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Knockdown of endogenous *SKIP* gene enhanced insulin-induced glycogen synthesis signaling in differentiating C2C12 myoblasts

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PI(3,4,5)P_3 produced by the activated PI3-kinase is a key lipid second messenger in cell signaling downstream of insulin. Skeletal muscle and kidney-enriched inositol phosphatase (SKIP) identified as a 5'-inositol phosphatase that hydrolyzes PI(3,4,5)P_3 to PI(3,4)P_2, negatively regulates the insulin-induced glycogen synthesis in skeletal muscle. However the mechanism by which this occurs remains unclear. To elucidate the function of SKIP in glycogen synthesis, we employed RNAi techniques to knockdown the *SKIP* gene in differentiating C2C12 myoblasts. Insulin-induced phosphorylation of Akt (protein kinase B) and GSK-3β (Glycogen synthase kinase), subsequent dephosphorylation of glycogen synthase and glycogen synthesis were increased by inhibiting the expression of SKIP, whereas the insulin-induced glycogen synthesis was decreased by overexpression of WT-SKIP. Our results suggest that SKIP plays a negative regulatory role in Akt/ GSK-3β/GS (glycogen synthase) pathway leading to glycogen synthesis in myocytes. [BMB reports 2009; 42(2): 119-124]

INTRODUCTION

In pigs muscle glycogen concentration at the time of slaughtering is an important indicator of meat pH, which ultimately influences port quality characteristics (1). The glycogen synthesis in muscle tissues is activated by insulin (2). PI(3,4,5)P_3 is a key lipid second messenger in cell signaling processes downstream of insulin signaling (3). Consequently hydrolysis of PI(3,4,5)P_3 by lipid phosphatases may affect insulin signaling and thus, muscle glycogen concentration.

The skeletal muscle and kidney-enriched inositol phosphatase (SKIP) gene was identified as a 5'-inositol phosphatase of PI(3,4,5)P_3, and is predominantly expressed in the skeletal muscle, heart and kidney (4). It was reported that SKIP played a negative regulatory role in insulin signaling in CHO cells where exogenous expression of SKIP inhibited GLUT4 translocation in the cells and glycogen synthesis in L6 myocytes (5). However, it is unclear as to whether SKIP plays a similar role in insulin signaling within insulin-targeted tissues such as skeletal muscle, where excess amounts of glucose are stored as glycogen for the maintenance of glucose homeostasis (6).

Other genes/factors that may affect insulin-induced glycogen synthesis include *protein kinase B* (*Akt*) and *glycogen synthase kinase 3* (*GSK-3*). Insulin-induced glycogen synthesis is required to activate Akt via pre-generated phospholipids by PI3K (7). The rise in PI(3,4,5)P_3 concentrations and the subsequent activation of Akt are extremely important for exerting various metabolic actions of insulin (8, 9), including initiation of protein translation, glucose transport, glycogen and lipid synthesis. Since the insulin-induced increase in PI(3,4,5)P_3 is modulated by SKIP, it seems logical that SKIP can regulate insulin signaling through the Akt pathway. GSK-3, located downstream of Akt, is an important molecule in further transmitting insulin signal for glycogen synthesis in skeletal muscles. Inactivation of GSK-3 by Akt results in the activation of glycogen synthase, which is the rate-limiting enzyme for glycogen synthesis by insulin (10, 11).

In this study, the RNA interference (RNAi) approach was applied to investigate the role of *SKIP* in regulating insulin-induced glycogen synthesis in the skeletal muscle cells. We firstly constructed *SKIP* short-hairpin RNA (shRNA) expression plasmids and then defined the most effective RNAi shRNA plasmid. By knocking down *SKIP*, we were able to investigate the influence of *SKIP* on insulin-induced glycogen synthesis signaling. We aimed to develop an understanding of the quality traits of *SKIP*-regulated pork meat.

