Efficient Production of Offspring from Japanese Wild-derived Strains of Mice (*Mus musculus molossinus*) by Improved Assisted Reproductive Technologies[1]

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Summary sentence: Offspring from Japanese wild-derived mice (*Mus musculus molossinus*) were produced efficiently by devising assisted reproduction technologies including superovulation and embryo transfer protocols.

Keywords: *cryopreservation, embryos, inhibin, spermatozoon, superovulation*

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). About four to six times more oocytes were ovulated compared with eCG-hCG treatment in both strains, reaching ~25–30 oocytes per female. Consequently, the procedures for in vitro fertilization using these superovulated oocytes and cryopreservation of embryos and spermatozoa could be optimized for both of the wild-derived strains. However, MSM/Ms embryos, but not JF1/Ms embryos, failed to develop to term after embryo transfer because of intrauterine death at mid- to late-gestation. We could overcome this obstacle by cotransfer of these embryos with those from laboratory strains combined with treatment of recipient females with an immunosuppressant (cyclosporin A). Thus, a series of ARTs essential for efficient production and preservation of the wild-derived strains were devised successfully. These technologies will facilitate systematic studies on mouse genetics and epigenetics using a wider range of genetic diversity than currently available in the genus *Mus*.

INTRODUCTION

The laboratory mouse (*Mus musculus*) is one of the most important species used in current scientific studies on mammalian gene function, thanks to the availability of large amounts of information on 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, it is recognized by researchers that the present genetic diversity of laboratory mice is too small to permit comprehensive genetic mapping or effective allelic identification, which largely depend 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 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. m. 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 facility of the National Institute of Genetics, Japan, in 1987 [5]. Unlike MSM mice, JF1 mice are tractable and easy to handle and have a characteristic coat color with black spots on a white coat (like the giant panda) (Fig. 1B). They have been extensively used in the studies of genomic imprinting because of the high density of genetic polymorphisms in differentially methylated regions of imprinted genes. This enables the accurate and easy identification of the parental origin of alleles of imprinted genes [6].

Despite such usefulness and uniqueness, MSM and JF1 mice both have poor reproductive performance so it is difficult to generate large cohorts of offspring by natural mating. Even with the current assisted reproductive technologies (ARTs) available for laboratory mice, their poor reproductive efficiency cannot be overcome, because of the small number of oocytes ovulated from a single female and frequent prenatal death of fetuses after embryo transfer (see Results). These characteristics also make the preservation of these strains as frozen embryo stocks inefficient. As a result, they have been maintained as live mouse colonies at a high cost in mouse depository facilities and individual laboratories (see http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC00209&lang=en and http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC00639&lang=en). Therefore, in this study, we sought to develop ARTs for the efficient production of offspring in two Japanese wild-derived strains, MSM and JF1. We focused especially on the improvement of superovulation regimens and embryo transfer techniques, which are the biggest technical barriers to the common use of these mice in biomedical laboratories. For superovulation of females, we examined the feasibility of passive immunization against inhibin with an anti-inhibin serum (AIS), which is known to increase endogenous follicle-stimulating hormone (FSH) secretion [7]. It is expected that improvements of these technologies will not only facilitate mouse genetics using these wild-derived strains, but will also contribute to the safe and efficient

cryopreservation of embryos and/or gametes of these invaluable genetic models.

MATERIALS AND METHODS

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/Icr-*scid/scid* mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed under controlled lighting conditions (daily light period 07:00 to 21:00). 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.

Superovulation
Female MSM or JF1 mice at 4 to 12 weeks of age were injected intraperitoneally with equine chorionic gonadotropin (eCG, 2.5 IU) or AIS (50 µl to 200 µl), followed by injection with human chorionic gonadotropin (hCG, 2.5 IU) 48 h later. Mature metaphase II (MII) oocytes in cumulus were collected from the oviducts 16–17 h after the hCG injection. The AIS was obtained from a castrated goat immunized against [Tyr30]-inhibin α(l–30)-NH$_2$, conjugated to rabbit serum albumin, as described [8].

