Circulating Growth Differentiation Factor 11/8 Levels Decline With Age


Rationale: Growth differentiation factor 11 (GDF11) and GDF8 are members of the transforming growth factor-β superfamily sharing 89% protein sequence homology. We have previously shown that circulating GDF11 levels decrease with age in mice. However, a recent study by Egerman et al reported that GDF11/8 levels increase with age in mouse serum.

Objective: Here, we clarify the direction of change of circulating GDF11/8 levels with age and investigate the effects of GDF11 administration on the murine heart.

Methods and Results: We validated our previous finding that circulating levels of GDF11/8 decline with age in mice, rats, horses, and sheep. Furthermore, we showed by Western analysis that the apparent age-dependent increase in GDF11 levels, as reported by Egerman et al, is attributable to cross-reactivity of the anti-GDF11 antibody with immunoglobulin, which is known to increase with age. GDF11 administration in mice rapidly activated SMAD2 and SMAD3 signaling in myocardium in vivo and decreased cardiac mass in both young (2-month-old) and old (22-month-old) mice in a dose-dependent manner after only 9 days.

Conclusions: Our study confirms an age-dependent decline in serum GDF11/8 levels in multiple mammalian species and that exogenous GDF11 rapidly activates SMAD signaling and reduces cardiomyocyte size. Unraveling the molecular basis for the age-dependent decline in GDF11/8 could yield insight into age-dependent cardiac pathologies. (Circ Res. 2016;118:29-37. DOI: 10.1161/CIRCRESAHA.115.307521.)

Key Words: aging ■ Gdf11 protein, mouse ■ intercellular signaling peptides and proteins ■ Mstn protein, mouse ■ transforming growth factor-β

Both growth differentiation factor 8 (GDF8) and GDF11 are produced normally as inactive proproteins, which form a disulfide-linked homodimer after synthesis1 (Figure 1). Subsequent cleavage by one of the family of furin-type proprotein convertases generates a noncovalently associated latent complex, consisting of a ∼37-kDa aminoterminal inhibitory prodomain and 2 disulfide-linked ∼12.5-kDa carboxyterminal monomers, which form the mature, biologically active dimer.1,2 GDF8, or myostatin, is a well-studied regulator of skeletal muscle growth.1–3 Although both myostatin and GDF11 regulate skeletal muscle patterning, GDF11 is not redundant for anterior–posterior patterning of the axial skeleton.5 In addition, GDF11 has been implicated in governing islet progenitor cell number and maturation in the development of mouse pancreas.4

In 2013, we reported an apparent age-dependent decline in circulating GDF11 proteins in mice and demonstrated that restoring GDF11 to youthful levels reduces cardiac hypertrophy in older mice.6 However, GDF11 and GDF8 are highly homologous at the protein level, and recent publications from Egerman et al7 and Smith et al8 reported that key reagents that recognized GDF11 also recognize GDF8. Egerman et al7 also reported that GDF11/8 levels increase in mouse serum with age, based on the quantification of 2 Western analysis bands...
of different molecular weights (≈12.5 and ≈25 kDa), one of which (≈25-kDa band) showed a dramatic increase with age. A recent report by Smith et al also argues that GDF11 lacks an antihypertrophic effect in vitro and that raising circulating GDF11 levels in vivo has no effect on cardiac hypertrophy.

Here, we show that the ≈25-kDa band reported by Egerman et al is not GDF11 but predominantly immunoglobulin light chain; immunoglobulins have long been known to increase with age in C57BL/6 mice. We also show that circulating GDF11/8 levels decrease with age in mice and other mammalian species and that increasing GDF11/8 levels with exogenous GDF11 regulates cardiomyocyte size. In addition, new data from large human cohorts show that GDF11/8 declines with age, and low GDF11/8 levels are associated with an increased risk of pathological cardiovascular events and death.

These data suggest that the age-dependent decline in circulating GDF11/8 levels in mammals may have clinical relevance.

