Analysis of Cre-mediated genetic deletion of Gdf11 in cardiomyocytes of young mice


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Abstract

Administration of active growth differentiation factor 11 (GDF11) to aged mice can reduce cardiac hypertrophy, and low serum levels of GDF11 measured together with the related protein, myostatin (also known as GDF8), predict future morbidity and mortality in coronary heart patients. Using mice with a loxP-flanked (“floxed”) allele of Gdf11 and Myh6-driven expression of Cre recombinase to delete Gdf11 in cardiomyocytes, we tested the hypothesis that cardiac-specific Gdf11 deficiency might lead to cardiac hypertrophy in young adulthood. We observed that targeted deletion of Gdf11 in cardiomyocytes does not cause cardiac hypertrophy but rather leads to left ventricular dilation when compared to control mice carrying only the Myh6-cre or Gdf11-floxed alleles, suggesting a possible etiology for dilated cardiomyopathy. However, the mechanism underlying this finding remains unclear due to multiple confounding effects associated with the selected model. First, whole heart Gdf11 expression did not decrease in Myh6-cre;Gdf11-floxed mice, possibly due to upregulation of Gdf11 in non-cardiomyocytes in the heart. Second, we observed Cre-associated toxicity, with lower body weights and increased global fibrosis in Cre-only control male mice compared to flox-only controls, making it challenging to infer which changes in Myh6-cre;Gdf11-floxed mice were due to Cre toxicity versus deletion of Gdf11. Third, we observed differential expression of cre mRNA in Cre-only controls compared to the cardiomyocyte-specific knockout mice, also making comparison between these two groups difficult. Thus, targeted Gdf11 deletion in cardiomyocytes may lead to left ventricular dilation without hypertrophy, but alternative animal models are necessary to understand the mechanism for these findings.
We observed that targeted deletion of \textit{Gdf11} in cardiomyocytes does not cause cardiac hypertrophy but rather leads to left ventricular dilation when compared to control mice carrying only the \textit{Myh6-cre} or \textit{Gdf11-floxed} alleles. However, the mechanism underlying this finding remains unclear due to multiple confounding effects associated with the selected mouse model.

\textbf{Keywords:} Growth differentiation factor 11, myostatin, \textit{Myh6-cre}, cardiomyocytes, cardiomyopathy
**Introduction**

Growth differentiation factor 11 (GDF11) is a member of the transforming growth factor β (TGF-β) superfamily, best known for its morphogenic roles during development (14). The role of GDF11 in cardiac aging has been controversial (9, 21, 24, 25). We initially demonstrated that GDF11 is a circulating factor that when administered to aged mice can decrease age-related cardiac hypertrophy as indicated by a decrease in heart weight to tibia length ratios (12). Others have shown that GDF11 administration may be beneficial following myocardial infarction (6). However, following our initial report, contrasting work indicated that GDF11 administration to aged mice does not affect heart weight to body weight ratios (24), or, that GDF11 supplementation impairs cardiac function in concert with reducing cardiomyocyte size and cardiac mass (21). Furthermore, additional controversy has been raised due to a lack of specificity of antibody and aptamer reagents used to detect and quantify GDF11 separate from the closely related protein, myostatin (also known as GDF8 and encoded by the gene, *Mstn*).

Because GDF11 and GDF8 share approximately 90% identity in their active domains, it has been challenging to quantify levels of each protein independently (7).

Human clinical data indicate that low serum levels of the GDF11 + myostatin pool at study entry predict increased mortality in adult patients with heart disease over the subsequent eight years (16). These human data point to low levels of the circulating pool of GDF11 + myostatin as a potential pathogenic factor in human heart disease. However, conflicting human data have also been published suggesting that higher levels of GDF11 in adults with severe aortic stenosis are associated with an increased risk of adverse events following valve replacement surgery (22). Thus, further studies are needed to understand how perturbations to the GDF11 and myostatin system might contribute to cardiovascular risk.
The effect of GDF11 deficiency on the heart also remains unclear, but prior studies have evaluated the effect of myostatin deficiency on the heart. $Mstn^{/-}$ mice are viable and have increased skeletal muscle weight as well as increased heart weight and heart weight to tail length, but they show no increase in heart weight to body weight ratio (10). $Mstn^{/-}$ mice also have mildly increased left ventricular volumes and mildly reduced systolic function at baseline but have an enhanced response to isoproterenol stress with a higher percent change in fractional shortening compared to wild type mice (10). Inducible cardiomyocyte-specific deletion of $Mstn$ leads to transient chamber dilation and impairment of systolic function; however, upregulation of $Mstn$ in non-cardiomyocytes occurs leading to an unexpected overall increase in $Mstn$ expression in whole heart extracts of knockout mice (3). Conversely, overexpression of $Mstn$ leads to decreased heart weights in males but not females (20). Despite similarities in sequence between mature myostatin and GDF11, there are structural differences between the two proteins that could lead to distinct effects with deletion of $Gdf11$ in cardiomyocytes that have not previously been described with cardiomyocyte-specific $Mstn$ deletion (25).

We sought to test the effects of reduced levels of GDF11 in the heart during young adulthood in mice. Because systemic germline deletion in the mouse ($Gdf11^{/-}$) results in a number of developmental defects and is lethal by 1 day of age, likely due to renal agenesis (14), we generated a mouse model to specifically delete $Gdf11$ only within cardiomyocytes. We chose to delete $Gdf11$ from cardiomyocytes for our initial study as opposed to other cardiac cell types given the demonstrated phenotype seen with cardiomyocyte deletion of $Mstn$ and challenges of deleting $Gdf11$ only in the heart if targeting other cell types. By crossing a cardiomyocyte-specific $Myh6-cre$ allele with a $Gdf11$-floxed allele (13), we were able to induce genomic excision of the regions encoding mature GDF11 protein exclusively in cardiomyocytes. Our
experiment was designed to test the hypothesis that postnatal Gdf11 deficiency in cardiomyocytes would promote cardiac hypertrophy. We found instead that targeted cardiomyocyte deletion of Gdf11 during young adulthood, using the Myh6-cre system, does not result in cardiac hypertrophy and rather leads to progressive left ventricular dilation that is apparent in both females and males by 6 months of age. However, because of multiple adverse effects from Cre recombinase itself on the heart and potential differential expression of the cre gene across genotypes, we are unable to define the molecular mechanism of the dilated cardiomyopathy phenotype that develops when the Gdf11 gene is removed from cardiomyocytes using this mouse model.

