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Efficient Production of Offspring from Japanese Wild-Derived Strains of Mice (Mus musculus molossinus) by Improved Assisted Reproductive Technologies

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ABSTRACT

Because the genetic diversity of the laboratory mouse (Mus musculus) is very limited, wild-derived strains from this genus could provide invaluable experimental models for studies of mouse genetics and epigenetics such as quantitative trait locus analysis. However, such strains generally show poor reproductive performance under conventional husbandry conditions, so their use for large-scale analyses has been limited. This study was undertaken to devise assisted reproductive technologies (ARTs) for the efficient production of offspring in two wild-derived strains, MSM/Ms and JF1/Ms (Mus musculus molossinus). First, as females of these strains are poor responders to equine chorionic gonadotropin (eCG) stimulation, we examined the efficiency of superovulation by injecting anti-inhibin serum followed by human chorionic gonadotropin (hCG). Approxi-

INTRODUCTION

The laboratory mouse (Mus musculus) is one of the most important species used in current scientific studies of mammalian gene function, thanks to the availability of large amounts of information about its genetics and biology. Furthermore, as all the major gene modification strategies, including transgenesis and gene targeting, are applicable to mice with high reproducibility, this has prompted the use of a variety of mouse strains as models for human diseases and mammalian development. Another advantage of laboratory mice in research comes from their high tolerance to inbreeding depression, resulting in the availability of many inbred strains with normal reproductive performance [1]. Because of their defined genetic profiles, these inbred strains have played critical roles in sharing the same research results among different laboratories worldwide. However, researchers recognize that the present genetic diversity of laboratory mice is too small to permit comprehensive genetic mapping or effective allelic identification, which depends largely on the presence of intraspecies polymorphisms [2]. For example, in the case of genetic mapping, there are often substantial “blind spots” in genomes caused by the lack of genetic variation among the inbred strains involved in analysis. To circumvent this shortcoming associated with the existing strains of laboratory mice, wild-derived mice from different origins have been introduced into research laboratories, many of them established as inbred strains [3]. These strains can be used successfully as unique resources to discover new allelic variations and modifiers of spontaneous, randomly introduced or genetically engineered mutations [2].

The MSM/Ms (MSM) and JF1/Ms (JF1) strains are inbred mouse strains derived from Japanese wild mice belonging to the same subspecies of Mus musculus (M. musculus molossinus) [4, 5]. They carry a high level of polymorphisms that genetically distinguish them from laboratory mice, whereas their F1 progeny produced by breeding with laboratory strains are completely fertile, as far as they have been examined. Both strains have a smaller body weight than conventional laboratory mice and show several additional strain-specific characteristics. MSM mice were derived from wild-captured mice in Mishima, Japan [4], and are characterized by high locomotive activity, resistance to carcinogenesis, and resistance to diabetes induced by a high-fat diet (Fig. 1A). JF1 mice are thought to have originated from historical Japanese mouse fanciers. According to Japanese writings in the 1700s, they were bred widely with a variety of coat color mutations. They were probably extinct in Japan by the mid-1900s but were reimported from Denmark and introduced into the animal...
Animals

The MSM/Ms, JF1/Ms, and B6·chr17 (MSM) strains were provided by the RIKEN BioResource Center. B6D2F1 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and B6C3F1, BALB/cA, ICR, and C.B-17/scid/scid mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed under controlled lighting conditions (daily light period of 0700–2100 h). All animal experiments described here were approved by the Animal Experimentation Committee at the RIKEN Tsukuba Institute and were performed in accordance with the committee’s guiding principles.

MATERIALS AND METHODS

Animals

FIG. 1. The two Japanese wild-derived strains used in this study. Both strains have a smaller body weight than conventional laboratory mice (~10–15 g) and show several additional strain-specific characteristics. A) MSM/Ms mice have an agouti coat color and are characterized by high locomotive activity. B) JF1 mice are thought to have originated from Japanese mouse fanciers in the 18th century. They are tractable and easy to handle and have a characteristic coat color with black spots on a white coat. Bar = 1 cm.

