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Wt1 Is Involved in Leydig Cell Steroid Hormone Biosynthesis by Regulating Paracrine Factor Expression in Mice[1]

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ABSTRACT

Wilms tumor gene, *Wt1*, is abundantly expressed in testis Sertoli cells. Our recent study demonstrated that *Wt1* is involved in spermatogenesis by regulating Sertoli cell polarity. In the present study, we found that *Wt1* is also required for steroidogenesis in Leydig cells and that deletion of the *Wt1* gene resulted in defects in testosterone biosynthesis and downregulation of steroidogenic gene expression, including cytochrome P450 side-chain cleavage (*P450scc*), steroidogenic acute regulatory protein (*StAR*), 3beta-hydroxysteroid dehydrogenase I (*3beta-HSD*), and cytochrome P450 17A1 (*Cyp17a1*). The expression of *LHR* was significantly decreased in *Wt1*$^{-/flox}$; *Cre-ER*^{TM} testes after tamoxifen induction, whereas the luteinizing hormone level in serum was unchanged. Further studies revealed that desert hedgehog (*Dhh*) expression was regulated by *Wt1* in Sertoli cells and that its expression was significantly reduced in *Wt1*-deficient testes. In vitro study demonstrated that the defect in testosterone production and decreased expression of several steroidogenic genes in *Wt1*-deficient testis explants was partially rescued by smoothened agonist (SAG), a hedgehog pathway agonist. These results indicate that *Wt1* is most likely involved in Leydig cell steroidogenesis by regulating the expression of paracrine factors in seminiferous tubules. *Dhh* probably had important roles in this process, but we could not exclude the possibility that other factors were also required for Leydig cell steroidogenesis. Loss of *Wt1* leads to downregulation of paracrine factors, which in turn causes a decrease in steroidogenic enzyme expression and reduces testosterone production in Leydig cells. The results of this study further confirm that the cross talk between Sertoli cells and Leydig cells has important roles in Leydig cell steroidogenesis.

gene regulation, Leydig cells, spermatogenesis, testis, testosterone

INTRODUCTION

The primary function of testes is to produce sperm and testosterone. Testosterone is secreted by Leydig cells and is necessary for completion of spermatogenesis and maintenance of secondary sexual characteristics. Adult Leydig cells arise from stem Leydig cells postnatally, and they proliferate and differentiate to produce testosterone [1]. The proliferation and differentiation of postnatal Leydig cells are dependent on luteinizing hormone (LH) and its cognate receptor (*LHR*). Luteinizing hormone deficiency or inactivation of *LHR* results in Leydig cell hypoplasia and hypoandrogenemia [2–4]. In addition to the endocrine regulation by LH, paracrine factors are also involved in regulation of Leydig cell function. Coculture experiments demonstrated that Leydig cell function was influenced by coculture with Sertoli cell or Sertoli cell-conditioned media [5–7]. A recent study [8] found that testosterone production was significantly stimulated when Leydig cells were cocultured with peritubular cells of seminiferous tubules. These results suggest that direct interaction or the factors secreted by Sertoli cells or other cell types within seminiferous tubules are both required for steroidogenesis of Leydig cells; however, the exact mechanisms are largely unknown.

Wilms tumor gene, *Wt1*, encodes a nuclear transcription factor that is abundantly expressed in testis Sertoli cells. Previous investigations have demonstrated that *Wt1* is important for testis development, with inactivation of *Wt1* in Sertoli cells between Embryonic Days 12.5 and 14.5 resulting in testicular cord disruption and testis dysgenesis [9]. Our recent study [10] found that *Wt1* also has a critical role in spermatogenesis. Inactivation of *Wt1* in Sertoli cells of adult mice caused germ cell loss and reproductive defects in male mice. Further investigation revealed that *Wt1* is involved in spermatogenesis by regulating Sertoli cell polarity. Notably, we found that testosterone production was also dramatically reduced in *Wt1*-deficient mice compared with a control group. The expression of *LHR* and steroidogenic enzymes in Leydig cells was significantly decreased. Further research revealed that the expression of the paracrine factor desert hedgehog (*Dhh*) was significantly reduced in *Wt1*-deficient testes. In vitro experiments demonstrated that the hedgehog pathway agonist smoothened agonist (SAG) could partially rescue the defect in testosterone production in *Wt1*-deficient testis explants. These results indicate that *Wt1* is most likely involved in Leydig cell steroidogenesis by regulating the expression of paracrine factors in seminiferous tubules. However, the detailed mechanisms need further investigation.

MATERIALS AND METHODS

Mice

All animal work was carried out in accordance with institutional animal care and use committee regulations. All mice were maintained in a C57BL/6;129/SvEv mixed background. *Wt1*$^{+/flox}$ [9] mice were mated with mice carrying the *Wt1*-null allele (*Wt1*$^{+/-}$) [11] and *Cre-ER*^{TM} transgenic mice, in which *Cre-ER*^{TM} is ubiquitously expressed to permit temporally regulated

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Cre-mediated recombination in diverse tissues of the mouse [12], to produce $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ offspring. DNA isolated from tail biopsies was used for genotyping. Genotyping was performed by PCR as described previously [9, 13].

Tamoxifen Injection

Tamoxifen (Sigma) was dissolved in corn oil at a final concentration of 20 mg/ml. Two-month-old control ($Wt1^{+/flox}$; $Cre\text{-}ER^{TM}$, $Wt1^{-/flox}$) and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males were injected intraperitoneally with 9 mg per 40 g of body weight for 2 consecutive days to induce Cre activity and deletion of the *Wt1* gene as described previously [10].

Hormone Measurements

To collect blood samples, animals were lightly anesthetized, and approximately 50 to 200 μl of blood was withdrawn by puncturing the retro-orbital plexus. Serum samples were obtained by centrifugation at 3000 rpm for 10 min and stored at −20°C. Serum concentrations of testosterone, follicle-stimulating hormone (FSH), and LH were specifically measured by radioimmunoassay as described previously [14].

Tissue Collection and Histological Analysis

Testes were dissected from *Wt1*-deficient and control mice 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 stained with hematoxylin-eosin for histological analysis.

