

Mechanistic target of rapamycin (mTOR) pathway in Sertoli cells regulates age-dependent changes in sperm DNA methylation

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Abstract

Over the past several decades, a trend toward delayed childbirth has led to increases in parental age at the time of conception. Sperm epigenome undergoes age-dependent changes increasing risks of adverse conditions in offspring conceived by fathers of advanced age. Animal experiments also demonstrate that epigenetic transfer of maladaptive information of advanced age fathers is associated with reduced offspring health including reduced life span. The mechanism(s) linking paternal age with epigenetic changes in sperm remain unknown. The sperm epigenome is shaped in a compartment protected by the blood-testes barrier (BTB) known to deteriorate with age. Permeability of the BTB is regulated by the balance of two mTOR complexes in Sertoli cells. We hypothesized that this balance is also responsible for age-dependent changes in the sperm epigenome. Experiments with transgenic mice demonstrate that the shift of the balance in favor of mTOR complex two rejuvenates sperm DNA-methylome while the shift in favor of mTOR complex one accelerates aging of the sperm DNA-methylome and results in a reproductive phenotype concordant with older age. These results demonstrate for the first time that the balance of mTOR complexes in Sertoli cells regulates the rate of sperm epigenetic aging. Thus, mTOR pathway in Sertoli cells may be used as novel target of therapeutic interventions to rejuvenate the sperm epigenome in advanced-age fathers.

eLife assessment

This work shows for the first time that the balance of mTOR (mTORC1 and mTORC2) in Sertoli cells regulates the rate of sperm epigenetic aging. The manuscript presents **valuable** findings that have some theoretical and practical implications. The strength of the evidence is however **incomplete**: they are limited in some places and the aims are not always fully supported by the results.

Introduction

Over the past several decades, a trend toward delayed childbirth has led to increases in parental age at the time of conception. This delayed parenthood is attributed to secular and socioeconomic factors (Waldenström, 2016), reproductive technological advancement (Bray et al., 2006), and higher levels of post-graduate education (Mills et al., 2011). Emerging evidence suggests that higher male age contributes to poor pregnancy outcomes (Hassan & Killick, 2003), lower odds of live birth (Horta et al., 2019), and adverse health of offspring in later life (Montgomery et al., 2004; Puleo et al., 2012; Saha et al., 2009), including increased susceptibility to early development of cancer (Contreras et al., 2017), as well as neurodevelopmental, and psychiatric disorders such as schizophrenia (Gratten et al., 2016) and autism (Reichenberg et al., 2006). In experiments with mice it was demonstrated that higher male age is associated with reduced life span in offspring (Xie et al., 2018). Increasing evidence links advanced paternal age with altered offspring phenotype via age-dependent changes in the sperm epigenome (Ashapkin et al., 2022; Jenkins et al., 2014). Published data indicate that age is a powerful factor affecting DNA methylation and other epigenetic markers in mammalian sperm (Ashapkin et al., 2022), and age-dependent changes in these epigenetic mechanisms are involved in the regulation of key developmental pathways, including nervous system-related signaling, Wnt, Hippo, mTOR, and Igf1 (Guo et al., 2021; Ma et al., 2020; Pilsner et al., 2021; Suvorov et al., 2020; C. Wu et al., 2020; Xie et al., 2018; Yoshizaki et al., 2021). Despite this evidence, the mechanistic link between age and epigenetic changes in sperm remains unknown.

Final DNA methylation profiles of spermatozoa are a result of epigenetic events that occur during spermatogenesis (H. Wu et al., 2015), including meiotic divisions of spermatogonia and spermatocytes associated with passive loss of methylation and *de novo* methylation (Oakes et al., 2007). Global methylation drop of around 12-13% occurs in preleptotene spermatocytes and methylation is gradually reestablished during leptotene-pachytene stages so that the final DNA methylation patterns are obtained by the end of pachytene spermatocyte stage (Gaysinskaya et al., 2018; Loukinov et al., 2002; Oakes et al., 2007). A recent rat study identified the transition from elongating spermatids to late spermatids as another stage associated with changes in methylation of more than 5,000 DNA regions (Omri-Charai et al., 2023). After fertilization, most parental-specific epigenetic marks of gametes undergo reprogramming to establish totipotency in the developing embryo. However, imprinted loci, certain classes of repetitive sequences, and other genomic loci escape this reprogramming event and contribute to a non-Mendelian form of inheritance as demonstrated in humans (Atsem et al., 2016; Denomme et al., 2020) and rodents (Oluwayiose et al., 2021; Xie et al., 2018), suggesting that the final sperm epigenome acquired during spermatogenesis is a significant channel for the transfer of inheritable information to the next generation (Lisner & Kimmins, 2023).

Epigenetic events during spermatogenesis occur in the unique biochemical environment of the apical compartment of seminiferous epithelium, protected from body fluids by the blood-testis barrier (BTB) -the tightest blood-tissue barrier composed of coexisting tight junctions, basal ectoplasmic specializations, gap junctions, and desmosomes in Sertoli cells (Fig. 1). The BTB regulates the entry of chemical compounds, including nutrients, hormones, electrolytes, and harmful toxicants, thus creating a unique and highly selective environment for germ cells undergoing epigenetic reprogramming (Mok, Mruk, & Cheng, 2013). In rodents, age-dependent declines in fertility are caused by deterioration of the BTB (Levy et al., 1999; Paul & Robaire, 2013). Recent studies demonstrate that BTB integrity is determined by the balance between activities of the two complexes of the serine/threonine kinase mechanistic target of rapamycin (mTOR) in Sertoli cells, whereby mTOR complex one (mTORC1) promotes disassembly of the BTB and mTOR complex two (mTORC2) promotes its integrity (Li & Cheng, 2016; Mok, Mruk, & Cheng, 2013; Mok, Mruk, Lee, et al., 2013) (Fig. 1). mTOR expression is highest in testes out of all

tissues in humans (Fagerberg et al., 2014) and mice (Yue et al., 2014), and the mTOR pathway is recognized today as a major mechanism of longevity and aging regulation (Papadopoulos et al., 2019; Weichhart, 2018). Thus, we hypothesized that the balance of mTOR complexes in Sertoli cells may also play a significant role in age-dependent changes in the sperm epigenome. In the current study we test this hypothesis using transgenic mice with manipulated activity of mTOR complexes in Sertoli cells.

Material and Methods

Transgenic mice with Sertoli-specific inactivation of mTORC1 or mTORC2

To inactivate mTORC1, we used a Raptor (component of mTORC1) knockout model. To inactivate mTORC2, we used a Rictor (component of mTORC2) knockout model. To achieve Sertoli-specific knockouts, mice with floxed Rictor or Raptor (Rictor^{tm1.1Klg/SjmJ} and B6.Cg-Rptor^{tm1.1Dmsa/J} respectively) were crossed with mice with Cre recombinase controlled by the Amh Sertoli-specific promoter (129S.FVB-Tg(Amh-cre)8815Reb/J). All mice were purchased from Jackson Laboratories (Stock #: 020649, 013188 and 007915 respectively). In short, to generate Raptor Sertoli-specific KO mice, male B6.Cg-Rptor^{tm1.1Dmsa/J} mice were paired with female 129S.FVB-Tg(Amh-cre)8815Reb/J mice. In the next round of breeding F1 females were paired with the same B6.Cg-Rptor^{tm1.1Dmsa/J} males. In F2 offspring around 25% had genotype Raptor^{flox/flox}, and 25% had genotype Raptor^{flox/flox}, Amh^{cre/+}. Animals with these two different genotypes were paired together to produce F3 mice, which were used for sperm collection and reproductive phenotype assessment. F3 male mice were represented by 2 genotypes: Sertoli-specific KO of Raptor (suppressed mTORC1), and wildtype (WT) animals used as controls. The same breeding scheme was used to produce F3 male mice with Sertoli-specific KO of Rictor (suppressed mTORC2) and their corresponding WT siblings. At weaning all animals were identified via ear-punching, and ear tissue samples obtained from ear-punching were used for genotyping. Genotyping was done using RT-qPCR as recommended by the Jackson Laboratories. All animals were housed in a temperature (23 ± 2°C), and humidity (40 ± 10 %) controlled environment, with a 12-h light/dark cycle, and food and water available ad libitum. F3 male mice were euthanized at 4 time-points: 8, 12, 22 and 56 weeks of age (n ≥ 5 per genotype/timepoint). At each euthanasia testes were collected, weighed, and fixed in modified Davidson's solution and caudal epididymal sperm was collected via swim-up procedure (see below, Sperm collection and DNA extraction). All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the approval for this study was received from the Institutional Animal Care and Use Committee at University of Massachusetts, Amherst.

