Rictor Regulates Spermatogenesis by Controlling Sertoli Cell Cytoskeletal Organization and Cell Polarity in the Mouse Testis

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Maintenance of cell polarity is essential for Sertoli cell and blood-testis barrier (BTB) function and spermatogenesis; however, the signaling mechanisms that regulate the integrity of the cytoskeleton and polarity of Sertoli cells are not fully understood. Here, we demonstrate that rapamycin-insensitive component of target of rapamycin (TOR) (Rictor), a core component of mechanistic TOR complex 2 (mTORC2), was expressed in the seminiferous epithelium during testicular development, and was down-regulated in a cadmium chloride-induced BTB damage model. We then conditionally deleted the Rictor gene in Sertoli cells and mutant mice exhibited azoospermia and were sterile as early as 3 months old. Further study revealed that Rictor may regulate actin organization via both mTORC2-dependent and mTORC2-independent mechanisms, in which the small GTPase, ras-related C3 botulinum toxin substrate 1, and phosphorylation of the actin filament regulatory protein, Paxillin, are involved, respectively. Loss of Rictor in Sertoli cells perturbed actin dynamics and caused microtubule disarrangement, both of which accumulatively disrupted Sertoli cell polarity and BTB integrity, accompanied by testicular developmental defects, spermiogenic arrest and excessive germ cell loss in mutant mice. Together, these findings establish the importance of Rictor/mTORC2 signaling in Sertoli cell function and spermatogenesis through the maintenance of Sertoli cell cytoskeletal dynamics, BTB integrity, and cell polarity. (Endocrinology 156: 4244–4256, 2015)

In mammals, spermatogenesis takes place in the seminiferous epithelium, which is composed of Sertoli and germ cells, with the Sertoli cells serving as the “mother” or the “nursery” cells that support and nourish germ cells at different developmental stages (1, 2). The essential roles of Sertoli cells in spermatogenesis have been widely acknowledged, including nutrient supply, hormone regulation, paracrine interaction, and above all, construction of the blood-testis barrier (BTB) (1). The BTB is created by co-existing tight junctions (TJs), basal ectoplasmic special- ization (ES), and gap junctions between adjacent Sertoli cells in the seminiferous epithelium near the basement membrane, and provides a germ cell-friendly environment for the correct development and maturation of germ cells (3). The most distinctive and unique feature of the BTB is the presence of tightly packed actin filament bundles at the basal ES, which lie perpendicular to the Sertoli cell plasma membrane, conferring the unusual adhesive strength of

* H.D. and Z.C. contributed equally to this work.
Abbreviations: Akt, serine-threonine protein kinase B; AMH, anti-Mullerian hormone; Arpc2, actin-related protein complex 2; BTB, blood-testis barrier; CdCl₂, cadmium chloride; dUTP, deoxyuridinetriphosphate; ES, ectoplasmic specialization; FBXW7, F box and WD repeat domain containing 7; H&E, hematoxylin-eosin; IHC, immunohistochemical; IP, immuno- precipitation; MT, microtubule; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; Pi, propidium iodide; PKC, protein kinase C; Rac1, ras-related C3 botulinum toxin substrate 1; Rictor, rapamycin-insensitive component of TOR; siRNA, small interfering RNA; TBC, tubulobulbar complex; TJ, tight junction; TUNEL, terminal deoxy- nucleotidyltransferase-mediated dUTP nick end labeling; WT1, Wilms’ tumor 1; ZO-2, Zona occluden 2.
the BTB (3). The BTB undergoes cyclic restructuring during stage VIII–XI of the epithelial cycle to allow the transit and entry of preleptotene spermatocytes into the adluminal compartment, which is a prerequisite for meiosis and subsequent spermiogenesis (4, 5). Furthermore, Sertoli cells are highly polarized cells that extend from the basement membrane to the lumen of seminiferous tubules (6). These long apical extensions are in constant contact with germ cells and are required for germ cell development and differentiation, and their loss leads to premature germ cell loss (7). Cytoskeletal proteins are known to have numerous roles, such as determination of cell shape, cell motility, maintenance of cell junctions and intracellular trafficking, all of which help to maintain normal epithelial function and morphology. Thus, cytoskeletal networks are believed to serve as the foundation for the integrity of the BTB and its dynamic restructuring, as well as for the polarity of Sertoli cells and the entire seminiferous epithelium (8). Therefore, maintenance of Sertoli cell cytoskeletal dynamics is essential for BTB integrity, Sertoli cell function, and spermatogenesis. However, the mechanisms involved in the regulation of the integrity of the cytoskeleton and cell polarity in Sertoli cells remain to be elucidated.

