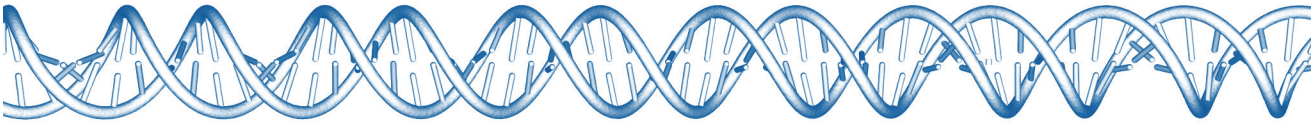

Cardiovascular Effects of GDF11

Biochemistry and Biology of GDF11 and Myostatin

Similarities, Differences, and Questions for Future Investigation

Ryan G. Walker, Tommaso Poggioli, Lida Katsimpardi, Sean M. Buchanan, Juhyun Oh, Sam Wattrus, Bettina Heidecker, Yick W. Fong, Lee L. Rubin, Peter Ganz, Thomas B. Thompson, Amy J. Wagers, Richard T. Lee

Abstract: **Growth differentiation factor 11 (GDF11) and myostatin (or GDF8) are closely related members of the transforming growth factor β superfamily and are often perceived to serve similar or overlapping roles. Yet, despite commonalities in protein sequence, receptor utilization and signaling, accumulating evidence suggests that these 2 ligands can have distinct functions in many situations. GDF11 is essential for mammalian development and has been suggested to regulate aging of multiple tissues, whereas myostatin is a well-described negative regulator of postnatal skeletal and cardiac muscle mass and modulates metabolic processes. In this review, we discuss the biochemical regulation of GDF11 and myostatin and their functions in the heart, skeletal muscle, and brain. We also highlight recent clinical findings with respect to a potential role for GDF11 and/or myostatin in humans with heart disease. Finally, we address key outstanding questions related to GDF11 and myostatin dynamics and signaling during development, growth, and aging. (*Circ Res*. 2016;118:1125-1142. DOI: 10.1161/CIRCRESAHA.116.308391.)**

Key Words: heart disease ■ ligands ■ myocardium ■ muscle

Growth differentiation factor 11 (GDF11), also known as bone morphogenetic protein 11 (BMP11), and its homolog myostatin (also known as GDF8) are closely related members of the transforming growth factor β (TGFβ) superfamily.[1,2] Myostatin plays an evolutionarily conserved role in antagonizing postnatal muscle growth, limiting both the number and size of individual muscle fibers.[1] Hence, disruption of the *myostatin* gene or targeted inhibition of myostatin protein triggers hypermuscular phenotypes in many mammals and fish.[3–5] Myostatin function also has been implicated in postnatal glucose metabolism and adipogenesis.[6] GDF11, in contrast, plays a broad role during mammalian development, regulating anterior/posterior patterning, formation of the kidney, stomach, spleen and endocrine pancreas, and olfactory neurogenesis.[2,7–11] GDF11's functions in postnatal tissues are less explored, partly because of the perinatal lethality of *Gdf11*-knockout mice,[2,7] which exhibit homeotic skeletal transformations, cleft palate, and renal agenesis (Table). Recent work identified GDF11 as a candidate hormonal regulator of aging in a variety of different organs. Consistent with this function, boosting levels of GDF11 protein in aged mice

Counterpoint, see p 1143
Response by Harper et al, see p 1142

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From the Department of Molecular Genetics, College of Medicine, University of Cincinnati, OH (R.G.W., T.B.T.); Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, Cambridge, MA (T.P., L.K., S.M.B., J.O., S.W., L.L.R., A.J.W., R.T.L.); Department of Neuroscience, Institut Pasteur, Paris, France (L.K.); Cardiovascular Division (T.P.), Department of Medicine, Brigham Regenerative Medicine Center, Brigham and Women's Hospital, Harvard Medical School, Boston, MA (Y.W.F., R.T.L.); Section on Islet Cell and Regenerative Biology, Joslin Diabetes Center, Boston, MA (J.O., S.W., A.J.W.); Division of Cardiology, Universitäres Herzzentrum, Zürich, Switzerland (B.H.); Department of Medicine, University of California, San Francisco (B.H., P.G.); and Division of Cardiology, San Francisco General Hospital, CA (P.G.).

*R.G. Walker and T. Poggioli are co-first authors.

Correspondence to Richard T. Lee, MD, Brigham Regenerative Medicine Center, 65 Landsdowne St, Cambridge, MA 02139. E-mail RLee@bwh.harvard.edu; or Amy J. Wagers, PhD, Department of Stem Cell and Regenerative Biology, Harvard University, 7 Divinity Ave, Cambridge, MA 02138. E-mail amy_wagers@harvard.edu

Nonstandard Abbreviations and Acronyms	
ALK	activin receptor-like kinase
BMP11	bone morphogenetic protein 11
FSTL3	follistatin-like 3
GASP	growth and differentiation factor–associated serum protein
GDF11	growth differentiation factor 11
rGDF11	recombinant GDF11
TGFβ	transforming growth factor β
TLD	tolloid-like

improves age-related phenotypes in the heart,[24] brain,[25] and skeletal muscle.[26] In addition, 2 studies recently implicated GDF11 as a negative regulator of erythroid differentiation in mouse aging and thalassemia models.[27,28]

Further highlighting the differences in myostatin and GDF11, *myostatin* mRNA is predominantly detected in skeletal and cardiac muscle, whereas *GDF11* mRNA is detected broadly in numerous tissues[12] and is most abundant in the kidney and spleen.[24] Both GDF11 and myostatin are found in the bloodstream, and while the functional implications of their circulation are still under investigation, their systemic presence implies that these proteins may act as hormonal signals. Given their high sequence similarity, it was expected that many of the features and functions of these 2 ligands should overlap. However, a growing number of studies have described disparities in their actions, sparking debate regarding their respective involvement in particular physiological processes. Here, we discuss the molecular properties of GDF11 and myostatin, their roles in regulating different organ systems, and the challenges encountered in studying these proteins, which have contributed to recent controversies about their biological roles.

Biochemical Regulation of GDF11 and Myostatin

The TGFβ family comprises >30 structurally related, yet functionally distinct ligands. This family can be subdivided into 3 subclasses: the TGFβs, BMPs, and activin/myostatins. GDF11 and myostatin belong to the activin/myostatin subclass and share 90% sequence identity within their mature, signaling domain. Similar to other TGFβ proteins, both GDF11 and myostatin are synthesized as precursor molecules where an N-terminal prodomain is cleaved from a C-terminal signaling or mature domain by a furin protease (Figure 1A). The mature ligands are propeller-shaped, disulfide-linked dimers that initiate signal transduction by engaging 2 type II receptors and two type I receptors using convex and concave surfaces, respectively[29] (Figure 2).

The molecular structure of myostatin has been extensively investigated, including 2 x-ray crystal structures of myostatin in complex with 2 known antagonists.[30,31] In contrast, GDF11 is less well characterized, and much of what is known for myostatin has been inferred for GDF11. However, the unbound x-ray crystal structure of GDF11 was recently determined revealing the classic propeller-shaped structure with subtle differences between myostatin and GDF11, particularly in receptor-binding epitopes.[32] Therefore, although many structural and regulatory mechanisms are shared between these 2 ligands, growing evidence also points to unique features of GDF11 and myostatin biology.

Role of the Prodomain in Latency and Activation

Although mature GDF11 and myostatin ligands share substantial sequence identity, their prodomains are only 52% identical (Figure 3). Like other TGFβ members, the GDF11 and myostatin prodomains aid in folding of the mature dimeric ligand.[34,35] However, unlike most TGFβ ligands, GDF11 and myostatin remain tightly bound to their prodomains after cleavage by furin-like proteases,[36–41] and are thereby held in a latent state, unable to bind receptors. Ligand activation requires additional cleavage of the prodomain by a tolloid-like (TLD) metalloproteinase.[38,39] Compared with other ligands, myostatin is inefficiently processed by furin, leaving a significant amount of unprocessed and presumably inactive protein.[36,37] However, a single-nucleotide polymorphism for the mutation K153R[42] dramatically improves furin processing, but has no effects on TLD activation[43] (Figure 3B). Interestingly, this allelic variant was found at higher frequency in 2 centenarian cohorts, when compared with controls,[44] although the implications of this polymorphism in terms of longevity and maintenance of muscle mass and strength have yet to be definitively established.[44–53] Although GDF11 has similar furin and TLD recognition sequences, it is not known if sequence variations, especially in the surrounding areas, which are more divergent, alter furin and/or TLD processing of GDF11 (Figure 3B).

Structural details of the latent state have yet to be described for GDF11 and myostatin. Still, despite differences in mechanism of activation (discussed below), the structure of TGFβ1 in complex with its prodomain[33] offers general insight into the molecular interactions driving latency. The latent structure of TGFβ1 has a ring-like appearance orchestrated by a centrally positioned mature dimer blanketed by the prodomains from each monomer[33] (Figure 3A). Ligand inhibition is mediated through interactions of the helical N terminus (α1) with the type I receptor site and a latency lasso where the prodomain wraps around the ligand fingertips toward the type II receptor site.[33] Consistent with this structure, myostatin prodomain residues 43–115 are necessary and sufficient to inhibit ligand activity.[54,55] Interestingly, this region contains the TLD proteolytic site[12,29] and is highly conserved with GDF11, suggesting that it serves a similar role in regulating GDF11 (Figure 3B).