Immunohistochemistry

Testes samples collected from 22-week-old mice were fixed in modified Davidson's fluid, dehydrated through a series of alcohols and xylene, and embedded with Paraplast X-tra paraffin (Leica Biosystems) using the Excelsior ES Tissue Processor (Thermo Fisher). Five-micrometer sections were cut on a Microm HM 355S microtome (Fisher) and mounted on Colorfrost Plus slides (Fisher). Immunohistochemistry (IHC) was performed on a DakoCytomation autostainer, using the envision HRP Detection system (Dako, Carpinteria, CA). Sections were deparaffinized in xylene, rehydrated in graded ethanols, and rinsed in Tris-phosphate-buffered saline (TBS).

Heat induced antigen retrieval was performed in a microwave for 20 minutes at 98°C in 0.01 M citrate buffer (pH6) for Rictor antibody and Tris-EDTA (pH9) for Raptor antibody. After cooling for 20 min, sections were rinsed in TBS and subjected to the primary rabbit polyclonal anti-Rictor (1:100, Abcam, ab70374), rabbit monoclonal anti-Raptor [EP539Y] (1:100, Abcam, ab40768) for 60 and 30 minutes respectively. After subsequent washes in TBS, slides were incubated with

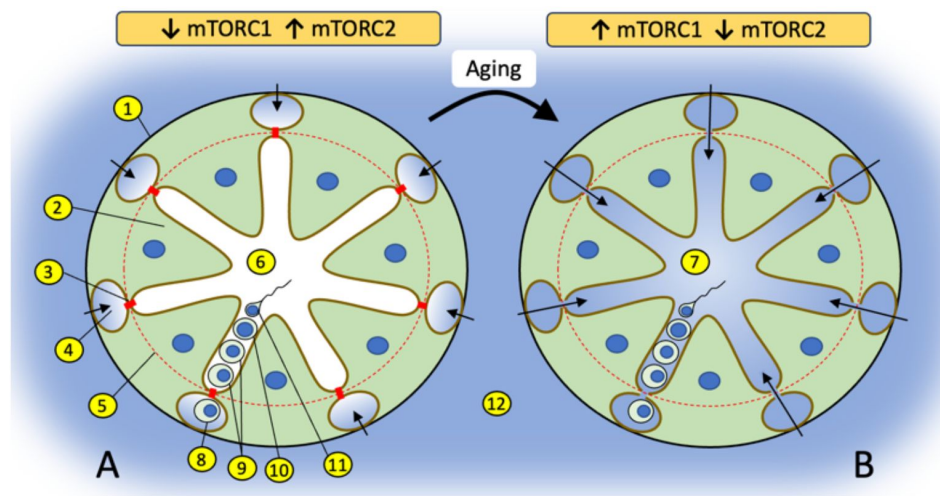


Fig. 1.

Changes in the balance of mTOR complexes and aging affect biochemical conditions of spermatogenesis in the apical compartment of seminiferous epithelium.

(A) Seminiferous tubule with tight BTB where interstitial environment (shown in blue) penetrates only to basal compartment of seminiferous epithelium as show by color and arrows. The tight state of the BTB is promoted by higher activity of mTORC2 over mTORC1 in Sertoli cells. **(B)** Seminiferous tubule with leaky BTB where interstitial environment penetrates to the apical compartment of seminiferous epithelium. BTB disassembly is promoted by higher activity of mTORC1 over mTORC2. (1) basal membrane of a seminiferous tubule; (2) Sertoli cell; (3) contact between Sertoli cells enforced by gap junction, tight junction and endoplasmic specialization (BTB); (4) basal compartment of the seminiferous epithelium; (5) the dotted red line illustrates structural separation of compartments by the BTB; (6) apical compartment of the seminiferous epithelium; (7) apical compartment in aged organism with biochemically “noisy” environment due to the leaky BTB; (8) spermatogonia; (9) spermatocytes; (10) spermatid; (11) spermatozoa; (12) interstitial environment.

secondary antibody (DAKO Envision Flex+ anti-rabbit polymer, K800921-2) for 20 minutes. Immunoreactivity was visualized by incubation with chromogen diaminobenzidine (DAB) (DAKO, K3468) for 10 min. Tissue sections were counterstained with Mayer's hematoxylin (Poly Scientific R&D Corp, S216), dehydrated through graded ethanols and xylene, and cover-slipped. Sections were viewed through a Zeiss Axio Observer Z1 inverted light microscope with ZEN imaging software. A minimum of 20 images per slide were captured at 88000 dpi using the AxioCam 506 color digital camera and analyzed for differences in antibody staining in seminiferous tubules at different stages.

BTB permeability analysis

The permeability of the BTB was assessed in all WT and KO mice euthanized at 22 weeks of age using a biotin tracer assay as described elsewhere (25). In short, animals were anesthetized with isoflurane, their testes were exposed, a small opening was created in the tunica albuginea of the right testes, and 50 μ l of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Life Technologies) in PBS containing 1 mM CaCl₂ was injected into the interstitium. After 30 min, mice were euthanized, and their testes were immediately removed and fixed in modified Davidson's solution. Testis were embedded in paraffin and 5- μ m sections. Deparaffinized slides were incubated with 5 μ g/ml Streptavidin Alexa Fluor 405 conjugate in PBS at 25°C for 1 hour, mounted using Diamond Antifade Mountant (P36965), and incubated for 24 hours at room temperature in dark room. The imaging was performed using A1R-SiMe confocal microscope (Nikon) at the light microscopy facility at the Institute of Applied Life Sciences, University of Massachusetts Amherst.

Sperm collection and DNA extraction

At each euthanasia, the right and left cauda epididymides were incised, cut three times, and incubated at 37°C for 30 minutes in 1 ml of sperm wash buffer (Cat. # ART1006, Origio, Denmark). After incubation the epididymides were removed, the tube was vortexed for 10 sec and 10 μ l of each sample was smeared evenly across the whole surface of the glass slide for microscopic analysis. The remaining samples were used for DNA extraction following the rapid method developed in J.R. Pilsner's laboratory (43). In short, sperm samples were subjected to a one-step density gradient centrifugation over 40% Pureception buffer (CooperSurgical Cat # ART-2100) at 600g for 30 min in order to remove possible somatic contamination. Sperm cells were homogenized with 0.2 mm steel beads at room temperature in a mixture containing guanidine thiocyanate lysis buffer and 50 mM tris(2-carboxyethyl)phosphine (TCEP). The lysates were column purified using QiaAMP DNA mini-Kit (Qiagen, Cat # 56304) and DNA quality of the eluate was determined using Nanodrop 2000 Spectrophotometer (#E112352; Thermo Scientific, Somerset, NJ). The DNA samples were stored at -80°C.

