miR-2337 induces TGF-β1 production in granulosa cells by acting as an endogenous small activating RNA

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INTRODUCTION
Transforming growth factor-β1 (TGF-β1) is an essential ligand of the TGF-β signaling pathway [1, 2]. As an extracellular signal molecule, the TGF-β1 signal first binds to the TGF-β receptors on the cell membrane, thereby activating R-SMADs in the cytoplasm, and ultimately enters the nucleus with Co-SMAD to control multiple cell functions [3]. TGF-β1 plays a vital role in cell growth, proliferation, and apoptosis of various normal cell types, as well as pathological cell types [1, 4]. In the ovary, TGF-β1 is highly expressed, as it is secreted from various follicular cells, and is detected in follicular fluid [5–7]. Increasing evidence has demonstrated that TGF-β1 is closely involved in ovarian functions, including follicular development [8, 9], ovulation [10], steroid production [11, 12], and GC apoptosis [13]. For instance, in female mice with knock out of the TGF-β1 gene, ovarian function was destroyed and the oocytes decreased by 40% [7]. TGF-β1 promotes estradiol release from mouse GCs [14], and inhibits the production of progesterone and prostaglandin E2 in the corpus luteum from bovine ovaries [15]. Our recent report demonstrated that TGF-β1 can inhibit porcine GC apoptosis [13]. In addition, TGF-β1 is involved in female fertility, including fertilization, early embryo development and litter size [2, 7].

As a multifunctional cytokine that is essential for mammalian health and disease, TGF-β1 regulation has always been a key focus of attention. Evidence suggests that TGF-β1 is regulated by various genetic and epigenetic factors [16, 17]. For genetic factors, more than a dozen mutations, including single nucleotide polymorphisms such as rs1800470 (C29T) and rs1800471 (G74C), and deletion/insertion polymorphisms such as c.–2389_–2391insAGG, as well as several transcription factors such as AP1 and SP1, have been demonstrated to control TGF-β1 levels in various tissues and cell types [18, 19]. The T allele at variant C29T is a risk factor for genetic susceptibility to myocardial infarction, and individuals with genotype TT showed low TGF-β1 concentration in the serum [20]. For epigenetic factors, DNA methylation, histone modification factors, and non-coding RNAs (ncRNAs) all control TGF-β1 regulation of the TGF-β1 signaling pathway and GC apoptosis. Taken together, our findings identify miR-2337 as an endogenous small activating RNA (saRNA) of TGF-β1 in GCs, while miR-2337 is identified as a small activator of the TGF-β signaling pathway which is expected to be a new target for rescuing GC apoptosis and treating low fertility.

RESULTS
Variants in Yorkshire TGF-β1 3′-UTR
A previous study demonstrated the involvement of two variants of sow fertility in the Yorkshire TGF-β1 3′-UTR [24]. To further investigate the full view of variants in the whole 3′-UTR of porcine TGF-β1, we screened this region using pooled-DNA sequencing in multiple pig breeds including Yorkshire, Suhuai, and Erhualian.

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Three completely linked SNPs, including two known variants named c.1583A > G and c.1587A > G, and the novel variant c.2074A > C, were identified in this region (Figs. 1A and S1). In a Yorkshire population (n = 325), there were three genotypes, AA/AA/AA, AG/AG/AC, and GG/GG/CC, of which the AA/AA/AA genotype was the dominant genotype (57.9%) (Fig. 1B, C). Two haplotypes A-A-A and G-G-C were identified in this population, of which A-A-A was the dominant haplotype (76.2%) (Fig. 1D). Association analysis revealed that the TNB of sows with the GG/GG genotype was the dominant genotype (57.9%) (Fig. 1B, C). Two AA/AA/AA, AG/AG/AC, and GG/GG/CC, of which the AA/AA/AA genotype was the dominant genotype (57.9%) (Fig. 1B, C). Two haplotypes A-A-A and G-G-C were identified in this population, of which A-A-A was the dominant haplotype (76.2%) (Fig. 1D). Association analysis revealed that the TNB of sows with the GG/GG genotype was the dominant genotype (57.9%) (Fig. 1B, C). Two AA/AA/AA, AG/AG/AC, and GG/GG/CC, of which the AA/AA/AA genotype was the dominant genotype (57.9%) (Fig. 1B, C).

