

lnc9141-a and -b Play a Different Role in Bovine Myoblast Proliferation, Apoptosis, and Differentiation

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Previously, our transcriptome sequencing revealed that lnc9141 was differentially expressed in muscles of fetal bovine, calf, and adult bovine, which is considered to provide the basis for raising the muscle mass. In this study, we identified lnc9141 characters. lnc9141 has different transcription start sites and 3' alternative splicing sites of exon 1, producing lnc9141-a and lnc9141-b transcripts that were highly expressed in the heart and lung. Moreover, neither lnc9141-a nor lnc9141-b had the ability to encode proteins. The functions of lnc9141-a and lnc9141-b were explored by cell cycle, 5-ethynyl-2'-deoxyuridine (EdU), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed that lnc9141-a or lnc9141-b overexpression decreased the number of myoblasts in the S phase and increased the proportion of cells in the G₀/G₁ phase. Furthermore, overexpressing lnc9141-a and lnc9141-b respectively downregulated the expression of *Cyclin D1*. However, lnc9141-a or lnc9141-b interference was found to increase the number of S-phase myoblasts, and upregulate *Cyclin D1* and *Cyclin E* expression. Through Annexin V-FITC/propidium iodide (PI) double staining and the expression of apoptosis marker genes (*Bax*, *Bcl2*, and *Caspase-3*), it was found that lnc9141-b could regulate the expression of *Bax* gene. Meantime, high expression of lnc9141-b could decrease *MyHC* expression. In addition, the intergenic region between *lnc9141* and *IRX5* was 2.3 kb, with a head-to-head orientation. The study also revealed the core regions of the *lnc9141* and *IRX5* promoter. Our study demonstrated that both lnc9141-a and -b expression inhibited bovine myoblast proliferation. However, lnc9141-b regulated *Bax* and *MyHC* expression. The regulatory mechanism of lnc9141-a and lnc9141-b needs to be further explored.

INTRODUCTION

Long non-coding RNAs (lncRNAs) are longer than 200 nucleotides and they do not encode proteins, which have been widely studied recently. In general, lncRNAs are divided into the following categories: promoter upstream transcripts (PROMPTs), enhancer RNAs (eRNAs), intergenic region lncRNAs (lincRNAs), and sense

or antisense lncRNAs. Studies have shown that these lncRNAs may be transcribed from the upstream region of promoters, enhancers, and intergenic regions and from the sense or antisense strand of the protein-coding gene.^{1,2} Recently, gene pairs positioned with a head-to-head orientation on opposite strands of the DNA were reported, including mRNA-lncRNA. The intervening sequences of less than 1 kb between the two genes have been termed "bidirectional promoters."^{3,4}

There are many functions attributed to lncRNAs, namely the ability to influence transcription, alternative splicing, and epigenetic modification. lncRNAs can bind specific proteins and change their activities and/or subcellular localizations.⁵ lncRNAs can also regulate transcription by binding and sequestering microRNAs (miRNAs) in complexes referred to as competing endogenous RNAs (ceRNAs).⁶ In recent years, certain lncRNAs have been reported to encode small, functional peptides.⁷ Studies have also shown that the secondary structure of lncRNA could be evolutionarily conserved. It is speculated that some lncRNAs with low or modest sequence conservation among species may possess similar functions, hinting at the importance of conserved secondary structures.^{8,9}

Myogenesis, the formation of muscle tissue, is divided into three stages that include satellite cells, myoblasts, and myofibers.¹⁰⁻¹² Myogenesis is regulated by specific transcription factors, such as the myogenic regulatory factors (Myf5, MyoD, MRF4, and Myogenin).¹³ These factors along with cell-cycle regulators coordinate the activities of myoblast proliferation and differentiation and control the expression of genes both transcriptionally and epigenetically.¹² The quiescent satellite cells are considered at G₀ phase. When they

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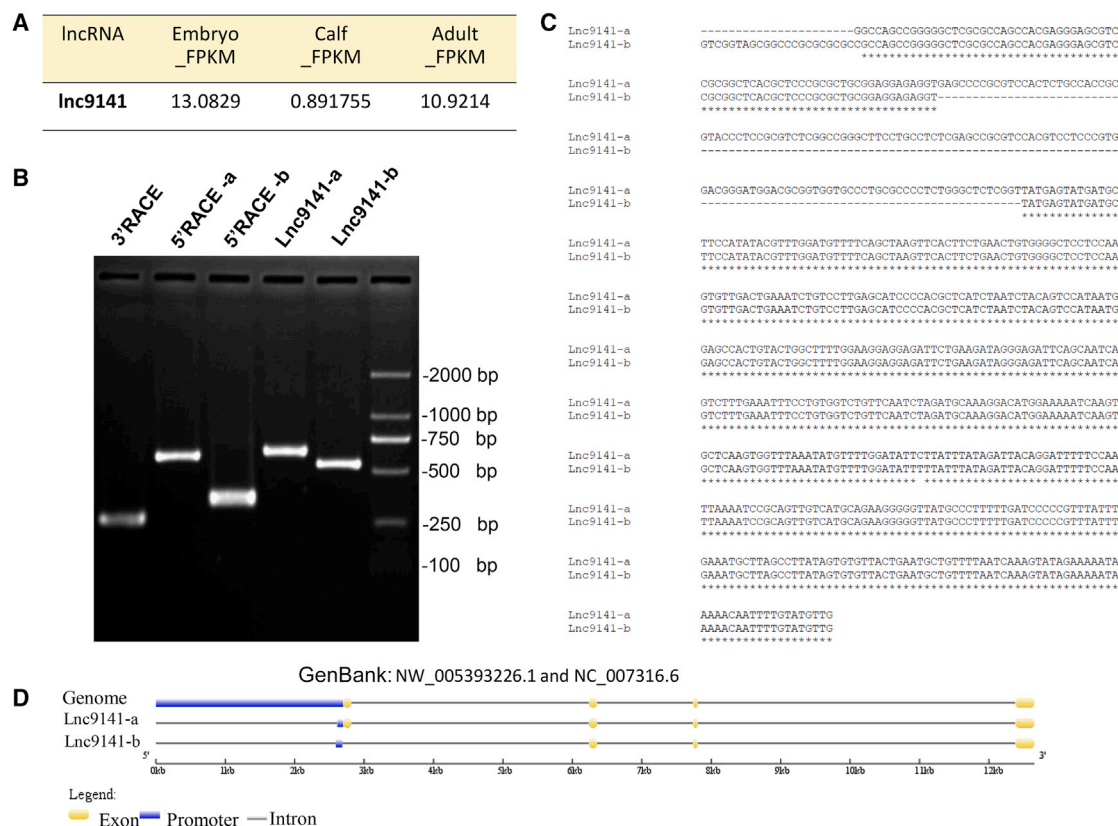


Figure 1. The Characters of lnc9141

(A) FPKM of lnc9141 from RNA-seq of three muscle development stages. (B) The 5' sequence, 3' sequence and full length of lnc9141. (C) The sequence comparison of two different transcripts (lnc9141-a and lnc9141-b). (D) The diagram of lnc9141-a and lnc9141-b location in bovine genome.

are regulated by cell-cycle genes, they re-enter into the cell cycle through S, G₂, and M phase to be activated satellite cells and myoblasts. Some activated satellite cells return to quiescence to maintain the satellite cell pool. It is commonly thought that myoblasts proliferate and fuse into existing myofibers with increasing expression of myogenic factors.^{13–16} Due to the fact that skeletal muscle contains multinuclear cells, it is suggested that individual myonuclear decay is a more frequent occurrence than wholesale myofiber death and skeletal muscle appears to possess an inherent resistance to apoptosis.¹⁷ However, a deeper exploration of the molecular mechanisms underlying myoblast character is necessary to improve our understanding of myogenesis.

