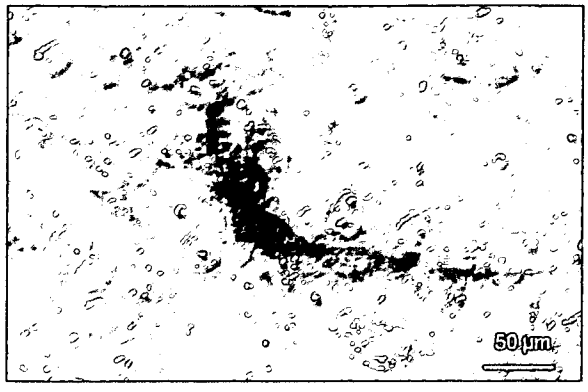

Figure 6 Hoffman modulation contrast image of a cell from the genital ridges and mesenteries of an embryo at day 33 postfertilization after 13 days of culture under conditions used to derive human embryonic germ cells. The cell (at center of photo) that was positive to alkaline phosphatase activity is stained red.

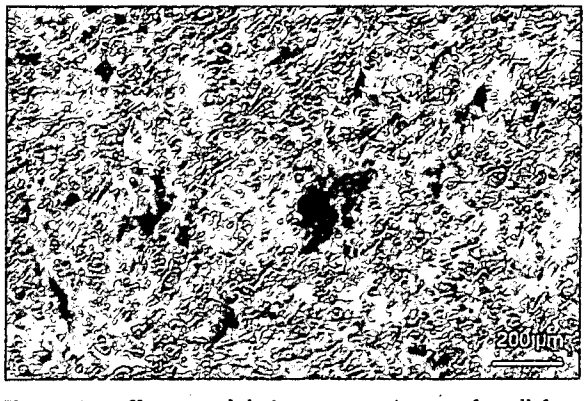

Figure 7 Hoffman modulation contrast image of a cell from the gonads of an embryo at day 66 postfertilization after 34 days of culture under conditions used to derive human embryonic germ cells. Cells that were positive to alkaline phosphatase activity are stained red.

arrived at the genital ridges via the mesenteries at approximately day 33 postfertilization, and finally colonized in the gonads by day 36 postfertilization. In mice, EG cells have been derived from PGC cultured from an embryo (aged between days 8.5 and 12.5 post coitus) under the presence of leukemia inhibitory factor, basic fibroblast growth factor and membrane-associated steel factor.[7,8] Based on our results about the formation status of the gonad and the location of PGC, the developmental stage of a cynomolgus monkey embryo at day 36 postfertilization apparently corresponds to that of a mouse embryo at day 12.5 post coitus (DPC). Stevens *et al.* reported that although more than 80% of mouse genital ridges at 12.5 DPC had the ability to form teratoma, this percentage dropped to approximately 8% at 13.5 DPC.[9] Labosky *et al.* reported that EG cells from a 12.5 DPC embryo did not have the ability to contribute to the germ line and that no EG cells could be derived from a 15.5 DPC embryo.[10] The PGC started meiosis at week 10 postconception in a human female embryo and in a rhesus monkey female embryo (same genus as cynomolgus monkey),[11] whereas PGC started meiosis at 13.5 DPC in a mouse female embryo. Although EG cell lines have been derived from 6 to 8-week postconception human embryos,[2,3] all cell lines were assumed to not have the ability to form teratoma. In conclusion, when the aim is to derive an EG cell line of a cynomolgus monkey, PGC should be collected from embryos earlier than day 33 postfertilization.

Genital ridges and mesenteries of a cynomolgus monkey embryo at day 33 postfertilization were cultured in this study according to the derivation and culture methods[2,3] of human EG cells. Namely, single cells to which the tissues were enzymatically dispersed were cultured on a STO feeder layer in culture medium supplemented with rhLIF, rhbFGF and forskolin. The cells were positive for ALP activity for a maximum of 13 days (Fig. 6). In contrast, cells cultured under the same conditions but derived from the gonads of an embryo at day 66 postfertilization were positive for ALP activity for 2 months (Fig. 7). This difference (i.e. the time that these PGC could be maintained by culture) is apparently related to the culture conditions and to the status of differentiation of PGC at collection. Derivation of an EG cell from a cynomolgus monkey was not achieved under the culture conditions used for humans. The PGC colonies obtained in the experiment disappeared with repeating passage. In the future, the successful derivation of EG cells will require research into the culture conditions or timing of passage for PGC from a cynomolgus monkey embryo collected earlier than day 33 postfertilization.

ACKNOWLEDGMENTS

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MONITORING OF GENE EXPRESSION IN DIFFERENTIATION OF EMBRYOID BODIES FROM CYNOMOLGUS MONKEY EMBRYONIC STEM CELLS IN THE PRESENCE OF BISPHENOL A

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ABSTRACT — An embryonic stem (ES) cell differentiation model would facilitate analysis of developmental processes at the cellular level and the effects of embryotoxic and teratogenic factors *in vitro*. We explored the use of differentiation of embryoid bodies (EBs) from cynomolgus monkey ES cells for embryotoxicity testing. We determined the mRNA expression of various genes using real-time RT-PCR. Oct-3/4 expression was almost completely suppressed on day 14, suggesting that ES cells reached differentiated status in around 14 days. mRNA expression of E-cadherin, connexin 43, caveolin-1, and argininosuccinate synthetase was reproducibly suppressed during EB differentiation in 7–32% of ES cells in three separate experiments. Although these may not be "general stemness marker genes" such as Oct-3/4, they could play a role in readying stem cells for differentiation in response to deletion of signals from feeder cells. Next, we examined the effects of bisphenol A (BPA) on the mRNA expression of several differentiation marker genes for ES cells. That of PAX-6, an ectoderm marker, with 0, 0.1, and 10 μM BPA in 21-day EBs was 3,500%, 6,668%, and 8,394%, respectively, compared with ES cells. The difference between doses of 0 and 10 μM BPA in 21-day EBs was statistically significant (p=0.049). Pax-6 activation in the presence of BPA may interfere with the development of eyes, sensory organs, and certain neural and epidermal tissues usually derived from ectodermal tissues. Differentiation of EBs from cynomolgus monkey ES cells could be a useful model for detecting gene expression changes in response to chemical exposure.

