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Review Article

Hylonome

Myostatin (GDF-8) as a key factor linking muscle mass and bone structure

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Abstract

Myostatin (GDF-8) is a member of the transforming growth factor-beta (TGF-β) superfamily that is highly expressed in skeletal muscle, and myostatin loss-of-function leads to doubling of skeletal muscle mass. Myostatin-deficient mice have been used as a model for studying muscle-bone interactions, and here we review the skeletal phenotype associated with altered myostatin signaling. It is now known that myostatin is a key regulator of mesenchymal stem cell proliferation and differentiation, and mice lacking the myostatin gene show decreased body fat and a generalized increase in bone density and strength. The increase in bone density is observed in most anatomical regions, including the limbs, spine, and jaw, and myostatin inhibitors have been observed to significantly increase bone formation. Myostatin is also expressed in the early phases of fracture healing, and myostatin deficiency leads to increased fracture callus size and strength. Together, these data suggest that myostatin has direct effects on the proliferation and differentiation of osteoprogenitor cells, and that myostatin antagonists and inhibitors are likely to enhance both muscle mass and bone strength.

Keywords: GDF-8, ActRIIB, Bone Density, Hypertrophy, Muscle-bone Interactions

Introduction

Myostatin (GDF-8), a member of the transforming growth factor-beta (TGF-β) superfamily of secreted growth and differentiation factors, is a negative regulator of skeletal muscle growth[1]. Loss of myostatin function is associated with an increase in muscle mass in mice, cows, and humans[2,3], and myostatin blockade improves muscle regeneration in dystrophin-deficient mdx mice, a model for Duchenne muscular dystrophy. The effect of myostatin is dose-dependent, as mice heterozygous for the disrupted myostatin sequence have intermediate muscle weights between normal mice and mice homozygous for the myostatin mutation[4]. During embryogenesis myostatin expression is restricted to developing skeletal muscles, but myostatin is still expressed and secreted by skeletal muscles during adult life[1]. Myostatin circulates in the blood in a latent form bound to a propeptide, which gets cleaved by BMP1/Tolloid matrix metalloproteinase releasing the active form. Follistatin and follistatin–related gene (FLRG) are other proteins that can bind and inhibit the activity of myostatin by maintaining its latency[2,5]. Active myostatin binds to its receptor, the type IIB activin receptor (ActRIIB), with high affinity and regulates the expression of its target genes through a TGF-β signaling pathway. Recent studies also show that myostatin can activate the p38 MAPK, Erk1/2, and Wnt pathways[6-8].

It has long been recognized that increased muscle mass and strength are associated with a resultant increase in bone mass[9-11]. Experimental investigations into this relationship have generally employed vigorous exercise or some other means of varying muscle-induced stimuli on bone[12-15]. The myostatin-deficient mouse model has provided a novel and unique approach for examining the effects of increased muscle mass on bone independent of the confounding variables associated with changes in physical activity. The body composition of mice lacking myostatin is now generally well characterized, and myostatin-deficient mice show an increase in muscle mass and decreased fat mass[4,16]. The myostatin-deficient mice referenced in this review are produced by homozygous deletion of the C-terminal region of the myostatin gene in embryonic stem cells[1]. We discuss below

