Leptin gene transfer regulates fibromuscular development and lipid deposition in muscles via SIRT1, FOXO3a and PGC-1α in mice *in vivo*

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Abstract. Leptin gene transfer in the liver by hydrodynamic-based gene delivery instead of peptide administration was used to investigate the effects of leptin on muscle mass accretion and lipid accumulation in muscles of wild-type mice. Food intake ($P<0.01$), body weight ($P<0.01$) and white adipose tissue ($P<0.01$) were significantly reduced in the leptin gene-treated group compared with the control group. Moreover, plasma leptin concentration was significantly increased after administration of the mouse leptin gene at a dose of 15 μg per mouse for 1 day ($P<0.01$) or 1 week ($P<0.05$). Furthermore, the mRNA abundance of myosin heavy chain type I (MyHC-I), myosin heavy chain type II (MyHC-IIa, MyHC-IIx), adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and lipoprotein lipase (LPL) genes in gastrocnemius muscle and extensor digitorum longus after administration of leptin for 1 week were significantly increased compared with the control group. Finally, we investigated the mechanism by which leptin gene transfer affects fibromuscular and fat deposition in muscle. Gene expression and protein levels of SIRT1, and proliferator-activated receptor-γ coactivator-1α (PGC-1α) were remarkably increased in extensor digitorum longus. On the other hand, PGC-1α and FOXO3a gene expression was observed to have significantly increased in gastrocnemius muscle. However, only changes in the protein levels of PGC-1α were observed ($P<0.05$). These results suggest that leptin may affect the growth and development of muscle, and fat deposition in wild-type mice via SIRT1 and FOXO3a and their downstream targets, including PGC-1α.

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Introduction

Increased fat deposition is one of the important issues that affect carcass quality in farmed animals and health in humans. Significant efforts are being made by pig husbandries to lower lipid deposition and increase the lean proportion of pigs. Regulation of energy metabolism has thus become a topic of great interest and extensive research in animal science. Nutrition and genetic factors are the major determinants of energy metabolism in pigs (1). In a previous study, we used microarray analysis to compare longissimus dorsi muscle gene expression profiles between Landrace and Jinhua swine, which is a Chinese breed known for good meat quality that is bright red, tender, juicy and rich in muscle fat (2). The result of microarray experiments showed that muscle fiber development and intramuscular lipid deposition was associated with adipokine genes such as leptin and adiponectin. Leptin is one of the genes we found to be upregulated in our microarray screen. Here, we used leptin gene transfer mice as a biomodel to better understand the relationship between adipokine genes and muscle development and lipid deposition.

Adipose tissue is not only an energy store but also an active endocrine organ that produces biologically active substances termed adipocytokines (3). These adipocytokines include leptin, adiponectin, resistin, apelin or visfatin (3,4). Leptin is a 16 kDa adipocyte-derived cytokine, encoded by the ob gene (5). The primary sites of leptin expression are adipocytes, although it is also expressed in gastric wall, vascular cells, placenta, ovary, liver and skeletal muscle (6-8). Leptin reduces body mass by acting on receptors in the hypothalamus (9,10) to decrease food intake and to increase sympathetic nervous system activity (3). During energy metabolism, leptin is the main factor taking part in regulation of lipid deposition and muscle mass accretion in skeletal muscle (11,12). The effect of leptin on fatty acid oxidation is due to a direct, rapid and transient stimulation of AMP-activated protein kinase

(AMPK) in skeletal muscle (13). The greatest effect of leptin is in oxidative fibres (14). Leptin (10 mg/ml) has been shown to increase free fatty acid (FFA) oxidation in isolated mouse soleus muscle by 42% (12). Also, leptin-induced increase in fatty acid catabolism have been demonstrated to be mediated by decreased skeletal muscle lipid deposition (15). Short-term leptin treatment (10 mg/kg/day for 10 days) in high-fat fed rats alters muscle fatty acid metabolism by increasing fatty acid uptake and oxidation compared with pair feeding alone (16). Leptin has been shown to increase glucose uptake in skeletal muscle tissues *in vivo* (17,18) and in a myotube cell line *in vitro* (19). However, it is not known whether leptin gene transfer has the same effects and mechanism of regulation. In a previous study, Xiang *et al* (20,21) reported successful leptin gene transfer by hydrodynamic-based gene delivery and investigated the effect of leptin on food intake and body weight. In this study, we focus on the effects of leptin gene transfer and the underlying mechanism on lipid deposition and muscle development.

