Decreasing maternal myostatin programs adult offspring bone strength in a mouse model of osteogenesis imperfecta

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During fetal development, the uterine environment can have effects on offspring bone architecture and integrity that persist into adulthood; however, the biochemical and molecular mechanisms remain unknown. Myostatin is a negative regulator of muscle mass. Parental myostatin deficiency (Mstn<sup>m<sup>tm1Sjl</sup></sup>) increases muscle mass in wild-type offspring, suggesting an intrauterine programming effect. Here, we hypothesized that Mstn<sup>m<sup>tm1Sjl</sup></sup> dams would also confer increased bone strength. In wild-type offspring, maternal myostatin deficiency altered fetal growth and calvarial collagen content of newborn mice and conferred a lasting impact on bone geometry and biomechanical integrity of offspring at 4 mo of age, the age of peak bone mass. Second, we sought to apply maternal myostatin deficiency to a mouse model with osteogenesis imperfecta (Col1a2<sup>omim</sup>), a heritable connective tissue disorder caused by abnormalities in the structure and/or synthesis of type I collagen. Femora of male Col1a2<sup>omim/omim</sup> offspring from natural mating of Mstn<sup>m<sup>tm1Sjl</sup></sup> dams to Col1a2<sup>omim/omim</sup> sires had a 15% increase in torsional ultimate strength, a 29% increase in tensile strength, and a 24% increase in energy in failure compared with age, sex, and genotype-matched offspring from natural mating of Col1a2<sup>omim/omim</sup> dams to Col1a2<sup>omim/omim</sup> sires. Finally, increased bone biomechanical strength of Col1a2<sup>omim/omim</sup> offspring that had been transferred into Mstn<sup>m<sup>tm1Sjl</sup></sup> dams as blastocysts demonstrated that the effects of maternal myostatin deficiency were conferred by the postimplantation environment. Thus, targeting the gestational environment, and specifically prenatal myostatin pathways, provides a potential therapeutic window and an approach for treating osteogenesis imperfecta.

In addition to regulating muscle growth postnatally, myostatin deficiency in the maternal environment increases muscle mass in offspring (5). Its effects are not related to lactation, as they persist after cross-fostering to wild-type (WT) dams (5). Although many studies have shown that the gestational environment affects adult metabolic and cardiovascular outcomes (6), there has been comparatively little focus on the role of the maternal environment in adult bone health. Suggestive evidence comes from epidemiologic cohort studies, which report correlations between weight at birth and 1 y of age with bone integrity later in life (7, 8). In animal models, gestational nutrition and hormone concentrations affect skeletal microarchitecture, bone mineral content, strength, growth plate morphology, and osteoblast differentiation capability (9–14). However, the mechanisms remain unknown.

In this study, we tested the hypothesis that decreasing myostatin in the uterine environment will confer a lasting positive effect on bone strength using three independent approaches. First we compared bone geometry and biomechanical integrity in WT offspring from WT and myostatin-deficient dams and sires. This strategy was then extended to Col1a2<sup>omim/+</sup> mice, which are heterozygous for a functional null mutation in the Col1a2 osteogenesis imperfecta | developmental origins of health and disease | fetal programming | myostatin | bone health

Osteogenesis imperfecta (OI) is a genetically and clinically heterogeneous heritable connective tissue disorder characterized by anomalies in type I collagen-containing tissues, particularly bone. Clinical manifestations include osteopenia/osteoporosis, fractures, skeletal deformities, short stature, and skeletal muscle weakness. Current therapies entail use of antiresorptive drugs and surgical interventions but have limited success (1). New approaches that decrease fracture risk while minimizing long-term damage to bone integrity are greatly needed. Myostatin, or growth and differentiation factor 8 (GDF8), is a TGF-β family member and negative regulator of muscle growth and development. It is highly conserved across species, primarily expressed in muscle cells, and participates in paracrine and endocrine signaling (2). Recently, we and others have shown that inhibiting myostatin, either genetically or pharmacologically, increases muscle mass and improves bone microarchitecture (3) and mechanical strength (4) in the OI murine (Col1a2<sup>omim</sup>) model.

