Research Article

Cell-autonomous requirement for mammalian target of rapamycin (*Mtor*) in spermatogonial proliferation and differentiation in the mouse[†]

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Abstract

Spermatogonial stem cells must balance self-renewal with production of transit-amplifying progenitors that differentiate in response to retinoic acid (RA) before entering meiosis. This self-renewal vs. differentiation fate decision is critical for maintaining tissue homeostasis, as imbalances cause defects that can lead to human testicular cancer or infertility. Little is currently known about the program of differentiation initiated by RA, and the pathways and proteins involved are poorly defined. We recently found that RA stimulation of the Phosphatidylinositol 3-kinase (PI3K)/AKT/Mammalian target of rapamycin (mTOR) kinase signaling pathway is required for differentiation, and that short-term inhibition of mTOR complex 1 (mTORC1) by rapamycin blocked spermatogonial differentiation *in vivo* and prevented RA-induced translational activation. Since this phenotype resulted from global inhibition of mTORC1, we created conditional germ cell knockout mice to investigate the germ cell-autonomous role of MTOR in spermatogonial differentiation. MTOR germ cell KO mice were viable and healthy, but testes from neonatal (postnatal day (P)8), juvenile (P18), and adult ($P > 60$) KO mice were smaller than littermate controls, and no sperm were produced in adult testes. Histological and immunostaining analyses revealed that spermatogonial differentiation was blocked, and no spermatocytes were formed at any of the ages examined. Although spermatogonial proliferation was reduced in the neonatal testis, it was blocked altogether in the juvenile and adult testis. Importantly, a small population of self-renewing undifferentiated spermatogonia remained in adult testes. Taken together, these results reveal that MTOR is dispensable for the maintenance of undifferentiated spermatogonia, but is cell autonomously required for their proliferation and differentiation.

Summary Sentence

Germ cell specific *Mtor* knockout mice exhibit a phenotype in which spermatogonia self-renew in adult mice, but fail to proliferate, differentiate, and complete spermatogenesis.

Key words: spermatogenesis, spermatogonia, testis, fertility, *Mtor*, mTORC1.

INTRODUCTION

Millions of spermatozoa are produced in the mammalian testis each day during steady-state spermatogenesis throughout the lengthy male reproductive lifespan. This sustained productivity depends on the action of spermatogonial stem cells (SSCs), which divide to produce daughter cells that replenish the SSC population (self-renewal) or become undifferentiated progenitor spermatogonia. These undifferentiated progenitor spermatogonia proliferate prior to initiating the weeklong process of differentiation in response to retinoic acid (RA) that precedes the formation of the first meiotic preleptotene spermatocytes [1–5]. Differentiation is essential as there have been no reports in the literature indicating that spermatogonia can initiate meiosis without first completing these differentiation divisions [6–8]. However, it is unclear what cellular and molecular changes must occur downstream of RA in spermatogonia to make them capable of entering meiosis [1].

One of the reasons so little is known about the proliferation and differentiation phases of spermatogonial development is that there are relatively few population-level changes in steady-state mRNA levels for specific genes during these processes [9, 10], which has prevented the identification of key molecules and pathways. Retinoic acid does indeed stimulate the transcription of a few genes in spermatogonia, including *Stra8* [6, 11–13] and *Rec8* [14], although neither gene encodes a protein required for spermatogonial differentiation [7, 15, 16]. However, our laboratory recently reported that the administration of exogenous RA to mice stimulated precocious expression, in prospermatogonia and undifferentiated spermatogonia, of protein markers (in addition to STRA8 and REC8) that are required for differentiation, including KIT, SOHLH1, and SOHLH2 [6, 17]. Retinoic acid increased the polyribosome occupancy for these mRNAs without concomitant increases in their abundance [6, 17]. We found that expression of these translationally suppressed mRNAs required the action of the PI3K/AKT/Mammalian target of rapamycin (mTOR) kinase signaling pathway [17, 18]. Taken together, it is apparent that RA can elicit two downstream responses: the transcriptional activation of a select few target genes and the enhanced translation of suppressed mRNAs via activation of kinase signaling pathways.

