Retinoblastoma Protein Plays Multiple Essential Roles in the Terminal Differentiation of Sertoli Cells

Roopa L. Nalam, Claudia Andreu-Vieyra, Robert E. Braun, Haruhiko Akiyama, and Martin M. Matzuk

Departments of Pathology (R.L.N., C.A.-V., M.M.M.), Molecular and Cellular Biology (R.L.N., M.M.M.), and Molecular and Human Genetics (M.M.M.), Baylor College of Medicine, Houston, Texas 77030; The Jackson Laboratory (R.E.B.), Bar Harbor, Maine 04609; and Department of Orthopaedics (H.A.), Kyoto University, Kyoto 606-8507, Japan

Retinoblastoma protein (RB) plays crucial roles in cell cycle control and cellular differentiation. Specifically, RB impairs the G1 to S phase transition by acting as a repressor of the E2F family of transcriptional activators while also contributing towards terminal differentiation by modulating the activity of tissue-specific transcription factors. To examine the role of RB in Sertoli cells, the androgen-depandent somatic support cell of the testis, we created a Sertoli cell-specific conditional knockout of \( Rb \). Initially, loss of RB has no gross effect on Sertoli cell function because the mice are fertile with normal testis weights at 6 wk of age. However, by 10–14 wk of age, mutant mice demonstrate severe Sertoli cell dysfunction and infertility. We show that mutant mature Sertoli cells continue cycling with defective regulation of multiple E2F1- and androgen-regulated genes and concurrent activation of apoptotic and p53-regulated genes. The most striking defects in mature Sertoli cell function are increased permeability of the blood-testis barrier, impaired tissue remodeling, and defective germ cell-Sertoli cell interactions. Our results demonstrate that RB is essential for proper terminal differentiation of Sertoli cells. (Molecular Endocrinology 23: 1900–1913, 2009)

The Sertoli cell is the somatic support cell of mammalian spermatogenesis. This mesenchymal epithelial cell operates uniquely and diversely to support the germ cells of the seminiferous epithelium. In the mouse, at embryonic d 10.5–12, Sertoli cells differentiate from their female counterpart, the granulosa cells, and begin expressing SOX9 and anti-Müllerian hormone (AMH) (1). Immature Sertoli cells continue to be proliferative until postnatal d 12–17, at which point they permanently exit the cell cycle (2). During this time, AMH is down-regulated just as p27 (CDKN1B), a cyclin-dependent kinase inhibitor (CDKI) and a marker of Sertoli cell maturation and mitotic quiescence, is up-regulated (1, 3). Sertoli cells also begin to produce secreted proteins important for germ cell development and establish the tight junctions of the blood-testis barrier (1). Thus, in Sertoli cells as in many other cell types, terminal differentiation is closely related to cell cycle exit (4).

The retinoblastoma protein (RB) pathway represents one of the most well-studied mechanisms of cell cycle regulation. In a simplified version of this model, G1 to S phase progression is mediated through the hyperphosphorylation of RB by cyclin/CDK complexes, preventing the binding of RB to E2F transcriptional activators (i.e. E2F1, E2F2, and E2F3a) (5). Loss of RB binding derepresses E2F proteins and allows the transcription of genes important for cell cycle progression (5). When cyclin/CDK complex activity is blocked by inhibitors like p27, RB remains hypophosphorylated and able to bind to E2Fs (5). Whereas hypophosphorylated RB is clearly a marker of cell cycle exit, its role in replicative senescence appears to be highly tissue specific (6).

The RB pathway has also been implicated in the terminal differentiation of a variety of cell types, which has led to the hypothesis that RB is a master regulator coordinating cell cycle exit with differentiation (4). Condi...

Abbreviations: AMH, anti-Müllerian hormone; AR, androgen receptor; BrdU, 5-bromo-2-deoxyuridine; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; GFP, green fluorescent protein; ID, inhibitor of DNA binding; RB, retinoblastoma; SV40, simian virus 40; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
tional knockout studies of \( Rb \) in skeletal myoblasts (7), osteoblasts (8), and preadipocytes (9) reveal that RB is essential for the proper differentiation of these progenitor cell types. It has been proposed that RB binding to tissue-specific and tissue-nonspecific transcription factors is necessary for proper differentiation of these and other cell types (6, 10). Studies in retinal interneurons also suggest that the inhibition of E2Fs, specifically E2F3a, by RB may be critical for their terminal differentiation and that this function is not related to cell cycle exit (11).

Due to the well-defined link between Sertoli cell maturity and mitotic quiescence, Sertoli cells represent a unique and interesting cell type to study the involvement of RB in the coordination of cell cycle exit and terminal differentiation. In mice, knockout of p27 with or without concomitant knockout of p21 (Cdkn1a), another CDKI, results in an overall increase in Sertoli cell number; however, the proliferative lifespan of Sertoli cells remains unchanged (12), suggesting that loss of CDKIs increased the rate of proliferation but does not abrogate replicative senescence. Other rodent models implicate the RB-E2F pathway in Sertoli cell cycle exit even more strongly. For example, transgenic mice expressing simian virus 40 (SV40) large T-antigen in Sertoli cells develop testicular tumors (13), and cellular division resumes in postmitotic rat Sertoli cell cultures when inhibitor of DNA binding 1 (ID1) or ID2 is overexpressed (14). Both SV40 T-antigen and the ID proteins are known to bind and inactivate RB. When replicative senescence is already overcome, as in the case of inhibin \( a \) (Inha) knockout mice that universally undergo Sertoli cell oncogenesis (15), perturbations of upstream regulators of RB can worsen (16) or lessen (17) tumor progression. Also, RB is a known transcriptional coregulator of androgen receptor (AR) (18, 19), a steroid hormone receptor that is indispensable for proper mature Sertoli cell function (20–22). Stage-specific staining of RB in Sertoli cells is closely related to that of AR as both are maximally expressed in stage VII–VIII (23), and changes are seen in this staining when androgens are suppressed (24). All of these observations, not to mention the importance of RB in the differentiation of granulosa cells (25), suggest that RB may be important for the coordination of cell cycle exit and terminal differentiation of Sertoli cells.

