Transgenic expression of myostatin propeptide prevents diet-induced obesity and insulin resistance

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Abstract

Obesity and insulin resistance cause serious consequences to human health. To study effects of skeletal muscle growth on obesity prevention, we focused on a key gene of skeletal muscle named myostatin, which plays an inhibitory role in muscle growth and development. We generated transgenic mice through muscle-specific expression of the cDNA sequence (5′-region 886 nucleotides) encoding for the propeptide of myostatin. The transgene effectively depressed myostatin function. Transgenic mice showed dramatic growth and muscle mass by 9 weeks of age. Here we reported that individual major muscles of transgenic mice were 45–115% heavier than those of wild-type mice, maintained normal blood glucose, insulin sensitivity, and fat mass after a 2-month regimen with a high-fat diet (45% kcal fat). In contrast, high-fat diet induced wild-type mice with 170–214% more fat mass than transgenic mice and developed impaired glucose tolerance and insulin resistance. Insulin signaling, measured by Akt phosphorylation, was significantly elevated by 144% in transgenic mice over wild-type mice fed a high-fat diet. Interestingly, high-fat diet significantly increased adiponectin secretion while blood insulin, resistin, and leptin levels remained normal in the transgenic mice. The results suggest that disruption of myostatin function by its propeptide favours dietary fat utilization for muscle growth and maintenance. An increased secretion of adiponectin may promote energy partition toward skeletal muscles, suggesting that a beneficial interaction between muscle and adipose tissue play a role in preventing obesity and insulin resistance.
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Obesity and its associated metabolic problem, insulin resistance, cause serious consequences to human health. Type 2 diabetes mellitus, which are typically associated with adult obesity, are expected to affect children and adolescents as the prevalence of overweight children in the US increased significantly in the past decades [1–3]. Obesity is simply the consequence of imbalanced energy intake and expenditure of the body from a metabolic point of view. As the main sites of energy utilization, skeletal muscle and adipose tissue play significant roles in the regulation of energy homeostasis. Skeletal muscles are one of the major organs responsible for insulin-mediated glucose disposal, maintaining glucose homeostasis of the body. Adipose tissue serves as an energy storage depot to maintain lipid homeostasis, thereby promoting the survival ability of human body [4]. Obesity occurs when the adipose tissue is overloaded with high-energy nutrients without subsequent expenditure. Insulin resistance, a defect in the ability of insulin to drive glucose into its major target tissue-skeletal muscle, is very common in obesity and type 2 diabetes mellitus. A plentiful body of evidence has indicated that high-fat diet contributes to obesity and insulin resistance in animal models and humans [5–8]. Excessive dietary fat intake contributes to insulin resistance of the body through disrupting adipocyte endocrinal function and glucose uptake in skeletal muscle [9]. The mechanisms by which adiposity promotes insulin resistance have been linked to several adipocyte hormones and protein factors, including leptin,

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adiponectin, resistin, tumor necrosis factor-α, and peroxisome proliferator-activated receptors [10–12]. Insulin resistance often precedes the development of type 2 diabetes mellitus. Increasing skeletal muscle activity can greatly improve insulin sensitivity, consequently, prevent the progression from impaired glucose tolerance in most type 2 diabetic patients [13]. The purpose of this study was to investigate whether the body will be better equipped to handle dietary fat if the skeletal muscles are well developed in adolescence.

The formation of muscle fiber occurs during the late stage of fetal development. Postnatal muscles growth is most dramatic during adolescence to attain the full functional capacity of skeletal system, then the rate of growth rapidly decelerates in adulthood, concurrently, adipose tissue gradually accumulates more fat when energy intake exceeds expenditure in the body. A key muscle-regulatory factor, myostatin, is known to play an inhibitory role in controlling muscle mass. Loss of myostatin activity through mutations is identified in several muscling cattle breeds [14–16] and in humans [17]. Myostatin-null mice showed a twofold increase in individual muscle mass over the wild-type mice, as well as suppression of body fat accumulation [18,19]. Like most members of transforming growth factor-β (TGF-β) superfamily, the mature form of myostatin is generated by cleavage of the precursor protein at the tetrapeptide (RSRR) site. The remaining N-terminal peptide is named propeptide (previously called prodomain). We and others have demonstrated that transgenic expression of the propeptide cDNA sequence successfully depresses myostatin function and promoted muscularity phenotype [20–22]. In our transgenic mice, generated by muscle-specific expression of the propeptide (the 5′-region 886 nucleotides) of myostatin, we observed significant muscling phenotypes: 20% faster growth rate and 44% more muscle mass than wild-type mice [21]. In comparison with myostatin-knockout mice, the propeptide transgenic mice still produce myostatin in the muscle and maintain normal adipose tissues. To determine whether enhanced muscle growth minimizes the incidence of diet-induced obesity, we studied the response of the propeptide transgenic mice to a high-fat diet.