**Materials and Methods**

**Animals – constitutively active Cre**

Mice expressing constitutively active Cre recombinase driven by the Myh6 promoter (B6.FVB-Tg(Myh6-cre)2182Mds/J) were obtained from The Jackson Laboratory. Mice containing a floxed (flanking loxP) Gdf11 allele with loxP sites flanking exons 2 and 3 of Gdf11 were generously provided by Dr. Se-Jin Lee (Johns Hopkins University) (13). Mice were bred to obtain three genotypes used in this study: Myh6<sup>cre/wt</sup>; Gdf11<sup>fl/fl</sup> (experimental genotype), Myh6<sup>cre/wt</sup>; Gdf11<sup>wt/wt</sup> (Cre-only control genotype, referred to as Myh6<sup>cre/wt</sup>), and Myh6<sup>wt/wt</sup>; Gdf11<sup>fl/fl</sup> (flox-only control genotype and littermate control, referred to as Gdf11<sup>fl/fl</sup>). All mice were on a mixed C57Bl/6J and 6N background.

Young adult male and female mice of all three genotypes were weighed weekly starting at 2 months of age. Echocardiograms were performed in all mice at 1-2 and 6 months of age in a...
blinded manner. Additional echocardiogram time points at 3 or 4 months of age were also performed in a subset of mice. Mice were observed for up to 6 months, and then animals were euthanized and serum and tissues harvested for further analysis. We also determined tibia length as a normalizing parameter because GDF11 administration has been shown to decrease body weight (17). Tissues for downstream DNA, RNA and protein analysis were flash frozen in liquid nitrogen and stored at -70°C until further processing was performed. All experiments were conducted according to the Guide for the Use and Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Harvard University Faculty of Arts and Sciences.

Animals – tamoxifen-inducible Cre

Mice expressing tamoxifen-inducible Cre recombinase (Mer-Cre-Mer, abbreviated MCM) driven by the Myh6 promoter (B6.FVB(129)-Alcflg(Myh6-cre/Esr1*)J/cf/J) were obtained from The Jackson Laboratory (backcrossed to a C57Bl/6J agouti background per vendor). Mice were bred with Gdf11fl/fl mice to obtain two genotypes used in this experiment: Myh6MCM/wt, Gdf11fl/fl (experimental genotype), and Myh6wt/wt; Gdf11fl/fl (flox control genotype). Male and female mice were stratified based on gender then randomized at 5 months of age to receive injections of either 4-hydroxytamoxifen (4OH-tamoxifen, 75 mg/kg) or vehicle (10% ethanol, 90% sunflower oil) via intraperitoneal (i.p.) injection for 5 days. Echocardiography was performed at baseline (at 5 months of age) and 4 weeks later (at 6 months of age) prior to harvest to evaluate left ventricular size and function. Tissues were harvested and processed for histology or qPCR.
Echocardiography

Mice were sedated with 0.1-0.5% inhaled isoflurane for echocardiography, with the dose titrated to maintain heart rates of >500 beats per minute for acquired images. Mice were placed on a heating pad, and echocardiograms were obtained with the Vevo770 (Visualsonics, Toronto, Ontario, Canada). M-mode was used to measure left ventricular (LV) interventricular septal (IVS) wall thickness, LV posterior wall thickness (LVPW), and LV internal diameter (LVID) during both systole and diastole. Fractional shortening (%), LV mass and LV volumes were calculated with the Visualsonics software package.

Cardiomyocyte and non-cardiomyocyte isolation from adult hearts

Cardiomyocytes and non-cardiomyocytes were isolated from adult male and female mice using a Langendorff-free method as previously described (1). We modified the original protocol to include blebbistatin (5 µM, Sigma) instead of 2-3-butanedione monoxime in the culture media. In addition, we used a peristaltic pump rather than hand injection to better control the flow rate of the perfused solutions. Briefly, mice were anesthetized with isoflurane then the chest cavity was opened. The descending aorta and inferior vena cava were cut followed by perfusion of 7 mL of EDTA buffer into the apex of the right ventricle. The aorta was then clamped and cut distal to the clamp, and the heart was removed. EDTA buffer (10 mL) was then perfused into the apex of the left ventricle followed by injection of 3 ml of perfusion buffer then 30-40 mL of collagenase buffer delivered into the left ventricular apex. The clamp was removed and the heart was manually dissociated. Stop buffer (perfusion buffer + 5% fetal bovine serum) was added, cells were passed through a 300 µm strainer then cardiomyocytes were allowed to gravity settle for 20 minutes. The supernatant containing non-cardiomyocytes and debris was plated into an
uncoated tissue culture plate in DMEM:F12/10% FBS for 3 hours. The cardiomyocyte fraction in
the pellet then underwent sequential gravity settling with low speed centrifugation (12 g x 3 min)
with calcium re-introduction followed by plating into laminin-coated plates for 3 hours. After 3
hours, both cardiomyocytes and non-cardiomyocytes were washed and harvested in Trizol.
Samples were frozen at -70 deg C until RNA extraction by QIAcube.

**PCR**

DNA was extracted from harvested tissue using the REDExtract-N-Amp Tissue PCR Kit
(Sigma). Table 1 shows the primers that were used to amplify *Myh6, Myh6-cre* and *Gdf11* PCR
products and their expected product sizes. PCR products were run on a 2% agarose gel in TAE
buffer at 100V for 45-60 minutes and gels were imaged in a Gel Doc EZ system (Bio-Rad).

**Quantitative PCR**

RiboZol reagent (VWR) and the E.Z.N.A. Total RNA I kit (Omega) were used to isolate
RNA from homogenized whole organ tissue, followed by the High Capacity cDNA Reverse
Transcription kit (Thermo Fisher Scientific) to reverse transcribe mRNA to cDNA according to
the manufacturers’ instructions. Quantitative PCR (qPCR) was performed using TaqMan probes
for *Nppb* (Mm01255770_g1), *Gdf11* (Mm01159973_m1, spanning exons 1-2), *Gdf15*
(Mm00442228_m1), *Inhba* (activin A, Mm00434339_m1), *Mstn* (Mm01254559_m1), and
*Tgfbr1* (Mm00436964_m1), with TATA-binding protein (*Tbp*) used as the housekeeping gene
(Mm01277042_m1) using a Bio-Rad CFX384 Real-Time System. Due to low yields of isolated
cardiomyocytes and non-cardiomyocytes from adult hearts, RNA was extracted using the Qiagen
QIAcube from Trizol (Thermo) followed by reverse transcription to cDNA using the
SuperScript VILO cDNA synthesis kit (Thermo) per the manufacturer’s instructions. The isolated cDNA (10-20 ng) was then pre-amplified using the Taqman PreAmp Master Mix (Thermo) for 14 cycles. The pre-amplified cDNA was then diluted 1:20 for quantification of Gdf11 expression in isolated cardiomyocytes and non-cardiomyocytes by qPCR on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems).

