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Identification of target genes for adenohypophysis-prefer miR-7 and miR-375 in cattle

B. Yuan[1], G.J. Sun[1], G.L. Zhang[2], J. Wu[2], C. Xu[1], L.S. Dai[1], J. Chen[1], X.F. Yu[1], Z.H. Zhao[1] and J.B. Zhang[1]

[1]College of Animal Sciences, Jilin University, Changchun, China
[2]Branch of Animal Husbandry, Jilin Academy of Agricultural Science, Gongzhuling, China

Corresponding author: J.B. Zhang
E-mail: zhangjb10066@163.com

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ABSTRACT. In this study, expression levels of miRNAs (miRNAs), miR-375 and miR-7, were detected in different tissues of cattle to determine whether adenohypophysis-prefer or exclusively expressed miRNAs, and target genes could be predicted by TargetScan, RNA22, and other software. Target genes related to pituitary function or reproductive traits were identified using a dual-luciferase assay. miR-375 and miR-7 were expressed differently in various tissues. miR-375 and miR-7 showed higher expression in the adenohypophysis, and there was a significant difference compared with expression in other tissues ($P < 0.01$). The binding sites for miR-7 were the mRNAs of bone morphogenetic protein receptor type II (BMPR2), prostaglandin F2 receptor negative regulator, gonadotropin-releasing hormone receptor, follicle-stimulating hormoneβ, somatostatin receptor 1, and interleukin-1β by bioinformatic analysis; similarly, the mRNAs of BMPR2 and leptin contained binding sites for miR-375, suggesting that these genes are affected by miR-7 or miR-375. Dual-luciferase reporter assays showed that miR-7 regulated

prostaglandin F2 receptor negative regulator expression, while miR-375 regulated BMPR2 expression. The mutated plasmid and miRNA mimics were used to co-transfect NIH3T3 cells; luciferase reporter assays showed that the inhibition of luciferase activity in the wild-type cells dramatically decreased from 75 to 26% with a 3-5-nucleotide mismatch mutation into the seed region of miR-7. miR-375 had nearly lost the ability to inhibit luciferase activity, suggesting that GTCTTCC is the site of interaction between miR-7 and the prostaglandin F2 receptor negative regulator sequence and that GAACAAA is the site of interaction between miR-375 and the BMPR2 sequence.

Key words: Prostaglandin F2 receptor negative regulator; miR-375; miR-7; Bone morphogenetic protein receptor type II; Bovine adenohypophysis

INTRODUCTION

Lee et al. (1993) discovered the gene lin-4, which is capable of regulating expression timing in *Caenorhabditis elegans*. lin-4 encodes no proteins, but it encodes and generates small RNA fragments. The RNA fragments combine with the 3'-untranslated region, which is transcribed by lin-14, at multiple regions through complementary base pairing. Therefore, the RNA fragments negatively regulate lin-14 on the post-transcriptional level, affecting the expression of the lin-14 protein and affecting the normal development of *C. elegans* larvae. lin-4 RNA is now known as miRNA (miRNA). miRNAs are a class of endogenous single-stranded non-coding small RNA molecules of approximately 22 nucleotides. They are complementary pair with gene sequences on the target mRNA, regulating mRNA expression levels and inhibiting protein translation on a post-transcriptional level. In the regulation of cellular activity of the body, miRNAs are important determinants of health and disease factors. miRNAs exist in many species, including bacteria, animals, plants, and even viruses.

The pituitary gland is the main endocrine organ of the body and is composed of the adenohypophysis and neurohypophysis. This gland regulates hormone production and secretion, affecting processes such as animal growth, bone metabolism, and cell cycle activity. Few studies have examined the relationship between miRNA and pituitary function. Studies have found that miR-26b plays a key role in the development of the pituitary. Additional studies showed that miRNA and tumor growth were closely related (Yu et al. 2012). For example, miR-15 and miR-16 were downregulated in pituitary adenomas and correlated with the secretion of the P43 protein. However, most miRNA functions in the pituitary remain unknown.

