

RESEARCH ARTICLE

Wt1 directs the lineage specification of sertoli and granulosa cells by repressing *Sf1* expression

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ABSTRACT

Supporting cells (Sertoli and granulosa) and steroidogenic cells (Leydig and theca-interstitium) are two major somatic cell types in mammalian gonads, but the mechanisms that control their differentiation during gonad development remain elusive. In this study, we found that deletion of *Wt1* in the ovary after sex determination caused ectopic development of steroidogenic cells at the embryonic stage. Furthermore, differentiation of both Sertoli and granulosa cells was blocked when *Wt1* was deleted before sex determination and most genital ridge somatic cells differentiated into steroidogenic cells in both male and female gonads. Further studies revealed that WT1 repressed *Sf1* expression by directly binding to the *Sf1* promoter region, and the repressive function was completely abolished when WT1 binding sites were mutated. This study demonstrates that *Wt1* is required for the lineage specification of both Sertoli and granulosa cells by repressing *Sf1* expression. Without *Wt1*, the expression of *Sf1* was upregulated and the somatic cells differentiated into steroidogenic cells instead of supporting cells. Our study uncovers a novel mechanism of somatic cell differentiation during gonad development.

KEY WORDS: *Wt1*, Sertoli cells, Granulosa cells, Steroidogenic cells, *Sf1*, Mouse

INTRODUCTION

In mammals, the development of a testis or ovary from the bi-potential gonads is regulated by sex-determining genes. The differentiation of gonadal somatic cells plays an essential role in this process. Transient expression of *Sry*, which encodes a transcription factor containing a high mobility group (HMG) domain, in the somatic cells of mouse XY embryos between E10.5 and E12.5 is essential for Sertoli cell differentiation from bi-potential supporting cells and testis formation (Gubbay et al., 1990; Sinclair et al., 1990; Koopman et al., 1991). In XX embryos, activation of the R-spondin 1 (RSPO1)– β -catenin pathway promotes the differentiation of granulosa cells and then the formation of the ovary (Chassot et al., 2008).

Leydig cells and theca-interstitial cells are the steroidogenic cells in male and female gonads, respectively. The steroid hormones produced by steroidogenic cells play essential roles in germ cell development and in maintaining secondary sexual characteristics. Leydig cells first appear in testes at E12.5, whereas theca-interstitial cells are observed postnatally in the ovaries along with the development of ovarian follicles. The origins of Leydig cells (Weaver et al., 2009; Barsoum and Yao, 2010; DeFalco et al., 2011) and theca cells (Liu et al., 2015) have been studied previously. However, the underlying mechanism that regulates the differentiation between supporting cells and steroidogenic cells during gonad development is poorly understood.

The Wilms' tumor (WT) suppressor gene *Wt1* encodes a zinc finger nuclear transcription factor that is initially expressed in the intermediate mesoderm at E9 in the area developing into the genital ridge, subsequently at E9.5 in the coelomic epithelium of the urogenital ridge and the underlying mesenchymal cells before sex determination (Pelletier et al., 1991b; Armstrong et al., 1993; Kreidberg et al., 1993). In adults, *Wt1* expression is maintained in ovarian granulosa cells and testicular Sertoli cells. Inactivation of *Wt1* gene causes embryonic lethality and the gonads fail to develop because of the apoptosis of genital ridge somatic cells (Kreidberg et al., 1993). WT1 has two major isoforms defined by the presence or absence of three amino acids (KTS) between the third and fourth zinc finger, denoted +KTS and –KTS isoforms. Mice lacking the +KTS isoform display male-to-female sex reversal. The –KTS isoform is not required for sex determination, but is important for the survival of the gonadal primordium (Hammes et al., 2001; Bradford et al., 2009).

Steroidogenic factor 1 (*Sf1*) is a nuclear hormone receptor that is expressed in the early adrenogonadal primordium from E9.5 (Ikeda et al., 1994). *Sf1* mutant mice show no gonad or adrenal development (Luo et al., 1994), suggesting that *Sf1* is essential for genital ridge development. In adults, *Sf1* is constitutively expressed in steroidogenic tissues, including the cortical cells in the adrenal gland, Leydig cells in the testis and theca cells in the ovary. *Sf1* activates the expression of steroidogenic enzymes by binding to shared promoter elements and is believed to be a key regulator of steroid hormone biosynthesis (Ikeda et al., 1993; Morohashi et al., 1993).

Our previous study showed that inactivation of *Wt1* in Sertoli cells after sex determination using *AMH-Cre* mice caused testicular cord disruption (Gao et al., 2006) and Sertoli cells can be reprogrammed into Leydig cells (Gao et al., 2006; Zhang et al., 2015). These studies indicate that *Wt1* plays important roles in testis development and Sertoli cell lineage maintenance. To investigate the exact function of *Wt1* in gonad somatic cell differentiation, in this study, the *Wt1* gene was inactivated using a tamoxifen-inducible Cre transgenic mouse model. Interestingly, deletion of *Wt1* after sex determination in the ovary caused

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ectopic formation of steroidogenic cells at E16.5. When *Wt1* was deleted before sex determination (approximately E10.5), unexpectedly, we found that the differentiation of the supporting cell lineage was blocked, with most somatic cells differentiating into 3 β -HSD-positive steroidogenic cells. Further *in vivo* and *in vitro* studies indicated that *Wt1* directs the lineage specification of Sertoli and granulosa cells by repressing *Sfl* expression. Without *Wt1*, *Sfl* was upregulated and the genital ridge somatic cells differentiated into steroidogenic cells instead of Sertoli and granulosa cells, and sex determination did not occur. These results demonstrated that *Wt1* is involved in gonad development by directing the lineage specification of Sertoli and granulosa cells.

RESULTS

Deletion of *Wt1* in ovaries after sex determination leads to ectopic development of 3 β -HSD-positive steroidogenic cells

Wt1^{-flox}; *Cre-ER*TM males were crossed with *Wt1*^{flox/flox} females and the pregnant females were injected with tamoxifen at E12.5. The ovaries from control and *Wt1*^{-flox}; *Cre-ER*TM embryos were collected at E16.5. 3 β -HSD (3 β -hydroxysteroid dehydrogenase), also known as HSD3B1, plays a crucial role in the synthesis of all classes of steroid hormone and can be used as a marker of steroidogenic cells. Numerous 3 β -HSD-positive steroidogenic cells were observed in *Wt1*-deficient ovaries (Fig. 1B) but not in control ovaries (Fig. 1A). Oil Red O staining was also found in steroidogenic cells in *Wt1*-deficient ovaries (Fig. 1F). To trace the origin of 3 β -HSD-positive cells in *Wt1*-deficient gonads, a WT1 and 3 β -HSD double staining experiment was performed. Our previous studies demonstrated that although the small truncated WT1 protein remaining after the deletion of two exons from the *Wt1*^{flox} allele had lost its function, it was still recognized by the antibody used in this study and could be used to trace the *Wt1* mutant cells (Gao et al., 2006; Hu et al., 2011; Wang et al., 2013). We found that most 3 β -HSD-positive cells were also positive for WT1 (Fig. 1D), suggesting they were transformed from *Wt1*-positive cells, and most *Wt1*-positive cells at this stage were pre-granulosa cells. The expression of other steroidogenic genes was also examined by real-time PCR analysis. As shown in Fig. 1G, the mRNA levels of *P450scc* (*Cyp11a1*), 3 β -HSD (*Hsd3b1*), *Cyp17a1* and *Star* were significantly increased in *Wt1*-deficient XX gonads compared with the levels measured in control ovaries. To test whether the steroidogenic cells come from the mesonephros, the ovaries from control and *Wt1*^{-flox}; *Cre-ER*TM mice were dissected at E13.5 and cultured *in vitro* with tamoxifen. A large number of 3 β -HSD-positive steroidogenic cells were observed in *Wt1*^{-flox}; *Cre-ER*TM ovaries but not in control ovaries (Fig. S1). These cells were also labeled with the anti-WT1 antibody, indicating that these 3 β -HSD-positive cells are derived from somatic cells inside the gonads, not from adrenal glands or mesonephros.

