

***Wt1* deficiency causes undifferentiated spermatogonia accumulation and meiotic progression disruption in neonatal mice**

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Abstract

Spermatogenesis is a complex process involving the regulation of multiple cell types. As the only somatic cell type in the seminiferous tubules, Sertoli cells are essential for spermatogenesis throughout the spermatogenic cycle. The Wilms tumor gene, *Wt1*, is specifically expressed in the Sertoli cells of the mouse testes. In this study, we demonstrated that *Wt1* is required for germ cell differentiation in the developing mouse testes. At 10 days *post partum*, *Wt1*-deficient testes exhibited clear meiotic arrest and undifferentiated spermatogonia accumulation in the seminiferous tubules. In addition, the expression of claudin11, a marker and indispensable component of Sertoli cell integrity, was impaired in *Wt1*^{-/*flox*}; *Cre-ER*TM testes. This observation was confirmed in *in vitro* testis cultures. However, the basal membrane of the seminiferous tubules in *Wt1*-deficient testes was not affected. Based on these findings, we propose that Sertoli cells' status is affected in *Wt1*-deficient mice, resulting in spermatogenesis failure.

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Introduction

Spermatogenesis is a continuous and highly organized process that requires the participation of multiple cell types. Sertoli cells, which interact directly with developing germ cells in the testis throughout spermatogenesis, play an indispensable role in germ cell development (Mruk & Cheng 2004). *Wt1* functions as a transcriptional regulator and is essential for the development of several organs, such as the kidney, heart, and gonads (Chamindrani Mendis-Handagama & Siril Ariyaratne 2001). Previous studies have demonstrated that *Wt1* is required during the earliest stage of gonad development, prior to sexual differentiation, in mice (Luo *et al.* 1994, Klattig *et al.* 2007). Studies have also shown that *Wt1* may be essential for later gonad development. The specific deletion of *Wt1* in Sertoli cells during E12.5–E14.5 resulted in testicular cord disruption and testis dysgenesis (Gao *et al.* 2006). Intriguingly, the *Wt1* mRNA levels in the testes still increased steadily after birth and reached their highest expression at 8 days *post partum* (dpp; Pelletier *et al.* 1991). Moreover, immunohistochemical studies confirmed that the WT1 protein is strongly expressed in the Sertoli cells associated with early spermatogonia, but not in the cells associated with late spermatogonia

(Suzuki & Saga 2008). The spatiotemporal expression pattern of *Wt1* suggests a potential role in the developing testis. However, the postnatal function of *Wt1* has not been elucidated due to embryonic stage lethality in *Wt1*^{-/-} mice (Bacon *et al.* 2003).

The objective of this study was to examine the function of *Wt1* in the neonatal testes. *Wt1*^{-/*flox*}; *Cre-ER*TM (tamoxifen-inducible) mice were treated with tamoxifen at 5 dpp to inactivate *Wt1* expression, and their phenotypes were examined at 10 dpp (5 days after tamoxifen treatment). To exclude the potential effects of other organs after *Wt1* inactivation, an *in vitro* testis culture model was employed to confirm the *in vivo* phenotype. The evidence showed that *Wt1* deficiency caused undifferentiated spermatogonia accumulation to undergo a meiotic progression disruption and that claudin11 expression was impaired in the *Wt1*^{-/*flox*}; *Cre-ER*TM testes. Therefore, we suggest that *Wt1* is essential for germ cell differentiation in the neonatal testes.

Materials and methods

Animals

All the mice were maintained on a C57BL/6; 129/SvEv mixed genetic background. *Wt1*^{*flox/flox*} (Gao *et al.* 2006) mice were

mated with mice carrying the *Wt1*-null allele (*Wt1*^{+/-}; Kreidberg *et al.* 1993) and *Cre-ER*TM (Hayashi & McMahon 2002) transgenic mice to produce *Wt1*^{-/*flox*}; *Cre-ER*TM offspring. Tail DNA was used for PCR genotyping, as described previously (Harada *et al.* 1999, Gao *et al.* 2006). The mice were housed in cages under a 12 h light:12 h darkness cycle. Two females were placed in a cage with one male in the evening and were examined for vaginal plugs the next morning. The day on which a vaginal plug was detected was designated as E0.5. All the animal procedures were approved by the committee of animal care at the Institute of Zoology, Chinese Academy of Sciences.

Tamoxifen treatment

Tamoxifen (Sigma) was dissolved in corn oil at a final concentration of 20 mg/ml. *Wt1*^{-/*flox*}; *Cre-ER*TM and *Wt1*^{-/*flox*} (control) mice at 5 dpp were treated with tamoxifen by i.p. injection at a dose of 9 mg/40 g body weight.

Immunohistochemistry and immunofluorescence

Wt1^{-/*flox*}; *Cre-ER*TM and *Wt1*^{-/*flox*} (control) mice at 5 dpp were treated with tamoxifen. Five days after tamoxifen treatment, the mice were killed by cervical dislocation. The testes were dissected and immediately fixed in 4% paraformaldehyde for up to 24 h. The samples were then stored in 70% ethanol and embedded in paraffin. The paraffin-embedded samples were sectioned at a 5 µm thickness and mounted on glass slides.