Sperm quality analysis

Sperm smears were fixed by immersing in 3% glutaraldehyde in PBS (PH=7.2) for 30 minutes, washed briefly in PBS (PH=7.2) and allowed to dry at room temperature. Smears were stained by 5% aniline blue in 4% acetic acid (PH=3.5) for 5 minutes, washed briefly in PBS (PH=7.2) and allowed to dry at room temperature. Slides were covered with a cover slip using mounting solution incubated at room temperature for 30 minutes and examined using an inverted light microscope at 40 X magnification. The sperm quantity was assessed by counting spermatozoa in 10 random windows on each slide. For sperm morphology analysis, 200 spermatozoa were counted, and number of abnormal spermatozoa was recorded.

Mitochondrial DNA Copy Number

Sperm DNA was used to measure mitochondrial copy number via real-time quantitative PCR using a published protocol (44). The primers used to calculate relative mitochondrial copy number include a primer pair for the mitochondrial genome (5'-CTCCGTGCTACCTAAACACCTTATC-3' and 5'-GACCTAAGAAGATTGTGAAGTAGATGATG-3') and another primer pair for nuclear genome for a

single copy Apob gene (5'-CACGTGGGCTCCAGCATT-3' and 5'-TCACCAGTCATTCTGCCTTTG-3'). Triplicate 5- μ l real-time PCR reactions, each containing iTaq Universal SYBR Green Supermix (Cat 172–5124, BioRad), primers, and cDNA template were loaded onto a 384-well plate and run through 40 cycles on a CFX384 real time cycler (Bio-Rad Laboratories, Inc). The data were analyzed using the manufacturer's CFX manager software, version 3.1. Relative quantification was determined using the $\Delta\Delta C_q$ method.

Library Preparation and Reduced Representation Bisulfite Sequencing (RRBS)

Sperm DNA methylation was analyzed at 8 and 22 weeks of age, with four randomly selected individual samples per age and genotype (Rictor KO, Rictor WT, Raptor KO, Raptor WT). Bisulfite conversion was performed on 100 ng of genomic DNA using the EpiTect Fast DNA Bisulfite kit from Qiagen (Cat.# 59824) and DNA libraries were constructed using the NUGEN Ovation RRBS Methyl-seq System (Cat.# 0353) according to the manufacturer's instructions. Libraries were sequenced on Illumina HiSeq 4000 at the Deep Sequencing Core Facility of the University of Massachusetts Medical School (Shrewsbury, MA) with an average of 22.7 million single-end reads (50-bp) per sample.

RRBS data analysis

Raw reads were trimmed using TrimGalore (v 0.6.6) and a NuGEN-specific adaptor trimming scripts available from GitHub (nugentechnologies/NuMetRRBS). Trimmed reads were mapped using Bismark-Bowtie2 with no mismatch allowed. Methylation counts were called using Bismark extract. Differentially methylated regions were identified using the Methyl kit (v 1.24.0) pipeline (45). In brief, the genome was tiled into sliding windows and a weighted methylation level was calculated for each window. To minimize error in base calling, we filtered out bases with less than 10x coverage and read counts more than 99.9th percentile and coverage values were normalized using default settings. We used a logistic regression model for *p*-value calculation subsequently adjusted for multiple comparison (FDR) using the SLIM method for final DMR identification. Individual DMRs were identified for a 100 bp sliding window with a minimum of 1 CpG in at least 3 samples out of 4 in an age/genotype group. DMRs with methylation difference at FDR < 0.05 were used to compare different age/genotype groups. To compare the overlap between DMRs induced by age and DMRs induced by KO we used Fisher's exact test. Genomic ranges were compared using the FindOverlapOfPeaks function available in the chiPeakAnno package (46). The total number of methylation regions used as a background was determined as a list of 100 bp tiles containing CpGs obtained by intersecting lists from two respective comparisons (e.g. WT8 vs WT22 and WT22 vs KO22 in mTORC1 experiment and WT8 vs WT22 and WT8 vs KO8 in mTORC2 experiment). Spatial genomic annotation was conducted using annotatr package (v1.24.0) (47) and annotated to the genomic features of the Ensembl genome. Genomic features were compared using the GenomicRanges package (v1.50.2) (48). Each DMR was assigned to the closest gene (<5 kb upstream transcription start site, promoter, 5'UTR, exon, or intron) or intergenic region, and graphs were plotted using ggplot2 package (v3.4.0). We used Metascape (29) to analyze biological categories associated with genes annotated to DMRs. For global methylation changes DMRs were analyzed for a 1000 bp sliding window with at least 3 CpGs and methylation difference > 10% at FDR < 0.05. The original RRBS data files (fastq) are available via Dryad (28).

Results

Approach to manipulate the balance of mTOR complexes

To study if shifting the balance of mTOR complexes activity in either direction in Sertoli cells will induce sperm DNA methylation and other reproductive changes concordant with accelerated or decelerated aging, we generated two transgenic mouse models with manipulated mTOR complexes

activity. We used the Cre-Lox approach to knockout (KO) critical components of mTORC1 or mTORC2 – Raptor and Rictor, respectively. In both experiments, Cre recombinase was controlled by the anti-Müllerian hormone (Amh) promoter to insure KO of Raptor or Rictor in Sertoli cells only (**Fig. 2**). We used immunohistochemistry with antibodies for Raptor and Rictor to test efficiency of our genetic manipulations. Indeed, staining of Sertoli cells with Raptor and Rictor antibodies was abolished in corresponding KO animals (Fig. S1). BTB permeability was measured in 22-week old KO mice and their wildtype (WT) siblings using a biotin tracer assay, where biotin is injected into the testis interstitium *in-vivo*, and its diffusion into the apical compartment of seminiferous epithelium is visualized in histological sections by streptavidin conjugated with a fluorescent dye (Meng et al., 2005). As predicted, in mice with suppressed mTORC2 the BTB was loose enough to allow biotin to migrate to the lumen of seminiferous tubules (**Fig. 3**).

Age dependent changes in sperm methylome affect methylation of developmental genes

We used reduced representation bisulfite sequencing (RRBS) to analyze DNA-methylation in sperm to test if manipulating mTOR complexes balance may accelerate or decelerate epigenetic aging of sperm. For this analysis we first identified regions of DNA that undergo significant changes in methylation in wildtype animals. To do so, we compared profiles of DNA methylation between 8 and 22-weeks old WT mice in each of the 2 experiments (**Fig. 2**). These ages correspond to young pubertal and late mature adult stages in humans (Bell, 2018; Flurkey et al., 2007). Methylation changes were analyzed for 100 bp windows containing a minimum of 1 CpG. Average number of CpGs per region was 10.18 ± 0.08 and 10.32 ± 0.09 (mean $\pm$ SE) in mTORC1 and mTORC2 experiments respectively (Fig. S2). Significant DMRs were defined as any 100 bp window with methylation difference at $FDR < 0.05$. Using this approach, we identified 1731 age-dependent DMRs in the mTORC1 experiment and 797 DMRs in the mTORC2 experiment, with 79 common DMRs identified in both experiments. In both experiments age-dependent changes were dominated by an age-dependent increase in methylation (Fig. S3A,B). We assigned each DMR to a gene with overlapping gene body or to a closest gene with transcription start site within 5000 bp downstream of the DMR (Amir et al., 2023) and used Metascape (Zhou et al., 2019) to analyze biological categories associated with differentially methylated regions. Furthermore, we determined genomic elements associated with significant DMRs (Fig. S4A,B). This analysis demonstrated that in both experiments, lists of genes associated with significant age-dependent DMRs are enriched with developmental categories (**Fig. 4A,B**), indicative of the possibility that age-dependent changes in sperm DNA methylation may be causative of the transfer of different developmental epigenetic information to the next generation. These findings are concordant with previous research (Pilsner et al., 2021; Suvorov et al., 2020; Xie et al., 2018). Specifically, common highly enriched categories across both experiments included: tube morphogenesis, brain development, cell morphogenesis, and other categories relevant to growth, embryo development, development of nervous system, muscles, and others. Enriched molecular mechanisms were dominated by small GTPase mediated signal transduction and regulation of kinase activity (protein phosphorylation, signaling by receptor tyrosine kinase, and other). The complete results of enrichment analysis can be found at Dryad (Amir et al., 2023).

