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*Original Article*

# miR-137 regulates testosterone secretion in Leydig cells of rats by Kiss-1

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**Abstract:** Objective: To determine the effect of miR-137 on secretion of testosterone in Leydig cells of rats by Kiss-1. Methods: Animal models of delayed hypogonadal hypofunction (LOH) were established. The techniques of qRT-PCR, Western blot, and ELISA were applied for detection of expression of miRNA-137 and Kiss-1 proteins, and testosterone in Leydig cells of animal models. Dual luciferase reporter assays were employed to predict the association between miRNA-137 and Kiss-1 protein. Results: Remarkably lower miRNA-137 expression ($t = 4.771$, $P = 0.001$) and considerably higher Kiss-1 protein expression ($t = 4.757$, $P = 0.002$) in Leydig cells of rats was observed in the control group than in the experimental group. Testosterone testing revealed that the control group was substantially higher than that of the experimental group ($t = 4.683$, $P = 0.002$). Correlation analysis demonstrated that miRNA-137 was negatively correlated with Kiss-1 in LOH rats ($r = -0.748$, $P = 0.001$), whereas miRNA-137 was negatively correlated with testosterone in LOH rats ($r = -0.637$, $P = 0.005$). In contrast, Kiss-1 was positively correlated with testosterone in LOH rats ($r = 0.774$, $P = 0.019$). Dual luciferase reporter assays indicated that miR-137 bound to the 3'UTR region of Kiss-1 gene. Conclusion: miR-137 may inhibit the secretion of testosterone in Leydig cells of rats by binding to Kiss-1.

**Keywords:** miR-137, Kiss-1, testosterone, late-onset hypogonadism

## Introduction

Late-onset hypogonadism (LOH), a male age-related disorder, is a clinical and biochemical syndrome presenting with clinically significant symptoms (tweet, underdeveloped muscle, and lower energy), and remarkably decreased serum testosterone levels [1, 2]. Studies indicate that the main pathogenesis of LOH is testosterone deficiency caused by aging, and which is secondary to LOH [3]. As far as the physiological mechanisms are concerned, aging also induces dysfunction of the hypothalamic-pituitary-gonadal axis, leading to hypothalamic secretion of gonadotropin-releasing hormone (GnRH) in the form of pulsed release and retarded expression. No matter whether the pituitary gonadotrope cells are thoroughly reactive or not, they decrease release of luteinizing hormone and follicle stimulating hormone, which eventually results in decrease of luteinizing hormone on the surface of Leydig cells. This contributes to androgen secretion and causes onset of LOH. Growing evidence also indicates that multiple clinical factors (age, obesity, smoking, and alcoholism) also give rise to the occurrence of LOH [4, 5].

microRNAs (miRNA), are small (19-22 nucleotides) non-coding single-stranded RNA molecules, that are a decisive class of non-protein-coding RNAs that function to initiate translational suppression or mRNA cleavage of target mRNAs by regulating the expression of other genes [6]. Studies in recent years demonstrate that miRNA-137 is strongly associated with the onset and progression of a wide range of diseases (cancers, nervous system diseases, and immune diseases). Nevertheless, few reports have so far been focused on LOH [7, 8]. Kiss-1 plays an important role in the irritation of GnRH secretion during puberty. GnRH released from the hypothalamus acts on the anterior pituitary triggering the release of luteinizing hormone (LH), and follicle stimulating hormone (FSH). Bioinformatics analysis revealed that miR-137

is closely related to the Kiss-1 gene. Therefore, this study was designed to elucidate the association between miR-137 and LOH by evaluating the levels of miR-137, Kiss-1 and testosterone expressed in serum of patients with LOH.

## Methods and materials

### *Animal sources*

Forty male SPF rats aged 60 weeks old and weighed 350-450 g were purchased from Western Technology, China and assigned to the experimental group. An additional 20 SDF Sprague Dawley male rats were purchased that were 20 weeks old and weighed 240-260 g. They were housed in specific pathogen-free (SPF) conditions at constant temperature of 22 ± 3°C and a humidity of 80 ± 2%. They were conventionally fed with adequate food and water before the experiment.

### *Main cells, reagents and instruments*

HEK293 cells were purchased from Guidechem, China; DMEM medium and 10% FBS from Gibco, USA; Percoll from Wikstroms, Sweden; RIPA lysis buffer was from Solarbio, China; BCA Protein Quantification Kit was from Beyotime Biotechnology, China; goat anti-mouse secondary antibodies, TRIzol, and RNA extraction kits were from Invitrogen, USA; Reverse transcription cDNA synthesis kits and PCR reaction kits were from Applied Biosystems, China; dual luciferase reporter assay kits were from Promega, China; UV-visible spectrophotometers were from Perkin-Elmer, China; and PCR gene amplifiers were from Bio-Rad, China.

