RECOMBINANT LENTIVIRUS VECTOR TARGETING BOVINE *LXRA* MRNA AND ITS SILENCING EFFECTS ON BOVINE MUSCLE SATELLITE CELLS

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ABSTRACT

Background: The liver X receptor α (*LXRα)* is a member of the nuclear hormone receptor superfamily which could regulate the transcription of the genes involved in cholesterol transportation. In this study, Lentiviral vector containing *LXRα*shRNAs were constructed and transferred in bovine muscle satellite cells. The results indicated that *LXRα* down regulated *PPARα*, *PPARγ*, *ABCA1*, *LPL*, *ApoE* genes after 48 h post-pLenti-03 viruses' infection. The silencing effects of bovine*LXRα* gene on related were significant. This work identifies bovine *LXRα* role in cholesterol metabolism.

Key word: bovine *LXRα* gene, lentivirus, vector construction, gene silencing.

INTRODUCTION

Obesity, hypertension**,** dysmetabolism are diseases caused by unhealthy diet custom which influenced human's living quality. Thus, a low-cholesterol lifestyle is necessary. Currently, people have paid much attention to balancing their diet by low-intake of cholesterol. Producing good quality meat with low-fat (beef, mutton, pork, etc.) is a vital way to provide people with healthy meals condition.

The liver X receptor α (*LXRα, NR1H3*) and *LXRβ* (*NR1H2*) are members of the nuclear hormone receptor superfamily. *LXRα* is abundantly expressed in the liver, intestine, adipose tissue, kidney and immune macrophages, whereas *LXRβ* is ubiquitously expressed (Thomas *et al*.2013; Savkur*et al*. 2004). LiverX receptors (*LXRs*) are activated in response to intracellular lipid accumulation, which could regulate transcription of an array of genes involved in the regulation of cholesterol homeostasis and reverse cholesterol transport (Zelcer*et al*. 2006; Lund *et al*.2003).Cholesterol homeostasis is intricately regulated by a battery of transcription factors among which *LXRs* are nuclear receptors that play a crucial role in transcriptional regulation of lipid metabolism and inflammation (Ostlund 1993) Activated *LXRs* form a heterodimer with the retinoid X receptor α (*RXRα*), which binds to *LXR* responsive elements(*LXREs*) and consequently promotes target gene expression (Zelcer2006). *LXR/RXR* heterodimers are characterized by the ability to be activated by ligand in an independent manner. Thus, *LXR/RXR* heterodimers are activated by the *RXR* ligand, e.g., 9-cisretinoic acid, and the *LXR* ligands, e.g. oxysterols, or are activated synergistically in the presence of ligands for both receptors (Willy *et al*.1995). Recent studies showed that *LXRα* is considered as a critical regulator in cholesterol homeostasis in macrophages, which could regulate several genes involved in cholesterol transport, such as the ATP-binding cassette trans-porters (*ABCs*), *ABCA1*, *ABCG1*, apolipoprotein E (*ApoE*) and lipoprotein lipase (*LPL*) (Vinoda*et al*. 2014).

ABCA1, *ABCG1* and *ApoE* are all target genes regulated by *LXR*(Ishimoto *et al*. 2006). Besides, peroxisome proliferator-activatedreceptor-alpha (*PPARα*) can activate the cytochrome p450 enzymes, resulting in some of the hydroxyl cholesterol as *LXRα* endogenous ligand further activate *LXRα*, which further activating *ABCA1* regulated cholesterol efflux. Recent studies indicated that*PPARγ* enhances cholesterol efflux by inducing the transcription of *LXRα*(Soumian*et al*. 2005).*PPARγ* also induces the expression of *ABCA1* and *ABCG1*, and promotes cholesterol efflux from macrophages through a transcriptional cascade mediated by *LXRα*(Chawla *et al*. 2001; Wong *et al*. 2008; Hu *et al*. 2013). *LXRα* can also activate *LPL*. Some studies have revealed that cholesterol-induced *LPL* gene expression in the liver is directly regulated by *RXR/LXR* heterodimers in a tissue-specific manner, which is mediated predominantly by *LXRα* in vivo (Zhang *et al*. 2006).

