SMAD4 activates Wnt signaling pathway to inhibit granulosa cell apoptosis

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Abstract
The TGF-β and Wnt signaling pathways are interrelated in many cell types and tissues, and control cell functions in coordination. Here, we report that SMAD4, a downstream effector of the TGF-β signaling pathway, induces FZD4, a receptor of the Wnt signaling pathway, establishing a novel route of communication between these two pathways in granulosa cells (GCs). We found that SMAD4 is a strong inducer of FZD4, not only initiating FZD4 transcription but also activating FZD4-dependent Wnt signaling and GC apoptosis. Furthermore, we identified the direct and indirect mechanisms by which SMAD4 promotes expression of FZD4 in GCs. First, SMAD4 functions as a transcription factor to directly bind to the FZD4 promoter region to increase its transcriptional activity. Second, SMAD4 promotes production of SDNOR, a novel IncRNA that acts as a sponge for miR-29c, providing another mean to block miR-29c from degrading FZD4 mRNA. Overall, our findings not only reveal a new channel of crosstalk between the TGF-β and Wnt signaling pathways, SMAD4–FZD4 axis, but also provide new insights into the regulatory network of GC apoptosis and follicular atresia. These RNA molecules, such as miR-29c and Inc-SDNOR, represent potential targets for treatment of reproductive diseases and improvement of female fertility.

Introduction
The canonical transforming growth factor-β (TGF-β) signaling pathway is of great physiological importance. The process of TGF-β signal transduction is quite simple: an extracellular TGF-β signal (ligands, mainly TGF-β1) first interacts with specific membrane receptors (TGFBR1/2) and activates cytoplasmic effector SMAD proteins (SMAD2/3), which further forms a regulation complex with SMAD4 and ultimately translocated into the nucleus, where they drive transcription. Among the SMAD proteins, SMAD4 is the only co-mediator. By acting as a central intracellular effector and final downstream component, SMAD4 plays an essential role in TGF-β signal transduction. Loss or deficiency of SMAD4 not only inactivates TGF-β signaling, but also impairs TGF-β-mediated transcriptional regulation and biological functions. Studies using conditional Smad4 knockout (Smad4-KO) mouse models have revealed that loss of SMAD4 can decrease the sensitivity of tissues or cells to TGF-β1.8

SMAD4 is not only a mediator and effector of the TGF-β signaling pathway in the nucleus, but is also a unique multifunctional modulator that regulates both transient and persistent cellular processes including cell proliferation, differentiation, autoimmunity, pluripotency and plasticity, and cell growth, apoptosis, autophagy, invasion, and metastasis. SMAD4 dysregulation is associated with embryonic developmental disorders, deficiencies in skeletal muscle differentiation and regeneration, loss of stem cell pluripotency, disorder and delay of nervous system development, female infertility, and other pathologies. Furthermore, deficiency or inactivation of SMAD4 diminishes innate immune responses to viral infection, and can even drive cancers. Accordingly, SMAD4 has been identified in quantitative studies as a biomarker of multiple cancers and probed extensively as a potential pharmaceutical target. In ovary, cell death and cell differentiation.
SMAD4 and SMAD4-dependent TGF-β signaling pathway have shown to be associated with follicular development, especially follicular atresia, the ultimate fate of most follicular development\textsuperscript{27,28}. Indeed, recent reports demonstrated that SMAD4 contributes to follicular atresia through inhibiting granulosa cell (GC) apoptosis, which is the main inducement of follicular atresia\textsuperscript{29}. However, the mechanism of SMAD4 regulating GC apoptosis is not completely understood.

