NORFA, long intergenic noncoding RNA, maintains sow fertility by inhibiting granulosa cell death

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Long intergenic non-coding RNAs (lincRNAs) have been proved to be involved in regulating female reproduction. However, to what extent lincRNAs are involved in ovarian functions and fertility is incompletely understood. Here we show that a lincRNA, NORFA is involved in granulosa cell apoptosis, follicular atresia and sow fertility. We found that NORFA was down-regulated during follicular atresia, and inhibited granulosa cell apoptosis. NORFA directly interacted with miR-126 and thereby preventing it from binding to TGFBR2 3′-UTR. miR-126 enhanced granulosa cell apoptosis by attenuating NORFA-induced TGF-β signaling pathway. Importantly, a breed-specific 19-bp duplication was detected in NORFA promoter, which proved association with sow fertility through enhancing transcription activity of NORFA by recruiting transcription factor NFIX. In summary, our findings identified a candidate lincRNA for sow prolificacy, and provided insights into the mechanism of follicular atresia and female fertility.
ong non-coding RNAs (lncRNAs) are defined as a class of RNAs whose transcripts are >200 nucleotides (nt) without open reading frame or protein-coding potential. After microRNAs (miRNAs), lncRNAs are rapidly emerging as important ncRNA, which have been proved to be distinctly involved in a wide range of biological and cellular processes in development and disease1-3. Compared with protein-coding RNAs, lncRNAs are often modest evolutionary conservation, unstable, relatively lower abundance in vivo, and tighter tissue-specific expression, which result in difficulties in lncRNAs research4-6. In recent years, thousands of lncRNAs have been identified to be actively transcribed from human genome and other organisms by high-throughput RNA sequencing (RNA-seq) technology and bioinformatics6-8, but only a small number of lncRNAs are screened in domestic animals, and well characterized in humans and rodents8,9. Therefore, the biological functions, mode of action, and regulation of most lncRNAs are still largely unknown.

It is known that mature miRNAs are mainly located in the cytoplasm, which function through degrading target mRNAs or inhibiting target mRNA translation at the posttranscriptional level8. In contrast to miRNAs, lncRNAs can be located either in the cytoplasm or nucleus, with 17% of lncRNAs showing relative enrichment in the nucleus and 26% relatively enriched in the cytoplasm1,12. Notably, different subcellular localization (nuclear or cytoplasm) of lncRNAs may participate in different mechanisms13,14, so the mechanism of lncRNAs action is more complex than that of miRNAs. For example, lncRNAs retained in the nucleus carry out their biological functions by interacting with chromatin-modifying complexes and transcriptional regulatory proteins15,16, directly controlling gene transcription in cis or trans17 and scaffolding of nuclear complexes18. Cytoplasmic lncRNAs are known to affect the activity or stability of mRNA through pairing with other RNA molecules such as mRNAs19 and miRNAs20, scaffolding of cytoplasmic complexes21, and decaying proteins and miRNAs away from its potential targets22.

miRNAs have been shown to target ovarian function-related genes and control various ovarian function such as steroidogenesis23, folliculogenesis24, ovulation25, and luteogenesis26. However, little is known of the role of lncRNAs in ovarian function27. In this study, we prove that NORFA (non-coding RNA involved in the follicular atresia), a long intergenic non-coding RNA (lincRNA), controls porcine granulosa cell apoptosis and follicular atresia by acting as a competing endogenous RNA (ceRNA) and inhibits endogenous miR-126. We also demonstrate that NORFA/miR-126 axis plays an important role in regulating granulosa cells apoptosis through targeting the transforming growth factor-β (TGF-β) signaling pathway. Furthermore, we identified a breed-specific 19-bp duplication in NORFA promoter, which could regulate NORFA transcription by altering the recruitment of NFIX to the promoter of NORFA.

Results

Identification and characterization of NORFA. Our recent study performed deep transcriptome sequencing in SMAD4-silencing porcine granulosa cells28, and a transcript that was highly expressed in granulosa cells was noted. We then isolated the full-length sequence of the transcript by using 5’ and 3’ rapid amplification of cDNA ends (RACE) (Supplementary Fig. 1) and identified the full length of this transcript to be 739 nt (Fig. 1a). Moreover, the transcript was found to be located between TRAF2 gene and EDI1 gene at pig chromosome 1, which consisted of 2 exons (Fig. 1b). The homologous sequence of this transcript was not detected in the genome of other mammals and the RNA structure is low conserved (Supplementary Fig. 2), suggesting that it is a pig-specific transcript. Both PhyloCSF and CPAT analysis showed that the transcript is a lincRNA, with the low coding potential, similar to other well-characterized lncRNAs such as HOTAIR and H19 (Fig. 1c, d).

The transcript had a low expression level in the duodenum, muscle adipose, and colon, while high expression was found in the lung, kidney, and ovary, more especially in the ovary (Fig. 1e). In porcine ovary, fluorescence in situ hybridization (FISH) showed that the transcript was mainly expressed in granulosa cells of follicles at different stages (preantral follicles, antral follicles, and mature follicles), while its expression was dramatically reduced in granulosa cells of atresia follicles (Fig. 1f). Moreover, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed that the transcript was down-regulated during follicular atresia of porcine ovary (Fig. 1g, h), indicating that the transcript might play an important role in follicular atresia of porcine ovary. Therefore, the transcript was termed as NORFA.

NORFA is involved in granulosa cell apoptosis. To further investigate the role of NORFA in follicular atresia, we synthesized NORFA expression vector and NORFA-specific small interfering RNA (siRNA) to overexpress and knockdown endogenous NORFA in granulosa cells cultured in vitro, respectively. We found that overexpression of NORFA enhanced NORFA level in granulosa cells (Fig. 2a). Besides, we also noticed that the expression level of pro-apoptotic gene BAX was decreased (Fig. 2b), and anti-apoptotic gene BCL-2 mRNA level (Fig. 2c) and BCL-2/BAX ratio (Fig. 2d) were upregulated after NORFA overexpression. In addition, NORFA overexpression decreased cell apoptosis rate (10.97 ± 0.58% vs 6.40 ± 0.57%) (Fig. 2e), indicating that NORFA is an anti-apoptotic factor in granulosa cells. By contrast, knockdown of NORFA attenuated NORFA levels (Fig. 2f) and BAX mRNA levels (Fig. 2g) but decreased BCL-2 mRNA levels (Fig. 2h) and BCL-2/BAX ratio (Fig. 2i). Furthermore, knockdown of NORFA increased cell apoptosis rate (9.03 ± 0.55% vs 13.86 ± 0.23%) (Fig. 2j). All our data suggest that NORFA is essential for inhibiting granulosa cell apoptosis and is involved in follicular atresia of pigs.

