

LncPrep + 96kb inhibits ovarian fibrosis by upregulating prolyl oligopeptidase expression

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Abstract. LncPrep + 96kb is a long non-coding RNA expressed in murine granulosa cells. The 2.2-kb fragment of LncPrep + 96kb inhibits aromatase expression and estrogen secretion in ovarian granulosa cells. In the present study, LncPrep + 96kb-knockout (KO) mice were generated, and significant ovarian fibrosis and reduced female fertility through fertility monitoring and superovulation. The augmentation of ovarian fibrosis was observed by Sirius red staining and western blot and *RT-qPCR*. Notably, LncPrep + 96kb was identified in conserved non-coding sequences adjacent to the prolyl oligopeptidase (POP) gene. Furthermore, POP expression was shown to be reduced in LncPrep + 96kb-KO mice, whereas overexpression of LncPrep + 96kb increased POP expression. Further studies revealed that POP regulated the expression levels of factors related to fibrosis, including matrix metalloproteinase 2 (MMP2), transforming growth factor β 1 (TGF- β 1) and peroxisome proliferator activated receptor γ (PPAR- γ). In conclusion, ovarian fibrosis was elevated in LncPrep + 96kb-KO mice, and POP may act as a target of LncPrep + 96kb, which mediates ovarian fibrosis through the regulation of PPAR- γ , MMP2 and TGF- β 1 expression.

Introduction

The ovary is a female reproductive organ with key functions including the production of mature eggs and the secretion of steroid hormones (1). Follicles serve as the fundamental

functional unit of the ovary and are comprised of oocytes surrounded by theca and granulosa cells (2). Follicles are classified into primordial, primary, secondary, antral and preovulatory follicles based on their developmental stage. Follicular growth begins with the transformation of flattened pregranular cells into cuboidal granulosa cells; these cells undergo continuous proliferation, causing follicles to expand from a single layer to multiple layers. As the antrum forms, granulosa cells differentiate into two subsets: Cumulus cells, which attach to the oocytes; and mural granulosa cells, which adhere to the follicular wall. The cumulus cells enclose oocytes to form the cumulus-oocyte complex (3). Notably, most follicles do not reach maturity, undergoing follicular atresia instead. Only a few follicles reach full maturity, migrate to the ovarian surface, and ovulate successfully (4).

The extracellular matrix (ECM), which comprises collagen, laminin and fibronectin, serves a critical role in follicle development (5-7). Notably, the ECM binds to growth factors and hormones, regulating the growth of granulosa cells and oocytes, in addition to providing structural support for growing follicles (8). During ovulation, luteinizing hormone surges trigger follicular rupture, cumulus expansion and oocyte maturation, with ECM remodeling being key to these processes (9). The inability to synthesize ECM components can lead to sterility or reduced fertility (10). Thus, the cyclical remodeling and degradation of the ECM are essential for follicle formation, maturation and ovulation.

In recent years, the pathological changes associated with ovarian fibrosis have garnered attention. With aging, excessive ECM accumulation in the ovarian microenvironment can induce fibrosis, contributing to ovarian aging (11). Factors such as surgery, inflammation and immune system disorders can also cause ovarian injury (12,13). During ovarian repair, cytokine interactions promote ECM production, leading to fibrosis and impaired ovarian function (14,15). Ovarian fibrosis exacerbates inflammation, disrupts angiogenesis and destabilizes ovarian homeostasis. Additionally, fibrosis in the ovarian cortex can reduce the populations of stromal cells, causing follicular obstruction, poorer oocyte quality and compromised reproductive outcomes (16). Ovarian fibrosis is also linked to various disorders, including endometriomas, polycystic ovary syndrome (PCOS) and premature ovarian failure (POF), ultimately decreasing or depleting ovarian

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function (17-22). Consequently, a fibrosis-induced decline in ovarian function poses a serious threat to reproductive health and overall quality of life.

Long non-coding RNAs (lncRNAs) were once considered 'noise' in gene transcription; however, it has been shown that they serve notable roles in both physiological and pathological cellular processes as components of the gene regulatory network (23). With advancements in high-throughput technology, numerous lncRNAs have been identified in mammalian ovarian somatic cells. Despite this, only a few lncRNAs have been thoroughly investigated, and revealed to be involved in follicular development and the regulation of female fertility (24). For example, lncRNA NEAT1, which is highly expressed in mammalian follicles, has been reported to be essential for the formation of the corpus luteum and normal fertility in mice (25). Another lncRNA, FDNCR, has been shown to be primarily expressed in the ovaries of Hu sheep with low proliferation capacity, and can induce granulosa cell apoptosis through the microRNA-543-3p/DCN axis (26). In addition, a previous study suggested that lncRNAs have a crucial role in ECM remodeling, with abnormal expression being closely linked to tissue fibrosis (27). Furthermore, lncRNA MEG3 promotes cardiac fibrosis by inhibiting matrix metalloproteinase 2 (MMP2) expression (28). lncRNA Erbb4-ir mediates transforming growth factor β 1 (TGF- β 1)-induced renal fibrosis (29) and lncRNA H19X is a key factor in TGF- β -driven fibrosis (30). Additionally, lncRNA PICSAR induces abnormal fibroblast proliferation, excessive ECM deposition and the formation of hypertrophic scarring through the regulation of TGF- β 1 (31). lncRNAs also contribute to tumor metastasis by regulating ECM remodeling (32).

Previous studies have highlighted the role of lncRNAs in ovarian follicle development, with dysregulated expression being closely linked to disorders characterized by abnormal follicular growth (33-35). Matsubara *et al* (36) identified lncPrep + 96kb within conserved non-coding sequences adjacent to prolyl oligopeptidase (POP). Notably, lncPrep + 96kb has two transcripts, 2.2kb and 2.8kb, which are specifically expressed in mouse ovarian granulosa cells; however, their function remains unclear. lncPrep + 96kb is a lncRNA that exhibits high expression in mouse granulosa cells. Using *in situ* hybridization, we analyzed the spatial and temporal specific expression of lncPrep + 96kb, revealing that lncPrep + 96kb is predominantly localized in the granulosa cells of primary and secondary follicles (37). Moreover, it has been shown to suppress aromatase expression and estradiol production in granulosa cells by inducing the translocation of endothelial differentiation-associated factor 1 (EDF1) from the nucleus to the cytoplasm (37). The present study successfully generated lncPrep + 96kb-knockout (KO) mice. The aim of the present study was to investigate the effects and underlying mechanisms of lncPrep + 96kb on ovarian fibrosis.

