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Review

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GDF11 and aging biology - controversies resolved and pending

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Abstract

Since the exogenous administration of GDF11, a TGF-ß superfamily member, was reported to have beneficial effects in some models of human disease, there have been many research studies in GDF11 biology. However, many studies have now confirmed that exogenous administration of GDF11 can improve physiology in disease models, including cardiac fibrosis, experimental stroke, and disordered metabolism. GDF11 is similar to GDF8 (also called Myostatin), differing only by 11 amino acids in their mature signaling domains. These two proteins are now known to be biochemically different both *in vitro* and *in vivo*. GDF11 is much more potent than GDF8 and induces more strongly SMAD2 phosphorylation in the myocardium compared to GDF8. GDF8 and GDF11 prodomain are only 52% identical and are cleaved by different Tolloid proteases to liberate the mature signaling domain from inhibition of the prodomain. Here, we review the state of GDF11 biology, highlighting both resolved and remaining controversies.

Keywords: GDF11, aging, systemic factor, heart, skeletal muscle, brain

INTRODUCTION

Aging plays an important role in almost all the most prevalent diseases afflicting modern humans. While there are "hallmark" mechanisms in the aging of many organisms[1], the molecular intersections of aging biology and human disease remain mysterious. As an example, one area of intersection is fibrosis and inflammation, both of which are associated with aging. In all tissues, an inflammatory response to damaged tissues provides key molecular signals for the activation of reparative cells[2]. Activation of this post-injury inflammatory response is necessary to promote adhesive interactions between leukocytes and endothelial cells and to drive the infiltration of these leukocytes to clear dead cells and initiate repair. To escape potential deleterious damage due to excessive inflammation, activation of an anti-inflammatory response is also essential. In parallel, tissue-resident fibroblasts proliferate and transdifferentiate into myofibroblasts and deposit extracellular matrix to restore and protect the structural integrity of the tissue. Among the many molecular pathways implicated in fibrosis and repair, members of the TGF-ß superfamily appear central. For example, TGF-ß family ligands participate in cardiac remodeling after infarction via regulation of inflammation and repair[3] and excessive TGF-ß signaling can also lead to cardiac fibrosis[4]. In many tissues, blockade of TGF-ß signaling is sufficient to blunt fibrotic responses, but genetic abolition of this signaling system, achieved by targeted disruption of its receptor or essential signal transducing proteins, has significant pathological consequences, including possible induction of systemic inflammation and a heightened risk of cancer[5].

In this review, we focus on GDF11, a member of the TFG-ß superfamily known to be required for the development of many organs but also suggested to have additional adult mammalian roles in the cardiovascular, musculoskeletal, and neurovascular systems. We discuss the molecular properties of GDF11 as well as its closely related ligand GDF8, also called Myostatin, and their reported effects and functions in regenerative tissues and aging diseases [Figure 1]. Over the past decade, several controversies have arisen around GDF11, but many of the major controversies have been systematically addressed. In addition, the biology of GDF11 is clearly relevant to humans given new reports of GDF11 loss-of-function genetic diseases that can affect the cardiovascular system, musculoskeletal, and nervous systems[6].

GDF11: BASIC BIOLOGY, STRUCTURE AND FUNCTIONS

Systemic factors during aging

Aging is associated with a decline of tissue and organ functions due to diverse biological processes including oxidative damage, mitochondrial dysfunction, and genome instability[7]. The regenerative capacity of some tissues, such as the brain or the skeletal muscles, may decline with aging in part due to a diminution of stem/progenitor cell numbers or dysregulation of stem cell responses. In adult mammals, new cardiomyocytes are formed from pre-existing cardiomyocytes rather than differentiating from a stem cell pool, but this capacity also declines with age in mammals including humans, and most adult cardiomyocytes appear to have limited ability to proliferate after mid-life[8]. The molecular mechanisms underlying the decline in cardiogenesis with aging in mammals are unclear, but, interestingly, voluntary exercise can restore youthful rates of new cardiomyocyte formation in old mice[9]. This shows that the age-dependent loss of new cardiomyocyte formation is reversible. The molecular mechanisms of cardiomyocyte aging remain mysterious, but as suggested by these exercise data, it is possible that systemic effects could be important.

Systemic mechanisms for organ aging have revealed multiple pathways, but unsurprisingly, no single mechanism. For example, declining skeletal muscle regenerative capacity with age is in part due to a defect of NOTCH pathway activation[10]. This age-related repair defect could be rescued after an injury in old mice by injection of a NOTCH activator directly in the muscle, to force NOTCH pathway activation. Moreover,

