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Construction of suppressor of cytokine signaling 2 (*SOCS2*) adenoviral overexpression vector and its impact on growth hormone-induced lipolysis in swine primary adipocytes

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ABSTRACT. We investigated the effect of overexpression suppressor of cytokine signaling 2 (*SOCS2*) on lipolysis in swine primary adipocytes (pAd) induced by growth hormone (GH). We constructed pAd-*SOCS2* adenoviral overexpression vectors to infect HEK293 cells for virus packaging and propagation. Cultured swine primary adipocytes were infected with virus particles; after 48 h the infected adipocytes were treated with 500 ng GH/mL in the growth medium. Lipometabolism-related gene expressions were detected at 0, 0.25, 0.5, 1, 2, and 4 h, by measuring mRNA and protein levels. The pAd-*SOCS2* overexpression vector was successfully constructed and the concentration of titrated virus was 1.2 x 10^9 PFU/mL. We found that virus infection significantly increased *SOCS2* mRNA and protein levels in swine primary adipocytes. Overexpression of *SOCS2* significantly inhibited the increase in fatty acid synthase, adipose triglyceride lipase mRNA, and protein expression at 0.5 h. However, after 0.5 h, this inhibition was not significant. We concluded that overexpression of *SOCS2* inhibited the

increase in lipolysis induced by GH in swine primary adipocytes; this could provide a basis for studies of lipometabolism.

Key words: Swine; *SOCS2*; Overexpression adenoviral vector; Primary adipocytes; Lipolysis

INTRODUCTION

As an important member of the overexpression suppressor of cytokine signaling (SOCS) family, *SOCS2* has a broad role in many physiological activities. Ouyang et al. (2006) used *SOCS2* plasmid vectors to transfect C2C12 mesenchymal precursor cells and investigated whether *SOCS2* could enhance cell proliferation and survival and inhibit spontaneous myotube formation. Zhang et al. (2010) demonstrated that *SOCS2* might play an important role in the immune defense of invertebrates by cloning the full-length cDNA of *SOCS2* from *Eriocheir sinensis* and analyzing the developmental status of *E. sinensis* SOCS2. On the other hand, *SOCS2* can regulate fat deposition and fat metabolism, but related research is lacking and regulatory mechanisms are unclear. The basal expression of *SOCS2* in adipocytes has been found to be low. Usually, *SOCS2* expresses at high levels when induced by growth hormone (GH) (Tollet-Egnell et al., 1999; Johansen et al., 2003; Berryman et al., 2004; Pasarica et al., 2007; Plockinger and Reuter, 2008).

GH, which is a peptide hormone secreted by the anterior pituitary, combines with GH receptor on the cell surface in target tissue and regulates growth. Specifically, GH could activate the hormone-sensitive lipase in subcutaneous adipose tissue to promote lipolysis and increase fatty acids in the blood. This function has been shown to be more effective in visceral fat (Ng et al., 2000; Sun et al., 2012a). However, *SOCS2* is an important negative factor of cytokine signal transduction, as growth is inhibited in *SOCS2*$^{-/-}$ mice (Metcalf et al., 2000). Besides, *SOCS2* overexpression in mice leads to a significantly bigger size than in wild-type ones (Greenhalgh et al., 2002). All these findings suggested that *SOCS2* might regulate the GH signal pathway 2-fold, but its biochemical mechanism is yet unknown. In this study, we overexpressed the *SOCS2* gene in swine primary adipocytes (pAd) and detected the lipometabolism-related genes by real-time PCR (RT-PCR) and Western blotting for a preliminary look at the role of *SOCS2* in the regulation of lipolysis in swine pAd induced by GH. A theoretical basis for the regulatory mechanism of *SOCS2* in the GH signaling pathway is discussed.

MATERIAL AND METHODS

Main materials

Three-day-old Large White pigs were purchased from Shaanxi Yangling Guangming farms; *Escherichia coli* DH5α was kept in our laboratory; pAdTrack-CMV, pAdEasy-1 plasmid and *E. coli* BJ5183 strain were donated by the College of Animal Science and Technology, Northwest A&F University, Pig Fat Deposition and Muscle Development Laboratory; human embryonic kidney cell line (HEK293) was purchased from the cell resource center of the Chinese Academy of Sciences.

