A novel positive feedback regulatory loop, TGF-β1/SMAD4/miR-184, controls the follicular development in sows

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ABSTRACT

TGF-β1, the core ligand of the transforming growth factor β (TGF-β) signaling pathway, is crucial for follicular development and female fertility. However, little is currently known about whether miRNAs, an important class of epigenetic regulators, mediate the anti-atretic effect of TGF-β1. Here, a joint transcriptomic analysis demonstrated that miR-184, an anti-atretic miRNA, was significantly elevated by TGF-β1 in sow granulosa cells (GC). Quantitative detection, correlation analysis, luciferase reporter assay and ChIP confirmed that TGF-β1 induces *miR-184* transcription in a SMAD4-dependent manner, while SMAD4 acts as a transcriptional activator to directly bind to *miR-184* promoter under TGF-β1 stimulation. Interestingly, RNA-seq and bioinformatics analysis found that the targets of miR-184 are enriched in the TGF-β pathway, with *TGF-β1* being feedback induced by miR-184. In vivo and in vitro mechanistic analysis revealed that miR-184, as a small-activating RNA (saRNA), activates *TGF-β1* transcription by binding to its promoter and forming a RNA-induced transcriptional activation complex with AGO2, CTR9, DHX9, and RNA polymerase II. Therefore, a novel positive feedback loop, TGF-β1/SMAD4/miR-184, has been identified. In vitro GC and follicle culture systems were applied and validated that this loop exerts anti-apoptotic/atretic functions. Moreover, comparative analyses showed that the levels of TGF-β1 and miR-184 in the follicles of high-fertility sows were significantly higher than those of low-fertility sows. Our findings highlight the critical role of the interaction between TGF-β1 and miR-184 in inhibiting GC apoptosis and follicular atresia, providing potential targets for improving follicular development and sow fertility.

Keywords: TGF-β1; SMAD4; miR-184; Feedback regulation; Sow GC apoptosis; Follicular atresia

INTRODUCTION

As the basic functional unit of ovarian tissue in female mammals, follicles are located within the ovarian cortex and are composed of theca cells, GCs and oocytes, which retain crucial hormonal and reproductive functions, and are the basis of female fertility (Yoshino et al., 2021). Based on the morphological features at different developmental stages, follicles can be categorized as pre-antral (primordial, primary, and secondary), antral, mature and atretic. Follicular atresia (FA), a common physiological phenomenon in the ovaries, is the final fate of the most follicles (> 99%), which is essentially a selection process of the dominant follicles (Zhou et al., 2019). However, excessive FA causes multiple reproductive diseases (e.g. Premature Ovarian Insufficiency and Polycystic Ovary Syndrome) and infertility (Shang et al., 2025; Shen et al., 2025), leading to the waste of biological resources and impairs the economic benefits of animal husbandry. Therefore, unveiling the regulatory mechanism of FA will contribute to the improvement in reproductive medicine and livestock production. Animal models and clinical data have highlighted that the apoptosis and non-programmed death of GCs are the main triggers of FA (Matsuda et al., 2012; Stringer et al., 2023), which are orchestrated by a multi-layered network that integrates various extracellular and intracellular factors. Of which, the TGF-β superfamily (a group of cytokines) and non-coding RNAs (a class of epigenetic factors) play critical roles in regulating follicular development (He et al., 2025; Liu & Fang, 2025).

TGF-β superfamily comprises 33 structurally related homo- and heterodimeric cytokines, which is the largest secreted signaling family in mammal and exerts versatile and fundamental functions in multiple cellular processes (Richardson et al., 2023). Despite its large membership, the signal transduction is quite simple. Upon extracellular ligand (e.g. TGF-β1) binding, the type II receptor (e.g. TGFBR2) phosphorylates the type I receptor (e.g. TGFBR1) via GS domain, which activates the receptor-regulated SMADs (R-SMAD, e.g. SMAD2/3). Activated R-SMADs form a complex with SMAD4 (the only co-SMAD), shuttle into the nucleus and bind to DNA, which finally regulates the transcription of target genes directly or along with co-factors (Hata & Chen, 2016; Hill, 2016). Solid evidence has revealed a close relationship between TGF-β superfamily and ovarian function, fertility, and reproductive diseases. In addition to GDF9 and BMP15, which are critical for GC proliferation, oocyte maturation and ovulation through autocrine and paracrine actions (Duan et al., 2024; Wang et al., 2025), TGF-β1 has been validated to induce folliculogenesis and oocyte meiosis, inhibit FA, and maintain angiogenesis during ovarian remodeling in humans and mice (Ingman et al., 2006; Patton et al., 2021; Trombly et al., 2009). However, it is still a challenge to unveil the full range of biological functions of TGF-β1 due to a lack of investigation in livestock

and insufficient identification of its functional targets.

Non-coding RNAs, a class of single-stranded RNAs widespread in organisms, mainly comprise five types of regulatory RNA: piRNA, miRNA, siRNA, lncRNA and circRNA. Among them, miRNAs are the most extensively studied, typically ranging from 19 to 25 nt in length and highly conserved among species (Shang et al., 2023), which are involved in all cellular processes by modulating the expression of target genes at multiple levels in a base-pairing manner (Kilikevicius et al., 2022). Nowadays, hundreds of miRNAs have been identified in mammalian ovaries with the advances in sequencing technology, exhibiting strong spatiotemporal specificity throughout follicular development, especially during FA (Zhang et al., 2024). Researches in recent decades with conditional knockout animal models, high-throughput sequencing, and gain- or loss-of-function highlight the pivotal role of miRNAs in female fertility by controlling estrous cycle, follicular development, oocyte maturation and ovulation (Hasuwa et al., 2013; Zhou et al., 2023). Mechanistic analyses have revealed that these functional miRNAs regulate crucial genes that play important roles in various stages of female reproductive axis (Tesfaye et al., 2018). Unfortunately, ~75% of the identified miRNAs are adverse as most studies have only focused on their classical regulatory mechanism (inhibit the expression of targets by binding to their 3'-UTR). Further investigation is needed to identify the generalized favourable miRNAs with anti-atretic effects, and clarify the universality of their targets and action modes.

Increasing evidence have shown that the interaction between TGF-β1 and miRNAs is involved in a variety of important biological processes, including follicular development (Butz et al., 2012; Du et al., 2018). However, the interaction mechanism and their biological effects remains largely unknown, particularly in domestic animals such as sows. Here, by integrating the transcriptome data from atretic follicles and TGF-β1-treated sow GCs, the candidate miRNAs mediating the anti-atretic function of TGF-β1 were systematically identified, including miR-184, a recently reported ovarian specifically highly-expressed miRNA which is down-regulated during FA and is critical for sow fertility (Shan et al., 2024). Additionally, RNA-seq revealed that *TGF-β1* was dramatically down-regulated after miR-184 knockdown, suggesting the existence of the positive feedback regulation between TGF-β1 and miR-184. This study aims to explore the formation mechanism of the novel feedback loop and elucidate its regulatory effects on FA in sows at the molecular, cellular and follicular levels. These findings will expand the regulatory mechanisms of FA mediated by the interaction between cytokines and non-coding RNAs, contributing to the inhibition of FA and the improvement of reproductive health in female mammals.

MATERIALS AND METHODS

Animals and ethics

The bilateral ovaries of healthy and sexually mature commercial sows at diestrus phase (*n*=180, 5.8 month and average mess 116.3 kg) were obtained from Zhushun (Nanjing, China) for the isolation and in vitro culture of follicles and GCs, as well as related experiments (Supplementary Table S1). Bilateral ovaries from diestrus Meishan (MS, *n*=5, 24.0 month and average mess 135.4 kg) and matched Large White (LW, *n*=10, 22.1 month and average mess 236.7 kg) sows with the records of three parities were provided by Muyuan (Zhenjiang, China) for comparative analysis. All animal-related experiments were reviewed, approved, and supervised by the Animal Ethics Committee of Nanjing Agricultural University, Jiangsu, China (NJAU No. 20223024059), and were conducted in accordance with the Regulation for the Administration for Affairs Concerning Experimental Animals.

Follicle classification and in vitro culture

Follicles were isolated and classified as healthy follicles (HF) and atretic follicles (AF) based on a comprehensive analysis including morphological observation, dissociative GCs counting and 17β-estradiol/progesterone (E2/P4) index detection as previously described (Guo et al., 2024). Only the follicles with their morphology in accordance with discrete GC density and steroid hormone levels were selected for experiments. In vitro culture and treatment of the isolated follicles were conducted according to the published study (Zhou et al., 2023).

Cell culture and treatment

Sow GCs isolated from healthy follicles with the diameter of 3-5 mm were seeded in culture plates filled with DMEM/F12 (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), and cultured at 37℃ with 5% CO_2. Once cells reached 80% confluence, transfection and treatment were conducted after detected mycoplasma-negative. For transfection, 50 nmol/L oligonucleotides and 3.2 μg plasmids were introduced into GCs using Lipofectamine 3000 reagent (ThermoFisher Scientific, USA). For treatment, GCs were cultured with FBS-free medium overnight and 10 ng/mL TGF-β1 (Novoprotein, China), 10 μmol/L SB431542 (Selleck, China), or 10 μmol/L LY2109761 (Selleck, China) were added for 24 h. The above specific concentrations were identified in previous studies (Du et al., 2016; Ma & Yi, 2022; Shan et al., 2024), and their effects on the activity of TGF-β signaling pathway and cytotoxicity in sow GCs were detected (Supplementary Figure 1). All the oligonucleotides involved in this study were synthesized by

GenePharma (China) and are listed in Supplementary Table S2.

Bioinformatics

High-throughput sequencing data involved in Supplementary Figure 2, tissue expression profile, and gene sequences were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov). Differentially expressed genes were identified with the criteria as $|\log_2(\text{fold change})|>1$ and FDR<0.05. The sequences of pri-, pre- and mature miR-184 were obtained from miRBase database (http://www.mirbase.org). Sequence alignment was conducted using DNAMAN software. The transcription factors (TF) and their motifs were jointly predicted by AnimalTFDB v.4.0 (http://bioinfo.life.hust.edu.cn), ALGGEN (http://alggen.lsi.upc.es), and JASPAR (https://jaspar.elixir.no). The miRNA responsive elements (MREs) in *TGF-β1* promoter were analyzed by miRDB (https://mirdb.org/), and the minimum free energy (MFE) was calculated by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Gene Set Enrichment Analysis (GSEA), and Kyoto Encyclopedia of Genes and Gnomes (KEGG) were conducted by RStudio v.4.3.2.

RNA extraction and RT-qPCR

Total RNA from GCs was extracted using TRIzol (Vazyme, China). After reverse transcribed into cDNA, the expression levels of interested genes (*TGF-β1*, *SMAD4* and *miR-184*) were quantified using a standard protocol with AceQ qPCR SYBR Green Master Mix (Vazyme, China), and normalized to the levels of *GAPDH* and *U6*, respectively. All qPCR reactions were repeated three times with three independent samples. The primers for qPCR were obtained from Tsingke (China) and are listed in Supplementary Table S3.

