miR-361-3p regulates FSH by targeting FSHB in a porcine anterior pituitary cell model

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Abstract

FSH plays an essential role in processes involved in human reproduction, including spermatogenesis and the ovarian cycle. While the transcriptional regulatory mechanisms underlying its synthesis and secretion have been extensively studied, little is known about its posttranscriptional regulation. A bioinformatics analysis from our group indicated that a microRNA (miRNA; miR-361-3p) could regulate FSH secretion by potentially targeting the FSHB subunit. Herein, we sought to confirm these findings by investigating the miR-361-3p-mediated regulation of FSH production in primary pig anterior pituitary cells. Gonadotropin-releasing hormone (GnRH) treatment resulted in an increase in FSHB synthesis at both the mRNA, protein/hormone level, along with a significant decrease in miR-361-3p and its precursor (pre-miR-361) levels in time- and dose-dependent manner. Using the Dual-Luciferase Assay, we confirmed that miR-361-3p directly targets FSHB. Additionally, overexpression of miR-361-3p using mimics significantly decreased the FSHB production at both the mRNA and protein levels, with a reduction in both protein synthesis and secretion. Conversely, both synthesis and secretion were significantly increased following miR-361-3p blockade. To confirm that miR-361-3p targets FSHB, we designed FSH-targeted siRNAs, and co-transfected anterior pituitary cells with both the siRNA and miR-361-3p inhibitors. Our results indicated that the siRNA blocked the miR-361-3p inhibitor-mediated upregulation of FSH, while no significant effect on non-target expression. Taken together, our results demonstrate that miR-361-3p negatively regulates FSH synthesis and secretion by targeting FSHB, which provides more functional evidence that a miRNA is involved in the direct regulation of FSH.

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Introduction

The gonadotropin FSH, which is secreted by the anterior pituitary gland, plays an essential role in animal reproduction: it promotes antral follicle development in females and stimulates spermatogenesis in the Sertoli cells in males (Bernard *et al.* 2010). FSH is a heterodimeric glycoprotein composed of a unique β subunit (FSHB) and a common α subunit (α -GSU) that is shared by chorionic gonadotropin (CG), LH and TSH. Many aspects of the transcriptional regulation of FSH synthesis and secretion have been revealed in recent decades. For example, FSH is mainly under the control of GnRH and TGF- β signaling (Wurmbach *et al.* 2001, Thackray *et al.* 2010). Recently, adiponectin was shown to play an important role in FSH secretion during the estrous cycle (Kiezun *et al.* 2014). However, little is known about the posttranscriptional regulation of FSH production.

microRNAs (miRNAs) are small non-coding RNAs (~22 nt) that regulate gene expression at the posttranscription level by suppressing translation or degrading their target mRNA (Ambros 2004, Karginov *et al.* 2010). The role of miRNAs in pituitary hormone regulation is increasingly evident, for example, miR-26b was shown to be involved in growth hormone regulation by targeting *LEF-1* (Zhang *et al.* 2010), while miR-375 was found to regulate pituitary pro-opiomelanocortin (POMC) expression (Zhang *et al.* 2013). To date, several studies have reported either direct or indirect role for miRNAs in the regulation of gonadotropins. Gonadotrope-specific deletion of *Dicer*, an endoribonuclease responsible for miRNA processing, results in severe suppression of gonadotropin production and fertility defects (Wang *et al.* 2014). *Let-7b/c* was found to enhance the stability of the common subunit α -GSU indirectly through downregulation of *KSRP* (Repetto *et al.* 2012).

