Androgen receptor and miRNA-126* axis controls follicle-stimulating hormone receptor expression in porcine ovarian granulosa cells

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Abstract

Androgen, which acts via the androgen receptor (AR), plays crucial roles in mammalian ovarian function. Recent studies showed that androgen/AR signaling regulates follicle-stimulating hormone receptor (FSHR) expression in follicles; however, the detailed mechanism underlying this regulation remained unknown. Here, we demonstrate that AR and miR-126* cooperate to inhibit FSHR expression and function in pig follicular granulosa cells (pGCs). In pGCs, overexpression of AR decreased, whereas knockdown increased, FSHR mRNA and protein expression; however, neither manipulation affected FSHR promoter activity. Using a dual-luciferase reporter assay, we found that the FSHR gene is a direct target of miR-126*, which inhibits FSHR expression and increases the rate of AR-induced apoptosis in pGCs. Collectively, our data show for the first time that the AR/miR-126* axis exerts synergetic effects in the regulation of FSHR expression and apoptosis in pGCs. Our findings thus define a novel pathway, AR/miR-126*/FSHR, that regulates mammalian GC functions.

Introduction

Follicle-stimulating hormone (FSH), a pituitary glycoprotein hormone, is an important component of the hypothalamic–pituitary–gonadal axis that is required for normal gonadal function and fertility (Valenti et al. 2013, El-Hayek et al. 2014, Miras et al. 2015). FSH binds to follicle-stimulating hormone receptor (FSHR) on the surface of granulosa cells (GCs) in ovaries and Sertoli cells in the testes, and ligand-bound FSHR then regulates downstream target genes (Zhang et al. 2012, Wu et al. 2015). In ovaries, FSHR plays a critical role in GC proliferation, differentiation, apoptosis, and hormones synthesis. Moreover, it is essential for overall follicular development and affects various aspects of female reproduction (Wei et al. 2013, Sriraman et al. 2014, Mazurkiewicz et al. 2015).

FSH can synergize with androgens to regulate FSHR expression in follicles (Sen et al. 2014, Walters 2015). Previous studies have shown that low-to-moderate doses of testosterone, a principal androgen, significantly increase FSHR mRNA levels in human luteinized GCs, whereas higher doses of testosterone do not further increase the FSHR mRNA levels and have no effect on FSHR protein expression (Garcia-Velasco et al. 2012). Sen et al. (2014) demonstrated that androgens, including testosterone and its metabolite dihydrotestosterone (DHT), regulate FSHR protein expression independent of transcription in primary mouse GCs. Androgens exert their biological activities via the androgen receptor (AR), a 110 kDa protein of the nuclear receptor superfamily (Li et al. 2015). However, it remains unclear whether AR regulates FSHR expression.

In mammals, AR-mediated androgen activities are crucial for normal ovarian function and female fertility (Shiina et al. 2006, Walters et al. 2008). Furthermore, data obtained in tissue-specific AR-knockout (ARKO) mice revealed that AR function is required for fully functional and healthy follicles (Sen & Hammes 2010). In small antral follicles of human, both the AR mRNA level in GCs and the androgen content in follicular fluid are positively correlated with the FSHR mRNA level in GCs (Nielsen et al. 2011). Likewise, FSHR and AR mRNA levels are positively correlated in GCs from both primate ovarian follicles and PCOS patients (Weil et al. 1999, Catteau-Jonard et al. 2008). In porcine ovary, FSHR and AR mRNA levels in follicles are negatively correlated during follicular atresia (Pan et al. 2012). Together, these observations suggest that levels of androgens and AR are closely correlated with FSHR expression during ovarian follicle development in mammals. However, the detailed mechanisms underlying the regulation of FSHR expression by AR in follicles remain unknown.

microRNAs (miRNAs), small non-coding RNAs of 20–22 nucleotides in length, play crucial roles in ovarian function and GC apoptosis (Imbar & Eisenberg 2014, Zhou et al. 2015, Liu et al. 2016). A recent study demonstrated
that androgens attenuate follicular atresia by increasing expression of miR-125b, which in turn suppresses the expression of proapoptotic proteins (Sen et al. 2014). Several studies also showed that androgens and AR control their target genes by regulating the expression of miRNAs (Ma et al. 2013, Lyu et al. 2014, Pasqualini et al. 2015). To date, however, the regulatory relationship between AR, miRNAs, and FSHR in GCs has not been fully elucidated. In this study, we chose miR-126*, an miRNA that significantly up-regulated during porcine follicular atresia (Lin et al. 2012), and a candidate regulatory miRNA of FSHR, which is sought to explore the molecular mechanism by which AR regulates FSHR expression through miRNA in porcine ovarian GCs (pGCs).

