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Research Article

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LncRNA-m18as1 competitively binds with miR-18a-5p to regulate follicle-stimulating hormone secretion through the Smad2/3 pathway in rat primary pituitary cells

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Abstract: Long noncoding RNAs (lncRNAs) are expressed in different species and different tissues, and perform different functions, but little is known about their involvement in the synthesis or secretion of follicle-stimulating hormone (FSH). In general, we have revealed lncRNA–microRNA (miRNA)–messenger RNA (mRNA) interactions that may play important roles in rat primary pituitary cells. In this study, a new lncRNA was identified for the first time. First, we analyzed the gene expression of lncRNA-m18as1 in different tissues and different stages by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and observed the localization of lncRNA-m18as1 with fluorescence in situ hybridization, which indicated that this lncRNA was distributed mainly in the cytoplasm. Next, we used RT-qPCR and enzyme-linked immunosorbent assay (ELISA) to analyze the regulation of FSH synthesis and secretion after overexpression or knockdown of lncRNA-m18as1 and found that lncRNA-m18as1 was positively correlated with FSH synthesis and secretion. In addition, mothers against decapentaplegic homolog 2 (Smad2) was highly expressed in our sequencing results. We also screened miR-18a-5p from our sequencing results as a miRNA that may bind to lncRNA-m18as1 and Smad2. We used RNA immunoprecipitation-qPCR (RIP-qPCR) and/or dual luciferase assays to confirm that lncRNA-m18as1 interacted with miR-18a-5p and miR-18a-5p interacted with Smad2. Fluorescence in situ hybridization (FISH) showed that lncRNA-m18as1 and miR-18a-5p were localized mainly in the cytoplasm. Finally, we determined the relationship among lncRNA-m18as1, miR-18a-5p, and the Smad2/3 pathway. Overall, we found that lncRNA-m18as1 acts as a molecular sponge of miR-18a-5p to regulate the synthesis and secretion of FSH through the Smad2/3 pathway.

Key words: Long noncoding RNA (lncRNA); MicroRNA (miRNA); Competitive endogenous RNA (ceRNA); Follicle-stimulating hormone (FSH); Mothers against decapentaplegic homolog 2/3 (Smad2/3)

1 Introduction

Follicle-stimulating hormone (FSH) is a key hormone in the regulation of mammalian reproduction, and is synthesized and secreted by the pituitary gland (Acevedo-Rodriguez et al., 2018). FSH is a heterodimeric glycoprotein hormone. In addition to FSH, the glycoprotein family also includes luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (CG) (Pierce and Parsons, 1981). As an intermediate factor of the hypothalamic–pituitary–gonadal (HPG) axis, FSH regulates downstream spermatogenesis and oogenesis by binding to FSH receptors located on testicular supporting cells and ovarian granulosa cells (Heckert and Griswold, 1993; George et al., 2011; Kaprara and Huhtaniemi, 2018; Kumar, 2018). Secretion of FSH is regulated by gonadotropin-releasing hormone (GnRH) synthesized by the hypothalamus as well as other factors, such as single-nucleotide polymorphisms (SNPs), follistatin, progesterone, activin, and microRNAs

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(miRNAs) (Popovics et al., 2011; Han et al., 2017b; Schubert et al., 2019; Stamatiades et al., 2019; Motta et al., 2020). MiRNAs represent a class of small biological molecules with a length of 19–23 nucleotides (Lei et al., 2017). There are reports in the literature that miR-7a2 acts as a positive regulator of FSH and LH synthesis (Ahmed et al., 2017) and that miR-132/212 mediates GnRH to regulate FSH expression (Lannes et al., 2016). In addition, several miRNAs, such as miR-21-3p and miR-433, downregulate expression of the *Fshβ* gene in rat anterior pituitary cells (Han et al., 2017b). However, to date, our understanding of the mechanisms regulating FSH secretion is still limited.

Long noncoding RNAs (lncRNAs), which represent a recently discovered type of regulatory RNA, are noncoding RNAs (ncRNAs) with a length of more than 200 nucleotides that can still function through a variety of mechanisms even though they cannot be translated into proteins (Ma et al., 2013; Cipriano and Ballarino, 2018). For instance, lncRNAs regulate the stability and/or translation of target messenger RNAs (mRNAs) to play roles at the posttranscriptional level (Noh et al., 2018). Some examples include the lncRNA β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) antisense (BACE1-AS), which can enhance the stability of BACE1 mRNA, and the lncRNA growth arrest-specific 5 (GAS5), which binds to c-Myc mRNA to regulate c-Myc translation through lncRNA–mRNA interaction (Faghihi et al., 2008; Hu et al., 2014). LncRNAs can also act as miRNA "sponges" or competitive endogenous RNAs (ceRNAs) to regulate target miRNA genes (Salmena et al., 2011; Xu et al., 2020). For example, lncRNA-RNA component of mitochondrial RNA-processing endoribonuclease (RMRP) regulates miR-206 as a molecular sponge to promote proliferation, migration, and invasion in bladder cancer (BC) cell lines (Cao et al., 2019); the lncRNA plasmacytoma variant translocation 1 (PVT1) serves as a ceRNA to negatively regulate the expression of miR-424 in cervical cancer cells (Gao et al., 2017); and lncRNA-maternally expressed gene 3 (MEG3) can upregulate B-cell lymphoma 2 (Bcl-2) expression by competitively binding with miR-181 family members to regulate the occurrence of gastric cancer (Peng et al., 2015). However, there have been relatively few studies on interactions between lncRNAs and on ceRNA mechanisms in the context of animal reproduction regulation.

Mothers against decapentaplegic homologs (Smads), which are eukaryotic transcription factors, play significant roles in the transforming growth factor-β (TGF-β) signaling cascade (Massague et al., 2005). In addition to regulating the transcription factor TGF-β, Smad proteins are affected by some ncRNAs. For instance, the lncRNA definitive endoderm-associated lncRNA 1 (DEANR1) induces activation of the endoderm factor forkhead box A2 (FOXA2) by interacting with Smad2/3 and positively regulating FOXA2 (Jiang et al., 2015). The lncRNA LINC00458 directly interacts with Smad2/3 to regulate the endothelial formation process (Chen et al., 2020). Overexpression of miR-155-5p can inhibit development of vascular calcification by inhibiting activation of the Smad2/3 signaling pathway (Zhao et al., 2020). The lncRNA oxidative stress responsive serine-rich 1-antisense RNA 1 (OSER1-AS1), a molecular sponge of miR-433-3p, promotes the occurrence and development of non-small cell lung cancer by increasing Smad2 expression (Liu et al., 2020). A role for Smad2 in regulating reproduction and development has also been reported (Bernard, 2004). However, there have been few studies on the mechanisms by which ceRNAs regulate FSH synthesis and secretion through the Smad2/3 pathway.