In this report, we explore the functions of RB in Sertoli cells by creating and studying a Sertoli cell-specific RB conditional knockout mouse model. Our findings show that mutant Sertoli cells are capable of supporting the first wave of spermatogenesis, but the function of these Sertoli cells rapidly declines, leading to infertility. Our results suggest that RB is dispensable for initial maturation of Sertoli cells but is essential for their sustained terminal differentiation, which includes maintenance of maturing germ cells and cell cycle quiescence.

**Results**

**Generation of Sertoli cell-specific Rb mutant mice**

To investigate the roles of RB in Sertoli cells specifically, we employed the Cre-loxP system. We used this conditional knockout system for two reasons. First, the homozygous null mutation of \( Rb \) uniformly causes lethality around embryonic d 14.5 (26–28). Second, although replacing RB in the placenta can rescue these pups allowing them to be viable until the perinatal period (29), RB is also expressed in spermatogonia (23), and we did not want to experience any secondary effects. To create a Sertoli cell-specific conditional knockout, we employed a transgenic mouse expressing Cre recombinase driven by the AMH promoter (\( Amb\)-Cre\( ^{\text{+}} \)), which has been shown to have high activity as early as embryonic d 14.5 (20). To obtain the most complete deletion possible, we intercrossed \( Rb^{\text{floxed/floxed}} \) mice (30, 31) with \( Rb^{\text{wt/wt}}-\ Amb\)-Cre\( ^{+} \) mice (26) so that Cre-mediated recombination would occur on a heterozygous background. Throughout this study, we refer to two different groups of mice generated by this cross: control (\( Rb^{\text{floxed/floxed}} \)) and Rb cKO (\( Rb^{\text{floxed<floxed}}-\ Amb\)-cre\( ^{+} \)).

The deletion of \( Rb \) occurred specifically in Sertoli cells and was very efficient. First, we showed that recombination occurred in whole-testis DNA (Fig. 1A) by performing PCR. If excision of exon 19 had occurred, a 260-bp product would be amplified (30, 31). DNA from Rb cKO testes at 4, 6, and 8 wk of age all contained this 260-bp product, whereas the age-matched control testis DNA was negative. Quantitative RT-PCR against the exon 19 to 20 junction of \( Rb \) showed a 63% reduction in RNA levels of Sertoli cells isolated from Rb cKO testes \textit{vs.} the control Sertoli cells (Fig. 1B). In addition, immunohistochemical staining of 6-wk-old testes showed that loss of RB staining in Rb cKO was specific to Sertoli cells and did not affect its expression in spermatogonia (Fig. 1, panel D \textit{vs.} panel C).

**Infertility in Rb cKO males is progressive**

Because mice reach sexual maturity by postnatal d 42, we set up 6-wk-old control and Rb cKO males with age-matched wild-type females to observe their fertility (Fig. 2). During the first month of breeding, there were no significant differences between control and Rb cKO mice in either the number of mice that produced litters (Fig. 2A, control \( = 1.0 \pm 0 \) (mean litters per mouse \( \pm \) se); Rb
The fertility of control mice (n = 6) was compared with Rb cKO mice (n = 9) over 6 months. Comparisons of litter size per month per mouse (A) and pups per litter per mouse (B) show the progressive nature of the infertility. **, P < 0.01.

FIG. 2. Sertoli cell-specific Rb ablation causes progressive infertility. The fertility of control mice (n = 6) was compared with Rb cKO mice (n = 9) over 6 months. Comparisons of litter size per month per mouse (A) and pups per litter per mouse (B) show the progressive nature of the infertility. **, P < 0.01.
maturity. At 6 wk of age, AR staining in Rb cKO mice (Fig. 4D) was comparable to the control (Fig. 4C) correlating to the results of Western blot analysis (Fig. 4B). By 8 wk of age, Sertoli cell sloughing could be observed as evidenced by AR-positive cells in the lumen of the seminiferous tubules (Fig. 4E, arrows). By 10 wk of age, although we identified a number of Sertoli cells that remained along the basement membrane, these Sertoli cells displayed profound variability in the intensity of AR staining (Fig. 4F, arrowheads). All of these results indicated that Sertoli cells in the Rb cKO mice had defects in sustained differentiation after the first wave of spermatogenesis.

Gene expression changes of Rb cKO Sertoli cells indicate improper differentiation

To better assess the mechanism leading to the Rb cKO phenotype, we obtained purified Sertoli cell samples. To do this, we crossed our mice with Sox9-GFP knockin mice, which, in the testis, express green fluorescent protein (GFP) exclusively in Sertoli cells (34). This allowed us to utilize fluorescence-activated cell sorting to enrich for Sertoli cells without culturing (supplemental Fig. S1, A–D, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). Also, we decided to use Sertoli cells that had been isolated from 6-wk-old mice, because this was the age at which control and mutant mice were histologically similar and Rb cKO Sertoli cells showed no signs of failure. RNA isolated from Sertoli cells of control (n = 3) and Rb cKO (n = 3) mice were used for microarray analysis (GEO accession no. GSE 17904).

By our analysis (see Materials and Methods), we discovered that deletion of Rb resulted in the down-regulation of 137 genes and the up-regulation of 372 genes (supplemental Fig. S1E and supplemental Table S1). Using the online bioinformatics tool DAVID (35, 36), we were able to categorize our results into a number of functional pathways (supplemental Table S2). We observed an up-regulation of many genes involved in cell cycle control, DNA synthesis, and apoptosis and the misregulation of genes that may be important for proper Sertoli cell function such as cell adhesion, tight junction formation, and tissue remodeling (i.e. proteases and their inhibitors).

