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Disruption of murine *Tenr* results in teratospermia and male infertility

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Abstract

Gametes rely heavily on posttranscriptional control for their differentiation. Translational control, alternative splicing, and alternative processing of the 3′ end of mRNAs are all common during spermatogenesis. *Tenr*, which encodes a highly conserved 72-kDa protein, is expressed solely in germ cells of the testis from the mid-pachytene stage until the elongating spermatid stage. TENR contains a double-stranded RNA binding domain, is localized to the nucleus, and is phylogenetically related to a family of adenosine deaminases involved in RNA editing. We show here that targeted mutation of the *Tenr* gene causes male sterility. *Tenr* mutant males have a reduced sperm count, and $Tenr^{-/-}$ sperm show a decrease in motility and an increase in malformed heads. Despite their sterility, some epididymal sperm from *Tenr* mutants have normal morphology. The ability of *Tenr* mutant sperm to fertilize zona pellucida-free oocytes and to bind to, but not fertilize, zona pellucida-intact oocytes suggests that the normal-appearing sperm are not able to penetrate the zona pellucida. These data suggest that TENR plays an essential function in spermatid morphogenesis.

Keywords: Spermatogenesis; Posttranscriptional control; RNA editing

Introduction

The differentiation of diploid spermatogonia into haploid spermatozoa is a complex process requiring elaborate transcriptional and posttranscriptional regulation. As much as 75% of the genome may be expressed at some point during spermatogenesis, and 4% of the genome is uniquely transcribed specifically in meiotic and haploid cells (Schultz et al., 2003). Novel germ cell-specific transcription factors and altered forms of the general transcriptional machinery all contribute to the exceptionally high transcript complexity in the testis (Eddy and O'Brien, 1998; Kimmins et al., 2004). Similarly, alternative spicing and translational control generate additional diversity and temporal control of protein synthesis, respectively (Braun, 2000; Kleene, 2003; Venables, 2002; Venables and Eperon, 1999). Translational control is essential for spermatogenesis because de novo protein production is required for the terminal stages of differentiation, which occur after transcription has ceased (Kleene, 2001; Monesi, 1964). Premature translation of even a single translationally controlled gene can cause sterility (Lee et al., 1995). Recent studies indicate that translational control is also important during meiosis, prior to transcriptional silencing (Tay and Richter, 2001).

Spermatid differentiation involves transcriptional silencing, nuclear condensation, elaboration of an acrosome, and formation of a flagellum. Fully differentiated spermatids shed their cytoplasm, and mature spermatozoa are released into the lumen of the seminiferous tubule, where they are transported to the epididymis and undergo further maturation. Sperm are stored in the cauda epididymis, where they are immotile and incapable of interacting with oocytes (Wasserman and Florman, 1997). They acquire the ability to fertilize eggs through the process of capacitation, which takes place during migration through the female reproductive tract (Harrison, 1996). Capacitated sperm penetrate the cumulous oophorous, contact the zona pellucida of the oocyte, and undergo the acrosome reaction, during which the contents of the acrosome are released, allowing the

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sperm to penetrate the zona pellucida. Following penetration, sperm contact and fuse with the plasma membrane of the oocyte, which result in oocyte activation, pronuclear formation, and syngamy (Evans and Florman, 2002).

TENR, a member of the double-stranded (ds) RNA-binding family of proteins, was originally identified in an expression screen for proteins that bound preferentially to the 3′ untranslated region (3′ UTR) of the protamine 1 (*Prm1*) mRNA (Schumacher et al., 1995). TENR is a nuclear protein expressed in a wagon-wheel-like pattern in meiotic spermatocytes and early haploid spermatids. The *Tenr* gene is highly tissue specific, having never been found to be expressed outside of the testis. To determine if it is required for spermatogenesis, we disrupted the *Tenr* gene in mice and examined the consequences on spermatogenesis and male fertility.

