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Efficient construction of recombinant adenovirus expression vector of the Qinchuan cattle *LYRM1* gene

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ABSTRACT. In this study, we cloned the coding DNA sequence (CDS) region of Qinchuan cattle LYR motif-containing 1 (*LYRM1*) and constructed a recombinant adenovirus expression vector to examine the function of LYRM1 on the cellular level. Total RNA was extracted from the adipose tissue of Qinchuan cattle, cDNA was obtained by reverse transcription, and polymerase chain reaction was used to amplify the CDS region of the *LYRM1* gene. The CDS-containing fragment was inserted into the shuttle vector pAdTrack-CMV to construct pAdTrack-CMV-LYRM1 vector. After linearization of pAdTrack-CMV-LYRM1 and the negative control vector pAdTrack-CMV by restriction endonuclease *Pme*I, the vectors were transformed into *Escherichia coli* BJ5183 containing pAdEasy-1 to obtain the recombinant adenovirus vector pAd-LYRM1 and pAd-CMV through homologous recombination. pAd-LYRM1 and pAd-CMV were then digested by *Pac*I and transfected into the 293A cell line. The recombinant adenovirus Ad-LYRM1 and Ad-CMV was obtained at a concentration of 7 x 10^8 and 1.3 x 10^9

green fluorescent units/mL, respectively. Preadipocytes derived from Qinchuan cattle were separately infected with Ad-LYRM1 and Ad-CMV. Quantitative real-time polymerase chain reaction demonstrated that the expression of LYRM1 was increased by approximate 28,000-folds after the infection with recombinant adenovirus for 48 h. In conclusion, we successfully cloned the CDS region of the Qinchuan cattle *LYRM1* gene, constructed the recombinant adenovirus expression vector, and obtained the adenovirus with high titer, providing valuable materials for studying the function of LYRM1 at the cellular level.

Key words: Qinchuan cattle; Adenovirus; LYRM1; Preadipocytes

INTRODUCTION

Obesity is a complex, common, and multifactorial disorder resulting from interactions between genetic, environmental, and psychosocial factors (Lee, 2009; Walley et al., 2009). It is associated with many severe diseases, including cardiovascular disease, heart disease, work disability, and diabetes (Gordon et al., 1977; Mokdad et al., 2003; Rahmouni, 2010). The global obesity prevalence is increasing at an alarming rate and is predicted to influence more than one billion people by 2020 (Flier, 2004). In the recent decades, obesity has become an important worldwide public health problem (Jeffery and Sherwood, 2008). Identifying the genes involved would help to illuminate the genetic basis of obesity. LYR motif containing 1 (*LYRM1*) is a highly expressed novel gene identified in the adipose tissue of obese patients. Previous studies have suggested that it can promote proliferation and inhibit apoptosis of preadipocytes (Qiu et al., 2009a; Zhu et al., 2010), indicating that it plays a significant role in human obesity. Over-expression of LYRM1 can induce mitochondrial impairment in 3T3-L1 adipocytes and inhibit glucose transport in rat skeletal muscles (Cao et al., 2010; Kou et al., 2011). Our previous study demonstrated that LYRM1 was related to the growth and meat quality traits of Qinchuan cattle (Li et al., 2013). However, few studies have examined the *LYRM1* gene in cattle. The molecular mechanism of LYRM1 regulating growth and fat deposition remains unclear.

Therefore, in the present study, we examined various aspects of the *LYRM1* gene. First, we successfully cloned the cDNA sequence of the Qinchuan cattle *LYRM1* gene. Next, we constructed a recombinant adenovirus expression vector and transfected the adenovirus vector into the 293A cell line. Virus containing the *LYRM1* expression vector with high titer was obtained. Finally, the virus was utilized to infect preadipocytes derived from Qinchuan cattle to verify the overexpression efficiency of the recombinant adenovirus vector. Our results provide valuable basic material for studying the molecular function of the *LYRM1* gene at the cellular level to determine potential mechanisms that could be used to improve growth and meat quality traits of cattle. Our results also provide valuable insights into the molecular mechanisms leading to human obesity.