Decreased activity of mTORC1 “rejuvenates” sperm methylome

At the next step, we analyzed if Sertoli-specific inactivation of mTORC1 affects methylation of age-dependent DMRs. Given that age-dependent changes in the sperm methylome are associated with age-dependent increase in the BTB permeability, we assumed that decreased permeability of the BTB due to genetic manipulation may result in sperm epigenome “rejuvenation”. Thus, our hypothesis predicts that DNA regions that undergo methylation changes in wildtype animals with age will undergo the opposite change in older animals with Sertoli-specific inactivation of mTORC1 as compared to WT mice of the same age (**Fig. 2**). In other words, DMRs that change with age

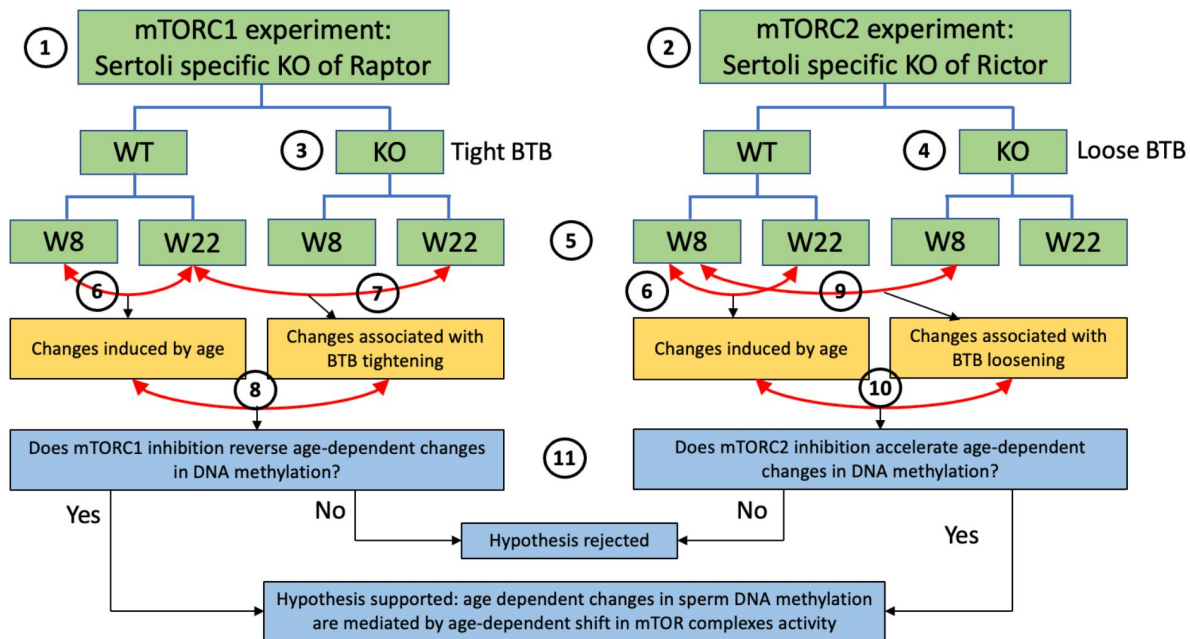


Fig. 2.

Scheme of experimental design for DNA methylation analysis.

This study utilized experiments with two transgenic mouse models: (1) one with mTORC1 suppression in Sertoli cells due to the cell-specific KO of Raptor, and (2) another with mTORC2 suppression in Sertoli cells due to the cell-specific KO of Rictor. Suppression of mTORC1 results in tightening of the BTB (3) and suppression of mTORC2 results in loosening of the BTB (4). DNA methylation changes were analyzed in sperm of each genotype on postnatal weeks 8 and 22 (5). In each experiment, we first identified physiological age-dependent changes in sperm DNA methylation by comparing epigenomes of WT 8-week old mice and WT 22-week old mice (6). We further used both experiments to test the hypothesis that age-dependent changes in the sperm epigenome are associated with the age-dependent increase in permeability of the BTB. Specifically, to test this hypothesis using the mTORC1 experiment, we compared physiological age-dependent changes (6) with changes induced by KO (tighter BTB) in older mice (7). Our hypothesis predicts that mTORC1 suppression in older mice will affect age-dependent DMRs in the direction opposite to the one induced by age (8). Similarly, to test our hypothesis using the mTORC2 experiment, we compared physiological age-dependent changes (6) with changes induced by KO (loose BTB) in younger mice (9). Our hypothesis predicts that mTORC2 suppression in young mice due to KO will produce similar effects on age-dependent DMRs as age itself (10). Outcomes of both experiments were used to support or reject our hypothesis (11).