To examine the role of miRNA in the regulation of pituitary development and reproduction, we use high-throughput sequencing to obtain the expression in 1- and 24-month-old adenohypophysis of Simmental to determine the tendency and specific expression of miRNAs in the bovine adenohypophysis, and real-time polymerase chain reaction (PCR) was used to detect part of miRNAs. We found that miR-7 and miR-375 were expressed in the bovine adenohypophysis. We predicted their target genes and selectively verified these genes through bioinformatic methods. We then analyzed the roles of these miRNAs in the regulation of gene expression in the adenohypophysis, laying a theoretical foundation for studying the cattle breeding mechanism.

MATERIAL AND METHODS

Preparation of different cattle tissues

The heart, liver, spleen, lung, kidney, brain, intestine, pituitary, and testis tissues were isolated from donor Yanbian cattle, which were then placed in liquid nitrogen for storage at -80°C.

Reagents

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). ReverTra Ace qPCR RT kit and SYBR® Green Real-Time PCR Master Mix (QPK-201) were purchased from Toyobo (Osaka, Japan). Ex Taq DNA polymerase, dNTP mixture, DNA marker, T4 DNA ligase, and the *Xho*I and *Not*I restriction enzymes were obtained from Takara (Shiga, Japan). DMEM high-glucose medium and trypsin were from Gibco (Grand Island, NY, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). miR-7, miR-375, and negative control mimics were from Zimmer in Shanghai. Lipofectamine 2000 was purchased from Invitrogen. psiCHECK-2 carrier and the Dual-Luciferase Reporter Assay System were from Promega (Madison, WI, USA). The plasmid extraction kit and PCR product purification kit were purchased from Axygen (Union City, CA, USA).

Collection of total RNA

Total RNA was isolated using the TRIzol method (Li et al., 2011).

Identification of tissue specificity

The pituitary-specific gene follicle-stimulating hormoneβ (FSHβ) was amplified by PCR and the product was detected by 1% agarose gel electrophoresis.

Primer design for the pituitary-specific gene FSHβ

Specific primers were used for amplification according to the published mRNA sequence as described in the GenBank database. Primers with the following sequences were used: 5'-CCAACATCACCATCACGG-3' and 5'-CAGTGGCTACTGGGTACGTGT-3'. The primers amplified 230 bp and were obtained from SangonBiotech Co., Ltd. (Shanghai, China).

Specific stem-loop reverse transcription (RT) primer for miRNAs

Specific stem-loop RT primers for 12 miRNAs were amplified according to the published miRNA sequence as described in miRBase. RT primers are shown in Table 1.

Prediction of target genes of miR-7 and miR-375 (Wang et al., 2012)

Target genes were predicted for the mature miR-7 and miR-375 sequences as described in miRBase and TargetScan (http://Www.targetscan.org/mmu_50/), miRDB (http://mirdb.org/miRDB/index.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), and other

biology software programs. Secondary structures of miRNAs were predicted by RNAm fold to selectively identify target genes.

Table 1. Primer sequences used in reverse transcription and real-time PCR.

Primer name	Primer sequence
miR-7	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAAC
	F: GAGCTTGGAAGACTAGTGAT
miR-375	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACGC
	F: GAGCATTTTGTTCGTTCGGC
Reverse primer	AGTGCAGGGTCCGAGG
	RT: CGCTTCACGAATTTGCGTGTCAT
U6	F: GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT

Construction of dual-luciferase reporter vector for candidate target genes of miR-7 and miR-375

For amplification of candidate target genes, the recombinant dual-luciferase reporter vector for candidate target genes was constructed, and the target genes were amplified by PCR using the cDNA from the bovine pituitary as a template. Upstream and downstream primers were designed for each candidate target gene with restriction sites of *Xho*I and *Not*I. The target gene was inserted downstream of the 3'-end in the *Renilla* luciferase gene of the psiCHECK-2 vector.

Base mutations of recombinant plasmids

The mutations in miRNA binding sites of the recombinant plasmid were introduced with 3-5 base mutation in the seed region. The mutagenic primers of approximately 40 bp with reverse complement sequences contained mismatched bases to the miRNA. The primers are shown in Table 2.

Table 2. Construct mutation primer sequence.