Immunohistochemistry Analysis

Immunohistochemistry (IHC) analysis of tissues from at least three mice for each genotype was performed using an avidin-biotin-peroxidase kit (Vectastain; Vector Laboratories) as recommended by the manufacturer and using antibodies to LHR (1:200, P2434Rbm; USCN Life Science Inc.), P450scc (1:400, ABS 235; EMD Millipore Corporation), 3β-HSD (1:400, sc-30820; Santa Cruz Biotechnology, Inc.), and StAR (1:200, sc-166821; Santa Cruz Biotechnology, Inc.). The IHC procedure was performed as described previously [15]. Stained sections were examined with a dissection microscope (Nikon Corporation), and images were captured by a charged-coupled device camera (DS-Ri1; Nikon Corporation).

Nucleic Acid Isolation and Quantitative RT-PCR

Total RNA was extracted from testes using a kit (RNeasy; QIAGEN) in accordance with the manufacturer's instructions. To quantify gene expression, real-time SYBR Green assay (Molecular Probes, Inc.) was performed with the isolated RNA. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as an endogenous control. All gene expression was quantified relative to *gapdh* expression. The relative concentration of the candidate gene expression was calculated using the formula $2^{-\Delta\Delta CT}$ as described in the SYBR Green user manual. Primers used for the RT-PCR are listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

Primary Sertoli Cell Isolation and In Vitro Culture

A modified method was used to isolate primary Sertoli cells from the testes of 6-wk-old mice [16]. Testes were decapsulated under the dissection microscope. The seminiferous tubules were pooled and washed with PBS three times. The tubules were incubated with 1 mg/ml of collagenase I (Sigma) and 0.5 mg/ml of DNase I (Sigma) in Dulbecco modified Eagle medium (DMEM) for 25 min at 33°C on a shaker, then washed twice with DMEM, and further digested with 1 mg/ml of collagenase I, 0.5 mg/ml of DNase I, and 1 mg/ml of hyaluronidase type III (Sigma) for 10 min at 33°C. The tubules were allowed to settle and were then washed twice with DMEM before being digested with 2 mg/ml of collagenase I, 0.5 mg/ml of DNase I, 2 mg/ml of hyaluronidase, and 1 mg/ml of trypsin for 20 min at 33°C. This final digestion step resulted in a cell suspension containing primarily Sertoli cells and type A spermatogonia. The dispersed cells were then washed twice with DMEM and placed into culture dishes in DMEM containing 10% fetal calf serum and incubated at 33°C and 5% carbon dioxide. Spermatogonia were unable to attach to the dish and were removed after the medium change on the next day. Tamoxifen (4-OH-tamoxifen, H7904; Sigma) was dissolved in ethanol to generate a 1 mM stock solution and further diluted to appropriate concentrations before use. Recombination was initiated by adding tamoxifen to cultured Sertoli cells at a final concentration of 1 μM. After 3 days of culture, total RNA was extracted.

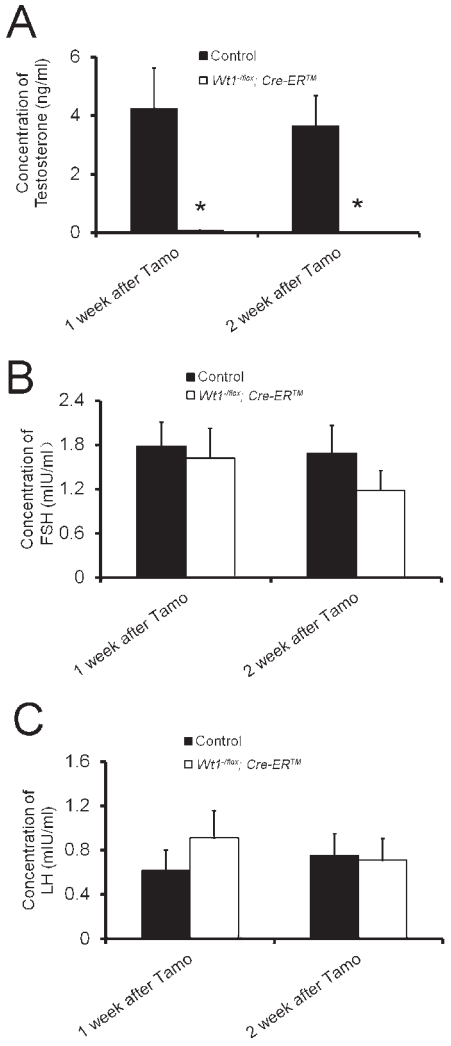

FIG. 1. The serum concentration of steroid hormones in control and *Wt1*-deficient males. **A**) The concentration of testosterone was dramatically reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males at both 1 and 2 wk after tamoxifen induction. **B**) The concentration of FSH in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males was not significantly changed after tamoxifen induction. **C**) The LH level was unaffected in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males after tamoxifen induction. Data are presented as mean ± SEM (n = 3). $^*P < 0.01$.

Organ Culture

The testes from 2-mo-old control ($Wt1^{+/flox}$; $Cre\text{-}ER^{TM}$, $Wt1^{-/flox}$) and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males were decapsulated and gently cut into several pieces 1 to 3 mm in diameter. The testis explants were cultured as described previously [17]. Briefly, 1.5% (w/v) agarose gel stands (10 × 10 × 5 mm in size and placed in six-well plates) were prepared 1 day before and incubated with culture medium for more than 24 h. Two to three testis explants were placed at the medium-air interface on each agarose gel stand. Each well contained 1.8 ml of DMEM/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum and 1 μM tamoxifen to induce Cre activity. The testis explants were cultured at 33°C and 5% carbon dioxide. Three days later, the medium was replaced with DMEM/F12 supplemented with 100 nM SAG (566661; Calbiochem) or vehicle only. The media were collected 72 hours later and stored at −20°C for subsequent hormone analysis.