For immunohistochemistry, the tissue sections (5 µm thick) were deparaffinized and rehydrated, followed by antigen retrieval in 10 mM sodium citrate buffer for 15 min. After washing the sections in PBS for 5 min, BSA was added to the sections to block nonspecific binding for 1 h. Next, specific primary antibodies were added and incubated overnight at 4 °C. The sections were then washed three times with PBS, and a secondary antibody was added to the sections for 1 h at room temperature. The sections were then washed three times in PBS prior to staining using a DAB substrate kit (Zhong Shan Technology, Beijing, China) and were counterstained with hematoxylin.

For immunofluorescence, 0.3% Triton X-100 in PBS was used as the blocking buffer. Specific primary antibodies were added and incubated overnight at 4 °C. The next day, the sections were washed three times with PBS and incubated with FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. The sections were then washed three times with PBS and counterstained with DAPI (Sigma) to visualize nuclei.

The following antibodies were used in this study: rabbit anti-*Wt1* (1:200; Epitomics, Burlingame, CA, USA; 2797-1), mouse anti-*Plzf* (1:200; Abcam, Cambridge, UK; ab104854), rabbit anti-claudin11 (1:200; Abcam, ab53041), rabbit anti-laminin (1:400; Abcam, ab11575), and mouse anti-vimentin (1:500; Abcam, ab20346).

Testis culture

Testes from *Wt1*^{-/*flox*}; *Cre-ER*TM and *Wt1*^{-/*flox*} (control) mice at 5 dpp were decapsulated and gently separated using two

forceps into four to six pieces of 1–3 mm in diameter. The tissue fragments were then positioned on stands made of 1.5% agarose gel that were placed in a 24-well plate, as described previously (Sato *et al.* 2011) and pre-balanced to soak in culture medium overnight. The culture medium consisted of DMEM/F-12 (1:1; HyClone, Logan, UT, USA; SH30023.01B) with 10% fetal bovine serum (for embryonic stem cell use; Gibco). Next, 4-OH tamoxifen (Sigma, H7904) was added to the culture medium at a final concentration of 1 µM. The culture medium was replaced every 2 days, and the cells were cultured in 5% carbon dioxide in air and maintained at 33 °C.

Real-time PCR

Wt1^{-/*flox*}; *Cre-ER*TM and *Wt1*^{-/*flox*} mice were injected with tamoxifen at 5 dpp. Five days later, the testes were dissected, and total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamers. Real-time PCR was performed using the SYBR Green Kit (Tiangen, Beijing, China) on a C1000 Thermal Cycler (Bio-Rad Laboratories). The primers used for real-time PCR amplification are listed in Table 1.

Western blotting

Wt1^{-/*flox*}; *Cre-ER*TM and *Wt1*^{-/*flox*} mice were injected with tamoxifen at 5 dpp. Five days later, the testes were lysed in radio immunoprecipitation assay lysis buffer containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche). The protein concentration in the supernatants was estimated using the Bradford assay (Bio-Rad Laboratories). The proteins were electrophoresed under reducing conditions in 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes. The blots were incubated overnight at 4 °C with primary antibody, followed by 1 h of incubation at room temperature with HRP-labeled secondary antibody. Specific signals were detected using the ECL western blotting detection system.

The following antibodies were used in this study: mouse anti-E-cadherin (BD, 610181), rabbit anti-N-cadherin (Santa Cruz, SC-7939), rabbit anti-β-catenin (Sigma, C2206), rabbit anti-occludin (Abcam, ab31721), and rabbit anti-PAR6β (Sigma, B8062).

Table 1 The primers used for real-time PCR amplification.

| | |
|----------------|--|
| <i>Wt1</i> | CCAGTGAAAACCTGTCCAGCGAAA ATGAGTCCTGGTGTGGGTCTTC |
| <i>Plzf</i> | CTGGGACTTTGTGCGATGTG CGGTGGAAGAGGATCTCAAACA |
| <i>Neurog3</i> | AGTGCTCAGTCCAATCCAC CGGCTTCTCGCTTTTGTCTG |
| <i>Gfra1</i> | GTACAGGTCCGCCTACATCAC GGCTGGAACCTTGTGCGAAGAA |
| <i>Ret</i> | GCATGTCAGACCCGAACCTGG CGCTGAGGGTGAAACCATCC |
| <i>Nanos2</i> | CTGCAAGCACAATGGGGAGT CGCTGGTAGAGAGACTGCTG |
| <i>GDNF</i> | CCAGTGACTCCAATATGCCTG CTCTGCGACCTTCCCTCTG |
| <i>GAPDH</i> | TTGTCTCCTGCGACTTCAACA ACCAGGAAATGAGCTTGACAAA |

