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Joint dysfunction and functional decline in middle age myostatin null mice

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Abstract

Since its discovery as a potent inhibitor for muscle development, myostatin has been actively pursued as a drug target for age- and disease-related muscle loss. However, potential adverse effects of long-term myostatin deficiency have not been thoroughly investigated. We report herein that male myostatin null mice (*mstn*^{-/-}), in spite of their greater muscle mass compared to wild-type (wt) mice, displayed more significant functional decline from young (3–6 months) to middle age (12–15 months) than age-matched wt mice, measured as gripping strength and treadmill endurance. *Mstn*^{-/-} mice displayed markedly restricted ankle mobility and degenerative changes of the ankle joints, including disorganization of bone, tendon and peri-articular connective tissue, as well as synovial thickening with inflammatory cell infiltration. Messenger RNA expression of several pro-osteogenic genes was higher in the Achilles tendon-bone insertion in *mstn*^{-/-} mice than wt mice, even at the neonatal age. At middle age, higher plasma concentrations of growth factors characteristic of excessive bone remodeling were found in *mstn*^{-/-} mice than wt controls. These data collectively indicate that myostatin may play an important role in maintaining ankle and wrist joint health, possibly through negative regulation of the pro-osteogenic WNT/BMP pathway.

Keywords

myostatin; ankle joint; grip strength; treadmill endurance; age

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Introduction

Since its discovery as an inhibitor of muscle growth, myostatin has been an attractive drug target in pharmaceutical as well as academic laboratories. Numerous studies have reported that inhibition of myostatin increases muscle mass and attenuates diet-induced metabolic dysfunction (1–8), supporting the potential therapeutic application of myostatin inhibitors for sarcopenia associated with aging and chronic illness as well as metabolic diseases. Besides muscle loss, aging is associated with major changes in the musculoskeletal system, including bone fragility, reduced tendon and cartilage strength, and joint elasticity, and connective tissue mineralization (9). Several prior studies have reported that older myostatin null ($mstn^{-/-}$) mice maintain their hypermuscularity, hypoadiposity, and superior insulin sensitivity (10, 11). However, $mstn^{-/-}$ mice experience more severe age-related decline of *in situ* muscle fatigue-resistance compared to wild-type (wt) controls (12). Lack of myostatin depletes mitochondria and impairs tolerance to chronic repetitive muscle contractions (13). In addition, $mstn^{-/-}$ mice have greater bone density but they also experience lumbar spine disk degeneration as well as weak and brittle hind limb tendons (14–18). Myostatin and its receptor are detected in tendons (19). Local administration of recombinant myostatin accelerates tendon repair and maintenance in rats (20), implying a direct effect of myostatin on tendon health. This treatment increased the volume and the contraction of the callus after 8 days, but did not improve its strength (20). Here, we investigated the effect of myostatin gene deletion on physical performance and ankle and wrist joint ultrastructure in young (3–6 mo) and middle age (12–15 mo) mice. Compared to wt controls, the $mstn^{-/-}$ mice displayed more significant age-related impairment of gripping strength and treadmill endurance. The ankle joint in middle age $mstn^{-/-}$ mice was severely deformed with characteristic degenerative changes in bone, tendon, and juxta-articular collagen-rich connective tissues. Concurrently, the $mstn^{-/-}$ mice also had increased expression of pro-osteogenic genes at the tendon-to-bone insertion area and altered plasma concentrations of selected cytokines and growth factors related to bone remodeling.

