

*Full Length Research Paper*

# The potential lipolysis function of musclin and its mRNA expression both in Pig adipose tissue and primary adipocyte

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Musclin is a newly discovered factor and its functions remain to be defined. This study investigated the tissue expression pattern of musclin gene and its potential effect on lipid metabolism. Musclin mRNA levels in adipose, muscle tissues and primary adipocytes were examined by quantitative PCR. The musclin gene expression in adult adipose tissue was significantly higher than that in muscle ( $p < 0.05$ ), and its expression in young adipose tissue was higher than old one. We further found that in adipose tissue, the expression level of musclin had a negative correlation with FAS gene ( $p = 0.01$ ), and positive correlation with PPAR gene ( $p < 0.05$ ). In adipocyte, the expression level of musclin gene had a positive correlation with LPL gene ( $p < 0.01$ ). Our results suggested that musclin is a potential factor that might play an important role in the regulation of adipogenesis.

**Key words:** Musclin, adipocyte, adipose tissue, lipid metabolism, expression.

## INTRODUCTION

Musclin gene was originally identified as another name "osteocrin" in mouse by Thomas et al. (2003). Since it was only detected in bone, this group named this protein as "osteocrin". They found that osteocrin was not only expressed in young bone cells, but also the expression was age-dependent; the expression level was higher in young animals than that in adults. Moreover, vitamin D was found to be able to inhibit the expression of osteocrin in primary osteoblastic cells (Thomas et al., 2003). One year later, musclin was identified from mouse skeletal muscle by Nishisawa et al. (2004). This group showed that musclin expression was regulated by nutritional changes and insulin. Musclin inhibited the insulin-induced glucose uptake and glycogen synthesis in myocyte (Nishizawa et al., 2004). And further research indicated

that musclin was mainly related to muscle fiber fast - glycolytic phenotypes (Staiger et al., 2006; Banzet et al., 2007). All these evidences indicated that the musclin might play an important role in glycogen metabolism.

The effect of musclin on lipid metabolism has not been systematically investigated, although Nishisawa et al. (2004) claimed that in their preliminary experiments, an adenovirus-mediated musclin expression significantly reduced fat mass in mice. Here, we aimed to investigate the potential correlation between musclin and lipid metabolism. This preliminary data suggested that musclin might be involved in lipid metabolism.

In this study, we examined the expression of musclin in pig fat tissue and primary adipocyte both *in vitro* and *in vivo*, and examined its effects on lipid metabolism and possible relationships with other lipogenetic genes.

## MATERIALS AND METHODS

### Experimental animals

Large White adult pigs, a local strain of Chinese pig, were provided by Shaanxi Guangming Pig farm. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Northwest Agriculture and Forestry. Subcutaneous fat

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**Abbreviations:** RT-PCR, Reverse transcription polymerase chain reaction; FAS, fatty acid synthesis; PPAR, peroxisome proliferator - activated receptor; LPL, lipoprotein lipase; TGH, triacyl glycerol hydrolase.

**Table 1.** PCR parameter of primers and conditions.

Gene	Primers	Amplified DNA (bp)	Tm°C	Mg <sup>2+</sup> (mmol/l)	Cycles
<b>Musclin</b>	F: ATGGACTGGAGACTGGCAAG R: CGGTTTCTACCAATCCGATC	374	56	3	34
<b>-actin</b>	F: ACTGCCGCATCCTCTTCCTC R:CTCCTGCTTGCTGATCCACATC	399	53.8	2.5	28
<b>FAS</b>	F: AGTGTCCACCAACAAGCG R: GATGCCGTCAGGTTTCAG	280	55.9	2.5	30
<b>PPAR</b>	F: ACCACTCGCATTCTTTGAC R: CCACAGACTCGGCACTCAAT	261	52.1	2.5	32
<b>TGH</b>	F: CTTGGCTCCTTGAGATTTG R: AGTTGGCAATGTTGTCTG	455	53.3	2.5	30
<b>LPL</b>	F: GCAGGAAGTCTGACCAATAAG R:GGTTTCTGGATGCCAATAC	183	54.3	2.5	30

Notes: F: Forward; R: Reverse

and muscle tissue were quickly excised right after the animal was slaughtered, then frozen in liquid nitrogen, and stored at 80 for later use.