Materials and methods

Cell culture

pGCs were collected from suitable porcine ovarian follicles (diameter: 3–5 mm) by aspiration using a syringe with a 20-gauge needle, filtered through a stainless steel filter (diameter: 3–5 mm) to remove cumulus–oocyte complexes, and seeded into T25 culture flasks containing Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco) supplemented with 15% fetal bovine serum (FBS) (Gibco) and 1% streptomycin/penicillin. The pGCs were harvested for extraction of total RNA and protein.

Overexpression of AR

To generate the AR expression vector, full-length AR cDNA was amplified from pGC cDNA using specific primers (Table 1). The PCR product was cloned into the KpnI/XhoI sites of vector pcDNA3.1 (+) (Invitrogen) and transformed into competent E. coli DH5α cells (Tigen, Beijing, China). The resultant recombinant plasmid, pcDNA3.1-AR, was verified by sequencing and double-digestion assay. For transfection of pGCs, cells were seeded in six-well culture plates at the appropriate density and incubated overnight. pcDNA3.1-AR or pcDNA3.1 (empty vector) was transfected into pGCs using 5 μL/mL Lipofectamine 2000 (Invitrogen). pGCs were harvested for extraction of total RNA and protein.

Western blot

After 48 h of transfection, plates were placed on ice, and pGCs were washed with 1 × PBS. Whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma). Total protein extracts were separated on 12% SDS-PAGE gels and blotted onto nitrocellulose membranes. After blocking with 5% skim milk at 4°C for 2 h, the membranes were incubated overnight with the following primary antibodies: monoclonal anti-AR (1:1500; Santa Cruz Biotechnology), monoclonal anti-FSHR (1:1000; Santa Cruz Biotechnology), or anti-GAPDH (1:2000; Cell Signaling Technology). Western blot assays were performed on samples from three independent experiments, and signal intensities were measured using the ImageJ software.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from pGCs using the TRIzol reagent (Invitrogen) and then reverse-transcribed into cDNA using the Super M-MLV RTase cDNA Synthesis Kit (Aogene, Shanghai, China). Quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara Bio) on an ABI StepOne System (Applied Biosystems). GAPDH mRNA was used as an internal normalization control. qRT-PCR of miRNAs (miR-126* and miR-143) in pGCs transfected with pcDNA3.1-AR were performed using the PrimeScript miRNA qPCR Starter Kit (Takara Bio), and U6 snRNA was used as the internal control. The primer sequences are summarized in Table 1.

Table 1: Primers used in this study.

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences (5′ → 3′)</th>
<th>Product size (bp)</th>
<th>Tm (°C)</th>
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**Promoter activity analysis**

The 1.9 kb 5′ proximal flanking region of pig FSHR was cloned using primers containing KpnI and HindIII sites (Table 1) and then inserted into vector pGL3 (Promega) to generate the luciferase reporter plasmid pGL3-FSHR1912. To determine whether AR regulates FSHR promoter activity, pGL3-FSHR1912 was co-transfected with pcDNA3.1-AR into pGCs using Lipofectamine 2000. After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega) on a Modulus Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA). Results are expressed as the ratio of firefly and Renilla luciferase activities.

**Bioinformatic analyses**

miRNAs targeting FSHR were predicted using three algorithms: TargetScan (Lewis et al. 2003), miRWalk (Dweep & Gretz 2015), and miRanda (Enright et al. 2003). The mature sequences of miR-126* genes from multiple vertebrate species were downloaded from miRBase (http://www.mirbase.org) and the GenBank database (http://www.ncbi.nlm.nih.gov), and sequence alignment was performed using ClustalW (http://www.ebi.ac.uk).

**pmirGLO-FSHR-3′-UTR construction and transfection**

A fragment of the porcine FSHR 3′ untranslated region (3′-UTR) harboring the predicted miR-126* binding sites was isolated and subcloned into the NheI/Xhol site of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Takara Bio) to yield pmirGLO-FSHR-3′-UTR. A mutant version of pmiR-GLO-FSHR-3′-UTR was generated by replacing UUAUUA of the putative miR-126* binding site with GCGCGC. 293FT cells were cultured in six-well plates and co-transfected with pmirGLO-FSHR-3′-UTR and miR-126* or negative control (NC) mimics. Luciferase activity assays were performed as described above in “Promoter activity analysis.” Sequences of miR-126* and NC mimics are summarized in Table 2.

