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Phosphatase of Regenerating Liver 2 (PRL2) Deficiency Impairs Kit Signaling and Spermatogenesis*

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Background: The PRLs are oncogenic when overexpressed but their physiological function is not well defined.
Results: PRL2-deficient mice exhibit testis hypotrophy, decreased sperm production, and impaired reproductive potential.
Conclusion: PRL2 promotes Kit signaling and germ cell survival by down-regulating PTEN.
Significance: The study reveals the biological importance of PRL2 in spermatogenesis and identifies PRL2 as a novel target for cancer and male contraception.

The Phosphatase of Regenerating Liver (PRL) proteins promote cell signaling and are oncogenic when overexpressed. However, our understanding of PRL function came primarily from studies with cultured cell lines aberrantly or ectopically expressing PRLs. To define the physiological roles of the PRLs, we generated PRL2 knock-out mice to study the effects of PRL deletion in a genetically controlled, organismal model. PRL2-deficient male mice exhibit testicular hypotrophy and impaired spermatogenesis, leading to decreased reproductive capacity. Mechanistically, PRL2 deficiency results in elevated PTEN level in the testis, which attenuates the Kit-PI3K-Akt pathway, resulting in increased germ cell apoptosis. Conversely, increased PRL2 expression in GC-1 cells reduces PTEN level and promotes Akt activation. Our analyses of PRL2-deficient animals suggest that PRL2 is required for spermatogenesis during testis development. The study also reveals that PRL2 promotes Kit-mediated PI3K/Akt signaling by reducing the level of PTEN that normally antagonizes the pathway. Given the strong cancer susceptibility to subtle variations in PTEN level, the ability of PRL2 to repress PTEN expression qualifies it as an oncogene and a novel target for developing anti-cancer agents.

The phosphatase of regenerating liver (PRL)[2] family of proteins are potential targets in oncology (1–3). Of the three PRLs, PRL2 is the most abundantly and ubiquitously expressed in adult human tissues while PRL1 has a somewhat more restricted pattern of expression and an overall lower level than PRL2 (4–6). PRL3 is primarily expressed in heart and skeletal muscle (5, 7). Unlike most protein phosphatases that function to reverse the action of protein kinases, the PRLs play a positive role in promoting cell proliferation and invasion (1–3). PRLs are found elevated in colorectal (8, 9), liver (10), prostate (11), pancreatic (12), and breast (13) cancers, as well as hematological malignancies such as multiple myeloma (14, 15) and acute myeloid leukemia (16, 17). Furthermore, elevated levels of PRL are detected almost exclusively in late stage and metastatic tumors, suggesting that they may play a role in malignancies (8, 9, 18–21). Experimentally, overexpression of PRL in both non-tumorigenic and tumor cell lines results in increased proliferation, anchorage-independent growth, and invasion (10, 22–25). When injected into the tail vein of mice, PRL-overexpressing cells induce rapid tumor growth and high levels of metastasis (10, 25). Conversely, down-regulation of PRL reduces oncogenic behaviors in melanoma, gastric, breast, and colon cancer cell lines, and results in smaller tumors with less metastases in mice (18, 26–29). Thus, the PRL phosphatases possess oncogenic potential when overexpressed.

Available biochemical data suggest that PRLs promote cell proliferation and migration through activation of a number of pathways, including those mediated by the Rho family of small GTPases, Src, ERK1/2, and PI3K (23, 24, 26, 30). However, our current understanding of PRLs came primarily from studies in cancer cell lines ectopically overexpressing PRLs. Little is known about the role of PRL in normal physiology. To define the biological function of the PRLs, we generated PRL2 knock-out mice to study the effects of PRL deletion in a genetically controlled, organismal model. We previously reported that PRL2 deficiency causes growth retardation in both embryos and adult mice (31). The embryonic growth retardation was attributed to placenta insufficiency. Decreased cell proliferation in PRL2$^{-/-}$ placenta appears to be caused by Akt inactivation as a result of up-regulation of the tumor suppressor PTEN. These results provide the first evidence that PRL2 is required for extra-embryonic development by promoting cell proliferation via the PI3K-Akt signaling pathway (31).

In addition to the placental phenotypes in females, PRL2-deficient male mice exhibit testis hypotrophy, suggesting possible defects in spermatogenesis. Spermatogenesis is a highly regulated process of proliferation and differentiation of sper-

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[2] The abbreviations used are: PRL2, phosphatase of regenerating liver 2; PTEN, phosphatase and tensin homologue deleted on chromosome 10; GDNF, Glial cell line-derived neurotrophic factor; SCF, stem cell factor.

matogonia, the germline stem cells in testis, leading to successive production of mature spermatozoa throughout the period of male sexual maturity (32). The balance between testicular stem cell proliferation and differentiation is regulated by a complex interplay of signaling events. While the self-renewal of stem cell is primarily stimulated by Glial cell line-derived neurotrophic factor (GDNF) produced by Sertoli cells (the somatic cells supporting germ cells within seminiferous tubules) (33), the differentiation of spermatogonia is heavily controlled by Kit signaling (34). Kit is a proto-oncogene encoding a protein tyrosine kinase in the PDGF receptor family (35). Kit is activated when bound to its ligand Stem Cell Factor (SCF) expressed in Sertoli cells, and plays crucial roles in regulating proliferation, migration, and survival of the Kit-positive cells, as well as the entry of meiosis (36).

In the present study, we analyzed the effect of PRL2 deficiency on male fertility and spermatogenesis at the cellular and molecular levels. We show that PRL2-deficient mice have atrophied testis and low sperm count, leading to impaired reproductive ability. Mechanistically, PRL2 deficiency results in elevated PTEN expression in testis germ cells. This attenuates the SCF-Kit stimulated Akt activation and increased germ cell apoptosis, causing decreased production of spermatozoa. Together, the data support an important role of PRL2 in spermatogenesis through regulation of the Kit signaling pathway.

EXPERIMENTAL PROCEDURES

Animals—Generation and genotyping of the PRL2$^{-/-}$ mice were previously described (31). Mice used in this study were all on a C57BL6/129P2 mixed genetic background. Experiments on mice were carried out in accordance with the regulations of The Institutional Animal Care and Use Committees at Indiana University. PRL2 monoclonal antibodies were generated as previously described (37).

Cell Culture and Stable Clone Selection—GC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (50 units/ml) and streptomycin (50 μg/ml) under a humidified atmosphere containing 5% CO_2. pCMV-Flag vector containing mouse PRL2 cDNA was co-transfected with puromycin selection vector into GC-1 cells using Lipofectamine 2000 (Invitrogen). 24 h after transfection, 2 μg/ml puromycin was added to the medium. Stable clones were isolated after 3 weeks selection. Overexpression of PRL2 was determined by Western blot.

Reproductive Performance Evaluation—Age matched wild-type and PRL2$^{-/-}$ males were each mated with 4 wild-type virgin females every day for 6 consecutive days. Vaginal plugs were monitored every morning, and plugged females were removed and replaced with virgin females. After 6 days, the total number of females plugged by each male was counted. Plugged females were kept for an additional month to determine the number of pregnant mice and their litter sizes. The percentage of pregnant mice among the plugged and the average litter size were calculated for each male.

Histology—Tissues were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, embedded in paraffin, serially sectioned (7 μm), and stained with H&E according to standard methods. For immunohistochemistry, de-paraffined and hydrated sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate for 20 min. Sections were then incubated with diluted antibodies (1:50 to 1:400) at 4 °C overnight. Signals were detected by VECTASTAIN Elite ABC kit and developed using DAB substrate from Vector laboratory (Burlingame, CA). Antibodies used were PLZF, Kit (Santa Cruz Biotechnology), Vimentin, PCNA and cleaved PARP (Cell Signaling Technology). TUNEL staining was performed using ApopTag fluorescein *in situ* apoptosis detection kit (Millipore) following the manufacturer's instructions. For LacZ staining, testis was fixed in 4% PFA on ice for 1 h, incubated in PBS/0.01%Nonidet P-40 for 4 h, and stained in β-gal substrate (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM EGTA, 0.01% Nonidet P-40 in 1× PBS) for 48 h at 37 °C. Testis was then embedded in paraffin and sectioned. Images were captured on a Leica DM2500 stereomicroscope. All images are representative of at least three samples.