KEY WORDS: Embryonic stem cell, Embryoid body, Differentiation, Monkey, Bisphenol A, Embryotoxicity

INTRODUCTION

Embryonic stem (ES) cells have great potential for cell therapy and regenerative medicine, but also represent a dynamic system suitable for identifying potential molecular targets for the development of novel

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drugs, providing an *in vitro* system to examine safety or potential toxicity in humans (Davila *et al.*, 2004; Wobus and Boheler, 2005). Particularly promising is the ES cell differentiation model, which includes developmental processes from early embryonic stages up to terminally differentiated cell types and which would enable us to analyze developmental processes at the cellular level and to determine the effects of embryotoxic and teratogenic factors *in vitro*. Establishing a reliable *in vitro* embryogenesis model would also contribute to a reduction in the number of animal experiments required for medical and pharmacological testing.

Experimental studies using non-human primate ES cells have advantages such as similar characteristics to human ES cells that are not observed in murine ES cells, and avoidance of the ethical problems caused by the use of human ES cells (Thomson *et al.*, 1995; Suemori and Nakatsuji, 2003; Adachi *et al.*, 2006; Byrne *et al.*, 2006). To date, however, the ES cell test for testing embryotoxicity primarily uses murine ES cells (Spielmann *et al.*, 1997; Scholz *et al.*, 1999; Imai and Nakamura, 2006). As a result, our knowledge on the molecular and cellular aspects of ES cells for embryotoxicity testing is based mainly on murine cells, and information on non-human primate ES cells is limited.

In the course of ES cell differentiation, genes encoding tissue-specific proteins are expressed in a developmentally controlled time pattern that closely resembles what is observed during embryogenesis. ES cells differentiate *in vitro* into embryoid bodies (EBs) comprising endoderm, mesoderm and ectoderm cell layers in the absence of the self-renewal signals provided by feeder layers (Weitzer, 2006). EB formation is considered to mimic embryo development during the stages of pre-gastrulation and early gastrulation. Using a mouse EB model, Wartenberg *et al.* examined anti-angiogenic agents in an *in vitro* assay system (Wartenberg *et al.*, 1998). However, to our knowledge, little information is available on the primate EB differentiation model for embryotoxicity screening.

Bisphenol A (BPA) is commonly used in various industries. Its monomer is used for polycarbonate plastic production, and its resin form is used as linings for most food and beverage cans, as dental sealant, and as an additive in other widely used consumer products. BPA is known to elicit weak estrogenic activity in *in vitro* and *in vivo* test systems. Although molecular mechanisms studies have revealed a variety of pathways in which BPA can stimulate cellular responses at very low doses in addition to the effects initiated by its binding to the classical estrogen receptors, there is little information available concerning the effect of BPA on early embryogenesis (vom Saal and Hughes, 2005; Kang *et al.*, 2006).

In the present study, to develop a model system for primate embryotoxicity testing, we used BPA as a model compound and examined changes of gene expression in response to exposure to BPA using cynomolgus monkey EB differentiation. We conducted experiments in three steps. Firstly, we determined the time necessary for EB differentiation in cynomolgus monkey ES cells, examining the expression of Oct-3/4, a POU-class transcription factor. This stemness marker gene was used since loss of pluripotency during spontaneous or induced differentiation has been correlated with progressive loss of Oct-3/4 expression (Niwa, 2001; Mitalipov *et al.*, 2003). In addition to morphological changes, we determined mRNA expression levels using real-time RT-PCR. This quantitative approach is rapid and sensitive and is considered suitable for pharmacological and cytotoxicity screening. Monitoring of ES cell differentiation with quantitative PCR has recently been reported (Noaksson *et al.*, 2005). Secondly, we examined the expression of several genes whose proteins were highly expressed in ES cells compared with EBs. These genes were selected on the basis of preliminary results obtained from a comparison of the protein-expression profiles of various genes in 12–16-day-old CMK-6 ES cells and CMK-6 EBs with Green Fluorescent Protein (Furuya *et al.*, 2003) using Power Blot™, a Western blot array analysis (unpublished result). In the third step, we examined the effects of BPA on mRNA expression of the following differentiation marker genes for ES cells: α-fetoprotein (AFP) and GATA-4 as endoderm markers; BMP-4 and Brachyury as mesoderm markers; and PAX-6 and N-CAM as ectoderm markers.

MATERIALS AND METHODS

Cell culture

The ES cell lines (CMK-6) used in this study were established from blastocysts of the cynomolgus monkey and were kindly provided by Dr Norio Nakatsuji of Kyoto University (Fig. 1A; Suemori *et al.*, 2001).

ES cells were grown on mouse embryonic fibroblast (MEF) feeder cells that were mitotically inactivated by mitomycin C in Dulbecco's Modified Eagle's Medium (DMEM/F12) (SIGMA) supplemented with

20% knock-out serum replacement (Invitrogen), 1% non-essential amino acids (SIGMA) and 2 mM L-glutamine (SIGMA) at 37°C in a humidified 5% CO_2 atmosphere.

