often difficult to prepare a large number of offspring simultaneously for precise and reliable analysis.

This study was undertaken to determine whether intersubspecific mouse hybrids between laboratory and wild-type strains could be produced efficiently by conventional ICSI. For this purpose, we injected B6D2F1 ([C57BL/6 × DBA/2]F1) oocytes with frozen-thawed spermatozoa from wild-derived strains, and assessed the developmental capacity of the intersubspecific embryos in vivo and in vitro. To assess the usefulness of these hybrid fetuses and placentas as research material, we examined them for the presence of genetic effects on their body and placental weights and for the inheritance of spermatozoan mitochondrial DNA (mtDNA).

In a second series of experiments, we produced intersubspecific F1 blastocysts by ICSI, and examined the feasibility of establishing embryonic stem (ES) cell lines from these hybrid blastocysts. We anticipate that these F1 ES cells will be efficient experimental material for the analysis of allelic expression patterns of imprinted genes or X-linked genes in vitro.

MATERIALS AND METHODS

Collection of Oocytes

Mature oocytes were collected from the oviducts of B6D2F1 or C57BL/6 (B6) female mice (SLC Co., Shizuoka, Japan) induced to superovulate with 7.5 IU of equine chorionic gonadotropin, followed 48 h later with 7.5 IU of human chorionic gonadotropin. The oocytes were placed in CZB medium (Chatot et al., 1989) and treated with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO) until the cumulus cells dispersed. The oocytes were placed in drops of CZB, covered with mineral oil (Nacalai Tesque, Kyoto, Japan), and maintained in plastic dishes (Falcon no. 1008; Becton Dickinson, Franklin Lakes, NJ) under 5% CO_2 in air at 37°C until use.

Collection and Freezing of Epididymal Spermatozoa

Mature spermatozoa were collected from the epididymides of JF1 (*Mus musculus molossinus*), HMI (*M. m. castaneus*), MSM (*M. m. molossinus*), and SWN (*M. m.* spp.) male mice at 60–90 days of age. These wild-derived mice were obtained from breeding colonies at the RIKEN Bioresource Center, Japan. Spermatozoa were frozen using a modification of the method developed by Nakagata and Takeshima (1993). The cryoprotective additives (CPA) consisted of 18% raffinose (Difco, Voigt Global Distribution LLC, Kansas City, MO) and 3% dehydrated skim milk (Difco). Fat and blood were removed from the cauda epididymis using filter paper. About 10 epididymal incisions were made with fine scissors under 100 ml of CPA in a 4-well plastic dish (no. 176740; Nunc, Roskilde, Denmark). The spermatozoa were dispersed by gentle shaking of the dish for 1 min, and the sperm suspension was divided into eight aliquots (10 µl each). Each aliquot was placed inside a 0.25 ml plastic straw (Cassou straw; IMV Technologies, L'Aigle Cedex, France) by sequentially aspirating about 100 µl of PB1, air (about 2 cm), the sperm suspension (about 0.8 cm), air (about 2 cm), and PB1 (about 1 cm) into the straw. The straw ends were sealed with straw powder (FHK straw powder; Fujihira Industry Co., Tokyo, Japan). The straws were cooled in a freezing canister (50 ml plastic syringe) floating on liquid nitrogen for 10 min, and were then immersed directly into liquid nitrogen.

Intracytoplasmic Sperm Injection

On the day of the ICSI experiments, the straws of cryopreserved spermatozoa were removed from the liquid nitrogen and immersed in a water bath at 37°C for 15 min. The spermatozoa were retrieved into NIM medium (Kuretake et al., 1996) and allowed to disperse. ICSI was performed at room temperature (23–26°C) as described in detail by Kimura and Yanagimachi (1995) and Wakyama et al., (1998), using a piezoelectric actuator (PrimeTech, Ibaraki, Japan).

In all ICSI experiments, except those involving sperm mtDNA analysis, the sperm head was separated from the midpiece and tail at the opening of an injection pipette by applying a piezoelectric pulse. The midpiece and tail were discarded, and the head was redrawn into the injection pipette and injected into an oocyte. ICSI was performed in HEPES-buffered CZB within 2–3 hr of oocyte collection. Injected oocytes were kept at room temperature for about 10 min before they were incubated at 37°C. Oocytes that survived injection were cultured in CZB at 37°C under 5% CO_2 in air.

Embryo Transfer

Embryos that had reached the two-cell stage at 24 hr in culture were transferred into pseudopregnant ICR females (8–12 weeks old) on day 0.5. On day 19.5, the recipient females were examined for the presence of fetuses, and live pups were nursed by lactating ICR females. Some females were killed at day 13.5 to collect the fetuses and placentas for the analysis of sperm-derived mtDNA.

Histological Examination

Placentas retrieved at term were fixed in buffered formalin for at least 3 days. After they had been dehydrated in a graded alcohol series, the placentas were embedded in paraffin. Sections of 4–5 µm thickness were stained with hematoxylin and eosin.

Analysis of Sperm-Derived mtDNA

Mitochondrial DNA was detected as follows. Tissue samples for mtDNA analysis were collected from the brains, livers, and muscles of fetuses at 13.5 days post-coitum and of 2-month-old adults. The tissues were treated with lysis buffer (50 ng/ml of proteinase K, 50 ng/ml of RNase, 1% SDS, 10 mM Tris/HCl [pH 8.0] and 10 mM EDTA) for 2 hr at 50°C. Genomic DNA was purified by phenol-chloroform extraction and ethanol precipitation. The allele-specific primers used for the

amplification of mtDNA were: DOM1, 5′-AGTACATTAAATCAATGGTTC-3′; MTJ2, 5′-AACAATTATCAACATAAACTG-3′; and COM1, 5′-TGGGCCCGGAGCGAGAAGAGG-3′. The DOM1 and COM1 primers were used to amplify the *M. m. domesticus* mtDNA and the MTJ2 and COM1 primers were used for the mtDNA of the wild-derived strains. PCR was carried out in a mixture (20 μl) containing 1× *Ex Taq* buffer (Takara, Shiga, Japan), 0.2 mM each dNTP, 0.5 U of *Ex Taq* HS DNA polymerase (Takara), 0.25 μM each primer, and 1 μl of 100 ng/μl total DNA solution. PCR was performed with an initial activation step of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 47°C for 30 sec, and extension at 72°C for 30 sec. After the reaction, the PCR products were applied to a 2% agarose gel and separated electrophoretically in 1× TAE buffer. The gels were stained with ethidium bromide to detect the PCR products.