Testicular Cell Isolation, Stimulation, and Western Blot Analysis—Testes isolated from wild-type or PRL2$^{-/-}$ males were de-capsulated and digested in DMEM containing 1 mg/ml collagenase I at 32 °C for 20 min with gentle agitation. Released interstitial cells were removed, and seminiferous tubules were washed twice with DMEM. Seminiferous tubules were then subjected to second enzymatic digestion in DMEM with 1 mg/ml collagenase I, 0.5 mg/ml trypsin, 50 units/ml hyaluronidase, and 100 μg/ml DNase I at 32 °C for 30 min with gentle agitation. Seminiferous tubules were pipetted up and down for 10 times to disassociate the cells. The cell clumps were removed by passing through a 70-μm nylon filter, and the single cell preparation was incubated in a culture dish in DMEM at 32 °C with 5% CO_2 for 3 h to allow Sertoli cells and peritubular cells to attach. Germ cells in the suspension were then counted and used immediately. For SCF stimulation, 1×10^6 cells were incubated with or without SCF for indicated amount of time, lysed in SDS protein sample buffer, separated by SDS-PAGE and subjected to Western blot analysis. All the antibodies used in Western blot analysis are from Cell Signaling Technology.

Sperm Count—Caudal epididymis were isolated from age-matched wild-type or PRL2$^{-/-}$ mice, minced in 10 ml BWW buffer (NaCl 5.54 g/liter, KCl 0.356 g/ liter, $CaCl_2 \cdot 2H_2O$ 0.250 g/ liter, KH_2PO_4 0.162 g/ liter, $MgSO_4 \cdot 7H_2O$ 0.294 g/ liter, $NaHCO_3$ 2.1 g/ liter, glucose 1.0 g/ liter, sodium pyruvic acid 0.03 g/ liter, BSA 3.5 g/ liter), and incubated at 32 °C for 15 min. After mixed by pipetting, the motile and total sperm numbers were counted using hemocytometer.

Statistical Analysis—All statistical significant differences were calculated using student's *t* test and represented by asterisks: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

RESULTS

PRL2$^{-/-}$ Male Mice Exhibit Impaired Reproductive Capacity due to Reduced Sperm Production—Anatomical examination revealed that the testis of PRL2$^{-/-}$ male are markedly smaller than that of the wild-type (47.2 ± 7.0 *versus* 103.0 ± 15.6 mg) (Fig. 1*A*). Since PRL2 deficient adult mice are ~20% smaller than wild-type littermates (31), we normalized the testis to body weight and found that the PRL2$^{-/-}$ testes were still ~30% smaller compared with those of the wild-type littermates (Fig.

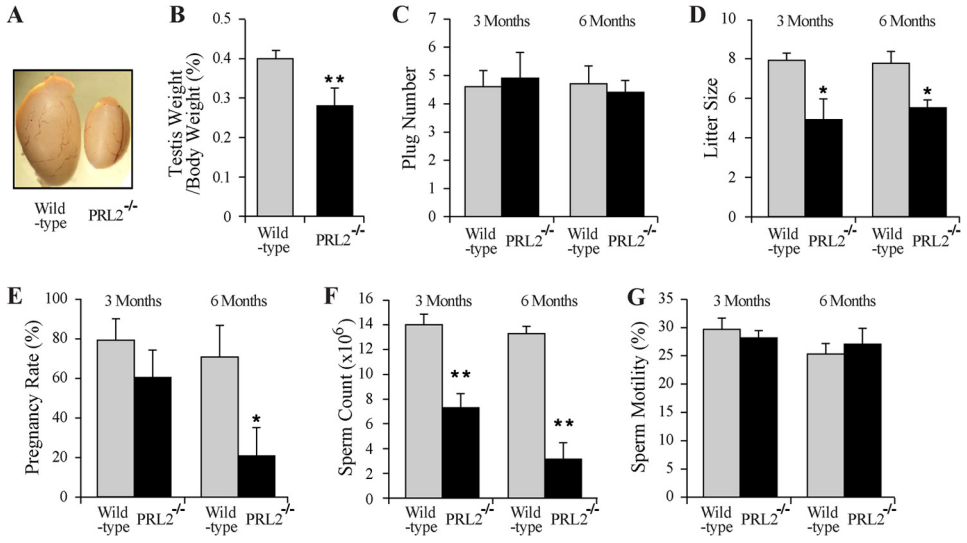

FIGURE 1. **PRL2$^{-/-}$ male has impaired reproductive ability due to reduced sperm number.** *A*, picture of testis from wild-type and PRL2$^{-/-}$ littermates at 3 months old. *B*, testis/body weight ratio of wild-type and PRL2$^{-/-}$ mice. $n = 5$ for each genotype. *C–E*, mating experiment to assess the reproductive performance of wild-type and PRL2$^{-/-}$ males. As described under "Experimental Procedures," each male was mated with 4 wild-type virgin females every day for 6 consecutive days. Plugged females were taken out and kept for pregnancy measurement. For 3 months old, WT: $n = 5$, KO: $n = 8$. For 6 months old, WT: $n = 4$, KO: $n = 4$. Data represent mean ± S.E. *F–G*, sperm counts of wild-type and PRL2$^{-/-}$ mice. Epididymis from one side of mice was removed, minced in BWW buffer, and incubated at 32 °C for 15 min. Sperm suspension was then added to hemocytometer to count total and motile sperm number. $n = 5$ for each genotype at each time point. Data represent mean ± S.E. *, $p < 0.05$, **, $p < 0.01$.

1*B*). To determine whether the reduced testis size affects PRL2$^{-/-}$ male reproductivity, wild-type and PRL2$^{-/-}$ males at 3 and 6 months old were mated with wild-type virgin females for 6 consecutive days. At both ages PRL2$^{-/-}$ males produced similar number of vaginal plugs as the matching wild-type animals (Fig. 1*C*), indicating that PRL2 deficiency did not affect the sexual drive in PRL2 mutant animals. However, the average litter size derived from PRL2$^{-/-}$ males was smaller than that from females mated to wild-type males (Fig. 1*D*). In addition, the percentage of pregnancy plugged by PRL2$^{-/-}$ males was lower than that by wild-type males (Fig. 1*E*). The difference in fertility was evident as early as 3 months of age and became more pronounced at 6 months of age, when only 20% of females plugged by PRL2$^{-/-}$ males became pregnant compared with 71% of females by the wild type (Fig. 1*E*). The reproductive performance was also evaluated in younger (2–2.5 month) and older (> 6 month) PRL2$^{-/-}$ and matched wild-type littermates. At younger age, there was no significant difference in fertility between the two genotypes (data not shown). PRL2$^{-/-}$ male mice (n>30) manifested a full-penetrance of sterility when they were beyond 9 months old while the wild-type at 1.5 year in our colony were still able to sire with normal liter size (data not shown). Thus, while the sexual drive appeared to be normal, the reproductive ability of the PRL2$^{-/-}$ male was compromised, and became progressively deteriorated as the mice aged. The age-dependent impaired fertility in PRL2$^{-/-}$ male is likely a cumulative effect due to a gradual decrease in spermatozoal production (see below).

Testosterone plays an essential role in testis development and function (38). Sertoli cell-specific deletion of androgen receptor (AR), the receptor for testosterone, results in reduced testis size and impaired spermatogenesis (39). The testicular hypotrophy and decreased reproductive capacity of PRL2$^{-/-}$ mice prompted us to examine whether testosterone level was affected by deficiency of PRL2. However, measurement of testosterone concentration in serum from 3 month old mice did not reveal significant difference between wild-type and PRL2$^{-/-}$ mice (data not shown), suggesting that the reduction of testis in PRL2$^{-/-}$ mice was not due to changes in testosterone level. The homeostasis of prostate and seminal vesicles also depends on proper testosterone level. Consistent with the normal level of blood testosterone in mutant mice, the prostates and seminal vesicles in PRL2-deficient mice were comparable in size to those in wild-type when normalized by their body weights (data not shown). Sperm counts were next measured to investigate the cause of reduced fertility in PRL2$^{-/-}$ male. Indeed, PRL2$^{-/-}$ epididymis contained significantly fewer spermatozoa than wild-type, especially at 6-month old (sperm count $3.1 \pm 1.3 \times 10^6$ for PRL2$^{-/-}$ and $13.3 \pm 0.6 \times 10^6$ for wild-type), although no significant difference in sperm motility was observed (Fig. 1, *F* and *G*). These results suggest that the impaired reproductive ability of PRL2$^{-/-}$ male is due to lower sperm production.

