The Creation of Mouse Models for Human Diseases Associated with Reproductive Disturbances by In Vitro Fertilization and Embryo Transfer

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Abstract: The induction of ovulation by hormone treatment, preparation of fertilized eggs by in vitro fertilization and recovery of offspring by embryo transfer were studied in five strains of mutant mice: C57BL/6-dy/dy progressive muscular dystrophy model, C57BL/6-ob/ob obesity model, and BALB/c-rl/rl, BALB/c-shi/shi and C57BL/6-mld/mld motor ataxia models. The homozygotes of these mutant mice are all affected with the disease about 2 weeks after birth, followed by reproductive disturbances. Ovulation could be induced by injection with PMSG-hCG in the females. Sperm was obtained from the cauda epididymis of males and used for in vitro fertilization. The success rate of the in vitro fertilization was as low as 71.6% in C57BL/6-dy/dy mice, but was over 85% in the other strains. When 2-cell embryos obtained by in vitro fertilization were transferred to the oviducts of pseudopregnant recipients, offspring were obtained from 39.2–57.7% of the transferred embryos. These offspring developed the expected diseases about 2 weeks after birth, and it was confirmed that the disease characters were reliably reproduced. These results demonstrate that the experimental system of in vitro fertilization and embryo transfer enables production of the offspring homozygous for a mutant gene and use of them for experiments before the onset of the disease which has been impossible.

Key words: animal models for human diseases, disturbance of reproduction, embryo transfer, in vitro fertilization, mutant mice

Introduction

Animal models play an important role in the research on human diseases, and the use of animal models which satisfy the objectives of such a research is an important factor in determining the results of the research. Vari-

ous methods are used in the development of animal models, including isolation of spontaneous mutants discovered by chance [3–5], selection of individual animals with specific characters [3–5] and creation of transgenic animals by introducing a gene into the animal for the purpose [2]. Among these animals, mutant strains

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which become infertile with the onset of the disease in the homozygous state of an autosomal recessive gene are usually obtained as homozygotes by mating heterozygous females and males [3–5]. However, since homozygotes, heterozygotes and normal animals are mixed in the same litters by this breeding method, it is impossible to differentiate homozygotes from the others before the onset of the disease. Therefore, experiments using clearly identified homozygotes during the fetal period and before the onset of the disease have been impossible.

We already reported that *in vitro* fertilization and embryo transfer are available in the production and maintenance of mutant strains of mice associated with reproductive disturbances (The 35th annual meeting of the Japanese Society of Experimental Animals: 1985). In the present study, ovulation by hormone injection, preparation of fertilized eggs by *in vitro* fertilization and production of offspring by embryo transfer were studied in homozygous mice of five mutant strains with reproductive disturbances which have been used as animal models for human diseases. Application of this experimental system of *in vitro* fertilization and embryo transfer makes it easy to maintain and produce such animal models with reproductive disturbances and additionally it possible to use homozygous animals for experiments before the onset of the disease signs, which was formerly impossible.

Materials and Methods

1. Animals: Five mutant strains of mice were used for the induction of ovulation and *in vitro* fertilization: C57BL/6-*dy/dy*, a model for progressive muscular dystrophy; C57BL/6-*ob/ob*, a model for obesity; and BALB/c-*rl/rl*, BALB/c-*shi/shi* and C57BL/6-*mld/mld*, models for motor ataxia. All mutant mice were produced in the Central Institute for Experimental Animals and used at 8 to 16 weeks of age. Female closed colony Jcl:ICR mice (CLEA Japan) were used as recipients for the transfer of the *in vitro* fertilized embryos and as foster mothers for pups.

2. Rearing conditions: The animal rooms were lighted for 14 hr (6:00 a.m.–8:00 p.m.) and darkened for 10 hr. The temperature was controlled at $24 \pm 2^\circ\text{C}$ and the humidity at 50–60%. Mice were kept in plastic cages bedded with wood chips. Pelleted-diet (CLEA

Japan; CA-1) was given to mice, but powder diet mixed with water was given as required to mutant strains with abnormal behavior after the onset of the disease. Water was provided *ad libitum*.

3. Induction of ovulation: Females of each strain were injected intraperitoneally with 5 i.u. of pregnant mare's serum gonadotropin (PMSG) and 5 i.u. of human chorionic gonadotropin (hCG) at a 48-hr interval to investigate the availability of this treatment to induce ovulation. The number of ovulated eggs was determined by counting the eggs obtained from the ampulla of oviducts 15 to 17 hr after the hCG injection, followed by *in vitro* fertilization.

