Journal of Reproduction and Development, Vol. 54, No. 2, 2008

—Full Paper—

A New ENU-Induced Mutant Mouse with Defective Spermatogenesis Caused by a Nonsense Mutation of the Syntaxin 2/Epimorphin (*Stx2/Epim*) Gene

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Abstract. *Repro34* is an *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation in mice showing male-specific infertility caused by defective spermatogenesis. In the present study, we investigated pathogenesis and molecular lesions in relation to spermatogenesis in the *repro34/repro34* homozygous mouse. Histological examination of the testis showed that the seminiferous epithelium of the *repro34/repro34* mouse contained spermatogonia and spermatocytes but no round and elongating spermatids. Instead of these haploid cells, multinucleated giant cells occupied the niche of the seminiferous tubules. Immunohistochemical staining for Hsc70t, an elongating spermatid specific protein, confirmed the absence of elongating spermatids. Furthermore, RT-PCR showed that there were significantly reduced expressions of the marker genes specifically expressed in the spermatid and that there was no difference in the expressions of the spermatocyte specific marker genes. These findings indicated interruption of the spermatogenesis during transition from the spermatocyte to spermatid in the *repro34/repro34* mouse. The *repro34* locus has been mapped on a 7.0-Mb region of mouse chromosome 5 containing the Syntaxin 2/Epimorphin (*Stx2/Epim*) gene, and targeted disruption of this gene has been reported to cause defective spermatogenesis. We therefore sequenced the entire coding region of the *Stx2/Epim* gene and found a nucleotide substitution that results in a nonsense mutation of this gene. The expression pattern of the *Stx2/Epim* gene during the first wave of spermatogenesis, increased expression at later stages of spermatogenesis, was in agreement with the affected phase of spermatogenesis in the adult *repro34/repro34* testis. We therefore concluded that the male infertility of the *repro34/repro34* mouse is caused by the interruption of spermatogenesis during transition from the spermatocyte to spermatid and that the nonsense mutation of the *Stx2/Epim* gene is responsible for the interruption of spermatogenesis.
Key words: ENU mutagenesis, Infertility, Mouse, Mutation, Spermatogenesis, Syntaxin 2/Epimorphin (*Stx2/Epim*) gene

(**J. Reprod. Dev. 54:** 122–128, 2008)

Mammalian spermatogenesis is one of the most dynamic processes of cell proliferation, differentiation and morphogenesis and involves numerous cellular and molecular steps. In order to understand the mechanisms underlying mammalian spermatogenesis, animal models with defective spermatogenesis are essential tools to identify the signaling pathways and molecules involved in regulation of spermatogenesis [1, 2]. So far, a large number of infertile genetic animal models have been established by spontaneous mutations and gene targeting technologies [2]. These animal models have contributed to understanding of the mechanisms involved in mammalian spermatogenesis [1–6]. They are also valuable for better understanding of the pathogenesis of human male infertility, which is mostly caused by defective spermatogenesis.

Recently, The Jackson Laboratory conducted the Reproductive Genomics Program in which a large number of mouse infertility models were systematically established by *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis [7]. Since ENU is one of the most effective alkylating mutagens that can randomly induce point mutations in genomic DNA, injection of ENU into the mouse induces mutations in germ cells 1,000-fold more often than those that occur spontaneously. Several large-scale ENU mutagenesis projects have been conducted for the mouse world wide to systematically produce a large number of mutant mice with particular phenotypes of interest [8, 9]. In the Reproductive Genomics Program, more than 30 mutants with reproductive abnormalities, including male and female infertility, have thus far been established by phenotypic screening of ENU-mutagenized mice [7, 10]. We have obtained one of these ENU-induced mutant mice, which shows male-specific infertility caused by defective spermatogenesis, and investigated its pathogenesis and molecular lesions. We report herein that the mutant mouse shows interruption of spermatogenesis during transition from the spermatocyte to spermatid and that a

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Accepted for publication: January 14, 2008
Published online: February 14, 2008
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Table 1. Nucleotide sequences of the primers used in semi-quantitative RT-PCR, sequencing and genotyping.