Statistical Analysis

Experiments were repeated at least three times. For Sertoli cell and testis explant culture, each experiment was repeated three times. At least three control

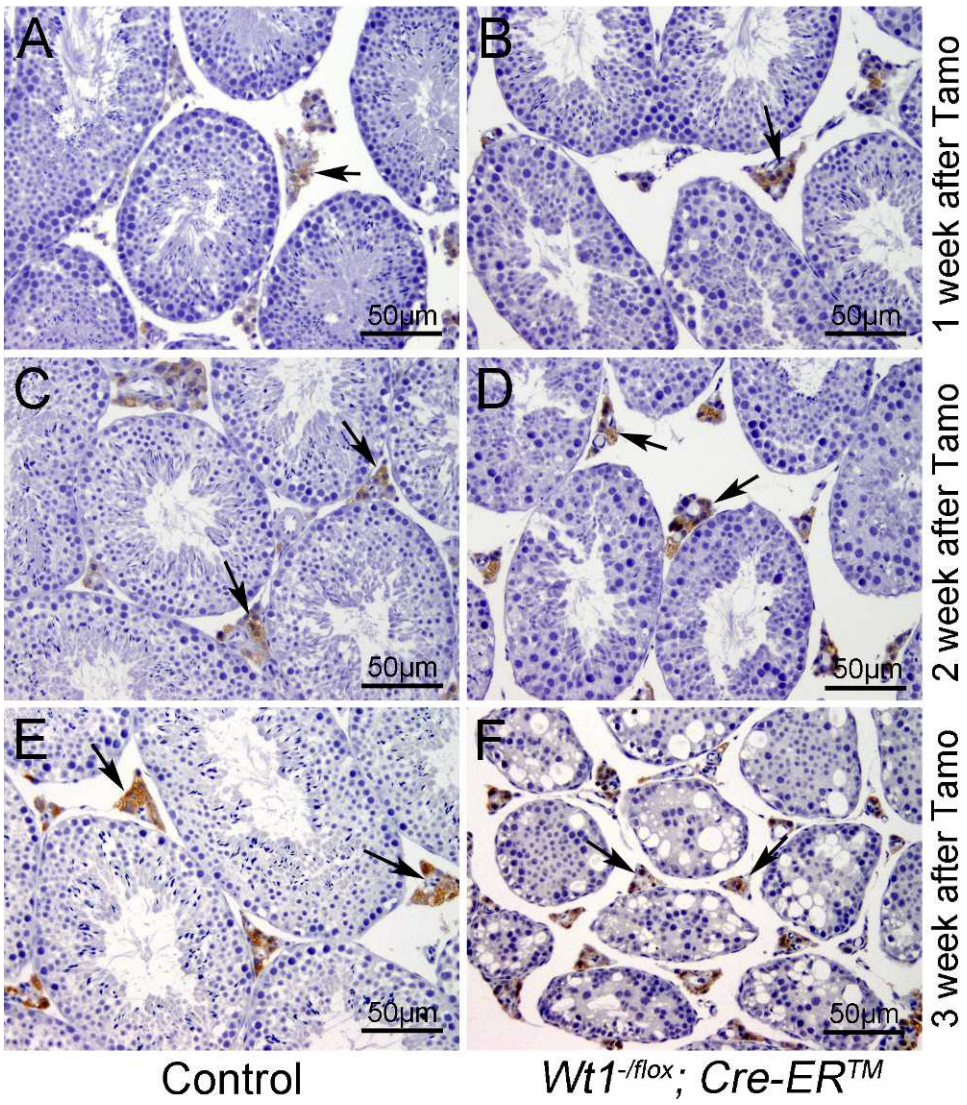

FIG. 2. The expression of *3β-HSD* in control and *Wt1*-deficient mice. 3β-HSD protein was detected by IHC in Leydig cells of both control mice (**A**, **C**, and **E** [arrows]) and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ mice (**B**, **D**, and **F** [arrows]) at 1, 2, and 3 wk after tamoxifen induction. No significant difference was noted between control and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ mice.

and mutant males at each time point were used for real-time PCR and IHC analysis. The quantitative results are presented as mean ± SEM. The data were evaluated for statistical differences using Student *t*-test and one-way ANOVA. $P < 0.05$ was considered significant.

RESULTS

Deletion of Wt1 *in Adult Mice Causes Reduced Testosterone Production*

To induce Cre activity, $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ and littermate control mice were injected with tamoxifen at age 2 mo. Our previous findings demonstrated that deletion of *Wt1* in adult testes by tamoxifen induction leads to germ cell loss that is due to the defect in polarity maintenance of *Wt1*-deficient Sertoli cells [10]. In the present study, we found that testosterone production is also dramatically reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males after tamoxifen induction. Testosterone (4.2 and 3.6 ng/ml) was detected in serum of control males at 1 and 2 wk after tamoxifen treatment. In contrast, only 0.06 and 0.03 ng/ml of testosterone were detected in serum of $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males (Fig. 1A). However, the concentrations of LH and FSH were unchanged between control and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ males at 1 and 2 wk after tamoxifen induction (Fig. 1, B and C). These results indicate that inactivation of *Wt1* leads to a defect in testosterone biosynthesis in Leydig cells, which is probably not due to the abnormality in gonadotropin secretion.

The Expression of Steroidogenic Enzymes Is Decreased in Wt1*-Deficient Testes*

To explore the underlying mechanisms that cause the defect in testosterone biosynthesis in *Wt1*-deficient males, the expression of enzymes required for testosterone biosynthesis was examined by IHC and real-time PCR. 3β-HSD protein was detected in Leydig cells of both control mice (Fig. 2, A, C, and E [arrows]) and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ mice (Fig. 2, B, D, and F [arrows]) at 1, 2, and 3 wk after tamoxifen induction. The protein levels of StAR and P450scc were dramatically reduced in the Leydig cells (arrows) of $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes (Fig. 3, B, D, F, and H [arrows]) compared with control testes (Fig. 3, A, C, E, and G) at 2 and 3 wk after tamoxifen treatment. To further quantify the expression of steroidogenic genes, real-