Aberrant differentiation of somatic cells in *Wt1*-deficient gonads

To further investigate the function of *Wt1* in genital ridge somatic cell differentiation during sex determination, pregnant *Wt1*^{flox/flox} females that were mated with *Wt1*^{-flox}; *Cre-ER*TM males, were injected with tamoxifen at E9.5. The morphology of gonads from *Wt1*^{-flox}; *Cre-ER*TM embryos was grossly normal at E12.5, but they were slightly smaller than control gonads (Fig. S2A,B) and no testicular cords were formed in *Wt1*-deficient XY gonads at E12.5 (Fig. S2E); this finding is consistent with previous studies (Hammes et al., 2001; Gao et al., 2006).

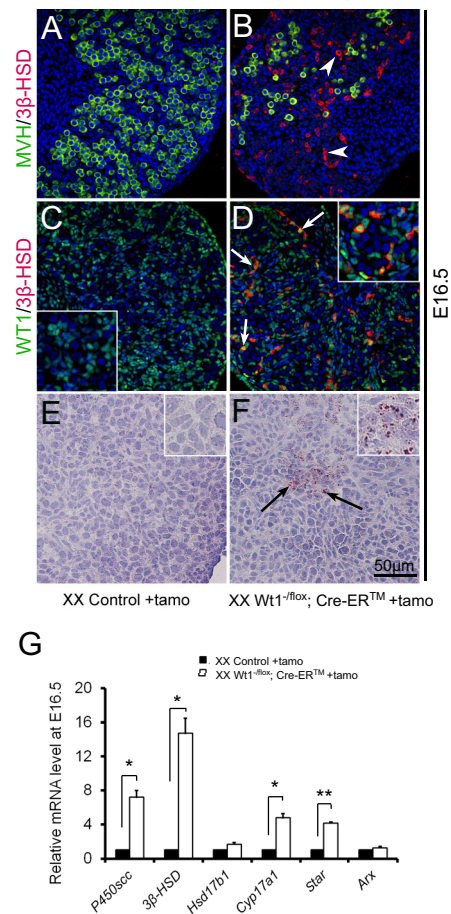


Fig. 1. Deletion of *Wt1* after sex determination results in ectopic development of steroidogenic cells in ovaries. Pregnant *Wt1*^{flox/flox} and control females were injected with tamoxifen (tamo) at E12.5 to induce Cre activity and the expression of steroidogenic genes in the embryonic gonads was examined at E16.5. Numerous 3 β -HSD-positive cells (B, red, arrowheads) are observed in *Wt1*^{-flox}; *Cre-ER*TM ovaries, and WT1 (green) is detected in 3 β -HSD-positive cells (D, arrows). Nuclei are stained blue with DAPI. Oil Red O-positive cells are observed in *Wt1*-deficient ovaries (F, arrows) but not in control ovaries (E). In E and F, the sections were counterstained with Harris hematoxylin. Insets show magnified views of control (C,E) and *Wt1*-deficient (D,F) gonads. (G) The mRNA levels of steroidogenic genes, such as *P450scc*, 3 β -HSD, *Cyp17a1* and *Star*, are significantly increased in *Wt1*-deficient XX gonads. Gonads with the same genotype were pooled for RNA preparation. The experiments were performed with three independent pools. The data are presented as means \pm s.e.m. **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

To examine the differentiation of somatic cells in *Wt1*-deficient gonads, the expression of testis- and ovary-specific genes was examined by immunostaining. The testis-specific gene *Sox9* was expressed in the Sertoli cells of control XY gonads at E12.5 and E13.5 (Fig. 2A,E). The ovary-specific gene FOXL2 was detected in granulosa cells of control XX gonads at E12.5 and E13.5 (Fig. 2C,G). The number of SOX9-positive cells was dramatically reduced in *Wt1*-deficient XY gonads. Interestingly, a few FOXL2-positive cells were also identified in *Wt1*-deficient XY gonads (Fig. 2B,F). The number of FOXL2-positive cells was also dramatically reduced in *Wt1*-deficient XX gonads, but a few SOX9-positive cells were observed in *Wt1*-deficient XX gonads (Fig. 2D,H). However, no AMH protein was detected and no testicular cords were formed in *Wt1*-deficient XX gonads (Fig. S3F).

Because most *Wt1*-deficient embryos died starting at E14.5, control and *Wt1*-deficient gonads were dissected at E13.5 and

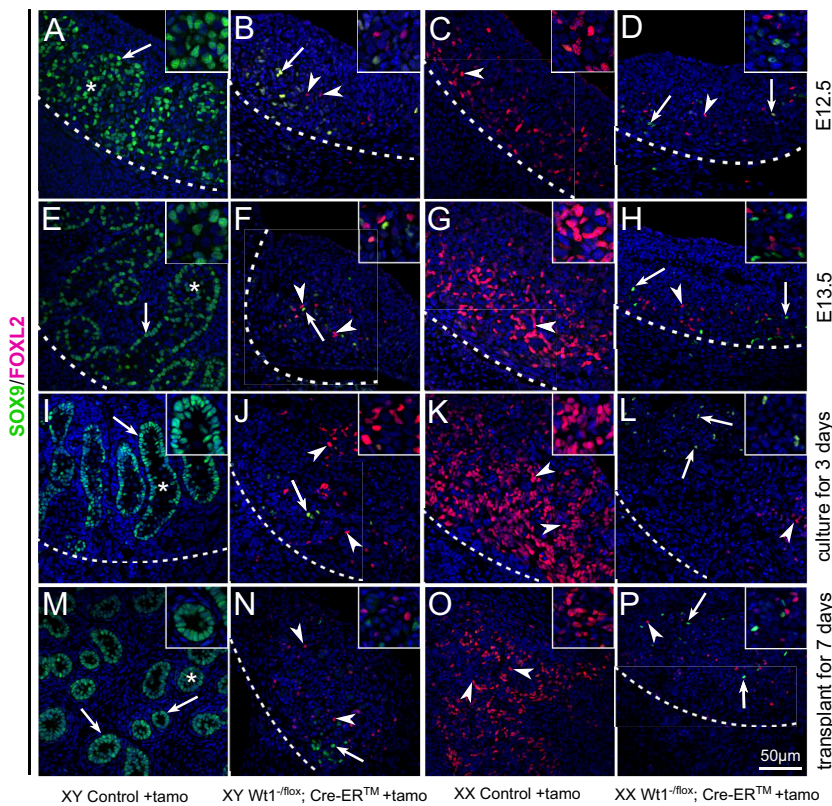


Fig. 2. Aberrant differentiation of somatic cells in *Wt1*^{-/-lox}; *Cre-ER*TM gonads. Pregnant *Wt1*^{fl/fl} females were injected with tamoxifen at E9.5 to induce Cre activity. SOX9 (green, arrows) and FOXL2 (red, arrowheads) are specifically expressed in the Sertoli cells of control XY gonads (A,E,I,M) and the granulosa cells of control XX gonads (C,G,K,O), respectively, from E12.5. SOX9-positive cells are dramatically reduced in number; however, a few FOXL2-positive cells are observed in *Wt1*-deficient XY gonads (B,F,J,N). By contrast, FOXL2-positive cells are dramatically reduced in number, and a few SOX9-positive cells are observed in *Wt1*-deficient XX gonads (D,H,L,P). No testicular cords are formed in *Wt1*-deficient XY gonads. Dashed lines denote the border between the gonads and mesonephros; asterisks indicate testicular cords. Insets show magnified views of supporting cells in the respective gonads. Nuclei are stained blue with DAPI.

cultured *in vitro* for 3 days or transplanted into the renal capsule for 7 days to examine gonadal development at later stages. Normal testicular cords were observed, and SOX9 was expressed in the Sertoli cells of control XY gonads after 3 days of *in vitro* culture (Fig. 2I) and 7 days after transplantation (Fig. 2M). Numerous FOXL2-positive cells were observed in control XX gonads (Fig. 2K,O). Similar to the findings at E12.5 and E13.5, only a few SOX9-positive and FOXL2-positive cells were noted in both *Wt1*-deficient XX (Fig. 2L,P) and XY (Fig. 2J,N) gonads, indicating that differentiation of supporting cells was affected in both XX and XY gonads when *Wt1* was inactivated before sex determination. Histologically, no significant difference was noted between *Wt1*-deficient XX and XY gonads.