Recently, studies on bovine myogenesis revealed the importance of lncRNAs, especially in myoblast differentiation and proliferation. Sun et al.¹⁸ showed that lncMD promoted primary myoblast differentiation through an interaction with miR-125b, resulting in an increase in *IGF2* expression. H19, a famous lncRNA, was required for bovine myoblast differentiation and suppressed the expression of the myoblast-inhibitory genes *Sirt1* and *FoxO1*.¹⁹ lncYYW was found to promote the expression of myoblast differentiation marker genes and to increase the number of bovine myoblasts by enhancing expres-

sion of *GHI* and its downstream genes.²⁰ Although these studies identified critical roles for lncRNAs in bovine myogenesis, the roles of many lncRNAs are still unknown.

The present study was undertaken to explore lnc9141 expression in three developmental stages (embryo, calf, and adult cattle). We found that *lnc9141* is located on bovine 18 chromosome and *lnc9141* and *IRX5* are positioned with a head-to-head orientation. The intervening sequence between them is 2.3 kb. And then through experiments, we identified the character of lnc9141, and assess the coding potential in this study. Overexpression and interference experiments were performed to explore its function in bovine primary myoblasts. Finally, this study also reveals the core promoter region of *lnc9141*. The objective of this study is to explore the function of lnc9141 in bovine myoblasts to provide a new direction for cattle breeding.

RESULTS

Identification of the Full Length of lnc9141-a and -b

Our previous study found that expression of NONBTAT009141 changes with development in bovine muscles.¹⁸ However, NONBTAT009141 is poorly studied. We named NONBTAT009141

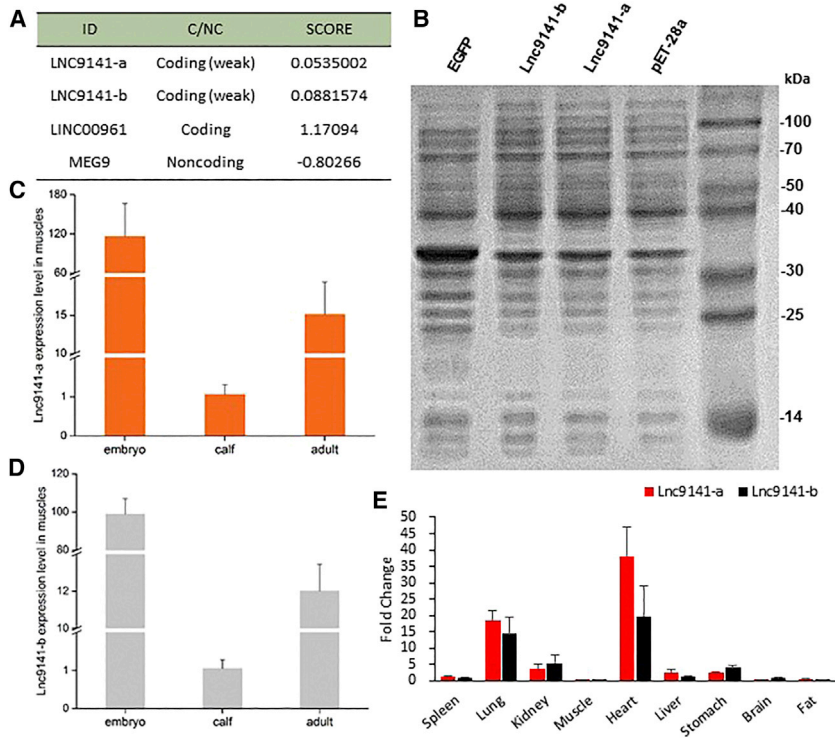


Figure 2. The Coding Potential and Expression Pattern of Lnc9141-a and Lnc9141-b

(A) The scores of coding capacity of Lnc9141-a and Lnc9141-b were given by CPC online software, and MEG9 and linc00961 were set as control. (B) The coding capacities of Lnc9141-a and Lnc9141-b were detected by prokaryotic expression, and the EGFP was a positive control. (C and D) The expression of Lnc9141-a (C) and Lnc9141-b (D) were detected in muscle of embryo, calf, and adult cattle. (E) The expression of Lnc9141-a and Lnc9141-b were detected in different organization of calf.

stomach, brain, and fat) have expression levels of Lnc9141-a and Lnc9141-b in calves, with the highest expression in heart and lung (Figure 2E).

Primary Myoblast Culture and Cytoplasmic Localization

After culturing primary myoblasts and then inducing them differentiation for 4 days, a large number of myotubes were observed under the microscope, which were validated by MyHC-immunofluorescence (Figures 3A and 3B). We separated the nuclear and cytoplasm of myoblasts and extracted the RNA from them respectively. Semi-

qPCR showed that Lnc9141-a and Lnc9141-b had existed in the cytoplasm (Figure 3C).

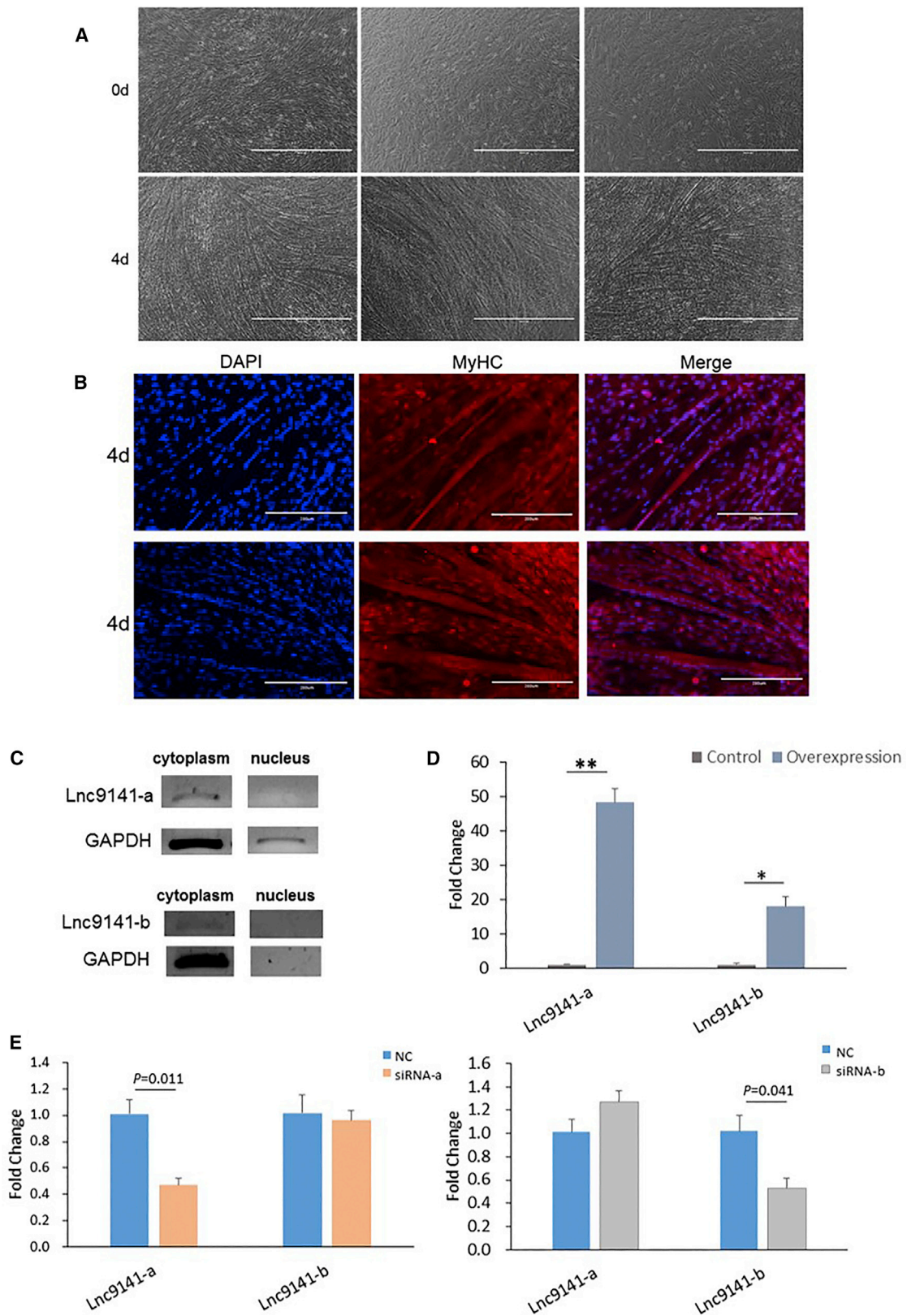
Lnc9141 Inhibited Primary Myoblast Proliferation

