Genetic disruption of myostatin reduces the development of proatherogenic dyslipidemia and atherogenic lesions in \textit{Ldlr} null mice

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**Objective:** Insulin-resistant states, such as obesity and type 2 diabetes, contribute substantially to accelerated atherogenesis. Null mutations of myostatin (Mstn) are associated with increased muscle mass and decreased fat mass. In this study, we determined whether Mstn disruption could prevent the development of insulin resistance, proatherogenic dyslipidemia, and atherogenesis.

**Research design and methods:** C57BL/6 Ldlr\(^{-/-}\) mice were cross-bred with C57BL/6 Mstn\(^{-/-}\) mice for >10 generations to generate Mstn\(^{-/-}/Ldlr^{-/-}\) double-knockout mice. The effects of high-fat/high-cholesterol diet on body composition, plasma lipids, systemic and tissue-specific insulin sensitivity, hepatic steatosis, as well as aortic atheromatous lesion were characterized in Mstn\(^{-/-}/Ldlr^{-/-}\) mice in comparison with control Mstn\(^{+/+}/Ldlr^{-/-}\) mice.

**Results:** Compared to Mstn\(^{+/+}/Ldlr^{-/-}\) controls, Mstn\(^{-/-}/Ldlr^{-/-}\) mice were resistant to diet-induced obesity, and had greatly improved insulin sensitivity, as indicated by 42% higher glucose infusion rate and 90% greater muscle \(^{[3]}H\)-2-deoxyglucose uptake during hyperinsulinemic-euglycemic clamp. Mstn\(^{-/-}/Ldlr^{-/-}\) mice were protected against diet-induced hepatic steatosis and had 56% higher rate of hepatic fatty acid \(\beta\)-oxidation than controls. Mstn\(^{-/-}/Ldlr^{-/-}\) mice also had 36% lower VLDL secretion rate, and were protected against diet-induced dyslipidemia, as indicated by 30-60% lower VLDL and LDL cholesterol, free fatty acids, and triglycerides. MR angiography and en face analyses demonstrated 41% reduction in aortic atheromatous lesions in Ldlr\(^{-/-}\) mice with Mstn deletion.

**Conclusions:** Inactivation of Mstn protects against the development of insulin resistance, proatherogenic dyslipidemia and aortic atherogenesis in Ldlr\(^{-/-}\) mice. Myostatin may be a useful target for drug development for prevention and treatment of obesity and its associated type 2 diabetes and atherosclerosis.
Heart disease and diabetes mellitus rank amongst the most prevalent disorders in most Western countries, and their incidence rates within the elderly population are particularly high (1). Aging is associated with decrease in muscle mass, increase in fat mass (2), insulin resistance and atherosclerosis progression, all conditions that predispose individuals to cardiometabolic diseases. Therefore, adiposity, sarcopenia and heart disease are inter-related consequences of aging that contribute substantially to morbidity and mortality among older humans.

Currently, most available therapies for heart disease are based on lowering of plasma cholesterol, such as statins. These interventions have little effect on adiposity and sarcopenia. We surmised that unlike available pharmacological therapies that are mostly targeted at cholesterol synthesis or metabolism, novel therapeutic strategies, such as myostatin inactivation, that directly target muscle and fat mass accumulation may be effective in protection against obesity and its metabolic ramifications.

Genetic disruption of myostatin, a TGF-β family member that functions as an endogenous inhibitor of muscle growth (3-5), leads to increased skeletal muscle mass and decreased fat mass in humans, cattle, mice, and other species (6; 7). Disruption of myostatin gene has also been shown to prevent the development of obesity (6). Inhibition of myostatin, either directly or through over-expression of myostatin propeptide, an endogenous myostatin inhibitor, has also been shown to prevent the development of obesity and insulin resistance (6).

In this paper, we show that the loss of myostatin attenuates the development of atherogenic lipid profile and the progression of atheromatous lesion growth in low density lipoprotein receptor-null (Ldlr−/−) mice, a widely used experimental model of atherogenesis. When Ldlr−/− mice, lacking the cell-surface transmembrane receptor that recognizes apolipoprotein B100 (apoB100), are fed western-type diet, they display hypercholesterolemia and atheromatous lesions resembling those observed in patients with familial hypercholesterolemia (8). Our data show that Mstn disruption in Ldlr−/− mice not only attenuates diet-induced fat accumulation and improves whole body insulin sensitivity, as has been reported recently (9; 10), but it also prevents the hepatic hypersecretion of proatherogenic lipoprotein, protects against the development of proatherogenic dyslipidemia, and reduces atherogenesis progression.

