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A Testicular Germ Cell-Associated Serine-Threonine Kinase, MAK, Is Dispensable for Sperm Formation

Yoichi Shinkai,[1]* Hideo Satoh,[2]† Naoki Takeda,[2]‡ Mikiko Fukuda,[1] Emiko Chiba,[2] Tomoya Kato,[3] Takashi Kuramochi,[4]§ and Yoshihiko Araki[3]

Institute for Virus Research, Kyoto University, Kyoto 606-8507,[1] Department of Oncology, Nippon Roche Research Center, Kamakura 247-0063,[2] Department of Immunology and Parasitology, Yamagata University School of Medicine, Yamagata 990-9585,[3] and Central Institute for Experimental Animals, Kawasaki 216-0001,[4] Japan

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A member of the mitogen-activated protein kinase superfamily, MAK, has been proposed to have an important role in spermatogenesis, since *Mak* gene expression is highly restricted to testicular germ cells. To assess the biological function of MAK, we have established MAK-deficient ($Mak^{-/-}$) mice. $Mak^{-/-}$ mice developed normally, and no gross abnormalities were observed. Spermatogenesis of the $Mak^{-/-}$ mice was also intact, and most of the mice were fertile. However, $Mak^{-/-}$ male-derived litter sizes and their sperm motility in vitro were mildly reduced. These data show that function of MAK is not essential for spermatogenesis and male fertility.

Spermatogenesis consists of three major stages: (i) a self-renewing stage of spermatogonia (stem cells) by mitosis, (ii) a meiotic division stage of spermatocytes, and (iii) a morphological maturation stage during which haploid spermatids become mature spermatozoa. To understand the molecular mechanisms of mammalian spermatogenesis, research approaches using other cellular systems or experimental results from other species (for example, invertebrate systems) sometimes provide useful information. Since stem cells from other types of tissues also self renew and proliferate like spermatogonia, it is quite possible that similar mechanisms control the cellular events of both reproductive and other stem cells. Indeed, similar molecular properties have been described for different stem cell systems. For example, both steel factor and its receptor, c-kit, is crucial for hematopoiesis and spermatogenesis (9). Furthermore, hematopoietic stem cells and spermatogonial stem cells express integrin molecules on the cell surface and can be isolated based on expression of specific integrins (12, 19, 22). Meiosis is a process unique to the germ lineage cell; however, all eukaryotes undergo meiotic cell division in special circumstances. It has been shown that molecules critical for meiotic recombination in yeast also exist in mammals and have similar functions (reviewed in reference 6).

Identification and characterization of specific molecules expressed within the testis is another approach to understanding spermatogenesis at a molecular level. Many testicular proteins have been identified and partially characterized (reviewed in reference 7). A serine-threonine kinase, MAK (male germ cell-associated kinase), is one such molecule. It was originally identified by weak cross-hybridization with a tyrosine kinase gene, *v-ros* (16). Since the expression of MAK protein was shown to be highly restricted in testicular germ cells at and after meiosis, it has been strongly speculated that MAK plays an important role(s) in cellular processes of spermatogenesis (10, 13, 16).

To assess the function of MAK in spermatogenesis and male reproductive physiology, we generated $Mak^{-/-}$ mice by homologous recombination in embryonic stem (ES) cells and characterized their reproductive processes, including spermatogenesis and fertility.

MATERIALS AND METHODS

Establishment of *Mak* knockout ES cells and mice. A DNA fragment containing the mouse *Mak* gene was isolated from a 129 genomic DNA library. A 1.1-kb *Hpa*I fragment and a 4.5-kb *Xho*I-*Not*I (vector origin) fragment 5′ to exon 5 and 3′ to exon 8 of *Mak*, respectively, were subcloned into the PLN-tk plasmid (5). The numbering of these exons was based on our mouse genomic DNA analyses and a published human *Mak* gene exon/intron structure (EMBL accession no. AL024498). Exons 5 to 8 of the mouse *Mak* gene were estimated to encode Lys^{127}-Val^{381} of MAK protein.