Primer name	Primer sequence
PTGFRN-F	TCGAGTCTTTGTCTCGGGTGAGATCCGTACCTGTTGCGAGTCAGAGC
PTGFRN-R	GGCCGCTCTGACTCGCAACAGGTACGGATCTCACCCGAGACAAAGAC
BMPR2-F	TCGAGGGTTTTGGAAGTTCGTAAGAGCACACATGATAGAGCAAGTGC
BMPR2-R	GGCCGCACTTGCTCTATCATGTGTGCTCTTACGAACTTCCAAAACCC

Dual-luciferase reporter assays

Transfection

NIH3T3 cells were co-transfected with the recombinant plasmid, miR-7, miR-375, and negative control mimics.

Luciferase reporter assays

The cell culture liquid was discarded and the cells were washed twice with phosphate-buffered saline. The cells were treated with 25 μL passive lysis buffer at room tempera-

ture for 15 min with slow shaking, and then the cell lysate was transferred to a detection plate. First, 100 μL luciferase assay substrate was added to the 2 syringes of the chemiluminescence analyzer. Subsequently, 50 μL Stop &Glo® Substrate was added, reading at intervals of 2 and 10 s.

RESULTS

Total RNA isolation

Total RNA from the cattle heart, liver, spleen, lung, kidney, brain, intestine, pituitary, and testis tissues was isolated using the TRIzol method. Next, 1% agarose gel electrophoresis was used to detect RNA. The ratio of A_{260}/A_{280} of total RNA was between 1.8 and 2.0 (Figure 1). This result demonstrated that total RNA was slightly degraded, but did not contain genomic DNA, proteins, or other impurities.

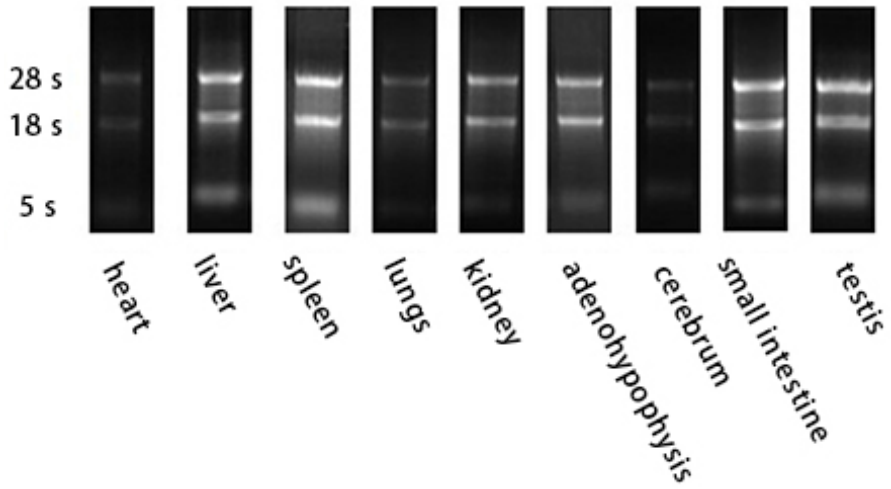

Figure 1. Electrophoresis result of total RNA.

Identification of tissue specificity

cDNA was obtained from total RNA by reverse transcription. The FSHβ gene was amplified by PCR and the product was evaluated by 1% agarose gel electrophoresis (Figure 2). A single amplification band was detected and the amplified fragment size was consistent with the expected fragment size (230 bp), indicating that total RNA was extracted from the pituitary tissue.

Fluorescent quantitative PCR

Expression of miRNA in cattle tissues

The relative expression levels of miRNA in different tissues were analyzed by reverse transcription-PCR. The levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the reference gene

U6 to reduce experimental error. Fluorescent quantitative PCR enabled detection of the levels of 2 miRNAs in 9 different tissues. Both miRNAs were expressed in every tissue, and there was a significant difference in the adenohypophysis compared to other tissues ($P < 0.01$), with most expression observed in the adenohypophysis (Figure 3).