RESEARCH DESIGN AND METHODS

Animals: C57BL/6 mice with homozygous deletion of Ldlr (Jackson Laboratory, Bar Harbor, ME) were cross-bred with C57BL/6 mice with homozygous deletion of Mstn. Their offspring were bred >10 times to generate Mstn−/−/Ldlr−/− double-knockout mice. All mice used in this study were males. Mice were provided food and water ad libitum and maintained on a 12 hour light/dark cycle. Mice were fed normal-chow (TD#2018, Harland Teklad, Madison, WI) until 4-months of age, then fed with high-fat/high-cholesterol (HF)-diet (TD#94059, Harland Teklad). The HF-diet consisted 15.8% w/w fat (primarily from cocoa butter), 1.25% w/w cholesterol, 41.2% w/w carbohydrate, and 19.7% w/w protein. Food consumption was measured using powdered HF-diet provided in glass jars with perforated metal tops to prevent scattering. Study protocols were approved by the Boston University’s Institutional Animal Care and Use Committee.

Body Composition and Metabolic Cage Measurements. Whole body fat and muscle mass were determined by NMR
Myostatin deficiency and atherosclerosis

Visceral and subcutaneous fat imaging were performed using low energy X-ray microCT scanner (LaTheta LCT-100A™, Echo Medical System). Energy expenditure and RER rates were measured using Oxymax system (Columbus Instruments, Columbus, OH) as previously described (11).

**Magnetic Resonance Angiography (MRA).** Mice were anesthetized using 1-2% (v/v) isoflurane and 1-2L/min oxygen flow through an anesthesia monitoring system (Smiths Medical PM, Waukesha, WI). Respiration was monitored by respiration sensor pillow (SA Instruments, Stony Brook, NY). Imaging was performed using a Bruker Avance 500 vertical bore spectrometer with 11.7T (Bruker, Billerica, MA). Data acquisition and reconstruction were performed with the ParaVision 3.0.2. 3D MRA were obtained using the FLASH_3D_ANGIO pulse sequence (flip angle = 20º, TE/TR = 2.18ms/20ms, FOV = 15mm×15mm×15mm). Four averages were obtained in each scan.

**En face detection of atheromatous lesions.** Preparation of aortas and quantification of atheromatous lesions were performed in animals after 12 weeks of HF-diet feeding, using procedures previously described (12). The heart with aorta was embedded in OCT (Sakura Finetech USA Inc, Torrance, CA), and serial 10µm-thick cytosections of aortic root were stained with Oil Red O by the Rodent Histopathology Core at Harvard Medical School (Boston, MA).

**Measurements of plasma lipids and other metabolites.** Blood glucose and ketone concentrations were determined using Glucometer Elite XL (Bayer, Tarrytown, NY) and Precision Xtra™ (Abbott laboratories, Bedford, MA), respectively. Plasma insulin and glucagon were measured using ultrasensitive ELISA (Alpco, Salem, NH). For lipid analyses, fasting (food removed for 12 hours) and non-fasting (food removed for 3 hours) blood samples were collected from the retro-orbital plexus under isoflurane anesthesia. Lipid analyses were performed using procedures previously described (13). Cholesterol and triglyceride distribution of the lipoproteins were performed by Cardiovascular Specialty Laboratories, Inc. (Atlanta, GA) using fast-performance liquid chromatography (FPLC) (14). Fractions 2-6 contain VLDL; fractions 7-11 contain LDL; and fractions 14-17 contain HDL.

**Glucose and insulin tolerance tests.** For glucose tolerance tests, non-fasting mice were given D-glucose (1g/kg) by intraperitoneal injection. For insulin tolerance tests, fasting mice were given insulin (0.75IU/kg) (Eli Lilly, Indianapolis, IN) by intraperitoneal injection. Blood glucose was measured using Glucometer Elite XL (Bayer, Tarrytown, NY).

**Hyperinsulinemic-euglycemic clamp studies.** Hyperinsulinemic–euglycemic clamps were performed by a modification of a described procedure (15). Briefly, right jugular vein of anesthesized mice (80-10mg/kg ketamine-xylazine IP) was catheterized with heparin-coated MRE-025 tubing (Becton Dickinson, Franklin Lakes, NJ). Animals were allowed to recover for 1-2 days. Insulin (10mU/kg/min) containing 0.3% BSA was infused at the rate of 1µL/min using a syringe pump (HA11D, Harvard apparatus, Holliston, MA). At 5min intervals, ~2µL of tail vein blood was drawn to measure blood glucose. Dextrose (25%) was infused at a rate sufficient to maintain blood glucose of 140mg/dL. Average glucose infusion rate (GIR) was measured over the final 30min of the 2hr clamp. Insulin was measured in blood samples from the last 3 collections.

**Muscle [³H]-2-deoxyglucose uptake.** Muscle uptake of the metabolically inert glucose analog, [³H]-2-deoxyglucose (DOG), was performed in mice at 30min before the end of 2hr euglycemic-hyperinsulinemic clamp experiments, with modification to
methods described previously (15). Briefly, DOG (30µCi) was administered intraperitoneally, and mouse hind-limb muscles were harvested, denatured, and the eluates were quantified using liquid scintillation analyzer (TriCarb 3100TR, PerkinElmer, Shelton, CT). The rate of glucose uptake was calculated by dividing the muscle DOG uptake over 30min interval (cpm/kg/min) by the mean blood glucose specific activity (cpm/mg glucose).