In this study, we identified the new lncRNA MSTRG.37997, which we have named lncRNA-miR-18a-5p sponge one (lncRNA-m18as1). We explored whether lncRNA-m18sa1, as a molecular sponge of miR-18a-5p, regulates FSH expression through the Smad2/3 pathway.

2 Materials and methods

2.1 Animals and cell culture

All Sprague-Dawley rats used in this experiment were of specific pathogen-free (SPF) grade and were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning, China). Samples of different tissues (heart, liver, spleen, lung, kidney, brain, pituitary, pineal, muscle, and testis) were taken from three 8-week-old male rats to detect gene expression of lncRNA-m18as1. Pituitary samples were taken from three male rats in different age groups (2, 4, 6, and 8 weeks old) to analyze gene expression of lncRNA-m18as1. Rat primary pituitary cells were

obtained as described in our previous study (Han et al., 2017b). The rat primary pituitary cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; HyClone, Logan, UT, USA) containing 100 U/mL penicillin/streptomycin (HyClone) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a 37 ℃ humidified incubator (Lishen, Shanghai, China) with 5% CO_2. The rat pituitary adenoma cell lines GH3 and MMQ were obtained from the National Infrastructure of Cell Line Resource (resource numbers: 3111C0001CCC000008 and 3111C0001CCC000081) and cultured under the same conditions.

2.2 siRNA synthesis and plasmid construction

LncRNA-m18as1 small interfering RNAs (siRNAs) and the negative control siRNA were designed and synthesized by GeneCreate Biological Engineering Co., Ltd. (Wuhan, China). LncRNA-m18as1 complementary DNA (cDNA) was inserted into the pReceiver plasmid (pcDNA3.1) to construct a stable expression plasmid carrying lncRNA-m18as1 (this plasmid was also synthesized by GeneCreate Biological Engineering Co., Ltd.). Plasmid extraction was conducted according to the protocol of an EndoFree Maxi Plasmid kit, which was obtained from Tiangen Biotech Co., Ltd. (Beijing, China).

2.3 Synthesis of miRNA mimic

The miR-18a-5p mimic and negative control were synthesized by RiboBio Biotech Co., Ltd. (Guangzhou, China).

2.4 Cell transfection

Rat primary pituitary cells were seeded on a 24-well plate at a density of 3×10^5 cells/well. A Lipofectamine 2000 Transfection kit was purchased from Invitrogen (Carlsbad, California, USA), and the recommended protocol was used to perform all transfection experiments in this study. The treatment concentrations of miR-18a-5p mimic, miR-18a-5p negative control, lncRNA-m18as1 siRNA, and siRNA negative control were 100 nmol/L. The final concentrations of the lncRNA-m18as1 overexpression plasmid were 500, 1000, and 1500 ng/mL. Serum-free basal medium was used throughout the transfection process. After transfection, the cells were incubated for 48 h for gene expression analysis.

2.5 Fluorescence in situ hybridization

Rat primary pituitary cells were seeded on a 24-well plate with cell slides at a density of 3×10^5 cells/well. Specific probes for lncRNA-m18as1 and miR-18a-5p were synthesized by Suzhou Genepharma Co., Ltd. (Suzhou, China). Fluorescence in situ hybridization (FISH) was performed in accordance with the manufacturer's recommended instructions to determine the localization of lncRNA-m18as1 and miR-18a-5p. Fluorescence microscopy (Nicon, Tokyo, Japan) was used to capture images.

2.6 Immunofluorescence analysis

Rat primary pituitary cells were seeded on a 24-well plate with cell slides at a density of 3×10^5 cells/well to determine the nuclear and cytoplasmic distribution of phosphorylated Smad4 after treatment. Notably, the whole process had to be carried out in a dark and humid box at room temperature to prevent the cells from drying out and to prevent fluorescence quenching. Fluorescence microscopy (Nicon) was used to capture images.

2.7 RNA immunoprecipitation-qPCR (RIP-qPCR) and luciferase reporter analysis

First, rat primary pituitary cells were transfected with the miR-18a-5p mimic and its negative control, and a lysate was prepared with RIP Lysis Buffer. Next, the cell lysate was centrifuged, and the supernatant was divided into two groups. An anti-argonaute 2 (anti-AGO2) antibody or a nonspecific immunoglobulin G (IgG) antibody (Abcam, Cambridge, MA, UK) was added, and the samples were incubated overnight at 4 ℃. Then, protein A/G magnetic beads were added, and the samples were incubated for 1 h at 4 ℃. After that, the pellets were centrifuged and washed using RIP Wash Buffer. Enrichment of lncRNA-m18as1 and miR-18a was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). More detailed experimental steps were carried out in accordance with the provided protocol of a Magna RIP RNA Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA).

We used RNAhybrid 2.2 (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and TargetScan (http://www.targetscan.org/vert_71) to predict the potential binding site between lncRNA-m18as1 and miR-18a-5p.

A sequence from approximately 200 base pairs upstream to 200 base pairs downstream of the lncRNA-m18as1 binding site for miR-18a-5p was cloned into the pmirGLO plasmid to obtain lncRNA-m18as1-WT; and a sequence with the lncRNA-m18a-5p binding site for miR-18a-5p was mutated to obtain lncRNA-m18as1-MuT. After cotransfection with miR-18a-5p mimic or its negative control, four experimental treatment groups were formed: the lncRNA-m18as1-WT+miR-control group, lncRNA-m18as1-WT+miR-18a-5p group, lncRNA-m18as1-MuT+miR-control group, and lncRNA-m18as1-MuT+miR-18a-5p group.

The same method was used to predict the binding site between miR-18a-5p and Smad2 mRNA and obtain Smad2-WT and Smad2-MuT. After cotransfection with miR-18a-5p mimic or its negative control, four experimental treatment groups were formed: Smad2-WT+miR-control group, Smad2-WT+miR-18a-5p group, Smad2-MuT+miR-control group, and Smad2-MuT+miR-18a-5p group. The intensities of firefly luciferin and renilla luciferin were detected with a Dual Luciferase Reporter Gene Assay kit (Beyotime, Shanghai, China).