Materials and methods

Animals. Myostatin propeptide-transgenic mice were generated by standard microinjection techniques using B6SJL F1 (Taconic, Germantown, NY) females as zygote donors using the transgene MLC-pro construct [21]. Transgenic and wild-type littermate mice ($n = 80$) were obtained from the offspring of propeptide transgenic mice mating with B6SJL F1. Animals were housed in cages with a constant temperature (22 °C) and 12-h light/dark cycle, and were weaned at 4 weeks of age, and given free access to a normal fat diet (10% kcal fat, D12450, metabolizable energy 3.85 kcal/g, Research Diets, New Brunswick, NJ) until 9 weeks of age. Based on the genotypes (transgenic and wild-type), male mice were randomly assigned to two types of diet: normal (low) fat (10% kcal fat, D12450) and high-fat diet (45% kcal fat, D12451,

metabolizable energy 4.73 kcal/g, Research Diets) from 9 to 18 weeks. These purified diets were formulated as a modification of two publications [22,23]. The 45% kcal high-fat diet contains more lard and maltodextrin, and less corn starch compared to normal fat diet. Diet compositions are available on the website of http://www.researchdiets.com.

The ages and the period of dietary treatment were selected to simulate human muscle growth. Nine weeks of age in mice represent adolescence, and 16–18 weeks of age in mice are equivalent to human adulthood. During the dietary treatment, mice were given free access to diet and water. Animals and food were weighed weekly. Energy intake was calculated individually by feed consumption multiplied by metabolizable energy content of the diet, expressed as kcal/day. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

Muscle fiber characterization. The left hind limbs from transgenic and wild-type mice ($n = 4$–6 per genotype) at 18 weeks of age were used for determination of muscle fiber type and size as described [21] using a combination of succinic dehydrogenase and acid myofibrillar ATPase staining procedure.

Glucose tolerance and insulin sensitivity tests. Both tests were done on 8–12 mice per treatment group at 16 weeks of age after an overnight fast, and they were separated by at least 1 week. For glucose tolerance test, mice received an intraperitoneal injection of 10% dextrose (1 g/kg body weight). Insulin sensitivity test were done by intraperitoneal injection of human insulin (Lily, Indianapolis, IN) at a dose of 0.75 U/kg body weight. Two blood drops from tail were taken at 0, 30, 60, and 120 min after dextrose/insulin injection for measuring blood glucose by Accu-Check glucose monitor (Roche Diagnostic, Indianapolis, IN) in duplicate. Glucose tolerance was expressed by actual blood glucose value and insulin sensitivity was expressed as the percentage of the initial (time 0) blood glucose value.

Western blotting. Mice ($n = 6$–8 per genotype group) fed with high-fat diet) at 18 weeks of age were used for detection of Akt protein and phosphorylation. Gastrocnemius muscle samples were collected 8 min after intraperitoneal injection of insulin (10 U/kg body weight). The tissue samples were homogenized in buffer [100 mM Tris, pH 7.4, 250 mM sucrose/protease inhibitor mixture (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg leupeptin, and 10 μg of aprotinin/ml, 1 μM microcystin, 1 mM PMSF, and 10 mM sodium fluoride)] and then centrifuged at 15,000g for 15 min at 4 °C [24]. Western blotting was performed by using a chemiluminescent method with antibodies against AKT and phosphor-AKT Ser[473] (Cell Signaling Technology, Beverly, MA). The relative density of the protein bands was quantified by using Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Muscle and fat pad weights. Mice were killed at 18 weeks of age after an overnight fast. Carcass, front and hind limb were determined as described [21]. Individual muscles shown in Table 1 were dissected and weighed. Three different fat pads were dissected and weighed, including subcutaneous, epididymal, and retroperitoneal adipose tissue fat pad.