The “mammalian, or mechanistic target of rapamycin” *Mtor* encodes a master regulatory protein kinase that modulates a diverse array of cellular processes such as growth, proliferation, metabolism, differentiation, autophagy, protein synthesis, and cytoskeletal regulation (reviewed in [19–24]). A wide variety of intracellular and extracellular stimuli can direct these events, which occur differentially in a cell-type-specific manner. MTOR acts through two distinct protein complexes: mTORC1 and mTORC2. As part of mTORC1, the main role is the stimulation of cap-dependent protein translation by phosphorylating two critical downstream substrates: “eukaryotic translation initiation factor 4E binding protein 1” (EIF4EBP1) and “ribosomal protein S6 kinase” (RPS6KB1/2) (reviewed in [21, 24]).

Reports from our laboratory and others have implicated MTOR signaling as an important molecular regulator of spermatogonial development [18, 25–28]. In recent studies, germ cell conditional knockouts were created for *Tsc1* and *Tsc2* [26, 29], which encode upstream negative regulators of mTORC1 and mTORC2 (reviewed in [30–34]). In these studies, *Ddx4*-Cre expression resulted in germline deletion of these genes as early as embryonic day (E)15.5 [35], resulting in upregulation of mTORC1 (and potentially mTORC2) in fetal prospermatogonia. In both reports, there was a partial phenotype with some amount of increased spermatogonial differentiation

that appeared to occur at the expense of SSC self-renewal in some tubules, while adjacent tubules had seemingly normal spermatogenesis [26, 29]. The loss of *Tsc1* resulted in ~50% reduction in cauda epididymal sperm and smaller litter sizes [29], and similar results would be expected in the *Tsc2* KO mice [26]. We also recently inhibited mTORC1 by systemic administration of rapamycin to neonatal pups, and reported that it prevented the RA-induced translation of KIT, SOHLH1, and SOHLH2; blocked spermatogonial differentiation; and prevented the appearance of preleptotene spermatocytes during the first wave of spermatogenesis [18]. This approach inhibited mTORC1 in germ cells and in all somatic cells throughout the body, leaving open the possibility that mTORC1 is not required in a germ cell-autonomous manner to direct spermatogonial differentiation.

Here, we extend those findings to directly test the cell-autonomous requirement for mTORC1 signaling in spermatogonial development by generating germ cell-specific deletion of *Mtor*, which is an essential component of mTORC1 and mTORC2. Our results indicate that neonatal prospermatogonia transition to spermatogonia and begin to proliferate in the absence of MTOR, but that these spermatogonia fail to differentiate. The proliferation of subsequent spermatogonia in *Mtor* KO mice is reduced, causing a dramatic decrease in overall germ cell numbers by P18 and in adult mice ($P > 60$). However, adult testes from *Mtor* KO mice retain small numbers of undifferentiated spermatogonia that likely represent the SSC population. Taken together, our results reveal that MTOR is not essential for spermatogonial survival and self-renewal, but is required for both the proliferation and differentiation of progenitor spermatogonia during steady-state spermatogenesis.

MATERIALS AND METHODS

Generation and care of experimental animals

All animal procedures were carried out in adherence with protocols adherent to the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of East Carolina University (AUP #A194). *Mtor* germ cell KO mice were created by crossing female mice carrying a floxed *Mtor* allele (#0011009, The Jackson Laboratory) with young (<P60) male mice carrying one floxed allele as well as the *Ddx4*-Cre transgene (#006954, The Jackson Laboratory). The resulting WT and KO pups were on a mixed background (C57Bl/6/129S4/SvJae/FVB). Floxed *Mtor* and Cre alleles were identified by PCR-based genotyping (primers: *Mtor* Forward 5'-TTATGTTTGATAATTGCAGTTTGGCTAGCAGT, *Mtor* Reverse- 5'-TTTAGGACTCCTTCTGTGACATACATTTTCT, Cre Forward 5'-CTAAACATGCTTCATCGTCGGTCC, and Cre Reverse 5'-GGATTAACATTCTCCACCGTCAG). The following numbers of mice were analyzed at each of these ages: P8 – 5 WT and 2 KO, P18 – 4 WT and 2 KO, $P > 60$ – 4 WT and 4 KO. Bands were resolved on 3% agarose gels. For BrdU incorporation assays, adult males were intraperitoneally injected twice daily for 8 days (1.5 mg of BrdU per day, Invitrogen).