2.8 RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the corresponding protocol. Reverse transcription of RNA was performed with a FastKing gDNA Dispelling RT SuperMix kit (Tiangen Biotech Co., Ltd.). RT-qPCR was performed using SuperReal PreMix Plus (SYBR Green, Tiangen Biotech Co., Ltd.), and the primers were designed on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and verified with Primer 3 website (https://primer3.ut.ee). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an endogenous reference for mRNAs, lncRNA and U6 were employed as endogenous references for miRNA. All primers used in this study are listed in Table S1.

2.9 ELISA

After 48 h of transfection, the culture supernatant was collected and the FSH secretion level was analyzed with a rat FSH enzyme-linked immunosorbent assay (ELISA) kit (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's protocol. The concentration gradient of the standard curve was 0, 0.75, 1.5, 3, 6, and 12 IU/L.

2.10 Western blot analysis

After 48 h of transfection, rat primary pituitary cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% (volume fraction) phenylmethylsulfonyl fluoride (PMSF) and 1% (volume fraction) protein phosphatase inhibitor Mix (Beijing Solarbio Science & Technology Co., Ltd., China). The concentration of each protein sample was determined with a bicinchoninic acid (BCA) assay kit (Millipore). After separation with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Then, the PVDF membranes were incubated in blocking solution prepared with 5% (volume fraction) bull serum albumin (BSA) dissolved in Tris-buffered saline Tween (TBST) for 1 h at room temperature and then incubated with specific primary antibodies at 4 °C overnight. On the second day, the membranes were washed three times in TBST for 10 min each before being incubated with secondary antibodies for 1 h at room temperature. The immunoreactive bands were detected using Millipore Sigma™ Immobilon™ Western Chemiluminescent horseradish peroxidase (HRP) Substrate (ECL) (WBKL0500, Millipore). The antibodies used in this experiment were anti-GAPDH (Cell Signaling Technology, Boston, MA, USA), anti-Smad4 (Cell Signaling Technology), anti-Smad2/3 (Cell Signaling Technology), anti-phospho-Smad2/3 (Abcam), and anti-phospho-Smad4 (Affinity, Golden, CO, USA). The primary antibody and blocking solution were diluted 1:1000 (volume ratio) for subsequent use, while the secondary antibody and blocking solution were diluted 1:5000 (volume ratio).

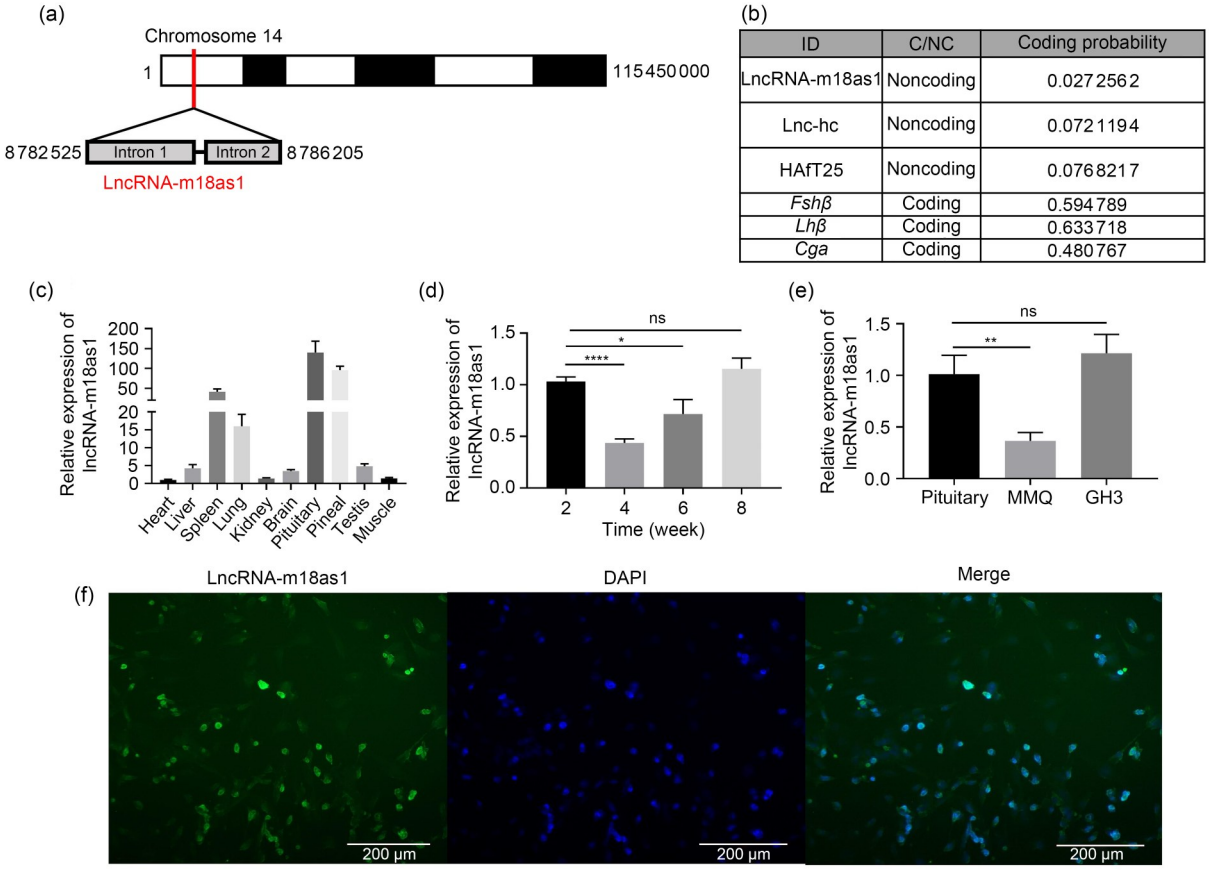
2.11 Statistical analysis

Western blot bands were grayscale-analyzed with ImageJ (Version 1.52a, National Institutes of Health, Bethesda, MD, USA) and the data from luciferase reporter analysis and western blot were normalized. Then all data were presented as mean±standard deviation (SD) of three independent experiments. Data were subjected to multiple comparisons for significant differences by SPSS 19.0 (SPSS, Chicago, IL, USA). The whole graphs were generated using GraphPad Prism 8 software (Prism for Mac OS X, GraphPad Software Inc., San Diego, California, USA). $P<0.05$ was considered to indicate statistical significance.