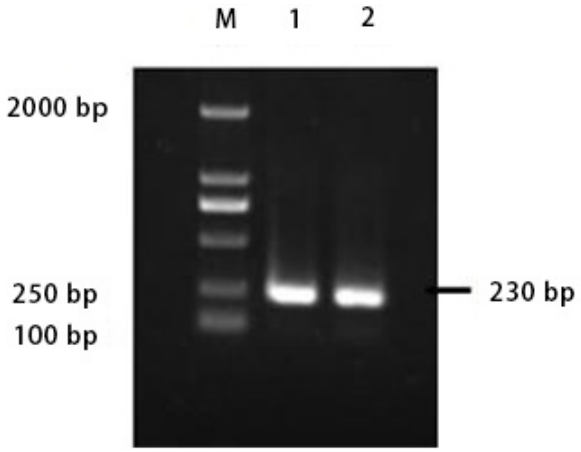

Figure 2. PCR of FSHβ gene of the adenohypophysis. *Lane M* = maker; *lanes 1* and *2* = amplicons.

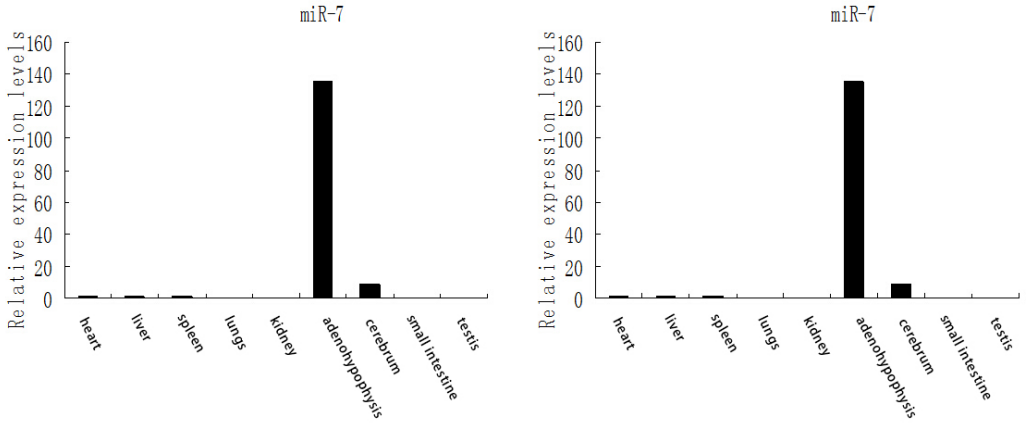

Figure 3. Relative expression levels of miRNAs in the different tissues of cattle.

Prediction of target genes for miR-7 and miR-375

Target genes were predicted and selected from hundreds of genes predicted by TargetScan and other database and the secondary structure of binding sites was predicted. The comprehensive predictions revealed binding sites for miR-7 in the mRNAs of bone morphogenetic protein receptor type II (BMPR2), prostaglandin F2 receptor negative regulator

(PTGFRN), gonadotropin-releasing hormone receptor, FSHβ, somatostatin receptor 1, and interleukin 1β, and binding sites for miR-375 in mRNAs of BMPR2 and leptin. Therefore, these genes were selected as the target genes for miR-7 and miR-375. The duplex structures of miR-7 and PTGFRN as well as miR-375 and BMPR2 are shown in Figure 4.

