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Original Research

WT1 influences apoptosis and proliferation of immature mice granular cells through regulation of the wnt/β-catenin signal pathway

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Abstract: It was to study the influence of Wilms tumor suppressor gene (WT1) on ovarian granular cells (GCs) in mice, and the molecular mechanism involved. LV-WT1 short hairpin ribonucleic acid (shRNA) vector was used to downregulate WT1 expression in granular cells (GCs). The effects of WTI on proliferation and apoptosis of GCs were investigated. Western blot and qRT-PCR were used to assay the mRNA and protein expressions of Bax/bcl-2 in GCs transfected with LV-WT1-RNAi. The expression levels of SUZ12, Wnt5a, Wnt11, Wnt4, Wnt3a, Wnt2 mRNA in GCs were also determined. LV-WT1-RNAi significantly reduced WT1 expression, increased apoptosis and inhibited proliferation of GCs. The inhibition of WT1 had no significant effect on the expression of bcl-2 in GCs. The expressions of Wnt2, Wnt4 and Wnt5a were augmented in WT1-knockdown GCs, relative to non-transfected cells. WT1 activation is necessary for maintaining early survival of GCs in follicles via activation of the Wnt/β-catenin signal pathway.

Key words: WT1; Apoptosis; Granulosa cells; Wnt/β-catenin signaling.

Introduction

During the process of growth and development, most follicles undergo a degenerative process known as follicular atresia, with less than 0.1% ovulation rate. Previous studies have shown that follicular atresia is caused by apoptosis of the ovarian GCs (1, 2), in pigs (3), ewes (4), cows (5), and mice (1). Excessive follicular atresia may lead to infertility. Apoptotic signal pathways mediated by death-receptor and mitochondria-mediated type I programmed cell death systems are active in mammalian ovaries of GCs (6-8). However, the intracellular signal pathway involved in the apoptosis of GCs has not been determined.

The Wilms tumor suppressor gene (WT1) was originally identified as a tumor suppressor involved in the formation of kidney Wilms tumor, and was subsequently found to be associated with genito-urinary disorders such as Denys Drash and Frasier syndromes (9-12). Lately, it has emerged as a powerful inhibitor of apoptotic cell death in the developing kidney and male germ cells (13). Furthermore, considerable evidence suggest that WT1, as a transcription factor, activates or inactivates genes involved in apoptosis, growth and differentiation of GCs (14-16). There is a dearth of information on the pro- or anti-apoptotic functions of WTI and the associated regulatory mechanisms in GCs, especially in the early FSH-independent follicular developmental phases (14).

Recently, Sanchez *et al*. reported that WNT4 up-regulation and WNT3 down-regulation of WNT pathway may specifically regulate follicular development, ovulation and Latinization, resulting in reduction of chemotherapy-induced follicular damage (17). The regulation of the WNT signaling pathway in bovine granulosa cells is associated with follicular growth and steroidogenesis (18). It has been reported that triptolide induces apoptosis in breast cancer cells via the WNT/β-catenin signaling pathway (19). Some studies have shown that WT1 inhibits WNT/β-catenin signaling (20). However, no direct experimental evidence have been reported for WT1-mediated inhibition of beta-catenin signaling pathways. It has been speculated that a dysregulated specific gene in the WNT/β-catenin signaling pathway may be involved in granulosa cell apoptosis in endometriosis (21). Studies have shown that the WNT/β-catenin signaling pathway plays an important role in ovarian development in embryonic mice (22). Thus, the WNT/β-catenin signaling pathway plays an important role in mammalian ovarian and ovarian granulosa cells.

To investigate whether WT1 affects the GCs' survival in the FSH-independent stages of early follicle development, cell proliferation and apoptosis were assayed through EdU and flow cytometry. Furthermore, the expression of several key genes involved in major pathways of Wnt signaling were measured. The results suggest that WT1 is essential in maintaining the development and survival of GCs through regulation of apoptosis via the Wnt/β-catenin signaling pathway.

Materials and Methods

Animals and chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis. MO, USA). Female ICR mice aged 21 d were purchased from Zhaoyan New Drug Research Center Co. Ltd. (Suzhou). Ethical approval was obtained from the Animal Ethics Committee of Soochow University, in compliance with the Experimental Animal Regulations of the National Science and Technology Commission, China.