Methods

Animal Care and Usage
All animal studies were performed as approved by the Harvard Committee on Animals. Aged (22-month old) C57Bl/6 mice and serum from 4-month-old and 18-month-old rats were obtained from the National Institute on Aging. Rag1 knockout male retired breeders (7-month old) and age/sex-matched wild-type retired breeders were obtained from Jackson Laboratories (Rag 1 knockout: 002216; wild-type: 000664). Serum from horses and sheep was obtained from BioChemed Services, VA.

Western Analysis
Serum and protein samples (Figures 2 and 3) were prepared with loading buffer containing Tris–HCl (50 mmol/L, pH 6.8), dithiothreitol (100 mmol/L), β-mercaptoethanol (4%), sodium dodecyl sulfate (2%), bromophenol blue (0.1%), and glycerol (10%). Heart tissues (Figure 4) were lysed in radioimmunoprecipitation assay buffer (25 mmol/L Tris–HCl [pH 7.6], 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) freshly supplemented with 1 mmol/L of phenylmethanesulfonyl fluoride (Sigma-Aldrich), proteinase inhibitor (Sigma), protein phosphatase inhibitors 2 and 3 (Sigma-Aldrich), and 1.25% β-mercaptoethanol (Sigma-Aldrich). All samples were boiled 5 minutes at 95°C, loaded on NuPAGE 4% to 12% Bis-Tris gels (LifeTechnologies) and run with NuPAGE MES sodium dodecyl sulfate running buffer (LifeTechnologies) supplemented with sodium metabisulfite (5 mmol/L). Proteins were then transferred on polyvinylidene fluoride (PerkinElmer Life Sciences) or nitrocellulose (Amersham Protran, GE Healthcare) membranes in transfer buffer containing 10 mmol/L of Tris–HCl, 200 mmol/L of glycine, and 20% methanol. After transfer, membranes were incubated with primary antibodies at 4°C overnight. Proteins were detected with horseradish peroxidase–conjugated antibodies and enhanced chemiluminescence (Amersham GE Healthcare, RPN2236).

Nonstandard Abbreviations and Acronyms

GDF growth differentiation factor

Figure 1. Growth differentiation factor 11 (GDF11)/8 processing schematic diagram. Schematic representation of GDF11/8 protein structure and the corresponding processing leading to the mature and active ligands. The active ligand is reduced to 2 ≈12.5-kDa monomers in the presence of reducing agent. SP indicates signal peptide.
For Coomassie staining, gels were incubated at room temperature for 15 minutes with fixing solution (25% isopropyl alcohol and 10% glacial acetic acid) and incubated overnight with PageBlue Protein Staining Solution (LifeTechnologies).

**IgG Depletion From Mouse Serum**

Old mouse serum (400 μL) was diluted 2-fold in modified PBS buffer containing 0.6 mol/L of NaCl and 0.02% of NP-40. IgG was depleted from mouse serum by protein G sepharose absorption. Diluted serum was incubated with 300 μL of Protein G sepharose for 1.5 hours to further remove residual IgG from the protein G–depleted serum. IgG captured by protein G sepharose was washed extensively with modified PBS and eluted by 0.1 mol/L of glycine, pH 2.8. Eluted IgG fractions were neutralized by adding 1/30 of a volume of 2 mol/L of Tris–HCl, pH 7.5.

**Primary Antibodies**

Anti-pSMAD2 (3108), anti-pSMAD3 (9520), anti-totSMAD (43B4), and anti-GAPDH (2118) are from Cell Signaling; anti-GDF11/8 (ab124721) and anticardiac troponin T antibody (ab8295) are from Abcam; anti-pSMAD3 from Millipore (07–1389) was used for immunostaining. Secondary antibodies: goat antirabbit IgG (H+L)-HRP conjugate, BIO-RAD 172 to 1019; goat antimouse IgG (H+L) secondary antibody, Alexa Fluor 594 conjugate (A-11032); goat antirabbit IgG (H+L) secondary antibody, Alexa Fluor 488 conjugate (A-11080); and wheat germ agglutinin, Alexa Fluor 647 conjugate (W-32466) antibodies are from LifeTechnologies.