**Apoptosis assay**

pGCs were transfected with miR-126* mimics, inhibitors (Table 2) or pcDNA3.1-AR, and co-transfected with miR-126* and pcDNA3.1-AR according to the methods described by Liu et al. (2014). pGC apoptosis was detected using the Annexin V-FITC/propidium iodide apoptosis kit (KeyGEN, Nanjing, China). pGCs were seeded into six-well plates at a density of 3–8 × 10^6 cells per well. After treatment, cells were digested with 0.25% EDTA-free trypsin, washed twice with cold PBS, and suspended in 500 mL binding buffer containing propidium iodide/Annexin V-FITC in the dark at a room temperature for 15 min. Stained cells were counted on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

**MTT assays**

Methyl thiazolyl tetrazolium (MTT) assays were conducted to evaluate pGC growth. pGCs were cultured in 96-well plates at a density of 3000 cells per well. At 0, 24, 48, and 72 h after transfection with pcDNA3.1 or pcDNA3.1-AR, cell viability was determined using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Optical density (OD) was measured at 560 nm on an ELISA microplate reader.

**RNA interference**

Small interfering RNA targeting AR (AR-siRNA) was designed based on the cDNA sequence of porcine AR (GenBank ID: AF331845). AR-siRNA or NC-siRNA was transfected into pGCs using Lipofectamine 2000. AR and FSHR mRNA levels were measured by qRT-PCR, and FSHR protein level was evaluated by western blot. siRNAs were synthesized by GenePharma (Shanghai, China) (Table 2).

**Statistical analysis**

Statistical analyses were performed using IBM SPSS Statistics v20.0 (SPSS). Unpaired two-sided Student’s t-test and one-way ANOVA were used to evaluate the significance of differences. All data are presented as mean ± S.E.M.

**Results**

**AR inhibits FSHR expression in pGCs**

To investigate the regulatory effect of AR on FSHR expression, we overexpressed or knocked down AR in cultured pGCs. As expected, overexpression of AR increased the level of AR protein in pGCs (Fig. 1A); mRNA and protein levels of FSHR were reduced in AR-overexpressing pGCs (Fig. 1B and C). However, AR-siRNA decreased AR protein expression in pGCs (Fig. 1D) and increased FSHR mRNA and protein levels (Fig. 1E and F). These results confirm that FSHR is a downstream target of AR and that AR can inhibit FSHR expression in pGCs.

**AR does not affect FSHR promoter activity**

To investigate the mechanism by which AR regulates FSHR expression, we isolated 1.9 kb of the 5′ proximal flanking region of the porcine FSHR gene. However, we did not detect the androgen response element (ARE) within the proximal FSHR promoter. To further explore the
possibility of direct transcriptional regulation, we created porcine FSHR promoter–reporter constructs (Fig. 1G). As expected from the absence of an ARE, AR overexpression had no effect on FSHR promoter activity (Fig. 1H), suggesting that AR inhibition of FSHR expression in pGCs did not through regulation of FSHR promoter activity.

**FSHR is a predicted target of miR-126*, which is regulated by AR**

To determine how AR regulates FSHR, we identified miRNAs that target FSHR and are regulated by AR in pGCs (Fig. 2A). A total of 112 candidate miRNAs targeting FSHR were predicted, of which 56 were predicted by at least two algorithms (Fig. 2B). miRNA microarray assays revealed two miRNAs, miR-126* and miR-143, that were significantly up-regulated during follicular atresia in porcine ovary; of the two, miR-126* was up-regulated to a greater degree (Fig. 2C) (Lin et al. 2012). In addition, after transfection with pcDNA3.1-AR, transcription of miR-126* was up-regulated in pGCs, whereas the level of miR-143 was unchanged (Fig. 2D). Therefore, we focused on miR-126* in our subsequent analyses.

**FSHR is a target of miR-126* **

Sequence alignment revealed that vertebrate miR-126* genes share a common seed sequence (AUUAUUA) (Fig. 2E). This seed sequence is complementary to sequences present in the 3′-UTRs of FSHR in humans, rats, and pigs (Fig. 2F). To determine whether the FSHR mRNA is a true target of miR-126*, we constructed luciferase reporters containing the putative miR-126* target sites of porcine FSHR 3′-UTR, as well as mutant forms of the target sites (Fig. 2G). Luciferase assays revealed that miR-126* significantly decreased the activity of the wild-type, but not mutant, reporter (Fig. 2H and I), indicating that FSHR mRNA is a bona fide target of miR-126*.