Western blotting and immunoprecipitation (IP)

Total protein from GCs was collected using cold RIPA lysis buffer and the concentration was detected with BCA method. Immunoblotting assays were performed as previously described (Guo et al., 2025). The original high-resolution images were obtained from a LAS-4000 ChemiDoc densitometer, quantified by ImageJ software (NIH, USA), and normalized to GAPDH protein level. Each group contained three independent biological replicates. For IP assay, total extracted protein was incubated with specific primary antibodies overnight in the IP binding buffer (#SB-IP011, Share-bio, China). Next day, protein A/G agarose beads (Bioworld, China) were added into the lysates and incubated at 4℃ for 6 h. After washed three times with IP buffer, the beads were eluted in protein loading buffer and boiled for 5 min. Finally, the interacted proteins were

resolved in SDS-PAGE gels and identified by immunoblotting. The involved antibodies are listed in Supplementary Table S4 and the original blot images are detailed in Supplementary Figure.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously (Du et al., 2020a). Briefly, after fixed with formaldehyde, the cross-linked protein-DNA complexes were then immunoprecipitated with the indicated antibodies and protein A/G agarose beads. After eluted and purified, the enrichment of protein-interacted DNA fragments were detected and quantified by PCR and qPCR. Anti-IgG was utilized as an internal control, and the genomic DNA was served as an input control.

In vitro biotinylated dsDNA pull-down

The dsDNA probes of *TGF-β1* promoter containing wild-type or mutant miR-184 responsive elements were obtained from Sangon (China), and biotinylated using the Biotin 3'-End DNA Labeling kit (Beyotime, China). 10 μg purified biotin-labeled dsRNA probes were incubated with 30 μg purified total RNA from sow GCs at room temperature for 4 h. Then, the biotinylated dsDNA/RNA complexes were pulled down with streptavidin magnetic beads (#LSKMAGT02, Merck Millipore, China). After isolation, enrichment of miR-184 was quantified by RT-qPCR. Unlabeled dsDNA probe was served as an internal control, and the total RNA was used as an input control. The sequence information of dsDNA probes are listed in Supplementary Table S3.

Chromatin isolation by biotinylated RNA pull-down (ChIbRP)

ChIbRP assay was performed according to the protocol (Portnoy et al., 2016) with modifications. In brief, biotinylated miR-184 was purchased from GenScript (China) and transfected into sow GCs cultured in vitro for 48 h. Cells were cross-linked and lysed in ChIbRP buffer on ice for 20 min, and nuclei were pelleted at 5 500 rpm for 5 min, which was then re-suspended in ChIbRP buffer containing 10 mM EDTA and 1% SDS. After sonicated, the lysates were pre-cleaned with protein G magnetic beads at 4℃ for 2 h, and were then incubated with streptavidin magnetic beads at 4℃ overnight. Next day, the beads were rotating washed sequentially with ChIbRP buffer twice, LiCl buffer once, and TE buffer twice. Each washing cycle last for 5 min. After removing buffer, the biotinylated miR-184-interacted DNA fragments and proteins were eluted from beads, which were further identified and quantified by qPCR and immunoblotting. Biotinylated scramble miRNA was served as an internal control, genomic DNA and total protein from sow GCs were served as input controls.

Apoptosis analysis

The apoptosis of GCs was detected by fluorescence-activated cell sorting (FACS) on a cell counter (Becton Dickinson, USA) with the double staining of Annexin V-FITC and propidium iodide according to the manufacturer's instruction (Vazyme, China). The apoptosis rate was analyzed using Flowjo v.10.0 software (TreeStar, USA).

Proliferation and viability detection

The proliferation and viability of GCs were analyzed using Cell Counting Kit-8 (CCK-8, Vazyme, China) following the manufacturer's instructions as previously described (Huo et al., 2023). The absorbance (OD value) was detected after adding 10 μL CCK-8 solution and incubating for 2 h at 450 nm wavelength on a microplate reader. Cell viability was calculated as $(OD_{trt}-OD_{ctrl})/OD_{ctrl}$. Each group had six independent replicates.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TGF-β1 in follicular fluid and cell culture medium, and the activities of Caspase3 in GCs were analyzed using ELISA kits (Beyotime, China). For TGF-β1 concentrations detection, 100 μL neutralized samples were added into the plate and incubated at room temperature for 2 h. Biotinylated antibody, horseradish peroxidase-labelled streptavidin, and TMB solution were sequentially added and incubated in dark room for 1 h. Then, 50 μL reaction termination solution was added and the OD values were measured immediately at 450 nm wavelength. For Caspase3 activity analysis, GCs were lysed on ice for 15 min and the supernatant was collected. Then, 10 μL Ac-DEVD-*p*NA was added and incubated at 37℃ for 2 h, followed by detection of the absorbance at 405 nm wavelength. Finally, TGF-β1 levels and Caspase3 activities were calculated according to the standard curve conversion.

H&E staining, IHC and RNA-FISH

Sow ovary samples were fixed in 4% paraformaldehyde and sent to Servicebio (China) for hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), and RNA fluorescence in situ hybridization (RNA-FISH) with the standard protocols previously described (Shan et al., 2024). Briefly, the embedded ovaries were cut into 4 μm-thick slices and analyzed by microscopic detection after H&E staining. For IHC, the slices were incubated with the anti-TGF-β1 antibody (#D121324, Sangon, China) at 4℃ overnight after dewaxing and hydration, merged into the

HRP-labeled secondary antibody and adding DAB solution to initiate reaction. Non-immune rabbit serum (#6117ES03, 1:100, Yeasen, China) was utilized as a negative control to evaluate the specificity of anti-TGF-β1 antibody. For RNA-FISH, the slices were hybridized at 65℃ with the following DIG-labeled antisense probe against miR-184: 5'-ACC CTT ATC AGT TCT CCG TCC A-3'. The specificity of RNA-FISH probe has been verified by both DIG-labeled scramble probe (5'-CAG UAC UUU UGU GUA GUA CAA-3') and inhibitor control (5'-CCU UAU CAG UUC UCC GUC-3'). Images were obtained from a Nikon Eclipse 80i fluorescence microscope with a DS-2 digital camera.

Plasmids and luciferase assay

The reporters containing *TGF-β1* and *miR-184* promoters were generated by Tsingke (China). Expression vectors pcDNA3.1-*SMAD4* was previously prepared in our lab. The luciferase activity assays were performed as previously described (Sheng et al., 2025). In short, the activities (*Firefly* and *Renilla*) in GCs after treatment for 24 h were measured using a Dual-Luciferase Reporter kit (Vazyme, China) on a GLOMAX detection system. The relative luciferase activity of each sample was calculated as the ratio of *Firefly*/*Renilla*.

Statistical analysis

Data are shown as mean ± SEM with at least three independent biological samples. Statistical analyses were conducted and visualized using GraphPad Prism v.9.0 and IBM SPSS Statistics v.26.0. Significance between two or multiple groups was analyzed by a two-tailed independent Student's *t*-test or one-way analysis of variance (ANOVA). **P*<0.05, ***P*<0.01.

RESULTS

miR-184 is transcriptionally activated by TGF-β1 in sow GCs

To identify the miRNAs that potentially mediate the anti-atretic effect of TGF-β1, a joint analysis with our previous reported RNA-seq data (Zhang et al., 2024; Li et al., 2021) revealed that 32 TGF-β1-regulated miRNAs were differentially expressed during FA (Figure 1A; Supplementary Table S5). Of these, six miRNAs (miR-136, miR-144, miR-184, miR-321, miR-623 and miR-626) were notably up-regulated in TGF-β1-treated GCs and markedly down-regulated in AFs (Figure 1B), which were validated by RT-qPCR (Figure 1C). In view of the fact that miR-184 exhibited the most dramatic alteration and has been demonstrated to exert anti-atretic function in our recent study (Shan et al., 2024), it was therefore selected for the following research. Correlation analysis unveiled a robust positive correlation between the expression of TGF-β1 and miR-184 in follicles

(Figure 1D). Consistent with miR-184, TGF-β1 was dramatically inhibited during FA (Figure 1E). Subsequent quantitative analysis confirmed that knockdown of endogenous TGF-β1 significantly suppressed miR-184 expression in GCs (Figure 1F; Supplementary Figure S1A, B), indicating that miR-184 is positively regulated by TGF-β1. To investigate whether the regulation is occurred at the transcriptional level, RT-qPCR and luciferase activity assays were conducted and revealed a significant up-regulation of pri- and pre-miR-184 levels, as well as its promoter activity upon TGF-β1 stimulation, while the opposite effects were yielded after TGF-β1 knockdown (Figure 1G, H), validating that *miR-184* is transcriptionally activated by TGF-β1. Notably, analysis of the publicly accessible high-throughput sequencing data revealed that TGF-β1 induces the expression of *miR-184* across species, tissues and cell types (Supplementary Figure S2), suggesting that the transcriptional activation of *miR-184* by TGF-β1 is conserved.

TGF-β1 induces the transcription of *miR-184* in a SMAD4-dependent manner

To ascertain the mechanism by which TGF-β1 regulates the transcription of *miR-184*, SB431542 and LY2109761, two well-known and widely-applied TGF-β signaling pathway inhibitors, were employed and showed that the levels of pri-, pre- and mature miR-184 were significantly reduced, and the activating effect of TGF-β1 on *miR-184* transcription was considerably disrupted (Figure 2A), highlighting that the activation of the TGF-β signaling pathway by TGF-β1 is imperative for *miR-184* transcription. In combination with three algorithms, 12 TFs potentially targeting the core promoter of *miR-184* were predicted (Figure 2B; Supplementary Table S6). Among which, POU5F1, SMAD4, and SREBF2 stand out due to their high expression levels in sow ovaries (Figure 2C). Subsequently, a multi-dimensional joint analysis showed that SMAD4, rather than POU5F1 and SREBF2, is notably inhibited in sow GCs during FA (consistent with the alteration pattern of miR-184), presents a significantly positive expression correlation with miR-184 in follicles, and its expression and enrichment on *miR-184* promoter are significantly up-regulated in response to TGF-β1 (Figure 2D, E; Supplementary Figure S3), implying that SMAD4, the only downstream co-SMAD in the classical TGF-β signaling pathway, potentially mediates the regulation of TGF-β1 to *miR-184* transcription. To address this, RT-qPCR was conducted and revealed that knockdown of SMAD4 significantly reduced miR-184 level, and effectively countered the activating impact of TGF-β1 on miR-184 expression, however, solely augmenting SMAD4 without TGF-β1 stimulation had no substantial effect (Figure 2F; Supplementary Figure S1C, D), indicating that SMAD4 activates the transcription of *miR-184* under the stimulation of TGF-β1. Analysis of the sequence feature revealed the presence of two candidate SMAD4 binding elements (SBE) in the promoter

of *miR-184*, termed as SBE1 (−713/−705 nt) and SBE2 (−361/−353 nt) (Figure 2G). Luciferase activity detection revealed that knockdown of SMAD4 or treatment of TGF-β1 could influence the activities of reporter vectors containing two wild-type SBEs, which was attenuated by either SBE mutation, and abolished after both SBEs mutation (Figure 2H). Furthermore, ChIP was performed and validated that SMAD4 directly binds to both two SBEs, and its enrichment was increased after TGF-β1 treatment, which was reduced by the addition of SB431542 or LY2109761 (Figure 2I). Collectively, these findings demonstrate that SMAD4 mediates the regulation of TGF-β1 to the transcription of *miR-184* in sow GCs by acting as a transcriptional activator.