Gene	Forward (5'–3')	Reverse (5'–3')	Size (bp)
*H1t**	CGGCCTCAAGTACCCTTGTTCC	TTTCTTGCCCTTGCCCTTGT	345
*Hspa2**	CAGACGCAGACCTTCACTAC	TTTTGTCCTGCTCGCTAATC	433
Ccna1	ATGAGTTTGTCTACATCACTGACG	GTTGGCCCCACTCTCAGAAACC	510
*Hspal1**	GGTGATGAGGGTCTGAAG	GGGTGGGGGTGTGAAAAG	421
*Gapdhs**	AACATCATCCCATCTTCCACT	TCCGTGATAGCCGAGTAAGAAG	167
Prm2	GCTACCGAATGAGGAGCCCCA	GTGATGGTGCCTCCTACATTTCC	293
Tnp1	AGCCGCAAGATAAAGACTCATGG	CACAAGTGGGATCGGTAATTGCG	158
GAPDH	CTTTGGCATTGTGGAAGGG	CCTCTCTTGCTGCAGTGTC	554
Stx2 1	AGCAAGAGGCGGCGATCG	GAACATCACCTTTGCCACCCTG	1,008
Stx2 2	TGGCTGTCATTGCCGTCCTG	ACAGGCTAACTCTAGACCAGG	1,377
Stx2 3	AGCTAGACACTGCCTTGCGAA	AGGCTATGAAGTCAGCAGAGAGATG	1,193
Stx2 4	GTGGGATCACGAGTCACTCACT	GAGTGAGTTCCACGACAGCCA	360
Stx2 5	TGGCTGTCATTGCCGTCCTG	AGGCAAGGCCACTGTTGTGAAATC	435
Piwil1 1	AGAAGCCTGCTACAGGGGGTG	TACCCAGGTTGCTGGCTCGC	285
Piwil1 2	ACCACGACGATCAGGGAGTG	CAGATTGTAAGATTCTTCCAGAGAAGGAC	1,476
Piwil1 3	CCTCATCGACTACATCCACAAGG	ATCCCGCTAGAACCTCATCCTATG	1,406
Piwil1 4	ATTGGCCTGGAGTCATCCGAG	TCTAGCCACTCTAAGCTGCTCTC	1,220

*Primer sequences for these genes were obtained from Dix *et al*. [15].

nonsense mutation of the gene encoding Syntaxin2/Epimorphin (*Stx2/Epim*) is responsible for the defect of spermatogenesis.

Materials and Methods

Mice and preparation of DNA and RNA

The *repro34* mutant mice with male specific infertility were produced and provided by the ReproGenomics Program at The Jackson Laboratory. The mice have a mixed genetic background consisting of C57BL/6J and C3HeB/FeJ. The autosomal recessive gene responsible for the male infertility in the mutant mice has been mapped on mouse chromosome 5 and tentatively designated as *repro34*. Information regarding this mutant mouse, including rough mapping data and a brief description of the pathology, is available on the website of The Jackson Laboratory (http://reprogenomics.jax.org/mutants/G1-536-1.html). The *repro34/repro34* homozygous mice were obtained by mating of +/*repro34* heterozygous mice. The genotypes of the *repro34* locus were determined by typing *D5Mit138* and *D5Mit97* microsatellite markers flanking the *repro34* locus. Mice were euthanized using CO_2, and tissues samples, including the testes and epididymis, were excised. Testes were also excised from C57BL/6J mice on different postnatal days. Genomic DNA was extracted from these tissue samples by phenol-chloroform extraction, and total RNA was prepared from the testis samples by the acid guanidinium-phenol-chloroform (AGPC) method.

Histological analysis of the testis

Mouse testes were fixed in Bouin's fixative (picric acid:formaldehyde:acetic acid: =15:5:1) for 16–24 h at room temperature. After dehydration, the testes were embedded in paraffin and sectioned at 4 μm thickness. Hematoxylin and eosin stained sections were observed under lightmicroscopy.

For immunohistochemistry, testes were fixed in Bouin's fixative, rinsed with phosphate-buffered saline (PBS, pH7.4), dehydrated, embedded in paraffin, and sectioned to 4 μm thickness. After they were dewaxed, the sections were treated with blocking solution [(10% bovine serum albumin (BSA) in PBS)] and then incubated with anti-3 β-HSD antibody (gift from Dr. Mason; 1:100 dilutions in PBS with 3% BSA) or anti-Hsc70t antibody (gift from Dr. Fujimoto; 1:5,000 dilutions in PBS with 3% BSA) overnight at 4 C. After incubation with an horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for 1 hour at room temperature, the sections were washed three times with wash buffer (100 mM Tris, 150 mM NaCl and 0.05% Tween 20, pH 7.5), and the resultant immunocomplex was visualized by incubation with diamino benzidine. The sections were counterstained with Mayer's hematoxylin.

