**Wt1 functions in ovarian follicle development by regulating granulosa cell differentiation**

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The **Wt1** gene encodes a nuclear transcription factor that is specifically expressed in ovarian granulosa cells. However, the physiological significance of **Wt1** in ovarian follicle development remains elusive. In this study, we found that **Wt1**+/**R394W** mice were grossly normal, however, the females displayed severe reproductive defects. Only ~15% of the **Wt1**+/**R394W** females became pregnant after mating with wild-type males, compared with 88.2% of control females. Further study revealed that the subfertility of **Wt1**+/**R394W** females was caused by aberrant ovarian follicle development. Compared with control females, the ovary size and the number of developing follicles was significantly decreased in **Wt1** mutant ovaries which was very similar to premature ovarian failure (POF) in human patients. The results of in vitro studies demonstrated that the expression of **follicle stimulating hormone receptor** (FSHR), 3β-hydroxysteroid dehydrogenase and **Aromatase** was inhibited by **Wt1** in granulosa cells, and mutation of **Wt1** resulted in the upregulation of these genes and in the premature differentiation of granulosa cells. We also found that **Wt1** was likely involved in granulosa cell development via the regulation of **E-cadherin** and **Par6b** expression. Mutation in **Wt1** caused defects in polarity establishment in granulosa cells, which also likely contributed to the observed aberrant follicle development. The results of this study provide new mechanisms for understanding the regulation of ovarian follicle development and potential pathological cause of POF in human patients.

**INTRODUCTION**

In mammals, ovarian follicular development is a continuous process. In mice, the primordial follicles are formed within days after birth, and the oocytes are surrounded by a single layer of squamous somatic cells termed pregranulosa cells. The primordial follicles remain in a dormant state until they are activated by endogenous and exogenous factors. Upon activation, the primordial follicles develop into primary follicles and secondary follicles before acquiring an antral cavity. Preovulatory follicles are formed with further growth, and the oocytes are released after follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulation. In fact, only a small percentage of follicles undergo this process and release oocytes. Most of the developing follicles are lost as a result of atresia at different developmental stages (1–3). The development of early stage follicles (primordial to primary stages) is independent of FSH, as demonstrated by the absence of follicle stimulating hormone receptor (FSHR) in granulosa cells. From the preantral follicle, the granulosa cells acquire gonadotropin receptors and the ability to synthesize estrogen, which is important for follicle development and oocyte maturation (4–6). Premature ovarian failure (POF) is defined as 6 months of amenorrhea which occurs before 40 years of age with elevated serum concentrations of FSH (7). It is one of the most common diseases that causes female infertility (8). The pathological causes of POF include genetic mutations, chromosomal abnormalities, autoimmune diseases, environmental factors, infections and iatrogenic agents (9–13).

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The Wilms’ tumors (WT) gene WT1 encodes a nuclear zinc finger transcription factor that is expressed in urogenital ridge mesenchymal cells during embryonic development (14–16). In adults, WT1 is expressed in kidney podocytes, ovary granulosa cells, testis Sertoli cells, oviduct epithelial cells and uterine stromal cells (17,18). WT1 was originally identified as a tumor suppressor gene associated with the development of WT and was subsequently identified as being mutated in patients with Denys–Drash syndrome (DDS) (19–23). The most common WT1 mutation found in DDS patients is a C-to-T transition, amino acid 394 (R394W) (24,25). To investigate the mechanism by which this missense mutation causes DDS in humans, we generated a mouse strain carrying a C1180T point mutation (R394W) previously. We found that the WT1 homozygous mutant mice were embryonic lethal, and WT1+/R394W mice faithfully mimics the renal failure observed in DDS patients (26).

Wt1 is first expressed in the somatic cells of the ovary after sex determination at E12.5 in mice and is subsequently expressed in the granulosa cells of primordial and primary follicles, suggesting that WT1 likely plays important roles in ovarian development or folliculogenesis. However, the precise function of WT1 in female reproduction remains unclear. A previous study found that WT1+/− females are infertile and that embryonic development is blocked due to defects in oviduct functions (27). In the present study, we found that WT1+/+R394W females were subfertile, but the embryonic development in oviduct was normal, and the subfertility was due to defects in follicle development. The ovulation rate of WT1+/+R394W females was dramatically reduced compared with that of control females. Further investigation revealed that the WT1 mutation resulted in premature differentiation and abnormal cell polarity establishment of granulosa cells, which in turn led to aberrant follicle development and female subfertility.