Significance

Osteogenesis imperfecta (brittle bone disease) is an incurable genetic disorder. We demonstrate that maternal deficiency of myostatin (a negative regulator of muscle growth) can enhance bone biomechanical strength and integrity in control and osteogenesis imperfecta mouse offspring, using three independent approaches. We provide evidence that bone is responsive to developmental programming and that myostatin can mediate these effects. Embryo transfer experiments show that the effects of maternal myostatin deficiency are conferred by the postimplantation environment. These studies represent a paradigm shift in understanding and treating osteogenesis imperfecta—a shift from believing only genetic and postnatal environmental factors control bone health to the inclusion of prenatal/perinatal developmental programming as a modifiable factor controlling adult bone health.


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gene of type I collagen (15) and model the milder type I clinical form of OI (15, 16). Thus, in this second approach, we tested if the maternal myostatin deficiency that strengthened WT offspring bone could also strengthen OI bone. Finally, in a third approach, we sought to determine the critical window during gestation when maternal myostatin deficiency programs adult offspring bone strength. Osteoblast differentiation and fetal bone development occur after implantation, but major epigenetic reprogramming events occur during oogenesis and pre-implantation development. Thus, we tested whether the effects of maternal myostatin deficiency on Col1a2-oim/+ offspring bone could be conferred on offspring exposed only after embryo implantation. Embryos were transferred on gestational day 3.5 postcoitum (d3.5) from Col1a2-oim/+ donors to either Col1a2-oim/+ or Mstntm1Sjl/+ recipients, and the effects on adult bone microarchitecture and biomechanical integrity were investigated.

Results

Approach 1: Effect of Parental Myostatin Deficiency on Bone Geometry and Biomechanics of WT Offspring. To test the effect of parental myostatin deficiency on WT offspring bone strength, WT and Mstntm1Sjl/+ males and females and their femoral (B) torsional ultimate strength (Tmax), (C) tensile strength (Su), (D) torsional stiffness (Ks), and (E) shear modulus of elasticity (G). (F) Energy to failure (U). Values are least-squares means (LSmeans) ± SE. *P < 0.05. †P = 0.06. n(offspring/dam): WT, n = 19; Mstntm1Sjl/+ n = 23.15 dams.

Effect of Parental Myostatin Deficiency on Perinatal Growth and Collagen Content of WT Offspring. To evaluate how maternal myostatin deficiency impacts fetal development, placental and fetal growth were examined in late pregnancy (d16.5) in WT and Mstntm1Sjl/+ dams. WT male fetuses weighed 25% more when the sire was also Mstntm1Sjl/+ (Fig. 2C). Col1a2 expression was increased in offspring of Mstntm1Sjl/+ dams only when the sire was also Mstntm1Sjl/+ (Fig. 2D). However, hydroxyproline content per gram of bone (an indirect measure of type I collagen) was similar in the WT offspring tibiae from either WT or Mstntm1Sjl/+ dams (Fig. 2E). Together these data suggest that maternal myostatin deficiency does not alter the overall collagen content or its synthesis per gram of bone in adult offspring but may impact osteoblast activity and/or number.

Effect of Parental Myostatin Deficiency on Tibial Microarchitecture of WT Offspring. The tibial trabecular microarchitecture of 4-month-old WT male offspring from WT and Mstntm1Sjl/+ dams was assessed by high-resolution μCT. The trabecular bone volume to total volume (BV/TV), number, thickness, and separation were equivalent among the four crosses, exhibiting no main parental effect or interactions (Table S2).