Twenty micrograms of the linearized targeting construct, Tk2p102, was transfected into 2×10^7 TT2 ES cells (23), and the targeted clones were enriched with 0.3 mg of G418/ml and 1.5 μM of ganciclovir. The drug-resistant colonies were then screened by PCR with the following primers: SCR4, 5′-AGCCATCTCGC CAGCACCTCATA-3′, and G3, 5′-GGGCCAGCTCATTCCTCCACTC-3′. The positions of these primer sequences are shown in Fig. 1A. The correctly targeted clones were further confirmed by Southern blot analysis (described below) and then were injected into ICR mice embryos in the morula stage. Established chimeric male mice were bred with C57BL/6 (B6) females, resulting in germ line transmission. To minimize the effect of the mixed genetic background, the $Mak^{+/-}$ females were backcrossed with either B6 or CBA/J (CBA) males over five generations. Each strain of F_6 animals from the F_5 $Mak^{+/-}$ interbreeding or their descendents were used in some experiments, including mating experiments. B6, CBA, and ICR mice were purchased from Japan SLC Inc. (Hamamatsu, Japan), and all the animals were maintained and bred at the animal facility in our institute under specific pathogen-free conditions.

Western blotting. Eighty micrograms of total testicular cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electro-

* Corresponding author. Mailing address: Department of Cell Biology, Institute for Virus Research, Kyoto University, 53 Shogoin, Kawara-cho, Kyoto 606-8507, Japan. Phone: 81-75-751-3990. Fax: 81-75-751-3991. E-mail: yshinkai@virus.kyoto-u.ac.jp.

† Present address: Environmental Health Science Laboratory, Sumitomo Chemical Co. Ltd., Osaka 554-8558, Japan.

‡ Present address: Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto 860-0811, Japan.

§ Present address: Mitsubishi Kagaku Institute of Life Sciences, Machida 194-8511, Japan.

