Published in final edited form as:

Growth Factors. 2011 December ; 29(6): 253–262. doi:10.3109/08977194.2011.599324.

Myostatin (GDF-8) Inhibits Chondrogenesis and Chondrocyte Proliferation *In Vitro* by Suppressing Sox-9 Expression

Moataz Elkasrawy^a, Sadanand Fulzele^b, Matthew Bowser^b, Karl Wenger^b, and Mark Hamrick^{a,b,c,*}

^aDepartment of Cellular Biology & Anatomy, Georgia Health Sciences University, Medical College of Georgia Augusta, GA USA

^bDepartment of Orthopaedic Surgery, Georgia Health Sciences University, Medical College of Georgia Augusta, GA USA

^cInstitute of Molecular Medicine and Genetics, Georgia Health Sciences University, Medical College of Georgia Augusta, GA USA

Abstract

Here, we investigate a possible direct role for myostatin in chondrogenesis. First, we examined the effects of myostatin on the proliferation of bone marrow stromal cells (BMSCs) and epiphyseal growth plate (EGP) chondrocytes isolated from myostatin-deficient mice. Results show that myostatin deficiency is associated with a significant ($P < .001$) increase in proliferation of both BMSCs (+25%) and EGP chondrocytes (+35%) compared to wild-type cells. Next, we examined the effects of myostatin treatment on chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSC). These experiments show that myostatin treatment starting at either 0 or 48 hrs induces a significant decrease in Col2 protein synthesis by 31% ($P < .001$) and 25% ($P < .05$), respectively. Real-time PCR reveals significant ($P < .01$) down regulation of Sox9 mRNA expression with 10- and 100ng/ml treatments. Together, these findings suggest that myostatin has direct effects on chondrogenesis, and may therefore represent a potential therapeutic target for improving bone repair.

Keywords

TGF-beta; endochondral ossification; bone marrow stem cells

Introduction

Chondrocyte differentiation, proliferation, and maturation play key roles in the process of endochondral ossification, in which a cartilaginous template precedes the formation of bony skeletal elements. This same process also characterizes bone healing and repair, where a cartilaginous soft-callus precedes the formation of a fully ossified hard callus (Gerstenfeld et al. 2003). During this process, mesenchymal stem cells are recruited and stimulated to differentiate into chondrocytes that synthesize cartilage-specific matrix proteins, including collagen type II (Col2) and proteoglycans (Barnes et al. 1999; Einhorn 1998; de Crombrughe et al. 2000). This stage is carefully orchestrated by the differential expression

*Address all correspondence to: Mark W. Hamrick, Ph.D. Department of Cellular Biology & Anatomy Georgia Health Sciences University Medical College of Georgia Cb1116 Laney Walker Blvd. Augusta, GA 30912 USA PH: 706-721-1958 FAX: 706-721-6120 mhamrick@georgiahealth.edu.

Conflict of Interest: None.

of members of the TGF- β superfamily of proteins. Myostatin (GDF-8) is a member of this superfamily, and is widely known as a negative regulator of skeletal muscle growth and development (McPherron et al. 1997). Although myostatin is most highly expressed in skeletal muscle, it is also expressed during the first twenty four hours of fracture healing (Cho et al. 2002). Furthermore, the myostatin receptor (ActRIIB) is expressed in proliferating chondrocytes of the fracture callus in rats (Nagamine et al. 1998) as well as in bone-marrow derived mesenchymal stem cells (Hamrick et al. 2007). We have found that myostatin-deficiency increases cartilage area and bone volume in the fracture callus following fibula osteotomy (Kellum et al. 2009), suggesting that myostatin may play a role in the normal process of endochondral ossification during bone healing.

Myostatin binds the activin type IIB receptor (ActRIIB, or Acvr2B) (Lee and McPherron 2001), a serine/threonine kinase receptor that recruits and phosphorylates the type I co-receptors activin-like kinase receptor 5 (ALK5) and ALK4 (Rebbapragada et al. 2003). This consequently activates and phosphorylates Smad2/3, which dissociate from the ligand/receptor complex to bind with the co-Smad (Smad4), allowing translocation of the Smad complex to the nucleus where it targets several DNA binding proteins to regulate transcriptional response (Lee 2004). Recent studies also indicate that myostatin can activate the p38 MAPK, Erk1/2, and Wnt pathways (Allendorph et al. 2006; Stellman et al. 2006; Ekaza and Cabello 2007). Sox9 and Sox5 are transcription factors that play a major role in mesenchymal stem cell condensation and chondrocyte differentiation and, thereby, in cartilage formation (Ikeda et al. 2005; Lefebvre et al. 2001; Smits et al. 2001). We have previously shown that myostatin deficiency increases Sox9/Sox5 expression levels in the fracture callus (Hamrick et al. 2007). We have also shown that myostatin inhibition using systemic injections of myostatin propeptide enhances bone formation in fracture healing (Hamrick et al. 2010).