RESULTS AND DISCUSSION

The construction of RNAi expression plasmids and the knockdown of *SKIP*

To identify effective targeting sequences for the mouse *SKIP* gene, we designed 3 shRNAs against mRNA following Reynolds method (12). Individual shRNAs were inserted into a pSIREN-

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RetroQ-ZsGreen vector, in which the interfering RNAs were driven by the human U6 promoter, followed by CMV IE promoter-driven ZsGreen. We obtained 5 recombinant plasmids, including two controls: the shRNA against ZsGreen (control) and scrambled sequence (scrambled). Recombinant plasmids yielded two bands at 4.1 kb and 2.5 kb after digestion with *Hind* III (Fig. 1A), which showed that *SKIP* gene fragments were successfully reconstructed into the RNAi expression vectors. The sequencing results showed that all the inserted fragments were similar to the ones designed in this study.

Three *SKIP* shRNA expression plasmids and two controls were transfected into C2C12 myotube cells (Fig. 1B). The cells that were transfected with treatment shRNA plasmids were cultured for 48 h. After 48 h, the numbers of green fluorescent cells decreased markedly in the control experiment (pSILENCE against ZsGreen), whereas the numbers in the scrambled experiment did not change. After 60 h and 72 h, the pSILENCE had also worked. This indicated pSILENCE started to work after 48 h of transfection and lasted for at least 72 h or more. The results from RT-PCR (reverse transcription-polymerase chain reaction) showed that the suppressive effects of three shRNAs on the expression of *SKIP* were different (Fig. 2A). The third shRNA was the most effective (about 90% depletion), while the expression of GAPDH was not influenced by the loss of *SKIP*. Based on the results of the RT-PCR experiments, whole cell lysates were immunoblotted with antibody against SKIP, and the results obtained were comparable to that of RT-PCR (Fig. 2B). We concluded that one effective shRNA was obtained by which the SKIP expression was almost inhibited. It was demonstrated that the DNA vector-based RNAi could specifically and efficiently suppress target genes (13). From our experiments, we found that green fluorescence protein was observable 12 h after transfection, and a complete suppressive effect had become apparent 48 h after transfection. This inhibitive effect lasted for at least 72 h.

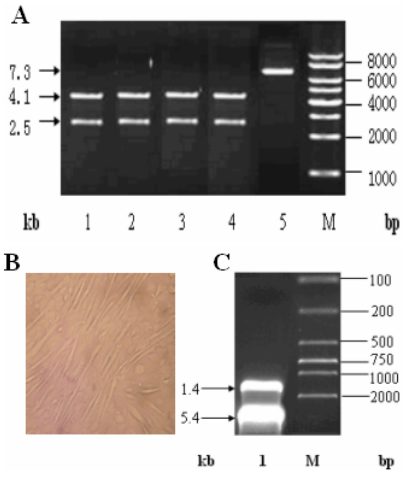

Fig. 1. (A) Agarose gel electrophoresis of recombinant plasmids digested with *Hind* III. 1: psiRNA-SKIP1; 2: psiRNA-SKIP2; 3: psiRNASKIP3; 4: scrambled control; M: 10 kb Marker; 5: RNAi-Ready pSIREN-RetroQ-ZsGreen (B) Differentiating C2C12 myoblasts on the 5th day after differentiation. (C) Restriction endonuclease analysis of pcDNA3.0-SKIP. M: Marker DL 2,000; 1: pcDNA3.0-SKIP digested by *Eco*R II and *Bam*H I.