PRL2 Deficiency Leads to Decreased Spermatogenesis—The structure of PRL2$^{-/-}$ testes was analyzed histologically to explore the cause for the reduced organ size and sperm count. Similar to wild-type controls, the seminiferous tubules of 3 month old PRL2$^{-/-}$ mice contained well-structured Sertoli cells, spermatogonia, primary and secondary spermatocytes, round spermatids and enlongated spermatids (Fig. 2, *A* and *B*). However, the diameter of seminiferous tubules of PRL2$^{-/-}$ mice is nearly 20% shorter than that of wild-type (Fig. 2*C*), suggesting a reduced cellularity in PRL2$^{-/-}$ tubules. In 3-month old mutant testes, abnormal clusters of cells could be found in the lumen of seminiferous tubules (Fig. 2, *A* and *B*). This is more frequently observed in 6-month old mutant testes (Fig. 2, *D* and *E*). In addition, seminiferous tubules either totally or partially devoid of germ cells were observed in 6-month old PRL2$^{-/-}$

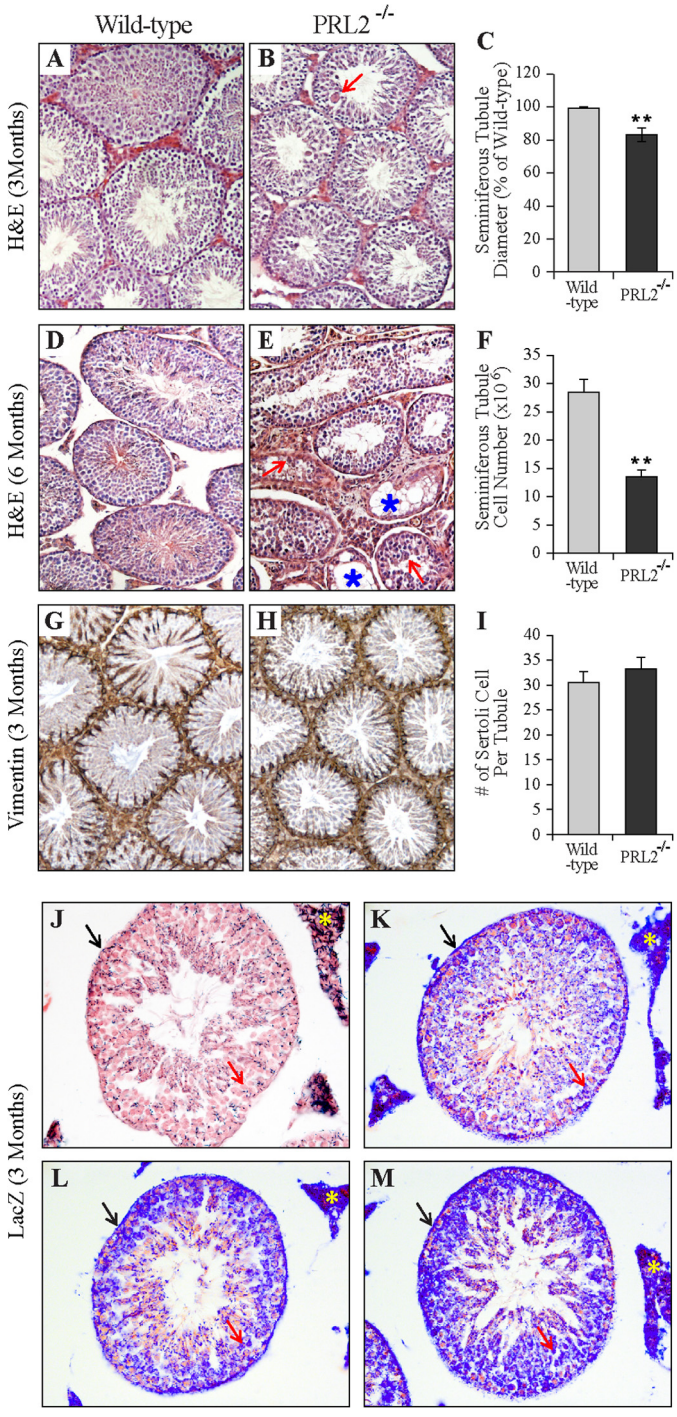

FIGURE 2. **PRL2$^{-/-}$ testis has hypotrophic seminiferous tubules.** *A* and *B*, H&E staining of wild-type (*A*) and PRL2$^{-/-}$ (*B*) testis sections at 3 months of age. The *black arrow* shows aberrant cell cluster. *C*, seminiferous tubule diameter of wild-type and PRL2$^{-/-}$ mice (% of WT). Seminiferous tubule diameters were measured on testis sections of wild-type and PRL$^{-/-}$ littermates. For each mouse at least 20 seminiferous tubules were measured. The average diameter of PRL2$^{-/-}$ seminiferous tubules was then calculated as a percentage of that of wild-type littermates and plotted. $n = 8$ for each genotype. Data represent mean ± S.E. *D* and *E*, H&E staining of wild-type (*D*) and PRL2$^{-/-}$ (*E*) testis sections at 6 months of age. *Red arrows* show aberrant cell clusters. *Blue stars* mark empty seminiferous tubules. *F*, seminiferous tubule cell numbers in wild-type and PRL2$^{-/-}$ testes. Seminiferous tubule cells from the testes of 3 month old wild-type or PRL2$^{-/-}$ mice were isolated and counted. $n = 3$ for each genotype. Data represent mean ± S.E. *G* and *H*, vimentin immunostaining on testis sections from wild-type (*G*) and PRL2$^{-/-}$ (*H*) mice of 3 months old. *I*, number of Sertoli cells per seminiferous tubule in wild-type and PRL2$^{-/-}$ testis. Cross sections of testes from 3 month old wild-type and PRL2$^{-/-}$ mice were immunohistologically stained for vimentin to label Sertoli cells. Sertoli cells in each seminiferous tubule were counted. At least 10 seminiferous tubules were counted for each mouse. Sertoli cell number per tubule was then averaged and plotted. $n = 5$ for each genotype. Data represent mean ± S.E. *J–M*, PRL2 expression indicated by LacZ expression under control of the endogeous PRL2 promoter. *J*, testicular tube section from wild-type serves as a negative control for LacZ activity-based staining (*blue*). *K–M*, testicular tube sections from PRL2$^{+/-}$ mice representing stages III (*K*), I (*L*), and XII (*M*) of spermatogenesis. PRL2 expression is specifically detected in PRL2$^{+/-}$ section in a ubiquitous manner, in interstitial tissue (*yellow* *), peritubular cells (*black arrow*), and spermatogenic cells (*red arrow*). While the staining intensity in the interstitial tissue and peritubular cells are comparable from stage to stage, the staining intensity in spermatogenic cells varies with spermatogenic stage. **, $p < 0.01$.

testes, indicating a severe cell loss in these tubules (Fig. 2, *D* and *E*).

To determine whether PRL2$^{-/-}$ seminiferous tubules were hypocellular, we counted the total number of cells isolated from seminiferous tubules of 3-month old PRL2$^{-/-}$ or wild-type testes. The results showed that the cell number in PRL2$^{-/-}$ seminiferous tubules was only 47% of the wild-type (Fig. 2*F*), which is consistent with the shorter diameter of seminiferous tubules as well as the smaller testis size in PRL2$^{-/-}$ mice. Seminiferous tubular cells consist of Sertoli cells, germ cells, and a small amount of peritubular myoid cells. Sertoli cells nourish and support the proliferation and differentiation of germ cell. Thus, a reduction of Sertoli cell number could result in loss of germ cells. To determine whether the development and growth of Sertoli cells were normal in PRL2$^{-/-}$ testis, we performed immunohistochemistry for vimentin, a Sertoli cell marker, to visualize Sertoli cells in wild-type and PRL2$^{-/-}$ mutant testis. Based on vimentin labeling, the number of Sertoli cells in each seminiferous tubule was counted, and the average cell number per tubule was found similar between the two genotypes (Fig. 2, *G–I*). This analysis revealed that Sertoli cell development was normal in PRL2$^{-/-}$ testes. Thus the hypo-cellularity observed in PRL2$^{-/-}$ seminiferous tubule is likely attributed to loss of germ cells, leading to impaired spermatogenesis in PRL2$^{-/-}$ testes.

