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miR-375 down-regulation of the rearranged L-myc fusion and hypoxia-induced gene domain protein 1A genes and effects on Sertoli cell proliferation

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Objective: This study aimed to screen and identify the target genes of miR-375 in pig Sertoli (ST) cells and to elucidate the effect of miR-375 on the proliferation of ST cells.
Methods: In this study, bioinformatics software was used to predict and verify miR-375 target genes. Quantitative polymerase chain reaction (PCR) was used to detect the relationship between miR-375 and its target genes in ST cells. Enzyme-linked immunosorbent assay (ELISA) of rearranged L-myc fusion (RLF) and hypoxia-induced gene domain protein 1A (HIGD1A) was performed on porcine ST cells, which were transfected with a miR-375 mimics and inhibitor to verify the results. Dual luciferase reporter gene assays were performed to assess the interactions among miR-375, *RLF*, and *HIGD1A*. The effect of miR-375 on the proliferation of ST cells was analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS).
Results: Five possible target genes of miR-375, including *RLF*, *HIGD1A*, colorectal cancer associated 2, POU class 3 homeobox 1, and WW domain binding protein 1 like, were found. The results of quantitative PCR suggested that mRNA expression of *RLF* and *HIGD1A* had a negative correlation with miR-375, indicating that *RLF* and *HIGD1A* are likely the target genes of miR-375. The ELISA results revealed that RLF and HIGD1A were negatively correlated with the miR-375 protein level. The luminescence results for the miR-375 group co-transfected with wild-type RLF and HIGD1A vector were significantly lower than those of the miR-375 group co-transfected with the blank vector or mutant RLF and HIGD1A vectors. The present findings suggest that RLF and HIGD1A are target genes of miR-375 and that miR-375 inhibits ST cell proliferation according to MTS analysis.
Conclusion: It was speculated that miR-375 affects cell proliferation through its target genes, which play an important role in the development of testicular tissue.

Keywords: miR-375; *RLF*; *HIGD1A*; Cell Proliferation

INTRODUCTION

miRNAs are short non-coding RNAs that are approximately 20 to 25 nucleotides long and have a regulatory role in eukaryotes [1]. Currently, most known miRNAs function by inhibiting mRNA translation and degrading mRNA. Mature miRNAs assemble into the RNA-induced silencing complex, which targets mRNA through complementary base pairing to regulate target genes by targeting mRNA degradation or repressing mRNA translation of target genes [2]. miRNAs participate in the regulation of a variety of functions, including gene expression, developmental timing, organ development, cell proliferation, cell apoptosis, cell differentiation, fat metabolism, the nervous system, and tumor biological processes [3,4].

miR-375 is a small non-coding RNA fragment that is transcribed from the fragments of

two genes, coiled-coil domain-containing protein 108-like and crystallin beta A2, in human chromosome 2. The mature miR-375 sequence is *UUUGUUCGUUCGGCUCGCGUGA*. The conserved sequence of miR-375 in different species is *CUUGUUU*. The aim of this study was to find a possible target gene of miR-375 that matches the *GAACAAA* sequence. In 2012, it was concluded that expression of miR-375 is significantly different between the mature and immature boar testis by Solexa deep sequencing [5] and it was hypothesized that miR-375 has an impact on boar reproduction. Previous studies have reported that miR-375 is a key factor that regulates E2 synthesis by mediating the corticotropin releasing hormone (CRH) signaling pathway to control the function of the animal reproductive system [6]. In bovine CCs, overexpression of miR-375 attenuated the proliferation ability and significantly increased the apoptosis rate of bovine CCs, whereas inhibition of miR-375 did not significantly change the proliferation ability or apoptosis rate. Based on the importance of ST cells and miR-375 functional analysis, it was speculated that miR-375 might play a role in the proliferation of ST cells.