The mechanistic target of rapamycin (mTOR) is a highly conserved Ser/Thr protein kinase that forms 2 distinct functional complexes termed mTOR complex 1 (mTORC1) and mTORC2 (9, 10). mTORC1 consists of the conserved components mTOR, rapamycin-sensitive adaptor protein of mTOR and mammalian lethal with sec13 protein 8, and is sensitive to rapamycin. It responds to the presence of nutrients and energy sources to control protein synthesis and ribosome biogenesis, and regulation of cell growth, proliferation, and metabolism (9, 10). In contrast, mTORC2 is insensitive to rapamycin. The essential core of mTORC2 comprises mTOR, mammalian stress-activated protein kinase-interacting protein 1, mammalian lethal with sec13 protein 8, and the rapamycin-insensitive subunit, rapamycin-insensitive component of TOR (Rictor) (11). It is known that mTORC2 regulates the activity of serine-threonine protein kinase B (Akt) and protein kinase C (PKC), controlling cell survival and cytoskeletal organization, respectively (11–13). It has also been shown that mTORC2 regulates serum- and glucocorticoid-induced protein kinase 1 to modulate sodium transportation (14). In contrast to mTORC1, for which many upstream signals and cellular functions have been defined, investigations into mTORC2 cellular functions and signaling are required.

Although the in vitro function of mTORC2 in the regulation of actin dynamics has been established, the underlying mechanisms involved in its role in maintaining Sertoli cell polarity and regulating spermatogenesis are not known. A recent study using Rictor-specific small interfering RNA (siRNA) reported the essential role of Rictor/mTORC2 in controlling BTB dynamics (15), but the functions and mechanisms of Rictor in modulating Sertoli cell function and spermatogenesis remain unclear. Therefore, using mice with Sertoli cell-specific deletion of Rictor, a core component of mTORC2, and a cadmium chloride (CdCl2)-induced BTB damaged mouse model, the current study aims to identify the roles and molecular mechanisms of Rictor/mTORC2 in the regulation of actin organization and cell polarity in Sertoli cells, and thus in spermatogenesis and male fertility.

Materials and Methods

Mice, husbandry, and genotyping

Anti-Mullerian hormone (AMH)-Cre mice (Jax number 007915) were from the Model Animal Research Center of Nanjing University (Nanjing, China). Rictor-loxp mice were kindly provided by Professor Mark A. Magnuson (Vanderbilt University), with the Rictor exon 3 flanked by 2 loxP sites. AMH-Cre mice were mated with homozygous Rictor-loxP (Rictor\textsuperscript{loxP/loxP}) mice to yield mice heterozygous for Rictor-loxP and homozygous for AMH-Cre, which were then bred with mice homozygous for Rictor-loxP and heterozygous for AMH-Cre. These mice carried a Sertoli cell-specific deletion of Rictor (AMH-Cre\textsuperscript{\textasciitilde}, Rictor\textsuperscript{loxP/loxP}), as the Rictor exon 3 was spliced out by the Sertoli cell-specific expression of Cre recombinase, leading to the complete loss of Rictor protein in Sertoli cells. These mice were termed SCRictorKO mice in this study, and the male mice homozygous for Rictor-loxP without AMH-Cre (AMH-Cre\textsuperscript{\textasciitilde}, Rictor\textsuperscript{loxP/loxP}) from the same litter were used as control mice. DNA isolated from tail biopsies was used for genotyping, which was performed using PCR as described in Supplemental Table 1. All animal experiments were approved by the Southern Medical University Committee on the Use and Care of Animals and were performed in accordance with the Committee’s guidelines and regulations.