miR-200b/miR-429 was found to promote LH secretion by targeting *ZEB1* (Hasuwa *et al.* 2013), while it was reported that miR-325-3p inhibits LH synthesis and secretion by targeting *LHB* (Nemoto *et al.* 2012). A recent study showed that miR-132/212 is involved in GnRH-induced FSH expression by targeting *SIRT1* and subsequently reducing the FOXO1-mediated inhibition of *FSHB* transcription (Lannes *et al.* 2015). In addition, Dang *et al.* showed that decrease of miR-22-3p is associated with FSH increase in the plasma of Han Chinese patients with premature ovarian failure (Dang *et al.* 2015). However, it is still unknown whether miRNAs can directly regulate FSH synthesis or secretion by targeting *FSHB*. miR-361-3p, an X-linked miRNA, is detected in reproductive organs, including testis (Wu *et al.* 2014), ovary (Sontakke *et al.* 2014) and exosome of follicular fluid (Sohel *et al.* 2013). Additionally, miR-361-3p was frequently differentially expressed in multiple reproduction processes including GnRH induction of FSH secretion (Ye *et al.* 2013), fetal ovarian development change (Veiga-Lopez *et al.* 2013) and atretic follicle (Sontakke *et al.* 2014), which are closely linked to FSH regulation. Most importantly, a bioinformatics study from our group predicted that miR-361-3p may be involved in FSH secretion by targeting *FSHB* mRNA (Ye *et al.* 2013). Accordingly, in the present study, we hypothesized that miR-361-3p regulates FSH by targeting *FSHB*, and examined the relationship between miR-361-3p and *FSHB* 3'-UTR using the luciferase reporter assay, and investigated intracellular *FSHB* synthesis and extracellular FSH secretion following overexpression or knockdown of miR-361-3p in pig anterior pituitary cells. Moreover, the miR-361-3p binding specificity to the target *FSHB* was further confirmed by co-transfection with miR-361-3p inhibitors and siRNAs.

Materials and methods

Animals

The animal experiments were conducted in accordance with the Review of Welfare and Ethics of Laboratory Animals guidelines approved by the Guangdong Province Administration Office of Laboratory Animals, and all experiments were conducted as described in the animal protocol (SCAU-AEC-2010-0416) approved by the Institutional Animal Care and Use Committee of South China Agricultural University.

Primary culture of porcine anterior pituitary cells

Six healthy 7-day-old male piglets (Landrace) were killed, and the primary anterior pituitary cell culture was conducted as previously described (Barb *et al.* 1990, Lin *et al.* 2003). Briefly, pituitary glands were removed under sterile conditions, and the anterior lobe was immediately dissected and washed with Dulbecco Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12; Life Technologies) supplemented with

100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mg/mL BSA. The glands were then pooled and minced to produce a suspension in the same medium and centrifuged for 10 min at 2000 rpm. After removal of the supernatant, the pellet containing the tissue fragments was incubated at 37°C in DMEM/F12 containing 0.25% trypsin-EDTA (Life Technologies) and 0.25% collagenase type α in a flask with constant stirring for 30 min. The enzyme-digested pituitary suspension was then centrifuged at 2000 rpm for 5 min, the supernatant was discarded and the cell pellet was resuspended in DMEM/F12 supplemented with antibiotics and BSA, filtered through a 75-µm nylon mesh to remove undigested tissue and cell aggregates, and centrifuged at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet was washed twice in DMEM/F12 supplemented with antibiotics and BSA and suspended at 3×10^5 live cells/mL in DMEM/F12 supplemented with antibiotics and 10% FBS (Life Technologies). Finally, six well plates were seeded with 2 mL/well of the cell suspension and cultured at 37°C in a humidified, 5% CO₂ atmosphere.

GnRH treatment

When 80–90% confluent, the pig anterior pituitary cells were serum-starved for 6 h in DMEM/F12 supplemented with antibiotics and BSA, and then incubated for 12 h or 24 h in fresh starvation medium supplemented with 100 nM GnRH (Okada *et al.* 2003, Yuen *et al.* 2009, Ye *et al.* 2013) (Gonadoliberin I; AnaSpec, Fremont, CA, USA). For the dose-dependent assay (10, 25, 100 nM), pituitary cell were treated for 12 h.

Anterior pituitary tissues collection

Eight anterior pituitary tissues were collected from eight male pigs (13–16 weeks). In brief, pituitary glands were removed and the anterior lobe was immediately dissected from each pituitary gland. Eight anterior pituitary glands were immediately frozen in liquid nitrogen until RNA extraction. Total RNAs were used to quantify *FSHB* mRNA and miR-361-3p expression by qRT-PCR analysis.