As a multifunctional intracellular signal transducer, SMAD4 has multiple functions, some but not all of which are dependent on its DNA-binding capacity, in various contexts it can behave as a transcriptional activator, co-regulator, or nuclear localization factor\textsuperscript{10,23}. SMAD4 is perhaps best known as a transcription factor, and in regulation of some of its target genes, it interacts with other cooperative factors such as co-activators and corepressors\textsuperscript{30,31}. SMAD4-binding elements (SBEs) in the promoter region are necessary for SMAD4 to recognize target genes and regulate transcription initiation\textsuperscript{32}. The best-characterized SBE motifs are GTCTG, CAGAC, and (CG)\textsuperscript{33,34}. In recent years, many direct targets of SMAD4, including both coding and noncoding genes (i.e., miRNAs and long noncoding RNAs), have been identified using chromatin immunoprecipitation (ChIP), ChIP-seq, and genome-wide mapping technology\textsuperscript{35,36}. In our previous study, we characterized the transcriptome of porcine GCs in which SMAD4 was silenced, and identified FZD4, a receptor of the Wnt signaling pathway and regulator of GC functions\textsuperscript{37}, as a new candidate target of SMAD4\textsuperscript{38}. In this study, we sought to confirm the regulatory effect of SMAD4 on FZD4 in GCs and to elucidate the molecular mechanism underlying this regulation, and their roles in regulating GC apoptosis.

**Materials and methods**

**Cell culture and treatment**

Porcine GCs were isolated and cultured as previously described\textsuperscript{39}. Briefly, GCs were collected from 3-5 mm of healthy follicles with 22-gauge needles and seeded into T25 flasks with Dulbecco’s Minimum Essential medium/nutrient F-12 (DMEM/F-12, Gibco) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin, and 100 mg/mL streptomycin (Gibco). HEK 293T cells were cultured in DMEM with 10% FBS at 37 °C in a 5% CO\textsubscript{2} incubator. Lipofectamine \textsuperscript{*} 3000 transfection reagent (#L3000015, Life Technologies, Carlsbad, CA 92008 USA) was used to transfect oligonucleotides or plasmids at a final concentration of 20 μM. The oligonucleotides used in this study are listed in Supplementary Table S1. Animal experiments were approved by the Animal Ethics Committee at Nanjing Agricultural University, Nanjing, P. R. China (SYXK 2017-0027) and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (No. 2 of the State Science and Technology Commission, 11/14/1988).

**Bioinformatic analysis**

The candidate SMAD4-binding sites in the promoter of FZD4 and SMAD4-dependent noncoding RNA (SDNOR) were predicted by JASPAR (jaspar.genereg.net/), a software for prediction the binding motifs of transcription factors. Potential miRNAs that target FZD4 were predicted by four different algorithms, miRWalk 3.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk3/), miRDB (http://www.mirdb.org/miRDB/), TargetScan (http://www.targetscan.org/), and miRTarBase (http://amp.pharm.mssm.edu/Harmonizome/resource/MiRTarBase). RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) was performed to predict miR-29c binding sites in pig FZD4 3′-UTR and SDNOR, miRBase (http://www.mirbase.org/) was used to obtain pre- and mature miRNAs sequences. The coding potential of SDNOR was predicted by two software, Coding Potential Assessment Tool and Coding Potential Calculator.

**Plasmids construction and dual-luciferase reporter assays**

To generate luciferase reporters, the fragments of FZD4 and SDNOR promoters were amplified from porcine genomic DNA and cloned into pGL3-basic vector. The fragments of SDNOR and the 3′-UTR of FZD4 that contained putative miR-29c binding sites were cloned into pmirGLO vector. Mutant vectors were generated using the TaKaRa MutanBEST Kit (#R401, TaKaRa, Beijing, China). All the recombinant plasmids were verified by Sanger sequencing. Primers used for plasmids construction are listed in Supplementary Table S2.

After transfection for 24–48 h, porcine GCs were harvested and the lysates were collected for dual-luciferase analysis by using the Dual-Luciferase Reporter Assay System (#E1910, Promega, Madison, USA) following the kit’s manual. The GLOMAX detection system (Promega) was conducted to measure the firefly and renilla luciferase activities in cell lysates.