**RESULTS**

**WT1+/+R394W female mice are subfertile**

To test fertility, WT1+/+R394W and control female mice were co-caged with wild-type males and checked for the presence of a vaginal plug the next morning. Plugged females were examined for pregnancy at E14.5. As shown in Table 1, 15 out of 17 (88.2%) plugged females in the control group were pregnant, whereas only 14.3% (3/21) of the plugged females in the WT1+/+R394W group were pregnant, representing a dramatic reduction in the ratio of pregnancy.

**Table 1. WT1+/+R394W females were subfertile**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total females</th>
<th>Pregnant females</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>15</td>
<td>88.2</td>
</tr>
<tr>
<td>WT1+/+R394W</td>
<td>21</td>
<td>3</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Both control and WT1+/+R394W females were mated with wild-type males, and only females with plugs were counted in this experiment. The females were sacrificed at E14.5, and the females with embryos were defined as pregnant mice. *a > b, P < 0.01.*

**Table 2. The ratio of ovulation was dramatically reduced in WT1+/+R394W females**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Natural ovulation (average)</th>
<th>Superoovulation (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.0 (n = 7)</td>
<td>64.1 (n = 9)</td>
</tr>
<tr>
<td>WT1+/+R394W</td>
<td>2.0 (n = 7)</td>
<td>4.8 (n = 9)</td>
</tr>
</tbody>
</table>

*a > b, P < 0.01; c > d, P < 0.01.*

stimulation; this difference was significant. These results indicated that the mutation in WT1 reduced the ovulation rate and that the subfertility of WT1+/+R394W females was due to this ovulatory defect.

**Histological analysis of WT1+/+R394W ovaries at different developmental stages**

Given the fact that WT1 was specifically expressed in granulosa cells, the morphology of follicles at different developmental stages was examined by H&E staining. The morphology of primordial follicles was grossly normal in WT1+/+R394W females (Fig. 1B and D) at D3, except that several primary follicles with cuboidal granulosa cells were observed in control follicles (Fig. 1C) but not in WT1 mutant ovaries (Fig. 1D). The total number of primordial follicles was analyzed in serial sections. As shown in Figure 1E, ~4000 primordial follicles were observed in each ovary, and no difference was noted between control and WT1 mutant mice, suggesting that the assembly of the primordial follicle is not affected in WT1 mutant ovaries. Primary follicles with multiple layers of granulosa cells were noted in both control and WT1+/+R394W ovaries of 1-week-old mice (Fig. 2A and B, black arrows); however, compared with control ovaries, the number of primary follicles was dramatically decreased. As shown in Figure 2E, ~205 developing follicles were noted in the control ovaries, whereas only ~44 developing follicles were found in WT1 mutant ovaries. We also found that the morphology of the developing follicle was abnormal in WT1+/+R394W ovaries. In control ovaries, the oocytes were surrounded by well-organized granulosa cells (Fig. 2C, arrows), whereas the granulosa cells in WT1+/+R394W ovaries were disorganized, and the oocytes were only partially surrounded by granulosa cells in some developing follicles (Fig. 2D, arrows). Aberrant follicle development in WT1+/+R394W mice was evident at 3 weeks of age, and the WT1+/+R394W ovaries were significantly smaller than the control ovaries at this stage (Fig. 3E). The total number of developing follicles was also significantly reduced (Fig. 3F) in WT1+/+R394W ovaries. This phenotype is very similar to POF in human patients. In control ovaries, most of the follicles developed to the preantral stage, with multiple...
layers of granulosa cells (Fig. 3A and C, arrowheads). In addition to the reduced follicle number, many aberrant follicles with undifferentiated squamous granulosa cells were observed in control follicles but not in Wt1 mutant ovaries. No difference in primordial follicle number was noted between control and Wt1 mutant mice (E).

Aberrant development of in vitro cultured primary follicles from Wt1+/R394W ovaries

To further examine the follicle development in Wt1+/R394W mice, primary follicles with 2–3 layers of granulosa cells were dissected from the ovaries of 2-week-old females. Follicles with normal morphology were cultured in vitro, and follicular development was examined microscopically. As shown in Figure 4, the control (A) and Wt1 mutant (C) follicles were morphologically indistinguishable at D1. After 11 days of culture, >80% of the control follicles developed to the preovulatory stage, with a notable antrum (Fig. 4B). In contrast, only ~11% of Wt1 mutant follicles were morphologically normal (Fig. 4F); most of them contained oocytes that were detached from the granulosa cells (Fig. 4E) or were relatively small (Fig. 4D). These results further confirmed that the Wt1 mutation causes defects in follicle development.