LXRα is important for cholesterol metabolism in human and mice. Bovine *LXRα* gene also controls the cholesterol level and the outflow of cholesterol efflux in muscle. Given that the importance of *LXRα* gene, regulating cholesterol in metabolism, exploring *LXRα* gene function in cholesterol metabolism in muscle cells to balance the cholesterol content and improving the quality of beef are of great significance. Packaging and proliferating lentivirus with RNA interference (RNAi) technique is a conserved biological response to double-stranded RNA (Hannon 2002), RNAi is a sequence-specific process that regulates genetic functions and

provides defense against virus at the post-transcriptional level in mammalian cells and animals(Song *et al*. 2003; Jacque *et al*.2002). Lentivirus vector is a kind of inactivated HIV virus with many advantages, such as high transfection rate, stable expression in target cells and good security, so it has been wildly used in transgenic research as a crucial tool.

Lentiviral expression vector is a kind of long-acting system which can infect host cells with high-efficiency. Thus, we use this mediation system to achievefunctional gene research on bovine muscle satellite primary cells. Considering the importance of*LXRα* gene and related study on bovine has not been reported yet.We hypothesized that*LXRα*might influence some cholesterol metabolism regulating genes. To address this hypothesis, wesuccessfully construct virus vector in order to transfect bovine cells and detect some cholesterol regulation genes. To our knowledge, this study first demonstrated the interference of *LXRα* on bovine muscle satellite cells *in vitro* to investigate*LXRα*gene's function and its target genes. Furthermore, it can provide us with valuable information for further studying of bovine *LXRα* gene mechanism.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals and biochemicals used in this study are tested for cell culture and are of molecular biology grade.

Cell culture: HEK 293T Cells, the bovine muscle satellite cells were given by NBCIC(National Beef Cattle Improvement Center, Northwest A&F University, Yangling, China), and identified with cell markers by Immunofluoresence staining, cultured in Dulbecco's modified Eagle's medium (DMEM, gibco) with stable L-glutamine supplemented with 20% fetal bovine serum (FBS, gibco), 10% horse serum (HS，gibco), 100 U/ml penicillin（sigma）, 100 μg/ml streptomycin（sigma）(complete growth medium)， incubated at 37 °C under 5% CO_2 atmosphere（Thermo）.

Design of shRNA sequence and system: The sequence of bovine *LXRα* (NM_001014861.1) in GenBank. Interference shRNA sequences were designed on online software. Four shRNA oligos and the shRNA-NC sequence oligo were shown in table 1.Synthetic single-stranded DNA oligo was diluted with ddH_2O. Then, we mixed 0.5μl of each of top strand oligo and bottom strand oligo and 2μl 10×oligo annealing buffer, adding 8μl ddH_2O up to a final volume of 20μl to anneal. Oligo mixture was heated at 95ºC for 5min,and then placed at room temperature for 20 min forming double-stranded DNA. Synthetic double-stranded DNA was diluted to10nM with ddH_2O. The reaction mixture containing 4μl 5× ligation, 2μl pcDNA 6.2-GW/EmGFP-miR, 4μl 10nM ds oligo, 1μl 1U/μl T4 DNA ligase and 9μl ddH_2O was placed for 30min at room temperature. The products were then transformed into *E.coli* DH5α competent cells. Monoclonal colony was selected. Next, plasmids were extracted and sequenced. The four plasmids (14MR0054-01~14MR0054-04) were respectively recombined into pDONR221 vector by Invitrogen BP carrier recombination system. 100μl of DH5α competent cells was transformed by 5μl recombination reaction solution. Positive clones were screened and sequenced. Purpose sequence was further restructured to pLenti6.3/V5-DEST vector by LP restructuring system. DH5α competent cells were transformed by recombination reaction solution again; positive clones were selected and sequenced. Our results proved that lentiviral vector carrying interference sequence has been successfully achieved. The names of the four lentivirus vectors were shown in table 2.