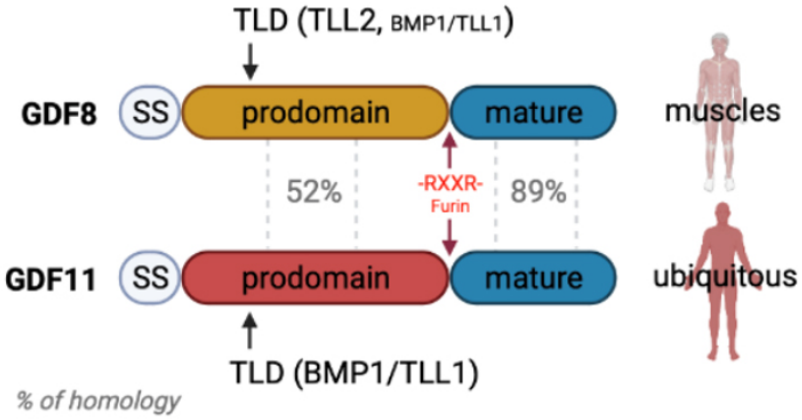


Figure 1. Distinct structure and expression patterns of GDF8 and GDF11. GDF8 and GDF11 share 89% amino acid identity in their mature domain, but only 52% in their prodomain. During processing, the signal sequence (SS) is removed, and the pre-pro-ligands are cleaved by Furin and Tolloid proteases (TLDs) to prepare the mature ligand for future signaling. GDF8, expressed predominantly in skeletal muscle, is cleaved by all TLDs and preferentially by TLL2 due to the availability of this TLD in the muscle, while the ubiquitously expressed GDF11 is cleaved preferentially by BMP1 and TLL1[6]. Figure made by @Biorender.

heterochronic transplantation experiments in which old muscles were grafted into young hosts, and vice versa, showed that old muscles in a young environment can regenerate after an injury while young muscles in an old environment regenerate no better than old muscles in an old host. These results indicated that the poor muscle regenerative capacity of old mice is due to the muscle environment[11]. One possible explanation for this observation, which has been under intense investigation for the past two decades, is that blood-borne circulating factors play a key role in regulating tissue regenerative responses and that variations in the level and/or activity of these factors with age are a key determinant of the observed differences in repair activity.

The aging process induces different structural and functional changes in the body suggesting changes between young and old subjects. To understand the possible influence of systemic factors in aging phenotypes, many studies have used parabiosis experiments. This technique consists of joining two mice surgically to share a common circulation. When an old mouse and a young mouse are joined in this manner, it is called heterochronic parabiosis; the old mice are exposed to factors present in young blood, and the young mice to factors present in old blood. Soluble factors and cells cross-circulate in heterochronic parabiotic mice, and many studies[12-18], demonstrated that this intervention can restore more youthful functions in the heart, skeletal muscle, bone, endocrine, and central nervous systems of old partners[12-23]. Conversely, heterochronic circulation has been shown to suppress healthy function in young animals in a subset of these systems[19-21,24]. Rando's lab showed that heterochronic parabiosis restores the activation of the NOTCH signaling pathway and improves skeletal muscle regeneration and stem cell activation in the old mice[17] but also induces heightened WNT signaling and fibrosis that suppresses stem cell function and regenerative myogenesis in the young mice[19]. Thus, in skeletal muscle, heterochronic parabiosis exerts clear bi-directional effects, remodeling muscle ultrastructure and enhancing repair in aged partners[12,13,17], and suppressing regenerative function and exacerbating fibrosis in young partners[19]. These results highlight the importance of blood-circulating factors during aging; vascular-active circulating factors in the context of aging and the brain have been recently reviewed by Bieri *et al.*[25].

GDF11 is a circulating factor that reduces cardiac hypertrophy in aged mice

Over the past 10 years, many studies, have sought to identify candidate molecular geronic (aging-related) factors, with studies implicating various hormones, cytokines, growth factors, and immune regulatory

proteins[25-28]. Heterochronic parabiosis was shown to reduce cardiac hypertrophy in old mice without a concomitant increase in heart size in young mice[15]. In that study, the effect of heterochronic parabiosis on the old heart was also apparent in the size of the cardiomyocytes themselves. An additional experiment was performed as a control, in which the mice were surgically connected but did not share a common cross-circulation. In these experiments, the reduction in size of the old heart was not observed in the absence of a shared circulation suggesting that young blood contains factors that regulate cardiac size in old mice. Taking a proteomics approach, 13 possible candidates were identified that significantly varied between young and old mice, but the study focused on GDF11 as it is a TGF-ß family member that is closely related-protein GDF8 was already known to regulate hypertrophy in the heart. Then a later study demonstrated that delivery of recombinant GDF11 (rGDF11) to old mice led to a decreased heart size[15]. An important point is that these experiments used an aptamer and monoclonal antibody against GDF11 that we believed at the time to be specific for GDF11, but both were later shown to cross-react with GDF8[29]. Thus, our experiments erroneously concluded that GDF11 levels decline in mouse blood with age, when in fact, our reagents were measuring both GDF11 and GDF8. Given the substantially greater abundance of GDF8 in circulation (50-100 times that of GDF11), it is not surprising that when reagents and assays capable of discriminating GDF11 from GDF8 eventually became available, they showed that GDF8 (not GDF11) was the age-dependent ligand[30] (and Karol *et al.*, under review). Nonetheless, the effects of exogenous GDF11 supplementation do appear clear and age-dependent.

An initial report showed that administration of exogenous GDF11 at 0.1 mg/kg to aged mice reduced cardiac hypertrophy, like the heterochronic parabiosis experiments[15]. But then, another lab performed a study in 2015 that did not reproduce this finding at 0.1 mg/kg with well-characterized recombinant GDF11[31]. During this time, quality control studies were performed and found that commercial preparations of GDF11 varied in protein quantity, and this was likely to be important when using GDF11 as an *in vivo* agent. This information was communicated to the companies that manufactured GDF11 and publicly stated that dose and protein quality may affect results. The Houser lab then showed, in 2016, with careful dose-response experiments that exogenous administration of GDF11 significantly reduced TAC-induced cardiac hypertrophy and improved cardiac function in a dose-dependent fashion[32]. However, mice receiving high doses of GDF11 (5 mg/kg) developed cachexia and premature death, showing that excessively high doses of GDF11 can cause deleterious effects at high doses, as others have confirmed[29,33,34].

