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Genetic Analyses of Fancy Rat-Derived Mutations

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Abstract: To collect rat mutations and increase the value of the rat model system, we introduced fancy-derived mutations to the laboratory and carried out genetic analyses. Six fancy rats were shipped from a fancy rat colony in the USA and used as founders. After initial crosses with a laboratory strain, TM/Kyo or PVG/Seac, inbreeding started and 6 partially inbred lines, including 2 sublines, were produced as Kyoto Fancy Rat Stock (KFRS) strains. During inbreeding, we isolated 9 mutations: 5 coat colors, American mink (*am*), Black eye (*Be*), grey (*g*), Pearl (*Pel*), siamese (*sia*); 1 coat pattern, head spot (*hs*); 2 coat textures, Rex (*Re*), satin (*sat*); and an ear pinnae malformation, dumbo (*dmbo*). Genetic analyses mapped 7 mutations to particular regions of the rat chromosomes (Chr): *am* to Chr 1, *sia* to Chr 1, *sat* to Chr 3, *Re* to Chr 7, *g* to Chr 8, *dmbo* to Chr 14, and *hs* to Chr 15. Candidate gene analysis revealed that a missense mutation in the tyrosinase gene, Ser79Pro, was responsible for *sia*. From mutant phenotypes and mapping positions, it is likely that all mutations isolated in this study were unique to the fancy rat. These findings suggest that fancy rat colonies are a good source for collecting rat mutations. The fancy-derived mutations, made available to biomedical research in the current study, will increase the scientific value of laboratory rats.

Key words: bioresource, coat color, genetic mapping, inbreeding, mutation

Introduction

Genetic analyses of common diseases in humans have revealed that gene mutations are involved in diseases. Genome sequencing projects of various mammalian species followed by comparative genome analyses have revealed that a large number of genes are shared among species. Thus, it is thought that mutations found in model animals and animals carrying such mutations can contribute to the better understanding of human diseases.

The laboratory rat (*Rattus norvegicus*) has been widely used as an animal model of human diseases, because its size is suitable for manipulation [1, 27]. Sequencing of the rat genome has shown that the rat has about 20,000 predicted genes and shares as many as 90% with humans [9]. So far, at least 70 mutations have been identified as causative genes of specific diseases and rat strains carrying such mutations can be used as good animal models for these diseases; however, considering the high number of rat genes predicted [9], more mutations will be required to investigate the full range of diseases. Thus,

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collecting rat mutations and making rats carrying these mutations available as bioresources would enhance the scientific value of rats as an animal model for human diseases.

There are several approaches to collecting rat mutations. They include discovering naturally occurring mutations and inducing mutations by random mutagenesis [21]. In addition, attempts have been made to collect mutations outside of the laboratory, from the field or fancy rat colonies; indeed, some inbred strains have been established from wild captured rats [11]. However, fancy rats have not been surveyed as a source of mutations, with a few exceptions [26].

Fancy rat colonies have potential as a source for collecting novel rat mutations, because various mutations are considered to persist only in fancy rats, largely coat and eye color mutations, and coat pattern mutations. Thus, when they are available in laboratory rats, most will provide opportunities to study the function of melanocytes, which are not only responsible for pigment synthesis in the skin and hair, but are also involved in inner ear and eye functions [30]. In addition, in human, dysfunctions of melanocytes result in skin disorders such as oculocutaneous albinism, piebaldism and skin cancers [12, 29], prompting us to introduce mutations found in the current fancy rat colonies to the laboratory and establish them as novel bioresources available for biomedical research.

In this study, we imported 6 fancy rats from a fancy rat colony in the USA to our laboratory. We tried to isolate fancy mutations and establish inbred strains carrying them. During inbreeding, we isolated 9 mutations, of which 7 were mapped to particular genomic regions of rat chromosomes. A coat color mutation, siamese, was identified as a missense mutation in the rat tyrosinase gene.

Materials and Methods

Animals

In July, 2005, 6 fancy rats were imported from a fancy rat colony named Spoiled Ratten Rattery (SRR) kept by Ms. E. Brooks in Kansas City, Missouri, USA (http://www.spoiledratten.com/index.html). These rats (SRR01-06) were used as founders to establish fancy-derived strains. SRR01 was female and the others were males. It was known that these founders carried the following mutations (Table 1): SRR01 carried dumbo (*dmbo*), Rex (*Re*), and satin (*sat*); SRR02 carried *sat* and siamese (*sia*); SRR03 carried American mink (*am*), grey (*g*), and Pearl (*Pel*); SRR04 carried dumbo (*dmbo*); SRR05 carried *Re*; and SRR06 carried Black eye (*Be*). TM/Kyo and PVG/Seac rats were selected as mating partners to obtain progeny from the founder fancy rats, because they are homozygous for nonagouti (*a/a*) and hooded (*h/h*) recessive mutations. SRR01, SRR05, and SRR06 were crossed with the TM/Kyo strain and SRR02, SRR03, and SRR04 were crossed with the PVG/Seac strain. Following caesarean operations, F_1 hybrids were introduced to specific pathogen-free (SPF) facilities in our institute. Brother-sister mating was carried out to establish fancy rat-derived strains for each founder. At each generation during inbreeding, rats showing the mutant phenotypes were selected. When different mutant phenotypes were found in an inbreeding line, sublines were separated.

To map the mutations isolated from fancy rats, a male rat representing each strain was used to make F_1 hybrids with BN/SsNSlc (BN) or WTC/Kyo (WTC) female rats.

Animal care and experimental procedures were approved by the Animal Research Committee, Kyoto University and were conducted according to the Regulation on Animal Experimentation at Kyoto University.

Genetic mapping

To map *sat* and *sia* mutations, SRR02 (F5) was mated with BN rats and 82 backcross progeny (BCP) were produced (cross 1). To map *am*, SRR03-*am* (F6) was mated with BN rats and 98 BCP were produced (cross 2). To map *g* and *Pel*, SRR03-*g*, *Pel* (F6) was mated with BN rats and 87 BCP were produced (cross 3). To map *dmbo* and *hs*, SRR04 (F5) was mated with BN rats and 99 BCP were produced (cross 4). To map *Re*, SRR05 (F3) was mated with BN rats and 50 BCP were produced (cross 5). To map *Be*, SRR06 (F6) was mated with BN and WTC rats, and 48 and 67 BCP were produced (crosses 6 and 7).

Genotyping was performed as described previously [16] with a set of highly informative simple sequence length polymorphism (SSLP) markers [20].