Reagents and antibody, Dulbecco's modified Eagle's medium (DMEM/F12) and type

I collagenase were purchased from Gibco (USA). Fetal bovine serum (FBS) was purchased from Hyclone (USA). Lipofectamine 2000 was purchased from Invitrogen (USA). Restriction enzymes *Pme*I and *Pac*I were purchased from New England Biolabs (USA). RNAiso Plus was purchased from Takara (Dalian, China). The RevertAid First Strand cDNA Synthesis kit and ExTaq DNA Polymerase were purchased from Fermentas (USA). Antibodies were purchased from Santa Cruz (USA).

Cloning of *SOCS2* and recombination of overexpression vector

Before cloning, cDNA was first synthesized. Next, based on the porcine *SOCS2* gene sequence (GenBank ID: NM_007706.3), the sequence tagged site and a pair of primers (*SOCS2*-*Bgl*II-F and *SOCS2*-Flag-*Xho*I-R) were designed to amplify the region by PCR. Later, the products were visualized following electrophoresis on a 1% ethidium bromide-stained agarose gel, and the unique band was cut out for fragment recovery and storage at -20°C for reuse (Bioflux Company).

pAdTrack-CMV plasmid and the *SOCS2* gene were double-digested with the enzymes *Bgl*II and *Xho*I, then the double-digested products were recycled and connected with T4 ligase at 16°C overnight. The scale of the *SOCS2* gene concentration and of the pAdTrack-CMV plasmid concentration was 3 to 5. After the transformation, screening and identification by PCR and double-digestion, pAdTrack-CMV-*SOCS2* was constructed and available for linearization with the enzyme *Pme*I. The linear fragments precipitated by ethanol were transformed into BJ5183 competent cells with pAdEasy-1 for homologous recombination. After screening on LB solid culture plates with 100 µg/mL kanamycin and identification, pAd-*SOCS2* was ready and stored at -20°C (Table 1).

Table 1. Primers used for the full-length amplification of *SOCS2* and detection of related gene.

Primer name	Primer sequence (5'-3')	Accession No.	Tm (°C)
SOCS2- *Bgl*II-F	GCAGATCTATGACCCTGCGGTGCCTGG	NM_001097461.1	57.0
SOCS2-Flag-*Xho*I-R	GCCTCGAGTTACTTATCGTCGTCATCCT TGTAATCTACCTGGAATTTATATTCTT		
SOCS2-F	ACTAACCTGCGGATTGAG	NM_001097461.1	53.8
SOCS2-R	CAGAGTGGGTGCTGATGT		
FAS-F	AGCCTAACTCCTCGCTGCAAT	NM_001099930.1	57.8
FAS-R	TCCTTGGAACCGTCTGTGTTC		
ATGL-F	CCTCATTCCACCTGCTCTCC	EU373817.1	59.3
ATGL-R	GTGATGGTGCTCTTGAGTTCGT		

Tm = melting temperature; AGATCT = *Bgl*II; CTCGAG = *Xho*I.

pAd-*SOCS2* package, propagation and virus titer determination

HEK293 cells were grown up to 70% confluence and were then transfected with adenovirus vectors of pAd-*SOCS2*, which were expanded and linearized with *Pac*I. After 24 h, cells were checked for green fluorescent protein (GFP) expression, and 8-10 days after the transfection, they were examined for cytopathic effect (CPE). Cells with CPE were scraped into vials for freezing in liquid nitrogen and thawing at 37°C, 5 times, and the vials were then centrifuged for 5 min at 12,000 rpm. Supernatant liquid containing the virus Ad-*SOCS2* was collected for subsequent transfection (when cells reached 90% confluence). At 24 h after trans-

fection, cell morphology and GFP expression were observed. After repeating this procedure 3 times, the virus titer was detected by the $TCID_{50}$ method and reached 1.2 x 10^9 PFU/mL.