GCs preparation

The mice were sacrificed via cervical spine dislocation. Mice ovaries were excised under sterile conditions. After washing thrice in PBS, the ovaries were placed in DMEM/F12 with 5 % fetal bovine serum (FBS) in cell incubator for 15 min. Ovarian follicle samples were taken through repeated needle puncturing, and were transferred into DMEM culture medium containing 0.1% hyaluronidase digestion for 2 min. The ovarian debris was removed by filtering through a 200-mesh sieve. After one rinse, the cells were cultured overnight for adhesion in DMEM/F12 medium supplemented with 10 % FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate.

Hematoxylin and Eosin staining

Coverslips with 80% cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing twice with PBS, the cells were stained with Hematoxylin and Eosin, and mounted with resin. Cell images were captured in Olympus FSX100 (Olympus, Tokyo, Japan).

Immunocytochemistry (ICC)

The coverslips with GCs were washed three times with PBS and fixed in 95 % ethanol for 30 min. The coverslips were treated with 3 % H_2O_2 and goat serum for 30 min. To block endogenous peroxidase and nonspecific binding sites, the coverslips were incubated with FSHR polyclonal antibody (22665-1-AP, Proteintech Biotechnology Inc.) at a working dilution of 1:100 overnight at 4°C. Then, the coverslips were incubated with anti-rabbit biotin-conjugated secondary antibody for 30 min at room temperature, and thereafter stained for 5 min with the substrate 3', 3-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin for 5 min. The coverslips were treated in triplicate. The results of ICC were evaluated under a light microscope.

Immunofluorescence (IF)

Coverslips with 80 % cells were fixed with 4 % paraformaldehyde, and were permeabilized for 10 min with 0.1% Triton in PBS. Then, the cells were blocked for 1 h with 4% BSA-PBS at room temperature and incubated overnight at 4°C with anti-Wilms tumor protein antibody (1:50 dilution; Abcam, Cambridge, MA). After washing the cells 5 times with PBS, they were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG in 1% BSA-PBS secondary antibody. The results of IF were examined under a fluorescence microscope.

Construction of lentivirus WT1 RNAi

The WT1-targeting oligonucleotides (WT1 (NM-144783)) are shown in Figure 1 and Table 1. The lentiviral suspension was purchased from GeneChem Biotechnology Co. Ltd (Shanghai, China). Prior to use, the titer of the lentiviral stock was 8×10^8 titer units (TU)/ml.

EdU assay

Cell proliferation was determined with 5-ethynyl-20-deoxyuridine (EdU) assay using Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Shanghai, China). Ovarian GCs (1.5×10^5) were seeded in 96-well plates and transfected with LV-WT1-RNAi or negative control oligonucleotides. After incubation at 37°C and 5 % CO_2 for 72 h, 50 μM EdU was added, and the mixture was incubated for another 24 h. Thereafter, the cells were fixed with 4 % paraformaldehyde and stained with Apollo Dye Solution. Nucleic acids were stained with Hoechst 33342. The degree of proliferation was calculated in accordance with the manufacturer's instructions. Images were obtained under a fluorescence microscope.

Flow Cytometry

Quantitative assessment of apoptosis was performed by determining the percentage of cells with highly condensed or fragmented nuclei. The cells were first

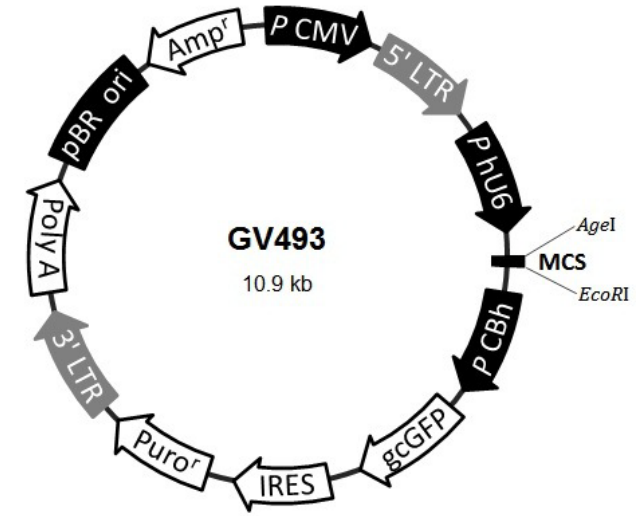

Figure 1. WT1-overexpressing lentiviral vector map.