Because Sertoli cell maturity is linked to mitotic quiescence, these results suggest an essential role for RB in the regulation of genes important for the maintenance of the terminally differentiated state of adult Sertoli cells. These points will be described in greater detail in the following sections.

Irregular cell cycle progression seen in Rb cKO Sertoli cells may be mediated by E2Fs

As mentioned above, our microarray analysis revealed the up-regulation of a number of genes involved in cell cycle control and DNA synthesis in Rb cKO Sertoli cells. We hypothesized that these gene changes may be related to E2F activity because RB is a known repressor of E2F
transcription factors. To investigate this possibility, we compared our gene changes with those seen in a study investigating the effect of overexpression of E2F1 or E2F3 in mouse embryonic fibroblasts (37); we will refer to this group of genes herein as “E2F-related.” Consistent with our hypothesis, we discovered that many of the genes that were up-regulated by E2F1 or E2F3 in those studies were also up-regulated in Rb cKO Sertoli cells (Fig. 5A and supplemental Table S2). In addition, there is a significant overlap between the genes that are E2F related and those involved in cell cycle control (Fig. 5A, 14 of 42; P < 0.01) and DNA synthesis (Fig. 5A, 16 of 26; P < 0.01). Although E2F3 and E2F4 are the only E2Fs that are normally expressed in adult Sertoli cells (38), we saw a significant up-regulation of E2f1 in Rb cKO Sertoli cells (Fig. 5B) that may have been the result of derepressed E2F3 transcriptional activity (39).

We used quantitative RT-PCR to determine the relative levels of Mcm2, Rrm2, and Ncaph in Rb cKO Sertoli cells (n = 5) vs. control (n = 6) because these were all putative E2F-target genes that were also involved in DNA synthesis/cell cycle control. We found that these genes were up-regulated in Rb cKO Sertoli cells by both microarray as well as quantitative RT-PCR (Fig. 5B). Minichromosome maintenance deficient 2 (Mcm2) is an indispensable component of the MCM2–7 complex that is loaded onto chromatin during G1 in preparation for S phase (40). Inappropriate expression of MCM2 protein has also been implicated as an early premalignant change (40). Transcription of ribonucleotide reductase M2 (Rrm2) is maximal during S phase (41), and small interfering RNA inhibition of this gene led to decreased proliferation of a variety of cell lines in vitro as well as in vivo (42). Transcription of non-SMC condensin I complex, subunit H (Ncaph) is thought to occur exclusively in proliferating cells with highest expression in the G2 phase of the cell cycle (43). Normally, adult Sertoli cells are postmitotic; therefore, the up-regulation of genes expressed in proliferating cells suggested that Rb cKO Sertoli cells might have been aberrantly cycling. To examine this, we injected 6-wk-old mice with 5-bromo-2-deoxyuridine (BrdU) and stained their testes for BrdU, the cellular incorporation of which is indicative of DNA

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Genotype</th>
<th>FSH (ng/ml)</th>
<th>LH (ng/ml)*</th>
<th>T (ng/dl)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Rb&lt;sup&gt;flox&lt;/sup&gt;−/−</td>
<td>34.0 ± 3.5&lt;sup&gt;a&lt;/sup&gt; (8)</td>
<td>0.26 ± 0.08 (7)</td>
<td>65.0 ± 13.6 (8)</td>
</tr>
<tr>
<td>6</td>
<td>Rb&lt;sup&gt;flox&lt;/sup&gt;−/−&lt;sup&gt;−&lt;/sup&gt;, Amh-cre&lt;sup&gt;+&lt;/sup&gt;</td>
<td>39.9 ± 4.2&lt;sup&gt;A&lt;/sup&gt; (12)</td>
<td>0.46 ± 0.11 (11)</td>
<td>93.3 ± 24.8 (11)</td>
</tr>
<tr>
<td>8</td>
<td>Rb&lt;sup&gt;flox&lt;/sup&gt;−/−</td>
<td>31.1 ± 2.5&lt;sup&gt;b&lt;/sup&gt; (10)</td>
<td>0.55 ± 0.20 (10)</td>
<td>88.1 ± 16.6 (10)</td>
</tr>
<tr>
<td>8</td>
<td>Rb&lt;sup&gt;flox&lt;/sup&gt;−/−&lt;sup&gt;−&lt;/sup&gt;, Amh-cre&lt;sup&gt;+&lt;/sup&gt;</td>
<td>51.8 ± 5.9&lt;sup&gt;a&lt;/sup&gt; (10)</td>
<td>0.99 ± 0.27 (9)</td>
<td>226.1 ± 88.4 (9)</td>
</tr>
</tbody>
</table>

Values are means ± se. Values in parentheses are the number of samples used per group.

<sup>a,b</sup> Differing superscript letters represent statistically different groups as determined Tukey-Kramer honestly significant difference test (P < 0.05).

Values in parentheses are not significant (P > 0.05 by one-way ANOVA).

* Values in parentheses are not significant (P > 0.05 by one-way ANOVA).
We never observed BrdU-positive Sertoli cells in control mice, but there were occasional Sertoli cells that labeled with BrdU in Rb cKO mice (Fig. 5, C–F). Performing similar studies in 4-wk-old and 8-wk-old mice revealed that Rb cKO Sertoli cells at both ages were also occasionally BrdU-positive (supplemental Fig. S2), suggesting that Rb cKO Sertoli cells do not completely stop proliferating at any time point in the period that is normally postmitotic.