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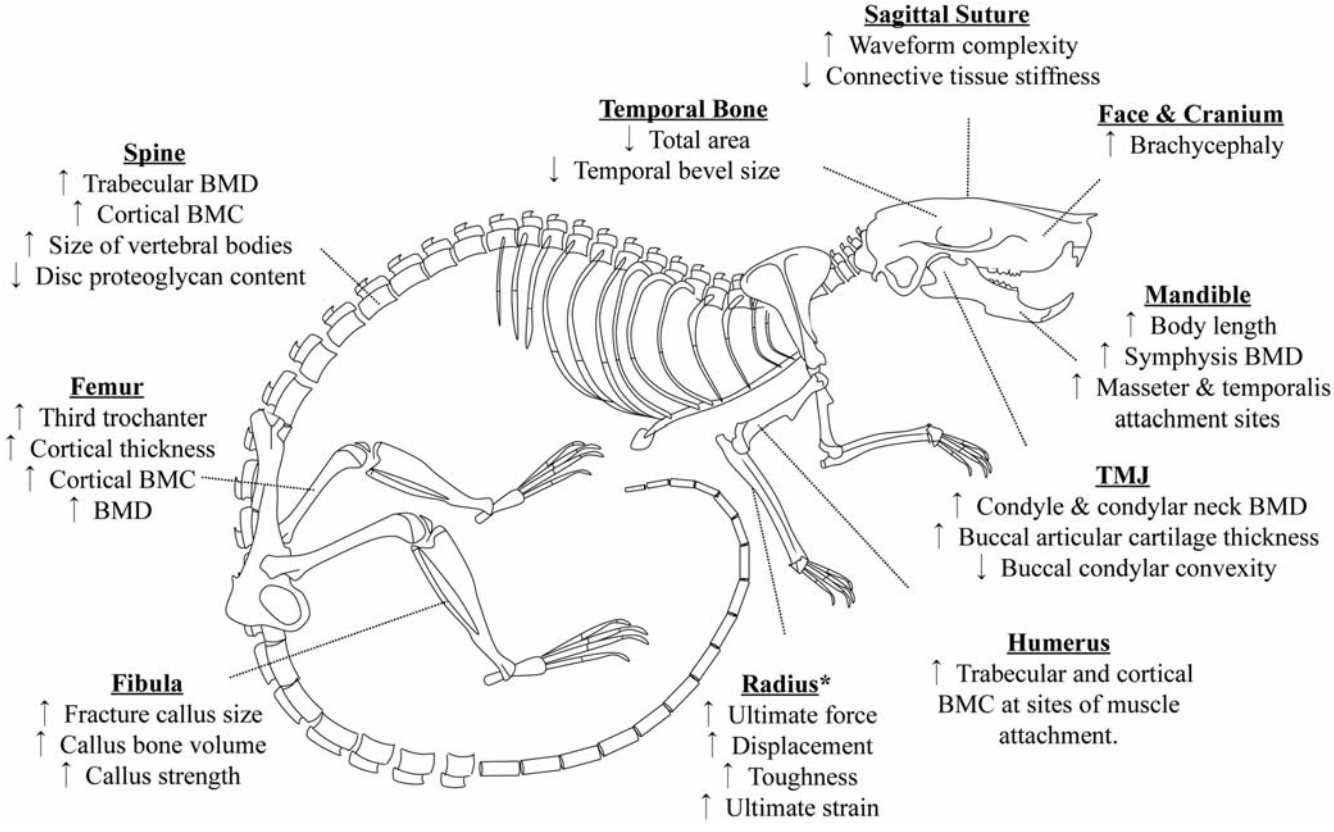

Figure 1. Summary of bony phenotypic changes associated with loss of myostatin function in mice. BMD: bone mineral density, BMC: bone mineral content, TMJ: temporomandibular joint, *: exercised knockout versus normal mice.

the different studies performed on myostatin-deficient mice in assessment of the effects of loss of myostatin function and increased muscle mass on bone mass, architecture, and regeneration, briefly summarized in Figure 1.

Morphology of the limb skeleton in mice lacking myostatin

To investigate the relationship between muscle and bone mass, the humerus of myostatin-deficient mice was analyzed using peripheral quantitative computed tomography (pQCT)[17]. Mice lacking myostatin showed increased mass of the triceps and deltoid muscles compared to controls, and myostatin-deficient mice showed significantly higher trabecular area and trabecular bone mineral content (BMC) in the proximal humerus as well as increased cortical area, cortical BMC, and periosteal circumference in the region of the deltoid crest. While no significant differences were observed in cortical bone mineral density (BMD) at any region of the humerus, these results suggest that the greater muscle mass of the mutant mice has a profound effect on regions of muscle insertion. The bony changes observed in the mutant mice are likely to reflect an increase in surface area required for the attachment of the correspondingly larger muscles (Figure 2a). However, it should be noted that the bony morphology may vary with types of muscle attachment (e.g., tendinous vs. fleshy), given that the tendons of myostatin-deficient mice were recently described as actually being smaller than those of normal mice[18].

