The novel cardiokine GDF3 predicts adverse fibrotic remodeling post-myocardial infarction

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SUPPLEMENTAL MATERIAL

Supplemental Methods

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Supplemental methods

Mouse MI model

Left anterior descending artery (LAD) permanent ligation was performed on male 8- or 13-week-old male C57BL/6J and PW1-reporter ($PW1^{nLacZ}$) mice, which were anesthetized in an induction chamber with 2% isoflurane mixed with 1.0 L/min 100% O_2 and placed on a supine position on a heating pad to maintain body temperature. Mice were intubated with endotracheal tube connected to a rodent ventilator (180 breaths/min and a tidal volume of 200 μL). During surgical procedure, anesthesia was maintained at 1.5-2% isoflurane with O_2. The heart was accessed via an intercostal incision through the left side of the chest, and a pericardial incision. The LAD was exposed and encircled with an 8.0 prolene suture at the proximal position. The suture was briefly snared to confirm the ligation by blanching the arterial region. A similar procedure was followed for SHAM mice except that the thread around the LAD was not ligated. Mice were analyzed 7 days after LAD permanent ligation. Blood samples were collected in heparin-coated Eppendorf tubes and immediately centrifuged at 200 ×*g* for 15 min at 4°C to separate the plasma, which was stored at −80°C until analysis. Hearts were excised and immediately digested for FACS sorting or qPCR analysis.

Cell isolation and fluorescence-activated cell sorting (FACS)

Small cell suspensions were prepared from total heart upon atria removal from 8-week-old $PW1^{nLacZ}$ mice. The ventricles were enzymatically digested with collagenase II and dissociated. The following antibodies were used for cell sorting: BUV737-tagged anti-CD31 (1:100 dilution; BD Bioscience), BUV395-tagged anti-TER119 (1:50 dilution, BD Biosciences), phycoerythrin-cyanin7-tagged anti-CD45 (1:500 dilution; eBiosciences). To detect β-gal reporter activity, cells were incubated with the fluorescent substrate 5-

dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) at 37°C for 1 h. The different populations were gated, analyzed, and sorted on a FACS Aria II cytometer (BD Biosciences).

CyQUANT™ Cell Proliferation Assays and functional screens

FACS-sorted $PW1^+$ and $PW1^-$ (FDG^-) cells were seeded in 24-well plates at a density of 15,000 cells/well and cultured under normal conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin and streptomycin (Sigma) for 5 days. The medium was collected and used to incubate serum-starved MEFs (cultured for 24 h under normal conditions and then serum starved for 24 h) for 24 h. The proliferation of MEFs was evaluated using the CyQUANT™ cell proliferation assay as per the manufacturer's instructions. Results obtained with $PW1^+$ cells isolated from SHAM mice were used as control.

cDNAs clones of the candidates were obtained commercially and were then sub-cloned into the pCMV6-entry mammalian expression vector (Origene, Rockville, MD, USA) that has a C-terminal Myc-DDK-Tag. We transfected these plasmids individually into HEK-293 cells (ATCC) with Lipofectamine 2000 (Life Technologies), and cultured the cells for 30 h in serum-free medium (DMEM, Gibco) to obtain conditioned supernatants. We also produced supernatants from HEK-293 cells transfected with an empty expression vector (pCMV6-entry, negative control). The presence of candidates in the conditioned supernatants was confirmed by western blotting the FLAG sequence. We tested all supernatants for proliferative effects on MEFs or adult cardiac fibroblasts using the CyQUANT™ cell proliferation assay as per the manufacturer's instructions. MEFs or adult cardiac fibroblasts incubated with pCMV6-empty conditioned supernatant served as the control.