To explore the effect of Lnc9141-a and Lnc9141-b on primary myoblast proliferation, we transfected overexpression vectors or small interfering RNA (siRNAs). The transfection efficiency was analyzed by qPCR, showing that the overexpression vectors and siRNAs significantly altered the expression of Lnc9141-a and Lnc9141-b (Figures 3D and 3E). The siRNA-a could reduce the expression of Lnc9141-a, while it did not influence Lnc9141-b expression, the siRNA-b could reduce the expression of Lnc9141-b, and it did not affect the Lnc9141-a (Figure 3E). Upon overexpression of Lnc9141-a or Lnc9141-b, *Cyclin D1* expression was reduced (Figures 4A and 4B). Furthermore, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that overexpression of Lnc9141-a or Lnc9141-b reduced cell vitality (Figure 4C). Flow cytometry was used to analyze the cell-cycle phase distribution. The results showed that when overexpressing Lnc9141-a or Lnc9141-b, the number of cells at G₀/G₁ phases decreased (Figure 4D). Additionally, the results of EdU assays showed that overexpression of Lnc9141-a reduced the number of primary myoblasts in S phases (Figure 4E). Further, to confirm the overexpression study, we perform siRNA assays and found that the mRNA or protein levels of *Cyclin D1*, *Cyclin E*, and *PCNA* were increased following transfection of siRNA-a or siRNA-b (Figures 4A and 4B). Interference of Lnc9141-a or Lnc9141-b enhanced cell viability by MTT assays (Figure 4C). Furthermore, when transfecting with siRNA-a or siRNA-b, the cell numbers

as Lnc9141 due to its gene identity (Figure 1A). To enable us to study the function of the Lnc9141, the sequence of the full length of Lnc9141 needed to be first identified. The 3'-rapid amplification of cDNA ends (3'-RACE) was performed to characterize the 3' UTR, and only one variant was obtained. However, the 5'-RACE assay identified two transcript variants (Figure 1B). These two variants were named as Lnc9141-a (657 nt) and Lnc9141-b (547 nt). Further, the sequence comparison between Lnc9141-a and Lnc9141-b showed that these two variants were transcribed from different start sites and had different 3' alternative splicing sites in exon 1 (Figures 1C and 1D).

Coding Potential and Expressing Profile of Lnc9141-a and -b

The coding potential of Lnc9141-a and Lnc9141-b was analyzed by CPC online software (http://cpc.cbi.pku.edu.cn/programs/run_cpc.jsp). This analysis illustrated that both Lnc9141-a and Lnc9141-b had weak coding potential compared with MEG9 and linc00961, which were set as control (Figure 2A). To verify whether both transcripts encode proteins, we constructed Lnc9141-a and Lnc9141-b into prokaryotic expression system and induced them to express in *E. coli*. No protein was found expressed in prokaryotic expression system when compared to the positive control that EGFP was inserted into the prokaryotic vector (Figure 2B). Further, it was observed with qPCR that both Lnc9141-a and Lnc9141-b were significantly higher in fetal and adult bovine skeletal muscle than that in calves, which is consistent with our RNA sequencing (RNA-seq) study¹⁸ (Figures 2C and 2D). Tissue distribution study revealed that all examined tissues (spleen, lung, kidney, muscle, heart, liver,



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at S and G₂ phases increased (Figure 4D). Interfering lnc9141-a or lnc-9141-b did not change the number of S phase primary myoblasts through EdU assays (Figure 4E). The change in cell number at different cell-cycle phases suggested that lnc9141-a and lnc-9141-b inhibited primary myoblast proliferation.

Effects of lnc9141-a and -b on Primary Myoblast Apoptosis

Overexpression or RNA interference of lnc9141-a did not affect the expression levels of apoptotic marker genes *Bcl-2* and *Bax*, whereas overexpression of lnc9141-b reduced *Bax* expression (Figures 5A and 5B). We also calculated the *Bcl-2/Bax* rate and found that the ratio increased when transfecting lnc9141-b overexpression vector; however, the ratio declined using siRNA-b (Figures 5A and 5B). As we know, *Bax* gene plays a crucial role in promoting cell apoptosis. However, FITC-Annexin V/propidium iodide (PI) staining assay was then used to detect the number of primary apoptotic myoblasts, showing that lnc9141-a and lnc9141-b did not influence the number of apoptotic myoblasts (Figure 5C). Thus, despite lnc9141-b causing decreased *Bax* expression, the net effect was no significant change in apoptosis.

Effects of lnc9141-a and -b on Primary Myoblast Differentiation

To determine the role of lnc9141-a and lnc9141-b in primary myoblast differentiation, we quantified the expression of both transcripts in primary bovine myoblasts after differentiation had been induced for 0, 1, 3, and 5 days. Both lncRNAs were upregulated during myoblasts differentiation (Figure 6A). qPCR was performed on RNA extracted from cells transfected with pcDNA3.1-lnc9141-a/lnc9141-b or siRNA-a/siRNA-b to analyze expression patterns of two marker genes of myoblast differentiation, *MyoG* and *MyHC*. The results revealed that lncRNA-a did not affect primary myoblast differentiation. However, the expression of *MyHC* was reduced significantly when overexpressing lnc9141-b ($p < 0.05$, Figure 6B), suggesting that a high expression level of lnc9141-b is potentially involved in primary myoblast differentiation.