Analysis of sperm motility
Epididymal spermatozoa freshly collected or frozen–thawed, as described [9], were preincubated in Human Tubal Fluid (HTF) medium [10] for 1 h at 37 °C under 5% CO_2 in air. The overall sperm motility, progressive motility, average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) were assessed using a Hamilton Thorn IVOS computerized semen analyzer (Hamilton Thorn, Beverly, MA). Motility was defined as any movement of the sperm head, and progressive motility was defined as the percentage of those spermatozoa that moved in a forward, linear direction at a speed of 50 µm/sec. These parameters were measured for >200 spermatozoa in at least three different fields.

In vitro fertilization
In vitro fertilization (IVF) was performed using a modified version of a published method [9]. HTF medium was used as the basal medium for sperm preincubation and fertilization. Mature male mice at 3–8 months of age were used for sperm collection from the epididymis. The caudae were punctured using a 26-G disposable needle to collect a sperm mass and motile spermatozoa were allowed to disperse into the preincubation medium: HTF containing 0.1 mg/ml polyvinyl alcohol (PVA) and 0.4 mM methyl-β-cyclodextrin [11,12], but lacking bovine serum albumin. The spermatozoa were incubated at 37 °C under 5% CO_2 in air for 1 h. At the time of insemination, the cumulus-enclosed oocytes, collected as described above, were placed into HTF medium and preincubated spermatozoa were inseminated at concentrations of 200–300 sperm/µl. Spermatozoa that had been frozen and thawed [9] were similarly used for IVF, but in some experiments, 1 mM reduced glutathione (GSH) was added to HTF medium for insemination. Four to five hours later, oocytes were removed from spermatozoa and cumulus cells by gentle pipetting, transferred into CZB medium [13] containing 5.6 mM glucose, 0.1 mg/ml PVA and 3.0 mg/ml bovine serum albumin and cultured at 37 °C under 5% CO_2 in air for about 24 h. Oocytes that developed into normal-looking 2-cell embryos were considered to be fertilized.

Embryo transfer
Embryos that reached the 2-cell stage after 24 h of culture in CZB medium were transferred into the oviducts of pseudopregnant females that had each been mated with a vasectomized male 1 day before. ICR females were used as recipients unless otherwise specified. Some recipient females were injected subcutaneously with 1 or 2 mg/kg cyclosporin A (Sigma-Aldrich, St Louis, MO) on day 5. In a preliminary study, we found that 2 mg/kg was the highest dose of this drug that did not compromise the systemic

condition of recipient females (data not shown). On days 19 or 20, the recipient female mice were examined for the presence of fetuses, and live pups obtained by Caesarian section were nursed by lactating ICR strain foster mothers. In some experiments, equal numbers of ICR embryos were cotransferred with MSM embryos bilaterally. In such cases, MSM fetuses were identified by their black eye color.

Embryo cryopreservation by vitrification
In some embryo transfer experiments, we used C.B-17/Icr-*scid/scid*, BALB/cA (Jcl) × JF1 or B6-Chr17(MSM) females as pseudopregnant recipients, but they often failed to mate with vasectomized males on schedule. To ensure the 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 employed an equilibrium vitrification method, as reported [14] with slight modifications. In brief, after embryos were equilibrated in a medium consisting of 5% dimethyl sulfoxide and 5% ethylene glycol (EG) in PB1 [15] for 3 min, they were transferred into a cryotube containing a vitrification medium: 42.5% (v/v) EG, 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's solution for at least 2 days and processed for paraffin wax embedding. Sections at 4-µm thickness 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 analysis of variance (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. The fertilization and birth rates were analyzed using Fisher's exact test. A probability of $P < 0.05$ was considered statistically significant.

RESULTS
Superovulation
We first examined the effect of the volume of AIS administered on the number of oocytes produced using MSM females at 12 weeks 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 about 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 used 50 µl AIS for injection per female. Next, we sought to find the best age of superovulation with AIS in the MSM and JF1 strains. We found that both age and superovulation regimen affected the effect of superovulation in both strains, with a significant interaction between these factors (Table 1). In MSM females, the best superovulation efficiencies were obtained with females injected at 5–7 weeks of age ($P < 0.05$; Fig. 2B), reaching about 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. The 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 weeks and 8 weeks of age, respectively (Fig. 2B). The rates of females that ovulated successfully were 75% (12/16) and 100% (8/8) at 5 weeks and 8 weeks 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. By contrast, in the eCG treatment group, there were only a few ruptured follicles within a section and atretic follicles or corpora lutea filled the most part 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.