Materials and methods

Experimental animals. Male ICR strain mice (n=20) at 5 weeks of age were used as experimental animals. Animals were maintained in a clean room at 23±1˚C with a 12:12 light-dark cycle and were fed a commercial diet (SLRC, Shanghai, China) *ad libitum*, and had free access to water during the experimental period. During the experimental period, the mice were cared for under the Guidelines of Animal Experimentation outlined by the Committee of Experimental Animal Care, Zhejiang University.

pVRmob construction. The construction of pVRmob was performed as previously described (20).

Hydrodynamics-based gene delivery. DNA of 15 µg (pVR empty vector, pVR leptin expression vector) and 20 µg GFP expression vector were injected into the tail vein as described in a previous study (20,21). After hydrodynamics-based gene delivery, the mice were maintained during the subsequent week to record food intake and changes in body weight. At designated time points during the experiment, blood was taken for analysis of the plasma leptin concentration. At the end of the experiment, treated mice were sacrificed by neck dislocation and liver, epididymal white fat and interscapular brown fat, *gastrocnemius* and extensor digitorum longus were removed quickly and weighed.

Enzyme-linked immunosorbent assay (ELISA). Plasma samples at 1 or 7 days after gene transfer by hydrodynamics-based gene delivery were obtained by centrifuging the blood at 12,000 rpm at room temperature for 15 min and stored at -20˚C until analysis. The plasma samples were assayed for leptin concentration using a commercially available mouse ELISA kit (Xitang, Shanghai, China) according to the manufacturer's protocol.

Real-time PCR. Approximately 200 mg each of gastrocnemius and extensor digitorum longus were obtained. Total-RNA was isolated using the TRIzol reagent (Invitrogen, CA, USA) and

RNA concentration was quantified by the NanoDrop ND-1000 spectrophotometer. For the first strand cDNA synthesis, constant amounts of 2.5 µg of total-RNA isolated from muscle were reverse transcribed using Oligo(dt)20 (Invitrogen) as primers and 10,000 units of M-MuLV reverse transcriptase (Fermentas, Shenzhen, China). Real-time PCR was performed using the LightCycler1.5 (MasterCycler EP gradient realplex4 Eppendorf, Germany) and the SYBR Premix Ex Taq™ (Takara, Otsu, Japan). The mouse SIRT1, proliferator-activated receptor-γ coactivator-1α (PGC-1α), FOXO3a, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), LPL, myosin heavy chain type I (MyHC-I), myosin heavy chain type II (MyHC-IIa), MyHC-IIb, MyHC-IIx and 18S primers used for the PCR were as follows: for SIRT1: sense, 5'-CAGACCCTCAAGCCATGTTT-3' and antisense, 5'-ACAC AGAGACGGCTGGAACT-3'; for PGC-1α: sense, 5'-CCGA GAATTCATGGACAAT-3' and antisense, 5'-GTGTGAG GAGGGTCATCGTT-3'; for FOXO3a: sense, 5'-ATGGGAG CTTGGAATGTGAC-3' and antisense, 5'-CCACATTCAA ACCAACAACG-3'; for ATGL: sense, 5'-AACACCAGCAT CCAGTTCAA-3' and antisense, 5'-GGTTCAGTAGGCCAT TCCTC-3'; for HSL: sense, 5'-TGAGATGGTAACTGTGA GCC-3' and antisense, 5'-ACTGAGATTGAGGTGCTGTC-3'; for LPL: sense, 5'-CTGGGCTATGAGATCAACAAGGT-3' and antisense, 5'-AGGGCATCTGAGAGCGAGTCT-3'; for MyHC-I: sense, 5'-ATAGGGGACCGTAGCAAGAAG-3' and antisense: 5'-TCCTCTCAGCCTTTAGCTGGA-3'; for MyHC-IIa: sense 5'-CGATGATCTTGCCAGTAATG-3' and antisense: 5'-ATAACTGAGATACCAGCG-3'; for MyHC-IIx: sense, 5'-GGACCCACGGTCGAAGTTG-3' and antisense: 5'-GGCTGCGGGCTATTGGTT-3'; for MyHC-IIb: sense, 5'-CAATCAGGAACCTTCGGAACAC-3' and antisense: 5'-GT CCTGGCCTCTGAGAGCAT-3'; for 18S: sense, 5'-TAACC CGTTGAACCCCATT-3' and antisense, 5'-CCATCCAATCG GTAGTAGCG-3'. The cDNA was amplified at the following conditions: 95˚C for 2 min, followed by 45 cycles of 15 sec at 95˚C and 15 sec at 57.4˚C, using the Takara SYBR Premix Ex Taq™. All results were normalized to the levels of 18S rRNA and relative quantification was calculated using the $\Delta\Delta Ct$ formula. Relative mRNA expression was expressed as fold-expression over the calibrator sample (average of gene expression corresponding to the wild-type group). All samples were run in triplicate and the average values were calculated.