Packaging lentivirus: When the 293T cells reached 70%-80% confluence, cells were plated at 1.0×10^6 cells per 10cm cell culture dish and cultured overnight. The media canbe removed directly before transfection. 9μg of Packaging Mix and 3μg of the lentiviral expression plasmids were added to 1.5mlOpti-MEM medium that was then preheated at 37ºC. 36μl lipofectamine 2000 was added to 1.5mlOpti-MEM medium and mixed gently; then, the mix was kept at room temperature for 5min. Next, we mixed plasmid solution and lipofectamine 2000 diluent and put them at room temperature for 20min. Then, 3ml of Plasmid liposome complex was added into cell dish and incubated at 37°C in 5% CO_2 for 6h,after which we removed the primary medium and replaced it with fresh DMEM with 10% FBS. The supernatant was collected at 48h by centrifugation at 3000 rpm/min for 10 min and filtrated by 0.45μm membrane filter unit. The virus stock solution was ultracentrifuged at 50000g for 2h; the supernatant was removed and resuspended in opti-MEM culture medium to determine the titer. Lentiviral stocks should be aliquoted and stored at -80°C.

Determination of viral titers: HEK293T cells were seeded into 96-well plate at a density of 8×10^3cells per well. Lentivirus was diluted with DMEM supplemented with 2% FBS and 8μg/ml Polybrene (DFP medium).The details of the viral stock dilution were shown in table 3. Then, we carefully removed the culture medium from 96-well plate and replaced it with 100μl diluted virus solution. The cells were incubated in at 37°C with 5% CO_2. After transfection for 24 hours, GFP expression was observed under fluorescence microscope, virus titer was calculated after transfection for 96 hours. Lentivirus vectors of pLenti-01~pLenti-04 were transduced into HEK293T cells, which were screened under the same view in fluorescent and bright fields of microscope(Fig.1).

Determination of MOI value: The bovine muscle satellite cells were maintained in DMEM supplemented with 20% of FBS and 10% HS. After the confluence, the cells were trypsinized and counted, 1×10^4 cells were seeded in 96-well plates with 2ml of medium, which were incubated overnight. According to MOI value of 2, 5, 10, 20, 50, 100,200, 300, we diluted lenti6.3-RNAi with DMEM supplemented with 2% FBS and 8μg/ml polybrene. Cells were lightly washed once with Phosphate Buffered Saline (PBS) and lentivirus liquid with different MOI values was added to the corresponding wells respectively. After 72 hours, the expression of GFP was observed.

RNA extraction: Total RNA was extracted from bovine muscle satellite cell samples using Trizol buffer, and the following reverse was performed using a High-capacity cDNA Reverse Transcription Kit.

qRT-PCR: The qPCR reaction system was 17.3μl ultra pure water, 2.5μl10×PCR buffer, 2μl magnesium ions, 0.2μl dNTPs, 0.5μl primers, 0.5μl 50×sybr, 1.2μl cDNA and 0.3μl of Taq DNA polymerase. The PCR amplification conditions were:2min at 95°C; 40cycles of 95°C for 10s, 30s at 60°C and 45s at 70°C.The expression level of mRNA was determined by cycle threshold (Ct) normalized with that of PPP1R11 using the $2^{-\Delta\Delta Ct}$ formula. Samples transfected with negative interference vector were used as a control.

Data Analysis:SYBR Green and cDNA were used for Quantitative Real-time PCR(RT-PCR).The expression level was quantified via $2^{-\Delta\Delta Ct}$. Analytical data was normalized to the mRNA expression level of endogenous control β-actin. Primer sequences were described in table 4.

RESULTS

Lentiviral packaging, the titer determination results: 293T cells were first transfected with lentivirus vectors for 24 hrs, and then observed under fluorescent (Fig. 1A-D) and light (Fig. 1 a-d) microscopes. Significant GFP could be found after transfection with virus in 100μL at 2×10^{-8} ml. The virus titers for Lenti-14MR0054-01, -02, -03 and -04 were 3×10^8, 2×10^8, 2.5×10^8 and 3.5×10^8 TU/ml, respectively.

LXRα mRNA expression: Bovine muscle satellite cells were infected with pLenti-01, -02, -03 and -04 lentiviruses for 48 h, and then these cells were collected qRT-PCR analysis. We found that the *LXRα* mRNA expression levels were decreased to 0.46, 0.23, 0.12 and 0.29 as compared to negative control lentivirus. The silencing efficiencies of the four viruses were 54%, 77%, 88% and 71%, respectively (Fig. 2).