For EB differentiation, entire ES cells colonies were loosely detached by 0.1% (w/v) collagenase (Wako Pure Chemical) from the feeder cells and transferred into a feeder-free 25 cm^2 flask (SUMILON) for floating culture. The medium was changed after the first day and half of it was changed every 7 days.

EBs (2×10^5/ml) were incubated for 7, 14 or 21 days with 0.1 μM or 10 μM BPA (SIGMA). In the control group, the same volume of DMSO was added to the media. Stock BPA (100 mM) was dissolved in DMSO and was stored at −20°C. The BPA was further diluted in culture medium just before use, and was sterilized through a filter with a pore size of 0.22 μm. The final DMSO concentration did not exceed 0.1% (vol/vol).

When harvesting ES cells and EBs for mRNA expression analysis, they were washed three times with PBS and then pooled at −80°C until RNA extraction.

Quantitative real-time RT-PCR

Total RNA from ES cells and EBs was isolated using a RNeasy Plus Mini kit (QIAGEN), which includes removal of genomic DNA contamination before cDNA synthesis. Samples were collected from three separate culture experiments.

cDNAs were synthesized from total RNA ranging from 100 ng to 1 μg using QuantiTect Reverse Transcription (QIAGEN) after elimination of genomic DNA contamination.

β-actin and differentially expressed genes were quantitatively detected with a LightCycler Instrument (Roche Diagnostics) using the LightCycler FastStart DNA Mater^{PLUS} SYBR Green I (Roche Diagnostics) according to the manufacturer's instructions.

The primers for each gene were designed and synthesized on the basis of respective information in NCBI or ENSEMBL using the software of Premiere Biosoft, so that the targets were 75–200 bp in length (SIGMA GENOSYS, Table 1).

PCR amplification was performed in a total volume of 20 μl containing cDNA and each primer (0.5 μM). The PCR cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec. The fluorescent product at the end of the 72°C extension period was detected. All PCR assays were performed in at least duplicate.

The data obtained were analyzed using the LightCycler analysis software. To confirm the amplification specificity, we subjected the PCR products to melting curve analysis. The results are given as the mean ± SE of samples from three separate culture experiments. The statistical analysis was conducted using Kruskal–Wallis test.

RESULTS

As shown in Fig. 1A and B, cynomolgus monkey ES cells spontaneously differentiated into EBs after separation from the MEFs. Fig. 2 shows the time-course of mRNA expression of Oct-3/4 in EBs in relation to that on Day 1 (ES cell). The average mRNA expression levels of Oct-3/4 in EBs were 3%, 0.9%, and 0.4% at 7 days, 14 days and 21 days, respectively, compared with ES cells. No significant effect of BPA on Oct-3/4 expression in EB differentiation was observed.

Next, we examined the mRNA expression of genes selected by the preliminary experiments

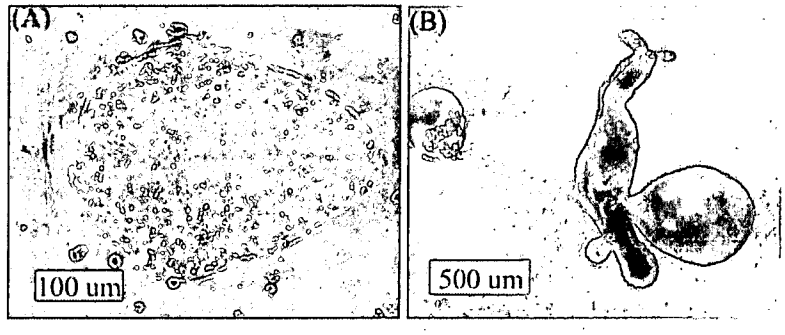

Fig. 1. A: Cynomolgus monkey ES cells, CMK-6.
B: EBs derived from CMK-6 ES cells on Day 14.

described in the Introduction. E-cadherin, connexin 43, caveolin-1, and ASS were consistently suppressed during EB differentiation. In the absence of BPA, the mRNA expression of E-cadherin was suppressed to 12%, 20%, and 21% at 7 days, 14 days and 21 days, respectively, compared with ES cells (Fig. 3A). In the absence of BPA, the mRNA expression of connexin 43 was suppressed to 32%, 32% and 27% at 7 days, 14 days and 21 days, respectively (Fig. 3B). In the absence of BPA, the mRNA expression of caveolin-1 was suppressed to 17%, 26% and 21% at 7 days, 14 days and 21 days, respectively (Fig. 3C). In the absence of BPA, the mRNA expression of ASS was suppressed to 10%, 9% and 7% at 7 days, 14 days and 21 days, respectively (Fig. 3D). There were no detectable differences on microscopy between the BPA treatment and non-treatment groups. In addition, no significant effect of BPA was observed on the expression of these genes in EB differentiation.

Table 1. Primer sequences for real timeRT-PCR.

Gene	Primers
β-actin	F: ACCCCGTGCTGCTGACC
	R: CCAGAGGCGTACAGGGATAGC
Oct-3/4	F: GCTCCTGAAGCAGAAGAGGATCACC
	R: GCCCTTCTGGCGCCGGTTACAGAAC
E-cadherin	F: AAGACCAAGTGACCACCTTAGAG
	R: AAACAGCAAGAGCAGCAGAATC
Connexin-43	F: TTCAATGGCTGCTCCTCACC
	R: GCTCACTTGCTTGCTTGTTGTA
Caveolin-1	F: CGGCTCAACTCGCATCTCAAG
	R: GCCAGGAACACCGTCAGGA
ASS	F: TGGCTGAAGGAACAAGGCTATG
	R: GCTGACATCCTCAATGAACACC
AFP	F: AGCTTGGTGGTGGATGAA
	R: CAGCCTCAAGTTGTTCCTCT
PAX-6	F: ACAGACACAGCCCTCACAAAC
	R: ATCATAACTCCGCCCATTCACC