**Mass Spectrometry**

Serum from a 22-month-old mouse was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gel as described, and the Coomassie stained gel was used to excise a fragment from the ~25-kDa region. The gel piece was washed with 100 mmol/L of ammonium bicarbonate (Sigma-Aldrich), reduced with 10 mmol/L of dithiothreitol, and alkylated with 55 mmol/L of iodoacetamide. Trypsin solution with 40 mmol/L of ammonium bicarbonate was added for an overnight incubation at 37°C to digest the sample. Peptides were then extracted from the gel slice by adding 0.5% trifluoroacetic acid and rinsed twice with 50% acetonitrile, and the sample volume was reduced by vacuum concentration. The sample was finally resuspended in 15 μL of 5% formic acid. Approximately half of the sample was used for matrix-assisted laser desorption ionization analysis (ABI model 4800 TOF/TOF; Applied Biosystems) after zip-tip clean up (Millipore), and the other half was submitted for liquid chromatography-mass spectrometry/mass spectrometry analysis (LTQ Orbitrap XT Mass Spectrometer (Thermo Fisher Scientific)). Data from both mass spectrometry platforms were searched using Matrix Science MASCOT search program against updated nonredundant database from National Center for Biotechnology Information.

**Immunofluorescence and Histology**

For paraffin sections (Figure 5C), mouse hearts were fixed with 4% paraformaldehyde and paraffin embedded. On deparaffinization and rehydration, samples were pretreated with boiling sodium citrate (10 mmol/L, pH 7.2) for 30 minutes, followed by incubation with primary antibodies at 21°C for 2 hours and then Alexa Fluor–conjugated secondary antibodies (Molecular Probes, Invitrogen). For cryosections (Figure 6B), mouse hearts were rinsed in 1× PBS and embedded in optimum cutting temperature compound and frozen. Sections (7 μm) were air dried and further processed through 4% paraformaldehyde and 0.5% Triton X-100. Blocking and incubation of primary and secondary antibodies were processed in 1% normal goat serum in 1× PBS. After counterstaining with 4',6-diamidino-2-phenylindole, sections were mounted and observed under fluorescence microscopy.
All quantitative analyses were performed in a blinded manner. All images were postprocessed in ImageJ (National Institutes of Health) software.

**Gene Expression Analysis**

Spleen, kidney, and quadriceps muscle were harvested from young (2 months) and old (24 months) C57Bl/6 mice and snap frozen in liquid nitrogen. Total RNA was extracted with Trizol (Life Technologies), and equal amounts of RNA were reverse transcribed to generate cDNA (Superscript III First Strand Synthesis SuperMix for qRT-PCR; Life Technologies). Real-time polymerase chain reaction analysis was performed on an Applied Biosystems 7900HT Real-Time PCR System using TaqMan primers for GDF11 (Mm01159973_m1, TaqMan Gene Expression Assays; Life Technologies). Relative quantification was calculated with the ΔΔCt method using hypoxanthine-guanine phosphoribosyltransferase (Mm01545399_m1, TaqMan Gene Expression Assay; Life Technologies) as a reference gene.

**Statistical Analysis**

Statistical comparison was performed by unpaired t test, ANOVA, or Mann–Whitney analysis for gene expression analysis. Statistical analysis was carried out with the Graphpad Prism 6 software, and statistical significance was assigned to differences with a P value of <0.05.

**Results**

**Age-Dependent Decrease of GDF11/8 Levels Across Multiple Species**

As GDF11 and GDF8 are highly homologous, they are difficult to distinguish biochemically and by most commercial antibodies, including the monoclonal antibody (Abcam) against GDF11 used by us and also by Egerman et al and Smith et al (data not shown). Thus, as in the study by Egerman et al, we refer to the proteins recognized by this antibody as GDF11/8 unless otherwise noted. To examine age-dependent variations in GDF11/8 levels, we compared circulating GDF11/8 protein levels across 4 different mammalian species at various ages using Western analysis (Figure 2A–2D). Western analysis under reducing conditions revealed that the ≈12.5-kDa band corresponding to the reduced monomer of GDF11/8 decreased dramatically with age in serum from mice (Figure 2A), rats (Figure 2B), horses (Figure 2C), and sheep (Figure 2D). We also identified the ≈25-kDa band described by Egerman et al, which showed an age-dependent increase in all 4 species analyzed.
Figure 4. IgG depletion from mouse serum reduces the 25-kDa band, but the 12.5-kDa band remains unchanged in old and young mice. A. The ±25-kDa band in serum from an old mouse is significantly removed after IgGs are depleted first with protein-G sepharose resin and further with a monoclonal antibody against mouse IgG (IgG depl.+anti-mIgG). Elution of IgG bound to protein G sepharose is shown in the far right lane. B. Western analysis showing that IgG depletion in sera from 3 pooled young mice (2-month old) was run on a reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and Coomassie stained. The protein band at 25-kDa was excised and submitted for mass spectrometry analysis.