Culture of primary adipocytes and infection with Ad-*SOCS2*

Subcutaneous fat from the neck and back of 3-day-old Large White pigs was harvested and washed with phosphate-buffered saline (PBS) 3 times. The samples were then sheared to 1 mm^3 and digested with collagenase I at 37°C for 1 h. Cells were filtered through a micron nylon membrane to remove tissue debris and concentrated by centrifugation. Isolated cell pellets were suspended in DMEM/F12. Finally, cells were seeded on culture plates at a density of 4.0 x 10^4 cells/cm^2 and grown in DMEM/F12, supplemented with 10% (v/v) FBS and 100 U mycillin, at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every other day. The cells were infected with Ad-*SOCS2* to choose the optimal multiplicity of infection (MOI). When 70% confluence was reached, the medium was changed to serum-free overnight, and the cells were induced with 500 ng/mL GH after 48 h. Cells were harvested at 0, 0.25, 0.5, 1, 2, and 4 h for extraction of RNA and protein.

RT-PCR

Primers of fatty acid synthase (*FAS*), adipose triglyceride lipase (*ATGL*) and *SOCS2* were designed by the Primer Premier 5.0 software for RT-PCR. RT-PCR amplifications were carried out on a Bio-Rad iQ5 (Hercules, CA, USA) by SYBR Premix Ex TaqTMII chemistry detection under amplification conditions. The $2^{-\Delta\Delta Ct}$ method was used for PCR data analysis. $\Delta\Delta Ct$ = (Ct, target gene in treated group - Ct, reference gene in treated group) - (Ct, target gene in control group - Ct, reference gene in control group).

Western blotting

Cells were harvested and lysed with 200 µL lysis buffer RIPA (20 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM sodium β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 10 µg/mL aprotinin) for 30 min at 4°C. After centrifugation at 12,000 g for 10 min at 4°C, the supernatants were removed and their protein concentrations were determined by the bicinchoninic acid method. Total protein extracts were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene membrane (60 V for 4 h). The membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween 20) at room temperature for 2 h, and probed overnight with unique (Santa Cruz) primary antibodies at 4°C. After washing with TBST, the membranes were probed with secondary antibodies (Santa Cruz) at room temperature for 1.5 h. Blots were visualized with a chemiluminescence reagent (Millipore, USA) and an imaging system (BioRad).

Statistical analysis

The SPSS 13.0 statistics software package was used for one-way ANOVA. Data are reported as means ± standard error. Differences were considered to be statistically significantly at $P < 0.05$ or $P < 0.01$.

RESULTS

Cloning of the *SOCS2* gene

We extracted RNA from pig subcutaneous adipose tissue for cDNA synthesis by reverse transcription. We then used cDNA to amplify the *SOCS2* gene by RT-PCR and ligate it to the pMd-18T vector for the construction of pMd-18T-*SOCS2*. After sequencing, pMd-18T-*SOCS2* was used as the template to amplify the *SOCS2* gene fragment with a tag and restriction sites and identified by electrophoresis, as described below. Figure 1A shows the representative gel picture of *SOCS2* (650 bp) amplification by PCR. The purified *SOCS2* gene PCR product is shown in Figure 1B. The *SOCS2* fragments amplified had a size of 650 bp, which matched the theoretical size.