In the third step, we examined the effects of BPA on the mRNA expression of the following differentiation marker genes for ES cells: AFP, GATA-4, BMP-4, Brachyury, PAX-6, and N-CAM. The results for two genes, AFP and PAX-6, which gave reproducible results in three separate cultures, are presented in Fig. 4A and B. The mean AFP mRNA expression in 14-day EBs was 204,132% in the presence of 10 μM BPA and 130,635% in the absence of BPA. The difference was

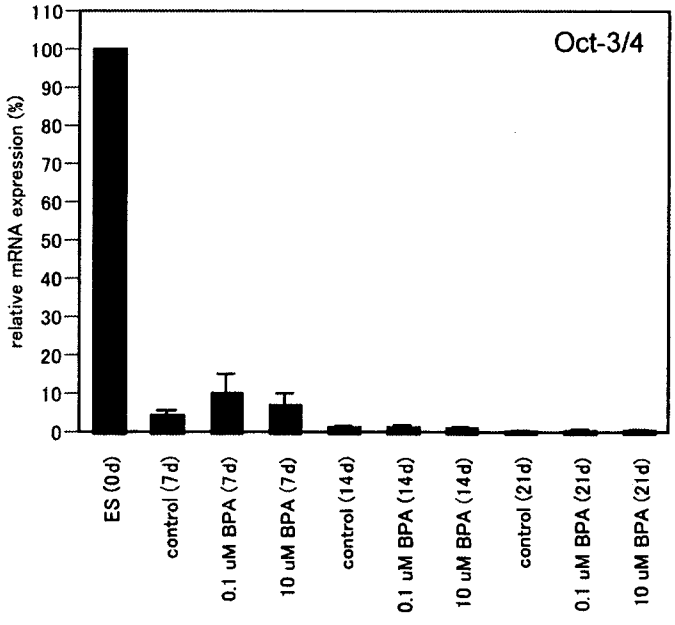

Fig. 2. mRNA expression of Oct-3/4 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

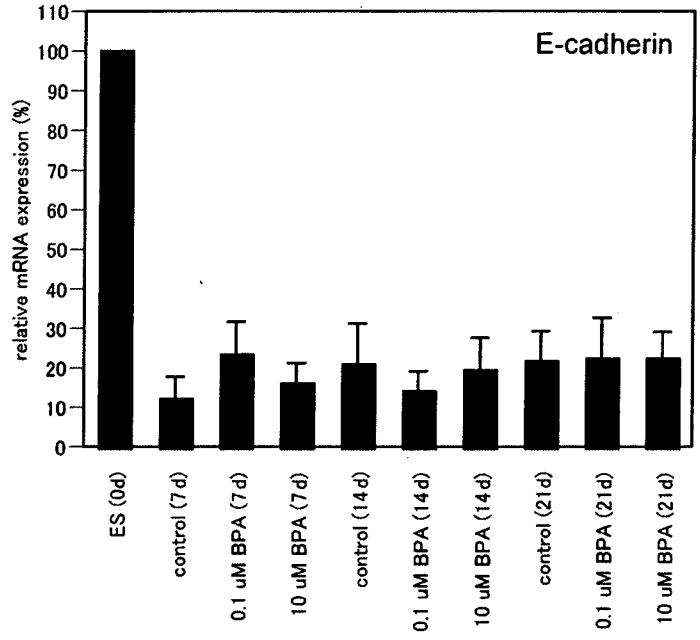

Fig. 3A. mRNA expression of E-cadherin in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

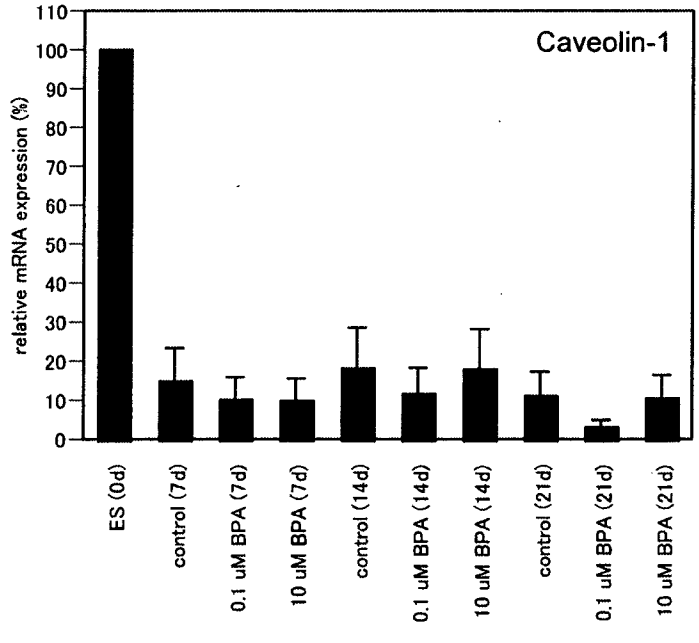

Fig. 3B. mRNA expression of caveolin-1 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