Basic biology of GDF8 and GDF11

GDF11, also called Bone Morphogenetic Protein 11 (BMP11), and its closely related protein GDF8, also known as Myostatin, are members of the TGFβ superfamily and share 89% of identity. GDF8 is only expressed in skeletal muscle and plays an evolutionarily conserved role in postnatal skeletal muscle growth, limiting both the number and size of individual muscle fibers[35]. Deletion of the Gdf8 gene or inhibition of GDF8 protein leads to muscle hypertrophy in many mammals and fish[36-38]. GDF11, in contrast, is ubiquitously expressed and plays different roles during mammalian development, regulating anterior/posterior patterning, and different tissue formations such as kidneys, endocrine pancreas, spleen stomach, and olfactory neurogenesis[39-46]. Due to the perinatal lethality of Gdf11-knockout mice[39,40], which exhibit homeotic skeletal transformations, cleft palate, and renal agenesis, the functions of GDF11 in postnatal tissues are less explored.

GDF8 and GDF11 are produced as unprocessed pre-pro complex proteins, and different cleavages are required to separate the mature signaling domain from the tight binding of the inhibitory prodomain. The critical cleavage of the prodomain is made by the Tolloid proteases (TLDs), which are zinc-dependent metalloproteinases that include 4 members: bone morphogenetic protein 1 (BMP1), mammalian tolloid (mTLD), tolloid-like 1 (TLL1) and TLL2. TLD substrates are wide-ranging and are essential for tissue

patterning and extracellular matrix assembly. GDF8 is cleaved by the four members of the TLD family, preferentially by TLL2, whereas GDF11 is cleaved by BMP1 and TLL1. *In vitro* experiments showed that a TLD cleavage-resistant mutation in the prodomain prevents ligand activation. *In vivo*, administration of a mutant GDF8 prodomain that is resistant to TLD cleavage increased muscle mass as GDF8 inhibitors do, but wild-type GDF8 prodomain does not inhibit in this manner. Thus, TLD cleavage of the prodomain is essential for ligand activation.

The mature domains of GDF8 and GDF11 are disulfide-linked homodimers with a propeller-like shape. This arrangement creates symmetrical concave and convex surfaces which are used for receptor binding. To signal, ligands assemble a combination of two Type II and two Type I Ser/Thr kinase receptors that have extracellular ligand binding domains. This complex allows the Type II receptor to phosphorylate the Type I receptor and initiates the downstream SMAD signaling cascade. While there are over 30 TGFβ family ligands, only 5 Type II receptors and 7 Type I receptors are available for signaling. GDF11 and GDF8 are members of the Activin subclass which signals through ALK4, ALK5, and ALK7 members of the Type I receptors. Signaling is differentiated at the receptor level where different combinations of Type I and Type II receptors can elicit different downstream responses.

Exogenous GDF11 improves brain vasculature

Aging is a well-known risk factor for many neurological disorders, including vascular dementia. With increasing age, there is a general loss in vascular density and blood flow, but also in the quality of the remaining vasculature[45-47]. There are also age-related changes in the blood-brain barrier, with a loss of endothelial junctional barrier proteins[48], a reduction in specific transport from blood to brain, a reduced transport of material from the brain to blood, and an increase in non-specific blood-brain transport[49]. With these changes, increased brain inflammation, a hallmark of aging, affects vascular integrity, probably leading to the increased entry of immune cells into the brain[50]. Thus, aging encompasses a constellation of vascular changes, many of which are also observed in diseases of the central nervous system. Numerous proteomics-type studies of young and old serum have been performed, identifying multiple blood-borne factors that influence brain function negatively or positively[16,17,20,51]. The circulating factors identified thus far derive from a variety of peripheral tissues and are secreted into the blood to signal at a distance, in an endocrine fashion.

The beneficial effects of heterochronic parabiosis were described in the brains of old animals[14,16,17,20,51,52]. Young blood can stimulate an increase in neurogenesis in both the subventricular zone and the hippocampus[16,20,51], associated with a greater vascular density in those regions. Accompanying these neural stem/progenitor and neuronal effects, the exposure to young blood led also to greater vascular density, not only in the neurogenic niches but throughout most of the brain. Brain endothelial cells appear to be particularly sensitive to heterochronic parabiosis, which may be expected as some circulating factors may not pass through the blood-brain barrier efficiently in the absence of injury[14,53]. Systemic administration of rGDF11 summarizes many positive effects of heterochronic parabiosis, including vascular remodeling of aged blood vessels and increased numbers of neural stem cells. rGDF11 also induces the proliferation of brain capillary endothelial cells and activates the SMAD2/3 pathway in these cells *in vitro*[14]. Although rGDF11 is unable to cross the blood-brain barrier, it likely binds to its receptors on endothelial cells, stimulating them to secrete pro-neurogenic factors[54-56].

