Reduced Circulating GDF11 Is Unlikely Responsible for Age-Dependent Changes in Mouse Heart, Muscle, and Brain

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Recent high-profile studies report conflicting data on the age-related change in circulating growth/differentiation factor 11 (GDF11) and myostatin as well as the former's influence on muscle regeneration. Both ligands bind and activate ActRIIB receptors with similar affinities and should therefore have similar actions, yet these studies suggest that GDF11 activates muscle regeneration whereas myostatin is well known to inhibit it. They also suggest that circulating GDF11 levels, but not those of myostatin, decline with age. We performed a careful assessment of the ELISA used to quantify circulating myostatin in these studies and determined that assay reagents significantly cross react with each protein, each of which is highly homologous. Circulating myostatin levels decreased with age and estimates of GDF11 levels using myostatin null mice indicate that they were almost 500 times lower than those for myostatin. This suggests that circulating GDF11 has little physiological relevance as it could not outcompete myostatin for ActRIIB binding sites. Together, these results further suggest that the previously reported aging muscle, heart, and brain phenotypes attributed to reduced circulating GDF11 should be reconsidered. **(*Endocrinology* 156: 3885–3888, 2015)**

A recent report in *Cell Metabolism* (1) questions three high-impact reports in *Cell* (2) and *Science* (3, 4), which suggest the age-related loss of circulating growth/differentiating factor 11 (GDF11) levels— but not those of another highly homologous ligand, myostatin— compromises cardiac and skeletal muscle function as well as vascular remodeling and neurogenesis. Sinha et al (4) specifically suggest that GDF11 "rejuvenates" aged muscle by restoring the levels and activity of satellite cells (ie, muscle stem cells). However, their results conflict with the very well-defined actions of myostatin (ie, growth/differentiation factor 8) which, like GDF11, binds the ActRIIb receptor, yet in contrast with data reported by Sinha et al (4), inhibits satellite cell proliferation (5, 6). Their results also conflict with Egerman et al (1) who further demonstrated that circulating GDF11 levels actually increase, not decrease, with age and that its suppressive actions in skeletal muscle are highly consistent with those of myostatin.

The age-related decline in circulating GDF11, but not myostatin (2, 4), is largely based upon assays (Western blots & ELISAs) using antisera that almost certainly cross react with both GDF11 and myostatin. Indeed, these antisera were generated against peptides that are 92% identical and according to the manufacturer's (R&D Systems) Web site; they have not been validated competitively in the presence of each competing homolog. There is no objective evidence, therefore, that circulating GDF11 levels actually decline with age despite the reported claims. We tested a critical assay used by Sinha et al (4) and additionally quantified circulating myostatin levels in mice of different ages. Our assessment suggests that circulating myostatin levels decline with age, that some critical reagents used in the previous studies (2–4) may have produced unreliable results and misleading conclusions and, more importantly, that circulating GDF11 has little if any physiological relevance.

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Abbreviation: GDF11, growth/differentiation factor 11.

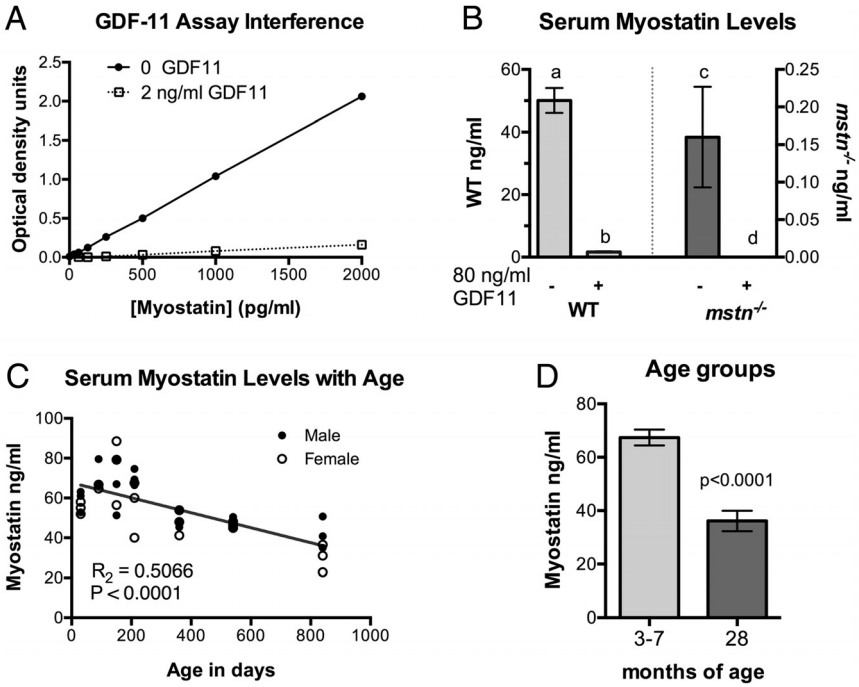

Figure 1. Circulating myostatin levels decrease in aging mice. A, A myostatin standard curve was generated in the absence or presence of recombinant GDF11. Serum myostatin levels were quantified in 3-month-old wild-type and *mstn*$^{-/-}$ male mice (n = 5/group), again in the absence or presence exogenous addition of GDF11 (B, $P < .05$ indicated by different letters) and in three 28 month-old wild-type mice of both sexes (C and D). Significant differences were determined by a regression analysis (C) and by a Student *t* test (D).

