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Muscle atrophy in transgenic mice expressing a human TSC1 transgene

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Abstract **Muscle mass is regulated by a wide range of hormonal and nutritional signals, such as insulin and IGF. Tuberous sclerosis complex (TSC) is an inherited hamartoma disease with tumor growth in numerous organs. TSC is caused by mutation in either TSC1 or TSC2 tumor suppressor genes that negatively regulate insulin-induced S6K activation and cell growth. Here we report that expression of human TSC1 (hTSC1) in mouse skeletal muscle leads to reduction of muscle mass. Expression of hTSC1 stabilizes endogenous TSC2 and leads to inhibition of the mTOR signaling. The hTSC1-mTSC2 hetero-complex and its downstream components remain sensitive to insulin stimulation and nutrition signals. This study suggests that an increase in the steady state level of resident TSC1–TSC2 complex is sufficient to reduce muscle mass and cause atrophy.**

Keywords: Muscular atrophy; Tuberous sclerosis 1; Mouse; Transgenesis

1. Introduction

Skeletal muscle atrophy accompanies various diseases, such as cancer, diabetes and AIDS [1]. Muscle atrophy could be induced by deregulation of different signaling pathways, including interleukin-1 (IL-1), tumor necrosis factor (TNF) and insulin/insulin-like growth factor (IGF) signaling, which lead to reduced protein synthesis or activated protein degradation [2–7]. Insulin/IGF signaling is one of the best-understood systems in growth regulation. Suppression of its downstream regulator mammalian target of rapamycin (mTOR), a positive regulator of protein synthesis, has been suggested to cause skeletal muscle atrophy [4–7]. Mice lacking both *AKT1* and *AKT2*, the downstream targets of insulin/IGF, have impaired mTOR activity and about 50% reduction in muscle mass [6]. Mice without ribosomal protein S6 kinase 1 (S6K1), a positive protein synthesis regulator downstream of mTOR, also display 20% reduction in muscle size without obviously affecting the number and type of muscle fibers [7].

Tuberous sclerosis complex (TSC) is an autosomal disorder characterized as benign tumors in multiple organs. The disease is caused by mutations in either the *TSC1* or the *TSC2* tumor suppressor genes [8]. The TSC1–TSC2 complex has been characterized in both *Drosophila* and mammals as a negative regulator of insulin/IGF induced S6K activation [9–12]. Germline disruption of *TSC1* or *TSC2* leads to embryonic lethality, demonstrating a general requirement for TSC genes in early development [13]. The C-terminal of TSC2 proteins contains a domain homologous to the GAP domain of Rap GTPase-activating protein (Rap GAP), which is responsible for the negative regulation of Rheb small GTPase and mTOR [14–16]. TSC2 proteins are suggested to be functional in a stable complex with TSC1 proteins through their coiled-coil domains [17,18]. This complex prevents ubiquitin-dependent degradation of TSC2 [18]. It can be destabilized by AKT1 dependent phosphorylation of TSC2, a process regulated by a kinase cascade originated from extracellular insulin/IGF molecules [9–11,19,20].

Cellular energy and nutrition levels regulate cell growth, cell size, and cell survival. Nutrition deprivation activates AMP-activated protein kinase (AMPK) in cultured mammalian cells, which in turn phosphorylates TSC2 on the T1227 and S1345 residues. AMPK phosphorylated TSC2 suppresses the activity of mTOR-S6K cascade. In *S6K1* knockout mice, skeletal muscle mass fails to respond to altered nutrition conditions, further suggesting a critical role for AMPK-TSC-mTOR in mediating nutrition signals [7,21,22].

To further understand the role of TSC genes in regulating muscle mass in vivo, we produced transgenic mice ectopically expressing hTSC1 in the skeletal muscle. These mice showed reduced muscle mass but otherwise were viable and fertile. Reduction of muscle mass is correlated with an increase of stable TSC2, presumably through its interaction with hTSC1 proteins. We further show that insulin and nutrition signals remain effective in regulating TSC1–TSC2 complex activity and the downstream events such as activation of S6K in the transgenic mice. Thus, hTSC1 transgenic mice represent an animal model for muscle atrophy, in which the muscle retains relatively normal response to insulin signaling.