### Increased death of Rb cKO Sertoli cells may be due to the p53 apoptotic pathway

Although we saw a profound up-regulation of cell cycle-related genes in Rb cKO Sertoli cells, the number of cycling Sertoli cells was quite low, and these mice never developed testicular cancer. Due to these findings and our observations of Sertoli cell sloughing (Fig. 4E), we hypothesized that Sertoli cell cycling triggered apoptotic signals that subsequently eliminated these abnormal cells from the testis. Rb cKO microarray pathway analysis showed the up-regulation of 40 genes related to apoptosis (Fig. 6A and supplemental Table S2), thus supporting our hypothesis. Interestingly, pathway analysis also revealed the up-regulation of genes related to the p53 pathway, and the correlation between apoptotic genes and p53-related genes was significant (Fig. 6A, 5 of 40; $P < 0.01$). When we verified select genes by quantitative RT-PCR, we noticed that although the mRNA level of *Trp53*, the gene encoding p53, was unchanged, the genes encoding its upstream activator, *Cdkn2a* (ARF), and its downstream targets, *Fas* (44), *Apaf1* (45), and *Bbc3* (46), were up-regulated, suggesting that p53 may have been activated posttranslationally in Rb cKO Sertoli cells (Fig. 6B). Despite the fact that a number of these p53-related genes were not contained in our “E2F-related” gene list, p53 activation may have still been E2F1 dependant because *Cdkn2a* is a direct transcriptional target of E2F1 (47).

The genes that we verified by quantitative RT-PCR (Fig. 6B) represented two main apoptosis-initiating pathways, the death receptor pathway and the mitochondrial apoptotic factor release pathway. In the death receptor pathway, the binding of Fas ligand by Fas death receptor (FAS) induces receptor trimmerization and recruitment and cleavage/activation of procaspase-8 (CASP8)(48). In the mitochondrial apoptogenic factor release pathway, BCL2 binding component 3 (BBC3/PUMA) indirectly promotes permeabilization of the mitochondrial membrane leading to the release of cytochrome c and apoptotic peptidase-activating factor 1 (APAF1), which form a holoenzyme complex with procaspase-9 (48, 49). These two pathways converge because procaspase-3 is a substrate of both active caspase-8 and caspase-9 (48), and the cleavage of caspase-3 is integral to its activation, which induces DNA fragmentation, a hallmark of apoptosis (48). To determine the functional significance of the up-regulation of these pathways, we examined apoptotic DNA fragmentation *in situ* by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis. Our results showed a significant increase in the apoptotic index of 6-wk-old Rb cKO testes as compared with controls, but the TUNEL-positive cells appeared to be a mixture of both Sertoli and germ cells (Fig. 6, C–E). To determine whether the apoptotic pathways that we saw up-regulated in our microarray were activated in Sertoli cells specifically, we performed cleaved caspase-3/GFP costaining and found that there
were indeed multiple Rb cKO Sertoli cells that stained positively for this key effector of apoptosis (Fig. 6, F–I). This once again suggested that tumor surveillance of the p53 pathway was responsible for preventing a cancer phenotype in Rb cKO testes.

**Relationship of Sertoli cell maturational defect to AR transcriptional activity**

Although increased apoptosis could partially explain the Rb cKO phenotype, it seemed an insufficient explanation for the widespread loss of maturing germ cells because many tubules still contained Sertoli cells (Fig. 3, M and N, and Fig. 4, E and F). Because RB is a known coregulator of AR transcriptional activity (18, 19), we hypothesized that defects in Sertoli cell function were due, in part, to misregulation of androgen-responsive genes by RB. Because we had ensured that AR mRNA (Fig. 7B) and protein (Fig. 4B) levels were comparable between 6-wk-old control and Rb cKO mice, we were able to test our hypothesis by comparing our microarray data to Sertoli cell gene lists from the literature that contained putative androgen-regulated genes (50–53), which we will refer to herein as “AR-related.” Through this comparison, we were able to identify 26 AR-related genes in our study, six of which were down-regulated and 20 of which were up-regulated (Fig. 7A and supplemental Table S2). Of those genes, five were related to cell adhesion molecules and tight junctions, and four were related to proteases and their inhibitors (supplemental Table S2). The overlap between these pathways and AR-related genes was significant (Fig. 7A, nine of 74; P/H11021 0.01), and the alteration of these pathways was also noteworthy because it showed that the histological changes seen in Rb cKO testes may be due to defects in tissue remodeling important for the maintenance of the dynamic interface of cell-cell junctions between Sertoli cells and other cells of the seminiferous tubule (51, 54).

One such cell-cell junction is the blood-testis barrier, a specialized occluding junction formed between neighboring Sertoli cells that must cyclically undergo remodeling to allow the passage of maturing germ cells from the basal to the adluminal compartment (54). Two tight junction components of the blood-testis barrier, claudin 3 (Cldn3) and occludin (Ocln), were down-regulated in Rb cKO Sertoli cells (Fig. 7B). Claudin 3 had previously been reported to be transiently associated with newly formed tight junctions at the time that germ cells move from the basal to the adluminal compartment, and its down-regulation was thought to be the main cause of increased permeability of the seminiferous tubules seen in Sertoli cell-specific AR knockout mice (55). To test whether this conclusion was

**FIG. 6.** RB deletion in Sertoli cells causes activation of apoptotic pathways. A, Venn diagram of microarray changes in E2F-related, p53-related, and apoptotic genes shows the overlap between these pathways. B, Quantitative RT-PCR of select (bold) genes from panel A, and Trp53 confirms the up-regulation of these genes in Rb cKO Sertoli cells (*, P < 0.05; **, P < 0.01; control, n = 6; Rb cKO, n = 5). RQ, Relative quantification. C–E, TUNEL (green) staining in 6-wk-old testes is increased in Rb cKO (D) as compared with control (C) as determined by apoptotic index (E) (**, P < 0.01; control, n = 3; Rb cKO, n = 4). F–I, Immunofluorescent analysis of cleaved caspase-3 (red) and Sox9-GFP (green) reveals the activation of caspase-mediated apoptosis in 6-wk-old Rb cKO Sertoli cells. Arrows (I) indicate Sertoli cells that did not stain for cleaved caspase-3. DAPI, 4',6-diamidino-2-phenylindole.
also true in our mouse model, we investigated the permeability of the Rb cKO blood-testis boundary by injecting a biotin tracer into the testicular interstitium. In 4-wk-old mice, biotin remained in the basal compartment of the seminiferous tubule in both the control and Rb cKO mice (supplemental Fig. S3, red), suggesting that the blood-testis barrier was normally established in both genotypes. In 6-wk-old mice, biotin remained in the basal compartment of the seminiferous tubule, close to the basement membrane in the control mice (Fig. 7C, red), but biotin staining pervaded the tubule in the Rb cKO mice (Fig. 7E, red). Loss of integrity of the Rb cKO blood-testis barrier suggested that RB was initially dispensable for the formation of the blood-testis barrier but might be indispensable for its remodeling as maturing germ cells crossed from the basal into the adluminal compartment and that this function might be directly related to the down-regulation of tight junction genes.