To corroborate the results obtained in serum, we also examined Gdf11 gene expression changes as a function of age in multiple mouse tissues (Figure 2E). Old mice showed significant reduction in Gdf11 gene expression in the spleen and kidney by quantitative real-time polymerase chain reaction analysis compared with young mice, but no differences were observed in skeletal muscle tissue (n=6–9 mice). Studies by other groups have similarly reported equivalent abundance of Gdf11 mRNA found in skeletal muscle in young versus aged mice, rats, and humans.2

The key 25-kDa protein recognized by anti-GDF11 antibodies increases with age in serum and, as shown here, is predominantly serum IgG light chain.

Egerman et al7 claimed that the ±25-kDa band identified by the anti-GDF11 antibody (Abcam) used in their studies, and ours, corresponds to the dimeric and active form of GDF11/8. On the basis of this statement, they argued that serum GDF11 levels increase overall with age, in contradiction with our previous reports.5,11 A close inspection of the Coomassie loading gel in the study by Egerman et al7 (Figure 1C of the study by
Egerman et al. strongly suggested that the ≈25 kDa-band represents a high abundance ≈25-kDa protein in serum and not GDF11, as their Coomassie loading control shows a clear correlation with their Western analysis at ≈25 kDa. Because this ≈25-kDa protein appeared to be very abundant in serum and failed to migrate at ≈12.5 kDa under reducing conditions, we hypothesized that this band was not GDF11/8 but represented immunoglobulin light chain. Immunoglobulin light chain has a molecular weight of ≈25 kDa, and it has long been known that plasma concentrations of immunoglobulins increase with age in mammals, including mice. Indeed, Western analysis demonstrated that the anti-GDF11/8 antibody (Abcam) recognized both heavy (≈50 kDa) and light (≈25 kDa) chains of purified mouse IgG (Figure 3A and 3B) and also purified rat, sheep, and horse IgG (Online Figures I and II). We also found that the ≈25-kDa light chain band comigrated with the band identified as the GDF11 dimer by Egerman et al. in the serum of old mice and old rats.

To further corroborate the conclusion that Egerman et al. quantitated IgG light chain as GDF11, we performed Western analysis using the same GDF11/8 antibody on sera from mice deficient in recombinase activating gene 1 (Rag1 knockout), which are genetically incapable of producing immunoglobulin. As shown in Figure 3C, the ≈25-kDa band was absent in Rag1 knockout mice compared with wild-type mice, further suggesting that the ≈25-kDa band was indeed immunoglobulin light chain. To investigate whether the ≈12.5-kDa band could also correspond to IgG, we performed IgG depletion from serum pooled from 3 old mice (Figure 4A; Online Figure III) or 3 young mice (Figure 4B) using protein G sepharose resin followed by further immunodepletion with a monoclonal antimouse IgG antibody. Protein G and the antimouse IgG antibody bind specifically to IgG. Removal of IgG from old mouse serum nearly eliminated detection of the band at ≈25 kDa, showing that this band is primarily composed of serum IgG light chain and not GDF11/8. The ≈25-kDa band was
recovered on elution of IgG captured by protein G-sepharose (Figure 4A; Online Figure III). Importantly, IgG depletion from young mouse serum showed similar results with depletion of the 12.5-kDa band and the 12.5-kDa band remaining unchanged (Figure 4B). We also performed mass spectrometry analysis on serum samples pooled from 3 old mice to identify protein abundance in the 25-kDa region. As expected, the majority of proteins identified within the 25-kDa band corresponded to immunoglobulin light chain (Figure 4C and 4D). Collectively, our results unequivocally demonstrate that the 25-kDa band measured by Egerman et al as GDF11 corresponds to IgG light chain and increases with age, whereas the 12.5-kDa band decreases with age.