miR-184 is a positive functional target of TGF-β1

The aforementioned findings prompted us to further investigate whether miR-184 mediates the biological functions of TGF-β1 in sow ovaries. To verify this, a series of in vitro experiments were designed and showed that knockdown of the endogenous miR-184 disrupted the anti-apoptotic, proliferative, and viability-maintaining functions of TGF-β1 in GCs (Figure 3A–C). Concurrently, it also markedly abrogated the inhibitory effect of TGF-β1 on Caspase3 activity (Figure 3D) and compromised its capacity to regulate the expression of apoptosis- and proliferation-related genes (Figure 3E), validating that miR-184 mediates the proliferative and anti-apoptotic functions of TGF-β1 in sow GCs. It is evident that the state of GCs directly determines the ultimate outcome of follicular development. To this end, an in vitro sow follicle model was applied and showed that addition of TGF-β1 could effectively maintain the morphology, clarity and vascular distribution of follicles, which was impeded after knockdown of miR-184, leading to pale and turbid follicles with severely deteriorated blood vessels (Figure 3F), revealing that the suppression of miR-184 impairs the capacity of TGF-β1 to maintain normal follicular development. Moreover, it also resulted in a substantial up-regulation of the internal GC apoptosis rate (Figure 3G) and a notable elevation of the Caspase3 activity (Figure 3H). Taken together, these findings demonstrate that miR-184 is a positive functional target of TGF-β1 and mediates its anti-atretic function.

miR-184 feedback activates the transcription of *TGF-β1* by acting as a saRNA

To identify the functional targets of miR-184, our previous RNA-seq data for the knockdown of endogenous miR-184 in GCs were systematically investigated, and the TGF-β signaling pathway was significantly enriched by KEGG analysis and GSEA (Figure 4A, B). Interestingly, detection of the expression pattern of the TGF-β pathway members revealed that in addition to *SMAD3*, a validated positive target of miR-184, the transcript level of *TGF-β1* was notably decreased after

miR-184 knockdown (Figure 4C). Subsequent quantitative analysis showed that inhibition of miR-184 significantly suppressed the expression and secretion of TGF-β1, while the opposite outcomes were observed in miR-184 over-expressed GCs (Figure 4D–G; Supplementary Figure S1E), confirming the feedback induction of *TGF-β1* expression by miR-184. Recent study has revealed that miR-184 functions as a saRNA in the nucleus, which made us investigate whether it regulates the transcription of *TGF-β1* through the same mechanism. To address this, the promoter of *TGF-β1* was characterized and 39 MREs were predicted (Supplementary Table S7), including two MREs for miR-184, designated as MRE1 (−951/−923) and MRE2 (−450/−426) (Figure 4H). Results from luciferase activity detection revealed that over-expression of miR-184 significantly up-regulated the promoter activity of *TGF-β1* with wild-type MRE2, while having no effect on the vectors containing wild-type MRE1, mutant MRE1 or mutant MRE2 (Figure 4I), suggesting that miR-184 recognizes and directly binds to the MRE2. To address this, in vitro biotinylated dsDNA pull-down was performed and showed that miR-184 was only pulled down by the biotinylated dsDNA probe with wild-type MRE2, rather than that with mutant MRE2 or unlabeled probe (Figure 4J). Besides, ChIbRP was conducted and confirmed in vivo that miR-184 directly binds to the MRE2 in *TGF-β1* promoter (Figure 4K, L). To further identify the activation mechanism of *TGF-β1* transcription by miR-184, the protein fraction of ChIbRP was analyzed and found that in addition to AGO2, three transcriptional regulators including CTR9, DHX9, and RNA polymerase II (Pol II) with DNA/RNA-unwinding/binding and transcription activity were also detected in the pull-down product of the biotinylated miR-184, rather than biotinylated scramble miRNA (Figure 4M). Interestingly, IP assays showed that AGO2 directly binds to CTR9, DHX9 and Pol II, while the binding capacity was strengthened by miR-184 mimics, but was weakened by miR-184 inhibitor (Figure 4N). Finally, ChIP assays revealed that the enrichment of AGO2, CTR9, DHX9, Pol II, H3K4me2, and H3K9ac on the promoter of *TGF-β1* was notably increased after over-expression of miR-184 (Figure 4O). These findings demonstrate that miR-184, as a saRNA, directly binds to the MRE2 in *TGF-β1* promoter, and forms a RNA-induced transcriptional activation complex with AGO2, CTR9, DHX9 and Pol II to feedback induces *TGF-β1* transcription. Moreover, a novel positive feedback regulatory loop was identified in sow GCs, that is TGF-β1/SMAD4/miR-184.

TGF-β1 is essential for the anti-atretic function of miR-184

In light of the established feedback regulation of *TGF-β1* by miR-184, it was hypothesized that TGF-β1 mediates the biological functions of miR-184. To make it clear, FACS and CCK-8 assays were performed and showed that knockdown of TGF-β1 dramatically inhibited the anti-apoptotic,

proliferative, and viability-maintaining functions of miR-184 in sow GCs (Figure 5A–C). Besides, according to the quantitative analysis, we noticed that inhibition of TGF-β1 resulted in miR-184 losing its capacity to suppress the activity of Caspase3 and to regulate the expression of apoptosis and proliferation-associated genes (Figure 5D, E), confirming that TGF-β1 mediates the biological functions of miR-184 in GCs. Further analyses with in vitro cultured sow follicles revealed that over-expression of miR-184 effectively maintained the normal morphology and clarity of follicles, as well as the blood vessels on their surface, which was disrupted after TGF-β1 inhibition, resulting in pale and turbid follicles without vascular distribution (Figure 5F), indicating that knockdown of TGF-β1 disrupts the maintenance effect of miR-184 on sow follicular development. Meanwhile, the apoptosis of internal GCs and the activity of Caspase3 were both significantly increased after treatment with TGF-β1 siRNA (Figure 5G, H). Taken together, the findings outlined above demonstrate that TGF-β1 is essential for the functional integrity of miR-184, particularly with regard to the anti-apoptotic/atretic effects.

TGF-β1 and miR-184 are highly expressed in the ovarian follicles of high-fertility sows

Considering the functions of TGF-β1 and miR-184 in the ovaries, we speculated that the feedback regulatory loop exerts a positive effect on sow fertility. To verify this assumption, Meishan (MS, a famous Chinese high-fertility pig breed) and Large White (LW, a Western lean pig breed) sows with the records of three parities were selected for following research (Figure 6A). Histochemical detection revealed that the proportion of antral follicles in the ovaries of MS and LW sows with high fertility (LW-H) was significantly higher than that in the ovaries of LW sows with low fertility (LW-L), while the percentage of atretic follicles was found to be lower in the MS sows (Figure 6B). IHC and RNA-FISH were conducted and revealed that the expression of TGF-β1 and miR-184 were elevated in pre-antral and antral follicles from MS and LW-H sows in comparison to those from LW-L sows (Figure 6C). To next verify the veracity of the above findings, follicles at different developmental stages (pre-antral, antral and atretic) were isolated, and the GCs with FF were collected. Quantitative comparison analysis revealed that the expression and secretion levels of TGF-β1 and miR-184 in pre-antral and antral follicles of MS and LW-H sows were significantly higher than those in follicles at the same stage of LW-L sows, however, no significant difference was observed in atretic follicles (Figure 6D–G). These findings demonstrate that TGF-β1 and miR-184 are highly expressed in the ovarian follicles of sows with high fertility, indicating that the positive feedback regulatory loop composed of TGF-β1 and miR-184 is essential for maintaining sow fertility.

DISCUSSION

Almost all biological processes, including follicular development, are regulated by complicated networks composed of multiple factors or genes across spatial and temporal scales (Dietlein et al., 2022), making it difficult to fully elucidate the formation mechanism of complex traits only by relying on a single omics. With the development of the high-throughput sequencing technology, multi-omics joint analysis has rapidly become the mainstream for identifying key functional regulators in physiological and pathological processes (Vankereyken et al., 2023). For example, Wang et al. (2024) unveiled hub genes and core networks contributing to egg-laying performance in hens via a multi-omics joint study. Here, six miRNAs that potentially mediate the anti-atretic effect of TGF-β1 were identified by integrating the comparative transcriptome of healthy and atretic follicles and the TGF-β1-involved GC transcriptome. In addition to miR-184, miR-136 and miR-144 have been reported to regulate ovulation, follicular development, steroid synthesis, and reproductive diseases in female mammals (Kitahara et al., 2013; Zhou et al., 2017). Although their functions in the ovaries remain unclear, miR-623 and miR-626 are involved in the regulation of cell apoptosis and injury (Xu et al., 2019; Zhong et al., 2021). Unlike miR-184, no potential SBE was predicted in the promoter of the other five miRNAs, and knockdown of SMAD4 did not inhibit their expression, nor did it impair the promotion effect of TGF-β1 (Supplementary Figure 4A, B), indicating that TGF-β1 induces their expression in a SMAD4-independent manner. Interestingly, we also noticed that TGF-β1 stimulation increased the expression of four miRNAs (except miR-321) to a great extent after miR-184 inhibition (Supplementary Figure 4C), highlighting their potential cooperative and compensatory effects with miR-184, which needs further validation. Moreover, 12 TGF-β1-inhibited miRNAs were also significantly up-regulated during FA, including miR-126, miR-130a, and miR-26b etc. which have been reported to induce GC apoptosis (Du et al., 2020b, 2020c), demonstrating that TGF-β1-induced miRNA network plays a significant role in the regulation of follicular development and female fertility.