To determine where PRL2 is expressed in the testis, we took advantage of LacZ expression that is under the control of endogenous *prl2* promoter in the mutant allele (31). Therefore, endogenous PRL2 transcription can be monitored by LacZ expression in heterozygous or homozygous PRL2 mutant mice. Since PRL2$^{-/+}$ testes were functionally and anatomically indistinguishable from those of the wild-type (data not shown), they were used to study endogenous PRL2 expression. As a specificity control for the assay, wild-type samples were performed in parallel. As expected, no X-gal staining was observed in wild-type testis (Fig. 2*J*). In heterozygous samples (Fig. 2, *K--M*), the interstitial tissue (yellow *), peritubular cells (black arrow), and germ cells (red arrow) are all positive for X-gal staining, revealing a ubiquitous expression pattern of PRL2 gene expression at the transcriptional level. The staining intensity within seminiferous tubule varies with spermatogenic stage as shown in Fig. 2, *K–M*, suggesting a spermatogenic stage-dependent expression pattern of PRL2 transcription. Because of the structural nature (being branching) of Sertoli cells and the punctated pattern of X-gal staining, whether PRL2 is expressed in Sertoli cells remains to be fully documented.

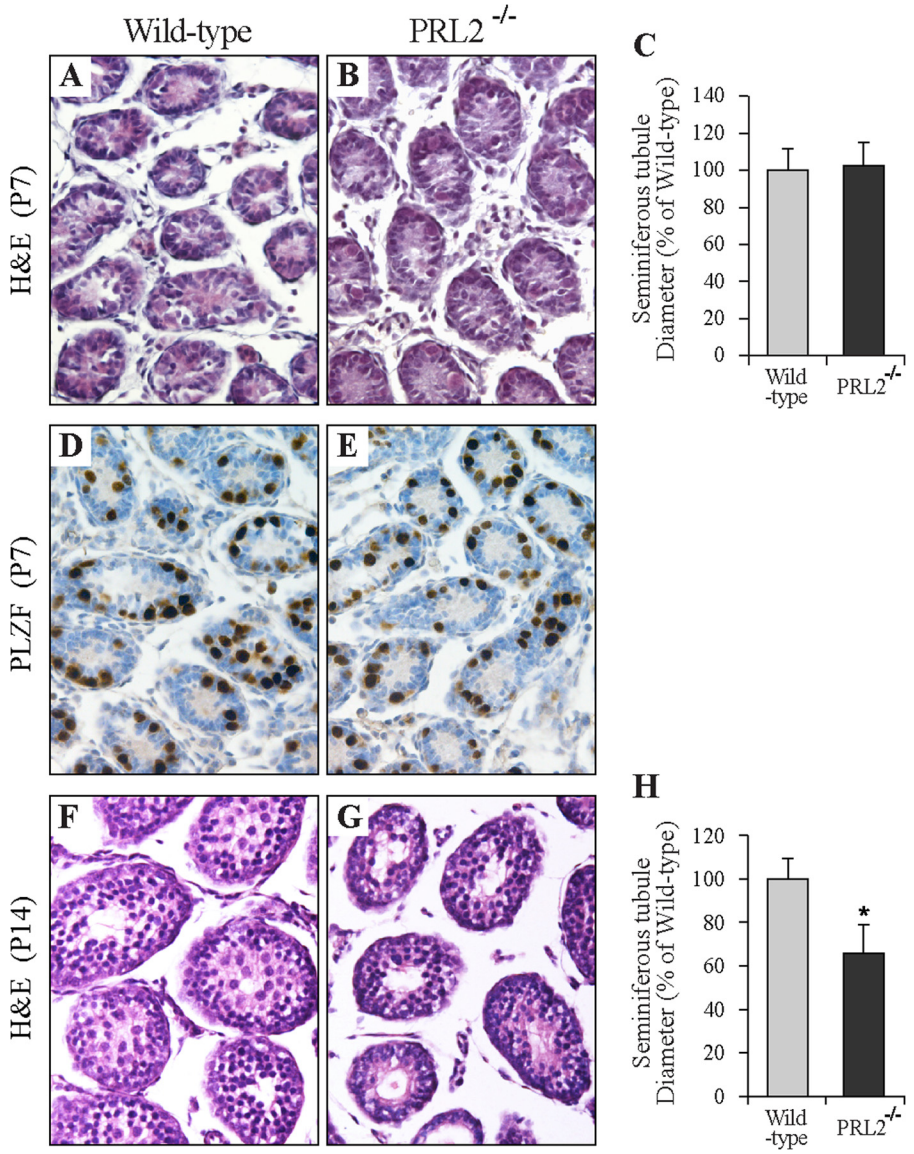

FIGURE 3. **Hypotrophy of PRL2$^{-/-}$ seminiferous tubules started at 2 weeks of age.** *A* and *B*, H&E staining of testis sections from 1 week old wild-type (*A*) and PRL2$^{-/-}$ (*B*) mice. *C*, seminiferous tubule diameter of wild-type and PRL2$^{-/-}$ testes at 1 week of age. $n = 3$ for each genotype. Data represent mean ± S.E. *D* and *E*, PLZF immunohistological staining of testis sections from 1-week old wild-type (*D*) and PRL2$^{-/-}$ (*E*) mice. *F* and *G*, H&E staining of testis sections from 2 weeks old wild-type (*F*) and PRL2$^{-/-}$ (*G*) mice. *H*, seminiferous tubule diameter of wild-type and PRL2$^{-/-}$ testes at 2 weeks of age. $n = 3$ for each genotype. Data represent mean ± S.E. *, $p < 0.05$.

PRL2 Deficiency Promotes Apoptosis in Germ Cells—In mice, spermatogenesis starts after birth (32). Along the process of the first round spermatogenesis, different types of germ cells appear in order of maturity, from undifferentiated spermatogonia to differentiating spermatogonia, primary spermatocyte, secondary spermatocyte, and lastly spermatid. To identify the cell type(s) and developmental stages affected by PRL2 deficiency, we analyzed wild-type and mutant testes at different stages of development to establish when the loss of cellularity occurs. At 1 week after birth, no structural difference could be detected between the wild-type and PRL2$^{-/-}$ testes (Fig. 3, *A* and *B*). The average diameter of the seminiferous tubules was similar between the two genotypes (Fig. 3*C*). At this stage, the majority of the cells inside the seminiferous tubules are Sertoli cells and undifferentiated spermatogonia with differentiating spermatogonia just starting to develop. We have shown that the development of Sertoli cell was normal in PRL2$^{-/-}$ testes. To determine the number of undifferentiated spermatogonia in wild-type and mutant seminiferous tubules, we performed immunohistological staining for PLZF, a transcription factor specifically expressed in undifferentiated spermatogonia (40). The result revealed that the number of PLZF-positive undifferentiated spermatogonia was comparable between the two genotypes (Fig. 3, *D* and *E*). This indicates that PRL2 deficiency does not affect the migration of primordial germ cells into the gonads during embryogenesis. Nor does PRL2 deficiency impair the proliferation of undifferentiated spermatogonia at this stage.