Table1. Sequences of lentivirus WT1 RNAi and Nc RNAi.

NO.	5'	STEM	Loop	STEM	3'
WT1-RNAi-a	CCGG	CACAGGTGTGAAACCATTCCA	CTCGAG	TGGAATGGTTTCACACCTGTG	TTTTTG
WT1-RNAi-b	AATTCAAAAA	CACAGGTGTGAAACCATTCCA	CTCGAG	TGGAATGGTTTCACACCTGTG	
Nc-a	CCGG	TTCTCCGAACGTGTCACGT	TTCAAGAGA	ACGTGACACGTTCGGAGAA	TTTTTG
Nc-b	AATTCAAAAA	TTCTCCGAACGTGTCACGT	TCTCTTGAA	ACGTGACACGTTCGGAGAA	

detached with 0.25% trypsin and collected after centrifugation at 1,200 g for 5 min. Then, they were washed twice with cold PBS and centrifuged at 1,200 g for 5 min. Following re-suspension, 100 µl solution ($1x10^5$ cells) was transferred to a 5-ml culture tube and 5 µl FITC Annexin V and 5 µl PI were added. After gentle vortexing, the cells were incubated for 15 min at room temperature in the dark. Thereafter, 400 µl 1×Binding Buffer was added to each tube. Apoptosis of the cells was analyzed using flow cytometry within 1 h.

Real-time PCR

Total RNA was extracted from the granulosa cells with TRIzol Reagent kits (Beyotime Institute of Biotechnology, Shanghai, China) in accordance with the manufacturer's instructions. After reverse transcription, qRT-PCR was performed on Applied Biosystems 7500 with specific SYBR Premix Ex TaqTM (TaKaRa, Otsu, Japan). Relative expression level was obtained with $2^{-\Delta\Delta CT}$ method. The primer pairs used for real-time PCR are shown in Table 2.

Western blot analysis

Cell lysates were prepared in RIPA buffer. Total protein content was determined with BCA quantitation kit. Equal amount of protein was first separated using SDS-PAGE and then transferred to PVDF membrane under ice-cold condition. The membrane was blocked with Western Blocking Buffer and then incubated overnight with corresponding primary antibodies against WT1, Bax and Bcl-2 (Abcam, Cambridge, MA) respectively, at 4°C. After rigorous washing with Western Wash Buffer, and incubation with secondary antibody for 1h at room temperature, the target bands were detected using Enhanced Chemiluminescence (ECL), and the intensities of the respective bands were quantified with densitometry.

Statistical Analyses

Data are presented as mean ± SEM of three biological replicates. Analyses of data were carried out using SPSS statistical software package (version 17.0). Comparisons of groups were done with Student's *t*-test or ANOVA. Values of $p < 0.05$ were considered statistically significant.

Results

Identification of mice ovarian GCs

Under the inverted phase contrast microscope, it was seen that the granule cells were spherical, and they adhered to walls after 10-h inoculation. The cells were irregularly polygonal or fusiform after 24 h of cultivation (Figure 2B). The GCs were spread over the dish bottom to form a single layer of cells after 5 days of cultivation. Part of the GCs appeared to aggregate to form colony-

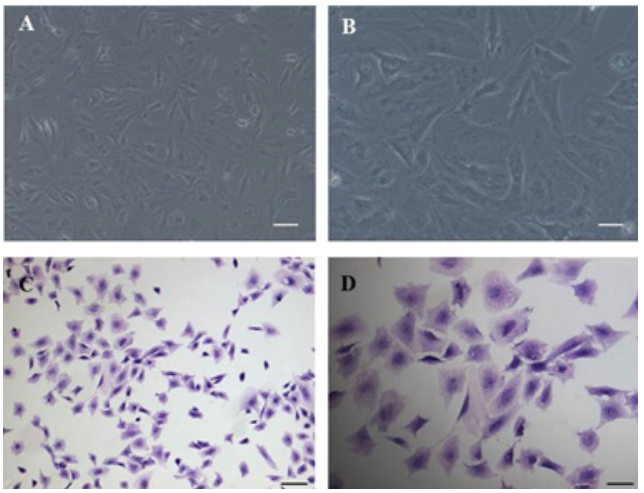

Figure 2. Morphological observation on primary culture of mice ovarian GCs. A, B: White Light; c, d: HE staining. A, C: Scale bar, 200µm; B, D: Scale bar, 100µm.