Effect of SKIP inhibition on insulin-induced Akt and GSK-3 phosphorylation

Phosphorylation of Akt on Ser473 residues, corrected by the loaded amount of Akt, increased with insulin-stimulation. Attenuation of SKIP expression levels was associated with a significantly enhanced phosphorylation of Akt on serine 473 ($P < 0.01$) in insulin-treated C2C12 myotube cells (Fig. 3A). The increased phosphorylation was observed across all doses of insulin that were employed. These results are similar with pre-

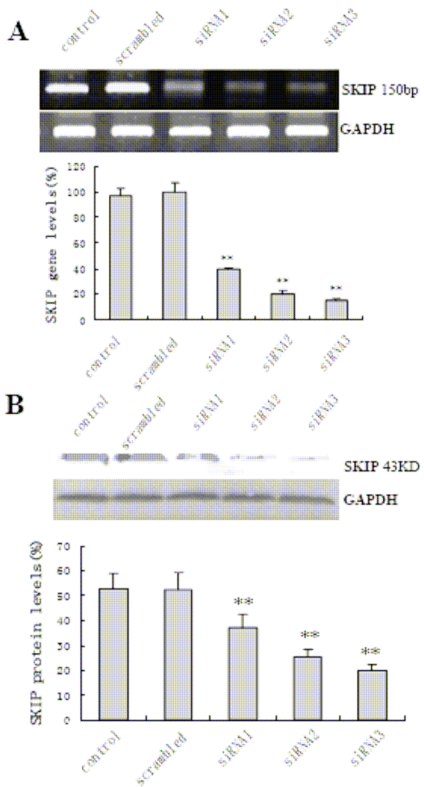

Fig. 2. Attenuation of SKIP expression by siRNA-mediated gene silencing in C2C12 myotubes. (A) Cells were transfected with ZsGreen siRNA (control), scrambled control (scrambled), psiRNA-SKIP1, psiRNA-SKIP2, psiRNASKIP3. The effective siRNA of SKIP, the third, was choosed by RT-PCR. (B) Cells were transfected with ZsGreen siRNA (control), scrambled control (scrambled), psiRNA-SKIP1, psiRNA-SKIP2, psiRNA-SKIP3, and cultured for 48 h before harvesting with lysis buffer, Protein (50 ug) of the total cell lysate was used for detection of SKIP and or GAPDH (loading control) by Western blot. The bottom panel depicts quantification of *SKIP* gene levels. Data are presented as mean ± S.E. of three independent experiments. $^{**}P < 0.01$.