At 2 weeks of age, however, loss of cellularity became evident in the PRL2$^{-/-}$ testes. The thickness of the seminiferous epithelium was reduced (Fig. 3, *F* and *G*), and the diameter of seminiferous tubules was shorter in PRL2$^{-/-}$ testes compared

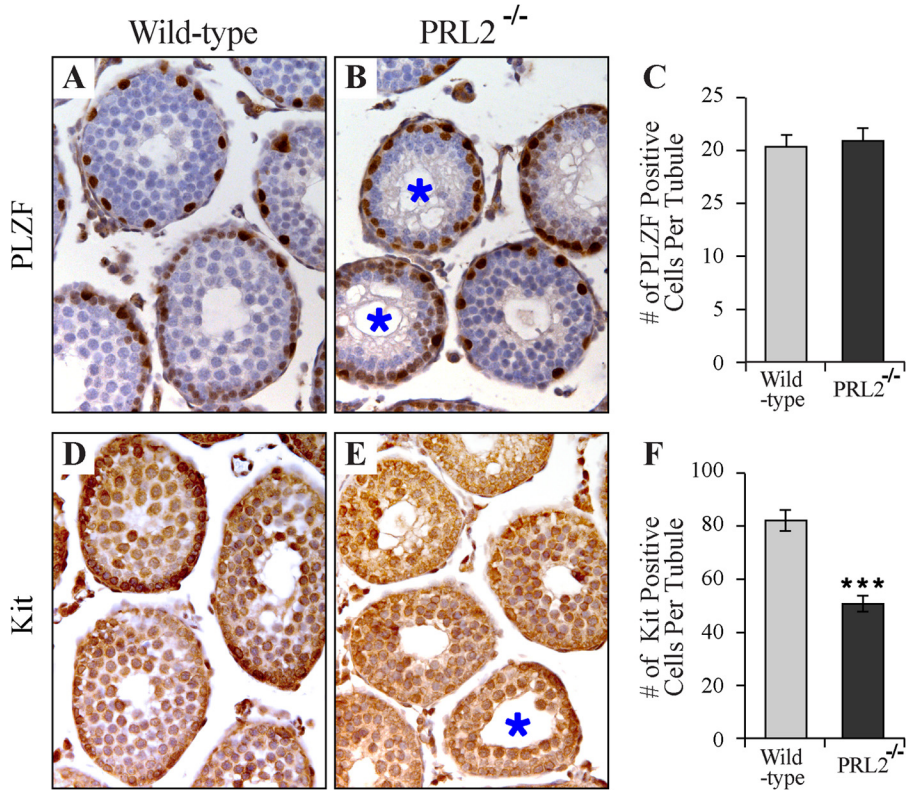

FIGURE 4. **PRL2$^{-/-}$ males have normal number of undifferentiated spermatogonia but lost Kit-positive cells at 2 weeks of age.** *A* and *B*, PLZF immunohistological staining showing undifferentiated spermatogonia in testis sections from 2-week old wild-type (*A*) and PRL2$^{-/-}$ (*B*) mice. Blue stars marked the PRL2$^{-/-}$ seminiferous tubules with very thin seminiferous epithelium. *C*, number of PLZF positive cells per seminiferous tubule in testis sections from 2 week old wild-type and PRL2$^{-/-}$ mice. For each mouse, at least 20 tubules were counted for PLZF-positive cells. $n = 3$ for each genotype. Data represent mean ± S.E. *D* and *E*, Kit immunohistological staining showing Kit-positive cells in testis sections from 2-week old wild-type (*D*) and PRL2$^{-/-}$ (*E*) mice. *Blue star* marked the PRL2$^{-/-}$ seminiferous tubule with very thin seminiferous epithelium. *F*, number of Kit-positive cells per seminiferous tubule in testis sections from 2 week old wild-type and PRL2$^{-/-}$ mice. For each mouse, at least 20 tubules were counted for Kit-positive cells. $n = 3$ for each genotype. Data represent mean ± S.E. ***, $p < 0.001$.

with that in the wild-type (Fig. 3*H*). We therefore conclude that the hypocellular phenotype of PRL2$^{-/-}$ testis started at 2 weeks after birth. The cell types inside seminiferous tubules at 2 weeks of age, in addition to Sertoli cells and undifferentiated spermatogonia, include differentiating spermatogonia and primary spermatocytes, both of which express Kit (41). To delineate which cell type was lost in PRL2$^{-/-}$ seminiferous tubule, we examined PLZF and Kit positive cells by immunohistochemistry in wild-type and mutant testes. The number of PLZF-positive undifferentiated spermatogonia was comparable between the two genotypes (Fig. 4, *A–C*). In contrast, the Kit-positive population was significantly reduced in PRL2$^{-/-}$ testes (Fig. 4, *D–F*). The difference was more pronounced in the mutant seminiferous tubules featuring a thin seminiferous epithelium (tubules with a blue star). Collectively, these analyses suggested that the primary cell type that is affected by PRL2 deficiency in 2 weeks old PRL2$^{-/-}$ testes are Kit-positive cells.

To determine whether the seminiferous hypocellularity was due to decreased proliferation, increased apoptosis, or a combination of both, we performed immunohistological staining for PCNA (Proliferating Cell Nuclear Antigen, a proliferation marker) and cleaved PARP (Poly ADP-Ribose Polymerase, an apoptosis marker) on testis sections of PRL2$^{-/-}$ and wild-type mice at 2 weeks of age. While the percentage of proliferating cells in the testis was similar between the two genotypes (Fig. 5, *A–C*), the labeling index for cleaved PARP was nearly 6-fold higher in the mutant germ cells, indicating that PRL2 deficiency primarily impair cell survival (Fig. 5, *D–F*). Consistent with the cleaved PARP staining, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay that detects the DNA breaks in apoptotic cells also showed a significantly increased number of TUNEL-positive cells in mutant testes (Fig. 5, *G* and *H*). Thus aberrant germ cell apoptosis provides a potential mechanism to explain the hypotrophy of seminiferous tubules and testes in PRL2$^{-/-}$ mice.

Increased apoptosis was also observed at later stages in PRL2$^{-/-}$ testis. Based on the cleaved PARP staining, the number of apoptotic germ cells was 6- and 9-fold higher in PRL2$^{-/-}$ testes at 3 and 6 months of age, respectively, than that in the wild-type animals (Fig. 6, *A–D*). This explained the progressively reduced sperm production in PRL2$^{-/-}$ males. Continuously elevated apoptosis will result in severe loss of germ cells, therefore producing fewer sperm. The apoptotic germ cells could be sloughed from the seminiferous epithelium and ultimately reach the caudal epididymis. Indeed, we found abnormal germ cell clusters in the lumen of the PRL2$^{-/-}$ testes. Cleaved PARP staining indicated that most of them were apoptotic cells (Fig. 6*B*, *red arrow*). Consistent with the histological presentation in the testis, examination of the caudal epididymis from 6 month old PRL2$^{-/-}$ mice revealed a scarcity of sperma-