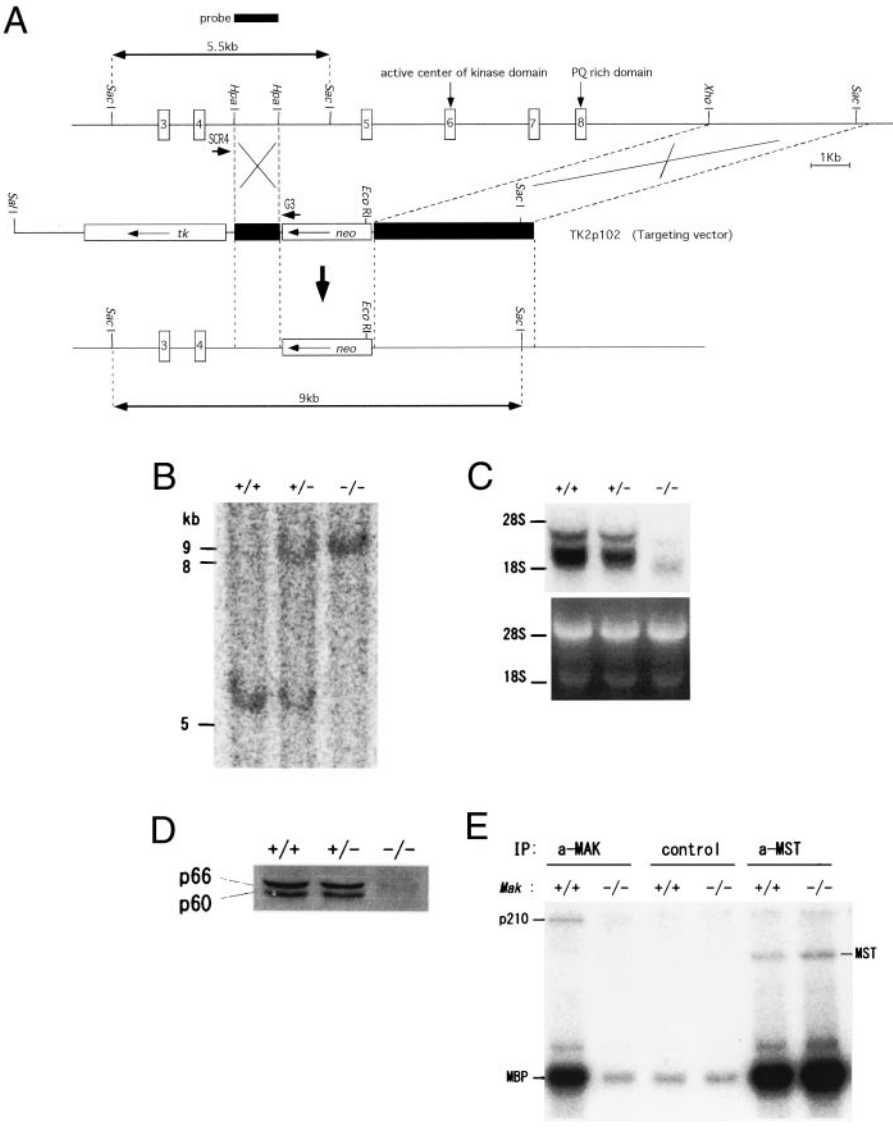

FIG. 1. Generation of a mouse line bearing a targeted disruption of the *Mak* gene. (A) Schematic representation of homologous recombination of the targeting vector, TK2p102, with the endogenous locus. TK2p102 was designed to replace the regions of exons 5 to 8 of the *Mak* gene, encoding a catalytic kinase domain (the activation loop region) and a PQ-rich domain with a PGK-Neo^r selection cassette (neo). Detection of homologous recombinants in ES cells was performed by PCR assay with SCR4 and G3 primers (small arrows). (B) Genotyping of $Mak^{-/-}$ mice by Southern blotting. DNA was digested with *Sac*I and hybridized with the probe shown in panel A. The 5.5-kb *Sac*I fragment corresponding to the wild-type allele is increased to 9 kb upon disruption of the *Sac*I recognition site located between exons 4 and 5 by integration of the Neo^r gene. (C) Northern blotting of testicular total RNA probed with the entire coding region of the *Mak* cDNA. Two distinct *Mak* transcripts (3.8 to 3.9 kb and 2.6 to 2.8 kb) were detected in the $Mak^{+/+}$ and $Mak^{+/-}$ testicular cell RNA. However, in the $Mak^{-/-}$ testicular RNA sample, the amount of these two transcripts was considerably diminished and sizes of both transcripts were reduced a little bit, probably due to the deletion of exons 5 to 8 in the targeted allele. The lower panel shows 18S and 28S rRNA stained with ethidium bromide on the same blot before probing. (D) Immunoblot of testicular cell lysates probed with a polyclonal Ab against a MAK carboxy terminus peptide (10). Both the p60 and p66 MAK proteins were missing from the $Mak^{-/-}$ testicular cell lysates. (E) Absence of MAK-associated protein kinase activity in the $Mak^{-/-}$ mice. Testicular cytoplasmic lysates were prepared as described in Materials and Methods and were incubated with the control Ab, Ab against MAK (α-MAK), or Ab against MST, a STE20-like serine/threonine kinase (α-MST). The immunoprecipitates (IP) were then applied to the in vitro kinase assay, with MBP as a substrate. p210 is a MAK-associated molecule and is efficiently phosphorylated by MAK (10).

transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antibody (Ab) against MAK (amino acid residues 603 to 622) (10).

Southern and Northern blotting. Ten micrograms of mouse genomic DNA was digested with *Sac*I, separated by 0.7% agarose–Tris-acetate-EDTA gel electrophoresis, transblotted to a nylon membrane, and probed with the ^{32}P-labeled 1.1-kb *Hpa*I fragment used in the *Mak* targeting vector. Eight micrograms of total RNAs from various mouse organs were separated by 1% agarose-formaldehyde gel electrophoresis, transblotted to a nylon membrane, and probed with the ^{32}P-labeled *Mak* cDNA (entire coding region). All the hybridized images were visualized with a BAS5000 Bio-Image Analyzer (Fuji Film, Tokyo, Japan).

