Table 2. Contribution of ES-Like Cells to Embryonic Development

Type of Cells	Number of Embryos Transferred	Number of Recipients	Number of Pups Born[a]	Number of Live Pups[b]	Chimera (%)	
					Male	Female
ES-like	193	11	54	36	9/22 (41)	4/14 (29)
ES	91	14	14	4	2/2 (100)	2/2 (100)
GS	124	7	28	16	0/8 (0)	0/8 (0)
4n rescue ES-like	92	4	0	NA	NA	NA
4n rescue ES	30	2	0	NA	NA	NA

NA, not applicable.
[a] In some experiments, fetuses were delivered by cesarean section at 19.5 dpc.
[b] Number of live pups on the next day after birth.

istics of germline cells. Possibly, the interaction with Sertoli cells normally directs germ cells to spermatogenesis and inhibits multilineage differentiation in the testis. However, when germline cells are continuously stimulated to expand in the absence of Sertoli cells, as in our culture conditions, germ cells may be released from this inhibition and some of the cells converted to pluripotent cells. Teratogenesis from germline cells is susceptible to environmental influences; for example, teratoma formation can be significantly enhanced (~10-fold) in vivo by ectopic transplantation of the fetal genital ridge (Stevens, 1984). As PGCs can become pluripotential only after in vitro culture and cytokine supplementation was also necessary for EG cell conversion (Matsui et al., 1992; Resnick et al., 1992), growth stimulation and release from somatic cells may modify the differentiation program of germline cells.

Several lines of evidence in our study provide support for the multipotential nature of spermatogonial stem cells. First, we did not find PGC-like germ cells in the neonatal testis, and we failed to induce mGS cells from neonatal testis in EG cell culture conditions (mSCF + LIF + bFGF). Therefore, the mGS cells arose through a different mechanism from that of EG cells, and the results suggest that PGC-like cells in neonatal testis, if any, are not responsible for the generation of mGS cells. Second, results of p53 knockout mouse experiments showed that mGS cells develop from GS cells. The use of the p53 knockout mouse was based on previous studies that showed an increased frequency of teratoma in this strain; it is estimated that loss of the *p53* gene results in a 100-fold increase in the susceptibility to testicular teratoma (Lam and Nadeau, 2003). Nevertheless, GS cells from this strain were phenotypically similar to wild-type spermatogonia and could produce normal-appearing spermatogenesis when transferred into seminiferous tubules. In this sense, they are indistinguishable from wild-type GS cells and fulfill the criteria for spermatogonial stem cells. Using this model, we found that the partial androgenetic imprint in mGS cells occurred with loss of the androgenetic imprint in GS cells. Perhaps the same is true of wild-type mGS cells; the partial androgenetic imprint patterns may not indicate the origin of mGS cells directly but rather reflect epigenetic instability in vitro, as reported for ES/EG cells (Labosky et al., 1994; Dean et al., 1998; Humpherys et al., 2001). Although these results are based on a mutant mouse model, they strongly suggest that GS cells are multipotential or can acquire multipotentiality by loss of a single gene. Spontaneous teratomas in mice occur almost exclusively in the 129/Sv background and are considered to develop from PGCs (Stevens, 1984). However, our results strongly suggest that spermatogonial stem cells are multipotential.

Interestingly, the acquisition of multipotentiality in mGS cells was concurrent with the loss of spermatogonial stem cell potential. Despite their testicular origin, mGS cells formed teratomas in the seminiferous tubules, indicating that this environment was no longer sufficient for spermatogenesis after the cells became pluripotent. This contrasts with GS cells, which produce spermatogenesis on transfer to the seminiferous tubules (Kanatsu-Shinohara et al., 2003a). Therefore, mGS cells are more closely related to ES/EG cells in terms of cell function. The reason for the loss of spermatogonial stem cell potential is unknown; however, we speculate that it may be related to the loss of responsiveness to GDNF during the course of the establishment of mGS cells, as GDNF is essential for the self-renewing division of spermatogonial stem cells (Meng et al., 2000). Another question that remains to be answered is why GS cells converted to mGS cells only at early passages. In our experiments, mGS cells appeared within 7 weeks of culture initiation but not at later stages. Once established, however, GS cells were stably committed to the germline, because we did not observe any mGS cell conversion when they were expanded in large-scale culture or transplanted in vivo. The loss of multipotentiality might be ascribed to the nonoptimal culture condition; it is widely known that ES cells differentiate easily and lose germline potential in the absence of LIF (Smith, 2001). Likewise, germline cells may tend to lose somatic cell potential in nonoptimal culture conditions. In this sense, it is interesting that, in contrast to mGS cells from wild-type mice, mGS cells developed in the long-term in p53 knockout mice. Although the mechanism for the maintenance or loss of multipotentiality of germline cells is currently unclear, the results suggest that this gene is involved in these processes, and GS cells from p53 knockout mice may be useful for analyzing how germline cells retain multipotentiality.

The most striking result from our experiments is the contribution of mGS cells to normal embryo development. Donor cell makers were present in various parts of the body, including the germline cells. These results demonstrate that mGS cells not only produce tumors but also can contribute to normal embryonic development. However, the function of the cells may not be completely

normal, because we could not recover live offspring in tetraploid complementation experiments, which indicates that mGS cells alone cannot produce a normal whole embryo. The failure is most likely related to the imprint status of mGS cells, since altered imprinted gene methylation causes fetal abnormalities with ES cells (Dean et al., 1998; Surani, 2001). Nevertheless, the imprint status of mGS cells did not influence the germline competence, and normal offspring were obtained from the chimeric animal. This agrees with the previous reports that both ES and EG cells can produce germline chimera (Robertson and Bradley, 1986; Labosky et al., 1994; Stewart et al., 1994), even with androgenetic imprint patterns (Narasimha et al., 1997).

The derivation of multipotent stem cells from the neonatal testis may have practical value for medicine and biotechnology. These cells are different from other reported multipotent cells in terms of morphology, marker expression, and capacity for differentiation (Verfaillie, 2002; Wagers and Weissman, 2004). While it is important to study the biology of individual cell types and assess their potential for clinical application, a major advantage of mGS cells is that techniques currently used to derive specific lineages of cells from ES cells are applicable directly. Clearly, the derivation of mGS cells has fewer ethical concerns than does the derivation of ES cells, because mGS cells can be obtained without sacrificing the conceptus or embryos. Furthermore, the availability of histocompatible, multipotent tissue for autotransplantation would circumvent immunological problems associated with ES cell-based technology. Although we failed to obtain mGS cells from mature wild-type animals, this was likely due to the low success rate of GS cell establishment. The results of the p53 knockout mouse experiment suggest that mGS cells can arise from mature testis. Development of more efficient systems to derive GS cells from mature testis is necessary at this stage of research, and suppression of p53 expression in GS cells, such as by RNA interference, may be useful for enhancing the frequency of derivation. Future studies should also be directed toward examining the effect of imprinting on the range and efficiency of differentiation. Such studies will provide important information for potential clinical applications.

Experimental Procedures

Cell Culture

Testis cells were collected from newborn (0–2 days old) ddY or DBA/2 mice (Japan SLC, Shizuoka, Japan). For some experiments, testis cells were collected from a newborn Green mouse (Kanatsu-Shinohara et al., 2003a) or p53 knockout mouse in ICR background (Tsukada et al., 1993). Testis cell culture was performed according to the previously published protocol (Kanatsu-Shinohara et al., 2003a), with slight modifications. In brief, testis cells were allocated to a gelatin-coated tissue culture plate (2×10^5 cells/3.8 cm^2). The next day, floating cells were recovered and passed to secondary culture plates. After 7 days in culture, the cells were passed to a fresh culture plate at a 1:2 dilution. When the cells were confluent (~7 days after the second passage), they were passed again (1:1 dilution). At the third or fourth passage, the cells were maintained on mitomycin C-inactivated MEF. ES-like cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% FCS, 5×10^{-5} M 2-mercaptoethanol, and 10^3 units/ml ESGRO (Invitrogen, Carlsbad, CA). To induce EG cells from neonatal testis, the same medium was also supplemented with 20 ng/ml human bFGF (Invitrogen), and cells were cultured on SIM-m220 (gift from Dr. T. Nakano, Osaka University).

For adult testis culture, 2×10^7 cells from 3- to 8-week-old wild-type and p53 knockout mice were used to recover spermatogonial stem cells with anti-CD9 antibody as described elsewhere (Kanatsu-Shinohara et al., 2004), and selected cells were plated on gelatin-coated plate (3×10^5 cells/9.5 cm^2). GS cell colonies were picked by micromanipulation and transferred to MEF for expansion.