Authors
The numbers of oocytes collected from MSM or JF1 females following a superovulation regimen using eCG or AIS. A) The effect of the volume of AIS injected on the number of ovulated oocytes in the MSM strain. AIS treatment significantly increased the number of ovulated oocytes compared with the eCG treatment. The best result was obtained with 50 μl of AIS. *P < 0.05 (Tukey-Kramer procedure). B) The effects of age and superovulation regimen on the numbers of ovulated oocytes. As there is an interaction between these two factors in both strains (Table 1), post hoc multiple comparisons were undertaken based on the groups of combinations of two factors. *P < 0.05 and *a,b; b P < 0.05 (Tukey-Kramer procedure). The numbers in parentheses indicate the numbers of females used.

Embryo Cryopreservation by Vitrification

In some embryo transfer experiments, we used C.B-17/scid/scid, BALB/cA (3cl) × JF1, or B6-Chr17 (MSM) females as pseudopregnant recipients, but they often failed to mate with vasectomized males on schedule. To ensure precise timing of embryo transfer experiments throughout, we used cryopreserved embryos for these recipient females. Cryopreserved embryos were also transferred into ICR-strain recipient females in some experiments. For cryopreserving IVF-derived 2-cell embryos, we used an equilibrium vitrification method as reported previously with slight modifications. Briefly, after embryos were equilibrated in a medium consisting of 5% dimethyl sulfoxide and 5% ethylene glycol in phosphate-buffered medium (PB1) [15] for 3 min, they were transferred into a cryotube containing a vitrification medium: 42.5% (v/v) ethylene glycol, 17.3% (w/v) Ficoll and 1.0 M sucrose in PB1. The cryotube was plunged directly into liquid nitrogen. On the day of embryo transfer, the cryotube was warmed to room temperature, and the embryos were exposed to 0.5 M sucrose/PB1 and then to 0.25 M sucrose/PB1 solutions. The surviving embryos were transferred to a drop of CZB medium and cultured as described above until embryo transfer.

Histology

For histological examination, ovaries were removed from females shortly after collection of oocytes from the oviducts. They were fixed with Bouin solution for at least 2 days and processed for paraffin wax embedding. Four-micrometer-thick sections were stained with hematoxylin and eosin.

Statistical Analysis

The numbers of oocytes collected from MSM or JF1 females and the sperm motility parameters were analyzed statistically by one- or two-way ANOVA, using SPSS software (SPSS Inc., Chicago, IL), as appropriate. The Tukey-Kramer procedure was used for multiple comparisons. Parameters calculated as percentages were transformed using arcsine transformation before ANOVA. Fertilization and birth rates were analyzed using the Fisher exact test. A P value of <0.05 was considered statistically significant.

RESULTS

Superovulation

We first examined the effect that the volume of AIS administered had on the number of oocytes produced, using MSM females at 12 wk of age. While the females responded poorly to conventional eCG treatment (3.6 ± 0.5 oocytes per female), they were induced to superovulate successfully with injections of AIS at volumes ranging from 50 to 200 μl, reaching approximately 10–15 oocytes per female (P < 0.05 vs. eCG treatment; Fig. 2A). Among the AIS groups, the 50-μl-treatment group showed the best result. Therefore, in the subsequent series of experiments, we injected 50 μl of AIS per female. Next, we sought to find the best age of superovulation with AIS in the MSM and JF1 strains. We found that both the age and the superovulation regimen affected superovulation in both strains, with a significant interaction between these factors (Table 1). In MSM females, the best superovulation efficiencies were obtained from females injected at 5–7 wk of age (P < 0.05; Fig. 2B), reaching approximately 25 oocytes per female. All (n = 20) females were induced to ovulate successfully during this age range. With the eCG injection, the number of oocytes ovulated was 11.1 ± 3.2 at best (Fig. 2B). These tendencies were also the case with JF1 females. Numbers of oocytes ovulated from AIS-injected females were 27.7 ± 4.6 and 23.3 ± 1.9 when they were induced to superovulate at 5 wk and 8 wk of age, respectively (Fig. 2B). The rates of females that ovulated successfully were 75% (12 of 16) and 100% (8 of 8) at 5 wk and 8 wk of age, respectively. These results were in accordance with the histology of ovaries collected from superovulated mice. In the AIS treatment group, the entire cortex of the ovary was filled with previously ruptured follicles or fully grown follicles. In contrast, in the eCG treatment group, there were only a few ruptured follicles within a section, and atretic follicles or corpora lutea filled most of the cortex (Fig. 3). This clearly indicated that more follicles started to grow synchronously in response to the AIS treatment than with the eCG treatment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age</th>
<th>Superovulation regimen</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSM</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>JF1</td>
<td>0.000</td>
<td>0.000</td>
<td>0.005</td>
</tr>
</tbody>
</table>