FSHB mRNA 3'-UTR plasmid construction

Based on the miR-361-3p seed sequence, wild-type (WT) or seed-mutated (MT) and seed-deleted (DT) *FSHB* 3'-UTR sequences, where the binding site (CTGGGG) of miR-361-3p was mutated or deleted, respectively, were chemically synthesized (Sangon, Shanghai, China). The sequences are as follows: WT sense oligo, 5'-CTAGAGGAGGAGCTCCAGGAA TGCAGAGTGCTGGGGCCTCAGTCCTATCACCACCTCGTT-3'; antisense, 5'-AACGAGTGGTGATAGGACTGAGGCCCCAGCA CTCTGCATTCTGGAGCTCCTCCT-3'; MT sense oligo, 5'-CTA GAGGAGGAGCTCCAGGAATGCAGAGTGgatatCCTCAGTC CTATCACCACCTCGTT-3'; antisense, 5'-AACGAGTGGTGATAG GACTGAGGGATATCCACTCTGCATTCTGGAGCTCCTCCT-3'; DT sense oligo, 5'-CTAGAGGAGGAGCTCCAGGAATGC AGAGTGCCTCAGTCCTATCACCACCTCGTT-3'; and antisense, 5'-AACGAGTGGTGATAGGACTGAGGCACTCTGCATTCT GGAGCTCCTCCT-3'. The complementary oligonucleotides

were resuspended at a 1:1 ratio (1 µg/µL each) in an annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl and 1 mM EDTA) and heated to 95°C for 10 min to remove secondary structures. The temperature was then gradually reduced until room temperature was reached. The annealed products were then cloned into the pGL3 control vector (Promega) downstream of the luciferase gene.

Luciferase reporter assay

CHO cells were seeded in 24-well cell culture plates (1.5 × 10⁵ cells per well), and cultured in RPMI-1640 (Life Technologies) supplemented with 10% FBS. The next day, the cells were transfected with 50 ng pRL-TK (Renilla luciferase control for normalization; Promega) and 500 ng recombinant luciferase *FSHB* 3'-UTR reporters, as well as miR-361-3p mimics or a negative control (75 pM, GenePharma, Shanghai, China) using Lipofectamine 2000 (Life Technologies). Cells were harvested 48 h after transfection, and luciferase activity was determined using a Dual-Luciferase Reporter Assay system, according to the manufacturer's recommendations (Promega).

Alteration of miR-361-3p expression in the porcine anterior pituitary cells

The porcine pituitary cells were cultured in 12-well plates as described above (see Primary culture of porcine anterior pituitary cells, 12-well plates were seeded with 2 × 10⁵ live cells/well). When 80–90% confluent, cells were transfected with 100 nmol/well of a synthetic RNA duplex (miRNA mimics; GenePharma) or a negative control (NC) to upregulate ssc-miR-361-3p expression. The sequences are as follows: mimics, 5'-CCCCCAGGUGUGAUUCUGAUUUGC-3' (sense), 5'-AAAUUCAGAAUCACACCUUGGGGGU-3' (antisense); NC, 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGGAATT-3' (antisense). Additionally, miR-361-3p inhibition was also achieved by transfecting cells with 100 nmol/well of 2'-O methylated single-stranded miR-361-3p antisense oligonucleotides (GenePharma). The sequences were as follows: ssc-miR-361-3p inhibitor,

5'-GCAAAUCAGAAUCACACUGGGGG-3'; negative control (i-NC), 5'-CAGUACUUUUGUGUAGUACAA-3'. At 24 h post-transfection, the supernatants were collected for FSH quantification by radioimmunoassay (RIA), while the cells were subjected to quantitative analysis to examine mRNA and miRNA expression, as well as Western blotting.

Radioimmunoassay

The supernatants of anterior pituitary cells were collected for FSH quantification by RIA using a double-antibody ¹²⁵I-FSH RIA detection kit (Nine Tripods Medical & Bioengineering Co Ltd., Tianjin, China). The intra- and interassay CVs were 5.5% and 8.7%, respectively. The minimum assay sensitivity was 1.0 mIU/mL, the primary data from RIA was further calibrated by total protein amount. At least six samples were used for each group.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from cells using the TRIzol reagent (Life Technologies) following the manufacturer's instructions. Total RNA samples were treated with recombinant DNase I (Ambion) to remove genomic DNA contamination. A minus-reverse transcriptase ('-RT') control was set to confirm genomic DNA was completely removed. To assay for either the miRNAs or mRNA, DNase I – treated total RNA (1 µg) was subjected to the reverse transcription reaction using M-MLV reverse transcriptase (Promega). Reverse transcription of pre-miR-361 was achieved by gene-specific primers. For miR-361-3p, reverse transcription was achieved using a stem-loop primer; for mRNAs, the first-strand cDNA was synthesized using oligo (dT)₁₈ primers, and the cDNA was diluted 5-fold with ddH₂O. A final 20-µL volume qRT-PCR was performed using the STRATAGENE Mx3005P sequence detection system. The PCR mix contained 2 µL cDNA, 10 µL 2× SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and 10 µM of each primer. The reaction cycle was as follows: 1 min at 95°C, 40 cycles of 15 s at 94°C and 15 s at the corresponding annealing temperature (T_m), and 72°C for 40 s, followed by a quick denaturation

Table 1 Primers used for reverse transcription (RT) and qRT-PCR.