Nonetheless, the mechanism for TGFβ activation differs from that of GDF11 and myostatin.[56,57] The TGFβ latent complex exists in the extracellular matrix covalently bound to the latent transforming growth factor β protein 1 (LTBP1) via N-terminal disulfide linkages, a feature not known to occur for GDF11 and myostatin.[58–60] In addition, the apex of the TGFβ latent complex interacts with $\alpha_V\beta_{VI}$ integrin.[56,57] The combination of these 2 interactions tether the latent TGFβ complex at both ends, such that cellular contractile forces release the mature TGFβ ligand from the prodomain.[33,56,57] Although there is no evidence that myostatin or GDF11 latent

Table. Comparison of Developmental Expression Patterns and Phenotypes in GDF11- and Myostatin-Deficient Mice

Tissue/Phenotype	MSTN	GDF11	
Predominant expression pattern	Developing and adult skeletal muscle[1]	Primitive streak and tail bud; Expressed in developing limb buds[2,7,12,13]	
	MSTN KO	GDF11 KO	MSTN/GDF11 DKO
Premature lethality	No[1,7]	Yes—perinatal[2,7]	Yes—born at expected ratio but none born alive[2]
Bone	NR	Anterior homeotic transformation of the axial skeletal (transformation of posterior vertebrae to anterior identity) via altered HOX gene expression on A/P axis[2]; increased frequency of cleft palate[7]	More severe homeotic transformations than GDF11KO; All have cleft palate; Additional skeletal defects, including limb defects (extra forelimbs, shortened limbs), Digit patterning defects (sixth digit)[7]
Kidney	NR	Most have Renal agenesis[14]	All have renal agenesis[7]
Pancreas	NR	Reduced pancreas size because of exocrine hypoplasia; 2- to 4-fold increase in endocrine progenitor cells by E18.[15] Increased number of islet progenitors[8]	NR
Olfactory epithelium	NR	Increased number of olfactory neurons and neuronal progenitors[10]	NR
Retina	NR	Increased number of retinal ganglion cells and reduced number of retinal amacrine cells and photoreceptors[11]	NR
Skeletal muscle	Myofiber hyperplasia and hypertrophy[1,16,17]	None reported (perinatal lethality)	NR (perinatal lethality)
Stomach	NR	Two-fold reduction in the thickness of gastric wall with reduced number of characteristic folds[8]	NR
Fat	Increased BW with decreased lipid content, decreased serum lipid and triglyceride levels[6,18,19]	None Reported—but analysis limited because of perinatal lethality	NR (perinatal lethality)
Heart	Increased HW and BW[20]	NR (perinatal lethality)	NR (perinatal lethality)
	Conditional MSTN KO in skeletal myofibers only (in MLC-cre X MSTN-flox)	Conditional GDF11 KO in skeletal myofibers only (in MLC-cre X GDF11-flox mice)	Conditional KO of GDF11 and MSTN in skeletal myofibers only
Adult skeletal muscle	Two-fold increase in young adult muscle mass (because of hyperplasia and hypertrophy); not apparent at birth (emerges postnatally); more glycolytic fibers (IIB)[7]	No increase in young adult muscle mass; no change in fiber type[7]	Same as MSTN conditional KO; no increase in phenotypic severity[7]
	Conditional MSTN KO in cardiac myocytes only	Conditional GDF11 KO in cardiac myocytes only	Conditional KO of GDF11 and MSTN in cardiac myocytes only
Cardiac myocytes	Did not prevent left ventricular decompensation after TAC.[21] Cardiac hypertrophy and heart failure, but cardiac function is restored after several weeks[22,23]	N/A	N/A

GDF11 and MSTN exhibit many similarities in their biosynthesis, regulation, receptor utilization, and intracellular signaling pathways. Yet, the consequences of loss of *Gdf11* or *MSTN* expression in mice are phenotypically distinct. Comparative analysis suggests only partial functional redundancy. See text for details. BW indicates body weight; DKO, double knockout; GDF11, growth differentiation factor 11; HW, heart weight; KO, knockout; MSTN, myostatin; NR, not reported; and TAC, transverse aortic constriction.

complexes are covalently bound to an LTBP, these latent complexes do interact with the extracellular matrix components LTBP3 and perlecan.[37,61] The purpose of these interactions remains unknown, but possibly relates to ligand activation, as binding to LTBP3 can prevent furin processing and overexpression of LTBP3 in skeletal muscle increase muscle mass.[37] Thus, interactions with extracellular matrix components may fine-tune the activity of myostatin and GDF11, and dissimilarities in GDF11 and myostatin prodomain sequences (Figure 3) could allow for unique extracellular matrix interactions across tissues.

Receptor Utilization by GDF11 and Myostatin

Similar to the activin-type ligands, both GDF11 and myostatin predominantly use the type II receptors activin receptor kinase II-A and type II receptors activin receptor kinase II-B and the type I receptors activin receptor-like kinase 4 (ALK4) and ALK5 to elicit signal transduction via SMADs 2 and 3[40,62,63] (Figure 2B). GDF11 also can signal through an additional type I receptor, ALK7, although its biological role remains undetermined.[62]

Unlike the BMPs, the TGFβ and activin/myostatin subclasses exhibit high affinity for the type II receptor and

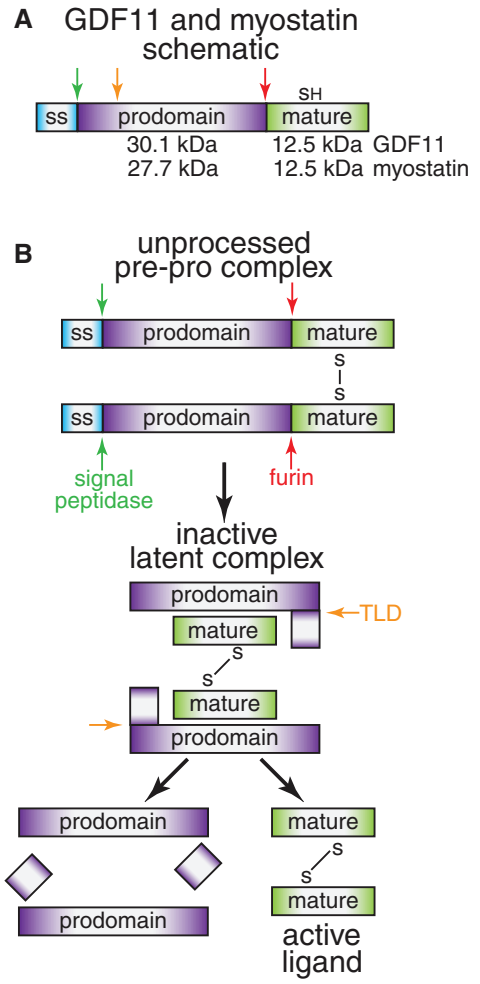

Figure 1. Biosynthesis and proteolytic processing of growth differentiation factor 11 (GDF11) and myostatin (MSTN). **A**, Schematic diagram of GDF11/MSTN monomer and relative position of proteolytic sites. **B**, Ordered proteolytic processing necessary to release an active dimer to elicit signaling.

low affinity for the type I receptor.[29] The type II receptors for the BMP and activin subclasses bind on the concave surface of the fingers, whereas TGFβs bind the type II receptor more distally, toward the fingertips.[29] This positioning facilitates a cooperative binding interaction between the type II and type I receptors, as shown by the structure of the TGFβ3:TBRII:ALK5 receptor complex.[64] In contrast, the receptors bind BMPs on opposite sides of the fingers, and thus are unable to interact with one another, as described in the BMP2: type II receptors activin receptor kinase II-B:ALK3 complex.[65] The ternary receptor configuration for activin/myostatin has yet to be determined, as detailed binding and structural studies have been hampered by the low affinity for their type I receptors.[29]

Using structural data as a guide to denote the approximate receptor interfaces,[64,66–68] it is likely that GDF11 and myostatin bind type II receptors similarly because residues in this location are identical (Figure 4). However, residues in the type I site, specifically the prehelix loop and wrist helix (Figure 4), are divergent between GDF11 and myostatin, suggesting that type I receptor binding might differ, especially in the utilization of ALK7. Supporting this notion, introduction of the myostatin prehelix loop into Activin A confers signaling through ALK5.[30] Similar chimeric protein studies should help to reveal the biological consequences of sequence differences between GDF11 and myostatin at the receptor interface. Furthermore, with growing evidence indicating the importance of coreceptors in assembling TGFβ ligand complexes,[69–76] further studies are needed to define their roles in GDF11 and myostatin signaling.