Pharmacological inhibition of ALK4/5/7 and ALK1/2/3/6 were respectively obtained with the use of AZ12601011 (10 μM, Sigma-Aldrich) and LDN193189 (5 μM, Stemcell). Serum-

starved MEFs were treated for 24h with GDF3 or conditioned media from FACS-isolated $PW1^+$ cells from ischemic mouse hearts or respective control supernatants in presence or absence of the ALK4/5/7 or ALK1/2/3/6 pharmacological inhibitors. Pharmacological inhibition of GDF3-related stimulation was obtained with the use of GDF3 neutralizing antibody (10 μg/mL, R&D Systems MAB57541), and a non-immune mouse IgG2b (10 μg/mL, BD Pharmingem 559530) was used as a control.

RNA-sequencing

In total, 300 ng of total RNA extracted from freshly isolated cells with SureSelect Strand-Specific RNA kit (Agilent) was used to prepare a library, according to the manufacturer's instructions. The resulting library was quality checked and quantified by peak integration on Bioanalyzer High sensitivity DNA labchip (Agilent). A pool of equal quantity of 12 purified libraries was prepared, and each library was tagged with a different index. The mRNA pool libraries were finally sequenced on Illumina Hiseq 1500 instrument using a rapid flowcell. The pool was loaded on two lanes of the flowcell. A paired-end sequencing of 2× 100 bp was performed.

HEK293 cell culture and transfection

HEK293 cells were cultured at 37°C in the presence of 5% CO_2 in DMEM (Life Technologies) supplemented into 10% FBS and 1% penicillin and streptomycin. HEK293 cells were plated at 600,000 cells well in 6 well-plates in a medium without antibiotics. After 24 h, transfection of expression plasmids (Origene and Genscript) was performed with Lipofectamine® 2000 (Life Technologies) according to the manufacturer's protocol using 2 μg of plasmids and 6 μL of Lipofectamine® 2000 diluted in Opti-MEM (Life Technologies). The cells were cultured for 2

days and then serum starved for 8 h prior to the collection of conditioned media and centrifugation at 200 ×*g* for 10 min. Supernatants were stored at −80°C.

qPCR analysis

RNA was extracted from cardiac cells isolated from MI and SHAM C57BL/6J mice 7 days after surgery using the RNAqueous Micro Kit (AM1931, Invitrogen), as per the manufacturer's instructions. Then, 500 ng of extracted RNA was subjected to reverse transcription using the SuperScript IV VILO kit (11756050, Invitrogen) as per the manufacturer's instructions. RNA was extracted from MEFs cells 24 h after conditioned media treatment using the NucleoSpin RNA Mini Kit (740955, Macherey-Nagel), as per the manufacturer's instructions. Then, 1 µg of extracted RNA was subjected to reverse transcription using the PrimeScript RT Reagent Kit (RR037A, Takara).

The resulting cDNA was subjected to qPCR using SYBR Select Master Mix (4472908, Applied Biosystems) on Quant Studio 3 Real-Time PCR system (Thermo Fisher) as per the following condition: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 95°C for 10 s and 60°C for 1 min. The expression of target gene was analyzed using the $-2^{\Delta\Delta CT}$ method following normalization to *RPL13* or *HPRT* expression. The primer pair used are listed in Supplemental Table 1.

Isolation of mouse embryonic fibroblasts

Primary MEFs were isolated from 13.5-days post-coitus C57bL/6J mouse embryos. The pregnant females were euthanized by cervical dislocation and embryos were surgically excised and separated from maternal tissues and the yolk sac in ice-cold phosphate-buffered saline (PBS). Embryos were then decapitated and eviscerated (removal of the heart, spleen, liver and intestine). The bodies were washed in ice-cold PBS to remove blood before being finely minced

in a Petri dish without PBS. Samples were incubated for 15 min at 37°C in the digestion solution (0.05% trypsin-EDTA solution [Life Technologies], 0.1 mg/mL DNase 1 [Sigma]). The suspension was allowed to settle. The supernatant was drawn off, mixed with MEF culture medium (DMEM 4.5 g/L D-glucose [Life Technologies], 10% FBS, 1% penicillin-streptomycin, 1% non-essential amino acid [Life Technologies]) and centrifuged for 5 min at 200 ×*g*. After centrifugation, the pellet containing MEFs was resuspended in MEF culture medium. The pellet from the tissue digestion was resuspended in the digestion solution and incubated for 15 min at 37°C. The cells were allowed to settle, the supernatant was drawn off and processed as previously described. The cells from the first and second digestion steps were pooled and then plated in Petri dishes. Each Petri dish received a volume of cell suspension equivalent to 1.5 embryos.