Flow cytometry

Flow cytometry was performed to quantify the percentage of cardiomyocytes in the cell population obtained immediately after the final step of the extraction procedure. In brief, the cardiomyocyte fraction was fixed with 1 mL of 70% ethanol then stored at -20 deg C. The non-cardiomyocyte fraction was plated into a six-well plate and allowed to expand for seven days in DMEM/F12 + 10% fetal bovine serum + 1% penicillin/streptomycin. Cells were dissociated with trypsin then fixed in 70% ethanol. The fixative was removed via low speed centrifugation (20 g x 3 min) and the cells were washed then permeabilized in 0.1% Triton in PBS. Cells were labeled overnight at 4 deg C using an antibody to cardiac troponin T (Abcam ab8295, diluted 1:250 in phosphate buffered saline (PBS) supplemented with 10% goat serum) to detect cardiomyocytes, and an antibody to vimentin (Abcam ab92547, diluted 1:200 in PBS supplemented with 10% goat serum), which is highly expressed in fibroblasts. After washing steps and incubation with the corresponding AlexaFluor 568 conjugated secondary antibody for 2h at room temperature (Thermo, Mouse IgG1 and Rabbit IgG, respectively), cells were analyzed by flow cytometry (MoFlo Astrios, Beckman Coulter, using the nozzle of 200µm). Data were analyzed with FlowJo software (version 10.0.8).
**Histology**

Harvested tissues were fixed in 4% paraformaldehyde for 24 hours then exchanged for 70% ethanol for 3 days prior to embedding in paraffin. Sections were stained with Masson’s trichrome staining as previously described (8). Global fibrosis was quantified using a Python script written to quantify the percentage of blue pixels out of the total pixels from a cross-sectional image of the heart (https://github.com/jgarbern/global-fibrosis). Average cardiomyocyte cross-sectional area for each mouse was quantified in a blinded manner with a total of 40 cardiomyocytes measured from multiple sections from each heart.

**Quantitative mass spectrometry**

Blood was obtained by retro-orbital collection at the time of harvest and transferred to serum separator tubes (SST). Tubes were spun at 2000 g for 5 min and serum was transferred to clean low binding microcentrifuge tubes and stored at -70 deg C until further processing. Samples (100 µL) were submitted to the Brigham Research Assay Core (BRAC) at Brigham and Women’s Hospital for quantitative mass spectrometry. Mouse serum was denatured, reduced and alkylated, followed by pH based fractionation using cation ion exchange solid phase extraction (SPE); appropriate elution fraction was digested with trypsin. After desalting and concentrating of tryptic digest, the peptide mixture was separated and eluted by liquid chromatography followed by mass spectrometric analysis operated in positive electrospray ionization mode. The most intensive and unique proteotypic peptides from GDF11 and myostatin as surrogated peptides along with heavy-labeled unique peptides as internal standards were used for quantitative determination of GDF11 and myostatin.
Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) unless noted otherwise. Data were evaluated with the D’Agostino and Pearson omnibus normality test; normally distributed data were evaluated with Student’s t-test or two-way ANOVA with Tukey post-hoc analysis, while data that were not normally distributed were analyzed with Mann-Whitney or Kruskal-Wallis with Dunn’s multiple comparisons test as indicated. Kaplan-Meier survival curves were evaluated the Mantel-Cox log-tank test. Body weight trends with time were analyzed with a two-way repeated measures ANOVA with Tukey multiple comparisons test. A p-value < 0.05 was considered statistically significant.

Results

Cardiomyocyte-specific deletion of Gdf11 leads to left ventricular dilation that progresses with age

Due to known sexual dimorphism in body weight and heart size in mice, we analyzed body weight, heart weight, and heart weight/body weight ratio data from males and females separately. Myh6^cre/wt; Gdf11^fl/fl mice had progressive left ventricular dilation with a significant increase in left ventricular end diastolic volume (Figures 1A (1-2 months), 1B (3-4 months), and 1C (6 months)), a significant decrease in septal thickness (Figure 1J) and a non-significant decrease in left ventricular posterior wall thickness (Figure 1L) by echocardiography that was apparent in both genders by 6 months of age. Male Myh6^cre/wt; Gdf11^fl/fl mice also had significantly increased left ventricular internal diameters at 6 months of age compared to sex-
matched Myh6<sup>cre/</sup>wt mice (Figure 1K). Cardiomyocyte-specific deletion of Gdf11 also led to a decrease in left ventricular function with decreased fractional shortening in Myh6<sup>cre/</sup>wt; Gdf11<sup>fl/fl</sup> females compared to sex-matched Gdf11<sup>fl/fl</sup> controls, and in Myh6<sup>cre/</sup>wt; Gdf11<sup>fl/fl</sup> males when compared to sex-matched Myh6<sup>cre/</sup>wt controls (Figure 1G). The chamber dilation and functional decline were not associated with differences in either estimated left ventricular mass by echocardiography, or heart weight compared to either the Cre-only control genotype (Myh6<sup>cre/</sup>wt) or the flox-only control genotype (Gdf11<sup>fl/fl</sup>) at 6 months of age (Figure 1H, 1I, 1F, respectively). Heart weight to body weight ratios were not different across groups at any age (Figures 1D (1-2 months), 1E (3-4 months), and 1F (6 months)). There were no significant differences in tibia lengths or heart weight to tibia length ratios at 6 months of age (Figures 1N and 1O, respectively).

