Peroxisome Proliferator-activated Receptor-D (PPARD) Coordinates Mouse Spermatogenesis by Modulating Extracellular Signal-regulated Kinase (ERK)-dependent Signaling*

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Background: The role of PPARD was examined in the testes because this receptor can regulate cell differentiation and proliferation.

Results: PPARD temporally represses ERK-dependent cell cycle signaling and increases tight junction proteins in Sertoli cells.

Conclusion: PPARD is essential for maturation of Sertoli cells to prevent testicular degeneration.

Significance: PPARD modulates Sertoli cell function and spermatogenesis.

Ppard−/− mice exhibit smaller litter size compared with Ppard+/+ mice. To determine whether peroxisome proliferator-activated receptor-D (PPARD) could possibly influence this phenotype, the role of PPARD in testicular biology was examined. Atrophic testes and testicular degeneration were observed in Ppard−/− mice compared with Ppard+/+ mice, indicating that PPARD modulates spermatogenesis. Higher expression of p27 and decreased expression of proliferating cellular nuclear antigen in Sertoli cells were observed in Ppard+/+ mice as compared with Ppard−/− mice, and these were associated with decreased Sertoli cell number in Ppard+/+ mice. Cyclin D1 and cyclin D2 expression was lower in Ppard+/+ as compared with Ppard−/− mice. Ligand activation of PPARD inhibited proliferation of a mouse Sertoli cell line, TM4, and an inverse agonist of PPARD (DG172) rescued this effect. Temporal inhibition of extracellular signal-regulated kinase (ERK) activation by PPARD in the testis was observed in Ppard+/+ mice and was associated with decreased serum follicle-stimulating hormone and higher claudin-11 expression along the blood-tissue barrier. PPARD-dependent ERK activation also altered expression of claudin-11, p27, cyclin D1, and cyclin D2 in TM4 cells, causing inhibition of cell proliferation, maturation, and formation of tight junctions in Sertoli cells, thus confirming a requirement for PPARD in accurate Sertoli cell function. Combined, these results reveal for the first time that PPARD regulates spermatogenesis by modulating the function of Sertoli cells during early testis development.

Spermatogenesis is a finely tuned process by which spermatogonial stem cells develop into mature spermatozoa. During spermatogenesis, different types of germ cells develop synchronously within the seminiferous tubule. Spermatogenesis is highly conserved among species and involves cell proliferation, differentiation, maintenance of a reserved germ cell population, and meiotic recombination (1). The molecular regulation of spermatogenesis is mediated by transcriptional, translational, and post-translational mechanisms (2,3).

Peroxisome proliferator-activated receptors (PPARs3: PPARα, PPARδ, PPARβ/δ, or NUC1), and PPARγ) are ligand-activated transcription factors that control a variety of biological processes in dynamic fashion (4). Previous studies showed that all three PPARs are expressed in both somatic and spermatogenic cells in the testis (5–7). However, whether PPARs have an important role in testis development is still unclear. Indeed, the physiological role of only PPARα has been critically examined to date in response to testicular toxicants (8).

Although Ppard−/− mice are viable and fertile, a previous study showed that they have an average litter size that is considerably smaller as compared with Ppard+/+ mice (9). The difference in litter size could be due to alterations in spermatogenesis.

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3 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PCNA, proliferating cellular nuclear antigen; FSH, follicle-stimulating hormone; PND, postnatal day; p-ERK, phospho-ERK; RAR, retinoic acid receptor; RXR, retinoid X receptor; TNF-α, transition nuclear protein 1.
genesis, which has not been critically examined to date. Thus, the hypothesis that PPARD modulates testis development by directly influencing germ cell maturation or indirectly affecting the function of Sertoli cells and Leydig cells, limiting their supportive capacity to germ cell development, was examined in the present study.

**Experimental Procedures**

*Mice—* Wild-type (*Ppard*+/+) and *Ppard*-null (*Ppard*−/−) mice (9) on a C57BL/6 genetic background were housed in a vivarium as described previously (10).

**Mouse Sertoli Cell Line—** The mouse Sertoli cell line TM4 was purchased from American Type Culture Collection (Manassas, VA). The TM4 mouse Sertoli cell line used was derived from mice aged 11–13 days, which closely models the developmental time frame examined in these studies. The TM4 mouse Sertoli cell line also allowed for complementary experiments to demonstrate the essential role of PPARD in the Sertoli cell in the regulation of proteins linked previously with proliferation and tight junctions in spermatogenesis by knockdown and/or overexpression of PPARD. Cells were cultured in a mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 (DMEM/F-12, 1:1) supplemented with 5% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin (Invitrogen) at 37 °C with 5% carbon dioxide. Cells were treated with or without GW0742 (a specific PPARD agonist (11)), DG172 (a specific PPARD inverse agonist (12)), or PD98059 (a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor). GW0742 was kindly provided by Drs. Andrew Billin and Timothy Willson (GlaxoSmithKline), and PD98059 was purchased (Cell Signaling Technology, Danvers, MA).

**Testicular Histopathology and Physiological Characterization of the Testis—** Six breeding pairs of male and female *Ppard*+/+ or *Ppard*−/− mice (6–8 weeks of age) were examined over a 10-month period. A total of 16 or 15 litters from *Ppard*+/+ or *Ppard*−/− mice were obtained using light microscopy. For evaluating testicular cross-sections per mouse and five mice in each age group per genotype were analyzed. 3,3′-Diaminobenzidine-positive cells were counted in 20 randomly chosen round seminiferous tubules in each testicular section. The relative intensity was determined using ImageJ software (version 1.47c) as described previously (10).