In this study, we use an *in vitro* strategy to investigate the role of myostatin in chondrogenesis to better understand its potential effects on the process of endochondral ossification and bone repair. The first experiments described here use mice lacking myostatin to determine the direct effects of myostatin on the proliferation of BMSCs and epiphyseal growth plate chondrocytes. We then use a cartilage aggregate culture model to determine the effects of myostatin on the chondrogenic differentiation of BMSCs. To better understand the effects of myostatin on TGF- β 1-regulated chondrogenesis *in vitro*, we also compared the action of myostatin to that of blocking two key molecules of the TGF- β 1 signaling pathway; TGF Receptor I Kinase (Alk5) and Smad3. Our results reveal that myostatin has potent anti-chondrogenic effects *in vitro*, and that these effects are mediated by myostatin's ability to suppress the chondrogenic factor Sox9.

Materials and Methods

Mouse strains

Collagen type 2-enhanced green fluorescence protein (Col2-eGFP) reporter mice (Grant et al. 2000) on a C57BL/6 background were used to isolate and culture BMSCs in chondrogenic conditions. Col2-eGFP mice were utilized because procollagen 2 protein levels in chondrocytes from these mice were previously shown to directly correlate with the relative degree of eGFP fluorescence intensity (Grant et al., 2000). Because a primary goal of this study is to assess the effects of myostatin on chondrogenesis, and type II collagen biosynthesis is a key marker of differentiated chondrocytes, this experimental approach is preferable over simply measuring col2 mRNA expression. Newborn pups positive for the transgene were selected at 1 day of age after scanning their digits under inverted fluorescence microscope. If the genotype was not easily visualized a subsequent round of genotyping was performed using DNA collected from the tails using the DNeasy Qiagen kit

(Cat#69506) and primers for eGFP listed in Table 1. Myostatin knockout mice were produced by homozygous deletion of the C-terminal region of the myostatin gene in embryonic stem cells (McPherron et al. 1997). These mice are from the colony previously described by our lab (Elkasrawy and Hamrick 2010). All procedures were performed under Institutional Animal Care and Use Committee (IACUC) approval.

Isolation and Purification of BMSCs from Col2-eGFP and myostatin knockout mice

BMSCs were isolated from bone marrow aspirates collected from long bones of eight Col2-eGFP reporter mice at 3–4 months of age using the general approach we have described previously (Zhang et al. 2008). Briefly, femora, tibiae, and humeri were dissected and cleaned from soft tissue. The bone heads were cut from both ends and bone marrow was flushed with α -MEM delivered with a 22-gauge syringe, then passed through a 70 μ m nylon mesh filter to form a single cell suspension. Non-adherent cells were removed by aspirating the media, washing adherent cells twice gently with PBS, then replacing it with new media, to reduce the contamination by hematopoietic cells. Cells reached 70–80% confluence after 3–4 weeks of incubation and media replacement every 3–4 days. At that point, cells were processed for negative selection from cell population positive for CD11b, CD45R/B220, and Pan DC using the Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set-DM (BD IMag) according to manufacturer's instructions.

Cells reached 70–80% confluence after 1–2 weeks of incubation and media replacement every 3–4 days at which point cells were processed for positive selection for Sca-1 (stem cell antigen-1) using the Anti-Sca-1 MicroBead kit (Miltenyi Biotec) according to manufacturer's instructions. Fluorescence-activated cell sorting (FACS) analysis was performed to evaluate the efficiency of antibody selection. A Becton Dickinson FACS Calibur Flow Cytometer at the Flow Cytometry Core Laboratory at the Medical College of Georgia was used.

Harvesting and culture of epiphyseal growth plate (EGP) chondrocytes

One week-old myostatin knockout and wild-type mice were used to harvest epiphyseal growth plate chondrocytes from the immature femur, tibia, and humerus using a protocol modified from Gosset et al. (2008). Six mice were euthanized using CO₂ overdose followed by thoracotomy. The femoral and humeral heads and condyles, and tibial plateau, were removed from the long bones and translucent growth plate cartilage collected, cut into small pieces, placed in 1X PBS, and then rinsed. Chondrocytes were isolated by cartilage digestion using collagenase D (Roche, Cat. No. 11 088 858 001).