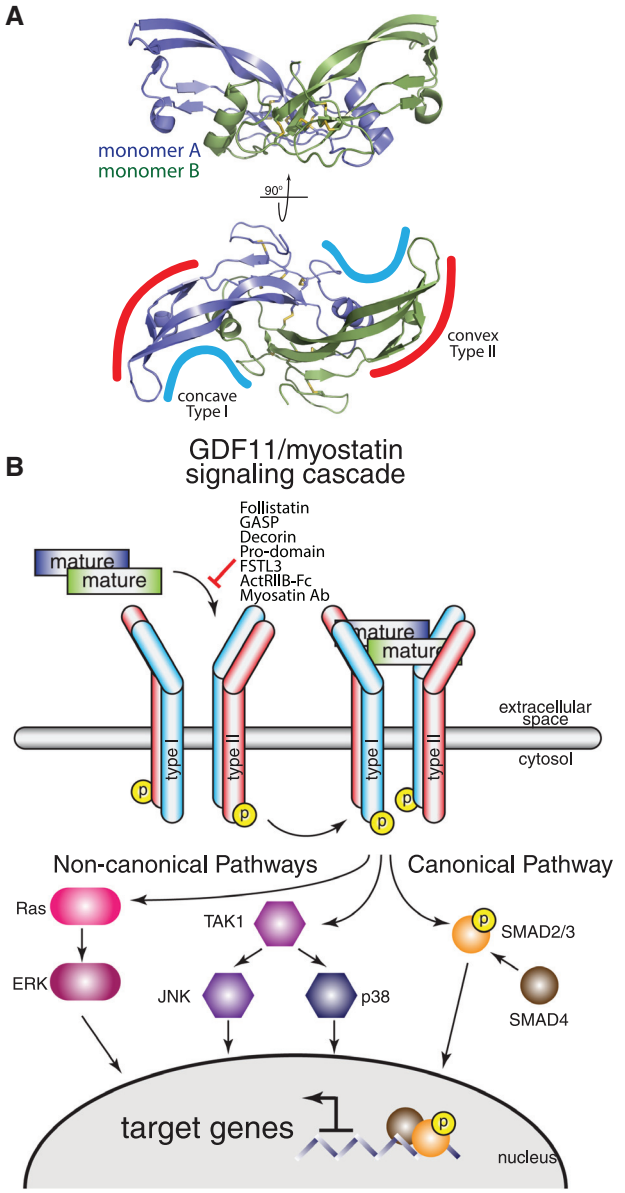

Figure 2. Structure of myostatin (MSTN) and reported elements of growth differentiation factor 11 (GDF11)/MSTN. **A**, The symmetrical MSTN dimer forms 2 distinct interfaces, concave, and convex, for receptor binding (PDB 3HH2[30]). **B**, GDF11 and MSTN induced canonical and noncanonical signaling. Known extracellular regulators and pharmacological inhibitors of GDF11 and MSTN are listed. FSTL3 indicates follistatin-like 3; and GASP, growth and differentiation factor–associated serum protein.

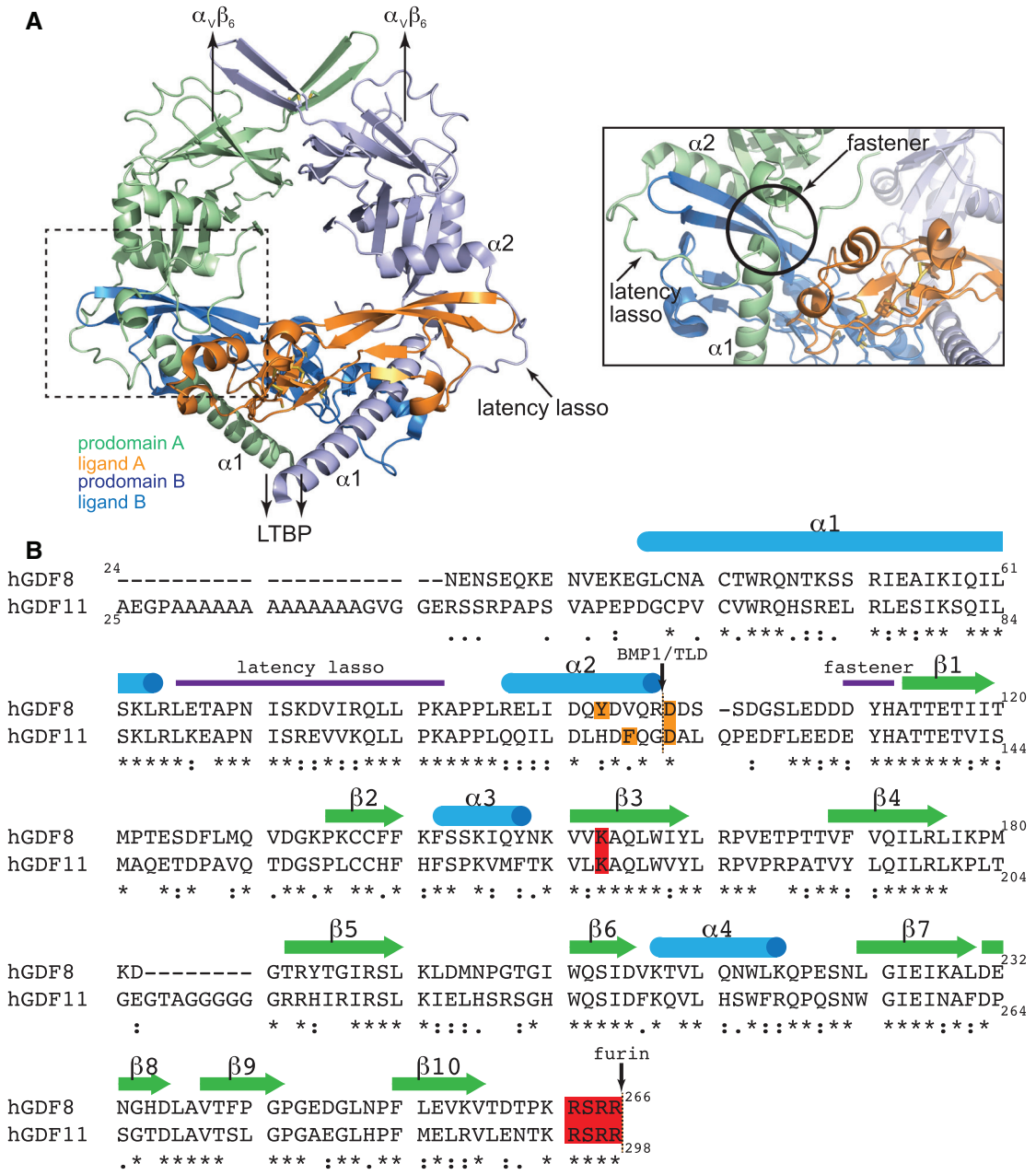

Figure 3. Structural organization of the transforming growth factor β1 (TGFβ1) prodomain and comparison of growth differentiation factor 11 (GDF11) and myostatin (MSTN) prodomains. A, Structure of the TGFβ1-prodomain latent complex (PDB 3RJR[33]). Key regions identified in the TGFβ prodomain for conferring inhibition of the mature domain are highlighted (dotted box on the left is shown as the inset). **B**, Sequence alignment of human GDF11 and myostatin prodomains with topology based on the TGFβ structure. Known proteolytic sites and residues important for each proteolytic event are highlighted (furin: red and TLD: orange).

Regulation of GDF11 and Myostatin by Extracellular Binding Proteins

Signaling by GDF11 and myostatin is regulated by extracellular-binding proteins that are typically thought to function as antagonists. These include follistatin, follistatin-like 3 (FSTL3), decorin, and growth/differentiation factor–associated serum proteins 1 and 2 (GASP1 and GASP2).[40,77–81] Structural studies indicate that 2 follistatin or FSTL3 molecules symmetrically embrace the ligand to block both receptor epitopes.[30,31] FSTL3 and follistatin similarly contain an N-terminal domain followed by tandem follistatin domains. The N-terminal domain binds in the concave type I receptor slot where GDF11 and myostatin show the highest divergence.[30–32] However, mutagenesis studies and comparison with other follistatin-ligand structures indicate that the follistatin N-terminal domain is highly plastic and can accommodate diverse type I interfaces.[30,31,82–84] Therefore, sequence differences likely have minimal impact on GDF11 and myostatin antagonism by follistatin. Sequence differences are also unlikely to impact the increased binding to cell surface–localized heparin/heparin sulfate, and the subsequent acceleration of ligand degradation that occurs when follistatin is bound to myostatin.[30] This

