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

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Abstract: Sperm epigenome undergoes age-dependent changes increasing risks of adverse conditions in offspring conceived by fathers of advanced age. The mechanism(s) linking paternal age with epigenetic changes in sperm remains 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.

One-Sentence Summary: The balance between mTOR complexes in Sertoli cells regulates sperm methylome aging.

Main Text:

Over the past several decades, a trend toward delayed childbirth has led to increases in parental age at the time of conception. Emerging evidence suggests that higher male age contributes to poor pregnancy outcomes (*1*), lower odds of live birth (*2*), and adverse health of offspring in later life, including increased susceptibility to early development of cancer (*3*), as well as neurodevelopmental, and psychiatric disorders such as schizophrenia (*4*) and autism (*5*). Increasing evidence links advanced paternal age with altered offspring phenotype via age-dependent changes in the sperm epigenome (*6*). Published data indicate that age is a powerful factor affecting DNA methylation and other epigenetic markers in mammalian sperm (*6*), and age-dependent changes in these epigenetic mechanisms are involved in the regulation of key

developmental pathways (*7–9*). 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 (*10*), including meiotic divisions of spermatogonia and spermatocytes associated with passive loss of methylation and *de novo* methylation (*11*). 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 (*12*, *13*) and rodents (*9*, *14*), suggesting that the final sperm epigenome acquired during spermatogenesis is a significant channel for the transfer of inheritable information to the next generation (*15*).

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 reprograming (*16*). In rodents, age-dependent declines in fertility are caused by deterioration of the BTB (*17*, *18*). 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 (*16*, *19*, *20*) (Fig. 1). mTOR expression is highest in testes out of all tissues in humans (*21*) and mice (*22*), and the mTOR pathway is recognized today as a major mechanism of longevity and aging regulation (*23*, *24*). 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.

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-Mullerian 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 florescent dye (*25*). 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 (*26*, *27*). 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±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 (*28*) and used Metascape (*29*) 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 (*7–9*). 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 (*28*).

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 will be returned to their younger state by mTORC1 inactivation. We identified 2738 significant DMRs induced by mTORC1 inactivation in mature mice (*28*) 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 ($r(224) = -0.48, p = 2,1e-14$), 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 ($r(28) = -0.68, p = 2,3e-4$, 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 (*28*). 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 ($r(116) = 0.81, p < 2.2e -16$), 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 ($r(27) = 0.83, p < 2.6e - 8$, 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 (*28*)).

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 (*30*, *31*). Additionally, mitochondria DNA copy number (mtDNAcn) increases with age in human sperm (*32*) and it was shown recently that sperm mtDNAcn is a novel biomarker of male fecundity (*33*, *34*). 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 (*26*, *27*). 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 (*35*), physical activity (*36*), stress (*37*), metabolic status (*38*, *39*), and environmental exposures (*40*), among others. Age is the most powerful known factor affecting DNA methylation in mammalian sperm (reviewed at (*6*)), and age-dependent changes in epigenetic mechanisms in sperm are involved in the regulation of major developmental pathways, including nervous system-related signaling, Wnt, Hippo, mTOR, and Igf1 (*6*). Additionally, it was shown recently that exposure to environmental xenobiotics may affect the sperm epigenome (DNA methylation and small noncoding RNA) via changes in the rate of sperm epigenetic aging (*7*, *8*). 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. The balance of mTOR complexes in Sertoli cells was previously identified as the mechanism of the BTB permeability regulation (*16*, *19*, *20*). A significant body of research demonstrated that BTB regulation is the major function of the mTOR pathway in Sertoli cells (*16*, *19*, *20*, *41*, *42*). Therefore, BTB permeability is the likely modulator of sperm epigenetic aging and may be a novel target of therapeutic interventions to rejuvenate the sperm epigenome in advanced-age fathers.

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Formal analysis: SA, OA, EM

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Resources: AS, JRP

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Data and materials availability: Raw and processed DNA methylation data as well as complete results of Metascape enrichment analysis are available via Dryad (*28*). All other data are available in the main text or the Supplementary Materials.