The cross-talk between TGF-β1 and non-coding RNAs is deeply involved in multiple physiological and pathological processes (Cordero et al., 2024). Current studies have shown that their interactions depend on mutual regulation at different levels. Specifically, TGF-β1 regulates the transcription and post-transcriptional processing of non-coding RNAs by activating the TGF-β signaling pathway. For instance, transcriptome analyses have revealed that TGF-β1 induces 19 miRNAs and 72 lncRNAs in sow GCs (Li et al., 2021, 2023), as well as 36 circRNAs in PMCs (Lin et al., 2022). Besides, TGF-β1 also regulates miRNA maturation, lncRNA processing, and circRNA

circularization (Niu et al., 2021; Sun et al., 2021). In turn, non-coding RNAs influence *TGF-β1* expression at different levels through direct and indirect mechanisms. For example, miR-2337 induces *TGF-β1* transcription by directly binding to its promoter (Wang et al., 2021). Conversely, circPKM post-transcriptionally modulates the stability of TGF-β1 mRNA indirectly via STMN1 in ICC cells (Chen et al., 2023). In this study, we have unveiled the interaction mechanism between TGF-β1 and miR-184 in sow GCs, confirming that TGF-β1 activates the transcription of *miR-184* in a SMAD4-dependent manner, and miR-184 acts as a saRNA to directly target and activate *TGF-β1* transcription by forming a regulatory complex with AGO2, CRT9, DHX9, and RNA PII, which is consistent with the findings in previous saRNA studies (Meng et al., 2016; Portnoy et al., 2016). Although the miRNAs mediating the functions of TGF-β1 in female mammalian reproduction have been preliminary identified, most of which are unfavorable. Here, we have validated that miR-184 carries and amplifies the anti-atretic effect of TGF-β1. Unfortunately, it remains unclear whether this novel feedback loop is regulated by hormones which are essential for estrous cycle, ovarian development, and reproduction in female mammals. Further investigations should focus on the feasibility of other types of non-coding RNA mediating the favorable functions of TGF-β1, which will contribute to elucidating the TGF-β1-induced non-coding RNA regulatory network to inhibit FA and improve sow fertility.

As a general regulatory type, feedback regulation refers to the regulation of downstream factors to the expression or activity of upstream factors and is commonly observed in signaling pathways and the endocrine system, which is essential for intracellular environmental homeostasis and functional sustainability (Guo et al., 2025). It can be categorized as positive or negative based on the alteration pattern of upstream factors, or as direct, indirect, or auto feedback according to the action mode (El-Samad, 2021). At present, three feedback types were identified existed in the TGF-β signaling pathway (Yan et al., 2018). (i) Receptor-ligand feedback. For example, Enomoto et al. reported that TGFBR2 feedback induces the transcription of *TGF-β1* in AT-lineage cells (Enomoto et al., 2023). (ii) SMAD-receptor feedback. SMAD4 directly feedback activates the transcription of *ACVR1B* and *BMPR2* (Liu et al., 2021), and indirectly feedback induces *TGFBR2* expression by inhibiting miR-425 in GCs (Du et al., 2018). Meanwhile, SMAD2/3/4 complex positively feedback regulates *TGFBR1* expression in CSCs via TGFBRAP1 (Liu et al., 2024). (iii) Ligand auto-feedback. For example, TGF-β1 auto-feedback promotes its own expression via TET3 in HSCs (Xu et al., 2020). Recent studies have also shown that miRNAs mediate the negative auto-feedback of TGF-β1, such as miR-145/TGF-β1 loop in myofibroblast (Melling et al., 2018). In contrast, our findings identified the miR-184-mediated indirect and positive auto-feedback of

TGF-β1, highlighting the significance of feedback mode in regulating the activity and function of TGF-β signaling pathway. Another issue is that the potential negative regulators or checkpoints for this positive feedback loop remain unclear, and whether the recently-annotated regulators for miR-184 (SREBF2 etc.) or the classical negative regulators for TGF-β1 (SMAD7, c-Ski/SnoN, and TMEPAI etc.) are involved still needs further investigation. Of particular significance is the observation that SBEs in *miR-184* promoter and MRE in *TGF-β1* promoter are highly conserved among mammals (Supplementary Figure S5). Interestingly, the positive mutual-regulation between TGF-β1 and miR-184, and their anti-apoptotic functions have also been observed in KGN and mouse GCs in our previous and ongoing studies, implying the universality of the formation mechanism and functions of TGF-β1/miR-184 feedback loop in ovarian follicles across species.

It is well known that the TGF-β signaling pathway and miRNAs are both essential for female fertility. Firstly, dysfunction of TGF-β1 secretion or abnormal TGF-β pathway activity leads to blocked development of the reproductive system, defects in ovarian function, abnormal follicular development and ovulation, disorders of hormone secretion, reduced implantation rate, and fetal dysplasia (Ingman & Robertson, 2009; Wang et al., 2014). Similar phenomena have been observed after knocking out the genes related to miRNA processing, such as *DICER* and *DGCR8* (Luense et al., 2009; Kim et al., 2016). Secondly, both of them contain multiple validated mutations significantly associated with female mammalian fertility (Wu et al., 2010; Pang et al., 2019), which are also essential for the breeding of high-fertility livestock. Despite several preliminary studies, their effects on female fertility were mainly investigated in model animals (mice and rats) or at cellular level (GCs and oocyte), and the direct investigation on the regulation and mechanism at follicular level are rare. Here, in vitro follicle culture system was applied and indicated that the anti-atretic feedback loop formed by TGF-β1 and miR-184 is crucial for sow follicular development.In combination with our recent study, we demonstrate that the interaction between TGF-β signaling pathway and non-coding RNAs is pivotal for the early formation of sow fertility. Although systematic exploration was conducted at the levels of sow GC and follicle, a limitation is that the impact of TGF-β1/miR-184 loop on the follicular development and fertility output in live sows was not identified due to the lack of gene editing and precision targeted delivery techniques. Further studies should continue to excavate the positive effects of TGF-β1 analogues, activators, and endogenous miRNAs on follicular development and female fertility in non-model animals, including sows, at individual level or directly at the follicular level in vivo.

In summary, this study systematically investigated the crucial miRNAs mediating the anti-atretic function of TGF-β1 in sow GCs, identified a novel anti-atretic positive feedback regulatory loop,

that is TGF-β1/SMAD4/miR-184, and revealed its formation mechanism by using in vitro GC and follicle culture systems (Figure 7). These findings emphasize the essential role of the interaction between cytokines and non-coding RNAs in the regulation of GC state and follicular development, and also provide valuable insights and promising targets for inhibiting FA, maintaining ovarian healthy and improving sow fertility.

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DATA AVAILABILITY

All data generated during this study are included in this published article and its Supplementary Information files.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

Q.Q.L. and X.D. completed the experimental design and manuscript writing. Q.Q.L. and H.Y.L. conducted experiments and collected data. Q.Q.L., Z,N,G., L,C., and S.B.S. analyzed the data. X.L.S., S.F.W., and Q.F.L. assisted. All authors read and approved the final version of the manuscript.

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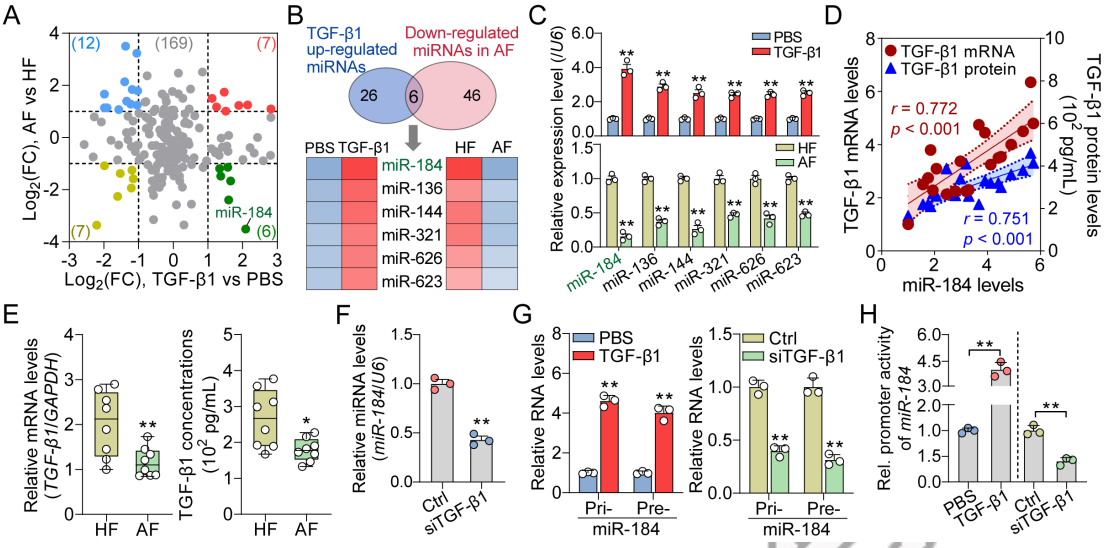
672 **FIGURES AND FIGURE LEGENDS**



673

674 **Figure 1 TGF-β1 induces the transcription of *miR-184* in sow GCs**

675 **A**: Dot plot showing the miRNAs that are regulated by TGF-β1 in sow CCs and are differentially
676 expressed during FA. **B**: The expression patterns of six miRNAs potentially mediating the
677 anti-atretic function of TGF-β1 under different conditions. Red and blue refer to high and low
678 levels, respectively. **C**: The expression of six candidate miRNAs in sow GCs treated with 10 ng/mL
679 TGF-β1 (upper) or from follicles in different states (lower) were detected by RT-qPCR (n=3). **D**:
680 Correlation analysis of miR-184 levels with TGF-β1 mRNA levels in follicles and TGF-β1 protein
681 levels in FF (n=20). **E**: The mRNA (left) and protein (right) levels of TGF-β1 in healthy follicles (HF,
682 n=8) and atretic follicles (AF, n=8) were measured by RT-qPCR and ELISA. **F**: The effect of TGF-β1
683 knockdown on the expression level of mature miR-184 was analyzed by RT-qPCR (n=3). **G**: The
684 expression levels of pri- and pre-miR-184 in sow GCs treated with TGF-β1 (left) or TGF-β1 siRNA
685 (right) were detected by RT-qPCR (n=3). **H**: The effects of TGF-β1 or TGF-β1 siRNA on the
686 promoter activity of *miR-184* were analyzed by luciferase activity assay (n=3). Data are shown as
687 mean ± SEM with at least three independent replicates. *: P<0.05; **: P<0.01.