Table 1. Mutations isolated from fancy rats

Mutation (symbol)	MP term (MP id)[a)]	Characteristic	Origin[b)]	KFRS strain	Mode of inheritance	Mapped position in rats		Candidate gene name (Gene symbol)	Mutant phenotype of candidate gene in mice
						Chr	Physical position[c)]		
American mink (*am*)	diluted coat color (0000371)	Light brown body hair [26]	Unknown. Different from the original mink described by Robinson	KFRS3A/ Kyo	recessive	1	95.5–103 Mb[d)]	Hermansky-Pudlak syndrom 5 (*Hps5*)	Mice Homozygous for *Hps5* mutation (*ruby-2*) have hypopigmented eyes and hair [33]
Black eye (*Be*)	diluted coat color (0000371)	Cream coat with pigmented eyes	Laboratory colony at Edinburgh University in Scotland in 1998 → Breeder in England	KFRS6/ Kyo	dominant	ND	ND		
dumbo (*dmbo*)	abnormal outer ear morphology (0002177)	Ears are set lower on the head, and are larger and rounder.	Fancy rats somewhere in the northwest US	KFRS4/ Kyo	recessive	14	79.0–84.7 Mb	H6 homeo box 1 (*Hmx1*)	Mice carrying *Hmx1* mutations exhibit enlarged ear pinnae with a distinctive ventrolateral shift [23]
grey (*g*)	diluted coat color (0000371)	Light grey body hair	Maybe Russian blue. From fancies of east coast US.	KFRS3B/ Kyo	recessive	8	57.3–95.2 Mb	RB27A, member RAS oncogene family (*Rab27a*) myosin VA (*Myo5a*)	Gene defects produce abnormal pigmentation and a gray or diluted coat color in ashen or dilute mice [22, 32] and dop rats [8].
head spot (*hs*)	head head spot (0002939)	White spotting on the head	Unknown	KFRS4/ Kyo	recessive	15	84.6–91.2 Mb	endothelin receptor type B (*Ednrb*)	Mice homozygous for the *Ednrbs* mutation show irregular white spotting, depending on the genetic background [25]
Pearle (*Pel*)	diluted coat color (0000371), embryonic lethality (0008762)	Lighter coat color expressed on mink or grey. Homozygotes die in the embryonic period (E10–E12)	English fancy [26]	KFRS3A/ Kyo KFRS3B/ Kyo	dominant	ND	ND		
Rex (*Re*)	wavy hair (0000410), nude (0003815), wavy vibrissae (0001279)	Heterozygotes show wavy body hair, while homozygotes lose body hair after the first molt. Both heterozygotes and homozygotes show wavy vibrissae.	England → Breeder in California	KFRS5/ Kyo	dominant	7	135–143 Mb[d)]	keratin 71 (*Krt71*)	Mouse mutations in the *Krt71* gene, caracul (*Ca*), cause wavy coat hairs in *Ca*/+ heterozygous mice [14]
satin (*sat*)	abnormal coat appearance (0001510), curly vibrissae (0001274)	Longer hair and shiny-looking "greasy" hair. Vibrissae are bent downward.	Fancy rats kept by a breeder in California	KFRS2/ Kyo	recessive	3	105.8–114.9 Mb	fibroblast growth factor 7 (*Fgf7*)	Mice lacking the *Fgf7* gene develop a matted coat [10]
siamese (*sia*)	diluted coat color (0000371)	Homozygotes show light body hair, but their ears, nose, tail, and scrotum are dark, as in Siamese cats. Eyes are slightly pigmented and appear red.	Laboratory in France in the 1980s → Breeder in UK → breeders in California	KFRS2/ Kyo	recessive	1	140.6–145.5 Mb	tyrosinase (*Tyr*)	Mice homozygous for $Tyr^{c\text{-}h}$ show light coat color and darkened ears, nose, and scrotum. [18]

[a)]: Mutant phenotypes are classified by mammalian phenotype ontology. [b)]: Provided by Ms. E. Brooks. [c)]: RGSC v3.4. [d)]: Expected theoretical maximum distance between *am* or *Re* and non-recombinant markers. Physical distance corresponding to 1 cM was expected to be 1 Mb.

Direct sequencing of the Tyr gene of Black-eyed and Siamese rats

Direct sequencing was performed as described previously [17]. Rat *Tyr* exons were amplified with the following 6 sets of primers: rTyr-1&2 (exon 1,463 bp), 5'-TGTTTGAGCAGATCTTGTACGG-3' and 5'-TGTTTTGCCAAAGTGAGGTAA-3'; rTyr-3&11 (exon 1,633 bp) 5'-GCGGAAACTGTAAGTTTGGA-3' and 5'-AAGGTTCCTTTTCTGTGCTGA-3'; rTyr-12&13 (exon 2,398 bp), 5'-TTTCATTCATATGTAAGTCCCTTG-3' and 5'-GCTTAGCATTGCAAAACTCACA-3'; rTyr-14&15 (exon 3,384 bp), 5'-TTGTTTATTTAAAATTAGGCTTACCTC-3' and 5'-TCTCAAAATAGAGAACCACCACAA-3'; rTyr-16&17 (exon 4,488 bp), 5'-AAAGTTTGAAGATAGTCAGCATTTGA-3' and 5'-TTTAGCTGTACAAAATATCCTTGAAA-3'; rTyr-18&10 (exon 5,489 bp), 5'-GCACTCAAACCCAAGCATCT-3' and 5'-TTCCTTAGAAACTGGGACGTG-3'.

Examination of fetuses at cesarean section

Six wild-type SRR03 females (+/+) and six *Pel*-heterozygous female SRR03 (*Pel*/+) rats were mated with the *Pel*-heterozygous SRR03 males (*Pel*/+). At P20, fetuses were removed by cesarean section. The numbers of corpora lutea, live fetuses, and embryo-fetal deaths were counted. Embryo-fetal deaths were categorized into early death (implantation sites, resorbed embryos, and placental remnants) and late death (early macerated fetuses, late macerated fetuses, and dead fetuses). The number of implantations was calculated from the sum of the number of live fetuses and the number of embryo-fetal deaths.

Statistical analysis

To determine the mode of inheritance and linkage relationship, chi-square tests were performed. When the *P* value of chi-square for 1:1 was more than 0.05, the mutation was thought to be an autosomal single gene. When the *P* value of chi-square for linkage was less than 0.05, the linkage relationship between loci was thought to be significant. For statistical analysis of embryo-fetal deaths found in the Pearl mutant, Student's *t*-test was performed using Microsoft Excel.