Hypoxia-induced gene domain protein-1a (HIGD1A) is referred to as hypoglycemia/hypoxia inducible mitochondrial protein1-a or hypoxia induced gene 1 and is a 10.4-kDa mitochondrial inner membrane protein [7]. Although Higd-1a is induced by hypoxia and promotes survival, its modes of action have not been defined. We showed that Higd-1a–transfected cells undergo significantly less apoptosis as the result of inhibition of the release of cytochrome c and reduction of caspase activity [8]. Reports have shown that Higd-1a depletion results in mitochondrial fission, depletion of mtDNA, disorganization of cristae, and growth retardation [9]. In addition, HIGD1A decreases tumor growth, but promotes tumor cell survival *in vivo* [10].

Rearranged L-myc fusion (RLF) is a protein coding gene. Diseases associated with RLF include left ventricular noncompaction. Zinc finger protein 292 is an important paralog of this gene. *RLF* is widely expressed in fetal and adult tissues, suggesting that it has a general role in transcriptional regulation [11]. Therefore, the relationship between miR-375 and its target genes can be verified and its effects on ST cell proliferation can be explored.

Therefore, it was hypothesized that miR-375 may regulate cell proliferation by regulating its target genes, thereby affecting animal reproduction. This study aimed to screen and identify the target genes of miR-375 in pig ST cells and to elucidate the effect of miR-375 on the proliferation of ST cells.

MATERIALS AND METHODS

Bioinformatics prediction of target genes

We found the miR-375 conservative sequence in miRBase (http://www.mirbase.org/) and determined the target genes of miR-375 by matching sequences from miRBase with the *GAACAAA* sequence. TargtScan (http://www.targetscan.org/vert_70/) was used to search for genes with the *GAACAAA* sequence. Then, the functions of the genes were analyzed by gene ontology.

Cells culture

Pig Sertoli (ST) cells were purchased from the Boster Biological Technology Company. ST cells were cultured in HIGH GLUCOSE (GIBCO, USA) with 10% fetal bovine serum (FBS; Gibco, Austria) and 1% penicillin-streptomycin (HyClone, USA) in an incubator at 37°C and 5% CO_2. A transfection plasmid was inserted using FuGENE HD (Roche, Switzerland) according to the manufacturer's instructions. Twenty-four hours after transfection, the cell morphology and expression level of the red fluorescent protein was observed under a fluorescence microscope (NikonTE2000, Japan).

Plasmid

The miR-375 mimic, miRNA-375 inhibitor and negative control mimic (NC) plasmids were purchased from the GenePharma Company in China. The sequences of the *RLF* and *HIGD1A* genes were amplified via PCR with *NotI* and *XhoI* restriction sites and then cloned into the *pmiR-RB-REPORT* vector to create the recombinant vectors *pmiR-RB-REPORT-RLF-WT* and *pmiR-RB-REPORT-HIGD1A-WT*.

The dual luciferase reporter gene primer sequence of *RLF*:

5'CCGCTCGAGTAGAAACAGACTGGCTCCAACAC 3'

5'GAATGCGGCCGCATCAATAAACCAACTGGAGAGCTAA 3'

The dual luciferase reporter gene primer sequence of *HIGD1A*:

5'CCGCTCGAGTAATGGAAGTGATTGGTTTATGAGC 3'

5'ATAAGAATGCGGCCGCTTGTGCTACTATGAAGAGTGATTTT 3'

The *pmiR-RB-REPORT-RLF-MUT* and *pmiR-RB-REPORT-HIGD1A-MUT* were purchased from the GENEWIZ Company in China. The recognition sequence of the miR-375 *CGAACAA* site was mutated to a non-recognition sequence, *CATGTAGC*, in the plasmid.