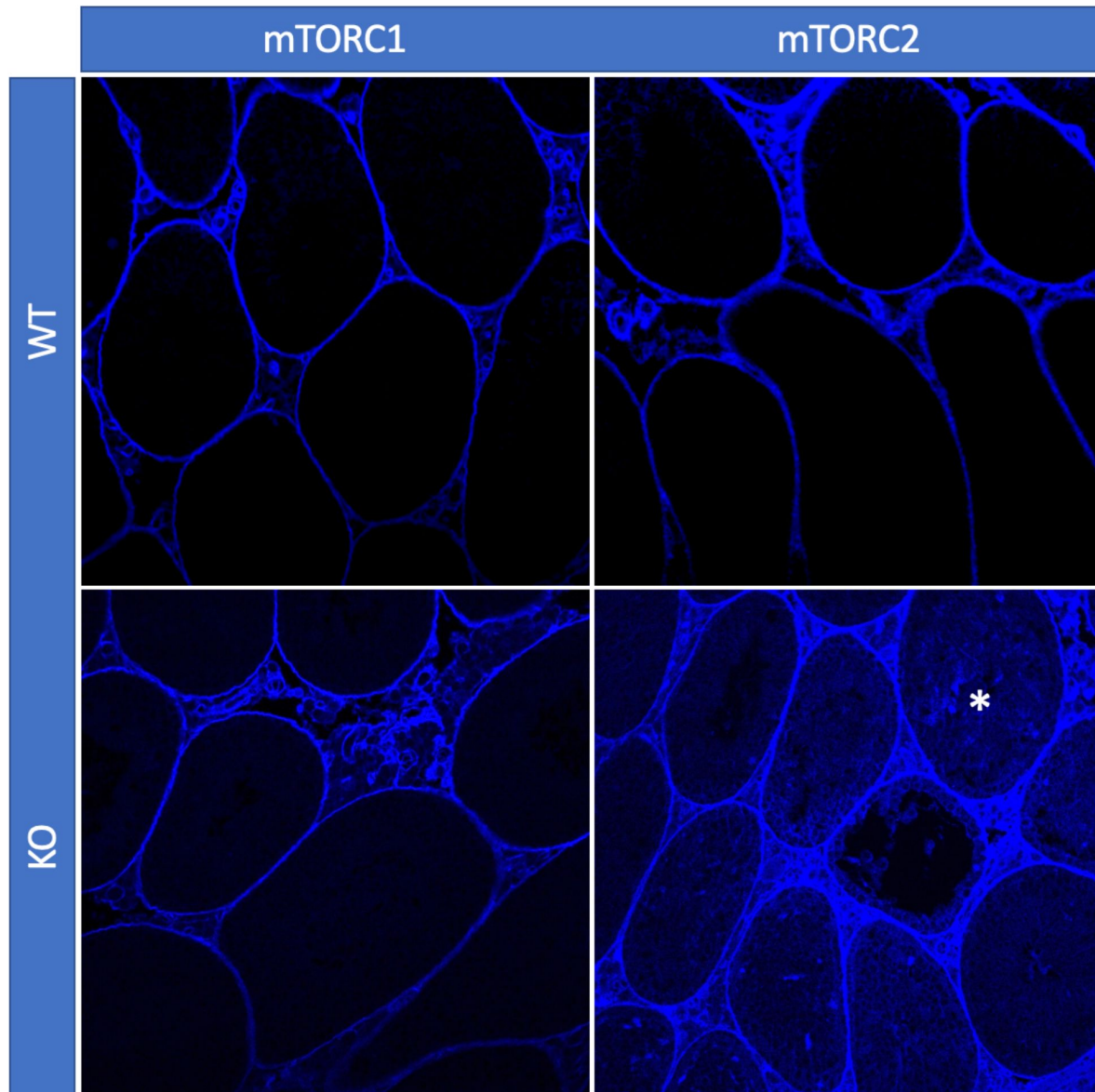


Fig. 3.

BTB permeability in 22-week old mice of different genotypes, representative images.

In mice with inactivated mTORC2 (Rictor KO), biotin tracer (blue) penetrated to the apical compartment of the seminiferous epithelium (asterisk) while in all other genotypes it did not cross the BTB.

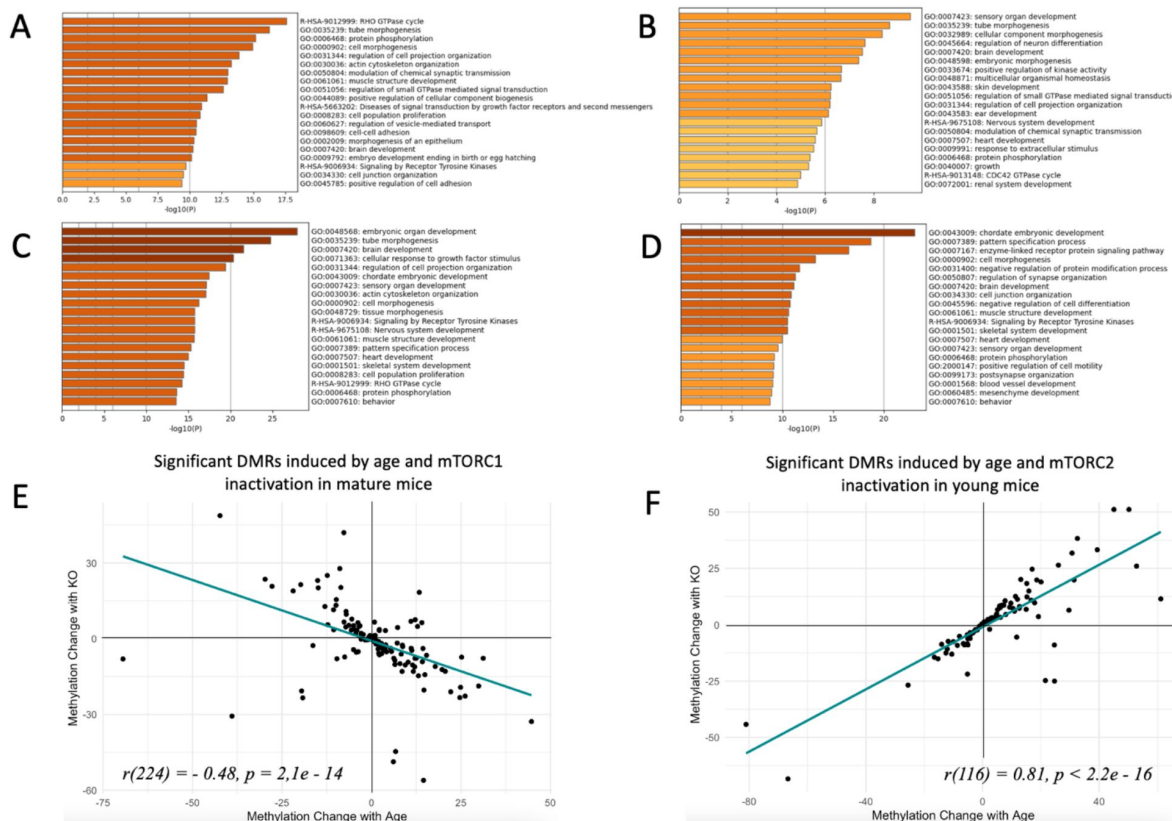


Fig. 4.

Changes in sperm DNA methylation induced by age and manipulation of mTOR pathway in Sertoli cells.

(A, B, C, D) Top biological categories enriched with genes associated with significant DMRs: (A) age-dependent DMRs in wildtype animals in the mTORC1 experiment; (B) age-dependent DMRs in wildtype animals in the mTORC2 experiment; (C) DMRs induced by mTORC1 inactivation in mature animals; (D) DMRs induced by mTORC2 inactivation in young animals. (E and F) Changes in methylation of significant DMRs induced by age and mTORC1 inactivation in mature mice (E) and by age and mTORC2 inactivation in young mice (F).

will be returned to their younger state by mTORC1 inactivation. We identified 2738 significant DMRs induced by mTORC1 inactivation in mature mice (Amir et al., 2023 [↗](#)) and compared them with age-dependent DMRs in WT animals. Most of DNA regions induced by genetic manipulation of Raptor undergo hypomethylation, -the direction of change opposite to the one induced by age (Fig. S3C). The distribution of genic elements associated with DMRs was similar in relation to age and genetic manipulation, suggesting that both factors affect the similar sets of elements (Fig. S4C). Next, we analyzed an overlap between significant age-dependent and KO-dependent DMRs in the list of all methylation regions identified in both comparisons. According to this analysis the two lists were highly overlapping with $p < 0.00001$, indicative that age and mTORC1 inactivation in Sertoli cells affect the similar sets of DMRs in sperm (Fig. S5A). Finally, we analyzed the direction of change of overlapping DMRs (Fig. 4E [↗](#)). We observed a strong negative correlation between methylation changes induced by age and changes induced by mTORC1 inactivation ($r = -0.48$, $p = 2.1e-14$), suggesting that in mature animals, mTORC1 inactivation in Sertoli cells returns age-dependent DMRs to their younger state. The correlation was even higher for DMRs that undergo 10% or higher change in relation to age or genetic manipulation ($r = -0.68$, $p = 2.3e-4$, Fig. S5C).