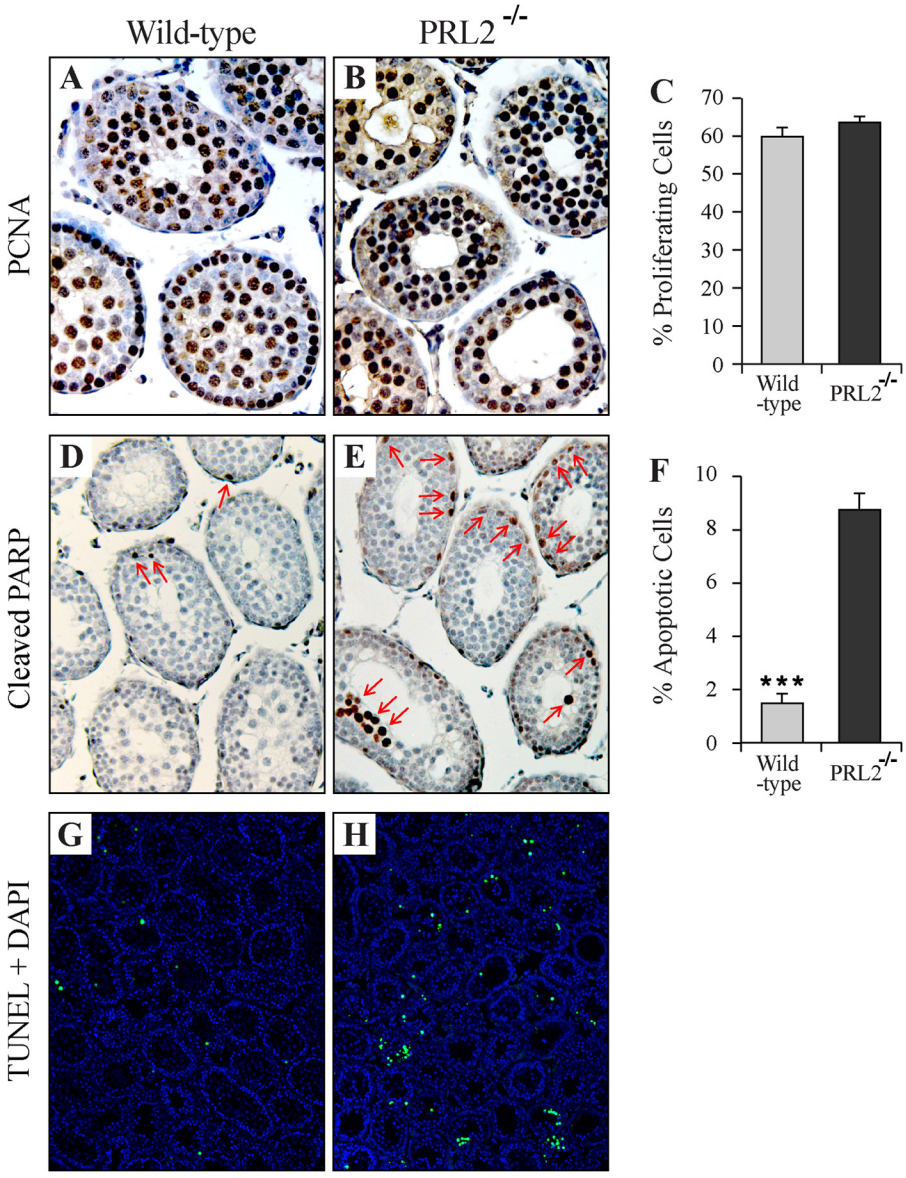

FIGURE 5. **PRL2 deficiency results in increased germ cell apoptosis at 2 weeks of age.** *A* and *B*, PCNA immunohistological staining showing proliferating cells in testis sections from 2 week old wild-type (*A*) and PRL2$^{-/-}$ (*B*) mice. *C*, percentage of PCNA-positive cells in testis sections from 2-week old wild-type and PRL2$^{-/-}$ mice. For each mouse, PCNA-positive and negative cells in 4 random fields were counted (~2,000 total), and the percentage of PCNA-positive cells were calculated. $n = 3$ for each genotype. Data represent mean ± S.E. *D* and *E*, cleaved PARP immunohistological staining showing apoptotic cells in testis sections from 2 week old wild-type (*D*) and PRL2$^{-/-}$ (*E*) mice. *Red arrows* mark the apoptotic germ cells. *F*, percentage of cleaved PARP positive cells in testis sections from 2 week old wild-type and PRL2$^{-/-}$ mice. For each mouse, cleaved PARP-positive and negative cells in 4 random fields were counted (~2,000 total), and percentage of cleaved PARP-positive cells were calculated. $n = 3$ for each genotype. Data represent mean ± S.E. *G* and *H*, TUNEL stain showing apoptotic cells in testis sections from 2 week old wild-type (*G*) and PRL2$^{-/-}$ (*H*) mice with DAPI counterstain of nucleus. *Green* fluorescence labels the apoptotic cells. ***, $p < 0.001$.

tozoa due to decreased spermatogenesis as well as an abundance of ectopically released germ cells (Fig. 6, *E* and *F*). Cleaved PARP staining showed that the majority of these ectopically released germ cells in the epididymal lumen were apoptotic (Fig. 6, *G* and *H*), which provided another indicator for the increased apoptosis in PRL2$^{-/-}$ testes.

PRL2 Deficiency Impairs Kit-mediated PI3K Signaling by Up-regulating PTEN—Testis development is primarily controlled by the GDNF-Ret and SCF-Kit signal pathways (42). GDNF-Ret pathway is responsible for maintaining the self-renewal of undifferentiated spermatogonia, while SCF-Kit pathway regulates the proliferation and survival of differentiating spermatogonia and spermatocytes. The data described above showed that in PRL2-deficient testes, the self-renewal of undifferentiated spermatogonia appeared to be normal, while the Kit positive cells underwent increased apoptosis, suggesting that SCF-Kit signaling might be compromised. To further substantiate this conclusion, we stimulated germ cells isolated from PRL2$^{-/-}$ and wild-type testes with SCF, and evaluated the activation status of down-stream effectors Akt and ERK1/2. As shown in Fig. 7, *A* and *B*, the basal level of Akt phosphorylation in PRL2$^{-/-}$ germ cells was only 47 ± 3% of that in the wild-type cells. As expected, SCF stimulation activated Akt in both genotypes. However, the levels of Akt phosphorylation in PRL2$^{-/-}$

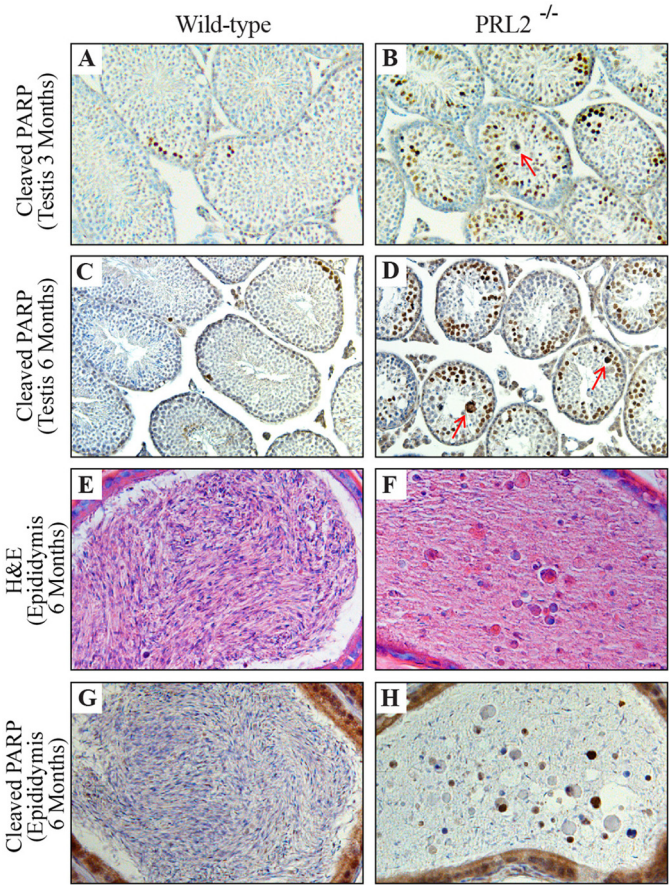

FIGURE 6. **PRL2 deficiency results in increased germ cell apoptosis at 3 and 6 months of age.** *A* and *B*, cleaved PARP immunohistological staining showing apoptotic cells in testis sections from 3 month old wild-type (*A*) and PRL2$^{-/-}$ (*B*) mice. *Red arrow* marks the apoptotic cell cluster. *C* and *D*, cleaved PARP immunohistological staining showing apoptotic cells in testis sections from 6 month old wild-type (*C*) and PRL2$^{-/-}$ (*D*) mice. *Red arrows* mark the apoptotic cell cluster. *E* and *F*, H&E staining of the epididymis sections from 6 month old wild-type (*E*) and PRL2$^{-/-}$ (*F*) mice. *G* and *H*, cleaved PARP immunohistological staining showing apoptotic cells in epididymis sections from 6 month old wild-type (*G*) and PRL2$^{-/-}$ (*H*) mice.

germ cells after 10 and 30 min of SCF stimulation were 33 ± 16% and 51 ± 6% of those in the wild-type (Fig. 7, *A* and *B*). The results indicate that PRL2 deficiency impaired both basal and Kit-mediated Akt activation. Interestingly, no significant change in ERK1/2 phosphorylation was observed upon SCF stimulation in either wild-type or PRL2$^{-/-}$ germ cells (Fig. 7, *A* and *B*).