**Variants in the MRE motif prevent target inhibition of miR-2337 on TGF-β1 3′-UTR**

Variants in MREs usually alter the interaction between miRNAs and the 3′-UTR [25]. Notably, two variants c.1583A > G and c.1587A > G were located at the MRE motif of miR-2337 (Figs. 2A, S2, and S3), and mutations led to the loss of the MRE motif. Furthermore, the MRE motif of miR-2337 was also detected in TGF-β1 3′-UTR of other mammals (Fig. 2B), indicating that TGF-β1 is a candidate target of miR-2337 in mammals. To investigate whether miR-2337 targets TGF-β1, we generated a reporter construct of the TGF-β1 3′-UTR containing miR-2337 MRE (Fig. 2C). This construct was co-treated in HEK293T cells with miR-2337 mimics, and it was found that the overexpression of miR-2337 inhibits the luciferase activity of this construct (Fig. 2D), suggesting that miR-2337 targets the porcine TGF-β1 gene. Then generated a reporter construct of TGF-β1 3′-UTR with haplotype G-G (Fig. 2C), and co-treated HEK293T cells with miR-2337 mimics. Luciferase assay revealed that miR-2337 did not affect the activity of the construct with haplotype G-G (Fig. 2E). Taken together, these results suggest that variants in the MRE motif influence target inhibition of miR-2337 on the TGF-β1 3′-UTR in pigs.

**miR-2337 induces, not reduces, TGF-β1 production in GCs**

To investigate the regulatory effect of miR-2337 on endogenous TGF-β1 expression, we analyzed TGF-β1 levels in miR-2337 mimics–treated GCs. After treating with miR-2337 mimics for 24 h, miR-2337 level was significantly increased in porcine GCs (Fig. S4), however, TGF-β1 mRNA levels were not altered (Fig. 3A). Notably, after miR-2337 mimics treatment for 48 h, TGF-β1 protein level was significantly increased, not decreased (Fig. 3B), indicating that in porcine GCs, miR-2337 is not an inhibitor of TGF-β1. In fact, it may be an activator, as miR-2337 may be a potential saRNA of TGF-β1 in GCs. It is well known that an important feature of saRNA activated gene is the delay in the onset of gene activation [26]. We therefore investigated the kinetics of TGF-β1 mRNA levels in GC treatment of miR-2337 mimics. As expected, induction of TGF-β1 transcription by miR-2337 initiate at 48 h (Fig. 3C), indicating that miR-2337 is a potential saRNA of TGF-β1 in GCs. In addition, we also detected TGF-β1 concentration in culture medium by ELISA, and found that miR-2337 induces upregulation of TGF-β1 production in GCs (Fig. 3D). These data suggest that miR-2337 functions as a potential saRNA of TGF-β1 to enhance TGF-β1 secretion in GCs.
in porcine GCs, which induces TGF-β1 expression via MRE motif in the promoter, not in the 3′-UTR.

miR-2337 binds to the MRE1 motif to alter histone modification of the TGF-β1 core promoter

To further investigate whether miR-2337 induces TGF-β1 transcription via the MRE1 motif, we also constructed a reporter vector of the TGF-β1 core promoter containing the mutant-type MRE1 motif (Fig. 5A). As expected, miR-2337 did not affect MRE1-mutated core promoter activity (Fig. 5B), indicating that miR-2337 activates TGF-β1 transcription via the MRE1 motif within its core promoter. It has been shown that sRNA induces target transcription by epigenetic changes in the core promoter region [26]. Therefore, we next tested whether miR-2337 influences histone modification of the TGF-β1 core promoter in GCs by ChIP assays. Overexpression of miR-2337 induces upregulation of H3K4me2, H3K4me3, and H3K9ac, whereas downregulation of H3K9me2 was induced at the MRE1 motif of the core promoter region (Fig. 5C). Our data suggest that miR-2337 directly binds to the MRE1 motif to alter histone modification of the TGF-β1 core promoter, thereby activating TGF-β1 transcription in GCs.