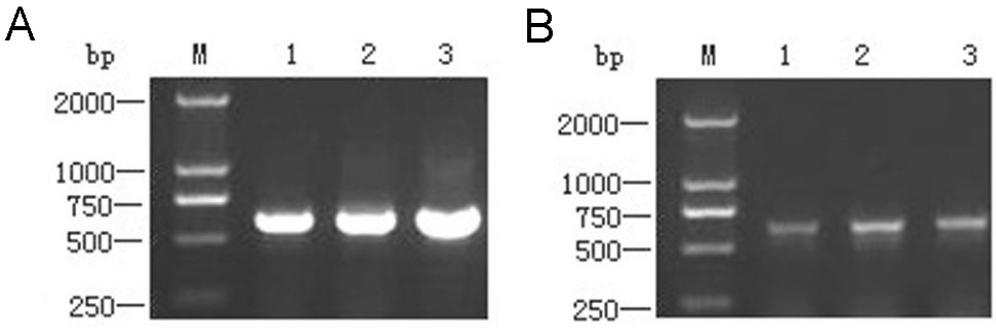

Figure 1. *SOCS2* gene electrophoresis analysis of PCR product and gel recovery product. **A.** Representative gel picture of *SOCS2* (650 bp) by PCR; **B.** Purified PCR product. *Lane M* = marker 2000; *lanes 1* to *3* = three repeats.

pAdTrack-CMV-*SOCS2* and Ad-*SOCS2*

The pAdTrack-CMV vector and the *SOCS2* target gene were digested with *Bgl*II and *Xho*I restriction enzymes. Then, the products were recycled for connecting with T4 ligase at 16°C overnight. After the transformation, screening and identification by PCR and double-digestion, the positive monoclonal were identified. Figure 2A shows the picked positive monoclonal of pAdTrack-CMV-*SOCS2*. It was verified by PCR (Figure 2B) and double-enzyme cleavage (Figure 2C), where the visible size was 650 bp and consistent with the theoretical size. pAdTrack-CMV-*SOCS2* was linearized with *Pme*I restriction enzyme and transformed into the BJ5183 competent cells containing pAd-Easy-1 for homologous recombination on LB plates incubated at 37°C for 16-20 h. Figure 2D shows the positive monoclonal of Ad-*SOCS2*. PCR identification is shown in Figure 2E. We used *Pac*I restriction enzyme to digest and release a 4.5-kb fragment, shown in Figure 2F. Verified by sequencing, the homology of the target *SOCS2* gene in the recombinant plasmid and deposited in GenBank (GenBank accession No. NM-007706.3) was found to be 100%, which showed that Ad-*SOCS2* was built successfully.

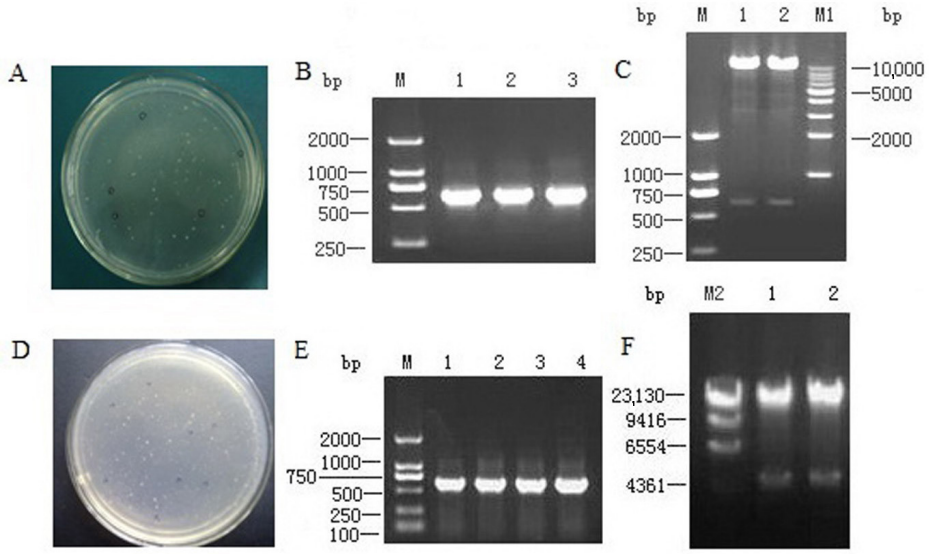

Figure 2. Identification of pAdTrack-CMV-*SOCS2* and recombinant adenovirus overexpression vector Ad-*SOCS2*. *Lane M* = marker 2000; *lane M1* = 1-kb marker; *lane M2* = λ*Hin*dIII marker; **A.** screening the positive monoclonal of pAdTrack-CMV-*SOCS2*; **B.** pAdTrack-CMV-*SOCS2* was verified by PCR; **C.** pAdTrack-CMV-*SOCS2* verified by double-enzyme cleavage; **D.** after homologous recombination, the positive monoclonal of Ad-*SOCS2* is picked; **E. F.** Ad-*SOCS2* verified by PCR and double-enzyme cleavage, respectively. *Lanes 1-3* (A), *lanes 1* and *2* (C, F), *lanes 1-4* (E) = three, two and four repeats, respectively.