**Histology.** Liver tissues were embedded in OCT. Serial 10µm-thick cytosections were prepared the Rodent Histopathology Core at Harvard Medical School (Boston, MA) and were stained with Oil Red O and Hematoxylin & Eosin (H&E).

**Intrahepatic lipid measurements.** Livers were lyophilized using Freeze Dry System (Freezone®, Labconco, Fort Scott, KS) to obtain liver dry weight. Liver lipids were extracted by the Folch method (16). Liver cholesterol and triglycerides were measured using Infinity® Cholesterol (Thermo Electron, Pittsburgh, PA) and T2449 Triglyceride Reagent (Sigma-Aldrich, St. Louis, MO), respectively.

**Mitochondrial fatty acid β-oxidation measurement.** Fresh liver tissue was homogenized in 250mM sucrose, 0.1mM EDTA, 50mM KCl, 10mM HEPES (pH 7.4) and centrifuged at 1000g for 5min. The supernatant was centrifuged at 10,000g for 15min, and pellets were dissolved in 50mM KCl, 70mM sucrose, 3.6mM MgCl₂, 7.2mM KHPO₄, 36mM Tris-HCl (pH 7.4). Protein concentrations were measured using Bradford reagent (Bio-Rad, Hercules, CA). 5µCi/mL [³H]-palmitate was incubated with 1mg of mitochondria in preincubation buffer with 2mM KCN at room temperature for 30min. Reaction was subsequently incubated for 60min in 5% perchloric acid, and supernatants were counted in a liquid scintillation counter.

**Hepatic VLDL (apoB100) secretion.** VLDL secretion rate was measured by modification of a previously described method (17). Briefly, fasted mice were injected with Triton WR 1339 (500 mg/kg; Sigma Chemical Co., St. Louis, Missouri, USA) via tail vein to block lipolysis. Blood samples were collected at baseline, 90min and 180min after injection. Plasma apoB100 was determined by western blot analysis using anti-apoB antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Hepatic VLDL secretion was calculated by the percentage increase in plasma apoB100 from baseline.

**Western Blot Analysis.** 1µL of plasma per lane was analyzed after treatment with sample buffer containing SDS and β-mercaptoethanol, followed by separation in 4-15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA). Liver and muscle tissue samples were extracted and processed as previously described (12). All primary, except anti-fatty acid synthase (Cell Signaling Technology, Danvers, MA), and secondary antibodies were purchased from Santa Cruz Biotechnology.

**Real Time Q-PCR.** Total RNA was extracted from frozen liver using RNeasy mini kit (Qiagen, Valencia, CA). For reverse transcription, 1µg of the total RNA was converted to first strand complementary DNA in 20µL reactions using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, La Jolla, CA), which was subsequently diluted 5 times. For PCR sample preparation, 5µL of cDNA was mixed in 20µL reaction volume with 10µM primer and SYBR master enzyme mix (SABiosciences, Frederick, MD). The reaction was initiated at 94°C for 10 min, followed by 40 cycles through 94°Cx15sec and 60°Cx1min. All reactions were run in duplicate. All reactions were run in duplicate. All Ct values were in the range of 20-30 cycles. Amplification curves were analyzed using SDS 1.9.1 software (Applied Biosystems, Foster City, CA). Hprt1 controls
provided relative gene expression levels. Refer to Supplemental Table 1 for primers.

**Statistical Analysis.** Experimental results are shown as mean ± SEM. The mean values from two independent groups were compared by using Student's *t*-test for independent samples. Differences among multiple groups were assessed using analysis of variance models. If ANOVA revealed a significant overall effect, then individual groups were compared by using Tukey’s procedure. Longitudinal data from multiple groups of animals were compared by using repeated measures analysis of variance, with a time-in-treatment factor and a treatment group factor. All statistical tests were one-tailed and *P*<0.05 were considered significant.