Establishment of ES Cell Lines

Blastocysts obtained by ICSI using B6 oocytes and MSM spermatozoa were used for the establishment of ES cells. Blastocysts were freed from the zona pellucida by treatment with acidic Tyrode's solution. Zona-free blastocysts were plated individually in four-well dishes (Nunc) covered with a mitotically arrested mouse embryonic fibroblast feeder monolayer. The culture medium consisted of Dulbecco's modified Eagle's medium, nonessential amino acids, β-mercaptoethanol, 15% Knockout Serum Replacement (Invitrogen, Carlsbad, CA), and 10^3 U/ml ESGRO (Invitrogen). The blastocysts were allowed to attach to the monolayer. After 7–10 days in culture, the outgrowing inner cell mass was removed from the remaining trophectoderm. At the first passage, primary explants were disaggregated into small clumps of cells by gentle pipetting, and then transferred onto feeder layers in single wells of four-well dishes. The ES cells were grown to subconfluence and gradually plated on to larger culture dishes. Culture dishes were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. The ES cells were passaged every 2–4 days onto freshly prepared feeder layers, with a daily change to fresh medium. The alkaline phosphatase activity of the ES cells was confirmed by staining with an alkaline phosphatase staining kit (Sigma-Aldrich, cat. no. 86-R) according to manufacturer's instructions.

Allelic polymorphisms were examined by PCR-RFLP (restriction fragment length polymorphism) for imprinted genes *H19* and *Zac1*, and by PCR-LP (length polymorphism) for a nonimprinted gene *Bing4*. Genomic DNA was isolated from two (B6 × MSM)F1 ES cell lines (#7 and #15). Feeder cells were removed by plating on a gelatin-coated dish for 4 hr followed by gentle pipetting. Floating cells (ES cells > 95%) were used for genomic DNA extraction as described above. Purified genomic DNAs were used as a template. PCR amplifications for *H19* and *Zac1* were carried out using specific primers (5′-AAAGCACCCGTGACTCTGTT-3′ and 5′-GGGGCAAAGGATGAAGTAGG-3′ for *H19*, 5′-GCTGGACCACCTCAAGTCTC-3′ and 5′-GGCAGCAAGCACTAGACCAT-3′ for *Zac1*). PCR was performed with an initial activation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, followed by an additional 7-min elongation step at 72°C. The amplified products were digested with the enzyme *Bgl*I (*H19*) or *Taq*1 (*Zac1*). For *Bing4*, the PCR fragment was amplified using the primers 5′-TGGAGGTCTTCCCTTGTGTC-3′ and 5′-CACCATGTCACAGGATGGAG-3′. The PCR condition was described above. The amplified products were analyzed by agarose gel electrophoresis.

Statistical Analysis

The efficiency of embryo development in vitro and in vivo was analyzed using the arcsine transformation followed by one-way ANOVA. The weights of offspring and placentas at term were analyzed using one-way ANOVA. A *P*-value of less than 0.05 was regarded as significant. When the factor (the strain of sperm) had significant effects on the parameters, a post hoc procedure using Scheffe's *F*-test was adopted for multiple comparisons between groups. Experiments were replicated 5–7 times for each strain. We used a computer program (SPSS for Windows, ver. 12.0; SPSS Inc. Chicago, IL) that was capable of performing factorial ANOVA with unequal replications.

RESULTS

Development of F1 Embryos In Vitro and In Vivo

About 70%–90% of oocytes survived injection, depending on the day of the experiment. The oocyte survival rate did not vary among the strains of sperm used, because spermatozoa from the wild-derived strains were the same in shape and size. Table 1 summarizes the in vitro development of embryos following ICSI using different strains of spermatozoa. There were no strain-dependent differences in the rates of development into two cells, morulae, or blastocysts ($P > 0.05$). No significant differences were observed in the birth rates following embryo transfer ($P > 0.05$) (Table 2). Normal ICSI offspring were obtained from all F1 combinations examined (Fig. 1) and the birth rates were consistently high (27%–34%).

TABLE 1. Development In Vitro of Embryos Following Microinsemination Using Sperm Heads from Different Wild-Derived Strains

Strain of sperm	No. embryos cultured	No. (%) 2-cells	No. (%) morulae	No. (%) blastocysts
JF1	199	148 (74.4)	11/18 (61.1)	10/18 (55.6)
HMI	316	266 (84.2)	29/46 (63.0)	21/46 (45.7)
MSM	371	302 (81.4)	47/80 (58.8)	35/80 (43.8)
SWM	193	163 (84.5)	26/41 (63.4)	22/41 (53.7)

ICSI embryos were randomly allocated to either embryo transfer at the two-cell stage or culture in vitro for 96 hr.
There are no statistical differences in the developmental rates among the groups.

TABLE 2. Development In Vivo of Embryos Following Microinsemination Using Sperm Heads From Different Wild-Derived Strains

Strain of sperm	Number of embryo transferred	Number of (%) implanted	Number of (%) term offspring
JF1	125	57 (45.6)*	36 (28.8)
HMI	215	103 (47.9)*	59 (27.4)
MSM	223	92 (41.3)*	61 (27.4)
SWM	128	84 (65.6)*	44 (34.4)

*$P < 0.05$.
There is no statistical difference in the birth rates among the groups.