Materials and methods

Animals. A total of eight lncPrep + 96kb-KO mice were obtained from GemPharmatech Co., Ltd (cat. no. AB849013, C57BL/6J, female mice, 20-25 g, 30 day old, GemPharmatech Co., Ltd). All animals were housed at the Laboratory Animal Center of the Medical College of Nanchang University (Nanchang, China) under controlled conditions, including a

stable temperature of $22\pm 2^{\circ}\text{C}$, relative humidity of $50\pm 10\%$ and a 12-h light/dark cycle, and free access to sufficient food and water. All animal care and procedures were approved by the Experimental Animal Center at Nanchang University (approval no. NCULAE-202209280023).

Genotyping. Genotyping of mice was performed using PCR amplification (2 X EasyTaq PCR SuperMix(+dye), cat. no. AS111-11, TransGen Biotech Co., Ltd.) of genomic DNA extracted from tail biopsies. Genotyping was performed using the following primers: lncPrep + 96kb wild-type (WT) mice, forward 5'-GGTAATCCTAACGCCACG-3' and reverse 5'-TACCGAGGCACAGTTTCCA-3'; lncPrep + 96kb-KO mice, forward 5'-GACACGCCTATTCATACAAGG C-3' and reverse 5'-GCAAGCGAGTCAGATTTGGCTTAG AAG-3'. Thermocycling conditions were as follows: 94°C for 10 min, followed by 36 cycles of 94°C for 30 s, 55°C for 45 s, and 65°C for 45 s, with a final extension at 65°C for 10 min. PCR products were separated on a 2% agarose gel (cat. no. BKM-AG-100, Shenzhen Bikamei Biotechnology Co., Ltd.) with 1 X Green fluorescent nucleic acid dye (10000X) (cat. no. G8140, Beijing Solarbio Science & Technology Co., Ltd.), and visualized under UV light. Wild-type mice showed a single band at 344 bp, while heterozygous mice showed bands at 390 bp and 344 bp, and homozygous mice showed a single band at 390 bp (Fig. S1)

Assessing female fertility for 6 months. Six female mice aged 21 days, both WT and KO female mice, were randomly selected. The body weight of the female mice was recorded every three days for a total of five times (Fig. S2). The female mice were mated with 12 proven fertile male mice (C57BL/6J, 20-25 g, 21 day old, Changsha Tianqin Biotechnology Co., Ltd.) at a 1:1 ratio. Successful mating was confirmed by the presence of a vaginal plug. After natural delivery, the pregnancy rate and the number of offspring were recorded.

Superovulation. Superovulation was induced in KO and WT female mice through intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; cat. no. P9970; Beijing Solarbio Science & Technology Co., Ltd.), followed by an additional 10 IU human chorionic gonadotropin (cat. no. YZ-150555; Beijing Solarbio Science & Technology Co., Ltd.) injection 48 h later. The mice were euthanized by cervical dislocation 13 h after the second injection. Subsequently, the ampulla of the fallopian tube was dissected under a stereomicroscope to collect and count oocytes for further analysis.

Hematoxylin and eosin (H&E) staining. The ovarian tissue were extracted from KO and WT mice and fixed in 4% paraformaldehyde (cat. no. P1110; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 24 h and then embedded in paraffin. Subsequently, 3-4- μm sections were prepared, followed by dewaxing and hydration. Using a H&E Staining Kit (cat. no. G1121; Beijing Solarbio Science & Technology Co., Ltd.), the nuclei were stained with hematoxylin for 15 sec and the cytoplasm was stained with eosin at room temperature for 30 sec. Finally, dehydration, clarification, neutral gum sealing and light microscopic observation were carried out sequentially.

Sirius-Red staining. Ovarian tissue sections (5 μ m) were depa-
raffinized and stained using the Sirius-Red Staining solution
(cat. no. G1472; Beijing Solarbio Science & Technology Co., Ltd.)
according to the manufacturer's instructions at room temperature
for 24 h. The slices were then immersed in xylene and alcohol
and mounted with synthetic resin. Three images from different
fields of view were randomly selected for each tissue section, and
images were captured using a light microscope (magnification,
x10). The images were exported in JPG format with a resolution of
1,920x1,080 pixels via NDP.view2 (Hamamatsu Photonics K.K.).
ImageJ (National Institutes of Health) was used to select areas
and collagen fiber content was semi-quantified by calculating the
ratio of staining intensity in the selected area to the total staining
intensity of the ovarian tissue.

Plasmid construction. PCDNA3.0-IncPrep + 96kb 2.2kb
and PCDNA3.0-IncPrep + 96kb 2.8kb plasmids (37), and a
pMigR1-POP plasmid (18) were constructed as previously
described. The IncPrep + 96kb 2.2kb and 2.8kb sequences were
amplified by PCR and inserted into the PCDNA 3.0 vector
(cat. no. V79020, Thermo Fisher Scientific Inc.) following
restriction enzyme digestion with *Kpn*I (cat. no. R3142M;
New England BioLabs, Inc.) and *Eco*RI (cat. no. R3101V;
New England BioLabs, Inc.). A guide RNA (gRNA) targeting
IncPrep + 96kb was cloned into the pSpCas9(BB)-2A-GFP
vector (PX458) (cat. no. 48138, Addgene, Inc.). The PX458
plasmid was digested with *Bbs*I and then annealed to the
gRNA, and successful insertion was confirmed by Sanger
sequencing. PCR primer sequences for IncPrep + 96kb 2.2kb
were: Forward, 5'-TTGGTACCAGCTTGTGTATTGCTCATA
T-3' and reverse, 5'-GGAATTCTTTGCTTTTAAATTTT
ATTTG-3'; and the sequences for IncPrep + 96kb 2.8kb were:
Forward, 5'-AGATCATGACAGGGGCTCCT-3' and reverse,
5'-AGCTGGCTGGTCCTCACAG-3'.

To construct the pMigR1-POP plasmid, the entire sequence
of POP was amplified and digested with *Xho*I (cat. no. R0146V;
New England BioLabs, Inc.) and *Eco*RI, and were subse-
quently cloned into the pMig plasmid vector (cat. no. MZ0431,
Shanghai Qiming Biotechnology Co., Ltd.). The successful
insertion was confirmed through Sanger sequencing. PCR
primer sequences for POP were: Forward, 5'-CCGCTCGAG
ATGCTGTCCTTCCAGTACCC-3' and reverse, 5'-CCGGAA
TTCTTACTGGATCCACTCGATGTT-3'.