2. Materials and methods

2.1. Transgenic mice

The 3.3 kb *MCK* promoter [23] was excised with *Eco*RI/*Xba*I and ligated into *Not*I/*Xba*I sites of pBluescript II KS+ vector. Myc-tagged full-length human *TSC1* cDNA was then cloned into the *Xba*I site with

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blunted-end ligation. The 2.1-kb *Spe*I/*Xho*I fragment of human growth hormone gene was inserted between the *Spe*I/*Xho*I sites to provide polyadenylation signal (hGH polyA) [24]. The transgene cassette was linearized by *Bss*HII, purified by agarose gel and microinjected into fertilized eggs from FVB/NJ mice. Transgenic offspring was identified by a 996-bp PCR product with the following primers: MCK5: 5′-CGAGCTGAAAGCTCATCTGCTCTCAGGG-3′; T1-3: 5′-TA-ATTCCGGATGAATTCGCACATGCTCC-3′. Animals were maintained on a 12/12 light/dark cycle and were allowed free access to food and water. For insulin stimulation test, eight-week-old mice were fasted overnight before receiving intraperitoneal (I.P.) injection of insulin next morning. Ten minutes after injection, tibialis anterior (TA) muscles were rapidly removed from sacrificed mice and processed for Western blot exactly as described below. For starvation and re-feeding experiments, five-week-old male mice were deprived of food for 2 days and then allowed free access to food and water for two days before analysis [7].

2.2. Histology

TA and extensor digitorum longus (EDL) muscles of 8-week-old male mice were weighed and embedded in OCT media (Leica CM) for frozen section. 7 μm thick transverse sections were collected and stained with haematoxylin/eosin solution (Sigma) to determine muscle and fiber size, or with NADH solution to distinguish fast and slow muscle fibers. Pictures were taken with a Leica dissecting microscope and a DC300 camera. Section areas were measured with Adobe Photoshop. Fiber size and fiber type were determined based on at lease 200 fibers counted in each muscle.

2.3. Western blot

Protein extraction was prepared with the RIPA lysis buffer (Santa Cruz) according to manufacturer's instruction and quantified with the BCA™ Protein Assay Kit (Pierce). Equal amounts of samples were separated by SDS/PAGE, transferred onto PVDF membranes (Millipore), and immunoblotted following standard protocols. Quantitative analysis was carried out with NIH ImageJ.

Goat anti-Myc antibody (Santa Cruz) was used to detect transgene expression. Rabbit anti-p-S6K (T389) antibody (Cell Signaling) was used to detect phosphorylation of Thr 389 residue of S6K proteins, while rabbit anti-S6K antibody (Cell Signaling) was used to determine total S6K proteins. Tubulin level usually served as loading control and was determined by Goat anti-tubulin antibody (Santa Cruz). Rabbit anti-TSC2 antibody (see acknowledgements) was used to detect endogenous mTSC2 proteins. HRP-conjugated bovine anti-goat antibody and anti-rabbit antibody were purchased from Santa Cruz.

2.4. Quantitative real-time RT-PCR

Total RNA extraction and reverse transcription was carried out with TRIzol (Invitrogen) and 9-bp random primers (Takara), respectively. Quantitative PCR was performed with the SYBRGreen PCR Master Mix system (ABgene) on an Mx3000p real-time PCR machine (Stratagene).

2.5. Statistical analyses

Results were expressed as the means ± S.D. Statistical significances of body weight, organ weight, and real-time results were determined using unpaired Student's *t*-test. Statistical significances of muscle size, fiber size and fiber type were determined using unpaired Student's *t*-test and two-tailed *Z* test. $P < 0.001$ is considered statistically significant in this study.

3. Results

3.1. Transgenic expression of hTSC1 in skeletal muscle

To investigate the function of TSC1–TSC2 complex in mouse skeletal muscle, we generated transgenic mice that expressed *hTSC1* cDNA driven by the promoter of mouse muscle creatine kinase (MCK) gene (Fig. 1A). Transgenes driven by MCK promoter express preferentially in skeletal muscle, with a higher level in fast-fiber enriched muscles, such as