Differentially expressed genes during follicle development

Ovarian follicle development is a complex process, and gene expression in granulosa cells varies at different developmental stages. As shown in Supplementary Material, Figure S1, Wt1 was highly expressed in the granulosa cells from the primordial (A, arrows) to the primary and secondary follicle stages (C, arrows); however, its expression was significantly reduced in large follicles (B, arrowheads). The expression of AMH, Wt1, FSHR, LHR and Aromatase was also assessed by real-time PCR analysis using RNA from the ovaries of 2-week-old, 3-week-old and PMSG-treated 3-week-old mice. As shown in Supplementary Material, Figure S2, most of the follicles in the ovaries of 2-week-old mice were primary and secondary (Supplementary Material, Fig. S2A), and the majority of follicles in the ovaries of 3-week-old mice had developed into preantral and antral follicles (Supplementary Material, Fig. S2B). Preovulatory follicles were present in the ovaries of PMSG-treated mice.
3-week-old mice (Supplementary Material, Fig. S2C). Real-time PCR showed that the expression of AMH and Wt1 was gradually reduced from primary to preovulatory follicles, whereas the expression of LHR and Aromatase was dramatically increased in preovulatory follicles compared with primary follicles (Supplementary Material, Fig. S2D). These results suggested that Wt1 likely repressed the expression of differential marker genes in granulosa cells. The expression of cell polarity genes (E-cadherin and Par6b) and Wnt/PCP-associated genes (Wnt4 and Wnt11) was also significantly reduced in preovulatory follicles compared with primary follicles (Supplementary Material, Fig. S2E), indicating that the granulosa cells likely lost their polarity during the course of follicle development.

Differentially expressed genes in Wt1+/R394W ovaries

To explore the molecular mechanisms underlying the defects in Wt1 mutant follicle development, the expression of genes that play important roles in follicle development was examined by real-time PCR and immunohistochemistry. 3β-Hydroxysteroid dehydrogenase (3β-HSD) protein was mainly expressed in the interstitium (Fig. 5A and C, arrowheads) of the ovaries of 3-week-old control mice, and no signal was noted in the granulosa cells (Fig. 5A and C, arrows). In Wt1 mutant ovaries, 3β-HSD protein was detected not only in the interstitium (Fig. 5B and D, arrowheads) but also in the granulosa cells of small follicles (Fig. 5B and D, arrows). The results of real-time PCR showed that the AMH mRNA level was significantly reduced in Wt1+/R394W ovaries, whereas the expression of LHR, FSHR, Aromatase and p450scc was significantly increased in Wt1+/R394W ovaries compared with control ovaries in 3-week-old mice (Fig. 5E).

The expression of FSHR, LHR and Aromatase is repressed by Wt1 in granulosa cells

To further examine the relationship between Wt1 and granulosa cell-specific marker genes, the differentiated granulosa cells isolated from PMSG-treated 3-week-old females were cultured in vitro and transfected with a Wt1-expressing adenovirus. As shown in Figure 6A, the expression of FSHR and Aromatase was significantly decreased in Wt1-transfected granulosa cells.
Wt1 transfection, but this difference was not significant. However, AMH expression was notably increased in Wt1-overexpressing granulosa cells. These results indicated that the expression of FSHR and Aromatase was repressed by Wt1, whereas the expression of AMH was induced by Wt1.

The expression of E-cadherin, Par6b and Wnt4 is induced by Wt1 in granulosa cells

Previous results (Supplementary Material, Fig. S2E) showed that E-cadherin, Par6b and Wnt4 were highly expressed in undifferentiated granulosa cells and dramatically reduced in differentiated granulosa cells of large follicles which was consistent with the expressing pattern of Wt1. To test whether the expression of these genes was regulated by Wt1, real-time PCR was performed. As shown in Figure 6B, the expression of E-cadherin, Par6b and Wnt4 was significantly increased in Wt1-overexpressing granulosa cells, indicating E-cadherin, Par6b and Wnt4 expression was also induced by Wt1. To examine whether cell polarity is associated with the differentiation of ovarian granulosa cells, in vitro cultured granulosa cells were transfected with a Wnt4-expressing adenovirus. As shown in Figure 6C, the expression of Par6b and E-cadherin was significantly increased in Wnt4-overexpressing granulosa cells; however, the mRNA levels of FSHR, LHR, Aromatase and AMH were unchanged, suggesting that the differentiation of granulosa cells is independent of the Wnt/PCP pathway.
The expression of Bmp2 is regulated by Wt1