Granulosa cell culture and transfection. A total of six female
mice (C57BL/6J, 25-30 g, 21 day old, Changsha Tianqin
Biotechnology Co., Ltd.) were injected with 5 IU PMSG.
After 48 h, the mice were euthanized by cervical disloca-
tion and their ovaries were harvested. The ovaries were then
placed in petri dishes containing sterile PBS (cat. no. P1020;
Beijing Solarbio Science & Technology Co., Ltd.) and follicles
were mechanically punctured with an injection needle to
release granulosa cells. The PBS containing granulosa cells
was collected in a centrifuge tube (cat. no. UFC910096;
Merck KGaA) and centrifuged at 2,500 x g for 5 min at room
temperature. After discarding the supernatant, the cells were
cultured in DMEM/F12 (cat. no. D6501, Beijing Solarbio
Science & Technology Co., Ltd.), 1% penicillin-streptomycin
(cat. no. FG101-01, TransGen Biotech Co., Ltd.) in a 6-well
culture plate (cat. no. CFE-F1006-01; TransGen Biotech Co.,

Ltd.) at 37°C in a humidified atmosphere of 5% CO₂. Once
cell confluence reached 70-80%, the cells were transfected
with 2 μ g plasmid using FuGENE® 6 Transfection Reagent
(cat. no. E2691; Promega Corporation). After 8 h at 37°C,
the medium was replaced with fresh medium. The cells
underwent RNA and protein extraction 48 h post-transfection,
and the effects of plasmid transfection were verified using
reverse transcription-quantitative polymerase chain reaction
(RT-qPCR) and western blotting (WB) (Fig. S3).

RT-qPCR. RNA was extracted from granulosa cells or ovarian
tissue using TransZol UP Enhanced RNA extraction kit
(cat. no. ET111-01-V2; TransGen Biotech Co., Ltd.), and its concen-
tration was measured using a NanoDrop 2000 spectrophotometer
(Thermo Fisher Scientific, Inc.). RT was performed using the
RT Kit (cat. no. AE311; TransGen Biotech Co., Ltd.) according
to the manufacturer's protocol. RT was carried out at 42°C for
30 min, with reverse transcriptase inactivation performed at 85°C
for 5 min. qPCR was conducted using the QuantiNova® SYBR
Green PCR Kit (cat. no. 208054; Qiagen GmbH). According to the
kit's instructions, the amplification system was prepared and run
on a PCR instrument (CFX96 Connect; Bio-Rad Laboratories,
Inc.). The reaction conditions were as follows: Initial denaturation
step at 95°C for 5 min, followed by 40 cycles at 60°C for 10 sec
and 95°C (+0.5°C/cycle) for 15 sec, and a final step at 95°C for
15 sec. β -actin was used as an internal control for standardiza-
tion, and relative quantification was calculated using the 2^{- $\Delta\Delta$ C_q}
method (38). Primer sequences, supplied by TransGen Biotech
Co., Ltd., are listed in Table I.

WB. Ovarian tissue or cells were lysed in RIPA buffer
(cat. no. PC101; Shanghai Yamei Biomedical Technology
Co., Ltd.) supplemented with 1X alkaline phosphatase
inhibitors 100X (cat. no. P1260; Beijing Solarbio Science
& Technology Co., Ltd.) and 1X protease inhibitors 100X
(cat. no. P6731; Beijing Solarbio Science & Technology Co.,
Ltd.). Protein concentration was measured using the Easy II
Protein Quantitative (BCA) Kit (cat. no. DQ111-01; TransGen
Biotech Co., Ltd.). A total of 20 μ g/lane protein samples were
separated by 12% sodium dodecyl sulfate-polyacrylamide gel
electrophoresis and were then transferred to PVDF membranes
(pore size, 0.2 μ m; cat. no. ISEQ00010; Merck KGaA). The
membranes were blocked with Blocker™ BLOTTO TBS solu-
tion (cat. no. 37530; Thermo Fisher Scientific, Inc.) for 1 h at
room temperature, followed by overnight incubation at 4°C with
primary antibodies. After an incubation with the secondary
antibodies at room temperature for 1 h, the protein bands were
visualized and semi-quantified using the EasySee Western Blot
Kit (cat. no. DW101-01; TransGen Biotech Co., Ltd.) and Image
Lab™ software 3.0 (Bio-Rad Laboratories, Inc.).

The following antibodies were used for WB:
Anti-PREP/POP (1:1,000; cat. no. A00298-2; Bioworld
Technology, Inc.), anti- β -actin (1:5,000; cat. no. 66009-1-Ig;
Wuhan Sanying Biotechnology), anti-peroxisome prolif-
erator activated receptor γ (PPAR- γ ; 1:1,000; cat. no. BA2120;
Boster Biological Technology Co., Ltd.), anti-TGF- β 1 (1:500;
cat. no. BA0290; Boster Biological Technology), anti-MMP2
(1:1,000; cat. no. A00286; Boster Biological Technology),
Mouse anti-Goat IgG (H+L) Cross-Adsorbed Secondary
Antibody, HRP (1:20,000; cat. no. HS101; TransGen Biotech

Table I. Oligonucleotides used for RT-qPCR.

Gene	Primer sequence
PPAR- γ	Sense: 5'-AGGACATCCAAGACAACCTG-3' Antisense: 5'-CTCTGTGACAATCTGCCTGA-3'
MMP2	Sense: 5'-GAACACCTTCTATGGCTGC-3' Antisense: 5'-GTTGTAGTTGGCCACATCTG-3'
TGF- β 1	Sense: 5'-CCAACTATTGCTTCAGCTCCA-3' Antisense: 5'-TTATGCTGGTTGTACAGGG-3'
β -actin	Sense: 5'-TAAAGACCTCTATGCCAACACAGT-3' Antisense: 5'-CACGATGGAGGGGCCGGAATCATC-3'
POP	Sense: 5'-CCGCTCGAGATGCTGTCCTTCCAGT ACCC-3' Antisense: 5'-CCGGAATTCTTACTGGATCCACTCGAT GTT-3'

PPAR- γ , peroxisome proliferator activated receptor γ ; MMP2, matrix metalloproteinase 2; TGF- β 1, transforming growth factor β 1; POP, prolol oligopeptidase.

Co., Ltd.), and Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP (1:20,000; cat. no. HS201; TransGen Biotech Co., Ltd.).