## Methods

Total RNA was extracted from adipose and muscle tissue with Trizol reagent by following the manufacturer's instructions (Tiangen Biothec Co.). First strand cDNA was prepared with Revert Aid TM First Strand cDNA Synthesis Kit (Bio-Tech). 20 L of reverse transcription polymerase chain reaction RT-PCR solution contained 6 L DEPC water, 5 g total RNA, 4 L 5x reaction buffer, 1 L random hexamer primers (0.2 µg/µL), 1 L RNase inhibitor (20 /µL), and 1 L MLV reverse transcriptase. The conditions for RT reaction were as follows: 25°C for 10 min, 42°C for 60 min, 70°C for 10 min (for enzyme inactivation), and 4°C for 5 min. The RT products were either used immediately for PCR or stored at 20°C. Since there was no swine musclin gene sequence available.

We used human and mice sequences as reference to design primers for amplification of pig *musclin* cDNA. The PCR reaction conditions were summarized in Table 1. PCR reaction was performed in a total volume of 25 L containing 1 L of tissue-specific cDNA, 3 L MgCl<sub>2</sub> (25 mmol / L), 0.25 L Taq DNA polymerase, 2 L dNTPs (2.5 mmol / L), 2.5 L 10 x buffer and 1 L of each primer (10 mol / L). The PCR product was examined by Agarose Gel Electrophoresis (AGE). PCR fragment was purified from Agarose Gel and cloned into Pmd - 18T vector (Takara Bio Inc.) for sequencing.

## Bio-informatics analysis of swine musclin gene

Several online programs were applied to analyze signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>) and sub-cellular localization (<http://www.cbs.dtu.dk/services/TargetP/> and <http://psort.nibb.ac.jp/form2.html>) of musclin protein.

## Pig preadipocyte and myocyte isolation and culture

Primary pig stromal vascular cell (preadipocyte) and myocyte were obtained from new born pig subcutaneous fat tissue and skeletal muscle respectively, grown and differentiated as previously described (Hong-mei et al., 2007). In brief, tissues were initially washed

with PBS containing high concentration of mycillin and then digested with collagenases at 37 for 1 h. Cells were then filtered through 40 micron nylon membrane to remove tissue debris and concentrated by centrifugation. Isolated cell pellets were resuspended in DMEM / F12. For each experiment, preadipocyte and myocyte were seeded in 12 well plates at a density of 10<sup>5</sup> cells per well. Primary preadipocyte was grown for preparation of total RNA at day 0, 2, 4, 6, 8 and 10 respectively.

## Quantitative PCR

Musclin mRNA from samples of adipose, muscle, primary preadipocyte (cultured for 0, 2, 4, 5, 6, 8 and 10 days) and myocyte (5 days) were determined by quantitative PCR. To determine the relationship between musclin and lipid metabolism, we examined the expression levels of fatty acid synthesis gene (FAS), peroxisome proliferators-activated receptor gene (PPAR ) and tricyclic glycerol hydrolase gene (TGH) in adipose tissue, and lipoprotein lipase gene (LPL) and the gene PPAR in primary adipocyte. To quantitatively determine the gene expression, we used -actins gene as the internal control. The primers and PCR amplification conditions were listed in Table 1.

Limited by a lack of real-time PCR equipment, we used a simple and similar real-time PCR technique. Briefly, for each sample, we prepared 6 tubes for PCR reaction, and the reaction was set as 26, 28, 30, 32, 34 and 36 cycles respectively. The PCR products were examined by agarose gel electrophoresis.