Expression analysis by semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To perform RT-PCR, first-strand cDNA was synthesized from 5 μg of the total testis RNA by reverse transcription using SpuerScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT primers (Invitrogen). The concentrations of cDNA were standardized by the level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression as a standard. The expression of marker genes and *Stx2/Epim* gene were examined by semi-quantitative RT-PCR under the following conditions: 25–30 cycles consisting of denaturation at 94 C for 30 sec, annealing at 50–55 C for 30 sec and extension at 72 C for 30 sec in a 10 μl reaction mixture with 2.25 mM $MgCl_2$, 100 nM of primers, 100 μM of each dNTP and 0.025 units/μl of *Taq* polymerase. The nucleotide sequences of the primers are shown in Table 1 (*H1t, Hspa2, Ccna1, Hspal1, Gapdhs, Prm2, Tnp1, GAPDH and Stx2 5*). The PCR products were electrophoresed through 2% agarose gel in TAE buffer and visualized with ethidiume bromide staining.

Table 2. Male-specific infertility of *repro34/repro34* mice

Male	Female	Litter number	Litter size
repro34/repro34	+/*repro34*	4	0
+/*repro34*	+/*repro34*	4	7.0 ± 1.5
+/*repro34*	*repro34/repro34*	3	6.7 ± 2.7

Sequence analysis

The entire coding regions of the candidate genes were amplified from the total testis RNA of normal and mutant mice by RT-PCR. First-strand cDNA was synthesized from 10 μg of the total RNA and used for PCR amplification with primer pairs for these genes. The nucleotide sequences of the primers are shown in Table 1 (Stx2 1–3, Piwil1 1–4). The PCR amplification was carried out under the same conditions as for semi-quantitative RT-PCR, and the nucleotide sequences of the amplified fragments were directly determined by the dideoxy chain termination method using an ABI310 automated DNA sequencer (Applied Biosystem, Foster, CA, USA.

Genotyping of the Stx2/Epim gene

The genotypes of the *Stx2/Epim* gene were determined by PCR-Restriction Fragment Length Polymorphism (RFLP). The genomic DNA samples obtained from the different genotypes of the mutant mouse stock and other inbred strains were used for genotyping. A 360-bp region of exon 3 of the *Stx2/Epim* gene containing the nucleotide substitution was amplified by PCR using a pair of primers (Table 1, Stx2 4). The PCR products were digested with *Taq*$^{\alpha}$I restriction endonuclease, and the digests were electrophoresed through 2.5% agarose gel in TAE buffer and visualized with ethidiume bromide staining.

Results

Interruption of spermatogenesis in repro34/repro34 mice

The breeding data confirmed male specific infertility in the *repro34* mutant mice. As shown in Table 2, mating between *repro34/repro34* homozygous males and +/*repro34* heterozygous females produced no offspring, while mating between *repro34/repro34* females and +/*repro34* males produced offspring with normal litter sizes. Examination of the anatomy of the *repro34/repr34* males showed significantly reduced testicular size with less than one-fourth that of the normal mouse. The other reproductive organs, including the epididymis and seminal vesicles, had no apparent abnormalities, but no sperm was observed in the lumen of the epididymis of the *repro34/repro34* mice, while a marked number of sperms accumulated in the normal mice (Fig. 1E and F). There were no abnormalities in the reproductive organs of the male and female +/*repro34* heterozygous mice

Histological examination of the testes of 10-week-old male mice revealed the pathogenesis of the defective spermatogenesis in the *repro34/repro34* mouse. At early stages of spermatogenesis (stages I-IV), the seminiferous epithelium of the +/+ and +/*repro34* mice consisted of spermatogonia, spermatocytes at the early pachytene stage and round and elongated spermatids (Fig. 1A). In contrast, the epithelium of the *repro34/repro34* mouse contained spermatogonia and spermatocytes, but no round or elongating spermatid (Fig. 1B). Instead of these haploid cells, multinucleated giant cells occupied the niche of the seminiferous tubules. The appearance of some of the nuclei in the multinucleated giant cells occasionally resembled those of the haploid nuclei of the round spermatid. In the epithelium at stages VII-X, the multinucleated cells were rarely seen, and most of the inner layer of the epithelium was occupied by pachytene spermatocytes with a normal appearance. At the later stages of spermatogenesis (stages XI-XII), multinucleated giant cells were frequently observed in the epithelium of the *repro34/repro34* mouse. In particular, those showing multiple metaphase plates with spindles were observed in this stage (Fig. 1D, inset). The occurrence of multiple metaphase plates in a single cell suggested that the germ cells of the *repro34/repro34* mice were able to differentiate through the meiotic prophase, but failed to complete the meiotic divisions (MI and MII) to generate haploid cells. The somatic cells of the mutant males, including the Sertoli cells and Leydig cells, were normal in appearance. Furthermore, immunohistochemical staining using anti-3 β-HSD antibody showed strong immunological reactivity in the interstitial cells of both the +/*repro34* and *repro34/repro34* testis (Fig. 2A and B). Since 3 β HSD is one of the crucial enzymes in the steroidogenesis that is specifically expressed in Leydig cells but not in other types of cells in the testis [11, 12], these finding indicated that the *repro34/repro34* mouse possesses a comparable number of steroidogenesis active Leydig cells. Taken together, it is likely that the infertility of the *repro34/repro34* mouse is caused by interruption of the germ cell differentiation that occurs later than the meiotic prophase and earlier than the round spermatid stage.