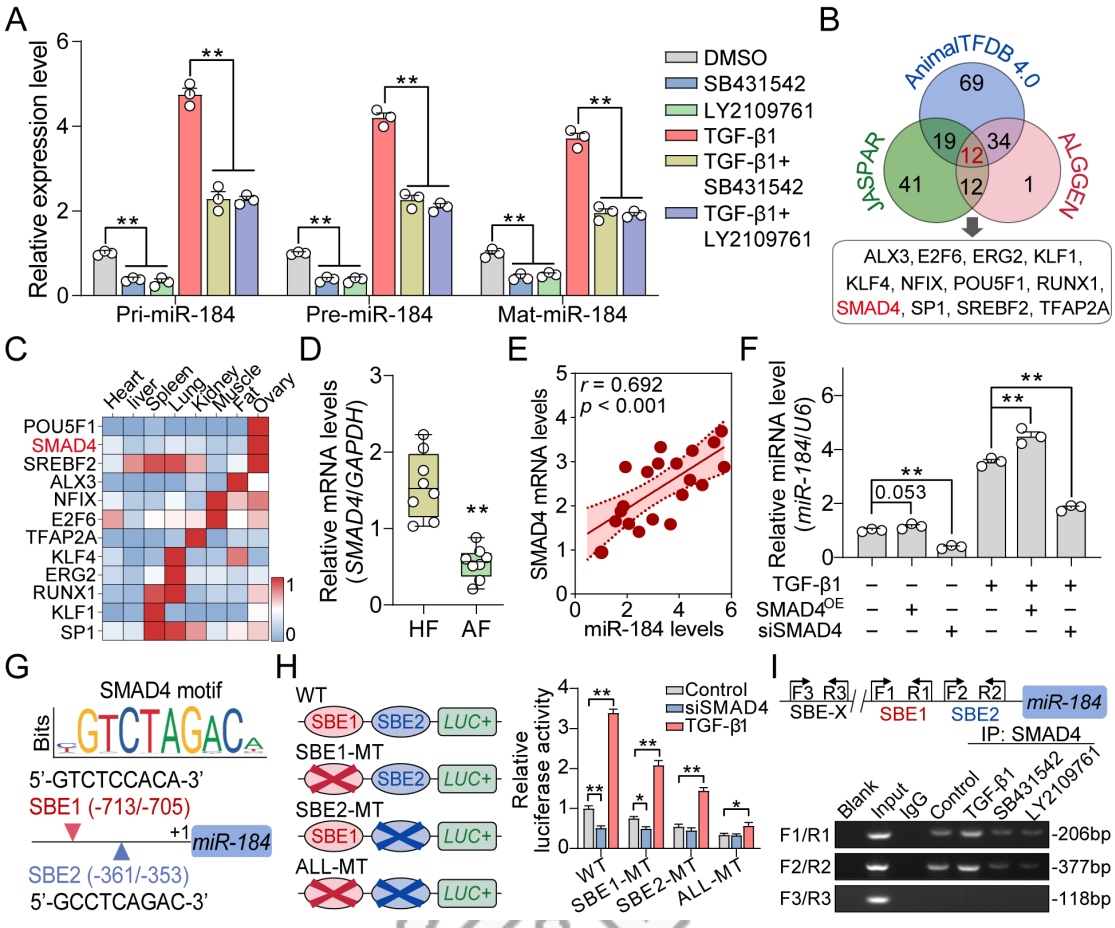


Figure 2 TGF-β1 induces *miR-184* transcription in a SMAD4-dependent manner

A: The effects of SB431542 (10 μmol/L) and LY2109761 (10 μmol/L) on the expression levels of miR-184 in sow GCs with or without TGF-β1 addition were detected by RT-qPCR (n=3). **B**: TFs potentially targeting *miR-184* promoter were predicted by ALGGEN, AnimalTFDB 4.0 and JASPAR database. **C**: Tissue expression profile of 12 candidate TFs in sows. **D**: The mRNA levels of SMAD4 in GCs from HFs (n=8) and AFs (n=8) were analyzed by RT-qPCR. **E**: Expression correlation analysis of SMAD4 and miR-184 in follicles (n=20). **F**: The expression levels of miR-184 in sow GCs under the indicated conditions were measured by RT-qPCR (n=3). **G**: Diagram depicting the sequence and location of two SBEs (SBE1 and SBE2) in *miR-184* promoter. **H**: Left panel: diagram showing the reporter vectors containing the *miR-184* promoter with the wild-type (WT) or mutant (MT) SBEs. Right panel: the activities of the reporter vectors in GCs under the indicated conditions were detected by luciferase assay (n=3). **I**: ChIP assay. Data are shown as mean ± SEM with at least three independent replicates. *: P<0.05; **: P<0.01.

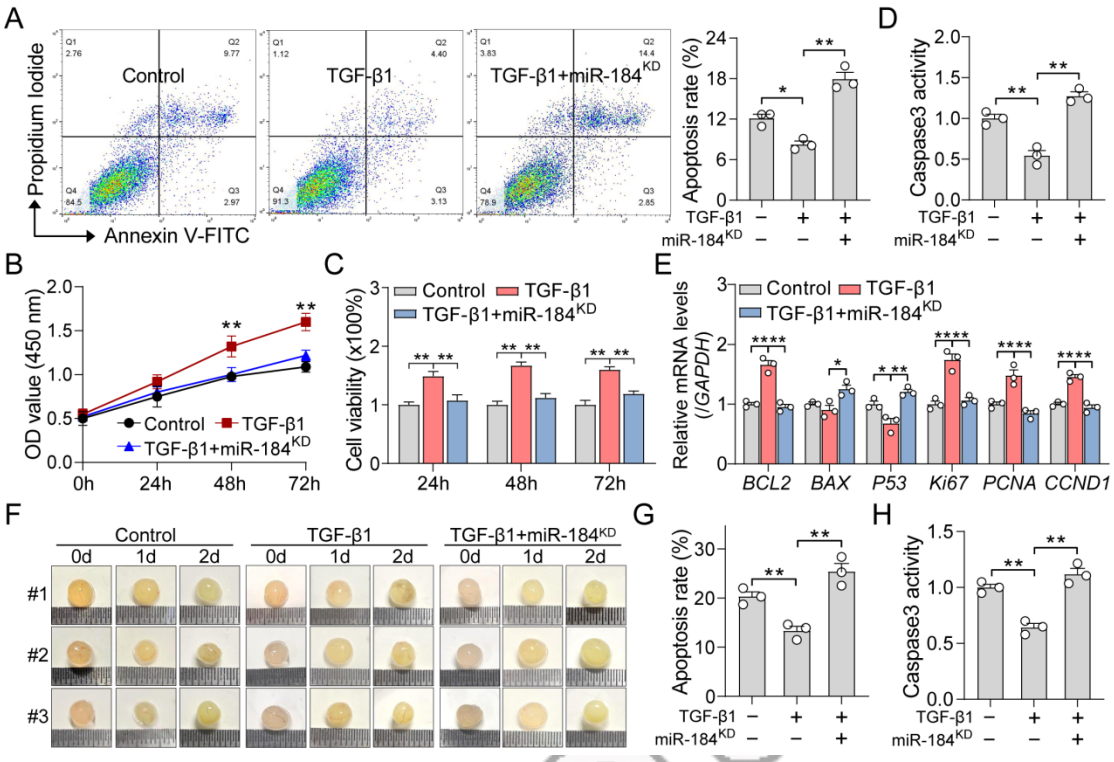


Figure 3 miR-184 is a positive functional target of TGF-β1

A–**E**: miR-184 was knocked down (KD) in TGF-β1-treated sow GCs. Cell apoptosis was detected by FACS (**A**, *n*=3), cell proliferation and viability were analyzed by CCK-8 (**B**–**C**, *n*=6), Caspase3 activity was measured by ELISA (**D**, *n*=3), and the levels of apoptosis- and proliferation-related genes were quantified by RT-qPCR (**E**, *n*=3). **F**: After the indicated treatment, the states of in vitro cultured sow follicles were detected by morphological analysis including morphological changes, clarity, and vascular distribution (*n*=6), **G**–**H**: Internal GCs were collected from follicles in **F**, the apoptosis rate was detected by FACS (**G**, *n*=3), and the Caspase3 activity was measured by ELISA (**H**, *n*=3). Data are shown as mean ± SEM with at least three independent replicates. *: $P<0.05$; **: $P<0.01$.

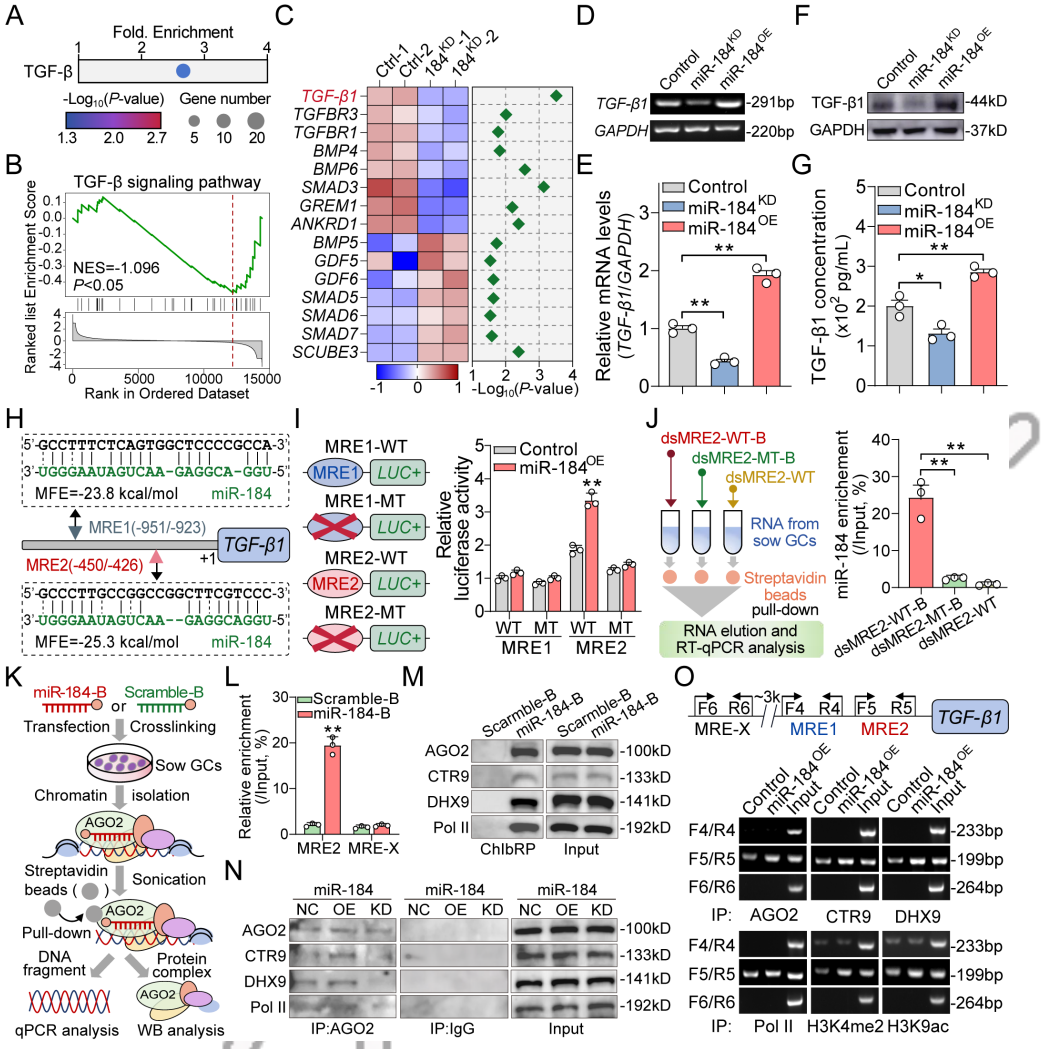


Figure 4 miR-184 feedback activates the transcription of *TGF-β1* by acting as a saRNA