Promoter Activity of lnc9141 and IRX5

According to the bovine genome sequence, *lnc9141* and *IRX5* are matched to bovine chromosome 18 in a head-to-head orientation. Notably, the intergenic region between *lnc9141* and *IRX5* was 2.3 kb, which could be considered a bidirectional promoter (Figure 7A). The *lnc9141* promoter was truncated into 5 segments and named Pro9-1, Pro9-2, Pro9-3, Pro9-4, and Pro9-5, based on length with Pro9-5 being the shortest. The length of the deletion constructs was 2,166 bp, 1,848 bp, 1,284 bp, 1,082 bp, and 720 bp, respectively. The *IRX5* promoter was shortened to five segments and named Pro-I1, Pro-I2, Pro-I3, Pro-I4, and Pro-I5, from longest to shortest. The length of the deletion constructs was 2,296 bp, 1,957 bp, 1,661 bp, 1,218 bp, and 604 bp, respectively (Figure 7A). The vectors

(pGL3-Basic, pGL3-Control, and pGL3-Pro) were co-transfected with the internal reference vector (pRL-TK) into HEK293T cells. The fluorescence activity of *lnc9141* deletion constructs showed that the activity of pGL3-Pro9-2 was significantly higher than that of the other truncated forms and pGL3-Basic ($p < 0.01$). As such, we defined the transcriptional start site of lnc9141-b as +1 position of *lnc9141* promoter. The region -1,447~-885 nt is the core region of the *lnc9141* promoter and responsible for transcription initiation of the *lnc9141* gene (Figure 7B). The results revealed that the fluorescence activity of the deletion constructs from *IRX5* promoter was not significantly higher than that of pGL3-Basic except for pGL3-Pro-I1. However, the fluorescence activity of pGL3-Pro-I1 was not significantly higher than that of the other deletion constructs, demonstrating that there was not a strong active region in the *IRX5* promoter (Figure 7C).

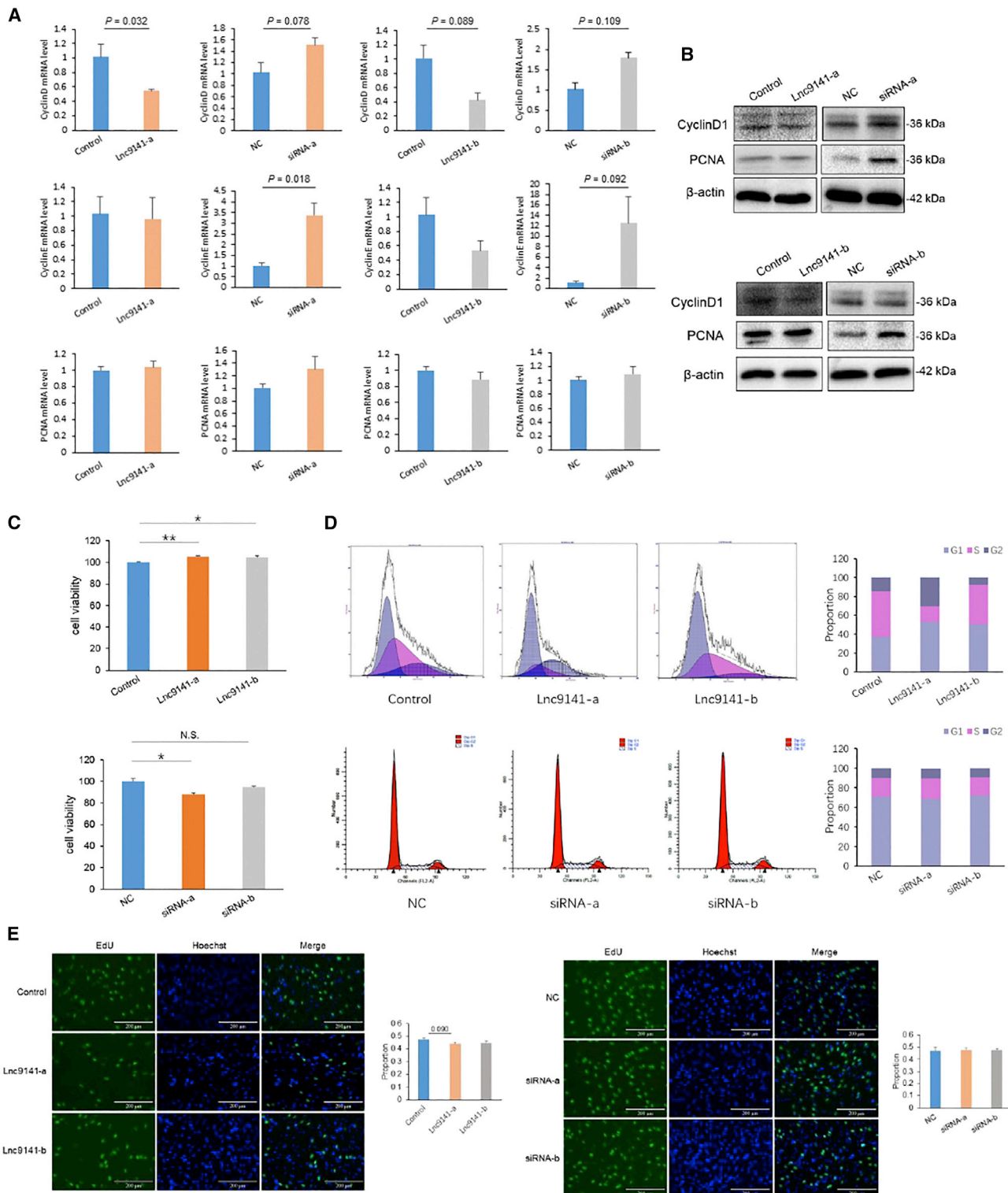
DISCUSSION

Since the development of comparative transcriptomics, RNA-seq is the routine technique used to assess gene expression in various development stages, organs, and treatment groups. For livestock, exploring differentially expressed genes is a common method to identify transcriptional differences that could be used to improve economic characteristics. Recently, 828 circular RNAs (circRNAs) were found to be significantly differentially expressed in embryonic and adult longissimus.²¹ It is reported that 584 defined long intergenic noncoding RNAs (lincRNAs) and 418 lincRNAs were found in nine muscle samples from Limousin bull calves.²² Liu et al.²³ identified 7,188 bovine lincRNAs from four different muscle tissues. Sun et al.¹⁸ found 401 differentially expressed lincRNAs in three development stages (embryo, calf, adult cattle). These studies provided detailed genetic information for bovine muscles using transcriptome sequencing. However, the functional roles of these lincRNAs in muscles are not clear. Verifying the functions of these differentially expressed genes is an important strategy to follow up on the results of the RNA-seq experiments.

In this study, we assessed lncRNA expression patterns at three different developmental stages to gain insights into the molecular mechanisms of myogenesis and later focused on the identification and function of *lnc9141* in myocytes.¹⁸ Two kinds of transcripts were identified, which we named lnc9141-a and lnc9141-b. Sequence analysis showed that *lnc9141* had different transcriptional start sites (TSSs) and 3' alternative splicing sites in exon 1 referring to GenBank: NW_005393226. It is suggested that the alternative splicing patterns of lncRNA were similar to those of the mRNA.²⁴ Furthermore, lnc9141-a and lnc9141-b seemed to follow "GT-AG" alternative splicing rules. Commonly found in genes, different TSSs produce distinct transcript isoforms, which can profoundly impact protein products and their activities. Certain transcriptional initiation sites can be used for compensatory transcription when other

Figure 3. The Primary Myoblasts Isolation and Vectors Construction

(A) The bovine myoblasts were separated and induced to differentiate. (B) Differentiated myoblasts were detected by MyHC immunofluorescence. (C) lnc9141-a and lnc9141-b were detected in cytoplasm and nucleus of bovine myoblasts. (D) The efficiencies of pcDNA-lnc9141-a and pcDNA-lnc9141-b was detected by qPCR. (E) The interference efficiencies of siRNA-a and siRNA-b were detected by qPCR.