RNA extraction and real-time quantitative polymerase chain reaction

ST cells transfected with the *miR-375* mimics, *miR-375* inhibitor and *miRNA-ShNC* were harvested at 24 h post-transfection. The reverse transcription primers and fluorescence-labeled primers for quantitative analysis of miR-375 and its target genes were designed using Primer 5.0. All of the primers were synthesized by Shanghai Sangon (Table 1). Total RNA was extracted using the innuPREP RNA Mini Kit (Analytikjena,

Table 1. Primers for qPCR detection

Symbol	Primer	Primer sequence (5′–3′)
miRNA-375-RT		GTCGTATCCAGTGCAGGGTCCGAGGTGCACTG GATACGACTCACGCG
miRNA-375	F-Primer	TGCGGTTTGTTCGTTCGGCT
	R-Primer	CAGTGCAGGGTCCGAGGT
U6-RT		AACGCTTCACGAATTTGCGT
U6	F-Primer	CTCGCTTCGGCAGCACA
	R-Primer	AACGCTTCACGAATTTGCGT
GAPDH	F-Primer	GTTTGTGATGGGCGTGAAC
	R-Primer	ATGGACCTGGGTCATGAGT
COLCA2	F-Primer	TCCTACCTTCAGGCAGAGACAC
	R-Primer	GCAGGAGAGTAACTACAGTCTAAGG
HIGD1A	F-Primer	TCCGAAAAGCAAAAGAGGC
	R-Primer	TACCAAGAGTCATTGCTCCCAC
POU3F1	F-Primer	AGTTGGAGACAGAGCTGAGGGA
	R-Primer	GCTTTGGATGGCAAGTCGATAT
RLF	F-Primer	TGTACGACTTACCTTACCCAGCAG
	R-Primer	GCGCTCCAAGAAAGTGTCTACAG
WBP1L	F-Primer	ACCGGGAAGCCCACAATTAC
	R-Primer	TAGCTGGAAGGCACTGTATGGT

Germany) according to the manufacturer's protocols. Based on the quality and concentration of RNA, cDNA was reversed transcribed with the PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed using a SYBR Premix Ex Taq (TaKaRa, Japan) on a stratagene Mx3005P (Agilent, USA) according to the instructions. The qPCR volume was 20 μL. The relative mRNA levels of *miR-375*, *RLF*, *HIGD1A*, colorectal cancer associated 2 (*COLCA2*), POU class 3 homeobox 1 (*POU3F1*), and WW domain binding protein 1 like (*WBP1L*) were normalized to U6 and *β-actin*. The data were analyzed using the SPSS 19.0 software, and the fold change of expression was calculated using the $2^{-\Delta\Delta CT}$ method according to the following formula:

$$\Delta\Delta Ct = [Ct\ (positive) - Ct\ (reference)] - [Ct\ (control) - Ct\ (reference)]$$

Here, $2^{-\Delta\Delta Ct}$ refers to the relative expression ratio.

Protein extraction and ELISA analysis

Total protein was extracted from cells transfected with the miR-375 mimics, miR-375 inhibitor and miRNA-ShNC using RIPA buffer (Boster, Wuhan, China) according to the manufacturer's instructions. The protein concentration was determined by a BCA Protein Assay Kit (keyGEN BioTECH, China). Then, the protein was tested by an ELISA kit (Unionhonest) according to the instructions.

Luciferase assays

ST cells were plated on 24-well plates for 24 h before transfection. miR-375 mimics plasmids (1 μg) containing the *RLF-WT* (1 μg), *HIGD1A-WT* (1 μg), *RLF-MUT* (1 μg), *HIGD1A-MUT* (1 μg) genes or luciferase vector (*pmiR-RB-Report*) (1 μg) were co-transfected with 2 μL of FuGENE HD (Roche, Switzerland) in triplicate for 36 h. The luciferase activities were detected using a SpectraMax M5 Microplate Reader (Molecular Devices, USA). The luciferase activities were expressed as the activity ratio of *Renilla*/luciferase.

Cell proliferation assay

Proliferation was examined using the 96 AQueous One Solution Reagent (Promega, Beijing, China) according to the manufacturer's instructions. Then, 100 μL cell suspensions (500 cells) were added to each well in a 96-well plate. After 12 hours, the miR-375 mimics, miR-375 inhibitor and miRNA-ShNC were transfected with FuGENE HD Transfection Reagent. Then, 20 μL of MTS (CellTiter 96 AQueous One Solution Cell Proliferation Assay) Reagent was added to each well and the absorbance values at 490 nm were recorded 0 h, 6 h, 12 h, and 24 h after transfection using a multiwall spectrophotometer. Each group was subjected to 3 Biological repeats, and the mean values were obtained.

Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as the mean±standard deviation. Statistical analysis was carried out by Student's t-test, and the values were considered significant at $p^{*}<0.05$, $p^{**}<0.01$.

RESULTS

RLF, *HIGD1A*, *COLCA2*, *POU3F1*, and *WBP1L* might be the target gene of miR-375

According to a bioinformatics website, the miR-375 binding sites in the 3 'UTR region was predicted and matched, and five possible target genes of miR-375 were found, *RLF*, *HIGD1A*, *COLCA2*, *POU3F1*, and *WBP1L* (Figure 1). All of the possible target genes had either one or two miR-375 binding sites in the 3 'UTR region.

miR-375 down-regulated the target genes *RLF* and *HIGD1A*

Expression of the GFP protein was observed in ST cells transfected with the *miR-375* mimics, *miR-375* inhibitor and *miRNA-ShNC*, suggesting that transfection was successful.

Expression of miR-375 in ST cells is shown in Figure 2. The qPCR results showed that expression of miR-375 in ST cells transfected with mimics exhibited a significant increase compared with cells transfected with inhibitor or shNC. In addition, expression of miR-375 was dramatically decreased in ST cells transfected with inhibitor compared with cells transfected with shNC. Expression of *RLF*, *HIGD1A*, *COLCA2*, *POU3F1*,