GDF11 is expressed in the developing mouse hippocampus, where it acts as an inhibitor of neurogenesis[43,44,57]. Lowering the expression of GDF11 in the adult hippocampus, using a Cre-inducible Gdf11 deletion mouse, increases the number of neural stem/progenitor cells[58] confirming that endogenous

GDF11 acts locally in the brain and acts as a negative regulator of neurogenesis even in adult mice. These results reveal that GDF11 can have different, and even opposite effects, when it is acting locally *vs.* hormonally, by targeting receptors on different cell types.

Potential therapeutic applications have arisen from studies of the effects of exogenous rGDF11 on the brain. An injection of rGDF11 twice per day, in Alzheimer's disease model mice with APP/PSEN mutations led to improvement of the cognitive performance of these mice associated with reduced inflammation, increased number of brain endothelial cells, and increased blood flow[59]. Other studies have found that infusion of rGDF11 after brain ischemia/reperfusion reduced mortality, improved behavior, reduced gliosis and inflammation, increased staining for myelin basic protein, and increased angiogenesis[60,61].

The mature domains of GDF11 and GDF8 are similar but not equivalent

Because GDF8 and GDF11 mature domains differ only by 11 amino acids, it has long been assumed that the GDF11 and GDF8 ligands would signal similarly. Their activities are similar when studied with *in vitro* assays[62] and in addition to their strong homology, these proteins are also inhibited by similar secreted proteins and bind to the same receptors[62]. Together, these data led to the previously popular concept that GDF8 and GDF11 were functionally interchangeable, with *in vivo* differences apparent due solely to variation in tissue-specific expression.

While it was shown that GDF8 and GDF11 signal through similar receptors, a direct rigorous comparison of the ligands had not been performed until we published a study demonstrating that GDF8 and GDF11 have significant differences in their signaling properties in multiple cell lines, showing that GDF11 is much more potent than GDF8. We also demonstrated that administration of GDF11 more potently induces SMAD2 phosphorylation in the myocardium compared to GDF8. A comparison of the GDF8 and GDF11 crystal structures revealed key structural differences between the two ligands and provided a potential basis as to why GDF11 is a more potent ligand than GDF8. To conclude, structural and biochemical experiments showed that GDF11 and GDF8 are not functionally equivalent, perhaps most importantly when ligand concentrations are low, as *in vivo*.

However, these studies did not address whether endogenous GDF8 and GDF11 are functionally equivalent *in vivo*. To assess this question, an *in vivo* study used different models in which the mature domains of GDF11 and GDF8 are genetically modified. They replace the entire mature domain of GDF8 with the mature domain of GDF11 ($GDF8^{GDF11MD}$) to investigate the interchangeability of the two ligand signaling domains. In this model, the entire Gdf11 mature domain replaces the Gdf8 mature domain in the Gdf8 locus, yielding $Gdf8^{Gdf11MD}$ mice entirely lacking GDF8 but with levels of GDF11 that are 25- to 50-fold higher than normal[63]. That showed that young $Gdf8^{Gdf11MD}$ mice exhibit modest reductions in muscle mass in some but not all muscles, with no apparent impact on total body weight, muscle regenerative potential, bone development, cardiac size, and function, or survival[63]. A similar version of this genetic modification was reported also by another lab[64], and both papers reported that chronically high levels of circulating GDF11 were surprisingly well tolerated in mice. In the same first study, two other mouse models were studied in which two specific amino acids of GDF11 were swapped with the corresponding GDF8 amino acids. That substitution of just two amino acids from GDF8 into GDF11 diminished GDF11 ligand potency and changed axial skeleton development. These substitutions resulted in a consistent phenotype with Gdf11-deficient mice, with axial skeletal defects without apparent perturbation of skeletal/cardiac muscle development or homeostasis. These combined experiments, uncover some distinctive features between the GDF11 and GDF8 mature domains *in vivo*, suggesting that the endogenous mature ligands are functionally different. Taken together, these findings provide direct evidence that structural and biochemical differences in the ligand mature signaling domains contribute significantly to their roles in mammalian development.