We also utilized a tamoxifen-inducible cardiomyocyte deletion model with administration of 4OH-tamoxifen or vehicle to 6 month old Gdf11<sup>fl/fl</sup> or Myh6<sup>MCM/</sup>wt; Gdf11<sup>fl/fl</sup> mice for 5 days at 75 mg/kg delivered intraperitoneally, but saw no significant differences in left ventricular end diastolic volume, estimated left ventricular mass, body weight, heart weight, or heart weight to body weight ratio with this treatment regimen (Figures 2E, 2F, 2G, 2I, 2J respectively) despite evidence of recombination by PCR (Figure 2C). We did not see significant changes in Gdf11 mRNA expression following this treatment regimen by qPCR (Figure 2D). However, we observed significant differences in survival across all groups in males (Figure 2B) but not females (Figure 2A). The etiology for the survival difference in 4OH-tamoxifen-treated males is unclear, although we presume it reflects tamoxifen toxicity given the acute nature of the deaths. Survival of only the “healthiest” mice may also confound our long-term results. 4OH-tamoxifen significantly increased tibia length, therefore we did not evaluate heart weight to tibia...
length ratios in this model (Figure 2H); the effect of tamoxifen on long bone length has been reported previously (26). We also found significantly increased activin A (Inbha) mRNA expression within the spleens of 4OH-TAM treated Myh6MCM/wt; Gdf11fl/fl male mice, and a similar non-significant trend in other TAM-treated groups (Figure 2K), consistent with prior reports that tamoxifen may activate TGF-β signaling (4, 5, 15). We did not study this model in further depth due to the absence of a cardiac phenotype at the age selected (6 months of age).

We note that the different findings observed between tamoxifen-inducible Myh6-MCM versus constitutively active Myh6-cre models may be due to the different ages at which Gdf11 knockdown occurs in the two models. In addition, application of stress such as with pressure overload could be considered to enhance any potential phenotype caused with tamoxifen-induced Myh6-MCM expression. In the context of increased mortality in mice injected with 4OH-TAM even without surgery, and concerns for confounding effects from 4OH-TAM administration on TGF-β signaling, we chose not to apply pressure overload stress (e.g. transverse aortic constriction model) or repeat this experiment at a younger age in the tamoxifen-inducible cardiomyocyte deletion model and instead focused on the constitutively active Myh6-cre model for the remainder of this study.

**Cardiomyocyte-specific deletion of Gdf11 is not sufficient to decrease Gdf11 mRNA expression in the whole heart**

Myh6 Cre-induced genomic recombination of Gdf11 was evident in the heart in neonatal mice (Figure 3A). Gdf11 recombination was observed only in the heart by PCR and, as expected, was not seen in the lung, liver, kidney, spleen, or skeletal muscle at 6 months of age (Figure 3B). However, despite evidence of DNA recombination, we did not observe differences in Gdf11...
mRNA expression in the whole heart at 6 months of age (Figure 3D), and in fact saw a significant increase in Gdf11 mRNA expression in Myh6\textsuperscript{cre/wt}, Gdf11\textsuperscript{fl/fl} male mice at 3 months of age (Figure 3C).

Since approximately half of the whole heart is composed of non-cardiomyocytes (including endothelial, fibroblast and smooth muscle cells) (2), we isolated cardiomyocytes and non-cardiomyocytes from adult (>2 months) mice to evaluate Gdf11 mRNA expression in the two cell populations. In a representative batch, the cardiomyocyte population consisted of 84% cTnT+ cardiomyocytes, while the non-cardiomyocyte population consisted of 95% vimentin+ fibroblasts, as quantified by flow cytometry (data not shown). Gdf11 expression shows substantial variability in Myh6\textsuperscript{cre/wt} mice in this analysis; however, we detected a decreased signal in cardiomyocytes from Myh6\textsuperscript{cre/wt}, Gdf11\textsuperscript{fl/fl} mice and a trend toward differences in Gdf11 expression in cardiomyocytes across genotypes (p-value 0.05 by Kruskal-Wallis test) (Figure 3E).

The high variability in Gdf11 expression in Myh6\textsuperscript{cre/wt} mice suggested that this genotype may be abnormal, which became evident in subsequent analysis. The Gdf11 signal in Myh6\textsuperscript{cre/wt}, Gdf11\textsuperscript{fl/fl} mice was greater than zero likely due to contamination from non-cardiomyocytes in the cardiomyocyte population, though it is possible this reflects incomplete recombination of the floxed alleles. We did not observe any differences in Gdf11 expression in non-cardiomyocytes across genotypes (Figure 3F). Direct comparison across cell types is challenging due to different amounts of RNA per cell in different cell types. If starting with an equal amount of RNA, there was a trend toward increased Gdf11 expression in non-cardiomyocytes compared to cardiomyocytes in all genotypes (data not shown). However, we obtained much lower total RNA after isolation from non-cardiomyocytes (predominantly cardiac fibroblasts) compared to an
equal number of cardiomyocytes, and therefore comparing Gdf11 expression from the same amount of total RNA may be suboptimal.

Due to challenges in quantifying protein levels of GDF11 with antibody-based approaches (7), we measured GDF11 and myostatin levels in serum by quantitative mass spectrometry and found that Gdf11^fl/fl females had significantly lower circulating levels of GDF11 than Myh6^cre/wt females; however, there were no significant differences in GDF11 serum concentrations between Myh6^cre/wt, Gdf11^fl/fl mice and their sex-matched Cre- or flox-only control groups (Figure 3G). Although the major source(s) of circulating GDF11 remains unclear, prior work from our group demonstrated that the spleen has the highest mRNA levels among organs studied (12); thus it is not unexpected that serum levels of GDF11 were not altered in Myh6^cre/wt, Gdf11^fl/fl mice, as only cardiomyocyte Gdf11 was targeted for deletion in these animals. Circulating myostatin levels did not differ across groups (Figure 3H).

**Mechanism of left ventricular dilation confounded by adverse effects from Myh6-driven Cre recombinase activity**

Myh6^cre/wt male mice were significantly smaller than both Gdf11^fl/fl and Myh6^cre/wt, Gdf11^fl/fl mice by 6 months of age (Figure 1M), with a plateau in the body weight curve in Myh6^cre/wt mice appearing at around 4 months of age in both genders (Figures 4C-D). In addition, there was a non-significant trend toward decreased survival in Myh6^cre/wt mice with death in several mice at around 4 months of age (Figures 4A-B).