Differentially expressed genes between control and *Wt1*-deficient gonads

To further verify the aberrant differentiation of somatic cells in *Wt1*-deficient gonads, the expression of genes that play important roles in gonad development were analyzed by real-time PCR at E11.5 and E13.5. As shown in Fig. 3A, the expression of ovary-specific genes *Wnt4*, *Rspo1*, *Foxl2*, *Bmp2* and *Fst* was significantly increased in *Wt1*-deficient XY gonads but decreased in *Wt1*-deficient XX gonads compared with expression in control gonads. By contrast, the expression of testis-specific genes *Sox9*, *Fgf9*, *Dhh*, *Amh*, and *Cyp26b1* was significantly decreased in *Wt1*-deficient XY gonads but increased in *Wt1*-deficient XX gonads at E13.5 (Fig. 3B), indicating that differentiation of male and female gonads was disturbed when *Wt1* was deleted before sex determination. The expression of *Sry* was also dramatically decreased in *Wt1*-deficient XY gonads at E11.5 (Fig. 3C), which is consistent with previous studies (Hammes et al., 2001; Bradford et al., 2009). The mRNA levels

of *Rspo1*, *Wnt4* and *Dax1* were downregulated in both *Wt1*-deficient XX and XY gonads at E11.5 (Fig. 3C), whereas 3 β -HSD expression was significantly upregulated in *Wt1*-deficient XY gonads at E11.5. These results indicate that sex differentiation is blocked in *Wt1*-deficient gonads.

Ectopic formation of the male-specific coelomic blood vessel and development of 3 β -HSD-positive steroidogenic cells in *Wt1*-deficient XX gonads

In the normal testis, a male-specific coelomic blood vessel is formed at E12.5 by endothelial cells migrating from the mesonephros. In the ovary, this migration is inhibited by *Wnt4* and *Fst*, and no coelomic blood vessel is formed (Jeays-Ward et al., 2003; Yao et al., 2004). In *Wt1*-deficient XX gonads, a coelomic blood vessel was also formed at E13.5 (Fig. 4B, white arrows). Considering the decreased expression of *Wnt4* and *Fst* as illustrated in Fig. 3A, the coelomic blood vessel was probably formed by the migration of endothelial cells from mesonephros.

To analyze the differentiation of steroidogenic cells, the expression of 3 β -HSD was examined by immunostaining. In normal testes, 3 β -HSD-positive Leydig cells were first observed at E12.5 (Fig. 5E), whereas these cells were observed as early as E11.5 (Fig. 5B) in *Wt1*-deficient XY gonads. Strikingly, numerous 3 β -HSD-positive steroidogenic cells were also noted in *Wt1*-deficient XX gonads at E12.5 (Fig. 5H) and E13.5 (Fig. 5L) but not in control ovaries (Fig. 5G,K). The number of 3 β -HSD-positive cells was dramatically increased in both *Wt1*-deficient XX (Fig. 5P) and XY (Fig. 5N) gonads after 3 days of *in vitro* culture, and most of the somatic cells in *Wt1*-deficient XX (Fig. 5T) and XY (Fig. 5R) gonads were 3 β -HSD positive 7 days after transplantation. To confirm these results, the expression of other steroidogenic cell-specific genes at 7 days after transplantation was

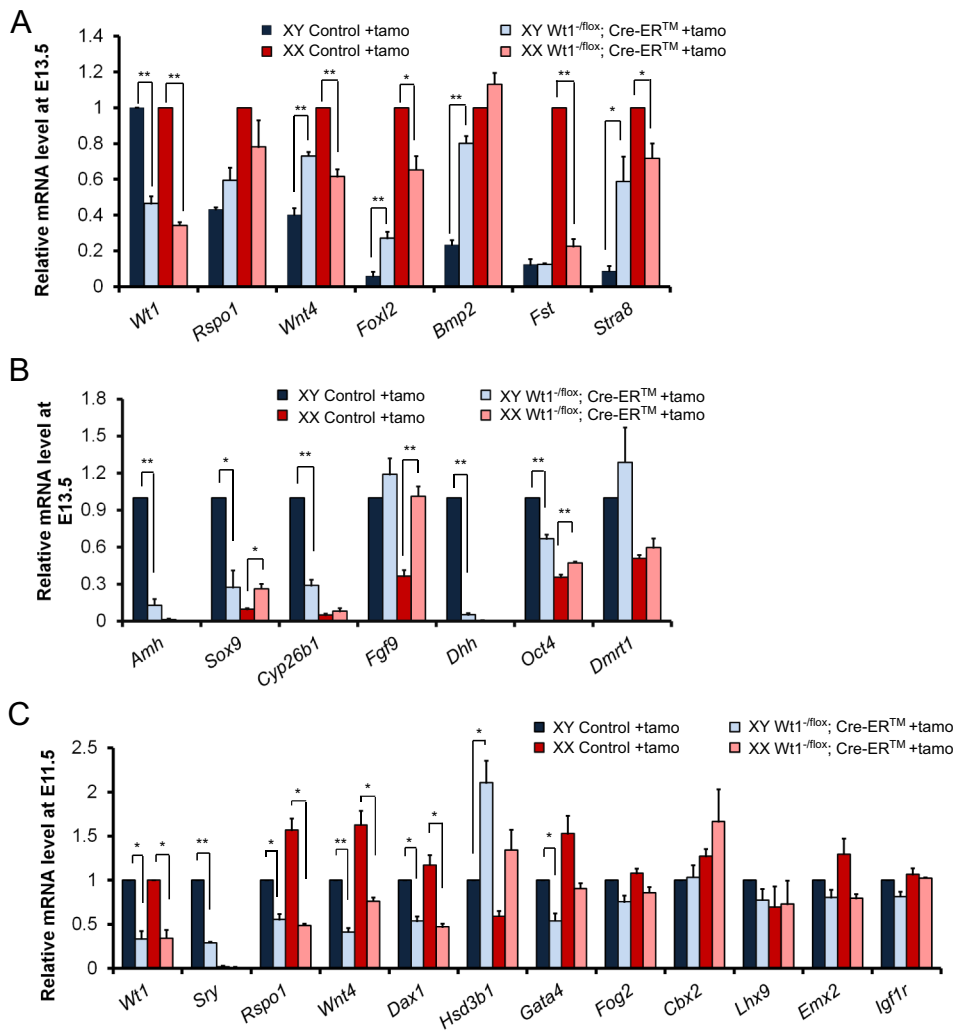


Fig. 3. Differentially expressed genes in *Wt1*^{-/-}; Cre-ERTM XX and XY gonads at E13.5 and E11.5. Pregnant *Wt1*^{flox/flox} females were injected with tamoxifen at E9.5 to induce Cre activity. The expression of (A) ovary-specific genes (*Wnt4*, *Rspo1*, *Foxl2*, *Bmp2* and *Fst*) and (B) testis-specific genes (*Sox9*, *Fgf9*, *Dhh*, *Amh* and *Cyp26b1*) at E13.5, and (C) several genes involved in early sex differentiation at E11.5 was evaluated by real-time PCR. Gonads with the same genotype at each time point were pooled for RNA preparation. The experiments were performed with three independent pools. The data are presented as mean±s.e.m. **P*<0.05, ***P*<0.01 (Student's *t*-test).

examined by immunofluorescence. INSL3, STAR and CYP17A1 were expressed in control testis (Fig. S4A,E,I) but not in control ovary (Fig. S4C,G,K). However, the number of cells expressing these three genes was significantly increased in *Wt1*-deficient XY (Fig. S4B,F,J) and XX (Fig. S4D,H,L) gonads 7 days after transplantation. The expression of other steroidogenic enzymes was also examined by real-time PCR. As shown in Fig. 4C, the mRNA levels of *P450scc*, *3β-HSD*, *Hsd17b1*, *Cyp17a1*, *Star* and *Arx* were significantly increased in *Wt1*-deficient XX gonads compared with the levels measured in control ovaries. After 3 days of *in vitro* culture, testosterone was detected in the medium of cultured control and *Wt1*-deficient XY gonads. A small amount of testosterone was also detected in the medium of cultured *Wt1*-deficient female gonads but not in the medium of cultured control ovaries (Fig. 4D). Collectively, these results demonstrated that many steroidogenic cells developed in both male and female gonads when *Wt1* was deleted before sex determination.