Table 1. Femoral geometry of WT male offspring

<table>
<thead>
<tr>
<th>Femoral parameter</th>
<th>WT dam</th>
<th>Mstntm1Sjl/+ dam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur length, mm</td>
<td>16.34 ± 0.06</td>
<td>16.58 ± 0.05*</td>
</tr>
<tr>
<td>Cortical bone width, mm</td>
<td>0.37 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Marrow diameter, mm</td>
<td>1.09 ± 0.03</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>Polar moment of area, mm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.83 ± 0.038</td>
<td>0.94 ± 0.033*</td>
</tr>
</tbody>
</table>

Values are LSmeans ± SE. *P < 0.05 compared with WT dam. n(offspring/dam): WT, n = 30; Mstntm1Sjl/+ n = 36.
bone formation, neonatal calvaria were harvested and evaluated for type I collagen expression and content. Calvaria harvested from neonatal WT pups from Mstntm1Sjl+/+ dams did not exhibit differences in the expression of the type I collagen genes Col1a1 or Col1a2 (Fig. 3 G and H), although they had 34% more hydroxyproline per gram of bone than calvaria harvested from WT dams (Fig. 3f).

Approach 2: Effect of Maternal Myostatin Deficiency on Bone Geometry and Biomechanics of Offspring. OL compromises bone material integrity and alters osteoblast and osteoclast activities. To determine if OL bone can respond to prenatal exposure to maternal myostatin deficiency, femora of Col1a2oim+/+ mice born to Col1a2oim+/+ and Mstntm1Sjl+/+ dams were evaluated (Fig. 4A). As in WT offspring, adult male Col1a2oim+/+ offspring body and muscle weights were not affected by maternal myostatin [sample size for females was insufficient (Table S1)]. Perinatal exposure to myostatin deficiency also did not alter femoral geometry (Table S3), as assessed by μCT.

By torsional loading to failure, femora of male Col1a2oim+/+ offspring from Mstntm1Sjl1/+ dams had increased femoral torsional ultimate strength (Tult) and tensile strength (Su) by 15% and 29% (Fig. 4B and C) relative to Col1a2oim+/+ offspring from Col1a2oim+/+ dams. These improvements are similar to those seen in WT offspring born to Mstntm1Sjl1/+ dams (Fig. 1). Femoral energy to failure (U) was not significantly increased in male Col1a2oim+/+ offspring from Mstntm1Sjl1/+ dams, although as in WT offspring (Fig. 1F), there was some evidence of an increase (Fig. 4F, P = 0.07). Femoral stiffness and shear modulus of elasticity were equivalent in male Col1a2oim+/+ offspring from Col1a2oim+/+ and Mstntm1Sjl1/+ dams (Fig. 4D and E). In contrast to males, femora from female Col1a2oim+/+ offspring did not exhibit differences in biomechanical strength based on maternal genotype (Fig. S1). Overall, bone strength in Col1a2oim+/+ offspring benefited similarly to that of WT offspring from being born to Mstntm1Sjl1/+ dams, although the geometric impact was not as robust.

Effect of Maternal Myostatin Deficiency on Bone Composition of Offspring. To determine whether the enhanced ability of Col1a2oim+/+ bone to resist fracture is dependent on changes in bone mineral, the hierarchical organization of the mineral to matrix composition was examined by Raman spectroscopy (Fig. 5). There were no differences in PO4/CO2 ratios between Col1a2oim+/+ offspring tibiae from Col1a2oim+/+ and Mstntm1Sjl1/+ dams (Fig. 5A). However, there was some indication of increased PO4/CH2 (mineral:protein) in tibiae of males born to Mstntm1Sjl1/+ dams (Fig. 5B) (P = 0.06). The PO4/amide I (mineral:collagen) ratio of Col1a2oim+/+ male offspring tibiae from Mstntm1Sjl1/+ was increased 27% compared with those from Col1a2oim+/+ dams (Fig. 5C). Tibial collagen cross-link ratios were similar in Col1a2oim+/+ male offspring born to Col1a2oim+/+ (1.80 ± 0.04) and Mstntm1Sjl1/+ (1.91 ± 0.04) dams (P = 0.16). Hydroxyproline content was also not altered (Fig. 5D), suggesting the increased ratio of mineral:collagen in offspring from Mstntm1Sjl1/+ is not due to an overall decrease in collagen content per gram of bone and therefore may reflect increased mineral content with altered structure.