4. *In vitro* fertilization and culture: *In vitro* fertilization was performed using the TYH medium as reported [9, 10]. Before use, 0.4 ml of the medium was placed in a plastic Petri dish (3.5 × 10 mm: Nunc), covered with paraffin oil, and equilibrated to the temperature and gas phase of 5% CO₂ in an incubator overnight. The eggs obtained by the induced ovulation were used. Sperm was obtained from the caudaepididymis of male homozygotes of each strain. After it was preincubated for 2 hr as a suspension in TYH medium covered with paraffin oil, insemination was performed by adding about 10 μl of the sperm suspension to 0.4 ml of TYH medium containing eggs so that the final concentration of spermatozoa was 100–300/ μl . The number of ovulated eggs was determined from 6 hr after insemination, and then the success of fertilization was observed on an inverted microscope using the male and female pronuclei and the second polar body released as indices. The eggs considered to be fertilized were cultured for another 20 hr under the same conditions or were transferred to m-WM medium [14] for development. The development into the 2-cell stage was examined.

5. Embryo transfer: Some of the embryos which developed into the 2-cell stage in culture were transferred to the oviducts of the pseudopregnant recipients (day when a copulation plug was observed: day 1) prepared by sterile mating with vasectomized males. The standard number of transferred embryos was 8 per unilateral oviduct or 16 on both sides. When the recipient females did not pup on the anticipated day after embryo transfer, the offspring were obtained by Caesarean section and then were reared by other foster mothers. The growth of the obtained offspring was observed, and the inheritance of the disease of each mutant strain

was examined by appearance of abnormal behaviors.

Results

1. *Induction of ovulation:* The proportion of the females in which ovulation could be induced by PMSG and hCG treatment and the number of ovulated eggs are shown for each mutant strain in Table 1. The proportion of ovulated females was as low as 65.8% (96/146) in C57BL/6-*dy/dy* but over 80% in the other strains. The average number of ovulated eggs per female was the smallest or 9.8 in C57BL/6-*dy/dy* and the largest or 20.4 in C57BL/6-*mld/mld*. It varied from 11 to 13 in the other mutant strains.

2. *In vitro fertilization:* Table 2 shows the results of *in vitro* fertilization for each strain. The fertilization rate 6 hr after insemination was as low as 71.6% in C57BL/6-*dy/dy* but consistently higher than 85% in the other mutants. Over 95% of these fertilized eggs developed into 2-cell stage in culture.

3. *Embryo transfer:* Table 3 shows the results of the transfer of 2-cell stage embryos obtained in culture after *in vitro* fertilization. The recipient females trans-

ferred with embryos showed a pregnancy rate different from 87.5 to 100% among mutant strains. As a result, offspring were obtained from 39.2 to 57.7% of the transferred embryos. Among the pregnant recipient females, 36 (76.6%) underwent spontaneous deliveries, but 11 (23.4%) did not deliver on the morning of the anticipated day, from which offspring were obtained by Caesarean section and then nursed by foster mothers.

4. *Rearing and verification of offspring:* More than 90% of the offspring obtained were weaned. At about 2 weeks of age, the disease characteristic of each mutant strain was confirmed to occur in all offspring by appearance of characteristic behaviors. Thus, the genetic characters of the mutant strains could be exactly reproduced by the experimental system of *in vitro* fertilization and embryo transfer.

Discussion

The mutant mouse strains of animal models used in the present study are all affected with a disease in homozygotes of an autosomal recessive mutation. The C57BL/6-*dy/dy* muscular dystrophy and C57BL/6-*ob/ob*

Table 1. Results of induced ovulation in mutant strains of mice with reproductive disturbances

Strain	No. of females used	No. of females with ovulation (%)	No. of eggs ovulated (Av $\pm$ S.D)
C57BL/6- <i>dy/dy</i>	146	96 (65.8)	939 (9.8 $\pm$ 9.2)
C57BL/6- <i>ob/ob</i>	22	18 (81.8)	231 (12.8 $\pm$ 7.1)
BALB/c- <i>rl/rl</i>	22	22 (100)	258 (11.7 $\pm$ 4.6)
BALB/c- <i>shi/shi</i>	29	28 (96.6)	357 (12.8 $\pm$ 5.2)
C57BL/6- <i>mld/mld</i>	18	18 (100)	368 (20.4 $\pm$ 14.6)

Table 2. Results of *in vitro* fertilization in mutant strains of mice with reproductive disturbances