To investigate the mechanism by which PRL2 attenuates Kit-mediated Akt activation, we first analyzed Kit expression in both wild-type and PRL2$^{-/-}$ germ cells. No change in Kit expression was observed as a result of PRL2 deletion (Fig. 7, *C* and *D*). We previously reported that in placenta, PRL2 deficiency led to elevated PTEN expression, an antagonist of PI3K signaling pathway, and therefore decreased Akt activation (31). To examine whether this is also the case in testis, we measured PTEN protein level in germ cells isolated from wild-type and PRL2$^{-/-}$ testes. As shown in Fig. 7, *C* and *D*, PTEN expression was 1.6 ± 0.2-fold higher in PRL2$^{-/-}$ samples, consistent with our observation in placenta. Therefore, impaired Akt activation in PRL2$^{-/-}$ germ cells was due to increased PTEN expression, which suppressed both the basal level and Kit-mediated Akt phosphorylation. Given the critical role of PI3K-Akt signaling in cell survival, it was not surprising that increased apoptosis, as evidenced by a 1.8 ± 0.1-fold increase in cleaved PARP protein level, was detected in PRL2 deficient germ cells (Fig. 7, *C* and *D*), which is in agreement with the immunohistochemistry data for cleaved PARP (Fig. 6, *B* and *D*) in PRL2$^{-/-}$ testes. Together, the data indicate that impaired spermatogenesis in PRL2$^{-/-}$ testis is primarily, if not solely, due to blockage of Kit-PI3K-Akt signaling, as a result of elevated PTEN expression.

If PRL2 deficiency elevates PTEN level, then excess amount of PRL2 should reduce PTEN expression. To test this hypothesis, we generated GC-1 cell lines stably overexpressing Flag-tagged PRL2. GC-1 cell was derived from mouse spermatogonia (43), which is physiologically relevant to the present study. As shown in Fig. 8, *A* and *B*, increased PRL2 expression (1–2-fold over endogenous PRL2) in GC-1 cell lines results in 28 ± 5% reduction in PTEN level compared with the GC-1 vector control cells. Accordingly, PI3K-Akt signaling was activated, as evidenced by the 3.0 ± 0.3-fold increase in Akt phosphorylation in PRL2 overexpressing GC-1 cells. Similar to results in germ cells, ERK1/2 phosphorylation was not affected when PRL2 expression was altered. To determine whether PRL2 controls PTEN at the level of protein stability as reported previously (31), GC-1 cells were treated with the protein synthesis inhibitor cycloheximide. As expected, PRL2 overexpression promotes PTEN degradation, and reduces PTEN's half-life from much greater than 30 h to 24 h in GC-1 cells (Fig. 8, *C* and *D*). Thus, PRL2 overexpression activates PI3K-Akt signaling by destabilizing PTEN, which is consistent with results obtained from germ cells lacking PRL2 showing elevated PTEN and decreased PI3K-Akt activity.

One mechanism by which PTEN protein could be stabilized is through formation of a complex with MAGI2 and β-catenin at the adherens junction, which prevents PTEN from degradation (44). The adherens junction component Vinculin plays an important role in maintaining the stability of the complex, because the complex is disrupted and PTEN level is reduced in cells lacking vinculin. We previously documented that Vinculin level is inversely correlated with PRL2 expression (31). In line with this observation, we found that Vinculin expression decreased 41 ± 13% in PRL2 overexpressing GC-1 cells (Fig. 8, *A* and *B*). Moreover, β-catenin expression was also reduced by 29 ± 13% upon increased PRL2 expression (Fig. 8, *A* and *B*), whereas a 30 ± 10% increase in β-catenin was observed in PRL2 null germ cells (Fig. 7, *C* and *D*). These observations support the notion that PRL2 down-regulates PTEN via inhibition of vinculin and β-catenin expression, which likely leads to disruption of the PTEN-MAGI2-β-catenin complex and exposes PTEN for degradation. Interestingly, the rate of β-catenin degradation was also increased in PRL2-overexpressing GC-1 cells (Fig. 8, *C* and *D*). More importantly, it appears that β-Catenin degradation ($t_{1/2}$ 16 h) precedes that of PTEN, indicating that the disassembly of the PTEN-MAGI2-β-catenin complex may be responsible for PTEN degradation. Taken together, these results are in agreement with studies in HEK293 cells overexpressing PRL2 (31) and suggest that PRL2 functions to destabilize the PTEN-MAGI2-β-catenin adherens junction complex to down-regulate PTEN leading to enhanced PI3k-Akt signaling.

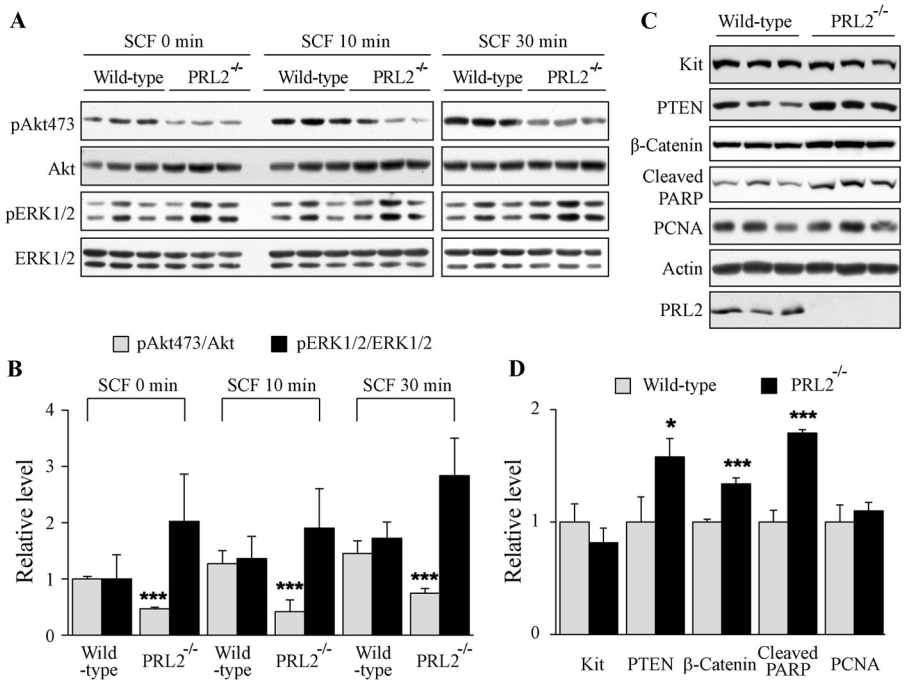

FIGURE 7. **PRL2 deficiency decreases Kit induced PI3K signaling by elevating PTEN level.** *A*, PRL2$^{-/-}$ germ cells showed reduced basal and SCF-induced Akt phosphorylation. Germ cells were isolated from 3 month old wild-type and PRL2$^{-/-}$ testes using stepwise enzyme digestion as specified under "Experimental Procedures." 1×10^6 cells were resuspended in 1 ml of serum-free DMEM media and stimulated with 10 ng/ml SCF for 10 min, 30 min, or left unstimulated. Cells were collected at the end of stimulation, lysed in SDS sample buffer and subjected to Western blot. *B*, quantification of blots from *A* was performed with ImageJ by measuring the intensity and calculating pAkt473/Akt and pERK1/2/ERK1/2 relative level. *C*, PRL2$^{-/-}$ germ cells expressed higher level of PTEN. 1×10^6 germ cells from wild-type or PRL2$^{-/-}$ testes were lysed in SDS sample buffer and subjected to Western blot for protein signals as indicated in the figure. *D*, quantification of blots from *C* was performed with ImageJ by measuring the intensity of Kit, β-catenin, PTEN, cleaved PARP, and PCNA, and normalized by actin.

DISCUSSION

This study reveals an important role for PRL2 in spermatogenesis. PRL2$^{-/-}$ male mice display testis hypotrophy, impaired reproductivity, and decreased sperm production as a result of compromised spermatogenesis. Ablation of PRL2 expression causes germ cell apoptosis, which primarily affects the Kit-positive cell population. Mechanistically, PRL2 deletion results in increased expression of PTEN, which antagonizes PI3K by degrading phosphatidylinositol (3, 4, 5) triphosphate (PIP_3). In line with this observation, we show that both the basal and SCF/Kit induced Akt phosphorylation are decreased in PRL2$^{-/-}$ germ cells compared with those in wild-type. The Kit-mediated PI3K-Akt pathway is known to play an essential role for the survival of Kit-positive cells (45). PRL2 deficiency, therefore, impaired the survival of Kit-positive cells by attenuating Akt activation. Together, the results suggest that PRL2 promotes Kit-mediated PI3K-Akt activation by down-regulating PTEN, and establish PRL2's critical and non-redundant role in promoting germ cell survival and maintaining normal level of spermatogenesis.