Materials and Methods

Animals

Original $mstn^{-/-}$ breeders were provided by Dr. Se-Jin Lee (Johns Hopkins University) and bred into C57BL/6 background for over 20 generations. Age-matched wt C57BL/6 mice were bred at the animal facility of Boston University School of Medicine. Mice of two age groups for each genotype were used for functional assessment in this work: young age (3–6 mo) and middle age (12–15 mo). The number of animals for each measurement is provided in the corresponding figure captions. For gene expression analysis, an additional neonatal age (8 days) group was also included for each genotype. Only male mice were studied in this work because the phenotype in female $mstn^{-/-}$ mice was generally not as significant as in the males. Animals were group-housed in a standard vivarium at the laboratory animal science center at Boston University School of Medicine and maintained on a standard chow-diet with a 12 hour light and dark cycle. All animal handling was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Physical Measurements

Body composition in live animals was measured by NMR as previously described (3). Treadmill endurance was measured using Exer 3/6 Animal Treadmill (Columbus Instrument). Animals were trained in the procedure by allowing a 15 minute of low speed walk (5 m/min) on the treadmill a day before the measurement. Acclimated mice were allowed to run on the treadmill at a speed of 10 m/min and grade of 5%, about 3 degrees incline (grade = tangent of the angle), for 4 minutes, and every subsequent 4 minutes, the speed was increased by 2 m/min until the mice were unable to remain on the treadmill despite an electrical shock stimulus.

Forelimb gripping force was measured by computerized gripping strength meter (Columbus Instruments). Seven measurements of total peak force were taken from each animal. The average of the values of the top five measurements was used for statistical analysis.

The range of passive motion of the ankle was measured by holding the un-anesthetized mouse in hand and gently pulling the hind foot until the surface of the foot aligned with the leg (range of 180°) or when the foot was locked at a certain angle and could not be moved any further. The angle between the surface of the foot and the leg was measured using a protractor as a measure of the range of motion.

Histology

Mice were sacrificed under deep anesthesia followed with cervical dislocation. One of the hind limbs was dissected from knee down, skinned, and fixed in PBS-buffered 10% (v/v) formalin, decalcified by 14% EDTA (wt/v), trimmed and embedded in paraffin. Serial sections were prepared and stained with hematoxylin and eosin using automated rodent pathology core service (<http://www.dfhc.harvard.edu/core-facilities/rodent-histopathology-pathology/>).

Tibialis Anterior muscle was cryo-sectioned to 6-μm-thickness. Sections were selected from the mid-belly region and fixed in 4% (v/v) formaldehyde for 25 minutes at 4°C, permeabilized with 0.5% (v/v) Triton X-100 for 15 minutes, and blocked in 5% (wt/v) normal goat serum (NGS) for 1 hour. Samples were incubated with rabbit anti-laminin (Abcam, ab11575) overnight at 4°C in 1% (wt/v) bovine serum albumin/1% (wt/v) NGS and then with the secondary antibody for 1 hour at room temperature. Pictures were acquired using a Nikon Eclipse TE2000-E microscope (Nikon Instruments Inc., Melville, NY).

Plasma analysis

Blood samples were collected from cheek vein under light anesthesia. Plasma samples were packed with dry ice and sent to an animal clinical laboratory (www.med.unc.edu/anclin/) for chemical analysis or to RayBiotech (<http://www.raybiotech.com/>) for cytokine array analysis.

CT scanning

Mice were anesthetized with ketamine and xylazine and scanned in a low-energy X-ray micro-computed tomography (CT) scanner (LaTheta LCT-100A, Echo Medical System), as previously described (3).

RNA isolation and qPCR analysis

Up on sacrifice, the Achilles tendon-bone insertion was carefully dissected off the muscle and fat tissue under a dissecting microscope. The isolated tissue sample was then frozen at -80°C before use. For adult mice, one of the hind limb was used for RNA analysis and one for histology. For the neonatal mice, both legs were used for RNA analysis. Three to five tendons, with the insertion region, were pooled and mixed with 1 ml ice-cold trizol and pulverizing together in liquid nitrogen. The mixture was then thawed on ice and homogenized. After centrifugation at $10,000\text{ g} \times 5\text{ min}$ at 4°C , the supernatant containing RNA was loaded onto a RNA binding column and purified following the manufacturer's instruction (Qiagen RNeasy Kit, #74104). Reverse transcription and real-time qPCR were performed as described in our previous studies (21). Intron-flanking PCR primers were designed using web-based software (Supplemental Table s1). The PCR results were normalized to the expression of house-keeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Statistics