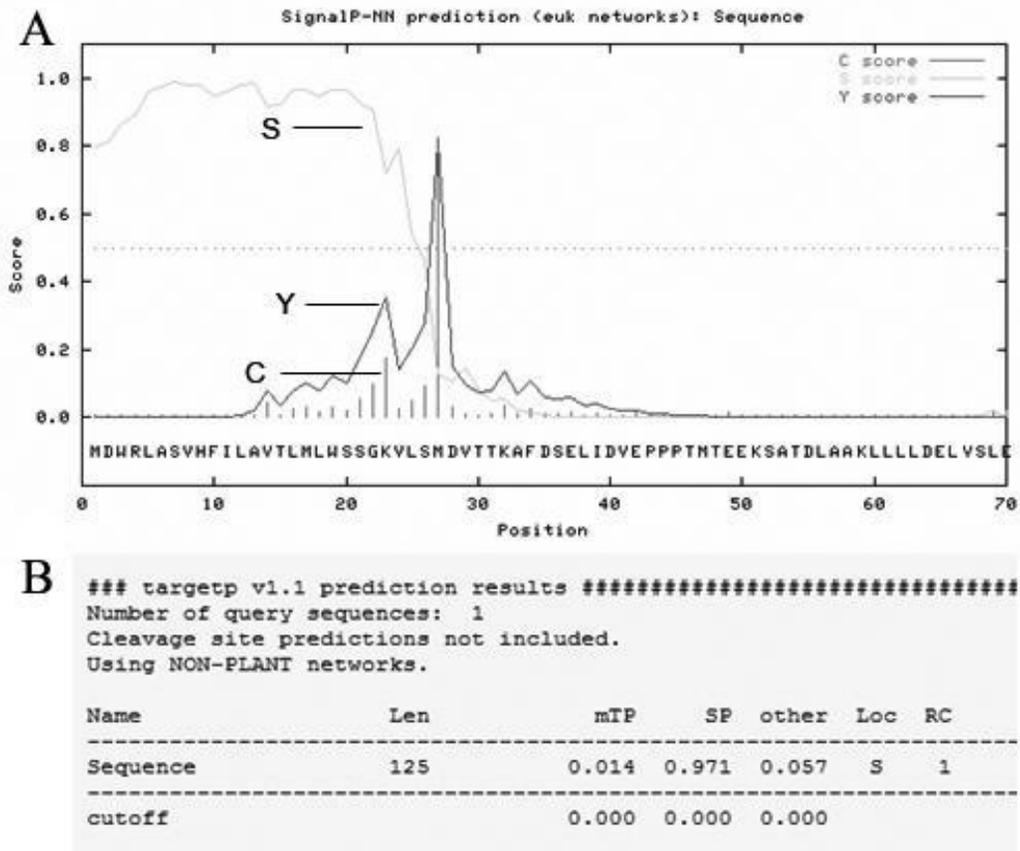
## Data analysis

Software SPSS 13.0 was used for statistical analysis. Musclin mRNA expression under standard conditions was analyzed with one-way ANOVA and LSD multiple comparison. Pearson's correlation coefficients were used to determine statistical linear associations between musclin and other genes involved in lipid metabolism. All data from samples was shown as means ± Standard Error (SEM).

## RESULTS

### Cloning of swine musclin gene

We amplified the swine musclin gene using RT-PCR.



**Figure 1.** Prediction results of musclin protein from online software. The signal peptide of musclin protein was predicted by neural network analysis from online server Signal P 3.0 (A). The *S*-score is reported for every single amino acid position, with high scores indicating that the corresponding amino acid is part of a signal peptide. *C*-score should only be significantly high at the cleavage site. *Y*-max is a derivative of the *C*-score combined with the *S*-score resulting in a better cleavage site prediction than the raw *C*-score alone, the high-peaking *C*-score is the true cleavage site. The *S*-mean and *D*-score is calculated separately for the length and the position of the predicted signal peptide. The subcellular localization of musclin protein was predicted by Target P sever (B) (mTP: mitochondria, SP: the signal peptide secretion path, other: positioning expressed in cells of other locations)

Agarose gel analysis of PCR product indicated a specific band and the sequencing result showed that the swine musclin cDNA consists of 375 bp. The swine musclin cDNA sequence was submitted to Gen Bank (accession no. EU122441). Aligned with other species, swine musclin cDNA shares the highest homology with human (86%), followed by cow (88%), sheep (87%), rat (78%), mouse (76%) and chicken (59%).

