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

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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) 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 cholesterol transport gene, ABCA1-LPL-ApoE genes after 48 h post-pLenti-03 viruses’ infection. The silencing effects of bovine LXRs gene on related were significant. This work identifies bovine LXRα role in cholesterol metabolism.

Key word: bovine LXRα gene, lentivirus, vector construction, gene silencing.
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 achieve functional 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, we successfully 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 Immunofluorescence 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), 100U/ml penicillin (sigma), 100µg/ml streptomycin (sigma) (complete growth medium), incubated at 37°C under 5% CO2 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 ddH2O. Then, we mixed 0.5µl of each of top strand oligo and bottom strand oligo and 2µl 10×oligo annealing buffer, adding 8µl ddH2O up to a final volume of 20µl to anneal. Oligo mixture was heated at 95°C for 5 min, and then placed at room temperature for 20 min forming double-stranded DNA. Synthetic double-stranded DNA was diluted to10nM with ddH2O. The reaction mixture containing 4µl 5× ligation, 2µl pcDNA 6.2-GW/Em GFP-miR, 4µl 10nm ds oligo, 1µl 1U/µl T4 DNA ligase and 9µl ddH2O 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 pLentis6.3/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 1.0×10⁴ cells per 1cm cell culture dish and cultured overnight. The media can be 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 37°C. 36µl lipofectamine 2000 was added to 1.5ml Opti-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% CO2 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 10min and filtered by 0.45µm membrane filter unit. The virus stock solution was ultra-centrifuged at 50000g for 2h; the supernatant was removed and suspended in opti-MEM culture medium to determine the titer. Lentiviral stocks should be aliquoted and stored at -80°C.

Determination of viral titers: HEK 293T cells were seeded into 96-well plate at a density of 8×10³ cells per well. Lentivirus was diluted with DMEM supplemented with 2% FBS and 8µg/ml Polybrene (DPF 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% CO2. 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, 1×10⁴ 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 and 300, we diluted lentivirus 6.3-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 ultrapure water, 2.5μl 10×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⁻ΔΔCt 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⁻ΔΔCt. 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 hours, 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 2×10⁸ ml. The virus titers for Lenti-14MR005A-01, -02, -03 and -04 were 3×10⁸, 2×10⁸, 2.5×10⁸ and 3.5×10⁸TU/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 and analyzed by 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 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 PPARα>PPARγ>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 was that 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, 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 transfec cells which have a high inflection 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 88% after lentivirus infecting bovine muscle satellite cells for 48h, 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 ABCA1 and 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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