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).

IVF
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, $69 \pm 7\%$ and $51 \pm 10\%$ 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 $66 \pm 6\%$ by addition of 1 mM GSH to the IVF medium (Fig. 5). By contrast, in the JF1 strain, higher fertilization rates were obtained even with frozen–thawed spermatozoa ($89 \pm 3\%$ and $92 \pm 2\%$, respectively) (Fig. 5).

Embryo transfer
In the first series of embryo transfer experiments, we followed our routine protocol using pseudopregnant ICR females as recipients. Embryos from the MSM strain were successfully implanted using this protocol, but none of them survived to term (day 19.5) irrespective of whether the embryos were fresh or vitrified (Fig. 6A; Table 2). No improvements were noted even in attempts to use females from other different strains as recipients (Table 2). Examination at Caesarian section revealed that at least some of the implanted embryos developed beyond mid-gestation, but all died *in utero* from unknown causes (Fig. 6B). By contrast, JF1 embryos implanted into recipient uteri survived to term at a rate similar to that for laboratory mice at rates feasible for laboratory applications (37% to 67%) in both fresh and vitrified groups (Table 2). Therefore, we sought to devise an embryo transfer protocol specifically for the full-term development of MSM embryos. Spontaneous abortion can occur due to inappropriate maternal–fetal interactions including maternal intolerance to the fetus and dysfunction of trophoblast cells. Therefore, we examined whether the intrauterine death of a MSM fetus could be rescued by treatment of recipient females with cyclosporin A, an immunosuppressant, or by cotransfer of 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 (1/37; 3%), but there was still frequent deaths of fetuses (Fig. 6A). Following cotransfer of laboratory mouse embryos (ICR strain), the live birth rate increased to 2/24 (8%) and no intrauterine fetal death was observed. Finally, when we combined these two protocols for embryo transfer, 7/24 (29%) or 5/31 (16%) survived to term (Fig. 6A, C). All 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±0.01 in females (n=9) and 0.96±0.03 in males (n=6), while those of the JF1 offspring were 1.25±0.03 in females (n=5) and 1.32±0.04 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 about 10 oocytes or more from a single female. In mice, treatment with an eCG/hCG combination is the most reliable and the most frequently used regimen 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 *Mus spretus* and *Mus spicilegus* are especially poor responders to this conventional superovulation regimen [17] and we confirmed in this study that females of the wild-derived subspecies, *Mus 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 (about 10 g for females) was advantageous because only 50 μl of AIS was sufficient for significant stimulation. By contrast, in laboratory mice (weighing about 20–25 g for females), 200 μl proved optimal for efficient superovulation [20]. Concerning the induction of ovulation of oocytes that had grown in response to AIS, conventional hCG treatment seemed to be effective irrespective of the strain or the age of animals, because only a few follicles remained unruptured at oocyte collection (Fig. 3). The quality of embryos was indistinguishable between the eCG and AIS groups in the ability to develop to term, at least in the JF1 strain: 40% (21/52) vs. 43% (24/56), respectively. With this successful superovulation regimen, we could proceed to perform the next series of ART experiments for producing embryos and offspring of wild-derived mice.

We could obtain practically effective fertilization rates of oocytes collected following AIS treatment using the conventional IVF procedure in both 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 the 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 in the 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 has an effect on 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 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 remain to be answered is why such contradictory results were obtained from the MSM and JF1 strains, even though they derive from the same original *M. m. molossinus* species. The ancestors of the MSM strain were directly introduced 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, not a wild-derived strain, although it is genetically considered to belong to the *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 (QTL) 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]. By contrast, the JF1 strain might be more suited for developmental biology and related epigenetic studies—especially genomic imprinting—where reproductive performance and easy accessibility to gametes, embryos and fetuses are 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.

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Figure legends

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.