Femora of mice lacking myostatin have a shaft that is compressed anteroposteriorly with a higher maximum moment of inertia[19] and significantly greater BMD[20] compared to normal animals (Figure 2b). The myostatin knockouts also showed visibly larger third trochanters than controls (Figure 2b), again suggesting a localized effect of the increased hindlimb muscle mass on bony sites of muscle insertion. This aspect of the bone phenotype in the knockout mice may arise from frequent stretching of the larger muscle during growth, which could propagate an osteogenic stimulus along its tendons to the periosteum promoting its growth. This might also explain the relative anteroposterior compression of the femoral shaft with myostatin deficiency. Femoral heads of the mice lacking myostatin are somewhat flattened superiorly with the articular surface extending towards the neck (Figure 2b), possibly indicating a chondral modeling adaptation in response to changes in hip structure or hindlimb posture associated with gross muscle hypertrophy.

The effects of altered myostatin signaling on exercise-induced bone modeling have been studied in the forelimbs of

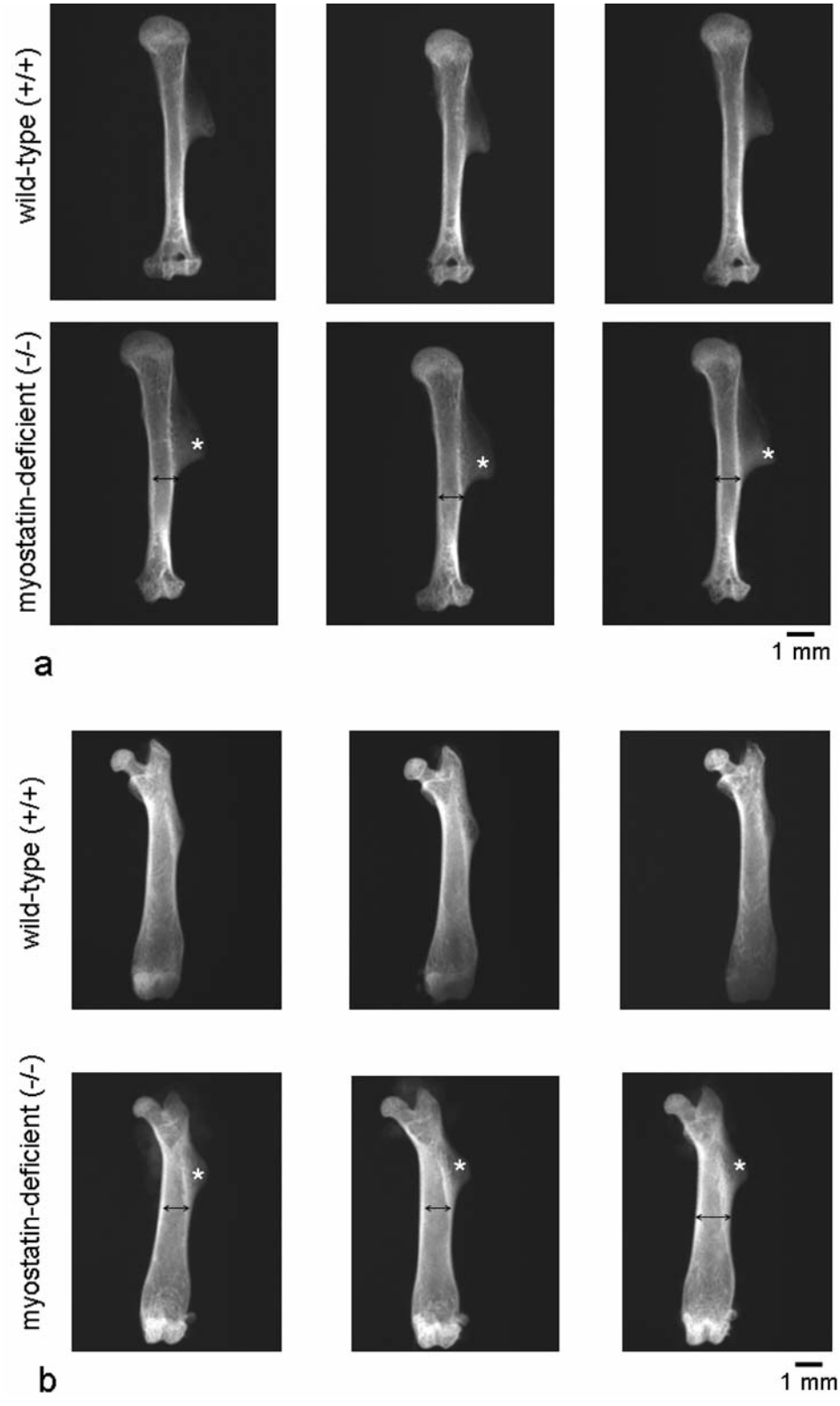

Figure 2. Radiographs demonstrating increased shaft diameter and increased muscle attachment site at the deltoid crest in humerus (a), and the third trochanter in femur (b) in Myostatin$^{-/-}$ mice compared to wild type. Notice the extension of the articular surface towards the neck of the femur (b).

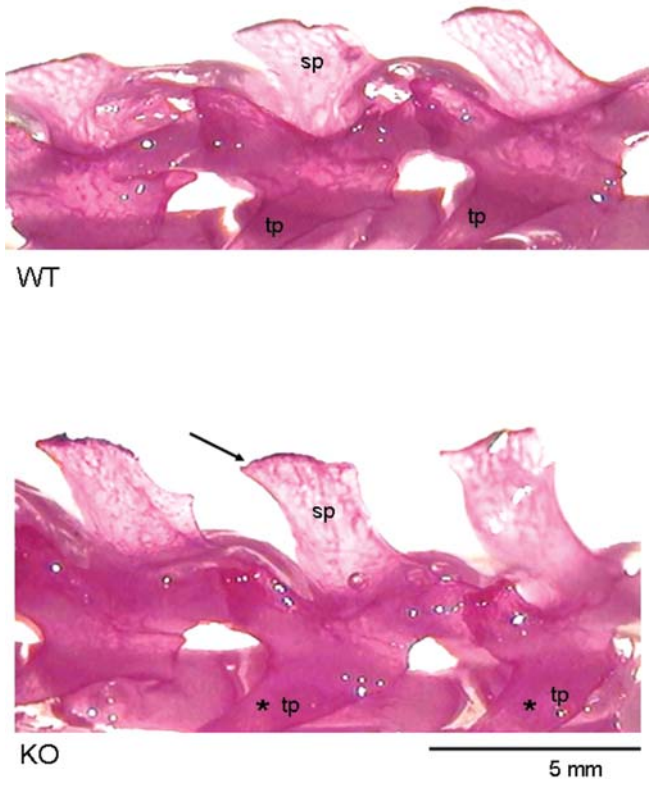