To confirm whether the mature spermatids were present in the *repro34/repro34* testis, we performed immunohistochemical staining of the testicular sections using anti-Hsc70t antibody. The Hsc70t protein is expressed in the cytoplasm of the elongating spermatid but not in less differentiated germ cells, including the spermatocyte and round spermatid [13]. As shown in Fig. 2C, the testis section of the +/*repro34* mouse showed strong immunological reactivity in elongating spermatids, but no signal was observed in spermatogonia, spermatocytes and round spermatids. On the other hand, the testis of the *repro34/repro34* mouse showed no positive signal in the seminiferous epithelium at various stages (Fig. 2D). These findings confirmed the absence of elongating spermatids in the *repro34/repro34* mice, and therefore it was evident that the spermatogenesis of the mutant mice did not proceed beyond the round spermatid stage.

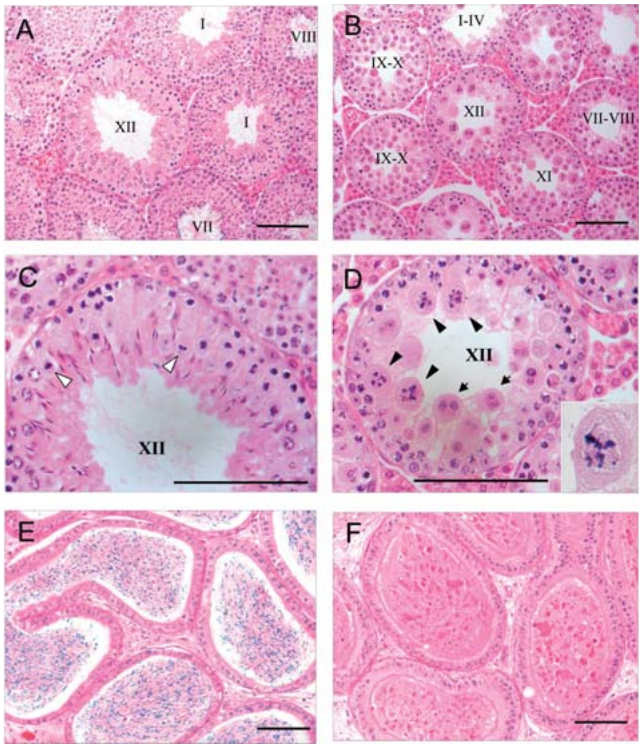

Fig. 1. Comparison of histology between +/*repro34* and *repro34/repro34* mice. Testes from 10-week-old +/*repro34* (A and C) and *repro34/repro34* (B and D) mice and epididymis from +/*repro34* (E) and *repro34/repro34* (F) mice were stained with HE. The seminiferous tubules of the *repro34/repro34* mouse contained no spermatids or spermatozoa; instead, multinucleated giant cells were observed (D; arrows). Note that the multinucleated giant cells with multiple metaphase plates was also observed in the stage XII seminiferous epithelium of the *repro34/repro34* mouse (D; arrowhead and inset), while normal metaphase cells were observed in that of the +/*repro34* mouse (C; open arrowhead). The epididymis of the mutant mouse contained no sperm (F). The Roman numerals in the tubular section indicate the stage of the seminiferous epithelium [31]. In the mutant testis, the stages were determined based on the morphology of spermatocytes and spermatogonia. Scale bars: 100 μm.