NORFA acts as a ceRNA of its nearby gene miR-126. To explore the functional mechanism of NORFA in porcine granulosa cells, we determined the effects of NORFA on the expression levels of nearby genes including 16 coding genes and 1 miRNA gene (miR-126) (Fig. 3a). The expression levels of four coding genes (EGFL7, PHPT1, TMEM141, and LCN10) were increased in granulosa cells after NORFA overexpression (Fig. 3b) but decreased after NORFA silencing (Fig. 3c). Interestingly, miR-126, an intrinsic miRNA transcript from EGFL729, was dramatically downregulated by NORFA overexpression (Fig. 3d) and upregulated by NORFA silencing in granulosa cells (Fig. 3e). These data suggested NORFA regulates the expression of its nearby gene including coding genes and the miRNA gene.

We next analyzed the cellular localization of NORFA in granulosa cells. High levels of cytoplasmic markers (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RPLP0) were detected in cytoplasm extraction, whereas the isolated nuclear fraction displayed a high level of nuclear marker (U6) (Fig. 3f). It should be noted that >75% of NORFA was located in the cytoplasm (Fig. 3f), indicating that NORFA was a cytoplasmic lncRNA, which was consistent with FISH assay (Fig. 3g; Supplementary Fig. 3a, b). Interestingly, an miRNA response element (MRE) of miR-126 was predicted at 556–578 nt of the NORFA transcript (Fig. 3h). To confirm whether miR-126 directly binds to NORFA, dual-luciferase reporters containing
Fig. 1 Identification and characterization of a transcript in pigs. a The full-length RNA sequence of the transcript. The sequence of the transcript was isolated from porcine granulosa cells by using 5’- and 3’-RACE (see also Supplementary Fig. 1). b Schematic representation of the transcript with associated UCSC Genome Browser tracks depicting mammalian conservation, GC percent, and CpG Islands. c The transcript was shown as green (Scale bars, 200 μm for lines 1, 2, and 4 and 100 μm for line 3). d The expression levels of the transcript in healthy follicles (HF) and atretic follicles (AF) were measured by qRT-PCR (n = 7). e The expression levels of the transcript in granulosa cells from healthy follicles (HF) and atretic follicles (AF) were measured by qRT-PCR (n = 7). The mature sequence of ssc-miR-126 (ucguaccggagaaauauccg) and the seed region (cguacc) are completely consistent with that in other vertebrates, respectively (Fig. 4a). Vertebrate miR-126 containing ssc-miR-126 is an intronic miRNA, which is embedded within the introns of gene encoding protein EGFL7 (Fig. 4b). To understand the potential function of miR-126, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to check out the pathways from miR-126 targets. The targets of miR-126 are enriched in the apoptosis, pathways in cancer, and phosphoinositide-3 kinase-AKT signaling pathway (Fig. 4c). Our previous study showed that miR-126 was upregulated during follicular atresia in porcine ovary identified by miRNA microarray assay50 (Fig. 4d), which is consistent with the present observation from qRT-PCR (Fig. 4e). The correlation analysis showed that the miR-126 levels in follicles had a negative correlation with NORFA levels (Fig. 4f). All the data clearly indicated that intronic miR-126 gene is highly evolutionary conserved in vertebrates and correlates with NORFA levels in follicles of porcine ovary.

miR-126 is relevant to NORFA expression in follicles. Owing to the unavailability of information on the characterization of the gene encoding miR-126 in pig, we first isolated and characterized the porcine gene encoding miR-126 (ssc-miR-126). The length of ssc-miR-126 gene is 73 bp, which shares high nucleotide identities with other mammals, such as humans, and other vertebrates, such as chicken and zebrafish (Supplementary Fig. 4). Furthermore, the expression level of NORFA expression in follicles of porcine ovary. However, there was no obvious changes in NORFA expression in follicles of porcine ovary (Fig. 3l, m). All the data are in line with the notion that NORFA acts as a ceRNA and sponges endogenous miR-126 in granulosa cells.

NORFA inhibits the pro-apoptotic activity of miR-126. Next, we analyzed the role of miR-126 in porcine granulosa cells. miR-126 was increased after treatment with miR-126 mimics (Supplementary Fig. 5a), then the rate of cell apoptosis was...
increased (9.08 ± 0.32% vs 16.75 ± 2.01%) (Fig. 5a) and BCL-2/BAX ratio was decreased (Fig. 5b). We also knocked down miR-126 inhibitor in vitro (Supplementary Fig. 5b). The data showed that the apoptosis rate of granulosa cells (15.59 ± 3.06% vs 8.16 ± 0.11%) was dramatically decreased (Fig. 5c) and BCL-2/BAX ratio was dramatically increased simultaneously (Fig. 5d) after miR-126 silencing. These data suggested that miR-126 is a pro-apoptotic epigenetic regulator in granulosa cells.

To further investigate whether miR-126 mediated the function of NORFA, miR-126 and NORFA were overexpressed or knocked down by co-transfecting a miR-126 mimic and pcDNA3.1-NORFA or miR-126 inhibitor and NORFA-specific siRNA. The results showed that miR-126 overexpression prevents NORFA-reduced cell apoptosis (6.39 ± 0.58% vs 12.19 ± 0.66%) and NORFA-induced BCL-2/BAX ratio (Fig. 5e, f). In contrast, inhibition of miR-126 rescued cell apoptosis (13.82 ± 0.61% vs 8.74 ± 0.75%) caused by NORFA-specific siRNA (Fig. 5g) and...
NORFA-siRNA reduces BCL-2/BAX ratio (Fig. 5h). These data reveal that NORFA inhibits cell apoptosis through sponging miR-126 in porcine granulosa cells.