Defects in Sertoli cell junction remodeling are both AR related and unrelated

Dynamic assembly of tight and adherens junctions of the seminiferous tubules are thought to be regulated by the balance between proteases and protease inhibitors (54), and protease inhibitors such as tissue inhibitor of metalloproteinase 1 (Timp1) and serine (or cysteine) peptidase inhibitor, clade A, member 3N (Serpina3n) were highly up-regulated in Rb cKO Sertoli cells (Fig. 7B). Although Timp1 is induced by a myriad of factors in the testis (56), one in vitro model suggests that when the collagen network of the basement membrane is perturbed by matrix metalloproteinases, Timp1 expression is induced by negative feedback to limit protease activity (57). Our microarray analysis showed that a variety of extracellular matrix components, such as isoforms of type IV collagen, were up-regulated in Rb cKO Sertoli cells (supplemental Table S2), which also supported the theory that the collagen network was perturbed. Because alterations in the basement membrane are thought to affect blood-testis barrier stability (57), we wanted to visualize the blood-testis barrier of Rb cKO mice. Ectoplasmic specializations are modified adherens junctions that are present between Sertoli cells (basal) and between germ cells and Sertoli cells (apical) (54). Basal ectoplasmic specializations colocalize with the blood-testis barrier (54); therefore, we visualized these structures by staining for espin, a marker of ectoplasmic specializations (58). The blood-testis barrier appeared very disorganized in 6-wk-old Rb cKO testes (Fig. 7F, green, white arrows), and any contact between the barrier and the basement membrane appeared to be lost (Fig. 7F, inset). Because direct contact between tight junctions and basement membrane components is related to junction stability (57), the increased permeability of the blood-testis barrier may be
secondary to disruption of the collagen network in addition to altered tight junction expression levels.

In contrast to matrix metalloproteinase inhibitors, serine protease inhibitors are thought to affect adherens junction dynamics between germ cells and Sertoli cells (54). The AR-related serine protease inhibitor SerpinA3n was up-regulated in Rb cKO Sertoli cells (Fig. 7B); therefore, to determine the significance of this gene change, we investigated the localization of apical ectoplasmic specializations. Apical ectoplasmic specializations are thought to be important for spermiogenesis, the process of forming the sperm head, and for the prevention of premature release of elongated spermatids from seminiferous tubules (54). Proper function and localization of apical ectoplasmic specializations may be dependent on constant remodeling because the shape and location of the head of elongating spermatids are constantly changing (54). Our studies showed that, similar to the basal ectoplasmic specializations, apical ectoplasmic specializations were also highly disorganized in Rb cKO mice (Fig. 7F, green, red arrows). These observations supported the notion that remodeling of germ cell-Sertoli cell junctions was also impaired and contributed to the most severe defect of Sertoli cell function in Rb cKO mice, the loss of maturing germ cells.

**Discussion**

Much work has been dedicated to the link between cessation of replication and terminal differentiation. This developmental work is invaluable to determining how cells can overcome replicative senescence and cause cancer. In our study of a Sertoli cell-specific knockout of Rb, we found that although RB was dispensable for initial maturation of Sertoli cells, it was essential for the full maturity of Sertoli cells. Initially, maturation of Rb cKO Sertoli cells was normal as evidenced by testis weights, germ cell maturation, and fertility that were comparable to control. After 6 wk of age, however, decreasing testis weights, loss of maturing germ cells, and infertility indicated a progressive decline in Sertoli cell function. When Sertoli cells were isolated and profiled for changes in gene expression, we observed an up-regulation of genes important for cell cycle progression and a misregulation of genes important for tubular remodeling of cell-cell junctions. These dysfunctional Sertoli cells were progressively eliminated from Rb cKO testes, and our data suggest that this process was mediated by p53-dependant apoptosis.

In our model of cell cycle control, RB is indispensable to maintenance of Sertoli cell terminal differentiation through a variety of interactions with transcription factors (Fig. 8). RB’s inhibition of E2F3a may be essential to preventing the up-regulation of E2F1, the expression of which, in turn, could be causing persistent cell cycling. This hypothesis is supported by observations in a variety of other tissues in which concomitant knockout of E2F1 and Rb has rescued abnormal cell cycle reentry (8, 11, 59). Recent findings revealed that Sertoli cells exit terminal mitosis and reenter the cell cycle when isolated and cultured in vitro (60), and other studies of Sertoli cell-specific knockouts of connexin 43 (Gja1), a gap junction protein important for cell-cell communication, also showed persistent Sertoli cell proliferation beyond the normal proliferative period (61). These results, taken together, indicate that the maintenance of adult Sertoli cells in a non-proliferative state in vivo may depend upon: 1) a fine balance between cell cycle inducers and inhibitors and/or 2) undisturbed Sertoli cell communication with other cells of the seminiferous tubule. Because abnormal Sertoli cell cycling is observed in both our knockout as well as the connexin 43 knockout model and because both knockouts also have impaired support of germ cell maturation, it will be important to ascertain whether deletion of E2F1 from Rb cKO Sertoli cells could prevent persistent Sertoli cell cycling and/or germ cell loss. The result of this double-knockout model may shed light on the possible role of cell cycle proteins in the maintenance of the cycling of the seminiferous tubule.