Serum measurements. Mice ($n = 9$–15 per treatment group) were fasted overnight for 8 h at 18 weeks of age before blood sampling. For measurement of insulin, leptin, resistin, and adiponectin, serum samples were prepared from whole blood collected by heart puncture using a syringe after mice were anesthetized with carbon dioxide [24]. Serum insulin, leptin, resistin, and adiponection were determined by using mouse ELISA kits with respective mouse counterpart (Crystal Chem, Downers Grove, IL) as a standard.

Statistical analysis. The data for mean comparisons were analyzed by subjecting to general linear model of SAS (SAS Inst., Cary, NC). In the studies of growth performance, body weights at all time points between transgenic and wild-type mice were analyzed by two-tailed Student's t tests. In the study of effects of the transgene on carcass, muscle and fat pad mass, significant differences between genotypes or dietary treatment plus genotypes were determined by a two-way ANOVA. No significant interaction between genotype and dietary treatment was present ($P > 0.05$). Differences within each set of means from glucose tolerance and insulin sensitivity tests were compared by

Table 1
Muscle mass of transgenic and wild-type mice fed a high-fat diet[*]

Genotype:	Weights (g)		% increase over wild-type
	Wild-type ($n = 12$)	Transgenic ($n = 10$)	
Carcass	12.63 ± 0.39	15.96 ± 0.28	26.4
Hind limb	1.80 ± 0.04	2.70 ± 0.10	50.0
Fore limb	0.90 ± 0.03	1.30 ± 0.03	44.4
Gastrocnemius/plantaris	0.17 ± 0.01	0.28 ± 0.01	64.7
Biceps femoris	0.22 ± 0.02	0.32 ± 0.01	45.5
Semitendinosus	0.20 ± 0.03	0.43 ± 0.02	115.0
Triceps brachii	0.11 ± 0.01	0.19 ± 0.01	72.7
Longissimus dorsi	0.22 ± 0.02	0.35 ± 0.02	59.1
Tibialias cranialis	0.08 ± 0.01	0.14 ± 0.01	75

[*] Significant difference between transgenic mice and wild-type littermates at $P < 0.0001$.

Student's t test, and least square means and their standard errors are reported. Significance was determined at $P < 0.05$.

Results

Muscle and adipose tissue mass of myostatin propeptide transgenic mice fed a high-fat diet

Both the propeptide transgene and endogenous myostatin mRNAs were detected in the muscle of transgenic mice (Fig. 1). Prior to the high-fat diet experiment, the transgenic mice grew significantly faster than their wild-type littermate mice when both groups were fed a normal diet: the body weights of transgenic and wild-type mice were significantly different at 9 weeks of age (30.87 ± 0.45 g vs 25.61 ± 0.29 g, $P < 0.001$). At 18 weeks of age, transgenic mice had increased muscle mass while wild-type littermates fed a high-fat diet developed adiposity with excessive adipose tissue pad mass (Fig. 2A).

The individual major muscles of transgenic mice were 45–115% heavier than those of wild-type mice (Table 1). The increases in skeletal muscle mass were widespread across major muscle groups that are primarily composed of fast-twitch muscle fibers. Muscle fibers appear normal in histochemical staining. Consistent with observations the promoter specifically directs transgene expression in type 2 (fast-twitch) muscle fibers [25,26], the size of fast-
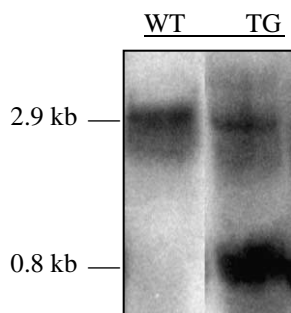