Proliferation assays

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation) was used to assess myostatin's effects on BMSC and EGPC proliferation utilizing cells derived from myostatin knockout and wild-type mice. 50 μ l of high glucose (HG) DMEM were added per well and the plate was equilibrated at 37°C under 5% CO₂. Cells were resuspended in DMEM to a final concentration of 6×10^4 cell/ml. 50 μ l of the cell suspension (3000 cell) was dispersed in the previously prepared plate bringing the total volume in each well to 100 μ l. The plate was incubated at 37°C under 5% CO₂ for one week with a media change every two days. At each time point, 20 μ l of CellTiter 96® Aqueous One Solution Reagent was added per well, then the plate was incubated at 37 °C for 1 hour. Optical densities of the samples were measured at 490nm using an automatic microplate reader (Model 550, Bio-Rad). Experiments were performed in triplicate.

Chondrogenic differentiation and aggregate culture of col2-eGFP BMSCs

To assess the chondrogenic potential of BMSCs, culture expanded BMSCs in high glucose DMEM were used at first passages to prepare three-dimensional (3-D) aggregate culture using the general protocol described by Penick et al. (2005). BMSCs were subcultured at 80–90% confluence to prevent contact inhibition of growth and spontaneous differentiation (Solchaga et al. 2004). To ensure equal number of cells per sample, one hundred microliters aliquots of the cell suspension (containing 0.25×10^6 cells) were seeded into the wells of a 96-well, V-bottom, 300 μ l polypropylene microplate (Thermo Scientific) using a multi-channel pipette. To better understand the effects of myostatin on TGF- β 1-regulated chondrogenesis *in vitro*, we compared the action of myostatin to that of blocking two key molecules of the TGF- β 1 signaling pathway; TGF Receptor I Kinase (Alk5) and Smad3. To that end we treated the aggregate cultures with recombinant myostatin (10ng/ml or 100ng/ml), 10 μ M TGF- β 1 Receptor I Kinase inhibitor II (Alk5i) (Calbiochem, Cat. No. 616452) or Smad3 inhibitor (SIS3) (Calbiochem, Cat. No. 566405) for 6 days.

Image Analysis

Aggregate cultures were embedded in OCT medium and frozen in liquid nitrogen. Cryostat sections of the aggregates were cut at 7 μ m and stained with DAPI. Images were then viewed using fluorescence microscopy and images captured using FITC filter. Images were converted to grayscale and brightness intensity threshold applied to segment GFP-positive regions from non-fluorescent areas. The area of GFP-positive pixels was quantified using NIH ImageJ software. Alternate sections were stained with hematoxylin and eosin or toluidine blue to examine cell morphology and cartilage extracellular matrix synthesis, respectively.

Analysis of gene expression

Quantitative RT-PCR was used to quantify the gene expression levels from aggregate cultures. Total cellular RNA was isolated from 5 combined aggregates replicates per sample for a total of 5–6 samples per treatment group using RNeasy Plus Micro Kit (Qiagen, Cat. No 74034), and samples homogenized using QIAshredder (Qiagen Cat. No 79654). Total RNA was quantified using NanoDrop™ 2000 spectrophotometer (Thermo Scientific), then equal amounts of RNA (0.25 μ g) were reverse transcribed using iScript cDNA Synthesis Kit (BioRad Cat. No 170-8891). Complementary deoxyribonucleic acid (cDNA) templates (50ng/gene) were amplified using Absolute SYBR Fluorocein Mix (Thermo Scientific, Cat. No AB-1220/A), iQ5 real-time PCR Detection System (BioRad), and custom designed primers specific to mouse genome as listed in Table 1. Data was analyzed as described previously (Hamrick et al. 2010) by the $\Delta\Delta C_t$ method using GAPDH and 18S as internal controls (Pfaffl 2001). Melting curve analysis of the RT-PCR products was performed on the real-time cyclers for the purpose of quality control.

Statistical Analysis

Results are plotted as mean $\pm$ SE. All statistical analysis was performed using Prism, version 5.0a. Comparisons between groups were assessed using the unpaired Student's *t*-test. Multiple group comparisons were performed using one-way ANOVA. Post hoc analysis was performed using Newman-Keuls multiple comparison test. In all cases, statistical comparisons were considered significant at $P < 0.05$.