Protein kinase assay. Testicular cell lysates were prepared based on the procedure described previously (10). Briefly, the testis was homogenized in modified LBK buffer containing 20 mM HEPES-NaOH (pH 7.5), 10% glycerol, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM β-glycerophosphate, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktails (Boehr-

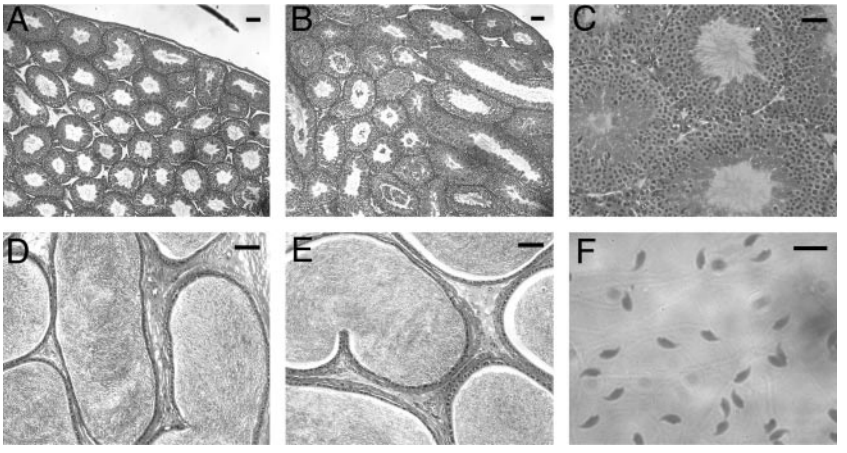

FIG. 2. Histochemical analysis of the *Mak*$^{-/-}$ mouse testis and epididymis. Hematoxylin-eosin staining of the testicular (A to C) and cauda of epididymal (D and E) tissue sections and a smear of the epididymal sperm are shown (F). (A) *Mak*$^{+/+}$; (D) *Mak*$^{+/-}$; (B, C, E, and F) *Mak*$^{-/-}$. The sizes of the scale bars in the panels are the following: 100 μM (A and B), 50 μM (C), 70 μM (D and E), and 10 μM (F).

inger Mannheim, GmbH, Germany), and they were centrifuged at 16,000 × *g* for 10 min at 4°C to obtain soluble cytoplasmic proteins. Cytosolic proteins (750 μg) were incubated with 1 μg of control Ab, Ab against MAK, or Ab against MST, a STE20-like serine/threonine kinase (14); the immune complexes were precipitated with protein A-Sepharose beads. The immunoprecipitates were suspended in 15 μl of the kinase assay buffer, which contained 20 mM HEPES-NaOH (pH 7.5), 10 mM $MnCl_2$, 0.25 mM β-glycerophosphate, 25 μM ATP, 5 to 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and 1 μg of myelin basic protein (MBP), and they were incubated for 30 min at 23°C. Protein phosphorylation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Histochemical analysis. Tissues from *Mak*$^{-/-}$ mice and their *Mak*$^{+/+}$ or *Mak*$^{+/-}$ littermates or age-matched control mice were rapidly dissected and placed in Bouin's fixative overnight. Excess fixative was removed with a 70% ethanol-NH_4OH solution. Tissues were then dehydrated and embedded in paraffin for microtome sections. Hematoxylin-eosin staining procedures were performed by a standard protocol.

Mating experiment. Two- to 5-month-old male *Mak*$^{-/-}$ mice and their *Mak*$^{+/+}$ or *Mak*$^{+/-}$ littermates or age-matched control mice were crossed with B6 females for at least 2 weeks. One male was bred to one or two reproductively active females.

Sperm motile activity assay. Mature sperm were prepared as described previously (8). Epididymides were removed from *Mak*$^{-/-}$ mice and their *Mak*$^{+/+}$ or *Mak*$^{+/-}$ littermates or age-matched control mice, and the vas deferens was cut close to the cauda epididymis. Mature sperm were expressed from the epididymis with forceps and were placed into human tubal fluid medium (1 epididymis per ml). After 30 min of incubation, sperm motility was monitored with a Sperm Quality Analyzer (Medical Electonic Systems Ltd., Santa Ana, Calif.) according to the manufacturer's instruction. The results are reported as the sperm motility index (SMI); SMI is the total value of sperm motility times sperm concentration (2, 11).