**Immunohistochemistry—** Expression of SOX9, p27, PCNA, claudin-11 (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1, cyclin D2, ERK, and phospho-ERK (p-ERK (Cell Signaling Technology) in the seminiferous tubule was determined by immunohistochemistry as described previously (10). Two testicular cross-sections per mouse and five mice in each age group per genotype were analyzed. 3,3′-Diaminobenzidine-positive cells were counted in 20 randomly chosen round seminiferous tubules in each testicular section. The relative intensity was determined using ImageJ software (version 1.47c) as described previously (10).

**Immunofluorescence—** Expression of PCNA, SOX9, or claudin-11 in the seminiferous epithelium was determined by immunofluorescence staining. Cross-sections (5 μm) of paraffin-embedded non-atrophic testes were deparaffinized, rehydrated, and heated in a 10 mM sodium citrate solution for antigen retrieval. Sections were incubated in 10% blocking serum followed by overnight incubation with primary antibodies at 4 °C. Sections were then incubated with Alexa Fluor-conjugated secondary antibodies (PCNA, Alexa Fluor 647; SOX9, Alexa Fluor 488; claudin-11, Alexa Fluor 488; Invitrogen) for 1 h and mounted with Vectashield mounting medium containing propidium iodide (Vector Labs, Burlingame, CA). Fluorescence signals were detected using excitation/emission wavelengths of 499/519 or 652/668 nm. All sections were imaged using laser-scanning confocal microscopy as described previously (16).

Quantification of Sertoli and Germ Cells—Sertoli cells in *Ppard*+/+ and *Ppard*−/− mouse testes were detected by assessing immunohistochemical expression of SOX9 because this protein is expressed exclusively in the nuclei of Sertoli cells in the seminiferous tubules (17). The average number of germ cells was determined by quantifying the number of hematoxylin-positive cells using ImageJ software (version 1.47c). The number of Sertoli cells per testis was determined as described previously (14). Twenty round seminiferous tubules in testicular cross-sections per mouse and five mouse testes in each age group per genotype were analyzed.

**Western Blot Analysis—** Quantitative Western blot analysis using radioactive detection techniques was performed as described previously (18). The relative expression level of each protein was normalized to the value of actin. A minimum of three mice per group were analyzed.

**Cell Proliferation Assay—** The xCELLigence system (ACEA Biosciences, Inc., San Diego, CA) was used for determining the changes in real time cell proliferation in response to activation of PPARD with an agonist (GW0742) or an inverse agonist (DG172) or the effect of inhibiting ERK signaling in TM4 cells as described previously (18).

**Transient Overexpression of Mouse PPARD in TM4 Cells—** TM4 cells (5 × 10⁵) were seeded in 6-well culture plates and transiently transfected with 10 μg of a pSG5-*Ppard* plasmid using Lipofectamine LTX reagent (Invitrogen) following the manufacturer’s recommended procedures. Twenty-four hours...
PPARD Regulates ERK Signaling in the Testis

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)

E

![Graph E](image5)

F

![Graph F](image6)

G

![Graph G](image7)

H

![Graph H](image8)

I

![Graph I](image9)

J

![Graph J](image10)

K

![Graph K](image11)

L

![Graph L](image12)

FIGURE 1. PPARD protects against reproductive failure and testicular degeneration in mice. A, the incidence of atrophic testis of Ppard+/+ and Ppard−/− mice was determined. Periodic acid-Schiff-hematoxylin-stained testicular cross-sections from adult Ppard−/− mice exhibit mild to severe degenerative phenotypes, including vacuolization of Sertoli cells (arrow; magnification, 40×) (B); testicular atrophy (magnification, 40×) (C); multinucleated giant germ cells (arrowhead; magnification, 100×) (D), and detachment of germ cells in the lumen (arrowheads; magnification, 40×) (E). Scale bars, 100 μm. The average body weight (F), atrophic testis weight (G), non-atrophic testis weight (H), atrophic testis weight/body weight ratio (I), average area of interstitial space in the tubule cross-section (J), average diameter of seminiferous tubules in the cross-section (K), and volume density of seminiferous tubules (L) of Ppard+/+ and Ppard−/− mice were determined over time. Values represent the mean ± S.E. (error bars). *, significantly different from Ppard−/−, p ≤ 0.05.

Serum Concentration of Follicle-stimulating Hormone (FSH), Inhibin B, and Testosterone—For FSH and inhibin B, serum was obtained from male Ppard+/+ and Ppard−/− mice on PND28 (n = 5) and PND56 (n = 5). Serum concentrations of FSH and inhibin B were measured by using a mouse FSH ELISA kit (TSZ ELISA, Waltham, MA) and an inhibin B enzyme immunoassay kit (Sigma-Aldrich) using the manufacturers’ recommended instructions, respectively. For serum testosterone, serum was collected at 1–2 p.m. to avoid circadian fluctuation (20) from male Ppard+/+ and Ppard−/− mice at peripubertal age (~4 weeks old; n = 4; housed in one cage) and at adult age (~15 weeks old; n = 10; housed in two cages). The serum concentration of testosterone was measured using a testosterone ELISA kit (Abcam, Cambridge, MA) following the manufacturer’s recommended instructions.

Statistical Analysis—The data were subjected to either Student’s t test or a parametric one-way analysis of variance followed by Tukey test for post hoc comparisons (Prism 5.0, GraphPad Software Inc., La Jolla, CA).