Results

Validation of col2-eGFP BMSCs

Newborn pups positive for the col2-eGFP transgene were selected at 1 day of age after scanning their digits under an inverted fluorescence microscope. Mice expressing col2-eGFP showed clearly defined GFP labeling in cartilage condensations representing the digits and metapodials of the fore- and hindlimb (Fig. 1a). FACS analysis was used to isolate BMSCs from the long bones of adult col2-eGFP mice 3–4 months of age. FACS analysis verified the degree of purity of isolated BMSCs positive for Sca-1 by percentage compared to unstained population as shown in Fig. 1b.

Myostatin deficiency increases the proliferation of BMSCs and chondrocytes in vitro

Myostatin deficiency significantly increased the proliferation of BMSCs ($P<0.001$) by 22% after 4 days in culture compared to wild-type BMSCs (Fig. 2A–C). Epiphyseal growth plate chondrocytes (EGPC) of 1-week-old myostatin knockout mice doubled in number after 2 weeks in culture ($P=0.001$) when compared to wild-type cells (Fig. 2D).

Myostatin reduces chondrogenic differentiation of BMSCs in vitro

Myostatin treatment induced a dose-dependent decrease in chondrogenic differentiation *in vitro* (Fig. 3A–B). 100ng/ml of recombinant myostatin decreased Col2 protein synthesis by 31% with treatment starting at 0 hour ($P<.01$; Fig. 3B). Since myostatin is expressed only during the first 24 hours after fracture, we examined the effects of early and late myostatin exposure on BMSC differentiation. We found that Col2 biosynthesis decreased by 25% with treatment starting at 48 hours ($P<.05$). No significant difference was found between exposure to myostatin at 0- versus 48 hours in chondrogenic media. At the molecular level, real-time RT-PCR quantification of Sox9 gene expression levels in aggregate cultures treated with 10 or 100ng/ml of recombinant myostatin, showed a decrease in Sox9 mRNA levels by 211% and 289% ($P<.01$, ANOVA), respectively, with myostatin treatment starting at 0 hour (Fig. 3C). Myostatin treatment was found to induce a qualitative decrease in the relative number of chondrocytes and degree of proteoglycan staining in the cartilage aggregates (Fig. 3D).

Suppression of chondrogenesis with myostatin treatment is similar to that observed by blocking TGF- β signaling pathway components

In the aggregate culture system, chondrogenic differentiation is facilitated by supplementing the chemically defined chondrogenic medium with 10ng/ml of TGF- β 1 (Johnstone et al. 1998). We blocked two key components of the Smad-dependent TGF- β signaling pathway; TGF Receptor I Kinase (Alk5), and Smad3, by treating the culture with 10 μ M of TGF Receptor I Kinase inhibitor II (Alk5i) or Smad3 inhibitor (SIS3) starting at 0 hour or 48 hours. Alk5i significantly decreased Col2 protein expression levels by 60% with treatment starting at 0 hour ($p=0.01$), and by 49.3% with treatment starting at 48 hours ($P<.05$) (Fig. 4A,B). No significant difference was found between treatments with Alk5i at 0- versus 48 hours in chondrogenic media (Fig. 5B). 10 μ M of Alk5i caused a 175% decrease in Sox5 mRNA levels, and a 117% decrease in Sox9 mRNA levels ($P=0.01$, ANOVA) (Fig. 4C). SIS3 significantly decreased Col2 expression levels by 99% with treatment starting at 0 hour ($P<.001$), and by 74.4% with treatment starting at 48 hours ($P=.001$) (Fig. 4D).

Discussion

Our experiments utilizing growth plate chondrocytes and bone marrow stromal cells indicate that congenital absence of myostatin enhances cell proliferation *ex vivo*. This result is consistent with previous data showing that myostatin expression suppresses myoblast

proliferation (Manceau et al. 2008), as well as the proliferation of uterine and myometrial cells (Ciarmela et al., 2009). In myoblasts and myometrial cells, the inhibitory effects of myostatin on proliferation appears to be regulated by phosphorylation of Smad-2 and expression of p21. On the other hand, myostatin enhances the proliferation of fibroblasts in mouse tendon (Mendias et al. 2008), as well as in fibroblasts isolated from human knee ligaments (Fulzele et al. 2010). Together, these studies suggest that myostatin is likely to have cell-specific effects that vary with tissue type, and future studies should be directed at defining the molecular mechanisms by which myostatin regulates gene expression in fibroblasts versus myoblasts. The evidence presented in this paper reveals that chondrocytes and bone marrow stromal cells respond to myostatin exposure in a manner that is more reminiscent of muscle-derived cells, in that myostatin appears to be an effective inhibitor of chondrocyte proliferation.