Statistical analysis. Data obtained from mating experiments were analyzed by the Mann-Whitney U test. The Student's *t* test was used to examine the significance of differences in the values of SMI. A probability of $P < 0.05$ was considered statistically significant.

RESULTS

Establishment of *Mak* knockout mice. To establish MAK-deficient mice, the *Mak* targeting construct Tk2p102 was transfected into the (B6 × CBA) F_1 ES cell line, TT2. Tk2p102 potentially introduced a deletion of exons 5 to 8 of mouse *Mak* that encodes about 45% of MAK amino acids, including a catalytic kinase domain (the activation loop region) and a PQ-rich domain (Fig. 1A). Of approximately 500 TT2 clones screened, 5 were identified as being targeted. One of the targeted clones, number 50, was germ line transmitted, and mice homozygous for the *Mak* deletion (*Mak*$^{-/-}$) were established by being mated with heterozygous mutant (*Mak*$^{+/-}$) mice. Southern blot analysis shows the status of the *Mak* deletion in the wild-type (*Mak*$^{+/+}$), *Mak*$^{+/-}$, and *Mak*$^{-/-}$ mice (Fig. 1B). *Mak*$^{-/-}$ mice were born at the expected Mendelian ratio and did not show any gross abnormalities (data not shown).

Development and function of male germ cells in the *Mak*$^{-/-}$ mice. To confirm the absence of MAK protein in the *Mak*$^{-/-}$ mice, we performed Northern and Western blot analyses. As shown previously (3, 10, 21) and in Fig. 1C, two distinct *Mak* transcripts (3.8 to 3.9 kb and 2.6 to 2.8 kb) were detected in the *Mak*$^{+/+}$ and *Mak*$^{+/-}$ testicular cells. In the *Mak*$^{-/-}$ testicular RNA sample, the amount of these two transcripts was severely diminished (the sizes of the two transcripts from the targeted allele were a little bit smaller, probably due to the deletion of exons 5 to 8 of *Mak*). Ab against MAK carboxy-terminus peptides (kindly provided by M. Shibuya) detected the two different sizes of *Mak* products, p66 and p60, in the *Mak*$^{+/+}$ and *Mak*$^{+/-}$ testicular cell lysates (Fig. 1D and reference 10). However, both products were totally absent in the *Mak*$^{-/-}$ testicular samples. In addition, we examined the protein kinase activity of immunoprecipitates from the *Mak*$^{-/-}$ testicular cell lysates with this anti-MAK Ab. As shown in Fig. 1E, kinase activity of the control serine/threonine kinase, MST (14), was present in both *Mak*$^{+/+}$ and *Mak*$^{-/-}$ testicular cell lysates, and MST itself and an artificial substrate, MBP, were phosphorylated. However, there was no MAK kinase activity in the *Mak*$^{-/-}$ testicular samples (no phosphorylation of MBP and a MAK natural substrate, p210 [10]).

To evaluate the impact of the *Mak* knockout on the development and function of male germ cells, we initially performed a histochemical analysis of the male reproductive organs. As shown in Fig. 2, spermatogenesis in most of the *Mak*$^{-/-}$ testes was normal, and mature sperm were present in the cauda (of 8 out of 9 animals). No significant differences were apparent with regard to size and weight of testis and epididymis among the age-matched *Mak*$^{+/+}$, *Mak*$^{+/-}$, and *Mak*$^{-/-}$ littermates (data not shown). These results allowed us to conclude that the *Mak* products are deficient in the *Mak*$^{-/-}$ mice, even though minor

truncated transcripts are present. The presence of spermatogenesis and viable sperm indicate that MAK is not essential for these processes.