**RESULTS**

*Mstn* deletion retards whole body, visceral and subcutaneous fat accumulation. *Ldlr*<sup>−/−</sup> mice with varying *Mstn* genotypes, wild-type (*Mstn*<sup>+/+Ldlr</sup><sup>−/−</sup>), heterozygous deletion (*Mstn*<sup>+/−Ldlr</sup><sup>−/−</sup>), and homozygous deletion (*Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup>), were fed HF-diet for 11-12 weeks. *Mstn*<sup>+/+Ldlr</sup><sup>−/−</sup> controls demonstrated substantial gains in body weight, whole body fat mass (measured by NMR), and visceral and subcutaneous fat mass (measured by microCT scan). In contrast, *Mstn*<sup>+/−Ldlr</sup><sup>−/−</sup> mice resisted weight gain and accumulation of whole body and visceral fat mass in response to HF-diet (Fig. 1a-b and Supplemental Fig. 1a-b). In agreement with the *in vivo* NMR data, the wet weights of the major fat depots - inguinal, epididymal, perirenal as well as the interscapular brown fat - were significantly lower in the *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> mice, as compared to wild-type, *Mstn*<sup>+/+/Ldlr</sup><sup>−/−</sup> and *Mstn*<sup>+/−/Ldlr</sup><sup>−/−</sup> mice (Fig. 1c). Thus, *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> mice are resistant to fat accumulation in all fat depots including the visceral and subcutaneous fat depot.

As expected, the *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> mice had significantly greater skeletal muscle mass, as indicated by the 2-fold higher mass of the *gastrocnemius* and the *quadriceps femoris* muscle groups (Fig. 1d and Supplemental Fig. 1c-d) than in the *Mstn*<sup>+/+/Ldlr</sup><sup>−/−</sup> controls. Throughout the course of HF-diet, *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> mice maintained significantly greater lean mass – assessed by NMR - compared to other genotype groups (Fig. 1d, and Supplemental Fig. 1c). The *soleus* muscle, a primarily oxidative muscle type, was not statistically significantly affected by *Mstn* disruption (Supplemental Fig. 1e). *Ldlr*<sup>−/−</sup> mice with heterozygous deletion of *Mstn* only displayed modest increase in muscle mass, but have similar fat mass accumulation compared to *Mstn*<sup>+/+Ldlr</sup><sup>−/−</sup> controls when fed HF-diet.

Despite the remarkable effects of *Mstn* deletion on body composition, neither the energy intake nor energy expenditure differed significantly among the various groups on normal chow (data not shown) or on HF-diet (Supplemental Fig. 2a). Ambulatory activity levels on HF-diet were assessed by infrared beam interruption, and notably, the horizontal ambulatory activity of *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> double-knockout group was 38% lower than that of controls (Supplemental Fig. 2b). However, despite this reduction in activity, analysis of whole body O<sub>2</sub> consumption (VO<sub>2</sub>) showed that energy expenditure was similar between the two groups (Supplemental Fig. 2c). If the data were expressed as a function of lean mass weight, *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> actually had lower VO<sub>2</sub> rate (13%) compared to controls. Furthermore, the respiratory exchange ratios (RER) were approximately 0.85 in *Ldlr*<sup>−/−</sup> mice with or without *Mstn* disruption, reflecting mixed utilization of carbohydrate and fatty acid oxidation in both animal groups (Supplemental Fig. 2d). Thus, the resistance to fat accumulation in *Mstn*<sup>−/−/Ldlr</sup><sup>−/−</sup> mice cannot be explained on the basis of reduced food intake, increased energy expenditure, or preference for fuel disposition.
Mstn deletion reduces diet-induced atherogenesis in Ldlr⁻/⁻ mice. To determine whether deletion of Mstn mitigates the development of vascular lesions, we first used in vivo MR angiography to evaluate lumen occlusion of the brachiocephalic artery, one of the most lesion-prone sites (Fig. 2a). After 8 weeks of HF-diet, Mstn⁺/⁺/Ldlr⁻/⁻ mice showed reduced lumen size and increased asymmetry (Fig. 2b-d); in contrast, brachiocephalic arterial lumen of Mstn⁻/⁻/Ldlr⁻/⁻ maintained patency without significant occlusion, as indicated by greater symmetry and significantly larger (1.7-fold) lumen area (Fig. 2c-d).

After 12 weeks of HF-diet, the animals were euthanized and aortas were dissected. Mstn⁺/⁺/Ldlr⁻/⁻ mice developed large plaques that were eccentric (partial circumference of arterial wall) and dispersed along the arch and in the descending aorta (Fig. 2e). Staining with Oil Red O Sudan IV revealed that the lesions were lipid rich (Fig. 2e). Analysis of the en face aorta demonstrated that atheromatous lesion area in Mstn⁻/⁻/Ldlr⁻/⁻ mice was 41% lower than in Mstn⁺/⁺/Ldlr⁻/⁻ controls (Fig. 2f-g). In correlation with the similar fat mass accumulation (Fig. 1b-c), Mstn⁺/⁺/Ldlr⁻/⁻ mice had similar lesion area compared to Mstn⁺/⁺/Ldlr⁻/⁻ controls (Fig. 2g), suggesting that heterozygous deletion of Mstn is not sufficient in providing protection against either fat accumulation or atherogenesis. The negative control, Mstn⁺/⁺/Ldlr⁻/⁻ mice group, had no detectable lesions. Thus, Ldlr⁻/⁻ mice with Mstn deletion were protected against the development/progression of atheromatous lesions.