Although myostatin is widely known as a potent inhibitor of muscle growth and development, there is increasing evidence that it also plays an important role in bone formation and repair. Several studies have shown that mice lacking myostatin show a general increase in bone density (Elkasrawy and Hamrick 2010) and myostatin deficiency causes an increase in osteogenic differentiation of BMSCs (Hamrick et al. 2007). Therapeutic modulation of myostatin activity in mice using a soluble decoy receptor (ActRIIb-fc) leads to a gain in bone mass and formation (Bialek 2008), and myostatin inhibition using systemic injections of myostatin propeptide can enhance bone formation in fracture healing (Hamrick et al. 2010). The findings presented in this paper expand upon these previous studies and shed new light on a plausible direct role for myostatin in the process of endochondral bone formation. Sox9 and Sox5 are transcription factors that play a major role in mesenchymal stem cell condensation and chondrocyte differentiation and, thereby, in cartilage formation (Ikeda et al. 2005; Lefebvre et al. 2001; Smits et al. 2001). We have previously shown that myostatin deficiency increases cartilage area, bone volume, and Sox9/Sox5 expression levels in fracture callus in vivo (Kellum et al. 2009). The decrease in chondrogenic differentiation associated with myostatin treatment reported here *in vitro* was observed at both the cellular and molecular levels. The synthesis of collagen type II was significantly suppressed with myostatin treatment during chondrogenesis, despite the identical starting number of BMSCs between treatment and control groups in the aggregate culture. Chondrocytes in the fracture callus express myostatin receptor (Nagamine et al. 1998) and are in close proximity to muscle. Although myostatin treatment during *in vitro* chondrogenesis caused a significant reduction in collagen type II biosynthesis independent of the timing of treatment (day 0 versus day 2), the effect was stronger with earlier treatment. This indicates that chondrogenesis is initiated soon after TGF- β stimulation, and suggests that the immediate timing of myostatin treatment *in vitro* as well as its expression during the first 24 hours of fracture may be interfering with TGF- β -mediated chondrogenesis.

TGF- β 1 is a major chondrogenic growth factor that is responsible for promoting cellular condensation, increasing cellular adhesion molecules, and stimulating extracellular matrix production during chondrogenesis (Barnes et al. 1999; James et al. 2009). TGF- β 1 primarily binds to T β RII and T β RI (ALK5), and activates both the Smad-dependent and -independent signaling pathways to regulate chondrogenesis (Li et al. 2005). TGF- β 1 is abundant in bone, among other tissues, and is constitutively expressed throughout the fracture healing process with the highest peak coinciding with that of myostatin during the early stage of fracture healing (Gerstenfeld et al. 2003). Crosstalk between TGF- β 1 and other members of the TGF- β superfamily, including myostatin, has now been reported by several authors (Zhu et al. 2007; Janssens et al. 2005). It is well established that TGF- β 1 and myostatin both bind the same co-receptor, ALK5 (Rebbapragada et al. 2003; Derynck and Feng 1997), and TGF- β signaling is known to up-regulate Sox9 and increase nuclear accumulation and stability of

beta-catenin during chondrogenesis (Zhou et al. 2004; Lorda-Diez et al. 2009; Kawakami et al. 2006). We report here that Sox9 was down regulated with myostatin treatment, and was also suppressed with ALK5 inhibition during TGF- β 1-induced chondrogenesis. These experimental findings suggest that myostatin might be capable of competitively inhibiting ALK5-mediated TGF- β 1 signaling, or alternatively that myostatin may stimulate the expression of certain factors that could actively inhibit TGF- β 1, such as Smad7 (e.g. Zhu et al. 2004; Iwai et al. 2008). Together, these findings reveal that there may be multiple pathways by which myostatin can alter TGF- β 1 signaling components during chondrogenesis.