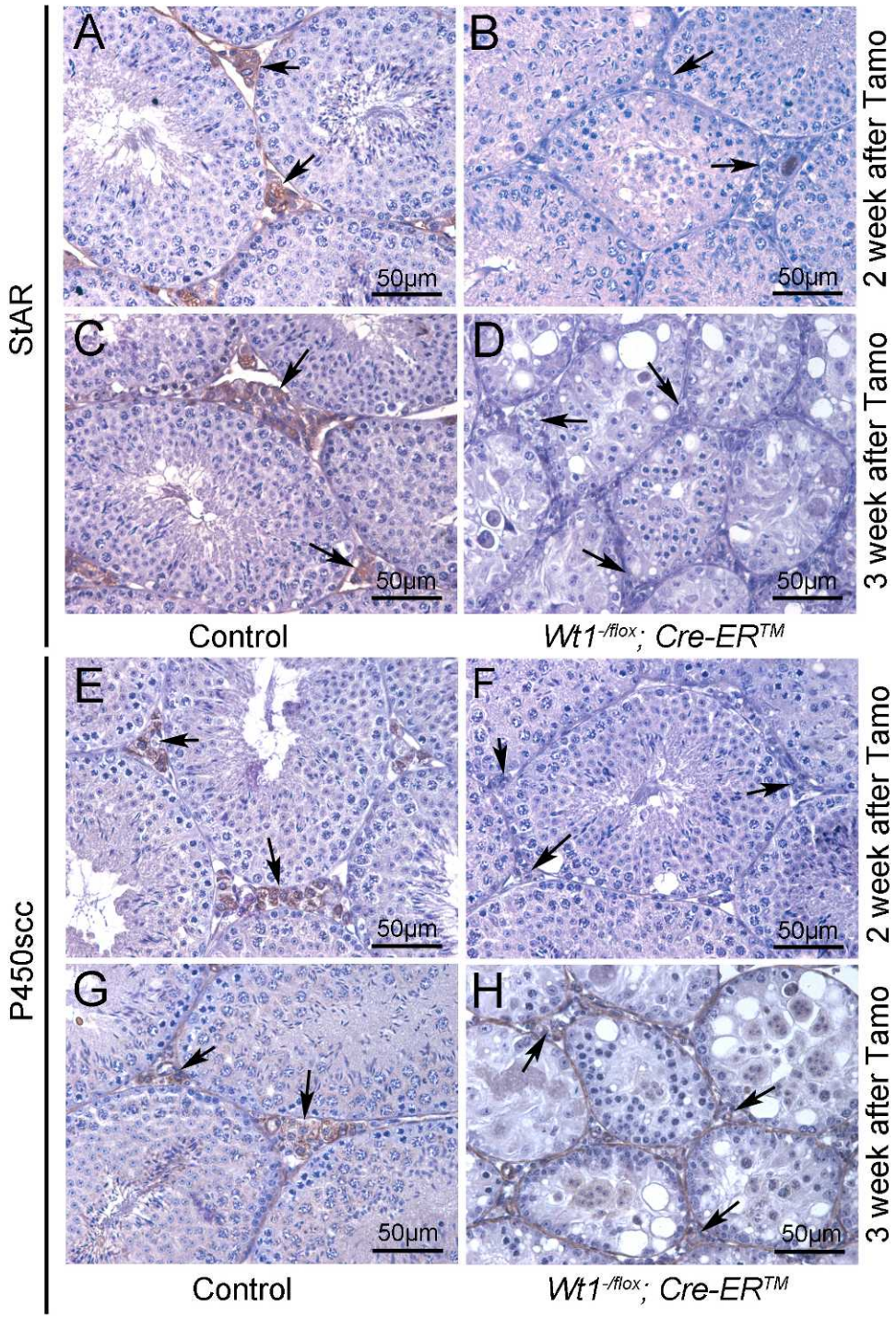

FIG. 3. The expression of StAR and P450scc in control and *Wt1*-deficient mice. StAR (**A** and **C**) and P450scc (**E** and **G**) protein was abundantly expressed in the Leydig cells (arrows) of tamoxifen-treated control testes. In contrast, the levels of StAR (**B** and **D**) and P450scc (**F** and **H**) protein were dramatically reduced in the Leydig cells (arrows) of $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes after tamoxifen treatment.

time PCR analysis was performed. As shown in Figure 4A, the mRNA level of *Wt1* was reduced approximately 50%, which was consistent with our previous study [10]. The mRNA levels of *StAR* and *P450scc* were significantly reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes at 1 wk after tamoxifen treatment, which was consistent with the results of IHC. We also found that the expression of *3β-HSD* and *Cyp17a1* was dramatically reduced in *Wt1*-deficient testes. In contrast, the expression of hydroxysteroid (17-beta) dehydrogenase 1 (*Hsd17b1*), hydroxysteroid (17-beta) dehydrogenase 3 (*Hsd17b3*), and cytochrome P450 19A1 (*Cyp19a1*) was unchanged in *Wt1*-deficient testes.

The Expression of LHR Is Decreased in Wt1*-Deficient Testes*

It has been demonstrated that pituitary LH regulates testosterone biosynthesis in Leydig cells via interacting with its cognate receptor (*LHR*) [18]. In this study, the expression of *LHR* was analyzed by IHC and real-time PCR. As shown in