Results

PPARD Modulates Testicular Development—To evaluate the effect of PPARD on testis development, body weight, testis weight, the diameter of seminiferous tubules, the volume density of seminiferous tubules, and the number of spermatid...
heads were examined. Interestingly, 46.7% of Ppard<sup>−/−</sup> male offspring (seven of 15 litters) exhibited uni- or bilateral testicular atrophy (Fig. 1A). These results indicate that PPARD has an important role in male reproductive development. In general, Ppard<sup>+/+</sup> mice showed normal testicular structure (Figs. 2 and 3). By contrast, abnormal testicular phenotypes in Ppard<sup>−/−</sup> mice were frequently observed. For example, Sertoli cell vacuolization was often found in the seminiferous tubules of Ppard<sup>−/−</sup> mice (Fig. 1B). Ppard<sup>−/−</sup> mice also exhibited mild to severe degeneration of spermatogenic cells in the seminiferous tubules over time, including testicular atrophy (Fig. 1C), multinucleated giant cells (Fig. 1D), and a marked germ cell depletion (Fig. 1E). No significant changes in body weight were observed between Ppard<sup>+/+</sup> and Ppard<sup>−/−</sup> mice before puberty (Fig. 1F). Atrophic testes were only observed in Ppard<sup>−/−</sup> mice from PND21 through PND56, and the average atrophic testis weight was gradually increased over time (Fig. 1G). The average non-atrophic testis weight/litter in Ppard<sup>−/−</sup> mice was larger than in Ppard<sup>+/+</sup> mice over time (Fig. 1H). This contributed to the increase in average non-atrophic testis/body.

**FIGURE 2.** PPARD prevents abnormal spermatogenesis during pubertal development (PND28). Periodic acid-Schiff-hematoxylin-stained testicular cross-sections of Ppard<sup>+/+</sup> and Ppard<sup>−/−</sup> mice were examined for the stages of spermatogenesis in the seminiferous tubule. A–E, representative photomicrographs of Ppard<sup>+/+</sup> mouse testes illustrating spermatogonia, spermatocytes, spermatids, and Sertoli cells found during normal spermatogenesis at various stages (VI–XII), F–O, representative photomicrographs of Ppard<sup>−/−</sup> mouse testes illustrating alterations in spermatogonia, spermatocytes, spermatids, and Sertoli cells found during spermatogenesis at various stages (II–XII). F, unable to determine the stage of spermatogenesis because of the abnormal phenotype. F and G, white arrowheads indicate multinucleated giant cells. F and J–O, yellow arrowheads indicate abnormal elongation or disorientation of spermatids from steps 9–12. I, the red arrowhead indicates multilayers of preleptotene spermatocytes. J and K, orange arrowheads indicate unidentified cells. H, the green arrowhead indicates a cell undergoing abnormal meiosis. Spg, spermatogonia; PL, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; Std, spermatid; SC, Sertoli cell; M, meiotic division. Magnification, 100×. Scale bars, 50 μm.
weight ratio in *Ppard*^−/−^ mice compared with *Ppard*^+/+^ mice (Fig. 1I). The average area of interstitial space in cross-sections of *Ppard*^−/−^ mice was similar to that in *Ppard*^+/+^ mice (Fig. 1J). An average larger diameter of seminiferous tubules and a greater volume density of seminiferous tubules in *Ppard*^−/−^ mice than in *Ppard*^+/+^ mice over time suggest that *Ppard*^−/−^ mice may produce more cells within the seminiferous tubules (Fig. 1, K and L).

**PPARD Regulates Spermatogenesis at Both Peripubertal and Adult Ages**—On PND28 and PND56, *Ppard*^+/+^ mice exhibited normal and well organized germ cell layers and stages of spermatogenesis that were easy to identify (Figs. 2, A–E, and 3, A–D). By contrast, abnormal spermatogenesis was observed in *Ppard*^−/−^ mice at the peripubertal stage (Fig. 2, F–O), and these effects were more severe at the adult stage where the staging of spermatogenesis was not feasible (Fig. 3, E–L). For example, on PND28, multinucleated germ cells (Fig. 2, F and G), abnormal meiosis (Fig. 2H), multilayers of preleptotene spermatocytes along the basement (Fig. 2I), and unidentified cells (Fig. 2, J and K) were frequently observed in *Ppard*^−/−^ mouse testes. Surprisingly, abnormal elongation of spermatids was widely found throughout the various stages of spermatogenesis, showing an irregular shape of acrosomal regions in step 9–12 spermatids, in *Ppard*^−/−^ mice as compared with *Ppard*^+/+^ mice (Fig. 2, J–O). In addition, step 9–12 spermatids were present in stage II-III tubules (Fig. 2, K and L), suggesting that spermatids are retained in the seminiferous epithelium in *Ppard*^−/−^ mice as compared with *Ppard*^+/+^ mice.

The abnormal phenotype in spermatogenesis persisted and was also observed on PND56 in *Ppard*^−/−^ mouse testes but not *Ppard*^+/+^ mouse testes (Fig. 3). The retention and abnormal elongation of spermatids remained throughout various seminiferous tubules (Fig. 3, E–L). Discontinued or mixed stages of spermatogenesis were also observed in *Ppard*^−/−^ mouse testes.
but not Ppard+/+ mouse testes (Fig. 3). For example, step 10–11 elongated spermatids were present with step 5–6 round spermatids, and step 16 elongated spermatids did not spermatize in Ppard−/− mouse testes, but these abnormalities were not observed in Ppard+/+ mouse testes (Fig. 3E). An unclear nuclear boundary (Fig. 3F), abnormal meiotic division (Fig. 3G), and unidentified cells (Fig. 3, H and I) were also found in Ppard+/− mouse testes but not in Ppard+/+ mouse testes (Fig. 3, A–D).