We next investigated the mechanisms by which disruption of Mstn retards atherogenesis progression. As circulating lipids are important contributors to atherogenesis, we determined the changes in plasma lipids and lipoproteins.

Mstn deletion in LDLR⁻/⁻ mice attenuates proatherogenic dyslipidemia. Even prior to HF-diet induction, Mstn⁻/⁻/Ldlr⁻/⁻ mice had significantly lower plasma triglycerides (TG), free fatty acids (FFA), and total cholesterol levels compared to Mstn⁺/⁺/Ldlr⁻/⁻ mice (Fig. 3a). After 10 weeks of HF-diet, Mstn⁺/⁺/Ldlr⁻/⁻ controls displayed marked increase in plasma TG (3.0-fold), FFA (2.3-fold), and cholesterol (3.8-fold) levels compared to baseline; the changes in plasma lipids were significantly greater than those observed in Mstn⁺/⁺/Ldlr⁻/⁻ mice after HF-diet. Plasma TG, FFA, and cholesterol levels after HF-diet were significantly lower in Mstn⁻/⁻/Ldlr⁻/⁻ than in Mstn⁺/⁺/Ldlr⁻/⁻ controls (Fig. 3b), suggesting that Mstn deletion protects against the development of proatherogenic dyslipidemia in Ldlr⁻/⁻ mice fed HF-diet.

We fractionated plasma lipoproteins by using fast phase liquid chromatography (FPLC). Plasma total cholesterol, very low density lipoprotein (VLDL)-cholesterol and low density lipoprotein (LDL)-cholesterol levels were lower in Mstn⁻/⁻/Ldlr⁻/⁻ mice than in Mstn⁺/⁺/Ldlr⁻/⁻ controls (Fig. 3c). TG contents of VLDL (VLDL-TG) and LDL fractions (LDL-TG) were also significantly lower in Mstn⁻/⁻/Ldlr⁻/⁻ mice than controls (Fig. 3d).

ApoB is an important predictor of cardiovascular risk; some patients with elevated apoB100 levels are at increased risk of cardiovascular disease despite having target cholesterol levels (18; 19). Hence, we analyzed the plasma apoB100 contents in our mice. Western blot analysis of plasma samples of Mstn⁺/⁺/Ldlr⁻/⁻ mice showed a significant increase in apoB100 after 11 weeks of HF-diet, and this diet effect was markedly attenuated in Mstn⁻/⁻/Ldlr⁻/⁻ mice (Fig. 3e). We also measured apoA1, the scaffold protein for high density lipoprotein (HDL). The ratio of apoB100 to apoA1, which is stoichiometrically related to the ratio...
of non-HDL to HDL particles, was lower in Mstn−/−/Ldlr−/− mice compared to controls (Fig. 3e), suggesting a more favorable lipoprotein profile in Mstn−/−/Ldlr−/− mice.

**Mstn−/−/Ldlr−/− mice have greater insulin-mediated glucose disposal.** Diet-induced obesity is strongly associated with deterioration of insulin sensitivity, clinically evidenced by increased circulating insulin, glucose, TG, and FFA (20). Insulin also serves as an important regulator of adipose lipolysis and hepatic VLDL production. Dysregulation of these metabolic functions of insulin could contribute to hyperlipidemia and accelerate atherosclerosis in mice fed with HF-diet (21; 22). Accordingly, we assessed the effect of Mstn deletion on insulin sensitivity in Ldlr−/− mice.

In addition to having lower plasma TG, FFA, VLDL, and apoB concentrations, Mstn−/−/Ldlr−/− mice also had significantly lower fasting blood glucose and insulin levels. These data suggested that Mstn−/−/Ldlr−/− mice were more insulin sensitive than Mstn+/+/Ldlr−/− controls even when fed HF-diet (Fig. 4a-c). Plasma insulin to glucagon ratio was also significantly lower (30%) in Mstn−/−/Ldlr−/− mice—Glucose tolerance test (GTT) did not reveal significant differences between rate of glucose disposal (Supplementary Fig. 3a), but this could be due to the compensatory increase in fasting insulin in Mstn+/+/LDLR−/− mice (Fig. 4b). When mice were injected with a fixed dose of insulin during insulin tolerance tests (ITT), Mstn−/−/Ldlr−/− mice maintained significantly lower blood glucose than their Mstn+/+/Ldlr−/− littermates (Supplemental Fig. 3b). Furthermore, Ldlr−/− mice with Mstn deletion had significantly lower phosphoenolpyruvate carboxykinase 1 (Pepck) expression (Supplemental Fig. 3c), the rate-limiting hepatic gluconeogenic enzyme (23), consistent with improved insulin sensitivity.