Mean and standard deviations were calculated for normally distributed data and median and interquartile ranges for skewed distributed data, respectively. Unpaired t-tests for independent samples were performed for simple two-group comparisons. Two-way analysis of variance was performed to assess combined age and genotype effects on outcomes. Models contained factors for age group, genotype and interaction term between them. If the interaction term was not significant then it was dropped from the model and analysis focused on overall main effects. Otherwise, interaction remained in the model and differences in outcomes were tested between factor levels using Tukey's multiple comparisons adjustment. Log-transformation was used for data with skewed distributions. All tests were performed at $\alpha=0.05$ level of significance.

Statistical analyses were conducted using SAS 9.3 software (SAS Institute, Cary NC) and Prism software (GraphPad Software Inc.).

Results

1. Myostatin null mice display compromised physical performance

Both young and middle aged $mstn^{-/-}$ mice in our cohort had greater lean mass and muscle mass than age-matched wt mice (supplemental Figure s1). At both young and middle ages, $mstn^{-/-}$ mice became exhausted earlier than wt controls for treadmill running and completed a shorter running time (Figure 1A) and distance (Figure 1B). Our findings are coherent with prior studies that reported homozygous lost-of-function myostatin mutation does not enhance racing performance in dogs (22), and myostatin null mice habitually display slower free walking speeds than wild type mice (23). Furthermore, we show that the $mstn^{-/-}$ mice,

but not the wt mice, also showed a significant age-related decline in both running time and running distance (Figures 1A and 1B).

At the young age, the absolute gripping force was greater in $mstn^{-/-}$ mice than wt controls (Figure 1C). Both groups showed an age-related decline, resulting in no difference in absolute gripping force at middle age between the two genotypes (Figure 1C). When normalized to body weight (Figure s2A) or forelimb cross-section (Figure s2B), the age-related decline in gripping strength remained significant but the difference between the two genotypes became insignificant. When normalized to lean body mass, age-related decline was significant only in $mstn^{-/-}$ group (Figure s2C). Notably, at middle age, the $mstn^{-/-}$ mice had larger muscle mass (Figure s1), forelimb cross-sectional area (Figure 1D), and muscle fiber size (Figure 1E) than age-matched wt mice as well as younger $mstn^{-/-}$ mice.

2. Myostatin null mice display ankle dysfunction at middle age

We examined whether the poor physical performance of the $mstn^{-/-}$ mice may be related to impairment of joint function. In wt mice, the wrist joint and ankle joint of wt animals remained fully flexible with the range of motion at 180 degree (not shown). At young age, $mstn^{-/-}$ mice also displayed a full range of passive motion at the forelimb wrist and hind limb ankle joints—a measure of joint flexibility. However, a significant number of middle-aged $mstn^{-/-}$ mice showed substantially reduced range of hind limb ankle motion, i.e. the ankle could not be passively extended beyond a certain angle (Figure 2A). The ankles of $mstn^{-/-}$ mice were also thicker as visually compared to the age-matched wt controls (Figure 2B, upper panel). However, no difference in the range of motion for the forelimb wrist joint was detected between ages or genotypes (data not shown).

We also performed CT scans for the ankle to show that the cross-sectional area (CSA) of the ankle at both ages was greater in the $mstn^{-/-}$ mice than in the wt controls (Figure 2C). The method is briefly illustrated in Figure s3A. When normalized to body weight, the difference between $mstn^{-/-}$ and wt mice was statistically significant for the middle-aged mice but not for the young age groups (Figure s3B). Notably, as animals grew older, the ankle CSA to body weight ratio decreased substantially in the wt mice (Figure s3B). This was in part because from young age to middle age, the ankle CSA increased only moderately (Figure 3A) but body weight increased substantially (Figure s1) in the wt mice. However, for $mstn^{-/-}$ mice, ankle CSA and body weight both increased substantially with age, and the ratio remained essentially unchanged. We also noticed that at middle age, $mstn^{-/-}$ mice displayed a considerable variation in ankle CSA. When the middle age $mstn^{-/-}$ mice were re-grouped between those with restricted ankle mobility versus those with still flexible ankles, the former group had larger ankle CSA compared to the latter (Figure 2D).