Inappropriate expression of E2F1 may also cause the activation of the p53-dependent apoptotic pathway. We have previously discussed the activation of the p53 pathway by CDKN2A/ARF. Additional gene changes that we discovered in our microarray analysis, such as the up-regulation of Cdt1 (chromatin licensing and DNA replication factor 1) and Cdc6 (cell division cycle 6 homolog), have also been linked to p53 stabilization, which is necessary for its activation because p53 normally has a very short half-life (62). Thus, although replicative senescence had been overcome, activation of p53-dependant apoptosis may be responsible for the prevention of tumor formation. In a variety of other Rb conditional knockout models of cancer, including ovarian cancer (63), small cell lung cancer (64), and medulloblastoma (65), concomitant p53 inactivation has been reported to be indispensable to precipitate oncogenesis. In addition, the p53 pathway is particularly intriguing to investigate especially when considering that a phenotype of progressive Sertoli cell loss, similar to that seen in our model, is observed in the knockout model of Bcl2L2/Bclw, a member of the BCL2 family of antiapoptotic proteins (66, 67). In Rb cKO mice, Bbc3/ PUMA up-regulation by p53 may be responsible for inhibiting the BCLW protein that would indirectly lead to activation of the proapoptotic protein, BAX, and the induction of apoptosis (68, 69). In the Sertoli cell-specific transgenic SV40 large T antigen model in which Sertoli cell proliferation leads to cancer (13), transformation...
could rely not only on RB inactivation but also the inactivation of p53, another known function of T antigen (70). In the future, it will be important to investigate whether a double knockout of Rb and Trp53 could lead to Sertoli cell oncogenesis.

Alternatively, loss of Sertoli cell expression of RB may only cause impairment of permanent cell cycle quiescence rather than the activation of cell cycle progression. Perhaps hyperplasia or cancer in our Rb cKO model cannot be achieved without a concomitant mitogenic signal (e.g., activins, FSH). It will be interesting to examine a double knockout of Inha and Rb to determine whether the combination of loss of Rb with increased activin signaling can precipitate early tumorigenesis, because these mice show the earliest signs of cancer at 4 wk of age (71). Conversely, although serum FSH, another known Sertoli cell mitogen, is increased in 8-wk-old Rb cKO mice, these cells do not exhibit tumorigenesis. It will be important to examine the activation of FSH signaling pathways in Trp53/Rb double knockouts to determine whether the mitogenic signaling of FSH is p53-dependent.

In addition to investigating the cell cycle and apoptotic defects, it will also be important to determine the ultimate cause of impaired terminal differentiation in our model. Although many of the gene changes relating to differentiation may be caused by misregulation of AR, a number of gene changes, such as the induction of Timp1, have not been reported to be regulated by AR. Still, our evidence suggested that RB may interact specifically with AR in Sertoli cells, meaning these cells may be regulated postmitotically like skeletal muscle in which RB is essential to modulate the tissue-specific transcription factors that control differentiation (72). Alternatively, is it the balance of cell cycle inducers (E2Fs) and cell cycle inhibitors (RB, p27) that perform an essential role in regulating Sertoli cells, specifically regarding their stage-specific functions? Future work should determine whether a double knockout of Rb and E2F3a would rescue the Rb cKO functional phenotype without rescuing the cell cycle phenotype as was seen in the elegant studies performed in retinal interneurons (11). If so, this would suggest that proper terminal differentiation of Sertoli cells is dependant more on the balance of the expression of cell cycle proteins rather than the actual maintenance of cell cycle quiescence. This hypothesis is also supported by the extensive amount of literature linking AR to cell cycle proteins both in Sertoli cells (23, 24) and prostate cancer cells (73, 74). However, it is also possible that activation of p53 alone is responsible for the progressive infertility, if the germ cell loss seen in the Bcl2I2 knockout is intrinsic to Sertoli cell function and not germ cell function as has been hypothesized (75).

In closing, our Rb cKO model that demonstrates Sertoli cell dysfunction has allowed us to arrive at two general conclusions. First, with regard to human infertility, understanding the function of Sertoli cells will lead to better in vitro methods of germ cell maturation that will not only help infertile couples conceive but will also provide a framework for better studies of human causes of male infertility. In addition, the contribution of Sertoli cell dysfunction to human infertility is unknown and has not been very carefully studied. Further understanding the function of Sertoli cells in mouse models will be of great benefit to the understanding of human infertility. Second, our studies attempt to dissect the essential role of RB in differentiation of Sertoli cells. Although we achieved the conclusions in this study by making careful comparisons to existing microarrays and mouse models, we propose that further delineation of these pathways using compound genetic models, as described above, will result in a better understanding of RB’s role in Sertoli cell function which, by comparative studies, will lead to a more universal understanding of how RB controls differentiation and cell cycle regulation in other tissue types as well.

Materials and Methods

Mouse lines and genotyping

Generation of mice containing a null (26) or floxed (65) mutation in the Rb gene and transgenic mice carrying a Cre

