Peer-Review Record

GDF11 and aging biology - controversies resolved and pending

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Academic Editor: Ali J. Marian

Reviewer 1: Anonymous

Reviewer 2: Anonymous

Reviewer 3: Anonymous

Round 1

Reviewer 1 Report

The review suggests changing the title. The current title of "post-natal biology" is rather too broad and non-specific. It does not seem to be within the scope of a cardiovascular journal, particularly a cardiovascular aging journal. The content of the article is also not exclusive to biology.

The review is structured to follow the temporal development of the findings, particularly the initial controversy surrounding the translation effects of GDF11 in muscle and brain aging. It might be better to restructure the article to discuss the basic biology of GDF11 before discussing the findings in translational studies.

The reviewer suggests introducing the GDF subfamily of proteins, their structures, expressions, and biological functions, before focusing on GDF11.

Several parts of the review read like a narrative, focused on the findings of the authors and others. It should be revised to read like an authoritative review article that extracts insights from the existing data without stating that X found this and Y found that.

The authors emphasize the putative role of the circulating factor in muscular aging but come short of specifics, except for GDF11. The reviewer suggests briefly introducing the circulation factors that are potentially involved in aging, before starting the discussion on GDF11.

Dr. Houser and colleagues have withdrawn a few papers due to scientific misconduct. The authors should make sure not to cite such papers in their review article.

There are a few places where the original articles should be cited (when describing specific findings) rather than a review article.

“Animal with age” should be rephrased.

gdf11 should be Gdf11 (line 216).

The section on GDF11 as a “regulator of metabolism” seems to be out of place. There is not much discussion on metabolism here.

Lines 241-242: “GDF11 and GDF8 have 89% amino acid identity in their mature domains, differing in their sequence only by 11 amino acids [72]. Is it 11% or 11 amino acids? It refers to the mature form of these proteins. The authors might wish to mention the significance of the differences in the pro-domains, which are much less similar (Figure 1).

Line 315: Bmp1 gene should be in italics.

Line 346: the authors state that it is unclear how common GDF11 mutations are. Such data should be available in several databases such as Gnomad, ClinVar, etc. The authors also should review the association of the GDF11 mutations with cardiovascular diseases (not much in the databases).

Figure 4 could be deleted and the data from the databases could be cited.

Authors’ Response

1#. Exact Reviewer comment:

“The review suggests changing the title. The current title of “post-natal biology” is rather too broad and non-specific. It does not seem to be within the scope of a cardiovascular journal, particularly a cardiovascular aging journal. The content of the article is also not exclusive to biology”

We changed the title according to your comment. Changes made to revised manuscript:

New title: GDF11 and aging biology – controversies resolved and pending.

2#. Exact Reviewer comment:

"The review is structured to follow the temporal development of the findings, particularly the initial controversy surrounding the translation effects of GDF11 in muscle and brain aging. It might be better to restructure the article to discuss the basic biology of GDF11 before discussing the findings in translational studies." And "The reviewer suggests introducing the GDF subfamily of proteins, their structures, expressions, and biological functions, before focusing on GDF11."

We thank the reviewer for the suggestion and have now added a part about GDF11 basic biology as suggested.

Changes made to revised manuscript: GDF11, also known as Bone Morphogenetic Protein 11 (BMP11), and its closely relative protein GDF8, also known as Myostatin, are members of the TGF β superfamily and share 89% of identity. GDF8 is only expressed in skeletal muscle and plays an evolutionarily conserved role in antagonizing postnatal skeletal muscle growth, limiting both the number and size of individual muscle fibers. Disruption of the Gdf8 gene or targeted inhibition of GDF8 protein triggers hyper-muscular phenotypes in many mammals and fish. GDF11, in contrast, is ubiquitously expressed and plays a broad role during mammalian development, regulating anterior/posterior patterning, formation of the kidney, stomach, spleen and endocrine pancreas, and olfactory neurogenesis. GDF11 functions in postnatal tissues are less explored partially due to the perinatal lethality of Gdf11-knockout mice, which do not survive with homeotic skeletal transformations, cleft palate, and renal agenesis.