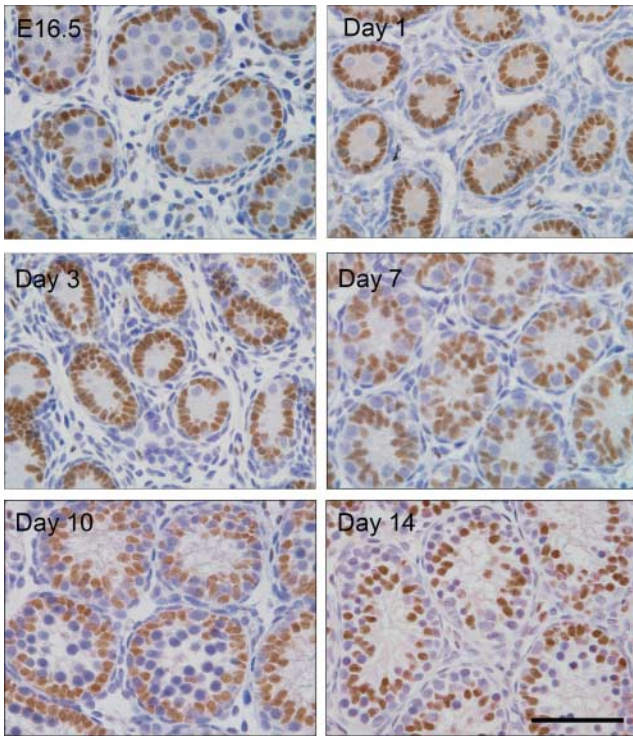


Figure 1 *Wt1* is specifically expressed in Sertoli cells in the seminiferous tubules of the developing testes. The testes of WT mice at E16.5, 1, 3, 7, 10, and 14 dpp were immunostained with anti-*Wt1* antibody. Scale bar=50 μ m.

Statistical analysis

The experiments were repeated at least three times. For the immunohistochemistry and immunofluorescence experiments, one representative image of the results of three independent experiments is presented. The data were evaluated for significant differences using Student’s *t*-test. A *P* value <0.05 was considered significant. Bar graphs were plotted using GraphPad Prism 6 (La Jolla, CA, USA).

Results

***Wt1* is specifically expressed in Sertoli cells in the seminiferous tubules of the developing testes**

To better understand the functions of *Wt1* in developing testes, we first determined the spatiotemporal expression pattern of *Wt1* protein in the WT mice from E16.5 to 14 dpp at six time points (E16.5, 1, 3, 7, 10, and 14 dpp). Testes from WT mice were embedded in paraffin and sectioned at a 5 μ m thickness. The tissue sections were then immunostained with anti-*Wt1* antibody. As shown in Fig. 1, *Wt1* is specifically expressed in Sertoli cells throughout the developmental stages.

***Wt1* is efficiently inactivated in *Wt1*^{flox}; *Cre-ER*TM testes after tamoxifen treatment**

To inactivate *Wt1* expression more efficiently, *Wt1*^{flox} mice were mated with *Wt*^{+/-}; *Cre-ER*TM mice to

generate *Wt1*^{flox}; *Cre-ER*TM mice (KO group) and *Wt1*^{flox} mice (control group). The littermate mice in both groups were indistinguishable, and the morphology and histology of the testes were normal compared with those of the WT testes (data not shown). To induce Cre-mediated recombinase activity, 5-dpp-old littermate mice from both groups were i.p. injected with tamoxifen at a dose of 9 mg/40 g (body weight). The use of the *Wt1*^{flox}; *Cre-ER*TM mice allowed disruption of the *Wt1* gene in an inducible manner, whereas the use of the *Wt1*^{flox} mice did not. The testes were collected 5 days after tamoxifen injection. After *Wt1* inactivation, the weight of both the body and the testis of the *Wt1*^{flox}; *Cre-ER*TM mice were significantly decreased compared with those of the control group (Fig. 2A and B), which was likely due to the negative effect of *Wt1* inactivation on cardiovascular tissue and the kidney. However, the ratio of the testis weight:body weight was not affected (Fig. 2E). *Wt1*-specific real-time PCR was used to examine the efficiency of tamoxifen-induced Cre recombination.