miR-2337 controls the TGF-β1-mediated TGF-β signaling pathway and GC apoptosis

TGF-β1 is both an activator of the TGF-β signaling pathway, and a repressor of porcine GC apoptosis [2]. We therefore tested whether miR-2337 controls the TGF-β signaling pathway and GC apoptosis. Levels of phosphorylated SMAD3 (p-SMAD3), a marker for the activity of this pathway, were significantly increased in miR-2337-overexpressing GCs (Fig. 6A). Flow cytometry showed that miR-2337 overexpression significantly inhibited the GC apoptosis rate (Fig. 6B). These data suggest that, similar to TGF-β1, miR-2337 is both an activator of the TGF-β signaling pathway, and a repressor of GC apoptosis. Furthermore, inactivation of the TGF-β signaling pathway by the TGF-β receptor-specific inhibitor SB431542 inhibited miR-2337 mimics-induced upregulation of p-SMAD3 levels in GCs (Fig. 6C). Consistent with this, miR-2337 could rescue the inactivation of TGF-β signaling pathway caused by TGF-β1 silencing in GCs (Fig. 57). In addition, the inhibitor SB431542 also reversed the downregulation of the GC apoptosis rate caused by miR-2337 mimics (Fig. 6D). These data suggest that miR-2337 controls the TGF-β signaling pathway and cell apoptosis by activating TGF-β1 in porcine GCs.

DISCUSSION

TGF-β1 is highly expressed in the reproductive system, which has been shown to be strongly related to female fertility by controlling important events in reproductive cycle, such as, ovulation, fertilization, implantation, early embryo development, and pregnancy [7, 28–30]. In sows, TGF-β1 is also an important candidate gene for reproductive traits [2, 24]. In this study, we identified three completely linked mutations (c.1558A > G and c.1557A > G, and c.2074A > C) in the porcine TGF-β1 3′-UTR, and the TNB of sows with the favorable GG/GG/CC genotype is 0.46 higher than that of other genotypes in the Yorkshire population (n = 325). Similarly, in another Yorkshire population (n = 567), the TNB of sows with the favorable GG/GG/CC genotype is 0.46 higher than that of other genotypes in the Yorkshire population (n = 325). Similarly, in another Yorkshire population (n = 567), the TNB of sows with the favorable GG/GG/CC genotype for mutations A > G at 2490 nt (i.e., c.1558A > G) and A > G at 2494 nt (i.e., c.1557A > G) was significantly higher than that of the AA/AA genotype [24]. Taken together, these findings suggest that variants in the TGF-β1 3′-UTR have a certain impact on the reproductive performance of Yorkshire sows. In the breeding of Yorkshire reproductive traits, these variants in the TGF-β1 3′-UTR can be used as potential molecular markers for marker-assisted selection (MAS), that is, to select individuals the favorable genotype GG/GG/CC, so as to quickly improve the fecundity of Yorkshire population.
Mutations in or near RNA regulatory elements have been shown to control the activity or function of the 3′-UTR by affecting the interaction between RNA binding proteins (RBPs), miRNAs and 3′-UTR [31–33], rs15705, for instance, is identified in the BMP2 3′-UTR as an osteoporosis-associated variant in Icelandic patients [34]. This variant disrupts a putative AU-rich element (ARE), which has been shown to influence 3′-UTR activity and posttranscriptional regulation of BMP2, perhaps through an ARE motif and RBP interaction mechanism [35, 36]. Interestingly, as a causant variant for natural clearance of infection with HCV infection, variant rs4803217 is located near the three classical ARE motifs and on the MRE motifs of two myomiRs in the IFNL3 3′-UTR, and strongly influences both ARE motif-mediated 3′-UTR activity, and miRNA regulation of 3′-UTR activity [31]. Herein we demonstrated that mutations c.1583A > G and c.1587A > G cause the loss of a miR-2337 MRE motif, thereby relieving the inhibition of miR-2337 on TGF-β1 3′-UTR activity. Recently, several mutations in MRE motifs that alter miRNAs interaction with the target 3′-UTR have been identified, such as rs78378222 in the miR-325-3p MRE motif of the TP53 3′-UTR [33], rs3802266 in a miR-181a-2-3p MRE motif of the ZHX2 3′-UTR [37], and rs41283642 in a miR-142-3p MRE motif of the TGFBR1 3′-UTR [38].

