. All cell measurements from the same heart were averaged as one averaged data point. At least three hearts from each group were studied.
Online Figure Legends

Online Figure I. Assessment of Antibody Reactivity and Function of rGDF11. A-B: Western Blot was used to determine the specificity of antibodies against GDF11 and Myostatin (MSTN) (100ng) in reduced vs non-reduced Samples. A. αGDF11 Abcam B. αGDF11 R&D Systems. The antibody from R&D specifically detected both reduced and non-reduced forms of GDF11. C: Functional activity of rGDF11 (R&D Systems) was determined by ability to activated Smad2/3 signaling in HepG2 reporter cells using a luciferase assay. rGDF11 induced Smad2/3 activity with EC50 and EC90 values of 1.9 nM and 8.6 nM respectively.

Online Figure II. Analysis of Cardiac Fibrosis. A. Percent fibrosis was determined in histological sections using Masson’s trichrome staining by measuring the percentage of collagen (stained in blue) out of the total myocardial area. There was no significant difference in fibrotic area between rGDF11 (n=6) and vehicle treated animals (n=6). NS= Non-Significant B. The effect of rGDF11 on fibroblast activation in vitro was examined by measuring fibronectin expression using normal human dermal fibroblast treated with a titration of TGF-β1, GDF-11, or GDF-8 at 1:3 dilutions, or medium. Fluorescence intensity values are plotted as a percent change from medium control (POC). GDF11 stimulated fibronectin expression with an EC50 of 176pM.

Online Table 1. Circulating Levels of GDF11 after Injection. Plasma was collected 1.5-2 hrs after injection of GDF11 at 0.1mg/kg or vehicle for peak levels (N=11) and 24 hours for trough levels (N=10). BQL=below quantification level (0.1ng/ml).

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Supplemental References

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B

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C

Recombinant Human GDF11 Activity
Smad 2/3 Signaling

![Graph showing RLU vs. log10 Concentration (nM)]
A

Cardiac Fibrosis

Fibrotic Area (%)

GDF11

Vehicle

NS

B

Fibronectin Expression

POC

log, stimuli [M]

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