Fig. 1. Northern blotting of skeletal muscle. Myostatin mRNA was detected at ~2.9 kb in TG and WT mice. The propeptide transgene mRNA was detected at ~0.8 kb only in TG mice.

twitch muscle fiber was significantly increased in transgenic mice compared to the wild-type mice (Fig. 2B). The histology results from mice fed high-fat diet are similar to our previous data with chow-fed mice [21]. The intramuscular fat from transgenic mice fed a high-fat diet appears normal. Muscle triglyceride content of the gastrocnemius muscle from transgenic mice fed either normal or high-fat diet was significantly lower than that from wild-type mice ($P = 0.014$). High-fat diet induced 170–214% more fat mass in subcutaneous, epididymal, and retroperitoneal fat pads in wild-type mice than in transgenic mice (Fig. 2C). Transgenic mice showed similar levels of fat deposition when they were fed a normal or a high-fat diet ($P > 0.05$). Consistent with the increased muscle mass, transgenic mice had significantly higher daily energy intake than wild-type mice feed either normal or high-fat diet ($P < 0.05$, data not shown).

Responses of insulin action of the transgenic mice to high-fat diet

We also measured the whole-body sensitivities to glucose and insulin after 8 weeks of dietary treatment. The fasting blood glucose levels were similar in transgenic mice fed a high-fat diet (116.3 ± 3.92 mg/dl) or a normal diet (113.3 ± 4.91 mg/dl), and did not differ significantly from wild-type mice fed a normal diet (108.4 ± 2.05 mg/dl, $P > 0.05$). The three groups of mice all showed a normal response in the glucose tolerance test. Their blood glucose concentrations returned to initial levels 2 h after glucose injection. However, wild-type mice fed a high-fat diet had significantly higher fasting blood glucose levels (134.2 ± 4.82 mg/dl) and grossly abnormal responses to glucose injection (Fig. 3A). At 2 h after glucose injection, the mean of blood glucose remained at 176.50 mg/dl, which was 32% higher than the initial value. Similar to the results of the glucose tolerance test, transgenic mice fed either a normal or a high-fat diet showed normal insulin sensitivity as measured by the relative glucose reductions in response to an insulin challenge. The blood glucose in transgenic mice at 90 min after insulin injection was on average
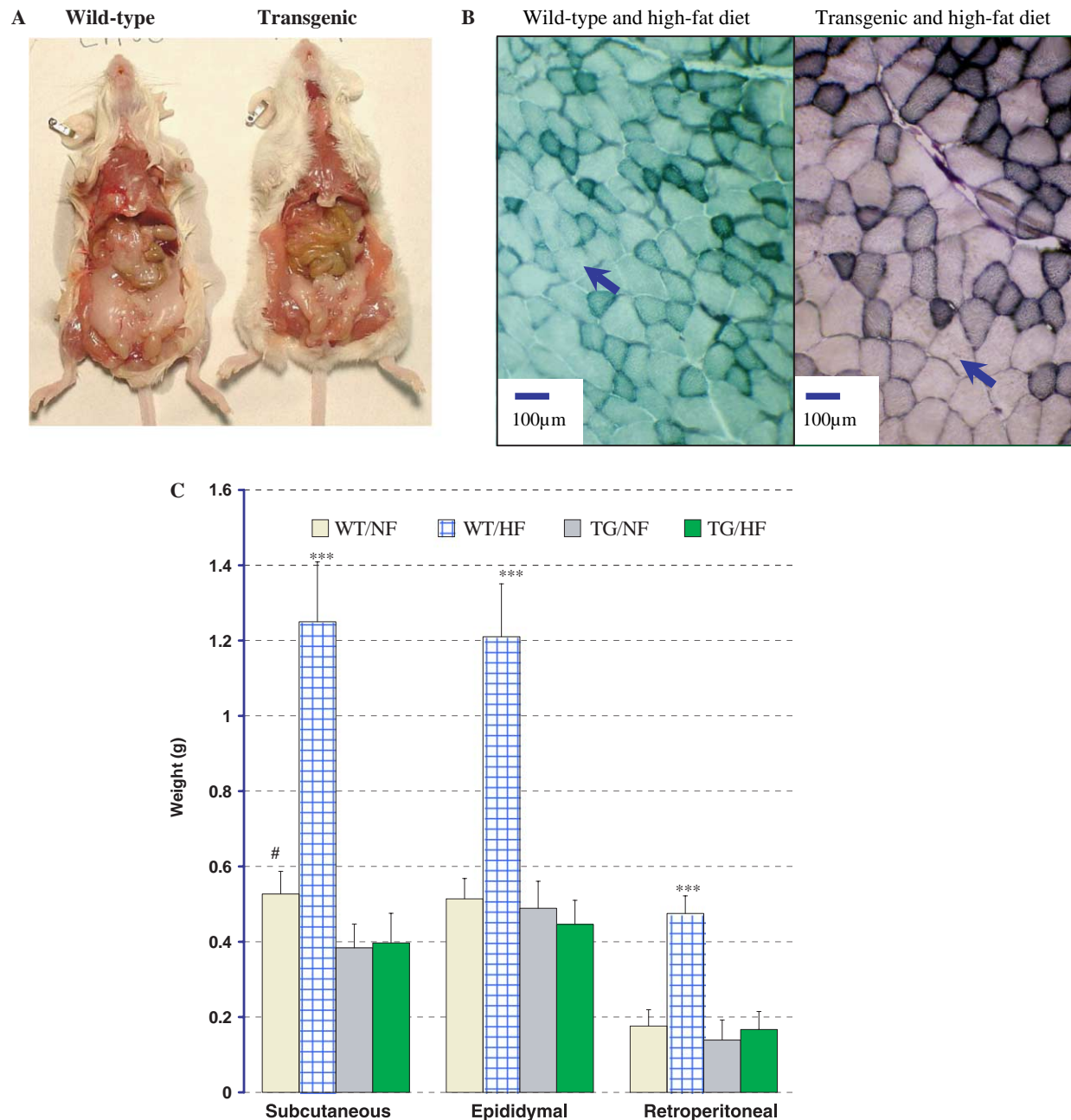
Fig. 2. (A) Gross carcass appearance of 18-week-old transgenic and wild-type littermate mice fed a high-fat diet. (B) Histochemical staining of gastrocnemius/plantaris muscle. The type 2 muscle fiber is pointed by an arrow. (C) Adipose tissue fat pad mass of transgenic (TG) and wild-type (WT) mice on normal (NF) and high-fat (HF) diets ($^{\#}P < 0.01$; $^{***}P < 0.001$).