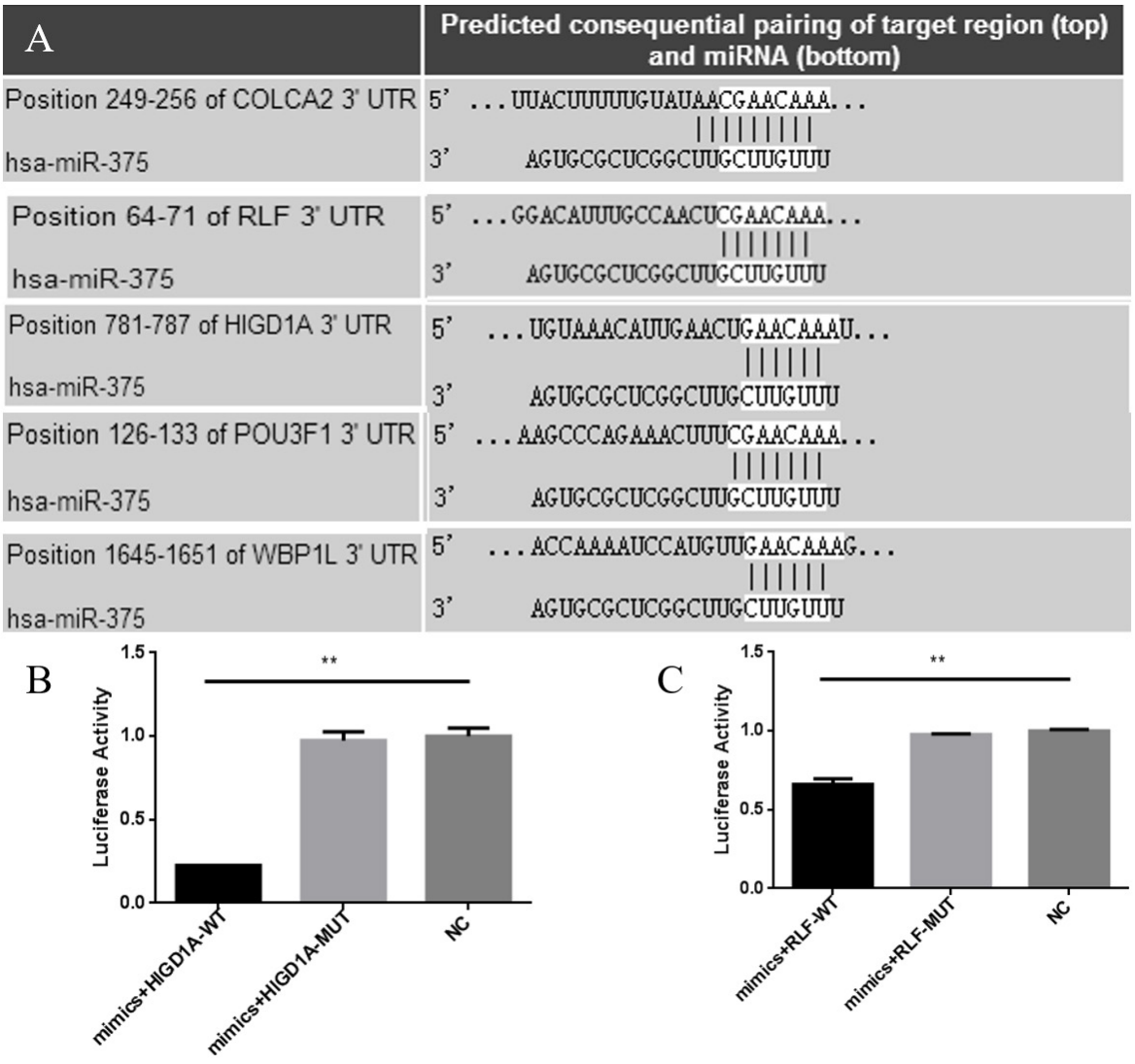

Figure 1. Target gene prediction and luciferase activity analysis. (A) Through analysis of a bioinformatics website, target genes complementary to the miR-375 conserved sequence were found. (B) Statistically significant differences between the experimental groups were determined using analysis of variance (ANOVA) with SPSS. p-values <0.05 were considered statistically significant. Luciferase activity of ST cells transfected with RLF-WT and HIGD1A-WT was significantly lower than cells transfected with RLF-mut, HIGD1A-mut and the negative control (p<0.01).

and *WBP1L* in ST cells was demonstrated in cells transfected with the mimics, inhibitor and shNC (Figure 2). Subsequent analysis of the target gene showed that expressions of *RLF* and *HIGD1A* were negatively correlated with miR-375. *COLCA2* has a positive correlation with miR-375. Both transfections with miR-375 mimics and the miR-375 inhibitor up-regulated the target gene *POU3F1*. ST cells transfected with miR-375 mimics up-regulated the target gene WBP1L. There was no significant difference between ST cells transfected with the miR-375 inhibitor and miRNA-shNC. Expression of *COLCA2*, *POU3F1*, and *WBP1L* in ST cells transfected with mimics, inhibitor and shNC is not negatively correlated with miR-375. Therefore, in the following experiment, we validated two possible target genes, *RLF* and *HIGD1A*.

Furthermore, the ELISA results were the same as those of qPCR analysis. Protein expression of *RLF* and *HIGD1A* were negatively correlated with miR-375 (Figure 3). Further evidence suggests that *RLF* and *HIGD1A* might be the target genes of miR-375.

Validation of the specific binding of *RLF* and *HIGD1A* with miR-375 by luciferase assays

Bioinformatics analysis revealed that there was one miR-375 conservative site on the 3'UTRs of *RLF* and *HIGD1A* (Figure 1). To verify whether miR-375 can bind to *RLF* and *HIGD1A*, we used the dual luciferase report assay. As shown in Figure 3, the luciferase activities of ST cells transfected with the *RLF-WT* or *HIGD1A-WT* vector significantly decreased compared with ST cells transfected with the *RLF-MUT* vector of *HIGD1A-MUT* vector. It was also observed that the luciferase activities