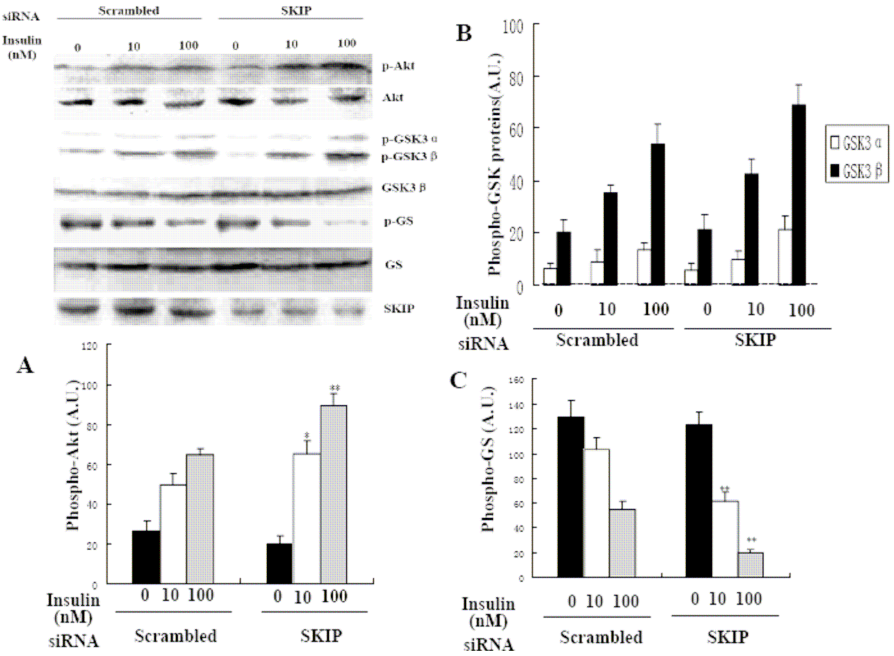

Fig. 3. Depletion of SKIP protein enhances insulin-induced Akt, GSK-3βphosphorylation and glycogen synthase dephosphorylation in C2C12 myotubes. Cultured myotubes were transfected with scrambled (4 μg), effective SKIP (4 μg) siRNA by Lipofectamine™ 2000 Reagent, cultured for 48 h, and serum-starved for 12 h before treatment with or without insulin for 30 min. Cells were then harvested with lysis buffer, and equal volume of total cell lysate was used for detection of phospho- (Ser^{473}) Akt, Akt, phospho-GSK3α/β, GSK3β, phospho-GS, GS and SKIP protein levels by Western blotting. The bottom panels depict quantification of phospho-Akt protein levels, phospho-GSK3α/β protein levels and phospho-GS protein levels. Data are presented as mean ± S.E. of three independent experiments. *P < 0.05, **P < 0.01.

vious reports in CHO cells (5).

It is known that both $PI(3,4)P_2$ and $PI(3,4,5)P_3$ bind with high affinity to the plekstrin homology (PH) domain of Akt leading to the recruitment of Akt to be phosphorylated on the plasma membrane (14). Since SKIP de-phosphorylates $PI(3,4,5)P_3$ to $PI(3,4)P_2$ via 5'phosphatase activity, the involvement of these phospholipids in the activation of Akt activity was considered to be complicated in vitro. However, our studies clearly showed that insulin-induced Akt activation by its phosphorylation was enhanced by knockdown of SKIP in C2C12 myotubes. $PI(3,4,5)P_3$, rather than $PI(3,4)P_2$, appeared to influence Akt activity on membranes in response to insulin in the cells. This idea is also supported by a recent report showing that $PI(3,4,5)P_3$ determined Akt activity on membranes, while cytosolic Akt activity correlated more closely with the level of $PI(3,4)P_2$ (15). The translocation of Akt to the cell membrane may not be affected due to the elevated $PI(3,4)P_2$, which can compensate for the reduced level of $PI(3,4,5)P_3$.

Given that a single event of SKIP depletion might enhance the phosphorylation of Akt, we investigated the other downstream target gene *GSK-3*. Our results showed that Akt phosphorylated GSK-3β and GSK-3α in response to insulin, in a dose-dependent manner. Interestingly, the expression level of phospho-GSK3β was 3 fold higher than phospho-GSK3α with or without insulin stimulation. This may account for the higher expression of GSK3 β compared to GSK3α in murine skeletal muscle cells. Attenuation of SKIP expression also increased phospho-GSK3β ($P < 0.05$) and phospho-GSK3α ($P < 0.05$) levels in response to insulin in C2C12 myotubes (Fig. 3B).

Because the activated GSK3 phosphorylates glycogen synthase, inactivation of GSK3 by Akt leads to the activation of glycogen synthase (16). Alternatively, glycogen synthase could be activated by protein phosphatase 1 (PP1), which is known to be localized downstream of PI3-kinase (17). Insulin-induced activation of PP1 dephosphorylates glycogen synthase, resulting in the activation of glycogen synthase. Thus, previous studies indicate two possible dephosphorylation mechanisms by which glycogen synthase is activated. However, the relative importance of Akt-GSK3 versus PP1 in insulin-induced glycogen synthase activation seems to be dependent on cell types. Previous studies have shown that insulin failed to induce activation of GS in skeletal muscle of the single $GSK3\beta^{9A/9A}$ knockin mice (10). These reports indicate that insulin stimulates GS in skeletal muscle by specifically inactivating GSK3β. Furthermore, if activation of a glycogen synthase phosphatase by insulin contributes to activation of glycogen synthase, then the activity of this enzyme would also have to be controlled by GSK3β. So we established that Akt-GSK3β was the key pathway in the regulation of insulin-induced glycogen synthase activation in skeletal muscle. And we thus conclude that *SKIP* is involved in the negative physiological regulation of insulin-induced Akt-GSK-3βsignaling, leading to glycogen synthesis in C2C12 myotubes.