On PND56, the total number of mature spermatid heads produced in Ppard−/− mice testes was higher than in Ppard+/+ mice (Fig. 4A). Spermatid heads of adult Ppard+/+ mice exhibited normal morphology, but abnormal spermatid head shapes were frequently observed in adult Ppard−/− mice (Fig. 4B), consistent with the results from the histopathological analysis described above (Figs. 2 and 3).

PPARD Modulates Serum Hormone Concentrations Associated with Spermatogenesis—There was no difference in serum FSH concentration observed between genotypes on PND28, but there was a marked increase in serum FSH on PND56 in Ppard−/− mice compared with Ppard+/+ mice (Fig. 5A). In contrast, the average serum concentration of inhibin B was lower on PND28 in Ppard−/− mice compared with Ppard+/+ mice, but there was no difference observed in serum inhibin B concentration between genotypes on PND56 (Fig. 5B). The average basal concentration of serum testosterone in male Ppard+/+ mice was significantly higher than in Ppard−/− mice at peripubertal age (Fig. 5C). Consistent with the age-dependent increased concentration of serum testosterone, the average serum testosterone concentration was higher in adult Ppard−/− mice (Fig. 5D). However, this average higher serum testosterone concentration was not observed in adult Ppard+/− mice (Fig. 5D).

PPARD Maintenance of Sertoli Cell and Germ Cell Numbers

PPARD differentially regulates serum FSH, inhibin B, and testosterone concentration. The average serum concentrations of FSH (A) or inhibin B (B) in Ppard+/+ and Ppard−/− mice on PND28 and PND56 were measured. C and D, the average serum concentration of testosterone in Ppard−/− and Ppard+/+ mice at peripubertal and adult ages, respectively. Values represent the mean ± S.E. (error bars). Values with different superscript letters are significantly different at p ≤ 0.05. *, significantly different from Ppard+/+, p ≤ 0.05.

Expression of p27 was present in Sertoli cells but not in germ cells (Fig. 7, A and B). Expression of p27 was higher in Sertoli cells from Ppard+/+ mice compared with Ppard−/− mice at both PND28 and PND56, suggesting that PPARD regulates the maturation of Sertoli cells (Fig. 7, C and D).

The cell cycle regulators cyclin D1 and cyclin D2 have important roles in spermatogenesis (21). Expression of cyclin D1 was found in spermatogonia in Ppard+/+ mice at both PND28 and PND56 (Fig. 8, A and B). In contrast, cyclin D1 was expressed in spermatogonia and preleptotene spermatocytes in Ppard−/− mice in particular on PND28 (Fig. 8, A and B). Spermatogonia in atrophic tubules were observed in Ppard−/− mice and expressed high levels of cyclin D1 (Fig. 8C). In addition, the number of germ cells that expressed cyclin D1 was lower in Ppard+/+ mice compared with Ppard−/− mice on PND28 and PND56 (Fig. 8D). Quantitative Western blot analysis of cyclin D1 was consistent with the changes observed with immunohistochemistry (Fig. 8E).
FIGURE 6. PPARD regulates the number of Sertoli and germ cells. A, representative photomicrographs of testicular cross-sections from Ppard^{+/+} and Ppard^{-/-} mice on PND14, PND21, PND28, and PND56. Testicular cross-sections were stained for SOX9 expression and counterstained with hematoxylin. The number of Sertoli cells was determined by counting SOX9-positive cells (brown color; black arrowheads) in the seminiferous tubules. The number of germ cells was determined by counting hematoxylin-stained cells (blue color). Magnification, 40×. Scale bars, 100 μm. Quantification of the average number of germ cells per tubule (B) or Sertoli cells per testis (C) from Ppard^{+/+} and Ppard^{-/-} mice from PND7-PND56 is shown. Values represent the mean ± S.E. (error bars). *, significantly different from Ppard^{-/-}, p < 0.05.

FIGURE 7. PPARD increases p27 expression in Sertoli cells. A and B, representative photomicrographs of testicular sections from Ppard^{+/+} and Ppard^{-/-} mice from PND28 and PND56, respectively, showing the expression of p27 in the seminiferous epithelium as assessed by immunohistochemistry. Testicular p27 was detected in Sertoli cells (arrowheads). Magnification, 40× (upper panels) and 100× (lower panels). Scale bars, 100 μm (upper panels) and 50 μm (lower panels). C, quantification of immunohistochemical intensity of p27. Values represent the mean ± S.E. (error bars). *, significantly different from Ppard^{-/-}, p < 0.05. D, quantitative Western blot analysis of p27 expression in whole testis homogenates of Ppard^{+/+} and Ppard^{-/-} mice from PND28 and PND56. The relative expression level of p27 was normalized to that of actin and represents the mean ± S.E. Values with different superscript letters are significantly different at p < 0.05.
FIGURE 8. PPARD represses expression of cyclin D1 in mouse testes. A and B, representative photomicrographs of testicular sections from Ppard+/+ and Ppard−/− mice from PND28 and PND56, respectively, showing the expression of cyclin D1 in the seminiferous epithelium as assessed by immunohistochemistry. Cyclin D1 was primarily expressed in spermatogonia (black arrowheads) of Ppard+/+ mice on both PND28 and PND56. By contrast, cyclin D1 expression was found in spermatogonia (black arrowheads), preleptotene spermatocytes (black arrows), and Sertoli cells (white arrowheads) of Ppard−/− mice on PND28 and PND56 but was more prominent on PND28. Magnification, 20× (upper panels) and 100× (lower panels). Scale bars, 100 (upper panels) and 50 μm (lower panels). C, cyclin D1 expression was notably higher in atrophic testis, spermatogonia (black arrowheads), and pachytene spermatocytes (white arrowheads) of Ppard−/− mice. D, quantification of the average number of cyclin D1-positive cells in the seminiferous tubules. Values represent the mean ± S.E. (error bars). *, significantly different from Ppard+/+, p ≤ 0.05. E, quantitative Western blot analysis of cyclin D1 expression in whole testis homogenates of Ppard+/+ and Ppard−/− mice from PND28 and PND56. The relative expression level of cyclin D1 was normalized to that of actin and represents the mean ± S.E. Values with different superscript letters are significantly different at p ≤ 0.05.

