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[1]. 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 described[2-4] 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 protocols[3-5].

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-□1, 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 Safire^2 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'-GTAACCCGTTGAACCCCATT, 5'-CCATCCAATCGGTAGTAGCG; atrial natriuretic factor (ANF) 5'-GCCCTGAGTGAGCAGACTG, 5'-GGAAGCTGTTGCAGCCTA; brain natriuretic factor (BNP) 5'-CTGCTGGAGCTGATAAGAGA, 5'-AGTCAGAAACTGGAGTCTCC; alpha myosin heavy chain (αMHC) 5'-ACCTACCAGACAGAGGAAGA, 5'-ATTGTGTATTGGCCACAGCG; beta myosin heavy chain (βMHC) 5'-ACCTACCAGACAGAGGAAGA, 5'-TTGCAAAGAGTCCAGGTCTGAG. The following primer sets were used for rat samples (forward, reverse): GAPDH 5'- GACATGCCGCCTGGAGAAAC, 5'- AGCCCAGGATGCCCTTTAGT; ANF 5'-ATCTGATGGATTTCAAGAACC, 5'- CTCTGAGACGGGTTGACTTC; BNP 5'-ACAATCCACGATGCAGAAGCT, 5'- GGGCCTTGGTCCTTTGAGA.

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 activate 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.

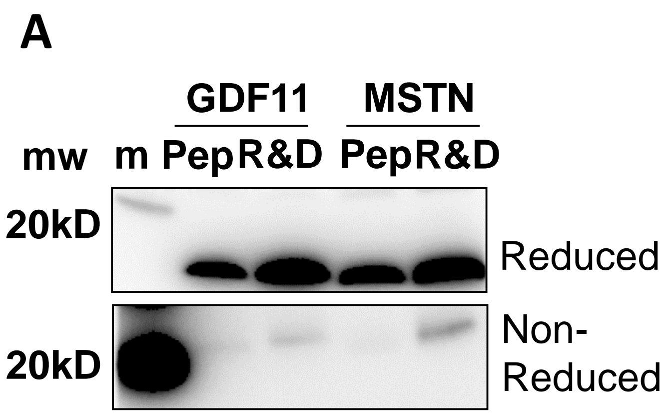
Online Figure II. Analysis of Cardiac Fibrosis. A. Percent fibrosis was determined in histological sections using Masson's trichrome staining by measuring the percentage of collagen (stained in blue) out of the total myocardial area. There was no significant difference in fibrotic area between rGDF11 (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).