Primer name	Primer sequence (5→3)	Gene accession	Product length (bp)	T _m (°C)
Pre-miR-361 RT primer	GAGGAGGAGGAAGCAAATCAGA	–	–	–
Pre-miR-361-F	AGAATCTCCAGGGGTAC	MI0013145	65	56
Pre-miR-361-R	GGAGGAAGCAAATCAGA	–	–	–
ssc-miR-361-3pRT primer	GTCGTATCCAGTGCCTGTCGTG GAGTCGGCAATTGCACTGGATAC GACGCAAATC	–	–	–
miR-361-3p-F	CCCCCAGGTGTGATTC	MIMAT0013934	55	58
miR-361-3p-R	ATCCAGTGCCTGTCGTGGA	–	–	–
U6-F	CTCGCTTCGGCAGCACA	NR_004394	71	58
U6-R	AACGCTTCACGAATTTGCGT	–	–	–
FSHB-F	CCATCTCCCAATCTGTCTC	NM_213875.1	177	58
FSHB-R	GCATTTAGTCCTTTCACCC	–	–	–
FOXL2-F	GAGAAGAGGCTCACGCTGTCCG	NM_001244665	112	58
FOXL2-R	TGAGGCTGAGGTTGTGGCGAAT	–	–	–
LHB-F	AGAGCTGAGCTTTGCCTCCATC	NM_214080.1	156	60
LHB-R	CGGTACAGGCCAAGGGTT	–	–	–
β-actin-F	CCAGCACGATGAAGATCAAGATC	AY550069.1	55	58
β-actin-R	ACATCTGCTGGAAGGTGGACA	–	–	–

at 95°C for 5 min, T_m , plus a slow ramp from T_m to 95°C to generate a melt curve to control the specificity of the amplified product. The no template control was set as the NC for both the miRNA or mRNA assays. U6 small nuclear RNA and β -actin were used for internal controls for miR-361-3p and *FSHB*, respectively. The optimization of primers was confirmed by standard curve construction (efficiency is 90%–105%, and $R^2 > 0.980$). Table 1 contains the information on all primers used for reverse transcription and qRT-PCR. The $2^{-\Delta Ct}$ method was employed to quantify and normalize the expression data.

Western blotting analysis

Proteins were extracted from the TRIzol-treated samples, as previously described (Chomczynski 1993). In brief, after removing the upper aqueous phase and interphase from Trizolysed supernatant, the lower organic phase was collected and precipitated using isopropanol at room temperature for 10 min. The samples were then centrifuged at 4°C, $12,000 \times g$ for 10 min and rinsed three times with 0.3 M guanidine hydrochloride. Next, absolute alcohol was added and the samples were centrifuged at 4°C, $7500 \times g$ for 5 min. After drying, the proteins were solubilized in 1% SDS containing 1 mM phenylmethyl sulfonyl fluoride, and the total soluble protein was quantified using a BCA protein assay. Protein aliquots (30 μ g) were separated on a 10% SDS-PAGE gel, electroblotted onto a polyvinylidene difluoride membrane and blotted with an anti-*FSHB* antibody (1:1000 dilution, Abcam). β -Actin (1:2000 dilution, Abcam) was used to normalize protein expression. Images were acquired using FluorChem M (ProteinSimple, Santa Clara, CA, USA), and analyzed using ImageJ software (NIH).

siRNA design and transfection

The siRNA oligos potentially targeting *FSHB*, siRNA1 (5'-CCAUGCAGACUCCUGUAU-3'), as well as the corresponding NCs were designed and synthesized by GenePharma. Primary anterior pituitary cells were transfected with either the siRNAs or NCs to select the optimal siRNA concentration. Finally, the siRNAs were co-transfected with the miR-361-3p inhibitor, and FSH levels in the culture supernatants were determined.

Statistical analysis

Data were presented as mean $\pm$ s.e.m., statistical analysis was performed by SPSS 17.0 software. Statistical significance was determined using the Student's *t*-test, whereas multiple comparisons were performed by analysis of variance and differences where $P < 0.05$ were considered significant. Pearson's correlation coefficient *R* was used to measure the product-moment coefficient of correlation between miR-361-3p and *FSHB* mRNA abundance.