Results

Fancy rat-derived strains

We isolated 9 mutations during inbreeding and assigned "Mammalian Phenotype terms (MP)" to their mutant phenotypes to make it easy to understand them [31] (Table 1). They involved 5 coat color mutations (*am*, *Be*, *g*, *Pel*, and *sia*), 1 coat pattern mutation (*hs*), 2 coat texture mutaions (*Re* and *sat*), and an ear pinnae malformation mutation (*dmbo*). The Pearl phenotype manifested in conjunction with homozygous status for *am* or *g*.

During inbreeding, the line originating from SRR01 became extinct. Although inbreeding was not fully completed, we tentatively named the derived lines Kyoto Fancy Rat Stock (KFRS). Each strain was defined with a number representing the names of the founder rats, and sublines were defined by the addition of a letter after the number. Six lines, including sublines, were produced and their strain names, mutations they carried, and generations at the end of February, 2010 were as follows: KFRS2/Kyo carrying *sat* and *sia* (F18), KFRS3A/Kyo carrying *am* and *Pel* (F19), KFRS3B/Kyo carrying *g* and *Pel* (F20), KFRS4/Kyo carrying *dmbo* and *hs* (F18), KFRS5A/Kyo carrying *Re* (F19), and KFRS6/Kyo carrying *Be* (F17) (Fig. 1 and Table 1).

Mode of inheritance and genetically mapped region of fancy mutations

In cross 1, 40 had satin-type body hair and 42 had normal body hair. Thirty-six had a siamese coat color, while forty-six had normal coat color. These findings indicated that both the *sat* and *sia* mutations were autosomal recessive. The linkage map including *sat* was *D3Got76* – 1.2 cM – *D3Got69*, *sat* – 1.2 cM – *D3Mco2*. The *sat* locus spanned the 9.1-Mb region defined by *D3Got76* and *D3Mco2*. The linkage map including *sia* was *D1Rat273* – 2.4 cM – *sia* – 2.4 cM – *D1Rat138*. The *sia* locus spanned the 4.9-Mb region defined by *D1Rat273* and *D1Rat138*.

In cross 2, 47 had American mink-type body hair and 51 had normal body hair, indicating the *am* mutation was autosomal recessive. The *am* showed no recombination with *D1Rat214* and *D1Mgh35* in 98 meioses, which indicated that *am* was located <3.0 cM away from these

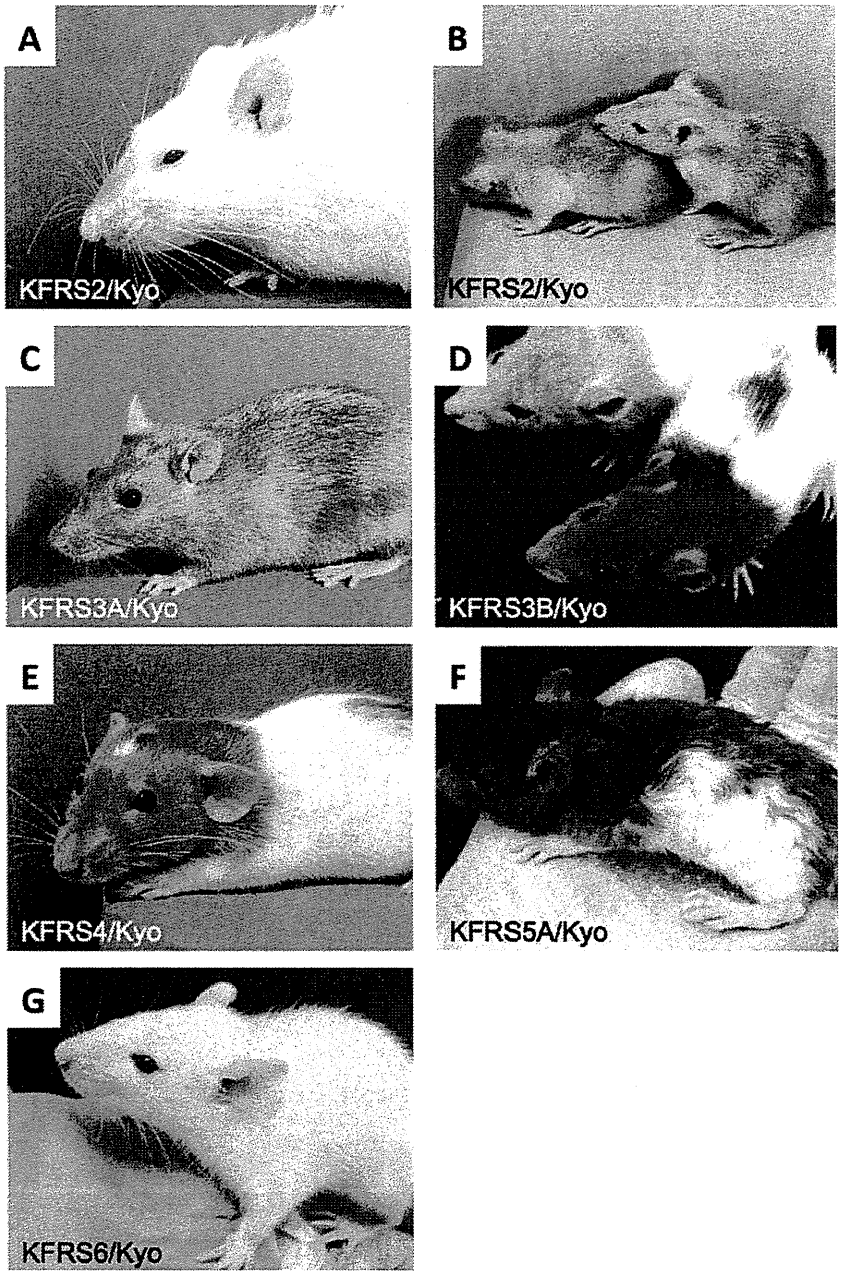

Fig. 1. Kyoto Fancy Rat Stock (KRFS) strains. (A) KFRS2/Kyo, 2 months of age, *sat/sat*, *sia/sia*. (B) KFRS2/Kyo, 3 weeks of age, Left; *sat/+*, *sia/sia*. Right; *sat/sat*, *sia/sia*. Note that siamese marking of the nose is apparent in the adult *sia/sia* rat, compared with the young rat. (C) KFRS3A/Kyo, *am/am*. (D) KFRS3B/Kyo, Upper; *g/g*, *Pel/+*. Lower; *g/g*. (E) KFRS4/Kyo, *dmbo/dmbo*, *hs/hs*. (F) KFRS5/Kyo, *Re/+*. (G) KFRS6/Kyo, *Be/Be*, *c/c*. All strains are homozygous for *a*.

markers with 95% probability [7].