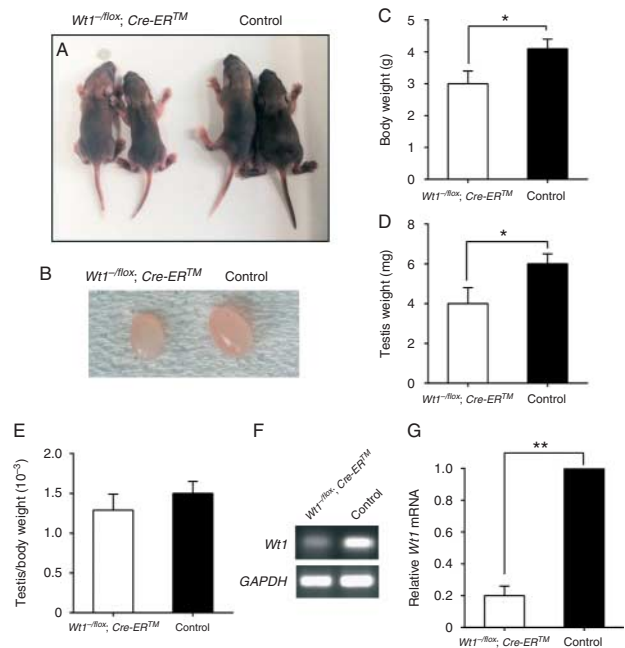


Figure 2 *Wt1* is mostly inactivated in *Wt1*^{flox}; *Cre-ER*TM testes after tamoxifen treatment. (A and B) Comparison of the body and testis size of *Wt1*^{flox} (control) and *Wt1*^{flox}; *Cre-ER*TM. (C and D) Quantitative analysis of the body weight and testes weight of *Wt1*^{flox} (control) and *Wt1*^{flox}; *Cre-ER*TM mice. Both the body and the testis weights of the *Wt1*^{flox}; *Cre-ER*TM mice were decreased compared with those of the control group **P*<0.05. (E) Quantitative analysis of the ratio of testis weight:body weight in *Wt1*^{flox} (control) and *Wt1*^{flox}; *Cre-ER*TM mice. (F) Real-time PCR was performed using RNA isolated from 10 dpp *Wt1*^{flox} (control) and *Wt1*^{flox}; *Cre-ER*TM (KO) testes and specific primer pairs against *Wt1* and *GAPDH*. Both the control and the KO mice were injected with tamoxifen at 5 dpp. For each sample, RNA from at least three animals was pooled. (G) Quantitative real-time PCR analysis of *Wt1* mRNA expression in control and KO testes. The mRNA levels were normalized to *GAPDH*, and the s.d.s from duplicate reactions are shown (***P*=0.006).

The primers were designed to target the eighth and ninth exons, and thus, normal *Wt1* mRNA could be detected, whereas disrupted *Wt1* mRNA could not. The real-time PCR results showed that only ~20% of normal *Wt1* mRNA was still present in the *Wt1*^{-/flox}; *Cre-ER*TM testes compared with the control testes (Fig. 2G), indicating that *Wt1* was mostly inactivated in the *Wt1*^{-/flox}; *Cre-ER*TM testes after tamoxifen treatment.

Undifferentiated spermatogonia accumulate in *Wt1*-deficient testes

Promyelocytic leukemia-associated protein (*Plzf*) is a zinc finger transcription factor involved in germline stem cell self-renewal. *Plzf* expression is restricted to the gonocytes and undifferentiated spermatogonia (Starz-Gaiano & Lehmann 2001, Santos & Lehmann 2004). Prior to tamoxifen treatment, the number and distribution of *Plzf*-positive cells was nearly identical in the *Wt1*^{-/flox}; *Cre-ER*TM and *Wt1*^{-/flox} testes at 5 dpp. After tamoxifen treatment, the number of *Plzf*-positive cells clearly showed a significant difference between the two groups (Fig. 3A). The number of *Plzf*-positive cells per tubule section was ~16 in the *Wt1*^{-/flox}; *Cre-ER*TM testes at 10 dpp, whereas only approximately eight were observed in the control group (Fig. 3B and C), indicating that undifferentiated spermatogonia abnormally accumulated after *Wt1* inactivation. Also, the number of *Plzf*-positive cells/1000 Sertoli cells was counted (Fig. 3D). Three control testes and three *Wt1*^{-/flox}; *Cre-ER*TM testes were used. Approximately 20 tubular cross sections were counted per animal. The average number of undifferentiated spermatogonia/1000 Sertoli cells was 208 in control testes, while in *Wt1*^{-/flox}; *Cre-ER*TM testes, the average number increased to 385.

Once *Wt1*^{-/flox}; *Cre-ER*TM mice were injected with tamoxifen, *Wt1* was inactivated in all organs because of universal *Cre* expression. *Wt1* has been reported to play an essential role in other organs, such as the kidney and heart. To eliminate the possible effect of other organs' *Wt1* loss on testicular spermatogonia *in vivo*, the *Wt1*^{-/flox}; *Cre-ER*TM testes were dissected at 5 dpp and cultured on 1.5% agarose gel with 4-OH tamoxifen to inactivate *Wt1* expression. This method has a higher knockout efficiency and allows the direct observation of any alterations in the development of the seminiferous tubules.

The intermediate filaments in the Sertoli cells of postnatal mice consist of vimentin (Aumüller *et al.* 1992). Thus, vimentin immunostaining should reflect the Sertoli cell distribution in the seminiferous tubules. It was observed that in the *Wt1*^{-/flox}; *Cre-ER*TM cultured testes, the number of *Plzf*-positive cells began to increase along the basal membrane from days 2–3. After 1 week in culture, the number of *Plzf*-positive cells exceeded the number in the control group (Fig. 4). The *in vitro* culture result confirmed our *in vivo* findings, with even more accumulation of undifferentiated spermatogonia.