After 12 h, the culture medium was changed to remove non-adherent cells and debris. The MEFs were passaged upon reaching 80% confluence. MEFs were harvested by trypsinization, centrifuged, and resuspended in a freezing medium (DMEM 4.5 g/L D-glucose, 1% penicillin-streptomycin, 10% dimethyl sulfoxide). Primary MEFs were cultivated between passage 0 and 4.

Western blot analysis

Proteins were extracted from frozen mouse heart tissues or from MEFs cells, using a Dounce-Potter homogenizer into ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.4, 150 mM sodium chloride, 1% IGEPAL CA-630, 50 mM deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with 1% anti-proteases (Sigma-Aldrich), 1% anti-phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2 and 3, Sigma-Aldrich), and 1 mM sodium orthovanadate. After 1 h incubation at 4°C, the homogenate was centrifuged for 15 min at 15,300 ×*g* and 4°C and the supernatant containing proteins was collected. Cardiac $PW1^{+}$ cells

from 22 mice and PW1^{-} cells from 16 mice were pooled, centrifuged at 500 ×*g* for 15 min at 4°C, and lysed in urea-thiourea buffer (5 M urea, 2 M thiourea, 50 mM dithiothreitol [DTT], and 0.1% SDS in PBS, pH 7.4). Proteins were extracted as described above. Protein concentrations for all samples were determined using a Bradford-based protein assay (Bio-Rad).

After isolation of cardiomyocytes and non-cardiomyocytes from the adult mouse hearts, as previously described[7], the proteins were denatured for 10 min at 70°C before loading on a NuPAGE™ Novex® 4-12% Bis-Tris gel (Life Technologies). After 3 h electrophoresis, proteins were transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) and stained with 0.1% Ponceau S (w/v in 5% acetic acid) to assess transfer quality and homogeneous loading. Membranes were blocked for 1 h in Tris-buffered saline with 0.1% Tween-20 (TBS-Tween) containing 1% skim milk with constant shaking and then incubated for overnight at 4°C with primary antibodies specific for GDF3 (1:1000 for tissue and 1:500 for plasma, Abcam), FLAG (1:1000, Sigma-Aldrich), Phospho-SMAD2$^{Ser465/467}$/3$^{Ser423/425}$ (1:1000, Cell Signaling), total SMAD2/3 (1:1000, Cell Signaling), Phospho-SMAD1/5$^{Ser463/465}$ (1:1000, Cell Signaling), total SMAD1 (1:1000, Cell Signaling), total SMAD5 (1:1000, Cell Signaling) and Alpha-Tubulin (1:10 000, Abcam) diluted in 1% skim milk/TBS-Tween. After washing, the membranes were incubated for 1 h at room temperature (23°C) with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 1% skim milk/TBS-Tween. Membranes were then washed and incubated for 5 min with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Life Technologies) before imaging with the Chemidoc® XRS+ camera (Bio-Rad) and analysis using the Image Lab™ software.