After the skin and muscle were dissected off, we found several abnormalities in the peri-articular region of the ankle joint in the middle age $mstn^{-/-}$ mice, especially at the site of tendon-calcaneus bone insertion (Figure 2B, lower panel). The Achilles tendon was thicker and shorter in the middle age $mstn^{-/-}$ mice than the wt controls (Figure 2B). Although not quantitatively measured in this work, we noticed that Achilles tendons in the $mstn^{-/-}$ mice were more likely to break during dissection than those in wt mice, in agreement with previous studies showing that tendon is “more brittle” in the $mstn^{-/-}$ mice (18, 20).

Histological examination revealed marked structural disorganization around the ankle in the middle age $mstn^{-/-}$ mice, including misshapen irregular morphology on bone and tendon, as well as synovial thickening with infiltration of inflammatory cells (Figure 3, right panel, A–D), in contrast to the normal morphology shown in the age-matched wt mice (Figure 3, left panel, A–D).

3. Myostatin null mice display altered plasma markers for bone remodeling

We measured the concentrations of selected plasma markers that are related to bone remodeling. Plasma calcium concentration did not differ between wt and $mstn^{-/-}$ mice at both young and middle ages (Figure 4A). Inorganic phosphate level was higher in young $mstn^{-/-}$ mice than in the wt controls (Figure 4B). Alkaline phosphatase (ALKP) level, a marker of bone cell activity (24), was higher in the $mstn^{-/-}$ mice than the wt controls at both young and middle ages (Figure 4C). In addition, we performed an exploratory proteomic analysis of 300 cytokines in plasma using the RayBio® Mouse Cytokine Antibody Arrays (G series). A complete data set for the array analysis is provided in supplemental Table s2. In comparison to wt mice, $mstn^{-/-}$ mice had significantly higher plasma concentrations of osteoactivin, tissue inhibitor of metalloproteinase-2 (TIMP-2), and osteopontin (Figure 4, D–F), all known to play a role for bone resorption (25–33). In addition, CD40 ligand (CD40L) was reduced in $mstn^{-/-}$ mice (Figure 4G). It is known CD40L is a direct target of TGF β /activin A (34, 35). CD40L may act to stimulate or inhibit osteoclastogenesis in a context-specific manner (36, 37). Our data suggest that myostatin, which shares the signaling pathways with TGF β /activin, may play a role to sustain CD40L expression. Collectively, these data indicate that bone homeostasis is dysregulated in $mstn^{-/-}$ mice.

4. Myostatin null mice display increased expression of pro-osteogenic genes in the Achilles tendon-bone insertion

To determine whether $mstn^{-/-}$ mice were predisposed to dysregulated bone remodeling of the ankle, we analyzed the mRNA expression for a panel of tendon and bone-related factors. At both young and middle ages, the $mstn^{-/-}$ mice had higher expression level than age-matched wt controls for bone morphogenetic protein 4 (BMP4), WNT co-receptor LRP5 and LRP6, WNT inhibitor Dickkopf-1 (DKK1), TGF β receptor 3 (TGFB3), bone-specific ALKP2, and tendon-enriched homeodomain transcription factor SIX1 (Figure 5, upper panel, A–G).

Because $mstn^{-/-}$ mice are characterized by hypermuscularity, the overgrown skeletal muscle in the adult animals may result in mechanical stress in tendon and bone as well as other connective tissues around the joints (38). To test whether myostatin deficiency alone results in altered gene expression, we analyzed the mRNA expression of tendon-bone insertion of the ankle at a neonatal age when muscle mass was not different between wt and $mstn^{-/-}$ mice. Even at this early age, $mstn^{-/-}$ mice displayed increased expression of BMP4, LRP5, LRP6, TGFB3, and SIX1 compared to age-matched wt controls (Figure 5, lower panel, A–E), similar to the pattern detected in young adult animals (Figure 5, upper panel). Expression of DKK1 (Figure 5, lower F) and ALKP2 (Figure 5, lower G), however, was found to be lower in $mstn^{-/-}$ mice than wt controls at this age. In addition, in the neonatal joints, $mstn^{-/-}$

mice displayed a large increase in tenomodulin (Figure 5, lower H), a differentiation marker of tenocytes (39), but this difference diminished at adult ages (data not shown).