Standard ES cell medium was used to culture D3 ES cells that ubiquitously express the EGFP gene under the CAG promoter (provided by Dr. M. Okabe, Osaka University; Niwa et al., 1991).

Antibodies and Staining

The following primary antibodies were used: rat anti-EpCAM (G8.8), mouse anti-SSEA-1 (MC-480), mouse anti-sarcomeric protein (MF20; Developmental Studies Hybridoma Bank, University of Iowa), rat anti-mouse Forssman antigen (M1/87), rat anti-human α6-integrin (GoH3), biotinylated hamster anti-rat β1-integrin (Ha2/5), biotinylated rat anti-mouse CD9 (KMC8), allophycocyanin (APC)-conjugated rat anti-mouse c-kit (2B8), rat anti-mouse CD31 (MEC 13.3), phycoerythrin (PE)-conjugated rat anti-mouse Ter119 (TER-119), biotinylated rat anti-mouse Mac1 (M1/70), biotinylated rat anti-mouse Gr1 (RB6-8C5), rat anti-mouse VE-cadherin (11D4.1), APC-conjugated rat anti-mouse CD45 (30-F11; BD Biosciences), rat anti-TDA (EE2; provided by Dr. Y. Nishimune, Osaka University), APC-conjugated rat anti-mouse Flk-1 (Avas 12α1; provided by Dr. S. Nishikawa, RIKEN), goat anti-mouse cardiac troponin-I (cTn-I) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human myosin light chain 2v (MLC2v) (Alexis Biochemicals Inc, Montreal, Canada), rabbit anti-mouse atrial natriuretic peptide (ANP) (Protos Biotech Corporation, NY), mouse anti-human myelin basic protein (MBP) (Pm43), rabbit anti-glial fibrillary acidic protein (GFAP), rabbit anti-mouse tyrosine hydroxylase (TH), and mouse anti-human β-tubulin III (Tuj) (SDL.3D10) (Sigma, St. Louis, MO). APC-conjugated goat anti-rat-IgG (Cedarlane Laboratories, ON, Canada), APC-conjugated streptavidin (BD Biosciences), Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 647-conjugated goat anti-rat IgM, Alexa Fluor 633-conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR), Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-rabbit IgG, ALP or peroxidase-conjugated donkey anti-mouse IgG, ALP-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA), ALP-conjugated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA), or ALP-conjugated goat anti-rat IgG (Chemicon) were used as secondary antibodies. The cell staining and analysis was carried out with a FACSCalibur system (BD Biosciences) (Kanatsu-Shinohara et al., 2003a). ALP or DAB staining was carried out using a VECTOR alkaline phosphatase substrate kit or DAB substrate kit (Vector Laboratories), respectively, according to manufacturer's protocol.

Differentiation into Specific Lineages In Vitro

For differentiation into mesodermal lineages, ES-like cells were cultured on OP9 feeder layers, and cell differentiation was induced as described (Nishikawa et al., 1998; Schroeder et al., 2003, Hirashima et al., 1999). Vascular cells were identified by the uptake of DiI-acetylated low-density lipoprotein (Molecular Probes). Methylcellulose culture was performed as described previously (Nishikawa et al., 1998). All cytokines were provided by Kirin Brewery (Tokyo, Japan). Neural cell differentiation was induced as previously described (Ying et al., 2003).

Analysis of Marker Gene Expression

RT-PCR for Nanog, Rex-1, ERas, Esg-1, Cripto, and ZFP57 were carried out using specific primers, as described (Mitsui et al., 2003; Goolsby et al., 2003; Takahashi et al., 2003; Tanaka et al., 2002; Kimura et al., 2001; Ahn et al., 2004). PCR amplifications for Oct-4, UTF1, and HPRT were carried out by using specific primers (5′-AGCTGCTGAAGCAGAAGAGG-3′ and 5′-GGTTCTCATTGTTGTCG GCT-3′ for Oct-4, 5′-GATGTCCCGGTGACTACGTCT-3′ and 5′-TCG GGGAGGATTCGAAGGTAT-3′ for UTF1, and 5′-GCTGGTGAAAAG GACCTCT -3′ and 5′- CACAGGACTAGAACACCTGC-3′ for HPRT).

Analysis of Imprinted Genes

Bisulfite genomic sequencing of DMRs of imprinted genes was carried out as described (Lee et al., 2002). PCR amplifications of each DMR region from bisulfite-treated genomic DNAs was carried out by using specific primers (5′-GGAATATTTGTGTTTTTGGAGGG-3′ and 5′-AATTTGGGTTGGAGATGAAAATATTG-3′ for *H19*, 5′-GGTTTGGTATATATGGATGTATTGTAATATAGG-3′ and 5′-ATAAAACACCAAATCTATACCAAAATATACC-3′ for *Meg3 IG*, 5′-GTGTAGAATATGGGGTTGTTTTATATTG-3′ and 5′-ATAATACAACAACAACAATAACAATC-3′ for *Rasgrf1*, 5′-TTAGTGGGGTATTTTTATTTGTATGG-3′ and 5′-AAATATCCTAAAAATACAAACTACACAA-3′ for *Igf2r*, 5′-GTAAAGTGATTGGTTTTGTATTTTTAAGTG-3′ and 5′-TTAATTACTCTCCTACAACTTTCCAAATT-3′ for *Peg10*, and 5′- GGTTTTTTAGAG GATGGTTGAGTG-3′ and 5′- TCCAACCCTACTAACCCATCACC-3′ for *Oct-4*). The DNA sequences were determined in both directions. For COBRA, PCR products were digested with restriction enzymes with a recognition sequence containing CpG in the original unconverted DNA (Xiong and Laird, 1997). Intensity of digested DNA bands was quantified with ImageGauge software (Fuji Photo Film, Tokyo, Japan).

Transplantation

For subcutaneous injections, approximately 2×10^6 cells were injected into KSN nude mice (Japan SLC). For microinjections into the seminiferous tubules, approximately 3×10^5 cells were injected into the seminiferous tubules of an immune-suppressed W mouse (Japan SLC) recipient through the efferent duct (Kanatsu-Shinohara et al., 2003b).

Chimera Formation and Microinsemination

Cells were injected into the blastocoel of 3.5 dpc blastocysts of C57BL/6 mice using a Piezo-driven micromanipulator (Kimura and Yanagimachi, 1995). The blastocysts were returned to the oviducts or uteri of 2.5 dpc pseudopregnant ICR foster mothers on the day of microinjection. Tetraploid embryo aggregation chimeras were produced using the method developed by Nagy et al. (1993), except that two-cell blastomeres were electrofused by applying an electric pulse (2500 V/cm, 10 μsec) in 300 mM mannitol solution. Microinsemination was carried out as described using BDF1 oocytes (Kimura and Yanagimachi, 1995). The embryos were transferred on the next day after culture.

Histology

Tissues were fixed in 10% formalin and processed for paraffin sectioning. Chimeric embryos were fixed in 4% paraformaldehyde and frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) for cryosectioning. Slices were analyzed with an Olympus confocal laser scanning microscope.

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Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis

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Summary

Primordial germ cells (PGCs) are derived from a population of pluripotent epiblast cells in mice. However, little is known about when and how PGCs acquire the capacity to differentiate into functional germ cells, while keeping the potential to derive pluripotent embryonic germ cells and teratocarcinomas. In this investigation, we show that epiblast cells and PGCs can establish colonies of spermatogenesis after transfer into postnatal seminiferous tubules of surrogate infertile mice. Furthermore, we obtained normal fertile offspring by microinsemination using spermatozoa or spermatids derived from PGCs harvested from fetuses as early as 8.5 days post coitum. Thus, fetal male germ cell development is remarkably flexible, and the maturation process, from epiblast cells through PGCs to postnatal spermatogonia, can occur in the postnatal testicular environment. Primordial germ cell transplantation techniques will also provide a novel tool to assess the developmental potential of PGCs, such as those manipulated in vitro or recovered from embryos harboring lethal mutations.