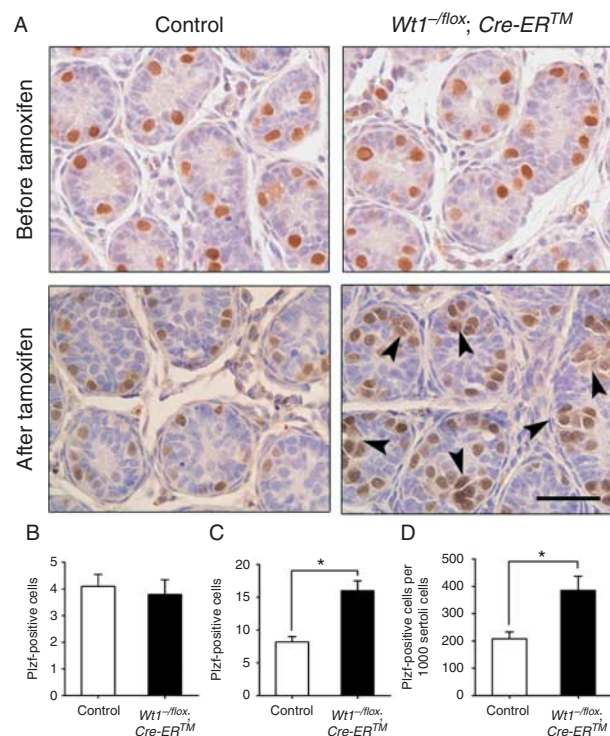


Figure 3 Undifferentiated spermatogonia accumulation in *Wt1*-deficient testes. (A) *Wt1*^{-/flox} (control) and *Wt1*^{-/flox}; *Cre-ER*TM testes were immunostained for *Plzf* prior to tamoxifen treatment at 5 dpp and 5 days after treatment (10 dpp). *Plzf*-positive cells accumulated and formed cell clusters (arrowhead). Scale bar=50 µm. (B and C) The number of *Plzf*-positive cells per seminiferous tubule section was quantified. The data expressed as the mean ± s.e.m. for at least three testes obtained from different mice (**P*=0.065). (D) The number of *Plzf*-positive cells/1000 Sertoli cells was counted. The data are expressed as the mean ± s.e.m. for three testes obtained from different mice.

The expression of undifferentiated spermatogonia genes is upregulated after *Wt1* inactivation

Based on the finding that the number of undifferentiated spermatogonia increased after *Wt1* deletion, we examined whether the expression of the undifferentiated spermatogonia-related genes *Plzf*, *Neurog3* (*Ngn3*), *Gfra1*, *Ret* (*C-Ret*), and *Nanos2* was also upregulated. Consistent with our anticipation, this gene expression increased remarkably compared with the expression in the control group (Fig. 5), indicating that the genes associated with undifferentiated spermatogonia were upregulated in the *Wt1*^{-/flox}; *Cre-ER*TM testes. Glial cell line-derived neurotrophic factor (*GDNF*) is a molecule secreted by Sertoli cells that promotes the proliferation of spermatogonial stem cells (Meng *et al.* 2000, Kubota *et al.* 2004, He *et al.* 2008). Although both the cell number and the related gene expression of undifferentiated spermatogonia increased in the *Wt1*^{-/flox}; *Cre-ER*TM testis, *GDNF* expression was maintained at the same level as in the control, indicating that the accumulation of undifferentiated spermatogonia may not have been caused by over-proliferation.

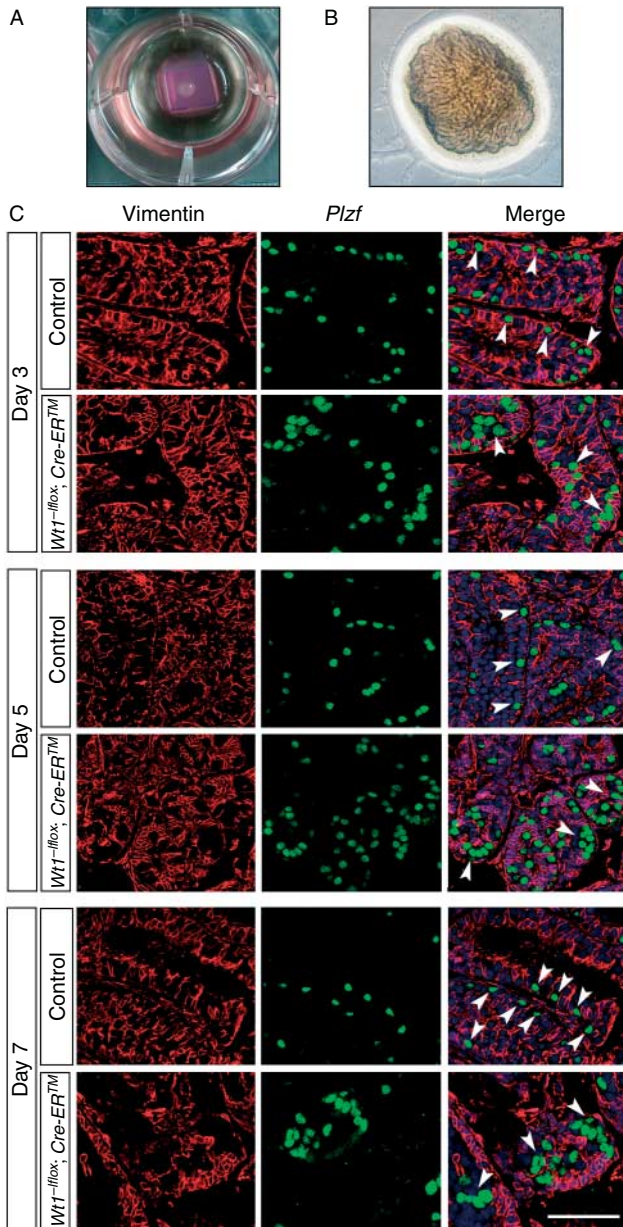


Figure 4 *Plzf*-positive cell accumulation in cultured testes after *Wt1* inactivation. (A) *In vitro* culture of a testis segment on 1.5% agarose gel in DMEM/F-12 medium in a 24-well plate. (B) Using an inverted microscope, the cultured testicular segment could be observed. (C) Immunostaining of vimentin (red), *Plzf* (green), and Hoechst (blue) in the cultured testis at days 3, 5, and 7. The arrowhead indicates undifferentiated spermatogonia. Scale bar = 50 μ m.