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transcriptional sites are occupied. Transcripts with alternative transcriptional start sites are often expressed in different development stages.^{25,26} Our results revealed that lncRNA possessed alternative TSSs, which is similar to the mRNAs. However, it is likely that lnc9141-a and lnc9141-b perform the same function in bovine primary myoblasts, with both contributing to maintain the content of *lnc9141* transcripts in cells. Our prokaryotic expression experiments showed that neither lnc9141-a or lnc9141-b encode protein. It is therefore likely that these lncRNAs function is based on their secondary structure or sequence, which could aid in their interactions with RNA or protein.

In this study, we demonstrated that lnc9141-a and lnc9141-b inhibited the proliferation of primary bovine myoblasts using MTT, EdU, and cell-cycle assays and altered *Cyclin D1* expression. Cyclin D1 is a major cell-cycle regulator, which drives progression through G₁ phase.²⁷ There are three homologous D-type cyclins—Cyclin D1, Cyclin D2, and Cyclin D3. Among these, Cyclin D1 is often highly expressed in cancer cells.²⁸ Cyclin D forms holoenzymes together with cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6). Cyclin D phosphorylates pRb and facilitates the expression of *Cyclin E*, a positive regulator of the G₁ checkpoint.^{29–31} In addition, Cyclin D1 binds the promoters of many genes and interacts with other transcriptional regulators of histone acetylation and methylation. Cyclin D belongs to signal-dependent transcription factors (SDTFs), which are expressed in response to internal and external signals.³² However, MyoD as a lineage-determining transcription factor (LDTF), drives myoblast-specific transcription programs.³² Different cell types are regulated by both SDTFs and LDTFs. LDTFs can interact with MLL complexes at cell-type-specific enhancers during muscle differentiation.³³ When satellite cells are activated to become myoblasts, *MyoD* is highly expressed and promotes the downstream expression of *MyoG*. From our results showing that lnc9141-b regulated *Cyclin D1*, *Cyclin E*, *PCNA*, *Bax*, and *MyHC* gene expression transcriptionally and post-transcriptionally, we speculated that lnc9141-b has a wide range of functions in cells. Possibly, lnc9141-b could act as a regulator of transcription or translation. The differentiation and apoptosis of skeletal muscle cells are complex, which need be regulated variously. The myoblast differentiation undergoes several different stages (Figure 8). First, quiescence satellite cells produce myoblasts through proliferation. And then, myoblasts differentiate and fuse into myotubes, which are multinuclear cells. In the myofiber, *Pax7* and *Myf5* expression declines and *MyoG* and *MyHC* expression increases, which is the specific expression in the terminal differentiation stage.^{12–14} Due to the fact that myotubes contain a variable mitochondrial content and distinct mitochondrial pools, they might be different from other cell death. However, the studies reported that myoblast or myofiber displays the characteristics

of apoptosis. During apoptosis, the Bcl-2 family proteins are important regulators to function, which contain pro-apoptotic and anti-apoptotic proteins.³⁴ Furthermore, the adenine nucleotide translocase (ANT), the voltage-dependent anion channel (VDAC), Bax, and cyclophilin D form the mitochondrial permeability transition pore (mtPTP), which facilitates the release of pro-apoptotic factors during apoptosis. Generally, *Bax* overexpression facilitates mtPTP formation and releases cytochrome *c*, whereas Bcl-2 overexpression has been shown to prevent mtPTP formation and inhibit cytochrome *c* release.^{17,35}

While bidirectional promoters have been extensively studied, recent work has shown that the expression of more than 10% of genes is controlled by bidirectional promoters.³⁶ According to the bovine genome, *lnc9141* and *IRX5* are positioned with a head-to-head orientation on opposite strands of the DNA. It was found by dual fluorescence assay that the active region of the *lnc9141* promoter was at –1,447~–885 nt, while the promoter of *IRX5* had no strongly active region. This suggested that the transcription of *IRX5* may be initiated through the recruitment of transcription factors by the *lnc9141* transcriptional event. The expression levels of *lnc9141* and *IRX5* at different tissues or stages may be determined by the methylation levels of CpG islands on both sides of the active region. Similarly, Lin et al.³⁷ demonstrated that chicken *CCDC152* is oriented in a head-to-head configuration with the antisense transcript of growth hormone receptor (*GHR*) gene.

CONCLUSION

In conclusion, our study revealed two transcripts of *lnc9141* and two isoforms that inhibited myoblasts proliferation. Furthermore, lnc9141-b regulated *Bax* and *MyHC* expression. Our results provide new insight into lncRNA function and lncRNA expression dynamics in myogenesis.

MATERIALS AND METHODS

Tissues Collection and RNA Isolation

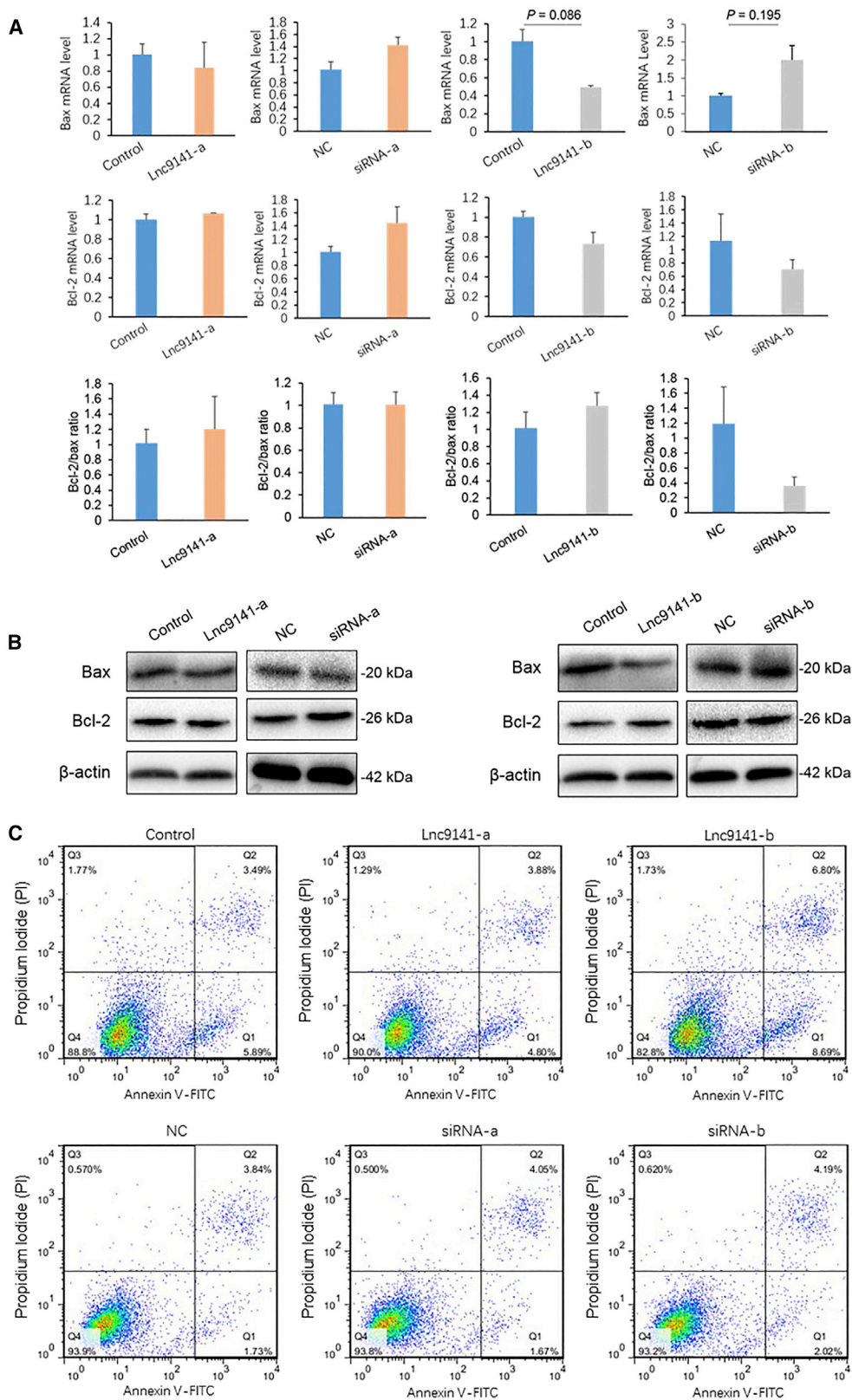
All experiments in this study were approved by the International Animal Care and Use Committee of the Northwest A&F University (IACUC-NWAFU). Fetal bovines (n = 3) and calves (n = 3) and adult bovines (n = 3) were in healthy condition. The animals were slaughtered quickly to collect the tissues, which were immediately frozen in liquid nitrogen and stored at –80°C prior to RNA isolation. Total RNAs were extracted using RNAiso Plus (Takara, Dalian, China).