Statistical analysis. Data were statistically analyzed using GraphPad Prism 7.00 (Dotmatics), and are presented as the mean $\pm$ SD. All experiments were repeated at least three times. Differences between two groups were evaluated using an independent samples Student's t-test. One-way ANOVA followed by Student-Newman-Keuls test was used for statistical comparisons among multiple groups. Two-way ANOVA followed by Tukey's HSD test was used to analyze the changes in body weight of mice. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LncPrep + 96kb-KO female mice have decreased fertility. The ovaries serve a crucial role in female reproduction and their abnormal development directly impacts fertility. To investigate the role of LncPrep + 96kb in ovarian function, the fertility of KO and WT mice were assessed. Over 6 months of female fertility testing, KO mice produced significantly fewer pups compared with WT mice (Fig. 1A). No significant differences were observed in body weight changes between the groups (Fig. S2). The number of litters born to KO mice was notably lower than the number born to WT mice (Fig. 1B), and the average litter size was also significantly reduced in the KO group (Fig. 1C). Additionally, the ovulation rate in immature superovulated mice was evaluated, and it was revealed that KO female mice ovulated fewer eggs (22 ± 12) compared with WT female mice (41 ± 21) (Fig. 1D, E).

LncPrep + 96kb-KO mice exhibit obvious ovarian fibrosis. Ovaries from 2-month-old WT and KO mice were subjected to H&E and Sirius-Red staining. H&E staining revealed no notable differences in follicular morphology between the two

groups (Fig. 2A). However, Sirius-Red staining highlighted a marked increase in ovarian fibrosis in KO mice compared with that in WT mice (Fig. 2B). To improve the clarity of fibrosis assessment, Sirius-Red staining was performed without hematoxylin counterstaining; the results showed a marked increase in collagen deposition in KO mice (Fig. 2C). Analysis of three randomly selected Sirius-Red staining sections using ImageJ software confirmed a higher collagen fiber content in KO ovaries compared with in WT ovaries (Fig. 2D).

TGF- β 1 expression is increased, whereas MMP2 and PPAR- γ expression is decreased in the ovaries of LncPrep + 96kb-KO female mice. Cytokines such as MMP2, TGF- β 1 and PPAR- γ are crucial in the development of tissue fibrosis (15,39). To further explore their roles in ovarian fibrosis, mRNA (Fig. 3A) and proteins (Fig. 3B) were extracted from the ovaries of KO and WT mice. The results revealed an upregulation of TGF- β 1 expression in KO mice, whereas MMP2 and PPAR- γ expression levels were significantly downregulated.

LncPrep + 96kb promotes POP expression. LncPrep + 96kb has been identified as a lncRNA transcribed from a conserved non-coding sequence of the mouse POP gene, which regulates POP expression in ovarian granulosa cells (36). Our previous study demonstrated that POP serves a role in androgen-induced fibrosis in an animal model of PCOS by regulating TGF- β 1 and MMP-2 expression (18). Analyses of both mRNA (Fig. 4A) and protein (Fig. 4B) levels revealed reduced POP expression in the ovaries of LncPrep + 96kb-KO mice. To further confirm this, granulosa cells were isolated, and it was confirmed that the mRNA expression levels of POP were lower in the KO group compared with those in the WT group (Fig. 4C). Conversely, POP expression was significantly increased in granulosa cells transfected with PCDNA3.0-LncPrep + 96kb 2.2kb and 2.8kb plasmids (Fig. 4D and E). These findings indicated that LncPrep + 96kb may modulate POP expression in the ovary.