A–B: KEGG analysis (**A**) and GSEA (**B**) showed that the targets of miR-184 were significantly enriched in the TGF-β signaling pathway. **C**: Heatmap showing the expression pattern of 15 TGF-β signaling pathway members in GCs after treatment with miR-184 inhibitor. **D–G**: The effects of over-expression or knockdown of miR-184 on the expression and secretion of TGF-β1 in GCs were analyzed by semi-qPCR (**D**, *n*=3), RT-qPCR (**E**, *n*=3), western blotting (**F**, *n*=3) and ELISA (**G**, *n*=3). **H**: Diagram showing the sequence and location of two potential miR-184 responsive elements (MRE1 and MRE2) in the promoter of *TGF-β1*. MFE indicates minimum free energy. **I**: Left panel: construction of the reporter vectors containing *TGF-β1* promoter with the wild-type (WT) or mutant (MT) MREs. Right panel: the effects of miR-184 over-expression (OE) on the activities of the reporter vectors were detected (*n*=3). **J**: In vitro biotinylated dsDNA pull-down assay. Enrichment of miR-184 in the pull-down products of different biotinylated dsDNA probes was quantified by RT-qPCR (*n*=3). **K**: Flowchart of ChIbRP assay. **L**: Enrichment of the *TGF-β1* promoter fragment containing MRE2 in the DNA fraction of ChIbRP product was detected by qPCR. **M**: Enrichment of AGO2, CTR9, DHX9 and Pol II in the protein fraction of ChIbRP product was analyzed by western blotting. **N**: Effects of miR-184 on the association of AGO2 with CTR9, DHX9 and Pol II in sow GCs were detected by IP. GAPDH protein level was detected as a loading control. **O**: ChIP assays. MRE-X served as a negative control. Data are shown as mean ± SEM with

732 at least three independent replicates. *: $P<0.05$; **: $P<0.01$.

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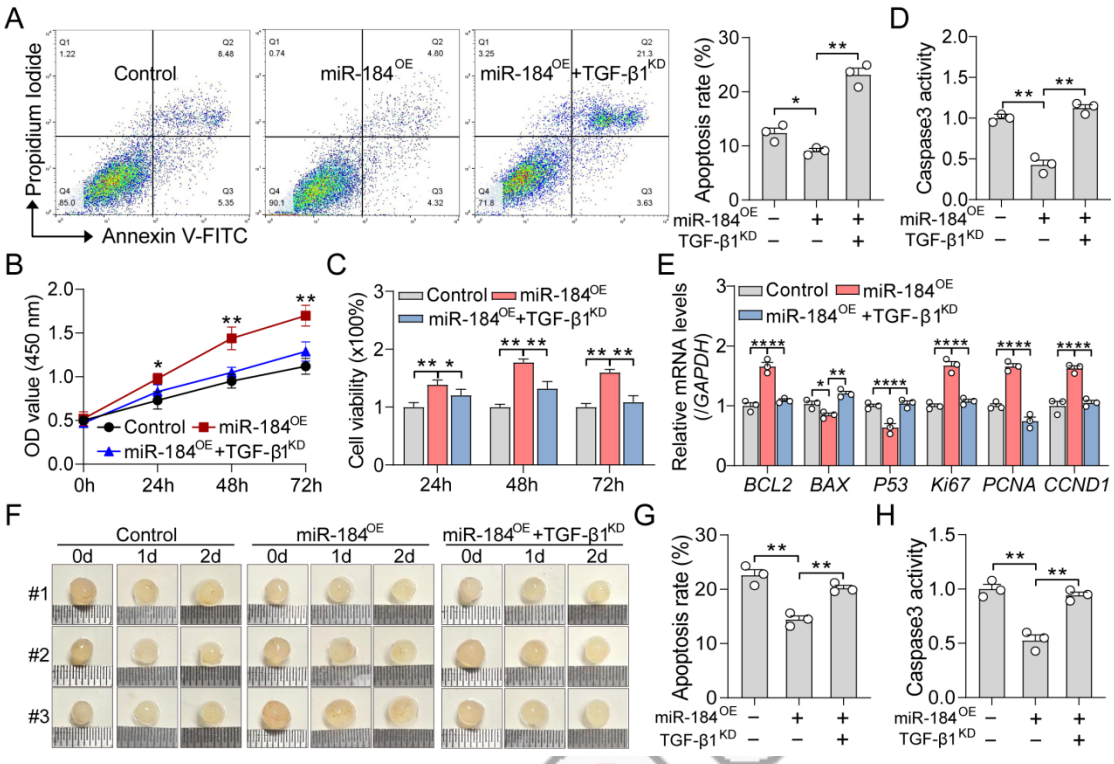


733

734 **Figure 5 TGF-β1 mediates the anti-atretic function of miR-184**

735 **A**–**E**: TGF-β1 was knocked down (KD) in miR-184-overexpressed sow GCs. Cell apoptosis was
736 detected by FACS (**A**, *n*=3), cell proliferation and viability were analyzed by CCK-8 (**B**–**C**, *n*=6),
737 Caspase3 activity was measured by ELISA (**D**, *n*=3), and the expression levels of apoptosis- and
738 proliferation-related genes were quantified by RT-qPCR (**E**, *n*=3). **F**: The states of in vitro cultured
739 sow follicles after treated as indicated were analyzed by morphological analysis including
740 morphological changes, clarity, and vascular distribution (*n*=6), **G**-**H**: The apoptosis rate of the
741 internal GCs from follicles in **F** was measured by FACS (**G**, *n*=3), and the Caspase3 activity was
742 detected by ELISA (**H**, *n*=3). Data are shown as mean ± SEM with at least three independent
743 replicates. *: $P<0.05$; **: $P<0.01$.

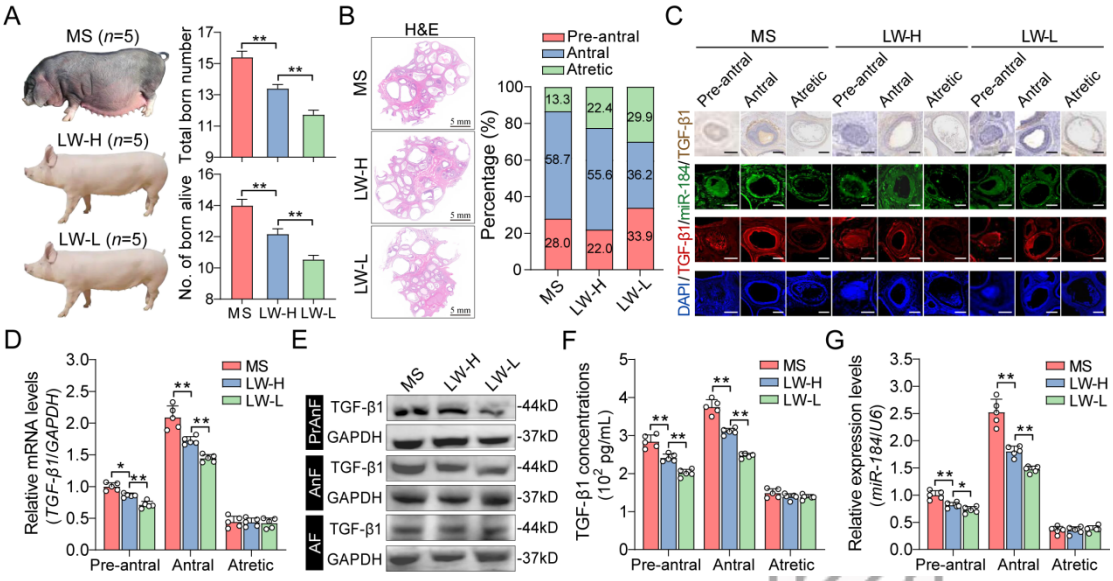


Figure 6 TGF-β1 and miR-184 are highly expressed in follicles of the high-fertility sows

A: The reproductive traits (total born number and number of born alive) of the studied MS and LW sows with different fertility (*n*=5). **B**: H&E staining and the statistical analysis of follicles at different developmental stages. **C**: The expression levels of TGF-β1 protein (brown), TGF-β1 mRNA (red) and miR-184 (green) of in follicles of the studied sows were assessed using IHC and RNA-FISH. Nuclei were stained with DAPI (blue). Scale bar: 1 mm. **D**–**E**: The expression levels of TGF-β1 in GCs from follicles at different developmental stages of the studied sows were analyzed by RT-qPCR (**D**) and western blotting (**E**). PrAnF, AnF, and AF in **E** indicate pre-antral, antral, and atretic follicles, respectively. **F**: The secretion levels of TGF-β1 in the fluids from follicles at different stages were quantified by ELISA (*n*=5). **G**: The expression levels of miR-184 in GCs from follicles at different developmental stages were detected by RT-qPCR (*n*=5). Data are shown as mean ± SEM with at least three independent samples. *: *P*<0.05; **: *P*<0.01.

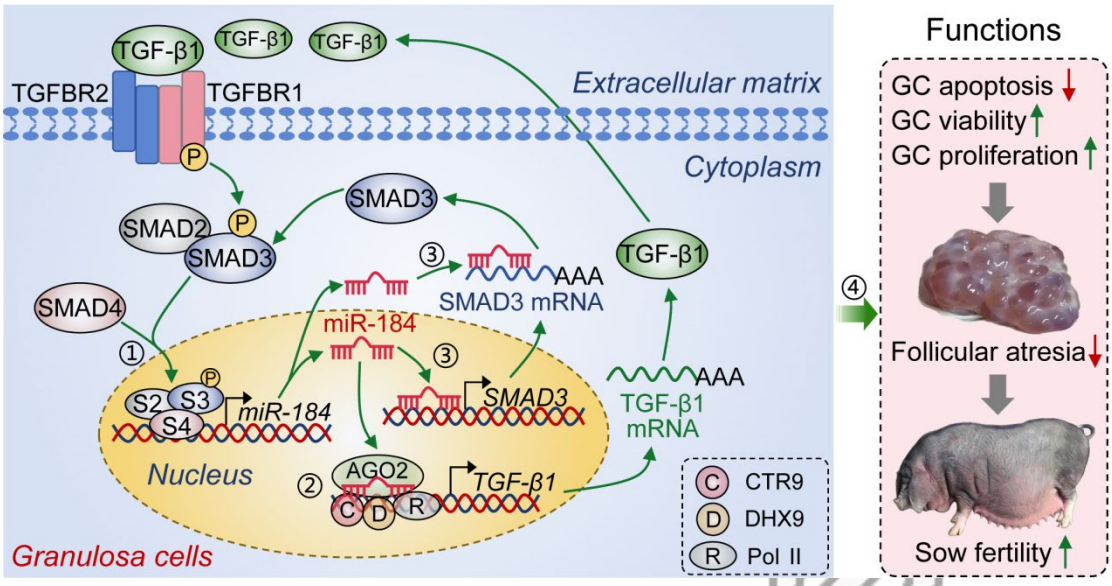


Figure 7 A working model of the TGF-β1/SMAD4/miR-184 feedback regulatory loop

① TGF-β1 induces the transcription of *miR-184* in a SMAD4-dependent manner, and SMAD4 is a direct transcription activator for *miR-184*. ② miR-184, as an saRNA, forms a regulatory complex with AGO2, CTR9, DHX9, and Pol II to activate the transcription of *TGF-β1* by directly binding to its promoter, which establishes a novel positive feedback regulatory loop. ③ Our recent study has demonstrated that miR-184 induces *SMAD3* expression in sow GCs at both transcription (as an saRNA in nuclei) and post-transcription (as an mRNA stabilizer in cytoplasm) levels (Shan et al., 2024). ④ The novel feedback regulatory network formed by TGF-β signaling pathway and miR-184 suppresses GC apoptosis, inhibits FA, and maintains sow fertility.