To explore the mechanism through which Wt1 regulates granulosa cell differentiation, the expression of bone morphogenetic protein 2 (Bmp2), Bmp6, Usf1 and Usf2 in Wt1 mutant ovaries and Wt1-overexpressing granulosa cells was examined. Real-time PCR results showed that the mRNA levels of these genes were not significantly altered in the ovaries of Wt1 mutant mice at 3 weeks of age (Supplementary Material, Fig. S3A). However, the expression of Bmp2 was significantly increased in Wt1-expressing adenovirus-transfected granulosa cells (Supplementary Material, Fig. S3B), suggesting that the expression of Bmp2 was regulated by Wt1.

DISCUSSION

Wt1 gene has been demonstrated to play important roles in heart (28) and testes (29) development. Mutation of Wt1 is associated with DDS syndrome (19–23), our recent study found that Wt1 mutation was associated with nonobstructive azoospermia (NOA) in human patients (30). Wt1 gene is also expressed in ovarian granulosa cells (17,18); however, the precise function of Wt1 in ovarian follicle development remains unclear. It has been reported previously that Wt1+/- females were infertile and the embryo development was blocked due to the defects of oviduct (27). In the present study, we found that mutation of the Wt1 gene resulted in subfertility in female mice. Histological study revealed that the subfertility of Wt1+/- females was caused by aberrant follicle development. The possible explanation for this discrepancy is that different mice strains were used. In our study, B6/129 mixed background mice were used. Whereas, mice with 129 background were used in Kreidberg’s study (27). The ovary size and number of developing follicles were significantly reduced in Wt1+/+R394W mice which was similar to POF in human patients. These results indicate that Wt1 plays important roles in ovarian follicle development.

Ovarian follicle development is a complex process that begins with the primordial follicles. In each estrus cycle, a cohort of primordial follicles is activated and sequentially gives rise to primary, secondary, preantral, antral and preovulatory follicles. After the oocytes are released in response to an LH surge, the residual follicle cells form the corpus luteum. During follicle development, the granulosa cells proliferate and differentiate. The early stages of follicle development are independent of pituitary gonadotropins such as FSH and LH because the corresponding receptors are absent from the granulosa cells (31,32). The granulosa cells begin to express FSHR at the preantral stage; then, in response to stimulation by FSH, these cells express steroidogenic enzymes (cholesterol side-chain cleavage cytochrome P450 (p450SCC), 3β-HSD and cytochrome P450 aromatase) and acquire the ability to synthesize steroid hormones (33–35). The luteinizing hormone receptor is expressed in the granulosa cells of preovulatory follicles that have been stimulated by FSH (36).

To explore the molecular mechanism of Wt1 in ovarian follicle development, the expression of genes that are important for follicle development was examined by immunohistochemistry and real-time PCR. Interestingly, the 3β-HSD protein was detected in granulosa cells of small follicles in Wt1 mutant ovaries. As a steroidogenic enzyme, 3β-HSD is typically only expressed in theca-interstitial cells and differentiated granulosa cells of antral and preovulatory follicles (37). Further study revealed that the mRNA levels of genes expressed in differentiated granulosa cells (FSHR, Aromatase and p450SCC) were also significantly increased in Wt1+/+R394W ovaries compared with control ovaries at 3 weeks of age. Wt1 was abundantly expressed in the undifferentiated granulosa cells of small follicles, and its expression was dramatically reduced in differentiated granulosa cells (18). Therefore, we speculate that Wt1 likely inhibits granulosa cell differentiation by repressing the expression of FSHR, Aromatase and p450SCC; additionally, mutation of Wt1 results in the early differentiation of granulosa cells.

To further confirm this hypothesis, Wt1 was overexpressed in the differentiated granulosa cells after PMSG treatment. We found that FSHR and Aromatase expression was significantly decreased in Wt1-overexpressing cells compared with control virus-transfected granulosa cells. These results confirmed that the expression of FSHR and Aromatase was inhibited by Wt1 in granulosa cells. Given that the expression of LHR, 3β-HSD and Aromatase is regulated by FSH, the differential expression of these genes was most likely due to a change in FSHR expression rather than direct regulation by Wt1.