Decreased activity of mTORC2 accelerates epigenetic aging of sperm

Similarly, we assumed that if age-dependent changes in the sperm methylome are associated with age-dependent increase in the BTB permeability, then increased permeability of BTB due to Sertoli-specific inactivation of mTORC2 may be associated with accelerated aging of the sperm epigenome. Thus, our hypotheses predicts that DNA regions that undergo methylation changes in wildtype animals with age will undergo similar changes in young animals with Sertoli-specific inactivation of mTORC2 as compared with same age young WT mice (Fig. 2 [↗](#)). We identified 1632 significant DMRs induced by decreased mTORC2 activity in Sertoli cells of young mice (Amir et al., 2023 [↗](#)). The majority of the DNA regions with methylation changes induced by genetic manipulation of Rictor undergo hypermethylation, - the direction of change concordant with accelerated sperm aging (Fig. S3D). The distribution of genic elements associated with DMRs was similar in relation to age and genetic manipulation, suggesting that both factors affect similar sets of elements (Fig. S4D). Further, we conducted an overlap analysis between significant age-dependent and KO-dependent DMRs in the list of all methylation regions identified in both comparisons. According to this analysis the two lists were highly overlapping with $p < 0.00001$, indicative that age and mTORC2 inactivation in Sertoli cells affect the similar sets of DMRs in sperm (Fig. S5B). Finally, we analyzed the direction of change of overlapping DMRs (Fig. 4F [↗](#)). We observed a strong positive correlation between methylation changes induced by age and changes induced by mTORC2 inactivation ($r = 0.81$, $p < 2.2e-16$), suggesting that in young animals, mTORC2 inactivation in Sertoli cells accelerates epigenetic aging of sperm. The correlation was even higher for DMRs that undergo 10% or higher change in relation to age or genetic manipulation ($r = 0.83$, $p < 2.6e-8$, Fig. S5D).

Manipulations of mTOR complexes affect methylation of developmental genes

Given our data demonstrate that DNA methylation changes induced by suppression of mTOR complexes affect age-dependent genes in sperm, it is not surprising that in both experiments (mTORC1 and mTORC2 suppression) the lists of genes associated with significant KO- dependent DMRs were enriched with developmental categories (Fig. 4C,D [↗](#)), indicative that age-dependent changes in mTOR pathway and in the BTB permeability may be causative of the transfer of altered developmental epigenetic information to the next generation. Specifically, enriched categories common for both experiments included brain development, heart development, chordate embryonic development, pattern specification process, cell morphogenesis, muscle structure development, signaling by receptor tyrosine kinases, skeletal system development, sensory organ development, behavior, and protein phosphorylation. Thus, there is a high concordance of

developmental categories enriched with genes associated with sperm methylation changes induced by natural aging (**Fig. 4A,B**) and induced by changes in mTOR complexes activity balance (**Fig 4C,D**) (see also Dryad dataset (Amir et al., 2023)).

Decreased mTORC2 activity is concordant with aged reproductive phenotype

We next analyzed if Sertoli-specific manipulation of the mTOR pathway may result in reproductive phenotypes concordant with age-related changes. It was demonstrated previously that testes weight and sperm counts decrease with age, while the percent of morphologically abnormal spermatozoa increases with age in humans and laboratory rodents (Bujan et al., 1988; Gunes et al., 2016). Additionally, mitochondria DNA copy number (mtDNAcn) increases with age in human sperm (Zhang et al., 2016) and it was shown recently that sperm mtDNAcn is a novel biomarker of male fecundity (Rosati et al., 2020; H. Wu et al., 2019). Thus, to analyze if manipulated mTOR pathway affects known age-dependent reproductive parameters, we analyzed testes size, sperm counts, sperm morphology and mtDNAcn in mice of all genotypes across four timepoints covering ages that are equivalent to critical reproductive stages in humans: 8 week old – young pubertal; 12 and 22 weeks old – beginning and ending of the mature adult stage; respectively; and 56 weeks old – advanced paternal age (Bell, 2018; Flurkey et al., 2007). Concordant with our hypothesis, mice with inactivated mTORC2 (permanently “leaky” BTB) demonstrated significant decreases in testis weight and sperm counts concomitant with increase in morphologically abnormal sperm and mtDNAcn (**Fig. 5A-D**). Inactivation of mTORC1 resulted in testes weight decrease and slight increase in mtDNAcn but did not affect sperm parameters significantly. These results suggest that although permanent changes of the mTOR complexes balance in either direction may have negative consequences for some reproductive parameters, mice with inactivated mTORC2 in Sertoli cells have a reproductive phenotype concordant with accelerated aging.

Discussion

An accumulating body of literature indicates that the sperm epigenome responds to a broad range of factors, including paternal diet (Watkins et al., 2018), physical activity (Benito et al., 2018), stress (L. Wu et al., 2016), metabolic status (Donkin et al., 2016; Wei et al., 2014), and environmental exposures (H. Wu et al., 2017), among others. Age is the most powerful known factor affecting DNA methylation in mammalian sperm (reviewed at (Ashapkin et al., 2022)), and age-dependent changes in epigenetic mechanisms in sperm are involved in the regulation of major developmental pathways (Ashapkin et al., 2022). Importantly, advanced paternal age at conception programs offspring life span for shorter duration via non-Mendelian transfer of inheritable information by spermatozoa (Xie et al., 2018). Additionally, the rates of sperm epigenetic aging (DNA methylation and small noncoding RNA) may be modified by exposures to environmental xenobiotics (Pilsner et al., 2021; Suvorov et al., 2020).

Despite this evidence, the mechanism(s) linking age or other paternal factors with epigenetic changes in sperm were unknown. The results of our experiments suggest that shifts of the balance of mTOR complexes activities in favor of mTORC1 or mTORC2 in Sertoli cells produce changes in sperm DNA methylation matching aging and rejuvenation respectively. Additionally, decreased activity of mTORC2 produced changes in reproductive parameters concordant with aging. Thus, mTOR pathway in Sertoli cells may be used as novel target of therapeutic interventions to rejuvenate the sperm epigenome in advanced-age fathers.

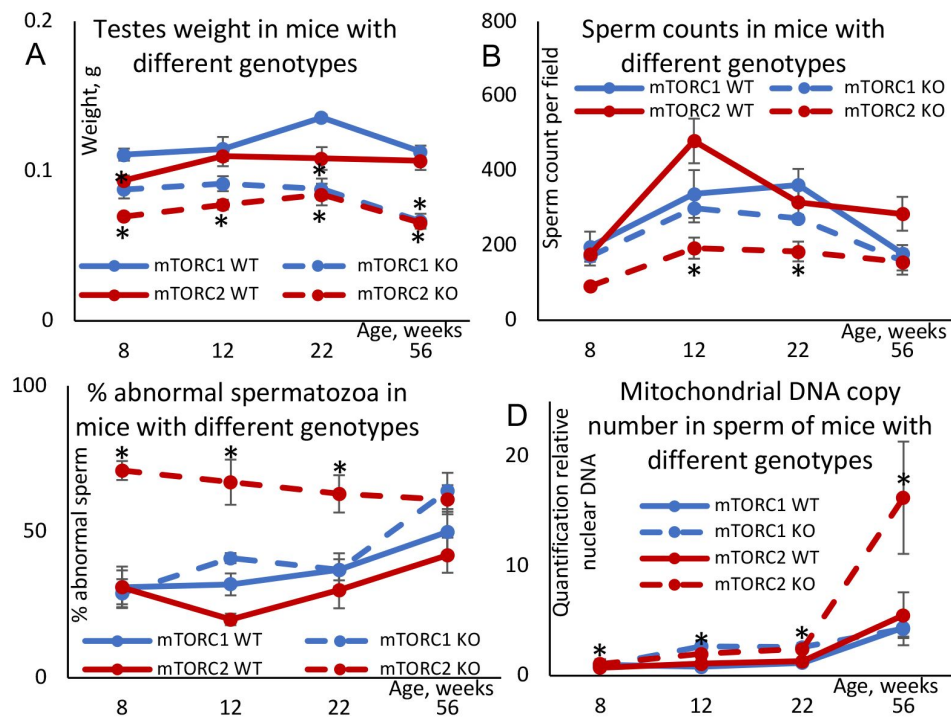


Fig. 5.