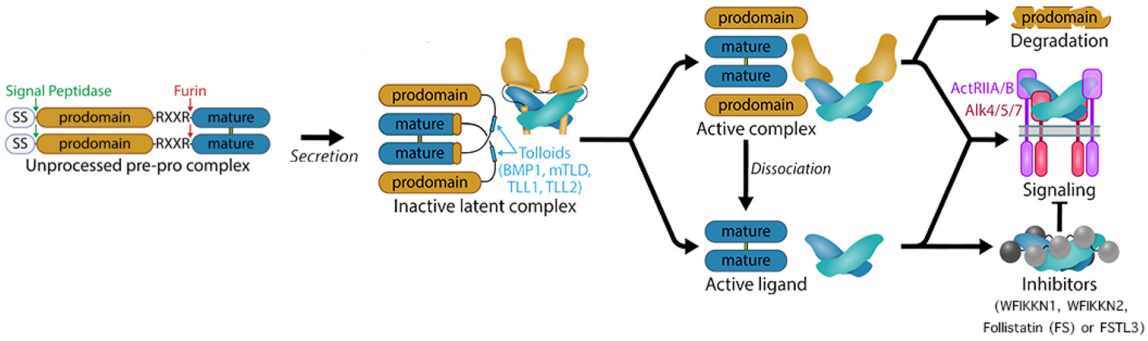


Figure 2. Proteolytic processing of GDF8 and GDF11 prodomains is a critical regulatory step. Proteolytic processing is necessary to pass from an unprocessed pre-pro complex protein to an active ligand able to signal. After a signal peptidase, the Furin protein recognizes and cleaves a specific motif -RXXR- between the prodomain and the mature domain. The inactive latent complex is then cleaved by the Tolloids family of protease to separate the prodomain from the active ligand. After cleavage, the prodomain is readily displaced when the mature ligand binds to the type II receptor and is likely degraded. Upon binding the type II receptor, a type I receptor is recruited and phosphorylated to activate downstream signaling pathways. The mature domain can also interact with inhibitors such as WFIKKN1, WFIKKN2, Follistatin (FS), or FSTL3.

Evidence for a "Triggered" prodomain-ligand state

After translation, both GDF8 and GDF11 are trapped in a non-signaling, latent complex by the N-terminal prodomain (termed the pro-complex) composed of a prodomain and a mature signaling domain. Activation of the pro-complex requires a second cleavage by TLD proteases at a highly specific location in the prodomain. The two pieces of the prodomain bind much weaker, allowing the mature ligand to be liberated and bind its receptors to signal. Thus, the synthesis of active GDF8 and GDF11 requires multiple steps. Step 1 - Two chains assemble and are connected in the mature region through a disulfide bond. Step 2 - The prodomain is cleaved from the mature domain via the protease Furin in the -RXXR- motif. Step 3 - The signal sequence is cleaved upon secretion of the pro-complex in the extracellular matrix. Step 4 - A TLD protease cleaves the prodomain, weakening its interaction with the mature ligand. Step 5 - The mature domain binds the cognate receptors and activates SMAD molecules. In addition to TLD, an activated state of the pro-complex can occur, where the prodomain is still attached to the ligand, but the ligand can signal without the addition of TLD [Figure 2]. This activated state is about 50% less active than the mature ligand alone suggesting that GDF8 and GDF11 exist in multiple states, ranging from the apo ligand which has the most activity to the latent pro-complex which has little to no activity. Because mass spectrometry measures total protein but not different molecular states, the different states in human blood are currently incompletely defined.

While latent GDF11 and GDF8 are known to be activated by the Tolloid proteases [Figure 2], which cleave the prodomains, activation can also be achieved under acid conditions in what is referred to as "acid-activation"[65]. This process was originally thought to dissociate the prodomain from GDF8; however, purification of the acid-activated form revealed that the prodomain remained in complex with the mature ligand[66]. This observation indicates that GDF8 can adopt both a latent state and an activated or 'triggered' state where the prodomain is still bound but GDF8 can nonetheless signal. Without activation, GDF8 retains a minor ability to stimulate signaling that is greatly enhanced following acid-activation. Structural analysis of the purified latent and acid-activated complexes using small angle X-ray scattering (SAXS) revealed that the acid-activated sample adopts a more open conformation than the latent prodomain-mature complex[66]. The idea of an open conformation was further supported by the crystal structure of the prodomain GDF8 complex[67]. Interestingly, point mutations have been identified in the prodomain that alleviate latency while the ligand is still bound to the prodomain[66,68]. These data support the concept that

GDF8, and likely GDF11, exist in multiple molecular states including (i) a tightly inhibited latent state where the prodomain mimics a similar mechanism to TGF-ß latency; (ii) a triggered state where the prodomain is bound in a more "open" state and the ligand is active; (iii) a Tolloid processed state which is active but not defined molecularly; (iv) an apo-state where the ligand is active and free of binding partners; and (v) an antagonist bound state where the ligand is neutralized by extracellular antagonists [Figure 3]. While the total GDF8 or GDF11 does not appear to change with age, it will be important to understand if the populations of each ligand are altered with age. For example, an increase in the antagonist FSTL3 would have a direct impact on GDF8 and GDF11[69].

Tolloid proteases cleave the inhibitory prodomain of GDF11

Tolloid proteases (TLDs) are zinc-dependent metalloproteinases. The four mammalian TLDs include two alternative splice forms of the Bmp1 gene - bone morphogenetic protein 1 (Bmp1) and mammalian tolloid (mTLD), and two related proteins, tolloid-like 1 (TLL1) and TLL2[70] [Figure 1 and 2]. TLDs have numerous proteolytic substrates that are essential for tissue patterning and extracellular matrix assembly[71]. TLDs also activate GDF8 and GDF11, both of which are secreted as latent precursors after cleavage by Furin which separates the large N-terminal prodomain from the C-terminal mature signaling domain[72]. Unlike other TGF-β superfamily proteins, the mature C-terminal domains of GDF8 and GDF11 remain tightly bound to their prodomains even after Furin cleavage and require TLD cleavage of the prodomain to convert from an inactive to a "triggered" state that is primed for subsequent activity of the mature, signaling-competent ligand[73-75]. As noted above, the proteolytic activation step appears to be essential for ligand function, as the introduction of TLD cleavage-resistant mutations into the GDF8 prodomain prevents ligand activation *in vitro*[74] and *in vivo*[76] and produces mice with significantly increased muscle mass, similar in phenotype to the phenotype elicited by genetic inactivation of GDF8 or administration of GDF8 inhibitors[76], despite dramatic elevation of GDF8 levels in the sera of these animals. *In vivo* administration of exogenous TLD-resistant GDF8-unprocess-pre-procomplex likewise increases muscle mass; however, similar administration of the native GDF8-unprocess-pre-procomplex does not block GDF8 activity in this manner[73]. Thus, it appears that TLD-mediated proteolysis serves a key regulatory function for GDF8 and GDF11 protein activity, an observation that explains why measures of total GDF8 and GDF11 protein are not sufficient, alone, to assess the *in vivo*, functional activity of this signaling system.