Discussion

Myostatin inhibitors are being developed for the prevention and treatment of functional limitations associated with aging and chronic illness, based on the premise that increases in skeletal muscle mass through myostatin blockade would increase muscle strength and physical performance. However, as reported here, the middle age *mstn*^{-/-} mice exhibited a substantial decline in physical performance compared to the wt controls. This functional decline was not associated with a loss of muscle mass. In contrast, middle age *mstn*^{-/-} mice were featured with a number of degenerative changes in the region of Achilles tendon-to-bone insertion at the ankle joint, including bone disorganization, thickening and calcification of tendons and periarticular connective tissue, synovial thickening and inflammatory cell infiltration. These changes might collectively contribute to the mobility restriction of the ankle and overall decline in physical performance.

We do not know whether the observed changes in ankle joints and juxta-articular collagen-rich soft tissue structures are the result of increased mechanical load due to hypermuscularity or the direct effects of myostatin deficiency on the juxta-articular collagen-rich soft tissues. Our findings of increased expression of genes of the WNT signaling pathway in the Achilles tendon-to-bone insertion of *mstn*^{-/-} mice from neonatal to middle age support the hypothesis that myostatin deficiency contributes to ankle dysfunction. For instance, myostatin promotes tenocytes proliferation and differentiation while inhibiting chondrocyte differentiation in cell culture, indicating that myostatin may mitigate calcification and degeneration of juxta-articular ligaments (40–42). Myostatin also inhibits expression of BMP2 and IGF1, decreases osteoblast differentiation, and reduces mineral formation in bone marrow-derived mesenchymal stem cells (43, 44). Furthermore, myostatin gene polymorphism is associated with variation in peak bone mineral density in humans (45, 46). These data together indicate that prolonged myostatin blockade may reduce the endogenous restraint on pro-osteogenic tissue remodeling, resulting in bone and soft tissue alterations and impaired the ankle joint function with aging. However, we cannot exclude the possibility that the observed phenotype could reflect the long-term effects of increased mechanical load on the joint and juxta-articular structures. Additional studies of the effects of myostatin on the osteoblast and osteoclast, tenocyte and/or chondrocyte activity in proper in vitro models are needed to allow examination of the direct effect of myostatin deletion in the absence of potential differences in muscle mass *in vivo*.

The biological effect of myostatin is mostly executed through the Smad3 signaling pathway (21, 47). Interestingly, targeted disruption of Smad3 gene in mice is associated with adult-onset osteoarthritis-like ankle joint phenotype (48), similar to what we found in the middle age *mstn*^{-/-} mice. Smad3 gene polymorphism is also associated with knee and hand osteoarthritis in humans (49, 50). These findings collectively suggest a role of myostatin/Smad3 signaling to balance the osteogenic activity in the skeleton and collagen-rich juxta-articular structures surrounding the ankle, a function that can be essential for the maintenance of the joint health. Since myostatin shares its cell surface receptor and receptor