Notably, we showed that, in porcine GCs, miR-2337 did not decrease TGF-β1 levels. Mechanistically, miR-2337 acts as an endogenous saRNA to induce TGF-β1 transcription and production. saRNAs refer to a double-stranded RNA (dsRNA) with a length of approximately 19–21 nt, which induces target transcription via an RNA activation (RNAa) mechanism by directly interacting with the promoter region of target genes [27, 39, 40]. Since 2006, scientists have found that 21 nt of dsRNA that are complementary to the promoter of targets, such as p21 and VEGF, can continuously induce target transcription in human cells. SaRNA has become a novel therapeutic, and has been used in clinical trials [41, 42]. Dozens of synthetic saRNAs have been identified to activate target transcription, for example, saRNAs KLF4-PR1, MYC-PR1, and MYC-PR2 activate KLF4 and c-MYC, two pluripotency reprogramming factors in mesenchymal stem cells [43]. Moreover PR11 [39] and PR-1611 [40] activate the PR gene in breast cancer cells, and CEBPA-saRNA activates the CEBPA gene in hepatocellular carcinoma cells [44]. Interestingly, in 2008, miRNAs, a class of naturally occurring endogenous small ncRNAs, have also been shown to function as endogenous saRNAs to directly activate target expression [45]. At present, several miRNAs that mediate the activation of targets have been identified, including miR-24-1 [46], miR-4281 [47], and miR-320 [48]. Taken together, our findings identified the first saRNA target TGF-β1 and TGF-β1 superfamily, which provides new insight into miRNAs regulation of TGF-β1. However, the mechanism of the variants in the TGF-β1 3′-UTR in terms of affecting sow reproductive performance requires further study.

Crosstalk between miRNAs and the TGF-β signaling pathway is one of the most important regulatory mechanisms of GC apoptosis in mammals [49, 50]. On the one hand, the TGF-β signaling pathway controls GC functions by regulating miRNA biosynthesis. For example, miR-224/320/383-mediated mouse GC proliferation and estradiol release [49, 51], miR-10a/10b-mediated GC states in humans and rodents [52], and miR-1306/143/29c/425-mediated porcine GC apoptosis [13, 50, 53, 54] are all regulated by the TGF-β signaling pathway. On the other hand, miRNAs directly target core members of other ligands, receptors, or SMADs of the TGF-β signaling pathway to regulate GC functions. For ligands, miR-130a induces GC apoptosis by reducing TGF-β1 in pigs [2]. For receptors, let-7g and miR-202-5p induce GC apoptosis by inhibiting pig TGF-β1 and goat TGF-β1, respectively [55, 56]. For SMADs, miR-4110 promotes goat GC apoptosis by inhibiting SMAD2 [57], and miR-224 controls mouse GC proliferation and estradiol release by inhibiting Smad4 [50]. Herein, we showed for the first time that miRNA activates the TGF-β1 signaling pathway to reduce GC apoptosis by target directly activating a core component of this pathway. Our findings defined the new function of miR-2337, and identify this miRNA as an activator of the TGF-β1 signaling pathway in GCs.

In conclusion, we screened the whole 3′-UTR of the porcine TGF-β1 gene and show the important role that TGF-β1 3′-UTR variants play on Yorkshire sow fertility. Importantly, mutations c.1583A > G and c.1587A > G that are located at the MRE motif of miR-2337 in