TGFBR2 (TGF-β receptor 2) is a direct target of miR-126 in granulosa cells. To evaluate the molecular mechanism by which miR-126 affects granulosa cell apoptosis and follicular atresia, four different algorithms were performed to predict the targets of miR-126. As a result, four common targets (PIK3R2, PEX5, TGFBR2, and TNFRSF10B) were identified (Fig. 6a). Of them, TGFBR2 is an important component of TGF-β signaling pathway that is involved in porcine granulosa cell apoptosis and follicular atresia.31 MRE motif of miR-126 was located at the residues 484–506 nt in the porcine TGFBR2 3′-untranslated region (UTR).
Fig. 3 NORFA acts as a ceRNA and sponges miR-126 in porcine granulosa cells. a Schematic showing the locations of NORFA (red) and its nearby coding genes on porcine chromosome 1. b, c The expression levels of NORFA nearby coding genes in porcine granulosa cells were detected by qRT-PCR after transfection with pcDNA3.1-NORFA (b) or NORFA-siRNA (c). d, e The expression levels of miR-126 in porcine granulosa cells were measured after pcDNA3.1-NORFA or NORFA-siRNA treatment. Level of miR-126 was detected by stem-loop qRT-PCR. f Subcellular localization of NORFA in porcine granulosa cells. Expression levels of NORFA and marker gene (GAPDH and RPLP0 for cytoplasm and U6 for nuclear) in isolated nuclear and cytoplasm fraction from porcine granulosa cells were detected by qRT-PCR. g Subcellular localization of NORFA in porcine granulosa cells was detected by FISH (Scale bars, 50 µm). Nucleus was dyed with DAPI (blue) and NORFA was dyed with NORFA-specific probe (green). h Schematic showing an miRNA response element (MRE) of miR-126 in exon 2 (E2) of porcine NORFA. i Schematic showing the interactions of miR-126 with wild-type NORFA (red) and the mutant version (green). j Porcine granulosa cells were co-transfected with dual-luciferase reporter vector containing the wild-type NORFA or the mutant version and miR-126 mimics, and luciferase activity was detected. k, l qRT-PCR analysis showing the expression levels of NORFA in porcine granulosa cells when miR-126 was overexpressed (k) or silenced (l). m Porcine granulosa cells lysates were incubated with biotin-labeled wild-type or mutated NORFA. After pull-down, miRNAs were quantified by qRT-PCR. miR-34c and miR-143 were used as negative controls. Experiments were conducted in triplicate. Data in b-e and j-m are shown as mean ± S.E.M. with three independent experiments. *P < 0.05.

Furthermore, miR-126 seed sequence was completely paired with the 3′-UTR of vertebrate TGFBR2 (Fig. 6b).

To investigate whether TGFBR2 is a target of miR-126, luciferase reporters containing the wild MRE motif or mutation were constructed (Fig. 6c) and co-transfected with miR-126 mimics into HEK293T cells. Luciferase assay showed that miR-126 decreased luciferase activity of the reporter containing the wt MRE motif (Fig. 6d) and with no change of reporter with the mutant MRE motif (Fig. 6e), indicating that TGFBR2 is a direct target of miR-126. We further examined the effects of miR-126 on the expression of TGFBR2 in granulosa cells cultured in vitro. It was found that ectopic expression of miR-126 attenuated the TGFBR2 expression in both the mRNA and protein levels (Fig. 6f, h), while inhibition of miR-126 enhanced the TGFBR2 expression (Fig. 6g, i). These results implicate that TGFBR2 is a functional target of miR-126 in porcine granulosa cells.

miR-126 regulates cell death by targeting TGFBR2. To test whether TGFBR2 is involved in follicular atresia of porcine ovary, we measured TGFBR2 levels in follicles during follicular atresia. Both mRNA and protein levels of TGFBR2 were downregulated during follicular atresia (Fig. 7a, b). Correlation analysis confirmed that TGFBR2 mRNA levels had a negative correlation with
miR-126 levels in follicles (Fig. 7c), indicating that TGFBR2 might have an opposite effect on follicular atresia compared to the function of miR-126.

Next, we analyzed the role of TGFBR2 in porcine granulosa cell apoptosis. The synthesized expression vector pcDNA3.1-TGFBR2 increased the TGFBR2 protein level (Fig. 7d) and reduced cell apoptosis rate (9.31 ± 0.47% vs 5.01 ± 0.33%; Fig. 7e). By contrast, TGFBR2-specific siRNA dramatically decreased the TGFBR2 protein level (Fig. 7f) and enhanced cell apoptosis (6.60 ± 0.32% vs 10.25 ± 0.13%) (Fig. 7g), suggesting that TGFBR2 is an anti-apoptotic factor in porcine granulosa cells.

To assess whether TGFBR2 mediated miR-126-induced granulosa cell apoptosis, miR-126 mimics or inhibitor and pcDNA3.1-TGFBR2 or TGFBR2-specific siRNA were co-transfected into cultured granulosa cells in vitro. We found that cell apoptosis (9.94 ± 1.08% vs 6.54 ± 0.41%) induced by miR-126 was recovered when TGFBR2 was overexpressed (Fig. 7h). In contrast, inhibition of TGFBR2 weakened the anti-apoptotic effect of miR-126
inhibitor (6.63 ± 0.30% vs 11.15 ± 0.13%; Fig. 7i). Together, these data provide strong evidence that miR-126 regulates granulosa cell apoptosis and follicular atresia by targeting TGFBR2.

NORFA activates TGF-β pathway by miR-126–TGFBR2 axis.

