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RNA interference of *leptin receptor* in chicken adipocytes

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ABSTRACT. In this study, chicken adipocytes were cultured to evaluate RNA interference by the *leptin receptor* gene. A small interfering RNA of the *leptin receptor* gene was synthesized, with a suppression rate of 60% being generated ($P < 0.01$). After the knockdown of the leptin receptor, the expression levels of certain genes decreased significantly; specifically, *peroxisome proliferator-activated receptor γ*, *fatty acid synthase*, *adipose triglyceride lipase*, and *lipoprotein lipase*. In addition, a significant increase in the expression of the *adiponectin* gene was documented. These results demonstrate that the *leptin receptor* gene might contribute to lipid metabolism by influencing the expressions of the *peroxisome proliferator-activated receptor γ*, *fatty acid synthase*, *adipose triglyceride lipase*, *lipoprotein lipase*, and *adiponectin* genes.

Key words: RNA interference; Leptin receptor; Chicken; Adipocyte; Lipid metabolism

INTRODUCTION

The control and prediction of fatness of farm animals is of high economic interest. Fat deposition, particularly intramuscular fat, affects the tenderness and juiciness of meat (Wood et al., 2008). However, the exaggerated development of adipose tissue in farm animals negatively affects whole-body metabolism, production efficiency, reproduction, and meat quality. The rapid growth rate of chickens leads to the deposition of excessive body fat and the associated impairment of total-body metabolism, in addition to disorders of reproductive function and muscular development, resulting in low performance with high mortality.

Leptin is an adipocyte-derived hormone that regulates the feeding behavior and energy homeostasis of mammals (Campfield et al., 1995). Leptin is present as a protein in chicken tissues (Ohkubo et al., 2007), and is primarily produced in adipose tissue. It has been proposed that circulating leptin exerts a profound effect on the appetite and body weight of animals by directly binding to receptors in the central nervous system (Lord et al., 1998). The reduction of food intake due to leptin has been demonstrated by the administration of recombinant leptin to both rodents and humans (Flynn and Plata-Salaman, 1999). Blood leptin levels are positively correlated with fat-pad size or adiposity. Therefore, leptin may be considered an "adipostat" that regulates and reflects body energy stores, linking fat deposits with the central nervous system. The leptin signal is mediated through a specific receptor that is localized in target tissues. The leptin receptor (OB-R) belongs to the class I cytokine superfamily (Tartaglia et al., 1995), which mediates multiple physiological functions in mammals. To obtain a complete understanding of the effects of leptin, it is necessary to study the functions of OB-R. Chicken OB-R, which exhibits 60% overall nucleotide sequence identity with mammalian OB-R, has been cloned in previous studies (Horev et al., 2000).

RNA interference (RNAi) is a process in which double-stranded RNA triggers the silencing of gene expression in a sequence-specific manner. This technique is becoming the method of choice for gene-function analysis of cells and whole organisms (Hannon, 2002).

Peroxisome proliferator-activated receptor γ (PPARγ) is the central regulator of adipogenesis. The regulatory role of PPARγ in adipogenesis has been illustrated more directly through both gain- and loss-of-function experiments. The ectopic expression of PPARγ in non-adipogenic cells, fibroblasts (Tontonoz et al., 1994), and myoblasts has been shown to promote the expression of a typical differentiation program, and adipogenesis upon treatment by specific agonists, such as thiazolidinediones. PPARγ is a master transcription factor of the adipocyte lineage. This factor induces the expression of a terminal differentiation program. fatty acid synthase (FAS) is a key enzyme of lipogenesis and may play an important role in the weight variability of abdominal adipose tissue (Marrube et al., 2004). Adipose triglyceride lipase (ATGL) hydrolyzes the first ester bond of stored triglycerides, which release non-esterified free fatty acids (Zimmermann et al., 2004). In chicken, ATGL is predominantly expressed in fractionated chicken adipocytes (Lee et al., 2009). Lipoprotein lipase (LPL) can catalyze the hydrolysis of the triacylglycerol component of circulating chylomicrons and very-low-density lipoproteins (Mead et al., 2002). Adiponectin is an adipokine that is abundantly expressed in the adipose tissues in mammals (Kadowaki and Yamauchi, 2005). It plays a dominant role in lipid and carbohydrate metabolism. Adiponectin is also involved in the regulation of energy balance and body weight (Fruebis et al., 2001).