Western blot analysis. Muscle samples (200 mg) were homogenized in lysis buffer (500 mM Tris-HCl, 10% SDS, 50% glycerol, and 500 mM DTT, pH adjusted to 6.8) supplemented with protease inhibitors. Soluble proteins were recovered after centrifugation at 12,000 x g for 20 min at 4˚C. Subsequently, protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, USA) spectrophotometrically at 595 nm. The sample corresponding to 50 µg protein was removed and added to a new tube, and was incubated at 100˚C for 20 min. An 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel was run at 200 V for 45 min. The proteins were transferred to a PVDF membrane and were blocked with 5% non-fat dry milk in 0.05% TBS-Tween-20 for 1 h at room temperature. Blots were then incubated with anti-SIRT1 (Tianjin Saier Biotechnology, China), anti-PGC-1α (Abcam, Hong Kong, China), anti-Foxo3a (R&D Systems, Shanghai,
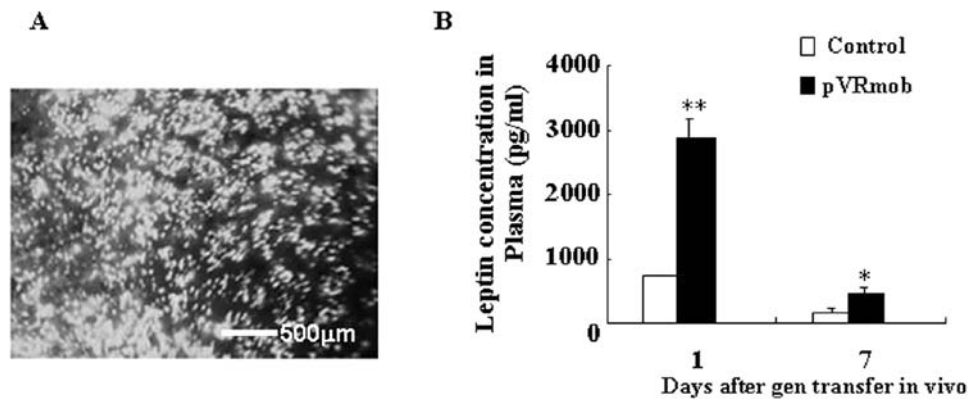
Figure 1. GFP gene expression in the liver (A) and the changes in plasma leptin concentration (B) after *in vivo* gene transfer by hydrodynamics-based gene delivery at a dose of 15 µg/animal. The GFP expression plasmid, pGFP-C1, was co-transferred at a dose of 20 µg/animal in combination with pVRmob. GFP-specific fluorescence signals were detected with a fluorescent microscope 1 day after hydrodynamics-based gene delivery. Scale is given by a horizontal bar. Vertical bars represent SEM of five mice. **Significantly different at P<0.01.

China) and β-actin (Abmart, Shanghai, China) antibodies in blocking buffer overnight at 4˚C. After washing with 0.5% TBS-Tween for five times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Beijing Cowin Biotechnology, Beijing, China) for 1 h at room temperature. The chemiluminescence ECL reagent (Enhanced Chemiluminescent System, Amersham Biosciences) was used to develop the bands, which were analyzed by density analysis using the ImageJ software.

Statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM). The significance of the differences between the groups in all of the experiments was determined by ANOVA, followed by two-tailed multiple t-tests with a Bonferroni correction using the biostatistics software SPSS 15.0. A P-value <0.05 was considered statistically significant.

Results

GFP and leptin gene transfer in the liver and their effects on plasma leptin concentration. GFP expression in liver after leptin gene transfer by hydrodynamics-based gene delivery is shown in Fig. 1A. Clear GFP-specific fluorescence signals were detected in hepatocytes. Leptin concentration in the plasma after *in vivo* leptin gene transfer by hydrodynamics-based gene delivery is given in Fig. 1B. At 2 days post-transfection, a remarkable increase of leptin concentration was detected in the pVRmob group compared with the control group, although the leptin concentration quickly decreased afterwards. Significant differences in plasma leptin were detected in the pVRmob group compared with the control counterpart at Day 2 (P<0.01) and 1 week (P<0.05). These results indicated that leptin gene transfer was successful and resulted in leptin production in the liver and subsequent secretion into the blood.

Effect of leptin gene transfer on food intake, body weight and adipose tissue. The changes in daily food intake and body weight after gene transfer by hydrodynamics-based gene delivery are displayed in Fig. 2A and B, respectively. A significant reduction in food intake was detected on Days 1, 5

and 7 after leptin gene transfer (P<0.01). The body weight of the leptin gene transfer group was significantly reduced and remained low after Day 4 (P<0.01). Weights of epididymal white fat and interscapular brown fat tended to be reduced at 1 week and the reduction in epididymal white fat weight reached statistical significance in the treated group at Day 7 compared with the control counterpart (P<0.01) (Fig. 2C). Thus, leptin produced in the transgenic mice had an effect on food intake, body weight and lipid accumulation in adipose tissue.

Effect of leptin gene transfer on the muscle fiber types of gastrocnemius muscle and extensor digitorum longus. The mRNA abundance of MyHC-I, MyHC-IIa, MyHC-IIb and MyHC-IIx in the gastrocnemius and extensor digitorum longus muscles as shown in Fig. 3. Leptin gene transfer led to a 118.75, 93.02 and 78.66% increase in the gastrocnemius muscle MyHC-I, MyHC-IIa and MyHC-IIx mRNA abundance, respectively (P<0.05, P<0.05 and P<0.01). However, MyHC-IIb mRNA abundance was reduced to 56.11% (P<0.01). In extensor digitorum longus, the mRNA abundance of MyHC-I, MyHC-IIa and MyHC-IIx were also higher by 140.05, 113.94 and 24.38%, respectively (P<0.05, P<0.05 and P<0.05), than those in the control counterparts. However, no significant change was observed in the extensor digitorum longus MyHC-IIb mRNA abundance. These results indicate that leptin may increase muscle accretion and red muscle types.