There was a significant increase in cardiac mRNA expression of Mstn in Myh6^cre/wt, Gdf11^fl/fl mice compared to Myh6^cre/wt mice at 2 months (Figure 5A), but not 6 months of age (Figure 5E). In contrast, at 6 months but not 2 months of age, Nppb, which encodes B-type
natriuretic peptide (BNP), a clinically used marker of heart failure, had significantly higher
expression in hearts of \textit{Myh6}^{cre/wt} mice compared to \textit{Gdf11}^{fl/fl} mice, with the experimental
genotype (\textit{Myh6}^{cre/wt}; \textit{Gdf11}^{fl/fl}) having an intermediate expression profile (2 months, Figure 5B;
6 months, Figure 5F). Expression of \textit{Tgfbr1}, which encodes one of the receptors for GDF11, was
also significantly lower in hearts of \textit{Gdf11}^{fl/fl} mice compared to both \textit{Myh6}^{cre/wt} mice and
\textit{Myh6}^{cre/wt}; \textit{Gdf11}^{fl/fl} mice at 6 months (Figure 5G) but not 2 months of age (Figure 5C). Finally,
expression of \textit{Gdf15}, which was reported to be upregulated following administration of
supraphysiologic doses of GDF11 (11), was significantly increased in hearts of \textit{Myh6}^{cre/wt} mice
compared to both \textit{Gdf11}^{fl/fl} mice and \textit{Myh6}^{cre/wt}; \textit{Gdf11}^{fl/fl} mice at 6 months (Figure 5H) but not 2
months of age (Figure 5D). These results suggest that cardiomyocyte deletion of \textit{Gdf11} has an
early effect on \textit{Mstn} expression, which precedes left ventricular dilation, and is followed later by
differential regulation on other proteins involved in TGF-\(\beta\) signaling. However, the differences
between the two control genotypes particularly at older ages suggest that the presence of Cre
recombinase has adverse effects on the heart and make it difficult to identify molecular
mechanisms to explain our echocardiographic findings in the experimental genotype.

\textbf{\textit{Myh6}-driven Cre recombinase expression leads to myocardial fibrosis and increased
cardiomyocyte size in males}

Cardiotoxicity has been previously described by Pugach et al. with prolonged \textit{Myh6}-
driven Cre expression in this strain (18), and our results are consistent with these prior findings.
We observed a significant increase in global myocardial fibrosis in males compared to females in
the Cre-only control genotype (\textit{Myh6}^{cre/wt}) at 6 months of age, with males more sensitive than
females to adverse effects from Cre, consistent with Pugach et al. (18) (Figures 6A and B). There
was a similar non-significant trend observed in $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice. In addition, there was a non-significant trend of increased global myocardial fibrosis in male mice expressing Cre recombinase compared to the flox-only control ($\text{Gdf11}^{\text{fl}/\text{fl}}$). Furthermore, the cardiomyocyte cross-sectional area was significantly increased in $\text{Myh6}^{\text{cre}/\text{wt}}$ male mice compared to either $\text{Gdf11}^{\text{fl}/\text{fl}}$ or $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice (Figure 6C).

**Cre mRNA expression is higher in $\text{Myh6}^{\text{cre}/\text{wt}}$ mice compared to $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice**

We evaluated whether Cre is differentially expressed across genotypes as a possible explanation for why $\text{Myh6}^{\text{cre}/\text{wt}}$ mice have a more pronounced phenotype in terms of body weight, myocardial fibrosis and expression of selected genes associated with heart failure such as $\text{Nppb}$. We found that cre mRNA expression is significantly greater in $\text{Myh6}^{\text{cre}/\text{wt}}$ mice compared to $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice (Figure 6D). In contrast to RNA levels, there were no differences noted in Cre protein expression in $\text{Myh6}^{\text{cre}/\text{wt}}$ mice compared to $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice (Figure 6E). No cre protein expression was detected in $\text{Gdf11}^{\text{fl}/\text{fl}}$ mice.

**Discussion**

We observed a significant increase in left ventricular end diastolic volume in mice with Cre-mediated genetic deletion of Gdf11 in cardiomyocytes. This finding was consistent in both males and females, with LV end diastolic volume significantly increased in $\text{Myh6}^{\text{cre}/\text{wt}}; \text{Gdf11}^{\text{fl}/\text{fl}}$ mice compared to both $\text{Myh6}^{\text{cre}/\text{wt}}$ and $\text{Gdf11}^{\text{fl}/\text{fl}}$ control mice. Left ventricular dilation was associated with a decrease in left ventricular systolic function, suggesting a possible role for GDF11 signaling in dilated cardiomyopathy. We observed that $\text{Mstn}$ and $\text{Gdf11}$ both transiently...
increase in RNA extracted from whole hearts of young adult Myh6^{cre/wt}; Gdf11^{fl/fl} mice prior to the onset of ventricular dilation, which suggests that there is a developmental component to the phenotype observed. However, our experimental design met numerous unanticipated challenges that prohibit clear interpretation of the underlying molecular mechanisms to explain these findings. First, we did not observe a significant decrease in Gdf11 expression in whole heart extracts from Myh6^{cre/wt}; Gdf11^{fl/fl} mice, suggesting that counter-regulation in non-myocytes may buffer cardiomyocyte-specific loss of Gdf11 in the heart. Second, mice expressing Cre recombinase had a different phenotype from mice not expressing Cre, with decreased body weights and increased global myocardial fibrosis in males, an effect only made clear because we utilized two control genotypes. Third, cre mRNA expression was different between the Cre control genotype and the Cre-containing experimental genotype. Although Cre protein levels were not different by western analysis, in the context of Cre-associated toxicity, further study of protein levels at different ages should be performed in future work to elucidate which perturbations to the cardiac system should be attributed to differences in Cre levels versus Gdf11 deletion.