Figure 3. Spines from normal (WT) and myostatin-deficient mice (KO) cleared and stained in alizarin red showing the large spinous process (arrow, sp) and transverse processes (asterisks, tp) in the knockout mice.

mice[21]. The exercise regimen involved 30 minutes of treadmill exercise 5 day/week for 4 weeks. Mechanical testing was carried out in three-point anteroposterior bending and structural parameters collected from histological sections. Biomechanical testing data showed that the ultimate force, displacement, energy-to-fracture, and ultimate strain increased significantly with exercise in myostatin knockouts, but not in wild type mice. Moreover, the cross sections of myostatin knockout mice became elliptical, expanded in the anteroposterior direction, in contrast to the rounded appearance of those in the wild type. The radii of the knockouts were curved longitudinally and the cross-sectional area of their medullary cavities decreased compared to wild type. Muscle mass was found to be a more powerful determinant of radius cross sectional moment of inertia (CSMI) in myostatin-deficient mice than body weight. These results suggest that myostatin deficiency may enhance the osteogenic response to exercise. This interpretation is consistent with our experimental data showing that compressive loading of bone marrow stromal cells *in vitro* increases the expression of osteogenic factors such as BMP-2 and IGF-1, but that myostatin treatment attenuates the osteogenic response to mechanical stimulation[22].