We next detected whether NORFA acted as an upstream molecule of miR-126 and affected the TGFBR2 expression. Correlation analysis revealed that NORFA level in follicles was positively correlated with the TGFBR2 mRNA level (Fig. 8a). In granulosa cells, we found that NORFA positively regulated the TGFBR2 expression at both the transcriptional level (Fig. 8b, c) and translational level (Fig. 8d, e), indicating that NORFA is a regulator of TGFBR2. To further determine whether miR-126 mediated this process, miR-126 was transfected into granulosa cells with pcDNA3.1-NORFA. Western blot showed that miR-126 inhibited NORFA-induced TGFBR2 expression (Fig. 8f). Besides, miR-126 inhibitor rescued NORFA-specific siRNA-reduced TGFBR2 expression (Fig. 8g). These data demonstrated that miR-126 mediated NORFA induction of TGFBR2 process in porcine granulosa cells.

To investigate whether NORFA–miR-126 axis interacts with TGF-β signaling pathway, we examined the total protein and phosphorylated levels of SMAD3, a core component of TGF-β signaling pathway and a downstream of TGFBR2. We found that NORFA overexpression increased p-SMAD3 (phospho-SMAD3) levels (Fig. 8h), whereas the p-SMAD3 was dramatically reduced by miR-126 inhibitor (Fig. 8i).

Fig. 6 TGFBR2 is a direct target of miR-126 in porcine granulosa cells. a Potential target genes of miR-126 were predicted by four different prediction algorithms (microRNA.org, PITA, Targetscan, and TarBase). b miRNA response elements (MREs) of miR-126 within TGFBR2 3′-UTR of four species (human, pig, mouse, and chicken) were predicted by RNAhybrid. mfe minimum free energy. c miR-126 MRE motif (red letters) and mutated type (blue letters) in the porcine TGFBR2 3′-UTR. d, e Porcine granulosa cells were co-transfected miR-126 mimics with dual-luciferase reporter vector containing the wild-type TGFBR2 3′-UTR (d) or the mutant version (e), and luciferase activity was detected. f, g TGFBR2 mRNA levels in granulosa cells after transfection with miR-126 mimics (f) or inhibitor (g) were measured by qRT-PCR. Western blotting was used to determine the change of TGFBR2 protein levels in granulosa cells after treatment with miR-126 mimics (h) or inhibitor (i). Data in d–i are shown as mean ± S.E.M. from three independent experiments. P values were calculated by a two-sided Student’s t test.
decreased after treatment with NORFA-specific siRNA (Fig. 8i). In addition, we noticed that NORFA silencing suppressed the sensitivity of granulosa cells to TGF-β1, which could be restored by overexpression of NORFA (Supplementary Fig. 6a–c). Besides, we also observed that miR-126 decreased p-SMAD3 levels (Fig. 8j), whereas p-SMAD3 levels was upregulated after treatment with miR-126 inhibitor (Fig. 8k). Furthermore, miR-126 suppressed NORFA-induced p-SMAD3 levels (Fig. 8l). On the other hand, miR-126 inhibitors had the ability to recover the levels of p-SMAD3, which was downregulated by NORFA silencing (Fig. 8m). However, pcDNA3.1-NORFA with the mutation type of miR-126-binding site (NORFA-mutOE) had no effect on the expression levels of miR-126, TGFBR2, and p-SMAD3 in granulosa cells (Supplementary Fig. 7a–c). These
above-mentioned observations demonstrate that miR-126–TGFBR2 axis is involved in the regulation of NORFA to TGF-β signaling pathway in porcine granulosa cells.

19-bp duplication in promoter influences NORFA expression.

To investigate the relationship between NORFA and pig prolificacy, variants of NORFA gene in three pig breeds, Erhualian (known as one of the most prolific pig breeds in the world), Landrace, and Yorkshire (two famous western pig breeds), were screened and genotyped by individual sequencing. Five point mutations (g.-12C>T in promoter, g.249A>G, g.269A>G, g.290G>A, and g.380G>C in intron 1) and a 19-bp duplication (g.-1223-GCC AGG GCG GCA GTC CCG G-g.-1193) were
Fig. 8 NORFA controls TGFBR2 and TGF-β signaling pathway through affecting miR-126 in porcine granulosa cells. a Relationship between NORFA and TGFBR2 expression levels in the follicles of porcine ovary was detected (n = 10). b–e Granulosa cells were transfected with pcDNA3.1-NORFA or NORFA-siRNA, and TGFBR2 mRNA (b, c) and protein (d, e) levels were determined by qRT-PCR and western blotting, respectively. f, g miR-126 mediated NORFA regulation of TGFBR2 protein levels in granulosa cells. h, i The protein levels of p-SMAD3 and SMAD3 were measured after NORFA overexpression (h) or silencing (i). j, k The protein levels of p-SMAD3 and SMAD3 were measured after miR-126 overexpression (j) or silencing (k). l, m The protein levels of p-SMAD3 and SMAD3 in porcine granulosa cells were detected after treatment with miR-126 and NORFA. Data in b–m are shown as mean ± S.E.M. from three independent experiments. Relative protein levels were calculated and shown below are immunoblots. P values were calculated by a two-tailed Student’s t test.

Fig. 9 Breed-specific 19-bp duplication affects NORFA transcription by altering NFIX recruitment. a Polymorphisms of the NORFA gene in Eruhalian (n = 106), Landrace (n = 94), and Yorkshire (n = 144) sows. Schematic showing the NORFA gene and the location of 5 SNPs and a 19-bp duplication. Genotype and allele frequency were calculated and are represented with columns and pie diagrams, respectively. b The luciferase activities of NORFA promoter containing 19-bp duplication alleles were detected. c, d The copy number (c) and expression levels (d) of NORFA in granulosa cells (different sizes) from A1/A1 and A2/A2 genotype sows (n = 3) were detected by qRT-PCR. e NFIX protein levels in granulosa cells after overexpression or knockdown of NFIX were detected by western blotting. f The expression levels of NORFA in granulosa cells after NFIX was overexpressed or silenced were measured by qRT-PCR. g The effects of NFIX on the luciferase activities of NORFA promoter containing 19-bp duplication alleles. FC indicates fold change. h Analysis of NFIX-binding motif by Jaspar prediction tools (upper) and ChIP-seq data (lower). i ChIP-qPCR assay. The binding enrichment of NFIX on NORFA promoter containing 19-bp duplication alleles were detected by ChIP-qPCR assay. FC means fold change. Beads indicates no antibody, IgG was used as negative control, and the enrichment was normalized to 0.2 input. Data in b–g and i are represented as mean ± S.E.M. with three independent experiments. P values were calculated by a two-tailed Student’s t test. *P < 0.05 and **P < 0.01.