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 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, which initiates the downstream SMAD signaling cascade. While there are over 30 TGF β family ligands, only 5 Type II receptors and 7 Type I receptors are available for signaling. Signaling is differentiated at the receptor level where different combinations of Type I and Type II receptors can elicit different downstream responses. GDF11 and GDF8 are members of the Activin subclass which signal through the Type I receptors ALK4, ALK5 and ALK7.

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. While it was shown that GDF11 and GDF8 signal through similar receptors, a direct rigorous comparison of the ligands had not been performed until we published a study demonstrating that GDF11 and GDF8 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. Comparison of the GDF11 and GDF8 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 GDF11 and GDF8 are functionally equivalent in vivo. An in vivo study highlighted distinct endogenous activities of GDF11 and GDF8 by genetically modifying their mature signaling domains. Full recoding of the GDF8 mature domain to that of GDF11 yielded mice lacking GDF8, with GDF11 levels ~50-fold higher than normal, and exhibiting modestly decreased muscle mass, with no apparent negative impacts on health or survival to adulthood. Substitution of the two specific amino acids in the fingertip region of GDF11 with the corresponding GDF8 residues resulted in prenatal axial skeletal transformations, consistent with Gdf11-deficient mice, without apparent perturbation of skeletal or cardiac muscle development or homeostasis. These experiments, uncover distinctive features between the GDF11 and GDF8 mature domains in vivo, and thus it is clear that the endogenous mature ligands are functionally different.

3#. Exact Reviewer comment:

"Several parts of the review read like a narrative, focused on the findings of the authors and others. It should be revised to read like an authoritative review article that extracts insights from the existing data without stating that X found this and Y found that."

We agree that an authoritative review can be easier to follow. Since some of the controversies involved work done by us and others, we felt that this part should be preserved. For example, we described data interpretation errors that we made, and we feel that perspective is important. We hope the reviewer will agree and allow us to keep some of this information.

Changes made to revised manuscript : We modified the text in this way (modifications in bold).

As an example: "Soluble factors and cells cross-circulate in heterochronic parabiotic mice, and studies from multiple labs, including ours [13-19], demonstrate that this intervention can restore more youthful functions in the heart, skeletal muscle, bone, endocrine and central nervous systems of old partners [13-24]"

Replaced by "Soluble factors and cells cross-circulate in heterochronic parabiotic mice, and many studies [13-19], demonstrated that this intervention can restore more youthful functions in the heart, skeletal muscle, bone, endocrine and central nervous systems of old partners [13-24]."

4#. Exact Reviewer comment:

"The authors emphasize the putative role of the circulating factor in muscular aging but come short of specifics, except for GDF11. The reviewer suggests briefly introducing the circulation factors that are potentially involved in aging, before starting the discussion on GDF11."

To address circulation factors levels and aging, we added text in the paragraph focused on parabiosis (sentence in bold)

Changes made to revised manuscript : The aging process induces different structural and functional changes in the body suggesting changes between young and old subject. To understand the possible influence of systemic factors in aging phenotypes, many studies have used parabiosis experiments. This technique joins two mice surgically so that they 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 from [13-19], demonstrated that this intervention can restore more youthful functions in the heart, skeletal muscle, bone, endocrine and central nervous systems of old partners [13-24]. Conversely, heterochronic circulation has been shown to suppress healthy function in

young animals in a subset of these systems [20-22, 25]. 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 [18] but also induces heightened WNT signaling and fibrosis that suppresses stem cell function and regenerative myogenesis in the young mice [26]. Thus, in skeletal muscle, heterochronic parabiosis exerts clear bi-directional effects, remodeling muscle ultrastructure and enhancing repair in aged partners [13, 14, 18], and suppressing regenerative function and exacerbating fibrosis in young partners [20]. 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. (27).

5#. Exact Reviewer comment:

"The section on GDF11 as a "regulator of metabolism" seems to be out of place. There is not much discussion on metabolism here."

We agree, and thus we deleted these paragraphs. Changes made to revised manuscript: Deletion of metabolism paragraph.

6#. Exact Reviewer comment:

"Lines 241-242: "GDF11 and GDF8 have 89% amino acid identity in their mature domains, differing in their sequence only by 11 amino acids [72]. Is it 11% or 11 amino acids? It refers to the mature form of these proteins. The authors might wish to mention the significance of the differences in the pro-domains, which are much less similar (Figure 1)."

We clarified that this referred to 11 of amino acids in the mature domain. We agree on the 52% identity in their prodomain as an essential point.