Effect of leptin gene transfer on gene expression related energy metabolism in gastrocnemius and extensor digitorum longus muscle. The expression of ATGL, HSL and LPL in gastrocnemius and extensor digitorum longus muscles at 1 week after leptin gene transfer are shown in Fig. 4. Leptin gene transfer caused a 114.79, 102.43 and 80% increase in gastrocnemius muscle ATGL, HSL and LPL mRNA abundance, respectively (P<0.05, P<0.01 and P<0.05). Similarly, leptin gene transfer had a positive effect on the mRNA abundance of ATGL, HSL and LPL in the extensor digitorum longus. The mRNA abundance of ATGL, HSL and LPL increased by 162.25, 124.69 and 110.13% respectively compared with the
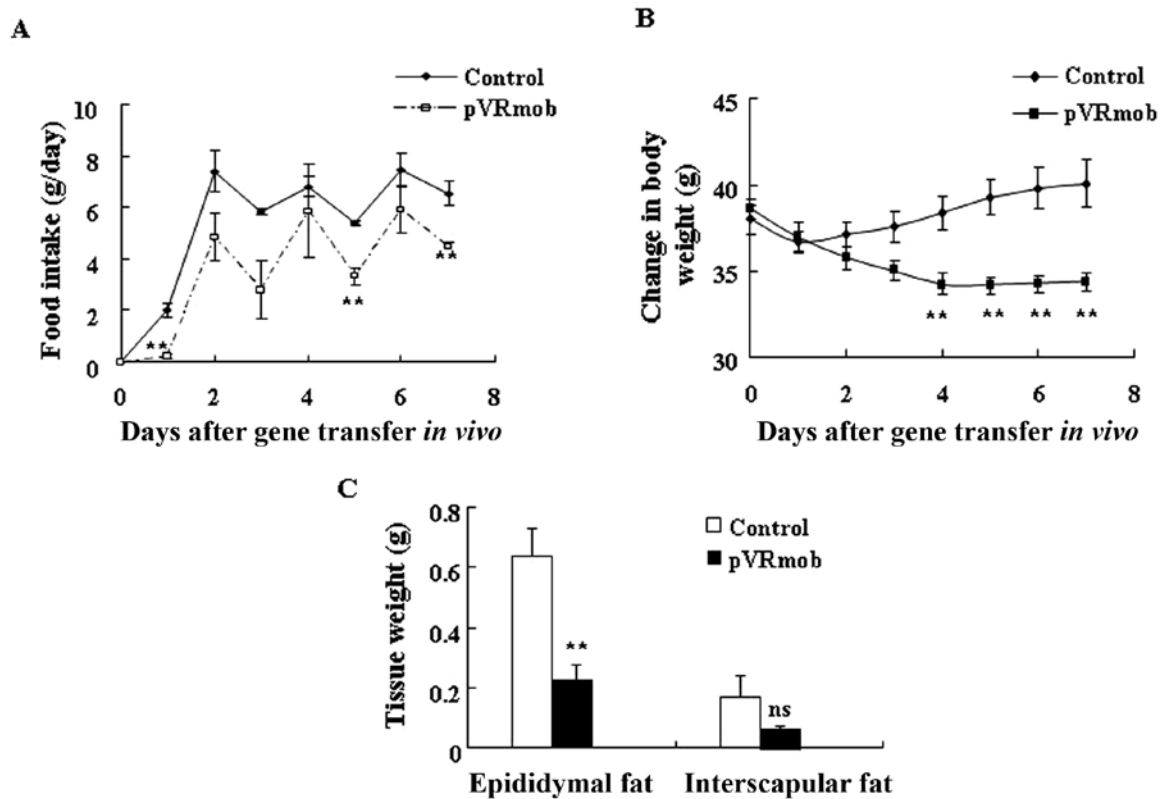
Figure 2. Changes in the food intake (A), body weight (B) and weight of epdidymal white fat and interscapular brown fat (C) of mice after transferring pVRmob gene *in vivo* by hydrodynamics-based gene delivery at a dose of 15 µg/animal. Each point represents the mean ± SEM of 5 mice. **Significantly different from the control at the same time point at P<0.01; ns, not significantly different (P>0.05).
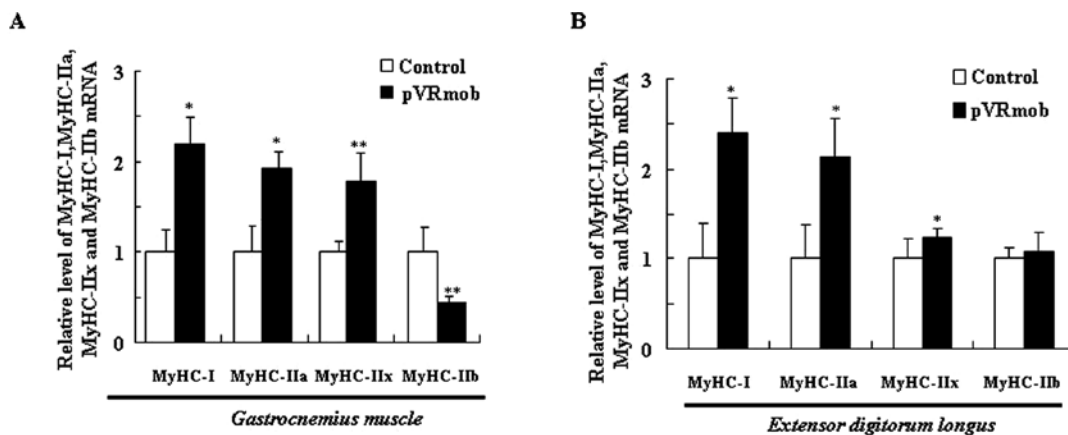