**GDF11 Activates SMAD Signaling in Cardiomyocytes**

GDF11 and GDF8 seem to activate the same receptors. The prodomain of GDF11 can inhibit its mature ligand function, whereas direct administration of mature functional GDF11 can activate downstream signaling, leading to phosphorylation of SMAD2. Exogenous GDF11 is expected to activate SMAD2 phosphorylation irrespective of whether the endogenous circulating protein is GDF11, GDF8, or both. To test the effects of GDF11 administration on intracellular signaling in vivo, we treated young and old mice with a single dose (1 mg/kg) of recombinant GDF11 via intravenous injection. As expected, SMAD2 phosphorylation was observed in the myocardium of young mice (Figure 5A and 5B) with nuclear localization of phospho-SMAD3 in both cardiomyocytes and nonmyocytes (Figure 5C; the patchiness observed in lower magnification is a fixation artifact) 1 hour after GDF11 injection. Aged mice showed higher basal levels of phosphorylated SMAD2, supporting previous studies suggesting that aged cells have reduced sensitivity to exogenous transforming growth factor-β attributable to constitutively active transforming growth factor-β signaling and to the increased presence of other transforming growth factor-β ligands.

**GDF11 Administration Reduces Heart Mass in Old and Young Mice**

We previously reported that administration of GDF11 at 0.1 mg/kg per day for 28 consecutive days reduced cardiac hypertrophy in old mice. To further characterize the antihypertrophic effect of GDF11, we performed a dose titration study in young and aged mice and observed a significant dose-dependent decrease of heart weight, normalized to tibia length, after only 9 days of treatment in mice receiving 0.5 or 1.0 mg/kg per day GDF11 (Figure 6A). Histological analysis of cardiac tissue (Figure 6B) revealed that GDF11 administration at 1.0 mg/kg per day for 9 days reduced cardiomyocyte cross-sectional area in young and old mice. Moreover, we observed that GDF11 significantly reduces body weight in young mice (1.0 mg/kg per d) and old mice (0.5 and 1.0 mg/kg per day; Online Table I). These results reveal that increasing circulating GDF11 levels with exogenous GDF11 can regulate cardiac hypertrophy in both young and old mice. However,
reductions in body weight suggest that in addition to direct signaling to the heart by exogenous GDF11, secondary signals from adipose tissue or other mechanisms may play a role in the antihypertrophic effect.

Discussion

In this study, we show that circulating GDF11/8 levels decline with age in multiple mammalian species, including mouse. Despite generating and testing dozens of custom monoclonal antibodies and commercial ELISA kits, we have been unable to identify precisely whether the decline is because of changes in GDF11, GDF8, or a combination of both. Similarly, an aptamer used in previous studies that recognizes an epitope of GDF11 also recognizes GDF8, but this aptamer against GDF11/8 also shows declining blood levels in humans with age.\(^10\) GDF11 and GDF8 are both processed to active mature ligands that are \(\approx 25\) kDa in total mass, but under reducing conditions, the active mature ligand is reduced to \(\approx 12.5\) kDa (Figure 1). Here, we demonstrate that the \(\approx 25\)-kDa band reported to increase with age in mouse serum and claimed to be the GDF11 dimer, as suggested by Figure 1 in the previous publication by Egerman et al,\(^7\) is actually IgG light chain. Our experiments revealed the affinity of the anti-GDF11/8 antibody (Abcam) for IgG light chain, as the signal dramatically diminished on IgG depletion. To corroborate this result, we performed mass spectrometry analysis and confirmed that IgG light chain is the predominant component of the \(\approx 25\)-kDa band (Figure 1). Comparison of the \(\approx 25\)-kDa band in old and young animals is consistent with the previously reported age-dependent increase in serum immunoglobulins,\(^9\) and thus, it is not surprising that Egerman et al\(^7\) confused the \(\approx 25\)-kDa band for GDF11. Given that the \(\approx 25\)-kDa band is immunoglobulin light chain, the data of Egerman et al\(^7\) actually show that GDF11/8 levels decline with age, as their data on the \(\approx 12.5\) kDa band and its decline with age are essentially identical to ours. A recent report from Rodgers and Eldridge\(^16\) suggested that levels of GDF11 in circulation may be too low to have physiological relevance; however, that conclusion should be viewed with caution, as it was based on an assay that shows selective detection of GDF8 (not GDF11), as confirmed by the study by Rodgers and Eldridge\(^16\) itself (Figure 1A). Moreover, previous studies applying mass spectrometry–based proteomics have confirmed the presence of GDF11 in the blood of both mice and humans.\(^17\)