![FIG. 8. A summary of the postulated role of RB in Sertoli cells. In wild-type (WT) Sertoli cells, inhibition of E2F3a by RB prevents expression of E2F1. In Rb cKO Sertoli cells, the aberrant activation of E2F3a and, thus, E2F1 leads to derepression of cell cycle and apoptotic genes. RB may also modulate the expression of AR-regulated genes in WT Sertoli cells; therefore, the lack of RB in Rb cKO Sertoli cells may directly lead to their misregulation. Repression of E2F3a by RB may be responsible for AR-independent gene changes involved in adult Sertoli cell function (i.e. Timp1 down-regulation).](https://academic.oup.com/mend/article-abstract/23/11/1900/2684179/Retinoblastoma-Protein-Plays-Multiple-Essential)
recombinase driven by the AMH promoter (Amh-Cre) (20) have been described previously. Generation of Sox9-GFP knock-in mice will be described in detail elsewhere (Akiyama, H., manuscript in preparation). Rbpre−/ Amh-Cre + Sox9-GFP+ mice were crossed to Rbfllox/+ mice to generate control and experimental animals. Tail DNA was used for PCR genotyping and was performed for all alleles according to the manufacturer’s protocol (New England Biolabs, Ipswich, MA). Primers for the Rb-null allele, adapted from Ref. 26, are as follows: RX3, 5′-GCATCTGACTCTTATTTGCGG-3′; RX3: 5′-CCACCATAGCGCAGGAGT-3′ and produced wild-type (724-bp) and mutant (400-bp) products. Primers for Rb−/− mice, described in Ref. 30, are as follows: Rb212, 5′-GGCGTGTGCCATCAATG-3′; Rb18, 5′-GGCGTGTGCCATCAATG-3′; PGK, 5′-GAAGAACGAGATGACGACC-3′ and produced a 443-bp product for the Rb−/− allele and a 260-bp product for the Rb−/+ allele. PCR for Amh-Cre: McreAMH, 5′-AGCTCAGGCTCTGAGCTAATG-3′ yielding a 748-bp product for the Rb−/− allele and a 260-bp product for the recombined allele. PCR for Amh-Cre: McreAMH, 5′-AGCTCAGGCTCTGAGCTAATG-3′; McreGene, 5′-AATCGCGAACTCTCAGCAGC-3′; producing a 443-bp product. PCR for Sox9-GFP: GfpF, 5′-CAAGATCCGCCACAACATCG-3′; GfpR, 5′-CAAGATCCGCCACAACATCG-3′; producing a 170-bp product. All primers designed by the laboratory were made using Primer3 online software (76).

Animal care and treatment

Mice were housed with unlimited access to food and water and exposure to 12-h light, 12-h dark cycles in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Baylor College of Medicine Institutional Animal Care and Use Committee. For BrdU incorporation studies, mice were injected with 125–155 mg BrdU/kg body weight (77). For serum collection, mice were anesthetized, and blood was collected by cardiac puncture. Microtainer tubes (BD Biosciences, Franklin Lakes, NJ) were used for serum isolation and sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (http://www.healthsystem.virginia.edu/internet/crr) for detection of FSH, LH, and testosterone. Immediately after cardiac puncture or 2 h after BrdU injection, the mice were euthanized, and the desired tissues were harvested. In general, one testis was fixed for histological analysis and the other was frozen for DNA extraction and quantitative PCR

Sertoli cell isolation, RNA extraction, microarray, and quantitative PCR

After removal of the tunica albuginea, testes were dissociated by sequential collagenase and trypsin treatment (78). Sox9-GFP+ Sertoli cells were isolated from this single-cell preparation in the Baylor College of Medicine Cytometry and Cell Sorting Core using a FACSAriaII cell sorter (BD Biosciences). Representative graphs showing gating of channels are displayed in supplemental Fig. S1. Collected cells were centrifuged, and RNA was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA). Microarray was performed by the Children’s Health and Nutrition Research Center Core of Baylor College of Medicine utilizing the MouseWG-6 v2.0 chip (Illumina, San Diego, CA). Results were analyzed using GeneSpring GX Version 9.0.0 (Agilent Technologies, Palo Alto, CA) by filtering for flags and then applying an unpaired t test (P < 0.05) on genes that had expression above the 20th percentile and were more than 1.5-fold different from control. For quantitative PCR applications, Sertoli cell RNA isolated from control (n = 6) and Rb cKO (n = 5) mice was converted to cDNA using SuperScriptIII (Invitrogen, Carlsbad, CA). Microarray data were deposited into National Center for Biotechnology Information’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE17904). Sybr Green primers were designed using PrimerExpress (Applied Biosystems, Foster City, CA) and are listed in Table 2 or were previously reported (25, 79). The remaining genes were assayed with Taqman probes (Applied Biosystems; Rrn2 (Mm00485881_g1), Ncapb (Mm00522764_m1), Bbc3 (Mm00519268_m1), Serpina3n (Mm00776439_m1)). Quantitative PCR were performed as described elsewhere (25) using Gapdh as an endogenous control for relative quantification.

Western blot analysis

Western blot analysis was performed as described in Ref. 80. Before storage at −80 C, tests samples were homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1.0% Nonidet P-40, 0.5% sodium deoxycholate (pH 8.0), and complete EDTA-free protease inhibitor cocktail tablets (Roche, Indianapolis, IN)). After determination of protein concentration, 100 μg of each sample was separated using 4–12% Bis-Tris gels (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (Whatman, Dassel, Germany) as instructed by the manufacturer. Membranes were probed at 4 C overnight with primary antibodies diluted in 5% milk/PBS (PBS with 0.05% Tween-20) followed by the appropriate peroxidase-conjugated secondary antibody (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Primary antibodies were diluted as follows: AR (sc-816; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:250; p27 (sc-528, Santa Cruz), 1:500; Sox9 (sc-20095, Santa Cruz), 1:500; and β-tubulin (T4026, Sigma), 1:3000. Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) and Kodak BioMax

### Table 2. Quantitative PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apaf1</td>
<td>AGCACAACCTCGGGCTGCAA</td>
<td>TGTGCCTCAAGGTTTTCTCTCT</td>
</tr>
<tr>
<td>Ar</td>
<td>GGAACCTCTCGTGAAGTAAAGAC</td>
<td>GATGGTCTATCAGAGTTCATCAAAGAA</td>
</tr>
<tr>
<td>Casp8</td>
<td>GAACTTGTATATTCAGTCAATTGTC</td>
<td>CCATGCAGGATGAAAGTCAAGAC</td>
</tr>
<tr>
<td>Cdkn2a</td>
<td>AGAGCAGCGGCGCATGCTT</td>
<td>TAGACTCAGTCCTGGGATTGG</td>
</tr>
<tr>
<td>Cldn3</td>
<td>GTCGGATCCACCTCCPTTCTC</td>
<td>TTPGTTCAATGGCCTGTTGGA</td>
</tr>
<tr>
<td>Fas</td>
<td>GCACCGCGTCTTCTTC</td>
<td>TTAAGCTGTAGCCCCAGCAAGCA</td>
</tr>
<tr>
<td>Mcm2</td>
<td>CACGATGAGGAGCTGAGAAGA</td>
<td>GGTGCAATGCTGGCAGAGGAT</td>
</tr>
<tr>
<td>Ocln</td>
<td>CTCGAGTAGCTACAGAGGAGGAGTGA</td>
<td>TTGACACTGACAGAGCGCATG</td>
</tr>
<tr>
<td>Timp1</td>
<td>CCACCCACAGACACGCTTCTC</td>
<td>GGTGAATGGCTCAGTGATTTC</td>
</tr>
</tbody>
</table>