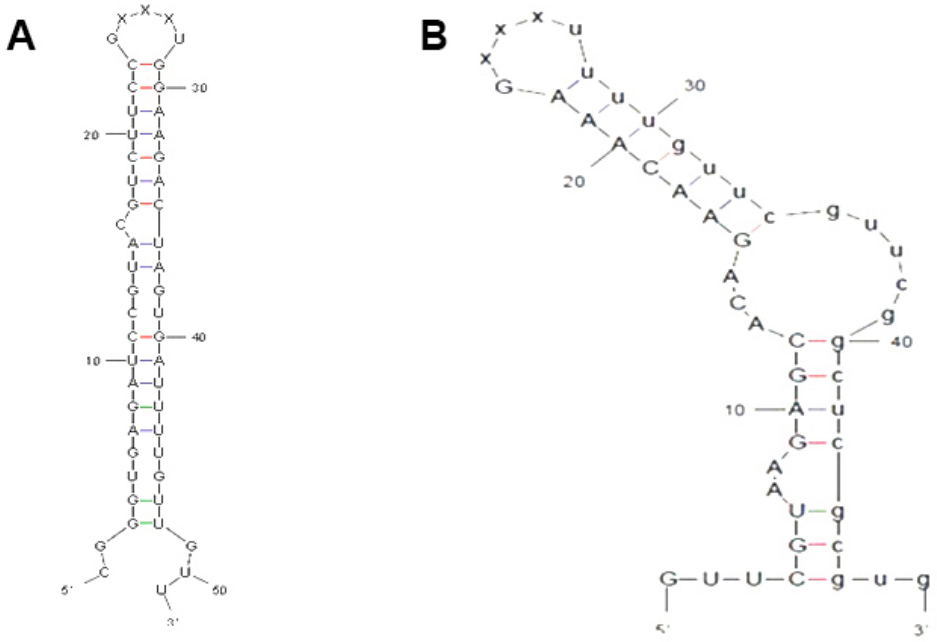

Figure 4. Duplex structure between miRNA and target gene. **A.** miR-7-PTGFRN. **B.** miR-375-BMPR2.

Identification of recombinant plasmid

The psiCHECK-2 recombinant plasmid containing a mutation from GGAAGAC to GCAACAG and psi-CHECK-BMPR2 recombinant plasmid containing a mutation from TTTGTTC to TATCATG were successfully constructed.

Identification of target genes for miR-7 and miR-375

miR-7 and miR-375 were selected for further study, and genes related to animal growth or reproductive traits were considered to be candidate target genes. The cloned binding sequence of the miRNA and target genes were inserted into the downstream of 3'-end in *Renilla* luciferase gene of the psiCHECK-2 vector, using the firefly luciferase reporter gene as an internal reference (Figure 5A). The binding of miRNA and cloned genes affects the activity of *Renilla* luciferase and reduces the expression rate of the 2 enzymes, indicating that candidate target genes may be substrates for the miRNA. The NIH3T3 cell line was co-transfected with a recombinant plasmid, miR-7, miR-375, and negative control mimics with a vector of plasmid 2000. Luciferase expression was detected at 36 and 48 h later. The results showed that with overexpression of miR-7 and miR-375, cell lines transfected with the PTGFRN and

BMPR2 plasmid showed significantly reduced expression of *Renilla* luciferase and firefly luciferase compared with the control group and other transfection groups ($P < 0.01$; Figure 5B). The control group showed no significant change; other transfection groups showed little change, but the difference was not significant ($P > 0.05$). Thus, PTGFRN and BMPR2 may be the target genes for miR-7 and miR-375.

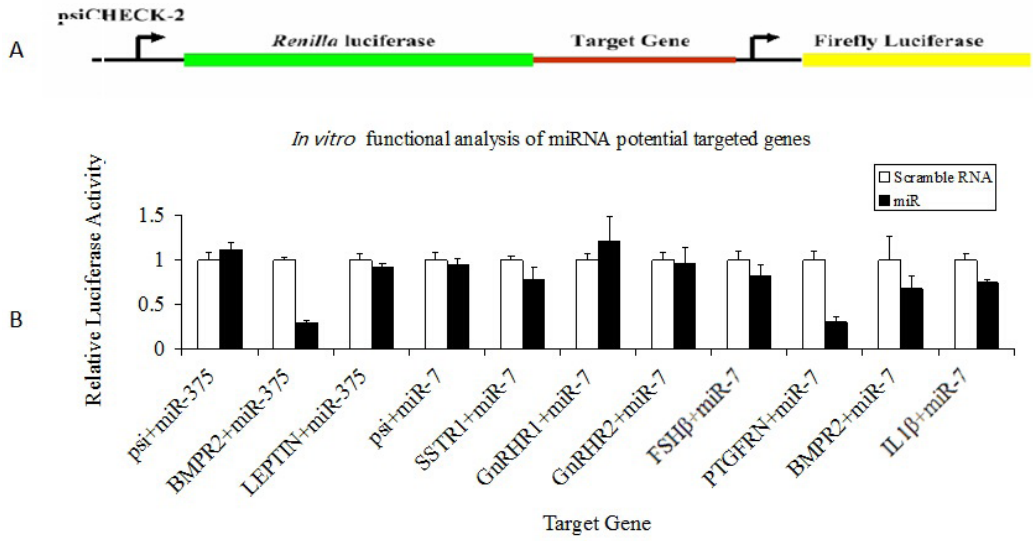

Figure 5. Identification of miR-7 and miR-375 target genes.