![Fig. 3 miR-2337 induces TGF-β1 expression in GCs. A, B GCs were treated with miR-2337 mimics, and mRNA (A) and protein (B) levels of TGF-β1 were measured at 24 h and 48 h, respectively. C GCs were treated with miR-2337 mimics, and TGF-β1 mRNA levels were determined at 24 h, 48 h, and 72 h. D TGF-β1 concentration in culture medium of GCs treated with miR-2337 mimics for 48 h, analyzed using ELISA. Data are represented as means ± S.E.M. (n = 3). *p < 0.05, ***p < 0.01.](image-url)
the TGF-β1 3′-UTR, relieves the inhibition of miR-2337 on 3′-UTR activity. However, in porcine GCs, miR-2337 does not reduce transcription and production of TGF-β1. We identified miR-2337 as a sRNA of TGF-β1 that induces TGF-β1 transcription through direct binding to the promoter and alterations in histone modification (Fig. 7). Furthermore, miR-2337 activates the TGF-β signaling pathway and reduces GC apoptosis by activating TGF-β1. Our findings implicate that miR-2337 might be an activator of the TGF-β signaling pathway, and a promising therapeutic target for GC apoptosis and low fertility.

**METHODS**

**Samples and ethics**

A total of 365 sows were included in the present study. The studied population was randomly selected from Jiangsu Kangle Pig Breeding Farm (Yorkshire sows, n = 325), Jiangsu Huaiyin Pig Breeding Farm (Suhuai sows, n = 20) and Changzhou Erhualian Production Cooperation (Erhualian sows, n = 20) for ear tissue sample collection and genomic DNA extraction. Their corresponding reproductive trait records were also collected for further association analysis. In addition, ovaries were isolated from 60 Duroc-Landrace-Yorkshire sows (sexually mature) for GC collection and culturing. In this study, all animal experiments were reviewed and supervised by the Animal Ethics Committee (AEC) of Nanjing Agricultural University, Jiangsu, China.

**Genomic DNA isolation and genotyping**

Using the phenol-chloroform method as previously described [2], the genomic DNA from ear samples of the studied Yorkshire sows was extracted and stored at −20 °C for further analyses. After detection of the concentration, purity and integrity of the DNA, a total of 325 DNA samples were selected for genotype validation. Using genomic DNA as a template, the variants in the 3′-UTR region of porcine TGF-β1 were amplified by PCR and identified using Sanger sequencing procedures (Sangon Biotech, Shanghai, China). The specific primers used for genotype validation are listed in Table S1.

**Cell culture and treatment**

Porcine GCs were extracted from small non-atretic follicles using a 22-gauge sterile syringe, and washed twice with 37 °C PBS. Next, the cells were seeded in plates filled with Dulbecco’s modified Eagle’s medium (DMEM/F12, #2177608, Gibco) containing 15% fetal bovine serum (FBS, #10270106, Gibco) and 1% penicillin/streptomycin solution (PS, #15140-122, Gibco), which was subsequently placed in a humid incubator at 37°C and 5% CO₂ aerobic conditions. KGN and HEK293T cells were seeded in plates filled with RPMI-1640 medium (#AF29477380, Hyclone) and DMEM medium (#AF29431640, Hyclone) containing 10% FBS and 1% PS, and cultured under the same conditions as porcine GCs. For transfection of recombinant plasmids and oligonucleotides, HighGene transfection reagent (#9619114TR, ABclonal) was used following the
promoter activity of XbaI and SacI restricted enzyme sites. To analyze the effects of miR-2337 on the promoter of porcine TGF-β1, different fragments of the promoter were amplified and inserted into pGL3-basic vectors between the XhoI and HindIII restriction enzyme sites. To detect whether miR-2337 directly targets TGF-β1, the 3′-UTR fragment of porcine TGF-β1 containing a potential MRE of miR-2337 was synthesized and cloned into the pmirGLO vector (#E1751, Promega) between XhoI and XbaI restriction enzyme sites. To analyze the effects of miR-2337 on the promoter activity of TGF-β1, the promoter of porcine TGF-β1 containing a potential MRE of miR-2337 was cloned into a pGL3-basic vector between the SacI and HindIII restricted enzyme sites. Treiler™ SoSoo Cloning kit (#TSV-S1, TsingKe) was used to generate mutant-type reporter vectors following the manufacturer's instructions. All recombinant vectors were verified by Sanger sequencing (Sangon Biotech, Shanghai, China). The primers used for plasmid construction are shown in Table S3. For dual-luciferase reporter assays, cells were lysed and the content was collected after treatment for 24 h. Next, the luciferase activities of Firefly and Renilla were measured using a Dual-Luciferase Activity Detection System kit (#E1751, Promega), and the ratio of Firefly/Renilla was used to determine the relative luciferase activity of each sample.