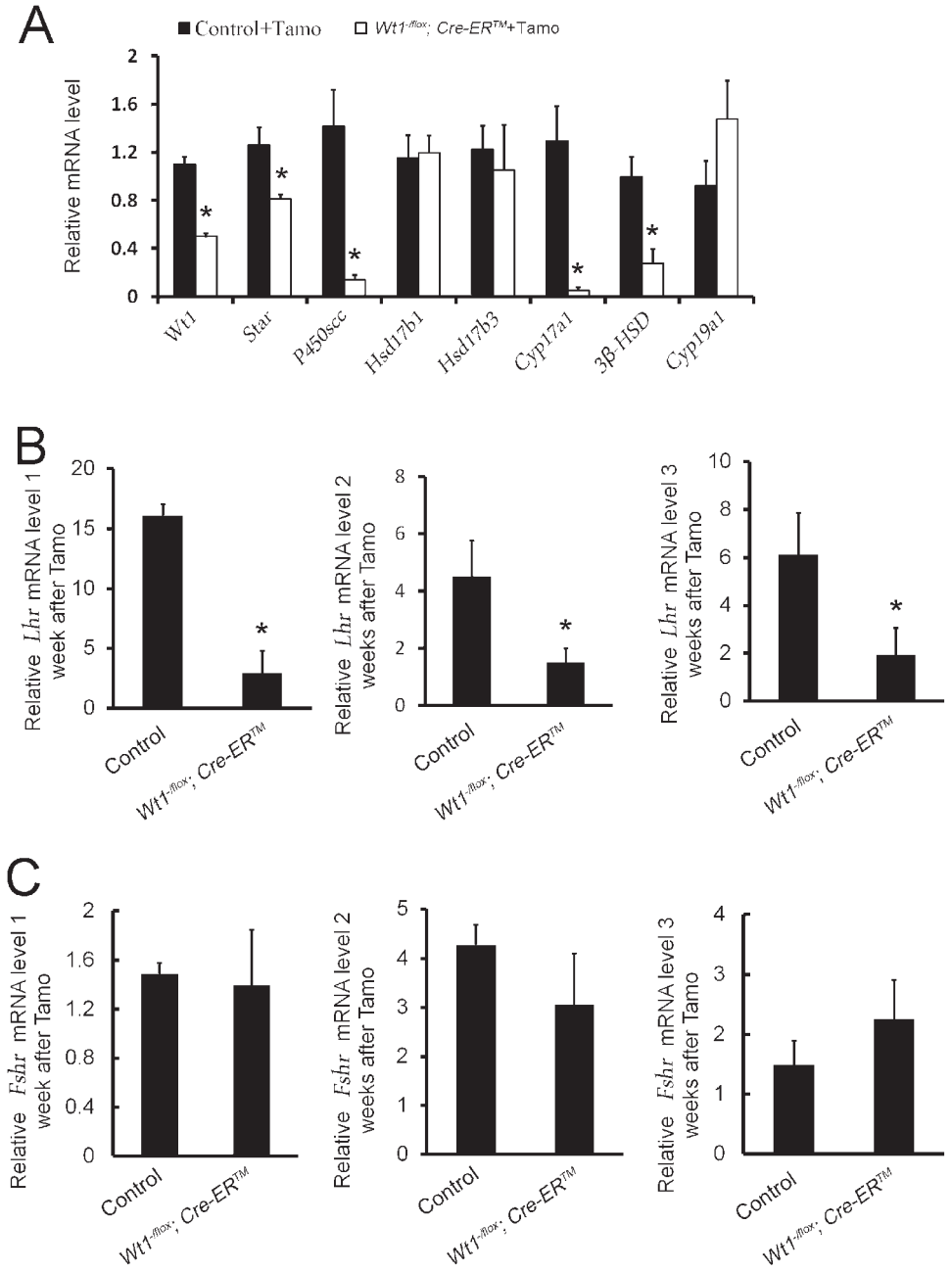

FIG. 4. The differential expression of steroidogenic enzymes and hormone receptors in control and *Wt1*-deficient testes. The mRNA levels of steroidogenic enzymes, *LHR*, and *FSHR* in tamoxifen-induced control and $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes were examined by real-time PCR. **A**) The mRNA levels of *StAR*, *P450scc*, *Cyp17a1*, and *3β-HSD* were significantly reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes at 1 wk after tamoxifen treatment, whereas the expression of *Hsd17b1*, *Hsd17b3*, and *Cyp19a1* was unchanged in *Wt1*-deficient testes. **B**) The mRNA level of *LHR* was dramatically decreased in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes at 1, 2, and 3 wk after tamoxifen treatment compared with control testes. **C**) The expression of *FSHR* was not altered in tamoxifen-treated $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes compared with the control groups. Data are presented as mean ± SEM (n = 3). $^*P < 0.05$.

Figure 5, LHR protein was detected in the Leydig cells (arrows) of control mice at 1 (A), 2 (C), and 3 (E) wk after tamoxifen treatment. In contrast, very weak signal of LHR protein was observed in the Leydig cells (arrows) of $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes (B, D, and F [arrows]). The results of real-time PCR also showed that the mRNA level of *LHR* was significantly decreased in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes after tamoxifen treatment compared with the control group (Fig. 4B). The expression of *FSHR* was unchanged in *Wt1*-deficient testes (Fig. 4C).

Dhh *Expression Is Regulated by* Wt1 *and Downregulated in* Wt1*-Deficient Testes*

It has been reported that *Dhh* is expressed by Sertoli cells and has important roles in regulating Leydig cell development [19, 20]. In this study, the expression of *Dhh* and its receptor (*Ptch1*) was analyzed by real-time PCR. As shown in Figure 6A, *Dhh* expression was significantly reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes at 1 wk after tamoxifen induction, whereas the expression of *Ptch1* was unchanged. To further examine the relationship between *Wt1* and *Dhh*, primary Sertoli cells were isolated from $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes and cultured in