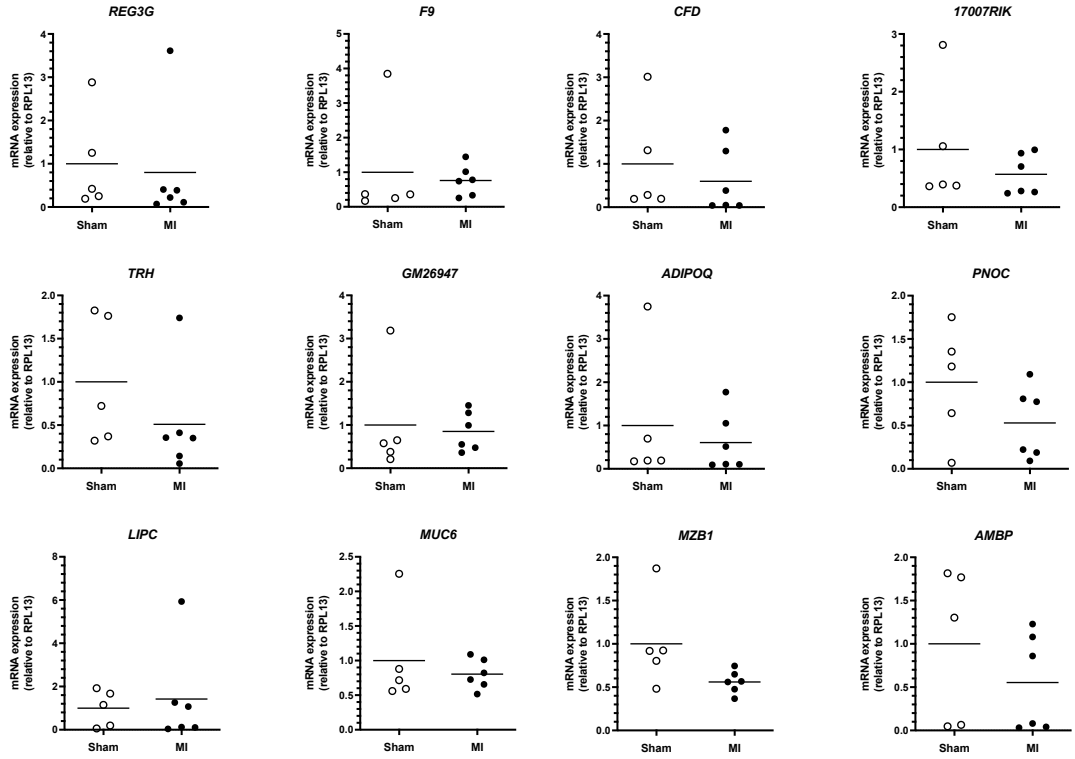
ELISA for GDF3 quantification

Mice GDF3 levels were measured by GDF3 sandwich ELISA assay (GenWay, GWB-KBBHW6) and human GDF3 levels were measured by Human GDF3 ELISA kit (Fine Test, EH3126) following the manufacturer's instructions. Briefly, standards and diluted samples (1:16 for mice and 1:2 for humans in standard diluent) were added to an anti-GDF3 microplate (pre-coated plate with an antibody specific for GDF3) and incubated for 60 minutes (mice) or 90 minutes (humans) at 37°C. After removing standards and samples, wells were washed and a biotinylated GDF3 detector antibody was applied. The plate was incubated for 1 h at 37°C. Wells were washed and incubated at 37°C with an avidin-HRP conjugate for 30 min for mice samples and with HRP-Streptavidin conjugate (SABC) for 30 min for human samples. Finally, after extensive washing, the wells were incubated with the 3,5,3′,5′-tetramethylbenzidine (TMB) substrate for 15 min in the dark at 37°C. The blue color product from the oxidation of TMB substrate changed into yellow after reaction termination with the addition of stop solution and incubation at 37°C for 15 min. Absorbance at 450 nm was quantitatively proportional to the amount of GDF3 captured in well and measured using microplate reader.