Approach 3: Effect of Myostatin Deficiency After Embryo Implantation on Femoral Geometry and Biomechanics. To begin to define the critical time point during gestation when myostatin deficiency programs offspring bone strength in the Col1a2oim+/+ mouse, we transferred embryos from a Col1a2oim+/+ to a Mstntm1Sjl1+ recipient dam so that offspring were exposed to the myostatin-deficient maternal environment only after d3.5 (Fig. 6A). We evaluated the femoral geometry and biomechanical strength of 4-mo-old male offspring using μCT and torsional loading to failure, respectively. Although WT and Col1a2oim+/+ offspring were also produced, the study was powered only to examine Col1a2oim+/+ offspring. At 4 mo of age, body and muscle weights were not affected by recipient genotype (Table S1). There were no differences in Col1a2oim+ male offspring femoral geometry resulting from maternal recipient genotype, although there was some evidence of increased marrow diameter in those born to Mstntm1Sjl1+ recipients (P = 0.10) (Table S4).

The torsional ultimate strength of the femora of male Col1a2oim+/+ offspring born to Mstntm1Sjl1+ recipients was 36% stronger than in those born to Col1a2oim+/+ recipients (Fig. 6B). There was...
insufficient evidence for an effect of maternal recipient myostatin status on tensile strength (P = 0.08), but Col1a2oim/+ male offspring from Mstntm1Sjl/+ recipient dams showed a 20% increase over those from control dams (Fig. 6C). As in the natural mating studies (approaches 1 and 2), femoral stiffness and shear modulus of elasticity (Fig. 6 D and E) were equivalent in males born to Mstntm1Sjl/+ and Col1a2oim/+ recipients. Energy to failure increased 83% in male Col1a2oim/+ offspring from Mstntm1Sjl/+ recipient dams (Fig. 6F). Although there was no Sex × Recipient Genotype interactions in the bone biomechanics, the female offspring did not respond as robustly as the males (Fig. S1). Thus, exposure to maternal myostatin deficiency only after d5.5 produced similar improvements in bone strength as exposure throughout oogenesis and gestation, and the maternal uterine environment is responsible.

Discussion

This study assesses the effect of maternal myostatin deficiency on bone development and maturity by characterizing bone strength, geometry, microarchitecture, and material in WT and Col1a2oim/+ offspring born to control versus Mstntm1Sjl/+ dams. Birth to a Mstntm1Sjl/+ dam improved torsional ultimate strength and tensile strength at the age of peak bone mass (4 mo) in both WT and Col1a2oim/+ male offspring in all three experimental approaches and produced significant or suggestive improvements in energy to failure. One caveat is that improvements were more equivocal in female offspring. Overall, we conclude that maternal myostatin deficiency improves bone biomechanical integrity in both WT and mice with mild OI (Col1a2oim/) and that this advantage is conferred during gestation after d5.5 (Table S5).

Alterations in both geometry and material properties contribute to increased bone strength, but they were more variable among the three experimental approaches than the improvements in biomechanical integrity. In WT offspring, gestation in a Mstntm1Sjl/+ dam increased the femoral polar moment of area (an estimate of the contribution of bone geometry to breaking strength). In contrast, Col1a2oim/+ offspring were more variable and did not show significant changes in femoral geometry. The Col1a2oim mouse model has compromised bone remodeling and osteoblast maturation and exhibits ER stress, in addition to the observed structural defects (18, 19). Cellular defects in osteoblast function may contribute to the less robust geometric response in Col1a2oim/+ offspring from Mstntm1Sjl/+ dams.