MATERIAL AND METHODS

Cloning and sequencing of the Qinchuan cattle *LYRM1* gene

cDNA was obtained using the iScript First Strand Synthesis System Kit (Bio-

Rad, Hercules, CA, USA) for reverse transcription-polymerase chain reaction (PCR) with 500 ng total RNA. Total RNA was extracted from the adipose tissue of Qinchuan cattle using the Trizol method (Li et al., 2009). High-fidelity polymerase KOD-Plus-Ver.2 (Toyobo Life Science Department, Shanghai, China) was used to perform the following PCR. According to the multiple cloning site of pAdTrack-CMV, the recognition sequence for the restriction endonucleases *Bgl*II and *Sal*I and their corresponding protective bases were respectively introduced into the forward and reverse primers (forward primer: 5'-GAAGATCTGCCACCATGACAATGGCAACACGACAAG-3'; reverse primer: 5'-ACGCGTCGACCTAGTTGGCTCATCATTCTCAG-3'). The recognition sequence is underlined, before which the corresponding protective bases of the 2 enzymes were included. PCR amplifications were performed in 20-μL reaction mixture containing 1.2 μL 50 ng/μL cDNA, 0.6 μL 10 μM primer, 1.2 μL 25 mM $MgSO_4$, 2 μL 2 mM dNTPs, 2 μL 10X PCR buffer, 04. μL 1 U/μL KOD-Plus, and 12 μL ddH_2O. The PCR protocol was as follows: 95°C for 5 min followed by 35 cycles at 95°C for 30 s, annealing at 60.1°C for 30 s, and 72°C extension for 40 s, followed by a final extension at 72°C for 10 min. The products for sequencing were first electrophoresed on 1.5% agarose gels, and then purified using Axygen kits (BMI Fermentas, Vilnius, Lithuania), and sequenced in both directions in an ABI PRIZM 377 DNA sequencer (Perkin-Elmer, Waltham, MA, USA). The sequence maps were analyzed using the SeqMan software.

Construction and identification of shuttle vector

The restriction endonucleases *Bgl*II and *Sal*I were used to digest the PCR products and the empty shuttle vector pAdTrack-CMV separately, and the fragments were then recycled from the gel and utilized for ligation. The ligation reaction included the following: 5 μL digested PCR products, 1 μL linear vector, 1 μL 10X T4 DNA ligase buffer, 1 μL T4 DNA ligase, and 2 μL ddH_2O. The reaction was incubated at 16°C for 16 h and then transformed into *Escherichia coli* DH5α competent cells. The plasmids were extracted by Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA, USA). Next, the recombinant plasmid was identified by endonucleases *Bgl*II and *Sal*I. The digested products were separated by 1.5% agarose gel electrophoresis. Simultaneously, the plasmid was sequenced in both directions to accurately clarify the construction. The positive recombinant plasmid was named pAdTrack-CMV-LYRM1.

Construction and identification of recombinant adenovirus expression vector pAd-LYRM1 and negative control pAd-CMV

pAdTrack-CMV-LYRM1 and pAdTrack-CMV were first linearized by endonuclease *Pme*I. The digested products were separately transformed into *E. coli* BJ5183 containing pAdEasy-1 to obtain the recombinant adenovirus vector pAd-LYRM1 and pAd-CMV through homologous recombination. Next, the plasmids were extracted and the recombinants were identified by *Pac*I using a follow-up sequencing method for precise identification. Finally, we acquired the recombinant adenovirus expression vector pAd-LYRM1 and the negative control pAd-CMV.