Changes made to revised manuscript: To clarify this part, "GDF11 and GDF8 have 89% amino acid identity in their mature domains, the active ligand of these proteins, differing in their sequence only by 11 amino acids", we modified the text for "GDF11 and GDF8 have

89% amino acid identity in their mature domains, differing in their sequence only by 11 amino acids”.

7#. Exact Reviewer comment:

“Line 346: the authors state that it is unclear how common GDF11 mutations are. Such data should be available in several databases such as Gnomad, ClinVar, etc. The authors also should review the association of the GDF11 mutations with cardiovascular diseases (not much in the databases).”

After exploring the Gnomad database and CVD software, we now include the following sentences.

Changes made to revised manuscript: “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)”

Reviewer 2 Report

The manuscript by Driss and colleagues seek to review the field of GDF11 biology and its role in human health and disease, while highlighting the controversies that have plagued this field based on animal studies and the use of the recombinant GDF11 protein. Overall, this is an outstanding review that does an excellent job in trying to reconcile the data that is within the field in light of new studies that are coming out in the field, which relate to potential functional domain/potential states(subforms)/activity, tissue-specific actions, exogenous versus endogenous actions of GDF11 versus GDF8. Further comments are provided in order to strengthen the manuscript.

Major Comments:

1. Abstract: The abstract could be more reflective and encompassing of why GDF11 is not functionally identical to GDF8 versus leaving it open ended. Inclusion of the areas of discussion such as functional domains/potential states (subforms)/activity should be generally referenced to provide a better overview of what is to be expected in the review.

2. It would be helpful if the authors put together a Table based on their studies highlighted in the Controversies section, to provide an overview of the blood/tissue types that are at the heart of the controversies, outcomes that were at the heart at the controversies and

related papers, and if these have been solved or what future directions will shed light on solving these controversies.

3. Could the authors shed further highlight on what studies focused on exogenous versus endogenous GDF11 and GDF8, and how this may be important in shedding light on the controversies, especially as it relates to the brain/skeletal muscle studies.

4. Figure 3: Colors do not relate to the images provided to depict the states of GDF11 and the domains. Please be congruent with color schemes.

5. Figure 4: What were the cardiac abnormalities reported as the legend focuses on skeletal muscle abnormalities. Please further describe what is meant by Ribs. Many of the follow-up studies related to the human mutations were done in non-mammalian studies. Were any studies done in mammalian cells or settings?

6. Reference 76 is not peer-reviewed. Is there any other data that supports the idea that domain replacement moderates the phenotypes?

7. The authors highlight the many subforms of GDF11; however, which ones are the most important (are they all equally important?)? It is not clear what forms would be detected using conventional strategies? Is this an area where investigators should be mindful of overinterpreting the activity and functions of GDF11?

Authors' Response

1#. Exact Reviewer comment:

"Abstract: The abstract could be more reflective and encompassing of why GDF11 is not functionally identical to GDF8 versus leaving it open ended. Inclusion of the areas of discussion such as functional domains/potential states (subforms)/activity should be generally referenced to provide a better overview of what is to be expected in the review."

We agree that in the first version of our abstract we focused on the variation of GDF11 levels in aging without any explanation about what is GDF11 itself. We modified the abstract according to this comment.

Changes made to revised manuscript:

New 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 more potently induces SMAD2 phosphorylation in the myocardium compared to GDF8. GDF8 and GDF11 prodomains inhibit the mature domains, and the prodomains are only 52% identical. The prodomains 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."

2#. Exact Reviewer comment:

"It would be helpful if the authors put together a Table based on their studies highlighted in the Controversies section, to provide an overview of the blood/tissue types that are at the heart of the controversies, outcomes that were at the heart at the controversies and related papers, and if these have been solved or what future directions will shed light on solving these controversies."