**RNA extraction and quantitative real-time PCR**

Total RNA from porcine GCs under different treatments was extracted using TRIzol reagent (#R-51001A, Angle Gene, China), and cDNA was synthesized using PrimeScript™ RT Master Mix (#RR036A, Takara, China) with 1 μg of total RNA as the template according to the manufacturer's instructions. qRT-PCR was performed using the Power SYBR Green PCR Master Mix (#Q111-02/03, Vazyme) and the reactions were detected on a StepOne Plus System (Applied Biosystems) as previously published [13]. The fold changes of the genes of interest were calculated using the 2^ΔΔCT method, and the relative expression levels of coding and non-coding genes were normalized to the levels of GAPDH and U6, respectively. The primers designed for the qPCR analysis are listed in Table S4.

**Bioinformatics analysis and association analysis**

The full length of the porcine TGF-β1 nucleotide sequence was downloaded from the Ensembl database (http://asia.ensembl.org/index.html). To predict the putative promoter of porcine TGF-β1, two online software, namely Promoter scan and Promoter 2.0, with different algorithms were utilized according to their corresponding instructions. In addition, the candidate miRNAs that potentially target porcine TGF-β1 were predicted using the miRBase database (http://www.mirbase.org/search.shtml), and the MREs of miR-2337 within the promoter and the 3′-UTR region of porcine TGF-β1 were analyzed using RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/mnahybrid). The genome location and sequence of mature miR-2337 were obtained from the miRBase database (https://www.mirbase.org/search.shtml=miR-2337). Furthermore, association analyses were performed in this study to detect the correlation between polymorphism of the variants within the 3′-UTR of porcine TGF-β1 and reproductive traits, such as the total number of piglets born (TNB) and the number of piglets born alive (NBA). SAS software was utilized according to their corresponding instructions. In addition, the samples were uploaded and separated by 4–20% SDS-PAGE. After electrophoresis for 1 h at 140 V, the separated proteins were transferred to PVDF membranes. The PVDF membranes were then incubated with blocking buffer containing 5% BSA and probed with the primary antibodies overnight. The next day, the membranes were re-rinsed with TBST buffer three times to remove excess primary antibodies, and then incubated with HRP-conjugated secondary antibodies. The high-solution images were obtained using a chemiluminescence imaging system, and the grayscale of each protein blot was quantified using ImageJ software. GAPDH protein expression served as an internal control. The primary antibodies used were TGF-β1 (#bs-0086R, Bioss, 1:1000 dilution), SMAD3 (#D15S234, Sangon, 1:1000 dilution), p-SMAD3 (#D15S153, Sangon, 1:1000 dilution), and GAPDH (#D198662, Sangon, 1:2000 dilution).

**ELISA**

Porcine GCs were lysed using RIPA lysis buffer (#BD0031, BioWorld) with 1% PMSF and protease inhibitor, and the total protein was extracted and collected for western blotting. The concentration of each protein sample was detected using the BCA method according to the manufacturer’s instructions. In brief, equal amounts of protein (~20 μg) from different samples were loaded and separated by 4–20% SDS-PAGE. After electrophoresis for 1 h at 140 V, the separated proteins were transferred to PVDF membranes. The PVDF membranes were then incubated with blocking buffer containing 5% BSA and probed with the primary antibodies overnight. The next day, the membranes were re-rinsed with TBST buffer three times to remove excess primary antibodies, and then incubated with HRP-conjugated secondary antibodies. The high-solution images were obtained using a chemiluminescence imaging system, and the grayscale of each protein blot was quantified using ImageJ software. GAPDH protein expression served as an internal control. The primary antibodies used were TGF-β1 (#bs-0086R, Bioss, 1:1000 dilution), SMAD3 (#D15S234, Sangon, 1:1000 dilution), p-SMAD3 (#D15S153, Sangon, 1:1000 dilution), and GAPDH (#D198662, Sangon, 1:2000 dilution).