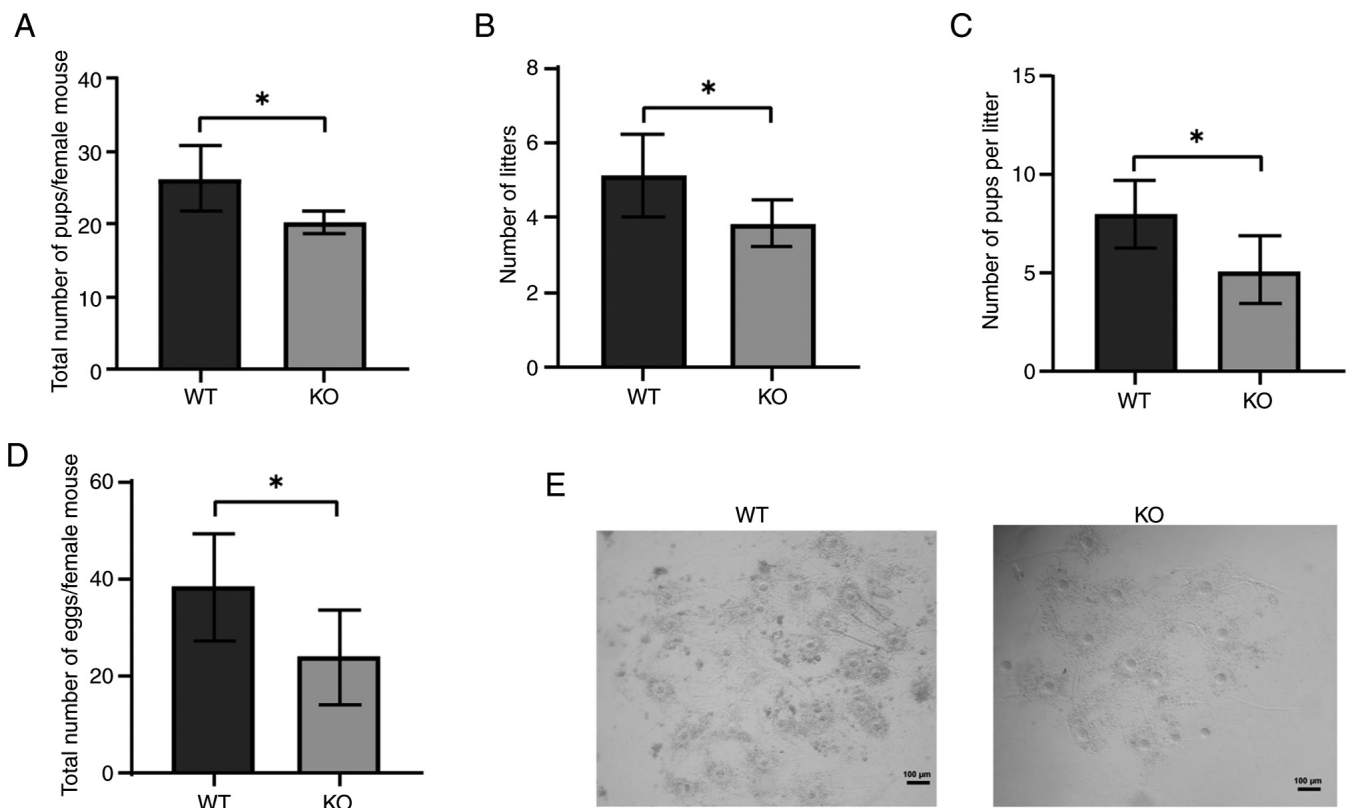


Figure 1. LncPrep + 96kb-KO female mice have decreased fertility. (A) Total number of pups/female mouse. (B) Number of litters, (n=6). (C) Number of pups per litter, (n=6). (D) Total number of eggs/female mouse, (n=3). (E) Egg was inspected under an optical microscope. Scale bar, 100 µm. *P<0.05, as determined by independent samples Student's t-test. KO, knockout; WT, wild-type.

Overexpression of POP inhibits TGF-β1 expression and increases MMP2 and PPAR-γ expression. To further investigate the role of in ovarian fibrosis, granulosa cells were cultured and transfected with the pMigR1-POP plasmid. The results showed that TGF-β1 expression was decreased, and MMP2 and PPAR-γ expression was increased at both the protein (Fig. 5A) and mRNA (Fig. 5B) levels. These findings suggested that POP may modulate the expression of fibrosis-related factors in granulosa cells.

Discussion

Ovarian fibrosis is characterized by the excessive proliferation of ovarian fibroblasts and accumulation of the ECM. Previous studies have shown that various ovarian disorders, including PCOS, ovarian endometriomas and POF, exhibit differing degrees of ovarian fibrosis (40,41). The ovaries of patients with PCOS exhibit abnormally elevated levels of TGF-β1, which promotes ECM production in mesenchymal cells and the synthesis of enzymes that inhibit ECM degradation. This results in excessive ECM accumulation in the ovary, contributing to ovarian interstitial fibrosis (42). Furthermore, systemic and localized chronic low-grade inflammation can increase oxidative stress within the ovary, accelerating the development of ovarian fibrosis (43-46). POF is characterized by fibrous tissue infiltration in the ovarian cortex or stroma and thickening of the ovarian capsule. Zhang *et al* (47) demonstrated that transplantation of human amniotic epithelial cells can inhibit ovarian granulosa cell apoptosis, activate the

TGF-β/Smad signaling pathway, and support the restoration of damaged ovarian vascular structures and functionality, thereby improving the symptoms of ovarian fibrosis. The present findings revealed pronounced fibrosis in the ovarian tissues of LncPrep + 96kb-KO mice, suggesting that LncPrep + 96kb may influence ovarian ECM remodeling and thereby affect ovarian function.

The growth, maturation and ovulation of ovarian follicles depend on the cyclical breakdown and restructuring of the ECM. This remodeling process is particularly crucial during cumulus expansion and follicular rupture (48,49). Ovarian fibrosis, marked by excessive ECM deposition, can directly impact follicular development and female fertility (50,51). In the current study, LncPrep + 96kb-KO mice exhibited reduced fertility and ovulation, which may be linked to increased ovarian fibrosis.

The primary pathological features of ovarian fibrosis include increased connective tissue within the mesenchyme, and a reduction or absence of follicles. Research has identified the involvement of various cytokines in the development of tissue fibrosis, including TIMPs, MMPs, ET-1, TGF-β1, VEGF, PPAR-γ and CTGF. These factors interact to disrupt the balance between ECM and degradation, leading to the excessive proliferation of ovarian mesenchymal fibroblasts (15). TGF-β1 serves a critical role in fibrosis by inhibiting MMP expression and activation, upregulating protease inhibitors such as TIMPs and plasminogen activator inhibitors, and promoting ECM component synthesis while suppressing its degradation (52,53). PPAR-γ, a ligand-activated transcription