### *Cell culture*

Human embryonic kidney epithelial (HEK293) cells were cultured in DMEM medium containing 10% FBS and penicillin-streptomycin double antibodies at 37°C in a 5% $CO_2$ incubator.

### *Dual luciferase reporter assay*

The Kiss-1 luciferase vector and the mutant vector, the miR-137 mimics, and irrelevant sequences were respectively transferred into HEK293 cells using LipofectamineTM 2000 kits. The luciferase activity in HEK293 cells in each group was detected following the manufacturer's instructions on the dual luciferase reporter assay kits. Samples were assayed for luciferase activity with the use of the kits for Promega dual luciferase reporter assay. The media in 24-well plates was pipetted 48 hours after co-transfection of the miR-137 inhibitor and Kiss-1 3'UTR regions. The plates were washed with PBS twice. After PLB 100 ul lysis buffer had been added to each well following the instructions, the plates were shaken on a plate shaker for 15 min, followed by collection of the lysates from the plates. Relevant reagents were ready for testing on the instrument. An automatic luminescence detector was used, with the program read-ahead set to 2 s, and the reading value to 10 s. Each time, 100 ul of LARII and stop & Glo reagent were added. First, 20 ul of lysate was added to the luminescent plate and placed in the bioluminescence detector. The configured LARII and Stop & Glo reagent were added to the abovementioned luminescent plate, and then the operating program was performed for detection. Fluorescence values were read and the data stored. The results of detection included firefly luciferase (F) and Renilla luciferase (R). The formula for calculating multiple of activity is as follows: Multiple of activity = (F/R) sample/(F/R) control.

### *Animal modeling*

The median serum testosterone levels of 20 male SD rats aged 20 weeks old was analyzed according to the method developed by Zhang et al., and the 95% confidence interval (CI) was calculated [9]. It was confirmed that the elderly SD rats were similar to elderly people regarding significant decreases in blood testosterone and free testosterone, and blood testosterone and free testosterone in adult SD rats (95% CI) were applied as a standard value. The lower limit of 95% CI was determined to be 2.04 ng/mL. Taken the value as a benchmark, after stimulation (The rats were kept under the condition of artificially reversed day and night, and restricted to food and water intermittently). Rats with blood testosterone and free testosterone levels lower than this benchmark were classified as successful LOH rat models. Of the 40 rats, 25 met the standard, so the 25 rats were successfully modeled. Among the 25 rats, 10 were randomly assigned to the experimental group and 10 to the control group.

### *Leydig cells extraction*

After being euthanized by cervical dislocation, the left testis of each rat was taken and the

**Table 1.** Primer sequences

| Gene | Forward primer | Reverse primer |
|---|---|---|
| miRNA-137 | 5'-TTATTGCTTAAGAATACGCGTAG-3' | 5'-TGGTGTCGTGGAGTCG-3' |
| U6 | 5'-GCTTCGGCAGCACATATACTAAAAT-3' | 5'-CGCTTCACGAATTTGCGTGTCAT-3' |

**Table 2.** Target gene prediction

| | mimics NC | miRNA-137 | t | P |
|---|---|---|---|---|
| pMIR-KISS1-WT | 1.02 ± 0.15 | 0.37 ± 0.05 | 7.120 | 0.002 |
| pMIR-KISS1-MUT | 1.01 ± 0.12 | 1.03 ± 0.22 | 0.138 | 0.896 |

testis capsule was removed. The testis capsule was reduced in size to 1 mm * 1 mm * 1 mm using scissors, placed in digestion buffer containing 1 g/L collagenase Type II and incubated at 37°C in a water bath for 30 min. The digested tissues were collected using a 200-mesh nylon sieve. The collected tissues were filtered at 2500 r/min for 5 min, and then the cells were harvested. After precipitation, the cells were re-suspended in PBS buffer and added to Percoll solution for gradient centrifugation. In this way, Leydig cells were finally obtained.

*qRT-PCR*

Total RNA was extracted from cells of each group with TRIzol reagent. The purity, concentration, and integrity of RNA were measured by a UV spectrophotometer and 1% agarose gel electrophoresis. Reverse transcription to cDNA was performed on the extracted total RNA according to the manufacturer's instructions on the miRNA transcription kits. qRT-PCR was conducted using the Taqman qRT-PCR kits. The miRNA-137 internal control was designed and synthesized by Shanghai Sangon Biotech, China, and U6 was set as the reference gene. The primer sequences are shown in **Table 1**. qRT-PCR was carried out under the following cycling conditions: 95°C for 3 min, 95°C for 15 s, 60°C for 30 s, 72°C for 15 s for a total of 40 cycles. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

*Western blot*

Total protein of the cells was extracted with the use of the RIPA lysate on the RIPA kits, and the concentrations of the protein samples were detected using the BCA protein quantification kits. Protein was loaded at 60 ug per well, separated by SDS-PAGE electrophoresis and transferred to the PVDF membranes. The membranes were blocked with 5% skim milk powder for 2 hours, incubated with primary antibodies (diluted at 1:1000) overnight at 4°C for 12 hours, then incubated with HRP-conjugated secondary antibodies (diluted at 1:5000) in a greenhouse, and visualized using the ECL chemiluminescence reagents in a darkroom. GAPDH was used as a control.