approximately 40% of the initial level; this result was not significantly different from that observed in wild-type mice fed a normal diet ($P > 0.05$, Fig. 3B). However, high-fat diet induced the wild-type mice developed insulin resistance, the mean of blood glucose at 90 min after insulin injection was 64% of the initial level, much higher than for the other three groups of mice ($P < 0.001$).

To elucidate the mechanism by which over-expression of myostatin propeptide enhances insulin sensitivity in animals fed a high-fat diet, we examined muscle protein kinase Akt, an obligate intermediate in the insulin signaling pathway. Akt links insulin receptor-mediated activation of phosphatidylinositol (PI) 3-kinase to the regulation of glycogen synthesis and glucose transport [27]. Insulin resistance is associated with impaired Akt phosphorylation [28,29]. Muscle tissue samples were obtained from transgenic and wild-type mice fed a high-fat diet. As shown in Fig. 3C, the propeptide transgene significantly increased insulin-induced phosphorylation of Akt at Ser[473]. The phosphor-Akt (Ser[473]) levels in skeletal muscle were 144% higher in the transgenic mice than in the wild-type mice.
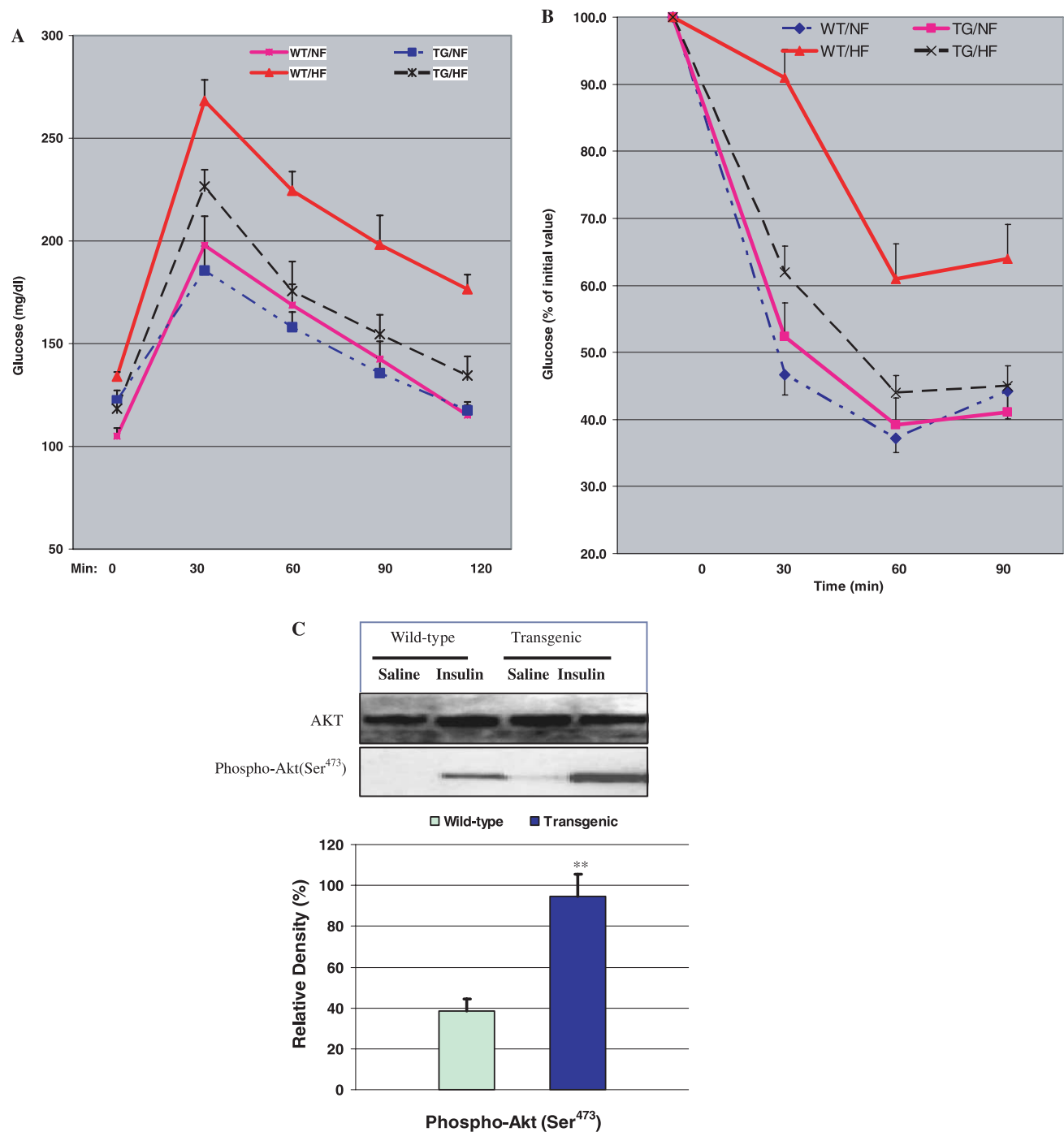
Fig. 3. (A) Glucose-tolerance tests for mice at 16 weeks of age. Mouse tail vein blood glucose was assayed at 0, 30, 60, 90, and 120 min after dextrose injection. $N = 8$–12 mice per treatment group. (B) Insulin-sensitivity test. Data represent percent of blood glucose level at time 0; $n = 8$–12 mice per group. (C) Effect of transgenic expression on Akt protein (AKT) and phosphorylation in gastrocnemius muscle. Mice at of 18 weeks of age were given an injection of saline or insulin. The blots from Western blotting are representative of three independent experiments. The relative density values of phospho-Akt (Ser[473]) to the total Akt protein are means of five to nine mice for each genotype from the three experiments, and the asterisks indicate a significant genotype difference ($P < 0.01$).