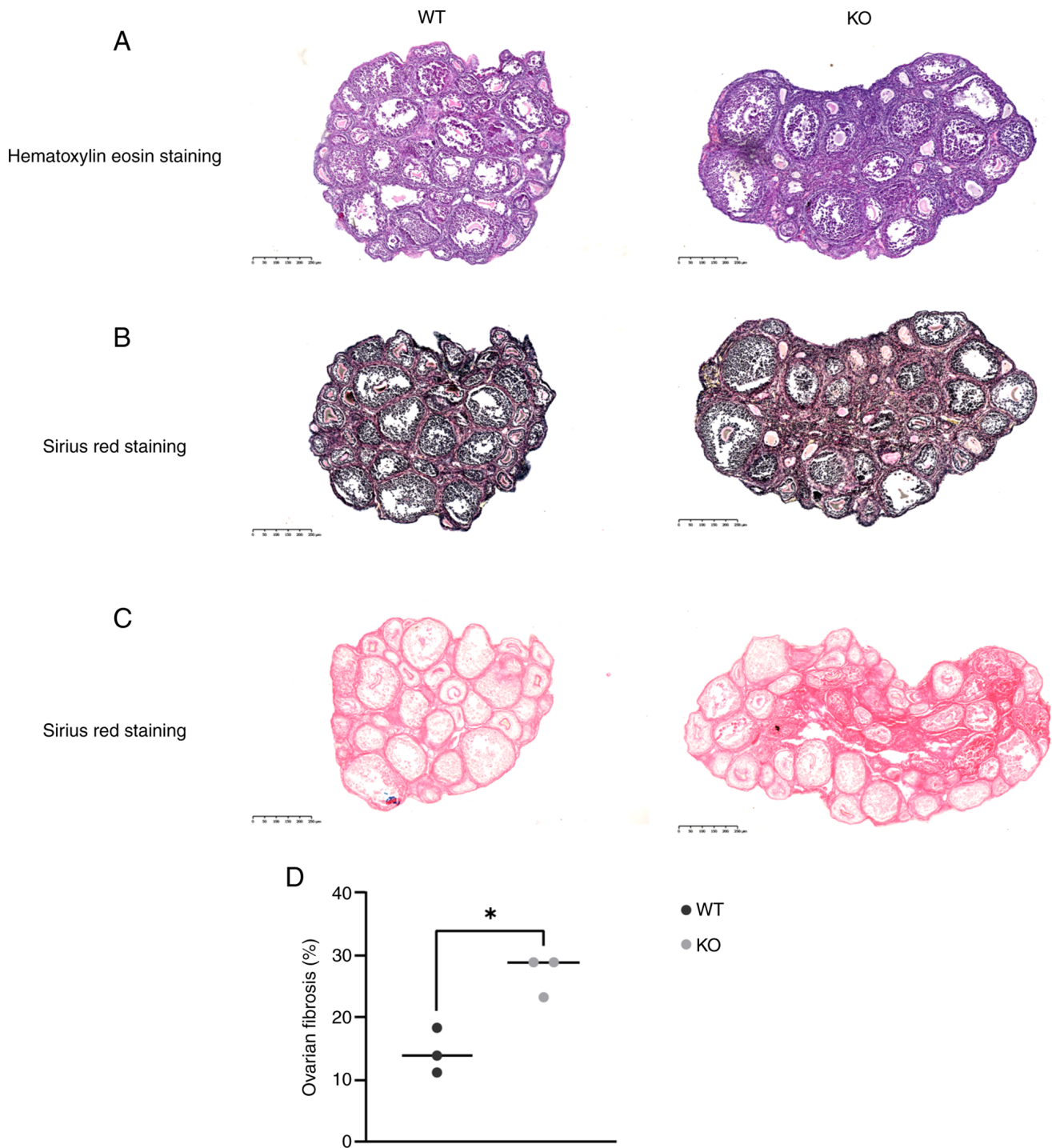


Figure 2. Ovarian fibrosis in LncPrep + 96kb-KO mice. (A) Hematoxylin eosin staining of ovarian sections of 2-month-old mice. Scale bar, 250 μ m. (B) Sirius red staining of ovarian sections of 2-month-old mice. (C) Sirius red staining of ovarian sections without hematoxylin counterstaining. Scale bar, 250 μ m. (D) Ovarian fibrosis as a percentage of ovarian tissue. Data are presented as the mean $\pm$ SD (n=3 mice/group). *P<0.05, as determined by independent samples Student's t-test. KO, knockout; WT, wild-type.

factor, is crucial in regulating glycolipid metabolism, immune system function, cell differentiation and apoptosis, and inflammatory responses. It has been shown that PPAR- γ agonists block TGF- β signaling, thereby impeding the progression of fibrosis (54). By regulating TGF- β and other pathways, PPAR- γ can inhibit the activation and proliferation of hepatic stellate cells, reduce ECM production and exert anti-fibrosis effects (55,56). MMP2 is present in all cell types, and degrades degenerated collagen I, collagen IV and other

ECM proteins. In animal models of diabetic cardiomyopathy, reduced MMP2 activity has been shown to be associated with increased myocardial fibrosis (57). Similarly, in the ovary, both endogenous (such as reactive oxygen species and inflammatory mediators) and exogenous (for example, high-fat or high-sugar diets) stimuli can elevate TGF- β 1 levels, leading to excessive ECM accumulation, disrupted MMP balance and the promotion of ovarian interstitial fibrosis (15,58,59). Yang *et al* (60) reported that abnormal TGF- β 1 expression and follicular fluid

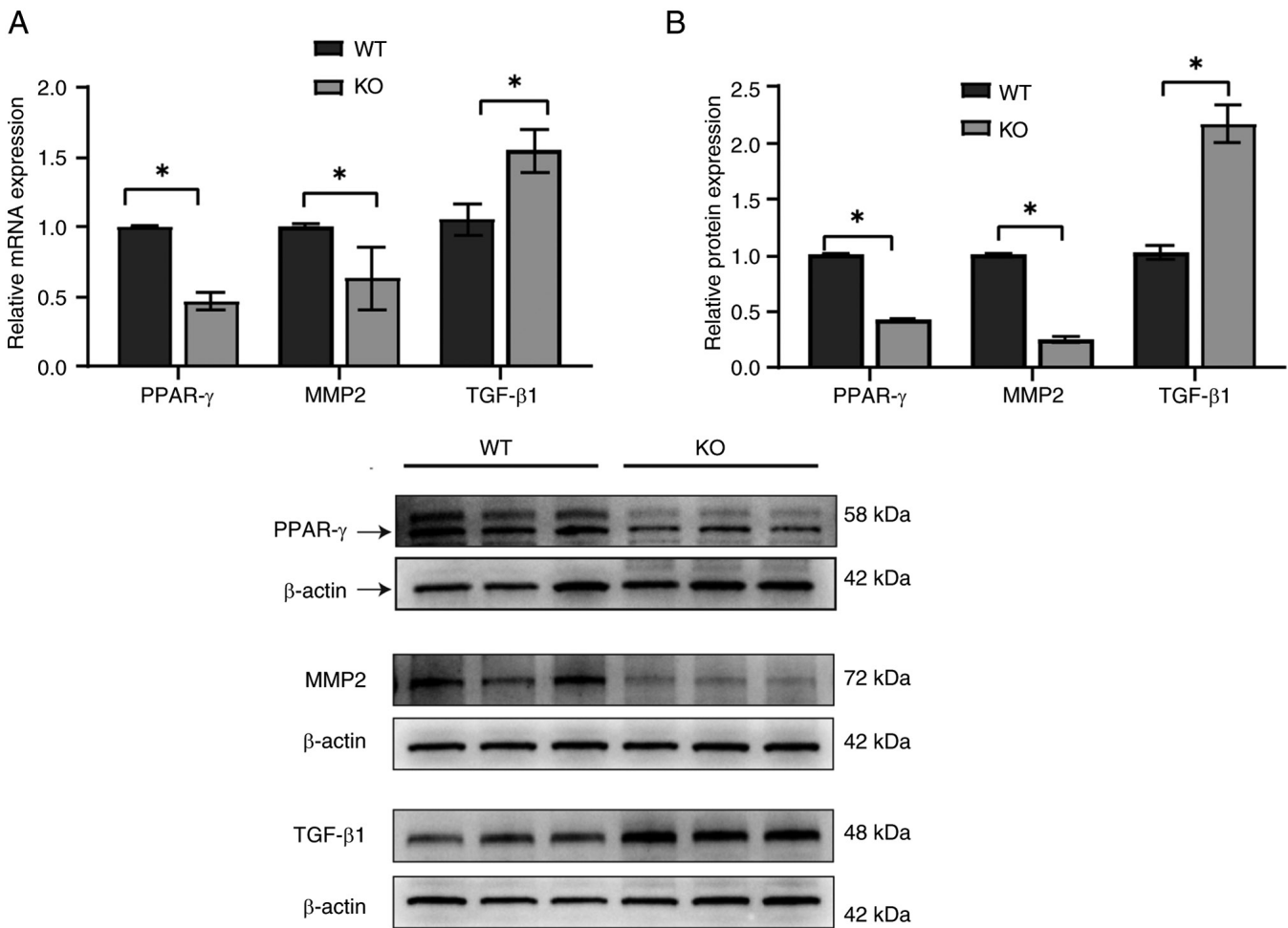


Figure 3. Change in fibrosis-related factors in IncPrep + 96kb-KO mice. (A) mRNA and (B) protein expression levels of TGF- β 1, PPAR- γ , and MMP2. * $P < 0.05$, as determined by independent samples Student's t-test. KO, knockout; WT, wild-type; TGF- β 1, transforming growth factor β 1; MMP2, matrix metalloproteinase 2; PPAR- γ , peroxisome proliferator activated receptor γ .