As an additional experimental approach for exploring the role of myostatin in bone development, a fibula osteotomy model was employed to determine the role of myostatin in fracture callus morphogenesis and bone regeneration[23]. Fracture callus size was increased significantly in myostatin deficient mice compared to wild type controls at both two and four weeks following osteotomy. Total bone area and callus strength in three-point bending were significantly higher in myostatin-deficient mice compared to wild type mice at four weeks following osteotomy. These results suggest that the early expression of myostatin in the fracture callus might be responsible for suppressing the proliferation and recruitment of progenitor cells to the fracture site. This interpretation is consistent with other studies (e.g., [24]) indicating that myostatin is highly expressed in bone immediately following fracture, during the very earliest phases of fracture healing.

Phenotype of the spine, skull, and jaw

Similar to morphology of the femur and humerus, the sites of muscle attachment on the spine are expanded in myostatin knockout mice, as evidenced by the larger spinous processes on L1-4 and broader transverse processes on L1-2 [25] (Figure 3). Although the breadth of the vertebral bodies of normal mice does not differ from the knockouts, a significant increase in the length of the vertebral bodies was observed in L3-5 of knockouts (Figure 3). Moreover, pQCT scans through L5 indicate that the knockout mice have significantly greater cortical BMC, a 50% increase in trabecular bone mineral density (BMD), and a 30% increase in trabecular bone volume. The increase in cortical BMC and trabecular BMD was more highly correlated with increased muscle mass than with body mass. The skeletal phenotype of mice lacking GDF11, a member of the TGF-β superfamily that is highly homologous to myostatin and shares similar signaling pathways[26], has also been described. These mice show dramatic anterior homeotic transformations of the axial skeleton, such as short or absent tails and an increased number of lumbar and thoracic vertebrae[27]. As was the case with GDF-8 deficiency, the effect of the GDF11 mutation is dose dependent, with $Gdf11^{+/-}$ mice presenting a milder phenotype than $Gdf11^{-/-}$ mice[27]. Alterations in the axial skeleton were even more extensive in myostatin$^{-/-}$ GDF11$^{-/-}$ double mutants, where the number of thoracic and lumbar vertebrae was further increased[26]. Other reports show that congenital absence of the myostatin receptor ActRIIB results in the formation of additional thoracic vertebrae[28]. While these results are generally suggestive of a role for myostatin in patterning of the axial skeleton, Gdf11 expression in limb bud mesenchyme around sites of chondrogenesis and its role in inhibiting chondrogenesis (and myogenesis) in chick limb micromass cultures *in vitro*[29,30], suggest it might be directly involved in limb bone development.

Craniofacial growth is influenced by the mechanics of mastication, and the role of masticatory forces in the modeling of the skull and jaw bones has frequently been studied by manipulating dietary food properties[12,31]. This approach is very useful but is often compromised by relatively short treatment durations[32]. The myostatin-deficient mouse has recently been employed as a new animal model for studying the relationship