Germ cell differentiation is severely impaired after *Wt1* inactivation

At 10 dpp, the first wave of spermatogenesis had already entered the stage of meiosis in the normal mouse testis, as revealed by the *Scp3* staining. By contrast, in the *Wt1*^{-/*flox*}; *Cre-ER*TM testes, the germ cells lacked or had very low levels of *Scp3* expression (Fig. 6A).

Scp3, *Dmc1*, and *Pgk2* are expressed in spermatocytes during meiosis (Robinson *et al.* 1989, Yuan *et al.* 2000,

Griswold *et al.* 2012). To examine the state of differentiation of the germ cells in the mouse testis after *Wt1* inactivation, these marker genes were analyzed by real-time PCR (Fig. 6B). The statistical results showed that all the examined meiotic markers were markedly decreased in the *Wt1*^{-/*flox*}; *Cre-ER*TM mice compared with the controls. Together, with *Scp3* staining, the results demonstrated that normal germ cell differentiation in the testis is severely impaired after *Wt1* inactivation.

The expression of claudin11 is severely impaired, whereas the integrity of the seminiferous tubules is not affected, in *Wt1*-deficient testes

Laminin expression in the basal laminae of the seminiferous tubules has been reported during the development of the mouse testis (Gelly *et al.* 1989, Enders *et al.* 1995). Our study showed that the expression and distribution of laminin in the seminiferous tubules did not obviously differ between the control and the *Wt1*-deficient mice (Fig. 7A). This finding indicated that the integrity of the seminiferous tubules is not affected by *Wt1* loss in the developing testis. The phenotype observed in our study is different from that of the embryonic stage, during which a loss of *Wt1* results in a disruption of seminiferous tubule development and a subsequent progressive loss of Sertoli and germ cells (Gao *et al.* 2006, Chen *et al.* 2013). This discrepancy might be explained by different effects of *Wt1* at various stages of testicular development.

Claudin11 is an obligatory protein in Sertoli cells for tight junction formation and blood–testis barrier integrity in the testis. Studies have shown that spermatogenesis does not proceed beyond meiosis in the absence of claudin11, resulting in male sterility (Gow *et al.* 1999, Mazaud-Guittot *et al.* 2010). Claudin11 was nearly absent in the *Wt1*-deficient testis (Fig. 7C and E),

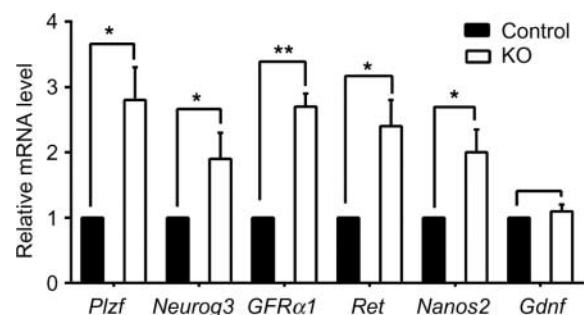


Figure 5 Expression of undifferentiated spermatogonia marker genes is increased after *Wt1* inactivation. Real-time PCR was performed using RNA isolated from 10 dpp *Wt1*^{-/*flox*} (control) and *Wt1*^{-/*flox*}; *Cre-ER*TM (KO) testes and specific primer pairs against *Plzf*, *Neurog3*, *Gfrα1*, *Ret*, *Nanos2*, *Gdnf*, and *Gapdh*. Mice from both the control and the KO groups were injected with tamoxifen at 5 dpp. Each of the combined samples was pooled from at least three animals. The mRNA levels were normalized to GAPDH, and the s.d.s from duplicate reactions are shown (**P* < 0.05, ***P* < 0.01).

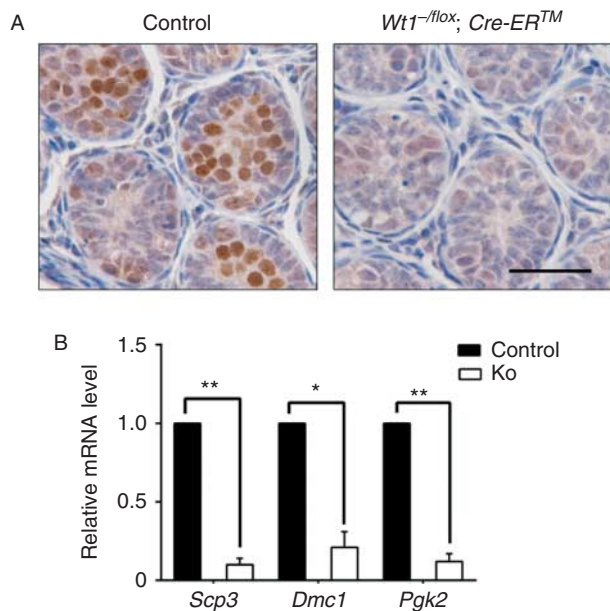


Figure 6 Meiotic progression disruption in *Wt1*-deficient testes. (A) Testes from *Wt1*^{-/*lox*} (control) and *Wt1*^{-/*lox*}; *Cre-ER*TM mice at 10 dpp after tamoxifen treatment were immunostained using antibodies against *Scp3*. Scale bar = 50 μ m. (B) RT-PCR was performed using RNAs isolated from 10 dpp *Wt1*^{-/*lox*} (control) and *Wt1*^{-/*lox*}; *Cre-ER*TM (KO) testes and specific primer pairs against *Scp3*, *Dmc1*, *Pgc2*, and *Gapdh*. Mice from both the control and the KO groups were injected with tamoxifen at 5 dpp. For each sample, RNA from at least three animals was pooled. The mRNA levels were normalized to *Gapdh*, and the s.d.s from duplicate reactions are shown (* $P < 0.05$, ** $P < 0.01$).