Packaging and propagation of Ad-*SOCS2*

Photographs of HEK293 cells infected with the adenoviral vector containing Ad-*SOCS2* were taken at 24 h when GFP could be first observed (Figure 3A).The fluorescence was observed as bundles for 7 days (Figure 3B), and after 10 days, most cells showed the CPE phenomenon, which meant that cells could be harvested for virus that were used for packaging (Figure 3C). Figure 3D, E and F show the progress of virus packaging; GFP in Figure 3D showed that almost 60% of the cells were infected in the first multiplication of virus, and this ratio increased more than 90% in the third multiplication (Figure 3E). After 24 h, the expression of cell GFP was apparent when the cells turned round and wrinkled-like, as shown in Figure 3F, and it was then appropriate to harvest the virus. The virus particles obtained had a titer of 1.2 x 10^9 PFU/mL.

MOI of Ad-*SOCS2* virus liquid

Figure 4A and B show the mRNA and protein expressions of *SOCS2* in swine primary adipocyte infected with Ad-*SOCS2* at different MOI. Expression of *SOCS2* in infected adipocytes was significantly higher than in the uninfected control group ($P < 0.01$). *SOCS2* in adipocytes infected with MOI 20 and 25 expressed nearly the same levels, but expression in both was significantly higher than that in cells infected with MOI 15. Figure 4C and D show that mRNA and protein expression of *SOCS2* in adipocytes induced with GH was significantly higher compared with Ad-null groups ($P < 0.01$). As a result, MOI 20 was the most suitable for further experiments.

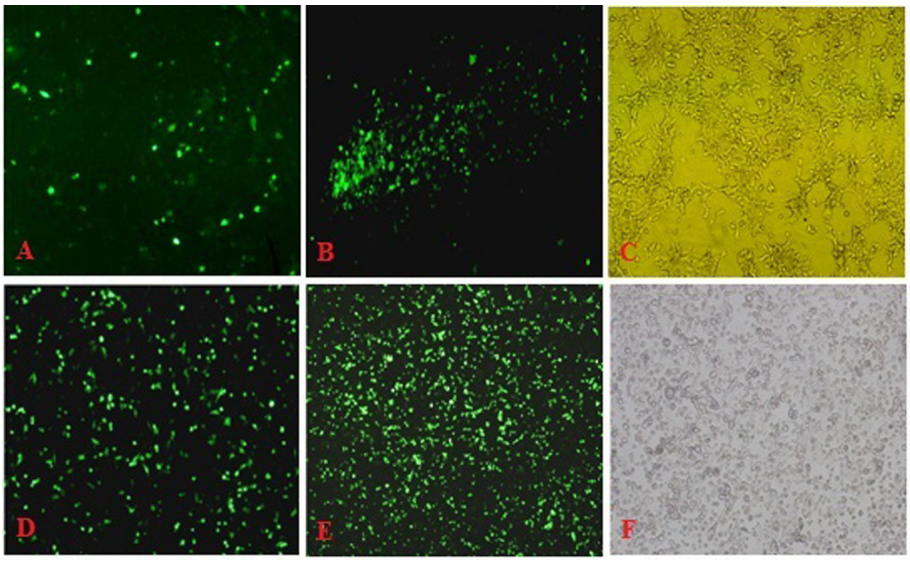

Figure 3. Ad-*SOCS2* packaging and multiplication (100X); HEK293 cells were infected with Ad-null or Ad-*SOCS2*. **A.** HEK293 cells infected with Ad-*SOCS2* for 24 h; **B.** HEK293 cells infected with Ad-*SOCS2* for 7 days; **C.** HEK293 cells infected with Ad-*SOCS2* for 10 days and then harvested; **D.** the first time multiplication of Ad-*SOCS2* virus; **E.** the third time multiplication of Ad-*SOCS2* virus; **F.** HEK293 cells are an apparent cytopathic effect phenomenon.