3 Results

3.1 Identification of lncRNA-m18as1

In our previous study, we identified lncRNAs in immature and mature rat pituitary glands (Han et al., 2017a). Based on these data, we selected the lncRNA MSTRG.37997 as our candidate research object and renamed it lncRNA-m18as1. LncRNA-m18as1 is an intergenic lncRNA on chromosome 14 composed of two introns (Fig. 1a). We used Coding Potential Calculator (CPC) 2.0 (http://cpc2.gao-lab.org) to prove that lncRNA-m18as1 did not have coding ability (Fig. 1b). We further proved this lack of coding potential through related tools on the NCBI and University of California Santa Cruz (UCSC) websites (data not shown). We further investigated the expression of lncRNA-m18as1 in different tissues of male rats. The results suggested that lncRNA-m18as1 was indeed expressed in these tissues. The pituitary had the highest expression of lncRNA-m18as1, followed by the pineal gland, spleen, lung, testis, liver, and brain, while the heart, kidney, and muscle had similar expression that was significantly lower than the expression in other tissues (Fig. 1c). In addition, we detected the gene expression of lncRNA-m18as1 in the pituitary tissues of rats of different ages (2, 4, 6, and 8 weeks old). When the expression of lncRNA-m18as1 in 2-week-old rats was used as a control, the expression of lncRNA-m18as1 in 4-week-old rats was decreased in comparison, and then expression gradually



ID	C/NC	Coding probability
LncRNA-m18as1	Noncoding	0.0272562
Lnc-hc	Noncoding	0.0721194
HAfT25	Noncoding	0.0768217
Fshβ	Coding	0.594789
Lhβ	Coding	0.633718
Cga	Coding	0.480767

Fig. 1 Identification of lncRNA-m18as1. (a) LncRNA-m18as1 chromosomal information; (b) Prediction of coding potential by CPC 2.0; (c) Expression of lncRNA-m18as1 in different tissues of male rats; (d) Expression of lncRNA-m18as1 in the pituitaries of rats at different developmental stages; (e) Expression of lncRNA-m18as1 in different pituitary cells; (f) LncRNA-m18as1 localization in rat primary pituitary cells as detected by FISH. The data are shown as mean±SD of three independent experiments. Statistical significance was analyzed by one-way ANOVA and Student's *t*-test (* $P<0.05$; ** $P<0.01$; **** $P<0.0001$; ns: not significant). LncRNA: long noncoding RNA; CPC: Coding Potential Calculator; FISH: fluorescence in situ hybridization; SD: standard deviation; ANOVA: analysis of variance; ID: identification; lnc-hc: lncRNA hc; HAfT25: hepatic aflatoxin transcript 25; *Fshβ*: follicle-stimulating hormone β; *Lhβ*: luteinizing hormone β; *Cga*: glycoprotein hormone α chain; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

increased with increasing age (Fig. 1d). Quantitative analysis of lncRNA-m18as1 in different pituitary cells showed that lncRNA-m18as1 expression was lowest in the MMQ cell line and was almost the same in primary pituitary cells and the GH3 cell line (Fig. 1e). The FISH results indicated that lncRNA-m18as1 was located mainly in the cytoplasm (Fig. 1f).

3.2 Effect of lncRNA-m18as1 on secretion of FSH by upregulating expression of *Fshβ*

To determine transfection efficiency, we transfected siRNAs and overexpression plasmids coupled with a fluorescent marker into pituitary cells and found that the cells were marked with red fluorescence, indicating that they were successfully transfected (Fig. S1). To determine which siRNA had the best knockdown effect, we transfected three siRNAs into rat primary pituitary cells and tested the expression of the lncRNA-m18as1 gene. The three siRNAs downregulated lncRNA-m18as1 to a similar degree, so we ultimately chose siRNA-3 for follow-up experimental treatment according to the RT-qPCR result (hereafter referred to as the siRNA) (Fig. 2a). We transfected the lncRNA-m18as1 overexpression plasmid into rat primary pituitary cells at three different concentrations (500, 1000, and 1500 ng/mL) and screened 500 ng/mL as the optimal concentration (Fig. 2b).

To determine whether lncRNA-m18as1 regulated the *Fshβ* gene, we transfected cells with the lncRNA-m18as1 siRNA and overexpression plasmid and analyzed *Fshβ* regulation. We also detected the effects of the above treatments on *Lhβ* and *Cga*. The results suggested that knockdown of lncRNA-m18as1 suppressed the mRNA expression of *Fshβ* (P<0.01), *Lhβ* (P< 0.05), and *Cga* (P<0.05) and reduced secretion of FSH (P<0.01) (Figs. 2c and 2d). However, overexpression of lncRNA-m18as1 had the opposite effects: it increased the mRNA expression of *Fshβ*, *Lhβ*, and *Cga*, as well as secretion of FSH (Figs. 2e and 2f).

3.3 Prediction and confirmation of the lncRNA-m18as1 target gene

LncRNA-m18as1 was highly expressed in our sequencing data, and Smad2 was also highly expressed. Therefore, to explore the mechanism mediated by lncRNA-m18as1 and Smad2, we used RNAhybrid 2.2 and TargetScan to predict miRNAs that might be able to bind with lncRNA-m18as1 or Smad2 in our