Effect of SKIP on insulin-induced glycogen synthesis

Activation of glycogen synthase by its dephosphorylation is known to be a rate-limiting step in insulin-induced glycogen synthesis of skeletal muscle. Ser641 is one of the most important phosphorylation sites for the regulation of glycogen synthase. Since SKIP was involved in the regulation of GSK-3β, glycogen synthase activity might also be regulated by SKIP.

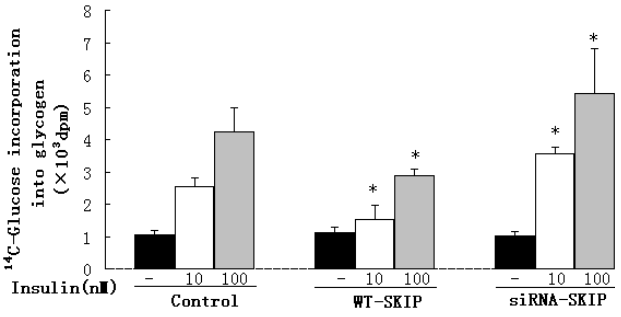

Fig. 4. Insulin-induced glucose incorporation into glycogen in transfected C2C12 myoblasts. Cells expressing WT-SKIP or siRNA-SKIP were stimulated with insulin for 1 h. Results are means of three separate experiments. *$P < 0.05$.

Therefore, we examined the effect of SKIP inhibition on insulin-induced glycogen synthase dephosphorylation on Ser641. Treatment with insulin resulted in an increase in glycogen synthase dephosphorylation accordingly, and suppression of SKIP increased insulin-induced glycogen synthase dephosphorylation remarkably ($P < 0.01$) in C2C12 cells (Fig. 3C). In order to clarify the relationship between SKIP and glycogen accumulation, we further examined the effect of SKIP on insulin-induced glycogen synthesis by measuring [^{14}C] glucose incorporation into glycogen in a dose-dependent fashion. A 100-nmol/L-insulin stimulation resulted in a 4-fold increase in [^{14}C] glucose incorporation into glycogen, in control C2C12 myotubes (Fig. 4). In the experiments that followed, we used a pcDNA3.0-SKIP overexpression vector (Fig. 1C). The overexpression of WT-SKIP decreased insulin-induced [^{14}C] glucose incorporation into glycogen, while this was increased by siRNA-SKIP. At 100 nmol/L insulin concentration, [^{14}C] glucose incorporation into glycogen was inhibited by ~32% via expression of WT-SKIP, whereas it was enhanced by ~28% via expression of siRNA-SKIP compared to that of the control C2C12 cells. These results were comparable with previous reports in L6 myotubes (5).

Our results indicate that SKIP is involved in the negative regulation of insulin-induced glycogen synthesis in C2C12 myotubes. This is further supported by recent studies showing that insulin-induced uptake of 2-deoxyglucose into the isolated soleus muscle was significantly enhanced in *SKIP*-mutant mice (18). It was speculated that insulin promoted glycogen synthesis by accelerating the rate of glucose entry into the cell in addition to glycogen synthase activation in skeletal muscle; meanwhile SKIP could negatively control glycogen storage. Reduced glycogen storage may reduce the formation of lactic acid during the post-slaughter conversion of muscle to meat, and thus the extent of the pH fall.