In cross 3, 46 had grey-type body hair and 41 had normal body hair, indicating the *g* mutation was autosomal recessive. The linkage map including *g* was *D8Rat36* – 6.9 cM – *D8Rat182*, *g* – 14.9 cM – *D8Rat131*. The *g* locus spanned the 37.9-Mb region defined by

D8Rat36 and *D8Rat131*.

In cross 4, 55 had dumbo-type ears and 44 had normal ears. Forty-five had white spots on their head and fifty-four had no head spots. These findings indicated that both *dmbo* and *hs* mutations were autosomal recessive. The linkage map including *dmbo* was *D14Arb10* – 1.0 cM – *D14Rat37*, *dmbo* – 6.1 cM – *D14Rat57*. The *dmbo* locus spanned the 5.7-Mb region defined by *D14Rat10* and *D14Rat57*. The linkage map including *hs* was *D15Got78* – 5 cM – *hs* – 12 cM – *D15Rat26*. The *hs* locus spanned the 6.6-Mb region defined by *D15Got78* and *D15Rat26*.

In cross 5, 24 had Rex-type body hair and 26 had normal body hair, indicating that the *Re* mutation was autosomal dominant. *Re* showed no recombination with *D7Mit1* and *D7Rat80* in 50 meioses, indicating that *Re* was located <5.8 cM from these markers with 95% probability [7].

Using crosses 6 and 7, we carried out genetic analysis of the *Be* mutation. In rat fanciers, it is known that the *Be* mutation masks the coat color only in combination with the albino mutation. This combination produces rats with a pale creamy white coat color and black eyes. To elucidate the inheritance pattern of the black eye, we first crossed a SRR06 male with BN/SsNSlc (*a/a*, *b/b*, *C/C*) rats. Since all (BN/SsNSlc × SRR06)F_1 rats had a black coat and pigmented eyes, we backcrossed F_1 females to SRR06 males. In cross 6, 27 had a white coat with black eyes, and 21 had a colored coat with black eyes. The phenotype of the white coat with black eyes was completely cosegregated with a missense mutation at *Tyr*, Arg299His, found in the albino Wistar rat [4]. Direct sequencing of the *Tyr* gene of the SRR06 genome demonstrated that SRR06 also harbored the Arg299His missense mutation (data not shown).

To elucidate the inheritance pattern of the black eye on the *albino* background, we crossed a SRR06 male with albino WTC/Kyo (*a/a*, *B/B*, *c/c*) rats. All (WTC/Kyo × SRR06)F_1 rats had a white coat and black eyes. We then backcrossed the F_1 females to WTC/Kyo males. In cross 7, 29 had a white coat with black eyes, and 38 had a colored coat with black eyes. These findings indicated that the *Be* mutation was a single autosomal mutation and manifested dominantly only in the presence of the albino mutation in the homozygous state.

Table 2. Number of embryo-fetal deaths found in Pearl mutants

Stage of embryo-fetal death	Cross to produce embryos	
	+/+ × *Pel*/+	*Pel*/+ × *Pel*/+
Implantation site	0.0 ± 0.0	0.0 ± 0.0
Resorbed embryo	0.2 ± 0.4	3.0 ± 0.6**
Placental remnant	0.0 ± 0.0	0.5 ± 0.5*
Early macerated fetus	0.0 ± 0.0	0.0 ± 0.0
Late macerated fetus	0.0 ± 0.0	0.0 ± 0.0
Dead fetus	0.0 ± 0.0	0.0 ± 0.0
Total	0.2 ± 0.4	3.5 ± 0.8**

*: $P<0.05$, **: $P<0.01$.

Embryonic lethality of the Pearl (Pel) mutation

There were no significant differences in the numbers of corpora lutea [12.5 ± 1.6 vs. 12.7 ± 0.8 (mean ± SD), P=0.42] and implantations (12.2 ± 1.6 vs. 11.7 ± 0.5, P=0.25) between wild-type (+/+) and Pearl (*Pel*/+) females both crossed with Pearl (*Pel*/+) males. Meanwhile, embryo-fetus deaths were significantly higher in (*Pel*/+ × *Pel*/+)F_1 embryos than in (+/+ × *Pel*/+)F_1 embryos: 3.5 ± 0.8 vs. 0.2 ± 0.4, $P<0.01$ (Table 2). Embryo-fetus deaths found in (*Pel*/+ × *Pel*/+)F_1 embryos included resorbed embryos (3.0 ± 0.6) and placental remnant (0.5 ± 0.5). The proportion of embryo-fetus deaths with regard to the number of corpora lutea in (*Pel*/+ × *Pel*/+)F_1 was 27.5%, which agreed with 25% embryo-fetus death when homozygous lethality occurred in *Pel/Pel* embryos.

Identification of siamese as a missense mutation in the Tyrosinase gene

Tyrosinase (*Tyr*) was thought to be a good candidate for *sia*, because mouse *himalayan* mutation (*h*) at the *Tyr* locus showed an extremely similar coat color phenotype to the siamese rat. Direct sequencing revealed a missense mutation (c. 235 T>C, p. Ser79Pro) in exon 1 of the *tyrosinase* gene in the *sia/sia* homozygous genome (Fig. 2). This missense mutation was completely cosegragated with the siamese coat phenotype in 82 (BN × SRR02)F_1 × SRR02 BCP and was not found among 34 rat inbred strains (data not shown).

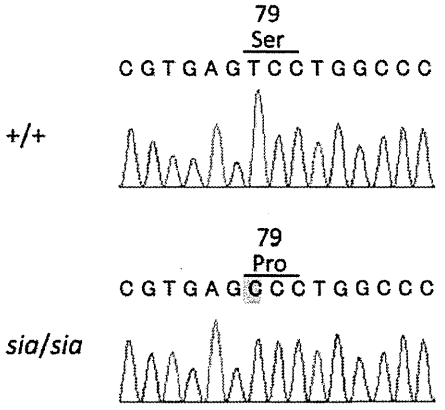

Fig. 2. Identification of siamese mutation. Sequence analysis of *Tyr* cDNA from wild-type and *sia/sia* rats. In the *sia/sia* rat, a nucleotide conversion T to C (red) was found at the position of nucleotide 253 of rat *Tyr* cDNA. The *sia* mutation converts the amino acid residue at codon 79 from serine (Ser) to proline (Pro).