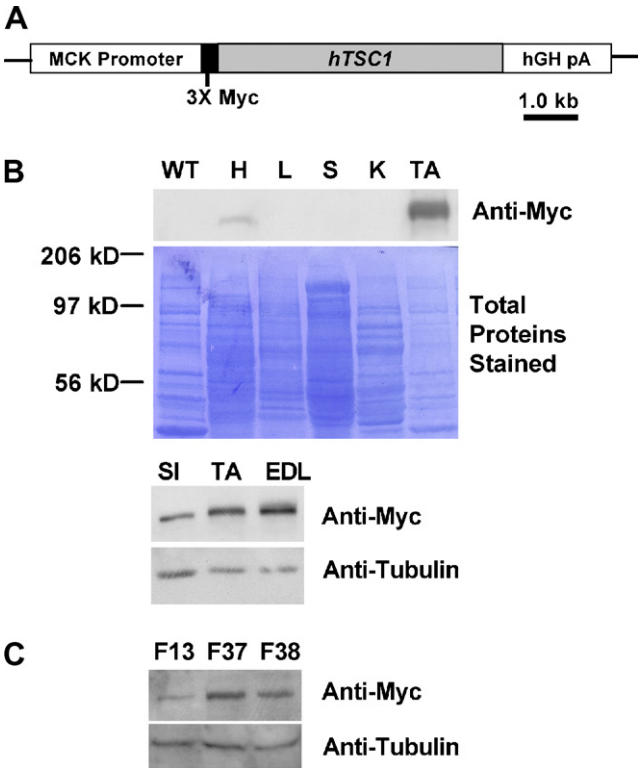

Fig. 1. MCK-driven *hTSC1* is preferentially expressed in mouse skeletal muscle. (A) The *MCK-hTSC1* transgenic construct. A 3.5 kb human *TSC1* cDNA (*hTSC1*) was fused in frame with a 3× myc tag sequence at the 5′ end and placed between the 3.3-kb MCK promoter and the 2.1 kb human growth hormone gene fragment containing the polyadenylation signal (hGH pA). (B) Expression pattern of hTSC1 in various organs (top panel) and different muscles (bottom panel). Transgene expression level was detected with anti-myc antibody. Amount of loaded proteins was judged by Coomassie blue staining or anti-tubulin antibody staining. H, heart; L, liver; S, spleen; K, kidney; TA, tibialis anterior; EDL, extensor digitorum longus; Sl, soleus; WT, TA sample from a wild type littermate. (C) Western blot shows transgene expression in TA muscles from different transgenic lines.

EDL, than in slow-fiber enriched muscles, such as soleus. However, a low-level leaky expression in cardiac muscle was also reported [23,25]. We produced 11 independent MCK-hTSC1 transgenic lines and characterized three of them (F13, F37 and F38) in this study. Consistent with previous studies of the MCK promoter, the hTSC1 transgene was found highly expressed in skeletal muscle such as tibialis anterior (TA). Among all other major organs analyzed, only heart showed a low-level transgene expression. Further analysis demonstrated that soleus expressed less hTSC1 proteins than EDL did (Fig. 1B). The level of hTSC1 expression in line F38 is 96 ± 21% of that in line F37 ($P = 0.37$), while hTSC1 expression in line F13 is only 26 ± 12% of that in line F37 ($P < 0.001$) (Fig. 1C).

3.2. The transgenic mice display skeletal muscle atrophy

All three transgenic lines had normal physical appearance at birth. However, F37 mice and F38 males exhibited reduced body weight than their wild type littermates since four to five weeks after birth (Fig. 2A). At the age of eight weeks, F37 males and females were 3.0 g (12.2%) and 2.0 g (9.8%) lighter than their wild type littermates, respectively, while F38 males