Most 3β-HSD-positive steroidogenic cells were derived from genital ridge somatic cells in *Wt1*-deficient XX and XY gonads

To trace the origin of steroidogenic cells in *Wt1*-deficient gonads, WT1 and 3β-HSD, SOX9 and 3β-HSD, and STAR and GATA4 double staining was performed. In control gonads (Fig. 6A,C), only

Sertoli and granulosa cells were labeled by the WT1 antibody at E12.5, and no WT1 signal was detected in 3β-HSD-positive Leydig cells (Fig. 6A, white arrowheads). Surprisingly, a small number of 3β-HSD-positive cells were also labeled with the WT1 antibody (white arrows) in both *Wt1*-deficient XY (Fig. 6B) and XX (Fig. 6D) gonads at E12.5. Interestingly, SOX9 and 3β-HSD double positive cells were also observed in *Wt1*-deficient XY and XX gonads. In control gonads (Fig. S5A,C), SOX9 was specifically expressed in Sertoli cells but not in granulosa cells and 3β-HSD was expressed in Leydig cells. By contrast, a small number of 3β-HSD and SOX9 double positive cells were observed in *Wt1*-deficient XY (Fig. S5B) and XX gonads (Fig. S5D).

GATA4 was expressed in most genital ridge somatic cells and abundantly expressed in testes Sertoli cells and ovarian granulosa cells (Fig. S5E,G) at E13.5. Many STAR-positive steroidogenic cells in both *Wt1*-deficient XY and XX gonads (Fig. S5F,H, white arrows) were also GATA4 positive. The above mentioned results indicate that many steroidogenic cells in *Wt1*-deficient gonads are derived from genital ridge somatic cells; they are most likely the progenitors of Sertoli and granulosa cells. The WT1, 3β-HSD and SOX9, 3β-HSD double positive cells may be the only cells undergoing transition, and the cells may lose WT1 and SOX9 signals after transition.

To further identify the origin of 3β-HSD-positive cells in *Wt1*-deficient gonads, lineage-tracing experiments were performed using

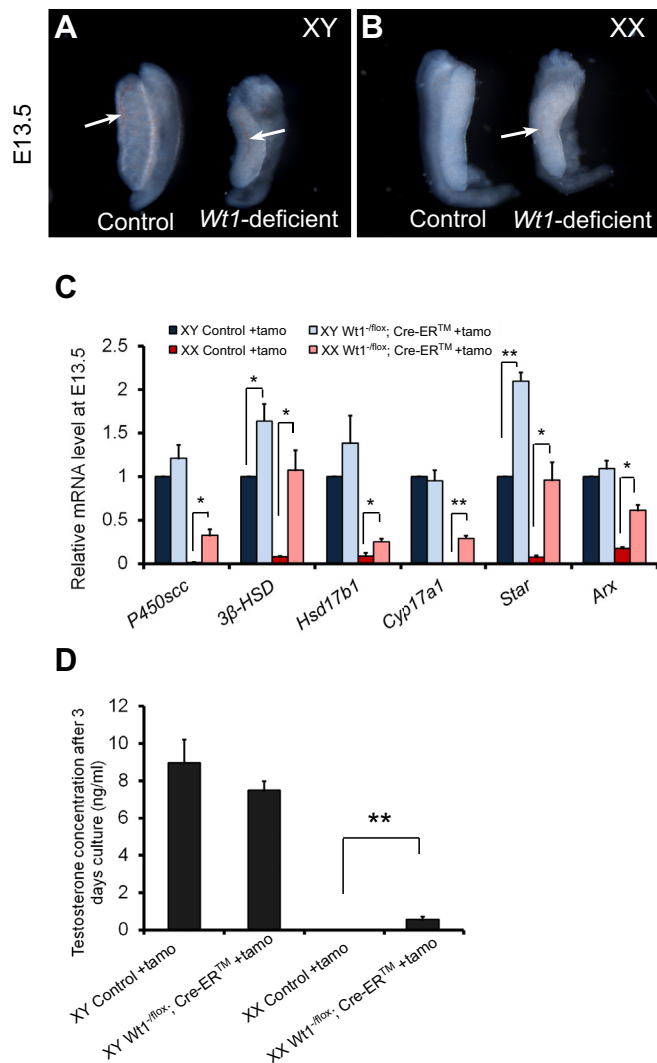


Fig. 4. Formation of coelomic blood vessel and secretion of testosterone in *Wt1*^{-flox}; Cre-ERTM XX and XY gonads. Pregnant *Wt1*^{flox/flox} females were injected with tamoxifen at E9.5 to induce Cre activity. A coelomic blood vessel formed in *Wt1*-deficient XX gonads (B, arrow) as well as in control and *Wt1*-deficient XY gonads (A, arrows) at E13.5. The mRNA levels of some steroidogenic genes were dramatically increased in *Wt1*-deficient XX gonads compared with levels measured in control ovaries (C). Gonads with the same genotype were pooled for RNA preparation. Experiments were performed with three independent pools. (D) A small amount of testosterone was detected in the medium of cultured *Wt1*-deficient XX gonads. Media from at least four pairs of gonads cultures of each genotype were used. Data are presented as mean±s.e.m. **P*<0.05, ***P*<0.0 (Student's *t*-test).

Wt1^{CreERT2/+} mice. In this mouse model, the cDNA encoding a Cre-modified estrogen ligand binding domain (CreERT2) was knocked in to the *Wt1* locus and the first *Wt1* coding exon was removed (Zhou et al., 2008). *Wt1*^{flox/flox}; *Rosa-EGFP*^{flox/flox} mice were crossed with *Wt1*^{CreERT2/+} mice and pregnant mice were injected with tamoxifen at E9.5 to initiate recombination. As shown in Fig. 6E,F, numerous 3β-HSD-positive cells in *Wt1*^{CreERT2/flox}; *Rosa-EGFP*^{flox/flox} XY and XX gonads were also stained with GFP at E13.5, indicating these 3β-HSD-positive cells were derived from *Wt1*-expressing precursor cells in the undifferentiated genital ridge, and these cells developed to steroidogenic cells when *Wt1* was inactivated.

SF1 expression was dramatically upregulated in *Wt1*-deficient XX and XY gonads

Nuclear receptor *Sf1* plays a key role in the regulation of steroid hormone biosynthesis by inducing the expression of steroidogenic enzymes in steroidogenic cells. Therefore, the expression of *Sf1* in control and *Wt1*-deficient gonads was examined by immunofluorescence. In control E13.5 testes, SF1 was expressed in both Sertoli and Leydig cells (Fig. 7A, green), but the signal in Leydig cells (Fig. 7A, arrows) was much stronger than that in Sertoli cells (Fig. 7A, arrowheads). In tamoxifen-treated *Wt1*^{-flox}; Cre-ERTM XY gonads, the number of cells with a high level of SF1 expression (Fig. 7B, white arrows) was dramatically increased. A low level of SF1 expression was also detected in pre-granulosa cells (Fig. 7C, white arrowheads) of control ovaries at E13.5. Strikingly, numerous cells with a high level of SF1 expression (Fig. 7D, white arrows) were noted in tamoxifen-treated *Wt1*^{-flox}; Cre-ERTM XX gonads at E13.5. Most of these cells were also 3β-HSD positive (Fig. 7D, red). However, a small portion of them were only SF1 positive (Fig. 7D, inset). These results suggest that the expression of SF1 is activated when *Wt1* is deleted.