Importantly, the potential relevance of declining circulating GDF11/8 levels in animals reported here has now been demonstrated in patients with coronary disease.\(^9\) Olson et al\(^10\) described 2 large, independent cohorts of patients with stable ischemic heart disease, in whom lower levels of circulating GDF11/8 were associated with higher risk of cardiovascular events, higher prevalence of left ventricular hypertrophy, and death.\(^10\) Furthermore, even in the relatively narrow age range of coronary patients compared with the general population, GDF11/8 levels declined with age.

Our group has described the potential role of exogenous GDF11 as an antihypertrophic factor. We previously demonstrated the antihypertrophic effect of GDF11 in mice by showing that administration of 0.1 mg/kg per day of GDF11 for 28 days reduced cardiac mass in aged mice.\(^6\) However, a recent article by Smith et al\(^8\) did not observe similar findings using the same dose of GDF11 (0.1 mg/kg per day) over the same period of 28 days. After our previous publication, but before this study, we became aware of batch-to-batch variations in the concentration of recombinant GDF11 protein. We reported this to the manufacturer, which confirmed with their own experiments that there was indeed substantial deviation of the actual amount of GDF11 from what was indicated by the company. After correction of this variation, we confirmed that the protein concentrations in the current lots are more reliable, and thus, we performed a more thorough study of dose-dependent effects of GDF11 on cardiac hypertrophy over a period of 9 days. As shown in Figure 6A, it seems that a minimum dose of 0.5 mg/kg per day leads to rapid reduction in cardiac mass. We speculate that differences between our initial study\(^1\) and the study by Smith et al\(^8\) could be attributable to variations in commercial protein lots that occurred before more rigorous quality control. Differences between our initial observations, the present results, and the results reported by Smith et al\(^8\) could be explained by a combination of different protein sources, protein refolding efficiencies, and concentrations used. As GDF11 is now being studied as an in vivo pharmacological agent rather than a cell biology reagent, attention to dose response and bioactivity is clearly important, as differences in protein refolding and preparation will affect any experiment.

It will be important to investigate the mechanisms causing the observed decrease in body weight induced by GDF11 (Online Table 1). Preliminary data from our laboratory show that administration of 0.5 mg/kg per day of GDF11 for a period of 9 days induces significant reduction in adipose tissue in old mice, whereas skeletal muscle tissue mass remains unchanged (unpublished data). These data suggest a potential indirect effect of adipose tissue on cardiac tissue initiated by exogenous GDF11. Thus far, it seems that circulating GDF11/8 levels in a given species and at a specific age are in a relatively narrow range. Because adding GDF11 to the systemic circulation has clear effects, but in a specific dose range, it is plausible that both hyperactivity and hypoactivity of this signaling system could lead to abnormal physiology. We speculate that sustained overexpression of GDF11/8 signaling could lead to adverse effects in some tissues, even if other tissues tolerate increases beyond the normal range. This scenario is common in mammalian hormonal systems, including thyroid, adrenal, and pituitary hormones.