FIG. 2. The numbers (± S.E.) 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 AIS. $^{a,a\prime}P < 0.05$ (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 (see Table 1), *post hoc* multiple comparisons were undertaken based on the groups of combinations of two factors. $*P < 0.05$ and $^{a,b;\ a\prime,b\prime}P < 0.05$ (Tukey–Kramer procedure). The numbers in parentheses indicate the numbers of females used.

FIG. 3. Histology of ovaries from JF1 mice treated with eCG or AIS. **A**) In the eCG treatment group, there were a few ruptured follicles (arrow) while atretic follicles or corpora lutea (asterisks) filled the most part of the cortex. **B**) In the AIS treatment group, the entire cortex of the ovary was filled with many fully grown follicles, most of which had completed ovulation (arrows). Bar = 0.1 mm.

FIG. 4. Results of computer-assisted sperm analysis (mean ± S.E.). **A**) There were no significant differences between MSM and JF1 spermatozoa in the rates of overall motile or progressively motile (P. motile) spermatozoa ($P > 0.05$). **B**) By contrast, all velocity parameters including average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) were significantly higher in MSM than in JF1 spermatozoa, as indicated by square lines ($P < 0.05$).

FIG. 5. Fertilization *in vitro* of oocytes inseminated with fresh or frozen–thawed spermatozoa in MSM and JF1 strains (mean ± S.E.). In the MSM oocytes, the fertilization rate using frozen–thawed spermatozoa was

significantly lower than that using fresh spermatozoa, but the rate was improved by addition of GSH to the fertilization medium. By contrast, JF1 oocytes showed high fertilization rates even with frozen spermatozoa. [a,a']$P < 0.05$ by Fisher's exact test. Oocytes were corrected after a superovulation treatment with eCG or AIS. The number within each bar indicates the number of oocytes observed. The number in parenthesis under each bar indicates the number of males used.

FIG. 6. The full-term development of embryos following embryo transfer in the MSM strain showing the birth rates of MSM pups and the ratios of live/dead fetuses. Cryopreserved embryos were used throughout the experiments except for one group indicated as "Fresh". All embryos were transferred into ICR strain recipient females. The number on each bar indicates the number of MSM embryos transferred. Without any treatment, no live fetus reached term due to their intrauterine death before term, as shown in the photograph on the upper right. The incidence of fetal death was partially rescued by the combination of cyclosporin A (CsA) treatment and cotransfer of embryos from the ICR strain. As a result, healthy MSM pups were born alive, as shown in the photograph on the lower right.

TABLE 1. Effects of age and superovulation regimen on the numbers of ovulated oocytes in MSM and JF1 females.

		MSM	JF1
Main effect	Age	<u>0.000</u>	<u>0.000</u>
	Superovulation regimen	<u>0.000</u>	<u>0.000</u>
Interaction		<u>0.000</u>	<u>0.005</u>

Results were obtained by two-way ANOVA; $P < 0.05$ was considered statistically significant (underlined). 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 Fig. 1B.

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

| Strain of		Vitrification	No. of embryos		No. of fetuses	
Embryos	recipient female		transferred	implanted (%)	dead (%)	alive (%)
MSM/Ms	ICR	–	39	29 (74)	9 (23)	0 (0)
		+	19	11 (58)	5 (26)	0 (0)
	B6C3F1	–	39	18 (46)	4 (10)	1 (3)
		+	27	15 (56)	1 (4)	0 (0)
	C.B-17/Icr-*scid/scid*	+	40	21 (53)	0 (0)	0 (0)
	BALB/cA × JF1	+	54	18 (33)	0 (0)	0 (0)
	B6 x B6-Chr17(MSM)*	+	44	0 (0)	0 (0)	0 (0)
JF1/Ms	ICR	–	90	67 (74)	1 (1)	35 (39)
		+	56	45 (80)	8 (14)	24 (43)
	B6D2F1	–	65	54 (83)	6 (9)	32 (49)
		+	24	20 (83)	0 (0)	16 (67)
	B6C3F1	–	39	36 (92)	0 (0)	18 (46)
		+	27	25 (93)	0 (0)	10 (37)