Cell quantitation from frozen testis sections

Immunostaining was performed to mark the entire germ cell population using antibodies against DDX4 (cytoplasmic pan germ cell marker in the neonatal testis [8]) or TRA98 (nuclear pan germ cell marker at all developmental stages [36, 37]). All Sertoli cells were marked using an antibody against GATA4. Antibody information

Table 1. Antibodies

Protein	Vendor (Catalog Number)	Dilution
STRA8	Abcam (ab49602)	1:3000
ZBTB16	Santa Cruz (sc-22839)	1:500
c-PARP1	Cell Signaling Technology (#9544)	1:100
DDX4	R&D Systems (AF2030), Abcam (ab13840)	1:800, 1:1000
GATA4	Santa Cruz (sc-1237)	1:100
GFRA1	Proteintech (AF560)	1:800
KIT	Cell Signaling Technology (#3074)	1:1000
MTOR	Cell Signaling Technology (#2983P)	1:400
p-RPS6	Cell Signaling Technology (#5364P)	1:800
TRA98	Abcam (ab82427)	1:1000

is provided in Table 1. Quantitation of germ cells expressing fate marker genes was carried out as previously described [38]. Briefly, all germ cells detectable within distinct microscope fields were counted from testes of two *Mtor* germ cell KO mice at P8 and two *Mtor* KO mice at P18. Labeled cells were deemed positive or negative for a marker using the threshold tool in Image J (U.S. National Institutes of Health) using the program's default algorithm. Thresholds used were as follows: DDX4 = 100–255, KIT = 40–255, GFRA1 = 90–255, STRA8 = 70–255, ZBTB16 = 50–255, TRA98 = 25–255. The numbers of cells deemed positive for each marker were divided by the total numbers of germ cells labeled by DDX4 or TRA98, and the percentage was obtained by multiplying by 100. The total numbers of DDX4+ germ cells and GATA4+ Sertoli cells were reported per testis cord cross section. DDX4+ and GATA4+ cells were counted from >20 cord cross sections across distinct microscopic fields. Apoptotic germ cells were identified by staining for cleaved PARP1 and the pan germ cell marker DDX4 and quantitated across two discrete microscope fields (at $\times 200$) per animal. Images for quantitation were acquired with a Fluoview FV1000 laser scanning confocal microscope (Olympus America).

Cauda epididymal sperm counts

Cauda epididymides were dissected immediately following euthanasia. Each epididymis was placed into 1 ml of room temperature 1X PBS, cut into small pieces, and repeatedly mixed by pipetting. Samples were diluted 1:10 with distilled water to immobilize sperm, and duplicate counts were obtained per epididymis using a hemocytometer.

Histological analysis and indirect immunofluorescence

Histological analyses were carried out on sections of paraffin-embedded Bouin's-fixed testes. Nuclear and cytoplasmic structures were visualized following standard hematoxylin and eosin (H&E) staining. Images were captured using an Axio Observer A1 microscope (Carl Zeiss Microscopy) equipped with an XL16C digital camera and Exponent version 1.3 software (Dage-MTI). Testes were immersion-fixed in fresh 4% paraformaldehyde, washed in 1X PBS, incubated overnight in 30% sucrose at 4°C, and frozen in O.C.T. Five micrometer sections were incubated in blocking reagent (1X PBS containing 3% BSA+ 0.1% Triton X-100) for 30 min at room temperature. Primary antibodies were diluted with blocking reagent and incubated on tissue sections for 1 h at room temperature. The primary antibodies used are listed in Table 1. Primary antibody was omitted from one section in each procedural replicate to serve as a negative control. Following three stringency washes in 1X PBS + 0.1% Triton X-100, sections were incubated in blocking reagent containing secondary antibody (Alexa Fluor-405, -488, -555, or -

559 at a 1:1000 dilution, Invitrogen) and/ or phalloidin-635 (1:500, Invitrogen) for 1 h at room temperature. Stringency washes were performed as above. To detect BrdU incorporation, an additional 1-h incubation in 2M HCl was performed prior to immunostaining. Labeling of BrdU+ nuclei was accomplished using a fluorescently conjugated Alexa Fluor 488 Anti-BrdU antibody (1:50, Life Technologies). Coverslips were mounted using Vectastain containing DAPI (Vector Laboratories), except in cases when acid treatment or Alexa Fluor-405 secondary was used. Images of stained tissues were obtained using a Fluoview FV1000 laser scanning confocal microscope (Olympus America).