5'- and 3'-RACE

5'- and 3'-RACE experiments were performed to determine the full length of *lnc9141*. RNA was isolated from bovine skeletal muscle. 5'-RACE experiments were performed using the SMARTer RACE

Figure 4. The Effect of lnc9141 on Myoblast Proliferation

(A–E) When overexpressing or interfering with lnc9141-a and lnc9141-b, respectively, (A) mRNA expression of *Cyclin D1*, *Cyclin E*, and *PCNA* were detected; (B) protein levels of Cyclin D1 and *PCNA* were detected by western blot; (C) the cell vitality was detected by MTT; (D) cell cycle was detected by FCM flow cytometry; and (E) the number of proliferation cells was counted.



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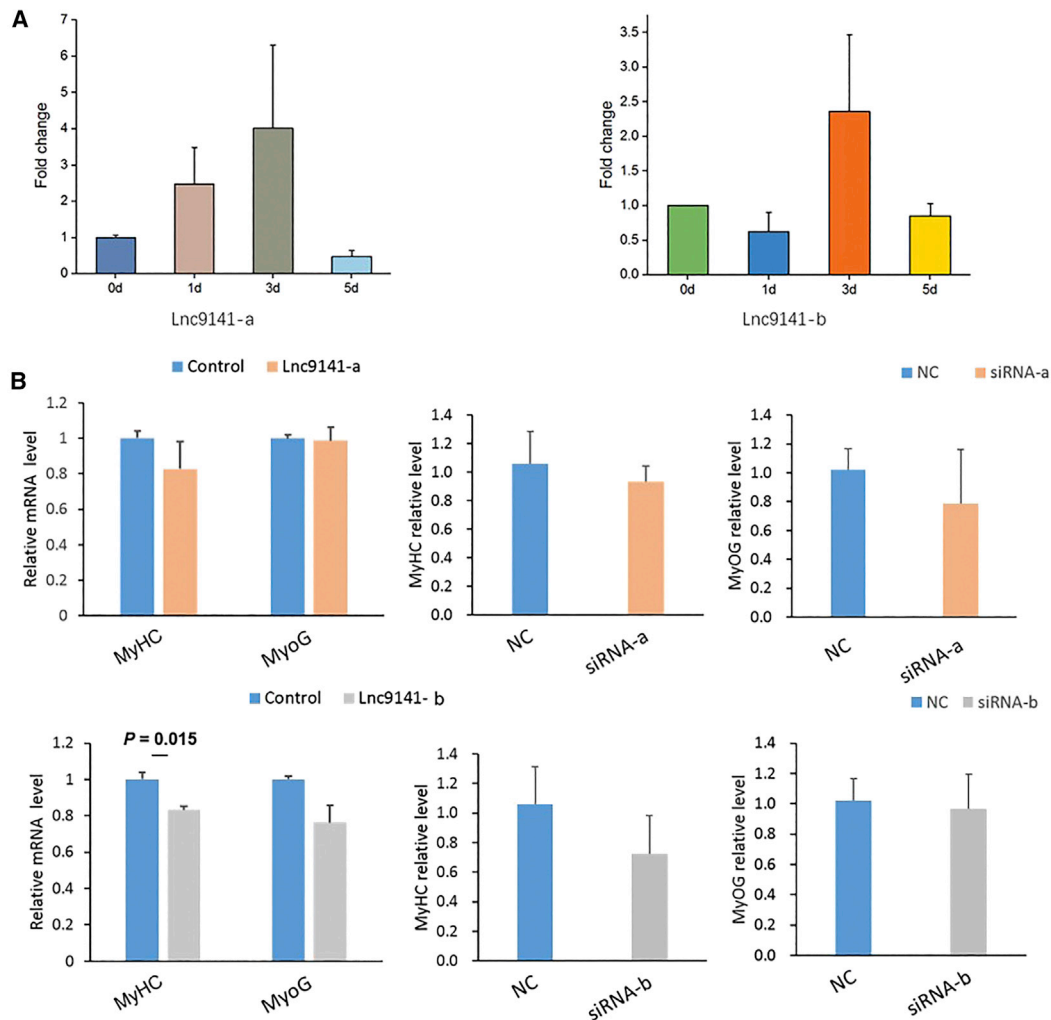


Figure 6. The Effect of lnc9141 on Myoblast Differentiation

(A) After primary myoblasts were induced to differentiate for 0d, 1d, 3d, and 5d, the expression of lnc9141-a and lnc9141-b were detected. (B) The mRNA levels of MyHC and MyoG were detected in 4-day differentiated myoblasts where lnc9141-a or lnc9141-b was overexpressed or inferred.

cDNA Amplification Kit (Clontech, CA, USA) according to the manufacturer's protocol. 3'-RACE experiments were carried out using classic RACE according to Scotto-Lavino et al.³⁸ The gene-specific primers for RACE are listed in Table 1.

Vectors Construction

The full lengths of lnc9141 were cloned into vectors to generate expression plasmids. lnc9141-a was cloned into pcDNA3.1 and pET-28 using KpnI/NotI. lnc9141-b was cloned into pcDNA3.1 and pET-28 using HindIII/XhoI. Deletion constructs of lnc9141 and IRX5 promoters

were cloned into pGL3-basic using HindIII/NheI. T4 DNA ligase was used to ligate the cloned fragments into each vector. The siRNAs of lnc9141-a and lnc9141-b were synthesized by Shanghai GenePharma. The siRNA-a for lnc9141-a is 5'-GGGCUCUCG GUAUGAGUATT-3'. The siRNA-b for lnc9141-b is 5'-GGAGGA GAGGUAUGAGUATT-3'.

Cell Culture

Primary myoblasts isolated from the muscle tissue of 3-month-old fetal bovine using collagenase digestion were cultured *in vitro* in

Figure 5. The Effect of lnc9141 on Myoblast Apoptosis

(A-C) When overexpressing or inferring with lnc9141-a and lnc9141-b, respectively, (A) mRNA expression of Bax and Bcl-2 were detected and Bcl-2/Bax ratio was calculated; (B) protein levels of Bax and Bcl-2 were detected by western blot and the gray level was analyzed by Image Lab; (C) the number of apoptosis cells was counted.