Discussion

From mapped positions and phenotype resemblances to existing mutations of rats or mice, we selected candidate genes for the fancy mutations (Table 1). For *Myo5a* and *Ednrb*, rat mutations have been identified: dilute-opisthotonus (*dop*) mutation in *Myo5a* [8] and spotting lethal (*sl*) mutation in *Ednrb* [15]. We confirmed the absence of these mutations in *grey*-homozygous KFRS3B/Kyo and *head spot*-homozygous KFRS4/Kyo rats (data not shown). Therefore, all the fancy mutations isolated were likely to be unique and our study has made them available to the laboratory.

The Ser79Pro missense mutation was completely cosegregated with the siamese phenotype and was specific to KFRS2/Kyo. Missense mutations around the 79th amino acid of TYR provoke albinism in mice and humans, suggesting that this region plays an important role in hair and skin pigmentation [3, 24]. Therefore, we concluded that the S79P missense mutation is responsible for the siamese phenotype in rats. Tyrosinase is the key enzyme involved in the melanin biosynthetic pathway and is responsible for the rate limiting step [5]. Mutations in the *TYR* gene cause human oculocutaneous albinism 1 (OCA1) [24]. Although there are more than 100 mutations in the mouse *Tyr* locus, such as albino (Arg77Leu), himalayan (His420Arg), and chinchilla (Ala482Thr) [3], increasing the range of *Tyr* mutations will provide a wealth of information on the biology of tyrosinase and lead to better understanding of the pathogenesis of OCA1.

In addition to previous work on the Pearl phenotype [26], we revealed that approx. 25% embryos were largely resorbed, suggesting that *Pel/Pel* embryos die in the early stage of organogenesis (gestation days 10 to 12) [6]. There is a close relationship between *Pel* and agouti (*A*) [26]. In the current study, we carried out preliminary genetic analysis using 46 *g*-homozygous rats from cross 3. However, we failed to find a linkage relationship between *Pel* and *D3Mit2*, a SSLP marker located 2 cM apart from *A*, which suggests that multiple genetic determinants might be involved in the expression of *Pel*.

To our knowledge, this study is the first report on the systemic introduction of fancy-derived mutations to the laboratory. Fancy rats are considered to be a good source for developing a new bioresource of rats. They allow us to isolate rat mutations effectively. Usually, the rate at which new mutations arise spontaneously is exceedingly low: it is known that, on average, only one gamete in 100,000 is likely to carry a detectable mutation at any single locus naturally occurring mutation rate [28], which means that the discovery of mutations depends on chance. In this study, we could isolate 9 unique mutations from only 6 founder rats, and it took only a few generations to isolate them. Moreover, fancy rats are usually kept by outbreeding, so when they are subjected to inbreeding in the laboratory, hidden mutations sometimes manifest. Actually, we observed the cataract and sterile phenotypes, which were unknown in the SRR, at several generations after starting inbreeding (data not shown).

Fancy rat colonies are thought to be maintained relatively independent of laboratory rats and have unique breeding histories different from the laboratory rats [2]. Therefore, it is expected that the fancy-derived KFRS strains will retain their unique genetic background different from laboratory rats, although almost half of them are derived from laboratory rats. The IS/Kyo strain originates from a cross of a wild captured male rat with Wistar female rats [13] and shows a clearly different

genetic background from other strains [20]. Systematic phenotypic analysis of IS/Kyo rats uncovered their unique traits, such as hypotension and high body temperature, which implies that a wild-derived genome might confer these unique traits [19]. Following several generations, all KFRS strains will be established as full inbred strains. Thus, we consider that the systematic genotype and phenotype analyses of these KFRSs will reveal their genetic background and untapped unique traits, which make them potential disease models. Finally, phenotypically annotated KFRSs will contribute to increase the scientific value of rats.

Acknowledgments

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Chapter 22

Techniques for In Vitro and In Vivo Fertilization in the Rat

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Abstract

Although in vitro and in vivo fertilization are powerful tools for restoring conserved sperm as well as stocked males in the rat, the techniques have progressively gained importance. However, the techniques are not used extensively for efficient production of rat offspring, because the techniques require a great deal of skill. This chapter describes the protocols for in vitro and in vivo fertilization in the rat. Namely, sperm collection, sperm cryopreservation, pre-incubation of sperm, and insemination (co-culture with sperm and oocytes) for in vitro fertilization and intrauterine insemination for in vivo fertilization with fresh or frozen/thawed spermatozoa are provided.

Key words: Intrauterine insemination, In vitro fertilization, Rat sperm, Sperm cryopreservation

1. Introduction

The laboratory rat has been used extensively in many fields of medical and biological research because of body size, ease of manipulations and breeding characteristics. In addition, there is a lot of unique and invaluable rat models that resemble human diseases such as cardiovascular disease, diabetes, arthritis and many behavioral disorders (1). Recently, the rat genome sequence of the Brown Norway that is a well characterized inbred strain and the founder strain for several important genetic panels was released (2). Although gene targeting in the rat is still limited, recent advances in molecular and reproductive technologies (3) have enabled generating significant transgenic rats by DNA microinjection (4), sperm mediated gene transfer (5), lentivirus method (6) and *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (7). To collect, preserve and supply unique and invaluable rats for medical

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and biological research at the international and local research communities, the national rat genetic resources have been established (3).

Techniques for in vitro and in vivo fertilization in the rat provide the genetic resources and its users with efficiency of restoring conserved sperm as well as stocked males, and also enable generation of fertilized ova and embryos for various purposes such as ENU-mutagenesis, DNA-microinjection and cryopreservation. The techniques in the rat therefore have progressively gained importance.

This chapter provides detailed description of the protocols for in vitro and in vivo fertilization in the rat. Namely, sperm collection, sperm cryopreservation, pre-incubation of sperm, and insemination (co-culture with sperm and oocytes) for in vitro fertilization (IVF), and intrauterine insemination for in vivo fertilization with fresh or frozen/thawed spermatozoa are provided.

2. Materials

2.1. Collection of Epididymal Sperm

1. Modified R1ECM for fertilziation (F-mR1ECM) (*see* Table 22.1: 110 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 10.0 mM sodium lactate, 0.1 mM glutamine, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (50×; Gibco BRL, Grand Island, NY), 1% (v/v) MEM nonessential amino acid solution (100×; Gibco BRL) and 4 mg/mL of fatty acid free bovine serum albumin (BSA)) (8, 9).
2. For dissection of cauda epididymis: fine scissors, fine forceps, watchmaker's forceps, filter papers (about 5 cm×5 cm), a 26-gauge needle, a 35-mm plastic dish (Falcon 35-1008, Becton Dickinson Labware, Franklin Lakes, NJ) supplemented with 500 µL F-mR1ECM.
3. Fertile male rats (We use over 15 weeks of aged rats, although around 7–8 weeks aged rats are used as sperm donor in general).
4. For sperm quality evaluation: examination chamber on a warm-plate at 37°C and a hemocytometer.