Statistics

Statistical analyses were performed using the Student *t*-test, and the level of significance was set at $P < 0.05$. Error bars indicate one standard deviation.

Results

Mtor is ablated specifically in germ cells

We generated germ cell-specific *Mtor* KO mice by *Ddx4*-cre-mediated deletion, resulting in excision of exons 1–5 [39] as early as E15.5 in fetal prospermatogonia [35]. This approach should ensure sufficient time for loss of encoded protein in quiescent precursor prospermatogonia (~E15.5-P2). We first verified that MTOR protein was indeed absent in germ cells of KO mice. Although MTOR was readily detectable in the cytoplasm of WT spermatogonia and Sertoli cells at P8, no MTOR was detectable in spermatogonia of the germ cell KO testis (Figure 1A–F). We further verified loss-of-active MTOR by assessing the phosphorylation status of RPS6, an established downstream substrate that is phosphorylated by RPS6KB1 in response to activated mTORC1 (reviewed in [20–22]). There was no detectable p-RPS6 in KO spermatogonia, in contrast to those from WT littermate controls (Figure 1G–L). Taken together, these results confirm that germ cell-specific *Ddx4*-Cre-mediated deletion of *Mtor* occurred with high specificity and efficiency.

Germ cell development is disrupted in Mtor KO animals

Next, we determined the effect that loss of *Mtor* had on germ cell development. Germ cell *Mtor* KO mice appeared healthy, and were grossly indistinguishable from littermate controls. There were no significant differences in body weight between WT and KO animals at P8, P18, and $P > 60$ (Figure 2A). There was, however, a statistically significant decrease in testis weights at each of these ages (Figure 2B). This decrease was reflected in the gross morphology of KO testes at P18 and $P > 60$ (Figure 2C). P8 *Mtor* KO testes appeared grossly normal (data not shown). There was no significant difference in paired seminal vesicle weights from $P > 60$ mice (171.53 ± 28.13 mg in KO, 183.80 ± 27.71 mg in WT).

Dramatically reduced testis size and weight during development reflects a loss in cellularity, which typically affects the expanding germ cell population. To assess this, we performed histological analysis of H&E-stained testis sections from P8, P18, and $P > 60$ *Mtor* WT and KO mice. We first examined testes from KO mice at P8, a time when WT testes normally contain type A_{undiff}, A_{diff}, intermediate, and type B spermatogonia as well as the first meiotic preleptotene spermatocytes [40]. At P8, *Mtor* KO testes contained abundant spermatogonia located adjacent to the basal aspect of the tubules, but there were no apparent preleptotene

Figure 1. Confirming MTOR deletion in KO testes. (A–F) Immunostaining for MTOR (in green) reveals a clear signal in DDX4+ spermatogonia (in red) from WT but not KO mice. Co-stained cells are shown in yellow. (G–L) Phosphorylated RPS6 (in green) is present in P8 WT DDX4+ spermatogonia (in red), but not in KO spermatogonia. Scale bar (in A) = 50 μ m.

Figure 2. Deletion of *Mtor* results in reduced testis size and weight and apparent loss of differentiating germ cells. (A) Average body weight (in g) of *Mtor* WT and KO mice at P8, 18, and $P > 60$ (adult). (B) Average testis weights (in mg) of *Mtor* WT and KO in P8, 18, and $P > 60$ mice. (C) Images of WT and KO littermate testes at P18 and $P > 60$. (D–I) Hematoxylin and eosin-stained representative sections are shown from *Mtor* WT and KO testes at P8 (D, E) P18 (F–G), and $P > 60$ (H, I). Preleptotene spermatocytes are encircled (in D) with a white dashed circle. Double asterisks denote statistical significance at $P < 0.01$. Error bars indicate one standard deviation. Scale bar (in D) = 50 μ m.