Tissue collection and histological analysis

Mouse testes and epididymis were removed and weighed, fixed in 4% paraformaldehyde, and then processed using paraffin wax and standard methods. Five sections of each testis and epididymis (5 µm, taken 200 µm apart) were stained with hematoxylin-eosin (H&E) for regular histological analysis. Immunohistochemical (IHC) staining was performed following the standard procedure, using horseradish peroxidase-conjugated anti-IgG secondary antibodies (Jackson Immuno Research) and was visualized by Diaminobenzidine. Slides were finally counterstained with hematoxylin. Immunofluorescent staining was performed as for IHC, except for the use of Alexa Fluor 488 dye-labeled secondary antibodies and 4',6-Diamidine-2'-phenylindole dihydrochloride (Invitrogen) for visualization of the nucleus. Immunofluorescent images were obtained using Fluoview FV1000 confocal microscopy (Olympus). The antibodies used in this study are summarized in Table 1.
Cell apoptosis was evaluated in testis sections using a terminal deoxynucleotidyltransferase-mediated deoxyuridinetriphosphate nick end labeling (TUNEL) assay, for in situ visualization of DNA fragmentation with a commercial kit (Promega). The number of apoptotic cells in each seminiferous tubule was counted and normalized against the area of the tubules, which were calculated according to the formula: π/4 × long diameter × short diameter (16). All tubules on 3 sections from each testis (200 μm apart) were analyzed.

**Western blot analysis**

Whole testes were triturated and lysed on ice, and then boiled in sodium dodecyl sulfate loading buffer. The protein extracts were then subjected to 6%–12% SDS-PAGE and subsequent processing following a standard protocol. The antibodies used for Western blot analysis are summarized in Table 1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vendor</th>
<th>Catalog Number</th>
<th>Application</th>
<th>Working Dilution for WB or (IF/IHC) or [IP]</th>
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<td>Rabbit anti-Rictor</td>
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Antibodies used in this study cross-reacted with the corresponding proteins in rats as indicated by the manufacturers. WB, Western blotting; IF, immunofluorescence; IP, Co-IP.

**Propidium iodide (PI) staining and flow cytometry analysis**

A testicular germ cell suspension was prepared following an enzyme-based process with collagenase and trypsin (17). PI staining was performed as described previously (17). DNA content in stained cells was analyzed using a FACScan flow cytometer (BD Immunocytometry Systems).

**Pull-down assay and immunoprecipitation (IP) experiment**

In vivo Rac1 activity in Sertoli cells was quantified by measuring GTP-bound Rac1 (activated form) in the whole testis. A kit was used to specifically monitor the pulled down GTP-bound Rac1 (Millipore). Briefly, testes were triturated on ice and then lysed with magnesium lysis/wash buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor mixture (Sigma). Activated Rac1 was pulled down by beads that specifically recognize GTP-bound Rac1. After washing, the beads were subjected to immunoblotting using an anti-Rac1 antibody (Millipore).

An IP experiment was performed as described previously (18). Briefly, the testes were triturated on ice and then lysed with 3-[3-Cholamidopropyl] dimethylammonio] propane sulfonate-containing lysis buffer. After centrifugation, β-actin in the supernatant was immunoprecipitated using an anti-β-actin antibody (Sigma) and an IgG as the control. After incubation with protein G Sepharose and subsequent washing, IPs were subjected to immunoblotting for β-actin, occludin (Epitomics), and β-catenin (Cell Signaling Technology).

**CdCl₂ treatment**

Two-month-old wild-type C57 male mice were treated with CdCl₂ (Sigma) at 5 mg/kg/dip for 3 days. One testis was collected for Western blot analysis, 1 epididymus for sperm counting, and the other testis and epididymus were processed using paraffin wax for histological analysis as described above.

**Transient transfection of testes with siRNA in vivo**

Two-month-old wild-type C57 males were used in this experiment. One testis from each mouse was injected with 20-μL transfection mix containing 2.5OD FBXW7-specific siRNA oligo (GenePharma Co) and 5-μL Entranster-in vivo transfection reagent (Engreen Biosystem), via a microliter syringe (Hamilton), and the other testis of the same mouse received nontargeting siRNA. The sequences for FBXW7-specific siRNA were...

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**Table 1. Antibodies Used for Different Experiments in This Study**

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as follows: sense, 5'-CUCAGGUGUUUGGAAUUTT-3' and antisense, 5'-TTGAGAGUCCCAAACCCUAUA-3', both with cholesterol-2'-OMe modification at 5'-end and phosphorothioate modification at 3'-end. The injection was performed 3 times on every other day. For the RNAi plus CdCl₂ treatment group, the mice were administered CdCl₂ 12 hours after the last injection of siRNA. Three days later, testes were collected with one half of each testis for H&E staining and the other half for Western blot analysis.

Statistical analyses

All experiments were carried out in triplicate. For morphological evaluation, at least 5 sections (100 μm apart) from each testis or epididymis were analyzed. For Western blot analysis, 1 representative set of data is shown. Data are expressed as mean values ± SE, and differences between groups were analyzed using the t test (SPSS 13.0). P < .05 was considered statistically significant.