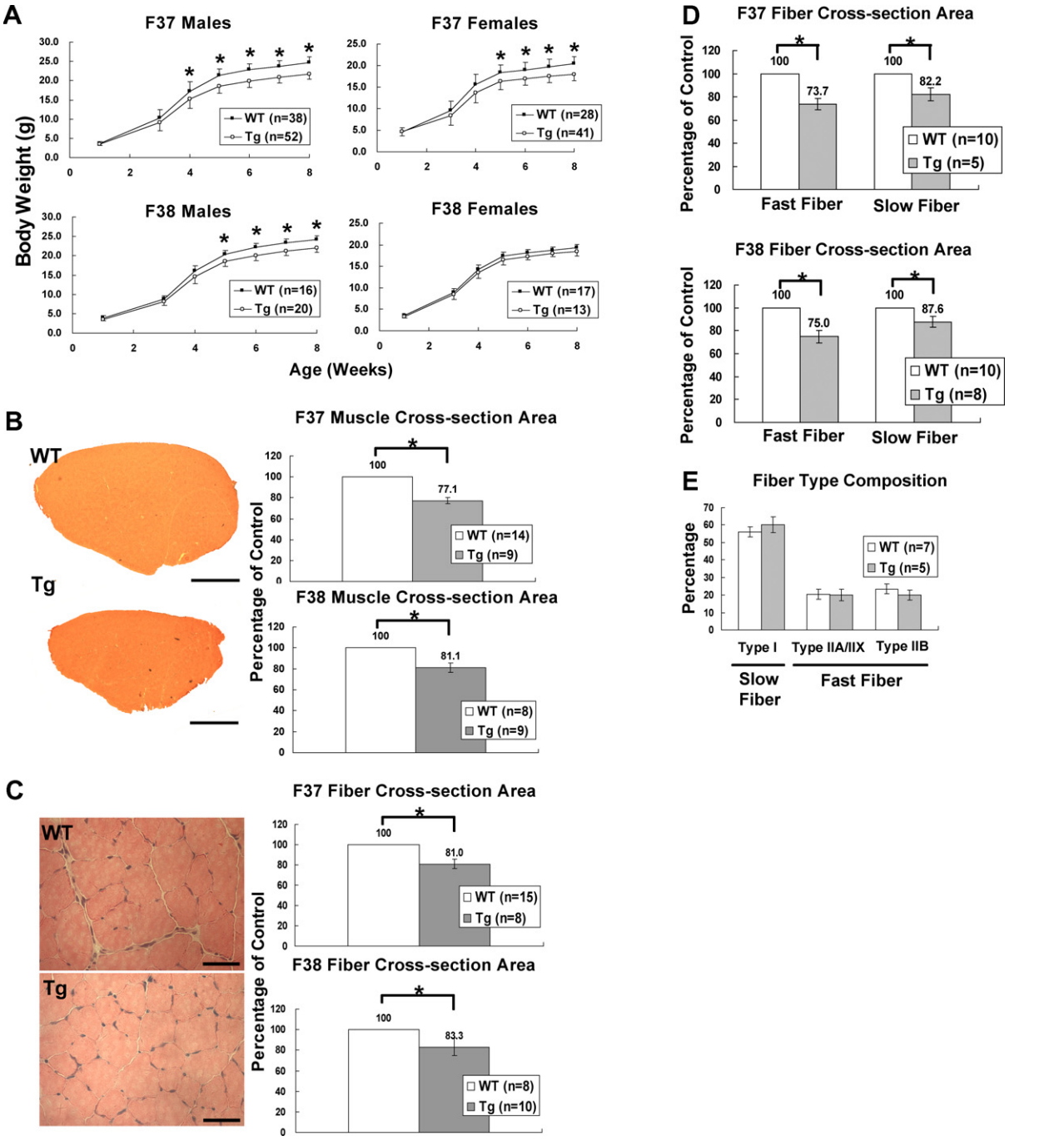

Fig. 2. Ectopic expression of *hTSC1* causes skeletal muscle atrophy in mice. (A) Body weight reduction in transgenic mice. (B) Muscle size reduction in transgenic mice measured with H&E staining of cross-sections of TA and EDL from adult male mice. Left panel showed the section from a F37 transgenic mouse and its wild type littermate, bar = 1 mm. Quantification of different lines is shown in the right panel. (C) TA fiber size reduction in transgenic mice. Left panel showed the H&E staining of the cross-section of TA from a F37 transgenic male mouse and its wild type littermate, bar = 50 μm. Quantification of different lines is shown at the right panel. (D) Size reduction of fast fibers and slow fibers in transgenic mice. NADH staining of the cross-section of TA from adult male mice was performed to measure the average size of different fibers. (E) Composition of slow (type I) fibers and fast (type II) fibers did not change in transgenic mice. TA muscles from adult F37 transgenic males and their wild type littermates were analyzed. Tg, transgenic mice; WT, wild type littermates; n, number of the samples, error bar shows the standard deviation; $^{*}P < 0.001$.

of the same age were 2.5 g (10.3%) lighter. F38 females and F13 showed no significant body weight reduction (Fig. 2A and data not shown).