*B6-based consomic strain with Chromosome 17 derived from the MSM strain [22]. All embryos were produced by IVF using fresh or frozen spermatozoa and from oocytes produced using superovulation with eCG or AIS. Two to five females were used for each recipient strain.
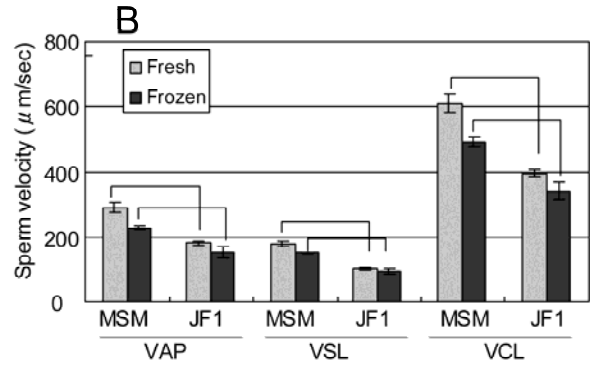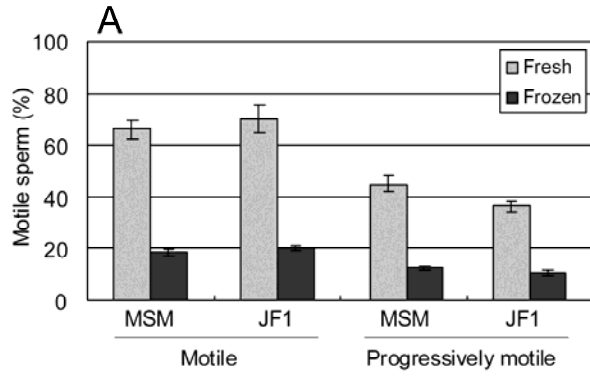