Results

Rictor is expressed in Sertoli cells during testicular development and down-regulated in a CdCl₂-induced BTB damage model

To determine the role of Rictor/mTORC2 in spermatogenesis, we first characterized the expression of Rictor in the seminiferous epithelium of mice at different developmental ages. IHC analysis showed that Rictor was firstly expressed at the center of the seminiferous tubule at the age of 2 weeks (Figure 1A). As testicular development proceeded, Rictor was predominantly expressed near the basement membrane of the seminiferous tubules, consistent with the localization of Sertoli cells and BTB (Figure 1A), indicating the important role of Rictor in testicular development and function.

To examine the correlation between Rictor and both BTB function and spermatogenesis, we established a CdCl₂-induced BTB disruption mouse model. BTB construction was damaged after 3 days of treatment with CdCl₂, with an excess of vacuoles near the basement membrane of seminiferous tubules (Figure 1B). No visible spermatozoa were observed in the epididymis of CdCl₂-treated mice (Figure 1C). In addition, a decrease in occludin and N-cadherin protein levels was observed in CdCl₂-treated testes (Figure 1D). These data indicated the successful generation of the BTB disruption model. Interestingly, IHC staining showed that Rictor was significantly reduced after CdCl₂ treatment (Figure 1E), indicating the association of Rictor with BTB integrity. Down-regulation of Rictor by CdCl₂ treat-
ment was further confirmed by the immunoblotting results of Rictor and p-Akt (S473) (Figure 1F). Moreover, we observed significant up-regulation of F box and WD repeat domain containing 7 (FBXW7) protein level in CdCl₂-treated testes (Figure 1F), in line with a recent report, which showed that Rictor undergoes FBXW7-mediated ubiquitination and proteasomal degradation (19). To verify the possible FBXW7-mediated regulation of Rictor, FBXW7 was knocked down to test whether CdCl₂ could still down-regulate Rictor. FBXW7 protein level was markedly decreased after the introduction of FBXW7-specific siRNA into testes, but did not cause obvious developmental defects in testes or epididymis (Supplemental Figure 1). Interestingly, down-regulation of FBXW7 protected Rictor from degradation and damage in testicular tubules caused by CdCl₂ (Supplemental Figure 1). These results demonstrate that Rictor is important for maintaining BTB integrity and spermatogenesis.

**Generation of mutant mice with Sertoli cell-specific deletion of Rictor**

To further investigate the roles of Rictor/mTORC2 in Sertoli cells with regard to BTB integrity and spermatogenesis, we generated mutant mice with Sertoli cell-specific ablation of Rictor, by crossing Rictor<sup>loxP/loxP</sup> mice with transgenic mice with AMH promoter-mediated Cre (Figure 2A), which has been widely used to delete target genes in the Sertoli cells of testes (20–22). IHC analysis showed a significant decrease in Rictor expression in 1-month-old SCRictorKO mice, suggesting successful Cre-mediated recombination of the floxed Rictor gene (Figure 2B). Immunoblotting of Rictor and p-Akt (S473) also showed a significant decrease in mTORC2 signaling in mutant mice (Figure 2, C and D). Taken together, these results demonstrate successful deprivation of the Rictor gene in Sertoli cells in SCRictorKO mice.

**Rictor in Sertoli cells is required for testicular development**

Next, we focused on seminiferous tubule development in SCRictorKO mice. There were no obvious differences in physical appearance and body weight between SCRictorKO mice and control littermates at any age (Figure 3A). The testes of 1-month-old SCRictorKO mice were not significant decreased but were reduced by 70% compared with control mice at 3 months old (Figure 3, B and C).