We next investigated whether the body weight reduction was mainly due to the loss of muscle mass. Significant decrease in muscle mass of both TA and EDL was observed in F37 mice and F38 males ($P < 0.001$, Table 1). In line F13, muscle weight reduction was approximately 8% ($P < 0.005$, data not shown). A less dramatic weight reduction was also observed in heart, liver, spleen, and kidney of the transgenic mice (Table 1). These results indicate that muscle mass reduction is a major contributor to weight loss in the transgenic mice.

Table 1
Organ weight of wild type and transgenic mice

	Heart (mg)	Liver (mg)	Spleen (mg)	Kidney (mg)	TA (mg)	EDL (mg)	Body weight (g)
F37 Male							
WT ($n = 16$)	123.2 ± 15.2	1388.2 ± 187.4	98.6 ± 14.0	212.4 ± 31.1	44.8 ± 4.5	9.3 ± 1.4	24.6 ± 1.9
Tg ($n = 9$)	111.2 ± 9.0$^{\#}$	1263.1 ± 166.7	92.5 ± 7.8	194.7 ± 21.1	32.4 ± 2.4*	7.2 ± 1.6*	21.6 ± 1.1*
F37 Female							
WT ($n = 12$)	96.1 ± 6.8	1061.9 ± 144.6	100.1 ± 11.0	142.3 ± 12.0	38.2 ± 3.2	8.2 ± 0.6	20.4 ± 1.1
Tg ($n = 7$)	101.5 ± 10.4	1051.3 ± 113.7	99.2 ± 17.3	129.7 ± 17.7	29.4 ± 1.7*	6.7 ± 0.4*	18.4 ± 1.1*
F38 Male							
WT ($n = 8$)	119.0 ± 11.2	1252.2 ± 90.3	95.4 ± 5.5	195.8 ± 17.5	42.7 ± 1.8	9.3 ± 0.7	24.3 ± 0.9
Tg ($n = 10$)	109.4 ± 7.3	1207.9 ± 75.5	91.9 ± 10.2	190.7 ± 14.1	33.9 ± 1.5*	7.6 ± 1.0*	21.8 ± 1.0*

Eight-week-old mice and their wild type littermates were analyzed. Data are presented as means ± S.D.
Data of transgenic animals were also compared with those of wild type individuals to calculate the P values. *, $P < 0.001$; $^{\#}$, $P = 0.042$; unmarked, $P > 0.05$.

The skeletal muscle TA and EDL of the transgenic mice was further examined by histological analysis. In F37 and F38 male mice, the combined cross-section of TA and EDL displayed a 23% and 20% reduction in size, respectively (Fig. 2B). Correspondingly, the average of fiber size in TA had a 19% and 17% reduction, respectively (Fig. 2C). Although both slow (type I) and fast (type II) muscle fibers showed smaller sizes in these two lines, the change of fast fibers was more severe than that of slow fibers ($P < 0.001$, Fig. 2D). This was likely due to higher transgene expression in fast fibers driven by the MCK promoter. F13 males displayed about 12% decrease in both muscle and fiber size (data not shown). These observations support that hTSC1 expression resulted in skeletal muscle atrophy in a dosage-dependent manner. Consistent with previous studies in *AKT1* transgenic and *S6K*1 mutant mice, analysis of fiber type composition did not reveal any significant changes in *hTSC1* transgenic mice when compared with wild type littermates (Fig. 2E) [7,26].