whereas the protein's expression and localization were normal in the control testis (Fig. 7D and F). Because of blood–testis barrier formation at ~ 2 weeks after birth, a loss of claudin11 will result in the failure of Sertoli–Sertoli tight junction formation in *Wt1*-deficient testes, eventually leading to meiosis failure.

E-cadherin is downregulated in Wt1-deficient testes

To investigate the underlying mechanism underlying the phenotype of *Wt1*^{-/*lox*}; *Cre-ER*TM testes, several potential target proteins of *Wt1* were measured by western blotting. All the molecules examined, except E-cadherin, showed no obvious change at the protein level within 5 days. Downregulation of E-cadherin after *Wt1* loss was observed in *Wt1*^{-/*lox*}; *Cre-ER*TM testes (Fig. 8A), whereas the expression of N-cadherin, β -catenin, Par6b, and occludin remained the same in control testes.

Discussion

Sertoli cells are the only cell type that interacts with developing germ cells in the seminiferous tubules during testis development. Hence, the status of Sertoli cells is closely related to spermatogenesis. Here, we showed that *Wt1* is specifically expressed in Sertoli cells in the

seminiferous tubules of the developing testes. This observation is consistent with the results of previous reports on fetal and newborn testes (Pelletier *et al.* 1991, Mundlos *et al.* 1993). The spatiotemporal expression of *Wt1* in Sertoli cells indicates the essential role of *Wt1*. However, the exact role of *Wt1* during testis development has not been illuminated. In this study, we carefully observed the phenotype of *Wt1*-deficient mice and found that *Wt1* deficiency causes undifferentiated spermatogonia accumulation and meiotic progression disruption in neonatal mice.

The characterization of *Wt1*^{/lox}; *Cre-ER*TM mice

Wt1 has been reported to play an important role in gonad genesis and spermatogenesis (Gao *et al.* 2006, Klattig *et al.* 2007). Deletion of *Wt1* in Sertoli cells at an early embryonic stage causes disruption of the testicular cord. Our recent study showed that inactivation of *Wt1* in adult mice resulted in massive germ cell death and only Sertoli cells were present in most of the seminiferous tubules (Wang *et al.* 2013).

Immunohistochemical studies confirmed that the WT1 protein is strongly expressed in the Sertoli cells

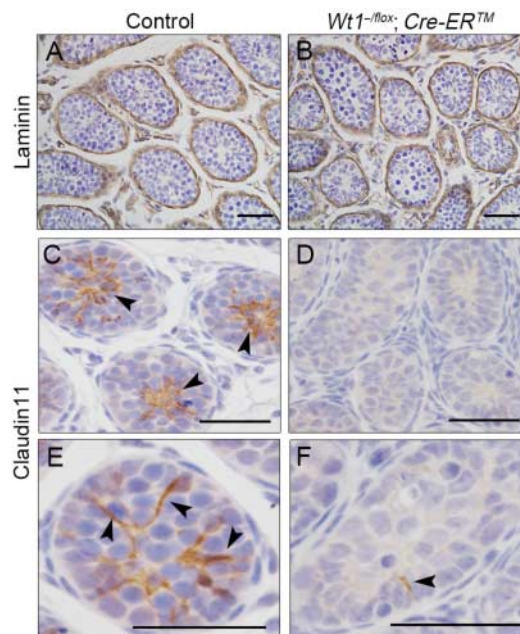


Figure 7 Expression of laminin and claudin11 in the testes from *Wt1*^{-/*lox*}; *Cre-ER*TM and *Wt1*^{-/*lox*} (control) at 10 dpp after tamoxifen treatment. (A and B) The testes of *Wt1*^{-/*lox*} and *Wt1*^{-/*lox*}; *Cre-ER*TM mice at 10 dpp after tamoxifen treatment were immunostained using antibodies against laminin. Laminin was expressed in the basal laminae of the seminiferous tubules, with no clear difference in the seminiferous tubules between *Wt1*^{-/*lox*}; *Cre-ER*TM and *Wt1*^{-/*lox*} mice. (C, D, E and F) The testes from *Wt1*^{-/*lox*} and *Wt1*^{-/*lox*}; *Cre-ER*TM mice at 10 dpp after tamoxifen treatment. Claudin11 was mainly expressed in the Sertoli cells' junctions in the *Wt1*^{-/*lox*} testis, whereas only very little claudin11 (arrowhead) could be detected in the *Wt1*^{-/*lox*}; *Cre-ER*TM testis. Scale bar = 50 μ m.