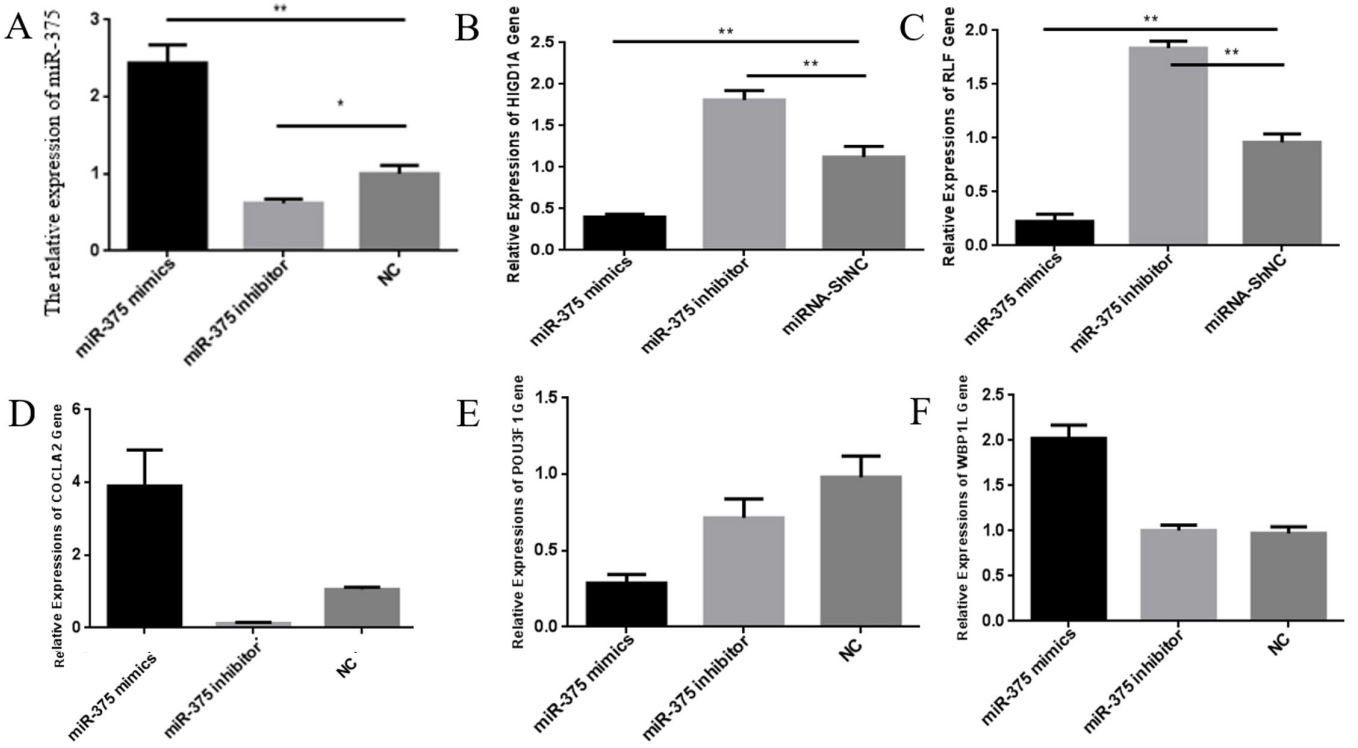

Figure 2. Relative expression of miR-375 and target genes in ST cells. (A) miR-375 was up-regulated in Sertoli (ST) cells when transfected with miR-375 mimics ($p<0.01$). miR-375 was down-regulated in ST cells when transfected with the miR-375 inhibitor ($p<0.01$). (B) The relative expression of *HIGD1A* in ST cells showed a significant increase when transfected with miR-375 mimics compared with the negative control ($p<0.01$). The relative expression of *HIGD1A* in ST cells was significantly higher after transfection with the miR-375 inhibitor compared with the negative control ($p<0.01$). (C) The relative expression of *RLF* in ST cells showed a significant increase when transfected with miR-375 mimics compared with the negative control ($p<0.01$). The relative expression of *RLF* in ST cells was significantly higher after transfection with the miR-375 inhibitor compared with the negative control ($p<0.01$). (D) The relative expression of *COLCA2* in ST cells had no negative correlation with miR-375. (E) The relative expression of *POU3F1* in ST cells had no negative correlation with miR-375. (F) The relative expression of *WBP1L* in ST cells had no negative correlation with miR-375.

