**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 adrenogenital 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...
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 3β-HSD-positive steroidogenic cells. Further \textit{in vivo} and \textit{in vitro} studies indicated that \( Wt1 \) directs the lineage specification of Sertoli and granulosa cells by repressing \( Sf1 \) expression. Without \( Wt1 \), \( Sf1 \) 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 3β-HSD-positive steroidogenic cells

\( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) males were crossed with \( Wt1^{\text{flox/fflo}} \) females and the pregnant females were injected with tamoxifen at E12.5. The ovaries from control and \( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) embryos were collected at E16.5. 3β-HSD (3β-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 3β-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 3β-HSD-positive cells in \( Wt1 \)-deficient gonads, a WT1 and 3β-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 \( Wt1 \) 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 3β-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), 3β-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 \( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) mice were dissected at E13.5 and cultured \textit{in vitro} with tamoxifen. A large number of 3β-HSD-positive steroidogenic cells were observed in \( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) ovaries but not in control ovaries (Fig. S1). These cells were also labeled with the anti-WT1 antibody, indicating that these 3β-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 \( Wt1^{\text{flox/fflo}} \) females that were mated with \( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) males, were injected with tamoxifen at E9.5. The morphology of gonads from \( Wt1^{-\text{flox}}; \text{Cre-ERT}^\text{TM} \) 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 (Hamnes et al., 2001; Gao et al., 2006).

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
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 3β-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 3β-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 3β-HSD was examined by immunostaining. In normal testes, 3β-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 3β-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). The number of 3β-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 3β-HSD positive 7 days after transplantation. To confirm these results, the expression of other steroidogenic cell-specific genes at 7 days after transplantation was
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...
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; AMH-Cre mice were used to examine the expression of SF1 in Wt1-deleted Sertoli cells. In Wt1+/flox; Rosa-EGFP+/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; AMH-Cre testes (Fig. 7F, arrows) was significantly increased compared with that in control testes (Fig. 7E, arrowheads), which was comparable to the expression observed in Leydig cells (Fig. 7F). 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

Wt1CreERT2/+ 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). Wt1CreERT2/fllox; Rosa-EGFPfllox/fllox mice were crossed with Wt1CreERT2/+ 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 Wt1CreERT2/fllox; Rosa-EGFPfllox/fllox 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.
mutation in any one of the four WT1 binding sites did not significantly affect the repressive function of WT1 with respect to Sf1 expression in Leydig cells (Fig. 8E). By contrast, when WB2 and WB3 were simultaneously mutated, Sf1 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. 5. Ectopic development of 3β-HSD-positive steroidogenic cells in Wt1−/flox; Cre-ERTM gonads. Pregnant Wt1flox/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, arrows). No steroidogenic cells are observed in control XX gonads, 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 (C,G,K,O,S) and 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.

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−/flox; Cre-ERTM gonads at E12.5. Pregnant Wt1flox/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 Wt1CreERT2/flox; Rosa-EGFP+/flox XY and XX gonads are also stained with GFP (arrows) at E13.5. Pregnant Wt1CreERT2/flox; Rosa-EGFP+/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.
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-ERT2+ gonads at E12.5 and the GFP, 3β-HSD double positive cells in WT1CreERT2+/flox, Rosa-EGFP+/flox gonads indicate that steroidogenic cells in WT1-deficient gonads were derived from the precursors of supporting 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 all the WT1-expressing cells in undifferentiated genital ridges develop into supporting cells or steroidogenic cells. This needs further investigation.

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 Sox9 (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
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
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, genital ridge somatic cells differentiate into steroidogenic cells and sex differentiation will not occur. Wt1 is involved in gonad somatic cell differentiation by repressing Sf1 expression.

**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−/−; Cre-ERTM and control (Wt1fl/+; Cre-ERTM) embryos were obtained by crossing Wt1−/−; Cre-ERTM males with Wt1fl/+; Cre-ERTM females. Pregnant Wt1fl/+ 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−/−; Rosa-EGFP+/fl mice were obtained by crossing Wt1−/−; AMH-Cre mice with Wt1fl/+; Rosa-EGFP+/fl. Further details of strains used can be found in supplementary Materials and methods.

**Plasmids and adenovirus**

A Sf1 promoter fragment encompassing nucleotides −589 to +85 (Woodson et al., 1997; Wilhelm and Engert, 2002) was cloned into the SacI and HindIII sites of the pG3basic luciferase reporter vector (Promega). A QuickChange 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 Sf1 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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