—Research Note—

Leydig Cell Hyperplasia in an ENU-induced Mutant Mouse with Germ Cell Depletion

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Abstract. *Repro22* is an *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation in mice showing depletion of both male and female germ cells. In the present study, we investigated the male phenotypes of the mutant mouse at the adult stage. The *repro22/repro22* homozygous mice showed reduced body weights as well as markedly reduced testis weights. Histological examination of the testes at 4 and 10 months of age showed no germ cells in the seminiferous tubules of the affected testis while a number of Sertoli cells were observed in the tubules. In addition to the germ cell depletion, the testes of the affected mouse contained expanded intertubular spaces that were filled by Leydig cell-like interstitial cells. These interstitial cells were confirmed to be Leydig cells by immunohistochemical staining using anti-3 β -HSD antibody. The estimated number of Leydig cells in the affected testes at 10 months of age increased approximately 2 fold compared with those of normal testes. Furthermore, the plasma testosterone levels of the affected mice at 10 months of age were significantly higher than those of the normal mice. These findings indicated that the *repro22/repro22* mouse developed hyperplasia of Leydig cells that was presumably caused by the absence of germ cells in the seminiferous tubules.

Key words: ENU mutagenesis, Germ cell, Infertility, Leydig cell, Mouse, Testosterone

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Infertility is a major health problem worldwide that influences more than 10% of couples, and approximately 50% of cases of infertility are caused by male factors. In particular, Sertoli cell-only syndrome, a form of non-obstructive azoospermia, is the most serious male infertility, as patients with this disorder show germ cell depletion and are untreatable using assisted reproduction techniques. Although a significant portion of male infertilities is believed to be caused by autosomal recessive mutations [1], the genetic factors that underlay most nonsyndromic male infertilities have not yet been identified, with a few exceptions [2, 3]. Mutant mice established by gene targeting technology, chemical mutagenesis and spontaneous mutations [4–7], showing various features of infertility are expected to be useful animal models for investigating the pathogenesis and genetic factors of human infertilities. However, only a limited number of mouse models for Sertoli cell-only syndrome or germ cell depletion have been reported [8–11]. *Repro22* is a mutation in mice recently produced by *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis at The Jackson Laboratory, showing depletion of germ cells in both the male and female. In the present study, we investigated male phenotypes of the mutant mouse at the adult stage and found that the mutant mouse

showed Leydig cell hyperplasia and an elevated plasma testosterone level in addition to germ cell depletion.

Materials and Methods

The mice bearing the *repro22* mutation were produced and provided by the ReproGenomics Program at The Jackson Laboratory. The mice have a mixed genetic background of C57BL/6J and C3HeB/FeJ. The autosomal recessive gene responsible for the germ cell depletion in the mutant mice has been mapped on mouse chromosome 4 and tentatively designated as *repro22*. Information about this mutant mouse is available on the website of The Jackson Laboratory (<http://reprogenomics.jax.org/mutants/G1-453-41.html>). The *repro22/repro22* homozygous mice were obtained by mating between *+repro22* heterozygous mice. The genotypes of the *repro22* locus were determined by typing *D4mit54* and *D4mit42* microsatellite markers flanking the *repro22* locus. Mice were euthanized by CO₂, body and testis weights were recorded and their testes were collected. Blood samples were also collected and the plasmas were stored at –20 C for subsequent hormone assay.

Testes of mice were fixed in Bouin's solution, embedded in paraffin and sectioned at 4 μ m thickness. Hematoxylin and eosin stained sections were observed under light microscopy. For immunohistochemistry, testes were fixed in Bouin's solution, embedded

in paraffin, and sectioned at 4 μ m thickness. After they were dewaxed, the sections were treated with blocking solution (10% BSA in PBS) and then incubated with anti-3 β -hydroxysteroid dehydrogenase (3 β -HSD) rabbit IgG (gift from Dr. I Mason, 1:100 dilutions in PBS with 3% BSA) overnight at 4 C. After incubation with an HRP-conjugated goat anti-rabbit antibody for 1 hour at room temperature and washing three times with wash buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20), the resultant immunocomplex was visualized by incubation with diaminobenzidine. The sections were counterstained with Mayer's hematoxylin. The total number of Leydig cells in testes was estimated from the numbers of 3 β -HSD positive cells in the sections and total volume of the testes.

The concentration of testosterone in blood plasma was determined with enzyme immunoassays as described previously [12]. In brief, samples were incubated with anti-testosterone antiserum (1:20,000 dilution); (gift from Dr. B Szafranska) at room temperature for 18–24 h, horseradish peroxidase-labeled testosterone (1:20,000 dilution) was added to them and then they were incubated for 4 h at 4 C. After addition of substrate buffer with 0.05% 3,3',5,5'-tetramethylbenzidine, the samples were further incubated at 36 C for 40 min, and the reaction was stopped by addition of H₂SO₄. The absorbency was measured at 450 nm with a plate reader (Bio-Rad, Hercules, CA, USA).