	Peak	**Trough**
GDF11	12.8±8.6 (ng/ml)	0.6±0.5 (ng/ml)
Vehicle	BQL	BQL

Supplemental References

1. Loffredo FS, Steinhauser ML, Jay SM, Gannon J, Pancoast JR, Yalamanchi P, Sinha M, Dall'Osso C, Khong D, Shadrach JL, Miller CM, Singer BS, Stewart A, Psychogios N, Gerszten RE, Hartigan AJ, Kim MJ, Serwold T, Wagers AJ and Lee RT. Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell*. 2013;153:828-39.
2. Zhang H, Makarewich CA, Kubo H, Wang W, Duran JM, Li Y, Berretta RM, Koch WJ, Chen X, Gao E, Valdivia HH and Houser SR. Hyperphosphorylation of the cardiac ryanodine receptor at serine 2808 is not involved in cardiac dysfunction after myocardial infarction. *Circ Res*. 2012;110:831-40.
3. Duran JM, Taghavi S, Berretta RM, Makarewich CA, Sharp Iii T, Starosta T, Udeshi F, George JC, Kubo H and Houser SR. A characterization and targeting of the infarct border zone in a swine model of myocardial infarction. *Clin Transl Sci*. 2012;5:416-21.
4. Taghavi S, Duran JM, Berretta RM, Makarewich CA, Udeshi F, Sharp TE, Kubo H, Houser SR and George JC. Validation of transcatheter left ventricular electromechanical mapping for assessment of cardiac function and targeted transendocardial injection in a porcine ischemia-reperfusion model. *Am J Transl Res*. 2012;4:240-6.
5. Duran JM, Makarewich CA, Sharp TE, Starosta T, Zhu F, Hoffman NE, Chiba Y, Madesh M, Berretta RM, Kubo H and Houser SR. Bone-derived stem cells repair the heart after myocardial infarction through transdifferentiation and paracrine signaling mechanisms. *Circ Res*. 2013;113:539-52.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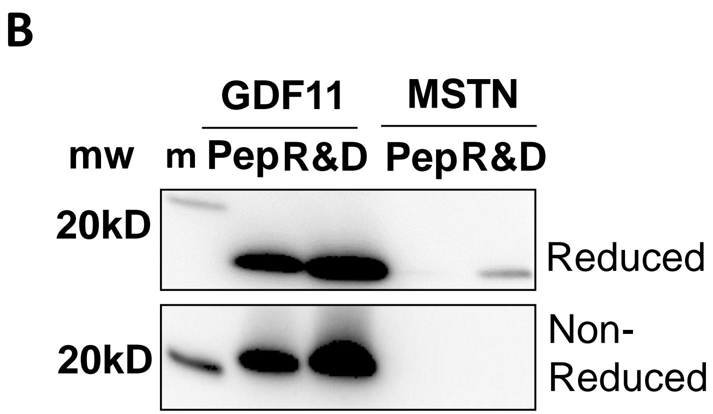
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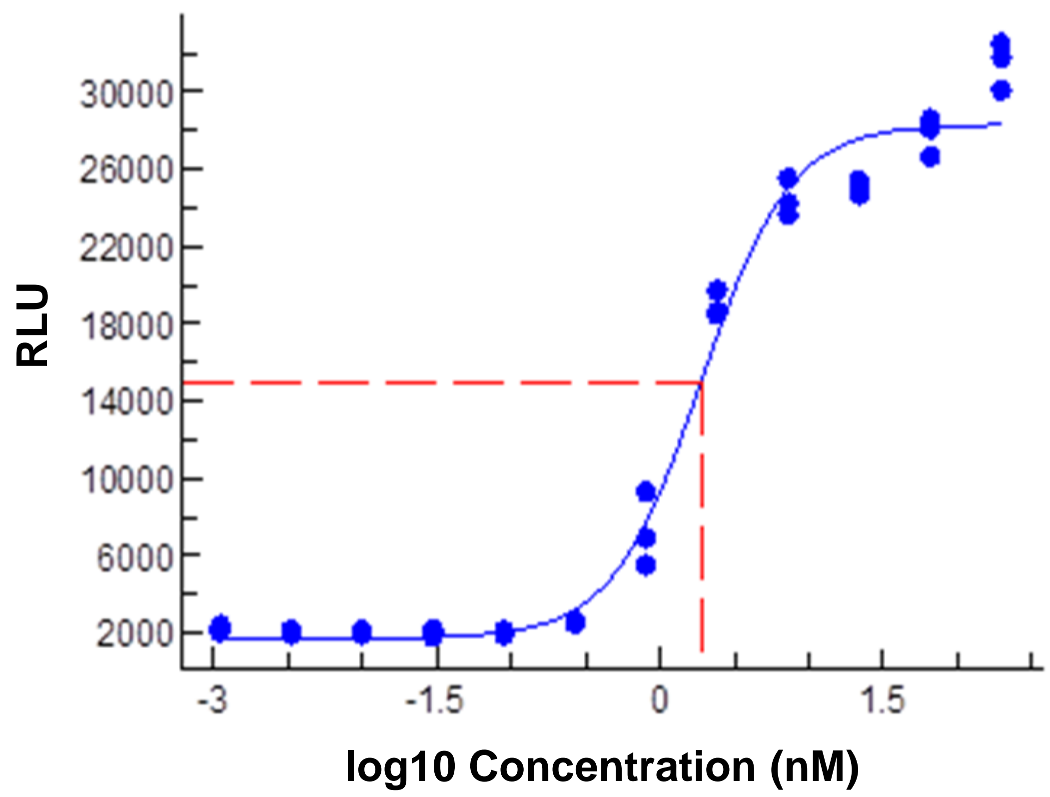
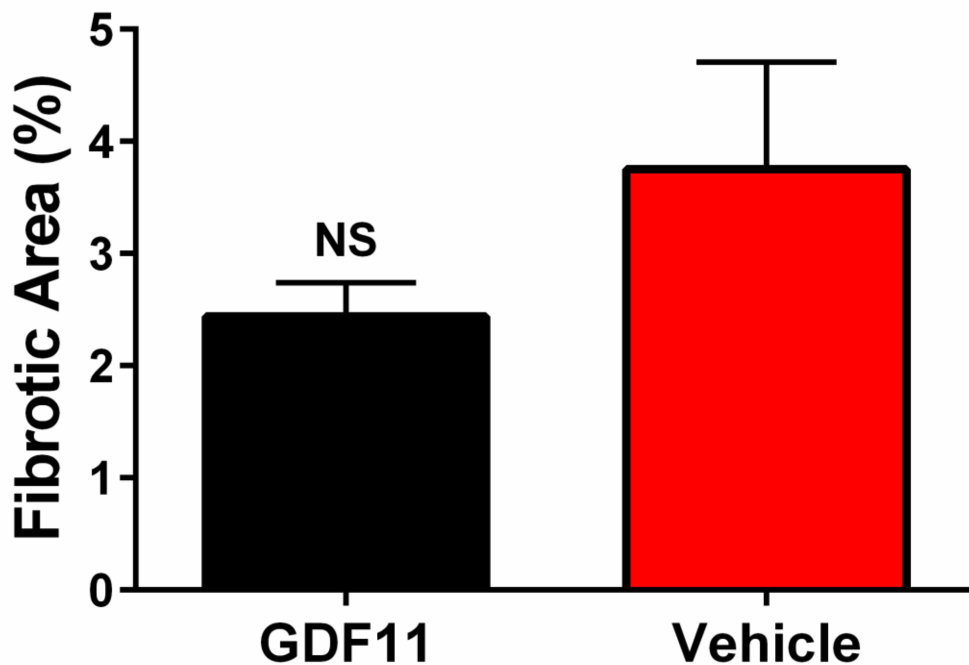
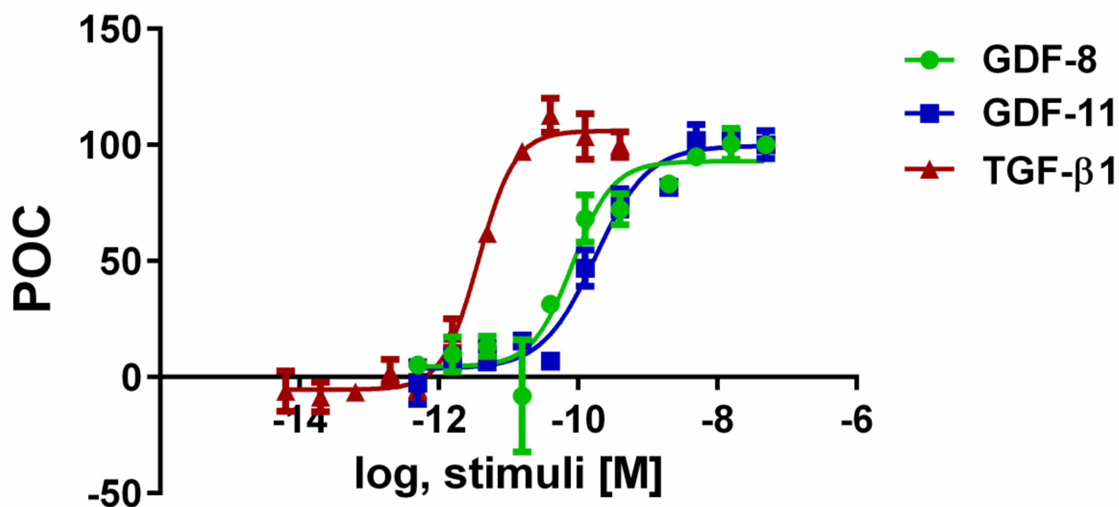

C
Recombinant Human GDF11 Activity
Smad 2/3 Signaling
RLU
30000
26000
22000
18000
14000
10000
6000
2000
-3
-1.5
0
1.5
log10 Concentration (nM)

A **Cardiac Fibrosis**



B **Fibronectin Expression**



	GDF-8	GDF-11	TGF-β1
HillSlope	1.497	1.147	1.662
EC50	8.334e-011	1.755e-010	3.700e-012