Key words: Germ cell, Epiblast, Primordial Germ Cell (PGC), Pro-spermatogonia, Spermatogonia, Testis, Spermatogenesis, Transplantation, Microinsemination

Introduction

Mammalian germ cells undergo unique genetic and cellular changes as they develop and differentiate to form functional gametes. A population of pluripotent epiblast cells at around 6.5 days post coitum (dpc) gives rise to primordial germ cells (PGCs), which become identifiable as a cluster of cells at the base of the allantois at 7.25 dpc (Ginsburg et al., 1990; Tam and Zhou, 1996; Tsang et al., 2001). During development, the number of PGCs increases from 40 cells at 7.5 dpc to 25,000 cells at 13.5 dpc, and they migrate through the developing hindgut and mesentery to reach the urogenital ridge (UGR) at around 10.5 dpc. By 13.5 dpc, PGCs in the male genital ridge enter into mitotic arrest and become pro-spermatogonia, while germ cells in the female arrest at meiotic prophase I (reviewed by McLaren, 2003). Primordial germ cells show different features at different developmental stages. For example, migratory-stage PGCs exhibit a higher frequency of conversion into embryonic germ cells, pluripotent cells that resemble blastocyst-derived embryonic stem cells, than do PGCs in the gonads (Matsui et al., 1992; Resnick et al., 1992; Labosky et al., 1994). In addition, epigenetic changes characteristic to germline cells also occur in PGCs. Erasure of parental genomic imprints on both paternal and maternal alleles in PGCs commences near the time of their settlement in the UGR at 10.5 dpc, and new imprints are imposed in pro-spermatogonia before birth (Szabo and Mann, 1995; Ueda et al., 2000; Surani, 2001; Hajkova et al., 2002). Therefore, the characteristics of PGCs change during development before they mature into postnatal germ cells.

Spermatogenesis is initiated shortly after birth (Russell et al., 1990; Meistrich and van Beek, 1993). Pro-spermatogonia resume mitosis as spermatogonia, at around postnatal day 5, then enter into meiosis as spermatocytes and produce spermatids, which develop into spermatozoa. Spermatogonial stem cells are a subpopulation of spermatogonia and have the unique ability to self-renew as well as to differentiate to produce spermatozoa (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). These cells continue to divide throughout the life of the animal, and can be identified by their ability to generate and maintain colonies of spermatogenesis following transplantation into the seminiferous tubules of infertile recipient testes (Brinster and Zimmermann, 1994). Using this assay, several groups have shown that pro-spermatogonia in developing fetal testes can differentiate into spermatogonial stem cells when transferred into the adult testis (Ohta et al., 2004; Jiang and Short, 1998). However, it is unknown if germline cells at earlier stages of development can produce spermatogonial stem cells or spermatogenic colonies after transplantation.

In this investigation, we sought to determine the potential of germline cells from earlier embryos to develop into spermatogonial stem cells, using immature recipient animals.

Epiblast cells or PGCs were transplanted into infertile mouse testes and examined for their ability to re-populate the seminiferous tubules.

Materials and methods

Collection of donor cells

Donor cells were collected from pregnant C57BL/6 mice that were maintained in a controlled environment with 12:12 light:dark cycles from 08.00 h to 20.00 h (SLC, Shizuoka, Japan). The day when a copulation plug was found was designated as 0.5 dpc. In some experiments, we used a transgenic mouse line C57BL/6 Tg14 (act-EGFP) OsbY01 (designated Green) provided by Dr M. Okabe (Osaka University, Osaka, Japan) (Okabe et al., 1997). The spermatogonia, spermatocytes and round spermatids of these mice express the enhanced green fluorescent protein (EGFP) gene, which gradually decreases after meiosis. The sex of embryos was determined using genotyping based on the *Ube1* PCR method (Chuma and Nakatsuji, 2001), and only male embryos were used in this study. Due to the limitations of cell recovery and the number of animals that could be injected per day, the sex check was not performed in experiments using 6.5 dpc embryos. Cells for transplantation were obtained from whole embryonic ectoderm with primitive endoderm at 6.5 dpc, or germ-cell-containing tissues by dissection of the posterior thirds of 8.5 dpc embryos, the mesenteries and guts of 10.5 dpc embryos, the UGRs of 10.5 dpc embryos, and the genital ridges of 11.5, 12.5, 14.5 and 16.5 dpc embryos. Tissues from each developmental stage were dissociated by enzymatic digestion using 0.25% trypsin with 1 mmol/l EDTA (Invitrogen, Carlsbad, CA) for 10 minutes. Cells were suspended in Dulbecco's modified Eagle's medium, supplemented as previously described (Ogawa et al., 1997).

Transplantation into recipient testes

Donor cells were transplanted in histocompatible W/W^v or W^v/W^v mice (*W* mice, obtained from SLC, Shizuoka, Japan). Only 5- to 10-day-old male mice were used as recipients. *W* mutants lack endogenous spermatogenesis (Silvers, 1979), because of mutations in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991). Recipient animals were placed on ice to induce hypothermic anesthesia, and returned to their dams after surgery (Shinohara et al., 2001). Approximately 2 μl of cell suspension were introduced into each testis by injection via the efferent duct (Ogawa et al., 1997).

Histological analysis

Three to four months after transplantation, the recipient testes were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and processed for paraffin sectioning. Sections were stained with hematoxylin and eosin. Two histological sections were prepared from the testes of each animal and viewed at 400× magnification to determine the extent of spermatogenesis. The numbers of tubule cross-sections with or without spermatogenesis (defined as the presence of multiple layers of germ cells in the seminiferous tubule) were recorded for one histological section from each testis. Meiosis was detected by immunofluorescence staining using anti-synaptonemal complex protein 3 (SCP3) antibody (Chuma and Nakatsuji, 2001) and Alexa 488-conjugated anti-rabbit immunoglobulin G antibody (Molecular Probes, Eugene, USA). Periodic acid Schiff (PAS) staining (Muto Pure Chemicals, Tokyo, Japan) was carried out to examine acrosome formation in spermatids. In experiments using Green mice, recipient testes were recovered 10 to 11 weeks after donor cell transplantation, and analyzed by observing EGFP signals under fluorescence microscopy. Donor cells were identified specifically because host testis cells had no endogenous fluorescence. A cluster of germ cells was defined as a colony when it occupied the entire circumference of the tubule and was at least 0.1 mm long (Nagano et al., 1999). Cryosections of the testes fixed in 4% paraformaldehyde in PBS were stained with Rhodamine-conjugated Peanut agglutinin (PNA) (Vector, Burlingame, CA) for acrosomes, and with Hoechst 33258 (Sigma, St Louis, MO) for nuclei.

Microinsemination

Microinsemination was undertaken by intracytoplasmic injection into C57BL/6 × DBA/2 F1 oocytes (Kimura and Yanagimachi, 1995). Embryos that were constructed using spermatozoa or elongated spermatids derived from 8.5 dpc or 12.5 dpc PGCs were transferred into the oviducts of pseudopregnant ICR females after 24 or 48 hours in culture, respectively. Live fetuses retrieved on day 19.5 were raised by lactating ICR foster mothers.

Genotyping of offspring and bisulfite sequencing of imprinted genes

PCR fragments of the *Kit* gene encompassing the *W* point mutation or the W^v mutation (Nocka et al., 1990; Hayashi et al., 1991) were amplified using genomic DNA from mice derived from PGC transplantation, or from a W/W^v mouse as a control heterozygote for both mutations. PCR primers were 5′-CATTTATCTCCTCGACAAC-CTTCC-3′ and 5′-GCTGCTGGCTCACAATCATGGTTC-3′ for *W* genotyping, and 5′-AGATGGCAACTCGAGACTCACCTC-3′ and 5′-TGCCCCCACGCTTTGTTTTGCTAA-3′ for W^v genotyping. Amplified products were gel extracted and directly sequenced.

Bisulfite genomic sequencing of differentially methylated regions (DMRs) of the *Igf2r* and *H19* imprinted genes was carried out as described (Ueda et al., 2000; Lee et al., 2002; Lucifero et al., 2002). Briefly, genomic DNAs were isolated from the offspring derived from PGC transplantation, and treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils, but does not affect 5-methylated cytosines. Polymerase chain reaction amplification of each DMR from bisulfite-treated genomic DNAs was carried out using primer sets as described (Ueda et al., 2000; Lucifero et al., 2002), and DNA sequences were determined.