Packaging and proliferation of adenovirus

pAd-LYRM1 and pAd-CMV were digested by *Pac*I. Next, we gel-extracted the digested products. The recycled products were separately transfected into the 293A cell line

with 80-90% confluence using TurboFect™ *in vitro* Transfection Reagent (Fermentas). Twenty-four hours later, we observed the cell morphology as well as the count and distribution of the green fluorescence using a fluorescence microscope. Adenovirus packaging and proliferation processes were observed each day. We collected the cell suspension containing the adenovirus until 50% of the cells had dropped off from the cell culture plate. Through repeated freezing and thawing of the suspension at -80° and 37°C, we obtained the virus supernatant by centrifuging the suspension at 3000 r/min for 5 min; the Ad-LYRM1 and Ad-CMV vectors were obtained.

The virus supernatant was used to infect the 293A cells with 90% confluence. When 50% of the cells had detached from the plate, we collected the cell suspension and obtained the virus with higher titer as described above. Next, we repeated the infection-freezing and thawing-collection process; adenovirus with higher titer was retrieved. The virus titer was measured according to the method of Lybarger et al. (1996) and Hitt et al. (2000).

Identification of adenovirus efficiency

The virus supernatant Ad-LYRM1 and Ad-CMV were used to infect Qinchuan cattle preadipocytes with 90% confluence, which was cultured in DMEM/F12 medium containing 10% fetal bovine serum. After 4 h, we replaced the culture medium with complete medium. Forty-eight hours later, we collected the cells, from which we extracted the RNA. Next, we performed reverse transcription to obtain cDNA. Quantitative real-time PCR were performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Hilen, Germany) according to the manufacturer instructions to detect the expression level of the target gene *LYRM1*. Glyceraldehyde 3-phosphate dehydrogenase was used as the control gene. Three technical replicates were performed for each product. Primer sequences are listed in Table 1.

Table 1. Primers for quantitative real-time PCR.

Gene	Sequence of primers	Size (bp)
LYRM1	F: TCAGGCTTGCGAAGAAATGG R: ATCAGGTCTGTGTCTGTGAG	130
GAPDH	F: CCAACGTGTCTGTTGTGGAT R: CTGCTTCACCACCTTCTTGA	80

RESULTS

Qinchuan cattle *LYRM1* gene cloning and sequencing

After total RNA was extracted from the adipose tissue of Qinchuan cattle, cDNA was obtained using reverse transcription. We amplified 410-bp fragment containing the coding DNA sequence (CDS) region of the *LYRM1* gene from the cDNA, which was used as the PCR template (Figure 1). The sequencing results revealed that the CDS region of the *LYRM1* gene was amplified successfully.

Construction and identification of recombinant adenovirus vector

The PCR products and the empty shuttle vector pAdTrack-CMV were separately di-

gested by the restriction endonucleases *Bgl*II and *Sal*I. The CDS region of the *LYRM1* gene was inserted into the linearized pAdTrack-CMV vector. The recombinant vectors pAdTrack-CMV-LYRM1 and pAdTrack-CMV were digested with *Bgl*II and *Sal*I, respectively; pAdTrack-CMV-LYRM1 showed 2 bands containing the target 410-bp fragment; pAdTrack-CMV, as a blank control, only displayed 1 band (Figure 2).

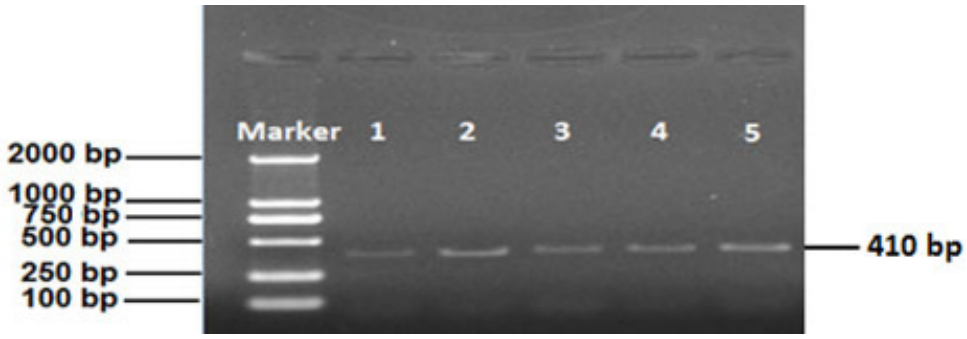

Figure 1. PCR products of the *LYRM1* gene. *Lanes 1-5* = Agarose gel electrophoresis of *LYRM1* gene PCR products.