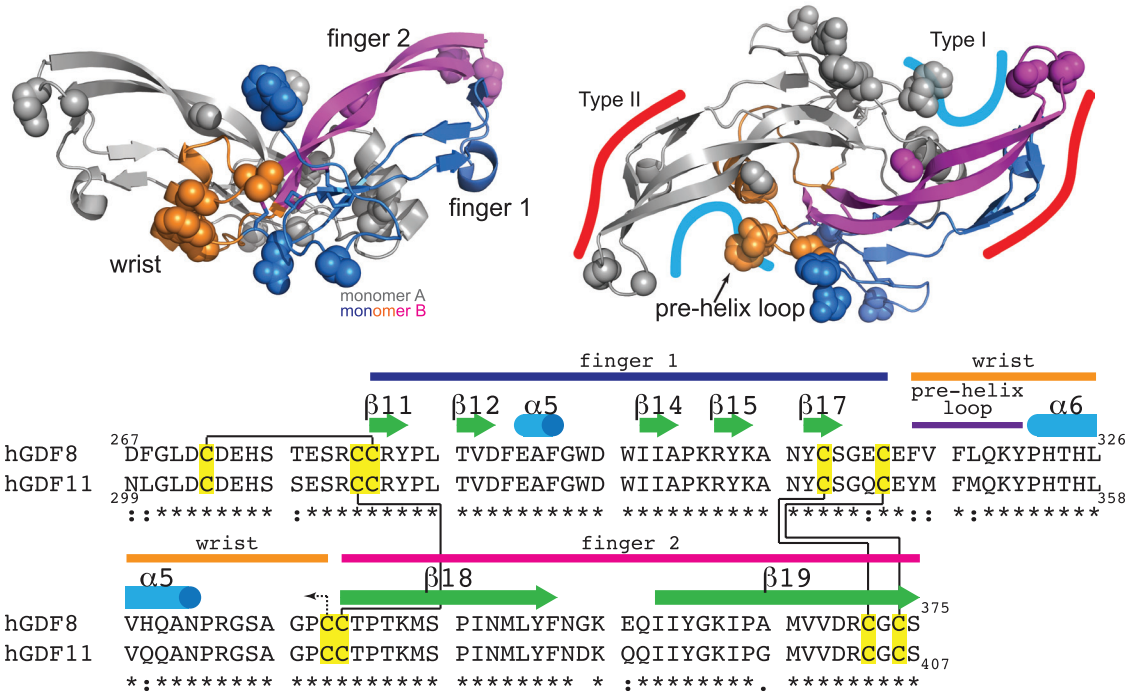

Figure 4. Structural organization and comparison of growth differentiation factor 11 (GDF11) and myostatin (MSTN) mature domains. MSTN dimer is shown where 1 monomer colored to show three subdivisions of the ligand (finger 1: blue; wrist: orange; finger 2: magenta), which correspond to the colors in the sequence alignment below. Residues that differ between GDF11 and myostatin are shown as spheres. Differences are localized predominantly to the type I site. Topology in the sequence alignment is an extension of the topology shown in Figure 3 and delineated by the structure of myostatin (PDB 3HH2[30]). Cysteines (yellow) and corresponding intramolecular (solid black line) and intermolecular (dotted line) disulfide linkages are shown.

interaction is known to regulate myostatin signaling within skeletal muscle[85,86] and a similar mode of regulation may exist for GDF11. Interestingly, FSTL3 does not bind heparin and, therefore, readily escapes the cell surface to enter circulation.[87] Thus, the distinct localizations of follistatin-type antagonists provide an intriguing mechanism for differentially modulating the actions of circulating versus locally produced GDF11 and myostatin.

In contrast to broad TGFβ ligand antagonism by follistatin-type molecules, GASP1 and GASP2 selectively inhibit GDF11 and myostatin.[77,88–91] GASP proteins contain 6 domains, a whey acidic protein domain, follistatin domain, immunoglobulin-like domain, 2 tandem kunitz domains, and a netrin-like domain.[77,92] The follistatin domain is the primary driver of ligand antagonism.[91] Despite a similar domain layout, GASP1 binds myostatin in a unique 1:1 ratio, forming an asymmetrical complex, whereas GASP2 binds symmetrically in a 2:1 ratio,[79] similar to follistatin or FSTL3.[30,31,88] Interestingly, C-terminal truncation of GASP1 induces 2:1 binding and weaker affinity for myostatin, similar to that of GASP2.[88,90,91] GASP proteins antagonize signaling by preventing ligand binding to the type II receptor,[90,93] an intriguing mechanism given that GASP maintains unique specificity for GDF11 and myostatin, despite conservation of the type II receptor epitope among the activin/myostatin subclass. This observation suggests that steric forces and/or additional molecular contacts (eg, in the type I epitope) are likely important in defining this unique ligand–antagonist relationship.

In summary, GDF11 and myostatin are regulated by specific and nonspecific interactions at nearly every step from biosynthesis to engagement with their cognate receptors. Given that sequence divergence exists between the GDF11 and myostatin prodomains, and to a lesser extent in the mature domains, it remains important to determine if the distinct biological functions of these proteins are driven, in part, by these molecular differences.

Signaling by GDF11 and Myostatin

Canonical TGFβ signaling is mediated through a series of SMAD proteins. Receptor binding by either GDF11 or myostatin induces phosphorylation and activation of the receptor-regulated SMAD (R-SMAD) proteins SMAD2 and SMAD3. Subsequently, the phosphorylated R-SMAD proteins assemble to form oligomeric complexes with the common SMAD (coSMAD) SMAD4, and this complex accumulates in the nucleus to regulate gene expression through direct and indirect DNA binding (Figure 2B). Cellular responses to SMAD2/3 activation are highly context dependent, and the presence or the absence of particular transcriptional cofactors, DNA-binding partners and chromatin modifiers can dramatically alter the ultimate output of ligand binding.[94,95] GDF11 and myostatin may also signal through noncanonical (ie, non-SMAD) pathways, including ERK, JNK, and p38 MAPK (Figure 2B),[22,96–98] adding further complexity.

The transcriptional targets of GDF11 and myostatin signaling remain incompletely defined. A recent study compared gene-expression changes after stimulation of human primary muscle cells with GDF11 or myostatin.[98] Only a few

differentially represented transcripts were identified, suggesting that GDF11 and myostatin gene regulation may be essentially identical.[98] However, this analysis was limited to a single cell type and neither ligand activated a robust gene-expression signature (the highest observed Fold Change values were only ≈4-fold for either ligand). Thus, further experiments are needed to clarify potential differences in the transcriptional output of GDF11 and myostatin signaling in different cell types and physiological contexts.

GDF11-Related Pathways in the Heart

The incidence of myocardial infarction and heart failure increases with age,[99] and aging increases mortality risk with any given infarction event.[100] TGFβ signaling regulates responses to myocardial ischemic injury,[101,102] with recent studies suggesting possible roles for GDF11, myostatin, and FSTL3 in the heart.

Myostatin in the Heart

Myostatin is best known for inhibiting skeletal muscle growth,[1,3] but genetically engineered models have demonstrated an additional function in cardiac tissue. Myostatin is expressed in fetal and adult hearts,[103] and its expression increases in patients with decompensated heart failure[104] and congenital heart disease.[105] Myostatin protein levels rapidly increase after ischemia,[106] and its circulating levels increase in mice after transverse aortic constriction–induced hypertrophy.[21] Germ-line inactivation of myostatin does not cause cardiac hypertrophy and does not attenuate cardiac fibrosis in dystrophin-deficient mice, indicating that myostatin does not function in cardiac muscle in a manner similar to skeletal muscle.[107] However, other experiments reveal that myostatin-null mice develop increased heart and body weights. Myostatin$^{-/-}$ mice (28- to 30-month old) were reported to have increased normalized heart mass at death compared with myostatin$^{+/+}$ and myostatin$^{+/-}$ mice.[108] Aged mice with myostatin deletion have improved fractional shortening, smaller left ventricle diastolic diameters, and less fibrosis compared with aged wild-type mice.[109] Heineke et al[21] compared deletion versus overexpression of myostatin in cardiomyocytes. Myostatin deletion in cardiomyocytes did not prevent left ventricular decompensation under pressure overload, whereas transgenic overexpression of myostatin in the heart inhibited cardiac growth.[21] Inducible genetic deletion of myostatin in adult mouse cardiomyocytes leads to dramatic deterioration of cardiac function and high mortality, as well as increased glycolysis and glycogen storage, revealing the importance of endogenous myostatin for adult cardiomyocyte metabolism.[23] Thus, myostatin likely participates in cardiac growth and metabolism, although the experimental findings have not been as consistent in the heart as in skeletal muscle.

Cardiac Effects of GDF11

GDF11 is expressed in cardiac tissue[18] but at lower levels compared with spleen, kidney, and skeletal muscle in mice.[24] We reported an antihypertrophic effect of GDF11 in aging mice.[24] Using heterochronic parabiosis, we reported reduced cardiac hypertrophy in aging mice that shared a common circulation with young mice. We further devised a sham parabiosis procedure wherein mice were joined but did not share a chimeric circulation. With heterochronic sham parabiosis, hypertrophy in old mice was not reduced, implying the presence of a circulating factor that regulated cardiac size. Proteomic studies identified GDF11 as a candidate for this antihypertrophic effect, and supplementing blood levels in old mice by daily intraperitoneal injection of recombinant GDF11 (rGDF11) protein reduced cardiomyocyte size and heart mass during 4 weeks.[24]

In contrast, Smith et al[110] reported recently that injections of the same quantity of rGDF11 used by us in old mice (0.1 mg/kg, injected daily) did not alter cardiac structure or function. These apparently contradictory results may be explained, in part, by the different sources of rGDF11 and by a potential dose-dependent effect of GDF11. After the study by Smith et al,[110] we performed a dose–response analysis and demonstrated that for a given protein preparation, the reduction in cardiac mass by GDF11 is dose dependent; for recent protein preparations with improved quality control of protein concentration, a dose of 0.5 mg/kg reduced cardiac mass in 9 days.[111] Injection of rGDF11 rapidly activates SMAD signaling in cardiac tissue of both young and old mice,[111] which together indicate that exogenous GDF11 can regulate cardiomyocyte size and hypertrophy and highlight that doses and protein preparations used could affect the results of in vivo studies.[112]

Given observations reported previously for genetic manipulation of myostatin expression,[22,107,108] it is likely that administration of recombinant myostatin could achieve a similar antihypertrophic effect, but this has yet to be tested. Furthermore, it remains to be determined if a similar effect can be achieved with other ligands that likewise activate the SMAD2/3 pathway, such as members of the TGFβs or activin subclasses. Finally, although most studies to date have focused on SMAD activation as a readout of signaling activity, it will be interesting to determine if cross talk with noncanonical pathways may achieve ligand-specific effects.