**Rapid amplification of cDNA end (RACE)**

The full-length sequence of the SDNOR transcript and the 5′-flanking sequence of FZD4 were obtained by using the SMARTer	extsuperscript{*} RACE 5′/3′ Kit (#634858, Clontech Laboratories, Inc, CA94043, USA). Briefly, total RNA from porcine GCs was reverse-transcribed into first-strand cDNA using SMARTer reverse transcriptase. cDNAs were then amplified, ligated to adapters, and cloned into pUC19 vector. The full-length sequence of SDNOR and the 5′-flanking sequence of FZD4 were confirmed by Sanger sequencing. The primers used in this process are listed in Supplementary Table S3.
Quantitative real-time PCR assay

In brief, total RNA was isolated from cells using the High-Purity RNasy Mini Kit (#74104, Qiagen, Beijing, China) and reverse-transcribed into cDNA by using HiScript II Q RT SuperMix for qPCR (#R223-01, Vazyme Biotech Co., Ltd, Nanjing, China) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) analysis was performed using the StepOnePlus System (Applied Biosystems) with AceQ qPCR SYBR Green Master Mix (#Q111-02, Vazyme Biotech Co., Ltd, Nanjing, China). Fold changes of interested genes were computed using the 2−ΔΔCt method. qRT-PCR was conducted in triplicate, and the results are presented as mean ± S.E.M. after normalization to GAPDH and U6 for coding and noncoding genes, respectively. Primers used for real-time PCR are listed in Supplementary Table S4.

Subcellular localization

Nuclear and cytoplasmic were extracted from porcine GCs using the method as previously described40. Briefly, porcine GCs were lysed in cold lysis buffer and placed on ice for 10 min. Then, cells were centrifuged at 12,000 × g for 3 min at 4°C. The supernatant (cytoplasmic extract) was immediately frozen (−80°C) for subsequent analysis. The nuclear pellet was resuspended with cold DEPC water containing 1 mM RNase inhibitor and placed on ice for 5 min, and then centrifuged at 10,000 × g for 5 min. The supernatant (nuclear extract) was removed and the remainder was frozen (−80°C) for subsequent analysis.

Western blotting

For western blotting analysis, protein lysates from whole cells were prepared using RIPA buffer with protease inhibitors and phosphatase inhibitors. After incubation on ice for 30 min, the supernatant was collected and purified and placed on ice for 15 min at 4°C. The BCA Protein Assay Kit (#P0012, Beyotime, Jiangsu, China) was used to detect the concentration of total protein. Western blotting was conducted as described previously41. Primary antibodies were anti-FZD4 (Sangon Biotech, #D121422, 1:1000), anti-β-catenin (Sangon Biotech, #D260137, 1:1000), anti-caspase-3/cleaved caspase-3 (Proteintech, #19677-1-AP, 1:1000), and anti-GAPDH (ORIGENE, #TA802519, 1:5000). HRP-conjugated secondary antibodies were diluted in 0.25% BSA/TBST. The original high-resolution western blotting images were obtained by a high-sensitivity chemiluminescence imaging system (Bio-rad, #Chemiluminescence touch) and the densitometry of each blotting image was analyzed by Quantity One software with Gauss Model Trace method. House-keeping protein GAPDH was used as an internal control.

Flow cytometry

Porcine GC apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection Kit (#A211-01, Vazyme Biotech Co., Ltd, Nanjing, China), and flow cytometry was performed as previously described40. Cells were counted by flow cytometry (Becton Dickinson), and the rate of apoptosis was analyzed using the FlowJo software (TreeStar). Specifically, the rate of apoptosis was calculated based on the percentage of cells in the Q2 and Q3 quadrants, representing early- and late-stage apoptotic cells, respectively.