**The AR/miR-126* axis regulates FSHR expression in pGCs**

We next confirmed that miR-126* can regulate FSHR expression in pGCs. qRT-PCR revealed that the FSHR mRNA level was significantly reduced in pGCs transfected with miR-126* mimics (Fig. 3A). Western blots showed
AR and mRNA-126* axis controls FSHR expression

Overexpression of AR promotes pGC apoptosis

A flow cytometry analysis showed that overexpression of AR significantly increased the rate of apoptosis (Fig. 4A). In addition, AR overexpression significantly increased the mRNA level of BAX, a proapoptotic gene (Fig. 4B), but did not affect the mRNA level of BCL-2, an anti-apoptotic gene (Fig. 4C). MTT assays revealed that AR overexpression significantly decreased pGC proliferation (Fig. 4D). Consistent with this, the proliferation marker PCNA was markedly down-regulated in pGCs transfected with pcDNA3.1-AR (Fig. 4E). These results indicate that AR induces apoptosis and decreases viability in pGCs.

The AR/miR-126* axis promotes pGC apoptosis

Next, we investigated whether miR-126* is involved in pGC apoptosis. Overexpression of miR-126* significantly increased the rate of apoptosis (Fig. 5A) and the level of BAX mRNA (Fig. 5B), but had no effect on the BCL-2 mRNA level (Fig. 5C). Inversely, inhibition of miR-126* significantly decreased the rate of apoptosis (Fig. 5D). Flow cytometry revealed that inhibition of miR-126* blocked the AR-induced increase in the rate of pGC apoptosis (Fig. 5E). Taken together, these data demonstrate that miR-126* promotes pGC apoptosis in cooperation with AR.

Discussion

The results of this study demonstrated that FSHR is a downstream target of AR and that AR acts as an inhibitory regulator to suppress FSHR expression in pGCs. Bioinformatic analysis and luciferase reporter assays revealed that FSHR is a functional target of miR-126* in pGCs and that miR-126* is in turn regulated by AR. Moreover, AR and miR-126* exerted a synergistic effect on pGC apoptosis. These data strongly suggest that AR plays an important role in FSHR expression and apoptosis in pGCs through the novel AR/miR-126*/FSHR pathway.

As the most physiologically important nuclear receptor and cell surface receptor in ovarian follicles, respectively, AR and FSHR are considered to be essential regulators of GC biology (Wang et al. 2015). Many recent publications demonstrated that AR and FSHR mRNA levels are tightly correlated (Catteau-Jonard et al. 2008, Nielsen et al. 2011, Pan et al. 2012) and that androgens can regulate FSHR expression in GCs (Sen et al. 2014, D), providing further support for the idea that FSHR mRNA is a target of miR-126* in pGCs. Furthermore, in pGCs transfected with pcDNA3.1-AR, AR-mediated suppression of FSHR protein levels was rescued by co-transfection with miR-126* inhibitors (Fig. 3E). Together, these results show that AR inhibits FSHR expression in pGCs via miR-126*.
Walters 2015), including pGCs (Durlej et al. 2011); however, no study to date has examined regulation of FSHR expression via AR in GCs.

We found that AR overexpression inhibits FSHR expression at the mRNA and protein levels, whereas knockdown of AR induces FSHR expression. In keeping with this result, Pan et al. (2012) reported that AR and FSHR mRNA levels are negatively correlated in ovarian follicles during porcine follicular atresia. AR is involved in the regulation of FSH-induced functions in GCs and might through regulating FSHR expression (Lu et al. 2009). Similarly, Chen et al. (2015) recently demonstrated that AR-dependent DHT inhibits FSHR-dependent FSH-induced GC proliferation in rats, consistent with previous studies (Pradeep et al. 2002, Kayampilly & Menon 2012). However, FSH also reduces AR expression in GCs of rats (Lu et al. 2009) and sheep (Rivera et al. 2015). Conversely, AR and FSHR mRNA levels are positively correlated in GCs, and low and medium doses of AR-dependent testosterone increase FSHR expression in primates (Weil et al. 1999, Catteau-Jonard et al. 2008). Taken together, these observations indicate that AR acts as an inhibitory regulator to suppress FSHR expression in pGCs.