Supplementary Materials

Materials and Methods

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). 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. S4. Genic 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 5% DMRs induced by age and by mTORC1 inactivation in mature mice among all common methylation regions identified in both comparisons. **(B)** Overlap between 5% 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.

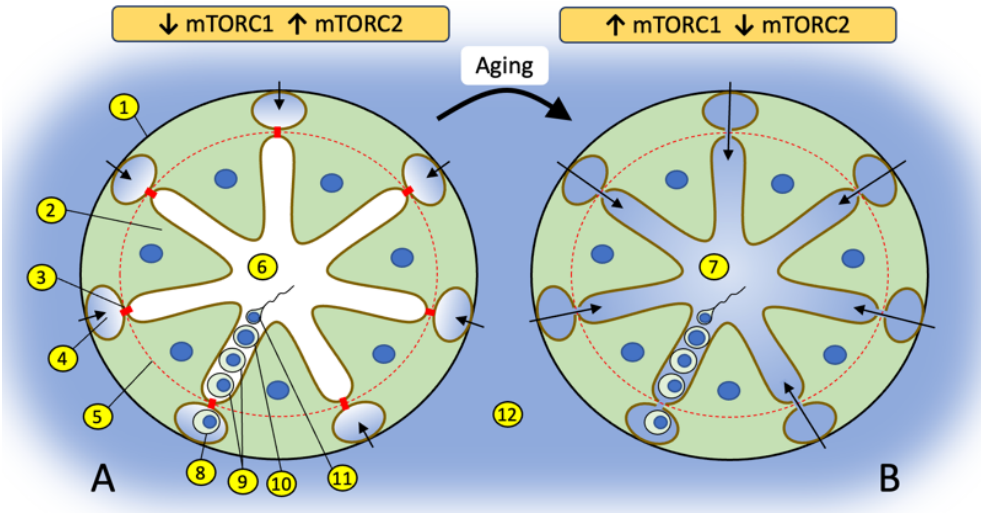

Fig. 1. Changes in the balance of mTOR complexes and aging affect biochemical conditions of spermatogenesis in the apical compartment of seminiferous epithelium. (A) Seminiferous tubule with tight BTB where interstitial environment (shown in blue) penetrates only to basal compartment of seminiferous 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.