FSTL3 in Cardiac Tissue

FSTL3 is expressed in cardiac tissue[113] and its expression increases in end-stage failing myocardium in humans.[114] Heart mass, left ventricular and systolic pressure, and systolic arterial pressure are increased in FSTL3-deficient mice compared with wild-type mice.[115] In addition, experimental cardiac injury induces myocardial expression of the prosurvival TGFβ ligand, activin A, and one of its antagonist regulators, FSTL3, where it is thought that the relative expression levels of these molecules dictate cell survival after insult. Interestingly, cardiomyocyte-specific deletion of FSTL3 reduces infarct size and apoptosis, suggesting a detrimental effect of endogenous FSTL3 on the heart, whereas overexpression of FSTL3 inhibits the prosurvival effect of activin A.[116] FSTL3 also regulates cardiac hypertrophy induced by pressure overload.[113] Although no differences were seen between hearts of cardiac-specific FSTL3$^{-/-}$ and wild-type mice in standard physiological conditions, FSTL3$^{-/-}$ mice[113] exhibited attenuated myocardial hypertrophy and reduced left ventricular dilatation, and systolic dysfunction and interstitial fibrosis were reduced[113,117] after transverse aortic constriction-induced pressure overload (transverse aortic constriction). These data suggest that endogenous FSTL3 regulates the heart in many circumstances

and that induced expression of FSTL3 may have deleterious effects. It remains to be determined how cardiac insult and upregulation of FSTL3 may affect the heart over time with respect to GDF11 and myostatin levels, especially in the case for older populations where evidence suggests that GDF11 and/or myostatin levels decline with age.[111] Nevertheless, because FSTL3 inhibits multiple TGFβ family ligands,[118] cardiac effects of FSTL3 cannot be attributed to a particular ligand interaction at this time.

GDF11-Related Pathways in Skeletal Muscle

Skeletal muscle is composed of multinucleated, nondividing fibers. Repair of muscle fibers after severe damage invokes the regenerative activities of a unipotent population of muscle stem cells, known as satellite cells, which reside immediately adjacent to myofibers in adult muscle. Aging impairs both the homeostatic maintenance of muscle mass and muscle regenerative potential, and recent studies have focused on the potential role of GDF11-related signaling pathways in these age-associated changes.

Myostatin in Muscle Homeostasis and Repair

Prevailing views on GDF11 function in skeletal muscle and satellite cells have been greatly influenced by analogy to myostatin because of the high homology of their mature, C-terminal ligand domains (Figure 4). Myostatin is expressed almost exclusively in mature and developing muscle and negatively regulates muscle mass. Targeted disruption of myostatin in mice produces a near doubling of adult muscle mass, with an increase in both the size and number of muscle fibers[1] and a shift toward more glycolytic fiber types.[1,16] Whether the hypertrophic and hyperplastic phenotype of myostatin-null muscle reflects, in part, a release of myostatin-mediated inhibition on muscle satellite cells remains unclear. Although some studies suggest that myostatin inhibits satellite-cell proliferation,[119,120] others have reported that it has no impact.[121,122] Likewise, some groups have found increased numbers of satellite cells in myostatin-null muscle,[123] whereas others report slightly lower numbers.[121] Myostatin overexpression and supplementation studies suggest that high levels of myostatin can drive rapid muscle atrophy,[124,125] although more moderate increases in myostatin do not detectably alter muscle mass.[126] Conversely, treatment of mice with the inhibitory myostatin propeptide induces muscle hypertrophy.[127]

GDF11 Effects in Muscle

In contrast to the profound effects of myostatin deficiency, muscle phenotypes are not prominent in GDF11-null mice, which exhibit defects in a variety of mesodermal, endodermal, and ectodermal lineages and die within 24 hours after birth.[2] Nonetheless, double mutants lacking both myostatin and GDF11 exhibit increased penetrance of renal, palatal, and skeletal abnormalities,[7] suggesting that myostatin can compensate to some degree for loss of GDF11 in GDF11-null mice. Studies to similarly assess possible redundancy of GDF11 and myostatin function in regulating muscle mass have been challenging because of the fact that GDF11-null mice die before the age at which myostatin-null mice begin to exhibit muscle phenotypes. However, analysis of mice with muscle fiber–specific deletion of GDF11 showed no changes in muscle mass or fiber type, and muscle-specific GDF11 deletion did not exacerbate the phenotype of myostatin-null animals.[7] These results suggest that GDF11 and myostatin may have distinct functions in skeletal muscle, or that GDF11's effects on muscle are mediated by GDF11 protein that is produced by nonmuscle tissues.

GDF11 has been implicated as a negative regulator of muscle development by studies in chick embryos and in cultures of differentiating C2C12 cells and primary human and mouse muscle myoblasts. In these systems, GDF11, much like myostatin, can block myogenic differentiation.[98,128–130] Yet, other data suggest that GDF11's role in myogenesis may be more complex. *Gdf11* mRNA levels in mouse skeletal muscle seem to peak during the most rapid phase of postnatal muscle growth and are higher in males than in females, despite males having greater muscle mass.[130] *Gdf11* expression is also increased in the muscles of myostatin-null mice, which nonetheless exhibit accelerated muscle growth and increased muscle mass.[130] These data raise the possibility that GDF11's actions in muscle may not fully overlap with those of myostatin such that GDF11 cannot compensate fully for the loss of myostatin in muscle. Whether this lack of functional redundancy reflects differences in the biochemical properties and signaling activities of GDF11 and myostatin, or differences in their absolute levels in muscle, remains to be determined. Interestingly, ectopic GDF11 can induce expression of follistatin,[128] which as discussed above inhibits both GDF11 and myostatin signaling. Thus, GDF11 can initiate a negative feedback loop that may antagonize its own activity, as well as that of other activin/myostatin ligands. The existence of such a feedback mechanism suggests the likely importance of maintaining signaling by these ligands within a tight physiological range.

Experiments using heterochronic parabiosis recently implicated GDF11 as a candidate regulator of aging phenotypes in the heart, muscle, and brain.[24–26] Consistent with this notion, satellite cells isolated from the uninjured muscle of aged mice that received daily intraperitoneal injection of rGDF11 for 4 weeks showed improved myogenic activity in ex vivo clonal assays and a reduced burden of DNA damage. In addition, aged animals supplemented with rGDF11 showed accelerated recovery from muscle injury in a cryoinjury model.[26] rGDF11 treatment in aged mice also improved neuromuscular junctions and myofibrillar and mitochondrial morphology, and increased average exercise endurance and grip strength without any detectable changes in muscle mass or fiber caliber.[26] Notably, the dosage of rGDF11 in these studies was similar to that reported previously to lack effects on muscle size in an in vivo rMSTN dosing study.[126] Interestingly, young mice in this study showed no differences in regenerative capacity after treatment with the same amount of rGDF11, suggesting that GDF11's beneficial effects on muscle were age dependent.

In contrast to the studies summarized above, a subsequent article questioned the beneficial effects of rGDF11 on muscle aging, reporting instead that supplementation of rGDF11 in aged mice has no effect and in young mice impairs muscle repair. Yet, it is important to note that the study design used by this group[98] differed significantly from that in the earlier

studies.[26] From a reagent standpoint, the authors used rGDF11 from a different protein source and used a different dosing regimen. Furthermore, they assessed muscle regeneration in a more severe, cardiotoxin-mediated damage model, which ablates the majority of resident satellite cells[131,132] and shows distinct temporal and spatial features of regeneration in comparison with cryoinjury.[133,134] Thus, the different outcomes obtained in the 2 studies may reflect differences in experimental design, and comparison of the methods used could provide an avenue for discovering key mechanisms that determine GDF11's in vivo effects. For instance, the inability of rGDF11 to accelerate repair in the satellite-cell depleting cardiotoxin injury model[98] suggests that sustaining a sufficient pool of regenerative satellite cells may be essential for a beneficial effect on regeneration in response to rGDF11. Likewise, the possible negative impact of higher doses of rGDF11 in young mice[98] could indicate that rGDF11 exhibits antagonistic pleiotropy in young versus old animals, or that the precise timing and dosage of rGDF11 administration is critical in determining its in vivo effects.