identified within the NORFA gene (n = 344) (Fig. 9a; Supplementary Fig. 8). Notably, the 19-bp duplication located in the core promoter region of NORFA is a breed-specific variant, of which two copies (A1) only occurs in Eruhalian pigs, while one copy (A2) exists only in Landrace and Yorkshire sows (Fig. 9a; Supplementary Fig. 9), suggesting that this variant may be involved in sow prolificacy. Luciferase assay showed that A1-type promoter has higher transcriptional activity than A2-type promoter (Fig. 9b). Similarly, the expression levels of NORFA in granulosa cells from A1/A1 ovarian follicles (2 ≤ d ≤ 9 mm, 5 ≤ d ≤ 9 mm, and 9 ≤ d ≤ 13 mm) are higher than that in granulosa cells from A2/A2 sows, respectively (Fig. 9c, d). All these data
indicate that 19-bp duplication in NORFA promoter strongly influences NORFA level by regulating its transcription activity.

Prediction of binding sites for transcription factors revealed that five additional binding motifs for transcription factors including NFIX, E2F4, E2F6, GABPA, and PAX2 were detected in A1-type promoter region (Supplementary Fig. 10). With the highest prediction score, transcription factor NFIX was chosen for further investigation. We found that expression level of NORFA in granulosa cells was dramatically suppressed after knockdown of NFIX, whereas enhanced by ectopic expression of NFIX (Fig. 9c, f), revealing that NFIX is a transcription activator of NORFA in granulosa cells. Furthermore, we noticed that both A1 and A2 promoter activities were increased after NFIX overexpression, but the increased activity of A1 promoter induced by NFIX was higher than that of A2 promoter (Fig. 9g), indicating that 19-bp duplication influences NFIX regulation of NORFA transcription activity. In addition, chromatin immunoprecipitation (ChIP)-qPCR assay confirmed that NFIX directly binds to NFIX-binding element (NBE) motifs in both A1- and A2-type promoters, but A1-type promoter could recruit more NFIX in comparison to A2-type promoter (Fig. 9h, i). Together, all data demonstrate that the 19-bp duplication influences NORFA transcription by altering the sensitivity of NORFA promoter to transcription factor NFIX.

**NORFA mediated NFIX inhibition of granulosa cell apoptosis.**

We noticed that NFIX mRNA level in granulosa cells from healthy follicles (HFs) was higher than that from atretic follicles (AFs; Supplementary Fig. 11), indicating that NFIX is involved in porcine follicular atresia. Fluorescence-activated cell sorting (FACS) showed that the apoptosis rate of porcine granulosa cells was increased after NFIX silencing (Fig. 10a), whereas ectopic expression of NFIX suppressed cell apoptosis (Fig. 10b), suggesting that, like its target NORFA, NFIX is also an inhibitor of granulosa cell apoptosis and follicular atresia in porcine ovary.

We next investigated whether NFIX inhibits granulosa cell apoptosis by enhancing NORFA. As expected, NORFA overexpression could restore NFIX-siRNA-induced upregulation of cell apoptosis rate (Fig. 10a), while knockdown of NORFA could suppress NFIX-induced downregulation of cell apoptosis rate (Fig. 10b), demonstrating that NORFA mediated the inhibition of NFIX to porcine granulosa cell apoptosis. Besides, we also found that TGFBR2 protein level and p-SMAD3 activity were inhibited after knockdown of NFIX, which could be abolished by ectopic expression of NFIX (Fig. 10c). Opposite results were observed after transfected with pcDNA3.1-NFIX and NORFA-siRNA (Fig. 10d). All the data demonstrate that NORFA mediates the regulation of NFIX to porcine granulosa cell apoptosis and the activity of TGF-β signaling pathway.

**Discussion**

Atresia and degeneration is the ultimate fate of most ovarian follicles (>99%) in mammalian ovary, only <1% of follicles undergo ovulation. Therefore, the study of the mechanism of follicular atresia is very important for decreasing the follicular atresia, increasing ovulation rate, and female fertility. It is now known that follicular atresia is triggered by granulosa cell apoptosis, which is thought to be involved in the death ligand receptor-induced and mitochondria-controlled apoptotic pathways. Previous studies have shown that follicular atresia and granulosa cell apoptosis were regulated by ncRNAs, especially by miRNAs such as miR-125b33 and miR-1275. However, IncRNA regulation of follicular atresia and granulosa cell apoptosis remains unclear. In this study, we proved that NORFA, a lncRNA, controls porcine granulosa cell apoptosis, demonstrating that lncRNA is involved in follicular atresia of mammalian ovary. Although several lncRNAs were recently identified in mammalian ovary or ovarian cells including oocytes, somatic cells such as corpus luteum (CL) cells, and cumulus–oocyte complexes, knowledge of the role of lncRNAs in reproduction is still largely unknown. Neat1 is one of the few IncRNA whose functions in mammalian ovary have been validated. Neat1 knockout (KO) mice are discovered to have prominent parasaclee formation, dysfunction of the CL, low progesterone levels in serum, ovarian defects, and decreased fertility, suggesting that Neat1 is required for CL formation and the establishment of pregnancy. Notably, NORFA is a pig-specific lncRNA and may be the breakthrough for us to reveal the mechanism that lncRNA regulates female reproduction in pigs. Further studies are needed to investigate the role of NORFA in regulating other functions of granulosa cells (e.g., steroidogenesis), follicular atresia in vivo, and reproductive performance such as litter size in pigs.