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**Figure 2.** Generation of mice with Sertoli cell-specific deletion of Rictor. A, Genotyping the F2 offspring after mating transgenic Cre and loxP mice. Genotyping was performed as described in Supplemental Table 1. Lane 1 indicates the negative loading control without DNA template. Lane 2 indicates the positive loading control with identified genotype. Lane 3 and 5 indicate the genotypes of control and SCRictorKO mice, respectively. B, Immunofluorescent staining of Rictor in the testes from 1- and 3-month-old SCRictorKO mice and control littermates. 1 m, 1 month; 3 m, 3 months. Scale bar, 25 μm. C, Immunoblotting of Rictor and p-Akt (S473) in SCRictorKO and control mice. Protein samples were extracted from the whole testis of 1-month-old SCRictorKO and control mice, at which time the testes had a large proportion of Sertoli cells. D, Quantification of the results in C. Protein levels were normalized against α-tubulin. Values indicate mean values ± SE. ***, P < .001.
Similar changes were observed in the epididymis (Figure 3, B and C). These findings indicate defective testicular development in SCRictorKO mice. To verify whether loss of Rictor in Sertoli cells impedes seminiferous tubule development, we compared the seminiferous tubules between control and SCRictorKO mice morphologically. Mild abnormalities were observed in the seminiferous tubules of 1-month-old SCRictorKO mice, as shown by the absence of long spermatids and the presence of some cell clusters. As age increased, more severe defects were observed, such as progressive lesions in seminiferous epithelium, characterized by the absence of seminiferous lumen and long spermatids, and loss of cell hierarchy in the whole seminiferous epithelium (Figure 3, D and E). Some tubules displayed clusters of cells, some severe vacuolization in seminiferous epithelium, and some complete loss of germ cells (Figure 3E). These results suggest that Rictor in Sertoli cells is essential for seminiferous tubule integrity and its deficiency leads to testicular developmental defects.

Ablation of Rictor in Sertoli cells impairs spermatogenesis and causes azoospermia

Testicular atrophy commonly causes spermatogenic defects. As expected, histological examinations showed that in contrast to the epididymis of control mice which were full of spermatozoa, there were no visible spermatozoa in the epididymis of SCRictorKO mice as early as 3 months old, only undefined round cell debris (Figure 4A). We then counted the sperm and found that SCRictorKO mice demonstrated azoospermia, and accordingly,
were sterile (Figure 4B). We next determined whether defects in spermatogenesis contributed to azoospermia seen in SCRictorKO mice. The relative distribution of germ cell populations in the testes of control and SCRictorKO mice was determined by quantifying the DNA content in PI-labeled germ cells via cytometric scanning. Three main histogram peaks of DNA content are usually observed, corresponding to haploid (1C, spermatids and spermatozoa), diploid (2C, spermatogonia, preleptotene primary spermatocytes, and secondary spermatocytes), and tetraploid cells (4C, spermatogonia, leptotene, zygotene, pachytene, and diplotene primary spermatocytes), respectively. The SCRictorKO mice exhibited a comparable number of tetraploid cells, a significant increase in diploid cells and a significant decrease in cells with haploid DNA content (Figure 4, C and D), indicating a partial meiotic

**Figure 4.** Defective spermatogenesis and azoospermia in SCRictorKO mice. A, No spermatozoa in the epididymis of adult SCRictorKO mice as shown by H&E. Scale bar, 100 μm for upper panels and 25 μm for lower panels. B, Sperm counting and fertility assessment. Sperm were released from the epididymis. For fertility assessment, 14 C57 females were analyzed for each group. C, Flow cytometry analysis of the DNA content in germ cells of after PI labeling. 1C, haploid cells; 2C, diploid cells; 4C, tetraploid cells. D, Quantification of the results in C; n = 7 for each group. E, Cell apoptosis by TUNEL illustrates progressive germ cell loss in the seminiferous epithelium of SCRictorKO mice. Scale bar, 25 μm. F, Quantitative data on apoptotic cell numbers in the SCRictorKO and control group. Data were analyzed as described in Materials and methods; n = 3 for each group. Unless specifically stated, all samples were from 3-month-old SCRictorKO and control mice. All values and bars indicate mean values ± SE. *, P < .05; **, P < .01; ***, P < .001.
arrest. Reduced postmeiotic spermatid survival may also have contributed to this observation as the TUNEL assay showed a progressive increase in the number of apoptotic germ cells in the SCRictorKO testes (Figure 4, E and F). Therefore, loss of Rictor in Sertoli cells causes partial meiotic arrest and excessive germ cell apoptosis, in turn contributing to azoospermia in SCRictorKO mice.