Chromatin immunoprecipitation

ChIP assays were performed as previously described39. The SMAD4–DNA complex was pulled down with rabbit anti-SMAD4 (Santa Cruz Biotechnology, #sc-1909-R) antibody. After decrosslinking, enrichment of interested DNA fragments was analyzed by semiquantitative PCR and qPCR. Antibody against IgG (Santa Cruz, #sc-2358) was used as the internal control, and unprocessed chromatin served as the input control. PCR primers used in these experiments are listed in Supplementary Table S5. ChIP-qPCR signals were calculated as fold enrichment relative to input control signals; experiments were performed in technical triplicates. Specific antibody ChIP signals were normalized against IgG control ChIP signals from the same samples.

RNA pull-down

SDNOR-WT (miR-29c MRE1, MRE2), SDNOR-MUT (miR-29c MRE1, MRE2), and NORFA-WT (miR-126 MRE) were transcribed from vector pSPT19-SDNOR-WT, pSPT19-SDNOR-MUT, and pSPT19-NORFA-WT in vitro, respectively. Five single-stranded RNA transcripts were biotinylated modified by Biotin-RNA Labeling Mix (#No. 11685597910, Roche) and T7 RNA polymerase (#EP0111, Thermo Fisher Scientific), which were then collected and purified with an RNeasy Mini Kit (#74104, Qiagen). The purified biotinylated transcripts (4 μg) were incubated with 15 μg porcine GC total RNA for 4 h at room temperature. Streptavidin magnetic beads (#LSKMACT02, Merck Millipore) were used to isolate the biotin-RNA/interested-RNA complex according to the manufacturer’s protocol. After isolation, the levels of target miRNAs presenting in the pull-down material were detected by qRT-PCR analysis.

Statistical analysis

All statistical evaluations were performed using GraphPad Prism v5.0 software (GraphPad software) and the Statistical Program for Social Sciences software v20.0 (SPSS). The Student’s t test (two groups) and one-way analysis of variance (ANOVA) were used for single comparison and multiple group comparisons, respectively.
Multiple comparisons between the groups after ANOVA test were confirmed by using S–N–K method. *p < 0.05 was considered statistically significant.

Results
SMAD4 is a strong inducer of FZD4 in porcine GCs
In a previous study, we used RNA-Seq to identify 1025 mRNAs that were differentially expressed (greater than twofold) in SMAD4 knockdown (SMAD4-KD) porcine GCs. Among them was FZD4, which encodes an important receptor in the Wnt signaling pathway. qRT-PCR demonstrated that the FZD4 mRNA level was dramatically reduced in SMAD4-KD GCs, but significantly induced in cells overexpressing SMAD4 (SMAD4-OE). Consistent with this, the level of FZD4 was markedly decreased by SMAD4-KD and significantly increased by SMAD4-OE, indicating that SMAD4 is a strong inducer of FZD4 in porcine GCs. Furthermore, β-catenin, the key downstream molecule of FZD4 and mediator of the Wnt signaling pathway, was also positively regulated by SMAD4, suggesting that SMAD4 controls the Wnt signaling pathway in porcine GCs. In addition, we noticed that expression of SMAD4 was positively correlated with FZD4 expression (Pearson correlation coefficient r = 0.793, p = 0.002) in follicles of porcine ovary in vivo. Together, these data suggest that SMAD4 induces FZD4 and the FZD4-dependent Wnt signaling pathway in porcine GCs.