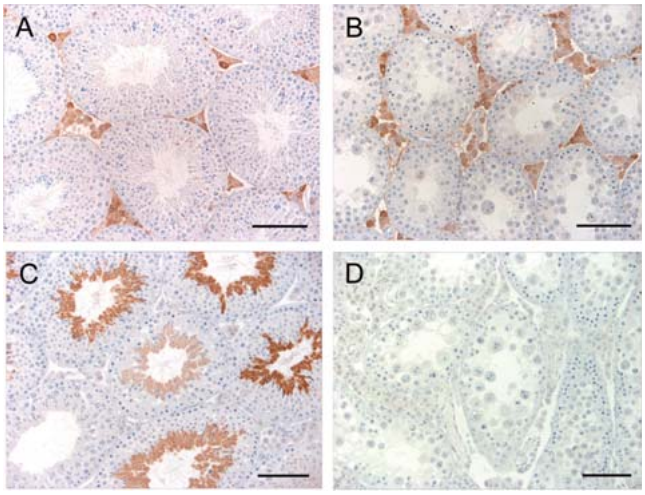

Fig. 2. Comparison of 3 β-HSD and Hsc70t positive cells between +/*repro34* and *repro34/repro34* mice. Testes from 10-week-old +/*repro34* (A and C) and *repro34/repro34* (B and D) mice were stained with anti-3 βHSD (A and B) or anti-Hsc70t (E, F) antibody. Strong immunological reactivity for 3 β-HSD was observed in the interstitial cells of both the +/*repro34* (A) and *repro34/repro34* (B) testes. Strong immunological reactivity for Hsc70t protein was observed in the cytoplasm of elongating spermatids in the +/*repro34* testis (C), but no reactivity was observed in the seminiferous epithelium of the *repro34/repro34* testis (D). Scale bars: 100 μm.

Expressions of the marker genes in specific stages of spermatogenesis

To further investigate the stage of spermatogenesis at which interruption of spermatogenesis occurred in the *repro34/repro34* mouse, the expressions of stage-specific marker genes were examined in *repro34/repro34* and +/+ mice by semi-quantitative RT-PCR. We employed genes for Histone H1t (*H1t*) [14], Heat shock protein 70-2 (*Hspa2*) [15], Cyclin A1 (*Ccna1*) [16], Heat shock protein 1-like (*Hspa1l*) [17], glyceraldehydes 3-phosphate dehydrogenase (*Gapdhs*) [18], Protamine 2 (*Prm2*) [19] and Transition protein 1 (*Tnp1*) [20]. The schematic expression patterns of these genes are indicated in Fig. 3. The results of semi-quantitative RT-PCR indicated no significant differences in *H1t* and *Hspa2* expression, slightly reduced expression of *Ccna1*, significantly reduced expressions of *Hspa1l*, *Gapdhs* and *Prm2*, and a trace level of expression of *Tnp1* in the *repro34/repro34* testis. The specific expressions of *H1t* and *Hspa2* in leptotene to diplotene spermatocytes, *Ccna1* in mid-pachytene to metaphase spermatocytes, *Hspa1l* and *Gapdhs* in the round and elongating spermatid stages and *Prm2* and *Tnp1* in the elongating spermatid have been reported previously (Fig. 3). Therefore, the observed expressions of these marker genes in the *repro34/repro34* testis, together with the histological observation of the testes, demonstrated that the spermatogenesis of the *repro34/repro34* mice was interrupted during transition from the spermatocyte to spermatid.

Sequence analysis and genotyping of the genes located in the critical region

The *repro34* locus has been mapped on a 7.0-Mb interval of mouse chromosome 5. According to the mouse genome database, this interval contains at least 60 genes. Among these 60 genes, we found two strong candidate genes for the *repro34* mutation, namely the piwi-like homolog 1 (*Piwil1*, also called *Miwi*) and Syntaxin 2/Epimorphin (*Stx2/Epim*) genes, since the targeted disruptions of these genes have been reported to cause the defective spermatogenesis [21, 22] that resembles a the phenotype of the *repro34/repro34* mouse. We therefore determined the nucleotide sequences of the entire coding regions of the *Piwil1* and *Stx2/Epim* genes of the *repro34/repro34* and +/+ litter mate mice by RT-PCR and direct sequencing of the amplified fragments. Comparison of the nucleotide sequences of these genes between the *repro34/repro34* and +/+ mice revealed a single nucleotide substitution of C to T at nucleotide 273 of the *Stx2/Epim* gene. This nucleotide substitution