### Bioinformatics analysis

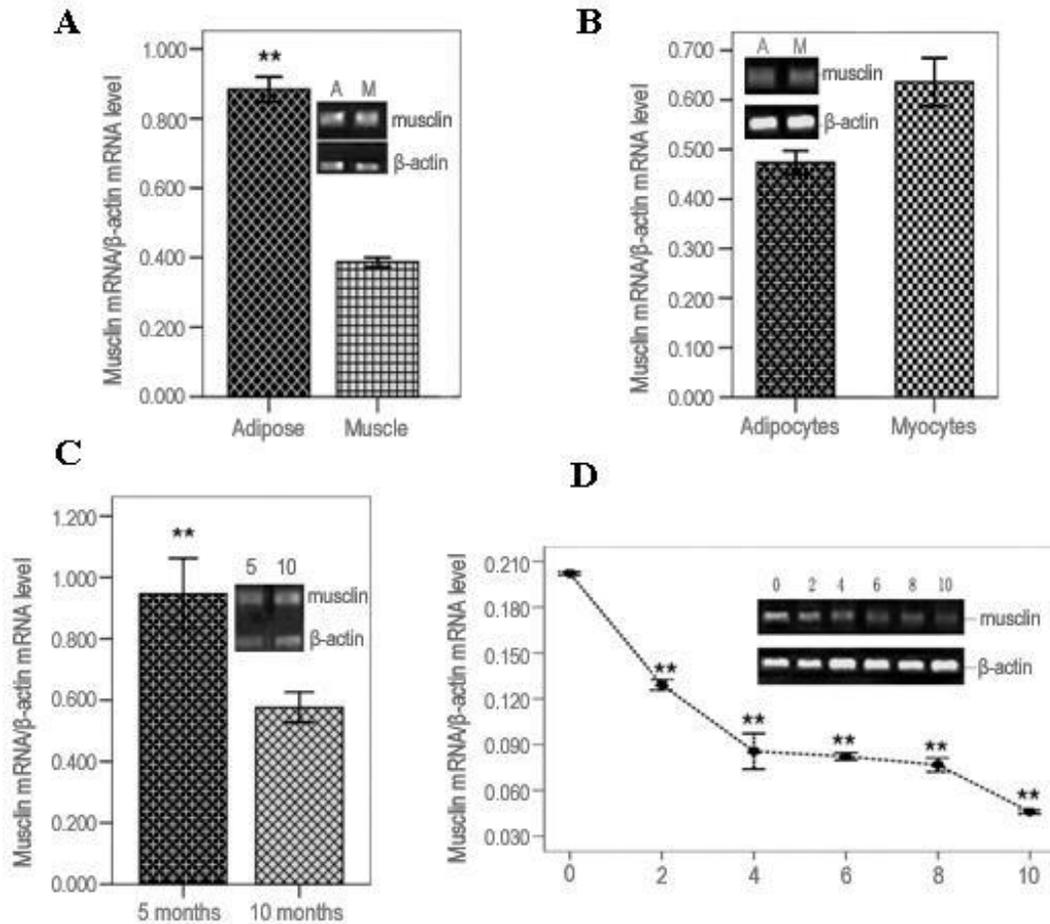
Using neural network analysis from online server Signal P 3.0, we found that swine musclin protein contains a signal peptide on the N-terminus, located from amino acid 1 to 26. A cleavage site was predicted between amino acid 26 - 27, which suggested that the musclin protein might be released by cell (Figure 1A).

As was shown in Figure 1B, the target P 1.1 program analysis further indicated that musclin was a secreted protein, since it was very low in mitochondria and mostly distributed in the outside of cells, including the cell surface. All these data suggested that swine musclin might function as a cytokine in outside the cell or both extra cellular and intra cellular.