LXRα related genes mRNA expression: To explore the effects of *LXRα* knockdown on *LXRα* related genes, the expression of *PPARα, PPARγ, ABCA1, LPL, RXRα*and *apoE* were also determined with by qRT-PCR analysis, and the results were summarized in fig. 3. As compared with negative control group, the mRNA expression levels of *PPARα, PPARγ, ABCA1, LPL* and *apoE*were decreased in bovine muscle satellite cells when *LXRα* was inhibited, whereas that of *RXRα* was increased (Fig. 3). *LXRα* played a negative role in regulating. The results showed that up-regulation of *RXRα* expression was involved in *LXRα* suppression; therefore, the expression of *LXRα* and *RXRα* were negatively correlated.

The interference efficiency of pLenti-03 vector was 88%, which was the best among all the vectors. The relative expression of the six genes regulated by pLenti-03 was shown in fig. 4.Compared with the negative control group, except *RXRα*, the expression level of the other five genes decreased less than one. The related genes decreased by *LXRα* were in the order of *PPARγ>PPARα >LPL>apoE>ABCA1*. The relative expression of *RXRα* gene was higher than the negative control and was up-regulated. Hence, the silencing effects of bovine*LXRα* gene on *PPARα, PPARγ, ABCA1, LPL, RXRα* and *apoE*were significant.

DISCUSSION

Bovine muscle satellite cells were used in our experiment. the positive rate of GFP was very high when cell was infected by adenovirus with MOI value 3000, but the positive of cellular fluorescence intensity was very low. The reason for this wasthat adenovirus cannot be applied to infect bovine muscle satellite cells. However, the positive rate of the cells was very high when lentivirus infecting bovine muscle satellite cells with MOI 300. Although the positive rate was low and cellular fluorescence intensity was weak,which also have an obvious effect on the cellular shape. Lentiviral vector was developed basis on the gene therapy of the human immunodeficiency virus and have the high efficiency for infection and stable silencing target gene, therefore, it was used widely (Wang *et al*. 2012; Zhao *et al*. 2013). Lentivirus, as gene transfer vector, was characterized by high transfection efficiency in non-dividing cells and dividing cells, was high titer, good stability in target cells and little immunoreactivity, and has been widely used in transforming into vector of genetic engineering (Koh*et al*. 2009).Compared to another carrier lentiviral vector, it has its unique advantages: for some difficult to transfect cells which has a high infection rate for primary cells, stem cells and undifferentiated cells, and the genetic recombination could not occur and the vector could be stably expressed.

In this study, we found that *LXRα* genes were down-regulated by eighty-eight percent after lentivirus infecting bovine muscle satellite cells for 48 h, the

expression of *ABCA1* and *ApoE* were also decreased. Meanwhile, *LXRα* could increase the expression of ATP-binding cassette transporter protein and promote cholesterol efflux. Additionally, after activating *LXRα* in macrophages, the expression of *ABCA1, ABCG1* and *ApoE* also increased. *LXR* and *PPAR* pathway coupling increased expression of ABCA1and regulated lipid intake and reversed transport (Tobin *et al*. 2000). *LXRα* genes might decrease the expression of*ApoE*. Compared with the control group, the expression of *PPARγ* was decreased in bovine muscle satellite cells. When the expression of *LXRα* gene is increased, more heterodimers could be formed by *PPARγ* and *RXR*, inhibiting *PPARγ*and *RXR* forming heterodimers and decreasing the expression of *PPARγ*(Yoshikawa*et al*. 2003).Therefore, when *LXRα* was down-regulated, *LXRα* and *PPARγ* could form a competitive mechanism, which caused decreased expression of *PPARγ*.However, the mechanism of up-regulated expression of *RXRα* has not been identified and the further study should be remained and investigated. It is of great significance to study intracellular lipid metabolic through interfering *LXRα* gene. Lentivirus interfering vector lays the foundation for further studying *LXRα* gene function in lipid metabolism in bovine muscle satellite cells.

Conclusion: lentiviral vector carrying the shRNA targeting *LXRα* gene was successfully constructed, and the lentiviral vector can be efficiently expressed in bovine muscle satellite cells. We also explored the effects of *LXRα* gene silencing on the metabolic associated genes. This study provides new insights into the regulation of bovine *LXRα* in cholesterol metabolism.

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