FSH signaling plays essential roles in follicle development, and the inactivation of FSHR results in female infertility with follicular development arrest in the pre-antral stages in a mouse model (38). FSH stimulates adenylyl cyclase activity and increases the production of cAMP by interacting with its receptors in granulosa cells (39), thereby activating the expression of target genes, which leads to granulosa cell differentiation. FSHR is expressed in granulosa cells, and the mechanisms regulating its expression have been studied extensively. In this study, no potential Wt1-binding site was identified in the promoter region of the FSHR gene, suggesting that Wt1 is not likely to interact directly with the FSHR promoter. Previous studies have demonstrated that upstream stimulatory factor 1 (Usf1) and Usf2 regulate FSHR expression by interacting with an E-box in the proximal promoter (40). However, real-time PCR showed that the expression of Usf1 and Usf2 was not altered in Wt1 mutant ovaries or Wt1-overexpressing granulosa cells, suggesting that Wt1 does not regulate FSHR expression via Usf1 and Usf2. It was previously reported that bone morphogenetic protein 2 (Bmp2) blocks FSH-induced FSHR expression by inducing the phosphorylation of SMAD1/5/8 in hen granulosa cells (41), whereas Bmp6 induces FSHR expression in these cells (42). In the present study, we found that the expression of Bmp2 was significantly upregulated in Wt1-overexpressing granulosa cells. These results suggest that Wt1 likely inhibits FSHR expression by inducing Bmp2 expression in granulosa cells; however, the detail mechanism needs to do further investigation.

In this study, we also found that the cell polarity genes (E-cadherin and Par6b) and Wnt/PCP-associated genes (Wnt4 and Wnt11) were abundantly expressed in undifferentiated granulosa cells and that this expression was dramatically reduced in the differentiated granulosa cells of preovulatory follicles, which is consistent with the pattern of Wt1 expression. In vitro studies confirmed that the expression of E-cadherin, Par6b and Wnt4 was induced by Wt1 in granulosa cells. These results suggest that the granulosa cells in small follicles are polarized epithelial cells with high levels of E-cadherin and Par6b expression. As follicle development progresses, the expression of
E-cadherin and Par6b decreases, and the granulosa cells lose their polarity in large follicles. Our in vitro study confirmed that the expression of E-cadherin and Par6b was significantly induced by Wt1 in cultured granulosa cells, suggesting that Wt1 is required for the maintenance of cell polarity in granulosa cells at an early developmental stage by inducing E-cadherin and Par6b expression. Another interesting question is whether the loss of cell polarity is associated with granulosa cell differentiation. To address this question, Wnt4 was overexpressed in differentiated granulosa cells. The results showed that the expression of E-cadherin and Par6b was increased significantly; however, the expression of granulosa cell differentiation-associated genes (AMH, FSHR, LHR and Aromatase) was not altered, indicating that a loss of cell polarity is not associated with granulosa cell differentiation.

Other than premature differentiation of granulosa cells, defect of primordial follicles activation was also noted in Wt1 mutant ovaries. Many primary follicles with asymmetric and disorganized granulosa cells were observed in Wt1 mutant ovaries at 1 week of age. The defect in granulosa cell development was evident even at 3 weeks of age, and many follicles without cuboidal granulosa cells were noted in Wt1 mutant ovaries. The granulosa cells undergo a transition from squamous epithelial cells into polarized cuboidal epithelial cells during primordial follicle activation; however, the mechanism that regulates this transition is unknown. Based on the results of this study, we speculate that Wt1 is also likely to be involved in cell polarity establishment or maintenance in granulosa cells by regulating the cell polarity genes E-cadherin and Par6b expression. Our recent study demonstrated that Wt1 gene was involved in Sertoli cell polarity maintenance by regulating E-cadherin and Par6b expression (unpublished data). Thus, a mutation in Wt1 causes a defect in the polarity establishment or maintenance of granulosa cells, which probably also contributes to the aberrant follicle development in Wt1+/R394W females.

In summary, the present study demonstrated that Wt1 played critical roles in ovarian follicle development, and a heterozygous mutation in the Wt1 gene resulted in reproductive defects in female mice. Further study revealed that Wt1 is involved in granulosa cell differentiation regulation via inhibiting FSHR expression and in granulosa cell polarity establishment or maintenance via regulating E-cadherin and Par6b expression. Mutation in Wt1 caused a defect in granulosa cell polarity establishment and premature differentiation, which in turn caused aberrant follicle development. The results of this study provide new mechanisms for understanding the regulation of ovarian follicle development and potential pathological cause of POF in human patients.