a Results were obtained by two-way ANOVA; a P value of <0.05 was considered statistically significant (boldface type).

b As there was an interaction between the females' age and the superovulation regimen, post hoc multiple comparisons were undertaken based on the groups of the (age × superovulation regimen) combinations. For results, see Figure 1B.
Analysis of Sperm Motility

There were no statistically significant differences between MSM and JF1 spermatozoa in the overall rates of motile spermatozoa and progressively motile spermatozoa, irrespective of the sperm treatment (fresh or frozen; \( P > 0.05 \); Fig. 4A). In both strains, frozen-thawed spermatozoa yielded significantly lower motility scores than fresh spermatozoa (\( P < 0.05 \); Fig. 4A). All velocity parameters including VAP, VSL, and VCL were significantly higher in MSM than in JF1 spermatozoa (\( P < 0.05 \); Fig. 4B).

Embryo Transfer

We then performed IVF experiments to examine whether oocytes obtained by the AIS treatment had normal fertilizability in vitro, using freshly prepared or frozen-thawed epididymal spermatozoa. In MSM females, 69% ± 7% and 51% ± 10% of oocytes were fertilized successfully with fresh and frozen-thawed spermatozoa, respectively (Fig. 5). The lower fertilization rate with frozen spermatozoa could be improved to 66% ± 6% by addition of 1 mM GSH to the IVF medium (Fig. 5). In contrast, in the JF1 strain, higher fertilization rates were obtained even with frozen-thawed spermatozoa (89% ± 3% and 92% ± 2%, respectively) (Fig. 5).

IVF

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embryos from laboratory mice, which might induce a normal immunotolerant status for recipients. With the cyclosporin A treatment, we obtained one live MSM pup at term (1 of 37; 3%), but there were still frequent deaths of fetuses (Fig. 6). Following cotransfer of laboratory mouse embryos (ICR strain), the live birth rate increased to 2 of 24 (8%), and no intrauterine fetal death was observed. Finally, when we combined these two protocols for embryo transfer, 7 of 24 (29%) or 5 of 31 (16%) survived to term (Fig. 6). All of these MSM offspring were successfully nursed by ICR foster mothers and grew into normal adults.

The body weights of the MSM offspring were $0.93 \pm 0.01$ g in females ($n = 9$) and $0.96 \pm 0.03$ g in males ($n = 6$), while those of the JF1 offspring were $1.25 \pm 0.03$ g in females ($n = 5$) and $1.32 \pm 0.04$ g in males ($n = 8$). As far as we examined, there were no effects of experimental conditions (e.g., the strain of recipient females) on these body weights in either strain.