### Different expression pattern of musclin in adipose tissue and muscle tissue, adipocyte and myocyte

We compared the musclin gene expression in pig muscle and adipose tissue. The data suggested that musclin expression in adipose tissue was much higher than in muscle tissue ( $p < 0.01$ ) (Figure 2A).

To further verify the musclin gene expression in muscle and adipose tissue, we next examined the expression of



**Figure 2.** The expression of musclin mRNA in different tissues, primary cells (A, B), adipose tissues from pigs of different ages (C) and adipocytes cultured for different days (D),  $\beta$ -actin was used as an internal control. The level of musclin mRNA in adipose tissue is remarkably higher than muscle (A) (A, adipose tissue; M, muscle tissue). The difference of musclin mRNA level between adipocyte and myocyte was not significant (B) (A, adipocyte; M, myocyte). The level of musclin mRNA in adipose tissue of ten-month-old pig indicated much lower than a five-month-old (C). The level of musclin mRNA in culture adipocytes of different days indicated an age-dependent manner (D). \*\*  $p < 0.01$ .

the musclin gene in primary adipocyte and myocyte. The primary adipocyte and myocyte were prepared from young pigs and maintained with DMEM / F12 medium for 5 days. The expressions of musclin gene in adipocyte and myocyte had no significant difference (Figure 2B). This result confirmed that the musclin gene was indeed expressed in adipose tissue.

### The expression pattern of musclin gene both in adipose tissue and adipocyte

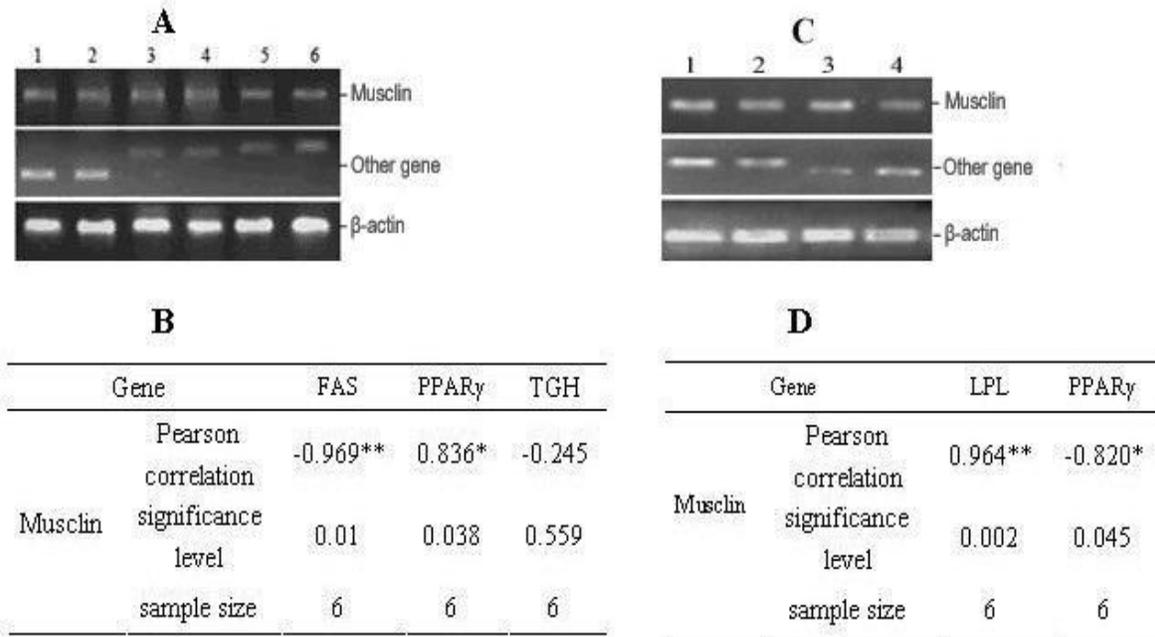
We next examined the musclin gene expression in adipose tissue from different ages of animals. As shown in Figure 2C, musclin gene expression in subcutaneous adipose of five-month-old. Large White pigs was significantly higher than that in ten-month-old ( $P < 0.05$ ), suggesting that musclin gene expression is higher in

young pig than that in old pig. This expression pattern was further supported by primary adipocyte experiments. The primary adipocytes were prepared from young pig adipose tissues and maintained in DMEM / F12 medium. As demonstrated in Figure 2D, the expression level of the musclin gene in primary adipocyte decreased with culture time.