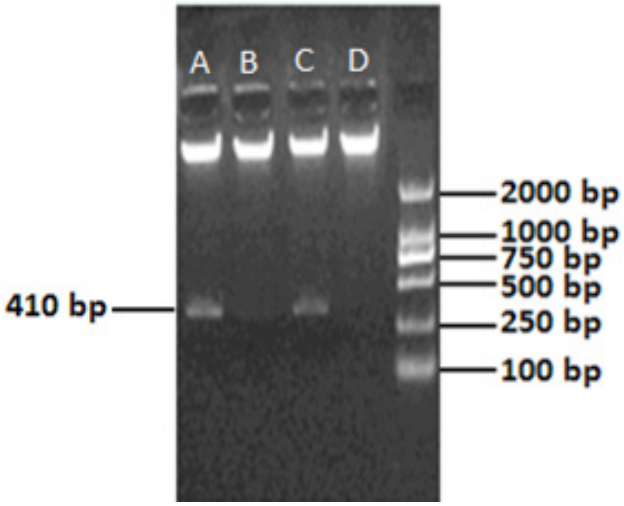

Figure 2. Identification of pAdTrack-CMV-LYRM1 and pAdTrack-CMV by restriction endonuclease *Bgl*II and *Sal*I. *Lanes A* and *C* = pAdTrack-CMV-LYRM1 digested by *Bgl*II and *Sal*I. *Lane B* and *D* = pAdTrack-CMV digested by *Bgl*II and *Sal*I.

After linearization by *Pme*I, pAdTrack-CMV-LYRM1 and pAdTrack-CMV were separately transformed into *E. coli* BJ5183 to obtain the recombinant adenovirus vectors pAd-LYRM1 and pAd-CMV. The recombinants were identified by digestion with *Pac*I, and both recombinants showed one 4.5- and one 30-kb bands (Figure 3). We also performed a follow-up sequencing method for precise confirmation of the results. Our results demonstrated that pAdTrack-CMV-LYRM1 and pAdTrack-CMV were successfully constructed.

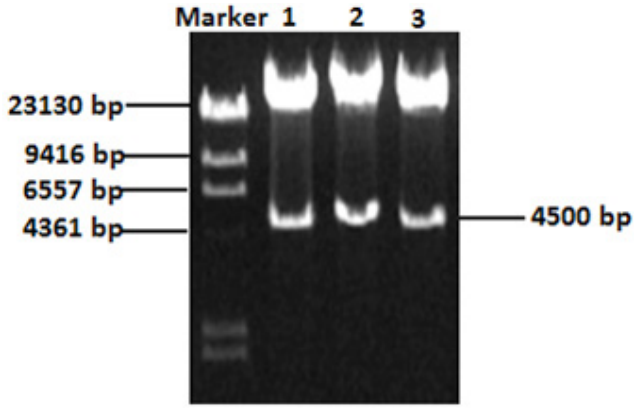

Figure 3. Identification of pAd-CMV and pAd-LYRM1 plasmid by *Pac*I. *Lanes 1-2* = Identification of pAd-LYRM1 by *Pac*I. *Lane 3* = Identification of pAd-CMV by *Pac*I.

Packaging and proliferation of the adenovirus

*Pac*I was used to linearize pAdTrack-CMV-LYRM1 and pAdTrack-CMV. We subsequently obtained the approximately 30-kb DNA fragments by gel extraction from the digested products, which were transfected into the 293A cell line to form adenovirus; 24 h later, the cells grew well with low green fluorescence (Figure 4A). Several days later, green fluorescence increased significantly (Figure 4B). After 10 days, additional green fluorescence was observed, with half of the cells detaching from the plate (Figure 4C). Next, we collected the virus suspension through repeated infection-freezing and thawing-collection; adenovirus with higher titer was obtained. The virus titers of pAdTrack-CMV-LYRM1 and pAdTrack-CMV were 7 x 10^8 and 1.3 x 10^9 green fluorescent units/mL, respectively. We utilized the adenovirus with high titer to infect 293A cells for 20 h, and green fluorescence was distributed over the entire culture plate (Figure 4D). Twenty-eight hours later, half of the cells had detached from the plate and we collected the virus.