It is also important to remember that rGDF11's impact on muscle repair in vivo likely represents the integrated effects of this growth factor on many different target cells. GDF11 signaling has been implicated in many biological processes, including vasculogenesis,[25] a process clearly documented to influence the efficiency of muscle repair.[135] Indeed, if the primary actions of GDF11 in vivo are on vasculature, this may explain the coordinated responses to systemic administration of this protein seen in skeletal muscle, heart, and brain, as each of these organs shows critical dependence on proper vascularization, a process which declines with increasing age.[136]

The potential effects of changing levels of myostatin on aging muscle have also been examined. As mentioned above, human genomics studies suggest an association of the K153R polymorphism, thought to increase myostatin activity by enhancing furin processing,[43] with longer lifespan.[44] Interestingly, homozygosity for the K153R allele is extremely rare, and most R allele-carrying centenarians are heterozygous for this variant, a genotype that does not seem to alter muscle phenotypes in aged individuals.[50,51,53] Studies in mice also suggest that alterations of myostatin signaling may affect lifespan. A recent study of male myostatin$^{+/+}$, myostatin$^{+/-}$, and myostatin$^{-/-}$ mice (n=38–42 mice per group) revealed an increase in both median and maximum lifespan of heterozygous animals, which showed a 30% decrease in circulating myostatin levels, when compared with wild-type controls.[108] In contrast, the median and maximal lifespan of myostatin$^{-/-}$ mice did not differ from wild-type. Replication of this study, with inclusion of female animals, should be illuminating, particularly as data from human subjects suggests sex-specific differences in the regulation of circulating myostatin levels with age.[137]

Analogous studies of lifespan in GDF11 mutant mice are complicated by the developmental phenotypes exhibited by both GDF11$^{-/-}$ and GDF11$^{+/-}$ mice. However, Zhou et al[138] recently reported that levels of circulating GDF11 are heritable in genetically diverse inbred mouse strains and can be used to predict median lifespan, with higher GDF11 levels at middle age associated with longer lifespan. In addition, an overall positive impact of higher GDF11 and/or myostatin levels on lifespan, as well as muscle function, has been corroborated in invertebrate models. For example, overexpression of *myoglianin,* the fly ortholog of both GDF11 and myostatin, delayed the onset of age-related neuromuscular dysfunction and extended lifespan, while its inhibition hastened neuromuscular decline and caused premature death.[139] Studies in shrimp likewise implicate the ancestral form of GDF11/myostatin in supporting muscle growth and survival.[140]

In summary, although more research is clearly needed, evidence across species implicates GDF11 as a candidate regulator of muscle homeostatic and regenerative function during aging and suggests that raising levels of circulating GDF11 might be helpful for some age-related muscle pathologies. Emerging evidence also implicates both GDF11 and myostatin in regulating longevity and suggests that even subtle variations in ligand activity can alter phenotypic outcomes. Although the mechanisms underlying these effects remain unclear at present, these data raise the possibility that systemic GDF11 could provide a useful biometric for mammalian aging.

GDF11 in the Brain

TGFβ family ligands have diverse and pleiotropic roles in the development and maintenance of the nervous system, and their effects can vary between ligands, target cell types, and an animal's developmental stage or age. Neurological effects of GDF11 have been best characterized in the developing olfactory epithelium, spinal cord, and retina, where GDF11 influences the timing and progression of neurogenesis as well as the ratios of different neural subtypes.[10,11,141] In the developing olfactory epithelium, GDF11 plays a critical role in an autoregulatory loop, wherein newly born olfactory receptor neurons and their immediate neuronal precursor cells signal to neighboring cells to limit the production of more olfactory receptor neurons.[10] Olfactory receptor neurons and immediate neuronal precursors secrete GDF11, which drives cell cycle exit by inducing upregulation of the cyclin-dependent kinase inhibitor p27^{Kip1}. Loss of GDF11 augments proliferation of immediate neuronal precursors and increases the number of differentiated neurons. Conversely, enhanced activation of GDF11 by genetic deletion of follistatin leads to sharp decreases in the numbers of both immediate neuronal precursors and olfactory receptor neurons. In the spinal cords of mice lacking GDF11, neural progenitor cells fail to exit the cell cycle, the rate of neurogenesis is slowed, the ratios of neural subtypes are altered, and the positional identities of motor neurons are disrupted.[141–143] Interestingly, in the retina, GDF11 also controls the timing of neurogenesis, but through a mechanism that does not involve proliferation.[11] Instead, GDF11 controls the length of time that progenitor cells are competent to produce retinal ganglion cells, and thereby regulates the ratio of RGCs to photoreceptors and amacrine cells.

Compared with its role in the developing nervous system, less is known about the role of endogenous GDF11 in the mature nervous system. Northern blot analysis of tissues from adult rats (3–4 weeks of age) showed high expression of *Gdf11* in dental pulp and brain.[12] In the same study, RNA in situ hybridization identified specific areas of *Gdf11* expression in the brain, including the dentate gyrus of the hippocampus, the

hypothalamus, and the Purkinje cell layer of the cerebellum.[12] It was recently demonstrated that systemic administration of rGDF11 in aged mice alters brain physiology.[25] Heterochronic parabiosis experiments showed that young systemic factors could reverse age-related neurogenic decline by enhancing neural stem cell production and, thereby increase adult neurogenesis and olfactory discrimination capacities. Furthermore, youthful blood factors induced remodeling of the aged vasculature, restoring cerebrovascular blood flow to its youthful levels. Systemic injection of rGDF11 recapitulated several beneficial effects of heterochronic parabiosis, including vascular remodeling of the aged blood vessels in the subventricular zone and increased numbers of neural stem cells in this area. rGDF11 also induced the proliferation of brain capillary endothelial cells and activated the SMAD2/3 pathway in vitro. The in vivo effects of rGDF11 were not as prominent as those seen with heterochronic parabiosis, suggesting that additional factors may be present in the young circulation that exerts these central nervous system effects.[144] Future studies testing different concentrations of rGDF11,[111] different treatment lengths, and coinjection of factors such as insulin-like growth factor 1 or epidermal growth factor that affect neural stem cell proliferation and/or vascular behavior may clarify this issue.

The central nervous system effects of rGDF11 are because of exogenous/circulating protein and not due to brain-derived GDF11, whose role in the adult/aged brain is not yet understood. It will be important to determine if systemically administered rGDF11 crosses the blood–brain barrier and acts directly on neurons and neural stem cells, or if its effects on the central nervous system are a by-product of its cerebrovascular effects. It will likewise be exciting to determine if an autoregulatory loop exists between circulating and endogenous GDF11 that could amplify its effects. Interestingly, the effects of systemic rGDF11 are consistent with a recent report that constitutive activation of ALK5, a type I receptor for GDF11 and multiple TGFβs, leads to increased adult neurogenesis and higher expression of c-Fos in newborn neurons in the adult hippocampus, more complex dendritic arborization, increased neural activity, and improved performance in memory tests.[145] In addition, TGFβ1 is neuroprotective in mouse models of Alzheimer disease and excitotoxicity,[146,147] and a recent study also reported that GDF10, which activates similar intracellular pathways as TGFβ1, β2, β3, and GDF11, improves brain vasculature, increases neurite outgrowth, and enhances performance in behavioral assays in a mouse model of stroke.[148]

Clinical Implications of GDF11 and Myostatin

Olson et al[149] investigated whether GDF11 and myostatin might have similar cardioprotective properties in humans to those demonstrated in mice. In 928 archived plasma samples in subjects with stable coronary heart disease from the Heart and Soul prospective observational cohort, they measured circulating levels of ligand using modified aptamers as binding reagents.[149] This assay does not discriminate GDF11 from myostatin; thus, the measured analyte is referred here as GDF11+myostatin. The aims of the study were to characterize the association of plasma levels of GDF11+myostatin in humans with (1) age, (2) left ventricular hypertrophy (LVH), and (3) cardiovascular events and all-cause mortality during a median follow-up of 8.9 years. The key findings were replicated in 971 subjects with stable coronary heart disease from the HUNT3 Norwegian cohort, with a median follow-up of 4.5 years.

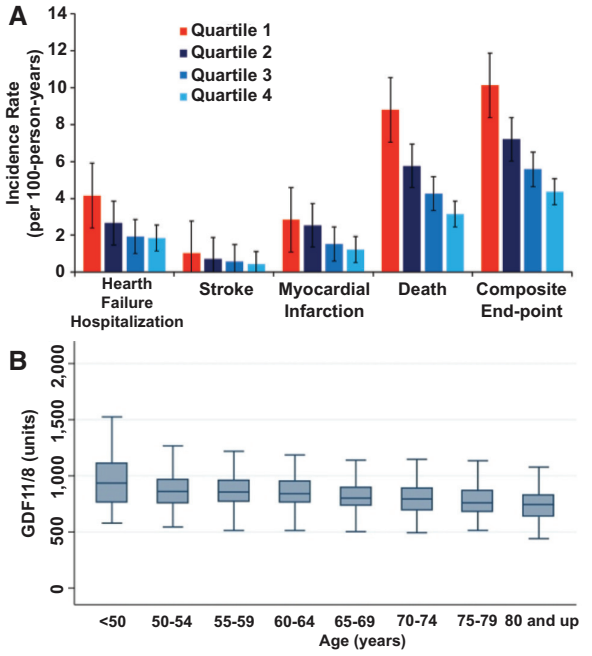

Figure 5. Growth differentiation factor 11 (GDF11)/myostatin (MSTN) in humans. A Incidence of heart failure hospitalization, stroke, myocardial infarction, all-cause death, and their composite end point in the HUNT3 cohort, unadjusted, stratified by quartile of GDF11+MSTN. Quartile 1 is the referent quartile with the lowest GDF11+8 concentrations. *P* values for trend are <0.001 for heart failure, death, and composite end point, 0.004 for stroke, and 0.02 for myocardial infarction. **B**, GDF11+MSTN levels by age in HUNT3 cohort, unadjusted. Inner line=median, box 25th to 75th percentile, outer whiskers denote adjacent value 1.5× height of box. Units=relative fluorescence units. *P*<0.001. Reproduced from Olson et al.[149] Copyright © 2016, the authors.