were not significantly different after transfection of the *RLF-MUT* and *HIGD1A-MUT* vectors (Figure 4). The luciferase assay results proved that *RLF* and *HIGD1A* can bind specifically to miR-375.

miR-375 inhibited proliferation of ST cells

The MTS assay results suggested that ST cells transfected with the *miR-375* inhibitor have a faster proliferation rate after 24 hours than ST cells transfected with miR-375 mimics (Figure 3).

DISCUSSION

MicroRNAs (miRNAs) are a general class of endogenous non-coding RNAs that are 22 nucleotides long, are found in diverse species and play important roles in the initiation and progression of malignancies. miRNAs are essential to many *in vivo* biological processes, such as cell proliferation, apoptosis, the immune response and tumorigenesis [12].

miRNAs regulate expression of the target genes in animals and plants at the post transcriptional level and reduce the mRNA or protein translation level of target genes. Especially regarding the translation repression effect, miRNA combines with mRNA rather than directly degrading it, resulting in translational repression [13]. Therefore, regulation of this gene does not significantly reduce the mRNA level a gene, which makes it necessary to detect expression at the protein level to understand the regulatory effects of miRNA. However, by using few experiments, it was shown that miRNA can regulate the expression of target genes [14]. In summary, the relationship between miRNA and expression of the target genes at the cellular mRNA and protein levels can be used to determine whether there is a relationship with a target gene. In this study, it was found that the expression level of miR-375 in ST cells was negatively correlated with the mRNA and protein levels of the possible target genes *RLF* and *HIGD1A*, suggesting that *RLF* and *HIGD1A* might be targeted by miR-375.

It is widely known that the classical miRNA gene silencing mechanism suggests that the body forms mature miRNA and a silencing complex that contains Argonaute and other proteins. This strategy makes use of the principle of complementary base specific recognition in combination with the 3′ UTR to predict the target mRNA. The translation efficiency or stability of mRNA was regulated, and expression of specific genes in