Changes in age-dependent reproductive parameters in mice with inactivated mTORC1 or mTORC2 in Sertoli cells.

(A) Testes weight. **(B)** Sperm counts in sperm smears. **(C)** Percent abnormal spermatozoa. **(D)** Mitochondrial DNA copy number. All data are Mean $\pm$ SE, $n = 5-6$ /age/genotype, ANOVA followed by Dunnett's test, * $p < 0.05$ when compared with corresponding WT.

Conflict of interest

AS is a CEO of ReGENE LLC. All other authors declare that they have no conflict of interest.

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Author contributions

Conceptualization: AS

Methodology: SA, OA, EM, JM, JRP, OO, OS, AS

Software: SA, OA, EM, OO Validation: SA, OA, EM Formal analysis: SA, OA, EM

Investigation: SA, OA, EM, JM, JRP, OO, OS, AS Resources: AS, JRP

Data Curation: SA, OA, EM Visualization: SA, OA, EM, JM, OO Funding acquisition: AS

Project administration: AS Supervision: JRP, AS Writing – original draft: AS

Writing – review & editing: SA, OA, EM, JM, JRP, OO, OS, AS

Data availability

Raw and processed DNA methylation data as well as complete results of Metascape enrichment analysis are available via Dryad ([Amir et al., 2023](#)). All other data are available in the main text or the Extended Data.

Supplementary Materials

Fig. S1. Immunohistochemistry analysis of the efficiency of Rictor and Raptor KO in Sertoli cells. Representative images illustrate lack of staining for Rictor and Raptor proteins in Sertoli cells in corresponding KO models. Arrows show staining by Rictor or Raptor antibodies respectively in Sertoli cells.

Fig. S2. Distribution of CpG per 100 bp regions. (A) All overlapping regions identified in age and genotype groups in mTORC1 inactivation experiment. (B) Significant ($q < 0.05$) overlapping regions identified in age and genotype groups in mTORC1 inactivation experiment. (C) All overlapping regions identified in age and genotype groups in mTORC2 inactivation experiment. (D) Significant ($q < 0.05$) overlapping regions identified in age and genotype groups in mTORC2 inactivation experiment.

Fig. S3. Methylation changes per chromosome in mice of different genotype and age. (A and B). Percent DMRs altered by age in WT animals in mTORC1 (A) and mTORC2 (B) inactivation experiments. (C) Percent DMRs in mature mice altered by mTORC1 inactivation. (D) Percent DMRs in young mice altered by mTORC2 inactivation.

Fig. S4. Genomic elements enriched with DMRs associated with age and inactivation of mTOR complexes. Changes induced by age in WT animals in mTORC1 (A) and mTORC2 (B) inactivation experiments. (C) Changes induced in mature mice by mTORC1 inactivation. (D) Changes induced in young mice by mTORC2 inactivation.

Fig. S5. Changes in methylation of significant DMRs induced by age and genetic manipulation of the mTOR pathway in Sertoli cells. (A) Overlap between significant DMRs induced by age and by mTORC1 inactivation in mature mice among all common methylation regions identified in both comparisons. (B) Overlap between significant DMRs induced by age and by mTORC2 inactivation in young mice among all common methylation regions identified in both comparisons. (C) Comparison of age-dependent changes and changes induced by mTORC1 inactivation in mature mice. (D) Comparison of age-dependent changes and changes induced by mTORC2 inactivation in young mice.

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Editors

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Reviewer #1 (Public Review):

In the manuscript "Mechanistic target of rapamycin (mTOR) pathway in Sertoli cells regulates age-dependent changes in sperm DNA methylation", the authors proposed to test if the balance of mTOR complexes in Sertoli cells may play a significant role in age-dependent changes in the sperm epigenome. The paper could be of interest and has a good scientific aim but there are too many drawbacks that hamper the initial enthusiasm. All sections need extensive revision. The paper is mostly descriptive without a mechanistic-orientated explanation for the observed results.

Specific comments:

1. The abstract is poorly written. There is a lot of unnecessary introduction that does not provide a rationale for the work. It is not possible to understand the experimental approach or the major data just by reading the abstract. It does not clearly represent the work.
2. The introduction is somewhat vague and does not provide a clear rationale for the hypothesis. There should be more focus more on the role of mTOR in Sertoli cells that goes far beyond BTB. That will give more focus on mTOR. Then it is important to focus on BTB and mTOR: what is known? What is the gap and how can it be solved? Several relevant references are missed concerning mTOR and Sertoli cells.
3. The Material and Methods section needs improvement. There is much important information missing. For instance: how many animals were used per group and how was the breeding done? At what age? Statistical analysis should be explained in detail.
4. The results description could be improved. It is vague without highlighting how much difference was detected. The results should be numerically described when possible and the differences should be highlighted. A 10% difference may be significant but not biologically relevant. To correctly evaluate the differences it is important to describe them with some degree of detail.
5. There is no discussion of the data. The authors just summarize their findings without a comprehensive analysis of the literature and how the effects can be mediated. mTOR interacts with different pathways (mTORC1 and mTORC2 are even mediators of distinct pathways). This would be very relevant to discuss. In addition, there are many study limitations not discussed. There is no clear mechanistic explanation of the way by which the mTOR pathway in Sertoli cells regulates age-dependent changes in sperm DNA methylation. The paper seems preliminary.
6. Figure 1 is too simple and does not provide any schematic support for the text.
7. Figure 2 lacks some detail. For instance, how many animals were used for each step?

8. Taking into consideration the roles of mTOR on sperm, particularly mTORC1, it is not clear whether there were any differences in sperm motility.

- <https://doi.org/10.7554/eLife.90992.1.sa2>

Reviewer #2 (Public Review):

In this study, the authors hypothesized that the balance of mTOR complexes in Sertoli cells may also play a significant role in age-dependent changes in the sperm epigenome. To test this hypothesis, the authors use transgenic mice with manipulated activity of mTOR complexes in Sertoli cells. These results suggest that the mTOR pathway in Sertoli cells may be used as a novel target of therapeutic interventions to rejuvenate the sperm epigenome in advanced-age fathers.

The authors attempt to demonstrate that the balance of mTOR complexes in Sertoli cells regulates the rate of sperm epigenetic aging. The authors have effectively met their research objectives, and their conclusions are supported by the data presented.

- <https://doi.org/10.7554/eLife.90992.1.sa1>

Reviewer #3 (Public Review):

Summary and Strength:

The manuscript by Amir et al. describes that Sertoli-specific inactivation of the mTORC1 and mTORC2 complex by KO of either Raptor or Rictor, respectively, resulted in progressive changes in blood-testis-barrier (BTB) function, testis weight, and sperm parameters, including counts, morphology, mtDNA content and sperm DNA methylation.