in patients with PCOS may be closely linked to development of the disease. Furthermore, in animal models of PCOS, the protein levels of TGF- β 1 in both the serum and ovarian tissue have been shown to be significantly higher compared with those in the controls (61). According to Miao *et al* (19) rosiglitazone can reduce ovarian fibrosis by lowering TGF- β 1 levels in the blood and ovarian tissues of rats with PCOS. In the current study, an increase in TGF- β 1 expression was observed in IncPrep + 96kb-KO mice, alongside downregulated PPAR- γ and MMP2 expression. These findings suggested that alterations in these fibrosis-related factors may drive the ovarian fibrosis observed in IncPrep + 96kb-KO mice.

POP is a serine endopeptidase that serves a vital role in physiological processes through its proteolytic activity. Abnormal POP expression has been reported to be associated with Alzheimer's and Parkinson's diseases, and inhibiting of POP is considered a promising strategy for treating neuropsychiatric disorders (62). Cavašin *et al* (63) reported that POP can also participate in the synthesis of the anti-fibrotic peptide N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP); Ac-SDKP inhibits renal fibrosis by suppressing TGF- β signaling (64). Additionally, POP can reduce liver cell activation by blocking the TGF- β signal transduction pathway (65) and inducing PPAR- γ , which offers certain potential for liver fibrosis. The

present study demonstrated that POP expression was decreased in the ovaries of IncPrep + 96kb-KO mice, whereas the overexpression of IncPrep + 96kb in granulosa cells promoted POP expression. Given that IncPrep + 96kb is located within a conserved non-coding sequence of the POP gene, it may be hypothesized that POP is the target gene of IncPrep + 96kb. Furthermore, overexpression of POP led to a significant decrease in TGF- β 1 expression, coupled with increased levels of MMP2 and PPAR- γ , suggesting that POP may mediate the ovarian fibrosis observed in IncPrep + 96kb-KO mice by regulating the expression levels of TGF- β 1, MMP2 and PPAR- γ .

In conclusion, the present study generated IncPrep + 96kb-KO mice and observed that IncPrep + 96kb KO was associated with increased ovarian fibrosis. As a target of IncPrep + 96kb, POP serves a key role in regulating TGF- β 1, PPAR- γ and MMP2 expression, contributing to the development of ovarian fibrosis. Despite the progress made in the current study, there are still limitations that need to be addressed. Firstly, the present study focused solely on the regulation of ovarian fibrosis by IncPrep + 96kb via POP; however, ovarian fibrosis is a complex process, and other mechanisms are likely involved. Future research should investigate additional pathways to gain a more comprehensive understanding of the regulatory mechanisms underlying