In the current study, small interfering RNA (siRNA) was synthesized to determine the

function of OB-R in the adipocytes of chicken. To investigate the function of the OB-R gene in chicken adipocytes, the gene expression levels of *PPARγ*, *FAS*, *ATGL*, *LPL*, and *adiponectin* were evaluated after knockdown of the OB-R gene.

MATERIAL AND METHODS

Cell culture

Chicken preadipocytes were prepared by the method of Ramsay and Rosebrough (2003), with some modifications. Abdominal adipose tissue was collected from 10-day-old broiler chicks by sterile dissection following rapid decapitation. The adipose tissue was minced into fine sections by using scissors and incubated in 10 mL digestion buffer (PBS[-], 0.1% collagenase) for 1 h at 37°C in a water bath. After incubation, growth medium [Dulbecco's modified Eagle's medium (DMEM/F12), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and streptomycin] was added to the digestion flask. The flask contents were mixed and filtered through 100- and 25-μm mesh nylon screens to remove undigested tissue and large cell aggregates. The filtered cells were centrifuged at 300 *g* for 10 min, to separate floating adipocytes from the pellets of stromal-vascular cells. The stromal-vascular cells were then seeded on 6-well culture plates and in a 50-mL culture flask (Corning, CA, USA) at a density of 1 x 10^4 cells/cm^2, and cultured in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C until confluence (3-4 days), at which point differentiation was initiated and the experiments were started.

Synthesis of siRNA

siRNA was designed based on the OB-R sequence (GenBank Accession No. AF169827.2) by using the Ambion siRNA Design tool (http://www.ambion.com/techlib/misc/siRNA_tools.html). Sequences were selected to be in the coding sequence of the *OB-R* gene, and to contain a GC content between 30 and 52%. The gene-specific oligonucleotide sequences for *OB-R* are shown in Table 1.

Table 1. Sequence of siRNA.

Primer name	Primer sequence (5'-3')
Ob-R-1F	GGCUCUGUCUCCUUGAUAA
Ob-R-1R	UUAUCAAGGAGACAGAGCC
GAPDH-positive control sense	GUAUGACAACAGCCUCAAGTT
GAPDH-positive control anti-sense	CUUGAGGCUGUUGUCAUACTT
Negative control sense	UUCUCCGAACGUGUCACGUTT
Negative control anti-sense	ACGUGACACGUUCGGAGAATT

Transfection

The Lipofectamine 2000™ reagent (TransGen) was used as the transfection reagent. Twenty-four hours before transfection, chicken adipocytes were seeded on a 24-well plate containing DMEM/F12 and 10% FBS without antibiotics, to obtain 80 to 90% confluence on the day of transfection. Opti-MEM Reduced Serum Medium (50 μL) was used to dilute 20 pmol siRNA, and then 50 μL translipid dilution (containing 1 μL translipid, which was

incubated at room temperature for 5 min before mixing) was added to this mixture. The mixture containing the diluted siRNA and the translipid dilution was added to the cultured cells after being incubating at room temperature for 20 min. Mock-transfected cells (i.e., cells only transfected with the Lipofectamine reagent) served as negative control for the experiment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control to assess transfection efficiency. The Lipofectamine complexes were removed after 6 h, and fresh medium containing antibiotics and FBS was added to the plates.

Real-time RT-PCR analysis

Twenty-four hours after transfection, total RNA was extracted from chicken adipocytes using Trizol reagent (TaKaRa, Tokyo, Japan). From 10 μL RNA, cDNA was prepared using a reverse transcriptase (TaKaRa, Tokyo, Japan). The expression levels of *OB-R*, *PPARγ*, *FAS*, *LPL*, *ATGL*, and *adiponectin* in chicken adipocytes were determined using quantitative RT-PCR. Real-time RT-PCR (ABI Prism 7300) was performed using the power SYBR green kit (Toyobo). The protocol for RT-PCR was 95°C for 10 s, 95°C for 15 s, and 60°C for 30 s (40 cycles). The primers used for quantitative RT-PCR are shown in Table 2.