To confirm our findings of improved insulin sensitivity in Mstn−/−, Ldlr−/− mice, we performed hyperinsulinemia-euglycemic clamp studies, the accepted gold standard for assessing insulin sensitivity (24). Mstn−/−/Ldlr−/− required significantly higher glucose infusion rates (GIR) to maintain euglycemia (Fig. 4d-e) than in controls. These data demonstrate that Mstn deletion improves whole-body insulin sensitivity through the enhancement of insulin-stimulated glucose disposal in peripheral tissues. It is possible that the increased GIR is simply due to greater muscle mass and the resulting increase in metabolic demand of the double-knockout mice. To explore this further, we measured the rates of [3H]-2-deoxyglucose uptake in skeletal muscle during the clamp procedure. In the Mstn−/−/Ldlr−/− mice, glucose uptake in quadriceps muscle was significantly higher compared to that of the Mstn+/+/Ldlr−/− controls (Fig. 4f) in a similar hyperinsulinemic state (Fig. 4g). Furthermore, phosphorylation of serine-473 in Akt, the pleiotropic kinase essential for many metabolic actions of insulin, was significantly higher in muscle tissues of Mstn−/−/Ldlr−/− mice compared to that of the controls. Phosphorylation of serine-21/9 in GSK3, one of Akt substrates, was also significantly higher, providing further evidence of improved insulin action in the skeletal muscle (Fig. 4h). Together, these data demonstrate that HF-diet induces an insulin-resistant state in Mstn+/+/Ldlr−/− mice and that disruption of Mstn prevents the development of insulin resistance, as consistent with other reports (9; 10).

**Mstn−/−/Ldlr−/− are protected against hepatic steatosis, and have higher hepatic fatty acid β-oxidation and lower VLDL secretion rates.** The liver plays a leading role in systemic lipid homeostasis (25). In response to HF-diet feeding, the livers of Mstn+/+/Ldlr−/− mice displayed substantial fat accumulation (Fig. 5a). In contrast, Mstn−/−/Ldlr−/− mice had significantly lower hepatic TG content, as well as total wet and dry liver weights (Fig. 5a, Supplemental Fig. 4a). This
protection against diet-induced hepatic fat infiltration in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice was associated with significant reduction in plasma adipose-derived FFA (Fig. 3a), glucose and insulin (Fig. 4a-b), as well as with diminished expression of lipogenic master gene, sterol regulatory element binding transcription factor 1 (Srebf1), and its key downstream target, fatty acid synthase (Fasn) mRNA and protein (Fig. 5b-d).

Hepatic fatty acid β-oxidation has been shown to prevent diet-induced obesity, fatty liver, and hyperlipidemia (26). In our \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice model, hepatic fatty acid β-oxidation rates in isolated hepatic mitochondrion were significantly greater (1.7-fold, Supplemental Fig. 4b) than those from controls. In addition, we have previously shown that \( \text{Mstn} \) deletion resulted in a 25% increase of liver mitochondrial DNA (manuscript in revision), implying that these mice have greater mitochondrial oxidation capacity. Plasma level of the ketone body β-hydroxybutyrate, an indirect marker of hepatic fatty acid β-oxidation, was also significantly higher in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) (Supplemental Fig. 4c) than in controls.

Decreased hepatic TG in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice was associated with reduced secretion of hepatic TG (23%) and VLDL (Fig. 5e). Suppression of hepatic fat accumulation in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice was associated with enhanced insulin action in the liver. The mRNA expression levels of insulin receptor substrate (Irs)1 and Irs2, two key players in the regulation of hepatic insulin signaling (27), were significantly higher in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice compared to \( \text{Mstn}^{+/}/\text{Ldlr}^{-/} \) controls (Supplemental Fig. 4d). Western blot analyses also showed significantly higher Akt and GSK phosphorylation in isolated liver tissues of \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice (Fig. 5f). As emerging data suggest a causative role of intracellular hepatic TG accumulation in the pathogenesis of hepatic insulin resistance and hypersecretion of VLDL (23; 28-30), these findings provide a mechanistic link between the protective effects of \( \text{Mstn} \) deletion on obesity, insulin resistance, and atherosclerosis (Supplemental Fig. 5).

**DISCUSSION**

In this report, we provide the evidence that \( \text{Mstn} \) disruption in \( \text{Ldlr}^{-/} \) mice reduces the development of proatherogenic dyslipidemia and atherogenesis progression. The \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice had lower plasma TG, FFA, and non HDL-cholesterol than controls. \( \text{Mstn}^{-/}/\text{LDLR}^{-/} \) mice also had lower hepatic VLDL secretion rates. While we cannot exclude direct actions of myostatin on the blood vessel wall, it is likely that the more favorable plasma lipid profile was the key contributor to reduced atherogenesis progression observed in \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice.

\( \text{Mstn} \) deletion prevented the development of diet-induced insulin resistance in \( \text{Ldlr}^{-/} \) mice. Hyperinsulinemic-euglycemic clamp studies revealed that \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice had significantly higher peripheral glucose disposal rates. Furthermore, decreased circulating insulin, as well as decreased glucose and adipose-derived FFA influx to the liver of the double-knockout mice may contribute to the protection against hepatic lipogenesis and hepatic insulin resistance (23; 25; 28).