Previous data suggest an autocrine source of myostatin expression in the fracture callus (Cho et al., 2002), but myostatin is also secreted by muscle and can circulate throughout the body (Zimmers et al. 2002). Catabolic conditions such as prolonged bedrest or unloading, trauma or burns, AIDS and cancer-related cachexia elevate circulating myostatin (Lang et al. 2001; Zhou et al. 2010). Any of these conditions that elevate circulating myostatin could therefore potentially impair bone healing through the mechanisms described above. Several myostatin inhibitors such as myostatin propeptide, decoy soluble receptor, myostatin antibody, and follistatin have been shown to enhance muscle regeneration, increase myofiber hypertrophy, and decrease fibrosis in healing muscle (Wagner 2005). Inhibiting myostatin signaling in cases of severe musculoskeletal injury may not only improve muscle regeneration but might also enhance bone healing in cases of orthopaedic trauma.

Acknowledgments

Funding for this study was provided by the National Institutes of Health (AR049717), the Office of Naval Research (N000140810197), and the Department of the Army (USAMRMC PR093619). Dr. William A. Horton generously provided the col2-eGFP mice utilized in this study, and Drs. Alexandra McPherron and Se-Jin Lee provided the myostatin-deficient mice. We are grateful to Drs. Xingming Shi and William Hill for their assistance with the isolation and culture of BMSCs, to Donna Kumiski and Penny Roon for their expertise in sectioning of the cartilage aggregates, and to Matthew Bowser and Phonepasong Arounleut for assistance in the lab.

References

- Allendorph GP, Vale WW, Choe S. Structure of the ternary signaling complex of a TGF-beta superfamily member. *Proc Natl Acad Sci U S A*. 2006; 103:7643–8. [PubMed: 16672363]
- Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repair. *J Bone Miner Res*. 1999; 14:1805–15. [PubMed: 10571679]
- Bialek P. Mice treated with myostatin/GDF-8 decoy receptor, ActRIIB-Fc, exhibit a tremendous increase in bone mass. *Bone*. 2008; 42(S1):S46.
- Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res*. 2002; 17:513–20. [PubMed: 11874242]
- Ciarmela P, Wiater E, Smith S, Vale W. Presence, actions, and regulation of myostatin in rat uterus and myometrial cells. *Endocrinology*. 2009; 150:906–14. [PubMed: 18845635]
- de Crombrughe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol*. 2000; 19:389–94. [PubMed: 10980415]
- Derynck R, Feng XH. TGF-beta receptor signaling. *Biochim Biophys Acta*. 1997; 1333:F105–50. [PubMed: 9395284]
- Einhorn TA. The cell and molecular biology of fracture healing. *Clin Orthop Relat Res*. 1998; 355(Suppl):S7–21. [PubMed: 9917622]
- Elkasrawy MN, Hamrick MW. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. *J Musculoskelet Neuronal Interact*. 2010; 10:56–63. [PubMed: 20190380]