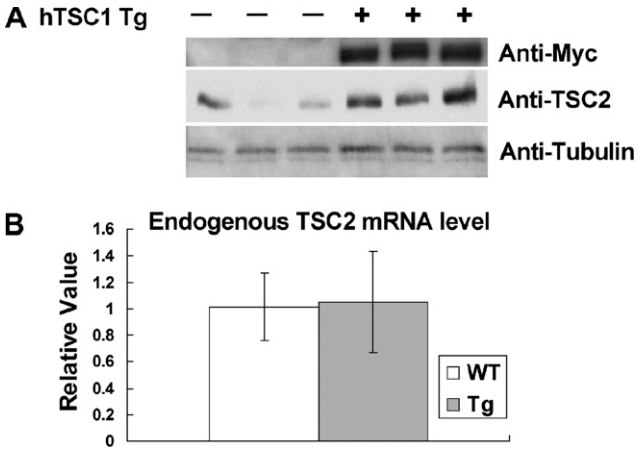

Fig. 3. Ectopic expression of hTSC1 stabilizes endogenous TSC2 proteins. (A) Western blot of TAs from three F37 transgenic individuals and their wild type littermates. Anti-myc antibody was used to detect transgene expression. Anti-TSC2 antibody shows endogenous TSC2 protein level. Tubulin level served as the loading control. (B) *TSC2* mRNA levels were not changed in transgenic animals. *TSC2* mRNA was quantified by real-time RT-PCR. TA samples were from F37 transgenic males and their wild type littermates. Each group has three individuals. Results were normalized with endogenous GAPDH mRNA level. Error bar shows the standard deviation.

3.3. Increased endogenous TSC2 protein level in hTSC1 transgenic mice

TSC1 and TSC2 form a functional complex to suppress insulin/IGF signaling cascade. Separation of TSC1 from TSC2 leads to ubiquitin-dependent degradation of the latter [18]. To study the mechanism of skeletal muscle atrophy in *hTSC1*

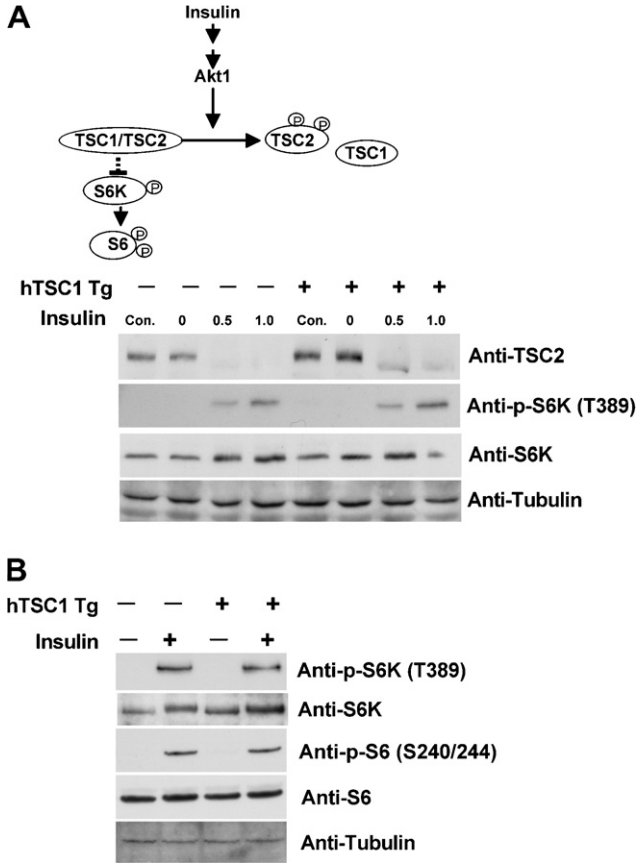

Fig. 4. TSC hetero-complex is regulated by insulin signaling. (A) Insulin stimulation decreased TSC2 protein levels and increased S6K phosphorylation in TA muscle. TA samples from adult female mice were used for Western blot. Each fasted mouse was stimulated with PBS containing 0, 0.5, or 1.0 IU of insulin as described above. Free feeding mice (Con.) were also sampled as the control. (B) S6K and S6 phosphorylation after insulin stimulation. All procedures were the same as in (A), except 5 IU of insulin was used for each mouse.

transgenic mice, we examined mRNA and protein expression levels of endogenous *TSC2* in TA muscles. Despite of increased protein level (Fig. 3A), the abundance of *TSC2* mRNA in the muscle sample of F37 transgenic mice was not affected (Fig. 3B). Similar results were obtained from F38 mice (data not shown). This observation implies that hTSC1 can stabilize endogenous mouse TSC2 (mTSC2) in vivo and the hTSC1-mTSC2 hetero-complex may be responsible for the reduction of muscle mass in the transgenic mice.