Figure 3. Changes in the expression of MyHC-I, MyHC-IIa, MyHC-IIb and MyHC-IIx genes in gastrocnemius muscle (A) and extensor digitorum longus (B) after transferring pVRmob gene for 1 week *in vivo* at a dose of 15 µg/animal. In real-time PCR analysis, amplification of 18S ribosomal RNA was used to normalize relative levels of MyHC-I, MyHC-IIa, MyHC-IIb and MyHC-IIx mRNA. Vertical bars represent the mean ± SEM of 5 mice. *P<0.05, ** P<0.01 significant different from control.

control group (P<0.05, P<0.05 and P<0.05). These results demonstrate that leptin gene transfer reduced lipid deposition in muscles via regulation of key genes such as ATGL, HSL and LPL that are related to energy metabolism.

Effect of leptin gene transfer on SIRT1, FOXO3a and PGC-1α gene expression and protein level in gastrocnemius muscle and extensor digitorum longus. The mRNA abundance and protein level of SIRT1, FOXO3a and PGC-1α in extensor digitorum and longus gastrocnemius muscles are presented

in Figs. 5 and 6, respectively. A significant increase in SIRT1 mRNA and protein level was observed in extensor digitorum longus (P<0.05, P<0.01), but not in gastrocnemius muscle. Interestingly, expression of FOXO3a was increased only in gastrocnemius muscle (P<0.05). PGC-1α mRNA abundance and protein level were both increased in gastrocnemius muscle and extensor digitorum longus (P<0.01, P<0.05). These results suggest that leptin gene transfer increases the gene expression of transcription factors such as SIRT1, FOXO3a and PGC-1α in extensor digitorum longus and gastrocnemius muscles.
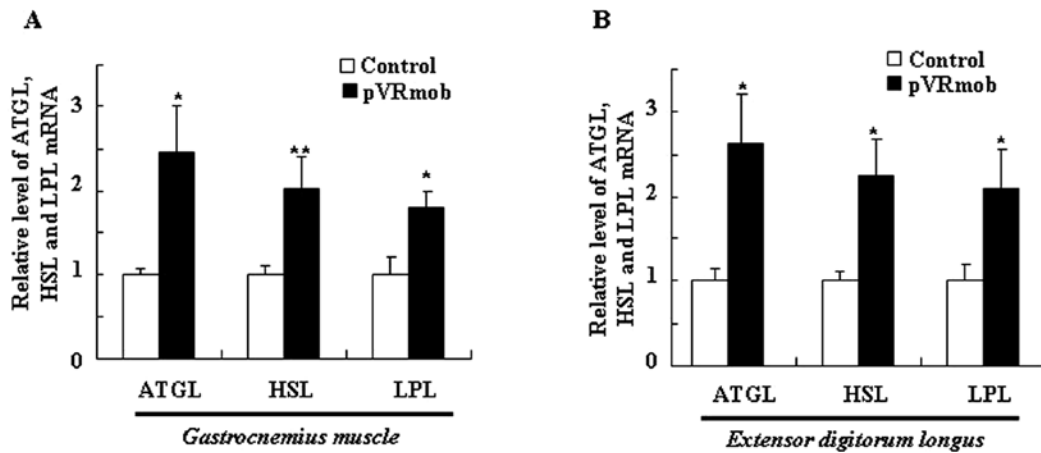
Figure 4. Changes in the gene expression of ATGL, HSL and LPL in gastrocnemius muscle (A) and extensor digitorum longus *muscle* (B) after transferring the pVRmob gene for 1 week *in vivo* at a dose of 15 µg/animal. In real-time PCR analysis, amplification of 18S ribosomal RNA was used to normalize the relative levels of ATGL, HSL and LPL mRNA. Vertical bars represent the mean ± SEM of 5 mice. *P<0.05, **P<0.01 indicate significant differences from the control.
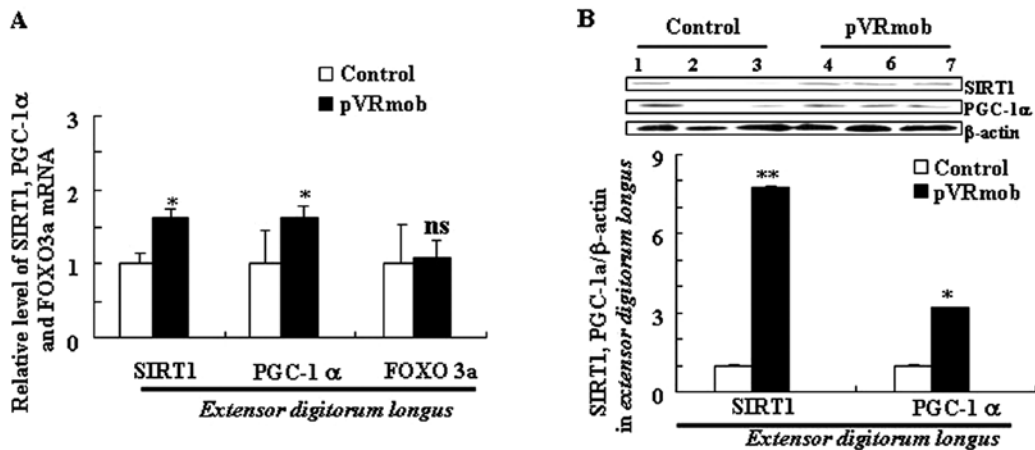