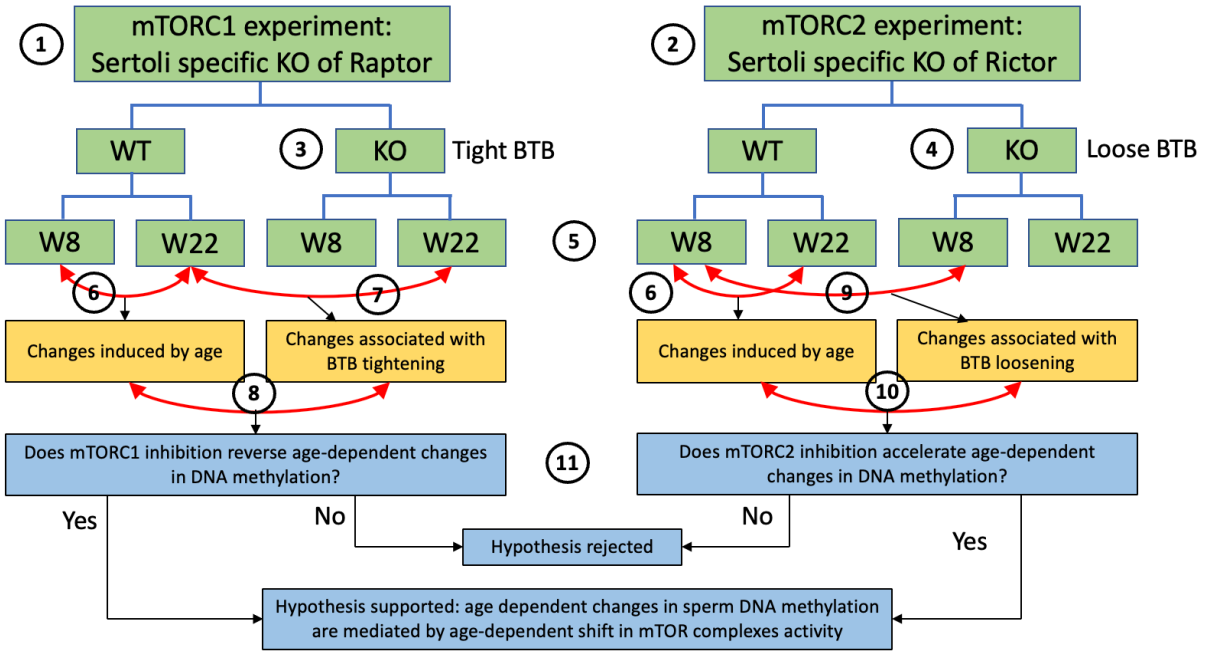

Fig. 2. Scheme of experimental design for DNA methylation analysis. This study utilized experiments with two transgenic mouse models: (1) one with mTORC1 suppression in Sertoli cells due to the cell-specific KO of Raptor, and (2) another with mTORC2 suppression in Sertoli cells due to the cell-specific 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-weak 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).