Results

Inverse relation between miR-361-3p expression and FSH synthesis

Previous findings from our group (Ye *et al.* 2013) indicated that FSH secretion was accompanied by a decrease in miR-361-3p levels. In this study, we directly tested if miR-361-3p modulated GnRH-induced FSH synthesis. Firstly, to test the dose-dependent effect of

Figure 1 Inverse relation between miR-361-3p expression and FSH synthesis. (A) Primary pig anterior pituitary cells were treated with 10, 25 and 100 nM GnRH for 12 h and subjected to qRT-PCR to examine miR-361-3p precursor (pre-miR-361) expression. (B) Examination of miR-361-3p expression by qRT-PCR. (C) Examination of the *FSHB* mRNA levels by qRT-PCR. (D) Examination of the FSH secretion in cell supernatant by RIA. (E) Primary pig anterior pituitary cells were treated with 100 nM GnRH for 12 and 24 h and subjected to qRT-PCR to examine pre-miR-361 expression. (F) Examination of miR-361-3p expression by qRT-PCR. (G) Examination of the *FSHB* mRNA levels by qRT-PCR. (H) Examination of the FSH secretion in cell supernatant by RIA. (I) FSHB protein expression after 24 h GnRH treatment by Western blotting. (J) Comparison of the relative expression of FSHB following densitometric analysis of blots shown in (I), densitometric analysis was conducted by ImageJ software. (K) Correlation analysis between miR-361-3p and *FSHB* mRNA abundance in pig anterior pituitary tissues by qRT-PCR. * $P < 0.05$; ** $P < 0.01$; ns, not significant.

GnRH on miR-361-3p expression, pig anterior pituitary cells were treated with 10, 25 and 100 nM GnRH, respectively, and then evaluated FSH expression as well as miR-361-3p abundance at 12 h after GnRH treatment. We observed that GnRH dose-dependently decreased miR-361-3p precursor pre-miR-361, (83%, 88%, 95% decrease, respectively, $P < 0.01$, Fig. 1A) and mature miR-361-3p expression (21%, 40%, 42% decrease, respectively, $P < 0.01$, Fig. 1B), but increased *FSHB* expression (1.95-fold $P < 0.05$; 4.46-fold, $P < 0.01$; 5.87-fold, $P < 0.01$, respectively, Fig. 1C) and FSH hormone secretion (2.06-fold, 2.16-fold, 2.59-fold, respectively, Fig. 1D). Furthermore, we test miR-361-3p expression and *FSHB* expression with GnRH exposure time. Pig anterior pituitary cells were treated with 100 nM GnRH, miR-361-3p and FSH expression were measured at 12 and 24 h after treatment. The results indicated that both 12 and 24 h treatment led to an obvious decrease in pre-miR-361 (95%, 90% decrease, respectively, $P < 0.01$, Fig. 1E) and mature miR-361-3p (78%, 37% decrease, respectively, $P < 0.01$, Fig. 1F). It also led to significant increase in *FSHB* mRNA expression (3.90-fold, 4.80-fold over the control group, respectively, $P < 0.01$, Fig. 1G) and FSH secretion (2.40-fold, 4.10-fold as the control group, Fig. 1H) and an increase tendency of *FSHB* protein (25% increase, $P = 0.064$, Fig. 1I and J). Interestingly, similar to the previous study (Yuen *et al.* 2009, Lannes *et al.* 2016), we also observed long GnRH exposure time (24 h) tended to restore pre-miR-361 and miR-361-3p expression. Regardless, these data supported that the GnRH-mediated downregulation of miR-361-3p was involved in FSH synthesis *in vitro*.

To further test if miR-361-3p has a potential role in FSH synthesis *in vivo*, a correlation analysis between miR-361-3p level and *FSHB* mRNA abundance was performed, the result showed miR-361-3p were

negatively correlated with FSH mRNA abundance ($r = -0.69$, $P = 0.029$) (Fig. 1K).