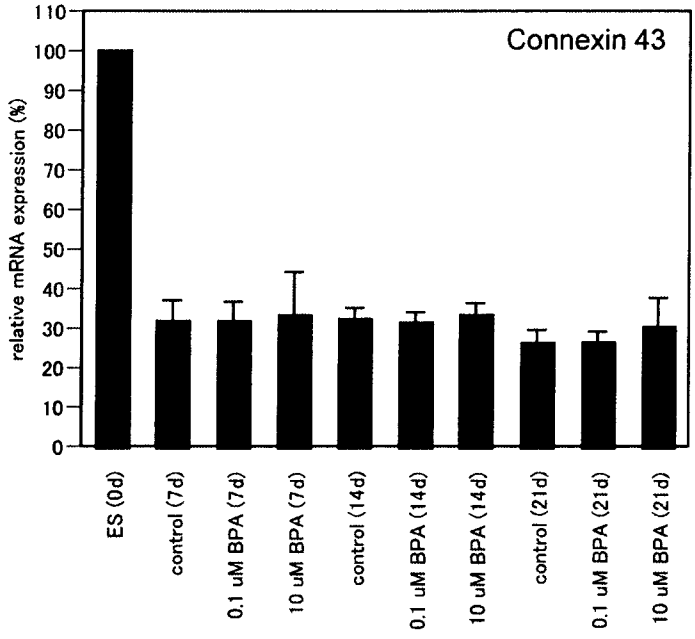

Fig. 3C. mRNA expression of connexin-43 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

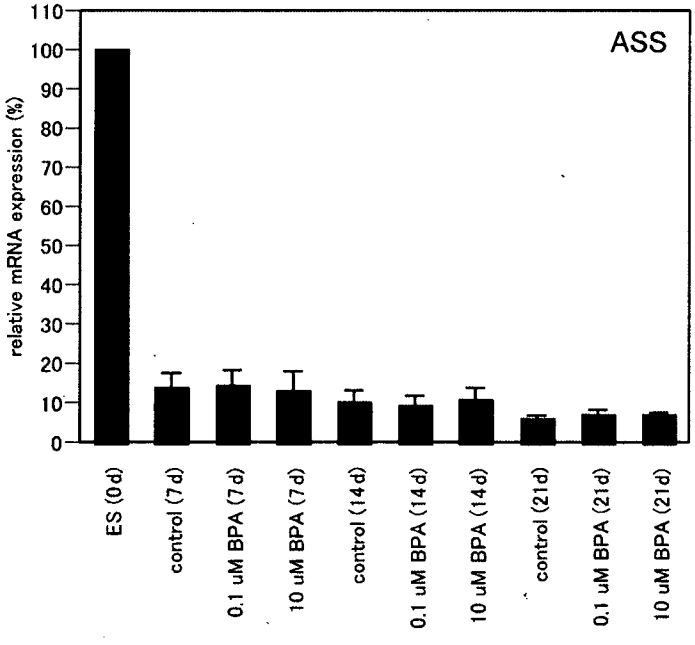

Fig. 3D. mRNA expression of ASS in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

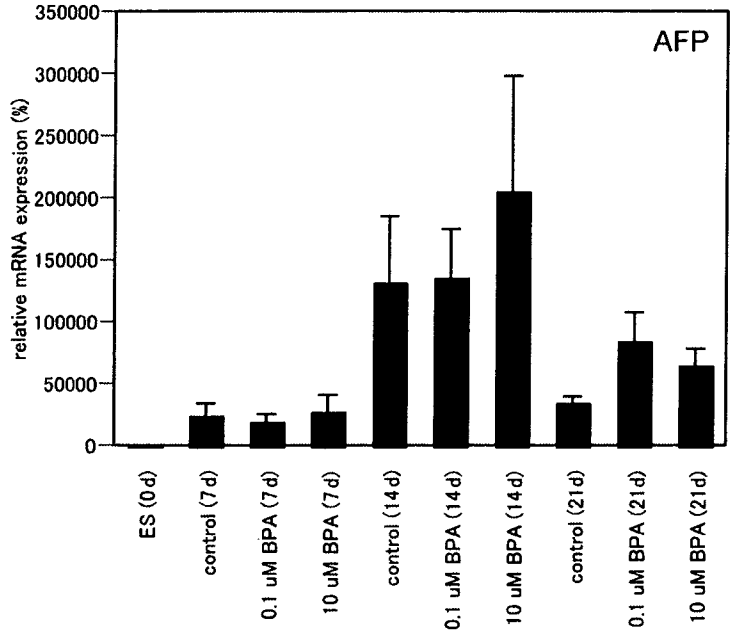

Fig. 4A. Effect of BPA on AFP mRNA expression in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

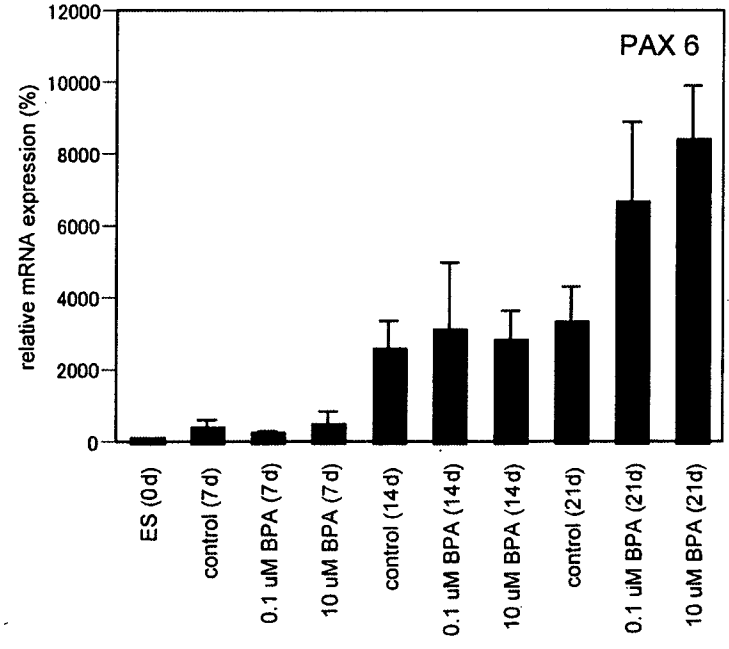

Fig. 4B. Effect of BPA on PAX-6 mRNA expression in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

not statistically significant (p=0.513). In 21-day EBs, the average mRNA expression in the presence of 0, 0.1 μM and 10 μM was 33,418% (reference category for p-value calculation), 80,100% (p=0.049) and 63,787% (p=0.049) respectively. The averages of three separate experiments of PAX-6 mRNA expression in the presence of 0, 0.1 and 10 μM BPA in 21-day EBs were 3,500%, 6,668% and 8,394%, respectively, compared with ES cells. The difference between doses of 0 and 10 μM was statistically significant (p=0.049). The difference between doses of 0 and 0.1 μM was not statistically significant (p=0.275). On Days 7 and 14, BPA did not show any evident effects on PAX-6 mRNA expression.