SUPPLEMENTARY FIGURES

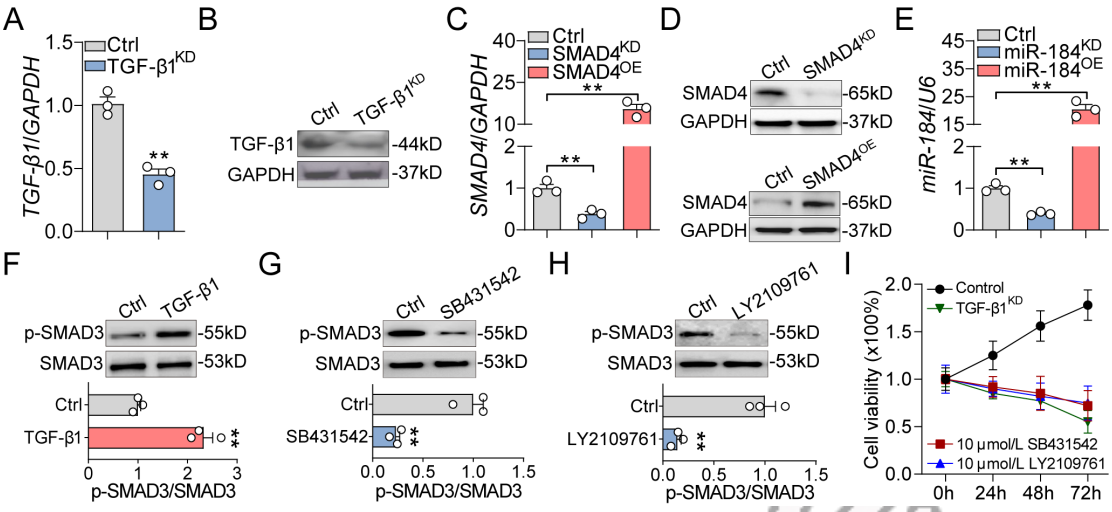


Figure S1 Detection of the inhibitory and over-expression efficiency and cytotoxicity

A–**B**: The inhibitory efficiency of TGF-β1-siRNA was detected by RT-qPCR (**A**, *n*=3) and western blotting (**B**, *n*=3). **C**-**D**: The inhibitory effect of SMAD4-siRNA and the expression efficiency of pcDNA3.1-SMAD4 were analyzed by RT-qPCR (**C**, *n*=3) and western blotting (**D**, *n*=3). **E**: The inhibitory and expression efficiency of miR-184 in sow GCs were measured by RT-qPCR (*n*=3). **F**–**H**: The effects of 10 ng/mL TGF-β1 (**F**), 10 μmol/L SB431542 (**G**), and 10 μmol/L LY2109761 (**H**) on the protein levels of p-SMAD3 (the activation marker of the TGF-β signaling pathway) in sow GCs were analyzed by western blot (*n*=3). **I**: The effects of 10 μmol/L SB431542, 10 μmol/L LY2109761, and TGF-β1-siRNA on GC viability were detected by CCK-8 assay (*n*=6). Data are shown as mean ± SEM and the significance were calculated by a two-tailed Student's *t*-test and ANOVA. **: $P<0.01$.

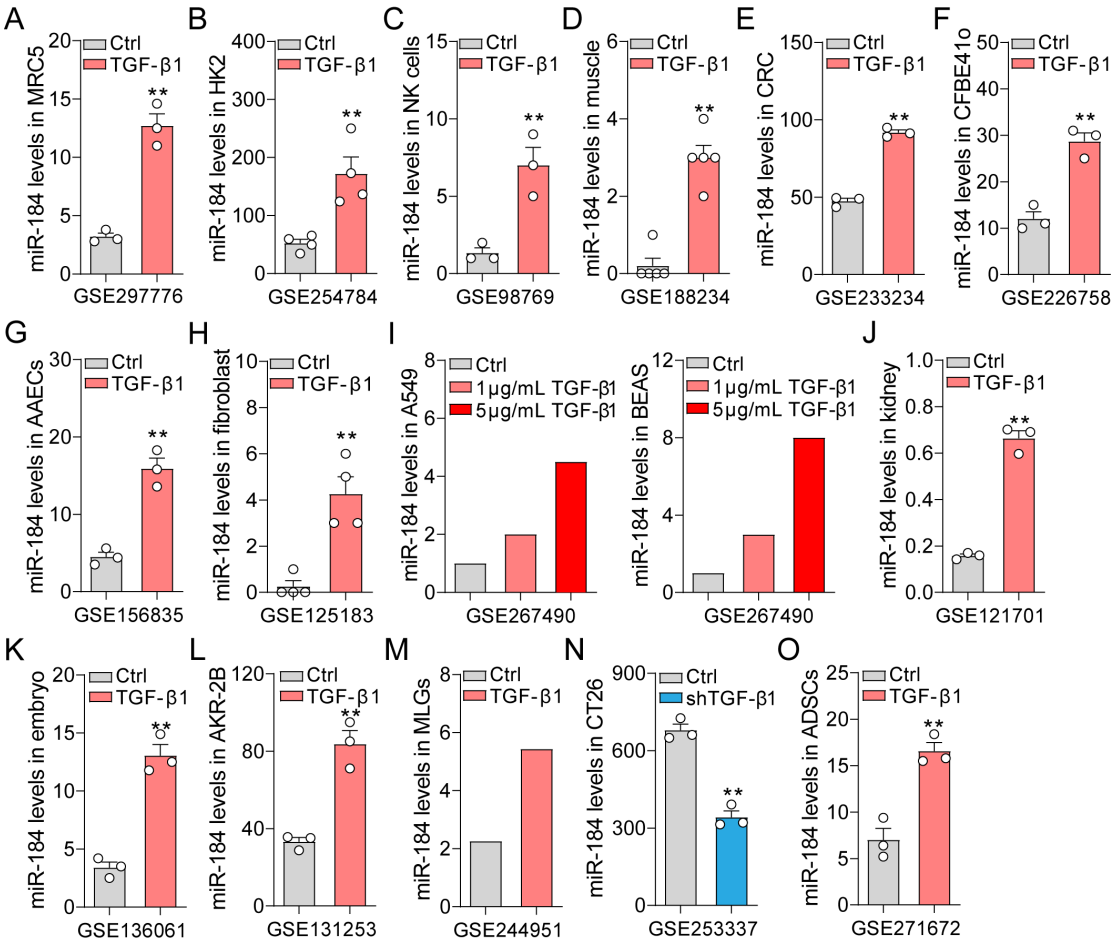


Figure S2 The induction of *miR-184* expression by TGF-β1 is highly conserved

A–**I**: The effects of TGF-β1 on the expression of *miR-184* in human tissues and cell types were obtained from NCBI GEO datasets. **A**: 10 ng/mL TGF-β1, *n*=3. **B**: 10 ng/mL TGF-β1, *n*=4. **C**: 5 ng/mL TGF-β1, *n*=3. **D**: 1 ng/mL TGF-β1, *n*=5. **E**: 10 ng/mL TGF-β1, *n*=3. **F**: 15 ng/mL TGF-β1, *n*=3. **G**: 5 ng/mL TGF-β1, *n*=3. **H**: 10 ng/mL TGF-β1, *n*=4. **J**–**N**: The effects of TGF-β1 or shTGF-β1 on *miR-184* expression in mouse tissues and cell types were obtained from NCBI GEO datasets. **J**: 10 ng/mL TGF-β1, *n*=3. **K**: 1 ng/mL TGF-β1, *n*=3. **L**: 10 ng/mL TGF-β1, *n*=3. **M**: 5 ng/mL TGF-β1. **N**: 5 μg shTGF-β1, *n*=3. **O**: The effect of 10 ng/mL TGF-β1 on *miR-184* expression in rabbit ADSCs was obtained from GSE271672 datasets, *n*=3. Data are shown as mean ± SEM. Significance were calculated by a two-tailed Student's *t*-test. **: $P<0.01$.

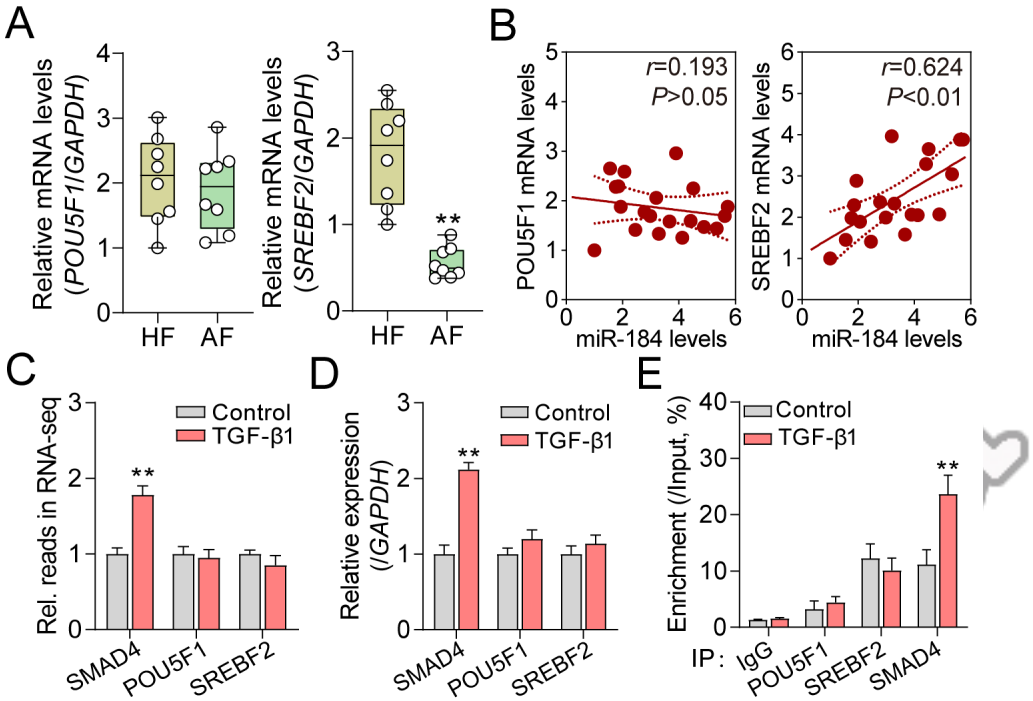
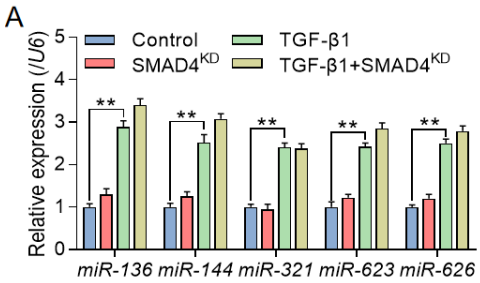