spermatocytes, in contrast to WT littermate controls (Figure 2D and E). This result resembles previous work from our laboratory, in which we showed that global inhibition of mTORC1 by rapamycin blocked spermatogonial differentiation in the neonatal testis in vivo [18]. The most advanced germ cells present in P18 WT testes are pachytene spermatocytes (Figure 2F, [40, 41]). However, seminiferous tubules in P18 *Mtor* KO testes contained only Sertoli cells and spermatogonia (Figure 2G). This phenotype was maintained in the adult, as the only remaining germ cells were identifiable as undifferentiated spermatogonia (Figure 2H and I). Not surprisingly, no sperm was present in the cauda epididymides of adult *Mtor* KO animals (0.0 ± 0.0 in KO, $13.18 \pm 6.66 \times 10^6/\text{ml}$ in WT controls, $P < 0.01$). Because there were no sperm present, we did not perform fertility trials.

We next assessed the size of the germ cell population at P8, and determined there was a 4-fold reduction within *Mtor* KO testes as compared to those from WT littermate controls (Figure 3A and B). Of note, a significant number of these DDX4+ germ cells in the P8 WT testes were preleptotene spermatocytes, which were absent in the KO (see Figure 2E). There were no significant differences in

the numbers of GATA4+ Sertoli cells in WT and KO testis sections (Figure 3C and D). There was no increase in the numbers of cleaved PARP1 (c-PARP1)+ spermatogonia in *Mtor* KO testes, revealing no significant increase in apoptosis (Figure 3E and F). We also determined that the proportion of germ cells expressing the proliferation marker MKI67 was not different in P8 WT or KO testes, indicating that similar numbers of spermatogonia were proceeding through the cell cycle (e.g. not in G_0) in WT and KO mice (Figure 3G and H).

Germ cell differentiation is impaired in neonatal P8 *Mtor* KO mice

Histological analysis revealed an absence of preleptotene spermatocytes as early as P8, and a clear reduction in numbers of spermatogonia in P18 and adult testes (see Figure 2F–I). Therefore, we next assessed whether germ cell loss of MTOR altered the proportions of undifferentiated and differentiating spermatogonia. We first performed immunostaining for ZBTB16 (also termed PLZF), which we previously reported was a marker of most spermatogonia in the

Figure 3. Deletion of *Mtor* reduces the P8 germ cell population without increasing quiescence or apoptosis. In each row, representative images of immunofluorescence for specific proteins and accompanying quantitation are shown for P8 WT and KO testes. (A, B) The germ cell population is labeled by DDX4 (green) and nuclei are stained with DAPI (blue). (C, D) GATA4+ Sertoli cells are labeled in red and nuclei are stained blue with DAPI. (E, F) The germ cell population is labeled by DDX4 (in blue) and apoptotic cells are labeled by c-PARP1 (in green). (G, H) Proliferating germ cells in any stage of the cell cycle other than G_0 are positive for MKI67 protein, which is labeled in green. All germ cells are labeled for DDX4 (in red) and nuclei are labeled with DAPI (in blue). Double asterisks denote statistical significance at $P < 0.01$. Error bars indicate one standard deviation. Scale bar (in A) = 50 μ m.

neonatal testis at P7 (both A_{undiff} and $\sim 80\%$ of A_{diff} spermatogonia), but became restricted to A_{undiff} spermatogonia by P10 [8]. Similar to those previous results [8], we found that $\sim 60\%$ of DDX4+ germ cells were ZBTB16+ in the WT testis (Figure 4A). In contrast, $\sim 96\%$ of P8 KO germ cells were ZBTB16+, indicating that they were A_{undiff} spermatogonia (Figure 4B). We next assessed GFRA1, which is a more restricted A_{undiff} marker in the developing testis [8]. A much larger percentage of P8 KO germ cells were GFRA1+ (40% in KO versus only 12% in WT testes) (Figure 4C and D, $P = 0.001$). To assess the extent of differentiation, we immunostained for the receptor tyrosine kinase KIT (also termed c-KIT), which is activated by RA, is required for differentiation, and is the only bona fide marker of differentiating spermatogonia [17, 42–49]. Less than 10% of spermatogonia in P8 KO animals were KIT+, and those exhibited faint staining; this is in stark contrast to the 50% of spermatogonia containing readily detectable KIT protein in WT P8 testes (Figure 4E and F). *Stra8* is also normally transcribed in response to RA, and is readily detectable at the protein level in differentiating spermatogonia and especially in preleptotene spermatocytes [6, 8, 11–13, 15, 17]. STRA8 was readily detectable in $\sim 13\%$ of germ cells in P8 *Mtor* KO animals, which was a noticeable reduction from values observed in WT littermates in which $\sim 28\%$ of germ cells contained detectable STRA8 (Figure 4G and H). These results reveal that a smaller proportion of *Mtor* KO germ cells activates *Stra8* in response to RA, but that *Mtor* ablation does not block germ cells' ability to respond to RA in this manner. The significant reduction in KIT+ differentiating spermatogonia and absence of spermatocytes in KO testes indicates that spermatogonial differentiation is disrupted in neonatal *Mtor* germ cell KO mice.