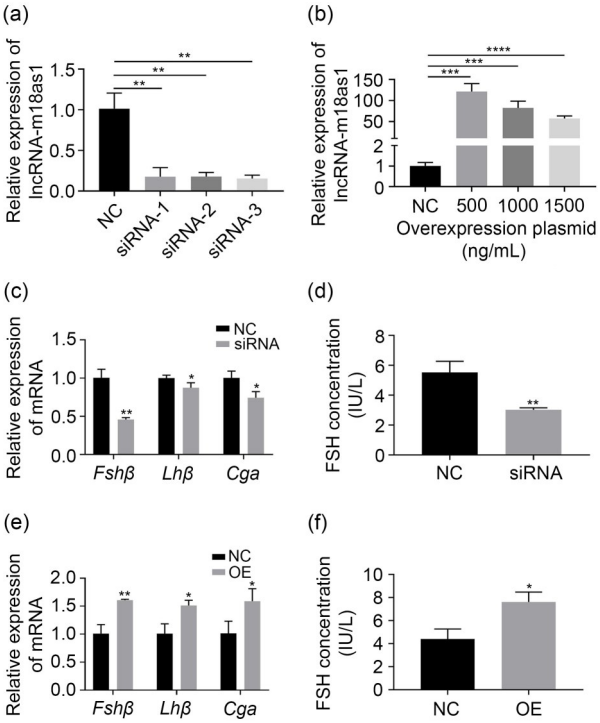


Fig. 2 Effects of lncRNA-m18as1 on secretion of FSH by upregulating expression of *Fshβ*. (a) Screening of siRNAs; (b) Screening of optimal concentration of overexpression plasmid; (c, d) RT-qPCR and ELISA after knockdown of lncRNA-m18as1; (e, f) RT-qPCR and ELISA after overexpression of lncRNA-m18as1 (OE). Data are shown as mean±SD of three independent experiments. Statistical significance was analyzed by one-way ANOVA and Student's *t*-test ($^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001; $^{****}P$<0.0001). LncRNA: long noncoding RNA; FSH: follicle-stimulating hormone; siRNA: small interfering RNA; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; OE: overexpression; SD: standard deviations; ANOVA: analysis of variance; NC: negative control; *Fshβ*: follicle-stimulating hormone β; *Lhβ*: luteinizing hormone β; *Cga*: glycoprotein hormone α chain; mRNA: messenger RNA.

miRNA sequencing results. Interestingly, miR-18a-5p had a base pair that matched lncRNA-m18as1 and a base pair that matched Smad2 (Figs. 3a and 3b). Therefore, we hypothesized that lncRNA-miRNA-mRNA network existed, which could regulate the synthesis and secretion of FSH, namely, an lncRNA-m18as1/miR-18a-5p/Smad2 network.

To confirm the interaction between lncRNA-m18as1 and miR-18a-5p, we detected the enrichment of miR-18a-5p and lncRNA-m18as1 in cells transfected with the miR-18a-5p mimic or negative control by AGO2 RIP-qPCR. In the treatment group, lncRNA-m18as1 was enriched along with miR-18a-5p (Fig. 3e).

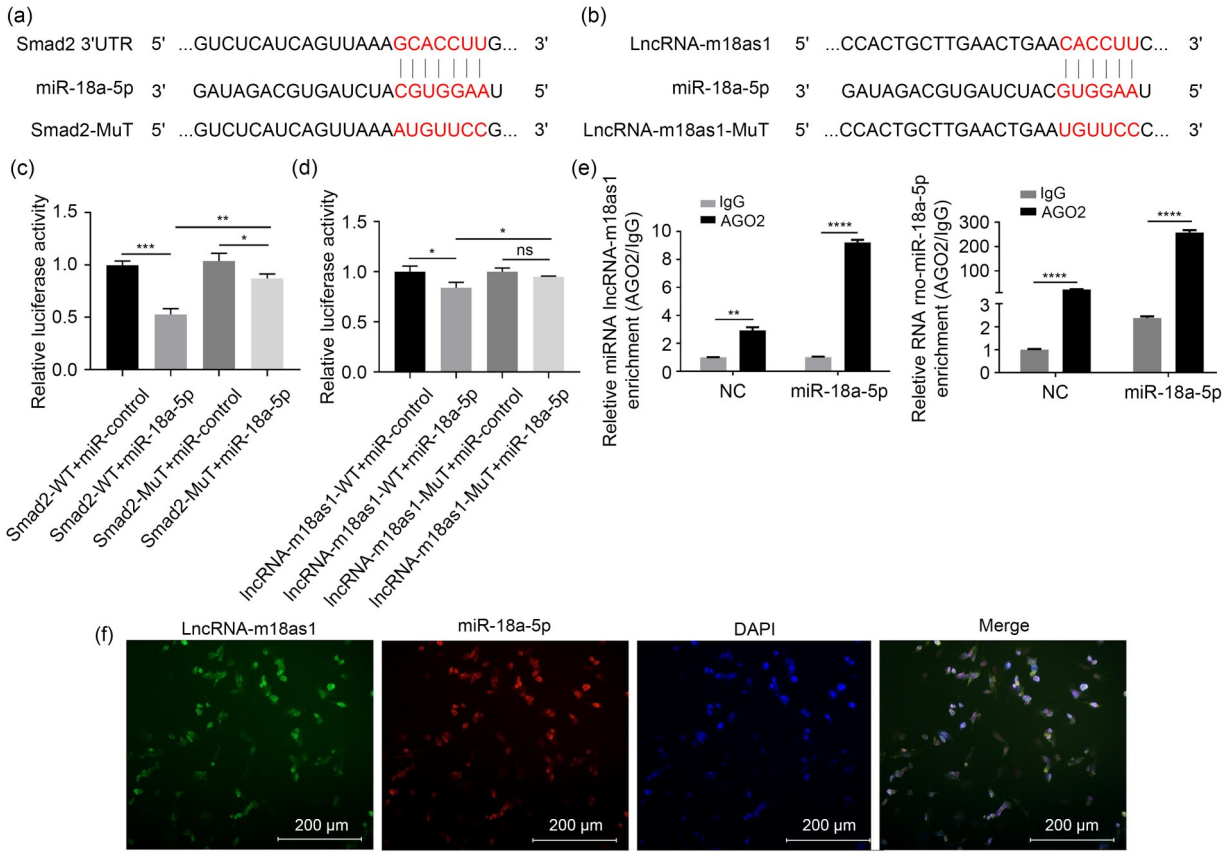


Fig. 3 Prediction and confirmation of the lncRNA-m18as1 target gene. (a, b) Sequences of the potential miR-18a-5p binding site and mutant sequence; (c) Luciferase assays were used to confirm that miR-18a-5p interacted with Smad2 mRNA; (d, e) Dual luciferase assays and AGO2 RIP-qPCR were used to confirm that lncRNA-m18as1 interacted with miR-18a-5p; (f) The colocalization of lncRNA-m18as1 and miR-18a-5p was observed. Data are shown as mean±SD of three independent experiments. Statistical significance was analyzed by one-way ANOVA and Student's *t*-test (* P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001; ns: not significant). LncRNA: long noncoding RNA; miR: microRNA; Smad2: mothers against decapentaplegic homolog 2; AGO2: argonaute 2; RIP-qPCR: RNA immunoprecipitation-quantitative polymerase chain reaction; SD: standard deviation; ANOVA: analysis of variance; WT: wild type; MuT: mutant; IgG: immunoglobulin G; NC: negative control; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

The dual luciferase reporter assay indicated that the binding site was no longer capable of binding miR-18a-5p after mutation (Fig. 3d). In general, these results suggested that lncRNA-m18as1 and miR-18a-5p had the ability to bind. Additionally, we observed the colocalization of lncRNA-m18as1 and miR-18a-5p in cells by FISH, which showed that these molecules were located mainly in the cytoplasm (Fig. 3f).