There are redundant pathways that regulate the PI3-kinase product PI(3,4,5)P_3 as well as SKIP; it is known that SHIP2 also possesses 5'-phosphoinositol phosphatase activity toward PI(3,4,5)P_3 (19). Insulin-induced glycogen synthesis via GSK3β and PP1 was inhibited by the expression of wild-type SHIP2, whereas it was enhanced by expression of the dominant-negative SHIP2 in L6 myocytes and 3T3-L1 adipocytes (20, 21). However, enhanced phosphorylation of Akt in the skeletal muscle and liver does not apparently affect the glucose homeostasis in SHIP2-knockout mice (22), probably because of the environment differences between *in vitro* and *in vivo* studies. We suggest that SKIP plays a compensatory role, at least in part, in the regulation of glycogen synthesis during the absence of SHIP2, especially in skeletal muscle. PTEN is an alternative lipid phosphatase to hydrolyse PI(3,4,5)P_3 too (23). Attenuation of PTEN expression by RNAi markedly enhanced insulin-stimulated Akt and GSK3α phosphorylation, as well as deoxyglucose transported in 3T3-L1 adipocytes (24). But PTEN does not seem to be the physiologically important lipid phosphatase in C2C12 myotube cells, as glucose tolerance and muscle insulin signaling were unchanged in muscle-specific *PTEN*-knockout mice fed on a normal chow diet (25). So SKIP may perform a similar function to PTEN in skeletal muscle.

CONCLUSIONS

In summary, we have clarified the molecular mechanism by which SKIP is involved in the negative regulation of insulin signaling leading to glycogen synthesis in C2C12 myotubes. Our results suggest that SKIP controls insulin-induced glycogen synthesis, at least in part, by negatively regulating Akt-GSK-3β-GS pathway via SKIP 5'-phosphatase activity. Thus, controlled reduction of muscle glycogen stores, obtained through up-regulation of the *SKIP* gene by some methods in the live animal at the time of slaughter, might be a potential method of reducing the extent of the pH decline postmortem.

MATERIALS AND METHODS

Materials and chemicals

Bovine pancreas insulin was obtained from Sigma-Aldrich (Gillingham, UK). Goat anti-SKIP polyclonal antibody (sc12071) was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho-Akt (Ser-473), phospho-GSK-3α/β (Ser-21/9), phospho-Glycogen Synthase (641), anti-Akt, anti-Glycogen Synthase and anti-GSK3β were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH monoclonal antibody was from Abcam (Cambridge, MA). The horseradishperoxidase-conjugated goat anti-rabbit IgG, anti-mouse IgG and rabbit anti-goat IgG were from Millipore (Bedford, MA). D-[U-^{14}C] glucose (specific activity 9.25-13.32 GBq/mmol) were purchased from NEN Life Science Products (Boston, MA).

Construction of recombinant pSIREN expressing siRNA

RNAi-Ready pSIREN-RetroQ-ZsGreen (BD Bioscience, CA) was used for DNA vector-based siRNA synthesis under the control of U6 promoter *in vivo*. There are two control siRNAs in this kit; Luciferase siRNA annealed oligonucleotide (control)

and the negative control, siRNA annealed oligonucleotide (scrambled). SKIP (accession numbers NM_008916.2) siRNAs were designed according to Ambion web-based criteria and BLAST searching showed no significant homology with other genes (psiRNA-SKIP1: CTCCAACAACTATGACACC, psiRNA-SKIP2: CTATGACACCAGTGAGAAA and psiRNA-SKIP3: CTC TTACTGGTCTTTGCCA). Three RNAi expression vectors were constructed to interfere SKIP mRNA expression. In order to accredit these vectors, a *Hind* III site was inserted into the vector. The oligonucleotides (100 pmol of sense strand and 100 pmol of antisense strand in 40 μL ultrapure water) were annealed by incubating at 95°C for 5 min followed by slow cooling at room temperature. The double-stranded hairpin siRNA templates were inserted between *Bam*H I and *Eco*R I sites of the pSIREN-RetroQZsGreenRNAi plasmid and transfected to *E. coli* DH5α competent cells. Plasmid DNA isolated from the positive clones was digested with *Hind* III to confirm the presence of the insert fragment and sequenced using a primer: 5'-ATGGAC TATCATATGCTTACCGTA-3'.