3.4. The TSC1–TSC2 complex is regulated by the insulin signal

Activity of the TSC1–TSC2 complex is regulated by AKT and its upstream growth signals. In mammalian cells and *Drosophila* insulin/IGF signals trigger AKT mediated phosphorylation of TSC2, and consequently inhibit functions of the TSC1–TSC2 complex. The TSC1–TSC2 complex is able to suppress the phosphorylation of Thr 389 in S6K, which is an essential step to further phosphorylate S6 (Fig. 4A) [10,11]. We evaluated the effect of insulin signaling on the stability of mTSC2 in fasted *hTSC1* transgenic mice. Insulin stimulation resulted in degradation of most endogenous mTSC2 in both control and transgenic individuals (Fig. 4A). Furthermore, phosphorylation of S6K and S6 in response to insulin stimulation occurred similarly in both *hTSC1* transgenic and the wild type littermates (Fig. 4A and B). These results indicate that the activity of hTSC1-mTSC2 hetero-complex is regulated by insulin signal in a manner similar to endogenous mouse TSC1–TSC2 complex.

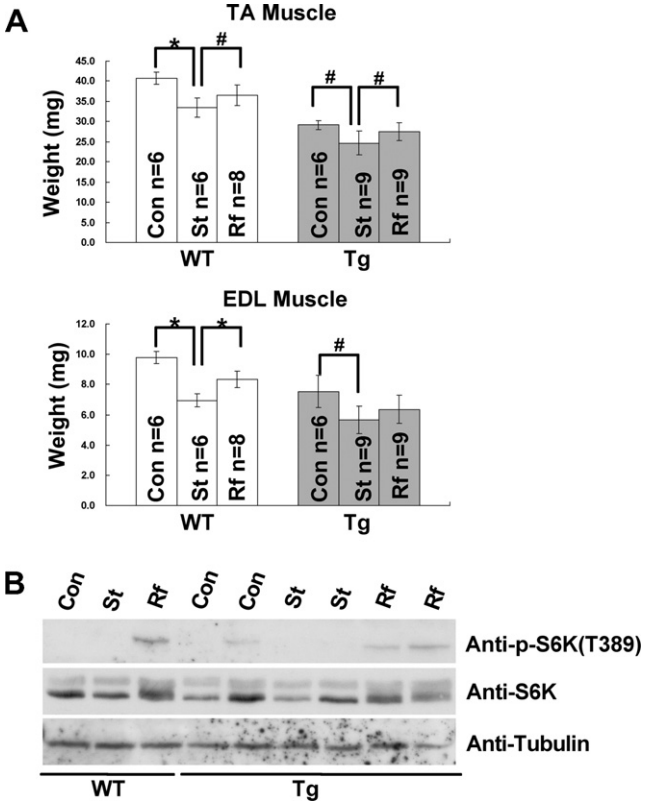

Fig. 5. Skeletal muscle of the transgenic mice responds to nutrition condition alterations. (A) TA and EDL mass changes after starvation and re-feeding processes. (B) Phosphorylation of S6K during starvation and re-feeding. Western blots of protein extracts from TA muscles were shown. Tg, transgenic group; WT, wild type littermates; Con, free fed mice; St, starved mice; Rf, re-fed mice. $^{\#}P < 0.05$; $^{*}P < 0.001$.

3.5. Nutrition regulates the TSC1–TSC2 complex in the transgenic mice

The TSC1–TSC2 complex is able to mediate cellular nutrition responses. To determine whether the hetero-complex is also subject to nutrition regulation, we evaluated the response of skeletal muscle to starvation and re-feeding conditions.

Both transgenic and wild type littermates lost about 30% of the body weight after 48-h starvation, and recovered to approximately 90% of the original weight after re-feeding for another 48 h (data not shown). In both groups of mice, TA and EDL muscle mass showed the same trend of reduction and recovery responding to the starvation and re-feeding processes, respectively (Fig. 5A). Both transgenic and wild type littermates had phosphorylated S6K level increased following nutrition recovery (Fig. 5B). These data indicate that the hTSC1-mTSC2 hetero-complex could respond to changes in nutrition conditions.