Figure 5. Changes in gene expression (A) and Western blot analysis (B) of SIRT1, PGC-1α and FOXO3a in extensor digitorum longus muscle after transferring the pVRmob gene for 1 week *in vivo* by hydrodynamics-based gene delivery at a dose of 15 µg/animal. In real-time PCR analysis, amplification of 18S ribosomal RNA was used to normalize the relative levels of SIRT1, PGC-1α and FOXO3a mRNA. Vertical bars represent the mean ± SEM of 5 mice. *P<0.05, **P<0.01 indicate significant differences from control. Western blot analysis was performed to examine the effects of leptin gene transfer on SIRT1, and PGC-1α protein levels in the extensor digitorum longus muscle; muscle samples were homogenized in lysis buffer (500 mM Tris-HCl, 10% SDS, 50% glycerol and 500 mM DTT, pH adjusted to 6.8) supplemented with protease inhibitors. β-actin protein level was used to normalize the relative levels of SIRT1 and PGC-1α protein. Vertical bars represent the mean ± SEM of 3 mice.
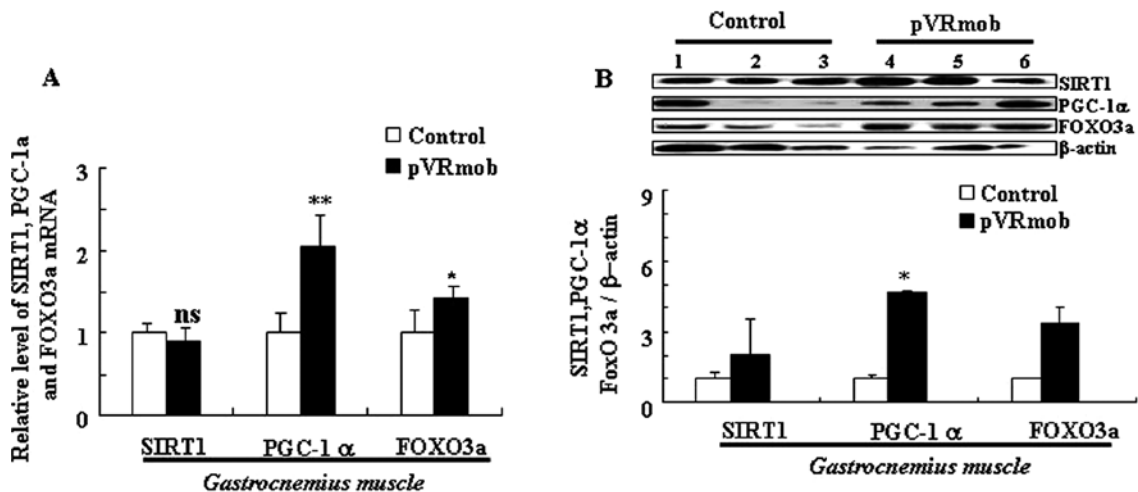