DISCUSSION

mRNA expression of Oct-3/4 was almost completely suppressed on Day 14, suggesting that ES cells reached differentiated status in around 14 days. E-cadherin, connexin 43, caveolin-1 and ASS were also consistently suppressed during EB differentiation (Table 2). The time patterns of their mRNA expression were similar to that of Oct-3/4. The cadherins are a class of transmembrane proteins that play important roles in cell adhesion. E-cadherin is first expressed in the 2-cell stage of mammalian development, and becomes phosphorylated in the 8-cell stage, where it causes compaction (Halbleib and Nelson, 2006). Connexin 43 is known to be related to gap junction-related protein, and gap junctions play significant regulatory roles in embryonic development (King and Lampe, 2005). Caveolin-1 has been shown to be the structural protein of plasmalemmal invaginations, termed caveolae, and functions as a tumor suppressor gene (Sotgia *et al.*, 2006). The tyrosine-phosphorylated form of caveolin-1 co-localizes with focal adhesions, suggesting that caveolin-1 plays a role in migration. Down-regulation of caveolin-1 leads to less efficient migration *in vitro*. ASS is an enzyme that catalyzes argininosuccinate synthesis from citrulline and aspartate, and is responsible for the third step of the urea cycle and one of the reactions of the citrulline-NO cycle (Husson *et al.*, 2003). ASS is highly conserved from bacteria to humans, and is present in large amounts in many tissues, including liver and kidney. It is difficult to discuss the significance of down-regulation of E-cadherin, connexin 43, caveolin-1, and ASS during EB differentiation, because they were selected by their ES/EB ratio in protein expression profiling. They may not be "general stemness marker genes" such as Oct-3/4, expression of which is always suppressed in any type of differentiation. However, they at least play a role, not only in maintaining the undifferentiated stem cell state, but also in readying stem cells for EB differentiation in response to deletion of signals from the MEFs. Reproducible results in some genes were obtained in separate experiments, indicating that this EB differentiation system could work as an embryotoxicity test.

Table 2. Average of mRNA expression of undifferentiated stem cell state-related genes.

	1d-ES	7d-EB	14d-EB	21d-EB
Oct-3/4	100%	3%	0.9%	0.4%
E-cadherin	100%	12%	20%	21%
Connexin 43	100%	32%	32%	27%
Caveolin-1	100%	17%	26%	21%
ASS	100%	10%	9%	7%

In response to BPA, expression of AFP andPAX-6 was increased at least temporarily. AFP is a glycoprotein that is produced principally in the fetal liver and gastrointestinal tract and is temporarily present during embryonic development. Estrogens were reported to modify AFP, exhibiting growth-suppressive properties (Vakharia and Mizejewski, 2000). BPA may interfere with the interaction between AFP and estrogen in EB differentiation. Recently, non-estrogenic effects of BPA on the central nervous system have been reported. In mice, prenatal and neonatal exposure to BPA induces a significant increase in the levels of dopamine D_1 receptor mRNA in the brain and increases central dopamine D_1 receptor-mediated activity (Suzuki *et al.*, 2003). In addition, expression of PAX-6 mRNA in embryos of *Xenopus laevis* was reported to be suppressed by treatment with 50 or 100 μM BPA from stage 10.5 to stage 35 (Imaoka *et al.*, 2007). PAX-6 is recognized as a master control gene for the development of eyes, sensory organs and certain neural and epidermal tissues that are usually derived from ectodermal tissues (Kondoh *et al.*, 2004). BPA may be related to one of the above functions in early development. The reason why the mRNA expression results for some genes were not reproducible may be that inappropriate primers were used for RT-PCR, that transfection with GFP may alter the characteristics of cells or that changes due to BPA may have occurred at the translational level rather than the transcriptional level.

In conclusion, our results indicated that this EB differentiation from cynomolgus monkey ES cells could work for detecting changes of gene expression in

response to BPA exposure, and could contribute to developing a primate ES embryotoxicity test in the near future.

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Efficient Production of Intersubspecific Hybrid Mice and Embryonic Stem Cells by Intracytoplasmic Sperm Injection