To confirm the interaction between miR-18a-5p and Smad2, we performed a dual luciferase reporter gene experiment. This experiment showed that fluorescence increased significantly after binding site mutation (P<0.05), indicating that this site was the target of miR-18a-5p (Fig. 3c). These results suggested that lncRNA-m18as1 may have competitively bound with miR-18a-5p to regulate expression of Smad2.

3.4 Competitive binding of lncRNA-m18as1 to miR-18a-5p to regulate the protein level of Smad2

To analyze the relationship between this ceRNA mechanism and Smad2, we treated rat primary pituitary cells with an overexpression plasmid or siRNA of lncRNA-m18as1. The results showed that overexpression of lncRNA-m18as1 decreased expression of miR-18a-5p (P<0.05) and increased the protein level of Smad2 (60 kDa) (P<0.05), as shown in Figs. 4a–4d. Knockdown of lncRNA-m18as1 exhibited the opposite result (Figs. 4e–4h). Then, we cotransfected lncRNA-m18as1 and miR-18a-5p in a rescue experiment. Overexpression of miR-18a-5p inhibited the lncRNA-m18as1 overexpression-induced increases in Smad2 protein levels (P<0.05; Figs. 4i and 4j).

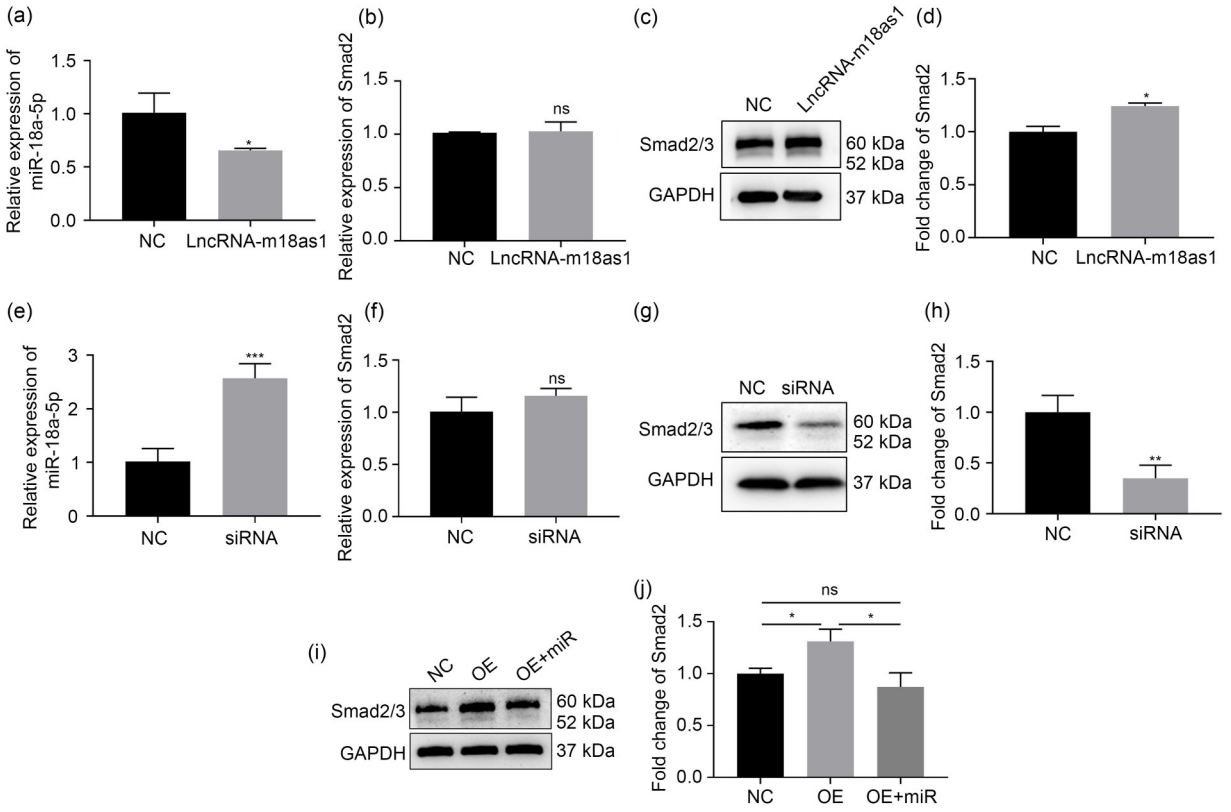


Fig. 4 Competitive binding of lncRNA-m18as1 to miR-18a-5p to upregulate the protein levels of Smad2. (a–d) RT-qPCR and western blot analysis were used to detect miR-18a-5p and Smad2 expression after transfection of the lncRNA-m18as1 overexpression plasmid; (e–h) RT-qPCR and western blot analysis were used to detect miR-18a-5p and Smad2 expression after knocking down lncRNA-m18as1; (i, j) Western blot analysis was used to assess expression of Smad2 protein after overexpression of lncRNA-m18as1 (OE) alone or overexpression of lncRNA-m18as1 and miR-18a-5p (OE+miR) together. Data are shown as mean±SD of three independent experiments. Statistical significance was analyzed by one-way ANOVA (* P<0.05; ** P<0.01; *** P<0.001; ns: not significant). LncRNA: long noncoding RNA; miR: microRNA; Smad: mothers against decapentaplegic homolog; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; SD: standard deviation; ANOVA: analysis of variance; NC: negative control; siRNA: small interfering RNA; OE: overexpression; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

These findings suggested that competitive binding of lncRNA-m18as1 to miR-18a-5p may have regulated secretion of FSH through the Smad2/3 pathway.