Spermatocytes are absent from P18 *Mtor* KO testes

We next examined WT and *Mtor* KO mice at P18, when WT testes contain all spermatogonial subtypes as well as more advanced germ cell types including preleptotene, leptotene, zygotene, and pachytene spermatocytes (Figure 2F, [40]). We examined the fate of the remaining spermatogonia in P18 *Mtor* KO testes. The percentage of germ cells positive for markers of A_{undiff} spermatogonia (ZBTB16 and GFRA1) was significantly higher in P18 *Mtor* KO testes than in those from WT littermate controls. In KO animals, $\sim 75\%$ were ZBTB16+ and $\sim 25\%$ of remaining germ cells were GFRA1+, whereas in WT littermates these proteins were detectable in less than 10% of the germ cell population. (Figure 5A–D). KIT was undetectable in P18 *Mtor* KO spermatogonia, indicating that remaining germ cells were A_{undiff} spermatogonia (Figure 5E and F). The number of c-PARP1+ apoptotic cells within seminiferous tubule cross sections was not statistically different between P18 WT and KO testes (Supplemental Figure S1). However, the number of MKI67+ proliferating germ cells was greatly reduced in P18 KO testes, with only 33% of P18 KO spermatogonia containing detectable MKI67 as compared to the 70% that were MKI67+ in WT littermate testes (Figure 5G and H). Together, these results indicate that only undifferentiated spermatogonia remain in P18 *Mtor* KO testes, and that many are KI67–, and likely in G_0 of the cell cycle.

Only small numbers of undifferentiated spermatogonia remain in $P > 60$ adult *Mtor* KO testes

We assessed the long-term consequences of loss of MTOR on steady-state spermatogenesis by examining adult *Mtor* KO mice. Histological analyses of H&E-stained $P > 60$ testis sections revealed that seminiferous epithelia of adult *Mtor* KO mice contained Sertoli cells and a

small number of spermatogonia with morphology resembling A_{undiff} spermatogonia (see Figure 2H and I) [50–52]. To assess whether these spermatogonia were maintained longer term, we immunostained P150 adult *Mtor* KO testes, and found that all remaining spermatogonia were ZBTB16+/GFRA1+ and KIT–, revealing that they were A_{undiff} spermatogonia (Figure 6A–F). Spermatogonia in P150 *Mtor* KO were not all quiescent (in G_0).

Steady-state spermatogenesis in adult mammals is directed by the actions of a slow-cycling pool of SSCs, and failure in spermatogonial self-renewal in adult mice results in exhaustion of the germ line [5]. The presence of MKI67+ spermatogonia in P150 KO testes suggested that *Mtor* KO germ cells can maintain their population through self-renewing divisions (see Figure 6G and H). To assess whether this population was simply maintained from a younger age or undergoing self-renewing divisions, we treated $P > 60$ adult KO and WT mice with the synthetic thymidine nucleotide analog BrdU for 8 days. This represents one cycle of the seminiferous epithelium (reviewed in [4, 53, 54]), and should provide sufficient time for dividing spermatogonia to progress through S-phase. Immunostaining revealed that *Mtor* KO germ cells were indeed BrdU+, indicating that they retain the ability to self-renew and maintain their population (Figure 7).