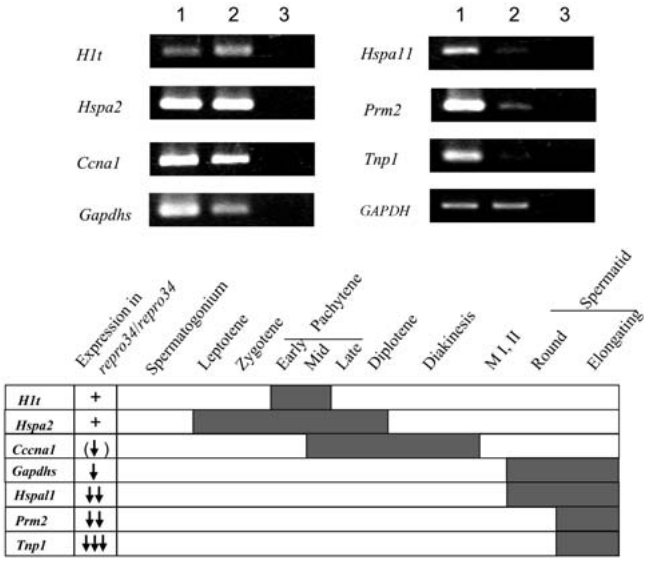

Fig. 3. Comparison of the expression levels of marker genes for particular stages of spermatogenesis by semi-quantitative RT-PCR. Expressions of seven marker genes in the adult testis were compared between +/+ (lane 1) and *repro34/repro34* (lane 2) mice, as well as a negative control (lane 3). *GAPDH* was used as a standard for the amount of RNA. Schematic expression patterns of these seven genes during spermatogenesis are shown at the bottom.

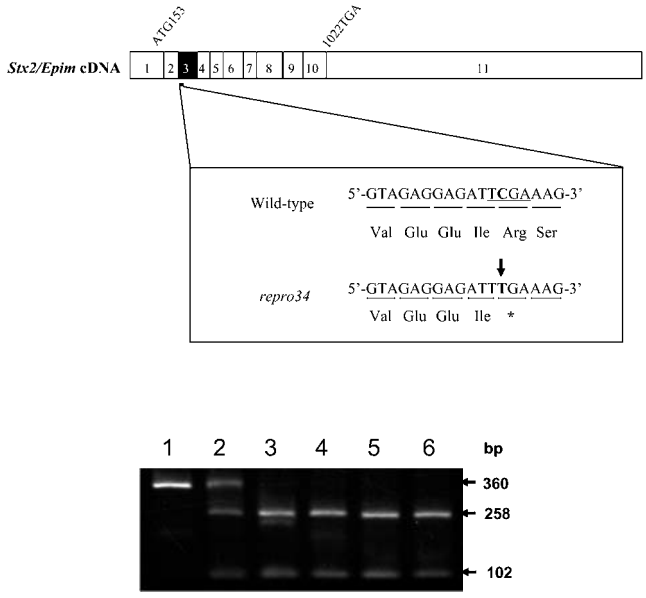

Fig. 4. A nonsense mutation of the *Stx2/Epim* gene in the *repro34* mutant allele. (Top) Partial nucleotide sequences of the *Stx2/Epim* gene showed a nonsense mutation in the *repro34* mutant allele. A nucleotide substitution of C to T in exon 3 results in a premature termination at codon 41 (*). (Bottom) PCR-RFLP of the *Stx2/Epim* gene for detecting the nonsense mutation. Since the substitution occurred in the recognition site of the *Taq*$^{\alpha}$I restriction enzyme (TCGA), the wild-type allele gave 102- and 258-bp digested fragments, while the mutant allele gave 360-bp undigested fragments. Lane 1: *repro34/repro34*, Lane 2: +/*repro34*, Lane 3: +/+ (C57BL6/J), Lane 4: +/+ (C3H/He), Lane 5: +/+ (A/J), Lane 6: +/+ (BALB/c).

causes a nonsense mutation, which results in premature termination at codon 41 of the gene (Fig. 4). On the other hand, no differences were observed in the *Piwil1* gene. These findings indicated that the nonsense mutation of the *Stx2/Epim* gene is responsible for the mutant mouse phenotype.

To confirm the concordance between the nonsense mutation and the defective spermatogenesis, genotypes of the *Stx2/Epim* gene regarding the C to T substitution at nucleotide 273 were examined in mice of the *repro34* stock and other inbred strains by the PCR-RFLP method. Since the nucleotide substitution occurred in a recognition site of *Taq*$^{\alpha}$*I* restriction endonuclease (Fig. 4), the *repro34* mutant allele has no *Taq*$^{\alpha}$*I* site in this region, although the wild type allele dose have it. Amplification of a 360-bp genomic region of the Stx2/Epim gene, including the nucleotide substitution, by PCR and digestion of the amplified fragment with *Taq*$^{\alpha}$*I* demonstrated that all the *repro34/repro34* mice had a 360-bp undigested fragment, while the +/+ and +/*repro34* mice had 102- and 258-bp digested fragments and both digested and undigested fragments, respectively. All the other strains of mice had the digested fragment (Fig. 4). Therefore, the nucleotide substitution of the *Stx2/Epim* gene was confirmed to be specific to the *repro34* mutation.