Table2. Sequences of gene Primers.

Primer	Primer (5'-3')	Product size (bp)
WT1	FP: AGCACGGTCACTTTCGACG RP: GTTTGAAGGAATGGTTGGGGAA	85
β-actin	FP: GAGACCTTCAACACCCCAGC RP: ATGTCACGCACGATTTCCC	263
Bcl-2	FP: GAGAGCGTCAACAGGGAGATG RP: CCAGCCTCCGTTATCCTGGA	108
Bax	FP: AGACAGGGGCCTTTTTGCTAC RP: AATTCGCCGGAGACACTCG	137
Wnt3a	FP: TCCACGCCATTGCCTCAG RP: CACCATCCCACCAAACTCG	120
β-catenin	FP: ATGGAGCCGGACAGAAAAGC RP: CTTGCCACTCAGGGAAGGA	108
Wnt5a	FP: CAACTGGCAGGACTTTCTCAA RP: CCTTCTCCAATGTACTGCATGTG	77
Wnt11	FP: GCACTGAATCAGACGCAACAC RP: CGACAGGGCATACACGAAGG	243
SUZ12	FP: GGCTGACCACGAGCTTTTC RP: TGGTGCGATAAGATTTCGAGTTC	85
Wnt2	FP: CTCGGTGGAATCTGGCTCTG RP: CACATTGTCACACATCACCCT	150
Wnt4	FP: AGACGTGCGAGAAACTCAAAG RP: GGAACTGGTATTGGCACTCCT	210

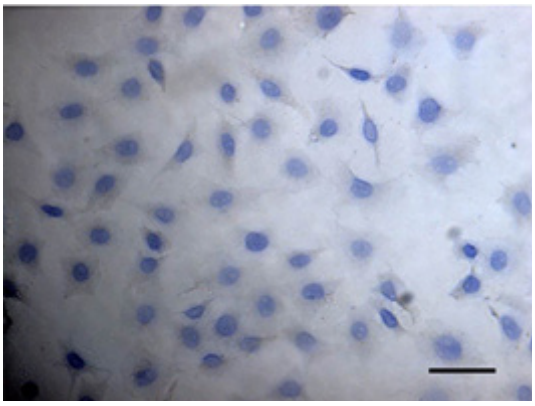

Figure 3. Granular cells (GCs) identification via the expression of FSHR. The expression of FSHR was detected by immunocytochemistry to identify GCs. GCs cytoplasm and membrane stained positive for FSHR. Scale bar, 100μm.

like embryonic stem cells. Results from H&E staining, consistent with microscope observations, showed that the cell morphology was intact. Cells were evenly distributed and polygon-shaped. The cytoplasm was pale red in color, and the nucleus was dark blue (Figure 2D).

The FSHR expression was analyzed through immunocytochemistry to identify GCs. As shown in Figure 3, the cytoplasm and membrane of the GC were visible, with more than 90 % positive FSHR staining. Therefore, the GCs were considered suitable for use in subsequent experiments.

WT1 expression in GCs

Immunofluorescence assays revealed that the expression of WT1 protein in GCs was more localized in the nucleus than in the cytoplasm (Figure 4). These results are in accordance with those reported in previous works which showed that the WT1 was expressed mainly in the nucleus of GCs (23).Total RNA was extracted from GCs for real-time PCR analysis (Figure 5B) , while WT1 protein levels were analyzed with Western blot (Figure 5C). These assays showed that WT1 was specifically expressed in GCs.

LV-WT1-RNAi downregulated WT1 expression

Since the GCs were not susceptible to infection, the MOI was adjusted to approximately 100. A 90% lentiviral infection efficiency was achieved after LV-WT1-RNAi transfection into GCs, as evidenced by GFP expression at 72 h post- infection (Figure 5A). As shown in Figure 5B , endogenous WT1 mRNA was significantly reduced, when compared to the control and the Nc groups ($p < 0.05$). Moreover, RNAi transfection was successful in producing noticeably reduced WT1 protein levels at 72 h post-infection, as determined by Western blot (Figure 5C). These data suggest that RNAi decreases the expression of WT1.