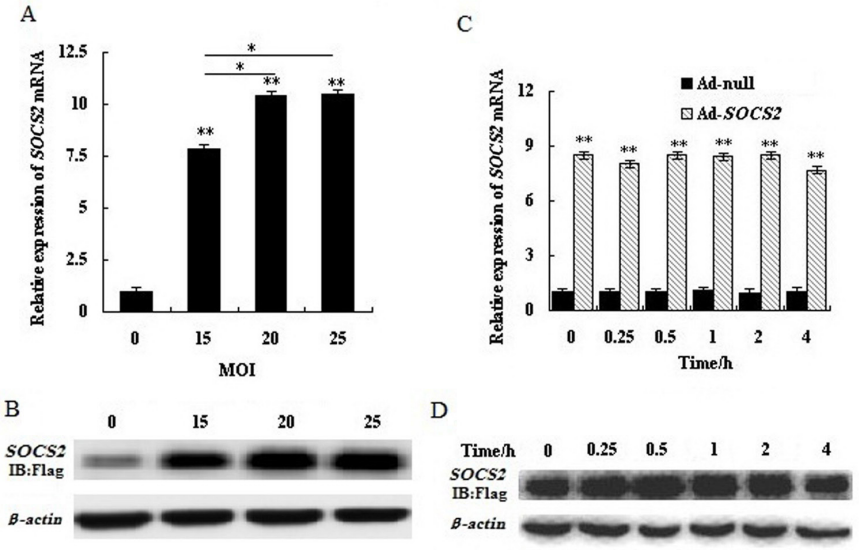

Figure 4. Measure of Ad-*SOCS2* multiplicity of infection (MOI) values and the effect of Ad-*SOCS2* on swine primary adipocyte. *Significant difference ($P < 0.05$). **Significant difference ($P < 0.01$). **A.** *SOCS2* mRNA expressions in different MOI values in swine primary adipocyte; **B.** *SOCS2* protein expressions in different MOI values in swine primary adipocyte; **C.** overexpressions of *SOCS2* could obviously increase the *SOCS2* mRNA expression; **D.** overexpressions of *SOCS2* could obviously increase the *SOCS2* protein expression.

Expressions of key enzymes in fat metabolism

First, we used 500 ng/mL GH to treat adipocytes for 0, 0.25, 0.5, 1, 2, and 4 h. PBS groups were as controls, which clarified the effect of GH on primary adipocytes. The cells were collected and expression of *FAS* and *ATGL* was determined by RT-PCR and Western blotting. Compared with control groups, 500 ng/mL GH increased *FAS* mRNA expression in primary adipose cells, and it was significantly higher within 0.5 h, but decreased afterwards, with a significant decrease 4 h later (Figure 5A). The same situation occurred with gene *ATGL* that is shown in Figure 5B. At the same, we determined the protein expression of these two genes, which showed the same trend with mRNA expression (Figure 5C, D, E, and F). These results indicated that 500 ng/mL GH could stimulate *FAS* expressions during an early stage (0.25-1 h), but with time, mRNA and protein expressions were notably reduced. At the same time, GH increased *ATGL* expression, both mRNA and protein, but the inhibitory action decreased with time.