*Statistical analysis*

All the findings collected from this study were analyzed using the SPSS software package, version 20.0 (Strong-Vinda, Beijing, China). Count data are described as percentages (%), and the Chi-square test was employed for comparisons between the two groups. Measurement data are presented as mean ± se; linear correlation analyses were performed for exploring correlations between variables. $P<0.05$ was considered to be statistically significant.

## Results

*Animal models*

Four weeks after modeling, the rats in the experimental group became depressed, dim-eyed, with less food and water intake, and some had reduced body weight. By contrast, the rats in the control group were in good conditions, lively and responsive, with normal food and water intake and gradual weight gain.

*Target gene prediction*

Targetscan prediction demonstrated that miR-137 acted on the Kiss-1 gene 3'UTR region, resulting in reduced synthesis and higher predicted values of both genes. Dual luciferase reporter assay revealed that miR-137 was bound to Kiss-1 gene 3'UTR region, as shown in **Table 2**.

*Testosterone expression levels in rats of the two groups*

After modeling, the testosterone expression level (3.84 ± 1.51 ng/mL) of rats in the control

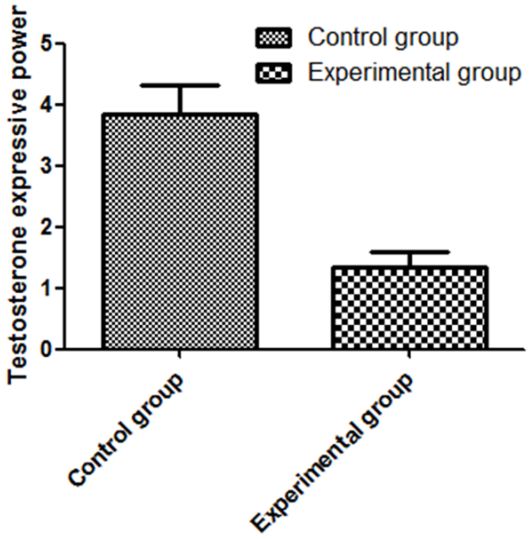


**Figure 1.** Testosterone expression. Testosterone expression in the blood of rats in the control group was substantively higher than that in the experimental group (t = 4.683, P = 0.002).

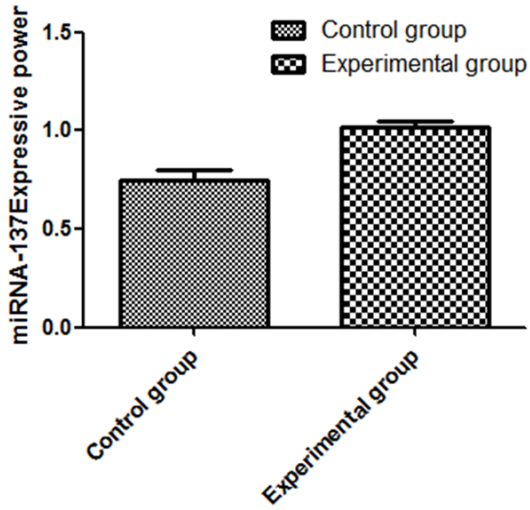


**Figure 2.** miRNA-137 expression. qRT-PCR detection revealed remarkably lower miRNA-137 expression in cells of rats in the control group than in the experimental group (t = 4.771, P = 0.001).

group was remarkably higher than that (1.350 ± 0.745 ng/mL) in the experimental group and there was a statistical difference between the two groups (t = 4.683, P = 0.002, **Figure 1**).