**Western blotting**

Porcine GCs were lysed using RIPA lysis buffer (#BD0031, BioWorld) with 1% PMSF and protease inhibitor, and the total protein was extracted and collected for western blotting. The concentration of each protein sample was detected using the BCA method according to the manufacturer’s instructions. In brief, equal amounts of protein (~20 μg) from different samples were loaded and separated by 4–20% SDS-PAGE. After electrophoresis for 1 h at 140 V, the separated proteins were transferred to PVDF membranes. The PVDF membranes were then incubated with blocking buffer containing 5% BSA and probed with the primary antibodies overnight. The next day, the membranes were re-rinsed with TBST buffer three times to remove excess primary antibodies, and then incubated with HRP-conjugated secondary antibodies. The high-solution images were obtained using a chemiluminescence imaging system, and the grayscale of each protein blot was quantified using ImageJ software. GAPDH protein expression served as an internal control. The primary antibodies used were TGF-β1 (#bs-0086R, Bioss, 1:1000 dilution), SMAD3 (#D15S234, Sangon, 1:1000 dilution), p-SMAD3 (#D15S153, Sangon, 1:1000 dilution), and GAPDH (#D198662, Sangon, 1:2000 dilution).

**Subcellular localization**

Nuclear and cytoplasmic isolation was performed using the method described by Du et al. [59]. Briefly, 1 mL of lystate buffer was added to a centrifuge tube containing porcine GCs, lysed for 5 min, and then centrifuged for 3 min (10,000 × g), and the supernatant containing cytoplasmic extract was absorbed and stored at −80 °C. The nuclear
pellet was resuspend and then centrifuge for 3 min (10,000 x g), discard the supernatant, and the nuclear extract is present in the pellet. RNA extraction and qRT-PCR was carried out by the above methods.

**ChiP assay**

ChiP assays were performed using Pierce Agarose ChiP Kit (#26156, Thermo) according to the manufacturer’s protocol. In brief, after transfection for 72 h, 1% formaldehyde was added to the medium and porcine GCs were cross-linked for 10 min, and then under 6U of micrococcal nuclease had been added in a 37 °C water bath for 15 min. H3K4me2 antibody (#9725 S), H3K4me3 antibody (#9751 S), H3K9me2 antibody (#4658 S) and H3K9ac antibody (#9649 S) were all obtained from Cell Signaling Technology (USA), and were used to pull down the immunoprecipitated complex. After pull down, the fragments of interest were detected by PCR and the amplified products were analyzed by 3% agarose gel electrophoresis, and the enrichment was normalized to the IgG group. IgG antibody (#2985 S, Cell Signaling Technology) was used as a negative control and unprocessed DNA served as the input control. The primers used for the ChiP assay are listed in Table S5.

**Apoptosis detection**

After 48 h of treatment, porcine GCs were collected and the cell apoptosis rate was detected using a Cell Apoptosis Detection kit (#A211-01/02, Vazyme). First, porcine GCs were harvested and rinsed with cold PBS twice after treatment for 48 h, then 100 μL of binding buffer were added to resuspend the cells for 10 min. Subsequently, 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) were added to dye cells for at least 10 min under dark conditions. Total GCs were detected using fluorescence-activated cell sorting (FACS) on a cell sorting machine, and the apoptosis rate was analyzed using FlowJo software.

**Statistics analysis**

In this study, data were analyzed using GraphPad Prism v5.0 software, and represented as mean ± S.E.M. with at least three independent replicates. The differences (p-values) between two different groups were determined by a two-tailed Student’s t test, and p < 0.05 was considered a statistically significant difference.

**DATA AVAILABILITY**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

L.W.: performed the experiments, writing - original draft and data curation. X.D.: data curation, visualization, and writing - review & editing. C.L.: methodology and validation. W.W. and Z.P.: funding acquisition. Q.L.: conceptualization, methodology, writing - review & editing, project administration, funding acquisition. All authors read and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

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