WT1 knockdown inhibited the proliferation of GCs

In order to study the correlation between WT1 expression and GCs, EDU kit was used to analyze the proliferation of GCs. As shown in Figure 6, there was no significant difference in the proliferation of GCs between the transfected negative control and the blank control. However, proliferation in transfected LV-WT1-RNAi group was significantly lower than that in NC group ($p < 0.05$), which indicates that WT1 facilitated the proliferation of GCs.

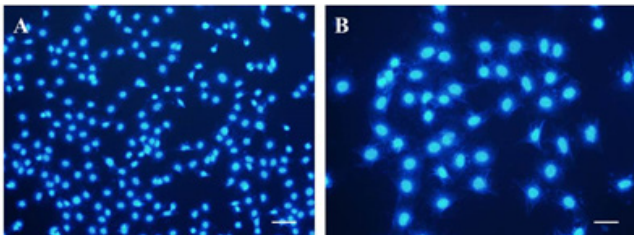

Figure 4. Localization of WT1 in GCs by immunofluorescence assay. GCs expression of WT1 protein was more localized in the nucleus compared to the cytoplasm. a: Scale bar, 400μm; b: Scale bar, 200μm.

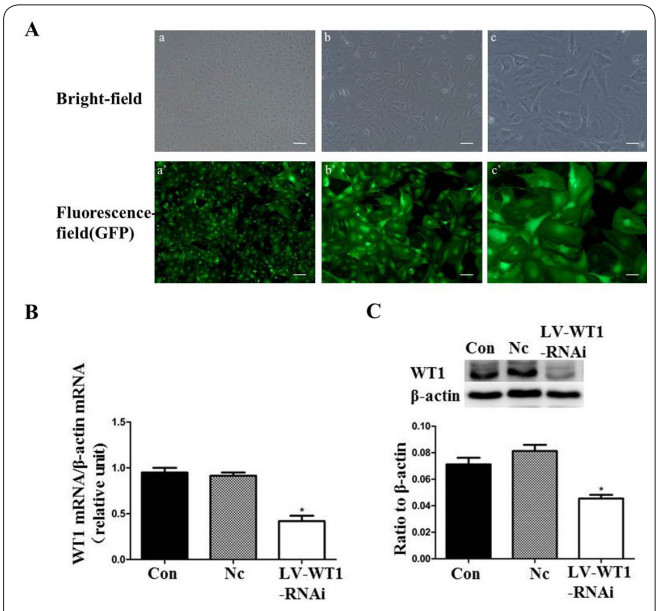

Figure 5. Targeted knockdown of WT1 via lentiviral-mediated RNAi in GCs. A. Images of lentiviral-infected GCs at a MOI of 100.a,a': Scale bar, 400μm; b, b': Scale bar, 200μm; c, c': Scale bar, 100μm. B, The relative expression of WT1 mRNA in GCs, as determined by RT-PCR. C, WT1 protein levels as detected by Western blot. *, $p < 0.05$, compared with Con and Nc. Con, not infected; Nc, negative control.

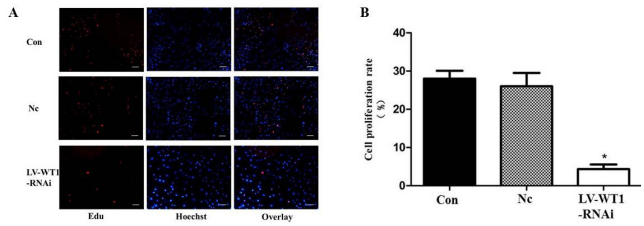

Figure 6. Effect of downregulation of WT1 on GCs proliferation. A. GCs proliferation was analyzed by EdU kits. The nucleus being replicated were fluorescently stained with EdU (red). Nuclei were stained with Hoechst 33342 (blue). B. There was a significantly lower rate of cell proliferation in transfected LV-WT1-RNAi group than that of the Nc group. Scale bar: 400μm. *, $p < 0.05$, compared with Con and Nc.