FIGURE 9. PPARD represses expression of cyclin D2 in mouse testes. A and B, representative photomicrographs of testicular sections from Ppard+/+ and Ppard−/− mice from PND28 and PND56, respectively, showing the expression of cyclin D2 in the seminiferous epithelium as assessed by immunohistochemistry. Cyclin D2 was primarily expressed in spermatogonia (black arrowheads), preleptotene spermatocytes (black arrows), and pachytene spermatocytes (white arrowheads) of Ppard+/+ mice on both PND28 and PND56. By contrast, cyclin D2 expression was found in spermatogonia (black arrowheads), preleptotene spermatocytes (white arrows), leptotene spermatocytes (white arrows), and pachytene spermatocytes (white arrowheads) of Ppard−/− mice on PND28 and PND56 but was more prominent on PND28. Magnification, 20× (upper panels) and 100× (two lower panels). Scale bars, 100 (upper panels) and 50 μm (two lower panels). C, quantification of the intensity of cyclin D2 expression in the seminiferous tubules. Values represent the mean ± S.E. (error bars). *, significantly different from Ppard+/+, p ≤ 0.05. D, quantitative Western blot analysis of cyclin D2 expression in whole testis homogenates of Ppard+/+ and Ppard−/− mice from PND28 and PND56. The relative expression level of cyclin D2 was normalized to that of actin and represents the mean ± S.E. Values with different superscript letters are significantly different at p ≤ 0.05.
Expression of cyclin D2 was observed in spermatogonia, preleptotene spermatocytes, and pachytene spermatocytes in Ppard+/−/− mice (Fig. 9, A and B). Expression of cyclin D2 was also observed in spermatogonia, preleptotene spermatocytes, and pachytene spermatocytes in some tubules of Ppard+/−/− mice, but leptotene spermatocytes in some tubules of Ppard+/−/− mice also expressed cyclin D2 (Fig. 9, A and B). Pachytene spermatocytes strongly expressed cyclin D2 in adult Ppard+/−/− mice compared with Ppard+/+/+ mice (Fig. 9, A and B). Quantitative Western blot analysis of cyclin D2 was consistent with the changes observed with immunohistochemistry (Fig. 9, C and D).

**PPARD Regulates Expression of Claudin-11 in Sertoli Cells**

The blood-testis barrier is a tight junction that separates the testis from the blood. Claudin-11 is a protein that plays a role in forming these tight junctions. The expression of claudin-11 was examined in Ppard+/−/− mouse Sertoli cells on PND14, and then more localized along the blood-testis barrier beginning from PND21 (Fig. 11, A and B). Although claudin-11 was also detected in Sertoli cells in Ppard+/−/− mice on PND14, its expression was greatly reduced on PND21 and PND28, indicating a delay in forming the blood-testis barrier (Fig. 11, A and B). Quantitative...
Western blot analysis shows results for claudin-11 expression in *Ppard*^-/-^ mouse Sertoli cells compared with *Ppard*^+/+^ mouse Sertoli cells on PND21 and PND28 (Fig. 11C). The mouse Sertoli cell line TM4 was used to further examine the functional role of PPARD in claudin-11 expression. Ligand activation of PPARD induced claudin-11 expression in the cytoplasm (Figs. 12A and 14A). Furthermore, transiently overexpressing PPARD also caused increased expression of claudin-11 in TM4 cells (Figs. 12A and 14A). By contrast, the PPARD inverse agonist DG172 or knockdown of PPARD by siRNA significantly reduced expression of claudin-11 in TM4 cells (Figs. 12B and 14B).

**PPARD-dependent Modulation of ERK Activity Regulates Tight Junctions and Proliferation in Sertoli Cells**—In *Ppard*^+/+^ mice, p-ERK expression in Sertoli cells increased postnatally with marked expression noted on PND28 but was diminished by PND56 (Fig. 13, A and B). Surprisingly, in Sertoli cells of *Ppard*^-/-^ mice, the expression of p-ERK was persistently higher from PND7 through PND56 (Fig. 13, A and B). Overexpression and/or ligand activation of PPARD strongly suppressed p-ERK expression in TM4 cells (Fig. 14A). In contrast, knockdown of PPARD showed the opposite effect as p-ERK expression was increased in TM4 cells (Fig. 14B). The downregulation of p-ERK expression by ligand activation and/or overexpression of PPARD correlated with increased expression of claudin-11 and p27 and decreased expression of cyclin D1 and cyclin D2 (Fig. 14A). Similarly, knockdown of PPARD correlated with decreased expression of claudin-11 and p27 and increased expression of cyclin D1 and cyclin D2 (Fig. 14B).