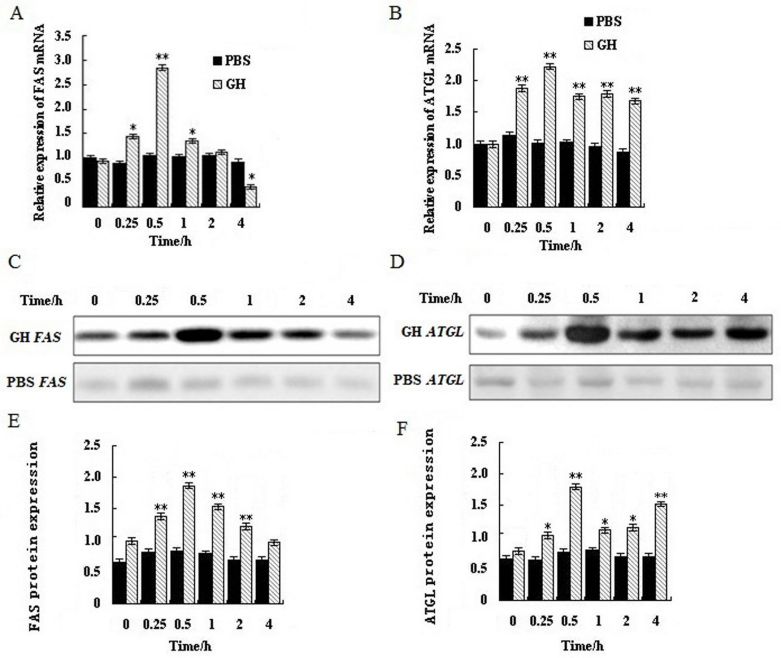

Figure 5. Growth hormone (GH) induced fatty acid synthase (*FAS*) and adipose triglyceride lipase (*ATGL*) expressions. **A.** and **B.** *FAS* and *ATGL* mRNA expressions in primary adipose cells; **C. D. E. F.** FAS and ATGL protein expressions in primary adipose cells. PBS = phosphate-buffered saline. *Significant difference ($P < 0.05$). **Significant difference ($P < 0.01$).

Next the swine primary adipocytes were infected with pAd-*SOCS2* for 48 h, Ad-null infection served as control. The adipocytes were then treated with 500 ng/mL GH for 0.25, 0.5, 1, 2, and 4 h after serum starvation overnight. By detecting the expressions of *FAS* and *ATGL* by RT-PCR and Western blotting, we found that overexpression of *SOCS2* could inhibit the increase in mRNA expression of both genes induced by GH at 0.25 h ($P < 0.05$) and 0.5 h ($P < 0.01$) (Figure 6A, B). In Figure 6C, D, E, and F, we see that overexpression of *SOCS2* significantly inhibited GH-induced *FAS* and *ATGL* protein expression, which was consistent with mRNA detection.