To further confirm these results, *Wt1*^{-flox}; *Rosa-EGFP*^{flox/flox}; *AMH-Cre* mice were used to examine the expression of SF1 in *Wt1*-deleted Sertoli cells. In *Wt1*^{flox/flox}; *Rosa-EGFP*^{flox/flox}; *AMH-Cre* testes, a GFP signal was only detected in Sertoli cells (Fig. 7E, green), indicating that *AMH-Cre* is only activated in Sertoli cells. A low level of SF1 (Fig. 7E, red) was expressed in Sertoli cells (Fig. 7E, arrowheads), and a much stronger signal of SF1 was detected in Leydig cells (Fig. 7E, arrows). The expression of SF1 in Sertoli cells of *Wt1*^{-flox}; *Rosa-EGFP*^{flox/flox}; *AMH-Cre* testes (Fig. 7E, arrows) was significantly increased compared with that in control testes (Fig. 7E, arrowheads), which was comparable to the expression observed in Leydig cells (Fig. 7E). These results indicate that inactivation of *Wt1* in gonad somatic cells leads to the upregulation of SF1 expression.

WT1 suppressed *Sf1* expression by directly binding to the promoter region

To further clarify the relationship between *Wt1* and *Sf1*, *Wt1* was overexpressed in primary Leydig cells in which *Sf1* was abundantly expressed. The results showed that the mRNA level of *Sf1* decreased to ~26% of that in the control group after infection with *Wt1*-expressing adenovirus (Fig. 8B).

An *Sf1* promoter fragment encompassing nucleotides -589 to +85 was reported to be sufficient to direct *Sf1* expression (Woodson et al., 1997; Wilhelm and Englert, 2002). To analyze the regulation of *Sf1* expression by *Wt1*, *Sf1* luciferase reporter plasmid was constructed with this promoter fragment. After overexpression of *Wt1* in primary Leydig cells, *Sf1* reporter activity was decreased to 30% of that in control Leydig cells (Fig. 8C), indicating that *Sf1* expression in Leydig cells is repressed by WT1. To verify whether *Sf1* is the direct target of WT1, a ChIP assay was performed using Sertoli cells from 2-week-old mice, in which a low level of SF1 was still expressed. The reason we used Sertoli cells instead of Leydig cells is that a large number of cells is required for the ChIP assay and the number of Leydig cells is very limited in testes. Four previously defined WT1 binding sites (WB1, WB2, WB3 and WB4) in the *Sf1* promoter region (Wilhelm and Englert, 2002) were analyzed. The ChIP assay results showed that WT1 bound strongly to WB1, WB2 and WB3+4 sites in the *Sf1* promoter (Fig. 8D), indicating that WT1 directs *Sf1* expression by binding to its promoter. To examine the function of each WT1 binding site, a point mutagenesis assay was conducted. The results of the luciferase assay showed that a

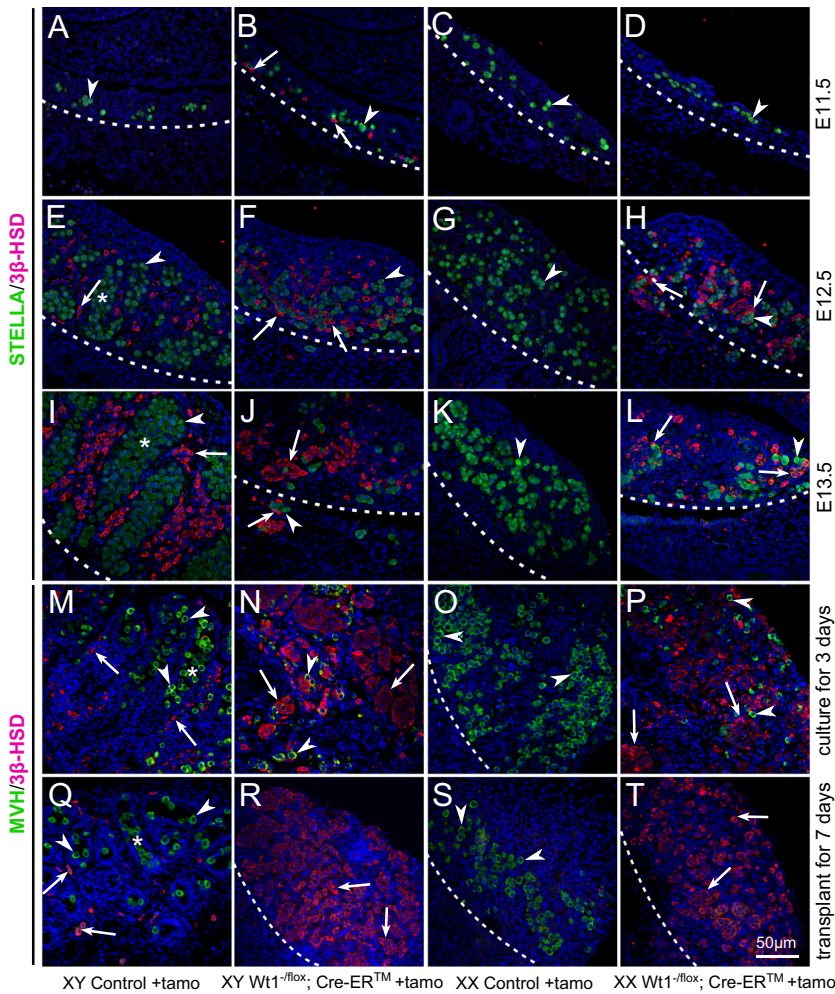


Fig. 5. Ectopic development of 3β-HSD-positive steroidogenic cells in *Wt1*^{-/-lox}, *Cre-ERTM* gonads. Pregnant *Wt1*^{flox/flox} females were injected with tamoxifen at E9.5 to induce Cre activity. Germ cells were labeled with STELLA or MVH (green, arrowheads) and steroidogenic cells with 3β-HSD (red, arrows). In control XY gonads, Leydig cells are observed from E12.5 (A,E,I,M,Q), whereas 3β-HSD-positive Leydig cells are first observed in *Wt1*-deficient XY gonads at E11.5 (B, white arrows) and increased at E12.5 and E13.5 (F,J, white arrows). No steroidogenic cells are observed in control XX gonads, whereas 3β-HSD-positive Leydig cells are first observed in *Wt1*-deficient XX gonads at E11.5 (D). However, 3β-HSD-positive cells are observed in *Wt1*-deficient XX gonad at E12.5 (H) and E13.5 (L). The number of 3β-HSD-positive cells significantly increases 3 days after *in vitro* culture and 7 days after renal capsule transplantation in both *Wt1*-deficient XX (P,T) and XY gonads (N,R). Dashed lines denote the border between the gonads and mesonephros. Nuclei are stained blue with DAPI.

mutation in any one of the four WT1 binding sites did not significantly affect the repressive function of WT1 with respect to *Sfl* expression in Leydig cells (Fig. 8E). By contrast, when WB2

and WB3 were simultaneously mutated, *Sfl* promoter activity was not affected (Fig. 8E, WB2+3 vs normal promoter without *Ad-Wt1*; WB1+2+3 vs normal promoter without *Ad-Wt1*), indicating that