miR-361-3p targets the *FSHB* 3'-UTR

We previously reported that *FSHB* has one putative binding site for miR-361-3p in the 3'-UTR (Ye *et al.* 2013), and RNAhybrid prediction showed miR-361-3p targets the *FSHB* 3'-UTR by perfectly binding this site via the seed sequence (a 2–7 nt sequence at the miRNA 5'-end, CCCCAG) and the adjacent sequences (Fig. 2A). Hence, we hypothesized miR-361-3p regulates FSH production by directly targeting *FSHB* mRNA. To prove this, the 3'-UTR sequence of *FSHB* harboring the predicted binding sites for miR-361-3p was inserted downstream of the luciferase gene in pGL3-control luciferase reporter vectors. *FSHB* 3'-UTR sequences with a mutation or deletion in the seed sequence were also designed and inserted. The constructs were named WT, MT and DT, respectively (Fig. 2B and C). CHO cells were co-transfected with the pGL3-control plasmid as well as the pRL-TK plasmid for normalization, along with miR-361-3p mimics or a NC. Measurement of the relative luciferase activity 48 h after transfection revealed that transfection with the miR-361-3p mimics reduced the luciferase activity of the WT plasmid significantly ($P < 0.01$), but did not affect that of the MT and DT-*FSHB* mRNA 3'-UTR plasmids (Fig. 2D). These results indicate that *FSHB* is a direct target of miR-361-3p.

miR-361-3p overexpression inhibits FSH synthesis and secretion in pituitary cells

To obtain further evidence that miR-361-3p regulates FSH, we transfected anterior pituitary cells with miR-361-3p mimics, which led to a remarkable increase in

Figure 2 miR-361-3p directly targets *FSHB*.

(A) Predicted secondary structure of miR-361-3p for binding to the *FSHB* 3'-UTR, generated using RNAhybrid. (B) Partial *FSHB* 3'-UTR sequence in wild-type (WT), mutant (MT) and deleted (DT) constructs. The miR-361-3p seed sequence binding sites were replaced or deleted in MT and DT, respectively. Ssc-miR-361-3p represents the pig miR-361-3p sequence. (C) Schematic of WT, mutant or deleted recombinant luciferase reporter plasmids. WT, MT or DT *FSHB* mRNA 3'-UTR sequences were cloned downstream of the luciferase reporter gene. (D) Dual-Luciferase Assay for CHO cells transfected with reporter plasmids containing *FSHB* mRNA 3'-UTR WT, MT or DT sequences. * $P < 0.05$; ** $P < 0.01$.

Figure 3 miR-361-3p overexpression significantly decreases FSH synthesis and secretion. (A) Examination of miR-361-3p levels following transfection of miR-361-3p mimics or a negative control (NC) into pig anterior pituitary cells. (B) Examination of *FSHB* mRNA expression in cells transfected with miR-361-3p mimics or the NC. (C) Western blotting to examine FSHB protein expression in cells transfected with miR-361-3p mimics or the NC. (D) Comparison of the relative expression of FSHB following densitometry analysis of the blots shown in (C), densitometry analysis was conducted by ImageJ software. (E) Quantification of FSH in the culture supernatants of cells transfected with miR-361-3p mimics or the NC. (F) Examination of *FOXL2* mRNA expression in cells transfected with miR-361-3p mimics or the NC. (G) Examination of *LHB* mRNA expression in cells transfected with miR-361-3p mimics or the NC. * $P < 0.05$; ** $P < 0.01$.

miR-361-3p abundance within the cell (65-fold increase, $P < 0.01$; Fig. 3A). Examination of the intracellular *FSHB* mRNA abundance as well as FSHB protein levels also indicated that the upregulation of miR-361-3p expression significantly decreased FSHB expression, both at the mRNA (41% decrease, $P < 0.01$, Fig. 3B) and protein level (81% decrease, Fig. 3D and E). To test whether miR-361-3p overexpression also affected FSH secretion, we examined FSH levels by RIA in the culture supernatants 24 h after transfection, and found a significant decrease (62% decrease, $P < 0.01$, Fig. 3C). Notably, overexpression of miR-361-3p in pituitary cells did not alter non-target genes (*FOXL2* and *LHB*) expression (Fig. 3F and G).

Confirmation of miR-361-3p function by miR-361-3p inhibition and target blockade

As the mimic-induced overexpression of miR-361-3p led to a significant decrease in FSH synthesis and secretion, we tested whether downregulation of miR-361-3p exerted the opposite effect. Porcine anterior pituitary cells were transfected with miR-361-3p inhibitors or an i-NC. As expected, miR-361-3p inhibitor transfection significantly decreased miR-361-3p abundance in the pituitary cells (7% of that in i-NC-transfected cells, $P < 0.01$, Fig. 4A). In contrast to treatment with miR-361-3p mimics (Fig. 3B, C, D and E), treatment with the miR-361-3p inhibitor significantly increased *FSHB* mRNA abundance (40% increase over that in the i-NC-transfected cells, $P < 0.05$, Fig. 4B) and protein secretion