- Fulzele S, Arounleut P, Cain M, Herberg S, Hunter M, Wenger K, Hamrick MW. Role of myostatin (GDF-8) signaling in the human anterior cruciate ligament. *J Orthop Res.* 2010; 28:1113–8. [PubMed: 20186835]
- Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem.* 2003; 88:873–84. [PubMed: 12616527]
- Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. *Nat Protoc.* 2008; 3:1253–60. [PubMed: 18714293]
- Grant TD, Cho J, Ariail KS, Weksler NB, Smith RW, Horton WA. Col2-GFP reporter marks chondrocyte lineage and chondrogenesis during mouse skeletal development. *Dev Dyn.* 2000; 218:394–400. [PubMed: 10842365]
- Hamrick MW, Shi X, Zhang W, Pennington C, Thakore H, Haque M, Kang B, Isaacs CM, Fulzele S, Wenger KH. Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone.* 2007; 40:1544–53. [PubMed: 17383950]
- Hamrick MW, Arounleut P, Kellum E, Cain M, Immel D, Liang LF. Recombinant Myostatin (GDF-8) Propeptide Enhances the Repair and Regeneration of Both Muscle and Bone in A Model of Deep Penetrant Musculoskeletal Injury. *J Trauma.* 2010; 69:579–83. [PubMed: 20173658]
- Ikeda T, Kawaguchi H, Kamekura S, Ogata N, Mori Y, Nakamura K, Ikegawa S, Chung UI. Distinct roles of Sox5, Sox6, and Sox9 in different stages of chondrogenic differentiation. *J Bone Miner Metab.* 2005; 23:337–40. [PubMed: 16133682]
- Iwai T, Murai J, Yoshikawa H, Tsumaki N. Smad7 inhibits chondrocyte differentiation at multiple steps during endochondral bone formation and down-regulates p38 MAPK pathways. 2008; 283:27154–64.
- James AW, Xu Y, Lee JK, Wang R, Longaker MT. Differential effects of TGF-beta1 and TGF-beta3 on chondrogenesis in posterofrontal cranial suture-derived mesenchymal cells in vitro. *Plast Reconstr Surg.* 2009; 123:31–43. [PubMed: 19116522]
- Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta1 to the bone. *Endocr Rev.* 2005; 26:743–74. [PubMed: 15901668]
- Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.* 1998; 238:265–72. [PubMed: 9457080]
- Joulia-Ekaza D, Cabello G. The myostatin gene: physiology and pharmacological relevance. *Curr Opin Pharmacol.* 2007; 7:310–5. [PubMed: 17374508]
- Kawakami Y, Rodriguez-Leon J, Izpisua Belmonte JC. The role of TGFbetas and Sox9 during limb chondrogenesis. *Curr Opin Cell Biol.* 2006; 18:723–9. [PubMed: 17049221]
- Kellum E, Starr H, Arounleut P, Immel D, Fulzele S, Wenger K, Hamrick MW. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. *Bone.* 2009; 44:17–23. [PubMed: 18852073]
- Lang CH, Silvis C, Nystrom G, Frost RA. Regulation of myostatin by glucocorticoids after thermal injury. *FASEB J.* 2001; 15:1807–9. [PubMed: 11481237]
- Lee SJ. Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol.* 2004; 20:61–86. [PubMed: 15473835]
- Lee SJ, McPherron AC. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci U S A.* 2001; 98:9306–11. [PubMed: 11459935]
- Lefebvre V, Behringer RR, de Crombrughe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis Cartilage.* 2001; 9(Suppl A):S69–75. [PubMed: 11680692]
- Li TF, O'Keefe RJ, Chen D. TGF-beta signaling in chondrocytes. *Front Biosci.* 2005; 10:681–8. [PubMed: 15569609]
- Lorda-Diez CI, Montero JA, Martinez-Cue C, Garcia-Porrero JA, Hurlé JM. Transforming growth factors beta coordinate cartilage and tendon differentiation in the developing limb mesenchyme. *J Biol Chem.* 2009; 284:29988–96. [PubMed: 19717568]

- Manceau M, Gros J, Savage K, Thome V, McPherron A, Paterson B, Marcelle C. Myostatin promotes the terminal differentiation of embryonic muscle progenitors. *Genes Dev.* 2008; 22:668–81. [PubMed: 18316481]
- Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, Glinka A, Niehrs C. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature.* 2002; 417:664–7. [PubMed: 12050670]
- McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature.* 1997; 387:83–90. [PubMed: 9139826]
- Mendias CL, Bakhurin KI, Faulkner JA. Tendons of myostatin-deficient mice are small, brittle, and hypocellular. *Proc Natl Acad Sci USA.* 2008; 105:388–93. [PubMed: 18162552]
- Nagamine T, Imamura T, Ishidou Y, Kato M, Murata F, ten Dijke P, Sakou T. Immunohistochemical detection of activin A, follistatin, and activin receptors during fracture healing in the rat. *J Orthop Res.* 1998; 16:314–21. [PubMed: 9671926]
- Penick KJ, Solchaga LA, Welter JF. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. *Biotechniques.* 2005; 39:687–91. [PubMed: 16312217]
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
- Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol.* 2003; 23:7230–42. [PubMed: 14517293]
- Solchaga LA, Welter JF, Lennon DP, Caplan AI. Generation of pluripotent stem cells and their differentiation to the chondrocytic phenotype. *Methods Mol Med.* 2004; 100:53–68. [PubMed: 15280587]
- Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, de Crombrughe B, Lefebvre V. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev Cell.* 2001; 1:277–90. [PubMed: 11702786]
- Steelman CA, Recknor JC, Nettleton D, Reecy JM. Transcriptional profiling of myostatin-knockout mice implicates Wnt signaling in postnatal skeletal muscle growth and hypertrophy. *FASEB J.* 2006; 20:580–2. [PubMed: 16423875]
- Wagner KR. Muscle regeneration through myostatin inhibition. *Curr Opin Rheumatol.* 2005; 17:720–4. [PubMed: 16224249]
- Zhang W, Ou G, Hamrick M, Hill W, Borke J, Wenger K, Chutkan N, Yu J, Mi QS, Isaacs CM, Shi XM. Age-related changes in the osteogenic differentiation potential of mouse bone marrow stromal cells. *J Bone Miner Res.* 2008; 23:1118–28. [PubMed: 18435580]
- Zhou S, Eid K, Glowacki J. Cooperation between TGF-beta and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. *J Bone Miner Res.* 2004; 19:463–70. [PubMed: 15040835]
- Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, Rosenfeld R, Chen Q, Boone T, Simonet WS, Lacey DL, Goldberg AL, Han HQ. Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell.* 2010; 142:531–43. [PubMed: 20723755]
- Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca MF, Huard J. Relationships between transforming growth factor-beta1, myostatin, and decorin: implications for skeletal muscle fibrosis. *J Biol Chem.* 2007; 282:25852–63. [PubMed: 17597062]
- Zhu X, Topouzis S, Liang LF, Stotish RL. Myostatin signaling through Smad2, Smad3, and Smad4 is mediated by the inhibitory Smad7 by a negative feedback mechanism. *Cytokine.* 2004; 26:262–72. [PubMed: 15183844]
- Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esqueda AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ. Induction of cachexia in mice by systemically administered myostatin. *Science.* 2002; 296:1486–8. [PubMed: 12029139]