2.2. Preparation of Recipient Females, and Vasectmized Males

1. Light cycle-controlled rat room.
2. Recipient females (10–14 weeks age; we use Crlj: Wistar rats) for embryo transfer and intrauterine insemination.
3. Vasectomized males (15–30 weeks age, Wistar (any strain)).

Table 22.1
Modified R1ECM media for rat sperm, oocytes and in vitro fertilization (IVF)[a]

Ingredients	Fw.	Product No.[b]	F-mR1ECM	C-mR1ECM
NaCl	58.44		110 mM	76.7 mM
KCl	74.55		3.2 mM	3.2 mM
$CaCl_2$			2.0 mM	2.0 mM
$MgCl_2$	95.21		0.5 mM	0.5 mM
$NaHCO_3$	84.1		25.0 mM	25.0 mM
Sodium lactate	72.06		10.0 mM	10.0 mM
Sodium pyruvate	110.04		0.5 mM	0.5 mM
Glucose	180.16		7.5 mM	7.5 mM
Glutamine	146.1		0.1 mM	0.1 mM
Polyvinyl alcohol	Av Mol wt 30,000–70,000	P8136	–	1.0 mg/mL
BSA[c]		A6003-10G	4.0 mg/mL	–
EAA[d] (Invitrogen)		11130-051	2% (v/v)	2% (v/v)
NEAA[e] (Invitrogen)		11140-050	1% (v/v)	1% (v/v)
Osmolarity			310 mOsM	246 mOsM

[a]According to the methods described in **refs.** 8 and 9
[b]The product No of the ingredients except BSA and amino acid solutions is on Sigma Life Science (St. Louis, MO)
[c]BSA; albumin, from bovine serum, essentially fatty acid free, Sigma-Aldrich (St. Louis, MO)
[d]Minimal essential medium (MEM) amino acid solution (50×; Gibco BRL, Grand Island, NY)
[e]MEM nonessential amino acid solution (100×; Gibco BRL)

2.3. In Vitro Fertilization (IVF)

2.3.1. Sperm Pre-incubation

1. F-mR1ECM (*see* Table 22.1). When using cryopreserved sperm, F-mR1ECM including 200 μM of 3-isobutyl-1-methyl-xanthin (IBMX) is superior (see Note 1) (10).
2. Paraffin or mineral oil (*see* Note 2).
3. Oil-covered droplets of 100 μL F-mR1ECM in 35-mm dishes (Falcon 35-1008).
4. Incubator (37°C, 5% CO_2 in air).

2.3.2. Superovulation

1. Immature female rats (4–5 weeks age; we use Crlj: Wistar rats).
2. Equine chorionic gonadotropin (eCG).
3. Human chorionic gonadotropin (hCG).
4. Physiological saline.
5. For injection: a 1-mL syringe and a 26-gauge needle.

2.3.3. Collection of Cumulus Oocyte Complexes (COCs) and Insemination (Co-culture with Sperm and COCs)

1. F-mR1ECM (*see* Table 22.1).
2. Paraffin or mineral oil (*see* Note 2).
3. For dissection of oviductal ampulla: fine scissors, fine forceps, and preparation needles.
4. For collection: sperm suspended 100 µL droplets of F-mR1ECM in a 35-mm plastic dish (Falcon 35-1008) (*see* Subheading 22.2.3.1).
5. Incubator (37°C, 5% CO_2 in air).

2.3.4. Culture of Putative Zygotes

1. Modified R1ECM for culture (C-mR1ECM) (*see* Table 22.1: 76.7 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 10.0 mM sodium lactate, 0.1 mM glutamine, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (50×; Gibco BRL), 1% (v/v) MEM non-essential amino acid solution (100×; Gibco BRL) and 1.0 mg/mL of polyvinyl alcohol).
2. Paraffin or mineral oil (*see* Note 2).
3. Oil-coverd droplets of 100 µL C-mR1ECM supplemented with 0.1% (w/v) hyaluronidase in a 35-mm plastic dish (Falcon 35-1008).
4. Incubator (37°C, 5% CO_2 in air).

2.3.5. Embryo Transfer of Zygotes (IVF embryos)

1. The zygotes (IVF embryos) in droplets of C-mR1ECM) (*see* Table 22.1).
2. Stereo microscopy with transmitted light base.
3. Anesthetic.
4. Seventy % Ethanol in squeeze bottle.
5. Surgical apparatus; scissors, forceps.
6. Sterile gauze.
7. Sterile Pasteur pipettes with cotton plug for embryo transfer capillary.
8. Mouthpiece.
9. Suture thread (silk, No. 2).
10. Clips for suture (Michel suture clips; 7.5 mm×1.5 mm, Harvard Apparatus, MA, US).

2.4. In Vivo Fertilization: Intrauterine Insemination

2.4.1. Sperm Freezing Medium

1. Components; Lactose, trishydroxymethylaminomethane (Tris), penicillin G, streptomycin sulfate, Orvus ES Paste (OEP; Equex STM™, Nova Chemical Sales Inc., Scituate, MA; *see* Note 3), chicken eggs, sterile distilled water or extra-pure water, sterile filter paper (about 15 cm×15 cm).
2. Preparation of primary sperm freezing medium (SFM-1):

2.1. Lactose (10.4 g) is dissolved with water, and the volume is adjusted to 100 mL.

2.2. One chicken egg is washed well, and disinfected with 70% alcohol. The egg-shell is broken carefully and the egg-yolk with small amount of egg-white is placed on the sterile filter paper. Then the egg-white is removed by rolling the egg-yolk. The surface membrane of the egg-yolk is ruptured, and the inner yolk is aspirated into a sterile syringe. Thirty mL of the inner yolk is added to the lactose solution (*see* item 2.1).

2.3. Penicillin G (1,000 unit/mL) and streptomycin sulfate (1 mg/mL) are added to the egg-yolk and lactose solution (*see* item 2.2).

2.4. The pH of the egg-yolk and lactose solution is measured using pH meter or BTB test paper. In general, the pH of the solution shows around 7.8.

