Inhibition of mammalian target of rapamycin signaling pathway decreases retinoic acid stimulated gene 8 expression in adult mouse testis

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Objective: To evaluate the expression of mammalian target of rapamycin (mTOR) pathway molecules in mouse spermatogenesis and as well as its role during proliferation and meiotic initiation of spermatogenic cells.
Design: Experimental animal study.
Setting: University.
Animal(s): C57Balb-C adult male mice.
Intervention(s): Expressions of mTOR signaling pathway proteins in adult testis were evaluated. Then the effect of inhibition of this pathway on proliferation and differentiation of spermatogonial stem cells was investigated using seminiferous tubule culture.
Main Outcome Measure(s): Immunohistochemistry was performed to evaluate the expressions of mTOR signaling pathway proteins. To inhibit mTOR signaling pathway by rapamycin, seminiferous tubule culture was done. Viability assay and terminal deoxynucleotidyl transferase dUTP nick end labeling was performed to evaluate the culture conditions and to examine cell death, respectively. Western blot was used to determine the expressions of the PCNA, STRA8, and VASA proteins.
Result(s): Our results showed that spermatogonial stem cells and preleptotene spermatocytes express total mTOR, p-mTOR, total p70S6K, p-p70S6K, and p-4EBP1. Expressions of p-p70S6K, p-4EBP1, PCNA, and STRA8 decreased significantly in the rapamycin-treated group, where no difference was observed in VASA expression. Cell viability and the number of apoptotic cells were similar for all groups.
Conclusion(s): Our findings suggest that the mTOR signaling pathway may have role in the proliferation and stimulation of meiotic initiation of spermatogonial stem cells. To the best of our knowledge, this is the first ex vivo study that reports the function of the mTOR pathway in adult mouse spermatogenesis. (Fertil Steril® 2014;102:1482–90. ©2014 by American Society for Reproductive Medicine.)
Key Words: mTOR, rapamycin, seminiferous tubule culture, STRA8

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Target of rapamycin (TOR) signaling is an evolutionarily conserved mechanism among eukaryotes. TOR forms two complexes, TOR-1 and TOR-2, which are larger proteins and are 70% identical (1). TOR orthologs have been identified in *Caenorhabditis elegans* (2) *Drosophila melanogaster* (3) mammals (4) and *Arabidopsis thaliana* (5). Mammalian TOR (mTOR) belongs to the phosphoinositide kinase-related family (PIKK) (6). MTOR

controls protein synthesis by phosphorylating downstream substrates, including p70S6 kinase ($p70^{S6K1}$) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) (7). P70S6K phosphorylates the 40S ribosomal protein S6, which leads to the translation of 5′terminal oligopyrimidine tract RNAs, which primarily encode ribosomal proteins and components of the translation apparatus (8). Phosphorylation of 4E-BP1 disrupts its binding to eIF4E, a protein that binds the 5′ cap structure of mRNA. Released eIF4E then forms a functional translation initiation complex with eIF4G, eIF4A, and eIF3 ribosomes, enhancing translation (9). The mTOR signaling pathway regulates cell cycle, growth, and metabolism via protein synthesis (10). Embryonic mutations in mTOR are lethal (11). MTOR activation is regulated by food amount, energy level, and growth factors and disregulated by stress and hypoxia.

MTOR inhibitors are being used in organ transplant patients to suppress the immune system. Rapamycin (sirolimus), which is a macrocyclic lactone isolated from a strain of *Streptomyces hygroscopicus*, was identified more than 20 years ago (12). Rapamycin is an antiproliferative agent (6) and inhibits mTOR via binding to its FK506 binding subunit (13). Recent case reports indicate that rapamycin administration impairs male gonadal function (14). Male patients treated with rapamycin have decreased testosterone (T) levels and sperm counts and increased follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (15–21). There are limited studies in the literature about the adverse effect of rapamycin on spermatogenesis. An in vitro study showed that rapamycin is a key regulator of spermatogonial proliferation (22). According to this study, stem cell factor (SCF)/c-kit uses a phosphoinositide 3-kinase (PI3K)/AKT/p70S6K/cyclin D3 pathway to promote spermatogonial stem cell proliferation. Although this in vitro study highlights a role for mTOR in spermatogonial stem cell behavior, the possible contribution of mTOR signaling to spermatogenesis is yet to be determined.