This time-dependent expression manner of musclin suggested that musclin might be involved in the regulation of cell differentiation or adipose tissue development.

### Correlation between musclin and some lipogenetic genes expressed in adipose tissue and primary cells

To support the hypothesis that musclin is involved in adipocyte differentiation and adipose tissue development, we next investigated the relationship between musclin



**Figure 3.** The statistical correlation between musclin and lipogenetic genes was analyzed by SPSS software. The electrophoresis result of musclin and lipogenetic genes mRNA level in adipose tissue, lanes 1 and 2: TGH (5-month-old and 10-month-old respectively), lanes 3 and 4 of second line: PPAR (5-month-old and 10-month-old respectively), lanes 5 and 6 of second line: FAS (5-month-old and 10-month-old respectively) (A). Correlation analysis result of musclin and lipogenetic genes in adipose tissue (B). Electrophoresis result of lipogenetic genes mRNA level in adipocytes, lanes 1 and 2 of second line: LPL (5 days and 10 days respectively), lanes 3 and 4 of second line: PPAR (5 days and 10 days respectively) (C). Statistical correlation analysis results of musclin and lipogenetic genes in adipocytes (D). \*\*  $p < 0.01$ , \*  $p < 0.05$ .

expression and the expression levels of other lipogenetic genes in adipose tissue and primary adipocytes. FAS, PPAR and TGH are the main genes that play an important role in lipid metabolism in adipose tissue. Applying Pearson's correlation analysis to the data obtained from the adipose tissue samples, we revealed that the expression level of musclin had a negative correlation with FAS gene expression ( $r = -0.969$ ,  $p = 0.01$ ), had a positive correlation with PPAR gene expression ( $r = 0.836$ ,  $p < 0.05$ ), and no correlation with TGH gene expression ( $r = -0.245$ ,  $p > 0.05$ ). In primary adipocytes, we examined the expression of LPL and PPAR genes and compared their expression levels with musclin gene expression (Figure 3c and 3d). Musclin expression showed a negative correlation with PPAR expression ( $r = -0.820$ ,  $p < 0.05$ ), and a positive correlation with LPL ( $r = 0.964$ ,  $p < 0.01$ ), suggesting a potential linkage between musclin and the Lipolysis function of LPL.

## DISCUSSION

The musclin gene was originally isolated from mouse bone and muscle tissue and therefore named "osteocrin" or "musclin". It was initially believed that musclin was exclusively expressed in bone and muscle tissue in

Thomas et al. (2003) and Nishisawa et al. (2004) research, separately. However, in this study, we found that musclin also expressed in adipose tissue and primary adipocytes. We cloned musclin gene from swine adipose tissue and examined its expression pattern in adipose tissues and primary adipocytes. It was found that musclin might influence the regulation of lipolysis (Dong et al., 2008). With regard to musclin expression and its possible roles in lipid metabolism, we have addressed four questions.

The first question concerned the tissue specificity of musclin gene expression. We found that the musclin expression level was higher in adipose tissue than in muscle. This discovery was completely different from the previous claim that musclin was exclusively expressed in muscle tissue (Nishisawa et al., 2004). They reported musclin expression in adipose tissue was not significant. This is probably due to the different expression of musclin between swine and mouse species.

The second question compared us *in vivo* versus *in vitro* experiments. We found that, between primary adipocyte and myocyte, the difference of musclin gene expression was not significant. This appeared inconsistent with our tissue test results. We hypothesized that the difference between the culture conditions *in vitro* and the physiological environments *in vivo* might contribute to the

musclin expression pattern in primary cells. The *in vitro* data strongly suggested that musclin expression in adipose tissue is regulated by other factors.