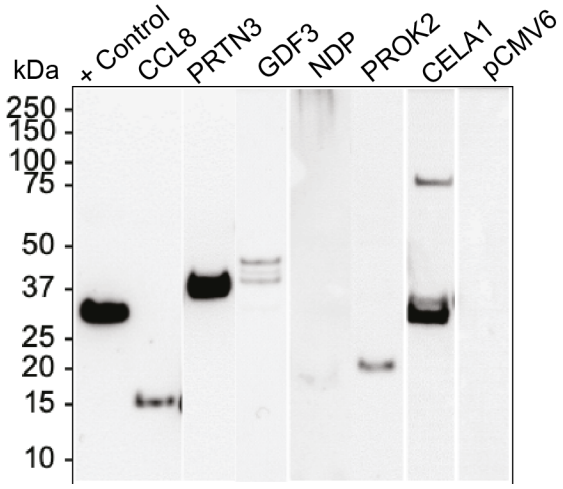
Supplemental Table 1. PCR primers

GENE	FORWARD PRIMER (5' TO 3')	REVERSE PRIMER (5' TO 3')
CCL8	TCTACGCAGTGCTTCTTTGCC	AAGGGGGATCTTCAGCTTTAGTA
PRTN3	ATGGCTGGAAGCTACCCATC	TGCCCACCTACAATCTTGGAG
F10	AGGACTCGGAGGGCAAACT	TCACGGACCTCTTCATAAGAACA
SAA3	TGCCATCATTCTTTGCATCTTGA	CCGTGAACTTCTGAACAGCCT
APOC4	TGTTCTTGGTCAGCTTTGTAGC	AGGCTGTGGGTCTTGTTTAGG
APOC2	ATGGGGTCTCGGTTCTTCCT	GTCTTCTGGTACAGGTCTTTGG
PROK2	TTGCGACAAGGACTCTCAGT	CCCATAGGTCAGATCCT
CIQTNF3	GCTGGTAAACATAGGTGGCG	GCGGTTCTTCATCAGCTTCA
CELA1	GGGGCTCCTCTGTGAAGAAT	AGCATACTGACCGTTCACCA
GDF3	TTCAGCTTCTCCCAGACCAG	CCTTTTCTTTGATGGCAGACAAG
DMKN	CGGGAGTCACACCTTCATCT	AACTTCAGCCACTTCAGCAG
NDP	AGTCTGAGAAGAAGGAGCCC	GCTTCTTTCACTTGCAACCG
POSTN	TGGTATCAAGGTGCTATCTGCG	AATGCCCAGCGTGCCATAA
RPL13	GGGTGGCCAGCTTAAGTTCT	GAGGAGGCGAAACAAGTCCA
HPRT	AGGGCATATCCAACAACAAACTT	GTTAAGCAGTACAGCCCCAAA

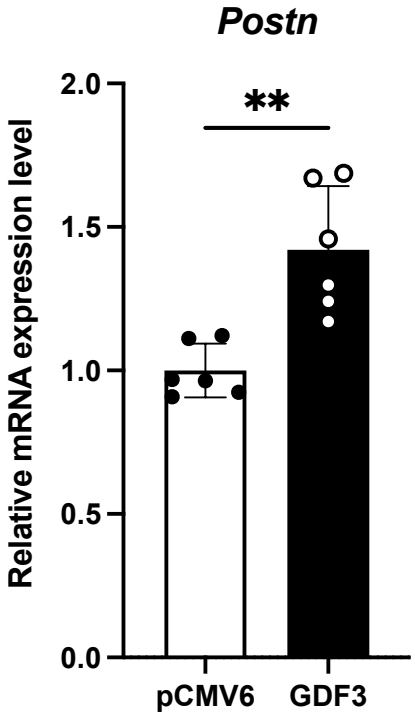
Supplemental Figure S1. Expression of candidates that were not increased in the ischemic heart. qPCR evaluation of *REG3G*, *F9*, *CFD*, *17007RIK*, *TRH*, *GM26947*, *ADIPOQ*, *PNOC*, *LIPC*, *MUC6*, *MZB1*, and *AMBP* expression in the infarcted area of MI hearts (N = 6) and the corresponding area of sham hearts (N = 5). Statistical significance was determined by Mann-Whitney U-test, all tests non-significant.