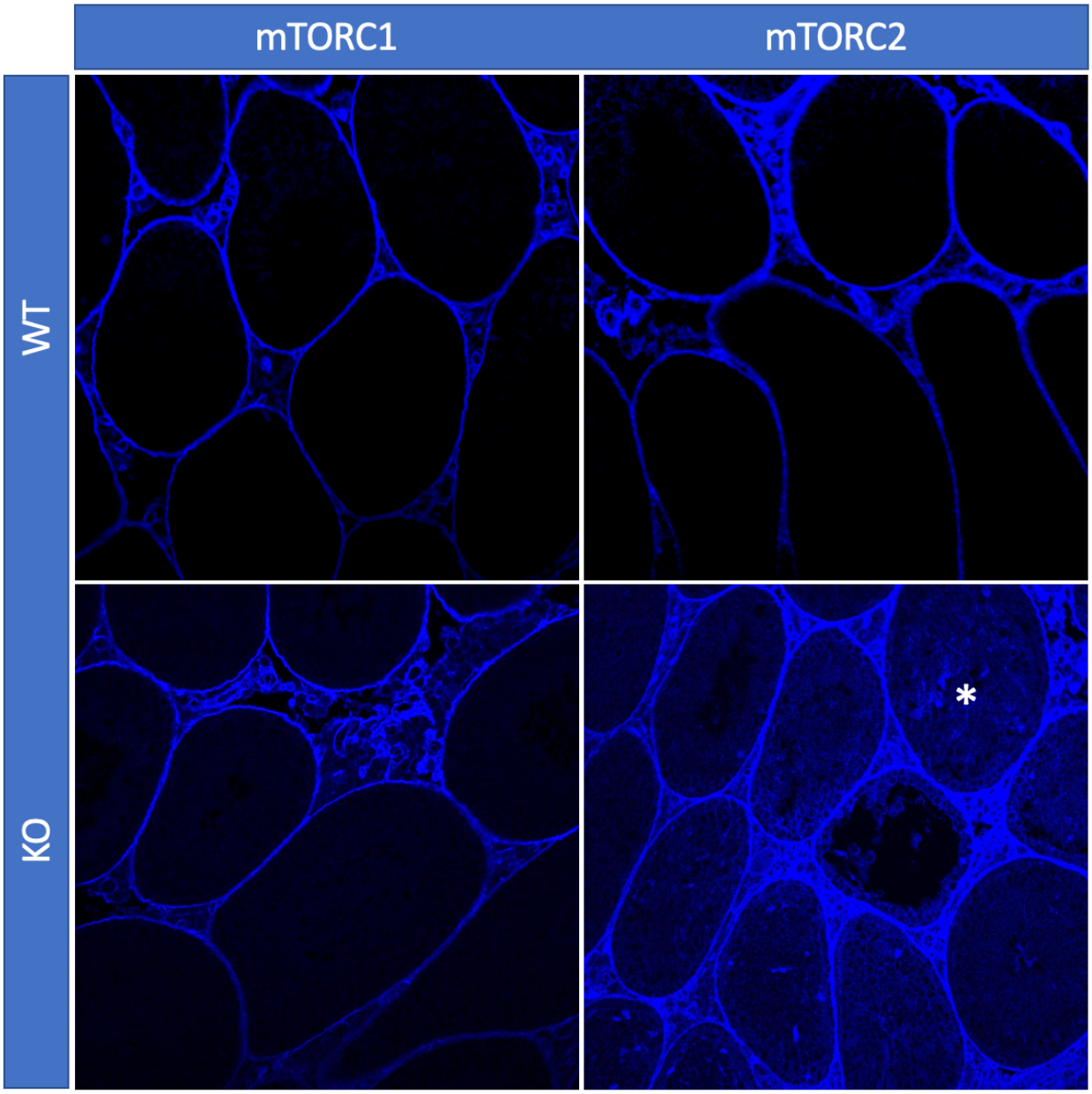

Fig. 3. BTB permeability in 22-week old mice of different genotypes, representative images. In mice with inactivated mTORC2 (Rictor KO), biotin tracer (blue) penetrated to the apical compartment of the seminiferous epithelium (asterisk) while in all other genotypes it did not cross the BTB.