Results

Epiblasts with primitive endoderms (6.5 dpc) or tissues containing fetal germ cells were collected from different stages of embryos (posterior third of 8.5 dpc embryos, mesenteries and guts of 10.5 dpc embryos, or gonads of 10.5 to 16.5 dpc embryos) (Fig. 1A). The cells were dissociated enzymatically and single cell suspensions were transplanted into the seminiferous tubules of recipient immature *W* mice. Although *W* mice have a very small number of spermatogonia (Ohta et al., 2003), spermatogenesis is arrested at the point of undifferentiated type A spermatogonia and no differentiating germ cells are found due to defects in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991) (Fig. 1B). Therefore, any spermatogenesis detected in the recipient testis must be derived from the donor cells. Although the concentration of cells injected varied due to the more limited recovery of cells from early-stage fetuses, the cell viability, or percentage of tubules filled with donor cells, was similar in all experiments. The recipient mice were sacrificed 3 to 4 months after transplantation, and the testes were examined histologically for the presence of spermatogenesis. This time period represents three to four spermatogenic cycles in mice (Meistrich and van Beek, 1993; de Rooij and Russell, 2000), which would allow sufficient time for the development of sperm from spermatogonial stem cells.

At least three experiments were performed using cells harvested from each stage of embryonic development, and the

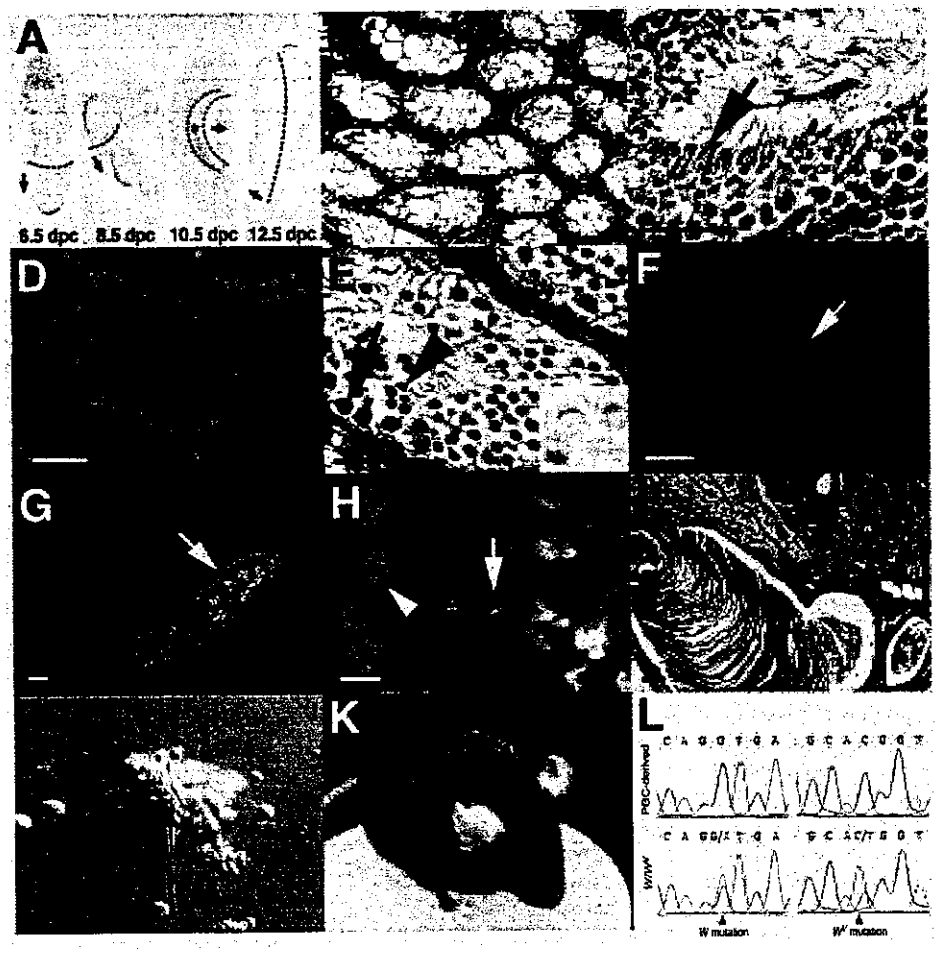

Fig. 1. Spermatogenesis and teratogenesis from fetal germ cells and epiblast cells. (A) Embryos at 6.5 and 8.5 dpc, a mid-part of 10.5 dpc embryo, and a male gonad and mesonephros at 12.5 dpc. Dotted lines demarcate regions used for transplantation. At 10.5 dpc, the urogenital ridges (asterisk) and mesentery with gut (arrow) were dissected separately. (B) A section of a *W* male testis (control recipient) stained with HE. Spermatogenesis is absent. (C) *W* mouse testis after transplantation of 8.5 dpc PGCs. Spermatozoa (arrow) are present in the center of the seminiferous tubule. (D) Anti-SCP3 immunostaining (green) of *W* testis after transplantation of 8.5 dpc PGCs, counterstained with Hoechst 33258 dye (blue). Inset, higher magnification view of the same sample. (E) Transplantation of epiblast cells at 6.5 dpc. Spermatocyte (arrow) and round spermatid (arrow head) were found. Inset shows acrosomes stained with PAS (red) in round spermatids. (F) *W* mouse testis transplanted with 8.5 dpc PGCs from Green mice embryos. Colonization of the recipient seminiferous tubule by EGFP (+) donor cells (green) was observed (arrow). (G) A section of the same testis as in (F), stained with Rhodamine-PNA (red) for acrosomes and with Hoechst 33258 dye (blue) for nuclei. Spermatogenenic colonies derived from EGFP (+) donor cells are present (arrow). (H) Higher magnification view of (G), showing spermatogonia (arrowhead) residing at the base of the seminiferous tubule, and elongated spermatids (arrow) with acrosomes (red), shedding the EGFP (+) cytoplasm. (I) Teratoma from epiblast cells at 6.5 dpc. Muscle, dermoid cyst and neuronal tissue are observed. (J) Spermatozoa (arrow) clustered around a Sertoli cell, released from a recipient testis of 8.5 dpc PGCs. (K) An offspring developed from an oocyte injected with a sperm derived from 8.5 dpc PGCs. (L) DNA sequences of the *Kit* gene around the *W* (left panels) and W^v (right panels) point mutations of an offspring derived from transplantation of 8.5 dpc PGCs (upper panels) and a W/W^v mouse as a control heterozygote (lower panels). Scale bars: 1 mm in F; 10 μm in H; 20 μm in J; 50 μm in others.

Table 1. Donor cell colonization in *W* recipient mice

Donor age (dpc)	Number of recipient testes	Number of transplanted cells ($\times 10^4$/testis)	Number of testes with spermatogenesis (%)	% tubule cross-section with spermatogenesis*	Number of testes with teratoma (%)
6.5	27	0.4±0.2	2 (7.4)	0.1±0.1	4 (14.8)
8.5	13	7.2±0.6	9 (69.2)	3.8±0.1	4 (30.8)
10.5 (mes+gut)	12	12.8±1.4	4 (33.3)	0.9±0.5	0
10.5 (UGR)	15	11.4±2.4	11 (73.3)	7.2±0.1	0
11.5	13	2.4±1.0	8 (61.5)	2.4±0.7	0
12.5	13	9.0±1.6	11 (84.6)	15.7±4.5	0
14.5	12	20	12 (100)	56.5±10.8	0
16.5	12	20	12 (100)	59.3±7.5	0

Values are mean±s.e.m. Results from at least three separate experiments. Testes were analyzed 3-4 months after transplantation. Mes+gut, mesentery and gut; UGR, urogenital ridge.

*Percentage of tubule cross-sections containing spermatogenesis/total tubule cross-sections examined in each testis.

results are summarized in Table 1. Overall, 69 of 117 (59%) recipient testes showed spermatogenesis, but no differentiating germ cells were found in control testes that did not receive an injection of donor cells (Fig. 1B). Spermatogenesis observed in the recipient testes originated from spermatogonial stem cells, because other spermatogenic cells do not have the

Table 2. Development of oocytes injected with spermatogenic cells derived from PGCs

Donor age (dpc)	Number of reconstituted eggs	In vitro development (%)			Number of eggs transferred	Implantation sites (%)	Number of offspring (%)
		One cell	Two cell	Four cell			
8.5*	186	78 (41.9)	105 (56.5)	NA†	105	47 (25.2)	20 (10.8)
12.5	182	8 (4.4)	25 (13.7)	130 (71.4)	130	88 (48.3)	29 (15.9)

Combined results using elongated spermatids and spermatozoa.
*Combined results from two different recipient testes.
†NA, not applicable because cells were transferred at the two-cell stage.

capacity for self-renewal and disappear by 35 days after transplantation (Russell et al., 1990; Brinster and Zimmermann, 1994; Shinohara et al., 2001). Spermatogenesis in the recipient testes that received fetal germ cells (8.5-16.5 dpc) was morphologically normal (Fig. 1C), and the kinetics of spermatogenic colonization were generally comparable to results obtained after transplantation of postnatal spermatogonial stem cells (Shinohara et al., 2001). All stages of spermatogenic cells, including mature spermatozoa, were found in recipient testes. Synchrony of meiosis in seminiferous tubules was confirmed by immunostaining for SCP3, a component of the synaptonemal complex (Fig. 1D).