To more definitively examine the role of ERK signaling in controlling tight junctions and proliferation of Sertoli cells, a specific MEK inhibitor, PD98059, was used to inhibit ERK activation in TM4 cells. Inhibition of p-ERK activity inhibited the proliferation of TM4 cells after 72 h of treatment. The PPARD inverse agonist DG172 enhanced TM4 cell proliferation (Fig. 15A) as observed previously (Fig. 10D). Co-treatment with PD98059 and DG172 attenuated the enhanced proliferation of TM4 cells observed in response to the PPARD inverse agonist DG172 (Fig. 15A). Inhibition of p-ERK activity by PD98059 also significantly induced claudin-11 and p27 expression but reduced cyclin D1 and cyclin D2 expression in TM4 cells (Fig. 15B). Inhibition of p-ERK activity by PD98059 reversed the effects on claudin-11, p27, cyclin D1, and cyclin D2 observed by treatment with the PPARD inverse agonist DG172 (Fig. 15B). These observations indicate that PPARD-dependent regulation of ERK activity is involved in tight junctions and proliferation of Sertoli cells.

**Discussion**

Testicular development and spermatogenesis are highly dependent on homeostatic control. Testicular dysgenesis can be caused by impaired germ cell differentiation, hormone insufficiency, and dysfunction of Leydig and/or Sertoli cells (which both provide paracrine support) (23). The present study revealed marked differences in the structure and morphology of testes between *Ppard*^+/+^ and *Ppard*^-/-^ mice and elucidated a cooperative role of PPARD in Sertoli cells/germ cell interactions, thus demonstrating for the first time that PPARD...
influences spermatogenesis. Because there was no apparent difference in interstitial space between \textit{Ppard}\textsubscript{H11001}/H11001/H11001 and \textit{Ppard}\textsubscript{H11002}/H11002/H11002 mice, the smaller diameter and volume density of seminiferous tubules coupled with the lower number of Sertoli cells or germ cells in \textit{Ppard}\textsubscript{H11001}/H11001/H11001 mice suggest that PPARD helps to regulate the number of Sertoli and germ cell populations by providing an inhibition of Sertoli cell proliferation through timely maturation during the prepubertal period. Sertoli cell immaturity and the delay in forming the blood-testis barrier observed in \textit{Ppard}\textsubscript{H11002}/H11002/H11002 mice may result in the disruption of seminiferous epithelium.

Normal spermatid head shape is required for male fertility as demonstrated by previous studies (24–28). Unexpectedly, the nuclear elongation in step 9–12 spermatids does not progress normally in \textit{Ppard}\textsubscript{H11002}/H11002/H11002 mice. The retention of elongated spermatids in the seminiferous epithelium is associated with more spermatid heads in adult \textit{Ppard}\textsubscript{H11002}/H11002/H11002 mice. This is the first report showing a correlation between PPARD expression and spermatid morphology, and it suggests that PPARD is required for spermatid formation. Similar phenotypes were reported in retinoic acid receptor (Rar)-\textit{a} and retinoid X receptor (Rxr)-\textit{b}/\textsubscript{-/-} mice carrying atrophic testes and abnormal spermatids (24, 29).

Because PPARs and RARs can both form heterodimers with RXRs, receptor competition among PPARD, RXR, and RAR could influence spermatogenesis and male infertility. However, the severity of the testicular degenerative phenotype is stronger in \textit{Rxrb}\textsubscript{H11002}/H11002/H11002 and \textit{Rara}\textsubscript{H11002}/H11002/H11002 mice because these mice are sterile. Nevertheless, further evaluation of how PPARD controls the process of forming spermatid heads and the spermiation is warranted.

The number of Sertoli cells determines the number of germ cells, testis size, and daily sperm production in the seminiferous epithelium (30–32). The proliferation and differentiation of Sertoli cells depend on hormone regulation, especially FSH released from the anterior pituitary (33). In the present study, the average serum FSH concentration was lower in \textit{Ppard}\textsubscript{H11001}/H11001/H11001 mice than in \textit{Ppard}\textsubscript{H11002}/H11002/H11002 mice, especially as adults. The mechanism by which PPARD causes this effect cannot be determined from the present study. However, mRNA encoding \textit{Ppard} can be suppressed by FSH treatment in rat Sertoli cells (34), suggesting the possibility of a positive feedback mechanism. By contrast, inhibin B regulates FSH through a negative feedback mechanism (35). Thus, the increased serum concentration of inhibin B observed in \textit{Ppard}\textsubscript{H11001}/H11001/H11001 mice may be associated with the decrease in serum FSH observed in this genotype. Com-

![FIGURE 12. PPARD mediates ligand-induced expression of claudin-11 in TM4 cells in vitro. A, representative immunofluorescence photomicrographs showing the effect of the PPARD agonist GW0742 or overexpression of PPARD on claudin-11 expression (green color) in TM4 cells. B, representative immunofluorescence photomicrographs showing the effect of the PPARD inverse agonist DG172 or knockdown of PPARD on claudin-11 expression (green color) in TM4 cells. Cells were counterstained with DAPI to stain the nuclei. Magnification, 60 ×. Scale bars, 10 μm. Middle panels, quantitative Western blot analysis of PPARD expression in TM4 cells. +, positive control (COS1 cell lysate from cells transfected with mouse \textit{Ppard} expression vector). The relative expression level of PPARD was normalized to that of actin and represents the mean ± S.E. Lower panels, quantification of the intensity of claudin-11 expression in TM4 cells. Values represent the mean ± S.E. (error bars). Values with different superscript letters are significantly different at \( p \leq 0.05 \).](https://example.com/figure12)
bined, these two possible mechanisms could explain why a higher serum concentration of FSH is found in Ppard−/− mice.