The data obtained were analyzed by Student's *t*-test. Differences were accepted as significant at *P*<0.05.

Results

Mating between +/*repro22* heterozygous male and female mice produced affected (*repro22/repro22*) mice at a ratio slightly lower than the expected Mendelian ratio (1:3). The sizes of the affected mice were apparently smaller than those of normal mice at weaning (data not shown). A comparison of body weight between the affected and normal mice at 4, 6 and 10 months of age (Fig. 1) indicated that the body weights of the affected mice were significantly less than those of normal mice at 4 months of age, but no significant difference was observed at 6 and 10 months. Examination of the anatomy of the affected mice showed markedly reduced testicular sizes, while no apparent visible differences were observed in accessory sex organs. The testis weights of the affected mice were approximately 10% of those of the normal mice at 10 months of age (Table 1).

Histological examination of the testes showed germ cell depletion in the *repro22/repro22* mouse. As shown in Fig. 2, no germ cells were observed in the seminiferous tubules of the affected testis, while germ cells of different stages, including spermatogonia, spermatocytes, spermatids, and spermatozoa in addition to Sertoli cells were clearly observed in the normal mouse. In the tubules of the affected mice, aggregates of unidentified cells, which were presumably Sertoli cells, were frequently observed in the lumen of the tubules, and isolated Sertoli cells were observed in the peripheral regions of the tubules. Most of the peripheral Sertoli cells were attached to the basement membranes of the tubules at 4 months age; however, at 10 months of age, only a few Sertoli cells were still attached to the basement membranes and cell aggregates were

more abundant. Vacuoles were frequently observed in tubules at both 4 and 10 months of ages. The diameters of the seminiferous tubules were reduced to approximately 50% of the normal tubules, and the intertubular region was apparently expanded. The expanded intertubular spaces of the affected testes were filled with interstitial cells that looked like Leydig cells. The interstitial cells were more abundant at 10 months of age (Fig. 2, D). To confirm whether the interstitial cells of the affected testes were Leydig cells, we performed immunohistochemical staining of testicular sections using anti-3 β -HSD antibody. Three- β -hydroxysteroid dehydrogenase is one of the crucial enzymes in steroidogenesis that is specifically expressed in Leydig cells but not in other types of cells in the testis [13, 14]. As shown in Fig. 3, the testes sections from normal mice showed strong immunological reactivity in Leydig cells in normal testes, and the signal was also observed in the abundant interstitial cells of the affected testes at 4 and 10 months of age. These findings confirmed that the interstitial cells in the intertubular spaces of the affected testes were steroidogenesis active Leydig cells. We then estimated the number of Leydig cells in affected and normal testes at 10 months of age by counting the 3 β -HSD positive cells. The estimated total number of Leydig cells in the affected testis was significantly higher than that of normal testis (Table 1). These findings indicated that hyperplasia of Leydig cells developed in the *repro22/repro22* mice at this stage.

Next, we measured the plasma testosterone levels in affected and normal mice at 10 months of age to reveal whether or not the increased number of Leydig cells affected the hormone level. The results showed significantly increased plasma testosterone levels in the affected mice compared with the normal mice (Table 1). The elevated plasma testosterone level might be a consequence of the increased number of Leydig cells in the *repro22/repro22* mouse.

Discussion

The most obvious phenotype of the *repro22/repro22* male mouse was depletion of germ cells in the testis. The affected mice showed complete absence of spermatogonia, spermatocytes, spermatids and spermatozoa in seminiferous tubules. In addition to germ cell depletion, we found an increased number of Leydig cells and elevated plasma testosterone level in the aged *repro22/repro22* mouse in the present study. Hyperplasia of Leydig cells associated with germ cell depletion has also been reported in human and mouse male infertilities. Klinefelter syndrome is the most common sex chromosome aneuploidy in humans with a 47, XXY karyotype. Patients of Klinefelter syndrome have small testes with azoospermia caused by depletion of germ cells, and they frequently show Leydig cell hyperplasia [15]. Therefore, the Leydig cell hyperplasia observed in the *repro22/repro22* mouse is likely caused by a secondary effect of the mutation mediated by germ cell depletion rather than direct effects of the mutation on proliferation of Leydig cells, although we could not exclude the latter possibility.

It should be noted that elevated plasma FSH and LH levels have been observed in conjunction with germ cell depletion in humans and mice [15, 16]. FSH and LH are known to stimulate Sertoli cells to accelerate spermatogenesis and Leydig cells to secrete testosterone, respectively, but both LH and FSH have also been

Fig. 1. Comparison of body weights between affected (*repro22/repro22*) and normal (+/+ or +/*repro22*) mice at 4, 6 and 10 months of age. The body weight was significantly lower at 10 months of age. Values represent means $\pm$ SEM (n=4-5). *Statistically significant ($P < 0.03$).