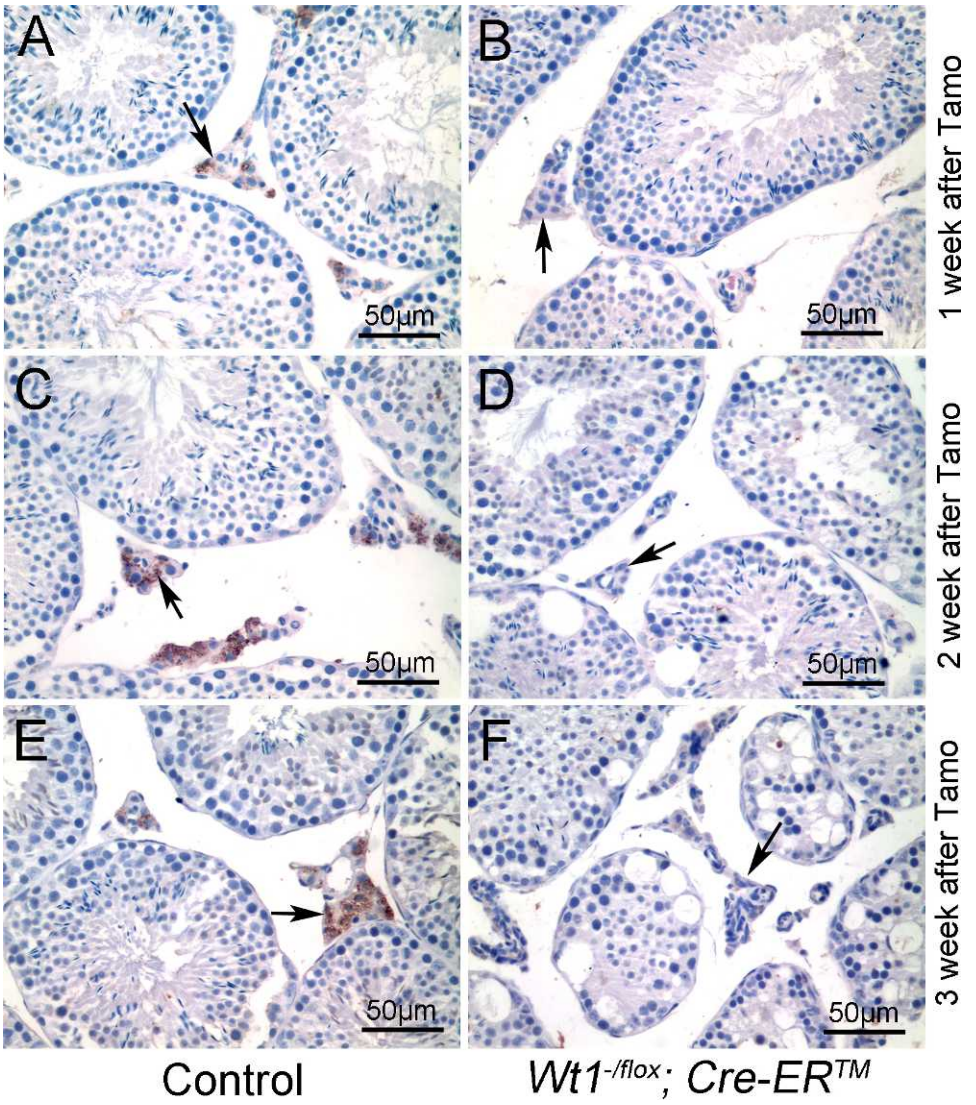

FIG. 5. The expression of LHR was dramatically reduced in *Wt1*-deficient testes. LHR protein was detected by IHC in the Leydig cells (arrows) of control testes (**A**, **C**, and **E** [arrows]) at 1, 2, and 3 wk after tamoxifen treatment. In contrast, very weak signal of LHR protein was observed in the Leydig cells of *Wt1*$^{-/flox}$*; Cre-ER*^{TM} testes (**B**, **D**, and **F** [arrows]) after tamoxifen treatment.

vitro; *Wt1* was inactivated by adding tamoxifen to the culture medium. As shown in Figure 6B, the expression of *Dhh* was significantly reduced in *Wt1*-deficient Sertoli cells, and *Dhh* expression could be rescued by transfection with *Wt1*-expressing adenovirus but not control virus. These results indicate that the expression of *Dhh* was regulated by *Wt1* in Sertoli cells and that deletion of *Wt1* results in downregulation of *Dhh*, which is probably a cause of the defect in Leydig cell steroidogenesis. *Pdgfa* [21–23], *Nr5a1* [24], and *Fgf9* [25] are also reported to be involved in Leydig cell development and function. The expression of *Pdgfa* and *Nr5a1* was decreased in *Wt1*-deficient testes, whereas the expression of *Pdgfrα* (the receptor of *Pdgfa*) and *Fgf9* was unchanged (Supplemental Fig. S1). The in vitro study results showed that *Pdgfa* expression was unchanged in *Wt1*-deficient Sertoli cells, suggesting that *Pdgfa* is probably not directly regulated by *Wt1* in Sertoli cells.

SAG Partially Rescues the Decreased Testosterone Production and Steroidogenic Gene Expressions in Wt1-*Deficient Testes*

To test the functions of paracrine factors in Leydig cell steroidogenesis, testis explants were cultured in vitro (Fig. 7A) and treated with SAG, a chlorobenzothiophene-containing hedgehog pathway agonist. We found that the expression of *Ptch1* and *Gli1* was significantly upregulated after SAG treatment (Fig. 7B), which indicates that hedgehog signaling was activated. Testosterone production from *Wt1*$^{-/flox}$*; Cre-ER*^{TM} testis explants was significantly reduced after tamoxifen induction. Testosterone (54.73 ng/ml) was detected in the medium of tamoxifen-treated control testis explants, and only 12.58 ng/ml of testosterone was detected in the medium of tamoxifen-treated *Wt1*$^{-/flox}$*; Cre-ER*^{TM} testis explants. These results were consistent with the in vivo study. Notably, we found that the concentration of testosterone was increased up to 31.48 ng/ml in the medium of *Wt1*-deficient testis explants after SAG treatment (Fig. 7C). This result indicates that the defect in testosterone production in *Wt1*-deficient testes was partially

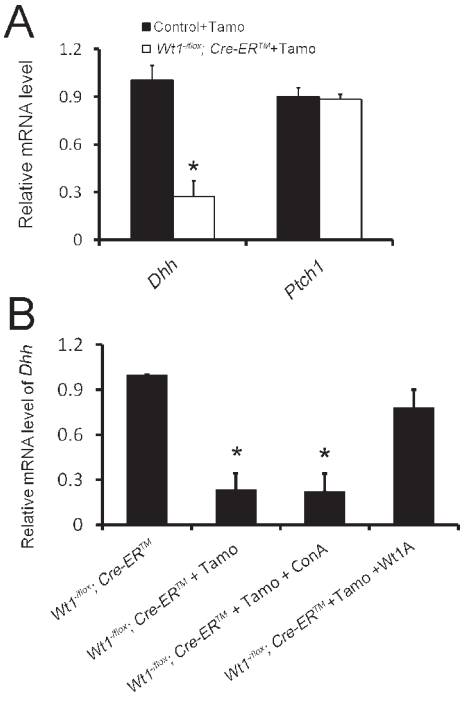

FIG. 6. The expression of *Dhh* was regulated by *Wt1* and downregulated in *Wt1*-deficient testes. **A**) The expression of *Dhh* was significantly reduced in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testes at 1 wk after tamoxifen treatment, whereas the expression of its receptor (*Ptch1*) was unchanged. **B**) The expression of *Dhh* was dramatically decreased in *Wt1*-deficient Sertoli cells and rescued by transfection with *Wt1*-expressing adenovirus. Data are presented as mean ± SEM (n = 3). $^*P < 0.05$.

rescued by SAG. The expression of *Wt1*, *Dhh*, *P450scc*, *Cyp17a1*, *3β-HSD*, *Nr5a1*, and *LHR* was decreased in tamoxifen-treated $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ testis explants. The expression of *P450scc*, *Cyp17a1*, *3β-HSD,* and *Nr5a1* was significantly increased in *Wt1*-deficient testis explants after SAG treatment, whereas *LHR* expression was not significantly increased with SAG treatment (Fig. 7D).