Materials and methods

Construction of targeting vector

A 129Sv/J mouse genomic library in bacteriophage lambda was screened with a *Tenr* cDNA probe. A 15.5-kb clone was isolated, mapped, and used to construct a targeting vector. A 4-kb *Aat*II–*Xho*I gene fragment of the *Tenr* genomic locus was cloned upstream of a promoterless *lacZ*/*Pgk* promoter-driven *Neo* cassette (*Pgk–Neo*). A 2-kb *Sal*I–*Hin*dIII fragment of the *Tenr* genomic locus was cloned downstream of the cassette and upstream of a *Pgk*-promoter-driven diphtheria toxin (*Pgk–Dta*) gene. Homologous recombination of this targeting vector into the genomic *Tenr* locus resulted in deletion of a region that included 82 bp 5′ of the *Tenr* transcription initiation site, the 5′ untranslated region (UTR), and 35 bp 3′ of the translation initiation site. The ES cell line AK7 was transfected by electroporation with 25 μg of *Not*I-linearized targeting vector and plated onto mitomycin C-treated SNL cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% bovine fetal serum, glutamine, penicillin and streptomycin, and 0.1 mM β-mercaptoethanol (BME). After 24 h of growth in DMEM, transfected ES cells were selected for in DMEM supplemented with 300 μg/ml G418. After an additional 10 days, ES cell colonies were isolated and treated with trypsin and EDTA to dissociate the cells. One-half of the cells were used for genotyping by PCR, and the other half was plated in 96-well plates. PCR was performed in a 50-μl reaction with 100 nM primers, 5 mM $MgCl_2$, 1 mM dNTPs, and 5 U Taq polymerase. The primers used were 5′CAGAACAGCACTGGAATCCTCAGAG3′ and 5′CCGCTATCAGGACATAGCGTTGGC3′. Cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 4 min, and finally 72°C for 5 min. Candidate clones were expanded and homologous recombination confirmed by Southern blot analysis. Extracted DNA was cut with *Hpa*I, electrophoresed, and transferred to Hybond-N membrane (Amersham Biosciences) in 20× SSC. The blots were probed with a ^{32}P-labeled probe generated from a 2-kb *Hin*dIII–*Hpa*I fragment of the *Tenr* genomic clone, washed, and exposed to X-ray film.

Generation and genotyping of Tenr mutant mice

Five independent ES cell clones carrying the *Tenr* disruption were used to generate chimeric mice. ES cells were injected into C57BL/6J blastocysts and implanted into pseudo-pregnant recipient C57BL/6J females. Progeny male chimeric mice were crossed to female C57BL/6J mice to obtain $Tenr^{+/-}$ animals. Those chimeric males that transmitted the 129Sv/J genotype were crossed to 129Sv/J females to generate $Tenr^{+/-}$ animals in an isogenic background. Genotype analysis was performed on isolated genomic DNA by Southern blot as described above or by PCR using the primer pair 5′CCGCTATCAGGACATAGCGTTGGC3′ and 5′CCCATGGCTACAAACAATCC3′ to detect the recombined allele and the primer pair 5′TGACCGCTCTAGGTTGTCCT3′ and 5′GGTCAGTCGCGTCTTTCAA3′ to detect the wild-type locus. The cycling profile used was 1 cycle of 5 min at 95°C, 40 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 2 min, and 1 cycle of 72°C for 7 min.

RNA isolation and Northern blot analysis

Total RNA was extracted from sexually mature mouse testes by homogenization in 1.5 ml guanidinium hydrochloride solution and precipitation overnight at 4°C with 10 ml 4 M lithium acetate, followed by resuspension, phenol/chloroform extraction, and ethanol precipitation (Cathala et al., 1983). Alternatively, RNA was extracted from testes by homogenization in Triazol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Northern analysis was performed using 10 μg of total RNA. The RNA was electrophoresed for 2.5 h at 100 V in a 15-cm 1.5% agarose formaldehyde gel and transferred to Hybond-N in 20× SSC. The blots were probed with ^{32}P-labeled DNA fragments corresponding to *Tenr* cDNA or *Prm1* cDNA and exposed to X-ray film.

In vivo and in vitro fertilization

For analysis of fertility after normal mating, males were housed with 129Sv/J females for 5 days or until a vaginal plug was noted, after which the female was replaced. At least four plugs were observed for each male, and the resulting litter sizes were recorded. In vitro fertilization was done according to Szczygiel et al. (2002). Briefly, caudal epididymal sperm were isolated and capacitated for 90 min in T6 media (97.84 mM NaCl, 1.42 mM KCl, 0.36 mM Na_2HPO_4, 0.47 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.78 mM $CaCl_2$, 24.9 mM sodium lactate, 0.47 mM sodium pyruvate, 5.56 mM glucose, 0.4% BSA). Oocytes were