Materials and Methods

Assay validation of the R&D System myostatin ELISA (ELISA, catalog No. DGDF80) was performed by running a standard curve in the absence or presence of 2 ng/mL GDF11, also acquired from R&D Systems (catalog No. 1958-GD-010). This assay (without additional GDF11) was then used to quantify serum myostatin levels in male and female mice of different ages. Serum was acquired from the National Institute on Aging, National Institutes of Health (see Acknowledgments) and from mice within breeding colonies established at the Washington State University. Handling and maintenance of mice was performed according to protocols preapproved by Institutional Animal Care and Use Committees at these institutions. In each experiment, assays were constructed and performed according to the manufacturer's protocol. Significant differences among mice of different ages were determined by a regression analysis and among mice from binned age groups, by a Student *t* test.

Results

Using the identical ELISA as Sinha et al (4), we quantified standard curves for myostatin in the absence and presence of competing GDF11 (Figure 1A). Our data clearly demonstrate that GDF11 significantly compromises the quantification of myostatin when both proteins are present as the recognition of myostatin standards was almost completely displaced by GDF11. These novel data complement those of Egerman et al (1) who documented nonspecificity of the GDF11 SOMAmer and Western blotting antiserum used by Sinha et al (4). The addition of excess GDF11, at concentrations slightly below the previously reported circulating levels of murine myostatin (7), also compromised myostatin quantification in serum from healthy adult mice (Figure 1B). Most importantly, myostatin was also detected in some serum samples from *mstn*$^{-/-}$ mice, which cannot be myostatin and most likely represents circulating GDF11. These levels are comparable to those reported by Egerman et al (1) using a novel, specific, and validated assay to measure serum GDF11 in mice, rats and humans.

We also quantified serum myostatin in mice of varied ages and determined that it gradually declined with age (Figure 1, C and D). This was evident when the sample population was assessed by regression analysis and in pairwise comparisons of binned age groups where serum levels in 24-month-old mice were just 58% of those in 3–7-month-old mice. These data conflict with Sinha et al (4), but are highly consistent with the previously reported age-related decline in circulating myostatin levels among human subjects (7). They are also consistent with Egerman et al (1) who reported reduced myostatin mRNA expression in aged muscle; that circulating GDF11 levels increase, not decrease; and that GDF11 inhibits rather than stimulates myogenesis.

Discussion

Although the R&D Systems myostatin ELISA cannot distinguish myostatin from GDF11, circulating levels of the latter are inconsequentially low and likely have little physiological relevance. Indeed, both myostatin and GDF11 share comparable picomolar affinities for the ActRIIb receptor (8), yet myostatin's circulating molar concentration is almost 500 times greater. Circulating GDF11 would, therefore, not be able to successfully compete for

ActRIIb binding sites. This does not imply, however, that autocrine and paracrine GDF11 is without effect, only that the cytokine functions primarily as a local rather than endocrine regulator and that changes in circulating GDF11 levels per se are mostly irrelevant. Indeed, another ActRIIb ligand, activin, circulates at levels that are similar to those of GDF11 or up to 10-fold higher, yet activin functions as an autocrine and paracrine factor (9, 10).

It follows that many conclusions presented by Loffredo et al (2), Sinha et al (4), and Katsimpardi et al (3) need revisiting as their underlying assumptions—that the differential change in circulating GDF11 and myostatin and a biological relevance of the former—are incorrect. In fact, several studies report results that directly conflict with Loffredo et al (2) and Sinha et al (4) including the demonstration that myostatin inhibits many growth processes in skeletal and cardiac muscle, both of which are hypertrophied by attenuating ActRIIb ligands and atrophied with transgenic mice overexpressing myostatin (6, 11–16).

An important distinction between Sinha et al (4) and Egerman et al (1) is the source of recombinant GDF11 used in each study. Both used recombinants generated in *E. coli*, although from different companies: Sinha et al (4) from PeproTech and Egerman et al (1) from R&D Systems. Post-translational modification of GDF11 requires proteolytic cleavage and stabilization of the mature dimer by a disulfide linkage, which cannot occur in bacteria. Thus, recombinant GDF11 made in *E. coli* must be refolded and validated using an appropriate bioassay and although it is possible that such recombinants are biologically active, there is evidence that recombinant myostatin generated in *E. coli* is either less active or may even function as a dominant negative and produce contrary effects depending on cell type and differentiation status (17). Thus, differences in the quality of recombinant peptides used in each study could have potentially contributed to the inconsistencies between the studies and between what is reported by Sinha et al (4) and what is well known of ActRIIb activation. Adding to the confusion are errors in the data analysis performed by Sinha et al (4) as many of the statistical analyses (*t* tests and Mann-Whitney *U* test tests with more than two independent variables; Figures 1–3, S2, S4, S6, S11, S14, S19) required a Bonferroni correction that was not applied and as a result, many of the noted comparisons would not meet the minimal significance threshold of $P = .0083$ or $P = .0042$, depending on the experiment/Figure, instead of $P = .05$. In other words, many of the reported differences are simply not different.

Myostatin is expressed more widely than presumed and particularly in muscle, heart, brain, and fat and has pleiotropic actions beyond simply inhibiting skeletal muscle growth (6, 18). Some evidence even suggests that myostatin maintains blood vessel integrity. Of note, attenuating ActRIIb ligands in circulation, which includes primarily myostatin and activin, induced bleeding and resulted in the premature termination of a clinical trial testing ACE-031, a ligand-trap composed from the ActRIIb extracellular domain (19). The demonstration that ActRIIb ligands and specifically recombinant GDF11, for example, can restore blood vessel volume in aged mice (3) may actually be indicative of endogenous myostatin action and not that of GDF11. In fact, this may be true for many of the actions previously described for circulating GDF11.

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