Genotype-Dependent Phenotypes in F1 Fetuses and Placentas

In contrast to the strain-independent efficient embryo development in vitro and in vivo, the mean body weights and placental weights at term varied significantly with the strain of spermatozoa used ($P < 5 \times 10^{-10}$ and $P < 5 \times 10^{-26}$, respectively). As shown in Figure 2A, the mean body weight of (B6D2F1 × HMI)F1 (hereafter referred to as HMI-F1) term fetuses was the greatest, and was significantly different from that of the JF1-F1, SWN-F1, and MSM-F1 term fetuses ($P < 0.05$). Genotype-dependency was more pronounced for placental weight. A multiple comparison test demonstrated that, among the six combinations (four strains) examined, five were significantly different ($P < 0.05$) (Fig. 2B). Here again, HMI-F1 placentas were largest, being about twice as heavy as those of the other F1 combinations. Histological examinations revealed that the HMI-F1 placentas had a characteristic hyperplastic phenotype with an enlarged basal layer (Fig. 3). An increased number of glycogen cells and an irregular boundary between the basal and labyrinthine layers were also noted (Fig. 3). We also produced HMI-F1 placentas by conventional IVF and confirmed that this enlarged phenotype was due to the intersubspecific cross. However, the ICSI technique might have reinforced the phenotype (data not shown). These differences in fetal and placental weights cannot be attributed to litter size, because litters were between one and seven pups in all groups and there were no strain-dependent differences.

All F1 offspring showed agouti coat color, as expected (Fig. 1). At least two adult pairs in each group were caged

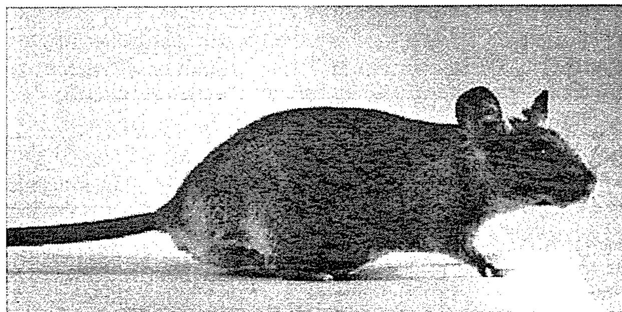

Fig. 1. A hybrid male mouse generated by ICSI using HMI sperm. All hybrid offspring generated in this study had agouti coat color, as expected. They appeared healthy and both males and females were proven to be fertile as far as tested.

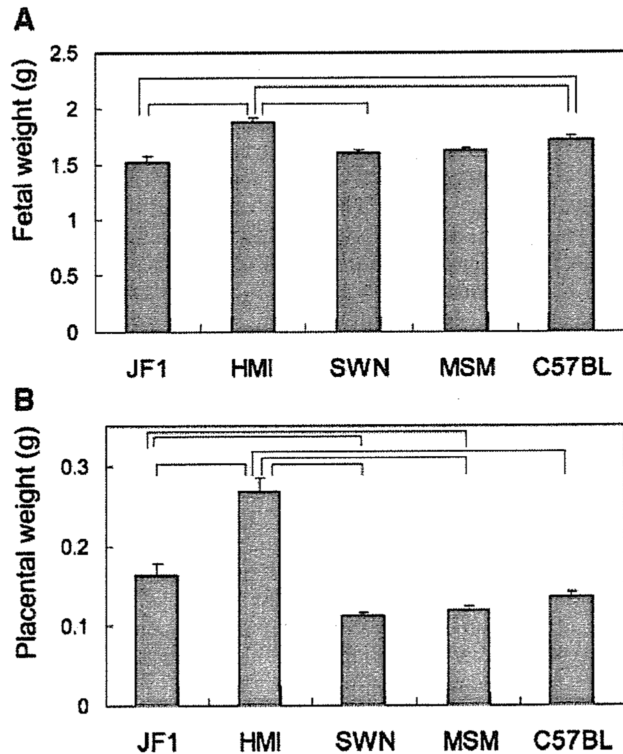

Fig. 2. The mean body (**A**) and placental (**B**) weights of F1 hybrid offspring at birth. The values for offspring generated from C57BL spermatozoa by ICSI in different experiments are shown for comparison. Statistically significant differences ($P < 0.05$) are denoted by the brackets above the bars. Error bar, SE.

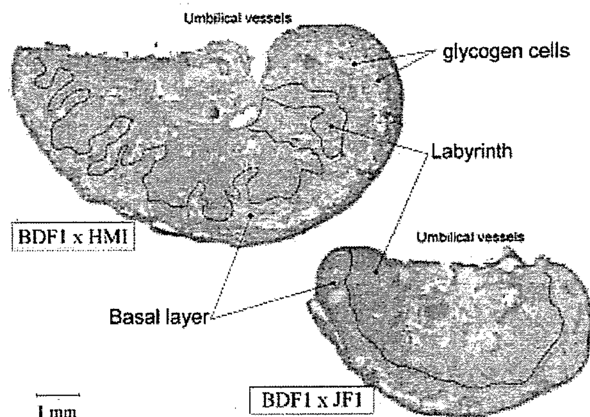

Fig. 3. Histology of placentas of HMI-F1 and JF1-F1 fetuses at term. Most HMI-F1 fetuses had an enlarged placenta with characteristic histological features (upper). As the labyrinthine and the basal layers are extensively interdigitated, their boundary is very irregular in cross-section (dotted line). The number of trophoblastic glycogen cells in the basal layer is significantly increased (pale cells). In contrast, a JF1-F1 placenta (lower) shows histology typical of normal mouse placentas.

together to examine their fertility. All F1 males and females gave birth to offspring within 3 months.