DISCUSSION

In testes, the steroid hormone biosynthesis by Leydig cells is a hormonally regulated, multistep process. Testosterone is synthesized through concerted action of carrier proteins and a cascade of steroidogenic enzymes. Mutations of any of these components result in aberrant steroidogenesis [26, 27]. It has been demonstrated that gonadotropin LH has essential roles in steroid hormone biosynthesis. The expression of steroidogenic enzymes in Leydig cells is regulated by LH; inactivation of LHR results in decreased testosterone levels and downregulation of steroidogenic enzymes, including *StAR*, *3β-HSD*, and *P450scc* [18]. Follicle-stimulating hormone is another important gonadotropin; however, its receptor is only expressed in Sertoli cells, and inactivation of FSHR does not affect steroidogenesis and male fertility [28].

Besides endocrine factors, it has been reported that Leydig cell function is also regulated by paracrine factors from seminiferous tubules. Coculture experiments indicated that the biosynthesis of steroid hormones by Leydig cells is regulated by secreted factors from Sertoli cells or peritubular cells of seminiferous tubules [5–8]. However, which factors are involved in this process is largely unknown. Efforts to characterize and purify these paracrine factors have been unsuccessful.

The *Wt1* gene encodes a nuclear transcription factor and is abundantly expressed in testis Sertoli cells. Our previous study [10] found that *Wt1* has an essential role in spermatogenesis; deletion of *Wt1* causes germ cell death, which is due to loss of polarity in Sertoli cells. In the present study, we found that inactivation of *Wt1* also caused defects in steroidogenesis in Leydig cells. Testosterone production was dramatically reduced in *Wt1*-deficient males. Further study revealed that the expression of enzymes that are essential for testosterone biosynthesis was significantly reduced in *Wt1*-deficient testes. These results indicate that loss of *Wt1* results in downregulation of steroidogenic enzymes, which in turn leads to the defect in steroidogenesis in Leydig cells. However, *Wt1* is expressed by Sertoli cells in testes, whereas the steroidogenic enzymes are expressed by Leydig cells. The downregulation of these enzymes in *Wt1*-deficient testes must be indirectly influenced by *Wt1* inactivation. Given that gonadotropins have a critical role in regulating steroidogenesis of Leydig cells, the concentrations of LH and FSH in serum were examined. We found that both FSH and LH levels were not significantly changed in $Wt1^{-/flox}$; $Cre\text{-}ER^{TM}$ mice, indicating that the production of gonadotropins was unaffected in *Wt1*-deficient males and that the defect in Leydig cell steroidogenesis was most likely due to the change in paracrine factors. To exclude the possibility that tamoxifen treatment caused the degeneration of Leydig cells, TUNEL assay was performed. As shown in Supplemental Figure S2, most apoptotic cells were observed within seminiferous tubules at 2 and 3 wk after tamoxifen injection; no TUNEL-positive cells were noted in the interstitium of *Wt1*-deficient testes, suggesting that decreases in testosterone in *Wt1*-deficient testes were not due to the degeneration of Leydig cells.

Dhh is one of the secreted factors specifically expressed in Sertoli cells following the expression of *Sry* in testes, and its receptor (*Ptch1*) is expressed in Leydig cells and peritubular myoid cells. Previous studies [19, 29] have demonstrated that the *Dhh* signaling pathway has important roles in regulating the proper development of fetal Leydig cells in testes. Deletion of *Dhh* in male mice results in pseudohermaphroditism, and testes from adult feminized mice lack adult-type Leydig cells [20]. Yao et al. [30] found that *Dhh* mutation also resulted in a defect in the differentiation of fetal Leydig cells, with P450scc and SF1 expression dramatically decreased in male gonads at Postcoitum Days 13.5 to 14.5. However, the role of *Dhh*/*Ptch1* signaling in regulating adult Leydig cell steroidogenesis has not been described to date. In the present study, we found that the expression of *Dhh* was regulated by *Wt1* in Sertoli cells and that *Dhh* expression was significantly reduced in *Wt1*-deficient testes, suggesting that the defect in steroidogenesis in the Leydig cells of *Wt1*-deficient mice was probably due to the downregulation of *Dhh*. To test this hypothesis, the testis explants were cultured in vitro and treated with SAG. We found that testosterone production and the expression of some steroidogenic enzymes in *Wt1*-deficient testis explants were partially rescued by SAG treatment. These results confirmed that *Dhh* had roles in regulating adult Leydig cell steroidogenesis. Other factors have also been reported to have roles in Leydig cell development such as *Pdgfa* [21–23]. *Pdgfa* is expressed in seminiferous epithelium and its receptor *Pdgfrα* is expressed in interstitial mesenchymal cells in mice. In contrast to *Dhh*, *Pdgfa* does not influence the fetal generation of Leydig cells, but it is essential for adult Leydig cell development. Inactivation of *Pdgfa* causes a complete lack of Leydig cells and spermatogenic arrest in adults [23]. In this study, we found that the expression of *Pdgfa* was also significantly reduced in *Wt1*-deficient testes; however, its expression was unchanged in