Our mechanism studies underlying NORFA regulation of porcine granulosa cell apoptosis and follicular atresia have revealed that NORFA acts as a ceRNA of nearby mir-126 gene, which was upregulated during ovarian follicular atresia. The ceRNA hypothesis posits that specific RNAs, such as miRNAs, pseudogene transcripts, circular RNAs, and lncRNAs, can impair miRNA activity through acting as molecular sponges for endogenous miRNAs, thereby enhancing miRNA target expression. Acting as a ceRNA is the main function mode for lncRNAs containing the same MREs with targets. For example, TGFβ2-OT1 sponges miR-3960, miR-4488, and mir-4459 to control autophagy and inflammation of vascular endothelial cells. In this study, miR-126 is an intragenic miRNA transcripted from the intron of EGFL7, which is a nearby coding gene of NORFA. Not unexpected, we found that mir-126 and EGFL7 were oppositely regulated by NORFA. However, some issues such as how NORFA regulates the expression of nearby coding genes and the molecular mechanism of how NORFA controls granulosa cell apoptosis and follicular atresia through these nearby coding genes are very interesting questions for future investigation.

As an intronic miRNA, miR-126 has been shown to regulate multiple important cell biological processes, such as physiological processes including cell proliferation, apoptosis, differentiation, adhesion, and intercellular interaction; and pathological processes including cell cycle, differentiation, and self-renewal of human acute myeloid leukemia stem cells; and cell proliferation, survival, apoptosis, migration, invasion, motility, and metastasis of cancer cells. In the present study, we have shown that miR-126 promotes porcine granulosa cell apoptosis and follicular atresia, demonstrating that miR-126 is required for the functions of granulosa cells in female mammals. In normal cells, KO mouse model showed that plasmacytoid dendritic cell apoptosis rate is increased in Mir126−/− mice, and miR-126 regulates the expression of innate response genes (e.g., Tlr7, Tlr9, Nkjb1, and Vegfr2) and directly targets the mammalian target of rapamycin pathway. In pathological cells, miR-126 widely regulated cell apoptosis of various cancer cells, such as mesothelioma cells and cervical cancer cells.

Increasing studies have demonstrated that miRNAs directly interact with TGF-β signaling pathway to control the state and function of granulosa cells. Meanwhile, few reports demonstrate that lncRNAs also directly interact with TGF-β signaling pathway, but hundreds of TGF-β-induced lncRNAs have been identified, mainly in cancer cells. Here we showed that NORFA governs TGF-β signaling pathway-mediated porcine granulosa cell apoptosis and follicular atresia by interacting with miR-126. TGF-β-activated Inc-ATB is involved in TGF-β signaling pathways.
pathway, which interacts with miRNAs to govern TGF-β signaling pathway in various cells, including enhancement of TGFBR2 and SMAD2 by inhibiting the endogenous miR-425-5p in hepatic stellate cells53 and directly binding to miR-141-3p in gastric cancer cells 54. In addition, TGF-β recruits the Smad proteins and the CCCTC-binding factor complexes on the H19 imprinting control region chromatin, and the complexes could function as insulator in cis and regulator of transcription and replication in trans55.

In recent years, lots of important trait-associated variants within lncRNAs have been identified using high-throughput methods such as genome-wide association studies (GWAS) and whole-genome...
and then prevents miR-126 binding to the target NORFA UTR, which further releases TGFBR2 and activates TGF-β signaling pathway, thereby inhibiting cell apoptosis and follicular atresia. Importantly, we also identify a sow prolificacy-associated variant, 19-bp duplication in the promoter region of lincRNA NORFA. This variant enhances NORFA transcription through recruiting more transcription factor NFIX to the promoter region of NORFA, which is involved in follicular atresia and granulosa cell apoptosis. Overall, our findings provide insights into the molecular mechanism of follicular atresia and the high prolificacy of Erhualian pigs and a target for improving the reproductive performance of other pig breeds (e.g., Landrace and Yorkshire) by using marker-assisted selection and gene editing technology.

Methods

Compliance with ethical regulations. All animal experiments were approved by Animal Ethics Committee at Nanjing Agricultural University, China (SYXK (Su) 2015-0036) and performed in accordance with the Regulations for Administration of Affairs Concerning Experimental Animals (No. 2 of the State Science and Technology Commission, 11/14/1988).

Cell culture and treatment. Porcine granulosa cells from healthy ovarian follicles (3–5 mm diameter) were collected and cultured as previously described. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C with a humidified air atmosphere containing 5% CO2. For transfection, granulosa cells and HEK293T cells were seeded into 6- or 12-well plates and Lipofectamine™ 3000 Transfection Reagent (#1300015, Life Technologies, CA92008, USA) was used for transfection. For TGF-β treatment, cells were treated with TGF-β1 (10 ng/ml) (R&D systems) followed by maintaining in medium without FBS for 12 h. The oligonucleotides used were synthesized by GenePharma (Shanghai, China) and listed in Supplementary Table 1.

Follicular classification. Follicles were isolated from ovaries and classified as HFs and AFs according to the morphological identification, granulosa cell density, and progesterone/estradiol (P4/E2) ratio. Brieﬂy, ovarian follicles with a red color having small blood vessels, low antral granulosa cell density (≤2500/μl), and a low P4/E2 ratio (<2.0) were categorized as HFs. While, the transparent colorless follicles with high antral granulosa cell density (>2500/μl) and a high P4/E2 ratio (>2.0) were categorized as AFs. Only follicles with morphology in accordance with P4/E2 values were selected for further investigation. For detection of the copy numbers of NORFA in granulosa cells, ovarian follicles of different size (2 ≤ d ≤ 5 mm, 5 ≤ d ≤ 9 mm and 9 ≤ d ≤ 13 mm) were isolated.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from granulosa cells using the High Purity Total RNA Extraction Kit (#RP5511, Biotek, China) and reverse transcribed into cDNA by PrimeScript™ RT Master Mix (#RR036A, TaKaRa). qRT-PCR was performed with Acteq™ qPCR SYBR® Green Master Mix (#Q111-02, Vazyme Biotech co., Ltd.), and the relative expression levels were calculated with 2-ΔΔCt method. GAPDH was used as endogenous control for NORFA and coding genes. To measure the miR-126 expression level, miR-126 stem-loop primer was designed to synthesize cDNA and the specific primers of miR-126 were designed for qRT-PCR. U6 small nuclear RNA acted as an internal control. The copy numbers of NORFA in granulosa cells from Erhualian purebred sows (A1/A1) and Yorkshire cross-bred sows (A2/A2) were measured by copy reference assay with qPCR. The standard curve was produced by using a 10-fold serial dilution of purified NORFA standards. Besides, reaction efficiencies were measured through establishing single and multiplex standard curves. Primers used here are listed in Supplementary Table 2.