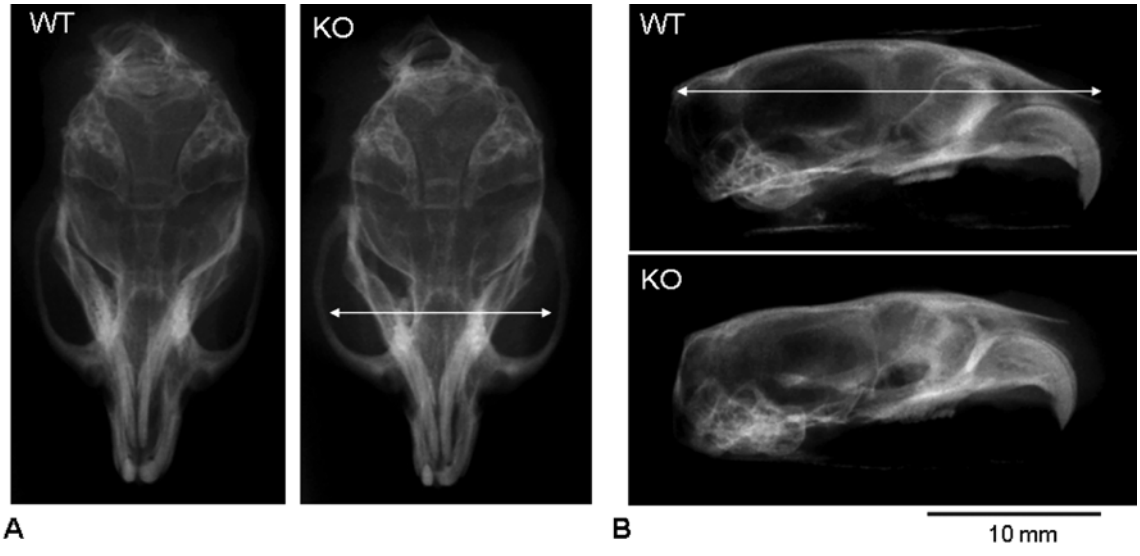

Figure 4. Radiographs of the skull from superior (A) and lateral (B) aspect in normal (WT) and myostatin-deficient (KO) mice showing the flaring zygomatic arches (arrow, A) in the knockout mice and the more elongate skull (arrow, B) in the wild-type mice.

between craniofacial morphology, hypermuscularity and increased bite force. Myostatin-deficient mice were found to have over 50% greater mass of temporalis and masseter muscles with a 33% higher bite force compared to controls[33]. The greater mass of the chewing muscles in the knockout mice was associated with a more elongate and curved mandibular corpus, referred to as a "rocker-type" mandible[34], and flaring of the zygomatic arches (Figure 4). Significant negative correlation coefficients were detected between masseter muscle weight and cranial vault length, with the myostatin-deficient mice having a shorter, more brachycephalic cranial vault and maxilla (Figure 4). Microcomputed tomography (microCT) has been used to demonstrate an increase in bone density at the mandibular symphysis and an increase in cortical bone mineralization at the mandibular corpus of knockouts compared to wild type controls[35,36]. Together, these studies indicate that congenital absence of myostatin alters not only the gross morphological appearance of the jaw and skull but also the microstructure of mandibular bone.

Mice lacking myostatin have also been shown to present distinctive features and morphologies of the individual cranial bones and the cranial sutures. Morphologic changes in the temporal bone and squamosal suture associated with loss of myostatin function include a decrease in total surface area of the temporal bone and reduced beveling of the temporal bone suture margins[37]. One possible explanation that was put forth to explain these findings is that the hypertrophied masseter muscle acting on the opposite side of the zygoma creates a lower strain environment within the bony sutures of knockout mice. Generally, the waveform complexity of the cranial sutures is increased in the myostatin-deficient mice but the stiffness of the sutures in tensile loading is decreased[33]. It has been hypothesized that higher tensile stresses at the sagittal suture in the knockouts induces the local connective tissue to become more compliant[33]. It should be noted here that the decreased stiffness reported in the sagittal sutures of the knockout mice is contrary to the increased tendon stiffness observed in the leg muscles of myostatin-deficient mice[18].

To reproduce the effects of the excessive, repetitive, and/or altered biomechanical loads associated with temporomandibular joint (TMJ) diseases, the myostatin-deficient mouse model has been used to study the role of increased mandibular elevators, and bite forces, on TMJ morphology[38]. MicroCT scanning revealed greater BMD in the outer condyle and the condylar neck of the knockouts compared to the control group, and the knockouts had an asymmetric suchondral bone surface with thicker articular cartilage. In contrast, most external mandibular dimensions were slightly smaller in knockouts than in wild type, which is again consistent with the brachycephaly of the cranial vault and the maxilla reported by Vecchione and colleagues[34]. A higher proteoglycan content and collagen type II expression in the TMJ articular cartilage has been reported in myostatin knockout mice[36], perhaps reflecting an adaptive change in the extracellular matrix to the increasing bite forces.