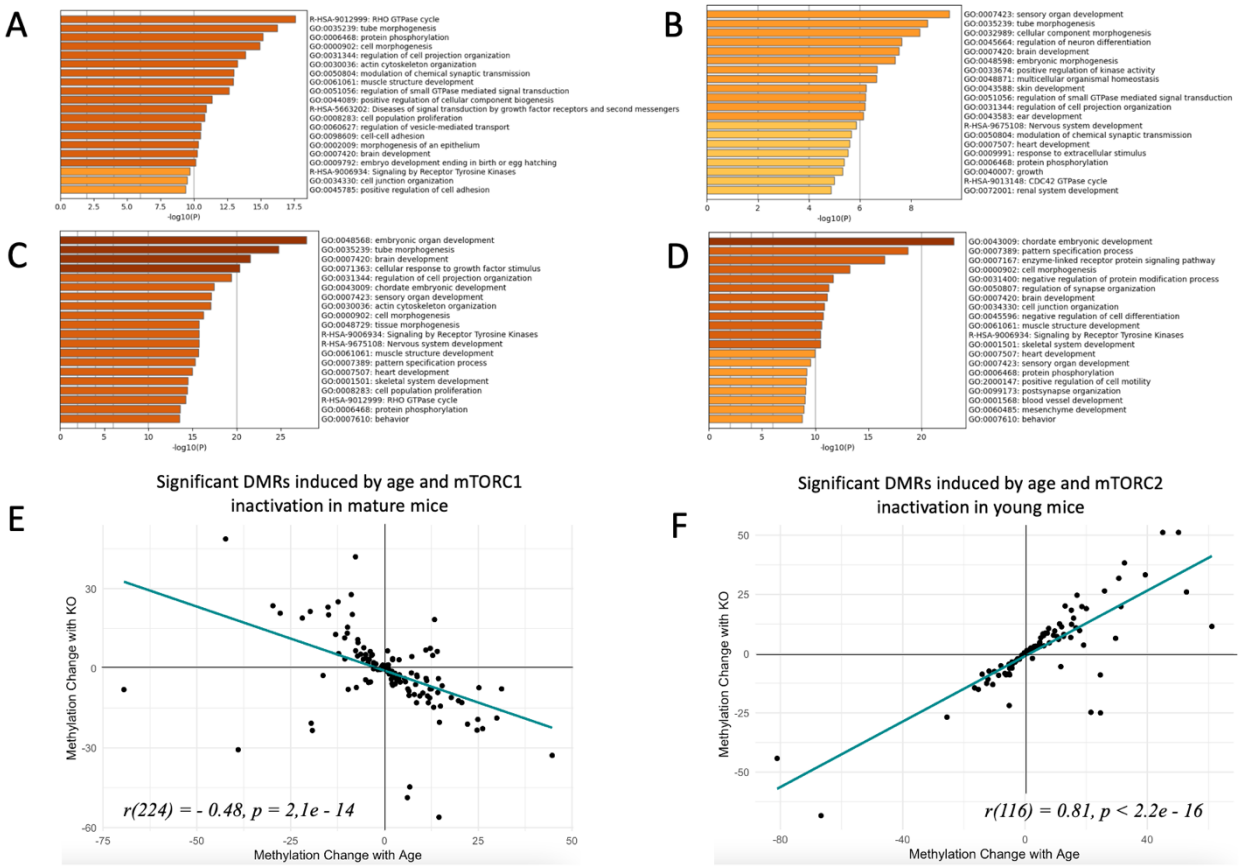

Fig. 4. Changes in sperm DNA methylation induced by age and manipulation of mTOR pathway in Sertoli cells. (A, B, C, D) Top biological categories enriched with genes associated with significant DMRs: **(A)** age-dependent DMRs in wildtype animals in the mTORC1 experiment; **(B)** age-dependent DMRs in 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)**.

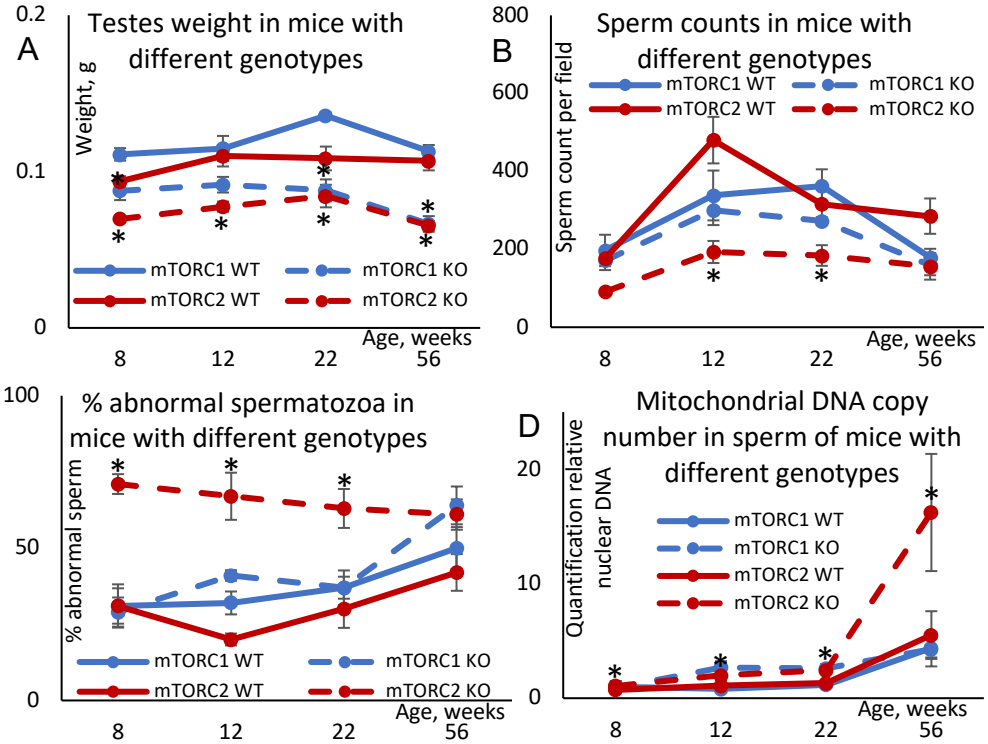

Fig. 5. Changes in age-dependent reproductive parameters in mice with inactivated mTORC1 or mTORC2 in Sertoli cells. (A) Testes weight. **(B)** Sperm counts in sperm smears. **(C)** Percent abnormal spermatozoa. **(D)** Mitochondrial DNA copy number. All data are Mean ± SE, n = 5-6/age/genotype, ANOVA followed by Dunnett's test, *$p < 0.05$ when compared with corresponding WT.