The third question is related to the musclin expression pattern. In the present study, we found that musclin expression in adipose tissue of young swine is higher than old swine. Its expression decreased as age increased, suggesting that musclin might be involved in adipocyte proliferation and adipose tissue development. This result is similar to the previous research, which showed that osteocrin /musclin could be used as a marker of early osteoblast maturation due to its strong correlation with early stage of bone formation (Thomas et al., 2003; Bord et al., 2005). Given the fact that fat mass gradually increases with animal growth, the musclin expression pattern implied that musclin might negatively regulate fat mass accumulation in adipose tissue.

The fourth question was with regard to the potential function of musclin in lipid metabolism. Previous research showed that musclin protein significantly inhibited fat mass accumulation in mice, suggesting musclin could be the underlying link between skeletal muscle and adipose tissue (Nishizawa et al., 2004). Moreover, the authors also found that insulin regulated the expression of musclin and musclin had feedback effect on insulin. Recent studies showed that insulin resistance in skeletal muscle decreased muscle glycogen synthesis, resulting in an increase in plasma triglyceride concentration (Petersen et al., 2007). Although it was well known that obesity was associated with insulin resistance, much of the relationship between obesity and insulin resistance actually came from visceral and ectopic lipid accumulation (Carr et al., 2004; Xiao – Rong et al., 2008). All these evidences implied that insulin-resistant induced fat mass and the abnormal process of fat accumulation in tissues other than fat tissue could increase the risk of obesity. How musclin is involved in insulin induced fat accumulation remains to be further investigated. Intramuscular fat development had become a popular target for many scientists. A recent paper showed that 14 differentially expressed genes might participate in the development of intra muscular fat (Lee et al., 2007). Additionally, considering that musclin was originally identified in muscle, our results provided a new insight with the interaction between adipose tissue and muscle tissue, also musclin might be a candidate gene that participate intra muscular fat development.

Furthermore, a recent paper reported Foxo1, a transcription factor involved in lipid metabolism, inhibited the expression level of the musclin gene (Yasui et al., 2007). To further understand the relationship between musclin and obesity, we evaluated the correlation between musclin and key genes of lipid metabolism, and found that musclin had a negative correlation with FAS, a key enzyme which regulates de novo lipogenesis and improves triglyceride accumulation in adipose tissue. Many studies have shown that insulin, bile acids and feeding could increase FAS mRNA and protein levels

(Moustaid et al., 1993; Matsukuma et al., 2006; Ranganathan et al., 2006). Musclin could suppress the function of insulin (Nishizawa et al., 2004) . Accordingly, we proposed that musclin might negatively regulate FAS to restrain lipid synthesis and decrease fat mass. Lipoprotein lipase (LPL) is an important enzyme which catalyses the hydrolysis of tricyclic glycerol. Thiazolidinediones affected adipocyte LPL production through activation of PPAR , promoting the hydrolysis of triglyceride (Schoonjans et al., 1996). Furthermore, insulin might increase the activity and expression of LPL (Albalat et al., 2007). Therefore, based on the observation that musclin expression was positively correlated with LPL, it suggested that musclin might be involved in lipid degradation. Since lipolysis function is a complicated procedure, further study on the interaction between musclin and other lipolysis genes will be conducted in our future experiment. PPAR is a member of the nuclear receptor superfamily of transcription factors and previous studies showed that PPAR responded to specific ligands by altering gene expression in a cell developmental and sex-specific manner (Burns and Heuvel, 2007). PPAR regulates gene expression in many functional pathways. In this study, the correlation between musclin and PPAR in adipose tissue was positive but in adipocyte was negative. This probably was due to some other factors involved in PPAR expression on musclin, whereas in *in vitro* adipocyte culture, these factors were not present. How musclin regulates lipid metabolism via PPAR remains to be further elucidated.

In conclusion, in the present study, we found that musclin was expressed in both adipose tissue and primary adipocytes, and had a positive effect on lipolysis. Our data suggested that musclin represented a novel potential factor which could be involved in lipid metabolism. Further elucidation of the function and activity of musclin might provide new avenue for obesity therapeutic approaches.

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