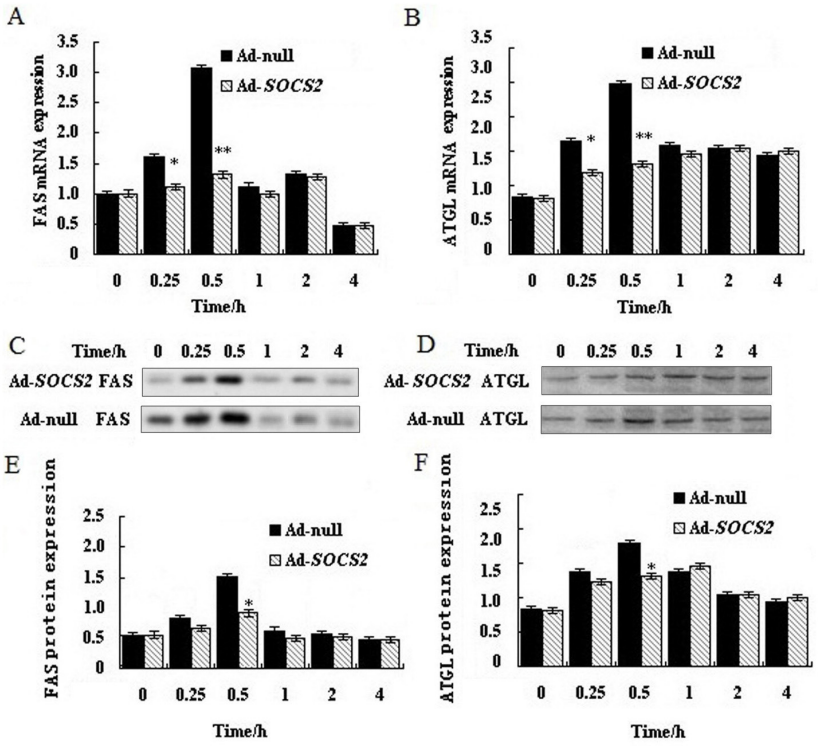

Figure 6. Overexpression of *SOCS2* could inhibit growth hormone (GH)-induced fatty acid synthase (*FAS*) and adipose triglyceride lipase (*ATGL*) expressions. **A.** and **B.** *FAS* and *ATGL* mRNA expressions in primary adipose cells; **C. D. E. F.** FAS and ATGL protein expressions in primary adipose cells. *Significant difference ($P < 0.05$). **Significant difference ($P < 0.01$).

DISCUSSION

Research on lipid metabolism is meaningful, as it is closely related to many diseases such as obesity, hepatic steatosis, coronary heart disease, and so on. With regard to the current study, it had already been found that GH significantly promotes lipolysis by reducing glucose transport and lipid synthesis, and repressing the transcription of *FAS* in both *in vivo* and *in vitro* experiments (Louveau and Gondret, 2004). It also has been reported that GH induces a high level of expression in the SOCS family, especially *SOCS2*, a stable longtime high level expression (Paul et al., 2000; Greenhalgh et al., 2005). Flores-Morales et al. (2001) and Rico-Bautista et al. (2004) confirmed this; they also found that GH could induce a stable high level of *SOCS2* expression and that other SOCS family members just showed a little increase in expression, which gradually weakened. Further studies have shown that *SOCS2* is a negative regulator of GH signaling, resulting in inhibition of intestinal epithelial cell proliferation, which is induced by GH and IGF-1 (Miller et al., 2004), the negative action of estrogen on GH signaling, which is caused by an upregulation of *SOCS2* (Leung et al., 2003), and the blocking of GH-dependent inhibition of neural stem cell differentiation (Turnley, 2005). It is also known that glucocorticoids have negative effects on somatic growth and enhance GH-induced *SOCS2*

expression in primary hepatocytes (Tollet-Egnell et al., 1999; Sun et al., 2012b), suggesting *SOCS2* as the mediator of the negative effects of glucocorticoids on GH action.

All these results are consistent with our study that GH improved expression of *SOCS2* steady (Figure 4C and D), while the *SOCS2* gene negative feedback regulated GH signaling pathway at the same time.

At present, the specific role of both the interaction between the *SOCS2* gene and the GH signaling pathway and its mechanism of fat metabolism are poorly researched. To further investigate the mechanism of *SOCS2* on GH regulation of fat cell lipid metabolism, we constructed an overexpression vector with pAd-*SOCS2* to study the impact of GH-induced fat metabolism. To facilitate a follow-up test, a flag tag sequence was added in the downstream primer of amplified *SOCS2* CDS. HEK293 cell density was required to be more stringent when pAd-*SOCS2* was packaged. We found that the HEK293 cells showed a higher infection efficiency when confluence was 60-70%. The degree of cell confluence should reach 90% when the virus is propagated for more efficient infection; the good state of cells was the main factors in the entire packaging and propagation process. The final virus titer was 1.2×10^9 PFU/mL.

Specifically, mRNA and protein expression of *FAS* and *ATGL* in swine primary adipocytes was significantly increased within 0.5 h after treatment with GH, which was in line with the research of Stewart et al. (2004) who showed that GH induced the expression of *FAS* in 3T3-L1 cells. However, another previous study showed that expression of *FAS* was not affected with GH treatment at either the mRNA or protein level (Kawai et al., 2007). This difference may be due to different cell types or detection times. Further results indicated that the *SOCS2* gene may regulate fat metabolism through the GH signal pathway, because overexpression of the *SOCS2* inhibited the expression of *ATGL* and *FAS*, which should have been increased by the treatment with GH. However, the specific mechanism of this inhibition is still uncertain.

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