Fig. 2. Comparison of histology between affected and normal mice. The testes of 4- and 10-month-old normal (+/+ or +/*repro22*; A and C) and affected (*repro22/repro22*; B and D) mice were stained with HE. The seminiferous tubules of affected mice contained no germ cell. Note that cell aggregates (arrows) and vacuoles (arrowheads) were frequently observed in the lumen of the seminiferous tubules of the *repro22/repro22* mouse. Scale bars: 20 μ m.

Fig. 3. Comparison of steroidogenesis positive Leydig cells between affected and normal mice. Testes of 4- and 10-month-old normal (+/+ or +/*repro22*; A and C) and affected (*repro22/repro22*; B and D) mice stained with anti-3 β HSD antibody. Immunological reactivity for 3 β HSD was observed in the cytoplasm of interstitial cells in both normal and affected testes and the expanded intertubular spaces of the *repro22/repro22* testis were filled with 3 β HSD-positive cells. Scale bars: 20 μ m.

reported to stimulate proliferation of Leydig cells [17, 18]. Therefore, the elevated levels of FSH and LH have been thought to be the cause of the hyperplasia of Leydig cells in Klinefelter syndrome. Interaction between germ cells and Sertoli cells in the testis is essential for the cellular activity of Sertoli cells, and disturbance of this interaction by absence of germ cells could result in dysfunction of Sertoli cells [16, 19]. In the present study, detachment of Sertoli cells from the basement membranes of seminiferous tubules was observed in the aged affected mice. These findings suggested dysfunction of Sertoli cells in the affected mice, since attachment of Sertoli cells to the basement membrane is indispensable for maintenance of their functions [20]. Dysfunction of Sertoli cells, including inhibin secretion, might in turn cause increased levels of FSH. Furthermore, positive correlation between the FSH and LH levels of males has been reported [21, 22], since secretion of these gonadotropins shares a common regulatory system. Taken together, the hyperplasia of Leydig cells in the *repro22/repro22* mouse might be caused by dysfunction of Sertoli cells that results in elevated secretion of both FSH and LH; however, measurement of the FSH, LH and inhibin levels in mutant mice is necessary to prove this hypothesis. On the other hand, disturbance of the inter-

Table 1. Comparison of testis weight, number of Leydig cells and plasma testosterone (T) level between normal (+/+ or +/*repro22*) and affected (*repro22/repro22*) mice at 10 months of age

Mice	Testis weight (mg)	Number of Leydig cells ($\times 10^5$ /testis)	Plasma T (ng/ml)
Normal (n=4)	200.3 $\pm$ 6.7	7.68 $\pm$ 1.21	0.91 $\pm$ 0.02
Affected (n=5)	22.8 $\pm$ 3.9**	16.6 $\pm$ 1.77**	4.30 $\pm$ 2.43*

Values represent means $\pm$ SEM. *Statistically significant ($P < 0.01$). **Statistically significant ($P < 0.001$).

action among germ cells and different types of somatic cells in the testis by germ cell depletion might directly affect proliferation of Leydig cells. For example, desert hedgehog (*Dhh*) secreted from Sertoli cells during testis development is essential for proliferation and differentiation of Leydig cell precursors [23]. Therefore, disruption of the signals from germ cells or aberrant signals from Sertoli cells can directly affect proliferation of Leydig cells.

The reduced body weight observed in the *repro22/repro22* mouse was similar to that observed in other mutant mice showing germ cell depletion. For example, mice with *gcd* mutation or targeted disruption of *Tiar* gene showing germ cell depletion have been reported to be significantly smaller than their littermates at embryonic stages [24, 25]. Furthermore, the proliferation activity of embryonic stem (ES) cells established from the *Tiar* deficient mouse is significantly lower than those of normal ES cells under certain conditions [25]. This evidence suggests that the genes required for germ cell development also have particular functions on proliferation of somatic cells during embryonic development. Therefore, it is of particular interest whether the reduced body weight of the *repro22/repro22* mouse is caused by the difference in embryonic development or not. On the other hand, while the reduced body weights of the mutant mice were apparent until 4-months of age, no significant difference was observed at 6 and 10-months of age (Fig. 1). Since testosterone have a positive effect on body weight [26], the elevated serum testosterone levels of the *repro22/repro22* mice might have some effects on the recovery of body weight of the mutant mice at the adult stage. It should also to be noted that those mutant mice with the germ cell depletion show normal numbers of primordial germ cells (PGCs) at the early embryonic stage, but the number of PGCs decreases significantly after migration of the cells to genital ridge [24, 25], indicating that the genes responsible for these mutations play important roles in proliferation, migration and survival of PGCs. The similar phenotype of the *repro22/repro22* mice suggested that the gene responsible for the *repro22* mutation is also involved in germ cell proliferation, migration and survival.

The *repro22* mutant mouse will be a useful animal model for human infertility caused by germ cell depletion. In particular, it might be valuable for evaluating the effects of germ cell depletion on the somatic cells of the testis, including Leydig cells and Sertoli cells, and on other physiological processes during development.

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