The age of the donors had a significant effect on the number of recipient seminiferous tubules with observable spermatogenesis. Whereas only 0 to 11% of the tubules showed spermatogenesis in testes that received 8.5 dpc PGCs, spermatogenesis derived from donor cells at later stages of gonadal development was generally more extensive, and recipient testes grew larger due to the increased production of germ cells. In one case, transplantation of 16.5 dpc pro-spermatogonia resulted in 96% of the tubules with evident spermatogenesis, and spermatozoa were transported to the epididymis (data not shown). Spermatogenesis was also found in the recipients that were injected with cells from epiblasts with primitive endoderms. Although we could not identify mature spermatozoa histologically, spermatogenesis was found in two different testes that received injections of 6.5 dpc epiblast and primitive endoderm cells. The spermatogonia, spermatocytes and round spermatids that developed in the recipient seminiferous tubules appeared morphologically normal (Fig. 1E), and acrosome formation in round spermatids was confirmed by PAS staining (Fig. 1E, inset).

To confirm the donor origin of spermatogenesis, we used Green mice that ubiquitously express the *EGFP* transgene. Donor cells were collected from the posterior thirds of 8.5 dpc embryos that showed EGFP signals. This allowed specific identification of donor cells, because endogenous host testis cells had no detectable fluorescence. Four experiments were performed, and a total of 16 testes were microinjected with donor cells. Approximately 6 to 12 × 10^3 cells were transplanted into each testis. When the recipients were analyzed 10 to 11 weeks after transplantation, 4 of 16 (25%) testes had spermatogenic colonies that showed EGFP signals (Fig. 1F). The average number of colonies per testis was 0.3 ± 0.1 (mean ± s.e.m.). Histological analysis of the EGFP (+) colonies showed the presence of apparently normal spermatogenesis (Fig. 1G,H).

While these results indicate that all types of donor cells differentiated into spermatogonial stem cells, differentiation of donor cells was not restricted to the germline lineage. Consistent with findings of previous studies (Illmensee and Stevens, 1979), testes receiving epiblast cells or 8.5 dpc PGCs formed teratomas (Fig. 1I). Epiblast cells produced larger tumors than did 8.5 dpc PGCs, and some of the seminiferous

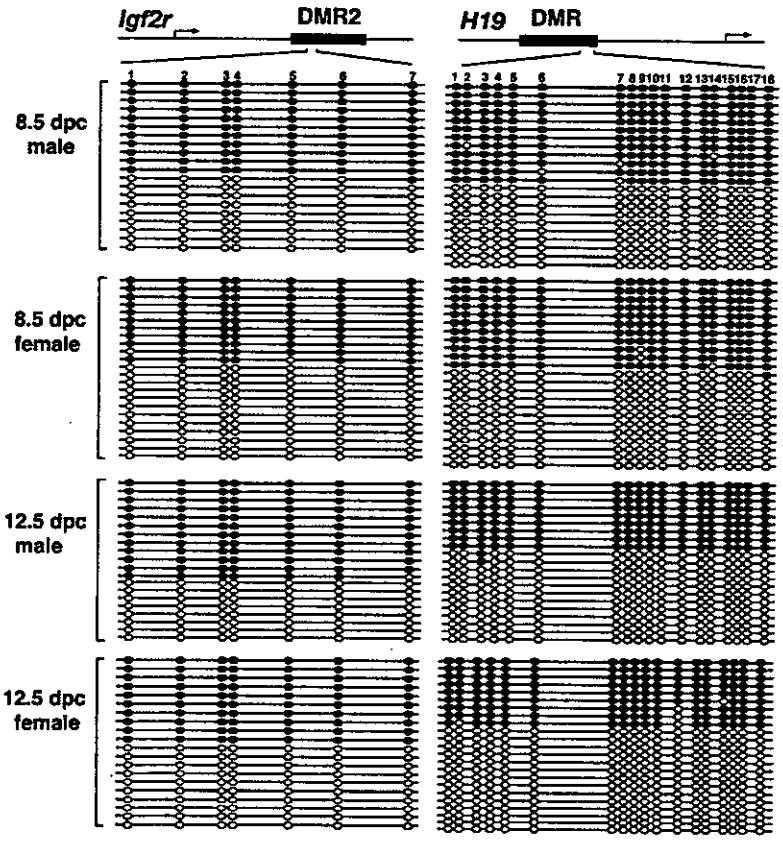

Fig. 2. DMR methylation of the *Igf2r* and *H19* genes in offspring derived from transplantation of 8.5 and 12.5 dpc PGCs. DNA methylation was analyzed by bisulfite genomic sequencing. Both male and female offspring of each stage of donor PGCs were analyzed. Individual lines represent sequenced clones. Black ovals indicate methylated cytosine-guanine sites (CpGs) and white ovals indicate unmethylated CpGs.

tubules were dilated and broken. Cells from three germ layers, including ciliated epithelium, muscle, neuron and bone, were found in these testes. Interestingly, spermatogenesis was occasionally observed in tubules not affected by tumorigenesis, indicating that the same population of donor cells could cause spermatogenesis and teratogenesis. No teratomas were found in the recipients of gonadal PGCs or pro-spermatogonia.

To examine whether germ cells generated from PGCs were fully functional, we performed microinsemination, a technique commonly used to produce offspring from infertile animals and humans (Kimura and Yanagimachi, 1995; Palermo et al., 1992). Donor PGCs were collected from 8.5 or 12.5 dpc embryos, and transplanted into the testes of *W* mice. Four months after transplantation, spermatozoa or elongated spermatids were collected from tubule fragments by mechanical dissociation (Fig. 1J), and microinjected into oocytes. The results of the microinsemination experiments are summarized in Table 2. Spermatogenic cells derived from 8.5 dpc PGCs appeared to be less competent for egg activation, because a significant number of eggs receiving elongated spermatid/spermatozoa from 8.5 dpc PGCs stayed at metaphase II or the metaphase II-anaphase transition and did not develop into the 2-cell stage after 24 hours. However, offspring were obtained from oocytes inseminated with spermatid/spermatozoa derived from both stages of PGCs (Fig. 1K). Genotyping of offspring showed that they did not carry *W* or W^v mutations in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991), demonstrating the offspring were derived from wild-type donor PGCs that had been transplanted into the testis (Fig. 1L). No apparent abnormality was seen in any of the offspring, and they were fertile. Bisulfite sequencing analysis of the offspring showed no obvious fluctuations in the methylation status of the DMRs of paternally methylated *H19* and maternally methylated *Igf2r* genes (Fig. 2).

Discussion

This study demonstrates that not only migrating PGCs but also epiblast cells can differentiate into spermatogonial stem cells and produce spermatogenesis after transfer into postnatal testis. The ability to initiate and maintain spermatogenesis after transfer into the infertile testis fulfills the criteria for the identification of spermatogonial stem cells (Brinster and Zimmermann, 1994). Developmental processes that occur during differentiation of spermatogonial stem cells from epiblast cells include induction of PGCs among epiblast cells, migration and proliferation of PGCs, erasure of parental genomic imprints, and G1 (G0) arrest in the developing male gonad. Nonetheless, our results demonstrate that these developmental events do not necessarily require embryonic somatic environments, and most processes of male germline differentiation can take place in postnatal testis. This flexibility of fetal germ cell differentiation may be related to, and partly account for, the recent success in the derivation of sperm from embryonic stem cells in vitro (Toyooka et al., 2003; Geijsen et al., 2004).