Recognition of human GDF11 genetic diseases

An initial GDF11 mutation with a dominant inheritance pattern and variable penetrance has been reported to cause cleft lip/palate and rib/vertebral hypersegmentation as observed in Gdf11$^{-/-}$ mice[40]. This mutation affects the RXXR motif that is essential for the cleavage by Furin and replaces the second Arginine with a Glutamine. The result of this mutation in humans is the absence of GDF11 cleavage and behaves as a dominant GDF11-loss-of-function variant[77] [Figure 4]. The phenotype in this family was Cleft lip with or without cleft palate. Through the Undiagnosed Disease Network, multiple other loss-of-function heterozygous Gdf11 mutations were subsequently identified in patients with multi-system defects including neurological, cardiovascular, or ocular phenotypes[6]. These new data reveal the importance of GDF11 function in humans. Exploration of the Gnomad database suggests that mutations in GDF11 are infrequent (pLOF: pLI = 0.98 o/e = 0.06), but mutations in GDF11 are associated with cardiac diseases (HuGE score: 4.28).

CONTROVERSIES

Prior work from our lab has identified GDF11 as a target of interest in aging-related dysfunction[12,14,15,80,81]. Some of our results were unexpected by the field, as they diverged from the previously established activities of GDF8[80,81], and so our initial studies generated substantial discussion and controversy[29,33,74,80,82]. Here we describe some of the issues and outcomes surrounding the GDF11 debate (summarized in Table 1).

Table 1. Summary of the various controversies surrounding GDF11 - status and conclusions

Controversies	Status	Conclusion	References
Total circulating GDF11 during aging	Resolved	The total amount of circulating GDF8 (but not GDF11) declines with aging.	[16,30,83-86]
GDF11 and geronic effects	Partially resolved	Supplementation on rGDF11 can reverse age-related deficits in different organs (incompletely resolved in skeletal muscles)	[13,15,16,30,32,78,79,94]
Cardiac hypertrophy	Resolved	Supplementation on rGDF11 reduces cardiac hypertrophy in aging	[16,29,32,33,84,100-102]
Exogenous GDF11 and toxicity	Resolved	Exogenous rGDF11 at high doses produces Myostatin-like effects	[33,70]

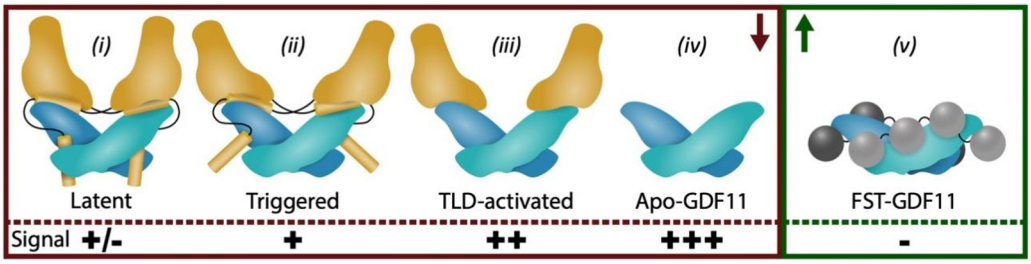


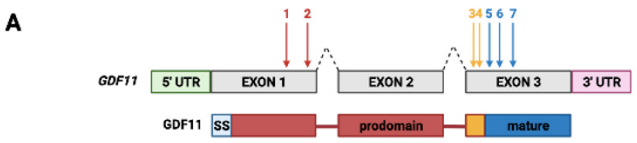
Figure 3. Different potential states of GDF11. (i) Latent, with green and purple dimer and brown prodomains; (ii) Triggered as realized through acid activation; (iii) Tolloid processed; (iv) free ligand; and (v) antagonist bound with red and pink antagonists. + denotes active signaling states. Arrows indicate the possibility that some specific forms may change with age.

Total circulating GDF11 does not decline with age

In 2013, we reported an important decline in systemic levels of GDF11 in aged compared to young mice[15]. This conclusion was based on results from a study using an aptamer-driven analysis of serum from young (2 months) *vs.* old (24 months) mice performed using Somalogics SomaMERs[74], as well as Western blotting using a monoclonal antibody from Abcam, which, at the time, was reported to be specific for GDF11. Subsequent reports from the Glass laboratory at Novartis, and others including us[29,83,84] revealed that the SomaMER and monoclonal antibody used in these initial studies cross-react with GDF8. Thus, while these studies were consistent with a reduction in the circulating pool of GDF11 + GDF8 in aged animals, our initial suggestion that systemic levels of GDF11 declined with aging was incorrect, as the GDF11 + GDF8 signal was dominated by GDF8, which has lower potency compared to GDF11 but circulates at substantially higher concentrations. A report by the Glass laboratory at Novartis argued that circulating levels of GDF11 might actually increase with age[29]; however, thus far, an increase in circulating levels of GDF11 has not been supported by mass spectrometry studies in mice or humans[85]. We are currently studying subforms of GDF11 and GDF8 in human blood, and it appears that specific subforms of GDF11 may change in abundance in an age-specific manner (unpublished observations).