WT1 knockdown promoted GCs apoptosis

Tunnel techniques used in previous studies inevitably had background interference and easily introduced experimental errors. In the present study, flow cytometry was used to investigate the effects of WT1-knockdown on cell apoptosis. As shown in Figure 7, there were no significant differences in the degree of apoptosis in GCs

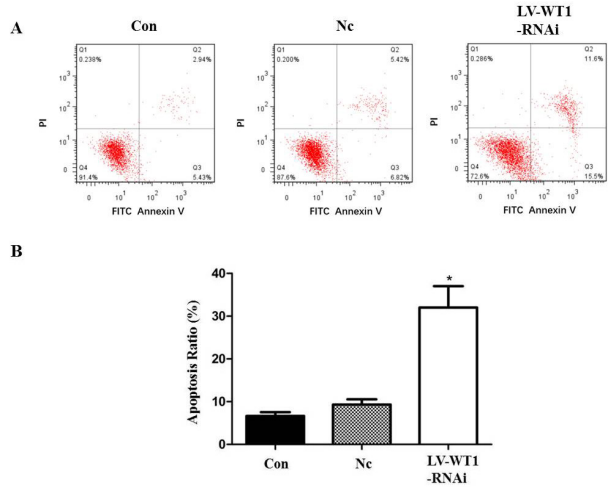

Figure 7. Effect of downregulation of WT1 on GCs apoptosis. A: Apoptosis was evaluated by Annexin V-FITC and PI staining followed by flow cytometry analysis. Percentage of necrotic cells (Q1: Annexin V-FITC−/PI+), late apoptosis cells (Q2: Annexin V-FITC+/PI+), early apoptosis cells (Q3: Annexin V-FITC+/PI−), living cells (Q4: Annexin V-FITC−/PI−). B: There was a greater number of apoptotic cells in WT1-downregulated group than observed in Nc group. *, $p < 0.05$, compared with Con and Nc.

between the control and negative control groups. However, LV-WT1-RNAi markedly promoted apoptosis in GCs, resulting in higher percentage apoptosis in GCs than in LV-Nc infected cells ($p < 0.05$).

Effects of WT1 downregulation on basal Bax and bcl-2 expressions in GCs

Cellular apoptosis was significantly higher in WT1-downregulated GCs than in the control group ($p < 0.05$; Figure 7). Therefore, studies were carried out to determine whether this phenomenon was reflected in changes in apoptosis-related genes by assaying the expressions of Bax (a pro-apoptotic protein) and Bcl-2 (an anti-apoptotic protein) 72 h after LV-WT1-RNAi transfection into GCs. As shown in Figure 8, the expression of Bax was significantly upregulated in WT1-knockdown GCs, when compared with non-transfected cells and negative control LV-transfected cells ($p < 0.05$). In contrast, no changes were seen in Bcl-2 expression, when compared with the control groups ($p>0.05$).

Effect of WT1 downregulation on the Wnt/β-catenin signaling pathway in GCs

Expression levels of a number of key genes that function in major pathways of Wnt signaling, including Wnt/Ca^{2+} pathway (SUZ12), non-canonical (Wnt5a, Wnt11 and Wnt4) planar cell polarity and canonical Wnt/β-catenin (Wnt3a, β-catenin and Wnt2) pathways were quantified. As shown in Figure 9, the expressions of Wnt2, Wnt4 and Wnt5a were upregulated in WT1-knockdown GCs, when compared with non-transfected cells and negative control LV-transfected cells ($p<0.05$).

Discussion

The initiation and progression of follicular atresia are driven by apoptosis of GCs which may happen at various stages of follicle development (1, 24). Despite the characterization of several factors that mediate ovarian