Interestingly, we also noticed that FZD4 is related to porcine follicular atresia in vivo. To further explore the biological implications of FZD4 in porcine GCs, we knocked down endogenous FZD4, resulting in attenuation of the Wnt signaling pathway in these cells. FZD4 depletion dramatically induced cleaved Caspase-3 (c-Caspase-3), a marker of apoptosis in porcine GCs. The SMAD4-dependent miRNA–mRNA interaction network suggested that two miRNAs, miR-29c and miR-10b, may target FZD4. We identified candidate miRNAs targeting FZD4 using four algorithms (miRWalk, miRDB, miRTarBase, and TargetScan), and identified miR-29c as one of two common miRNAs (the other was let-7g). Therefore, we selected miR-29c for further study. Using RNAhybrid, we identified a putative miRNA response element (MRE) for miR-29c in the 3′-UTR of porcine FZD4. miR-29c targeted porcine FZD4 via its 3′-UTR region.

miR-29c acts as an apoptotic factor by suppressing FZD4 expression in porcine GCs
The SMAD4-dependent miRNA–mRNA interaction network suggested that two miRNAs, miR-29c and miR-10b, may target FZD4. We identified candidate miRNAs targeting FZD4 using four algorithms (miRWalk, miRDB, miRTarBase, and TargetScan), and identified miR-29c as one of two common miRNAs (the other was let-7g). Therefore, we selected miR-29c for further study. Using RNAhybrid, we identified a putative miRNA response element (MRE) for miR-29c in the 3′-UTR of porcine FZD4. miR-29c targeted porcine FZD4 via its 3′-UTR region.
performed loss- and gain-of-function experiments (Fig. 3d, g). Ectopic expression of miR-29c dramatically decreased the levels of FZD4 mRNA (Fig. 3e) and FZD4 protein (Fig. 3f), as well as the level of β-catenin (Fig. 3f), in GCs. By contrast, the levels of FZD4 mRNA (Fig. 3h), FZD4 protein, and β-catenin protein (Fig. 3i) were significantly increased by miR-29c depletion. These data suggest that FZD4 is a functional target of miR-29c in GCs, and that miR-29c inhibits both FZD4 and the FZD4-dependent Wnt signaling pathway.

We next investigated the role of miR-29c in porcine GCs. FACS revealed that ectopic expression of miR-29c significantly induced GC apoptosis (Fig. 3j), whereas inhibition of miR-29c significantly decreased it (Fig. 3k), indicating that miR-29c acts as a pro-apoptotic factor in porcine GCs. Furthermore, miR-29c-induced GC apoptosis was prevented by FZD4-OE (Fig. 3j). Depletion of FZD4 restored GC apoptosis in cells treated with miR-29c inhibitor (Fig. 3k). In addition, miR-29c significantly elevated the level c-Caspase-3, and this effect was reversed
by ectopic expression of FZD4 (Fig. S5). Together, these results prove that miR-29c directly targets FZD4 and inhibits its expression, leading to apoptosis.

**SDNOR, a novel SMAD4-dependent IncRNA, is involved in follicular atresia and GC apoptosis**

To further investigate the mechanism underlying SMAD4 inhibition of miR-29c, we deduced a SMAD4-dependent IncRNA–miR-29c interaction network from our previous RNA-seq data38. Notably, a novel IncRNA not only interacts with miR-29c, but also maps near FZD4 on porcine chromosome 9 was identified. We performed a RACE assay to obtain the full-length sequence of this IncRNA, and found that it is 739 nt in length (Figs.4a, b and S6a). Based on bioinformatic prediction, this IncRNA has no protein-coding potential (Fig. S6b, c); accordingly, we named it **SDNOR**. Porcine **SDNOR** is located ~69.5 kb upstream of **FZD4** (chromosome 9) and consists of five exons and four introns that exhibit relatively low evolutionary conservation among mammals (Fig. 4c).

In addition, we identified an enrichment in H3K27 acetylation at the TSS of porcine **SDNOR**, suggesting that it has the features of an RNA polymerase II transcription unit (Fig. 4c).