No Detection of Paternal mtDNA in F1 Offspring by Polymorphic Analysis

It can be assumed that mtDNA is essentially maternally inherited, because sperm mtDNA is selectively destroyed after fertilization. We performed intersubspecific ICSI using whole spermatozoa and examined whether sperm mtDNA was eliminated as it is in normal fertilization. As shown in Figure 4A, no mtDNA from the spermatozoa of wild-derived strains was detected in any of the fetal or adult tissues (brain, liver, or muscles) after whole-sperm ICSI. This was also the case with head-only ICSI and conventional IVF (Fig. 4A). To ascertain the minimal level of mtDNA molecules detectable with our PCR experiments, we amplified serially diluted amounts of total DNA purified from JF1 tail tissue. As shown in Figure 4B, wild-type mtDNA was detected in dilutions of up to 10^{-5}. Thus, we may infer that the sperm DNA, if any, was present at less than 10^{-5} times the original amount in the tissues of F1 offspring.

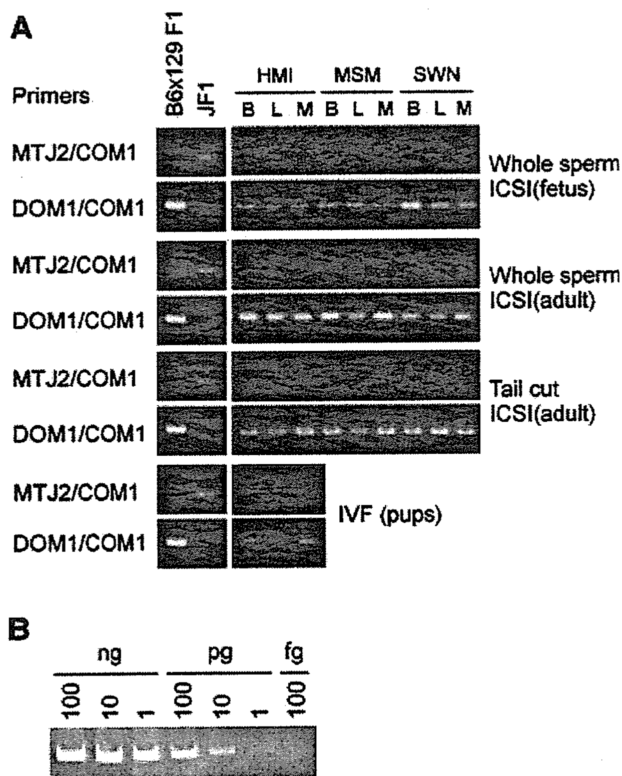

Fig. 4. Detection of parental mtDNAs in interspecific fetuses and adult mice by PCR. **A:** Paternal mtDNA was not detected in fetuses or adults derived from interspecific ICSI, indicating that sperm mtDNA is not transmitted to offspring, simulating normal fertilization. B6 × 129 F1 has the mtDNA sequence of laboratory mice. **B:** To determine the minimal detectable quantity of mtDNA in our system, we amplified serially diluted amounts of total DNA purified from JF1 tail tissue. Mitochondrial DNA from wild-derived mice was detected at dilutions of up to 10^{-5}. Thus, we infer that sperm mtDNA, if any, was present at less than 10^{-5} times the original amount in the tissues of F1 offspring.

Establishment of Intersubspecific F1 ES Cell Lines and their Polymorphic Analysis

Intersubspecific F1 ES cell lines were established from B6 × MSM hybrid blastocysts. For the production of the F1 blastocysts, B6 oocytes were injected with frozen-thawed MSM spermatozoa and cultured for 96 hr. Twenty (27%) blastocysts were obtained from 72 injected oocytes. After these blastocysts had been cultivated under conditions suitable for ES cells, nine ES cell lines were established. Thus, the efficiency of establishment was 45% from blastocysts and 12% from injected oocytes. All ES cell lines showed alkaline phosphatase activity (data not shown).

The allelic polymorphisms at the *H19*, *Zac1*, and *Bing4* loci were examined in two (B6 × MSM)F1 ES cell lines (#7 and #15). As expected, the MSM (paternal) allele and the B6 (maternal) allele were distinguished in both ES cell lines by PCR-RFLP or PCR-LP analysis (Fig. 5).

DISCUSSION

The ICSI technique has been extensively used to study the mechanisms of fertilization and in the rescue of male factor infertility in animals and humans (Ogura et al., 2005; Yanagimachi, 2005). One of the alternative practical applications of ICSI is the production of F1 hybrid embryos, which are often difficult to obtain by conventional IVF or natural mating. In our experiments, we used spermatozoa that had been collected from wild-derived mice and cryopreserved for later use. In general, the fertilizing capacity of frozen-thawed mouse spermatozoa is reduced or completely lost in vitro, and this is often the case with wild-derived mouse spermatozoa (Nakagata, 2000). Therefore, the use of ICSI in this study is also better than IVF in terms of rescuing cryodamaged spermatozoa from wild-derived strains. Our results clearly show that intersubspecific hybrid F1 mice can be efficiently produced by ICSI using cryopreserved wild-type mouse spermatozoa. There were no strain-dependent effects on embryo development in vitro or in vivo, indicating that the genetic combinations we tested do not adversely affect the development of F1 embryos. This is in contrast to the interspecies hybrids generated between laboratory mice and *M. spretus*, which often result in a significant decrease in the number of F1 hybrids produced. Furthermore, hybrid F1 males produced by that combination are sterile (Matsuda et al., 1992). In this study, both the males and females of our intersubspecific combinations were fertile. These differences in reproductive performance may be attributable to differences in the phylogenetic distances of the hybrid combinations.