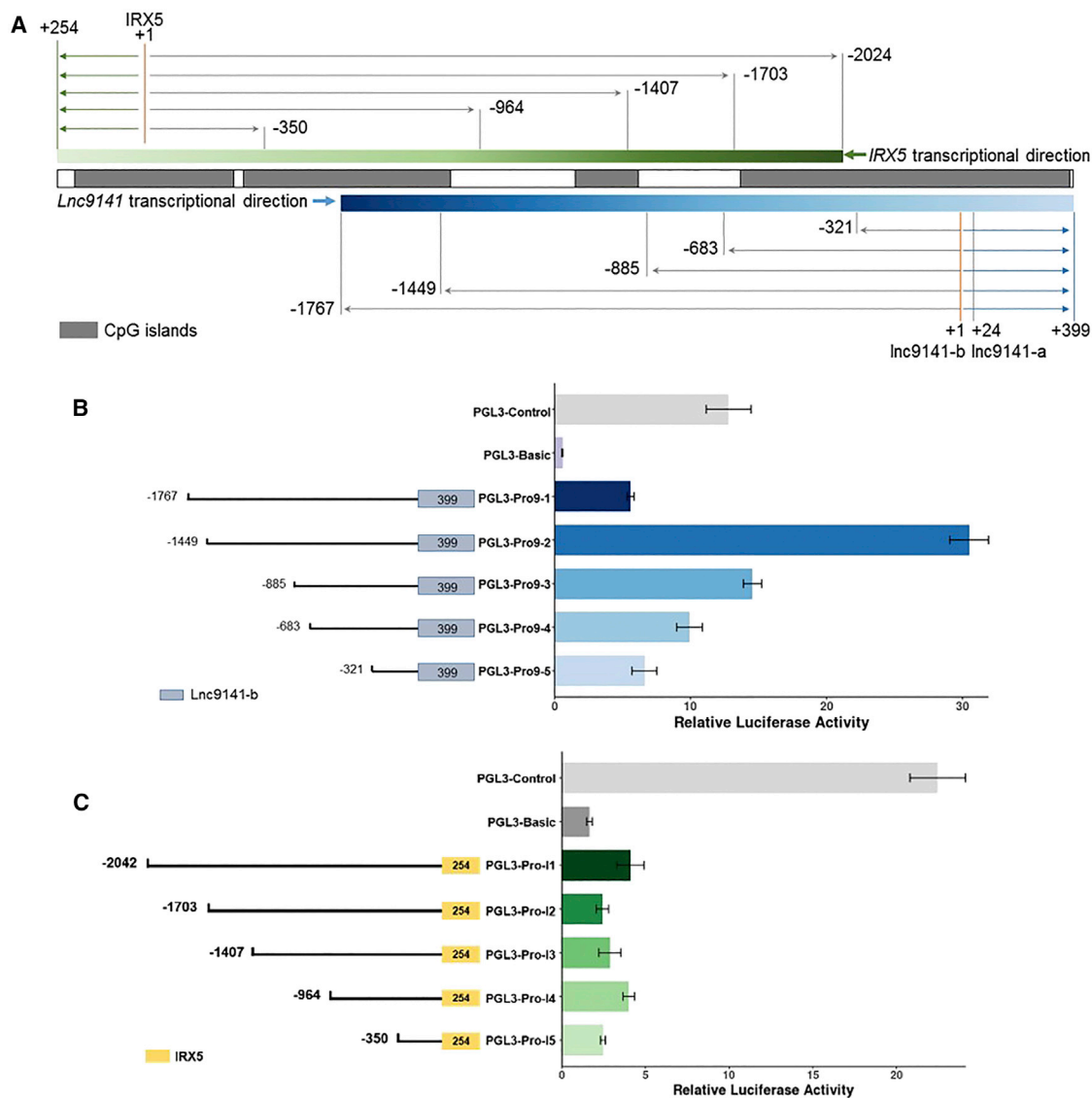


Figure 7. The Promoter Activities of *Inc9141* and *IRX5*

(A) The diagram of deletion constructs of *Inc9141* (blue) and *IRX5* (green); the white and gray line represents sequence in bovine genome (gray lines represent CpG islands). (B) The fluorescent activity of deletion constructs of *Inc9141* is shown. (C) The fluorescent activity of deletion constructs of *IRX5* is shown.

medium containing 20% fetal bovine serum (FBS) and 1% double antibiotics (penicillin and streptomycin). To induce differentiation, we changed the culture medium to differential medium (DMEM with 2% horse serum and 1% double antibiotics) when myoblasts were at 80% confluence. HEK293T cells (ATCC, USA) were cultured in medium including 10% FBS and 1% double antibiotics. All cells were cultured at 37°C with 5% CO₂ and transfected with different vectors or siRNAs using the transfection reagent (Dining, Beijing, China). Nuclear and cytoplasmic RNAs of primary myoblasts were separated and isolated using the PARIS kit (Life Technologies, Carlsbad, CA, USA).

qPCR and Semi-qPCR

RNA samples for qPCR and semi-qPCR were reverse-transcribed to cDNA using the PrimeSript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's recommendations. qPCR was performed in 12 μL reactions using the SYBR Premix Ex Taq kit (Takara, Dalian, China) and Bio-Rad CFX96 PCR System (Bio-Rad, USA). The qPCR amplification program was as follows: pre-denaturation at 95°C for 30 s, followed by 39 cycles of 95°C for 5 s and 60°C for 30 s. Relative expression was calculated using the 2^{-ΔΔC_t} method.³⁹ The semi-qPCR amplification program was as follows: 95°C pre-denaturation for 5 min, followed by 30 cycles of 95°C for 30 s,

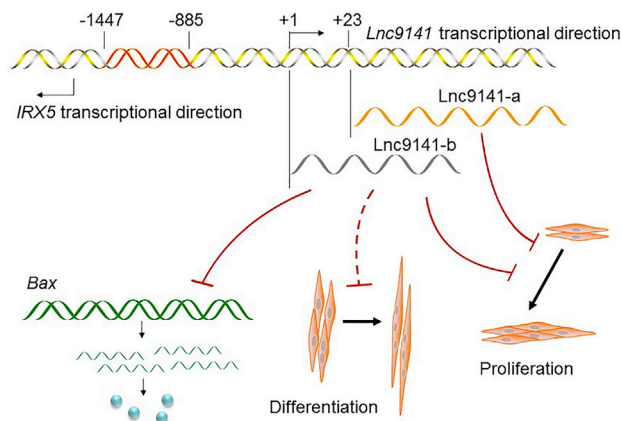


Figure 8. The Function of Lnc9141-a and Lnc9141-b and Regulated Region of Lnc9141 Promoter

Lnc9141-a and Lnc9141-b inhibit myoblast proliferation, Lnc9141-b inhibits *Bax* expression, and Lnc9141-b could inhibit myoblast differentiation in potential.

60°C for 30 s, and 72°C for 20 s, and ending with a 72°C extension for 10 min. The PCR products were resolved on 2% agarose gels, stained with ethidium bromide (EB), and then imaged. *GAPDH* was used as an internal control for qPCR and semi-qPCR. All primers are listed in Table 2.