Expression of the Stx2/Epim gene during the first wave of spermatogenesis

To examine the onset of *Stx2/Epim* gene expression in mouse spermatogenesis, we performed semi-quantitative RT-PCR using RNA samples obtained from the testis of prepubertal mice from days 2 to 32 after birth, in which the first wave of the spermatogenesis progress. As shown in Fig. 5, constitutive low level expressions of the *Stx2/Epim* gene were detected from days 2 to 16, but the expression gradually increased from days 18 to 26, and the highest level of expression continued until day 32. The highest expression was also observed in the adult testis. Since the germ cells in the first wave complete meiotic prophase after day 18 [23], the observed expression pattern suggested that the onset of *Stx2/*

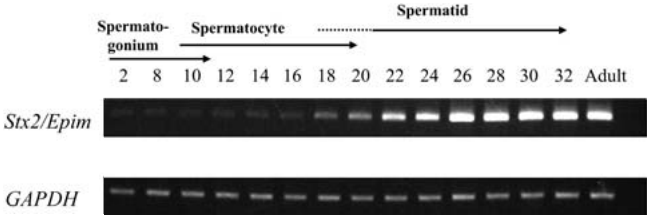

Fig. 5. Expression of the *Stx2/Epim* gene during the first wave of mouse spermatogenesis. The expression of the *Stx2/Epim* gene was examined in the testes of 2- to 32-day-old prepuberal and adult mice. The corresponding stages of the first wave of the spermatogenesis according to Bellvé *et al.* [23] are indicated at the top. The *GAPDH* gene was used as a standard for the amount of cDNA.

Epim gene expression in the germ cells of the testis is later than meiotic prophase.

Discussion

In the present study, we found a nonsense mutation of the *Stx2/Epim* gene in ENU-induced mutant mice exhibiting defective spermatogenesis. The mutation of the gene at codon 41 resulted in truncation of more than two thirds of the Stx2/Epim protein. Therefore, it is likely that the function of the *Stx2/Epim* gene is completely disrupted in the *repro34/repro34* mouse. Stx2/Epim is a member of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) family, which plays an essential role in membrane fusion at the terminal step of cytokinesis and vesicle fusion during exocytosis [24]. Stx2/Epim has also been also reported to be a mesenchymal derived cell surface-associated protein that is involved in various processes of epithelial morphogenesis, such as hair follicle growth and lung epithelial tubular formation [25]. Recently, Wang *et al.* [21] reported that targeted disruption of the *Stx2/Epim* gene in the mouse resulted in impaired spermatogenesis as well as increased intestinal growth and decreased susceptibility to chemical induced colitis. The testicular sections from the *Stx2/Epim* null mice showed depletion of spermatids and absence of mature spermatozoa in seminiferous tubules [21], which remarkably resemble the observations for the *repro34/repro34* mouse in the present study. The *Stx2/Epim* null mouse also showed no apparent abnormality in female reproductive traits as in the case of the *repro34/repro34* mouse. These phenotypic similarities between the *repro34/repro34* and *Stx2/Epim* null mice, in addition to identification of the nonsense mutation in the *Stx2/Epim* gene, indicated that the nonsense mutation of the *Stx2/Epim* gene is responsible for the defective spermatogenesis in the *repro34/repro34* mouse.