GDF11, Myostatin, and Age

Analysis in patients revealed an inverse relationship of plasma GDF11+myostatin to age in the HUNT3 cohort (Figure 5A), which was also observed in the Heart and Soul cohort.[149] Despite the narrower age range of these human subjects compared with the extremes of age reported in mice,[24] significantly lower levels of GDF11+myostatin were detected in older compared with younger individuals in both patient cohorts.[149] This cross-sectional analysis likely underestimates the decline in GDF11+myostatin with age because GDF11+myostatin is inversely associated with all-cause mortality; thus, surviving older individuals enrolled in these cohorts likely had higher GDF11+myostatin concentrations than those older individuals who had died and whose potentially lower levels could not be captured. Although GDF11+myostatin levels were higher in males compared with females enrolled in this study, an age-related decline in GDF11+myostatin levels was detected in both sexes.[149] In support, a recent study used mass spectrometry to quantify circulating myostatin specifically in human

subjects without chronic disease, and likewise reported higher myostatin concentrations in younger men compared with younger women, but found sex-specific differences in the effects of age on myostatin levels (higher myostatin concentrations in older versus younger women and lower myostatin concentrations in older versus younger men).[137] Circulating levels of the antagonist FSTL3 increased with age in both sexes.[137] Thus, although this latter study[137] analyzed a much smaller cohort (only 40 individuals of each age/sex), together these reports[137,149] highlight a potential influence of age, sex, and health status on GDF11+myostatin levels and are consistent with an age-related loss of function of these ligands in humans, either through diminished protein concentrations[149] or through increased inhibition of ligand activity.[137]

GDF11, Myostatin, and Cardiovascular and All-Cause Mortality

A relationship of plasma GDF11+myostatin levels to cardiovascular events (heart failure hospitalization, stroke, and myocardial infarction), all-cause deaths and their composite end point were also demonstrated in 971 subjects in the HUNT3 cohort (Figure 5B).[149] In both the HUNT3 and Heart and Soul cohorts, there was a strong, graded relationship between plasma GDF11+myostatin and cardiovascular and mortality outcomes.[149] Participants in the highest quartile of GDF11+myostatin had markedly lower risk of individual types of events and their composite end point than those in the lowest quartile, both unadjusted (Figure 5B) and after multivariable adjustment.[149]

GDF11, Myostatin, and LVH

Of the 928 participants in the Heart and Soul cohort, 368 had LVH by echocardiography.[149] There was a significant, inverse, graded relationship between GDF11+myostatin and LVH, with 31% of participants in the highest quartile of GDF11+myostatin having LVH compared with 46% in the lowest quartile.[149]

In sum, in 2 large independent cohorts of patients with stable coronary heart disease, lower levels of GDF11+myostatin were associated with notably higher rates of incident cardiovascular events and all-cause deaths as well as higher prevalence of LVH. Finally, GDF11+myostatin levels are lower among older compared with younger individuals. Taken in the context of mechanistic studies in mice, these findings in humans support the hypothesis that GDF11 and/or myostatin protect against adverse cardiovascular events and all-cause deaths.[149] More broadly, these findings support the hypothesis that age-related decline in GDF11+myostatin contributes to cardiovascular aging and mortality in humans[149] as it does in mice.

Future Directions for Clinical Research

A significant interaction was present in the multiracial Heart and Soul cohort between race and GDF11+myostatin for the composite cardiovascular and all-cause mortality outcome.[149] Whites compared with non-whites had both lower levels of GDF11+myostatin and a stronger link between plasma GDF11+myostatin and adverse outcomes. As noted previously, GDF11+myostatin levels also varied by sex.[149] Accordingly, future studies should investigate in greater detail any racial and sex differences in GDF11 and myostatin levels and their relationship to outcomes.

As discussed above, several endogenous inhibitors of GDF11 have been reported. These inhibitors may antagonize other ligands in the TGFβ family, including myostatin and activin.[78,79,90] Heidecker et al[150] investigated the association of GASP1, FSTL3, and follistatin with cardiovascular outcomes and all-cause mortality in the Heart and Soul and HUNT3 cohorts, using the same archived plasma samples as for the aforementioned GDF11+myostatin analyses. High levels of FSTL3 were associated with increased risk of adverse cardiovascular events and all-cause mortality, and the effect of low levels of GDF11+myostatin and high levels of FSTL3 were additive. Subjects in the least favorable quartile of GDF11+myostatin (lowest) and its inhibitor FSTL3 (highest) had nearly 7-fold increased risk of cardiovascular events and all-cause mortality.[150] Thus, any future studies of human diseases will need to consider the levels of GDF11+myostatin, levels of their inhibitors and their interactions.

Any therapeutic targeting of GDF11 or myostatin in humans must consider its potential adverse effects. GDF11 has been reported to exacerbate anemia in the setting of ineffective erythropoiesis.[151] Blocking GDF11 alleviates anemia in a mouse model of β-thalassemia and also in humans.[152] Analysis of observational cohort studies may provide much needed clarity whether levels of GDF11 and/or myostatin are associated with any additional adverse outcomes.

Current Controversies

The suggestion that GDF11 may serve as an evolutionarily conserved age-dependent hormone in multiple organ systems[24–26,139] raises possibilities for manipulating this signaling pathway to restore or preserve function in aging tissues. Yet, this provocative notion also presents a new and unexpected role for GDF11 within the TGFβ superfamily, and contrasts with studies of many other TGFβ family proteins in aging, which have frequently concluded that these molecules suppress healthy tissue function during aging, most notably by promoting tissue fibrosis and inflammation.[153–156] Thus, it is not surprising that there has been some skepticism and even controversy surrounding these results. It is likely that much of this controversy reflects the fact that GDF11 is a relatively understudied member of the TGFβ superfamily, particularly compared with its close relative myostatin, and thus the tools for studying GDF11 biology are still evolving. In addition, given the substantial sequence conservation of GDF11 with myostatin, many have assumed that the functions of these proteins should be identical.[7,98] Here, we discuss some of these controversies and suggest strategies to resolve the apparent discrepancies to advance our mechanistic understanding of GDF11 functions in organ physiology and aging.

Presence in Circulation and Direction of Change With Age of Circulating GDF11 Protein

In 2013, we reported a significant decline in systemic levels of GDF11 in aged, when compared with young mice.[24] This conclusion was based on an aptamer-driven analysis of serum from young (2 months.) versus old (24 months.) mice performed using Somalogics SomaMERs,[157] in combination with

Western blotting using a monoclonal antibody from abcam, which, at the time, was reported to be specific for GDF11. Subsequent reports from our group[111] and others[98,149,158] revealed that the SomaMER and mAb used in these initial studies cross react with myostatin, and so, although these studies are consistent with a reduction in the circulating pool of GDF11 and myostatin in aged animals, these data cannot discriminate the relative impact of aging on systemic levels of GDF11 specifically. A report from Egerman et al[98] argued that circulating levels of GDF11 might actually increase with age, based on the results of Western blot, RNA expression, and GDF11-specific immunoassay. However, the Western results reported by this group were based on quantification of a ≈25-kDa immunoreactive band, the approximate size of the disulfide-linked mature ligand dimer, which was present in samples that had been reduced and denatured before Western blot analysis. Puzzled by this observation (because the blotting conditions should have reduced the dimeric ligand to monomer), we reproduced these experiments and confirmed that the ≈25-kDa band does increase with age. However, we further demonstrated that this band is composed of serum immunoglobulin, known to increase with age,[159] and not GDF11 or myostatin.[111] As expected, under denaturing and reducing conditions, detection of GDF11 and myostatin in serum is limited to the ligand monomer of ≈12.5 kDa, which declines with age in the studies by Egerman et al,[98] as in previous reports.[24,26] In addition, the RNA expression analysis reported by Egerman et al[98] was limited to skeletal muscle, which expresses GDF11 at substantially lower levels (2- to 3-fold) than other tissues (eg, the spleen and kidney,[24] which may represent the predominant sources of circulating GDF11 protein). Finally, their immunoassay results in rats and humans were extremely underpowered (eg, only 9 individuals >60 years old were assessed in the human studies, with no evaluation of health status) and did not yield statistically significant differences for either species.