Construction of overexpression vector and identification
Wild-type *SKIP* (*SKIP*-WT) was generated by introduction of the relevant cDNAs into the pcDNA3.0 mammalian expression vector (Clontech). After extraction of the plasmid, identification with *Eco*R II and *Bam*H I digests were carried out.

Cell Culture, insulin treatment and Transfections
C2C12 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100-μg/ml streptomycin (Invitrogen). For differentiation, DMEM was substituted with differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% (v/v) horse serum, 2 mM L-glutamine, 100 U/ml penicillin). On the 4th or 5th day of differentiation, the cells were seeded onto 6-well plates and grown to 80% confluence before transfected with 4 μg of plasmid DNA using Lipofectamine™ 2000 Reagent, according to the manufacturer's instructions. After adding normal medium (DMEM with 10% FBS), cells were further incubated for 48 h before treating with insulin.

RNA isolation and Reverse transcription-PCR (RT-PCR)
Total cellular RNA was extracted from cell monolayers with Trizol resgent (Invitrogen). RNA was converted into first-strand cDNA by combining 2 μg of the RNA with 4 μl 5 × Transcription Reaction Mix, which includes Oligo (dT) and random hexamer primer (Invitrogen) according to the manufacturer's instructions. One-tenth or one-twentieth of the reaction mixture was used as a template for each PCR. For the amplification of the desired cDNA, the following gene-specific primers were used. GAPDH: sense 5'GCAGTGGCAAAGTGGAGATT3'; antisense 5'GTCTT CTGGGTGGCAGTGAT3'. SKIP: sense 5'ACACCCCGAACCTG AATC3'; antisense 5'GAGGT GGAGGAGACTTGC 3'. The PCR conditions were as follows: 94°C for 4 min, 28 cycles of 94°C for 45 s, annealing at 60°C, 72°C for 1 min and a final extension at 72°C for 7 min. Primer pair, which amplified the fragment spanning intron 8 of the GAPDH gene was applied to exclude the possibility of DNA contamination during all RT-PCR reactions.

Western blotting
Cells grown in 6-well plates were washed twice with ice-cold PBS, and then harvested. Cells from one well were solublilized in 100 μl SDS (sodium dodecyl sulphate) sample buffer, boiled for 5 min, and then ultrasonically homogenized for 3 min. Equal amounts of total protein were separated by electrophoresis on 10 or 12% SDS-polyacrylamide gels, and then transferred onto polyvinylidene difluoride membrane. The membrane was blocked in TBS-T with 3% BSA for 2 ~ 3 hours. Next, the membrane was incubated with indicated antibodies at 4°C overnight. After washing the membrane, the proteins were detected by peroxidase-conjugated immunopure rabbit anti-goat IgG (H+L).

Glycogen synthesis assay
Cells of 3 groups were seeded (1×10^5) onto 24-well plates, after the induced myotubes formation, incubated with MEM (1 ml) for 18 hours. MEM media containing 18.5 MBq/L D-[U-^{14}C] glucose was added into each well during experiment along with 0, 5 and 100 nmol/L insulin. After a further incubation for 90 min, 0.1 ml/well 30% KOH was added to terminate the reaction. Cells were completely lysed after incubation in 95°C bath for 30 min. The solution in the wells was applied to a Whatman GF/C chromatography filter-paper, oven dried and then fixed in alcohol (0.66 in volume fraction) overnight; washing with alcohol (0.66 in volume fraction) for twice then dried. Specific activity was measured by lipid scintillation method.

Statistical analysis
Data were analyzed using the SPSS statistical package (v.13.0). Intergroup comparison was carried out by the analysis of variance. The data are represented as means ± SE. p values were determined by unpaired Student's t test, and a p value of less than 0.05 was considered statistically significant.

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