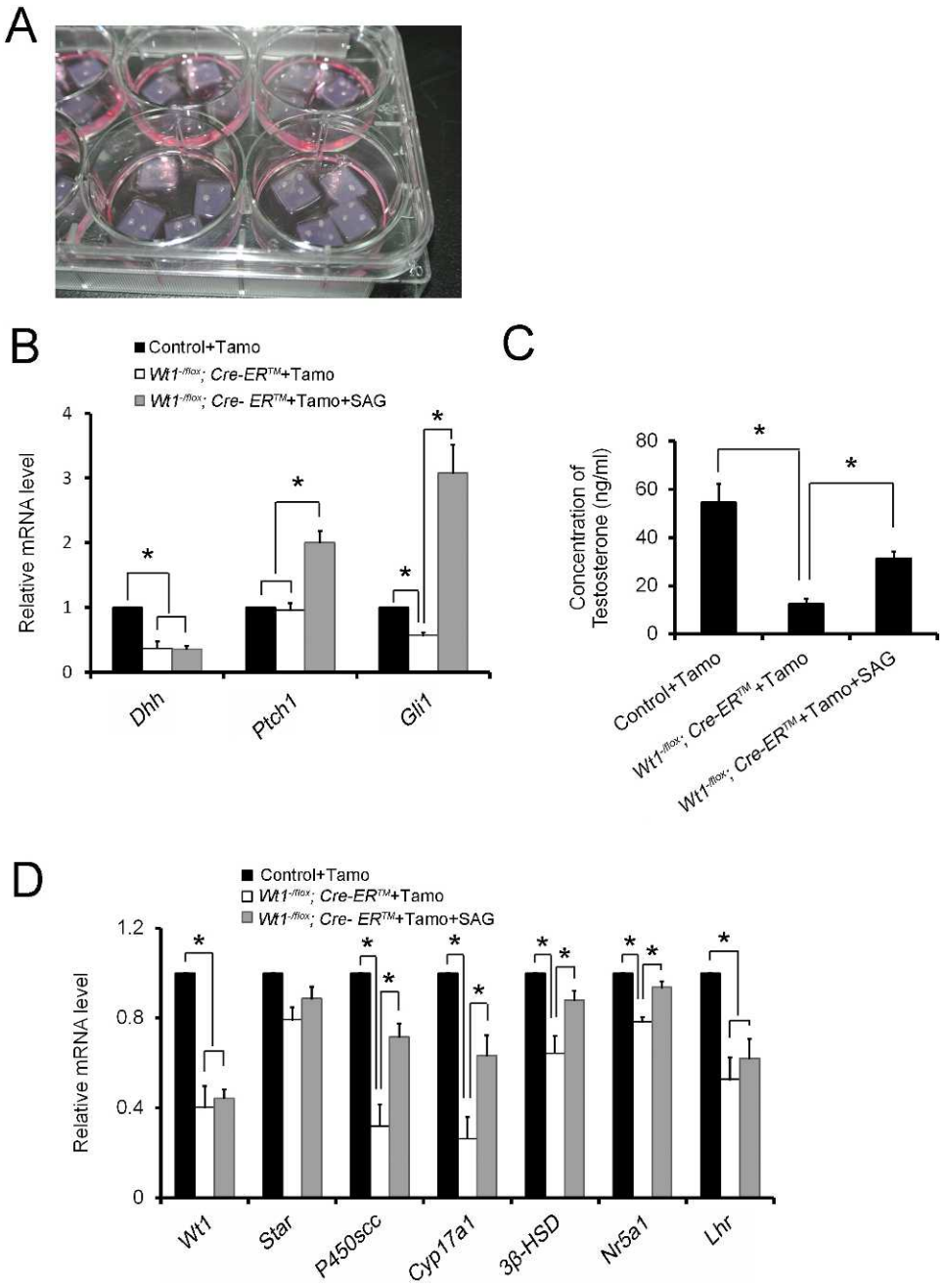

FIG. 7. The defect in testosterone production in *Wt1*-deficient testes was partially rescued by SAG treatment. **A**) Testis explants were cultured on agarose gel stands in six-well plates. **B**) The mRNA levels of *Dhh* and *Gli1* were significantly reduced in $Wt1^{-/flox}$; *Cre-ER*^{TM} explants after tamoxifen treatment; *Ptch1* and *Gli1* expression was significantly increased with SAG treatment. **C**) Testosterone production was significantly reduced in $Wt1^{-/flox}$; *Cre-ER*^{TM} explants after tamoxifen treatment and partially rescued by SAG treatment. **D**) The expression of *Wt1*, *P450scc*, *Cyp17a1*, *3β-HSD*, *Nr5a1*, and *LHR* was decreased in tamoxifen-treated $Wt1^{-/flox}$; *Cre-ER*^{TM} testis explants. SAG treatment partially rescued the expression of *P450scc*, *Cyp17a1*, *3β-HSD*, and *Nr5a1*. *LHR* expression was also increased after SAG treatment but not significantly. In **B**, **C,** and **D**, data are presented as mean ± SEM (n = 3). *$P < 0.05$.

Wt1-deficient Sertoli cells, suggesting that *Pdgfa* is not specifically expressed in Sertoli cells or that this gene is not regulated by *Wt1* directly. The decrease in *Pdgfa* is a secondary effect of *Wt1* deletion in Sertoli cells; this change probably also contributes to the defect in steroidogenesis in *Wt1*-deficient testes.

Besides steroidogenic enzymes, the expression of *LHR* was also significantly decreased in *Wt1*-deficient testes. Given that the expression of steroidogenic enzymes in Leydig cells is regulated by LH via interacting with its cognate receptor *LHR*, the downregulation of steroidogenic enzymes was probably due to the decrease in *LHR* expression in *Wt1*-deficient testes. However, SAG treatment did not significantly rescue LHR expression in in vitro cultured testis explants. These results suggest that the effect of *Dhh* on the steroidogenic gene expression is probably not through LHR. There might be other paracrine factors from seminiferous tubules regulating LHR expression.

Nr5a1 is mainly expressed in Leydig cells and is essential for Leydig cell differentiation by controlling the expression of

steroidogenic enzymes [31, 32]. Studies [33, 34] have reported that the hedgehog pathway induces Leydig cell development by upregulating *Nr5a1*. In the present study, *Nr5a1* expression was significantly reduced in *Wt1*-deficient testes; however, the decrease in *Nr5a1* was not so dramatic as that in steroidogenic gene expression. Whether *Nr5a1* is involved in this process remains unclear.

In summary, we demonstrated in this study that the *Wt1* gene is involved in Leydig cell steroidogenesis by regulating the expression of *Dhh*. Inactivation of *Wt1* causes downregulation of *Dhh* in Sertoli cells, which in turn leads to the decreased expression of steroidogenic enzymes and defects in testosterone biosynthesis in Leydig cells. Given the fact that regulation of steroidogenesis in Leydig cells is complicated, we could not exclude the possibility that other paracrine factors are also involved in this process. The detailed mechanisms need further investigation.

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