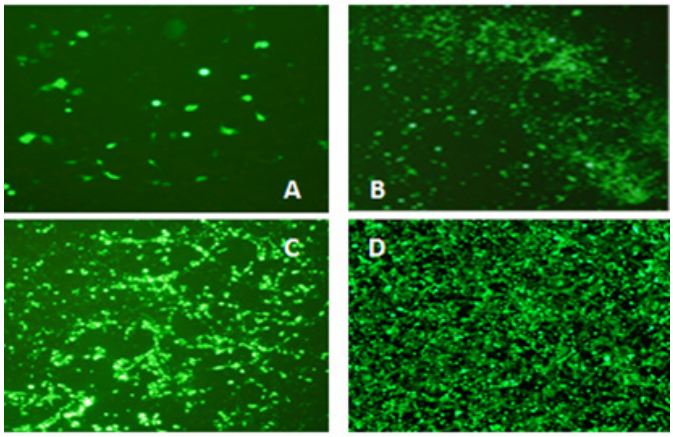

Figure 4. Package and propagation of adenovirus. **A.** Green fluorescence in 293A cells infected after 24 h. **B.** Green fluorescence increased and showed a grape-like gathering after 7 days. **C.** Green fluorescence covered the entire field of vision, and most cells were detached after 10 days. **D.** Twenty hours after infection by high titer adenovirus.

Efficiency of recombinant adenovirus vector

To determine the efficiency of the recombinant adenovirus vector, 200 μL high-titer adenovirus of Ad-LYRM1 and Ad-CMV was exploited to separately infect Qinchuan cattle preadipocytes with 95% confluence. Twenty-four hours later, we observed green fluorescence under a fluorescence microscope (Figure 5). After 48 h, we collected the cells, extracted the total RNA, and performed reverse transcription. Reverse transcription-PCR was used to detect the expression level of the target gene *LYRM1*, which suggested that expression in the cell infected by Ad-LYRM1 was approximately 28,000-fold higher than in the negative control (Figure 6). Our results demonstrated that the adenovirus was successfully packaged and could be effectively used for transcribing the Qinchuan cattle *LYRM1* gene.

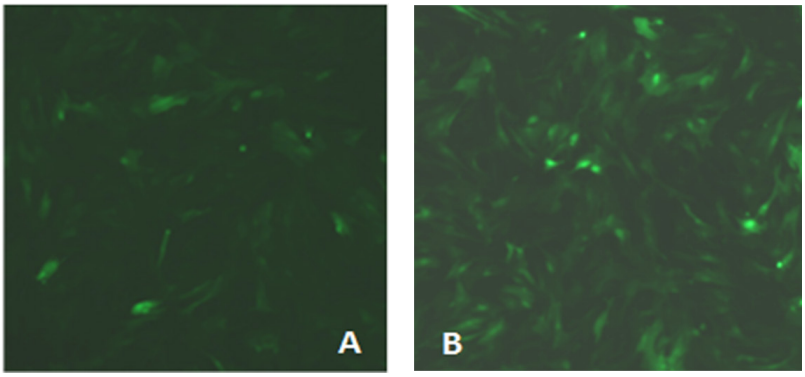

Figure 5. Green fluorescence in Qinchuan cattle preadipocytes infected by recombinant adenovirus. **A.** Infected by Ad-LYRM1. **B.** Infected by Ad-CMV.