In contrast to the efficient strain-independent embryo development observed in vitro and in vivo, the mean body weights and placental weights at term varied with the strain of spermatozoa used. This tendency was

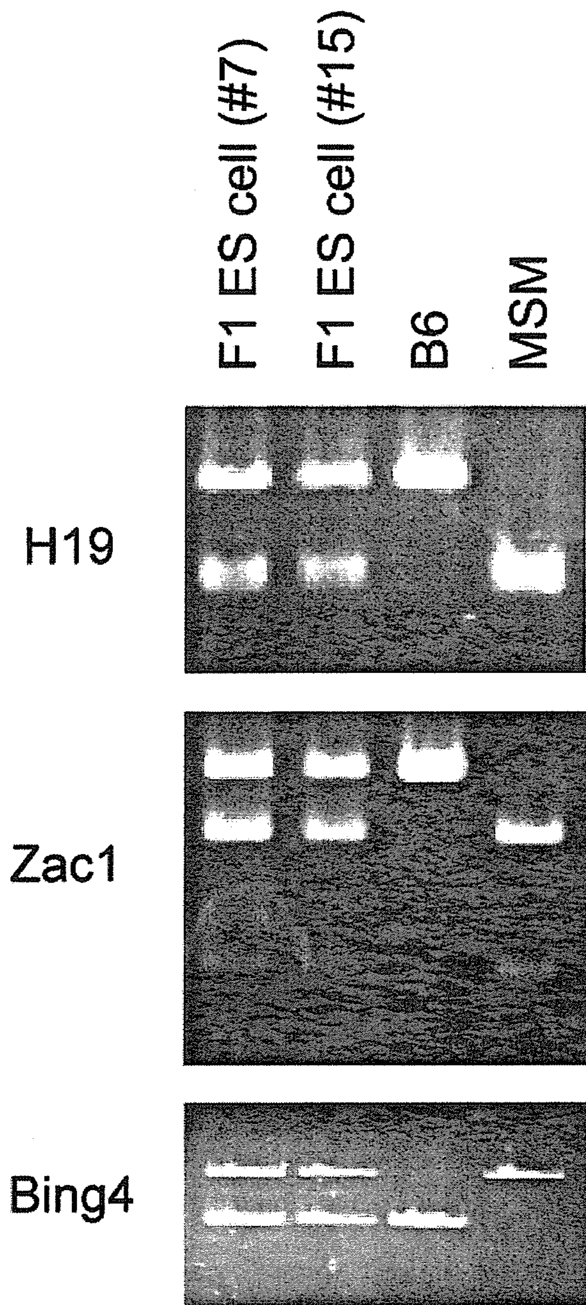

Fig. 5. Presence of allelic polymorphisms in the genome of (B6 × MSM)F1 ES cell lines. Both #7 and #15 ES cell lines showed heterozygosity at three loci examined. Control B6 and MSM genomes were obtained from the liver.

especially evident for placental weight (with a *P*-value of less than 5×10^{-26}, on one-way ANOVA). This great strain-dependency is mostly attributable to the hyperplastic alterations observed in HMI-F1 placentas. Their gross and histological features are very similar to those observed in interspecific hybrid mice (e.g., *M. musculus* × *M. spretus*) (Zechner et al., 1996) and are reminiscent of those produced from somatic cell cloning (Wakayama and Yanagimachi, 1999; Inoue et al., 2002) and in *Esx1* knockout mice (Li and Behringer, 2001). Because of the morphological features shared by these three types of placental abnormalities, they were thought to derive from some common pathological mechanisms. However, in a recent global gene expression analysis, only one gene of known function and one expressed sequence tag of unknown function were identified as common genes with changed expression levels (Singh et al., 2004). The number of genes thus identified was smaller than expected. More detailed morphological and biochemical analyses using early-stage placentas will facilitate a better understanding of the etiology of hyperplastic placentas. HMI-F1 embryos, which can be efficiently produced by ICSI, should provide a good model for genetic studies to address this question. The pups of JF1-F1 were the smallest in body and placental weights. This might have been due to some genetic effects in this strain, which has the smallest body weight among the wild-derived strains used in this study (Koide et al., 1998).

Although our main purpose in this study was to determine the efficiency of producing intersubspecific F1 hybrid mice by ICSI, it was also interesting to ascertain whether the F1 hybrids produced could be effectively used for genetic studies, especially those involving polymorphic analyses. Therefore, we investigated the fate and transmission of sperm mtDNA in fetuses and offspring produced by whole-sperm ICSI. Oocytes have a resident ubiquitin system that detects and eliminates sperm-derived mtDNA immediately after fertilization (Sutovsky et al., 2000). Unlike normal fertilization, which is achieved by sperm–oocyte membrane fusion, ICSI introduces a spermatozoon while the plasma membrane is thought to remain largely intact. Therefore, it is assumed that the timing of the exposure of sperm mitochondria to the ooplasm after ICSI is delayed relative to that of normal fertilization, and they would thus escape the ubiquitin degradation system (Sutovsky et al., 2004). However, as far as we could ascertain, no sperm-derived mtDNA was detected in any of the F1 fetuses or offspring. This is in sharp contrast to the results of interspecific crosses between *M. musculus* and *M. spretus*, in which sperm mtDNA was transmitted to a small group of offspring (Kaneda et al., 1995). Interestingly, somatic cell mtDNA from the JF1 strain introduced into BDF1 oocytes by nuclear transfer cloning was consistently transmitted to offspring, albeit in very small copy numbers, due to the injection of isolated nuclei (Inoue et al., 2004). These findings together strongly suggest that ICSI does not increase the likelihood of sperm mtDNA escaping the sperm-mtDNA degradation system, as shown previously by Cummins et al. (1997), and that this degradation system is effective in the intersubspecific combinations tested in this study. Human infertility clinics have reported that ICSI increases the risk of heteroplasmy in resultant embryos (St. John et al., 2000), but this is still controversial because paternal

mtDNA inheritance has not so far been confirmed in ICSI-derived babies (Marchington et al., 2002).