GDF11 and geronic effects

In prior publications[12,14,15,78], we reported that supplementation of circulating GDF11 can reverse age-related deficits in multiple major organ systems, recapitulating many of the effects seen with heterochronic parabiosis. However, some subsequent publications have challenged the notion that GDF11 may be beneficial in aging (anti-geronic), suggesting that it also possesses potential "pro-geronic" actions. Such contradictory impacts on aging phenotypes are not dissimilar to those of other aging-relevant regulators, including IGF1[86,87] metformin[88], and rapamycin[89], likely reflecting dose-dependent and context-specific functions. Still, a handful of studies[29,31,82,90] have directly challenged our reports[12,15] that supplementation of GDF11 can have beneficial effects on the heart and skeletal muscle. In this regard, as discussed above, it is important to note that subsequent work from our group demonstrated that at least some of the reported



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		Inheritance Pattern	Mutation Type	Mutation Site	Ribs	Cardiac abnormalities
	Gdf11 ^{KO}	N/A	Nonsense	N/A	+	?
1	N94Rfs*47	Autosomal Dominant	Nonsense	Prodomain	-	-
2	Q147Gfs*82	Autosomal Dominant	Nonsense	Prodomain	+	+
3	R295P	*De novo*	Missense	Furin cleavage site	+	+
4	R298Q	Genetic	Missense	Furin cleavage site	+	+
5	E306K	*De novo*	Missense	Mature domain	+	+
6	T319Nfs*5	*De novo*	Nonsense	Mature domain	+	-
7	Y336*	*De novo*	Nonsense	Mature domain	+	+

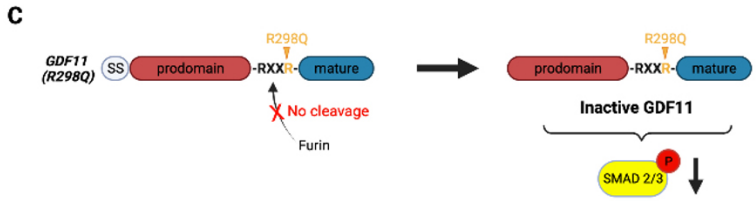

Figure 4. Human genetic diseases associated with GDF11 mutations. (A) 7 mutation sites have been identified in the GDF11 gene associated with several defects such as cleft palate and skeletal abnormalities. Mutations 1 and 2 are located in the prodomain, Mutations 3 and 4 in the Furin cleavage site, and Mutations 5-7 in the mature domain of GDF11. (B) Summary table of GDF11 mutations identified in humans. (+) indicates defects; (-) indicates no defect. + Ribs suggest skeleton defects. (C) Schematic depicting the functional impact of mutation 3, located in the Furin cleavage site, highlighting the importance of Furin cleavage for GDF11 activity.

discrepancies relate to variability in the specific activity of commercially available recombinant GDF11 protein and to critical differences in experimental design[74,78], and additional follow up studies from our labs[78] as well as studies from independent research groups[82,91-93], have confirmed the reproducibility of our published results. The most controversial aspects of GDF11's potential geronic activities relate to its impact on skeletal muscle biology, which we address below.

Effect of GDF11 supplementation on cardiac hypertrophy

Our studies published in 2013[15] and 2015[78] reported an anti-hypertrophic effect of rGDF11 administration by comparing heart weight-tibia length (HW/TL) ratio in treated and control aging mice, while a study by Smith *et al.* reported no effects on the heart[31]. As discussed in[74], this disagreement appears to reflect dose-dependent effects of rGDF11 on cardiac mass, and an additional study revealed a positive influence of GDF11 on cardiac function and infarct size after cardiac injury in aged mice[92]. The potential benefits of GDF11 in cardiac hypertrophy[94] have now been confirmed by multiple laboratories[32,95-97].

GDF11 and GDF8 - effects on skeletal muscle mass and regeneration

Initial studies from our labs indicated that rGDF11 supplementation reverses age-related muscle dysfunction and improves muscle strength, endurance, and regenerative potential in aged mice, with no discernable effects in young mice[12]. However, a subsequent paper from David Glass's lab, then at Novartis, pursuing therapeutics that would antagonize GDF8 and GDF11 to treat age-related muscle