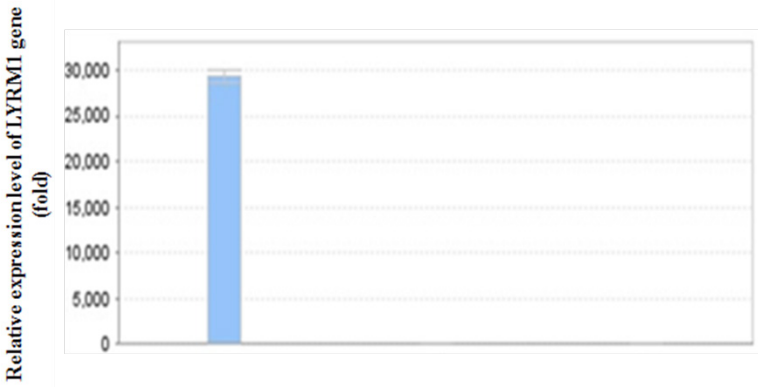

Figure 6. Relative expression of the *LYRM1* gene. mRNA abundance was normalized by glyceraldehyde 3-phosphate dehydrogenase. Expression of *LYRM1* in cells infected with Ad-LYRM1 was increased significantly compared with that in cells infected with Ad-CMV.

DISCUSSION

In recent decades, obesity has become an important worldwide health problem, which is a multifactorial disease regulated by the interactions between susceptibility genes and en-

vironmental factors (Basham and Luik, 2008; Qiu et al., 2009b). Obesity is associated with severe cardiovascular and metabolic complications (Kopelman, 2000; Spiegelman and Flier, 2001). Therefore, it is urgent to explore the mechanisms resulting in obesity, which can provide disease basis for treatment options. *LYRM1* was identified as a novel gene involved in regulating the size of the preadipocyte pool and affecting adipose tissue homeostasis (Qiu et al., 2009a). Previous demonstrated that the expression of *LYRM1* was higher in the adipose tissue of obese individuals. Additionally, *LYRM1* was found to induce insulin resistance and influence insulin-stimulated glucose uptake in adipocytes (Cao et al., 2010). It can also promote proliferation and inhibit the apoptosis of preadipocytes (Zhu et al., 2010). Our previous study suggested that LYRM1 was associated with growth and meat quality traits in Qinchuan cattle. Therefore, we hypothesized that *LYRM1* may be utilized to improve meat quality and growth traits in Qinchuan cattle.

Recombinant adenovirus can be used for gene therapy, vaccination, and gene transfer (Berkner, 1988; Graham and Prevec, 1991; Morgan and Anderson, 1993). It also provides a versatile system for gene expression and therapeutic applications; generating and producing the virus was simple and did not involve complicated procedures. Through homologous recombination in bacteria, the recombinant adenoviral plasmid can be obtained with fewer enzymatic manipulations. After transfection of the plasmid into a packaging cell line, the recombinant virus can be identified and acquired using green fluorescent protein (He et al., 1998). Generally, high titer virus and high level transgene expression can be obtained.

In the current study, we successfully cloned the complete CDS region of *LYRM1* and obtained the shuttle vector pAdTrack-CMV-LYRM1 and the negative control pAdTrack-CMV. Subsequently, the recombinant adenovirus expression vector pAd-LYRM1 and the negative control pAd-CMV were acquired through homologous recombination in *E. coli* BJ5183 cells. Next, we obtained the adenovirus Ad-LYRM1 and Ad-CMV vectors in 293A cells. Finally, the adenovirus was transfected into preadipocytes of Qinchuan cattle to identify the virus efficiency through reverse transcription-PCR. The results indicated that the expression level of LYRM1 in the preadipocytes infected by Ad-LYRM1 was improved by approximately 28,000-fold compared to the negative control.

Our results can be used to investigate the function of *LYRM1* on the cellular level in Qinchuan cattle, which would increase the understanding of the *LYRM1* gene for improving growth and meat quality traits. However, there were some limitations to our present study. We obtained effective adenovirus, but to accurately understand the role of *LYRM1* in regulating the development of cattle, extensive studies should be performed to examine the effects of the *LYRM1* gene on other genes in important pathways or networks. This will provide additional valuable information for enhancing the production performance of cattle.

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