Although we observed a non-significant trend toward decreased Gdf11 expression in isolated adult cardiomyocytes of Myh6^{cre/wt}; Gdf11^{fl/fl} mice (complete absence of Gdf11 could not be shown likely due to contamination from non-cardiomyocytes), we did not observe a significant decrease in Gdf11 expression in the whole heart despite constitutively active Myh6-driven Cre expression and PCR evidence of Gdf11 recombination. In fact, we observed a transient increase in Gdf11 expression in whole heart extracts from male Myh6^{cre/wt}; Gdf11^{fl/fl} mice compared to male Gdf11^{fl/fl} control mice at 3 months of age and a similar non-significant trend compared to male Myh6^{cre/wt} control mice. A similar effect was previously reported when
Mstn was targeted for deletion in cardiomyocytes using mice with tamoxifen-inducible Myh6-driven Cre expression, where Mstn mRNA expression increased in whole heart extracts due to upregulation in non-myocytes despite cardiomyocyte deficiency of myostatin (3). Due to different amounts of total RNA in cardiomyocytes versus non-cardiomyocytes, and low expression levels of Gdf11 in cardiomyocytes requiring pre-amplification of cDNA to detect a reliable signal by qPCR, we were unable to directly compare expression of Gdf11 in cardiomyocytes to non-cardiomyocytes. Nonetheless, in non-cardiomyocytes, we did not observe a difference in Gdf11 expression levels across genotypes. Taken together, this suggests that Gdf11 may act locally in cardiomyocytes, given the presence of a dilated phenotype even in the absence of expression differences at the organ level. In addition, we observed a significant increase in Mstn mRNA expression in whole heart extracts of 2 month old mice. This suggests that Cre-mediated cardiomyocyte deletion of Gdf11 leads to downstream signaling effects during development which precede the observed phenotype of left ventricular dilation starting at 3-4 months of age. We also did not examine other organs given the lack of serum differences in GDF11 at 6 months. Alternative models used in future work should focus on early downstream signaling changes in the heart and other organs (such as the spleen which has higher baseline mRNA expression) as well as measuring circulating GDF11 levels at younger ages to better understand how these changes affect cardiac phenotype at later time points.

Myh6<sup>cre/wt</sup> mice have previously been shown to develop progressive myocardial fibrosis and inflammation due to DNA damage at endogenous “loxp-like” or “pseudo-loxp” sites in the myocardium (18). In that study, the authors identified 227 loxp-like sites within genes that could be potentially recognized by Cre recombinase, when tolerating ≤4 mismatches in the canonical loxp sequence. Of these 227 degenerate loxp sites, 55 are expressed in the heart, leading to
numerous off-target effects from Cre recombinase including myocardial fibrosis and upregulation of apoptotic markers such as p53 and Bax (18). This previous study also found that males appear to be more sensitive to the off-target effects of Cre expression in cardiomyocytes, with an increased heart weight to body weight ratio in Myh6\textsuperscript{cre/wt} compared to wild type control (C57Bl/6J) male mice at 6 months of age (18). Although we did not have wild type mice as a control in our study, we also observed that Myh6\textsuperscript{cre/wt} males had significantly increased global fibrosis compared to females and Myh6\textsuperscript{cre/wt} males had significantly increased cardiomyocyte cross-sectional area compared to Gdf11\textsuperscript{fl/fl} control mice and Myh6\textsuperscript{cre/wt}; Gdf11\textsuperscript{fl/fl} knockout mice. It remains unclear whether the potency of Cre toxicity is identical in the presence or absence of loxP, or whether in the presence of loxP, there might be fewer off-target effects due to the stoichiometry of Cre:loxP versus Cre:loxP-like or pseudo-loxP sites. These results underscore the importance of inclusion of Cre control mice as well as evaluation of both genders when using Cre-lox technology.

We utilized both Myh6\textsuperscript{cre/wt} and Gdf11\textsuperscript{fl/fl} controls to attempt to account for adverse effects from Cre recombinase. Our results demonstrate that without inclusion of both controls, our data would have been easily misinterpreted with potentially incorrect conclusions drawn – a lesson that was learned from experience after we failed to include the Myh6\textsuperscript{MCM/wt} control line in the tamoxifen-inducible study described in Results above. For example, without Gdf11\textsuperscript{fl/fl} controls, we may have incorrectly concluded that deletion of Gdf11 leads to a decrease in Nppb expression. Conversely, without Myh6\textsuperscript{cre/wt} controls, we may have incorrectly concluded that deletion of Gdf11 leads to an increase in Nppb expression. However, with inclusion of both control genotypes, we see that there is actually a confounding effect with significant differences in expression of Nppb between Gdf11\textsuperscript{fl/fl} and Myh6\textsuperscript{cre/wt} control mice. It is challenging to
reconcile these differences among the different genotypes. It appears that the presence of Cre
induces stress on the mouse (with increased mortality, decreased body weight, increased Nppb
expression, and increased cardiomyocyte size). Comparing the Myh6^{cre/wt} control mice to
Myh6^{cre/wt}; Gdf11^{fl/fl} mice, one might conclude that cardiomyocyte deletion of Gdf11 is actually
cardioprotective (with increased survival, increased body weight, decreased Nppb expression and
decreased cardiomyocyte size). However, other explanations are possible as well, such as
differential off target effects in the presence or absence of true loxP sites, or differential
expression of Cre in the two genotypes. Given that comparison of Gdf11^{fl/fl} control mice to
Myh6^{cre/wt}; Gdf11^{fl/fl} mice leads to different conclusions than when comparing Myh6^{cre/wt} control
mice to Myh6^{cre/wt}; Gdf11^{fl/fl} mice, we are unable to definitively determine the mechanisms by
which Gdf11 deletion in cardiomyocytes leads to left ventricular dilation. Inclusion of multiple
control groups admittedly adds significant costs and time required to maintain a larger animal
colony, but careful selection of control groups is necessary to obtain meaningful results.

We observed higher cre mRNA but not protein expression in Myh6^{cre/wt} compared to
Myh6^{cre/wt}; Gdf11^{fl/fl} mice in this study. Variable cre RNA expression in different generations has
been reported in other Cre lines (19, 23). For example, in an albumin-Cre model, despite
transmission of albumin-cre in genomic DNA of successive generations, some mice did not
express cre in the liver (23). The authors speculated that that this could be due to multiple
homologous recombination events leading to segregation of inactive copies of the transgene,
binding of transcriptional inhibitors, or post-transcriptional silencing of cre (23). In addition,
cytosine methylation of loxP sites following Cre recombination of a parent can lead to inhibition
of Cre-mediated recombination in subsequent generations (19). Finally, although all mice were
on a mixed C57Bl/6J and 6N background, there may be generational differences due to different
degrees of backcrossing to the underlying genetic background. It is possible that there is a more complex interaction with Cre and the underlying genetic background which will be difficult to uncover. To try to address this confounding effect, we used littermate controls to compare Gdf11^{fl/fl} with Myh6^{cre/wt}; Gdf11^{fl/fl} mice. However, our breeding strategy paired Myh6^{cre/wt} with wild type (C57Bl/6J) mice and Gdf11^{fl/fl} with Myh6^{cre/wt}; Gdf11^{fl/fl} thus Myh6^{cre/wt} were not littermates with Myh6^{cre/wt}; Gdf11^{fl/fl} mice and may have had varying degrees of methylation or varying genetic backgrounds in the breeders. Future work to understand how Cre mRNA and protein levels change with age in both genotypes is necessary to interpret whether variable Cre levels might be confounding the phenotype seen with cardiomyocyte Gdf11 deletion.