The important factor that contributed to the results of our experiments is the use of immature postnatal testes as recipients. Recently, Ohta et al. (Ohta et al., 2004) showed that pro-spermatogonia from 14.5 dpc embryos completed spermatogenesis when transplanted in mature seminiferous tubules, while spermatogenesis did not occur from PGCs from 12.5 dpc embryos. Another group reported that germ cells from day 0-3 mouse pups did not show spermatogenic colonies after transplantation into adult testes (McLean et al., 2003). In our study, however, epiblast cells at 6.5 dpc and PGCs at 8.5 to 16.5 dpc produced spermatogenesis after transplantation into immature seminiferous tubules at postnatal day 5 to 10. This difference might simply be ascribed to structural differences; immature Sertoli cells lack tight junctions and may allow migrating transplanted cells easier access to the stem cell niches, which are distributed nonrandomly in the seminiferous tubules (Chiarini-Garcia et al., 2001). Alternatively, immature testis may express factors that support survival and differentiation of epiblast cells and PGCs, while mature testis may not. Because PGCs have chemotaxic activity (Godin et al., 1990), we speculate that some of the transplanted cells migrated into the niches of immature seminiferous tubules, where they could survive. PGCs and epiblast cells may have then switched their cell cycle fate to function as spermatogonial stem cells. Both male and female PGCs enter into meiosis in the absence of the male gonadal environment, but do not if they lodge there (McLaren and Southee, 1997; Chuma and Nakatsuji, 2001). Somatic cells in the fetal testis are assumed to produce a substance that inhibits the meiotic transition of PGCs. Given our results, it appears that seminiferous tubules of newborn mice may also have similar meiosis-inhibiting activity.

Erasure of parental genomic imprints commences in PGCs at around the time of their arrival in the UGR (Szabo and Mann, 1995; Surani, 2001; Hajkova et al., 2002). However, it has not been clear whether this epigenetic event depends on induction from the UGR or is programmed autonomously in PGCs. As the offspring from PGC transplantations were viable and apparently healthy, epigenetic modifications, including erasure of parental genomic imprints, should have occurred appropriately in transplanted PGCs. This was corroborated by the normal methylation patterns exhibited in the DMRs of the *Igf2r* and *H19* genes. Because the establishment of paternal methylation proceeds after birth in the normal testis (Ueda et al., 2000), it seems unlikely that the postnatal testis has the ability to erase parental methylation. Therefore, our results suggest that PGCs may have the autonomous program for the erasure of parental methylation before reaching the UGR.

Primordial germ cell transplantation will provide a new experimental approach for the study of PGC development. Primordial germ cells harvested from embryonic lethal mutants as early as gastrulation can be traced for their differentiation capacities by transplantation into recipient testis. Similarly, PGCs manipulated in vitro, such as those cultured with growth factors or transfected with vectors (De Miguel et al., 2002; Watanabe et al., 1997), can now be assessed for their effects on subsequent differentiation in vivo. Such functional studies would help elucidate factors that regulate male germline development in mammals.

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Original Article

Fertilization and preimplantation development of mouse oocytes after prolonged incubation with caffeine

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Background and Aims: Previous studies have shown that caffeine might cause artificial dephosphorylation at threonine-14 and tyrosine-15 of the p34cdc2 catalytic subunit of maturation-promoting factor (MPF), elevate MPF activity in metaphase II oocytes cultured for a prolonged period, and that caffeine decreases fragmentation in oocytes cultured for up to 96 h.

Methods: Studies were carried out on: (i) the effect of caffeine on the morphological status of oocytes cultured for 96 h; (ii) the parthenogenetic activation and the fertilization of oocytes incubated in a medium that contained caffeine, and (iii) the fertilization and preimplantation development ability of zona-intact and zona-free oocytes by *in vitro* fertilization (IVF) and by intracytoplasmic sperm injection.

Results: In parthenogenetic activation, the incidence of diploid parthenotes in 24-h caffeine-treated oocytes was significantly higher than 24-h non-treated oocytes. For fertilizability of these oocytes, a significant increase in the fertilization rate resulted from IVF after 12-h caffeine incubation. Although no fertilized eggs were observed after intracytoplasmic sperm injection in 24-h non-treated oocytes, fertilized eggs were observed in caffeine-treated oocytes. MPF activation occurs in relation to nuclear/spindle position, and mitotic spindles and actin filaments determine the site of cleavage during cytokinesis. Spindle disruption does not cause cytofragmentation, but does induce cell cycle arrest.

Conclusion: Based on our results, although caffeine might increase MPF activity, prolonged time in any incubation causes some disruption of cytoskeletal filaments, which might be responsible for the poor development of caffeine-treated oocytes. (Reprod Med Biol 2004; 3: 245–251)

Key words: caffeine, *in vitro* fertilization, intracytoplasmic sperm injections, maturation promoting factor, parthenogenesis.

INTRODUCTION

OOCYTES OF MOST mammals are ovulated at metaphase II, and remain at this stage until activation by penetration of spermatozoon or by artificial means such as parthenogenetic activation.[1,2] Investigations using amphibian oocytes and cytoplasmic transfer revealed that mature oocytes contain a large amount of maturation-promoting factor (MPF) in their cytoplasm that maintains the meiotic arrest of these oocytes.[3–5] MPF is a serine/threonine protein kinase composed of a catalytic subunit, p34cdc2, and a regulatory subunit, cyclin B,[6–8] and is a universal cell cycle regulator of both mitosis and meiosis.

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Cytoplasmic changes affecting the quality of the oocyte, such as decreased ability for normal fertilization and embryonic development, occur when the arrest period is prolonged.[9,10] Spontaneous oocyte activation[11,12] and subsequent fragmentation, and abnormal cleavage after activation characterized by unequal blastomeres,[10,12] have been observed in oocytes cultured for a prolonged period. Low MPF activity might be one cause of these changes. In general, p34cdc2 is phosphorylated at threonine-14 (T14) and tyrosine-15 (Y15) by the Myt1 and Wee1 kinases after association with cyclin B, and this inactive form, called pre-MPF, accumulates during G_2 phase. Therefore, activation of MPF at the G_2 to M transition depends on dephosphorylation at T14 and Y15 by Cdc25 phosphatase.[13] Kikuchi *et al.*[14] suggested that, in addition to the gradual decrease in cyclin B, this phosphorylation contributes to the decrease in MPF activities, and that artificial dephosphorylation of pre-MPF might increase MPF activity.

Control of oocyte aging could have many advantages for *in vitro* fertilization (IVF), sperm injection and cloning by nuclear transfer. Kikuchi *et al.*[14] found a significant elevation of MPF activity in aged oocytes after artificial dephosphorylation at T14 and Y15 of p34cdc2 as a result of caffeine treatment. Caffeine reportedly induces the T14 and Y15 dephosphorylated form of p34cdc2, resulting in elevation of MPF activity in mammalian cultured cells[15,16] and *Xenopus* oocytes.[17]

We examined the effect of caffeine on the morphological status of oocytes cultured for a prolonged period. Then, we studied the fertilization of oocytes incubated in medium that contained caffeine. Because age-dependent alterations in zonae pellucidae might reduce the fertilizability of an oocyte[18] and because no studies on the effect of caffeine on zonae pellucidae have been reported, we examined the fertilization and preimplantation development abilities of zona-intact and zona-free oocytes by IVF and by intracytoplasmic sperm injection (ICSI).

MATERIALS AND METHODS

Collection of metaphase II-arrested oocytes and treatment with caffeine

FEMALE BDF1 MICE, 2–3 months old, were maintained on a 10-h light cycle at the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Ibaraki, Japan. Females were induced to superovulate by intraperitoneal injections of 7.5 IU of equine chorionic gonadotropin followed 48 h later by 7.5 IU of human chorionic gonadotropin (hCG). Ovulated oocytes were collected from oviducts 14–16 h after the hCG injection. Oocytes were treated with hyaluronidase (1500 U/mL; Sigma, St. Louis, MO, USA) in TYH medium[19] for a few minutes to completely disperse cumulus cells. After being rinsed in TYH medium, oocytes were incubated at 37°C under 5% CO_2 for 0–96 h in 50 μL of TYH medium with and without 5 mM caffeine under mineral oil.

Observation of morphological changes in oocytes

At 0, 6, 12, 24, 48, 72 and 96 h incubation in the medium with caffeine (hereafter called 0, 6, 12, 24, 48, 72 and 96 h-caffeine group, respectively), the morphological status of metaphase II-arrested oocytes was observed and compared with that of oocytes incubated in the medium without caffeine at the same times (hereafter called 0, 6, 12, 24, 48, 72, and 96 h-no-caffeine group, respectively).

Parthenogenetic activation of oocytes

Oocytes were collected from BDF1 mice and incubated in TYH medium with 5 mM caffeine for 24 h or in TYH medium without caffeine for 0 or 24 h. Oocytes were activated by culturing for 6 h in Ca^{2+}-free TYH medium with 10 mM strontium (Sr^{2+}) and 6.6 μg/mL cytochalasin B (Sigma, St. Louis, MO, USA). Parthenotes with two pronuclei were washed with Whitten's medium[20] and then cultured for 96 h in 50-μL drops of Whitten's medium, covered with mineral oil, at 37°C under 5% CO_2 in air. The parthenotes were observed every 24 h after parthenogenetic stimulus.