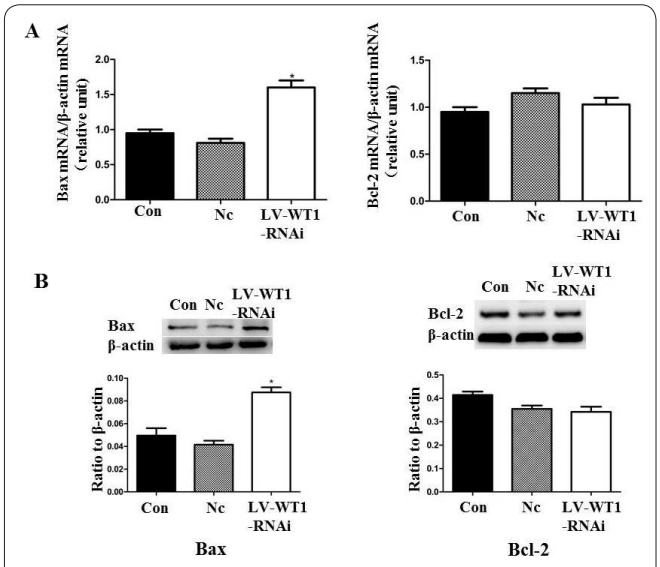

Figure 8. Expression of apoptosis-related gene in GCs. A. The mRNA expression of the apoptosis-related genes Bax and Bcl-2 72 hours post-transfection in control, Nc and WT1-knockdown granulosa cells, as quantified by RT-PCR using β-actin as an internal control. *, $p < 0.05$, compared with Con and Nc. B. The protein expression of Bax and Bcl-2 72 hours post-transfection in control, Nc and WT1-knockdown granulosa cells, as quantified by western blotting using β-actin as an internal control. *, $p < 0.05$, compared with Con and Nc.

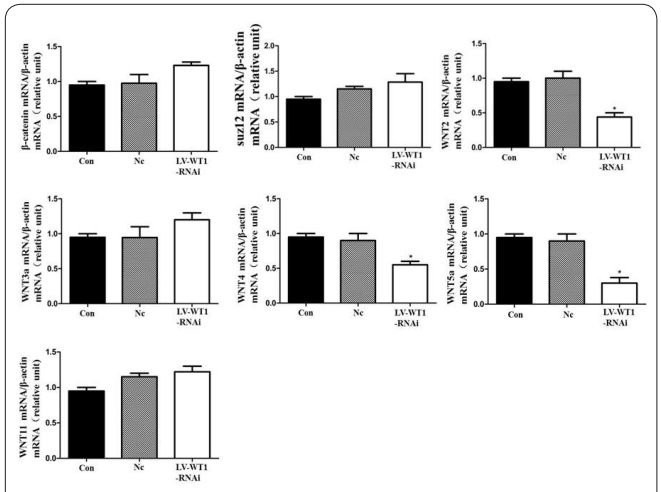

Figure 9. Expression of Wnt/β-catenin signaling pathway -related gene in GCs. Genes related to the Wnt/β-catenin signaling pathways were subjected to mRNA quantification at 72 hours post-transfection in the control, Nc and WT1-knockdown granulosa cells. mRNA expressions were determined via RT-PCR analysis, with gene expressions normalized to housekeeping gene β-actin. *, $p < 0.05$, compared with Con and Nc.

GCs apoptosis (25, 26), not much is known about the survival of preantral follicles in the FSH-independent stage of follicular development. The FSH- independent follicles are considered to be in the early stages of follicle development (27, 28). Therefore, an attempt was made to determine the mechanisms that protect and maintain the viability of early-stage follicles. To this end, an shRNA-based method was used to repress WT1 expression, so as to investigate immature GCs cell survival. The results suggest that WT1 down-regulation inhibited cell proliferation and led to significant apoptosis, which may be related to Wnt/β-catenin signaling pathway.

Studies have led to the view that FSHR is uniquely expressed in female GCs cells (29). Thus, it was used as a standard for the identification of GCs. In this study, data showed that the FSHR in the cultured GCs could reach more than 90 % positive expression. Besides, WT1 was highly expressed in GCs, which further supports the use of FSHR as an ideal marker for GCs.

The expression of WT1 is restricted to some tissues such as the kidney and gonads (30, 31). In the kidney, the WT1 protein has been shown to function as a transcriptional repressor of growth-related genes (32). In contrast, little is known concerning the function and regulation of WT1 in gonads. Studies have shown that WT1 mRNA is found in GCs of ovarian follicles and Sertoli cells of the testis (30, 31). It has also been reported that development of the gonadal ridge was abnormal in WT1-deficient mice (33), suggesting an important role for WT1 in embryonic gonad development. Moreover, it has been reported that WT1 was expressed in ovarian GCs and its expression remained strong during early stages of development, but progressively declined during gonadotropin-dependent follicular maturation in the human ovary (34). Another study showed during follicular growth in sheep ovary, WT1 was expressed in GCs from the type 1 (primordial) to the type 4 stages, but thereafter its expression was reduced in type 5 (antral) follicles (35). Another animal study revealed that the ovary size and number of developing follicles were significantly reduced in Wt1+/R394W mice which was similar to POF in human patients (14). These results indicate that WT1 plays important roles in ovarian follicle development. In the present study, an LV-WT1-RNAi vector was successfully constructed to investigate the role of WT1 in GCs. The LV-WT1 shRNA markedly repressed WT1 in both transcription and expression levels. These data also showed that downregulation of WT1 significantly inhibited cell proliferation and induced cell apoptosis in GCs. These results suggest that WT1 is essential for the GCs survival.