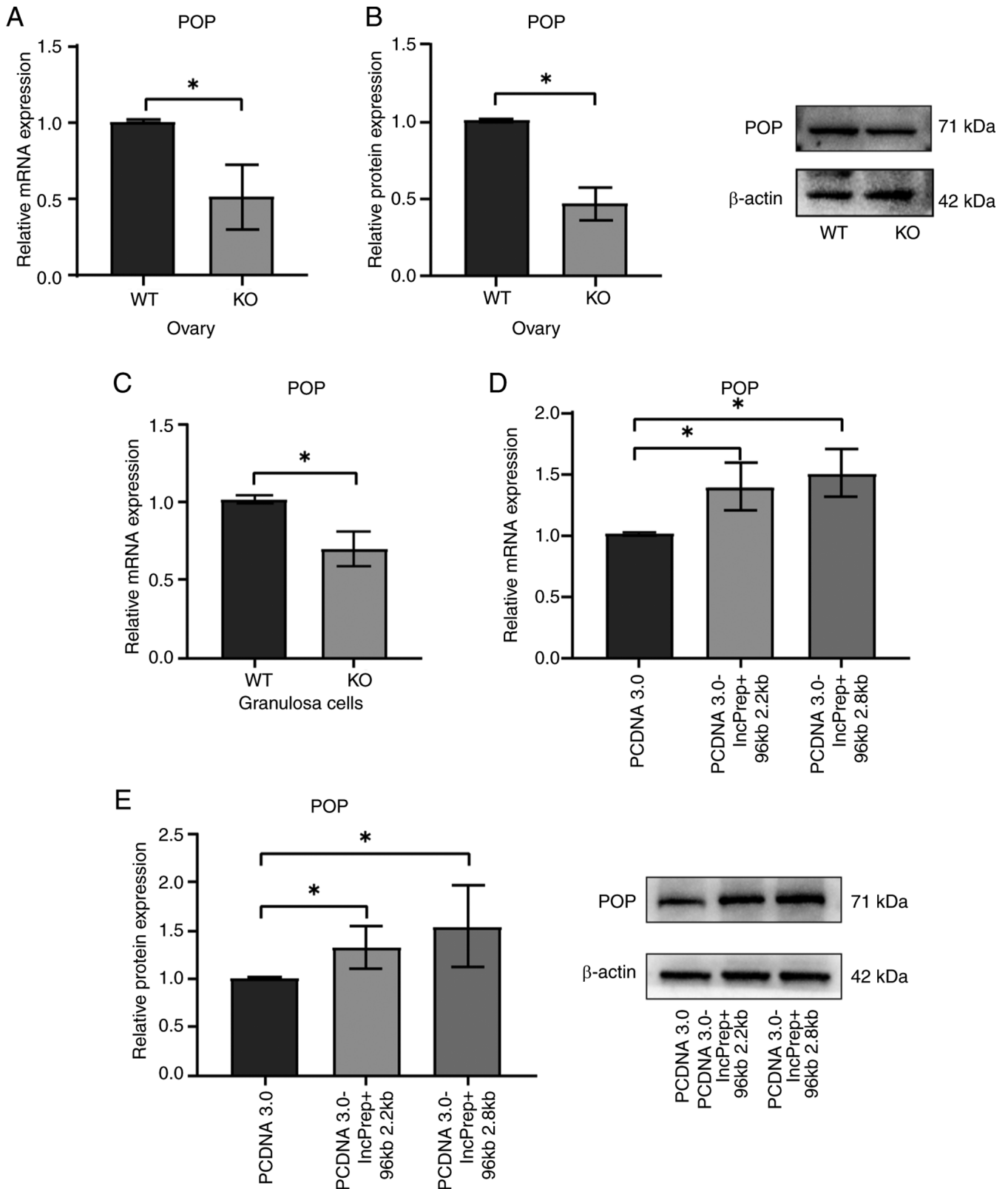


Figure 4. Impact of IncPrep + 96kb on POP expression. (A) mRNA and (B) protein expression levels of POP in the ovaries of mice. (C) mRNA expression levels of POP in granulosa cells. (D) mRNA and (E) protein expression levels of POP following overexpression of IncPrep + 96kb 2.2kb and 2.8kb. * $P < 0.05$, as determined by independent samples Student's t-test or one-way ANOVA followed by Student-Newman-Keuls test. KO, knockout; WT, wild-type; POP, prolyl oligopeptidase.

ovarian fibrosis. Since IncPrep + 96kb exhibits specific expression in granulosa cells and is crucial for follicular development, other mechanisms may also be responsible for

the observed reduction in fertility and ovulation. Further studies are needed to investigate how IncPrep + 96kb regulates ovarian function and impacts fertility.

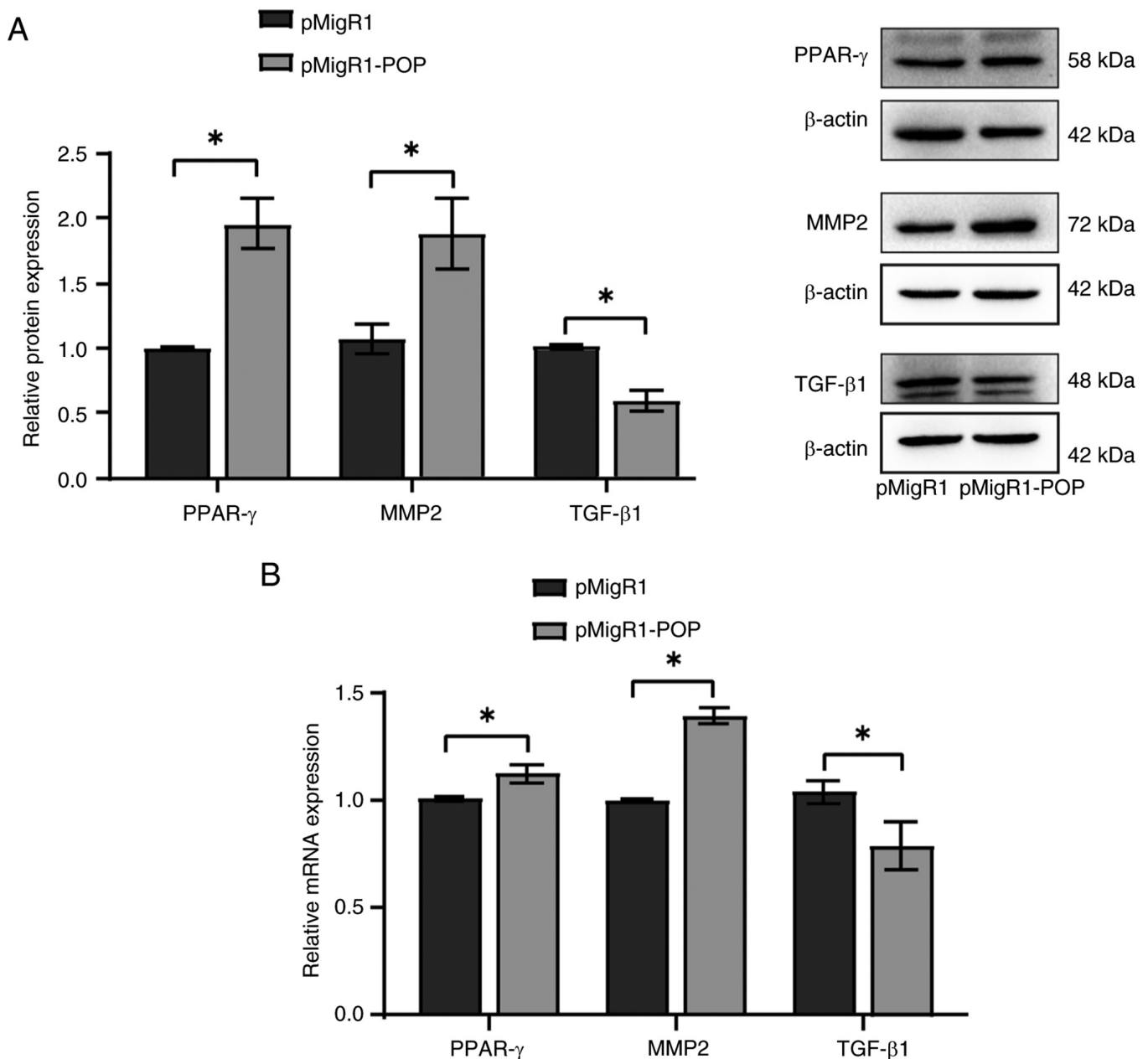


Figure 5. Effects of POP on the regulation of fibrosis-related factors. (A) Protein and (B) mRNA expression levels of PPAR-γ, MMP2 and TGF-β1 following overexpression of POP (pMigR1-POP). *P<0.05, as determined by independent samples Student's t-test. TGF-β1, transforming growth factor β1; MMP2, matrix metalloproteinase 2; PPAR-γ, peroxisome proliferator activated receptor γ; POP, prolyl oligopeptidase.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HZ, JW and CZ performed the experiments, analyzed the data and wrote the manuscript. XF, LX, YJ and XL analyzed the data. YH, SW and JL performed the experiments. HZ, CZ and QH designed the study and performed experiments. SW, LX and CZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee at Nanchang University (approval no. NCULAE-202209280023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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