Strain	No. of females used	No. of eggs examined	No. of fertilized eggs (%) ¹⁾	No. of embryos developed to 2-cell(%) ²⁾
C57BL/6- <i>dy/dy</i>	146	939	672 (71.6)	653 (97.2)
C57BL/6- <i>ob/ob</i>	22	231	209 (90.5)	202 (96.7)
BALB/c- <i>rl/rl</i>	22	258	221 (85.7)	215 (97.3)
BALB/c- <i>shi/shi</i>	29	357	314 (88.0)	305 (97.1)
C57BL/6- <i>mld/mld</i>	18	368	360 (97.8)	359 (99.7)

¹⁾: Percentage of examined eggs.

²⁾: Percentage of fertilized eggs.

Table 3. Results of transfer of embryos derived from *in vitro* fertilization in mutant strains of mice with reproductive disturbances

Strain	No. of embryos transferred	No. of pregnant/ No. of recipients (%)	No. of pups (%) ¹⁾	No. of young weaned (♀ : ♂)
C57BL/6- <i>dy/dy</i>	209	14/16 (87.5)	82 (39.2)	75 (34:41)
C57BL/6- <i>ob/ob</i>	84	6/6 (100)	46 (54.8)	44 (28:16)
BALB/c- <i>rl/rl</i>	126	8/9 (88.8)	60 (47.6)	56 (31:25)
BALB/c- <i>shi/shi</i>	111	10/10 (100)	64 (57.7)	64 (23:41)
C57BL/6- <i>mld/mld</i>	134	9/10 (90.0)	72 (53.7)	72 (31:41)

¹⁾: Percentage of transferred embryos.

ob obesity models show macroscopic abnormalities from 2 weeks after birth, and both males and females are sterile because of impossible copulation. The motor ataxia models, BALB/c-*rl/rl*, BALB/c-*shi/shi* and C57BL/6-*mld/mld*, develop motor ataxia at 2 weeks of age. Neither males nor females exhibit normal mating behavior and offspring can not be obtained by the mating of homozygotes [3–5]. Therefore, in these mutant strains, homozygotes are obtained from about one fourth of offspring by mating heterozygous females and males [3, 6]. Ovarian transplantation intended for obtaining homozygous offspring more efficiently from ovaries of homozygous females transplanted in normal females has also been reported [8].

In the present study, it was evident that ovulation could be induced with PMSG and hCG in homozygous females of mutant strains associated with reproductive disturbances. Among these strains, the ovulation-inducing rate was as low as 65.8% and the average number of ovulated eggs per ovulated female was only 9.8 in C57BL/6-*dy/dy*. The lower ovulation-inducing rate and the smaller number of ovulated eggs might be associated with the very poor growth due to muscular dystrophy reflected in the body weight of only about 15 g at 8 weeks of age when the mouse is mature. In the other strains, both parameters were comparable to or slightly lower than those of the normal BALB/c and C57BL/6 strains [11]. Three-to 4-week old immature mice are known to be most responsive to the hormones and ovulate the largest number of eggs [13, 15]. Therefore, it will be necessary to study induced ovulation in young mice of these strains. However, it was possible to obtain fertilizable spermatozoa from the cauda epididymis of the homozygous males of each strain. The *in vitro* fertilization rate was as low as 71.6% in C57BL/

6-*dy/dy*. Furthermore, it was as high as 85.7–97.8% in the other strains and did not differ from that in normal BALB/c and C57BL/6 strains [12]. It became evident that the embryos developed into the 2-cell stage in culture after *in vitro* fertilization produced offspring at a high frequency when transferred into the oviducts of pseudopregnant recipients. These offspring showed the strain-specific abnormalities, demonstrating that our method did not modify the features of the disease at all in the mutant strains.

Suzuki *et al.* [7] also reported that the *in vitro* fertilization technique is available for production and maintenance of obese diabetic C57BL/KsJ-*db/db* mice. The experimental system enables us to use fetuses or neonates homozygous for the mutant genes for experiments before the onset of the disease. A detailed histological study on the onset of muscular dystrophy and progress of the symptoms was already made using homozygous C57BL/6-*dy/dy* mice prepared by this *in vitro* fertilization and embryo transfer system, and the important results were obtained [1]. Since this experimental system can be applied to many strains in which eggs and spermatozoa can be used in *in vitro* fertilization, it should be effective in various types of animal experiments and breeding systems for mutant animals with reproductive disturbances in the future.

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