**DISCUSSION**

This study was undertaken to develop a series of ART procedures for the efficient use of wild-derived mouse strains as novel experimental models for studying genetics and epigenetics in mice. Among these technologies, the collection of as many mature oocytes as possible is the most essential technique for consistent animal experimentation, as well as for cryopreserving valuable animal resources. Laboratory inbred mice usually ovulate at least 5–10 oocytes periodically, as estimated from the numbers of littermates. Therefore, we may expect that successful superovulation can induce the release of approximately 10 oocytes or more from a single female. In mice, treatment with an eCG-hCG combination is the most reliable and the regimen most frequently used for inducing superovulation at any stage of the estrous cycle, although there is a great strain-dependent variation ranging from 5.4 to 39.5 oocytes per female [16]. Wild species such as *M. spretus* and *M. spicilegus* are especially poor responders to this conventional superovulation regimen [17], and in this study, we confirmed the fact that females of the wild-derived subspecies *M. musculus molossinus* were also poor responders. To circumvent the difficulty in collecting mature oocytes from these mice, we attempted to superovulate them by passive immunization with AIS [18–20], which is known to stimulate folliculogenesis in different mammalian species, including mice, rats, hamsters, guinea pigs, cows, mares, ewes, and goats [7]. As inhibin is a gonadal hormone that selectively inhibits FSH secretion from the pituitary gland, immunization against inhibin is expected to increase the endogenous FSH level, leading to the development of supernumerary follicles in a...
more natural way than the conventional eCG-hCG treatment [7]. Here, AIS treatment was very effective in inducing superovulation in both MSM and JF1 mice, although there was an apparent age-dependent effect. In a practical sense, the small size of these mice (approximately 10 g for females) was an apparent age-dependent effect. In a practical sense, the small superovulation in both MSM and JF1 mice, although there was [7]. Here, AIS treatment was very effective in inducing superovulation with eCG or AIS. Consequently, there is a high demand for developing effective methods for embryo production to further improve the IVF procedure in both wild-derived strains.

We could obtain practically effective fertilization rates of oocytes collected following AIS treatment by using conventional IVF procedures in both the wild-derived strains. However, the fertilization rates in MSM oocytes were generally lower than those in JF1 oocytes, and GSH treatment [21] was necessary when spermatozoa had been frozen-thawed. This was an unexpected result because the sperm motility parameters (VAP, VSL, and VCL) of MSM spermatozoa were significantly higher than those in JF1 spermatozoa, even after freezing-thawing (Fig. 4B). Thus, higher sperm motility did not always result in a higher fertilization rate under these in vitro conditions. It is known that GSH treatment primarily affects oocytes, especially the zona pellucida [21, 22]. Therefore, for further improvement of the IVF procedure in the MSM strain, it might be necessary to focus on the treatment of the spermatozoa, including optimization of preincubation medium, to attain a more effective induction of sperm capacitation.

Among the ART procedures we tested, the embryo transfer technique most clearly discriminated between the potential of MSM and that of JF1 embryos. No MSM pups were born alive because of intrauterine death of fetuses during late gestation, while most JF1 embryos surviving mid-gestation reached term. It is unlikely that these embryos had been genetically damaged, so their late developmental arrest might be attributed to endocrine insufficiency or immunological dysfunction. We found that this problem could be overcome by the combination of two treatments: administration of cyclosporin A to recipient females and cotransfer of laboratory mouse embryos. Interestingly, these treatments had a synergistic effect on the survival of MSM embryos. Cyclosporin A is an immunosuppressant and is known to increase the survivability of fetuses in CBA/J females mated with DBA/2 males, an immunologically mediated model of spontaneous abortion in mice [23]. Therefore, the cotransfer of laboratory mouse embryos might have modulated the maternal immune response to the fetus by a mechanism different from that of cyclosporin A and/or the systemic endocrine condition of recipient females. This problem associated with embryo transfer could not be ameliorated even when we used B6 × B6-Chr17 (MSM) recipients, which bear chromosome 17 from MSM mice [24]. As the major histocompatibility complex (MHC) in mice is encoded on this chromosome [25], this result suggested that intrauterine death of MSM fetuses cannot be attributed primarily to MHC-involved rejection.

One of the important questions that remains to be answered is why such contradictory results were obtained from the MSM and JF1 strains, even though they derive from the same original Mus musculus molossinus species. Ancestors of the MSM strain were introduced directly into the laboratory from the wild [4], while the JF1 strain has a long history as a Japanese fancy mouse breed since the 1700s [3]. The ratios of polymorphic markers that are different from common inbred strains are consistently higher in MSM mice than in JF1 mice [26]. In a recent review, therefore, the JF1 strain was classified as a fancier-derived strain and not a wild-derived strain, although it is genetically considered to belong to the M. molossinus subspecies [3]. The genetic relationship between the JF1 strain and common laboratory strains is currently under investigation (Dr. T. Shiroishi, personal communication).