Normalized luciferase activity (*Renilla*/Firefly) in NIH3T3 cells were co-transfected with reporter constructs in the presence of miR-7, miR-375, or scramble RNA mimics. Significant changes in the luciferase activity of pis-CHECK-PTGFRN and pis-CHECK-BMPR2 were observed compared with other groups in the presence of miR-7 or miR-375 overexpression, respectively. The results are reported as means ± SE from 3 independent experiments with 4 culture replicates each.

Identification of action sites of miR-7 and miR-375 on target genes

Three or 5 base mutations were introduced to the binding region between miR-7 and PTGFRN, miR-375, and BMPR2 to identify action sites. The NIH3T3 cell lines were co-transfected with recombinant plasmid, miR-7, miR-375, and negative control mimics and a vector of plasmid 2000. After 36 and 48 h, the luciferase assay was performed. The inhibition of the wild-type on luciferase activity was greatly decreased from 75 to 26% when a base mutation was introduced into the seed region of miR-7, and miR-375 nearly lost the inhibition ability in the luciferase activity, suggesting that the predicted binding site was the interaction site (Figure 6). The results also showed that regulation of mir-7 on PTGFRN was mediated by the interaction between the GGAAGAC sequence in mir-7 and the GTCTTCC sequence in PTGFRN. Similarly, the regulation of mir-375 on BMPR2 was mediated by the interaction between the TTTGTTC sequence in mir-375 and the GAACAAA sequence in BMPR2.

A indicates the PSI vector, psi-CHECK-PTGFRN and psi-CHECK-MUT-PTGFRN co-transfected with miR-7 mimics in NIH3T3 cells. B indicates the PSI vector, psi-CHECK-BMPR2, and psi-CHECK-MUT-BMPR2 co-transfected with miR-375 mimics in NIH3T3 cells. The results are reported as means ± SE from 3 independent experiments with 4 culture replicates each.

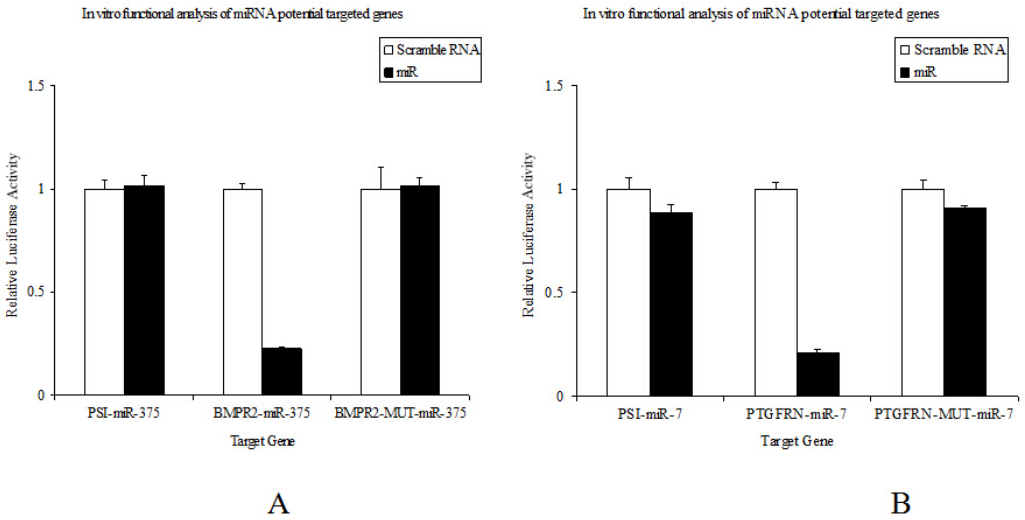

Figure 6. Mapping of miR-7 and miR-375 response element.