Bioinformatics analysis. The genomic lociation, conservation, and GC percent of NORFA were obtained from ensembl database (http://www.ensembl.org/index.html). The coding potential of NORFA was analyzed by PhyloCSF and CPAT (http://www.cbrc.jp/research/db/CPAT.html). The MRLEs of ssc-miR-126 were predicted by starbase v2.0 (http://starbase.sysu.edu.cn/mirLCiRNA) and miRcode (http://www.mircode.org/). The mature sequences of miR-126 from different species were obtained from mirBase (http://www.mirbase.org), and their genomic locations were analyzed by NCBI database (https://www.ncbi.nlm.nih.gov/). The candidate miR-126 target genes were analyzed using four different algorithms, microRNA.org (http://www.microrna.org/), TargetScan (http://www.targetscan.org/), PITA (http://genie.weizmann.ac.il/piba/mirn7/), and TarBase (http://diana. cslab.ecu.ntou.gr/tarbase/). Venn diagram indicating the intersected genes was generated using a Draw Venn Diagram online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). DAVID (Database for Annotation, Visualization and Integrated Discovery, version 6.7, https://david.ncifcrf.gov/) was used for KEGG pathways analysis of miR-126-target genes. A significance level of P < 0.05 and an enrichment score ≥ 2 were set as the thresholds. RNAhybrid was used to measure minimum free energy (http://bibiserv.techfak.uni-bielefeld.de/rna-hybrid/). The binding motifs of different transcription factors within NORFA promoter were predicted by JASPAR (http://jaspar.genreg.net/). Heatmap was generated by using R package (heatmap).
Rapid amplification of cDNA ends. The 5’ and 3’ ends of NORFA were identified by using the SMARTer® RACE 5’/3’ Kits (#634858, Clontech Laboratories, CA94043, USA). Briefly, 500 ng total RNA from porcine granulosa cells was used as template to synthesize 5’- and 3’-RACE Ready-first-strand cDNA. Specific primers were designed for RACE following the instructions. The 5’-RACE reversed primer was: 5’-GAT TAC GGC AAG CTT GTC GTC CGA CGT CTC CGC AG-3’. The 3’-RACE forward primer was: 5’-GAT TAC GGC AAG CTT TG TGG TCT CTC AAG ACG GGA CGT-3’. Amplification products were identified by electrophoresing on 2% agarose gel and cloned into T3 vectors for sequencing to get the full-length sequence of NORFA. The full length of porcine NORFA has been submitted to the NCBI Genbank database (#MK879596.1).

Histology analysis. The porcine ovaries were fixed in 10% formalin in phosphate-buffered saline (PBS). The fixed specimens were dehydrated through an ethanol series (70%, 80%, 90%, 100%), cleared in xylene, and embedded in paraffin according to standard techniques. Ovarian slices from the embedded specimens were cut into 4-μm-thick sections, stained with hematoxylin and eosin, and analyzed by microscopic examination.

RNA fluorescence in situ hybridization. RNA FISH for NORFA was performed by Goodbio technology (Wuhan, China). Briefly, porcine ovaries were collected and immediately frozen in liquid nitrogen. The sliced samples were postfixed in 4% paraformaldehyde, acetylated in 1% triethanolamine and 0.25% acetic anhydride, prehybridized, and hybridized at 65 °C using the following anti-sense probes: 5’-TGG TAC TGG GCC GCC GGG TGT CGT CGG AGG GGA GCT TCA GCT GCA CGG ACC TCT TCT TCT TGG AGG GAA GTA AAG TGA GAA AGT GCC CTT CGG TGA-3’. Nucleus was stained with 4,6-diamidino-2-phenylindole dye (blue). To detect the location of NORFA in porcine granulosa cells, cells were rinsed briefly in cold PBS and fixed with 4% formaldehyde for 20 min at room temperature. Then cells were permeabilized in PBS (0.5% Triton X-100) on ice for 5 min, and hybridization was performed using the probes above. Images were obtained from fluorescence microscopy using a Nikon Eclipse 80i microscope equipped with a Nikon DS-2 digital camera.

Subcellular localization. Porcine granulosa cells were collected for nuclear and cytoplasmic extraction using the method described by Wei et al.17 with slight modifications. Briefly, granulosa cells were lysed in cold lysis buffer (10 mM Tris, 0.1 mM EDTA, 1 mM NP-40, and 1 mM RNase Inhibitor) for 5 min on ice and then centrifuged at 10,000 × g for 3 min at 4 °C. The supernatant cytoplasm extract was immediately frozen (−80 °C) for subsequent analysis. The nuclear pellet was resuspended on ice with DEPC water with 1 mM RNase Inhibitor for 3 min and centrifuged at 10,000 × g for 3 min. The supernatant nuclear extract was removed and the remainder was frozen (−80 °C) for subsequent analysis.

Western blotting. Western blotting assays were performed as described previously31. The primary antibodies used here are anti-TGFBR2 (Sangon Biotech, #D155818, 1:1000), anti-NFIX (Affinity, #DF3250, 1:1000), anti-SMAD3 (Sangon Biotech, #D155153, 1:1000), anti-p-SMAD3 (Sangon Biotech, #D155234, 1:1000), and anti-GAPDH (ORIGENE, #TA002519, 1:3000). GAPDH was measured as an internal control.