Figure 1.

(A) Visualization of Col2-GFP in the digits of 1-day-old pups under an inverted fluorescence microscope. (B) FACS analysis verifying the degree of purity of isolated BMSC positive for Sca-1 by percentage compared to unstained population.

Figure 2. (A) Wild-type and (B) myostatin knockout BMSCs in culture. (C) BMSCs from myostatin knockout mice increase in number 22% more than wild-type after 4 days in culture. (D) Epiphyseal growth plate chondrocytes (EGPCs) from myostatin knockout mice doubled in number after 14 days in culture.

Figure 3.

(A) Myostatin decreases BMSC chondrogenic differentiation *in vitro*. Fluorescence microscopic images of aggregate culture of BMSC from Col2-GFP reporter mice after 6 days in chondrogenic media in absence or presence of myostatin starting at 0 hour. (B) Quantification of Col2 protein expression by surface area using ImageJ. 100ng/ml of recombinant myostatin significantly decreased Col2 expression levels by 31% with treatment starting at 0 hour. (C) Real-time PCR quantification of Sox9 gene expression levels in aggregate cultures treated with myostatin normalized to housekeeping genes GAPDH and 18S averages. 10- or 100ng/ml of recombinant myostatin caused a decrease in Sox9 mRNA levels by 211% and 289%. (D) Cartilage aggregates stained with hematoxylin and eosin or toluidine blue show decreased cell number and extracellular matrix staining following myostatin treatment.

D **Figure 4.**

Chondrogenesis is decreased by inhibiting TβRI (ALK5) and Smad3 signaling by Alk5i and SIS3 treatments, respectively. (A) *In vitro* fluorescence microscopic images of aggregate cultures of BMSC from Col2-GFP reporter mice after 6 days in chondrogenic media in absence or presence Alk5i or SIS3 starting at 0 hour. (B) Quantification of Col2 protein expression by surface area using ImageJ. 10μM of Alk5i significantly decreased Col2 expression levels by 60% with treatment starting at 0 hour and by 49.3% with treatment starting at 48 hours. (C) Real-time PCR quantification of Sox5 and Sox9 gene expression levels in aggregate cultures treated with Alk5i. 10μM of Alk5i caused a 175% decrease in Sox5 mRNA levels, and a 117% decrease in Sox9 mRNA levels. (D) 10μM of SIS3 significantly decreased Col2 expression levels by 99% with treatment starting at 0 hour, and by 74.4% with treatment starting at 48 hours.

Table 1

Genes and primer sequences used for real-time PCR analysis of chondrocyte gene expression.

Gene	Primer Sequence	Amplicon length (bp)	Accession number
SOX-9	5'-aaagttgatctgaagcgagagg-3' 5'-gaaggtcacaatcttgagatga-3'	123	NM_11448
SOX-5	5'-gtggaagaggaggagagtga-3' 5'-aaattcctcagagtggcctg-3'	87	BC110478
eGFP	5'-ctgtacagctcgtccatgccg-3' 5'-ggagaggggtccagcccagctac-3'	900	YP_003162718
GAPDH	5'-catggcctccaaggagtaaga-3' 5'-gagggagatgctcagtggttg-3'	105	M32599
18S	5'-agtgcgggtcataagcrtgc-3' 5'-gggcctcactaaacctcca-3'	90	V00851