DISCUSSION

miRNA plays an important role in regulating cellular activities, such as cell differentiation, proliferation, metabolism, and apoptosis (He et al., 2013). Since the first miRNA was discovered, its regulatory role in the post-transcriptional has been widely examined. With the development of biological technology, much has been learned about miRNAs (Dynoodt et al., 2013). The identification ability and regulatory mechanism of miRNAs have become a hotspot in scientific research (Guo et al., 2010).

In this study, real-time quantitative PCR was used to detect the expression level of miR-375 and miR-7 in cattle heart, liver, spleen, lung, kidney, brain, testis, pituitary, small intestine, and other tissues. The 2 ΔΔCt method was used for quantitative analysis (Schmittgen et al., 2000). The experimental results showed that the expression level of miR-375 and miR-7 differed in various tissues. The expression level of miR-375 and miR-7 were expressed in the pituitary. Compared with other tissues, the expression level of miR-375 and miR-7 show significant differences in the pituitary. Thus, we speculate that miR-375 and miR-7 play important roles in regulating the development and function of the pituitary gland. However, the function, regulatory mechanism, and influence on animals' reproduction traits of miR-375 and miR-7, such as mature gametes, embryonic development, secretion of pituitary hormones, and other processes, remain unclear (Shi et al., 2012; Dey et al., 2012).

In animals, miRNAs primarily exert their regulatory roles in the expression of target genes. Therefore, in this experiment, the expression of miRNAs in bovine pituitary was examined to determine which gene regulates pituitary development. Comprehensive analysis of

the results using various software programs can improve the prediction accuracy of candidate miRNA target genes (Zhou et al., 2012; Altmäe et al., 2012).

Quantitative analysis showed that the expression levels of miR-375 and miR-7 were high in the pituitary. This indicates that these genes may play important regulating roles in the development and secretion of hormones in the pituitary. Therefore, miR-7 and miR-375 were selected for further study. Target genes were predicted according to the mature miR-7 and miR-375 sequences as described in miRBase by TargetScan, RNA22, and other biology software. The secondary structures of target genes related to hormone secretion and pituitary growth were selectively predicted. The results showed that PTGFRN and BMPR2 were the target genes of miR-7 and miR-375, respectively. Thus, the target genes were suitable for screening (Li et al., 2012; Wei et al., 2013).

Some genes and miRNAs regulate hormone production and secretion, by either promoting or inhibiting these processes (Perez-Castro et al., 2012). In this study, the adenohypophysis-prefer miR-7 and miR-375 were studied, and genes related to hormone synthesis or secretion were predicted and analyzed. Seven candidate target genes were obtained: gonadotropin-releasing hormone receptor, FSHβ, somatostatin receptor 1, interleukin 1β, leptin, BMPR2, and PTGFRN. Gonadotropin-releasing hormone receptor can interact with gonadotropin-releasing hormone and thus regulate reproductive performance, playing a critical role in regulating the synthesis and intermittent release of the gonadotropin-luteinizing hormone and FSH (Norwitz et al., 1999). Somatostatin receptor 1 is a somatostatin receptor that mediates the effect of somatostatin, regulating somatostatin secretion in the pituitary.Leptin acts on the hypothalamus-pituitary-gonadal axis, which can regulate animals' reproductive performance and hematopoietic function and promote the proliferation and differentiation of cells, playing a catalytic role in genetics and breeding. Interleukin 1β can stimulate angiogenesis.

In the current study, PTGFRN and BMPR2 were identified to be the target genes of miR-7 and miR-375 based on the results of the *in vitro* dual-luciferase assay. BMPR2 plays an important role in the bone morphogenetic protein-signaling pathway. These proteins can affect embryonic development and the formation of bones and organs, playing a critical role in the early mammalian development and nervous system differentiation. Recent data showed that bone morphogenetic proteins stimulate FSHβ transcription (Lee et al., 2007; Ho and Bernard, 2009). PTGFRN can inhibit prostaglandin F2α by interacting with its receptor. Prostaglandin F2α is an important regulatory factor for luteal, uterine contractility, ovulation, and embryo implantation, which can stimulate the pituitary to release luteinizing hormone (Schauer et al., 2012). Thus, miR-7 and miR-375 may affect hormone synthesis and secretion by regulating PTGFRN or BMPR2. However, the mechanisms require further analysis.

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