3.5 Regulation of FSH by lncRNA-m18as1 through the Smad2/3 pathway

To determine whether lncRNA-m18as1 competitively bound miR-18a-5p through the Smad2/3 pathway to regulate FSH, we transfected cells with the lncRNA-m18as1 overexpression plasmid and added the Smad2/3 pathway inhibitor SB431542 (Giraldez et al., 2006; Krol et al., 2010). We found that treatment with the inhibitor SB431542 inhibited upregulation of phosphorylated Smad2 and phosphorylated Smad4 (Figs. 5a and 5b), and the ELISA results demonstrated that inhibition of the Smad2/3 pathway reduced secretion of FSH (Fig. 5c). We also observed nucleocytoplasmic distribution of phosphorylated Smad4. We found that phosphorylated Smad4 was distributed mainly in the nuclei of rat primary pituitary cells (Fig. 5d). These findings indicated that lncRNA-m18as1 sponged miR-18a-5p to regulate FSH through the Smad2/3 pathway.

4 Discussion

With the deepening of research, interest in lncRNAs is growing. Although lncRNAs are usually expressed at lower levels than other RNAs, they often exhibit more significant tissue specificity (Ransohoff et al., 2018). In this study, lncRNA-m18as1, an lncRNA

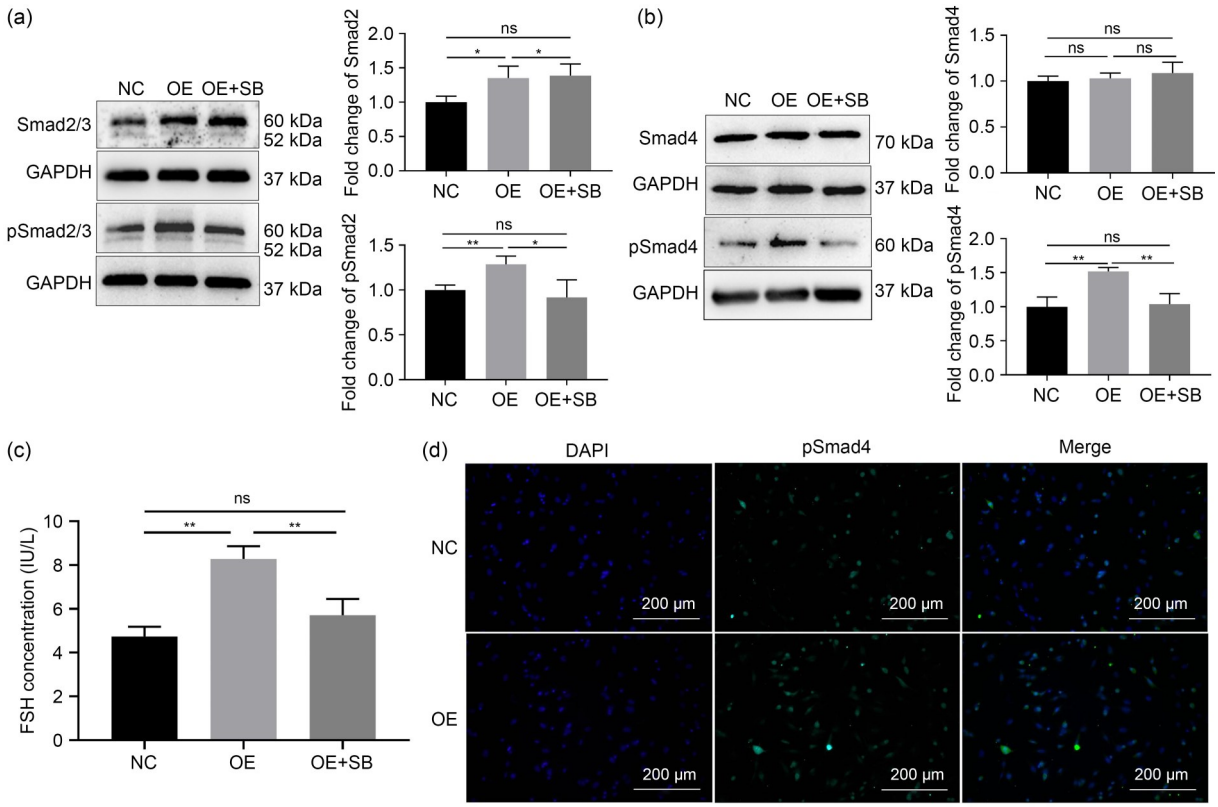


Fig. 5 Regulation of FSH by lncRNA-m18as1 through the Smad2/3 pathway. (a, b) Western blot analysis was used to assess the effect of overexpression of lncRNA-m18as1 (OE) alone or overexpression of lncRNA-m18as1 followed by treatment with the Smad2/3 pathway inhibitor SB431542 (OE+SB) on Smad2/3, phosphorylated Smad2/3 (pSmad2/3), Smad4, and phosphorylated Smad4 (pSmad4) protein levels; (c) ELISA was used to analyze FSH secretion after the same treatment; (d) Nuclear and cytoplasmic distribution of pSmad4 was observed with immunofluorescence. Data are shown as mean±SD of three independent experiments. Statistical significance was analyzed by one-way ANOVA (* $P<0.05$; ** $P<0.01$; ns: not significant). LncRNA: long noncoding RNA; Smad: mothers against decapentaplegic homolog; FSH: follicle-stimulating hormone; OE: overexpression; SB: SB431542; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation; ANOVA: analysis of variance; ns: not significant; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NC: negative control; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

located on chromosome 14, had the highest expression in the pituitary gland, while its expression in the heart, kidneys, and muscles was lower. Moreover, expression levels of lncRNA-m18as1 differed in two pituitary tumor cell lines, which suggests that this lncRNA exhibits cell type-specific expression. The detected levels of lncRNA-m18as1 in rats of different ages were consistent with the concept that lncRNAs have stage specificity (Wang et al., 2021). LncRNAs are also known to exhibit spatial specificity. It is this spatial specificity that enables lncRNAs to have different functions in the cytoplasm and the nucleus. In the nucleus, lncRNAs function as chromatin regulators to regulate transcriptional procedures and as scaffolds to participate in the establishment and spatial organization of nuclear compartments. In the cytoplasm, lncRNAs regulate the transduction and transformation of pathway signals and subsequent modification of gene expression (Yu and Li, 2020; Bridges et al., 2021). We observed through FISH that lncRNA-m18as1 was located mainly in the cytoplasm, suggesting that it may be involved in a mechanism regulating pathway activation or gene expression.