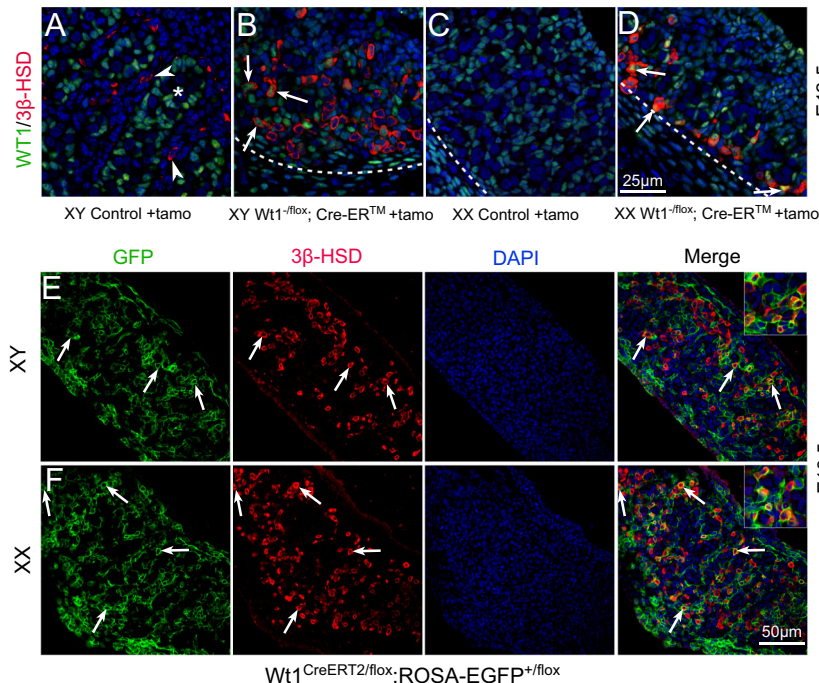


Fig. 6. Steroidogenic cells in *Wt1*-deficient gonads are derived from genital ridge somatic cells. (A-D) Double staining of WT1 and 3β-HSD in control and *Wt1*^{-/-lox}, *Cre-ERTM* gonads at E12.5. Pregnant *Wt1*^{flox/flox} females were injected with tamoxifen at E9.5 to induce Cre activity. Dashed lines denote the border between the gonads and mesonephros. (E,F) Many 3β-HSD-positive cells in *Wt1*^{CreERT2/flox}; *Rosa-EGFP*^{+flox} XY and XX gonads are also stained with GFP (arrows) at E13.5. Pregnant *Wt1*^{flox/flox}; *Rosa-EGFP*^{flox/flox} mice were injected with tamoxifen at E9.5 to initiate recombination. Arrowheads and asterisk in A indicate Leydig cells and testicular cord, respectively; insets in merged panels show magnified views of GFP and 3β-HSD double-positive cells. Nuclei are stained blue with DAPI.

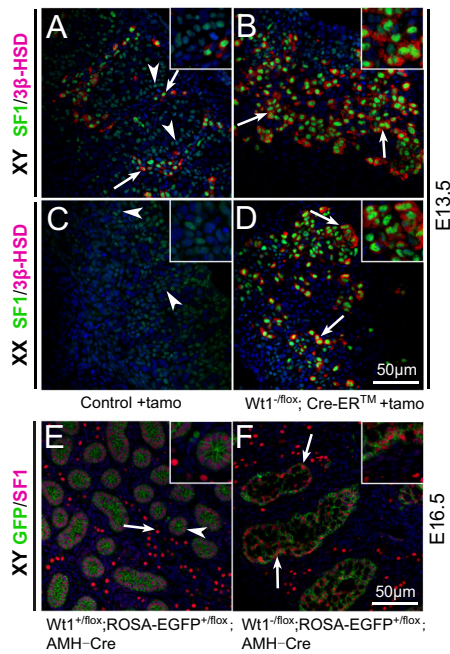


Fig. 7. SF1 expression was dramatically increased in *Wt1*-deficient XX and XY gonads. (A–D) Pregnant females were injected with tamoxifen at E9.5 to induce Cre activity. (A) In control E13.5 testis, SF1 (green) is expressed in both Sertoli (arrowheads) and Leydig cells (arrows). (B) In tamoxifen-treated *Wt1*^{-flox}; *Cre-ER*TM XY gonads, the number of cells with a high level of SF1 expression (arrows) was dramatically increased. (C) A low level of SF1 expression is detected in pre-granulosa cells (arrowheads) of control ovaries at E13.5. (D) Numerous cells with high levels of SF1 expression (arrows) are observed in tamoxifen-treated *Wt1*^{-flox}; *Cre-ER*TM XX gonads at E13.5. (E) In control E16.5 testes, a low level of SF1 (red) is expressed in Sertoli cells (arrowhead) and a much stronger SF1 signal is detected in Leydig cells (arrow). (F) Expression of SF1 in Sertoli cells of *Wt1*^{-flox}; *Rosa-EGFP*^{+/flox}; *AMH-Cre* testes (arrows) at E16.5 is significantly increased compared with Sertoli cells in control testis (E). Insets in A–F show magnified views of SF1-positive cells. Nuclei are stained blue with DAPI.

both WB2 and WB3 are indispensable for the interaction of WT1 with the *Sfl* promoter.

DISCUSSION

Mammalian gonads arise from the bi-potential gonads, which can develop into testes or ovaries depending on the differentiation of Sertoli cells and granulosa cells, which has been extensively studied previously. A number of genes have been shown to be required for the development of the genital ridge, such as *Emx2* (Yoshida et al., 1997), *Lhx9* (Luo et al., 1994), *Nr5a1* (Sadovsky et al., 1995) and *Wt1* (Kreidberg et al., 1993). Inactivation of these genes causes defects in bi-potential gonad development. Steroidogenic cells (Leydig and theca-interstitium) are another major cell type in gonads. However, the molecular mechanism regulating the differentiation of these cells during gonad development is still largely unknown.

Our recent study demonstrated that inactivation of *Wt1* in Sertoli cells after sex determination using *AMH-Cre* mice resulted in the transformation of Sertoli cells to Leydig cells, suggesting that Sertoli and Leydig cells are probably derived from same progenitor cells (Zhang et al., 2015). In control ovary, 3β-HSD-positive theca-interstitial cells are not observed during the embryonic stage. In the present study, inactivation of *Wt1* in ovaries after sex determination also caused ectopic development of steroidogenic cells during the

embryonic stage and some of them expressed the truncated WT1 protein. These results suggest that the differentiation of steroidogenic cells in male and female gonads is most likely regulated by a common mechanism. To further explore the functions of *Wt1* in gonad somatic cell differentiation, *Wt1* was deleted in genital ridge somatic cells before sex determination. Strikingly, we found that the differentiation of both Sertoli and granulosa cells was blocked and most somatic cells in both male and female gonads are 3β-HSD-positive steroidogenic cells. These results indicate that *Wt1* is indispensable for both the specification and maintenance of supporting cells, not only in male gonads but also in female gonads.

Further remaining questions to be answered are what happens to the progenitors of supporting cells and where do the steroidogenic cells come from in *Wt1*-deficient gonads? Ectopic development of steroidogenic cells has also been observed in *Wnt4* mutant XX gonads during the embryonic stage (Vainio et al., 1999). A subsequent study suggested that the 3β-HSD-positive cells are steroidogenic adrenal precursors that migrate from the mesonephros because of *Wnt4* inactivation (Jeays-Ward et al., 2003). In the present study, the expression of *Wnt4* was reduced by ~50% in *Wt1*-deficient gonads. However, many more steroidogenic cells were observed and the phenotypes observed in the two models were different (Vainio et al., 1999; Jeays-Ward et al., 2004). In addition, steroidogenic cells were also detected in ovaries *in vitro* when *Wt1* was inactivated after sex determination. These results exclude the possibility that the 3β-HSD-positive cells in *Wt1*-deficient gonads come from the adrenal glands.

Supporting cells and steroidogenic cells are two major types of somatic cells in gonad. In *Wt1*-deficient gonads, nearly all the supporting cells disappeared and a large amount of steroidogenic cells ectopically developed. These results raised the question of whether the steroidogenic cells in *Wt1*-deficient gonads developed from the precursors of supporting cells.