Spermatogonial stem cells undergo mitotic divisions by initiation of spermatogenesis and further differentiate to primary spermatocytes (23). Retinoic acid is the key regulator of spermatogonial differentiation and has been demonstrated to regulate meiotic initiation via a gene named retinoic acid stimulated gene 8 (*Stra8*) (24). In adult testis; type A and type B spermatogonia and preleptotene spermatocytes express *Stra8* (25). *Stra8* is required for spermatogenic cells to undergo the morphological changes that define meiotic prophase and for these cells to exhibit the molecular hallmarks of meiotic chromosome cohesion, synapsis, and recombination. *Stra8*-deficient preleptotene cells replicate their DNA but fail to enter meiotic prophase (26). Mouse VASA homologue is a molecular germ cell marker that has been expressed until the postmeiotic cell stage.

In the present study, we first evaluated the expression of mTOR signaling pathway proteins (total mTOR, p-mTOR, total p70S6K, p-p70S6K, p-4EBP1) in adult mice testis by immunohistochemistry. We found that essentially spermatogonia and preleptotene spermatocytes express mTOR signaling pathway proteins. On the basis of our findings, we suggested that the mTOR signaling pathway may have a role during proliferation and also in the stimulation of meiotic initiation of spermatogonial cells. Thus, after inhibition of mTOR in the ex vivo seminiferous tubule culture system, we evaluated the expressions of proliferation and differentiation markers, PCNA and STRA8, respectively.

MATERIALS AND METHODS

Animals

Three-month-old C57Balb-C male mice were used, and four groups were established; control (0 hour; n = 6), 24-hour culture (n = 6), rapamycin treated (n = 6), and ethanol treated as the vehicle group (n = 6). Animals were obtained from the Akdeniz University Animal Research Unit. Animals were anesthetized and killed by cervical dislocation. Testis tissues were removed. The experimental protocol was approved by the animal core and usage committee of Akdeniz University, protocol number 2010.10.01, and was in accordance with the Declaration of Helsinki and the International Association for the Study of Pain guidelines.

Adult Mouse Seminiferous Tubule Culture

Four groups were established: control (0 hour), 24-hour culture, rapamycin-treated, and ethanol-treated. To inhibit mTOR signaling pathway, 200 nmol of powder rapamycin (LC Laboratories, R-5000) dissolved in ethanol was used. An equivalent volume of ethanol was added to the vehicle groups. Dissected testes from mice were placed in Gibco RPMI 1640 (Invitrogen) medium and decapsulated using micro scissors. Seminiferous tubules were dissociated gently using fine forceps and then cut into small fragments. Tubule fragments were cultured in 30 μL hanging drops in RPMI medium (RPMI 1640) containing 0.1% albumin bovine fraction V solution of bovine serum albumin (Sigma catalog no. A8412) at 32°C for 24 hours with 5% CO_2/95% air. Up to five tubule fragments were cultured in each hanging drop.

Cell Viability Assay

After culturing, seminiferous tubules were treated with collagenase dispase (Roche) for 10 minutes and then homogenized by pipetting with Gibco tripsin-EDTA (Invitrogen) for 2–3 minutes. After homogenization, cells were counted, and equal numbers of cells were loaded to 96-well plates to evaluate the viability of cells in the culture system. A cell proliferation assay kit (Roche, 11 465 007 001) was used for the evaluation of cell viability. This assay is based on the cleavage of yellow tetrozolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide labeling reagent) to purple formazan crystals by metabolic active cells (27). The formazan crystals formed are solubilized, and the resulting colored solution is quantified using a scanning multiwall spectrophotometer. Briefly, MTT labeling reagent solution was added into each well (10 μL solution per 100 μL of media), and wells were incubated for 4 hours in the incubator with 32°C, 5% CO_2/95% air condition. After 4 hours, solubilization solution (100 μL solution per 10 μL MTT labeling reagent) was added into each well and incubated overnight. Finally, spectrophotometric absorbances of the samples were measured at 560 nm with a plate reader (Biotek Epoch).