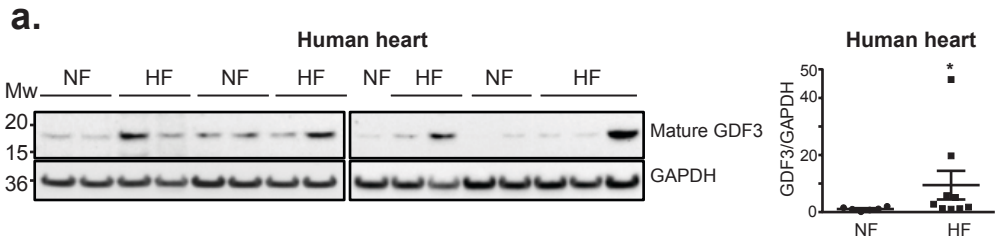
Supplemental Figure S2. Over-expression of secreted proteins in supernatants collected 48hours after transfection of the six candidate genes cDNAs (sub-cloned into the pCMV6-entry mammalian expression vector with a C-terminal Myc-DDK-Tag) in HEK-293 cells. The presence of candidate markers was confirmed by Western blotting of the FLAG sequence. pCMV6 corresponds to the supernatants collected in HEK293 cells transfected with an empty vector (negative control).



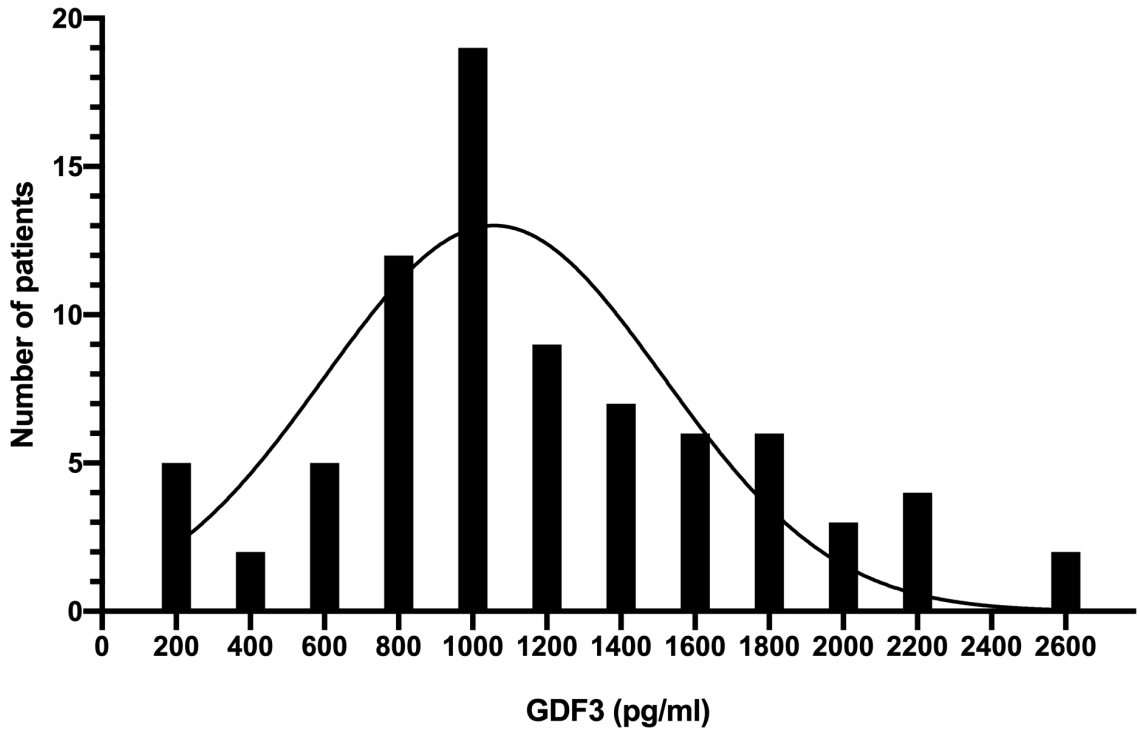
Supplemental Figure S3. Expression of periostin in MEFs treated with GDF3. Periostin (POSTN) mRNA levels in MEFs treated with control vs. GDF3 supernatants. N=6 in duplicate. **P < 0.01 as determined by Mann Whitney test