Immunofluorescence

Primary bovine myoblasts were induced to differentiate for 4 days and then washed three times with PBS and fixed in 4% paraformaldehyde for 30 min. The cells were incubated with primary antibody-MyHC (Abcam, Cambridge, MA, USA) (diluted in 1% BSA) at 4°C overnight. The cells were washed and then incubated with goat anti-rabbit IgG H&L secondary antibody (Abcam, Cambridge, MA, USA) (diluted in 1% BSA) at 25°C for 2 h. To visualize DNA, we added a 5 mg/mL DAPI solution. Finally, the cells were washed three times with PBS and observed under a fluorescence microscope (EVOS fl, AMG, USA).

Western Blot

Cells were collected and lysed in RIPA buffer with PMSF. Total protein was quantified using the Bradford method.⁴⁰ Proteins were separated by 12% SDS-PAGE and then transferred to a 0.2 μm polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with milk powder solution (5% skim milk in Tris saline with Tween [TBST]) for 2 h at 25°C. After blocking, the membrane was incubated overnight with primary antibodies specific for anti-PCNA (catalog #13110), anti-Cyclin D1 (catalog #2978), anti-Bcl-2 (catalog #3498), anti-Bax (catalog #2772) (Cell Signaling Technology, Boston, USA), and anti-β-actin (catalog WL01845, Wanleibio, Shenyang, China) at 4°C respectively. The membranes were washed with TBST and incubated with goat anti-rabbit IgG secondary antibody (Biosharp, Hefei, China) for 2 h. Protein bands were detected using hypersensitive ECL luminescence reagent (Dining, Beijing, China).

Table 1. Specific Primers of RACE

Primer Names	Primer Sequences
3' RACE-outer-F	5'-CAGCAATCAGTCTTTGAA-3'
3' RACE-inter-F	5'-CCTGTGGTCTGTCAATC-3'
5' RACE-outer-R	5'-AAGGGCATAACCTCCTTCTGC-3'
5' RACE-inner-R	5'-TCTGCATGACAACCTGCGGAT-3'
5' RACE-inner2-R	5'-CTGATTGCTGAATCTCCCTATC-3'

Cell Proliferation Assay

MTT assay was performed to examine cell vitality (Wolsen Biotechnology, Xian, China). For the MTT assay, cells were plated into 96-well plates with 100 μL culture medium per well and each treatment group had six independent replicates. After 24 h treatment, the culture medium was changed to MTT medium (MTT: DMEM = 1:9) and the cells were cultured for another 4 h. Then after adding to each well 100 μL DMSO and shaking for 10 min, the absorbance was detected with an Infinite 200 Pro (TECAN, Austria). Cell proliferation was also detected using the KeyFluor488 Click-iT EdU Kit (KeyGEN BioTECH, China). Primary myoblasts were cultured with 100 μL growth medium in 96-well plates. 24 h after transfection with vectors or siRNAs, the cells were incubated with EdU medium for 2 h. Detection was performed according to the manufacturer's protocol.

Cell-Cycle Assay

Primary myoblasts were cultured in 60 mm plates and then transfected with different vectors or siRNAs. The different treatment groups were washed three times with PBS, collected, and then examined using the Cell Cycle Testing Kit (BestBio, Shanghai, China) according to the manufacturer. After PI staining, the cell suspension was analyzed by flow cytometry (FACS Canto II, BD BioSciences, USA).

Cell Apoptosis Assay

Primary myoblasts were cultured in 60 mm plates, then transfected with different vectors or siRNAs. The different treatment cells were washed three times with PBS and collected for FITC-AnnexinV/PI staining assay. Based on the protocol of Cell Apoptosis Detection Kit (BestBio, Shanghai, China), the cells were washed twice with PBS and then resuspended in 400 μL 1 × Annexin V at a density of 1 × 10⁶ cells/mL. Cells were incubated with 5 μL Annexin V-FITC at 2°C–8°C in the dark for 15 min and then for 5 more min upon addition of 10 μL PI. Stained cells were immediately analyzed by flow cytometry (FACS Canto II, BD BioSciences, USA).

Dual Fluorescence Detection

HEK293T cells were cultured in 96-well plates. When the cell density reached 80% confluence, different recombinant plasmids and the internal reference plasmid (pRL-TK) were co-transfected into HEK293T cells. The total amount of transfected DNA per well was 400 ng, while the transfection ratio of pGL3-Pro and internal reference vector (pRL-TK) was 50:1. At the same time, pGL3-Control

Table 2. Quantitative Amplification Primers

Gene Names	Primer Sequences	Length (nt)	Reference Sequence
lnc9141-a	F: 5'-TCTGGGCTCTCGGTTATGA-3'	101	XR_001351305
	R: 5'-TTTCAGTCAACACTTGGAGGAG-3'		
lnc9141-b	F: 5'-GCGGAGGAGAGGTTATGAGTAT-3'	118	XR_001351305
	R: 5'-GGGATGCTCAAGGACAGATTTC-3'		
Cyclin D1	F: 5'-ATGAAGGAGACCATCCCCCT-3'	250	NM_001046273.2
	R: 5'-TGAACCTCACGTCTGTGGCA-3'		
Cyclin E	F: 5'-CTGCAGCCTAAAATGCGAGC-3'	252	NM_001192776
	R: 5'-TCCTGAACAAGCCCCATCTG-3'		
PCNA	F: 5'-CGGACACGTTGGCACTAGTAT-3'	187	NM_001034494
	R: 5'-ACAGCATCTCCAATGTGACTG-3'		
Bax	F: 5'-CGAGTTGATCAGGACCATCAT-3'	119	NM_173894.1
	R: 5'-ATGTGGGTGTCCCAAAGTAG-3'		
Bcl-2	F: 5'-TGGATGACCGAGTACCTGAA-3'	124	NM_001166486.1
	R: 5'-GAGACAGCCAGGAGAAATCAAA-3'		
MyoG	F: 5'-CAAATCCACTCCCTGAAA-3'	93	NM_001111325.1
	R: 5'-GCATAGGAAGAGATGAACA-3'		
MyHC	F: 5'-TGCTCATCTACCAAGTTCC-3'	150	NM_001101835.1
	R: 5'-CACTCTTCACTCTCATGGACC-3'		
GAPDH	F: 5'-TGAGGACCAGTTGTCTCCTGCG-3'	145	NM_001034034.2
	R: 5'-CACCACCCTGTTGCTGTAGCCA-3'		

and pRL-TK were co-transformed as a positive control, and pGL3-Basic and pRL-TK were co-transformed as a negative control. Three biological replicates were used for each treatment. After 24 h, the transfected cells were lysed in 100 μ L 1 \times passive lysis buffer (PLB) (Promega, Madison, WI, USA) at room temperature for 30 min. Dual-luciferase activity was measured and firefly luciferase activity was normalized to renilla luciferase activity (pRL-TK).

Statistical Analyses

All experiments were carried out at least three times. Data are shown as means \pm SE. The statistical significance of results was analyzed by homogeneity of variance test, and then tested with Student's *t* test using SPSS 22.0 (Chicago, IL, USA). **p* < 0.05 were considered statistically significant, while ***p* < 0.01.

AUTHOR CONTRIBUTIONS

X.L. conceived the project and designed the experiments, H.C. and C.L. provided suggestions for the project, and M.Z. performed the primary experiments and wrote the manuscript. B.L. and J.W. carried out the primary myoblasts isolation. S.Z. and H.L. contributed reagents and materials. L.M. analyzed the data. We thank Dr. Wei Guo at Animal Science Wyoming INBRE Program, University of Wyoming, for revising the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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