FSH secreted by the pituitary gland plays a vital role in reproduction and development in mammals, and many related articles have recently been published (Dewailly et al., 2016; Stilley and Segaloff, 2018; Santi et al., 2020). The network mechanism that regulates the synthesis and secretion of FSH is very complicated. For example, hormones including GnRH, steroid hormones, and pituitary adenylate cyclase activating polypeptide (PACAP) play a very

important role in regulating the synthesis and secretion of FSH (Marshall and Kelch, 1986; Fujii et al., 2002; Kanasaki et al., 2012). Studies have also shown that the activator protein-1 (*Ap-1*) gene, forkhead box L2 (*Foxl2*) gene, and others, play a certain regulatory role in the transcriptional stage of FSH synthesis and secretion (Coss et al., 2004; Lamba et al., 2009). Multiple pathways including the cyclic adenosine monophosphate (cAMP) signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway are constantly enriched with more extensive research (Garrel et al., 2010; Tsutsumi et al., 2010; Sun et al., 2015). However, there have been few studies on the lncRNAs that regulate the synthesis and secretion of FSH. In this study, we found that lncRNA-m18as1 could promote FSH synthesis and secretion. The regulatory mechanism was not specific, because the gene expression of *Lhβ* and *Cga* was similarly regulated. A complicated network may have caused this phenomenon.

MiRNAs, which are highly conserved ncRNAs composed of 19–23 nucleotides, control many developmental and cellular processes in animals, plants, and protozoa as posttranscriptional regulators (Krol et al., 2010). In recent years, explorations of the roles of miRNAs in embryonic development, tumor progression, metabolic diseases, and other processes have opened up new dimensions of miRNAs (Bhaskaran and Mohan, 2014). It has been reported that miRNAs, like lncRNAs, are spatially specific (Long et al., 2018). Therefore, while observing lncRNA-m18as1 by FISH, we also observed miR-18a-5p and found that miR-18a-5p was also located mainly in the cytoplasm. This finding hints that lncRNA-m18as1 and miR-18a-5p may interact. One of the most well-known mechanisms of lncRNAs is molecular sponging, in which lncRNAs bind to miRNAs to regulate downstream processes through the ceRNA mechanism (Chan and Tay, 2018). For example, lncRNAs can competitively bind to miRNAs to participate in the pathological development of diseases (Li et al., 2019; Wang et al., 2019). In our study, the results showed that lncRNA-m18as1 completely bound with miR-18a-5p, which is consistent with the view that lncRNAs regulate gene expression by binding to miRNAs (Cesana et al., 2011).

Our results demonstrated that Smad2 was the target gene of miR-18a-5p in rat primary pituitary cells, which means that lncRNA-m18as1 may completely bind with miR-18a-5p to regulate expression of Smad2. Indeed, miR-18a-5p weakened the upregulation of the total Smad2/3 protein level induced by lncRNA-m18as1, suggesting that lncRNA-m18as1 sponged miR-18a-5p to regulate the activity of the Smad2/3 signaling pathway. Then, we added SB431542, an inhibitor of the Smad2/3 pathway, together with lncRNA-m18as1 overexpression plasmid to rat primary pituitary cells. After inhibition of this pathway, the concentration of FSH in the culture supernatant was reduced, indicating that lncRNA-m18as1 and miR-18a-5p form a ceRNA network to regulate the synthesis and secretion of FSH by stabilizing the content of Smad2. Phosphorylated Smad4 was located mainly in the nucleus, and there was export from or import into the nucleus. Previous studies have also demonstrated the distribution of phosphorylated Smad4 (Massague et al., 2005; Arjunan et al., 2016).

In conclusion, we found for the first time that lncRNA-m18as1, as a molecular sponge of miR-18a-5p, regulates expression of FSH through the Smad2/3 pathway (Fig. 6). Our results add to the existing knowledge about the relationship between ceRNA mechanisms and mammalian reproduction.

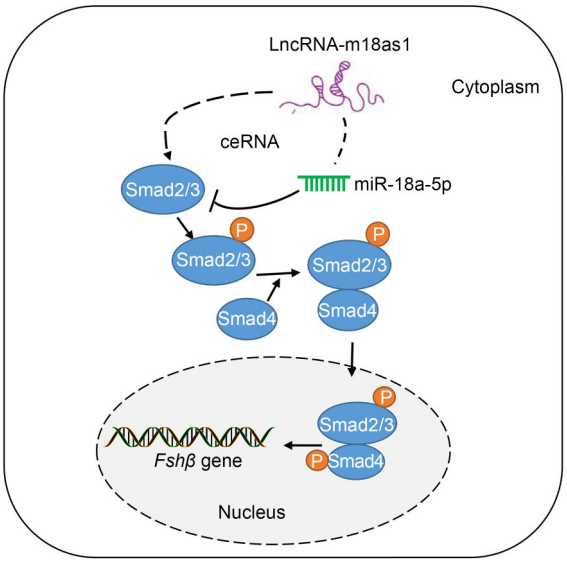


Fig. 6 Schematic of the proposed lncRNA-m18as1 mechanism in rat pituitary cells. lncRNA-m18as1 acted as a ceRNA to "sponge" miR-18a-5p, thus reducing the inhibitory effect of miR-18a-5p on *Fshβ* gene expression and promoting FSH secretion. LncRNA: long noncoding RNA; ceRNA: competitive endogenous RNA; miR: microRNA; *Fshβ*: follicle-stimulating hormone β; FSH: follicle-stimulating hormone; Smad: mothers against decapentaplegic homolog; P: phosphorylated.

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Author contributions

Wenzhi REN, Bao YUAN, and Wei GAO were responsible for the main experimental concept and design. Weidi ZHANG, Guokun ZHAO, Haoqi WANG, and Haixiang GUO performed rat pituitary gland dissection. Weidi ZHANG, Dongxu HAN, and Guokun ZHAO performed other experiments. Weidi ZHANG, Haoqi WANG, Yi ZHENG, and Zhonghao JI performed the data analyses. Weidi ZHANG and Bao YUAN wrote the manuscript. All the authors have read and approved the final version, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Weidi ZHANG, Wenzhi REN, Dongxu HAN, Guokun ZHAO, Haoqi WANG, Haixiang GUO, Yi ZHENG, Zhonghao JI, Wei GAO, and Bao YUAN declared that they have no conflict of interest.

All animals were fed at the Jilin Provincial Key Laboratory of Animal Models, and the animal procedures were carried out in accordance with the relevant guidelines of the Jilin University Guide for the Care and Use of Laboratory Animals. All animal procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (permit number: SY201912021), China.

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Supplementary information

Fig. S1; Table S1