2.5. The pH is adjusted to 7.4 by dropping of 10% Tris (v/v) solution.

2.6. The solution adjusted pH 7.4 is aliquoted to sterile 50-mL centrifuge tubes, and centrifuged at 1,250–1,750×*g* (around 3,000 rpm) for 15 min. The upper layer of the solution is recovered into sterile 15-mL tubes. After centrifugation, when a thin membrane is formed in the top of the solution, the membrane is removed using a sterile needle.

2.7. The recovered solution is used as SFM-1, and stored at below −20°C.

3. Preparation of secondary sperm freezing medium (SFM-2):

3.1. Using 1-mL syringe with needle, OEP (*see* **Note 3**) is added to SFM-1 at 1.4% (v/v), and the solution is mixed well using a sterile pipette.

3.2. The solution included OEP is aliquoted into sterile 15-mL tubes as SFM-2, and stored at below −20°C.

2.4.2. Freezing of Epididymal Sperm

1 Sperm freezing medium 1 and 2 (SFM-1 and SFM-2) (*see* Subheading 22.2.4.1) (11).

2 For dissection of cauda epididymis: fine scissors, fine forceps, watchmaker's forceps, filter papers (about 5 cm×5 cm), a 26-gauge needle, a 35-mm plastic dish (Falcon 35-1008) supplemented with 1.5 mL of SFM-1.

3 For sperm quality evaluation: examination chamber on a warm-plate at 37°C and a hemocytometer (*see* Subheading 22.2.1).

4 Incubator (15°C in air).

5 Refrigerator (5°C in air).

6 0.25-mL palastic straws for bull semen.
7 Programmable freezer.
8 Liquid nitrogen (LN_2) tank.

2.4.3. Intrauterine Insemination

1. Cryopreserved sperm (*see* Subheading 22.2.4.2).
2. Water bath (37°C).
3. Straw cutter (Fujihira Industry, Japan; Cryo Bio System/IMV, France).
4. Anesthetic.
5. Surgical apparatus; scissors, forceps.
6. Sterile 1.5-mL tubes.
7. Seventy % Ethanol in squeeze bottle.
8. Sterile gauze.
9. Sterile 21-gauge needles.
10. Sterile Pasteur pipettes with cotton plug for insemination.
11. Mouthpiece.
12. Suture thread (silk, No 2).
13. Clips for suture (Michel suture clips; 7.5 mm×1.5 mm, Harvard Apparatus).

3. Methods

3.1. Collection of Epididymal Sperm

1. Males as sperm donors are anesthetized appropriately, and sacrificed by cervical dislocation.
2. After disinfection, the abdomen is opened using scissors and forceps, then the bilateral testes and epididymides are excised.
3. The caudal epididymides are placed on sterile filter paper (about 5 cm×5 cm), and its fat adhered to the caudal region are removed thoroughly and carefully.
4. Cauda epididymides are excised from mature males under room temperature (22–24°C), and are placed on sterile filter paper (about 5 cm×5 cm), and its fat adhered the caudal region are removed thoroughly and carefully.
5. Both the caudal epididymides are sticked by a 26-gauge needle, a few droplets of sperm mass is collected and introduced into a 100 μL-drop of F-mR1ECM (Table 22.1).
6. After a few minutes, sperm motility and concentration of the drop of F-mR1ECM are microscopically assessed.

7. Sperm suspension was transferred into another 200 μL-drop covered with oil for sperm pre-incubation to adjust to 5×10^5 sperm/mL.

3.2. Preparation of Recipient Females and Vasectmized Males

1. A mature female confirmed to be proestrus by smear test (nucleated epithelia cells are noted) during the light-on period (in the morning) is selected as a candidate of recipient female (for embryo transfer of IVF embryos or intrauterine insemination).
2. The female is caged together with a vasectomized male that has copulation ability 2 h before the dark period (2 h before light-off) to induce pseudo-pregnancy.
3. On Next morning (for embryo transfer of IVF embryos) or at 2–5 h after light-off (for intrauterine insemination), vaginal plug(s) is (are) checked to confirm mating with the vasectomized male.

3.3. IVF

3.3.1. Sperm Pre-incubation

1. Spermatozoa are collected from cauda epididymides and transfered into another 200 mL-drop covered with oil for sperm pre-incubation to adjust to 5×10^5 sperm/mL (*see* Subheading 22.2.1).
2. The sperm suspension in a plastic dish is cultured for 5 h at 37°C under 5% CO_2 in air to induce sperm capacitation in vitro (*see* Note 1).

3.3.2. Superovulation

Animals are hosed with free access of water and standard chow under a 12-h day/night cycle, most often comprising a 6 a.m. to 6 p.m. light period. We have good experience using 4–5 week-age females of Crlj: Wistar and Slc: Sprague-Dawley for superovulation (12).

1. Preparations of hormones: lyophilized eCG and hCG were diluted to a final concentration of 166.7 inter unit (IU)/mL by physiological saline.
2. Three days before IVF, eCG of 300 IU/kg (body weight: BW) is intraperitoneally injected into the females.
3. After 48 h of the eCG injection (300 IU/kg (BW)), hCG is injected in the same way.

3.3.3. Collection of Cumulus Oocyte Complexes (COCs) and Insemination (Co-culture with Sperm and COCs)

1. Twelve to fourteen h after the hCG injection, the oviducts of the donors are excised, and placed on sterile filter paper (about 5 cm × 5 cm).
2. The fat adhered to the oviducts are removed thoroughly and carefully. The oviducts are placed into oil-covered F-mR1ECM drop (*see* Table 22.1, Note 4) in the dish for sperm pre-incubation, and moved to near the drop.

3. The ampullae are broken using a 26-gauge needle and fine forceps under stereoscopic microscope. When the ampullae are broken, cumulus-oocyte-complexes (COCs) are released.
4. The COCs are introduced into the drop containing sperm, by a 26-gauge needle, and then co-cultured for 10 h at 37°C under 5% CO_2 in air.

3.3.4. Culture of Putative Zygotes

1. After co-culture for 10 h, putative zygotes are transferred to C-mR1ECM (Table 22.1) supplemented with 0.1% hyaluronidase for 1 min.
2. The zygotes are washed three times in C-mR1ECM. The putative zygotes are transferred into oil-covered 100 µL droplets of C-mR1ECM in a 35-mm plastic dish (Falcon 35-1008), and cultured at 37°C under 5% CO_2 in air.