Tissue expression profiling of the reproductive system revealed that **SDNOR** is abundantly expressed in porcine ovary (Fig. S6d). In ovarian follicles, **SDNOR** was mainly present in GCs (Fig. 4d). Notably, we also observed that **SDNOR** was dramatically downregulated during follicular atresia (Fig. 4e), suggesting that it is somehow involved in this process. To further assess the role of **SDNOR** in regulating follicular atresia, we knocked down endogenous **SDNOR** in porcine GCs cultured in vitro (Fig. 4f). Silencing of **SDNOR** significantly increased GC apoptosis (Fig. 4g) and the level of c-Caspase-3 (Fig. 4h). These results suggest that IncRNA **SDNOR** is involved in GC apoptosis and follicular atresia in porcine ovaries.

RNA-seq and qRT-PCR confirmed that **SDNOR** was downregulated in SMAD4-silenced porcine GCs (Fig. S7a, b), indicating that SMAD4 inhibits **SDNOR** transcription in these cells. To investigate the mechanism by which SMAD4 regulates **SDNOR** transcription, we first cloned the 5′-regulatory region containing the putative promoter of porcine **SDNOR**. Four SBE motifs were identified at...
Fig. 3 (See legend on next page.)
984/988 nt (SBE1), 776/779 nt (SBE2), 364/467 nt (SBE3), and 341/344 nt (SBE4) (Fig. S7c). Luciferase assays proved that SMAD4 ectopic expression could significantly increase the activity of the SDNOR promoter, and that the SBE1 and SBE2 motifs are required to maintain this situation (Figs. 4i and S7d). In addition, ChIP assays...
confirmed that SMAD4 directly binds to SBE2 within the SDNOR promoter by functioning as a transcription factor (Fig. 4j, k). Together, these observations demonstrate that SMAD4 acts as a transcriptional regulator of SDNOR in porcine GCs.

**SMAD4 promotes expression of FZD4 by inducing SDNOR**

lncRNAs often exert their biological functions by controlling nearby genes[43-45]. We noticed that SDNOR depletion dramatically decreased the level of FZD4 mRNA, which is encoded by a gene near SDNOR, in porcine GCs (Fig. 5a). Similarly, the FZD4 protein level was significantly downregulated in SDNOR-KD GCs (Fig. 5b), indicating that SDNOR induces FZD4 expression in porcine GCs. To determine whether SDNOR regulates the FZD4-dependent Wnt signaling pathway, we monitored the β-catenin protein level in SDNOR-KD GCs. Knockdown of SDNOR dramatically decreased the β-catenin protein level (Fig. 5b). In addition, SDNOR-OE inhibited FZD4-KD-induced GC apoptosis (Fig. 5c) and production of c-Caspase-3 (Fig. 5d), whereas the opposite effect was observed in GCs co-treated with SDNOR-KD and FZD4-OE (Fig. S8a–c). These data demonstrate that SDNOR induces FZD4 and the FZD4-dependent Wnt signaling pathway, and controls FZD4-mediated apoptosis, in porcine GCs.

We next investigated whether SDNOR is involved in the maintenance of FZD4 expression in porcine GCs. Silencing of SDNOR suppressed the SMAD4-induced increases in FZD4 and β-catenin protein levels (Fig. 5b), indicating that SDNOR mediates SMAD4 activation of FZD4 and the FZD4-dependent Wnt signaling pathway in porcine GCs. Furthermore, SDNOR-KD inhibited the SMAD4-induced downregulation of GC apoptosis (Fig. 5e). In addition, c-Caspase-3 level was increased by SDNOR-KD in SMAD4-OE GCs (Fig. 5f), and the opposite effect was observed in GCs co-treated with SMAD4-KD and SDNOR-OE (Fig. S8d–f). Together, these findings reveal...
that SDNOR contributes to the maintenance of FZD4 expression in porcine GCs.