**Elimination of Rictor from Sertoli cells disrupts BTB dynamics**

To explore the possibility of BTB dysfunction in SCRictorKO mice, the expression and localization of several key component proteins constituting the BTB were examined by immunofluorescent analysis, including occludin and Zona occluden 2 (ZO-2) (TJ proteins), β-catenin and N-cadherin (ES) proteins, and actin-related protein complex 2 (Arpc2), a component of the Arp2/3 complex that plays a crucial role in inducing changes in the conformation of the actin network from its bundled to debundled state to facilitate TJ and basal ES remodeling (23). The results showed that all these proteins were uniformly and specifically localized near the basement membranes of seminiferous tubules in control mice, consistent with the localization of Rictor mentioned earlier (Figure 5, A–E). In contrast, in SCRictorKO mice, the expression and localization of these proteins were altered and scattered over the whole seminiferous epithelium (Figure 5, A–E). Interestingly, although their localization was aberrant, their protein levels remained relatively stable in SCRictorKO mice, except for an increase in occludin and Arpc2 (Figure 5, F and G). Furthermore, quantitative PCR analysis showed no significant changes in the mRNA levels of these molecules (Figure 5H), indicating that loss of Rictor in Sertoli cells alters the localization, but not the expression of BTB proteins, and the increased protein levels of occludin and Arpc2 may be due to lack of the Rictor/Cullin 1 complex, which was shown to promote its target protein ubiquitination and destruction (24). Together, these results suggest that Rictor in Sertoli cells is essential for the integrity and function of the BTB.

**Rictor maintains Sertoli cell polarity by regulating Rac1, Paxillin, and actin organization**

Sertoli cells are highly polarized mesoepithelial cells that extend from the basement membrane to the lumen of seminiferous tubules (6). The long apical extensions of Sertoli cells are synergistically framed by both highly organized actin and microtubule (MT) networks to direct germ cell migration towards the lumen of the seminiferous tubules (8). The morphological changes in seminiferous epithelium and the abnormal location of BTB proteins in SCRictorKO mice indicated the loss of apical extensions. As postulated, immunofluorescent examination of both actin and tubulin (Figure 6A) verified the disruption of organized actin and microtubular arrangement. Furthermore, Wilm’s tumor 1 (WT1), a marker for the nucleus of Sertoli cells, was scattered over the seminiferous epithelium in SCRictorKO mice, and not specifically localized near the basal membrane as in control mice (Figure 6B). This result also verified that the cluster of cells observed in SCRictorKO mice was not Sertoli cells, but germ cells. In addition, the abnormal location of Sertoli cells indicated loss of function, which was confirmed by the reduced expression of androgen-binding protein and glial cell line-derived neurotrophic factor in SCRictorKO mice, both of which are secreted by Sertoli cells and important for spermatogenesis (Supplemental Figure 2). Taken together, these results demonstrate loss of polarity in Sertoli cells and in the whole seminiferous epithelium in SCRictorKO mice.

Previously, it was shown that Rictor regulates organization of the actin cytoskeleton in a rapamycin-insensitive and rapamycin-sensitive adaptor protein of mTOR-independent pathway, in which PKCα is a mediator of this function (12, 13). Also, earlier findings in adipocytes showed that Rictor could regulate the actin cytoskeleton in an mTORC2-independent manner via Paxillin, a scaffolding protein that through phosphorylation on Y118 was found to be important in actin regulation (25). Thus, we determined the expression and/or activity of these key effectors participating in actin organization. In SCRictorKO mice, it was found that S657-phosphorylated-PKCα and Y118-phosphorylated-Paxillin levels declined significantly (Figure 6, C and D). In addition, the small GTPase, Rac1, was shown to be involved in modulation of the actin cytoskeleton (26). By using a pull-down assay that specifically recognized the GTP-bound Rac1 (the active form of Rac1), it was found that the activity of Rac1 was obviously decreased in SCRictorKO mice, with no reduction in total Rac1 (Figure 6, E and F), indicating that Paxillin and Rac1 are involved in Rictor/mTORC2-mediated actin homeostasis in Sertoli cells. Involvement of Rac1 was further confirmed by the decreased level of 2 classic target proteins of Rac1, p21-activated kinase 1 and WASP-family verprolin-homologous protein (Supplemental Figure 3). Furthermore, Co-IP experiments using whole testes tissue revealed a notable reduction in the interaction between β-actin and occludin, and between β-actin and β-catenin in SCRictorKO mice, indicating a reduced association between the actin cytoskeleton and BTB proteins after Rictor deletion (Figure 6, G and H). Taken together, these results demonstrate the central role of Rictor in the regulation of Sertoli cell actin dynamics, and BTB barrier damage in SCRictorKO mice was mainly attributed to the disruption of actin organization. Rictor
controls Sertoli cell actin organization through regulation of the small GTPase, Rac1, and phosphorylation of the actin filament regulatory protein, Paxillin.