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ABSTRACT Recently, mice and embryonic stem (ES) cells with allelic polymorphisms have been used extensively in the field of genetics and developmental biology. In this study, we examined whether intersubspecific hybrid mice and ES cells with these genotypes can be efficiently produced by intracytoplasmic sperm injection (ICSI). Frozen-thawed spermatozoa from wild-derived strains, JF1 (*Mus musculus molossinus*), MSM (*M. m. molossinus*), HMI (*M. m. castaneus*), and SWN (*M. m.* spp.), were directly injected into mature oocytes from laboratory mice ([C57BL/6 × DBA2]F1; *M. m. domesticus*). The in vitro and in vivo developmental capacity of F1 embryos was not significantly different among the groups ($P > 0.05$), and term offspring were efficiently obtained in all groups (27%–34% of transferred embryos). However, the mean body and placental weights of the offspring differed significantly with genotype ($P < 5 \times 10^{-10}$), with the HMI hybrid greatest in both body and placental weights. In an application study using these F1 offspring, we analyzed their mitochondrial DNA using intersubspecific polymorphisms and found the consistent disappearance of sperm mitochondrial DNA in the F1 progeny. In a second series of experiments, we generated F1 blastocysts by injecting MSM spermatozoa into C57BL/6 oocytes and used them to generate hybrid ES cell lines. The ES cell lines were established at a high efficiency (9 lines from 20 blastocysts) and their allelic polymorphisms were confirmed. Thus, ICSI using cryopreserved spermatozoa allows the efficient and immediate production of a number of F1 hybrid mice and ES cell lines, which can be used for polymorphic analysis of mouse genetics. *Mol. Reprod. Dev. 74: 1081–1088, 2007.* © 2007 Wiley-Liss, Inc.

Key Words: ICSI; mitochondrial DNA; placenta; polymorphism; ES cells

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) can bypass the process of sperm penetration of the cumulus cells and zona pellucida, and the process of fusion with the oolemma. ICSI was first introduced as an alternative assisted-reproduction technique in experiments with hamsters, and has subsequently advanced our understanding of the early events of fertilization (Yanagimachi, 2005; Ogura et al., 2005). This approach is now one of the most successful methods for achieving fertilization in severe cases of human male infertility. In laboratory species, including mice, ICSI has been used frequently for research purposes and the conservation of genetic resources (Ogura et al., 2001; Hirabayashi et al., 2002; Yamauchi et al., 2002; Ogonuki et al., 2003a,b; Ogonuki et al., 2005). When spermatozoa are unable to fertilize oocytes because of spontaneous mutations or gene modifications, experiments with ICSI (but not in vitro fertilization [IVF]) ensure the ability of the sperm genome to support embryonic development (Yanagimachi et al., 2004). The technique can also be advantageous in conventional IVF when mouse spermatozoa have been cryopreserved for genetic resource banking, because frozen-thawed mouse spermatozoa are occasionally unable to fertilize oocytes because of damage to the sperm plasma membrane (Szczygiel et al., 2002; Nishizono et al., 2004).

Interspecific or intersubspecific F1 hybrid mice are invaluable research material in mouse genetics, especially for genetic and gene expression analysis using polymorphisms between the parents. However, the production of F1 offspring by natural mating or conventional IVF is not always efficient or practicable, so it is

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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 *BglI* (*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

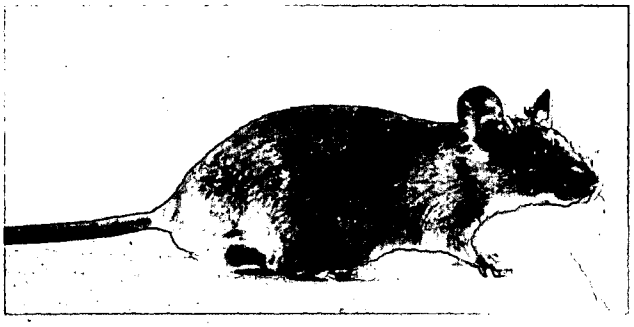

Fig. 1. A hybrid male mouse generated by ICSI using HMI sperm. All hybrid offspring generated in this study had agouti coat color, as expected. They appeared healthy and both males and females were proven to be fertile as far as tested.

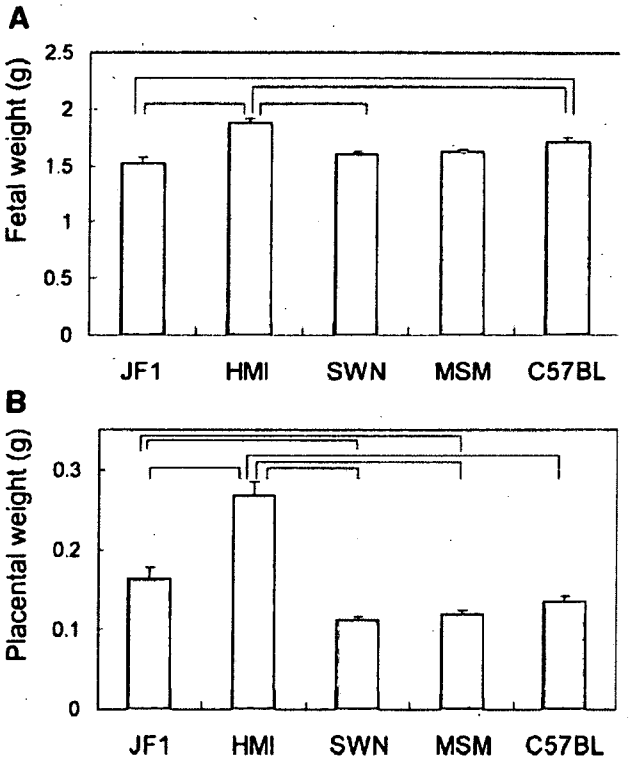

Fig. 2. The mean body (**A**) and placental (**B**) weights of F1 hybrid offspring at birth. The values for offspring generated from C57BL spermatozoa by ICSI in different experiments are shown for comparison. Statistically significant differences ($P < 0.05$) are denoted by the brackets above the bars.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

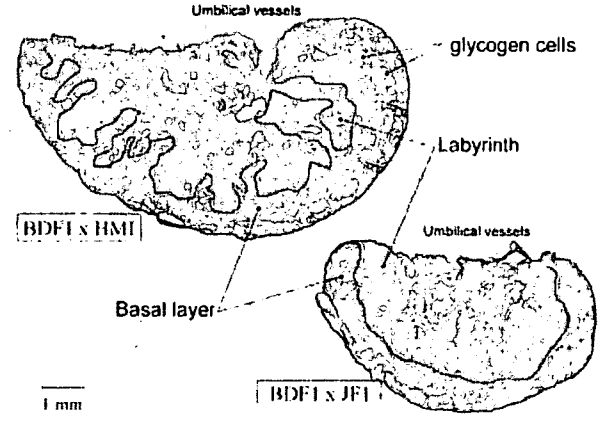

Fig. 3. Histology of placentas of HMI-F1 and JF1-F1 fetuses at term. Most HMI-F1 fetuses had an enlarged placenta with characteristic histological features (upper). As the labyrinthine and the basal layers are extensively interdigitated, their boundary is very irregular in cross-section (dotted line). 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.