In conclusion, our data show that circulating GDF11/8 levels decline with age in multiple mammalian species and that systemic administration of GDF11 protein rapidly stimulates SMAD signaling in the heart and reduces cardiac mass in both young and old mice. Because low circulating levels of GDF11/8 also predict future mortality in coronary patients, unraveling the specific contributions of circulating GDF8 versus GDF11 and determining how circulating levels of these ligands and their inhibitors affect myocardial biology are important goals. Potential approaches to distinguish GDF11 versus GDF8 have been taken by analyzing differences in the prodomains.\(^18,19\) Because the prodromains of GDF11 and GDF8 are substantially different, analyses of these prodromains and their
biological effects may yield significant differences that are not shown in studies of the mature GDF11 and GDF8 ligands.

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Disclosures
Harvard University and Brigham and Women’s Hospital have filed for intellectual property on Growth differentiation factor 11, listing Drs Wagers, Lee, and Loffredo and J.R. Pancoast as inventors. The other authors report no conflicts.

References

Novelty and Significance

What Is Known?

• Growth differentiation factor 8 (GDF8) and GDF11 share 89% amino acid identity and are secreted in a latent complex that becomes biologically active after proteolytic cleavage to a dimer consisting of 2 disulfide-linked ~12.5-kDa carboxyterminal monomers.
• Recent studies have questioned the antihypertrophic effects of increasing GDF11 in the circulation and the decline of GDF11 in the circulation with aging.

What New Information Does This Article Contribute?

• We show an age-dependent decline of circulating GDF11/8 in multiple species, including mouse.
• Daily injection of recombinant GDF11 leads to a dose-dependent reduction of cardiac mass (heart weight/tibia length ratio) and cardiomyocyte size with rapid activation of SMAD signaling.

Here, we show that the previously reported increase in GDF11 dimer with age is not GDF11 but predominantly immunoglobulin light chain; immunoglobulins have long been known to increase with age in C57BL/6 mice. Our data demonstrate that circulating GDF11/8 levels decrease with age in mice and other mammalian species and that systemic administration of GDF11 protein reduces cardiac mass in both young and old mice. Overall, this study provides evidence in support of the concept of GDF11 as an age-related antihypertrophic hormone.
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Supplemental Material

Supplemental Methods

Western blot analysis
Protein samples for Online Figure I and II run under reducing conditions were prepared with loading buffer containing Tris-HCL (50 mmol/L; pH 6.8), DTT (100 mmol/L), β-mercaptoethanol (4%), SDS (2%) bromophenol blue (0.1%) and glycerol (10%). Samples were boiled 5 min at 95°C, loaded on NuPAGE 4-12% Bis-Tris gels (LifeTechnologies) and run with NuPAGE MES SDS running buffer (LifeTechnologies) supplemented with sodium metabisulfite (5 mmol/L). For non-reducing gel, protein samples were prepared in the same loading buffer but without β-mercaptoethanol (4%), loaded on NuPAGE 4-12% Bis-Tris gels (LifeTechnologies) and run with NuPAGE MES SDS running buffer (LifeTechnologies). After the run, the non-reducing gel was incubated for 15 min at room temperature with running buffer, DTT (40 mmol/L) and β-mercaptoethanol (4%). Proteins form non-reducing and reducing gels were then transferred on polyvinylidene fluoride (PerkinElmer Life Sciences) or nitrocellulose (Amersham Protran, GE Healthcare) membranes in transfer buffer containing 10 mmol/L Tris-HCL, 200 mmol/L glycine and 20% methanol. Following transfer, membranes were incubated with blocking solution (5% non-fat dry milk in 10 mmol/L Tris-HCL pH 7.5, 0.5 mol/L NaCl and 0.1% Tween-20) for one hour and successively with primary antibodies over night. For Figure S1 (middle panel) no primary antibody was included in the overnight incubation. Proteins were detected with horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence (PerkinElmer Life Sciences). Band intensity was analyzed using ImageJ software. For Coomassie staining, gels were incubated at room temperature for 15 minutes with fixing solution (25% isopropyl alcohol and 10% glacial acetic acid) and incubated overnight with PageBlue Protein Staining Solution (LifeTechnologies). Purified immunoglobulin G protein samples were purchased from Abcam (mouse IgG), Rockland Antibodies & assays (rat IgG) and Bethyl Laboratories (sheep and horse IgG).
Online Figure Legends

Online Figure I. The Abcam antibody anti GDF11/8 recognizes immunoglobulins from multiple species. Western analysis on purified IgG from horse, sheep, rat, and mouse showing that the Abcam primary monoclonal antibody ab124721 anti-GDF11/8 reacts with all species of IgG light chain. Young and old serum is from mice (panel A). Panel B indicates that using the secondary antibody only gives negligible signal, indicating that the observed signal is contributed primarily by reactivity of the Abcam primary antibody to IgG. Panel A and B were processed in parallel. Coomassie stained gels (panel C) for the corresponding samples is also shown. M = molecular marker.