The testicular pathology of the *repro34/repro34* mouse has been described to be accumulation of meiotic division-phase spermatocytes and degenerating round spermatids in multinucleated cell bodies (http://reprogenomics.jax.org/mutants/G1-536-1.html). In the present study, we confirmed that the seminiferous epithelium of the *repro34/repro34* mouse contained spermatogonia and spermatocytes but no round and elongating spermatids, and a large number of multinuclear giant cells were observed in the tubules. The absence of elongating spermatids was confirmed by immunohistochemistry using anti-Hsc70t antibody. In addition to these phenotypes, we also found that giant cells with multiple metaphase plates were frequently observed in the epithelium at stage XII. Furthermore, significantly reduced expression of the marker genes expressed in round and elongating spermatids and trace level of expression of the genes expressed in the elongating spermatids were observed by semi-quantitative RT-PCR. These finding indicated that the spermatogenesis of the mutant mouse was interrupted during transition from the spermatocyte to spermatid and that it could not proceed beyond this stage. Since the SNARE function is associated with membrane fusion in cytokinesis, the *Stx2/Epim* gene may play an essential role in the final step, presumably in cytokinesis of meiosis. After completion of the prophase of meiosis I, the germ cells quickly undergo uninterrupted first and second cell divisions. Although the germ cells of the *repro34/repro34* mouse showed metaphase plates and haploid nuclei, no spermatids with normal appearance were observed in the seminiferous epithelium. Therefore, the germ cells of the mutant mouse likely underwent karyokinesis of meiosis I and II to form haploid nuclei but failed to complete cytokinesis to form haploid cells, thereby resulting in the formation of multinucleated cells. Although the cytoplasms of spermatids are still connected to each other by intercellular bridges after meiosis, the membrane fusion machinery might be required for cytokinesis of meiosis. The expression pattern of the *Stx2/Epim* gene in the testis during the first wave of spermatogenesis was in agreement with the abnormality observed in the mutant testis. In the prepubertal mouse, spermatogenesis starts just after birth and proceeds in a synchronized manner. During the first wave of the spermatogenesis, the expression level of the *Stx2/Epim* gene gradually increased after day 18 when the germ cells completed the meiotic prophase [23], suggesting that the *Stx2/Epim* gene functions in the germ cells later than the meiotic prophase.

The other possibility for the pathogenesis of the *repro34/repro34* mouse is that the interruption of spermatogenesis is mediated by dysfunction of the somatic cells in the testis. For example, expression of the *Stx2/Epim* gene has been observed in Leydig cells [21], and dysfunction of these cells can result in interruption of spermatogenesis since the secretion of testosterone from Leydig cells is essential for proper progression of spermatogenesis. However, a comparable number of 3 β-HSD-positive cells in the affected testis were observed in the present study, suggesting that the function of Leydig cells, at least the steroidogenesis of these cells, is unaffected in the *repro34/repro34* mouse. Further studies regarding the possible effects of the *Stx2/Epim* gene mutation on the function of somatic cells in the testis, including Leydig and Sertoli cells, and on the interaction between these somatic cells and germ cells are necessary to verify this possibility.

Stx2/Epim has also been reported to be localized in the acrosomal region of sperm [26, 27] and to be involved in the acrosome reaction in which calcium-stimulated exocytosis mediated by membrane fusion is required [28]. The present finding concerning the mutant mice did not provide any evidence regarding the involvement of Stx2/Epim in the acrosome reaction, as no mature sperm was obtained from them. However, the expression pattern of the *Stx2/Epim* gene during the first wave, which showed that there was the expression of the gene in post-meiotic gametes, was not contradictory to the possible function of Stx2/Epim in the acrosome reaction of mature sperm.

There are many spontaneous and targeted mutant mouse strains showing various types of defective spermatogenesis, and the most typical phenotype of them is arrest of spermatogenesis at the prophase of meiosis I by the pachytene checkpoint function [29]. However, the phenotype of the *repro34/repro34* mice showing interruption of spermatogenesis during transition from the spermatocyte to spermatid is definitively different from the typical phenotypes. One of the advantages of ENU mutagenesis for establishing animal models is the availability of various unique phenotypes by systematic screening, and we have actually obtained a mutant mouse showing the unique phenotype of defective sper-

matogenesis from the ENU mutagenesis program conducted by The Jackson Laboratory. Therefore, although targeting mutation of the *Stx2/Epim* gene has been reported, the results of the present study demonstrated the power of ENU mutagenesis to establish animal models of human infertility. The *repro34/repro34* mouse will be a unique animal model that is valuable for understating the pathogenesis of particular types of human male infertility. Furthermore, since a significant part of human male infertility is believed to be caused by autosomal recessive mutations [30], investigation of the human homologue of the *Stx2/Epim* gene in the patients showing interruption of spermatogenesis at the later stages of meiosis will reveal whether this gene is involved in particular types of male infertility.

Acknowledgement

We thank Drs. J. J. Eppig, M. A. Handel and J. C. Schimenti for providing the *repro34* mutant mice produced by the ReproGenomics Program at The Jackson Laboratory under the auspices of an NIH grant, P01 HD-42137. We also thank Drs. H. Fujimoto and T. Noce for the generous gift of antibodies. This work was supported by a grant (18650112) from the Japan Society for the Promotion of Science.

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