kinases with other TGF β family members (35, 44, 51, 52), in the absence of myostatin, the effects of pro-osteogenic factors may become less restrained. In addition, myostatin may inhibit WNT signaling that controls the bone development program (53, 54). Indeed, we found that *mstn*^{-/-} mice displayed increased expression of WNT co-receptor LRP5/6 from neonatal age to middle age. In addition, BMP4 expression was elevated in the *mstn*^{-/-} mice at neonatal and young adult age, although the effect became insignificant later in life, possibly a result of feedback inhibition. Cooperation between WNT and BMP signaling pathways can further enhance the pro-osteogenic effect of each other (55–57), which would predict more active tissue remodeling in the tendon-bone insertion region of the *mstn*^{-/-} mice than the wt controls. TGFBR3, also named as β -glycan, remained elevated in *mstn*^{-/-} mice at all ages studied, implying enhanced activation of bone and cartilage metabolism (58–60). Within this panel of genes, DKK1 is the only negative regulator that blocks WNT interaction with its co-receptor LRP5 and LRP6 (61). The reduction in DKK1 expression in *mstn*^{-/-} mice at neonatal age suggests that myostatin plays a role to induce DKK1 expression. Conversely, DKK1 expression was increased in adult *mstn*^{-/-} mice, likely also a result of feedback mechanism to counteract the pro-osteogenic activity in these mice.

The dysfunctional joint phenotype found in middle age *mstn*^{-/-} mice is relevant to the long-term safety of myostatin antagonists in older adults. However, the observations from developmental deletion of a particular growth factor should be interpreted cautiously because genetic disruption of signaling pathways during embryonic life may lead to adaptations that may not necessarily be observed when the same pathways are disrupted in adult life. However, two recent studies reported that a short-term injection of soluble form of a high affinity myostatin receptor (activin receptor IIB) results in a significant increase of bone mass in mice (62, 63). One of these studies also shows that short-term injection of a myostatin monoclonal antibody results a trend of increase in bone formation, implying that prolonged pharmaceutical myostatin blockade may indeed increase bone mass (63). To our knowledge, no studies have examined the long term effects of myostatin inhibition on the tendon, ligament, cartilage, and joint function. Studies of myostatin blockade on collagen-rich connective tissues are technically challenging, but nevertheless important. Generation of mouse models with tissue-specific deletion of myostatin receptor will be useful to elucidate the role of myostatin in the maintenance of normal bone and collagen-rich connective tissues.

In summary, our data suggest that *mstn*^{-/-} mice were intrinsically pro-osteogenic beginning at neonatal age. After reaching middle age, *mstn*^{-/-} mice displayed degenerative changes in the peri-articular structures surrounding the ankle joint, in association with substantial limitation of joint mobility and marked decline in the functional outcomes. Future studies are required to determine whether deleterious effect on joint function may occur with long-term pharmacological myostatin blockade in older adults and how myostatin signaling regulates the growth of bone and collagen-rich juxta-articular structures. The potential impact of long-term myostatin inhibition on articular and juxta-articular structures should be considered in the design of clinical trials of myostatin inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Myostatin null mice display greater functional decline at middle age than wild-type controls.
- Myostatin null mice exhibit misshapen of ankle joint structure, featured with osteoarthritis-like symptom at middle age.
- Myostatin null mice display altered gene expression at tendon-bone insertion region in favor of bone formation, starting at neonatal age and maintaining beyond middle age.

Figure 1. Myostatin deficiency accelerates age-related decline in physical performance independent of muscle hypertrophy

(A) Treadmill run time, (B) Run distance, and (C) Gripping strength. All results are shown as mean $\pm$ SD ($n = 9-10$ per group), w: wt, m: mstn^{-/-}. Results were analyzed with two-way ANOVA. There was significant interaction between age and genotype for run time ($p = 0.01$) and run distance ($p = 0.03$). There was no significant interaction between age and genotype for gripping force ($p = 0.2$), for which both main effects were significant: age ($p < 0.001$), genotype ($p = 0.004$). Results of multiple comparison adjusted Tukey's tests are shown in the figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Representative CT scan of upper arm cross-section is shown in (D) and laminin staining of muscle cross section in (E).