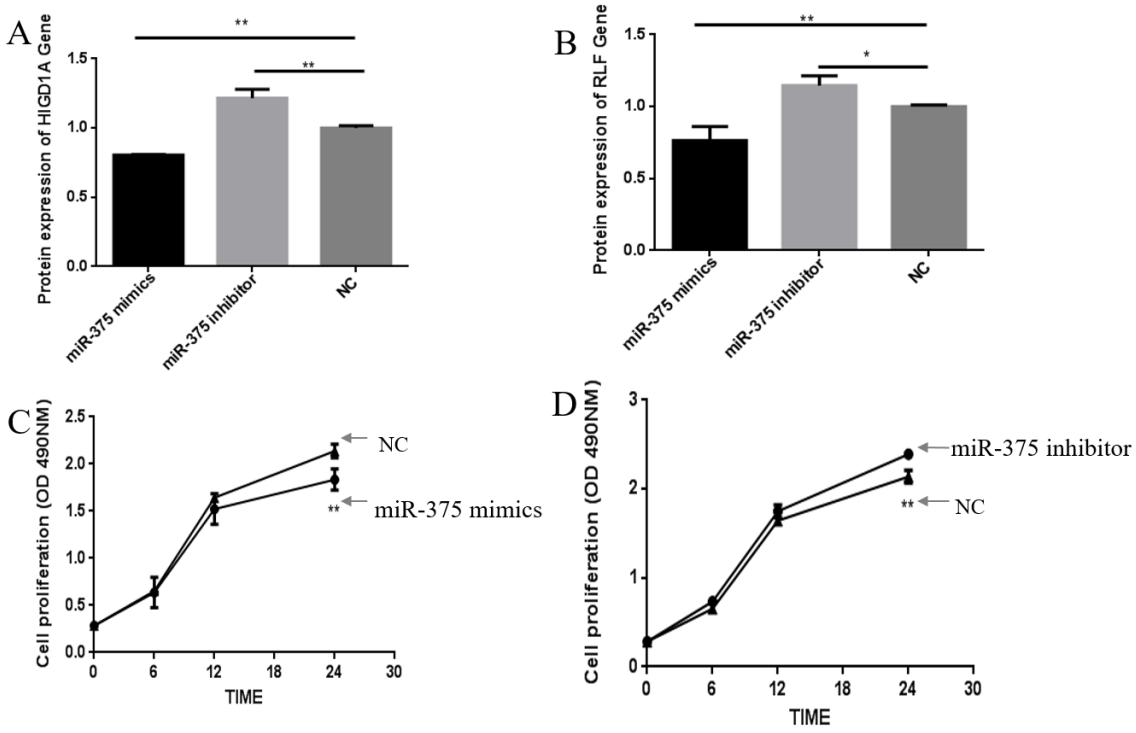

Figure 3. Protein expression of *HIGD1A* and *RLF* and their effects on cell viability in ST cells. (A) Protein expression of *HIGD1A* showed a significant increase when transfected with miR-375 mimics compared with the negative control ($p<0.01$). Protein expression of *HIGD1A* was significantly higher after transfection with the miR-375 inhibitor compared with the negative control ($p<0.01$). (B) Protein expression of *RLF* was significantly increased when transfected with miR-375 mimics compared with the negative control ($p<0.01$). Protein expression of *RLF* was significantly higher after transfection with the miR-375 inhibitor compared with the negative control ($p<0.05$). (C) Inhibition of cell growth was observed 24 h after transfection with miR-375 mimics ($p<0.01$). (D) The miR-375 inhibitor promoted ST cell growth ($p<0.01$).

the cytoplasm was controlled [15]. Possible target gene mRNA was predicted using the principle of specificity and the 3′ UTR.

miRNA is composed of small RNA of more than 20 bases, specifically, six to seven bases of seed regions that are specifically combined with the target gene 3′ UTR target site. miRNA can directly degrade or inhibit expression of target genes and regulate biological functions of an organism. However, when a base mutation or deletion occurs in the seed zone, alterations of biological function can result. The most effective way to detect the specific binding between miRNA and its target genes is to use the double luciferase reporter gene system. This system can detect degradation of the target gene by marker miRNA. The double luciferase reporter gene is expressed under the control of a promoter. If miRNA can specifically bind to the target 3′ UTR sequence and inhibit transcription, it will reduce expression of wild-type double luciferase. By contrast, if miRNA cannot bind to the target gene sequence, the luciferase reporter gene results will not change.

Bioinformatics analysis confirmed the existence of miR-375 target sites in the 3′ UTR of *RLF* and *HIGD1A*, and the dual luciferase reporter gene assay further validated that *RLF* and *HIGD1A* were targeted by miR-375. The results showed that *RLF* and *HIGD1A* are target genes of miR-375.

In this study, it was found that miR-375 inhibits ST cell proliferation. Sertoli cells provide structural and nutritional support for germ cell development. ST cells actively metabolize glucose and convert it to lactate, which is an important source of energy for germ cells. Furthermore, Sertoli cells can oxidize fatty acids, a metabolic process that is assumed to fulfill their own energy requirements [16]. ST cells play an important role in the development of germ cells and in providing the energy source for germ cells. Because miR-375 inhibited proliferation of ST cells, inhibition of miR-375 likely plays roles in the development of germ cells and energy metabolism.

CONCLUSION

In this study, we showed that miR-375 inhibited the proliferation of ST cells. miR-375 down-regulated its target genes *RLF* and *HIGD1A* at the mRNA and protein expression levels, suggesting that miR-375 affects the growth of the testicular tissue by targeting *RLF* and *HIGD1A*.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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