Maternal myostatin deficiency increased the calvarial collagen content per gram of bone in WT neonates. However, it did not do so in 4-mo-old WT or Col1a2oim/+ offspring tibia, suggesting that collagen content cannot account for the observed biomechanical differences. The significance of increased Col1a2 mRNA levels in 4-mo-old WT offspring tibia from only the Mstntm1Sjl/+ × Mstntm1Sjl/+ cross in the absence of increased Col1a1 mRNA levels or hydroxyproline is uncertain, as Col1a1 and Col1a2 gene expression are generally coregulated (20). The differences in calvarial neonate and adult tibia collagen content may reflect differences due to endochondral ossification versus direct mesenchymal intramembranous ossification and/or inherent differences in fetal bone modeling and/or adult bone remodeling. The WT offspring from Mstntm1Sjl/+ dams were larger at d16.5 than their counterparts from WT dams, and this may suggest that they are further along developmentally and thus in bone modeling.

Femoral PO43-/amide I (mineral:collagen) ratio was also increased in Col1a2oim/+ offspring of Mstntm1Sjl/+ dams relative to Col1a2oim/+ offspring of Col1a2oim/+ dams. Hypermineralization with reduced collagen content is believed to contribute to brittleness in OI (16, 21, 22), yet this did not occur here. Offspring of Mstntm1Sjl/+ dams displayed no differences in stiffness, shear modulus, or collagen cross-link ratios, despite increased mineral content. These findings were similar to a previous study examining the impact of myostatin deficiency in Col1a2oim/+ mice (4), suggesting that the mineral:matrix ratio alone does not predict bone strength and that further studies are needed to determine the mechanisms responsible for improved biomechanical strength of bone in response to maternal myostatin deficiency.

Bone and muscle are dependent upon the shared forces of the mechanostat unit, with bone strength being proportional to muscle mass (23), and bone responding to secretory factors released by muscle. Thus, one mechanism by which maternal myostatin deficiency strengthens bone may be via enhanced muscle loading. Previously, Lee demonstrated that offspring from Mstntm1Sjl/+ and Mstntm1Sjl/mstntm1Sjl dams had greater muscle mass at 10 wk of age than offspring from WT dams (5). Although we also detected some evidence of increased muscle mass in WT offspring from Mstntm1Sjl/+ dams (P = 0.12) (Table S1) and of increased relative contractility in the tibialis anterior muscle of Col1a2oim/+ offspring from embryo transfers to Mstntm1Sjl/+ dams (P = 0.09) (Fig. S2), we did not observe as clear an impact of maternal myostatin deficiency on muscle as on bone. This suggests

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**Fig. 4.** Femoral biomechanical integrity of Col1a2oim/+ offspring from natural mating of Col1a2oim/+ or Mstntm1Sjl/+ dams to Col1a2oim/+ sires. (A, approach 2) Col1a2oim/+ male offspring of Col1a2oim/+ and Mstntm1Sjl/+ dams were evaluated for (B) torsional ultimate strength (Tmax), (C) torsile strength (Su), (D) torsional stiffness (Ks), (E) shear modulus of elasticity (G), and (F) energy to failure (U). *P ≤ 0.05, †P = 0.07. Values are LSmeans ± SE. n (offspring dam), Col1a2oim/+, n = 10–11 dams; Mstntm1Sjl/+, n = 10–11 ± 3 g dams.

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**Fig. 5.** Tibial physiochemical composition of Col1a2oim/+ offspring from natural mating of Col1a2oim/+ or Mstntm1Sjl/+ dams to Col1a2oim/+ sires (approach 2). (A) Phosphate-carbonate (PO43-/CO32-) ratios as measured by Raman spectroscopy. (B) Hydroxyproline content. Values are LSmeans ± SE. *P = 0.03, †P = 0.06. n (offspring dam), Col1a2oim/+, n = 7 ± 4 dams; Mstntm1Sjl/+, n = 7 ± 4 dams.
that increased muscle loading is not sufficient to explain the increased torsional ultimate strength in offspring of Mstn+/- dams.