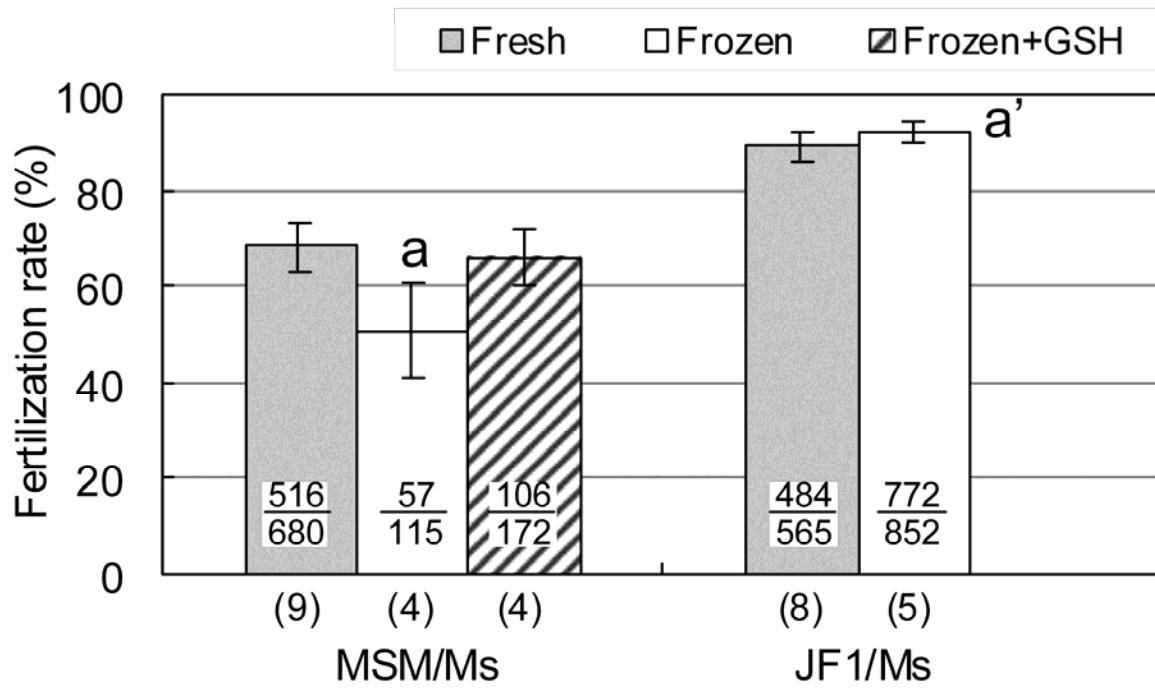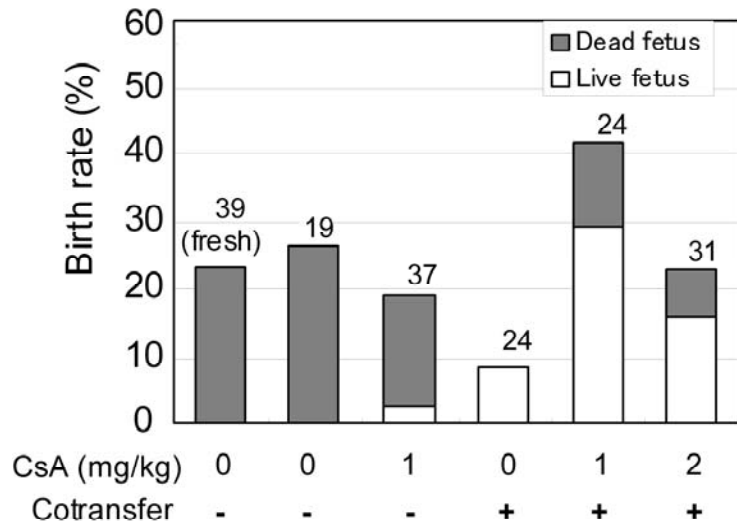Hasegawa et al. Fig. 1
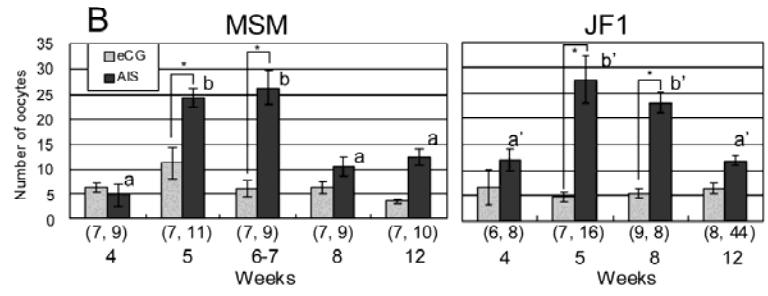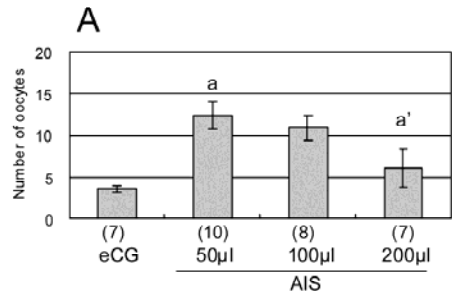
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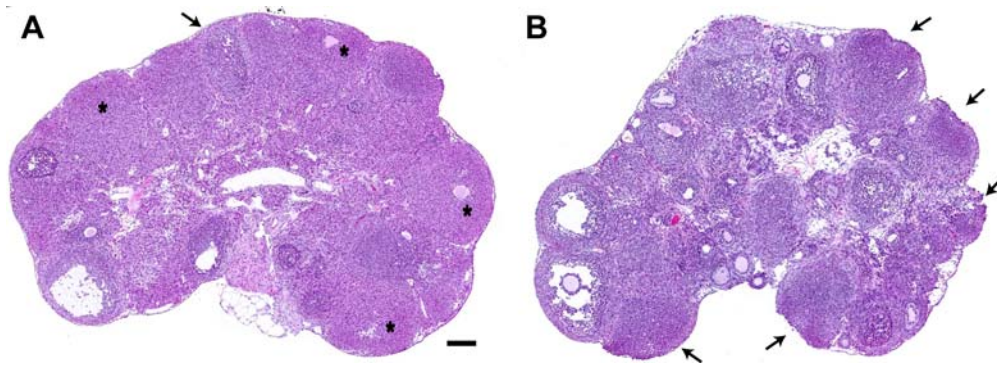
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