In vitro fertilization and development

In vitro fertilization of zona-intact and zona-free oocytes was accomplished as follows. Zona-free oocytes were obtained by treating oocytes in acidic Ringer's solution (137 mM NaCl, 26.8 mM KCl, 1.6 mM $CaCl_2$, 0.49 mM $MgSO_4$, 5.55 mM glucose, 4 mg/mL 40-kDa polyvinylpyrrolidone [PVP], pH 2.5) for a few seconds to dissolve the zona pellucida.[21] Zona-intact and zona-free oocytes were incubated for 0, 6, 12 or 24 h in 50 μL of TYH medium (covered with mineral oil) with and without 5 mM caffeine. Before insemination, all oocytes were placed in TYH medium for 15–30 min.

The spermatozoa used for insemination were collected from the cauda epididymis of mature BDF1 male mice and placed in TYH medium. After 2 h incubation at 37°C under 5% CO_2 in air, the sperm suspension was introduced into 50-μL drops of TYH medium containing 10–20 oocytes each. The final sperm concentration was 600 cells/μL for the zona-intact oocytes and 5 cells/μL for the zona-free oocytes. After 6 h insemination, eggs were washed in Whitten's medium. A Terasaki plate (SUMITOMO BAKELITE Co. Ltd, Tokyo, Japan) was used to culture the individual eggs. The eggs were then transferred individually into 10 μL of Whitten's medium at 37 C under 5% CO_2 in air, and then observed for formation of pronuclei. The embryonic development was observed at 24-h intervals up to 96 h after insemination.

Intracytoplasmic sperm injection and development

Oocytes cultured for 24 h in TYH medium, with and without 5 mM caffeine, were fertilized by ICSI. Microinjection was carried out using a micromanipulation system equipped with a piezo micropipette-drive unit (Prime

Tech, Ibaraki, Japan). The cover of a plastic dish (Falcon no. 1006; Becton Dickinson, Franklin Lakes, NJ, USA) was used as a microinjection chamber. Several small drops, each containing either HEPES-CZB medium for oocytes or 12% PVP for spermatozoa, were placed on the bottom of the dish and covered with mineral oil. A tail of spermatozoon showing apparently normal morphology was inserted tail first into an injection pipette. After the sperm head was separated from the tail by applying a few piezo pulses, it was injected into an oocyte.[22] After 6 h incubation at 37°C under 5% CO_2 in air, oocytes were washed in Whitten's medium and then cultured in Whitten's medium at 37°C under 5% CO_2 in air. The embryonic development was observed at 24-h intervals up to 96 h after sperm injection.

Statistical analysis

A χ^2-test was used for statistical evaluation of the results, as required. Differences giving a probability of $P < 0.05$ were considered to be significant.

RESULTS

TABLE 1 SHOWS the morphological status of metaphase II-arrested oocytes incubated for 0, 6, 12, 24, 48, 72 or 96 h in TYH medium, with and without caffeine. Up to 12 h, all oocytes showed normal morphology. At 24 h, although the rate of morphologically normal oocytes in the caffeine-treated group (100%) was higher than that in the non-treated group (89%), the difference was not statistically significant. At 48 and 72 h, the number of morphologically normal oocytes was significantly higher in the caffeine-treated group. At 96 h, neither group showed any morphologically normal oocytes.

Table 2 shows the observed parthenogenetic activation of non-kept (0 h) oocytes and oocytes incubated for 24 h in TYH medium with and without caffeine. In the 0 h-group, all oocytes showed two pronuclei, and 71% of these oocytes developed to blastocysts. The percentage of diploid parthenotes after 24 h in the caffeine-treated group was significantly higher than that in the non-treated group. In the caffeine-treated group, only 22% of the parthenotes developed to the two-cell stage, and no further development occurred. The diploid parthenotes in the non-treated group, however, did not develop even to the two-cell stage.

Table 3 shows the results of IVF and the pre-implantation development of cumulus-free or zona-free oocytes kept for 6, 12, or 24 h in TYH medium with or without caffeine. In the case of 0 h-groups involving cumulus-free and zona-intact oocytes and involving cumulus- and zona-free oocytes, fertilization rate was 35% and 82%, respectively. No significant difference in either the fertilization rate or development rate was evident between the

Table 1 Morphological status of metaphase II-arrested oocytes incubated in medium with and without caffeine

Medium with (+) or without (-) caffeine	Number of oocytes examined	Number of morphologically normal oocytes at different incubation times (%)						
		0 h	6 h	12 h	24 h	48 h	72 h	96 h
-	47	47 (100)	47 (100)	47 (100)	42 (89.4)	14 (29.8)†	2 (4.2)§	0 (0)
+	52	52 (100)	52 (100)	52 (100)	52 (100)	50 (96.2)‡	25 (48.1)¶	0 (0)

†, ‡, §, ¶Percentage of oocytes significantly differed between † and ‡($P < 0.05$) and between § and ¶($P < 0.01$).

Table 2 Parthenogenetic activation of oocytes incubated in medium with and without caffeine

Medium with (+) or without (-) caffeine	Incubation time (h)	Number of oocytes examined	Number of eggs (%)		Number of parthenotes developed to different stages (%)		
			With 2PN†	Without PN or fragmentation	2-cell	Morula	Blastocyst
-	0	34	34 (100)	0 (0)	34 (100)	33 (97.1)	24 (70.6)
	24	22	11 (50.0)‡	9 (40.9)	0 (0)	0 (0)	0 (0)
+	24	23	19 (82.6)§	4 (17.4)	5 (21.7)	0 (0)	0 (0)

†PN, pronucleus observed; 2PN, two pronuclei observed. ‡, §Percentage of diploid parthenotes significantly differed between ‡ and §($P < 0.05$).

Table 3 *In vitro* fertilization and embryonic development of oocytes incubated in medium with and without caffeine

Medium with (+) or without (–) caffeine	Oocytes	Incubation time (h)	Number of oocytes inseminated	Number of eggs with (%)				Number of embryos developed to different stages (%)		
				Pb2 + 1 or 2PN†	Pb2 + 0 PN	0Pb2 + 1 or 2PN	Total	2-Cell	Morula	Blastocyst
–	Cumulus-free, zona-intact	0	263	53 (20.2)	39 (14.8)	0 (0)	92 (35.0)	51 (19.4)	29 (11.0)	18 (6.8)
		6	46	4 (8.7)	2 (4.3)	0 (0)	6 (13.0)	3 (6.5)	2 (4.3)	0 (0)
		12	29	0 (0)	1 (3.4)	0 (0)	1 (3.4)	0 (0)	0 (0)	0 (0)
		24	135	2 (0.7)	8 (5.9)	2 (1.5)	12 (8.9)	1 (0.7)	0 (0)	0 (0)
	Zona-free	0	266	213 (80.1)	6 (2.3)	0 (0)	219 (82.3)	208 (78.2)	193 (72.6)	165 (62.3)
		6	50	36 (72.0)	2 (4.0)	0 (0)	38 (76.0)	35 (70.0)	25 (50.0)	13 (26.0)
		12	28	8 (28.6)	0 (0)	0 (0)	8 (28.6)‡	1 (3.6)¶	0 (0)	0 (0)
		24	117	1 (0.9)	4 (3.4)	2 (1.7)	7 (6.0)	1 (0.9)	0 (0)	0 (0)
+	Cumulus-free, zona-intact	6	56	4 (7.1)	3 (5.4)	0 (0)	7 (12.5)	2 (3.6)	2 (3.6)	1 (1.8)
		12	39	3 (7.7)	4 (10.3)	0 (0)	7 (17.9)	3 (7.7)	1 (2.6)	0 (0)
		24	136	2 (1.5)	10 (8.1)	3 (2.2)	15 (11.0)	2 (1.5)	0 (0)	0 (0)
	Zona-free	6	50	35 (70.0)	1 (2.0)	0 (0)	36 (72.0)	33 (66.0)	23 (46.0)	16 (32.0)
		12	40	16 (40.0)	0 (0)	0 (0)	16 (40.0)§	13 (32.5)††	2 (5.0)	0 (0)
		24	122	2 (1.6)	4 (3.3)	3 (2.5)	9 (7.4)	2 (1.6)	0 (0)	0 (0)

†Pb2, 2nd polar body; PN, pronuclei; ‡, §, ¶, ††percentage of eggs with PN or Pb2 significantly differed between ‡ and §($P < 0.05$) and 2-cell stage significantly differed between ¶ and ††($P < 0.01$).