In conclusion, deletion of the Gdf11 gene from cardiomyocytes may lead to left ventricular dilation and decreased systolic function, consistent with a dilated cardiomyopathy phenotype. However, due to numerous confounding factors associated with the selected, and commonly used, Cre-locx system as well as challenges in working with Gdf11 itself and its complicated regulatory system, we are unable to attribute a mechanism to this phenotype. These data highlight the importance of using appropriate control groups when using the Cre recombinase system, as opposite conclusions could have been drawn had only one of the two control genotypes been used for comparison. Further work to develop alternative animal models that avoid Cre toxicity as well as investigate alternative proteins involved in regulation of GDF11 expression are warranted.

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Disclosures

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Figure Captions

**Figure 1.** Targeted cardiac Gdf11 deletion leads to left ventricular dilation. (A-C) Left ventricular end diastolic volume by echocardiography at 1-2 months (A), 3-4 months (B), and 6 months of age (C). (D-F) Heart weight/body weight ratio at 1-2 months (D), 3-4 months (E), and 6 months of age (F). (G) Fractional shortening by echocardiography at 6 months of age. (H) Estimated left ventricular mass by echocardiography at 6 months of age. (I) Heart weight at 6 months of age. (J) Interventricular septal thickness during diastole at 6 months of age. (K) Left ventricular internal diameter during diastole at 6 months of age. (L) Left ventricular posterior wall thickness during diastole at 6 months of age. (M) Body weight at 6 months of age (just prior to harvest). (N) Tibia length at 6 months of age. (O) Heart weight to tibia length ratio at 6 months of age. Sample sizes at 1-2 months: Myh6cre/wt (n=5 females, n=9 males), Gdf11fl/fl (n=11 females, n=21 males), and Myh6cre/wt; Gdf11fl/fl (n=12 females, n=15 males) mice; n=4/group for heart weight to body weight ratio parameter. Sample sizes at 3-4 months: Myh6cre/wt (n=9 females, n=12 males), Gdf11fl/fl (n=9 females, n=13 males), and Myh6cre/wt; Gdf11fl/fl (n=13 females, n=14 males) mice for echocardiography; n=4/group for heart weight to body weight ratio parameter. Sample sizes at 6 months: Myh6cre/wt (n=6 females, n=16 males), Gdf11fl/fl (n=3 females, n=5 males), and Myh6cre/wt; Gdf11fl/fl (n=6 females, n=9 males) mice, *p<0.05, **p<0.01 by Kruskal-Wallis test with Tukey post-hoc analysis.

**Figure 2.** Survival curves, echocardiographic, heart weight, and body weight parameters in mice with tamoxifen-inducible Myh6-driven Cre expression (Myh6-MCM). (A and B) Kaplan-Meier survival analysis in Gdf11fl/fl (gray), and Myh6MCM/wt; Gdf11fl/fl (black) female (A) and male (B)
mice. Male mice have significant differences between groups with p<0.01 by the Mantel-Cox test. n=12-16/group at start of study. (C) Recombination of *Gdf11* gene seen in the heart but not liver, kidney, spleen or skeletal muscle following administration of 4-hydroxytamoxifen (75 mg/kg i.p. x 5 days) by polymerase chain reaction (PCR). (D) *Gdf11* expression in RNA extracted from whole heart 1 month after 4-hydroxytamoxifen administration in female and male mice by quantitative PCR (qPCR), n=3/group. Data are normalized to TATA-binding protein (*Tbp*) then to female *Gdf11*^fl/fl^ vehicle control. For echo and harvest data in (E) to (J), n=7-16/group, data collected 1 month after initiation of 4OH-tamoxifen injection (6 months of age). (E) Left ventricular end diastolic volume (µl) by echocardiography. (F) Estimated left ventricular mass (mg) by echocardiography. (G) Body weight (g) at harvest. (H) Tibia length, **p<0.01, ***p<0.001 by two-way ANOVA. (I) Heart weight and (J) Heart weight/body weight ratio. (K) Activin A mRNA expression in spleen is significantly increased in male *Myh6^MCM/wt; Gdf11^fl/fl^* mice 1 month after initiation of 4OH-tamoxifen injection (6 months of age), n=4-6/group, *p<0.05 by Kruskal-Wallis with Dunn’s multiple comparisons test. Data are normalized to *Tbp* then to female *Gdf11*^fl/fl^ vehicle control.

**Figure 3.** Targeted cardiomyocyte deletion of *Gdf11* does not decrease total *Gdf11* mRNA expression in mouse hearts. (A) Representative agarose gel image depicting *Myh6* (wild type band at 894 bp, *Myh6-cre* band at 300 bp) and *Gdf11* (wild type band at 359 bp, flox band at 393 bp, and Δ2-3 (post-recombination) band at 300 bp) alleles seen in the heart on day of life 0-1 by polymerase chain reaction (PCR) in *Myh6^cre/wt; Gdf11^fl/fl^* and *Myh6^cre/wt; Gdf11^fl/fl^* pups. (B) Recombination of *Gdf11* at 6 months of age in females and males in the heart but not in lung, liver, kidney, spleen or skeletal muscle by PCR. (C and D) *Gdf11* expression in RNA extracted...
from whole heart in (C) 3 month old (n=4/group) or (D) 6 month old (n=3-4/group) female and
male mice by quantitative PCR (qPCR). Data are normalized to TATA-binding protein (Tbp)
then to female Myh6^{cre/wt} control. (E and F) Gdf11 expression in RNA extracted from isolated
adult (>2 months old) cardiomyocytes (E) or non-cardiomyocytes (F), with each data point
(shown with open symbols) representing isolated cells from a single mouse of the same
genotype, n=3 per group (2 males, 1 female). Data normalized to Tbp then Myh6^{cre/wt} control.
Cardiomyocytes (E), p-value = not significant (n.s., 0.05); non-cardiomyocytes (F), p-value =
n.s. (0.3) by Kruskal-Wallis test. (G and H) Serum levels of GDF11 (G) and myostatin (H) as
determined by quantitative mass spectrometry in 6-month-old mice. n=3-4/group, *p<0.05 by
Kruskal-Wallis test followed by Tukey’s multiple comparisons test.