The mechanisms by which \( \text{Mstn} \) deletion improves insulin sensitivity are not fully understood. \( \text{Mstn}^{-/}/\text{Ldlr}^{-/} \) mice remained remarkably lean and resisted fat accumulation even when fed HF-diet for 12 weeks. Myostatin may exert complex effects on adipogenic differentiation (31; 32). In addition, it is possible that acquisition of hypermuscularity by itself could lead to leanness (11). Indeed, mice with increased muscle oxidation, such as those over-expressing IGF-1 (33) or Akt-1 (11) in skeletal muscles, have little fat deposition. In our myostatin-deficient model, the marked hypertrophy of glycolytic skeletal muscle was
associated with increased glucose uptake, lower blood glucose levels, and consequently lower circulating levels of insulin (11).

Increased hepatic fatty acid β-oxidation (FAO) and mitochondrial biogenesis with Mstn disruption may prevent the development of hepatic steatosis that was observed in Mstn+/+;Ldlr+/+ controls (25; 26). Consistent with the higher FAO rates, Mstn−/−;Ldlr−/− mice had higher ketone body production than controls. Improved insulin sensitivity might be expected to suppress FAO and lower ketone body production. However, insulin only effectively suppresses FAO in tissues, primarily muscle and fat, where glucose uptake is tightly regulated by insulin. Since hepatic glucose uptake is not regulated by insulin (34), improved insulin sensitivity in the Mstn−/−/Ldlr−/− mice does not pose limitation on liver FAO as it does in the skeletal muscle. This phenomenon has also been observed by others (11; 35). Our data suggest that the increased ketone body production in the double-knockout mice may be a result of physiological adaptation to limited adipose tissue storage for dietary fats from HF-diet.

Myostatin may also indirectly affect cardiometabolic risk by its effects on myokine secretion by the skeletal muscle. Similar to systemic loss of Mstn, inhibition of myostatin signaling exclusively in the skeletal muscle was sufficient to resist diet-induced obesity and insulin resistance (10). Myokines, such as IL-6, that can alter metabolic states (36). Other myokines, such as fibroblast growth factor-21 (Fgf-21) and follistatin like-1 (Fstl-1), may also have salutary effects on fat accumulation and insulin sensitivity (37; 38).

In summary, we have demonstrated here that Mstn deletion has beneficial cardiometabolic effects in an animal model of atherosclerosis. Mstn−/−/Ldlr−/− mice are partially protected against not only fat accumulation and insulin resistance, but also the development of proatherogenic dyslipidemia and atherosclerosis. These proof-of-concept studies raise the possibility that myostatin inhibitors may be useful agents for the prevention or treatment of atherosclerosis. Importantly, administration of myostatin inhibitors to humans have demonstrated specificity, safety, and effectiveness in increasing muscle mass and decreasing fat mass (39). To the extent that loss of skeletal muscle mass, adiposity, and atherogenesis occur contemporaneously during aging and in many chronic illnesses, new classes of promyogenic drugs, such as myostatin inhibitors, that increase skeletal muscle mass and retard fat accumulation are particularly attractive against these cardiometabolic disorders.

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Figure Legends

Figure 1 | Effects of Mstn disruption on body fat accumulation in Ldlr<sup>−/−</sup> mice. a, Representative gross appearance (top panel) and microCT image of visceral and subcutaneous fat (bottom panel) of mice after 12 weeks of HF-diet. c, cecum; vf, visceral fat; sf, subcutaneous fat. b, NMR analysis of total fat at baseline (time 0), and after 5 and 10 weeks of HF-diet. c, Inguinal, epididymal, perirenal, and intrascapular brown fat weights of mice after 12 weeks of HF-diet. d, Representative gross appearance of hind-limb muscles (left panel) and quadriceps muscle weights (right panel) of mice after 12 weeks of HF-diet. ++/++, Mstn<sup>+/−</sup>/Ldlr<sup>+/−</sup>. ++/−, Mstn<sup>+/−</sup>/Ldlr<sup>−/−</sup>. −/−, Mstn<sup>−/−</sup>/Ldlr<sup>−/−</sup>. Data are expressed as mean ± SEM (n=11-21). **P<0.01 compared to all other genotypes.