**Discussion**

In this study, using a CdCl$_2$-induced BTB-disruption mouse model, we found that Rictor expression in Sertoli cells was reduced when the BTB was disrupted. Using a mutant mouse model with Sertoli cell-specific deletion of Rictor, we further demonstrated that Rictor/mTORC2 in Sertoli cells is essential for spermatogenesis and male fertility. Developmental defects, incomplete meiotic arrest and excessive germ cell apoptosis were observed in the seminiferous epithelium in SCRictorKO mice, and mutant mice exhibited azoospermia and were sterile. Mechanistically, loss of Rictor perturbed cytoskeleton dynamics in Sertoli cells, disrupting cell polarity and BTB integrity. Our results also suggest that Rictor may regulate actin organization via both mTORC2-dependent and mTORC2-independent mechanisms, in which Rac1 and Paxillin are involved, respectively (Figure 7). Thus, we
conclude that Rictor/mTORC2 regulates spermatogenesis by controlling actin dynamics and cell polarity.

mTORC1 and mTORC2 are formed and function as 2 complexes within cells, and much more is known about mTORC1 than mTORC2 due to the availability of its natural specific inhibitor, rapamycin. However, it was recently shown that dual inhibition of mTORC1 and mTORC2 produces markedly better anticancer effects than inhibition of mTORC1 alone (27). Thus, there is growing evidence to suggest that mTORC2 deserves more attention. Additional cellular functions mediated by mTORC2 have begun to emerge in recent years. It was reported that increased mTORC2 activity by overexpression of Rictor promoted cell growth and motility via PKCα leading to the regulation of actin organization (28). Down-regulation of Rictor reduced cell adhesion function in podocytes, the cells that constitute the glomerular filtration barrier in the kidney (29). Of note, a recent study which introduced Rictor-specific siRNA into testes and primary Sertoli cells indicated that Rictor/mTORC2 regulates BTB dynamics via its effects on gap junction communications and the actin filament network (15). Of note, a recent study which introduced Rictor-specific siRNA into testes and primary Sertoli cells indicated that Rictor/mTORC2 regulates BTB dynamics via its effects on gap junction communications and the actin filament network (15).

Thus, we generated mice with Sertoli cell-specific deletion of Rictor, to determine the roles and underlying mechanisms of Rictor/mTORC2 in spermatogenesis. Based on the results of histological and molecular mechanism assays, our study demonstrated that loss of Rictor in Sertoli cells leads to disruption of BTB integrity and Sertoli cell polarity, which are mediated via alterations in actin and MT organization in Sertoli cells. In line with a previous report, our data showed reduced expression of phosphorylated-PKCα (S657) and activity of Rac1 after Rictor deletion, indicating the involvement of PKCα/Rac1 in mTORC2-dependent regulation of actin organization (15). However, our data on the notable reduction in p-Paxillin (Y118) indicate that the previous finding of no significant down-regulation of p-Paxillin (Y118) was due to an incomplete knockdown via siRNA in vivo (15), demonstrating an mTORC2-independent phosphorylation of Paxillin by Rictor. Our results also strengthen the previous finding that loss of Rictor disrupted the association between actin and BTB protein (15).

Our results also reveal that Rictor is crucial for actin organization in Sertoli cells during spermiogenesis. Quantification of the DNA content in PI-labeled germ cells