dysfunction[98-100], argued that supplementation with rGDF11 has no effect in aged mice and slows skeletal muscle repair in young mice[29]. As we discussed in a review published in 2016[74], it is possible that these conflicting results arose from critical differences in experimental design, particularly in the use of different muscle injury models, and differences in the dosage and bioactivity of the particular rGDF11 molecule that was administered in each study. Specifically, while our studies used a cryoinjury model[12], which causes limited damage to endogenous regenerative muscle stem cells, the Glass study[29] used a more severe cardiotoxin (CTX) model, which ablates > 85% of satellite cells[101,102]. Given this severe depletion of muscle regenerative cells in the CTX model, it might have been predicted that rGDF11 would fail to enhance regeneration in CTX-injured aged mice. In contrast, we reported enhanced regeneration after single cryoinjury in rGDF11-supplemented aged animals, which retain a largely intact pool of muscle satellite cells. The Glass lab's results in aged CTX-injured animals may reflect the severe depletion of regenerative stem cells in this model. This notion is further supported by data published by Sinha *et al.* demonstrating that when combined with satellite cell transplantation[12], rGDF11 supplementation does indeed enhance regenerative myogenesis in aged, CTX-injured muscle. Importantly, as muscle injury in older humans more often resembles the focal damage induced by cryoinjury, as opposed to the full muscle necrosis caused by CTX, we believe additional experimentation will resolve the discrepancy in prior results and encourage continued investigation of GDF11 as a target for aging muscles.

Exogenous GDF11 and toxicity

It is important to consider that the genetic loss of endogenous GDF11 and GDF8 function may not be the opposite of gain of function through exogenous mature ligands. There is extensive regulation of endogenous ligand activity through protease activation of the prodomains, for example. In addition, these ligands have several important endogenous inhibitors that can bind tightly and likely permanently inhibit signaling. Administration of mature GDF11 and GDF8 ligands bypasses the prodomain activation step and may lead to signaling before endogenous inhibitors can bind to the ligand. In the brain, endogenous GDF11 may have different functions depending on region, and exogenous GDF11 does not appear to appreciably penetrate the uninjured blood-brain barrier.

There also have been several reports suggesting that dosage of rGDF11 at very high levels in mice may drive muscle atrophy and fibrosis as well as death[32,69]. These data highlight the importance of understanding the biology of the GDF11 system, as articulated above. In particular, our studies clearly indicate a change in signaling when GDF11 is applied at very high levels, making it more "GDF8-like" and eliminating its normally pro-regenerative signaling activity. Thus, it is unsurprising that administration of rGDF11 at very high doses, which are neither physiologically nor therapeutically relevant, would produce Myostatin-like effects. Critically, such effects should not be taken as a true reflection of the normal biology of GDF11 *in vivo*, or its therapeutic potential. Indeed, data from our labs, and others, using much lower doses to achieve more modest increases in GDF11 levels have demonstrated a meaningful therapeutic window for GDF11 in numerous aging and disease models[66,78].

CONCLUSION AND PERSPECTIVES

As lifespan increases and the world's population grows, the need to develop more effective approaches to treat heart disease and other age-associated dysfunctions is greater than ever. GDF11 biology may play roles in the progression of age-related diseases, but the simple concept that GDF11 levels decline with aging and can be replaced like thyroid hormone is incorrect. Despite conflicting reports over the potentially divergent functions of the two ligands and continuing controversy over GDF11 function and potential effects in age-associated organ dysfunction, GDF11 and GDF8 continue to be pursued as important disease targets for the development of possible therapeutics. The new human *GDF11* genetic diseases show that understanding

GDF11 biology is important for humans, although the mutations may be rare. It is also unclear if GDF11 replacement could benefit patients with genetic loss of function mutations. For common diseases like coronary disease and other acquired diseases of aging, the role of GDF11 is unclear. The role of GDF11 measurements in human blood is incompletely defined, and a current conundrum is why mass spectrometry measurements of GDF11 in humans had not been revealing, while an aptamer measurement of GDF8/11 in humans was predictive of outcome in two moderate-size studies of coronary heart patients. More definitions of molecular mechanisms, including which target cells are activated, are needed to understand the effects of exogenous GDF11.

There are different major gaps in our understanding of how to leverage GDF11 as a potential therapeutic signaling molecule. First, the general coordination of cellular GDF11 signaling needs to be defined. There is a lack of spatial understanding regarding the series of processing, latency, and activation required for GDF11 signaling. Second, the best delivery mechanism for GDF11 therapy needs to be established. Current efforts have focused on injecting a bolus of mature GDF11. However, the half-life of GDF11 is ~12 h, and a significant amount of GDF11 needs to be injected, indicating a significant loss of the protein before it reaches its destination. Furthermore, injecting large amounts of GDF11 might deliver off-target effects, as was observed in mice with high doses of GDF11, which led to cachexia. Third, it is essential to understand how the recent discovery of GDF11 mutations impacts protein function and which methods of delivery (recombinant or viral expression) are capable of reintroducing GDF11 signaling in target cells.

DECLARATIONS

Author's contribution

Design of the figures: Ben Driss L, Howard JA

Manuscript conception, writing, and editing: Ben Driss L, Lian J, Walker RG, Thompson TB, Rubin LL, Wagers AJ, Lee RT

Availability of data and materials

No original data were generated for this review article.

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Conflicts of interest

Lee RT, Rubin LL, and Wagers AJ are cofounders and members of the scientific advisory board for and hold private equity in Elevian, Inc., a company that aims to develop medicines to restore regenerative capacity. Elevian also provided sponsored research support to the Lee RT, Rubin LL, and Wagers AJ labs. Lee RT, Rubin LL, Wagers AJ, and Thompson TB have filed patents related to GDF11 and GDF8 through their institutions.

Ethical approval and consent to participate.

Not applicable.

Consent for publishing.

Not applicable.

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