Figure 2 | Effects of Mstn disruption on atherogenesis progression in Ldlr<sup>−/−</sup> mice. a, Representative magnetic resonance angiograms (MRA) of aortic arch and its major branches. BCA, brachiocephalic artery. b, Cross sectional image of BCA. c-d, Symmetry coefficient (ratio of the largest to the smallest diameter) (c), and cross-sectional area (d) of BCA lumen (n=4). e, Oil Red O staining of atherosclerotic lesions in aortic root at the level of the aortic valves (top panel). Magnification 40x. Gross aortic arch and branches (bottom panel) (n=9-20). f-g, Sudan IV staining of en face aortas (f) and quantitative analyses of atherosclerotic lesion areas (percent of total aortic surface area) (g) (n=9-20). ++/++, Mstn<sup>+/−</sup>/Ldlr<sup>+/−</sup>. ++/−, Mstn<sup>+/−</sup>/Ldlr<sup>−/−</sup>. −/−, Mstn<sup>−/−</sup>/Ldlr<sup>−/−</sup>. Data are expressed as mean ± SEM (n=11-21). **P<0.01, *P<0.05.

Figure 3 | Atherogenic lipid profile in Ldlr<sup>−/−</sup> mice with Mstn deletion. a-b, Fasting plasma free fatty acids (FFA), triglycerides (TG), and cholesterol levels of mice at baseline (a) and after 10 weeks of HF-diet (b). c-d, Lipoprotein profile in Mstn<sup>+/−</sup>/Ldlr<sup>+/−</sup> (open circles) and Mstn<sup>−/−</sup>/Ldlr<sup>−/−</sup> (dark circles) mice after 11 weeks of HF-diet. Data are presented as average cholesterol (c) and TG (d) distribution for each group. e, Plasma apolipoprotein A1 (apoA1)- and apoB100-containing lipoprotein particles before and after induction of HF-diet. The graphs demonstrate the quantification of each molecule, displayed as apoB100/apoA1 ratio. Averages were taken from 4 different gels. Blood was drawn from mice after 10 weeks of HF-diet. ++/−, Mstn<sup>+/−</sup>/Ldlr<sup>−/−</sup>. −/−, Mstn<sup>−/−</sup>/Ldlr<sup>−/−</sup>. Data are shown as the mean ± SEM (n=7-10). *P<0.05, **P<0.01.

Figure 4 | Metabolic studies in Ldlr<sup>−/−</sup> mice with Mstn deletion. a-c, Fasting blood glucose (a) plasma insulin (b), and glucose (mg/dL) x insulin product (µIU mL) ratio (c) of Mstn<sup>+/−</sup>/Ldlr<sup>−/−</sup> and Mstn<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice after 8 weeks of HF-diet. d-g, Hyperinsulinemic-euglycemic clamp studies in mice after 12 weeks of HF-diet. Trace of blood glucose and glucose infusion rate (GIR) during the 2-hour clamp (d), average GIR (e), glucose uptake in quadriceps muscle (f), and average plasma insulin during clamp period (g). h, Akt serine-473 and GSKα/β serine-21/9 phosphorylation in the quadriceps muscle of mice. The graphs demonstrate the quantification of phosphorylated form of each molecule. AU, arbitrary units. Data are shown as the mean ± SEM (n=7-10). *P<0.05, **P<0.01.
Figure 5 | Effects of Mstn deletion on liver of Ldlr-/- mice. a, Hematoxylin and eosin (H&E) and Oil Red O staining of the liver of Mstn+/+Ldlr+/- and Mstn-/-Ldlr-/- mice after 12 weeks of HF-diet. b-c, mRNA expression of Srebf1 (b) and Fasn (c) in the liver of mice before and after 12 weeks of HF-diet. Values are expressed with respect to Mstn+/+Ldlr+/- controls. d, Protein expression of fatty acid synthase (Fasn). Averages were taken from 3 different gels. e, Plasma apoB100 at 0 and 180 minutes after injection of Triton WR1339, a lipoprotein lipase inhibitor. VLDL secretion is determined as percent increase of apoB100 from baseline. Averages were taken from 3 different gels. f, Akt serine-473 and GSKα/β serine-21/9 phosphorylation in the liver of mice after 12 weeks of HF-diet. The graphs demonstrate the quantification of phosphorylation of each molecule. +/+-- , Mstn+/+Ldlr+/-; --/--, Mstn-/-Ldlr-/- . AU, arbitrary units. Data are shown as the mean ± SEM (n=7-10). *P<0.05,**P<0.01.
Figure 2
Figure 3

(a) Basal
(b) HF-Diet

- FFA (mg/dL)
- TG (mg/dL)
- Cholesterol (mg/dL)

+++/-    ++/-    +/+++    ++/-

(c) Mstn<sup>+/+</sup>/Ldlr<sup>/--</sup>
Mstn<sup>/--</sup>/Ldlr<sup>/--</sup>

- Cholesterol (mg/dL)
- VLDL
- LDL
- HDL

(d) TG (mg/dL)

- VLDL
- LDL
- HDL

- Fraction

(e) Basal
HF-Diet

- apoB100
- apoA1
- albumin

+++/-    ++/-    +/+++    ++/-

- apoB100/apoA1

+++/-    ++/-    +/+++    ++/-
Figure 4
Figure 5