Figure 2. Myostatin deficiency results in dysfunctional ankle joint at middle age and increased ankle joint cross-section area (CSA)

(A) The range of free motion between the hind foot and the tibia for mstn^{-/-} at young (6 mo) and middle age (11 mo, 15 mo). One-sided t-test was performed for comparison between 6 months and 11 months and also between 6 months and 15 months: ###*p* < 0.001. Two-sided t-test was conducted for difference between two middle age groups (*p*=0.3). (B) Enlarged view of intact ankle in the middle age mice and the tendon and bone morphology after the removal of skin and muscle. The image of intact ankle is representative of *n* > 20 mice per group and the image of dissected ankle is representative of three animals per group. (C) Comparison of ankle joint cross-section area (CSA) between wt (*n* = 10) and mstn^{-/-} mice (*n* = 13), shown as box-and-whiskers plots, with box extending from 25th to 75th percentiles, and whiskers from min to max (w: wt, m: mstn^{-/-}). Data were log-transformed and results were analyzed with two-way ANOVA, which shows a significant age and genotype interaction (*p* < 0.001). Results of multiple comparison adjusted Tukey's tests are presented in the corresponding figures: ****p* < 0.001. (D) Comparison of ankle joint CSA of middle age myostatin between animals with restricted ankle motion (*n* = 10) versus those with free ankle motion (*n* = 3). # *p* < 0.05, unpaired t test.

Figure 3. Myostatin deficiency results in misshapen tendon and bony structures of the ankle at middle age

(Left panel) The ankle section of a wt mice illustrates intact limb bones (**A**), tendon-bone insertion points (**B**), and the IV tarsal bone (**C**) without significant synovium inflammation (**D**). **(Right panel)** The ankle section of $mstn^{-/-}$ mice illustrates misshapen limb bone (**A**), disorganized tendon-bone insertion (**B**), irregular mineralization (**C**, arrows), and inflammatory cells infiltrated into the synovium (**D**, arrows). Hematoxylin and Eosin; Scale bar A= 500 μ m, B, C = 200 μ m, D = 100 μ m.

Figure 4. Myostatin deficiency results in altered plasma concentration of selected markers of bone remodeling

Plasma concentration of (A) calcium, (B) phosphate, and (C) alkaline phosphatase (ALPK), at young and middle ages, as well as plasma concentrations of (D) osetoactivin, (E), TIMP-2, (F) osteopontin, and (G) CD40L for middle-age only. Results are shown as box-and-whiskers plots, with box extending from 25th to 75th percentiles, and whiskers from min to max (w: wt, m: mstn^{-/-}, n = 6) or as bar graph (mean ± SE, n = 3). Results in A–C were analyzed by two-way ANOVA with log-transformed data. For plasma calcium, there was no significant age and genotype interaction (p = 0.788), with main effect being significant for age (p = 0.019) but not for genotype (p = 0.937). For plasma phosphate, there was a significant age and genotype interaction (p < 0.001). For plasma ALPK, the age and genotype interaction was not significant (p = 0.546). The main effect was significant for age (p < 0.001) and genotype (p < 0.001). Tukey's multiple comparisons adjusted tests between subgroups are presented in the corresponding figures. *p < 0.05, ** p < 0.01, *** p < 0.001. Results in D–G were analyzed with unpaired t test with Welch's correction, #p < 0.05,).

Figure 5. Myostatin deficiency leads to altered mRNA expression in tissues of the tendon-bone insertion points

(Upper panel) Effects of genotype and age. **(A)** BMP4, **(B)** LRP5, **(C)** LRP6, **(D)** WNT inhibitor Dickkopf 1 (DKK1), **(E)** TGF β receptor 3 (TGFBR3), **(F)** ALPK2, **(G)** SIX-1. Results are shown as box-whiskers plots, with box extending from 25th to 75th percentiles, and whiskers from min to max, w: wt, m: *mstn*^{-/-}, and analyzed by two-way ANOVA. All data, except DKK1, were log-transformed. The main effect of genotype was significant for all ($p < 0.001$). The results of Tukey's tests with multiple comparisons adjustment were presented within the corresponding figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(Lower panel)**. Effects of genotype at neonatal age. The same panel of genes displayed in the upper panel with the addition of tenocyte differentiation marker tenomodulin (TNMD) was analyzed in wt and *mstn*^{-/-} mice, # $p < 0.05$, unpaired t-test for log-transformed data (no transformation for DKK1). Each sample was pooled from tissue isolated from three animals (9 animals per group).