Online Figure II. Comparison of non-reducing and reducing conditions on 25 kDa and 12.5 kDa species. Western analysis on a gradient of recombinant GDF11 protein, purified mouse IgG and sera from one young (2 month old) and one old (22 month old) mouse. Left panel shows results from a non-reducing gel and right panel represents reducing conditions. This shows that reducing the recombinant GDF11 leads to a lower molecular weight, consistent with reduction of the disulfide bond in the GDF11 dimer. M = molecular marker.

Online Figure III. IgG depletion from mouse serum reduces the 25 kDa band in young mice. In order to visualize the decrease in 25 kDa band upon IgG depletion, 5ul per sample was loaded and analyzed by Western blot. Western blot analysis showing that IgG depletion in sera from three pooled young mice (2 month old) significantly decreases the 25 kDa band. Sample order and treatment is the same as in Figure 4B. The 12.5 kDa band is not visible because of the low amount of sample loaded compared to Figure 4B. M = molecular marker. * = empty well.

Online Table I. Heart weight and body weight dynamics in young and aged mice.
Heart weight (HW) and body weight (BW) were analyzed at time of sacrifice after 9 days of daily administration of GDF11 or saline (n = 6-12/group). Delta body weight (%) represents change in body weight from pre-treatment to time of sacrifice (day 9), normalized to pre-treatment weight. All data presented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 vs saline group in the correspondent age group.
Online Figure I

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1\textsuperscript{v} Ab Abcam + 2\textsuperscript{v} Ab anti-Rabbit (5 min exposure)

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2\textsuperscript{v} Ab anti-Rabbit only (5 min exposure)

C

<table>
<thead>
<tr>
<th>GDF11</th>
<th>Purified IgG</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Horse</td>
<td>Young</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>Old</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td></td>
</tr>
</tbody>
</table>

Coomassie
Online Figure II

Non-reducing gel

Reducing gel

GDF11 (ng)  Purified mouse IgG  Young  Old

GDF11 (ng)  Purified mouse IgG  Young  Old

100 50 25 12.5 6.25 100 50 25 12.5 6.25
Online Figure III

<table>
<thead>
<tr>
<th>Purified mouse IgG</th>
<th>Input 5μl</th>
<th>IgG depl. + anti mlG</th>
<th>Elution 5μl</th>
</tr>
</thead>
</table>

![Image of gel electrophoresis](image-url)
## Online Table I

| Group                  | Heart weight
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg)</td>
</tr>
<tr>
<td>Young saline</td>
<td>139.3 ± 11.14</td>
</tr>
<tr>
<td>Young GDF11 (0.1mg)</td>
<td>135.5 ± 11.48</td>
</tr>
<tr>
<td>Young GDF11 (0.2mg)</td>
<td>139.7 ± 8.43</td>
</tr>
<tr>
<td>Young GDF11 (0.5mg)</td>
<td>122.8 ± 12.29*</td>
</tr>
<tr>
<td>Young GDF11 (1mg)</td>
<td>128.8 ± 12.83</td>
</tr>
<tr>
<td>Old saline</td>
<td>164.5 ± 15.88</td>
</tr>
<tr>
<td>Old GDF11 (0.1mg)</td>
<td>173.6 ± 19.18</td>
</tr>
<tr>
<td>Old GDF11 (0.2mg)</td>
<td>168.6 ± 17.68</td>
</tr>
<tr>
<td>Old GDF11 (0.5mg)</td>
<td>149.6 ± 15.39</td>
</tr>
<tr>
<td>Old GDF11 (1mg)</td>
<td>147.2 ± 10.34</td>
</tr>
</tbody>
</table>