AR is a member of the nuclear receptor superfamily, and androgen-bound AR functions as a nuclear transcription factor to directly regulate its part of target genes by binding to AREs in their promoter regions (Wu et al. 2011, Kroiss et al. 2015). In this study, we found that AR had no effect on FSHR promoter activity in pigs and that no ARE was

![Figure 3](image-url) miR-126* mediates AR-induced repression of FSHR in pGCs. (A) Overexpression of miR-126* decreases FSHR mRNA levels. (B) Overexpression of miR-126* decreases FSHR protein levels. miR-126* or NC mimics were transfected into cultured pGCs. FSHR mRNA and protein levels were detected and normalized to GAPDH. (C) Inhibition of miR-126* increases FSHR mRNA levels. (D) Inhibition of miR-126* decreases FSHR protein levels. (E) miR-126* mediates AR-induced repression of FSHR in pGCs. All data are represented as mean ± s.e.m. (n = 3). *P < 0.05; **P < 0.01.

![Figure 4](image-url) Overexpression of AR promotes pGC apoptosis. (A) Overexpression of AR increases the rate of pGC apoptosis. (B) Overexpression of AR increases BAX mRNA level in pGCs. (C) Overexpression of AR does not affect BCL-2 mRNA level in pGCs. (D) AR inhibited pGC proliferation. (E) AR represses PCNA mRNA expression in pGCs. Values are mean ± s.e.m. from at least three independent experiments. *P < 0.05; **P < 0.01.
present in the FSHR regulatory region of pigs, humans, mice, rats, cow, or sheep, suggesting the effect of AR on FSHR expression in pGCs by affecting its promoter activity.

In addition, several recent studies showed that AR can indirectly regulate the expression and function of its target gene also via mechanisms involving miRNAs (Ma et al. 2013, Lyu et al. 2014, Mishra et al. 2014, Pasqualini et al. 2015). In this study, we found that miR-126* could decrease FSHR expression and increase the rate of apoptosis in pGCs by binding directly to the 3′-UTR of FSHR. Because miR-126* expression is regulated by AR, we postulated that AR regulates FSHR expression via miRNAs. One downstream target of AR is miR-125b, which represses p53 expression; this miRNA cooperates with AR to synergistically inhibit the p53 function in mGCs (Sen et al. 2014). Similarly, Ribas et al. (2009) showed that androgen-bound AR up-regulates expression of miR-21, and the AR/miR-21 axis exerts oncogenic effects in prostate tumors by down-regulating TGFBR2 (Mishra et al. 2014). Therefore, we concluded that the coordinated action of AR and miR-126* plays a critical role in inhibiting FSHR expression in pGCs.

We next evaluated the relationship between the AR/miR-126* axis and the regulatory function of FSHR during GC apoptosis. Although the regulation of GC apoptosis by androgens has been extensively studied (Sen et al. 2014), the regulatory mechanisms of AR in particular remained unclear. Here, we showed that AR promotes pGC apoptosis, consistent with the elevated expression of AR during porcine follicular atresia observed in our earlier work (Pan et al. 2012), as well as the fact that DHT induces follicular atresia (Bagnell et al. 1982).

Figure 5 The AR/miR-126* axis promotes pGC apoptosis. (A) miR-126* promotes pGC apoptosis. (B) miR-126* up-regulates BAX mRNA in pGCs. (C) Effect of miR-126* on BCL2 mRNA level in pGCs. (D) Inhibition of miR-126* decreases pGC apoptosis. (E) miR-126* mediates the proapoptotic effect of AR in pGCs. All data are represented as mean ± S.E.M. (n = 3). *P < 0.05; **P < 0.01.
and reduces GC proliferation (Chen et al. 2015) in rat ovary. miR-126* (also known as miR-126-3p) is the complement of miR-126, and both miRNAs contribute to cellular functions such as apoptosis and proliferation (Meister & Schmidt 2010, Schober et al. 2014). To date, however, no study had addressed the role of miR-126* in GCs. Our results provide the first evidence that miR-126* promotes GC apoptosis. Hosokawa et al. (2015) demonstrated that miR-126* knockdown promotes proliferation and increases interferon-γ and granzyme B production in CD4+ and CD8+ T cells. In addition, overexpression of miR-126* significantly inhibits cell proliferation in thyroid cancer (Xiong et al. 2015) and breast cancer cells (Rohde et al. 2015). In this study, inhibition of miR-126* rescued AR overexpression-induced pGC apoptosis. Therefore, we conclude that the coordinated activities of AR and miR-126* play a critical role in inhibiting FSHR function in pGCs.

In summary, we showed that FSHR is a common target of AR and miR-126* in pGCs and that AR and miR-126* synergistically inhibit FSHR expression and pGC apoptosis. miR-126* is the first miRNA shown to target FSHR in mammalian ovary. In addition, we uncovered a novel signaling pathway, the AR/miR-126*/FSHR axis, as the mechanism underlying AR-driven control of GC function in mammalian ovary.

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 and fully declare any financial or other potential conflict interest.

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