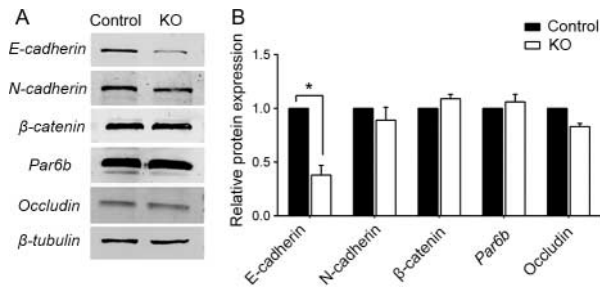


Figure 8 E-cadherin expression is decreased in *Wt1*-deficient testes. (A) Western blot analysis using specific antibodies for E-cadherin, N-cadherin, Par6b, and β -tubulin among the proteins isolated from 10 dpp *Wt1*^{-/*flox*} (control) and *Wt1*^{-/*flox*}; *Cre-ER*TM (KO) testes. The control and KO mice were injected with tamoxifen at 5 dpp. (B) Quantification of the relative levels of E-cadherin, N-cadherin, and Par6b (corrected to the total levels of their respective proteins) is shown (**P*<0.05).

associated with the development of early spermatogonia, but not at a later stage of spermatogenesis (Suzuki & Saga 2008). Therefore, we decided to inactivate *Wt1* at 5 dpp. Tamoxifen-inducible Cre recombination was effective as soon as 12 h, and the null allele in the *Wt1*^{-/*flox*}; *Cre-ER*TM mice resulted in a high inactivation efficiency. Real-time PCR analysis showed that the *Wt1* mRNA level decreased by only 20% at 10 dpp. However, the *Wt1*^{-/*flox*}; *Cre-ER*TM mice revealed a low survival rate after tamoxifen treatment due to *Wt1* loss-of-function in the kidney and heart. We injected tamoxifen at 5 dpp, and over 50% of *Wt1*^{-/*flox*}; *Cre-ER*TM mice (10 of 19) did not survive for more than 1 week. Thus, it was difficult to examine the phenotype after 12 dpp. To overcome this lethality due to the effects of *Wt1* on other organs, we performed an *in vitro* testis culture on an agarose gel using a method developed by Sato *et al.* (2011).

A tissue-specific RNAi study revealed that mice depleted of WT1 in Sertoli cells suffered from increased germ cell apoptosis, loss of adherens junctions, and impaired fertility (Rao *et al.* 2006). In Rao's study, the mouse Pcm (*Rhox5*) gene-driven promoter started expressing at 8 dpp, and *Wt1* was inactivated thereafter. We speculate that the difference between our study and Rao's is mainly caused by different inactivation time. The first ten neonatal days are very crucial for Sertoli cell maturation and spermatogonia differentiation. Also, the phenotype in Rao's study was mainly observed at adult stage while our study focused on the developmental stage.

The accumulation of undifferentiated spermatogonia is likely due to germ cell differentiation failure but not over-proliferation

The undifferentiated spermatogonia increased in number and formed clusters after *Wt1* inactivation. There might be two potential reasons for this phenomenon after *Wt1* loss: differentiation arrest or over-proliferation of spermatogonia. Data obtained from our experiments showed that differentiation arrest might be the main reason. First,

the total germ cell numbers within each tubule section showed no significant difference between the two groups. Secondly, there was clear meiotic arrest in the *Wt1*-deficient testes, which may have led to the increased number of undifferentiated spermatogonia. Moreover, the expression of *GDNF* (SSC self-renewal factor) in the *Wt1*^{-/*flox*}; *Cre-ER*TM testis was comparable with the expression in the controls. These findings suggested that undifferentiated spermatogonia accumulation might be due to a failure in spermatogonia differentiation, rather than the cells' over-proliferation.

***Wt1* may regulate Sertoli cells' mesenchymal–epithelial balance via E-cadherin**

Many phenotypes in cancer and *Wt1*-deficient mice may be attributed to the proteins that regulate the mesenchymal–epithelial balance (Hohenstein & Hastie 2006). In the first two neonatal weeks, Sertoli cells undergo a polarity establishment process for tight junction formation between cells, during which an apical–basal polarity is formed. Consequently, Sertoli cells adopt a more epithelial phenotype in the mesenchymal–epithelial balance during this period.

Cadherins are Ca²⁺-dependent cell adhesion molecules that play an essential role in organogenesis (Raz 2004). E-cadherin is one of the cadherin family members that is involved in epithelial–mesenchymal transitions during cell development and tumor invasion (Donovan *et al.* 1986, Enders & May 1994). Although E-cadherin is usually regarded as a specific marker for undifferentiated spermatogonia (Raz 2004, Kanatsu-Shinohara *et al.* 2008), E-cadherin expression in Sertoli cells is highest at 1 week after birth (Raz 2004). Previous studies have demonstrated that E-cadherin is a *Wt1* target gene that regulates E-cadherin expression in NIH 3T3 cells and epicardial cells (Shinohara *et al.* 1999, Sharpe *et al.* 2003).

Wt1 might also regulate the mesenchymal–epithelial balance of Sertoli cells through E-cadherin in the neonatal testis given that upon *Wt1* deletion, the mesenchymal–epithelial transition of Sertoli cells was impaired in the developing testis, resulting in meiotic arrest. Thus, we propose that *Wt1* may affect Sertoli cells' mesenchymal–epithelial balance via upregulation of E-cadherin. However, the precise role of *Wt1* in the developing testis needs to be further studied.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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