(60% increase over that in the i-NC-transfected cells, Fig. 4C). Additionally, to test whether the miR-361-3p effect on FSH was specific, siRNA candidate (siRNA-1) targeting *FSHB* was designed, FSH production was blocked (both at the mRNA and secreted protein level; 91%, 69% decrease, respectively, $P < 0.01$, Fig. 4D and E) in the siRNA-transfected cells. Then, porcine anterior pituitary cells were co-transfected with siRNA-1 and the miR-361-3p inhibitor, and we found that siRNA blocked the miR-361-3p inhibitor-induced upregulation of both *FSHB* mRNA synthesis (Fig. 4F) and FSH secretion (Fig. 4G), while both siRNA and inhibitor have no significant effect on non-target genes (*FOXL2*, *LHB*) expression (Fig. 4H and I), indicating that this miRNA specifically regulates FSH by binding *FSHB*.

Discussion

The role of miRNAs in pituitary hormone regulation is becoming increasingly evident, with recent studies indicating an involvement in gonadotropin regulation from the perspective of both miRNA biogenesis and specificity. In the present study, we found miR-361-3p could regulate FSH synthesis by targeting *FSHB* in pig pituitary cells. MiR-361-3p is an X-linked miRNA, and is annotated only in certain mammals (humans, mouse, rat and the pig) thus far, with the evidence indicating that the encoding gene (miR-361) is conserved in these species. In addition to the pituitary gland, miR-361-3p is reported to be expressed at other sites, including skeletal

Figure 4 Confirmation of miR-361-3p function by miR-361-3p inhibition and blockade of its target. (A) Pig anterior pituitary cells were transfected with miR-361-3p inhibitors or an inhibitor negative control (i-NC), and the relative expression of miR-361-3p was examined by qRT-PCR. (B) Examination of *FSHB* mRNA expression in cells transfected with miR-361-3p inhibitors or the i-NC. (C) Examination of secreted FSH levels following transfection with miR-361-3p inhibitors or the i-NC. (D) Examination of *FSHB* mRNA expression in cells transfected with the optimal FSH-targeted siRNA (siRNA-1, 60 pmol siRNA in 2 mL culture media) or the i-NC. (E) Examination of secreted FSH secretion following transfection of cells with siRNA-1 or the i-NC. (F) Examination of *FSHB* mRNA expression following transfection of cells with siRNA-1 or siRNA control (NC) in combination with the miR-361-3p inhibitor or the corresponding controls. (G) Examination of *FSHB* mRNA expression following transfection of cells with siRNA-1 in combination with the miR-361-3p inhibitor or the corresponding controls. (H) Examination of *FOXL2* mRNA expression. (I) Examination of *LHB* mRNA expression. * $P < 0.05$; ** $P < 0.01$; ns, not significant.

muscle (Nielsen *et al.* 2010), blood (Roth *et al.* 2012) and the central nervous system (Wang *et al.* 2011). Additionally, abnormal expression has been reported in multiple cancers, including lung cancer (Du *et al.* 2013), acute lymphoblastic leukemia (Schotte *et al.* 2011) and human oral cancer (Tanaka *et al.* 2011).

To date, little is known about miRNA-361-3p function. It was first identified as a potential regulator of FSH by our group, and was found to be downregulated in the primary porcine pituitary cells after GnRH treatment, with alterations in its abundance leading to changes in FSH secretion (Ye *et al.* 2013). In this study, we comprehensively examined FSH (*FSHB*) as well as miR-361-3p expression at different time points and different doses of GnRH treatment. We observed that miR-361-3p expression was negatively related to FSH expression *in vitro*, pituitary cells in response to GnRH and *in vivo* pituitary tissues. GnRH showed a dose-dependent increase in FSH but decrease in miR-361-3p abundance. Interestingly, time course of GnRH treatment showed that the miR-361-3p expression was recovered at 24 h after 100 nM GnRH, compared to 12 h, while its target protein *FSHB* showed less significant decrease. The recovery of miR-361-3p in response to GnRH was

quite similar to the observations reported in several studies (Yuen *et al.* 2009, Lannes *et al.* 2016), in which the expression of miR-132/212 and miR-125b tended to restore to steady-state level after 24 h GnRH treatment, where miR-125b recovery was mediated by inactivating the methylation effect of NSun2 methyltransferase on miR-125b gene. Obviously, it is possible that a similar negative feedback is involved in the recovery of miR-361-3p abundance in response to long-time GnRH stimulation. Nevertheless, a more detailed study on how GnRH regulate pre-miR-361 and miR-361-3p expression will be needed in the future.