In support of a role for PRL2 in promoting Kit-mediated spermatogenesis, the testicular phenotypes seen in *prl2*-null mice are reminiscent to those of animal models with Kit pathway component mutation or functional impairment. Germline homozygous-null mutations of either SCF or Kit cause infertility in male mice owing to failure of germ cell development (46, 47). Spermatogenesis is reduced in Kit$^{W\text{-}lacZ/+}$ mice: increased apoptosis, a smaller seminiferous tubule diameter and a lower ratio of differentiating spermatogonia to undifferentiated spermatogonia were observed in testes of the heterozygous mice due to Kit haplodeficiency (48). Application of a Kit-blocking antibody was shown to deplete the differentiating spermatogonia from the testis (49). The importance of Kit-mediated PI3K activation in the spermatogenic process is also well established. Mice bearing a point mutation in Kit to specifically disrupt p85 binding exhibit male sterility (50, 51). The mutant males failed to produce sperm due to decreased proliferation and increased apoptosis of spermatogonia. Knock-in male mice with inactive p110β, an isoform of PI3K, are sub-fertile due to severe loss of Kit-positive cells (52). In addition, since Kit-mediated cell survival is carried out by Akt (53), it is understandable that Akt1 knock-out male mice are also sub-fertile with decreased seminiferous tubule diameter, attenuated spermatogenesis and increased germ cell apoptosis (54, 55). Akt1 has also been reported to protect germ cells from radiation-induced apoptosis in testis (56), demonstrating an important survival function of Akt1 in spermatogenesis. Taken together the data support an essential role for the Kit-PI3K-Akt pathway in regulating the growth and survival of spermatogonia. Our results reveal that PRL2 promotes Akt activation in the testis by down-regulating PTEN. Thus it is not surprising that PRL2 deficiency attenuates the Kit-PI3K-Akt pathway leading to germ cell apoptosis and testis hypotrophy.

Consistent with our finding that increased PTEN expression in PRL2-deficient mice leads to apoptosis of spermatogonia, loss of heterozygosity in PTEN$^{+/-}$ mice causes testicular germ cell cancer (57). Thus, the biochemical mechanism for PRL2 function in testis is similar to that in the placenta, where PRL2

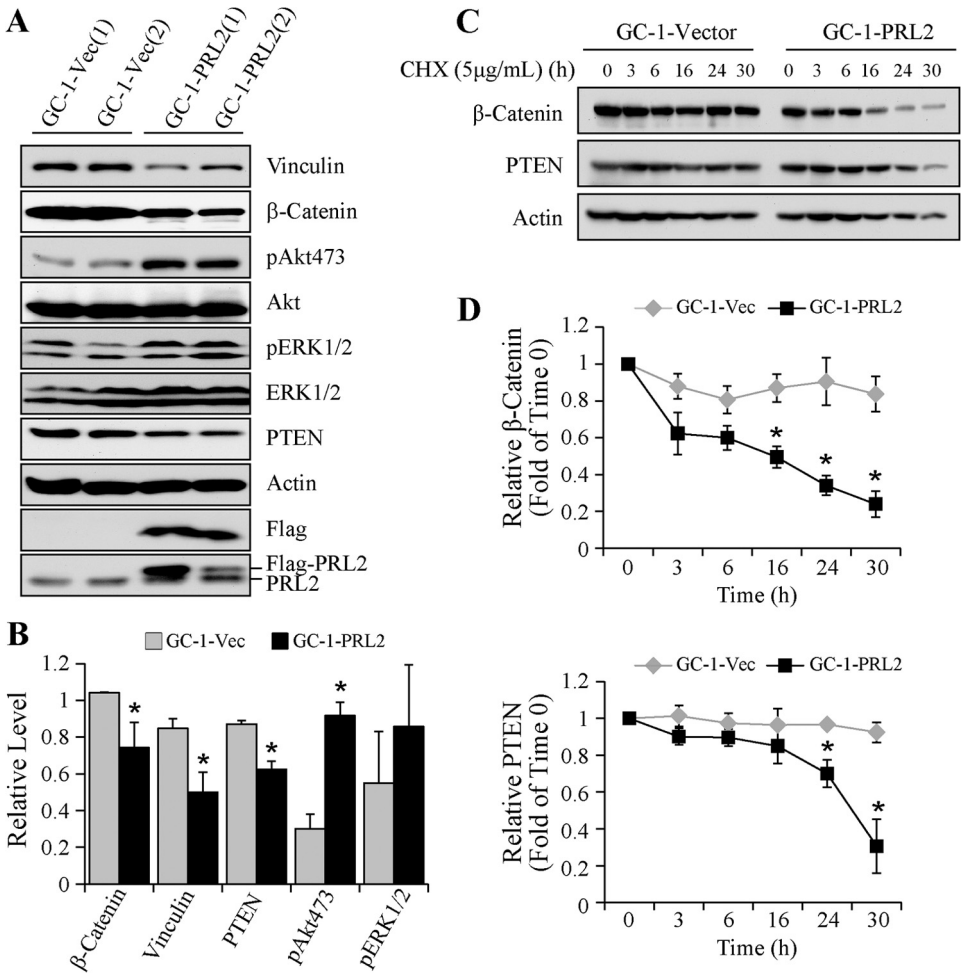

FIGURE 8. **Overexpression of PRL2 in GC-1 cells increases PTEN degradation by down-regulating adherens junction proteins vinculin and β-catenin.** *A*, PRL2-overexpressing GC-1 stable cell lines have decreased PTEN expression and increased PI3K/Akt signaling. Stable cell lines were generated as described under "Experimental Procedures." Control cells and PRL2-overexpressing cells were collected and analyzed by Western blot. *B*, quantification of blots from *A* was performed with ImageJ by measuring the band intensity and calculating vinculin/actin, β-catenin/actin, PTEN/actin, pAkt473/Akt, and pERK1/2/ERK1/2 relative level. *C*, GC-1-PRL2 stable cell line and its vector control cell line were treated with cycloheximide (CHX) at 5 μg/ml for indicated times, and cell lysates were analyzed for β-catenin, PTEN and actin proteins by Western blot. Data represent three independent experiments. *D*, quantification of blots from *C* was performed with ImageJ by measuring the intensity and calculating β-catenin/actin and PTEN/actin relative level at different time point, and plotted as *Fold of time 0* for each cell line.

down-regulates PTEN and promotes PI3K/Akt signaling for proper placenta development (31). The fact that the same mechanism was identified in two very different organs suggests a general role for PRL2 to suppress PTEN protein levels. PTEN is one of the most frequently mutated tumor suppressors, and notably, PTEN expression is also commonly down-regulated in cancer (58). Loss of function mutations in PTEN or even a modest reduction in PTEN (as low as 20%) amplify PI3K signaling and promote tumorigenesis in a variety of experimental models of cancer (59). Our finding that PRL2 can down-regulate PTEN offers a plausible explanation for the oncogenic potential of PRL2 to promote cell proliferation, migration, and survival. Furthermore, it identifies PRL2 as a potential drug target to manipulate PTEN expression. Adenoviral-mediated PTEN expression significantly inhibits tumor growth in multiple animal models (60–63), indicating that restoration of PTEN level may be an effective strategy for tumor therapy. While effective gene delivery system is not clinically available, inhibition of PRL2 with small molecule inhibitors may increase PTEN expression. Unlike inhibitors of the PI3K-Akt pathway, PRL2 inhibitors may restore not only the canonical PTEN function but also tumor-suppressive nuclear activities of PTEN that are unrelated to its lipid phosphatase activity (64). Furthermore, PRL2 inhibitors may also serve as new therapeutic approaches to male contraception as well as a number of malignancies associated with gain-of-function mutations in the Kit gene. Finally, given the role of Kit in stem cell maintenance and the ability of PRL2 to promote Kit-PI3K signaling by reducing the level of PTEN that normally antagonizes the pathway, we predict that PRL2 may also regulate the self-renewal and/or survival of other stem cells in addition to spermatogonia.

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