In addition to FSH, Sertoli cells also require testosterone to fully establish their supportive capacity by producing essential factors for germ cell development (36). Sertoli cells are the only cells that express androgen receptor in the seminiferous tubules (36). Testosterone produced from Leydig cells acts on Sertoli cells to indirectly influence spermatogenesis by regulating the integrity of the blood-testis barrier and the processes of spermiogenesis and spermatiation (36). Thus, the lower serum testosterone concentration detected in Ppard−/− mice are possibly associated with PPARD-dependent reduction of testosterone production. Interestingly, the negative feedback loop from testosterone dynamically regulates FSH production (37, 38), consistent with the differential level of serum FSH and testosterone concentrations observed in Ppard+/+ and Ppard−/− mice in the present study.

Although Ppard−/− mice produce more spermatids, the malformation of spermatid heads in Ppard−/− mice possibly impairs sperm mobility and fertility and could be due to the fact that PPARD promotes terminal differentiation in many somatic cell types (39, 40). That malformed spermatids could impact male fertility is supported by the phenotype of transgenic nuclear protein 1 (Tnp1)-null mice (28). TNP1 is a spermatid-specific protein responsible for histone replacement and chromatin condensation during spermiogenesis. Although Tnp1−/− mice produce more spermatids in the testis, the abnormal sperm shape and the lower motility of sperm in the epididymis contribute to a reduced litter size in Tnp1−/− mice (28). Thus, altered hormone production, abnormal spermatid head shape, and spermiation failure observed in Ppard−/− mice in the present study are indicative of impaired spermatogenesis. Whether this impairment of spermatogenesis contributes to the decrease in average litter size previously observed in Ppard−/− mice cannot be determined from the present study as this could be influenced by maternal factors. These possibilities should be examined in greater detail in the future.
ISOFORM OF P-ERK TO THE P42 OR P44 ISOFORM OF ERK ARE SHOWN WITH PPARD PRODUCTION, AND LARGER TESTES. ASSOCIATED WITH INCREASED SERTOLI CELL NUMBER, ENHANCED SPERM PROLIFERATION RE-ENTRY INTO THE CELL CYCLE DURING DEVELOPMENT. DECREASED PPARD IS REQUIRED FOR THE MATURATION OF SERTOLI CELLS BY BLOCKING RE-ENTRY INTO THE CELL CYCLE DURING DEVELOPMENT. DECREASED EXPRESSION OF p27 OBSERVED IN p27−/− MICE MAY IMPAIR SERTOLI CELL DIFFERENTIATION AND MATURATION, LEADING TO A DIMINISHED ABILITY TO SUPPORT SPERMATOGENESIS.

PREVIOUS STUDIES SHOW THAT p27−/− MICE HAVE INCREASED SERTOLI CELL NUMBER AND DAILY SPERM PRODUCTION, RESULTING IN LARGER TESTES COMPARED WITH WILD-TYPE MICE (41, 43). SIMILAR RESULTS WERE OBSERVED IN Ppard−/− MICE IN THE PRESENT STUDY COMPARED WITH P27−/− MICE BECAUSE LOWER EXPRESSION OF P27 WAS ASSOCIATED WITH INCREASED SERTOLI CELL NUMBER, ENHANCED SPERM PRODUCTION, AND LARGER TESTES. P27−/− MICE ALSO EXHIBIT A MIXED ATROPHIC PHENOTYPE IN TESTES (41) SIMILAR TO THE PHENOTYPE OF Ppard−/− MICE. THE INCREASE IN NON-ATROPHIC TESTIS WEIGHT IN Ppard−/− MICE IS CONSISTENT WITH ENHANCED PROLIFERATION OF SERTOLI CELLS. MOREOVER, DECREASED SERUM TESTOSTERONE CONCENTRATION AND INCREASED SERUM FSH CONCENTRATION WERE ALSO DETECTED IN BOTH ADULT Ppard−/− AND p27−/− MICE (44, 45). COMBINED, THESE OBSERVATIONS SUGGEST THAT PPARD-DEPENDENT REGULATION OF p27 SIGNALING RE Presses THE NUMBER OF SERTOLI CELLS AND IS REQUIRED TO MODULATE SPERMATOGENESIS. INTERESTINGLY, IMMATURE SPERM IN THE EPIDIDYMIS WERE OBSERVED IN p27−/− MICE (41). WHETHER PPARD-DEPENDENT p27 SIGNALING IS CRITICAL FOR GERM CELL MIGRATION AND SPERM MATURATION IN THE EPIDIDYMIS SHOULD BE EXAMINED IN GREATER DETAIL BECAUSE SIGNIFICANT SPERM MATURATION AND DIFFERENTIATION OCCUR IN THE EPIDIDYMIS.