DISCUSSION

Here, we report the cell-autonomous role for MTOR in normal spermatogonial development. Surprisingly, we find it is not essential for the transition of prospermatogonia to spermatogonia, proliferation of the first spermatogonia in the neonatal testis, or the self-renewal of spermatogonia in the adult testis. Together, this reveals a cell-autonomous requirement for MTOR function in the continuous proliferation and differentiation of spermatogonia in the developing testis.

These results are consistent with those from our previous work, in which we showed that systemic administration of rapamycin to neonatal mice led to a complete block of spermatogonial differentiation [18]. The similarity between the phenotypes (global inhibition of mTORC1 with rapamycin and deletion of *Mtor* in prospermatogonia reported here) is intriguing. Rapamycin preferentially inhibits mTORC1, although in prolonged treatments with high dosages can also inhibit mTORC2 [55]. In contrast, *Mtor* deletion inactivates both mTORC1 and mTORC2 [20, 21]. The fact that loss of mTORC1 and mTORC2 did not result in a more severe phenotype than rapamycin treatment alone suggests that MTOR acts primarily through mTORC1 in spermatogonia, and that mTORC2 function may be dispensable for early male germ cell development. Other recent reports support this important role for mTORC1 in spermatogonial differentiation. Two groups independently created male germ cell KO models (also using *Ddx4*-cre, [35]) for TSC1 and TSC2, upstream negative regulators of mTORC1 activity [26, 29]. They found that loss of TSC1 and TSC2 led to hyperactivation of mTORC1, which increased the proportion of differentiating spermatogonia at the expense of the undifferentiated population. Surprisingly, the reproductive phenotype of these mice was incomplete, with testes of KO mice containing a mixture of normal-appearing tubules adjacent to degenerating tubules missing germ cells and germ cell layers. Adult TSC1 germ cell KO mice had a 2-fold reduction in cauda epididymal sperm, but were fertile [29]. The fertility of TSC2 germ cell KO mice was not evaluated, but based on the similarities of TSC1/2 function in the mTORC1 pathway and the observed phenotype it is likely they exhibit similarly reduced but not lost fertility

Figure 4. Spermatogonial differentiation is blocked in *Mtor* KO mice at P8. In each row, representative images of immunofluorescence for specific spermatogonial fate marker proteins (colors indicate the specific marker on each panel), including ZBTB16 (A, B), GFRA1 (C, D), KIT (E, F), and STRA8 (G, H) along with DDX4 to mark all germ cells (in red, A, B and E–H). Nuclei are labeled (in A–F) with DAPI, in blue. The accompanying quantitation for WT and KO testes is presented alongside each panel. Asterisks denote statistical significance (* $P < 0.05$, ** $P < 0.01$). Error bars indicate one standard deviation. Scale bar (in A) = 50 μ m.

Figure 5. Only undifferentiated spermatogonia are retained in P18 *Mtor* KO testes. In each row, representative images of immunofluorescence for specific spermatogonial fate marker proteins (colors indicate the specific marker on each panel), including ZBTB16 (A, B), GFRA1 (C, D), and KIT (E, F) as well as the proliferation marker MKI67 (G, H) along with TRA98 to mark all germ cells. Nuclei are labeled with DAPI, in blue. The accompanying quantitation for WT and KO testes is presented alongside each panel. Asterisks denote statistical significance (** $P < 0.01$). Error bars indicate one standard deviation. Scale bar (in A) = 50 μ m.

Figure 6. Adult (P150) *Mtor* KO testes contain only undifferentiated spermatogonia. Spermatogonia are labeled for the undifferentiated markers GFRA1 (A, B) and ZBTB16 (C, D), the differentiating marker KIT (E, F), or the proliferating marker MKI67 (A, B). Examples of spermatogonia positive for each marker are indicated by white arrowheads. TRA98 (in red) was used to label all germ cells in (A, B, E–H). Nuclei are labeled (in blue) with DAPI, and the seminiferous tubules are outlined with dashed white lines. Scale bar (in A) = 50 μ m.