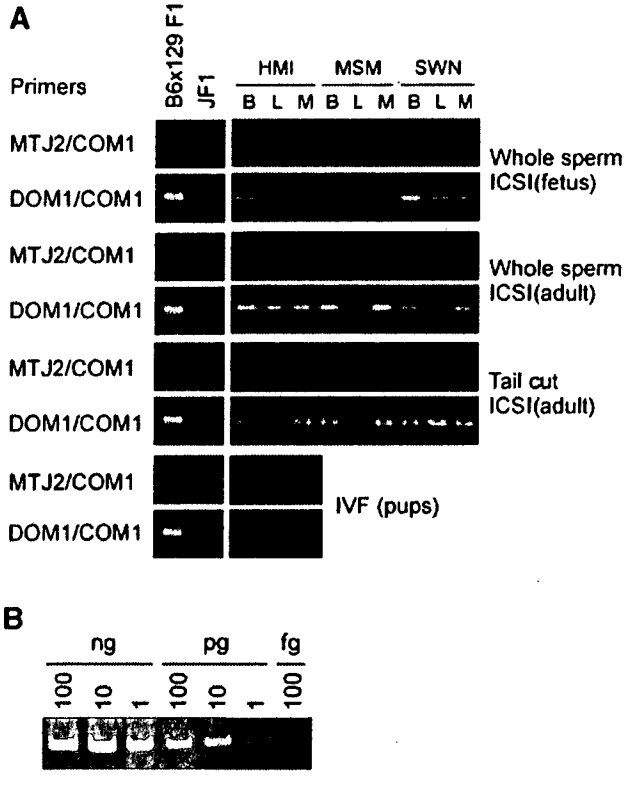

Fig. 4. Detection of parental mtDNAs in interspecific fetuses and adult mice by PCR. **A:** Paternal mtDNA was not detected in fetuses or adults derived from interspecific ICSI, indicating that sperm mtDNA is not transmitted to offspring, simulating normal fertilization. B6 × 129 F1 has the mtDNA sequence 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

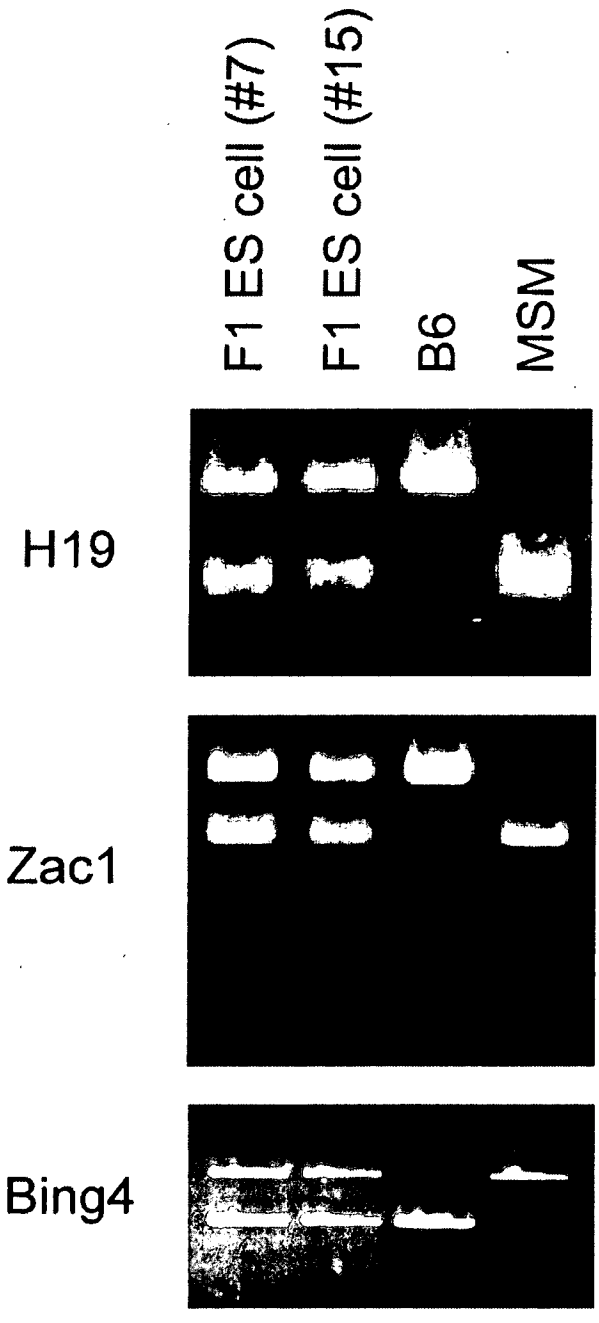

Fig. 5. Presence of allelic polymorphisms in the genome of (B6 × MSM)F1 ES cell lines. Both #7 and #15 ES cell lines showed heterozygosity at three loci examined. Control B6 and MSM genomes were obtained from the liver.

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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