Apopotosis analysis. Apoptosis Detection Kit (#A211-01, Vayzme biotech co., Ltd, China) was used to measure cell apoptosis rate. Briefly, 20,000 cells were stained with Annexin V-fluorescein isothiocyanate/propidium iodide and then sorted by FACS using a cell counting machine (Becton Dickinson). The apoptosis rate was analyzed using the Flowjo software (TreeStar) and calculated using the following equation: number of cells (in the right upper quadrant + number of cells in the right lower quadrant)/(total number of cells)×100.18

Plasmids. To identify the MRE of miR-126 in NORFA or TGFBR2 3’-UTR, porcine NORFA or TGFBR2 3’-UTR fragments containing potential miR-126 MRE motif were isolated and inserted into pmirGLO Dual-luciferase miRNA Target Expression Vector between Nhel and Xhol enzyme sites (#E1330, Promega). For NORFA overexpression vector construction, full-length cDNA sequence of NORFA was synthesized and cloned into pcDNA3.1 expression vector (#P790-20, Invitrogen) between Nhel and Xhol enzyme sites. To detect the effects of NFIX on the activity of NORFA promoter, porcine NORFA promoters with −1193 ins allele and del allele (which contains 2 and 1 NFIX binding motif, respectively) were cloned into pGL3-Basic reporter vector (#E1751, Promega) between KpnI and Xhol enzyme sites. To generate the NFIX expression vector, full-length cDNA sequence of porcine NFIX was amplified by using total RNA extracted from porcine granulosa cells and cloned into pcDNA3.1 expression vector within KpnI and Xhol enzyme sites. For RNA pull-down assays, psP19T-NORFA and psP19-NORFA-miR-31-3p were designed and transcribed by GeneScript. The biotinylated miRNA-31-3p plasmids were generated by using a TaKaRa MutanBEST Kit (#R401, TaKaRa) according to the manufacturer’s protocol. All of the recombinant plasmids were confirmed by Sanger sequencing. The primers used here are shown in Supplementary Table 3.

Dual luciferase assay. After transfection for 24 h, cells were collected and luciferase activities of each sample were detected by using Dual-Luciferase Reporter Assay System (#E1910, Promega) according to the manufacturer’s protocol. GLOMAX detection system (Promega) was conducted to measure the luciferase activity in cell lysates. Relative luciferase activity of each sample was calculated as the ratio of Firefly/Renilla.

RNA pull-down assay. NORFA and NORFA-mut (mir-126) were transcribed from vector pSPT19-NORFA and pSPT19-NORFA-mut (mir-126) in vitro, respectively. After biotinylated modification with the Biotin RNA Labeling Mix (#No.1168597910, Roche) and T7 RNA polymerase (#EP0111, Thermofisher Scientific), two single-stranded RNA transcripts were purified with an RNeasy Mini Kit (#74104, Qiagen, Valencia, USA). The purified biotinylated transcripts (5 μg) were incubated with 20 μg porcine granulosa cells total RNA for 4 h at room temperature. Streptavidin magnetic beads (#1LSK8175702, Merck Millipore) were used to isolate the biotin-NORFA/RNA complex according to the manufacturer’s protocol. After isolation, the levels of target miRNAs present in the pull-down material were detected by qRT-PCR analysis.

Chromatin immunoprecipitation. ChIP assays were performed as previously described31. Briefly, Rabbit anti-NFIX (Affinity, #DF3230) antibody was used to pull down NFIX-DNA complex and a nonspecific antibody against IgG (Santa Cruz, sc-2358) was used as an internal control, and the chromatin before immunoprecipitation was used as an input control. The specific PCR primers used to detect NFIX-binding motifs within NORFA promoter were listed in Supplementary Table 4. Each specific antibody ChIP sample was normalized to relative IgG antibody ChIP signals obtained from the same sample. ChIP-qPCR signals were calculated as fold enrichment of input control signals with technical triplicates.

Animal selection, sample collection, and DNA extraction. A total of 344 sows were randomly selected for NORFA single-nucleotide polymorphism (SNP) screening (106 Erhualian sows from Jiaoxi Erhualian Breeding Farm, Jiangsu Province, China; 144 Yorkshire sows from Yongkang Farming Technology Co., Ltd., Jiangsu Province, China; 94 Landrace sows from Huaiyin Breeding Farm, Jiangsu Province, China). Ear tissue sample (~2 g per sow) from each sow was collected in a cryotube and kept in 75% ethyl alcohol (v/v). All efforts were made to minimize animal suffering during ear tissue collection. The genomic DNA from each ear sample was extracted according to the standard phenol–chloroform method and the purified DNA was stored at −20 °C for further experiments.

Polymorphism analysis. Six pairs of primers were designed to analyze the polymorphism of NORFA according to the porcine NORFA sequence. The genomic DNA mentioned above as substrates, PCR was performed by using 2× Vazyme LAmp Master Mix (#P312-01, Vazyme biotech co., Ltd, China), and the products were confirmed by Sanger sequencing. Subsequently, the variants were analyzed by using a DNA sequencing software Chromas. Linkage disequilibrium and haplotypes were calculated and identified by using SHEsis (http://analysis.bio-x.cn/myAnalysis.php) and haploview 4.2 software with the SNP information of porcine NORFA gene. The primers used here are listed in Supplementary Table 5.

Statistics and reproducibility. All experiments were repeated at least three times and the data are presented as means ± S.E.M. Statistical analyses were performed using IBM SPSS Statistics v20.0 (SPSS Inc.). An unpaired two-tailed Student’s t test was used to evaluate the significance. Significance between conditions are denoted as *P < 0.05 and **P < 0.01. All the figures were built by the GraphPad Prism v7.0 software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The majority of the data in this study are included in this published article. The DNA sequence of NORFA is available in the NCBI Genebank database (#MK879596.1). The source data underlying the graphs and charts presented in the main figures are shown as Supplementary Data 1. The original images of gels and blots are shown in Supplementary Fig. 12. The newly constructed plasmids have been deposited into Addgene (deposit number: 77871). Other information supporting the findings of this study is available from the corresponding author on reasonable request.

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Author contributions

X.D. and Qifa Li conceived and designed the project. X.D. performed the experiments. L. L. and Qiqi Li assisted with experiments. X.D., L.Z., and Z.P. analyzed the data. X.D. wrote the manuscript with input from all other authors. All authors critically reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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