Decreased myostatin in the maternal environment may act by altering the differentiation capacity of progenitor cells to favor an osteoblast lineage. Maternal nutritional status during gestation can impact osteoblast differentiation (24), showing that developmental programming of bone occurs via this mechanism. The increase in calvarial hydroxyproline content per gram of bone in the absence of increased collagen expression per cell at birth suggests a greater number of osteoblasts in offspring of Mstn+/- dams. This could also account for the evidence of increased serum osteocalcin in 4-mo-old offspring of Mstn+/- dams. Muscle, bone, and fat are derived from the same precursor, mesenchymal stem cells (25). Addition of myostatin increases commitment of the mesenchymal stem cell [C3H 10T(1/2)] to an adipogenic fate, and mesenchymal stem cells from myostatin-deficient mice show increased osteogenic potential in vitro (25). However, as maternal myostatin did not enter the fetal circulation and fetal size was increased overall, an indirect action of maternal myostatin on fetal bone is likely. Additional studies are necessary to determine whether the lack of myostatin in the uterine environment increases osteoblast number or activity and whether this occurs via alterations in mesenchymal stem cell progenitors.

The fetal growth advantage at d16.5 may have programmed the growth trajectory of offspring from Mstn+/- dams to lead to the improved adult bone biomechanical integrity. In humans, birth weight and weight at 1 y correlate positively with adult bone integrity (7, 8). A Finnish cohort hospitalized for hip fracture were found to be shorter at birth but of average height at 7 y of age compared with individuals who did not fracture (26), suggesting an effect of childhood growth rate on adult bone strength. Bone strength did not differ among offspring at 1 mo of age, but serum osteocalcin levels were lower in male offspring from Mstn+/- dams (Fig. S3). At 1 mo of age, the bone is in its active growth trajectory, and the decrease in osteocalcin may indicate that offspring from Mstn+/- dams reached their peak growth acceleration earlier than offspring from WT dams.

The mechanism by which maternal myostatin deficiency promotes fetal growth is yet to be determined, but it may involve the placenta and the uterus. The effect of myostatin on placental glucose transport is mixed, with decreased glucose transport in a choriocarcinoma cell line (27) and increased transport in human placental explants (28). Myostatin also promotes extravillous trophoblast migration in vitro, which may alter placental development in vivo (29). Uterine knockout of myostatin receptor ALK4 profoundly alters placental organization (30), as does knockout of ALK5, which additionally disrupts implantation, spiral artery remodeling, and uterine natural killer cell localization (31). Determining whether myostatin regulates placental establishment and organization or transport of key nutrients by the uterus and placenta in vivo will be critical to understanding the mechanisms by which it promotes fetal growth.

The embryo transfer data (approach 3) begin to define the critical window in which myostatin deficiency alters programming of offspring bone geometry and integrity. During maternal germ cell development, DNA methylations are removed and reacquired during oogenesis (32). Second, the embryo undergoes demethylation following fertilization with remethylation beginning around the time of blastocyst formation (32). These events make the periconceptional window a critical time during which the maternal environment can alter the epigenetic landscape and program fetal and adult outcomes. Here, the periconceptional period did not prove to be the time at which maternal myostatin deficiency programs bone health. In the embryo transfer experiment, offspring exposed to the Mstn+/- maternal environment only after d3.5 showed similar improvements in adult bone biomechanical integrity as offspring exposed throughout oogenesis and embryonic development. This implicates the postimplantation uterine environment in programming of musculoskeletal outcome by maternal myostatin deficiency.

Although our data have consistently shown that maternal myostatin deficiency increases offspring bone strength in male offspring, the effect on females was less robust. When evaluating offspring muscle mass, Lee detected increases in muscle mass in both male and female offspring from Mstn+/- dams compared with those from WT parents (5). Although myostatin deficiency affected both sexes, more of the muscle groups were affected in WT male offspring than in female offspring (5), suggesting a more robust phenotype in the males. Sex differences are commonly observed in developmental programming models and often times more drastically affect males than females, although the molecular mechanisms driving the sexual dimorphic nature of developmental programming outcomes remain largely unknown (33).