In addition, bioinformatic prediction indicated that miR-361-3p may potentially target *FSHB*, as the mRNA contained a highly conserved binding site for miR-361-3p in the 3'-UTR. A Dual-Luciferase Reporter Assay showed miR-361-3p significantly decrease ($P < 0.01$) *FSHB* 3'-UTR luciferase activity, although only 20% decrease in activity were observed, which may be related to the different response capability of different cell model. The relationship between miR-361-3p and *FSHB* was further confirmed by a Dual-Luciferase Reporter Assay using reporter plasmids containing either a mutation or deletion in the miR-361-3p-binding site.

These results suggest that mutations in *FSHB* 3'-UTR (such as SNPs) may alter the affinity to miR-361-3p, and thus exert effects on fertility. This could possibly in a manner similar to that described by Clop *et al.* (Clop *et al.* 2006), where mutations in the myostatin 3'-UTR altered binding to miR-1 and miR-206 and resulted in translational repression of the myostatin gene.

In the present study, we show that miR-361-3p overexpression significantly decreased *FSHB* expression at the mRNA and protein level, and reduced the levels of the secreted hormone. Conversely, miR-361-3p blockade upregulated FSH production (at the mRNA as well as secreted protein levels), and this effect was blocked by an FSH-targeted siRNA, without altering other non-target genes (*FOXL2*, *LHB*) expression. Those results further confirmed a direct effect of miR-361-3p on FSH. Interestingly, miR-361-3p blockade using an inhibitor appeared to exert a less powerful effect than overexpression. This may be attributable to an offset effect due to endogenous miR-361-3p and iso-miRNAs, or other yet unidentified miR-361-3p family members (Cloonan *et al.* 2011). Alternately, miR-361-3p knockdown in pituitary cells may be compensated by other pathways. As described by Wang *et al.*, the pituitary gonadotrope expression of miR-361-3p as well as other miRNAs potentially targeting *FSHB* was significantly suppressed in Dicer-CKO mice, but that of *FSHB* was also decreased (Wang *et al.* 2014). It is possible that GnRH treatment alters the expression of other miRNAs; several studies have shown that GnRH induces miR-132/212 expression in gonadotrope L β T2 cells (Yuen *et al.* 2009). Recently, GnRH-induced FSH synthesis in both rat pituitary and mouse L β T2 cells was shown to be dependent on miR-132/212 (Lannes *et al.* 2015). Supporting a role for other miRNAs in the regulation of FSH synthesis, the present study reveals that miR-361-3p plays an inhibitory role in FSH biosynthesis by targeting *FSHB*.

In addition to miR-361-3p effect on pituitary FSH, miR-361-3p potentially involved in other reproduction processes was also detected in testis (Wu *et al.* 2014), ovary (Veiga-Lopez *et al.* 2013) and bovine follicular fluid (Sohel *et al.* 2013). Recent study (Sontakke *et al.* 2014) showed miRNA-361-3p was differentially expressed in ovarian development-related processes. Compared to large healthy follicles, miR-361-3p was significantly upregulated in large atretic follicles that involved the depletion of FSH (Ginther *et al.* 1999, Ilha *et al.* 2015). Taken together, miR-361-3p may participate in multiple fertility processes across HPG axis, although many details (such as whether miR-361-3p directly regulate ovary or testis development and how miR-361-3p regulate animal reproduction across HPG axis) remain to be elucidated in the future.

In summary, this study provides strong evidence to support that miR-361-3p directly targets *FSHB* and

negatively regulates its expression in porcine primary anterior pituitary cells. Our findings thus identify a new posttranscriptional regulator of FSH synthesis. As both miR-361-3p and its binding site on *FSHB* mRNA are conserved among humans, mice, rats and pigs, these findings also provide a therapeutic candidate strategy for treating FSH-secreting adenomas and enrich the mechanism underlying animal reproduction regulation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R Y, M L and C L performed experiment and data analysis; Q Q, T C, X C, S W, G S, L W and X Z participated in sample collection and data analysis; R Y, C L and Y Z participated in drafting the manuscript; and Q J, Q X and Y Z conceived of the study, participated in its design and coordination, and helped to draft the manuscript.

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