Table 2. Primers for RT-PCR.

Primer name	Primer sequence
OB-R	F: 5'-GCATCTCTGCATCTCAGGAAAGA-3'
AF169827.2	R: 5'-GCAGGCTACAAACTAACAAATCCA-3'
PPARγ	F: 5'-CACAAGCGGAGAAGGAGAAG -3'
AF163811	R: 5'-TCAGCGGGAAGGACTTTATG -3'
FAS	F: 5'-CTTCGGTGCCTGTGGTTTAT-3'
J04485	R: 5'-ACCTCCTGAGCCAGAGTGAA-3'
LPL	F: 5'-ACCTGGTTCCTGGACAGATG-3'
NM_205282	R: 5'-TTGGGACCCAGCTTTCATAC-3'
ATGL	F: 5'-CCAAAGAAGCACGAAAGAGG-3'
EU240627	R: 5'-AATACCCAAACGTCCTGCTG
Adiponectin	F: 5'-GCCAGGTCTACAAGGTGTCA-3'
AY523637.1	R: 5'-CCATGTGTCCTGGAAATCCT-3'
β-actin	F: 5'-CTGTGCCCATCTATGAAGGCTA -3'
L08165.1	R: 5'-ATTTCTCTCTCGGCTGTGGTG-3'

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS. The results are reported as means ± SE, and a P value of <0.05 was considered to be significant, while a P value of <0.01 was considered to be highly significant.

RESULTS

Morphology of adipocytes and observation of transfection rates

The chicken adipocytes were observed under an inverted microscope (Nikon Eclipse TE-2000U, Tokyo, Japan) and cells were photographed, and the images are shown in Figure

1A. The transfected adipocytes were observed under a fluorescence microscope, and the images are shown in Figure 1B. As shown in Figure 1B, the transfection rate was high.

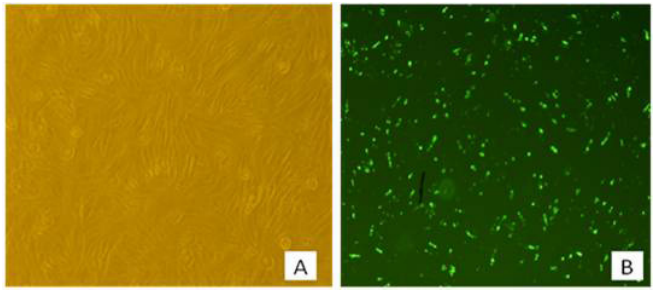

Figure 1. A. Chicken adipocytes before transfection (200X). **B.** GAPDH-positive control (200X) after transfection.

Expression levels of lipid metabolism-related genes after interference of the *OB-R* gene

The expression level of the OB-R mRNA decreased by 60% ($P < 0.01$; Figure 2), indicating the successful interference of the *OB-R* gene. Expression levels of *PPARγ*, *FAS*, *LPL*, and *ATGL* decreased by 19% ($P < 0.05$), 52% ($P < 0.01$), 39% ($P < 0.05$), and 51% ($P < 0.05$), respectively (Figure 2). The expression level of *adiponectin* increased by 69% ($P < 0.01$; Figure 2).