Studies from this group and one other[110] also suggested that circulating levels of GDF11 may be low compared with myostatin, leading a third group[160] to argue that changes in circulating GDF11 levels are mostly irrelevant. However, published mass spectrometry data clearly demonstrate the presence of GDF11 protein in both human and mouse sera,[161] and many critically important bioactive hormones are present at low levels (ie, pg/mL) in circulation, including glucagon, interleukin 6, and tumor necrosis factor α.[162] Thus, it is difficult to infer biological relevance from protein concentration alone. Future studies to develop highly specific reagents for measuring systemic GDF11 levels using either quantitative mass spectrometry or antibody-based immunoassays that can reliably distinguish GDF11 from myostatin in the blood of both humans and mice, something that has not yet been accomplished[98,110] will be essential to clarify the overall abundance and age-related changes of circulating GDF11 and myostatin proteins. Adding to the complexity, current methods[137,160,163,164] only measure the total amount of protein and cannot account for the status of the ligand in the serum (eg, free, latent, or in complex with an extracellular antagonist). Finally, it is still unclear if GDF11 and myostatin act predominantly as systemic hormones or if they may exert their most potent effects locally through autocrine/paracrine mechanisms, which complicates interpretation of the impact on peripheral tissues of changing levels of these proteins in the blood. Thus, it will be important to clarify which cell types produce GDF11 and what pathophysiological stimuli may alter GDF11 and/or myostatin expression.

Effect of GDF11 Supplementation on Skeletal Muscle Regeneration in Old and Young Mice

Studies investigating the effects of exogenous rGDF11 on muscle regeneration have reported apparently discrepant conclusions. Although we reported[26] that rGDF11 reverses age-specific muscle phenotypes and improves muscle strength, endurance and regenerative potential, with no discernable effects in young mice, Egerman et al[98] argued instead that rGDF11 supplementation has no effect in aged mice and may slow regeneration in young mice. As discussed above, these different conclusions likely arise from differences in protein source and experimental design. Of particular importance is the use by Egerman et al[98] of a more severe cardiotoxin injury model,[131,134] when compared with the milder cryoinjury model applied in our studies[26] and those that originally demonstrated rejuvenation of muscle repair by heterochronic parabiosis.[165] Furthermore, in their studies of young mice, Egerman et al[98] used a markedly different dosing schedule, shortening the pre-injury treatment window from 28 to 3 days and lengthening post injury treatment from 7 to 14 days. They also used an unconventional analysis strategy that selectively focused on tiny fibers that lacked apparent nuclei. The reported in vitro studies, also, were different in design, with one group analyzing cells purified from uninjured muscle,[26] and the other cells purified from cardiotoxin-damaged muscle,[98] with distinct culture conditions and time points of analysis. Thus, discrepancies in the reported effects of rGDF11 on muscle repair and muscle satellite cells may reflect differences in the recombinant protein itself, in protein dosage and treatment schedule, in the severity or method of muscle damage and the consequent size of the residual muscle stem cell pool, or in the particular culture conditions and analysis strategies used. Future studies to compare these experimental conditions side-by-side are necessary to identify the key experimental variables that underlie the different outcomes. In addition, the development and use of genetic gain- and loss-of-function models, as well as careful dose-titration assays in mice of different ages and different muscle injury paradigms will be helpful in establishing phenotypic thresholds for the possible context-specific effects of rGDF11 in skeletal muscle.

Effect of GDF11 Supplementation on Cardiac Hypertrophy

Our studies published in 2013[24] and 2015[111] reported an anti-hypertrophic effect of rGDF11 administration by comparing heart weight/tibia length ratio in treated and control aging mice, whereas the study by Smith et al[110] reported no effects on the heart. As discussed above, this disagreement may relate to dose-dependent effects of rGDF11 on cardiac mass, but it is also important to emphasize that Smith et al[110] did not observe changes in body weight in rGDF11 injected mice, whereas we reported a significant decrease in body weight in

aging mice with exogenous rGDF11.[111] Observations from our laboratories indicate that exogenous rGDF11 decreases body weight in old mice in a dose-dependent manner (unpublished data and studies by Sinha et al,[24] and Poggioli et al[111]). Although rGDF11 directly activates SMAD signaling in cardiomyocytes,[111] it is also possible that the reduction in cardiac size with exogenous rGDF11 in vivo is because of an indirect effect of rGDF11. A systemic signal, possibly initiated by a reduction in body size or a change in a specific tissue, is plausible. For example, there is a clear evidence for cross talk between adipose tissue and cardiac[166,167] and skeletal[139,168,169] tissues. Thus, any interpretation of a direct effect of rGDF11 on the myocardium must be considered in the context of its systemic effects.

In conclusion, GDF11 has emerged as an intriguing candidate in the regulation of vertebrate aging and considerable progress has been made in the analysis of its role in the progression of age-associated disease. Future investigation of GDF11 and myostatin biology and biochemistry will clarify the similarities and differences in the functions of these proteins and advance our understanding of organismal aging and disease.

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Disclosures

Harvard University and Brigham and Women's Hospital have filed for intellectual property on GDF11, listing Drs Wagers, Rubin, and Lee as inventors. The other authors report no conflicts.

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Response to Walker et al

Shavonn C. Harper, Andrew Brack, Scott MacDonnell, Michael Franti, Bradley B. Olwin, Beth A. Bailey, Michael A. Rudnicki, Steven R. Houser

Our comments in this response, as in our review, are primarily focused on the studies that have examined the Lee/Wagers hypothesis that circulating GDF11 levels in the blood fall with disease-free aging and that restoring youthful levels of GDF11 in the blood of old animals with recombinant(r)-GDF11 reverses pathological cardiac hypertrophy and improves deranged skeletal muscle repair and function.

Does Circulating GDF11 Decrease With Age?

We agree with the Lee/Wagers groups that the controversy related to whether GDF11 levels fall with age involves the reagents used. The reagents used in their studies do not reliably detect GDF11.[1–3] Therefore, their conclusions related to this topic are not well supported. However, new GDF11-specific assays have been developed and published by both the Glass groups[4] and by Boehringer–Ingelheim.[5] Data from the Glass laboratory[4] using multiple methods, including a GDF11-specific immunoassay, suggests that GDF11 protein accumulates with age, but more studies are warranted.

Does rGDF11 Therapy Improve or Depress Skeletal Muscle Repair in Old Mice?

There is not much agreement between the data in the Lee/Wagers experiments[2] and those from the Glass group.[4] Possible reasons for what seem to be mutually exclusive data sets are discussed in both of the current reviews. There is an agreement between groups that myostatin (GDF8, a closely related GDF11 family member) depresses skeletal muscle repair in both young and old mice.[6–9] There is also an agreement among many independent laboratories[10,11] that both myostatin and GDF11 activate identical signaling pathways. Although myostatin and GDF11 may engage their target receptors slightly differently, a comprehensive analysis of signaling in myoblasts including relevant signaling pathways, cellular responses, and unbiased gene expression analysis has revealed nearly identical activities.[4,11–13] Furthermore, the Wagers and Lee groups have not documented distinct signaling by GDF11 versus GDF8. It is therefore highly unlikely that the reported beneficial effects of rGDF11 on aging skeletal muscle are because of activation of distinct signaling pathways. In the review from the Lee/Wagers groups, they now suggest that rGDF11 may affect other cell types and thus be providing cell nonautonomous antiaging effects. This idea warrants further study but, if true, invalidates the claims that GDF11 improves satellite cell function via some unknown signaling mechanism.

Does rGDF11therapy Reverse Age-Related Cardiac Hypertrophy and Improve Cardiac Function in Old Mice?

There is some agreement between the results from the Houser laboratory and the Lee/Wagers laboratories. In our opinion, the data from both the groups do not support the idea that there is any form of cardiac hypertrophy in old C57Bl6 mice. The old mice are heavier, and their hearts weigh more. There is no difference in their heart weight/body weight (BW) ratio. The Houser group also showed that cardiac function was not abnormal in old mice. The parabiosis experiments in the original Lee/Wagers studies[1] clearly show that the reduction in heart size in the old animals is readily explained by a reduction in BW. Readers should know that they must get these BW data (Online Data Supplement 3) from the NCBI Pubmed Central site (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3677132/) because they were not included in the Cell paper.[1] What remains unexplained is how sham parabiosis in old animals can reduce their BW (again, see the NCBI Pubmed Central version of the study) by 25+% and have no corresponding effect on heart mass? Hopefully, this concern is explained in the response from the Lee/Wagers groups.

Using a carefully characterized lot of GDF11, the Houser laboratory showed that rGDF11 therapy (0.1 mg/kg) in old mice elevated circulating GDF11 levels but had no effect on heart or BW. The original report[1] from the Lee/Wagers groups, using a poorly characterized lot of rGDF11 (0.1 mg/kg), reported a decrease in heart weight with no change in BW.[1] The review by the Lee/Wagers groups does not clearly relate this aspect of their initial work.[1] In their review, they suggest that rGDF11 therapy might only reduce heart size when it reduces body size, as reported in their second study in *Circulation Research*.[3] It is unclear how rGDF11 effects on heart size can be both independent of[1] and dependent on[3] changes in body mass. If indeed the rGDF11 effects on heart size in both young and old animals are secondary to an effect on BW, this would invalidate an antiaging effect of rGDF11 on the heart. Finally, concerns with statistical analysis in the second cardiac/GDF11 report[3] will hopefully be explained. The data from the Lee/Wagers groups, and the associated media coverage, have given hope to aged individuals with cardiac, skeletal muscle, and central nervous system dysfunction. However, there is now sufficient concern about these data and we hope that any proposed rGDF11 clinical trials will do no harm.

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