Downloaded from https://academic.oup.com/mend/article-abstract/23/11/1900/2684179/Retinoblastoma-Protein-Plays-Multiple-Essential by guest on 15 September 2017
XAR film (Sigma, St. Louis, MO). Band densitometry was determined using ImageJ software (National Institutes of Health, Bethesda, MD).

**Histology, immunohistochemistry, and immunofluorescence**

Tissues were fixed in Bouin’s fixative or 10% neutral buffered formalin before paraffin embedding. Tissue embedding, sectioning, and staining for periodic acid Schiff and hematoxylin were performed by the Histology Core of the Department of Pathology of Baylor College of Medicine. Immunohistochemistry and immunofluorescence were performed on tissues fixed in formalin as described elsewhere (79, 81) with minor modifications. Briefly, 5-μm sections were cleared and rehydrated. Antigen retrieval was performed by boiling the samples in 10 mM citrate buffer (pH 6.0). Samples were blocked (3% BSA/10% serum in PBS) for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. This was followed by incubation in the appropriate biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA; 1:200) or fluorescent secondary antibody (Molecular Probes, Inc., Eugene, OR; 1:500). Primary antibodies were diluted as follows: AR (sc-816, Santa Cruz), 1:200; RB (G3–245, BD Biosciences), 1:100; BrdU (Bu20a, DAKO Corp., Carpinteria, CA), 1:100; GFP (A11122, Santa Cruz), 1:200; RB (G3–245, BD Biosciences), 1:100; GFP (A11122, Invitrogen), 1:50; and GFP (CLONTECH Laboratories, Inc., Palo Alto, CA), 1:50. Vectastain ABC kit (Vector Laboratories) followed by dianaminobenzidine incubation was used to visualize biotinylated secondary antibodies for immunohistochemistry which were counterstained with hematoxylin. Fluorescent samples were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)**

TUNEL analysis was performed on 6-wk-old testis sections as described previously (25) utilizing the ApopTag Plus fluorescein in situ apoptosis detection kit (Chemicon, Temecula, CA) as per the manufacturer’s guidelines. Apoptotic index was determined by counting the fraction of tubules that contained one or more apoptotic cells and was assessed in at least 10 high-powered fields (×100) for at least three biological samples per genotype.

**Biotin tracer assay**

This assay was performed as described previously (55) with minor modifications. Animals (6 wk of age) were anesthetized, and after their testes were exposed, a small opening was made in the tunica albuginea. Twenty-five microliters of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockland, IL) that had been freshly prepared in PBS containing 1 mM CaCl2 was injected into the interstitium. The contralateral testsis was injected with calcium chloride solution alone and served as a negative control. After 30 min had elapsed, the animals were euthanized, and the testes were harvested and fixed in 10% neutral buffered formalin. Testis sections were probed sequentially with streptavidin-Texas Red (1:400 in PBS; Thermo Scientific) to detect biotin and then anti-episp antibody (1:400, BD Biosciences) followed by Alexa Fluor 488 donkey anti-mouse secondary antibody (Molecular Probes).

**Statistical analysis**

Statistical analysis used JMP 7.0.1 software (SAS institute). Statistical significance was determined by one-tailed t test assuming unequal variance for two-sample comparison and by one-way ANOVA followed by Tukey honestly significant difference test for multiple sample comparisons. X2 test was used to determine the significance of association between groups in the Venn diagrams of the microarray (i.e. if the overlap between groups was at an expected frequency or at a higher frequency than would be predicted if there was no association).

**Acknowledgments**

We thank Dr. Tyler Jacks and Dr. Anton Berns for generously depositing the Rb-null and Rb-floxed mice, respectively, in the National Cancer Institute’s Mouse Repository of the Mouse Models of Human Cancer Consortium. We also thank Dr. Richard Behringer for providing us with access to the Sox9-GFP mice and Dr. Yi-Nan Lin and Dr. Jing Meng for assistance with experimental protocols.

This work was supported by National Institutes of Health Grant CA60651 (to M.M.M.) and the Edward J. and Josephine G. Hudson Scholar Fund (to R.L.N.). R.L.N. is also supported by the Baylor College of Medicine Medical Scientist Training Program. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver National Institutes of Child Health and Human Development/National Institutes of Health (Specialized Cooperative Centers Program in Reproduction and Infertility Research) Grant U54-HD28934. These funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Address all correspondence and requests for reprints to: Martin M. Matzuk, M.D., Ph.D., Stuart Wallace Chair and Professor, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Room S217, Houston, Texas 77030. E-mail: mmatzuk@bcm.tmc.edu.

Disclosure Summary: The authors have nothing to disclose.

**References**

6. Goodrich DW 2006 The retinoblastoma tumor-suppressor gene, the exception that proves the rule. Oncogene 25:5233–5243
8. Berman SD, Yuan TL, Miller ES, Lee EY, Caron A, Lees JA 2008 The retinoblastoma protein tumor suppressor is important for appropriate osteoblast differentiation and bone development. Mol Cancer Res 6:1440–1451
entiation into white versus brown fat through repression of PGC-1α. Cell Metab 2:283–295