Figure 4. Cre recombinase has adverse effects on survival and body weight. (A and B) Kaplan-
Meier survival analysis in (A) female and (B) male Myh6^{cre/wt} (dark gray, dashed line, n=12
females, n=24 males), Gdf11^{fl/fl} (light gray, dotted line, n=5 females, n=11 males), and
Myh6^{cre/wt}; Gdf11^{fl/fl} (black solid line, n=12 females, n=15 males) mice. (C and D) Body weight
versus age in weeks in (C) female and (D) male Myh6^{cre/wt} (gray circles with dashed line, n=5
females, n=13 males), Gdf11^{fl/fl} (gray squares with dotted line, n=5 females, n=11 males), and
Myh6^{cre/wt}; Gdf11^{fl/fl} (black tringles with solid line, n=12 females, n=15 males) mice. *p<0.05,
**p<0.01 by two-way repeated measures ANOVA with Tukey’s multiple comparisons test.

Figure 5. Gene expression analysis of in 2 month old (A-D) and 6 month old (E-H) from whole
hearts of (A and E) Mstn, (B and F) Nppb, (C and G) Tgfbr1, and (D and H) Gdf15 by qPCR.
Data are normalized to Tbp expression then to Myh6^{cre/wt} control. No significant differences were
observed between genders therefore data represent combined male and female data. n=7-8/group, *p<0.05, **p<0.01, ***p<0.001 by Kruskal-Wallis analysis with Dunn’s multiple comparisons test.

**Figure 6.** Cre mRNA expression is higher in Myh6^{cre/wt} compared to Myh6^{cre/wt}; Gdf11^{fl/fl} mice and is associated with myocardial fibrosis and increased cardiomyocyte size. (A) Representative histology sections of male and female Myh6^{cre/wt}, Gdf11^{fl/fl}, and Myh6^{cre/wt}; Gdf11^{fl/fl} mice stained with Masson’s trichrome stain. (B) Global fibrosis (% blue pixels) shows increased fibrosis in male (n=5-9/group) Myh6^{cre/wt} mice compared to females (n=3-5/group). (C) Cardiomyocyte cross-sectional area is increased in male Myh6^{cre/wt} mice compared to male Gdf11^{fl/fl} and Myh6^{cre/wt}; Gdf11^{fl/fl} mice. Cross-sectional area from 40 cardiomyocytes per mouse were averaged for each mouse then average cardiomyocyte cross-sectional area by mouse data were analyzed with n=3-6 female mice, n=5-10 male mice, *p<0.05, **p<0.01 by two-way ANOVA with Tukey post-hoc analysis. (D) Cre expression by qPCR from male and female 3-6 month old mice. No significant differences were detected between genders therefore data depict combined male and female data. Data are normalized to Tbp then to Myh6^{cre/wt} control. n=16/group, *p<0.05 by Mann-Whitney test. (E) Cre protein detected by Western analysis in Myh6^{cre/wt}, Gdf11^{fl/fl}, and Myh6^{cre/wt}; Gdf11^{fl/fl} 4 month old male mice. Band densitometry analysis comparing Myh6^{cre/wt} mice to Myh6^{cre/wt}; Gdf11^{fl/fl} (not shown) is not significant with p-value 0.2 by Mann-Whitney test.
Figure 1. Targeted cardiac Gdf11 deletion leads to left ventricular dilation

Left ventricular end diastolic volume

A. Age 1-2 months

B. Age 3-4 months

C. Age 6 months

Heart weight/body weight ratio

D. Age 1-2 months

E. Age 3-4 months

F. Age 6 months

Additional echocardiographic and morphometric parameters at 6 months

G

H

I

J

K

L

M

N

O
**Figure 2.** Survival curves, echocardiographic, heart weight, and body weight parameters in mice with tamoxifen-inducible Myh6-driven Cre expression

A. **Females**

- Gdf1^fl/fl^ vehicle
- Gdf1^fl/fl^, 4OH-TAM
- Myh6^floxed/fl^; Gdf1^fl/fl^, vehicle
- Myh6^floxed/fl^; Gdf1^fl/fl^, 4OH-TAM

B. **Males**

- **p<0.01 by Mantel-Cox**

C. **C**

D. Relative mRNA expression of GDF11

E. LV end diastolic volume (µl)

F. Estimated LV mass by echo (mg)

G. Body weight at harvest (g)

H. Tibia length (mm)

I. Heart weight (mg)

J. Heart weight/body weight ratio (mg/g)

K. Relative mRNA expression of Activin A

Downloaded from www.physiology.org/journal/ajpheart at Biblio der Rijksuniversiteit (129.125.166.190) on June 13, 2019.
Figure 3. Targeted cardiomyocyte deletion of Gdf11 does not decrease Gdf11 mRNA expression in mouse hearts.
Figure 4. Cre recombinase has adverse effects on survival and body weight.

A

Females

B

Males

C

Females

D

Males

Percent survival

Months

Percent survival

Months

Body weight (g)

Age (weeks)

Body weight (g)

Age (weeks)

- Myh6<sup>cre/wt</sup>
- Gdf11<sup>fl/fl</sup>
- Myh6<sup>cre/wt</sup>; Gdf11<sup>fl/fl</sup>

- Myh6<sup>cre/wt</sup>
- Gdf11<sup>fl/fl</sup>
- Myh6<sup>cre/wt</sup>; Gdf11<sup>fl/fl</sup>
Figure 5. Gene expression analysis of Mstn, Nppb, Tgfr1, and Gdf15 by qPCR

Age 2 months

A  Mstn  B  Nppb  C  Tgfr1  D  Gdf15

Age 6 months

E  Mstn  F  Nppb  G  Tgfr1  H  Gdf15
Figure 6. Cre mRNA expression is higher in Myh6\textsuperscript{cre/WT} compared to Myh6\textsuperscript{cre/WT}, Gdf11\textsuperscript{flo/flo} mice and is associated with myocardial fibrosis and increased cardiomyocyte size.
Table

Table 1. Primers for genotyping by PCR

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<th>Primers</th>
<th>Product size (bp)</th>
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*Gdf11* primers from McPherron et al.(13)