The WT1, 3β-HSD double positive cells in *Wt1*^{-flox}; *Cre-ER*TM gonads at E12.5 and the GFP, 3β-HSD double positive cells in *Wt1*^{CreERT2/flox}; *Rosa-EGFP*^{+/flox} gonads indicate that steroidogenic cells in *Wt1*-deficient gonads were derived from *Wt1*-expressing cells. In undifferentiated genital ridges, most of the somatic cells are WT1 positive and a large proportion of *Wt1*-expressing cells will develop into Sertoli and granulosa cells. After sex determination, *Wt1* is only expressed in testicular Sertoli cells and ovarian granulosa cells. In the present study, we also found that a small number of 3β-HSD-positive cells expressed the Sertoli cell-specific gene *Sox9*. Our recent study demonstrated that inactivation of *Wt1* in Sertoli cells after sex determination results in Sertoli to Leydig cell transformation (Zhang et al., 2015). Based on these results, we speculated that many of the 3β-HSD-positive cells, if not all, in *Wt1*-deficient gonads are derived from the progenitors of supporting cells. Other cells, such as the precursors of the steroidogenic cells, may also be an origin. Theca cells were recently reported to be derived from *Wt1*-positive cells indigenous to the ovary and *Gli1*-positive mesenchymal cells that have migrated from the mesonephros (Liu et al., 2015).

We also found many GFP-positive but 3β-HSD-negative cells. The possible explanation for this phenomenon may be that these cells have not yet transformed into steroidogenic cells at this stage, because the number of 3β-HSD-positive cells was dramatically increased in *Wt1*-deficient gonads 7 days after transplantation compared with the level at E13.5. Another possibility is that not all the *Wt1*-expressing cells in undifferentiated genital ridges develop into supporting cells or steroidogenic cells. This needs further investigation.

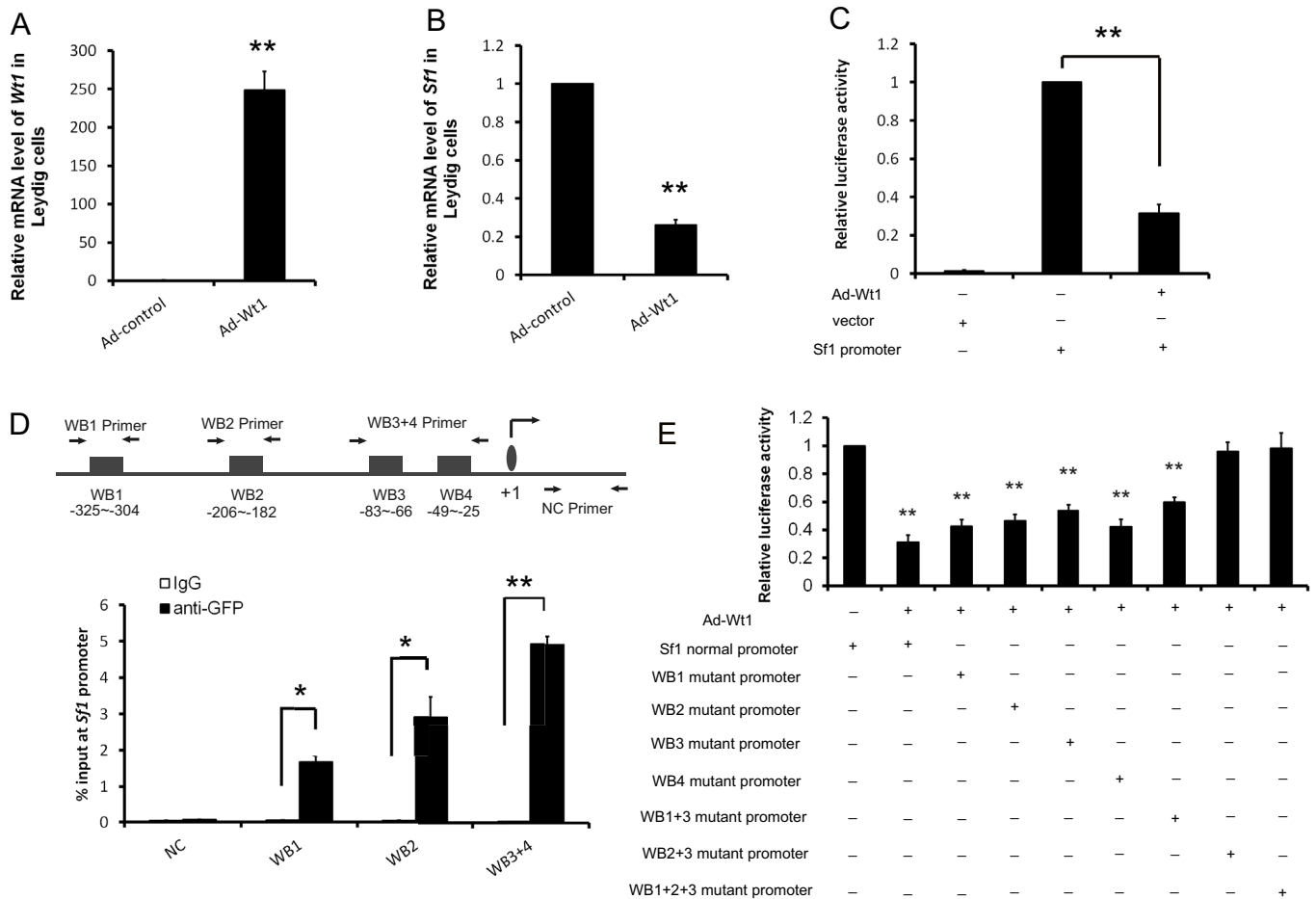


Fig. 8. WT1 suppresses *Sf1* expression by directly binding to the promoter region. *In vitro* cultured primary Leydig cells were infected with *Wt1*-expressing adenovirus. The *Wt1* mRNA level dramatically increases (A), whereas the mRNA level of *Sf1* significantly decreases (B) 24 h after infection. (C) The luciferase activity of the *Sf1* promoter reporter in Leydig cells is significantly reduced after *Wt1* overexpression. (D) ChIP analysis for potential WT1 binding sites on the promoter region of *Sf1* gene. The schematic image shows the predicted WT1 binding sites (WB1-WB4) within the *Sf1* promoter and the primers used for the ChIP assay. NC, negative control. Percentages of immunoprecipitated DNA compared with input are shown. (E) The activity of the *Sf1* promoter in Leydig cells is not significantly repressed by WT1 with mutation of any one of the WT1 binding sites. By contrast, the repressive function of WT1 on *Sf1* promoter activity is completely abolished when WB2 and WB3 are simultaneously mutated. ** $P < 0.01$, compared with results of the respective promoter without infection of *Wt1* adenovirus. In A-E, the mean \pm s.e.m. of three biological replicates measured in triplicate are shown. * $P < 0.05$, ** $P < 0.01$ (A,B,D: Student's *t*-test; C,E: one-way ANOVA).

It has been reported that *Sf1* is required for the development of steroidogenic cells and that loss of *Sf1* results in agenesis of gonads and adrenal glands (Luo et al., 1994; Sadovsky et al., 1995). The relationship between *Wt1* and *Sf1* has been studied previously using TM3 (Takasawa et al., 2014), TM4 (Wilhelm and Englert, 2002) and C2C12 (Val et al., 2007) cell lines. These results suggest that the expression of *Sf1* was activated by WT1. Surprisingly, in our study, *Sf1* expression was remarkably increased in gonad somatic cells when *Wt1* was inactivated. The subsequent *in vitro* studies using primary Leydig cells demonstrated that WT1 suppressed *Sf1* expression by directly binding to the promoter region. The discrepancy between our results and those reported in previous studies is most likely due to the use of primary cells in our study instead of cell lines. Another possibility is that *Wt1* plays different roles in *Sf1* expression at different developmental stages. Both *Wt1* and *Sf1* were expressed in genital ridge somatic cells before sex determination. However, *Sf1* expression was significantly decreased in the *Wt1*-positive Sertoli cells and significantly increased in *Wt1*-negative Leydig cells, suggesting that the expression of *Sf1* is antagonized by *Wt1*. *Wt1* is probably required for the basal level

expression of *Sf1* in the undifferentiated genital ridge, whereas *Sf1* expression is antagonized by *Wt1* in the supporting cells after sex determination. We speculate that the high level of *Sf1* expression in steroidogenic cells is likely to be induced by another factor that is antagonized by *Wt1*. In the absence of *Wt1*, high *Sf1* expression is induced by an unknown factor, which leads undifferentiated somatic cells to develop into steroidogenic cells. Interestingly, a recent study also showed that the differentiation of steroidogenic cells in the adrenal gland is repressed by *Wt1* (Bandiera et al., 2013), implying that *Wt1* plays a role in the differentiation of gonad somatic cells, most likely by inhibiting the development of steroidogenic cells.