Figure 7. Adult *Mtor* KO spermatogonia incorporate BrdU. Immunostaining was done to localize the pan germ cell marker TRA98 (in red) and the synthetic nucleotide analog BrdU (green) in testes of adult *Mtor* WT (A) and KO (B) animals. Examples of BrdU⁺ germ cells are indicated by white arrows and the boundaries of seminiferous tubules are outlined with dashed white lines. Scale bar (in A) = 50 μ m.

[26]. Mice with global loss of REDD1, another negative regulator of mTORC1, were also fertile [56]. Taken together, studies using these KO models reveal that increased mTORC1 activity in the male germline does alter the distribution of spermatogonial subtypes in the seminiferous epithelium, but does not affect all spermatogonia and does not result in infertility. It is possible that partial compensation by remaining mTORC1 inhibitors preserves a portion of the undifferentiated spermatogonial pool. Nonetheless, the results presented here as well as in the literature clearly indicate that control of mTORC1 signaling is cell autonomously required for normal spermatogonial development.

Our results demonstrate that MTOR is not required for the prospermatogonia-to-spermatogonia transition, which involves reentry into the cell cycle and movement from the center to the periphery of the testis cords [52, 57–60]. Also, undifferentiated spermatogonia are abundant in KO testes at P8, indicating that the initial expansion of the neonatal spermatogonial pool therefore occurred independently of MTOR. The significant ~4-fold decrease in the DDX4⁺ germ cell population is largely due to missing differentiating spermatogonia and preleptotene spermatocytes, which are generally

positioned near the center of the testis cords at this age (see Figures 2D and E, 3 and 4).

However, the continued proliferation of spermatogonia does not happen in *Mtor* KO testes after P8, as there were dramatic reductions in spermatogonial numbers in testes of P18 and $P > 60$ mice. The initial population of proliferating and differentiating spermatogonia during the first wave of spermatogenesis in the neonatal mouse testis is thought to form directly from precursor prospermatogonia. Evidence in support of this comes from studies using morphological characterization [50–52, 61–64], analysis of KO models [65–72], and lineage tracing [73]. Together, these studies reveal an increased reliance upon SSCs as spermatogenesis proceeds in the developing testis. The results presented here reinforce that model by providing evidence that the first spermatogonia formed in the neonatal testis behave differently (proliferating in the absence of MTOR) than those at P18 and $P > 60$. It is currently unclear why there is a differential requirement for MTOR in spermatogonia from these later ages.

MTOR plays a central role in maintaining homeostasis within multiple tissues, and can variously regulate stem cell self-renewal, progenitor proliferation, and/or differentiation. These disparate

effects depend on the cell type examined, as can be seen from excellent examples in the literature. In chondrocytes, mTORC1 is required for proliferation and initiation of differentiation [74]. In human embryonic stem cells and mouse osteoblasts, mTORC1 is essential for proliferation and for repression of differentiation [75, 76]. Similar to the results in spermatogonia, mTORC1 was shown to be required for the proliferation and differentiation of CD4⁺ T lymphocytes into effector T cells [77, 78]. The activation of nearly quiescent naïve T lymphocytes is accompanied by DNA replication and increased biosynthesis of proteins, lipids, and other macromolecules in order to produce the building blocks required for cell growth and proliferation (reviewed in [79]). These anabolic functions place an increased energetic demand on proliferating and differentiating cells, which may signal through mTORC1 to increase glycolysis and/or oxidative phosphorylation.

It is increasingly clear that tight control over mTORC1 activation is required for normal spermatogonial proliferation and differentiation in the developing testis. Our results presented here show that MTOR is not essential for spermatogonial survival and self-renewal in the adult testis, but that it is required for normal proliferation in the neonate, for any proliferation in the adult, and for differentiation at all stages of development. Future studies will elucidate the specific downstream cellular events mediated by MTOR that are necessary for coordination of survival, proliferation, and differentiation of spermatogonial populations in the mammalian testis.

Supplementary data

Supplementary data are available at [BIOLRE](http://biolre.onlinelibrary.com) online.

Supplemental Figure S1. Apoptosis is not increased in P18 testes. Immunostaining was done to localize c-PARP1 (in green) in P18 testes, and sections were costained with fluorescently conjugated phalloidin to label F-actin (in red). C-PARP1⁺ germ cells are indicated by white arrows. Scale bar (in A) = 50 μ m.

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