Discussion & Conclusions

Although the role of myostatin in muscle growth regulation has been widely investigated, its role in regulating bone mass, architecture and regeneration is becoming an area of increased interest. Genetic studies in human populations have shown that myostatin gene polymorphisms are associated with variation in peak bone mineral density[39], and transgenic overexpression of myostatin propeptide, which inhibits myostatin signaling *in vivo*, increases BMD in mice[40]. Thus, there is evidence from both

human studies and animal models to suggest that myostatin is an important regulator of both muscle mass and bone density. The mechanisms by which myostatin regulates bone formation are not well understood, but it is clear that myostatin has direct effects on the proliferation and differentiation of mesenchymal stem cells[41-43], and that myostatin and its receptor are expressed during bone regeneration[41,44]. Myostatin is also reported to inhibit cell proliferation in muscle cells *in vitro*, and induce apoptosis[45,46]. Myostatin therefore appears to be a potent anti-osteogenic factor that may function to suppress the proliferation and possibly the survival of osteo- and chondroprogenitors. Hence, the increased bone mineral density and biomineralization seen in many aspects of the skeleton described are, we believe, likely to be direct effects of myostatin deficiency on bone rather than mechanical responses or adaptations to increased muscle mass. Although certain features of the myostatin knockout phenotype, such as the increased proteoglycan content in the TMJ cartilage, are likely to due to changes in biomechanical loading and other features, such as the increased size of bony muscle attachment sites, may arise from a combination of developmental and mechanical factors, much of the data presented here and elsewhere indicates a direct role for myostatin in regulating bone formation and osteogenesis.

Surprisingly, certain tendons of the myostatin knockout mice have recently been described as smaller, more brittle, and hypocellular compared to those of normal mice[18]. Tendons of the mutant mice also exhibit a 14-fold increase in stiffness that might be attributed to an increase in cross-linking between type I collagen molecules. The exact mechanisms responsible for such microstructural changes are not known, and further studies are necessary to evaluate the regulatory role of myostatin on the development and adaptation of tendons and ligaments. Studies of muscle regeneration[47,48] and tendon fibroblasts[18] suggest that myostatin is a pro-fibrotic factor, in the sense that myostatin increases the expression of TGF beta and type 1 collagen. A more recent report found that local delivery of exogenous myostatin during tendon healing increased tendon cross-sectional area and extracellular matrix production[49]. Additional studies are needed to better define the basic molecular mechanisms by which muscle, tendon, and bone are functionally and developmentally integrated, and how the various TGF beta ligands such as myostatin and activin mediate these processes.

If indeed myostatin has direct anti-osteogenic effects in bone, then myostatin inhibitors would be expected to have therapeutic potential for increasing bone density and strength as well as muscle mass. This prediction is supported by recent data showing that a recombinant decoy myostatin receptor significantly increased bone formation and bone mass in mice[50]. These results, while not conclusive that direct binding of myostatin to its receptor accounts for the reported changes in bone formation, suggest that myostatin may compete with other ActRIIB-binding osteogenic factors, thus influencing bone formation. We have shown that a recombinant myostatin propeptide increased bone volume in healing osteotomies of the fibula[51,52]. It is well established that blocking myostatin signaling increases muscle mass and improves muscle regeneration, and it appears these same inhibitors have similar effects in bone, underscoring the tight coupling between muscle and bone anabolism. The direct effect of myostatin on osteoblasts remains to be confirmed, but would represent an invaluable piece of evidence for distinguishing indirect "muscle-determined loading" effects on bone from direct effects of myostatin. Future studies utilizing conditional knockout mouse models with targeted deletion of either myostatin or its receptor in osteoblasts, chondrocytes, or bone marrow stromal cells may shed the light on the underlying molecular mechanisms by which myostatin influences bone and cartilage formation. Furthermore, while systemically administered myostatin is known to cause muscle wasting[53], and transgenic mice overexpressing myostatin have reduced muscle mass[54], the skeletal phenotype associated with chronically elevated myostatin has not been described. It is likely that myostatin levels are increased in clinical conditions such as bedrest and disuse atrophy, anorexia nervosa, and HIV- and cancer-related cachexia, conditions in which significant loss of both muscle and bone occurs. A better understanding of myostatin-induced osteopenia and sarcopenia is likely to be a novel and promising area for therapeutic intervention in the future.

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