**Figure 6.** Disruption of cell polarity and actin organization in Sertoli cells in SC-RictorKO mice. A, Immunofluorescence of β-actin and α-tubulin in the testes of control and SC-RictorKO mice. Scale bar, 25 μm. B, IHC of WT1, a marker for the nucleus in Sertoli cells. Black arrowheads indicate normal WT1 localization near the basal membrane. Red arrowheads indicate aberrant localization scattered throughout the whole epithelium. Black arrows indicate cluster of cells. Red arrows indicate vacuolization. Scale bar, 25 μm. C, Immunoblotsting of S657-phosphorylated-PKCα and Y118-phosphorylated-Paxillin. Total PKCα and total Paxillin levels are also shown. Protein samples were from 1-month-old mice. D, Quantification of the results in C. Protein levels were normalized against α-tubulin. E, Reduced activity of Rac1 (GTP-Rac1) in SC-RictorKO mice. Activated Rac1 was pulled down by beads that specifically recognize GTP-bound Rac1. Elutions were subjected to immunoblotting using an anti-Rac1 antibody. Total Rac1 and tubulin were detected in the cell lysates before adding the beads. F, Quantification of the results in E. Protein level was normalized against total Rac1. G, Co-IP assay showed decreased association between actin and BTB proteins in SC-RictorKO mice. An IgG was used as the control. Cell lysates were immunoprecipitated using an anti-β-actin antibody and were subjected to immunoblotting using an anti-β-actin, occludin, and β-catenin antibody. H, Quantification of the results in G. Protein levels were normalized against β-actin. Unless specifically stated, all samples were from 3-month-old SC-RictorKO and control mice. All bars indicate mean values ± SE. *, P < .05; **, P < .01; ***, P < .001.
showed a significant decrease, but still a presence of haploid cells, indicating that a small portion of germ cells progressed beyond meiosis, thus, this incomplete meiotic arrest cannot solely lead to azoospermia in SCRictorKO mice. Otherwise, immunofluorescence analysis would have shown that actin was abundant at sites of cell-cell contact in the testis, including the basal ES and tubulobulbar complex (TBC) at the BTB formed between Sertoli cells, and the apical ES and TBC at the Sertoli cell-spermatid interface in control mice, but was completely disordered in SCRictorKO mice (Figure 6A). Therefore, it is likely that disarrangement of actin caused by Rictor deletion also decreased the association between actin and TJ and ES proteins, disrupting the dynamic reconstruction of apical ES and TBC at the Sertoli cell-spermatid interface, which is critical for spermiogenesis. Thus, both the finding of only round, but no long spermatids in seminiferous epithelium and of azoospermia in SCRictorKO mice are expected. During spermatogenesis, the MT system has frequent interactions with actin and participates in various cellular processes, such as signaling, cell adhesion, cell motility, maintenance of cell polarity, and protein targeting (8). For example, translocation of the apical ES in both directions in the seminiferous epithelium is thought to be mediated by MT-based motors using MTs as tracks. In some cases, MT minus ends may interact with junction protein complexes at the apical ES. Although we also observed aberrant distribution of tubulin in the testicular tubule in SCRictorKO mice, it is not clear whether this was directly caused by disarrangement of actin, or just a secondary phenomenon caused by a massive loss of germ cells and would not necessarily reflect any defects in MT function. Further studies are needed to address this issue.

Several previous case reports showed the important role of mTOR in spermatogenesis and male fertility, as organ transplant recipients treated with sirolimus (rapamycin), as an immunosuppressant, exhibited impaired spermatogenesis and reduced male fertility (30–33). The underlying mechanisms were not specifically elucidated until a recent study with Sertoli cell-specific deletion of Tsc genes showed that overactivation of mTORC1 disrupted Sertoli cell polarity and BTB integrity (34). Nevertheless, as activation of mTORC1 is usually associated with enhanced cell proliferation, it could be argued that changes in cell polarity and BTB complexes are caused by excessive proliferation, or excessive apoptosis due to endoplasmic reticulum stress induced by overactivation of mTORC1 (35). Indeed, germ cell loss was far more severe in the Tsc mutant mice than in the Rictor-SCRictorKO mice, and most seminiferous tubules in the Tsc mutant mice exhibited Sertoli cell-only phenotype. On the other hand, overactivation of mTORC1 always causes inhibitory feedback on mTORC2 via the Ribosomal protein S6 kinase/insulin receptor substrate axis (36, 37) and phosphorylation of Rictor (Thr1135) (38, 39). It cannot be ruled out that similar inhibition of mTORC2 occurs in Tsc mutant mice, although the activity of mTORC2 was not examined in that study. Our results collectively demonstrate that mTORC2 beyond mTORC1 regulates actin dynamics, BTB integrity, and Sertoli cell polarity. This notion is further supported by the finding that the mutant mice with Sertoli cell-specific deletion of Pten had a normal testicular phenotype (34), as both mTORC1 and mTORC2 are activated after Pten deletion and overactivation of mTORC1 may be compromised by mTORC2 via the promotion of cell survival.

In summary, we have shown the importance of Rictor/mTORC2 in Sertoli cell biology and spermatogenesis. The
SCRictorKO mice with Rictor deletion in Sertoli cells exhibited azoospermia and sterility. Loss of Rictor disrupted cytoskeleton dynamics, accompanied by disruptions in BTB integrity and cell polarity, which caused testicular developmental defects, spermiogenic arrest and excessive germ cell apoptosis in seminiferous epithelium. Further clarification of the mTOR pathway in Sertoli cell function and spermatogenesis may facilitate the development of improved treatments for spermatogenic defects and male infertility.

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