**SDNOR induces FZD4 by sponging miR-29c**

The mechanism of action of a lncRNA usually depends on its subcellular localization. To elucidate the mechanism by which SDNOR upregulates FZD4, we first determined the subcellular localization of SDNOR in porcine GCs. SDNOR was enriched in both the nucleus and cytoplasm (Fig. 6a). Function as molecular sponges, is common mechanism of both nuclear and cytoplasmic lncRNAs; interestingly, two putative binding sites (TGGTTC, 417–422 nt; TGGTG, 640–642 nt) for miR-29c, a miRNA that targets FZD4, were predicted within SDNOR (Fig. 6b). Accordingly, we hypothesized that SDNOR induces FZD4 by acting as a sponge for miR-29c. To test this hypothesis, we first investigated the interaction between SDNOR and miR-29c. Luciferase assays revealed that miR-29c dramatically decreased the activity of a SDNOR reporter vector containing miR-29c binding sites, but had no effect on a control vector containing mutated binding sites (Fig. 6c), confirming that miR-29c binds to its putative binding sites in SDNOR. Furthermore, RNA pull-downs confirmed that SDNOR physically associates with miR-29c in porcine GCs (Fig. 6d). Together, these data reveal that SDNOR acts as a molecular sponge for miR-29c in porcine GCs.

In porcine follicles, the levels of SDNOR and miR-29c were negatively correlated (Fig. 6e). Consistent with this, the miR-29c level was significantly elevated in SDNOR-KD GCs (Fig. 6f), suggesting that SDNOR is a determinant
of miR-29c level in porcine ovary cells both in vivo and in vitro. Furthermore, miR-29c decreased both SDNOR and FZD4 expression levels in a dose- and time-dependent manner; SDNOR was more sensitive than FZD4 to miR-29c (Figs. 6g, h and S9a, b), indicating that SDNOR induces FZD4 in porcine GCs by competitively binding miR-29c.

We also investigated whether miR-29c mediates the regulation of downstream molecules and GC apoptosis by SDNOR. miR-29c depletion rescued the decrease in FZD4 and β-catenin protein levels caused by siSDNOR (Fig. 6i), indicating that miR-29c mediates the regulation of SDNOR on FZD4 and the FZD4-dependent Wnt signaling pathway in porcine GCs. We also observed that depletion of miR-29c inhibited GC apoptosis and c-Caspase-3 production induced by SDNOR silencing (Fig. 6j), indicating that miR-29c mediates the regulation of GC apoptosis by SDNOR. Together, these data demonstrate that SDNOR physically associates with miR-29c, thereby alleviating the inhibitory effect of miR-29c on its target FZD4, and subsequently activates the Wnt signaling pathway and inhibits apoptosis in porcine GCs.

Discussion

In this study, we have clarified the role of SMAD4 in regulating FZD4 expression in ovarian GCs. We found that SMAD4 acts as a strong inducer of FZD4, controlling FZD4 transcription, the FZD4-dependent Wnt signaling pathway, and FZD4-mediated GC function. In addition, we showed that SMAD4 induces protects FZD4 through two molecular mechanisms: direct interactions with the promoter region of FZD4 and upregulating a novel lncRNA, SDNOR, which acts as a sponge for miR-29c.

The TGF-β signaling pathway is multifunctional, and controls many biological functions related to development and diseases, usually via interactions with other important signaling pathways such as BMP, ERK, Hippo, JAK/STAT, Notch, NF-κB, MAPK, and Wnt signaling pathways. In particular, crosstalk between the TGF-β and Wnt signaling pathways has been extensively studied. However, the regulatory relationship between these pathways is still unclear. Here, we demonstrated that SMAD4 directly binds to the SBE motifs of the FZD4 promoter by acting as a transcription factor, thereby inducing FZD4 and the Wnt signaling pathway in GCs. These findings establish a direct link between the TGF-β and Wnt signaling pathways in GCs.