We added the following table. Changes made to revised manuscript:

Controversies	Status	Conclusion	References
Total Circulating GDF11 during aging	Resolved	The total amount of circulating GDF8 (but not GDF11) declines with aging.	[8] [9] [10] [11-13]
GDF11 and Geronic Effects	Partially resolved	Supplementation on rGDF11 can reverse age-related deficits in different organs (incompletely resolved in skeletal muscles)	[8, 14-16] [11, 21-23]
Cardiac hypertrophy	Resolved	Supplementation on rGDF11 reduces cardiac hypertrophy in aging	[8] [10] [21] [29] [30-33]
Exogenous GDF11 and toxicity	Resolved	Exogenous rGDF11 at high doses produces Myostatin-like effects	[30, 34]

3#. Exact Reviewer comment:

“Could the authors shed further highlight on what studies focused on exogenous versus endogenous GDF11 and GDF8, and how this may be important in shedding light on the controversies, especially as it relates to the brain/skeletal muscle studies.”

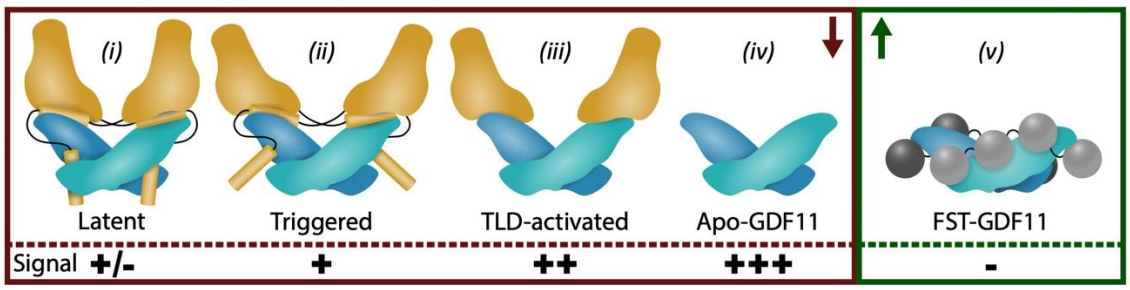
We added the following paragraph: Changes made to revised manuscript:

“It is important to consider that 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 ligand 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.”

4#. Exact Reviewer comment:

“Figure 3: Colors do not relate to the images provided to depict the states of GDF11 and the domains. Please be congruent with color schemes.“

To help the reader, we modified the colors to homogenize figure 2 and figure 3 based on the GDF11 states. Changes made to revised manuscript:



5#. Exact Reviewer comment:
“Figure 4: What were the cardiac abnormalities reported as the legend focuses on skeletal muscle abnormalities. Please further describe what is meant by Ribs. Many of the follow-up studies related to the human mutations were done in non-mammalian studies. Were any studies done in mammalian cells or settings?”

The abnormalities reported in the legend focused on the skeleton (not skeletal muscle) according to the gdf11-KO mice phenotype. The deletion of Gdf11 in mice leads to more ribs and a cleft palate; this is the reason why we focused on these two parameters.

Changes made to revised manuscript: We modify the figure legend to clarify the skeletons alterations. These mutations have not been rigorously studied in mammalian cells or settings thus far.

6#. Exact Reviewer comment:
“Reference 76 is not peer-reviewed. Is there any other data that supports the idea that domain replacement moderates the phenotypes?”

Reference 76 is now published in Life Science Alliance. Changes made to revised manuscript: We modified the reference.

7#. Exact Reviewer comment:
“The authors highlight the many subforms of GDF11; however, which ones are the most important (are they all equally important?)? It is not clear what forms would be detected using conventional strategies? Is this an area where investigators should be mindful of overinterpreting the activity and functions of GDF11?”

To clarify this, we added the paragraph below. Changes made to revised manuscript :

We added the following paragraph: 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. Step 3 - The signal sequence is cleaved upon secretion of the pro-complex in the extracellular matrix. Step 4 - 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. 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.

Reviewer 3 Report

GDF11 controversy is an important topic in ageing research and a comprehensive review to highlight the current progress in resolving these issues would bring clear interest from the field.

Authors should be commended for a fairly balanced and critical review that point out past mistakes and current state of understanding to some of key controversial issues. What would be much more interesting and beneficial to the field is to dedicate a section on the critical knowledge gap in GDF11 biology: biochemistry and regulation of its biogenesis and activation, receptor-specificity and downstream signaling, et al. It would be even better if authors can offer their perspective on the potential solutions for future studies, model systems, structural insights, species specific studies? These additional information would help the field to focus on future.

Authors' Response

Changes made to revised manuscript: We added the following paragraphs:

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 hours, 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 it was observed in mice with high doses of GDF11 leading 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.