SERTOLI CELLS IN Ppard+/− MICE STOP REPLICATING WHILE APPEARING PUBERTY, WHEREAS IMMATURE SERTOLI CELLS IN PUBERTAL Ppard−/− MICE CONTINUE TO PROLIFERATE. ALTHOUGH A SERTOLI CELL-SPECIFIC Ppard-null mouse line would be useful to examine the role of this receptor in Sertoli cell/spermatogenesis, the use of the TM4 mouse Sertoli cell line provided an alternative approach to demonstrate the essential role of PPARD IN THE SERTOLI CELL IN THE REGULATION OF PROTEINS LINKED PREVIOUSLY WITH PROLIFERATION AND TIGHT JUNCTIONS IN SPERMATOGENESIS. FUNCTIONAL ANALYSIS IN TM4 CELLS CONFIRMS THE ROLE OF PPARD IN SERTOLI CELLS AS THE DATA DEMONSTRATE THAT LIGAND ACTIVATION OF PPARD NEGATIVELY REGULATES CELL PROLIFERATION BY INCREASING p27 AND DECREASING CYCLIN D1 AND CYCLIN D2 EXPRESSION. SIMILARLY, OVEREXPRESSION OF PPARD IN TM4 MICE SERTOLI CELLS RESULTED IN THE SAME CHANGES IN EXPRESSION OF THESE PROTEINS. MOREOVER, KNOCKING DOWN EXPRESSION OF PPARD IN TM4 MICE SERTOLI CELLS MITIGATED THESE CHANGES IN EXPRESSION OF p27, CYCLIN D1, AND CYCLIN D2. THIS IS CONSISTENT WITH PREVIOUS FINDINGS THAT LIGAND ACTIVATION OF PPARD SUPPRESSES CYCLIN D1 OR INCREASES p27 EXPRESSION, CAUSING INHIBITION OF CELL PROLIFERATION IN CANCER CELLS AND PRIMARY KERATINOCYTES (16, 46, 47). FURTHERMORE, THE PRESENT STUDIES ALSO DEMONSTRATED THAT LIGAND ACTIVATION OF PPARD ALTERS EXPRESSION OF CELL CYCLE REGULATORS BY MODULATING ERK ACTIVATION IN SERTOLI CELLS IN VIVO AND IN VITRO, CONSISTENT WITH PREVIOUS STUDIES (16, 48–50). COLLECTIVELY, THESE FINDINGS INDICATE THAT PPARD-DEPENDENT REPRESSION OF ERK ACTIVITY MAY BE THE KEY TO THE BALANCE BETWEEN PROLIFERATION AND MATURATION OF SERTOLI CELLS. MOREOVER, THE OBSERVATION THAT p-ERK EXPRESSION INCREASES WITH AGING AND REACHES THE HIGHEST LEVEL AT PUBERTY FOLLOWED BY A MARKED DECREASE AT ADULTHOOD IN Ppard−/− MICE CORRELATES WITH THE CHANGES IN SERUM FSH LEVELS IN THIS GENOTYPE. THESE EFFECTS ARE NOT FOUND IN Ppard−/− MICE, INDICATING THAT PPARD IS ALSO REQUIRED FOR THESE CHANGES. THIS IS ALSO CONSISTENT WITH A PREVIOUS STUDY SHOWING THAT FSH TRIGGERS ERK-DEPENDENT PROLIFERATION IN NEONATAL SERTOLI CELLS BUT INHIBITS ERK ACTIVATION IN SERTOLI CELLS AFTER PUBERTY (49).

THE INTEGRITY OF DIFFERENT CELL JUNCTIONS AND THE REGULATION OF DYNAMIC JUNCTION PROTEINS ARE IMPORTANT FOR NORMAL SPERMATOGENESIS, ESPECIALLY IN CONTROLLING GERM CELL MIGRATION (51–53). INTERESTINGLY, ACTIVATION OF ERK ALSO FUNCTIONS AS A MEDIATOR TO SUPPRESS CLAUDIN-11 EXPRESSION IN RESPONSE TO HORMONE STIMULATION OR ENVIRONMENTAL TOXICANT EXPOSURE (54). RESULTS FROM THE PRESENT STUDY ALSO INDICATE THAT PPARD-DEPENDENT INHIBITION OF ERK ACTIVITY MODULATES CLAUDIN-11 EXPRESSION, WHICH IS CRITICAL FOR TIGHT JUNCTIONS IN SERTOLI CELLS. RECENT STUDIES SUGGEST THAT FSH/CAMP DOWN-REGULATE CLAUDIN-11 mRNA LEVELS IN MOUSE SERTOLI CELLS, LEADING TO A RESTRUCTURING OF TIGHT JUNCTIONS (55, 56). THIS IS CONSISTENT WITH THE CHANGES IN SERUM FSH OBSERVED IN THE PRESENT STUDY. ADDITIONALLY, THE INTEGRITY OF THE
The blood-testis barrier is responsible for the migration of preleptotene spermatocytes but also affects the ectoplasmic specialization, which controls the release of mature spermatids (57). Several studies have demonstrated that abnormal spermatogenesis and/or spermatid retention is associated with dynamic structures of cell junctions (58–60). Thus, this suggests that PPARD-dependent regulation of ERK/claudin-11/tight junctions in Sertoli cells contributes to the differentiation and elongation of spermatids. Combined, results from these studies demonstrate for the first time that PPARD regulates ERK signaling, which impacts Sertoli cell function, and germ cell development in mouse testes (Fig. 16).

**Author Contributions**—P.-L. Y., J. M. P., and F. J. G. conceived and coordinated the study and wrote the paper. L. C. performed and analyzed the experiments shown in Figs. 2 and 3. R. A. H. designed the analysis shown in Figs. 1 and 6 and performed histopathological analysis shown in Figs. 2 and 3. R. M. designed and interpreted the data shown in Figs. 10, 12, 14, and 15. All authors reviewed the results and approved the final version of the manuscript.

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