Cellular apoptosis is a tightly controlled process that is mediated by a range of external and internal signals which regulate death-receptors and mitochondrial pathways. Two members of Bcl-2 family, Bcl-2 and Bax, are involved in regulating the release of pro-apoptotic factors, a universal event during the apoptotic process (36). To a large extent, the expression of the bcl-2 hinders apoptosis, while Bax opposes the effect of bcl-2 (37). A study has revealed the presence of abnormal follicles with excessive number of GCs in Bax-deficient mice (38). Moreover,.Bax expression in atresia follicles was stronger than that of human healthy ovarian follicles (39). Moreover, Bcl-2-deficient mice have been shown to have reduced number of follicles (40), and overexpression of Bcl-2 resulted in decreased follicular apoptosis and atresia (41, 42). In a previous study, over-expression of WT1 was shown to prevent apoptosis of GCs from immature 25-day-old rats, through the regulation of Bax (43). The results from the present study suggest that downregulation of WT1 inhibits cell proliferation and lead to significant apoptosis through the regulation of Bax. However, further investigations to delineate the exact mechanisms that lead to Bax downregulation are necessary to fully understand this process. In this study, there were increased apoptosis and decreased proliferation in WT1 knockdown GCs, and decreased expressions of Wnt2, Wnt4, and Wnt5a. These data suggest that WT1 regulates the development of GCs by activating different branches of the Wnt/β-catenin signaling pathway. The Wnt4 protein is a critical player during prenatal and postnatal follicular development, maturation, and survival. Murine GCs subjected to targeted Wnt4 deletion brought about atrophied ovaries in mice, leading to decreased fertility and less healthy antral follicles at the 42nd day of life, when compared with mice with intact, unaltered GCs (44). The major phenotypic outcome of the conditional Wnt4-knockout mouse models is POF (45). Experiments where a heterozygous WT1 gene mutation caused female mice to develop reproductive impairments have highlighted the critical role of WT1 in modulating the development of ovarian follicles (14). The WT1 protein is known to induce Wnt4 expression in GCs (14). It has been proposed that WT1 carries out its regulatory effects on the Wnt4 gene through direct binding to a distant enhancer or promoter region on this gene (46). These findings indicate the importance of local GC expression of Wnt4 in stimulating follicular development.

It has been demonstrated that Wnt2 is expressed only in follicular GCs, suggesting that it may be a modulator of ovarian function and follicular development (47). Mice with Wnt2 gene mutations developed defects in placenta formation, although the surviving offspring hadd normal fertility (48). It has also been suggested that the effects of Wnt2 on GCs proliferations may be mediated by β-catenin (49). Nevertheless, the results obtained in the present study show that the effect of Wnt2 on GCs proliferation and apoptosis was mediated by WT1, and was not related to β-catenin. This study reports for the first time, the role of Wnt5a in follicular development, a phenomenon regulated by WT1. The Wnt5a and Wnt11 proteins are highly expressed in the GCs of mice (50). Moreover, Wnt5a transcripts have been detected in human luteinized GCs and cumulus cells (21, 51). Studies performed utilizing conditional gene targeting to attenuate Wnt5a expression (while preserving Wnt11) demonstrated that a lack of Wnt5a resulted in subfertility in female mice, accompanied by reduced ovulation rates and higher incidence of follicular atrophy (52).

The results obtained in this study have demonstrated that WT1 is essential in maintaining the development and survival of GCs. The knockdown of WT1 led to inhibition of the GCs proliferation and increased apoptosis, which may be related to the Wnt/β-catenin signaling pathway. These findings enable a better understanding of the regulatory mechanism of ovarian follicle development.

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Conflict of Interest

There are no conflict of interest in this study.

Author's contribution

All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Caiping Mao; Juan Wang, Jianping Qiu, Le Bo, Zhinan Wu, Anwen Zhou, Wendan Xu, Caiping Mao collected and analysed the data; Juan Wang and Jianping Qiu wrote the text and all authors have read and approved the text prior to publication.

Juan Wang and Jianping Qiu contributed equally to this work and should be considered as co-first authors.

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