Effects of the propeptide transgene on metabolic hormones

To investigate the hormonal changes caused by high-fat diet and the propeptide transgene, we measured insulin and several adipocyte hormones. As shown in Table 2, the concentrations of insulin, leptin, and resistin were similar in transgenic mice fed either a normal or a high-fat diet; these levels were slightly higher but not significantly different from those seen in wild-type mice fed a normal diet. However, the concentrations of insulin, leptin, and resistin were much higher in the wild-type mice fed a high-fat diet than in all other groups. High-fat diet increased serum insulin,

Table 2
Effects of transgene and diets on blood glucose and serum hormones[A]

Genotype:	Wild-type		Transgenic	
Diet treatment:	Normal fat	High-fat	Normal fat	High-fat
Fasting glucose (mg/dl)	108 .4 ± 2.1[a]	134.2 ± 4.8[b]	113.3 ± 4.9[a]	116.3 ± 3.9[a]
Insulin (ng/ml)	0.410 ± 0.052[a]	1.179 ± 0.154[b]	0.482 ± 0.082[a]	0.598 ± 0.116[a]
Leptin (ng/ml)	2.79 ± 0.364[a]	14.22 ± 2.42[b]	2.35 ± 0.932[a,c]	2.981 ± 0.403[a]
Resistin (ng/ml)	4.60 ± 0.395[a]	6.27 ± 0.509[b]	4.39 ± 0.627[a]	4.77 ± 0.465[a]
Adiponectin (ng/ml)	7.90 ± 0.778[a]	7.522 ± 0.627[a]	4.35 ± 0.602[b]	9.44 ± 0.787[c]

Mean ± SEM with different superscript letters within a row differ ($P < 0.01$).
[A] Transgenic and wild-type littermate mice were randomly assigned for diet treatments with each group of 9–15 mice.

leptin, and resistin by 97%, 377%, and 32%, respectively, in wild-type mice compared to transgenic mice. In contrast, the effects of high-fat diet on the adipocyte hormones of transgenic mice were minimal. Interestingly, high-fat diet induced much higher levels of serum adiponectin concentration in the transgenic mice than that found in the wild-type mice (9.44 ± 0.79 ng/ml vs. 7.90 ± 0.68 ng/ml, $P = 0.008$) while transgenic mice fed a normal diet had significantly lower serum adiponectin levels (4.35 ± 0.602).

Discussion

Myostatin is highly conserved among invertebrate species. The amino acid sequence of human myostatin is identical to that of the mouse myostatin [14]. Studies have demonstrated that myostatin is specifically expressed in skeletal muscle throughout all stages of muscle fiber formation, development, growth, and degeneration [18,31–34]. The muscling phenotypes of myostatin-deficient mice results from both increased number and size of fibers [18]. We showed that the enhanced muscle mass by disruption of myostatin function through its propeptide was caused by increased muscle fiber size [21], suggesting the effects of the MLC promoter-driven transgene predominantly on postnatal muscle growth. Here we showed that the propeptide transgene not only enhanced muscle growth, but also prevented dietary fat-induced obesity and insulin resistance. While wild-type mice on a high-fat diet for 2 months developed serious adiposity, impaired glucose tolerance, and insulin resistance, the transgenic mice are normal and healthy, accommodating a metabolic regulatory system that utilizes dietary fat for muscle growth and maintenance. An immediate indication from these findings is that muscle development and buildup play a fundamental role in maintaining a balanced utilization and expenditure of energy resource. The results support our hypothesis that well-developed skeletal muscles in early stage of life increase the flexibility of the body in utilizing dietary fat or other energy resource, so that the body would be better equipped to prevent obesity and its associated metabolic disorders in adulthood or later stages of life.