*miRNA-137 expression in Leydig cells of rats*

qRT-PCR indicated that the miRNA-137 expression level (0.747 ± 0.153) in the cells of rats of the control group was strikingly lower than that

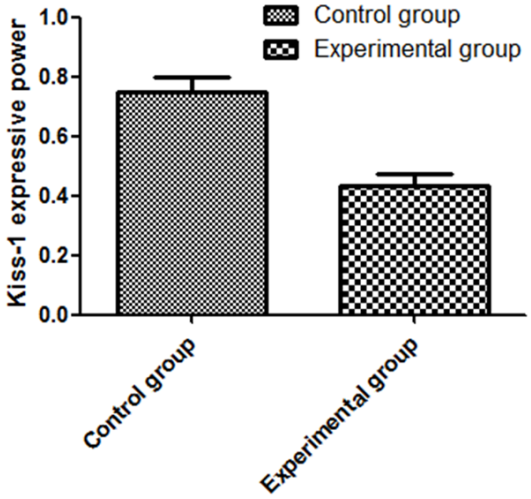


**Figure 3.** Kiss-1 protein expression. Western blot detection demonstrated Kiss-1 protein expression in cells of rats in the control group was considerably higher than that in the experimental group (t = 4.757, P = 0.002).

(1.021 ± 0.095) in the cells of rats in the experimental group (t = 4.771, P = 0.001, **Figure 2**).

*Kiss-1 protein expression in Leydig cells of rats*

Western blot analysis of Kiss-1 protein in Leydig cells of rats showed significantly elevated Kiss-1 protein expression (0.747 ± 0.164) in the control group than that (0.435 ± 0.127) in the control group (t = 4.757, P = 0.002, **Figure 3**).

*Correlation analysis of testosterone, miRNA-137 and Kiss 1 in LOH rats*

Correlation analyses on the indicators revealed that miRNA-137 was negatively correlated with Kiss-1 in LOH rats (r = -0.748, P = 0.001); miRNA-137 was also reversely correlated with testosterone in LOH rats (r = -0.637, P = 0.005). In contrast, Kiss-1 was positively correlated with testosterone in LOH rats (r = 0.774, P = 0.019).

## Discussion

The Kiss-1 gene was first thought to be a tumor metastasis suppressor. It is a relatively mature gene and is expressed in normal tissues (placenta, skeletal muscle, brain, and kidney), and prominently expressed in the hypothalamus and placenta [10-13]. In recent years, some scholars have found that Kiss-1 plays an essential role in the human reproductive system.

Ohtaki et al. found from a quantitative PCR analysis that Kiss-1 mRNA was expressed in testis, pancreas and other tissues with the exception of placenta tissue [14]. In tumors in particular, regulation of the Kiss-1 gene in urothelial cell neoplasms lowers the binding of NF-kB to the MMP-9 promoter, which inhibits the transcription of MMP-9 and results in reduced synthesis of MMP-9 and inhibited proliferation and invasion of tumor cells [15]. Other studies have reported that the Kiss-1 gene is an important member of the reproductive regulatory system, and it directly activates hypothalamic GnRH neurons to trigger puberty, and regulates GnRH expression by receiving signals of blood sex hormone levels [16, 17].

In the current study, prediction of kiss-1 target genes by the online prediction software demonstrated that miRNA-137 closely correlated with Kiss-1 gene. In order to prove its association, we further performed *in vitro* culture. The results of dual luciferase reporter assay indicated that miRNA-137 could regulate Kiss-1 gene, suggesting that Kiss-1 can serve as a downstream target gene of miRNA-137. Then the expression of miRNA-137, Kiss-1 protein and testosterone in LOH rat models was detected by qRT-PCR, Western blot, and ELISA. The results demonstrate that expression of miRNA-137 is remarkably higher in the experimental group than in the control group, and Western blot detection of Kiss-1 protein expression indicated that Kiss-1 protein expression in the experimental group was considerably lower than that in the control group. Furthermore, a negative correlation was observed between the miRNA-137 and Kiss-1 by the correlation analyses, indicating that miRNA-137 elevation targeted reduced Kiss-1 protein expression. Subsequently, expression of testosterone in Leydig cells was determined by the ELISA and the result indicated the expression of testosterone in the experimental group was substantively lower than that in the control group. Correlation analysis revealed that the protein expression of Kiss-1 was also positively correlated with the expression of testosterone. Multiple reports state that the changes in Kiss-1 protein give rise to different expression of testosterone, and we have further verified this point in our experiments [18-20]. Therefore, through the above experiments we preliminary speculated that miRNA-137 may regulate the secretion of testosterone via Kiss-1 to induce the onset of LOH.

However, there are still some limitations in this study. This experiment is a basic animal experiment, but it was not carried out in a clinical setting. The model selection was limited as most animals died due to old age, which might result in differences in the results of this experiment. Moreover, we still do not understand how Kiss-1 protein regulates the expression of testosterone and whether Kiss-1 protein regulates other pathways. Additional clinical studies with larger sample sizes are required to identify more associations.

In conclusion, miR-137 may inhibit the secretion of testosterone in Leydig cells of rats by binding to Kiss-1, and miR-137 is expected to become a new target for the management of LOH.

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## Disclosure of conflict of interest

None.

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