The differences in ART-related phenotypes and the genetic characteristics between the MSM and JF1 strains might indicate their differential suitability for mouse genomics. The MSM strain might be more suitable for positional cloning and quantitative trait locus analysis than the JF1 strain, because these analyses need as many polymorphic markers as possible for generating high-density linkage maps. These mice have been used extensively for behavioral analysis [3, 4, 27]. Germline-competent embryonic stem cell lines from the MSM strain are also available [28]. In contrast, the JF1 strain might be more suited for developmental biology and related epigenetic studies, especially genomic imprinting, where

### TABLE 2. Development in vivo of JF1 and MSM embryos after transfer into females of different strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recipient female</th>
<th>Vitrification</th>
<th>Transferred</th>
<th>Implanted (%)</th>
<th>Dead (%)</th>
<th>Alive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSM/Ms</td>
<td>ICR</td>
<td>–</td>
<td>39</td>
<td>29 (74)</td>
<td>9 (23)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>19</td>
<td>11 (58)</td>
<td>5 (26)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>B6C3F1</td>
<td>–</td>
<td>39</td>
<td>18 (46)</td>
<td>4 (10)</td>
<td>1 (3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>27</td>
<td>15 (56)</td>
<td>1 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>C.B-17/lcr-sc-id/sc-id</td>
<td>+</td>
<td>40</td>
<td>21 (53)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>BALB/c × JF1</td>
<td>+</td>
<td>54</td>
<td>18 (33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JF1/Ms</td>
<td>ICR</td>
<td>–</td>
<td>90</td>
<td>67 (74)</td>
<td>1 (1)</td>
<td>35 (39)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>56</td>
<td>45 (80)</td>
<td>8 (14)</td>
<td>24 (43)</td>
</tr>
<tr>
<td></td>
<td>B6D2F1</td>
<td>–</td>
<td>65</td>
<td>54 (83)</td>
<td>6 (9)</td>
<td>32 (49)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>24</td>
<td>20 (83)</td>
<td>0 (0)</td>
<td>16 (67)</td>
</tr>
<tr>
<td></td>
<td>B6C3F1</td>
<td>–</td>
<td>39</td>
<td>36 (92)</td>
<td>0 (0)</td>
<td>18 (46)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>27</td>
<td>25 (93)</td>
<td>0 (0)</td>
<td>10 (37)</td>
</tr>
</tbody>
</table>

*All embryos were produced by IVF using fresh or frozen spermatozoa and from oocytes produced using superovulation with eCG or AIS.

a Two to five females were used for each recipient strain.

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reproductive performance and easy accessibility to gametes, embryos, and fetuses is critically important. In these studies, dense genetic polymorphisms are not always necessary and a few markers identifying the parental origin of the allele are sufficient for reproducibility. Since the first report on the introduction of JF1 mice into a laboratory in 1998, this strain has played a critical role in a number of analytical studies in the field of genomic imprinting of oocytes [29], spermatogenic cells [30], sperm injection [31], and nuclear transfer [32].

The ART protocols established in this study will enable us to cryopreserve embryos and produce normal pups of MSM and JF1 mice on a larger scale than before. This is critically important for mouse depository facilities, including our center, because maintaining many live mouse strains under genetically and microbiologically controlled conditions is very expensive. We are now testing the feasibility of applying these ART protocols for other wild-derived strains maintained as live mouse colonies in our center (>40 strains), to shift our maintenance strategy to the embryo/sperm cryopreservation system. We have confirmed that the long-term preservation of at least some strains can rely solely on the cryopreservation of embryos in liquid nitrogen tanks.

ACKNOWLEDGMENT

We thank Dr. Kazuo Moriwaki, Dr. Toshihiko Shiroishi, and Mr. Yoichi Mizushima for their invaluable discussion on the genomic characters and embryo transfer regimens in wild-derived strains of mice. The wild-derived strains (nSM/Ms [code no.: RRBC00209] and JF1/Ms [code no.: RRBC02554]) used in this study were developed by Drs. Kazuo Moriwaki, Tsuyoshi Koide, and Toshihiko Shiroishi and were provided by RIKEN BioResource Center with the support of the National BioResource Project of MEXT, Japan.

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