3.3.5. Embryo Transfer of Zygotes (IVF Embryos)

Only the general techniques for embryo transfer are described in this chapter. The putative zygotes (IVF embryos) are transferred into both oviducts of the recipient on 0.5-day post coitum. The recipient is naturally mated with a vasectomized male to induce pseudo-pregnancy.

1. Anesthetize the recipient female for embryo transfer.
2. The zygotes (IVF embryos) are divided to two groups in droplets of C-mR1ECM.
3. The female is retained in the prone position, and the coat is removed using hair-clipping scissors in the bilateral lateral abdominal regions (about 3 cm × 3 cm) corresponding to the positions of the ovaries located caudal to the last costales.
4. The clipped surgical areas are disinfected with 70% ethanol.
5. A small incision (about 1 cm) is cut in the skin of the surgical area.
6. The ovary and oviduct are carefully pulled out by pinching the ovarian fat tissue with forceps, and retained on sterile gauze.
7. Pinching the ovarian bursa with fine forceps, the membrane is insiced along equatorial plane of the bursa (*see* Note 5).
8. The capillary already containing the zygotes (IVF embryos) is carefully introduced into infundibulum (the top end of oviducts).
9. The zygotes (IVF embryos) are expelled from the capillary in the infundibulum.
10. The ovary and oviduct are carefully returned to abdomen.
11. The muscle tissue is sutured corresponding to the size of the incision, and then the skin closed with auto clips or suture. The remaining half zygotes (IVF embryos) are transferred into the opposite oviduct in the same way.

3.4. In Vivo Fertilization: Intrauterine Insemination

3.4.1. Freezing of Epididymal Sperm

1. Before sperm collection, SFM-1 is warmed at room temperature (22–24°C), and SFM-2 is also kept at 5°C.
2. Cauda epididymides are collected (*see* Subheading 22.2.1).
3. Both the caudal epididymides are put into 1.5 mL of SFM-1 (*see* Subheading 22.2.2) in a 35-mm plastic dish (Falcon 35-1008), then cut about 15 times in each using minisize scissors, and kept for 5 min (sampling time) under room temperature.
4. After the cutting of caudal epididymides, sperm motility and concentration are micrscopically checked, and recorded.
5. After the sampling time, the dish contained sperm samples are transferred into a 15°C incubator for 30 min.
6. The dish is moved into a refrigerator or incubator at 5°C, and kept for 30 min.
7. After cooling to 5°C, 2.0 mL of the cooled SFM-2 is gently dropped into the sperm sample in SFM-1 using a micropipette.
8. The sperm suspension is loaded into 0.25-mL plastic straws, and exposed to vapor of LN_2 around –150°C (at about 4 cm above the level of LN_2) for 15 min (pre-freezing).
9. The straws contained sperm are then plunged into LN_2, and stored at –196°C.

3.4.2. Intrauterine Insemination

1. For thawing cryopreserved sperm, the straw is immediately immersed in 37°C water for 15 s.
2. The surface of thawed straw is completely wiped off with tissue paper.
3. The straw is cut using straw cutter for bovine artificial insemination, the sperm suspension is transferred into 1.0 mL of F-mR1ECM (Table 22.1) in a 1.5-mL tube kept at 37°C.
4. Sperm sample in the tube is kept at 37°C, and motility of the sample is checked as mentioned above (*see* Subheading 22.2.1 and Note 6).
5. After confirming vaginal plugs (mating), the female is appropriately anesthetized.
6. The female is retained in the prone position, and the coat is removed using hair-clipping scissors in the bilateral lateral abdominal regions (about 3 cm × 3 cm) corresponding to the positions of the ovaries located caudal to the last costales.
7. The clipped surgical areas are disinfected with 70% ethanol.
8. A small incision (about 1 cm) is cut in the skin of the surgical area.
9. The ovary and upper uterine horn are carefully pulled out by pinching the ovarian fat tissue with forceps, and retained on sterile gauze.

10. In the bilateral uterine horns, a 21-gauge needle is inserted into the uterine wall at a site 1.0–1.5 cm lower (vaginal side) from the oviduct-uterine junction to make a small hole penetrating into uterine lumen.
11. On bilateral sides, about 50 μL of sperm suspension which is contained $1–5 \times 10^6$ sperm and prepared beforehand is aspirated into a injection pipette made from a Pasteur pipette, using a mouth-piece (the degree of inhalation that suck 50 μL is to be investigated beforehand when washing the pipette with the sperm suspension).
12. The pipette tip is precisely inserted into the uterine lumen via the small hole, and the sperm suspension is gently injected into the uterine lumen.
13. After injection, the muscle tissue is sutured corresponding to the size of the incision, and then the skin closed with auto clips or suture. This insemination is performed on the opposite uterine horn in the same way.

4. Notes

1. When using cryopreserved (frozen/thawed) sperm, F-mR1ECM including 200 μM of 3-isobutyl-1-methyl-xanthin (IBMX) is suitable to induce in vitro capacitation of cryopreserved sperm. Recently we found that treatment with IBMX dramatically increased cAMP and tyrosine phosphorylation levels in frozen/thawed rat sperm. When the IBMX-treated frozen/thawed sperm was used for IVF, the proportions of pronuclear and blastocyst formation were significantly higher than those of frozen/thawed sperm treated without IBMX. The IVF embryos can develop to term at a high success rate equivalent to the rate obtained with IVF using fresh rat sperm (10).
2. We recommend high quality of paraffin or mineral oil (mouse embryo tested) for covering micro-drops of medium, because of toxic contamination or deterioration of oil quality. If you use standard quality oil, before use you had better check the toxicity of oil using mouse embryo culture. When using high quality oil, mouse embryos show more than 70% as blastocyst formation rate (13).
3. Before adding OEP into SFM-2, when the OEP does not look transparent, the container of OEP is kept in about 50°C for 30 min to remove air bubbles. After this treatment, the OEP shows transparent amber.

4. There is a genetic difference between Wistar and Sprague-Dawley rats in fertilization rates of IVF. In Sprague-Dawley rats, the F-mR1ECM containing 140 mM NaCl (360 mOsm) is superior to that containing 110 mM NaCl (310 mOsm) in fertilization rates during insemination (co-cultuture with sperm and oocytes) (14).
5. When making an incision of the bursa, Noyes scissors is preferable. It is key for embryo transfer into oviducts to diminish bleeding in the rat.
6. Even if sperm motility of inseminated sample showed less than 5%, there is a possibility that the recipient become pregnant through intrauterine insemination on the basis of our previous data. In the study (15), we obtained nine pups derived from sperm sample whose motility showed 2%, from three pregnancies of five inseminated recipients.

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