Figure S3 SMAD4, rather than POU5F1 or SREBF2, potentially mediates the regulation of TGF-β1 to the transcription of *miR-184*

A: The expression of POU5F1 and SREBF2 in HFs (n=8) and AFs (n=8) was detected by RT-qPCR. **B**: Expression correlation of POU5F1 or SREBF2 and miR-184 in follicles (n=20) was conducted by Pearson correlation analysis. **C**–**D**: The effects of TGF-β1 on the expression of SMAD4, POU5F1, and SREBF2 in sow GCs were analyzed by RNA-seq (**C**) and RT-qPCR (**D**, n=3). **E**: The enrichment of POU5F1, SREBF2, and SMAD4 on the promoter of *miR-184* was detected by ChIP (n=3). IgG was utilized as a negative control. Data are shown as mean ± SEM and the significance were calculated by a two-tailed Student's t-test. **: P<0.01.



B

Multi-algorithm prediction of SBE in the promoter

	ALGGEN	AnimalTFDB 4.0	JASPAR
miR-136	X	X	X
miR-144	X	X	X
miR-321	X	X	X
miR-623	X	X	X
miR-626	X	X	X

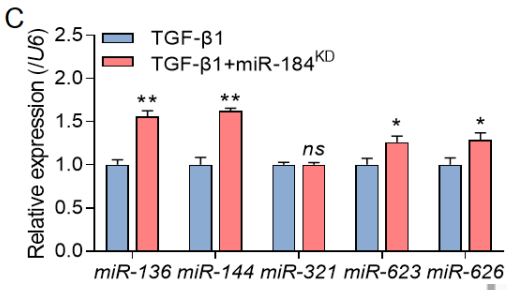


Figure S4 Four miRNAs have potential cooperative and compensatory effects on miR-184

A: The expression levels of five miRNAs (miR-136, miR-144, miR-321, miR-623, and miR-626) in sow GCs after the indicated treatment were detected by RT-qPCR (*n*=3). **B**: The potential SBEs in the promoter of *miR-136*, *miR-144*, *miR-321*, *miR-623*, and *miR-626* were analyzed by ALGGEN, AnimalTFDB 4.0, and JASPAR prediction tools. **C**: The effects of TGF-β1 on the expression of miR-136, miR-144, miR-321, miR-623, and miR-626 in miR-184-silenced sow GCs were analyzed by RT-qPCR (*n*=3). Data are shown as mean ± SEM and the significance were calculated by a two-tailed Student's *t*-test. *: $P<0.05$; **: $P<0.01$; *ns*: Not significant.

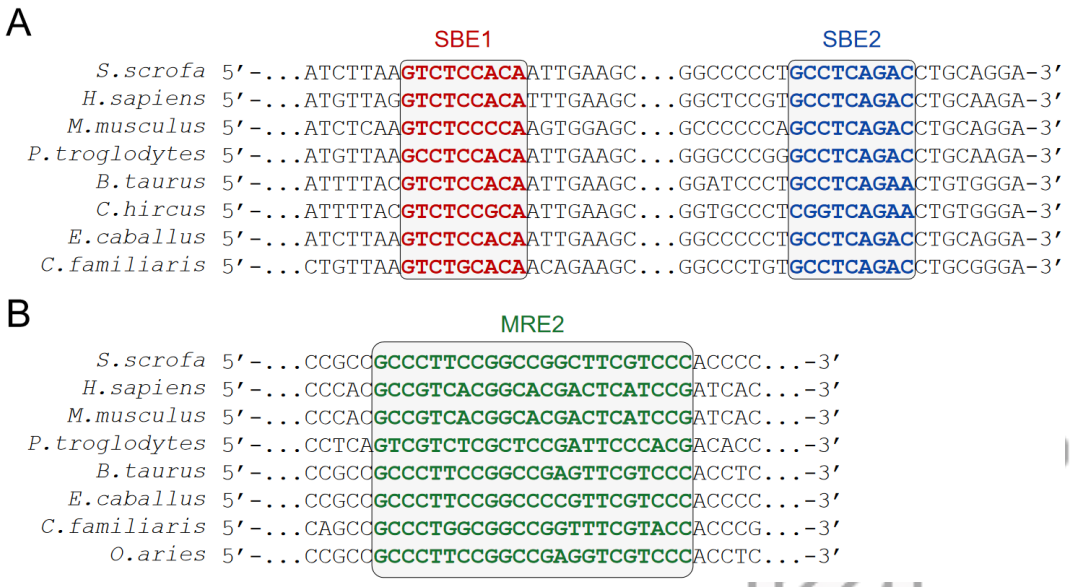


Figure S5 Interspecies conservation analysis of the SMAD4 binding elements (SBE) and the miR-184 responsive element (MRE).

A: The homology of SBE1 (red) and SBE2 (blue) in the promoter of *miR-184* from eight vertebrates (pig, human, mouse, chimpanzee, cattle, goat, horse and dog) was identified by sequence alignment. **B**: The homology of MRE2 (green) in the promoter of *TGF-β1* from eight vertebrates (pig, human, mouse, chimpanzee, cattle, sheep, horse and dog) was identified by sequence alignment.

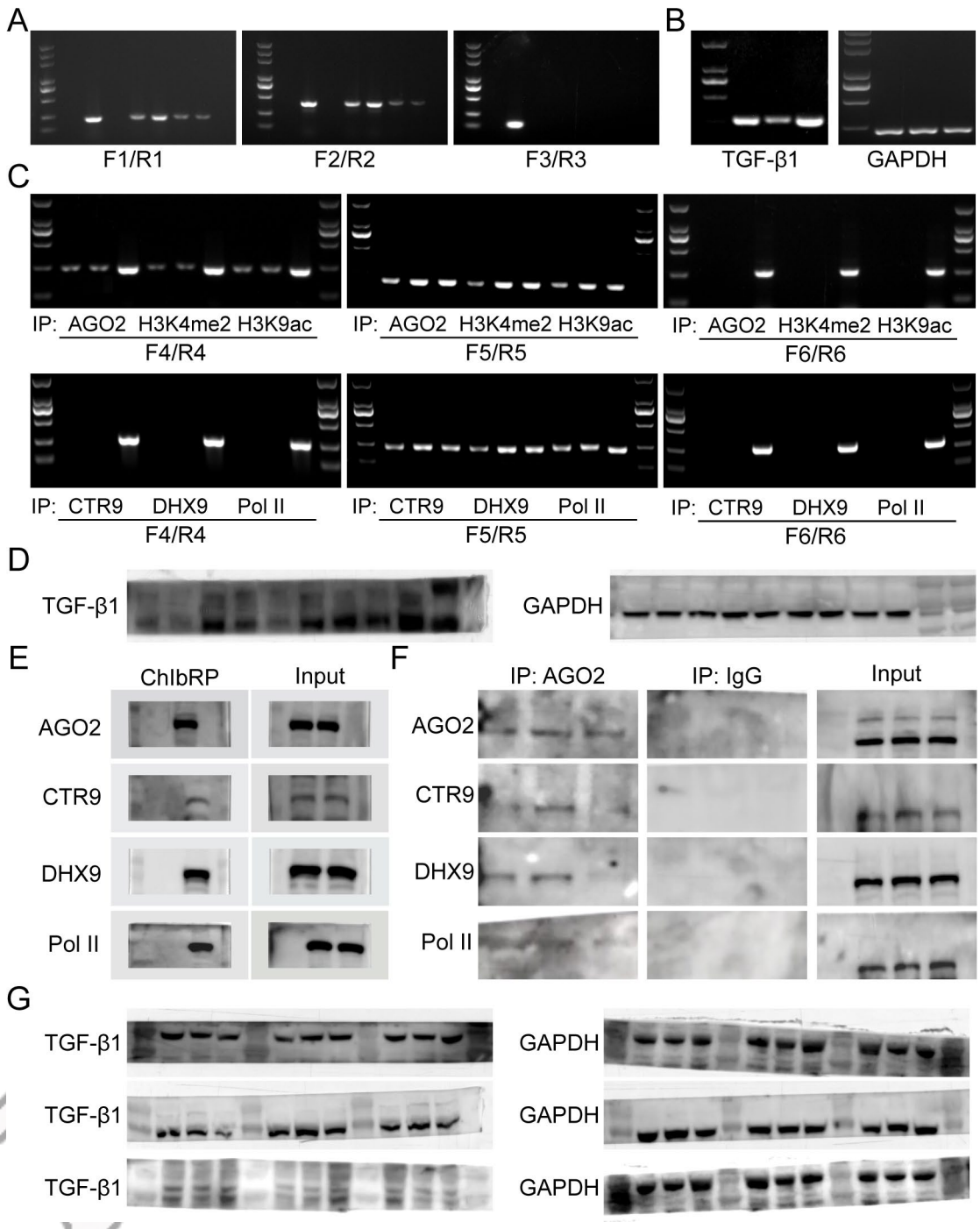


Figure S6 The original blot and gel images in this study

A: The original images of the gels in Figure 2I. **B**: The original images of the gels in Figure 4D. **C**: The original images of the gels in Figure 4O. **D**: The original images of western blotting in Figure 4F. **E**: The original images of western blotting in Figure 4M. **F**: The original images of western blotting in Figure 4N. **G**: The original images of western blotting in Figure 6E.

SUPPLEMENTARY TABLES

Supplementary Table 1 The sows involved in this study and related experiments.

Supplementary Table 2 The oligonucleotides used in this study.

Supplementary Table 3 The primers and probes involved in this study.

Supplementary Table 4 The antibodies utilized in this study.

Supplementary Table 5 The miRNAs that are regulated by TGF-β1 and differentially expressed during FA.

Supplementary Table 6 The TFs potentially targeting the core promoter of *miR-184*.

Supplementary Table 7 miRNAs that potentially target the promoter of *TGF-β1*.

中文摘要

TGF-β1/SMAD4/miR-184，一个控制母猪卵泡发育的新正反馈调控环路

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关键字：TGF-β1; SMAD4; miR-184；反馈调控；猪卵泡颗粒细胞凋亡；卵泡闭锁