ES cells with allelic polymorphisms are valuable research material for the study of genomic imprinting and X-chromosome inactivation. We found that the efficiency of establishing hybrid ES cells between B6 and MSM strains was very high (45%; 9/20). This efficiency is similar to or higher than that of ES cell establishment in laboratory mouse strains reported previously (Kawase et al., 1994; Suzuki et al., 1999). We confirmed that they maintained their allele-specific imprinting status, at least for the paternal imprinting of the *H19* gene (unpublished). The maternal allele of the *H19* gene in these ES cells was susceptible to methylation in vitro, as reported previously by Dean et al. (1998). The aberrantly imprinted status of ES cells is thought to compromise the development of fetuses generated by nuclear transfer (Humpherys et al., 2001) or tetraploid-complemented aggregation (Dean et al., 1998). The analysis of such relationships between imprinting status and fetal developmental capacity could be performed precisely using the intersubspecific ES cells we have produced by ICSI.

In conclusion, our study clearly demonstrates that intersubspecific F1 hybrid mice and ES cell lines can be generated efficiently by conventional ICSI. A large number of F1 mice and ES cell lines will allow us to perform detailed investigations and exact statistic analyses, providing us with precise information on mouse genetic hybrid effects on their phenotypes.

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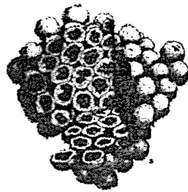

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Differential developmental ability of embryos cloned from tissue-specific stem cells

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Tissue-Specific Stem Cells

Differential developmental ability of embryos cloned from tissue-specific stem cells

(Running title: Cloning neural stem and mesenchymal stem cells)

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ABSTRACT

Although cloning animals by somatic cell nuclear transfer is generally inefficient, the use of certain nuclear donor cell types may significantly improve or deteriorate outcomes. We evaluated whether two multipotent stem cell lines produced *in vitro*—neural stem cells (NSCs) and mesenchymal stem cells (MSCs)—could serve as nuclear donors for nuclear transfer cloning. Most (76%) NSC-derived embryos survived the two-cell to four-cell transition, the stage when the major zygotic gene activation occurs. Consistent with this observation, the expression patterns of zygotically active genes were better in NSC-derived embryos than in fibroblast clone embryos, which arrested at the two-cell stage more frequently. Embryo transfer experiments demonstrated that at least some of these NSC embryos had the ability to develop to term fetuses (1.6%, 3/189). In contrast, embryos reconstructed using MSCs showed a low rate of *in vitro* development and never underwent implantation *in vivo*. Chromosomal analysis of the donor MSCs revealed very frequent aneuploidy, which probably impaired the potential for development of their derived clones. This is the first demonstration that tissue-specific multipotent stem cells produced *in vitro* can serve as donors of nuclei for cloning mice; however, these cells may be prone to chromosomal aberrations, leading to high embryonic death rates. We found previously that hematopoietic stem cells (HSCs) are very inefficient donor cells because of their failure to activate the genes essential for embryonic development. Taken together, our data lead us to conclude that tissue-specific stem cells in mice, namely NSCs, MSCs and HSCs, exhibit marked variations in the ability to produce cloned offspring and that this ability varies according to both the epigenetic and genetic status of the original genomes.

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INTRODUCTION

Cloning animals by somatic cell nuclear transfer depends on many factors, most of which remain unknown. Cloning studies in different animal species have shown that the donor cell type is one of the most important factors determining the success of cloning [1, 2]. Laboratory mouse strains provide the best models for this kind of study, because they allow rigorously controlled experiments using genetically defined animals. Our previous statistical analysis revealed that development of embryos *in vitro* and *in vivo* was better in nuclei from neonatal Sertoli cells than in embryos produced from adult cumulus oophorus cells [3, 4]. This donor cell-dependent difference may arise because of the undifferentiated status of the donor genome, as neonatal Sertoli cells are small, round, immature cells, unlike the large cells in the mature testis. This assumption is consistent with evidence showing that undifferentiated embryonic stem (ES) cells are the best donor cells for mouse cloning, leading to about 20% birth rates per embryo transfer in optimal conditions [5–7]. However, we found previously that hematopoietic stem cells (HSCs), the most undifferentiated cells of the hematopoietic lineage, are very inefficient donor cells compared with other differentiated cells of the same lineage [8, 9]. Development of HSC-derived cloned embryos is characterized by frequent developmental arrest at the two-cell stage. This is caused at least partly by failure to activate the gene for histone deacetylase 1, *Hdac1*, the key to regulating subsequent zygotic gene activation [10]. Because low *Hdac1* expression level is an inherent character of HSCs and is assumed to be related to their stem cell characters, the poor development of HSC-derived cloned embryos may be unique and not common to other stem cell

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clones. We were interested in investigating the developmental ability of embryos cloned from other stem cell types.

For reliable nuclear transfer experiments, the donor cells for cloning must be identified precisely by their morphology or should be prepared as a suspension with nearly 100% purity. At present, the mouse stem cells that fulfill this requirement are neural stem cells (NSCs) and mesenchymal stem cells (MSCs), both of which can be established by selective culture *in vitro* and are fully capable of differentiating *in vitro*. Here we used NSCs and MSCs as nuclear donors for cloning experiments and examined the developmental potential of the resultant embryos *in vitro* and *in vivo*. We also performed gene expression analysis of the cloned embryos and karyotyped the donor cells to clarify the results.