**MATERIALS AND METHODS**

**Animals**

All animal work were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) regulations. The mouse strain carrying the Wt1 R394W point mutation was generated in the laboratory of Dr Vicki Huff (26). The mice were maintained on the B6/129 genetic background. DNA isolated from tail biopsies was used for genotyping. The presence of the Wt1 R394W mutant allele was determined by PCR amplification as described previously (26).

**Tissue collection and histological analysis**

The ovaries were dissected immediately following euthanasia. Tissues were fixed in 4% paraformaldehyde for up to 24 h, stored in 70% ethanol and embedded in paraffin, after which sections (5 μm thickness) were cut and mounted on glass slides. Following deparaffinization, the slides were stained with H&E for histological analysis.

**Ovarian follicle isolation and in vitro culture**

Follicle culture was carried out as previously described (43,44). Ovaries of 14-day-old mice were dissected aseptically and transferred to L15 medium (Leibovitz) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. The ovaries were mechanically dissected using the beveled edges of two syringe needles. Follicles with 2–3 layers of granulosa cells, a centrally placed oocyte, an intact basal membrane and attached theca cells were collected and cultured individually in 10 μl droplets of culture medium (a-MEM supplemented with 5% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium and 100 mIU/ml recombinant FSH). The culture dishes were maintained in an incubator at 37°C with 100% humidity and 5% CO₂ in air. The medium was replenished every other day by removing and replacing 10 μl. The morphology of the follicles was recorded daily under a microscope.

**Granulosa cell isolation and culture**

Three-week-old female mice were injected with 5 IU PMSG, and the ovaries were collected 46 h later. The granulosa cells of preovulatory follicles were collected as previously reported (45). After mechanical dissection, follicles were digested in medium containing 1 mg/ml collagenase, 0.025% trypsin and 0.02 mg/ml deoxyribonuclease I for 5 min at 37°C. Following deparaffinization, the slides were stained with H&E for histological analysis. Tissue collection and histological analysis

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**Immunohistochemistry**

The ovaries were fixed with 4% paraformaldehyde for 24 h, washed with PBS and stored in 70% ethanol. The samples were embedded in paraffin, and 5-μm sections were prepared. After deparaffinization and rehydration through a graded ethanol series, the slides were incubated with 5% BSA for 30 min at room temperature and incubated with anti-α-HSD (Santa Cruz, sc-30820) and anti-Wt1 (Santa Cruz, sc-192) antibodies overnight at 4°C. After washing with PBS, the sections were incubated with biotinylated secondary antibody for 1 h. Then, the slides were incubated with avidin–biotin–peroxidase complex for 1 h at room temperature. The color was developed with 3,3-diaminobenzidine for 2 min and counterstained with hematoxylin for 2 min. The images were captured with a Nikon Microscope and a DP71 CCD camera.
RNA extraction and real-time PCR
Ovaries and granulosa cells were lysed with Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer’s instructions. A 2-μg sample of total RNA was reverse transcribed in a final volume of 25 μl containing random primers, Moloney murine leukemia virus reverse transcriptase, reaction buffer, deoxynucleotide triphosphates and ribonuclease inhibitor. The sequences of the specific primers used are listed in Supplementary Material, Table S1. Real-time PCR was performed using an ABI PRISM 7000 sequence detection system (PE Applied Biosystems, Foster City, CA, USA). The mRNA expression level was normalized against glyceraldehyde-3-phosphate dehydrogenase and analyzed using the comparative cycle threshold method.

Construction of adenovirus vectors
The adenoviruses containing Wt1 and Wnt4 cDNA were generated using the Gateway Expression System (Invitrogen). Candidate genes were amplified by PCR and inserted into the pENTR 3C vector (Invitrogen). The desired plasmids were then generated by homologous LR recombination. Viral constructs were transduced into a 293A cell line, and a high titer (10⁸ UI/ml) of viral particles was obtained by four rounds of amplification. The virus titer was determined as previously described (46).

Statistical analysis
Experiments were repeated at least three times. The data were evaluated for significant differences using Student’s t-test. A P-value of < 0.05 was considered significant. Bar graphs were plotted in MS Excel.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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