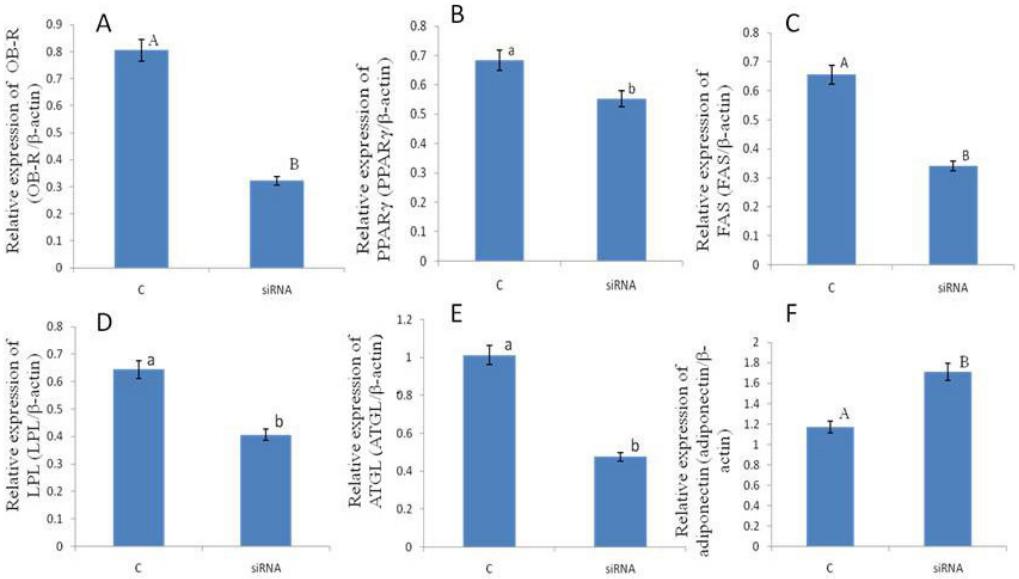

Figure 2. Effect of OB-R gene interference on abundance of OB-R. **A.** PPARγ, **B.** FAS, **C.** LPL, **D.** ATGL, **E.** adiponectin, **F.** mRNA. Relative expression levels of OB-R, PPARγ, FAS, LPL, ATGL, and adiponectin were determined by quantitative RT-PCR, normalized to that of β-actin. Each column represents means ± SE from three replicates. Different lowercase letters mean $P < 0.05$; different uppercase letters mean $P < 0.01$.

DISCUSSION

Leptin exerts its effects via OB-R on various bodily processes, such as energy metabolism, procreation regulation (Cassy et al., 2003), and nervous system development (Bouret, 2008). However, studies remain limited about the function of OB-R on chicken adipocytes. Therefore, in this experiment, RNAi was used to investigate the function of OB-R on chicken adipocytes. After the knockdown of the *OB-R* gene, expression levels of *PPARγ*, *FAS*, *LPL*, and *ATGL* decreased significantly. PPARγ is an important nuclear regulatory factor. Both cultures of adipocytes *in vitro* and studies using animals have shown that free fatty acids act in combination with PPARγ in adipocytes to increase the expression levels of acetyl-coenzyme A, fatty-acid transporter protein, LPL, and fatty acid-binding protein (Lapsys et al., 2000). The results of the present experiment showed that the interference of *OB-R* might suppress PPARγ activation, causing it to influence cell differentiation and further suppress the expression of *FAS*, *LPL*, and *ATGL*. PPARγ increases the mRNA levels of ATGL in subcutaneous and visceral white adipose tissue by directly acting on gene expression (Festuccia et al., 2006). In this experiment, the expression of the *ATGL* gene decreased with a decline in the expression of the *PPARγ* gene. OB-R might influence the expression of *FAS*, *LPL*, and *ATGL* through changes in the expression level of *PPARγ*.

Adiponectin is a cytokine that is secreted by adipocytes. In mammals, adiponectin causes insulin supersensitivity, increases the oxidation of fatty acids and the absorption of glucose in skeletal muscle cells, and suppresses the synthesis of glucose in the liver. The expression level of adiponectin is negatively correlated with that of leptin, which is an important regulatory factor in lipid metabolism and of blood glucose levels. In this study, the suppression of the *OB-R* gene significantly increased the gene expression of *adiponectin* in chicken adipocytes. The overexpression of adiponectin has been shown to decrease the amount of free fatty acids and triglycerides in mouse blood (Yamauchi et al., 2003). However, because studies on this subject remain limited, further research is required to investigate the function of OB-R in chicken metabolism. In conclusion, OB-R might have an important role in lipid metabolism by influencing the expression of *PPARγ*, *FAS*, *LPL*, *ATGL*, and *adiponectin*.

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