In mechanism terms, we discovered two important and unexpected ways that SMAD4 promotes FZD4 transcription in ovarian GCs. First, SMAD4 directly controls FZD4 transcription by acting as a transcription factor, the best-characterized manner in which SMAD4 regulates its target genes. The SMAD4 protein affects the transcriptional activity of target genes, usually by interactions between its DNA-binding domain and SBE motifs within the promoter region of target genes. Intriguingly, the regulatory effect of SMAD4 on target genes can act in one of two opposing ways: it can promote target gene transcription by acting as a transcriptional activator, or inhibit the transcription of target genes by acting as a transcriptional repressor. In this study, we showed that SMAD4 is a new transcriptional activator of FZD4, and that the two factors form a novel regulatory axis that mediates crosstalk between the TGF-β and Wnt signaling pathways in porcine GCs. A recent study has demonstrated that Wnt signaling pathway is the most inactivated pathway in gastric tumors from a Smad4-KO mouse model produced by using a transposon mutagenesis method. Moreover, several components of the Wnt signaling pathway, including Wnt3A52, β-catenin, Fzd1, Fzd2, Dkk1, and Sfrp1, as well as downstream targets such as c-Myc and Axin2, are regulated by SMAD4. CTNNB1 (also known as β-catenin), for instance, is induced by SMAD4 in human Tenon's capsule fibroblasts; this factor is involved in the promotion of cell proliferation and activation by TGF-β signaling pathways. Conversely, nuclear β-catenin and other signature genes of the Wnt signaling pathway are upregulated in epithelial tumors harboring Smad4-KO. However, the mechanism by which SMAD4 directly regulates the components of the Wnt signaling pathway had been unclear. Here, we demonstrated that SMAD4 directly binds to the SBE motifs of the FZD4 promoter by acting as a transcription factor, thereby inducing FZD4 and the Wnt signaling pathway in GCs. These findings establish a direct link between the TGF-β and Wnt signaling pathways in GCs.
expression in cis or trans with various mechanisms. As with miRNAs, many lncRNAs are regulated by the TGF-β signaling pathway. A recent study revealed a new mechanism by which TGF-β signaling regulates miRNA biogenesis: lncRNA nc886, which is induced by TGF-β, binds to Dicer, a key enzyme involved in miRNA biogenesis, thereby inhibiting miRNA maturation in ovarian cancer. However, the mechanism underlying regulation of lncRNA expression by the TGF-β signaling pathway remains largely unknown. In this study, we identified a novel lncRNA related to follicular atresia, SDNOR, and showed that SMAD4 induces SDNOR expression in GCs through an interaction with its promoter. In addition to providing a potential nonhormone therapeutic drug for improving follicular atresia and female fertility in pigs, our findings also provide a new insight into the mechanism by which the TGF-β signaling pathway regulates lncRNA expression in mammals. We also showed that SDNOR helps to maintain FZD4 expression by acting as a sponge for endogenous miR-29c, thereby eliminating the inhibition of FZD4 by miR-29c. LncRNA–miRNA interactions are already known to participate in regulation of the Wnt signaling pathway. LncRNA–miRNA systems not only act directly on the core members of the pathway, such as WNT2B, FZD3, and FZD5, but also indirectly on upstream regulators such as GSK3β, SOX9, and APC, thereby affecting the activation of this pathway. Overall, our findings demonstrate that a novel lncRNA–miRNA system, SDNOR-miR-29c, mediates positive regulation of FZD4 by SMAD4, establishing a novel molecular link between the TGF-β and Wnt pathways.

In summary, we have shown that the TGF-β and Wnt signaling pathways engage in crosstalk to regulate GC apoptosis. Specifically, SMAD4 helps to maintain FZD4 expression at both the transcriptional and posttranscriptional level, via both direct and indirect mechanisms, through a complex regulatory network consisting of miRNA, lncRNA, and protein-coding genes (Fig. 7). These coding proteins, especially FZD4, represent promising therapeutic targets for treatment of female reproductive diseases and improvement of female fertility. The noncoding RNAs involved in this pathway, including miR-29c and SDNOR, could be developed as nonhormone drugs to regulate these targets.

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Conflict of interest
The authors declare that they have no conflict of interest.

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