The described studies are based on the hypothesis that a decline of BTB function with increasing chronological age of a male contributes to the DNA methylation changes that are known to occur in sperm DNA of old males when compared to sperm DNA from isogenic young males. In order to demonstrate the relevance of a functioning BTB for the maintenance of sperm methylation patterns, the authors generated mice with genetically disrupted mTORC2 complex or mTORC1 complex in Sertoli cells and determined sperm methylation patterns in comparison to isogenic wild-type males. In line with previously published scientific literature (e.g. Mok et al., 2013; Dong et al, 2015; and others), the manuscript corroborates that a Sertoli-cell specific deletion of mTORC2 caused a loss of BTB function and a progressive spermatogenic defect. The authors further show that sperm DNA is differentially methylated (DMRs) as a consequence of either a mTORC2 disruption (associated with a loss of BTB function) or following a mTORC1 disruption (BTB function either increased or not leaky) when compared to their isogenic age-matched wt controls. Those DMRs overlap partially with changes in sperm DNA methylation that were found when comparing sperm from 8-week males with sperm isolated from 22-week-old male mice.

The authors interpret the observed changes as representative of the sperm DNA methylation changes that occur during normal chronological aging of the male. For an aged control group, the authors use sperm DNA of 22-week-old wild-type males from the mTORC2 and mTORC2 KO breeding and compare the sperm methylation patterns found in sperm from those 22-week males to 8-week young males, that are intended to represent an old and a young cohort, respectively. DNA methylation analysis indicates that a disruption of mTORC2 (& decrease of BTB function) results in increased DNA methylation of sperm DNA, while a disruption of mTORC1 (and proposed increase of BTB tightness, not shown in the manuscript, though) resulted in increased hypomethylation.

Weaknesses:

While the hypothesis and experimental system are interesting and the data demonstrating the relevance of the mTORC2 complex for BTB function is convincing, several open questions limit the evidence that supports the hypothesis that the sperm DNA methylation changes seen in old males are caused by BTB failure following an imbalance of mTOR signaling complexes. The major critique points are the lack of a chronologically old group and the choice of 8 weeks & 22 weeks age of age:

- Data illustrating the degree of BTB decline and sperm DNA methylation changes from chronologically "old" male mice is missing. 22-week-old mice are not considered old but are of good and mature breeding age, equivalent to humans in their mid-late twenties. (In the manuscript, the 22-week-old wildtype mice show no evidence of BTB breakdown (Figure 3), so why are their sperm used to represent "aged" sperm?
- Adding a group of "old" wild-type mice of 12-14 months of age, which is closer to the end of effective reproduction in mice, more equivalent to 45-59 year-old humans) could be used to illustrate that (a) aging causes a marked decrease in BTB function at this time in mouse life, and that this BTB breakdown chronologically aligns with the age-associated DNA hypermethylation seen in old sperm. Age-matched "old" mTORC1 KO, with a (supposedly) tighter BTB barrier, could then be expected to have a sperm DNA methylation profile closer to that of younger wild-type animals. Such data are currently missing. While the progressive testicular decline observed in the mTORC1 KO (Fig.5) could make it difficult to obtain the appropriately aged mTORC1 KO tissues, it is completely feasible to obtain data from chronologically old wild-type males. (The progressive testicular decline further raises the question of what additional defects the KO causes, and how such additional defects would influence the sperm DNA methylation profile.) The addition of data from an old group to the currently included groups could strengthen the interpretation that the observations in the BTB-defective mTORC2 KO mice are modelling an age-related testicular decline, provided that the DMRs seen in the chronologically old group significantly overlap with the BTB-defective changes.
- In the current form, the described differences in sperm DNA methylation are based on comparisons between pubertal mice (8 weeks) and mature but not old adult males (22 weeks), while a chronologically "old" group is missing from the data sets and comparisons. Thus, it appears that the described sperm methylation changes reflect developmental changes associated with normal maturation and not necessarily declining sperm quality due to aging. (Sperm obtained from 8-week-old mice likely were generated, at least in part, during the 1st wave of spermatogenesis, which is known to differ from the continuously proceeding spermatogenesis during the remainder of the mature life. During the 1st wave of spermatogenesis, Sertoli cells are known to undergo gene expression changes which could contribute to varying degrees of BTB function, and thus have effects on the sperm DNA methylation profiles of such 1st wave sperm.)
- It is unclear why the aging-related DMRs between the 8 and 22-week-old wild-type mice vary so dramatically between the two wild-type groups derived from the mTORC1 and the mTORC2 breeding (Fig. S4). If the main difference was due to mTORC1 or mTORC2 activity, both wildtype groups should behave very similarly. Changes seen in a truly "old" mouse (e.g. 20 weeks to 56 weeks), changes in "young mTORC1" and in "old mTORC2" are missing. How do those numbers and profiles compare to the shown samples?

Some general comments regarding the chosen age of animals:

- As mentioned, sperm from 8-week-old mice represent many sperm that were produced in the 1st wave of spermatogenesis; 22-week-old mice are not considered chronologically old mice, but mature and "relatively" young animals. 18-24 month-old mice are considered to be

equivalent to 56-69 year-old humans, and might be more suitable to detect aging effects. "Old mice" for study purposes should be at least 12-14 months of age, ideally >18 months of age. 22 weeks (5 months of age) are mice at good breeding age, but still considered mature adults, not old males, and therefore are not expected to show typical aging health problems (like declining fertility).

Even the cited reference (Flurkey et al. 2007) defines that "... mice used a reference group for "young mice" should be at least 3 months of age (~ 13 weeks), i.e. fully sexually mature. The authors specifically state: " The young adult group should be at least 3 months old because, although mice are sexually mature by 35 days, relatively rapid maturational growth continues for most biologic processes and structures until about 3 months. The upper age range for the young adult group is typically about 6 months. ... For the middle-aged group, 10 months is typically the lower limit.... The upper age limit for the middle-aged group is typically 14-15 months, because at this age, most biomarkers still have not changed to their full extent, and some have not yet started changing. For the old group, the lower age limit is 18 months because age-related change for almost all biomarkers of aging can be detected by then. The upper limit is 22-26 months, depending on the genotype." According to this reference, mice up to 6 months of age are generally considered "mature adults" (equivalent to humans 20-30 yrs), mice of 10-14 month are "middle-aged adults" (equivalent to ~38-47 human years) and 18-24 month mice are "old" (equivalent to human of 56-69 yrs.).

Going on these commonly used age ranges, it is unclear why the authors used 8-week-old mice (generally considered pubertal to late adolescent age) as young mice and 5-month-old mice as "old mice".

Differences seen between these cohorts most likely do not reflect aging, but more likely reflect changes associated with normal developmental maturation, since testis and epididymides continue to grow until about 10-11 weeks of age.

- The DMRs identified between 8 and 22-week-old animals could represent DMRs that are dependent on developmental maturation more than being changed in an "age-dependent" manner (in the sense of increased chronological age). This interpretation is congruent with the fact that those DMRs are enriched for developmental categories.

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Author Response

We are very thankful to the reviewers for a thorough review of our manuscript, and we are confident that we can address all identified weaknesses in the revised version. At the current point, we believe that it is important to mention the following:

1. The review by reviewer 1 contains factual errors. For example, the reviewer writes "There is much important information missing. For instance: how many animals were used per group and how was the breeding done?" Both animal numbers and the breeding scheme are described in detail in the manuscript.

2. Reviewer 3 criticizes our choice of animal ages used for the analysis of sperm DNA methylation aging. The reviewer suggests that the sperm of our younger group may contain spermatozoa from the 1st wave of spermatogenesis, while our older group cannot be considered chronologically old mice. We have experimental data that demonstrate that DNA regions that undergo methylation change with age have a linear association between methylation levels and age across the mouse lifespan (including ages used in our study). Thus, age-dependent changes in DNA methylation may be analyzed using any two ages as soon as they are different enough to detect the changes. We will include this experimental data in our resubmitted manuscript.