Next we examined the fertility of the male *Mak*$^{-/-}$ mice in vivo. Due to the B6 × CBA mixed genetic background of the established animals, we performed two different mating experiments. As the initial experiment, we used these mixed-background *Mak*$^{-/-}$ males and their *Mak*$^{+/-}$ littermates. In other experiments we back-crossed *Mak*$^{+/-}$ mice with either B6 or CBA mice and then used F_6 male mice established from F_5 B6 or CBA *Mak*$^{+/-}$ animals. In either mixed-background or F_6 (B6 or CBA) animals, most of male *Mak*$^{-/-}$ mice were fertile (7 out of 9 for mixed background *Mak*$^{-/-}$ male mice and 3 out of 3 and 4 out of 4 for B6 and CBA F_6 *Mak*$^{-/-}$ male mice, respectively).

The data indicate that the fertilizing ability of *Mak*$^{-/-}$ males is within normal limits. To confirm this observation, another set of mating experiments was designed to examine the average litter size from the *Mak*$^{-/-}$ male × B6 female breeding pairs. Results obtained from these in vivo experiments demonstrated that the average litter size from the *Mak*$^{-/-}$ male × B6 female breeding pairs (7 male mice, 12 replicates; litter size, 5.4 ± 2.8 [$P = 0.017$]) was approximately 35% reduced compared to that from the control pairs (8 male mice, 14 replicates; litter size, 8.3 ± 2.6).

The motility of mature sperm obtained from the cauda epididymis in the B6 background *Mak*$^{-/-}$ mice was studied in vitro. Sperm motility of the *Mak*$^{-/-}$ mice was mildly reduced compared to that of *Mak*$^{+/+}$ mice, and the SMI for the *Mak*$^{-/-}$ males was about 80% (for experiment 1, $n = 2$ and SMI = 174 ± 24 [this result was inconclusive due to the small number of replicates]; for experiment 2, $n = 5$ and SMI = 220 ± 24 [$P = 0.039$]) that of the control *Mak*$^{+/+}$ males (for experiment 1, $n = 2$ and SMI = 225 ± 19; for experiment 2, $n = 6$ and SMI = 266 ± 20).

DISCUSSION

MAK kinase activity has been shown to be dominant in spermatocytes of the late pachytene stage and to dramatically decrease in postmeiotic haploid cells (10). As described previously (16, 18), MAK possesses a TY motif that is common to cell cycle regulatory molecules of the Cdc2/Cdk2 family of proteins and a TXY motif that is characteristic of mitogen-activated protein (MAP) kinases. Cyclin-dependent kinases and MAP kinases are two major superfamilies of serine/threonine kinases, and both kinase superfamilies are of critical importance in modulating various aspects of cellular processes, such as proliferation, differentiation, apoptosis, or effecter functions. It has been speculated, therefore, that MAK has an important role in such cellular processes during the meiotic division to morphologically mature male germ cells (10, 13, 16).

Morphologically, spermatogenesis in the *Mak*$^{-/-}$ mice was intact, and *Mak*$^{-/-}$ males were mostly fertile (*Mak*$^{-/-}$ females were also fertile as expected; data not shown). However, the average litter size of the *Mak*$^{-/-}$ male mice and their sperm motility in vitro were mildly reduced. Therefore, we concluded that function of MAK is not essential for spermatogenesis and male fertility. Our data may suggest that MAK is involved in cellular function(s) of the mature sperm linked to efficient fertilization, such as energy transfer or cellular survival. So far, propidium iodide staining shows no clear induction of cell death in the *Mak*$^{-/-}$ epididymal sperm (Y. Shinkai, unpublished results). Further studies will be needed to address this issue.

It has been reported that three other members of the large MAP kinase superfamily, MRK (1), MOK (17), and ICK (20), show significant homology to MAK on their kinase domains. Two of them, MOK and ICK, are ubiquitously expressed, including in testicular cells. Therefore, it is still possible that the function of MAK in spermatogenesis is redundant, and these kinases may substitute for the role(s) of MAK. Indeed, many MAP kinases have been shown to have redundant functions and cross-talk on their signaling pathways (reviewed in references 4 and 15). In the near future, analyses of the *Mak*$^{-/-}$/*Mok*$^{-/-}$ or *Mak*$^{-/-}$/*Ick*$^{-/-}$ double knockout mice may provide more clear answers to this question.

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