MATERIALS AND METHODS

Preparation of donor cells

We used male (C57BL/6 × 129/Sv-ter) F1 strain mice (called B6 × 129F1 for brevity) to prepare the donor cells. NSCs were obtained from the brains of fetuses at 12.5 days postcoitum (dpc) as described previously [11–13]. Briefly, cells were dispersed by repeated pipetting in phosphate-buffered saline (PBS; pH 7.6), and were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 0.6% glucose, 100 μg/ml bovine transferrin (Invitrogen), 25 μg/ml bovine insulin (Sigma, St. Louis, MO), 10 μg/ml putrescine (Sigma), 30 nM sodium selenite

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(Sigma), 20 nM progesterone (Sigma), 20 ng/ml human epidermal growth factor (EGF, Sigma), and 20 ng/ml human fibroblast growth factor (FGF; Peprotech, Rocky Hill, NJ). Cells were cultured for one month by changing the medium every week until neurospheres formed. They were further cultured for more than one month until other contaminating cells were depleted from neurospheres.

MSCs were obtained from bone marrow cells according to the method of Sun et al. [14] with slight modifications. About 7.6×10^7 bone marrow cells were collected from four-week-old male mice and cultured in α-MEM (Invitrogen) containing 10% fetal bovine serum. The medium was changed every three days. After four passages, nonhematopoietic cells were collected using a fluorescence-activated cell sorter (FACS) Vantage SE (BD Biosciences, San Jose, CA) as a cell population that was negative for an anti-CD45.2 antibody (eBioscience, San Diego, CA). Single cells were seeded onto wells of a 96-well plate and putative MSCs were allowed to proliferate clonally. The cells were used for nuclear transfer shortly after cell line establishment (< 2 weeks in culture).

The ability of the NSCs and MSCs to differentiate was tested *in vitro* before they were used for nuclear transfer experiments. For NSC differentiation, neurospheres were allowed to adhere to poly-L-ornithine (Sigma P3655)-coated plates (Lab-Tec chamber slides; Nunc, Roskilde, Denmark) in EGF/FGF-free medium containing 2% bovine calf serum for four days [11, 13]. The NSCs proliferated and extended their neurites, and differentiated into neurons. Differentiated cell types were identified by staining using specific antibodies. NSC-derived

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differentiated cells were fixed in 4% paraformaldehyde in PBS at 25 °C for 30 minutes and washed thoroughly with PBS. The cells were permeabilized in 0.3% Triton X-100 in PBS for five minutes, washed in PBS, and treated with 10% normal goat serum in PBS for one hour. The primary antibodies used were as follows (dilutions in parentheses): rabbit anti-mouse MAP-2 polyclonal antibody (1:500–1:1000; AB5622, Chemicon, Billerica, MA); mouse anti-GFAP rabbit polyclonal IgG_1 (1:500; MAB360, Chemicon); and mouse anti-O4 monoclonal IgM (1:73; MAB345, Chemicon). After washing in PBS, the cells were treated with secondary antibodies as follows: Alexa Fluor 488-anti-rabbit IgG (1:400; A-11034, Invitrogen); Alexa Fluor 594-anti-mouse IgG_1 (1:400; A-21125, Invitrogen); and Alexa Fluor 350-anti-mouse IgM (1:400; A-31552, Invitrogen). After washing in PBS, the cells were observed under a fluorescent microscope.

MSCs were induced to differentiate *in vitro* using methods reported previously (osteoblasts, adipocytes [15], and chondrocytes [14]). To identify the specific cell types, the differentiated cells were stained with a reaction mixture for alkaline phosphatase for osteoblasts (Substrate kit 415161; Nichirei Biosciences Inc, Tokyo, Japan), Oil red O for adipocytes (Sigma), and Alcian blue (Sigma) for chondrocytes.

Fibroblasts as sources of control nuclei were obtained from the tail tips of adult (2–3 months old) male mice by confluent culture as described [16].

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Oocyte collection

Female B6D2F1 strain mice, 7–10 weeks old, were superovulated with 7.5 IU PMSG and 7.5 IU hCG at 48-hour intervals, and killed 16 hours after hCG injection. Mature meiosis stage II (MII) oocytes were collected from their oviducts. Cumulus cells were released in KSOM medium [17] containing 0.1% hyaluronidase and washed several times with fresh medium. Oocytes were cultured in KSOM medium at 37.5 °C in an atmosphere of 5.5% CO_2 in air until enucleation.

Nuclear transfer

Nuclear transfer was carried out as described [4, 8, 18]. MII oocytes were placed in Hepes-buffered KSOM medium including 7.5 μg/ml cytochalasin B (Calbiochem, San Diego, CA), and nuclei were removed with a small amount of cytoplasm. Enucleated oocytes were cultured in KSOM medium in an incubator (as above) for 30–60 minutes to allow the cell membrane to recover. NSCs and MSCs were enucleated using glass micropipettes and the nuclei of donor cells were injected into the ooplasm using a Piezo-driven micromanipulator (PrimeTech, Tsuchiura, Japan).

Adult fibroblasts were prepared from tail tips as reported [16]. Their nuclei were transferred into enucleated oocytes by electrofusion [19]. After nuclear transfer, reconstructed oocytes were cultured with KSOM medium for 1–2 hours and transferred into Ca^{2+}-free KSOM medium including 3 mM $SrCl_2$ and 5 μg/ml cytochalasin B. One hour later, activated oocytes were transferred into KSOM medium containing only 5 μg cytochalasin B and cultured further

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for five hours. After washing, the oocytes were cultured in fresh KSOM medium at 37.5 °C in an atmosphere of 5.5% CO_2 for 48 hours.

Embryo transfer

Reconstructed embryos that reached the 4–8-cell stage after 48 hours of culture in KSOM were transferred into the oviducts of pseudopregnant ICR strain females mated with vasectomized males the day before. On day 20, the recipient females were examined for the presence of fetuses, and live pups were nursed by lactating ICR females.