This study provides additional evidence that bone is responsive to developmental programming and the finding that myostatin can mediate these effects. Bone geometry, physiochemical composition, and cellular activity are programmed in offspring of Mstn+/- dams at 4 mo of age, with differences in fetal size as early as d16.5. Cumulatively, these changes increased bone biomechanical integrity at the age of peak bone mass. Importantly, our study provides evidence that OI bone, despite containing compromised material, cellular function, and biomechanical strength, is responsive to the developmental programming effects of maternal myostatin. Although research in developmental programming has largely focused on modifying harmful prenatal exposures, this finding opens up an avenue of research to explore the application of developmental programming to a congenital bone disorder and begins to define a potential in utero therapeutic window for treating OI.

Methods

Animals. All mouse models were backcrossed into a C57BL/6J congenic background from Jackson Laboratories, and homogeneity was confirmed using microsatellite markers (34). Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at the University of Missouri–Columbia with ad libitum access to water and food (5008 Formulab Diet; Purina Mills Inc.). This study was performed under an approved University of Missouri Animal Care and Use Protocol. All tissue collections and data analysis were performed blinded.

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Approach 1: Maternal Myostatin-Deficient Model. WT and heterozygous myostatin (Mstntm1Sjl/+ ) mice were bred to generate four genetic crosses (Fig. 1A). At d16.5, a set of dams were killed by CO2 and exsanguinated via carotid puncture. Placental and fetal weights were recorded and bone harvested from each fetus. Pups 1–2 d old from a second set of dams were killed by decapitation and the calvaria harvested and frozen. From a third set of dams, WT male offspring were killed at 1 or 4 [peak bone mineral density (35)] mo of age for collection of femurs, tibiae, and hindlimb muscles.

**Approaches 2 and 3: Maternal Myostatin Deficiency in COL1a2oim/+ Mice.** To test the effect of maternal myostatin deficiency on COL1a2oim/+ offspring bone integrity, we used two additional approaches. In approach 2, the natural mating model, COL1a2oim/^-/- and Mstntm1Sjl/^-/- dams were bred to COL1a2oim/^+/- sires and the resulting COL1a2oim/-/- offspring were evaluated (Fig. 4). In approach 3, the embryo transfer model, to determine if the effect of maternal myostatin deficiency on offspring musculoskeletal outcome was conferred pre- or post-implantation, d3.5 embryos were transferred from COL1a2oim/^-/- females mated to COL1a2oim/^-/- sires into pseudopregnant COL1a2oim/^-/- and Mstntm1Sjl/^-/- recipients as described in SI Methods (Fig. 6A). Offspring were killed at 4 mo of age and their hindlimb muscles, tibiae, and femora evaluated.

**Tibial µCT.** Right tibiae were thawed to room temperature before assessing cortical bone and trabecular bone properties, as described in SI Methods.

**Femoral Geometry and Torsional Loading to Failure.** Femoral geometry and torsional loading to failure, as described in ref. 35, mo of age for collection of femurs, tibiae, and hindlimb muscles.

**Diabetic puncture.** Placental and fetal weights were recorded and blood harvested by cardiac puncture. Placental and fetal weights were recorded and blood harvested by cardiac puncture. Histological sections of placental and fetal tissues were stained with hematoxylin and eosin (H&E) and counterstained with Alcian blue or periodic acid–Schiff (PAS). Images were captured using a Leica DM2500 microscope equipped with a cooled charge-coupled device camera (Hamamatsu). ImageJ (NIH, Bethesda, MD) was used for quantitative analysis of placental and fetal weights.

**Collagen Content and Raman Spectroscopy.** Collagen content was determined by a indirect assay quantifying hydroxyproline, as described in SI Methods and previously (36). Tibial cortical bone cross-sections were evaluated by Raman spectroscopy, as described in SI Methods.

**Statistical Analysis.** All statistical analyses were performed using SAS (SAS Institute Inc.). Experiments were evaluated by analysis of variance (ANOVA) and Fisher’s Protected Least Significant Difference with litter as the experimental unit and multiple offspring per litter as subreplicates, as detailed in SI Methods. Differences were considered to be significant at P < 0.05.

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