Figure 6. Changes in gene expression (A) and Western blotting analysis (B) of SIRT1, PGC-1α and FOXO3a in gastrocnemius muscle after transferring the pVRmob gene for 1 week *in vivo* by hydrodynamics-based gene delivery at a dose of 15 µg/animal. RT-PCR and Western blot analysis was performed as described in the Fig. 5 legend.

Discussion

In this study, we used leptin gene transfer mice as a biomodel to investigate the effects of leptin on lipid deposition, myofiber types in muscles and the underlying mechanism for those effects *in vivo*. We observed reduced food intake and decreased body weight in leptin gene transfer mice, which is in agreement with previous studies in which the effect of leptin administration in mice was investigated (20,22-25). These results validate the leptin gene transfer mice as a good biomodel to study the effect of leptin on mice physiology and energy metabolism *in vivo*. Moreover, the changes in plasma leptin concentration and body weight presented here indicate that a high dose of leptin by peripheral administration was required to lead to significant reductions in food intake and body weight in normal mice, and the potency of the weight-reducing effects of leptin was directly related to the relative rise in plasma leptin concentration after gene transfer *in vivo*. Furthermore, the significant reduction in epididymal white fat we observed after gene transfer by hydrodynamics based transfection implied that an important role of leptin produced in the liver might be to increase lipid catabolism through sympathetic neuron stimulation primarily in white, and possibly brown adipose tissue, as has been suggested previously (26). Taken together, our results show that hydrodynamics-based gene transfer can be an effective approach for systemic delivery of recombinant leptin to the central nervous system and peripheral tissue.

In a previous study, muscle mass accretion had been observed upon leptin administration in *ob/ob* mice (12). We hypothesized that leptin gene transfer in liver has similar effects on muscle mass growth in wild-type mice. The expression increases of MyHC-I, MyHC-IIa, MyHC-IIb in extensor digitorum longus and gastrocnemius muscle (Fig. 3) confirm our hypothesis and are consistent with previous findings (12,13).

ATGL, HSL and LPL are the key genes related to energy metabolism (27). ATGL and HSL take part in lipolysis and LPL is involved in fatty acid uptake. Therefore, the increase in gene expression of ATGL, HSL and LPL in gastrocnemius and extensor digitorum longus muscles (Fig. 4) suggest that leptin produced in the liver can directly target muscle tissues to reduce lipid deposition via increase in ATGL, HSL and LPL expression.

SIRT1 and FOXO3a play important roles in metabolic function and longevity in mammals (28,29). SIRT1, an NAD^+-dependent deacetylase, has been shown to have an important role in determining the state of FOXO acetylation and inducing nuclear localization of FOXO in response to oxidative stress. Activation of SIRT1 enhances mitochondrial oxidative function to regulate energy balance. Both AMPK and SIRT1 act in concert with the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), to regulate energy homeostasis in response to environmental and nutritional stimuli (30,31). PGC-1α interacts with multiple transcription factors to stimulate mitochondrial metabolic capacity (32). Recently, it has been reported that PGC-1α also constitutes an important mediator of muscle mass by inducing a down-regulating of the expression levels and activity of FOXO3a, hence, inhibiting muscle atrophy

(32). For these reasons, we decided to analyze the expression of SIRT1, FOXO3a and PGC-1α. We observed a increase in SIRT1, FOXO3a and PGC-1α expression at both the mRNA and protein levels (Figs. 5 and 6), suggesting that leptin regulates energy metabolism and fibromuscular development via SIRT1, FOXO3a and PGC-1α in gastrocnemius and extensor digitorum longus muscles.

Taken together, our results reveal that leptin can regulate fibromuscular development and lipid deposition *in vivo* in mice via activation of SIRT1, FOXO3a and their downstream targets, including PGC-1α, in skeletal muscle.

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