A previous study showed that the deletion of the *Wt1* +KTS isoform caused male-to-female sex reversal and reduced Sry expression, whereas the development of XX gonads was not significantly affected. The -KTS isoform is not required for sex determination, but is important for the survival of the gonadal primordium (Hammes et al., 2001). In this study, the development of both XX and XY gonads was affected after deletion of *Wt1*. The different phenotypes observed in this study were probably caused

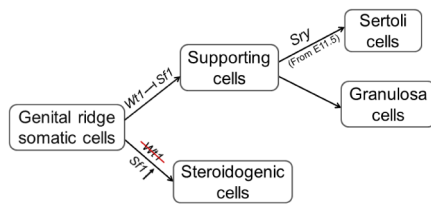


Fig. 9. A model of *Wt1* function in regulating gonadal somatic cell differentiation. Physiologically, genital ridge somatic cells will differentiate into *Wt1*-expressed granulosa or Sertoli cells in female and male gonads, respectively, with *Wt1* expression. Without *Wt1* expression, genital ridge somatic cells differentiate into steroidogenic cells and sex differentiation will not occur. *Wt1* is involved in gonad somatic cell differentiation by repressing *Sry* expression.

by the deletion of all *Wt1* isoforms in this study, which indicates that different isoforms of the *Wt1* gene function coordinately during gonad development. Another possible reason for the discrepancy is that the *Wt1* gene was inactivated at approximately E10.5 in our mouse model, whereas conventional knockout models were used in the previous study (Hammes et al., 2001).

In summary, we demonstrated that *Wt1* plays a key role in regulating the differentiation of somatic cells during gonad development. As proposed in the model illustrated in Fig. 9, genital ridge somatic cells will differentiate into granulosa or Sertoli cells in female and male gonads, respectively, with *Wt1* expression. Without *Wt1* expression, the somatic cells differentiate into steroidogenic cells, and sex differentiation will not occur. This study also demonstrated that *Wt1* is involved in gonad somatic cell differentiation by repressing *Sry* expression. Our study uncovered a new type of cell fate determination before the differentiation of Sertoli and granulosa cells, which provides a new concept for cell lineage determination in gonadal development. *WT1* was originally identified to play an etiological role in patients with Denys–Drash syndrome (DDS), which consists of a triad of phenotypes: WT predisposition, pseudohermaphroditism and early-onset renal failure (Drash et al., 1970; Call et al., 1990; Gessler et al., 1990; Huff et al., 1991; Pelletier et al., 1991a; Bruening et al., 1992; Coppes et al., 1992). The results obtained in this study imply that disorders of sex development (DSD) in human patients with *Wt1* mutations are likely to be caused by the aberrant differentiation of supporting cells and steroidogenic cells.

MATERIALS AND METHODS

Mice

All animal studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences (CAS). *Wt1*^{-flox}; *Cre-ER*TM and control (*Wt1*^{flox/flox}; *Wt1*^{-flox}) embryos were obtained by crossing *Wt1*^{-flox}; *Cre-ER*TM males with *Wt1*^{flox/flox} females. Pregnant *Wt1*^{flox/flox} females were injected intraperitoneally with 6 mg/40 g body weight tamoxifen at 9.5 or 12.5 days post coitum to induce Cre activity. *Wt1*^{-flox}; *Rosa-EGFP*^{+flox}; *AMH-Cre* mice were obtained by crossing *Wt1*^{+/-}; *AMH-Cre* mice with *Wt1*^{flox/flox}; *Rosa-EGFP*^{flox/flox} mice. Further details of strains used can be found in supplementary Materials and methods.

Plasmids and adenovirus

A *Sry* promoter fragment encompassing nucleotides –589 to +85 (Woodson et al., 1997; Wilhelm and Englert, 2002) was cloned into the *SacI* and *HindIII* sites of the pGL3basic luciferase reporter vector (Promega). A QuikChange kit (Stratagene) was used to introduce mutations using primers listed in Table S1. The adenoviruses containing *Wt1*-A cDNA were generated using the Gateway Expression System (Invitrogen).

Immunofluorescence and immunohistochemistry analysis

Gonads were dissected from *Wt1*-deficient and control embryos immediately after euthanasia, fixed in 4% paraformaldehyde for up to 24 h, stored in 70% ethanol and embedded in paraffin. Five-micrometer-thick sections were cut and mounted on glass slides. After deparaffinization, sections were processed for immunohistochemistry and immunofluorescence analyses of at least three embryos for each genotype using antibodies described in supplementary Materials and methods.

Nucleic acid isolation and quantitative reverse transcription PCR

Gonads with the same genotype were pooled for RNA preparation. The experiments were performed with three independent pools using *Hprt1* as an endogenous control, as described in supplementary Materials and methods.

Organ culture and renal capsule transplantation

After tamoxifen injection at E9.5, gonads with mesonephroi were dissected at E13.5 and cultured on agarose stands for 3 days or implanted under the renal capsule of recipient mice for 7 days as detailed in supplementary Materials and methods.

Oil red O (ORO) staining

After fixation in 4% paraformaldehyde and rinsing with 60% isopropanol, sections were stained with freshly prepared ORO solution, washed with isopropanol, counterstained with Harris Hematoxylin and mounted in glycerol–PBS (9:1) for further analysis, as described in supplementary Materials and methods.

Isolation of Leydig cells, transient transfection, infection and luciferase assay

Percoll-purified Leydig cells from 19-day-old mice were transfected with *Sry* normal or mutant promoter luciferase reporter plasmids (120 ng) using Lipofectamine 3000 transfection reagent when cells were ~70% confluent. After 12 h, the medium was removed and cells were infected with the control or *Wt1* adenovirus. Cells were harvested and lysed 24 h later. Luciferase activity was measured using a dual luciferase reporter assay system (Promega). Further details of all procedures are given in supplementary Materials and methods.

Chromatin immunoprecipitation (ChIP) assay

Sertoli cells from 2-week-old mice were isolated as previously described (Li et al., 2013). After the cells were infected with *Wt1* adenovirus for 24 h, ChIP assays were performed with the SimpleChIP Enzymatic Chromatin IP kit (magnetic beads, CST 9003) as described in supplementary Materials and methods. DNA from the immunoprecipitates and input DNA were analyzed by real-time PCR using primers listed in Table S1.

Hormone measurements

Media from organ cultures were collected and centrifuged at 3000 r.p.m. for 10 min. The supernatants were sent to Beijing North Institute of Biological Technology for testosterone measurement using Iodine [¹²⁵I] Testosterone Radioimmunoassay Kit according to the manufacturer's instructions.

Statistical analysis

Experiments were repeated at least three times. Three to five control or *Wt1*-deficient XY or XX embryos at each time point were used for immunostaining. For gonad culture or transplantation, at least four pairs of XY or XX gonads of each genotype were used. Quantitative results are presented as the mean±s.e.m. For the luciferase and ChIP assay, the mean±s.e.m. of three biological replicates measured in triplicate were calculated. The data were evaluated for significant differences using Student's *t*-test and one-way ANOVA. *P*-values <0.05 were considered to be significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.G. conceived and designed the experiments. M.C. and L.Z. performed the experiments. X.L., C.H. and D.C. analyzed the data. F.G., M.C. and L.Z. wrote the manuscript. All authors discussed the results and edited the manuscript.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.144105.supplemental>

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