Chromosomal analysis

NSC and MSC cell lines established as described above were subjected to chromosomal analysis. NSCs and MSCs in culture dishes were treated with 25 ng/ml colcemide for 30 minutes, and the round cells composed mostly of cells in metaphase were collected, spread onto clean glass slides, and allowed to dry in air. Q-banding staining was performed by a combined quinacrine-33258 Hoechst method [20]. Metaphase images were observed under a fluorescent microscope (Axio Photo 2, Carl Zeiss Co., Ltd., Germany) and karyotype analysis was performed using an Ikaros karyotyping system (Carl Zeiss).

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Gene expression analysis

We selected six zygotic genes, *Dppa2, Dppa3* (*Stella* or *PGC7*), *Dppa4, ERV-L, Hdac1*, and *eIF-1A*, based on previous studies on global or specific gene expression [21–23]. Embryos were individually analyzed by a quantitative reverse transcriptase- polymerase chain reaction (quantitative RT-PCR) technique. Cloned or *in vitro*-fertilized (IVF) two-cell embryos 24–26 hours after activation or 26–29 hours after insemination [24] were treated with acid Tyrode's solution to remove the zona pellucida, and cDNA was extracted using Cell-to-cDNA II kits (Ambion, Woodward, TX). PCR products amplified with the primers in Table 1 were diluted serially and used as external standards for quantitative real-time PCR. Measurements of gene expression levels were carried out using an ABI7900HT Sequence Detection system (Applied Biosystems, Foster City, CA) with QuantiTect Syber Green or QuantiTect Probe PCR kits (Qiagen, Hilden, Germany).

Statistical analysis

Development rates of embryos *in vitro* and *in vivo* were compared between groups using Fisher's exact probability test. The relative transcription levels of embryos or donor cells determined by quantitative real-time PCR were analyzed by one-way ANOVA followed by a post-hoc procedure using Scheffé's *F* test for multiple comparisons between groups where appropriate.

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All animals were maintained and used for experiments in accordance with the guidelines of the RIKEN Institute, Japan.

RESULTS

Determination of pluripotency of the donor NSCs and MSCs

Before the cloning experiments, we characterized the donor NSC and MSC lines for their ability to differentiate *in vitro*. Under appropriate culture conditions, NSCs differentiated into neurons, astrocytes, and oligodendrocytes, and MSCs differentiated into adipocytes, osteoblasts, and chondrocytes (Fig. 1). Thus, the stem cell lineages used here had differentiation potentials similar to those reported elsewhere [11–15].

Development of NSC and MSC cloned embryos

Cloned embryos reconstructed with NSC or MSC nuclei were cultured *in vitro* for 48 hours until they should have reached the four-cell stage. As shown in Table 2, more than half of the reconstructed embryos developed to the two-cell stage, whereas the remaining embryos did not divide. Because these one-cell-arrested embryos had formed pseudopronuclei successfully from the donor nuclei, the likely cause of their arrest was cell cycle asynchrony between the donor cell nucleus and the recipient ooplasm, as reported for cloning with ES cells and immature Sertoli cells [3-5].

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Among the two-cell embryos, those derived from NSCs showed a higher rate of growth to the four-cell stage (75.9%) than those from MSCs (45.9%) or fibroblasts (41.7%) ($P < 0.05$) (Table 2). After transfer of these four-cell embryos into recipient females, 5.3% (10/189) reached term in the NCS group. Two normal-looking pups and one dead pup were obtained, whereas the remaining seven were placenta-only conceptuses (Fig. 2). The two offspring lived for 3 weeks and 1 year, respectively, and the latter was proved to be fertile. One pup (3%, 1/33) was obtained in the fibroblast group. In contrast, no MSC embryos developed to term because of complete implantation failure as revealed by examination of the recipient females at the time of the Caesarian delivery.

Chromosomal analysis of donor cells

We examined the chromosome constitutions of the MSC line used in this study. Fifty-two (67%) of the 78 metaphase chromosome spreads examined had 41 chromosomes because of monosomy 4, trisomy 6, and two Y chromosomes (Fig. 3A). The remaining 26 spreads showed the normal number of chromosomes (2n = 40 in the mouse), but they also had aneuploidy of monosomy 4 and trisomy 6 (Fig. 3B). Heteromorphisms were often observed on chromosome 16.

We investigated further five other MSC lines and found highly frequent abnormal ploidy in all (Fig. 3C). Their chromosomal patterns were more severely affected as compared with that of the donor MSC line (Figs. A and B) probably due to their longer culture in vitro (additional one to two months in culture). In contrast, the NSC line had relatively normal ploidy levels (Fig. 3D).

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Gene expression patterns in NSC embryos

As zygotic genes are programmed to activate at specific stages during preimplantation development, their expression pattern is a good indicator of the success of genomic reprogramming by nuclear transfer. We analyzed the expression levels of six genes by real-time quantitative PCR using two-cell NSC-derived cloned embryos, control fibroblast-derived cloned embryos, control IVF embryos, and MII oocytes. As shown in Figure 4, the gene expression patterns of NSC-derived embryos were similar to those of IVF embryos in all genes examined except *Hdac1*, which was more actively expressed in the NSC-derived embryos than in IVF embryos ($P < 0.001$). By contrast, fibroblast-derived clones tended to show lower expression patterns for *eIF-1A*, *Dppa3*, and *Dppa4* than IVF embryos, although the trend was not significant for *Dppa3* ($P = 2.9 \times 10^{-6}$ for *eIF-1A*; $P = 0.172$ for *Dppa3*; and $P = 0.00035$ for *Dppa4*).

DISCUSSION

We aimed to evaluate whether tissue-specific stem cells, NSCs and MSCs, could be used as nuclear donor cells for cloning mice. Since we undertook detailed analysis for the effects of the donor cell type and genotype on cloning efficiency [4], we have assumed that a male genotype produced by hybridization between C57BL/6 and 129 strains (B6 × 129F1) might give us relatively high birthrates for clones. Based on this assumption, we recently cloned hematopoietic

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