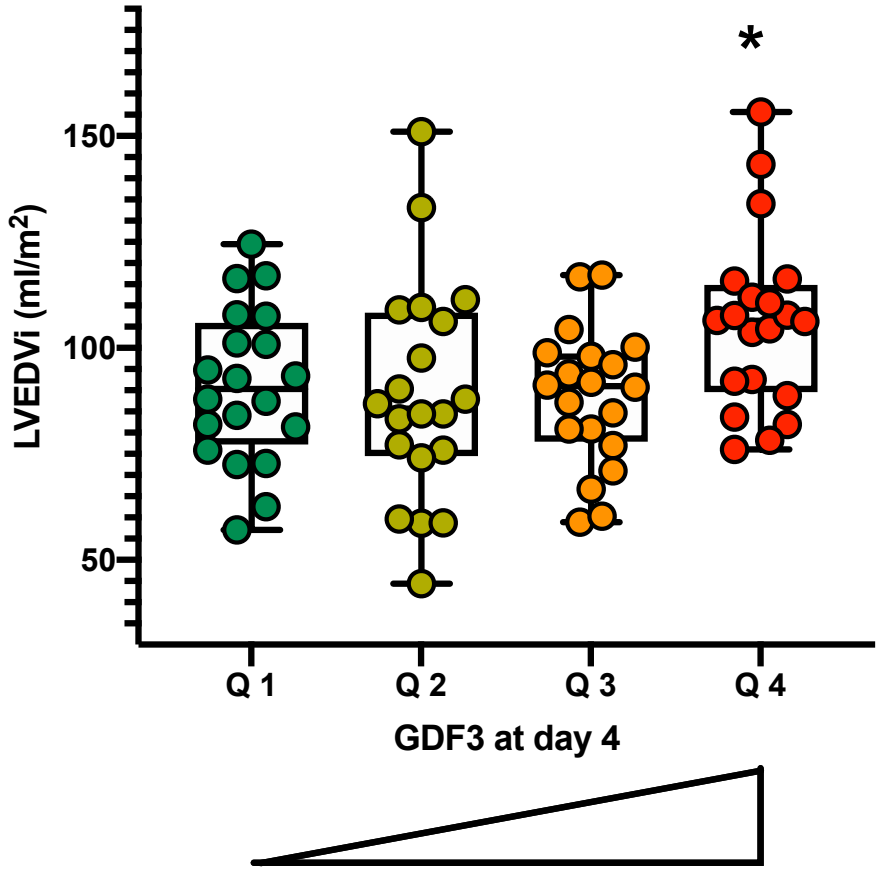
Supplemental Figure S4. Expression of GDF3 in human hearts. Representative Western blots and quantification of mature GDF3 in non-failing (NF) (n = 6) and failing hearts (HF) (n = 9) of patients. *P < 0.05 as determined by Mann Whitney test



Supplemental Figure S5. Distribution of plasma GDF3 levels. GDF3 was measured by ELISA in human plasmas collected at day 4±2 post-PCI.