The mechanism by which myostatin function is disrupted by its propeptide is not yet well defined. A large, latent form of myostatin complex was detected in the muscle of transgenic mice (data not shown), raising the possibility that the activity of myostatin, like that of TGF-β1, is regulated primarily by dissociation of its latent complex [35]. The molar ratio between propeptide and mature myostatin in serum is approximately 1:1 [36], and no data are available about the molar ratio in muscle. As the propeptide and mature myostatin are generated from the same precursor peptide, we assumed their molar ration is close to 1:1 in muscle tissue. A transgenic over-expression of the propeptide may promote forming of a more myostatin–latent complex, therefore resulting in less biologically active mature myostatin in muscle or even in circulation. Studies have indicated that myostatin inhibits adipocyte differentiation in vitro [37], and its mRNA was detected in the adipose tissue, which may suggest that myostatin has a direct effect on adipose tissue [37]. Myostatin-deficiency in mice reduces fat accumulation with increasing age on a regular rodent chow [19]. Individual fat pad of wild-type mice weighed on average 2.4–4.4 times those of myostatin-deficient mice at 5–6 months of age and increased up to a much higher fold by 7–9 months. This was thought to be primarily due to the myostatin-deficient mice remaining extremely lean with relatively little fat pad gain over the period of 7- to 8-month experiment. The authors speculated both direct and indirect effects of myostatin mutation on adipose tissue to suppress fat accumulation [19]. In the propeptide transgenic mice, transgene mRNA was not detected in the adipose tissue, excluding its possible direct effects on adipose tissue. We believe that enhanced muscling induced by the transgene during the early growing stage significantly changed nutrient partitions in the body. Energy demand for muscle growth and maintenance in the transgenic mice was so prominent that less energy was available for fat accumulation consequently, they maintained normal adipose tissue and a high insulin sensitivity.

High-fat diet induced wild-type mice developed adiposity and insulin resistance, but transgenic mice did not develop such metabolic problems on a high-fat diet. The data of serum hormones are in concert with the hypothesis that the body developed a metabolic regulatory system that utilizes triglyceride for muscle growth and maintenance. As we expected, the propeptide transgenic mice had dramatic muscling, as well as normal adipose tissue because they maintained a normal serum concentration of insulin, leptin, and

resistin on the high-fat diet compared with wild-type mice fed a normal diet. Deficiencies of leptin and its receptors in mice have well been associated with obesity and metabolic syndrome [38–40]. Resistin is an adipocyte hormone that potentially links obesity to diabetes, and dietary fat increase circulating resistin level and may cause insulin resistance of skeletal muscle [41]. The findings from wild-type mice were consistent with earlier observations that obesity leads to elevated levels of insulin, leptin, and resistin [30]. Increased levels of serum leptin and resistin in the wild-type mice fed a high-fat diet underline their metabolic problems associated with obesity and insulin resistance.

The result of serum adiponectin, a significant increase in transgenic mice fed a high-fat diet, shed a light on the mechanism of improved triglyceride utilization by skeletal muscle. Adiponectin secretion is lower in obese states than in normal body weight and improves insulin sensitivity [42,43]. Studies also showed that adiponectin can directly increase fatty-acid transport, oxidation, and dissipation in skeletal muscle, thus reducing the levels of intramyocellular lipids and improving insulin sensitivity in muscle cells and hepatocytes [44]. Adiponectin secretion from adipose tissue is increased by activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR-γ) and reduced by caloric excess [45]. In the transgenic mice, energy supplies to adipose tissue may become limited as a result of increased energy demand from skeletal muscles. Therefore, we speculate that transgenic mice would increase the activity of fatty acid oxidation in the liver and skeletal muscle to support muscle growth, which is probably driven by an increased activity of PPAR-γ action in the skeletal muscles. However, circulating adiponectin concentrations are negatively correlated with blood triglyceride, and strongly correlated with plasma HDL concentrations [43,46], and a receptor mediating adiponectin activity in skeletal muscle had not been reported [47]. Further experiments are needed for further clarifications.

Nevertheless, our findings provide evidence that disruption of myostatin by its propeptide prevents high-fat diet-induced obesity and insulin resistance. Enhanced muscling during early growth stages can significantly mobilize energy partition in favor of dietary triglyceride utilization for muscle growth and maintenance, limiting adipose tissue fat deposition. The increased levels of adiponectin observed in transgenic mice fed a high-fat diet also suggest that a better understanding of metabolic interactions between muscle and adipose tissue has an important application in preventing obesity and insulin resistance.

Acknowledgments

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