4. Discussion

TSC1 and TSC2 proteins can form stable complexes and function as suppressors of insulin/IGF pathway in *Drosophila* and cultured mammalian cells [9,11,14,19,20]. Inhibition of insulin/IGF signaling in mouse skeletal muscle could cause muscle atrophy [6,7,27,28]. In our study, ectopic expression of wild type hTSC1 enhanced the intracellular level of TSC2 proteins and caused a reduction in muscle mass. Considering the high degree of similarity among mammalian TSC protein sequences and functional conservation across species, these results suggested that the hTSC1 transgene induced an increase of functional TSC1–TSC2 complexes in muscle cells.

Skeletal muscle constitutes approximately 30% of total body mass [29]. In our experiments, hTSC1 expression in the skeletal muscle leads to more than 20% reduction of the muscle mass (Table 1), which accounts for nearly two thirds of total body weight loss (about 10%) in corresponding lines. In addition to muscle atrophy, a less dramatic weight reduction was also observed in other major organs (Table 1). This is not unexpected since skeletal muscle also takes part in energy and protein metabolism in vivo. Thus, muscle mass changes may induce metabolic alterations and weight reductions in other tissues.

In addition to growth control, insulin/IGF signaling is known to be involved in regulating glucose homeostasis. Inhibition of this pathway in mouse skeletal muscle usually causes impaired glucose homeostasis [27,28]. However, hTSC1 transgenic mice showed normal level of blood glucose in either fasting or random-feeding conditions, and behaved normally in glucose and insulin tolerance tests (data not shown). These results imply that increase of TSC1–TSC2 complex level in skeletal muscle does not affect glucose homeostasis.

In contrast to the above model that puts TSC proteins downstream of insulin signaling, a parallel model suggesting independent function of TSC proteins and insulin in regulating cell growth has also been proposed in *Drosophila* [9,30]. Our data showed that the level of TSC2 protein in skeletal muscle is subject to insulin regulation in both transgenic and wild type

mice (Fig. 4A). This result supports the linear relationship between insulin and TSC1–TSC2 complex.

TSC proteins suppress insulin-stimulated S6K activity with a dosage-dependent manner in cultured mammalian cells [11]. In *hTSC1* transgenic mice, we observed normal activation of S6K after insulin stimulation even though TSC1–TSC2 complex is increased (Fig. 4A and B). Transiently transfected cells are likely to produce a much higher level of TSC proteins than in the transgenic mice. In addition, TSC1 and TSC2 were co-transfected in the cell culture experiment whereas only TSC1 was used in producing the transgenic mice. These differences may lead to the resistance to insulin mediated TSC protein degradation in cultured cells but not in the transgenic mice.

S6K is a positive regulator of protein synthesis. It needs to be phosphorylated to be functional. However, S6K phosphorylation in mouse skeletal muscle is too low to be determined accurately under normal conditions. Thus we used starvation-refeeding assay to stimulate S6K phosphorylation. Although increased phosphorylation of S6K was observed in both transgenic and wild type littermates after refeeding, we did notice an approximately 36% reduction of phosphorylation in the transgenic group (Fig. 5B). Block of S6K activation, although not as dramatic as reported in the tissue culture system, may account for the reduced muscle mass in transgenic animals. This is consistent with the observations in *Drosophila* that S6K affects cell mass through insulin-TSC signaling [9,10].

An important observation is that muscle mass change of *hTSC1* transgenic mice in the starvation-refeeding test is in proportion to that of the wild type mice (Fig. 5A). This indicates that muscle mass is subject to regulation by an intrinsic genetic factor which differentiates the transgenic mice from the wild type controls. Intracellular TSC1–TSC2 complex has been shown to control cell size in a cell autonomous manner [9,10]. Mutations in *Drosophila* and mammalian *TSC1* or *TSC2* result in a cell autonomous increase in cell size, while overexpression of TSC1 and TSC2 leads to the opposite effect [9,10,21,31,32]. Our result is consistent with the idea that the level of TSC1–TSC2 complex is involved in setting the final size of muscle fibers.

In conclusion, our studies suggest that the TSC1–TSC2 complex plays an important role in determining the fiber size of skeletal muscles in mice. An increase of the TSC1–TSC2 complex leads to reduction of muscle mass and atrophic phenotypes. In addition to explore the utility of TSC1 expression as animal models for muscle atrophy, it would be interesting to investigate whether elevated TSC1–TSC2 complex level also responsible for a subset of muscle atrophy among human population.

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