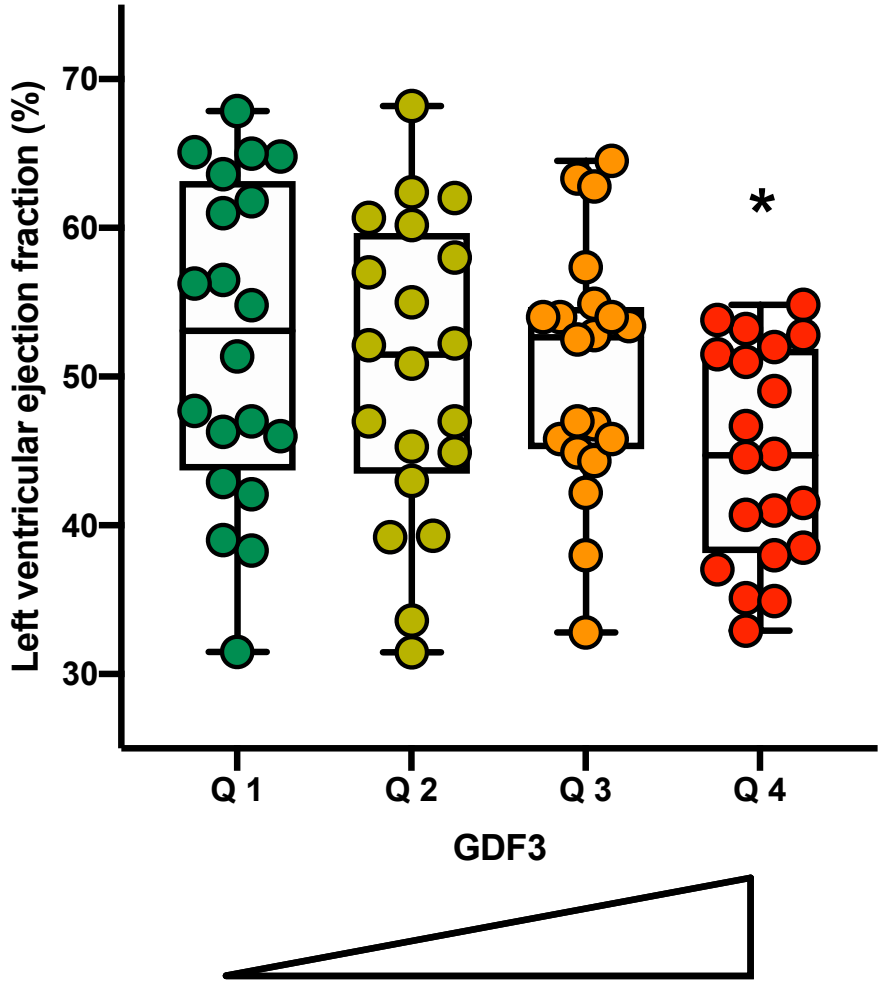


Supplemental Figure S6. Left ventricular end-diastolic volume indexed for body surface area measured on cardiac MRI 6 months after MI, according to GDF3 quartiles. GDF3 was measured by ELISA in human plasmas collected at day 4±2 post-PCI and patients were classified into 4 quartiles according to GDF3 levels. Statistical significance was determined by one way ANOVA followed by Tukey multiple comparison tests, *P < 0.05



	GDF3 levels (pg/mL)			
	Quartile 1 [150-807]	**Quartile 2 [825-1040]**	**Quartile 3 [1050-1510]**	**Quartile 4 [1550-2570]**
LVEDVi (ml/m^2) **Mean**	91.0	89.1	88.3	105.9
SD	18.2	25.9	16.3	20.9

Supplemental Figure S7. Left ventricular ejection fraction measured on cardiac MRI 6 months after MI, according to GDF3 quartiles. GDF3 was measured by ELISA in human plasmas collected at day 4±2 post-PCI and patients were classified into 4 quartiles according to GDF3 levels. Statistical significance was determined by one way ANOVA followed by Tukey multiple comparison tests, *P < 0.05



	GDF3 levels (pg/mL)			
	Quartile 1 [150-807]	**Quartile 2 [825-1040]**	**Quartile 3 [1050-1510]**	**Quartile 4 [1550-2570]**
LVEF (%) **Mean**	52.4	50.5	50.6	44.7
SD	10.7	10.1	8.3	7.2