Table 4 Intracytoplasmic sperm injection and development of oocytes incubated in medium with and without caffeine

Medium with (+) or without (−) caffeine	Incubation time (h) injected	Number of oocytes surviving	Number of oocytes (%)	Number of eggs (%) fertilized†	Number of eggs (%) unactivated‡	Number of eggs (%) fragmented	Number of embryos developed to stage (%)		
							2-cell	Morula	Blastocyst
−	0	31	26 (83.9)	26 (100)	0 (0)	0 (0)	26 (100)	21 (80.8)	16 (61.5)
	24	31	28 (90.3)	0 (0)	22 (78.6)	6 (21.4)	0 (0)	0 (0)	0 (0)
+	24	30	29 (96.7)	11 (37.9)	12 (41.4)	6 (20.7)	6 (20.7)	0 (0)	0 (0)

†Eggs showed two pronuclei, and released a 2nd polar body; ‡eggs did not fragment and did not release a 2nd polar body.

groups kept for 6 h in medium with and without caffeine. In contrast, both the fertilization and development rates to the two-cell stage significantly differed between the 12 h-caffeine and 12 h-no-caffeine groups of zona-free embryos. No development to blastocyst was observed in any 12 h or 24 h group, regardless of caffeine addition.

Table 4 shows the results of ICSI and the preimplantation development of oocytes kept for 24 h in medium with or without caffeine. The fertilization rate in the caffeine group (38%) was significantly lower than that in the 0 h-group (100%). Although the caffeine group had a higher fertilization rate than the no-caffeine group (0%), the two-cell stage embryos of the caffeine group showed no further development.

DISCUSSION

IN THIS STUDY, using IVF and ICSI examination methods, we examined the fertilization and preimplantation development abilities of oocytes incubated with caffeine for a prolonged period.

In IVF, the 6 h-caffeine and 6 h-no-caffeine groups showed similar fertilization rates, indicating that the oocytes in both groups had identical fertilizability. The fertilization rate of 12 h-caffeine oocytes was higher than that of 12 h-no-caffeine group, but further development beyond two-cell stage was poor. The percentage of morphologically normal oocytes in the 24 h-caffeine group was similar to that in the non-kept (0 h) group. The fertilization rates of these oocytes were lower than that in the 0 h-group, indicating identical morphology, although the quality was quite different. After accounting for the age-related changes observed in zonae pellucidae and plasmalemmae by ICSI, the percentage of fertilized eggs in the 24 h-caffeine group was higher than that in the 24 h-no-caffeine group, although their embryonic development was poor.

Within the cytoplasm, Cdc2 and cyclin B associate with microtubules and centrosomes, particularly during late interphase and M phase.[23–25] Observations of locally regulated microtubule dynamics in maturing starfish oocytes[26] and in mitotic ctenophore eggs[27] indicate that regionalized MPF activation occurs in relation to nuclear/spindle position. Nuclei and microtubule asters have independent but additive effects on MPF activation, and cooperate to trigger MPF activation within the egg.[28] Although MPF can be activated in the absence of nuclei, centrosomes and microtubules in *Xenopus* eggs, these structural components are not merely effectors but are active protagonists in controlling cell cycle progression.[28]

Mitotic spindles determine the site of cleavage.[29] The positions of microtubule asters also determine the location for formation of the cleavage furrow.[30,31] Microtubule asters signal the cell cortex to initiate a cleavage furrow, for which actin filament organization is involved. Depolymerization of actin filament inhibits both cleavage and cytofragmentation.[32]

The IVF experiments carried out in our study revealed no significant difference in the zona-intact oocytes between caffeine and no-caffeine treatments, suggesting that caffeine has no discernible effect on zonae pellucidae and plasmalemmae.

Our results suggest that disruption of the meiotic spindle and disorganization of the actin filament might be responsible for the poor preimplantation development. Disruption in the cytoskeleton might be responsible for the abnormal shapes of embryos derived from oocytes treated with caffeine. Spindle disruption does not cause cytofragmentation, but does induce cell cycle arrest during mitosis.[32] Therefore, in our parthenogenetic activation experiments and ICSI experiments, 17% and 41% of the eggs, respectively, remained at the one-cell stage without fragmentation, possibly due to a defect in the entry to mitosis.

Kikuchi *et al.*[14] added caffeine to the culture medium of aged oocytes, which have a high pre-MPF level, whereas we added caffeine to the medium of non-aged

oocytes. In our experiments, we did not determine the exact time when the MPF level became low or when the caffeine elevated the MPF level in the caffeine-treated oocytes. Future study should include measurement of the MPF level and examination of chromosomes and cytoskeletal filaments in oocytes and embryos treated with caffeine.

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Zona-Float Method for Separating Mouse Eggs from Other Cells

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Abstract: *We have developed a new method for separating mouse eggs from other cells, such as cumulus cells, using centrifugation with Percoll. Solutions of 45, 22.5, 11.3, and 5.6% Percoll were tested. With the 22.5% solution, 99% of whole eggs obtained by* in vitro *fertilization were collected from the upper part of the Percoll solution, and 98% of 2-cell embryos collected from these eggs developed to the blastocyst stage. Offspring were obtained after transfer of collected embryos to female mice. The greatest advantage of this method is that undamaged eggs are separated from other cells in one simple operation, regardless of the number of eggs.*

Key words: *cumulus cells, egg, mouse, Percoll, sperm*

Introduction

The study of reproduction at the molecular level has progressed in recent years [1]. In this field, it is often necessary to use many eggs simultaneously. Therefore, it is imperative that a simple technique for collecting the eggs and separating them from other cells be developed. In our laboratory, we often need to simultaneously prepare hundreds of mouse eggs that have been obtained by *in vitro* fertilization. The traditional method of manually separating eggs from other cells by micropipette is extraordinarily labor-intensive. Moreover, because the procedure takes a very long time, the delay may hurt the eggs. As the egg is very large in comparison with the other cells and has a unique extracellular matrix (zona pellucida), we presumed the specific gravity of an egg to be different from that of other cells. Therefore, we developed a method that takes advantage of the specific gravity difference to collect and separate eggs which eliminates the problems of the traditional method. We also investigated the normality of the eggs collected by this method and were able to verify the developmental potency of the eggs in culture, the cell number after culture, and the developmental potency to birth after embryo transfer.

Materials and Methods

Animals

Female and male BDF1 mice aged 8 weeks, purchased

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from Charles River Japan, Inc. (Yokohama, Japan) were used. The mice were housed in cages (35 × 29 × 18 cm) in an air-conditioned room in which the temperature (20 to 25°C) and lighting (14:10 h, light:dark) were controlled. A commercial diet (Type MF, Oriental Yeast Co., Ltd. Tokyo) and water were provided *ad libitum*.

Protocols were in compliance with the guidelines of the National Institute of Infectious Diseases for the care and use of laboratory animals and for biological hazard countermeasures.

Preparation of media and Percoll

TYH and Whitten's medium were used for *in vitro* fertilization and embryo culture, respectively. These media were prepared as described previously [2, 3] and stored at 4°C until use.

Isosmotic 90% Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was prepared beforehand by mixing 1 volume of 9% (wt/vol) NaCl solution with 9 volumes of Percoll and stored at 4°C until use. The 45, 22.5, 11.3 and 5.6% Percoll solutions were prepared by diluting 90% Percoll to 2, 4, 8, and 16 times the volume, respectively, with Whitten's medium just before use.

Investigation of Percoll solutions

A female BDF1 mouse was superovulated by treatment with equine chorionic gonadotropin (eCG) (Serotropin, 7.5 IU, intraperitoneal injection) followed by human chorionic gonadotropin (hCG) (Gonatropin, 7.5 IU, intraperitoneal injection). The female was caged with a male of the same strain, and a vaginal plug was taken as an indication of successful mating. The female mouse was euthanatized by cervical dislocation at 1.5 days post coitum, and the oviducts were removed. Two-cell embryos were collected from the oviducts by flushing with Whitten's medium. The embryos were separated into three groups: zona free, zona intact, and zonae pellucidae. For zona-free embryos, the zonae pellucidae were removed by immersing for 10 min in Whitten's medium containing 0.5% (wt/vol) pronase. Zona-intact embryos were simply kept in Whitten's medium. For zonae pellucidae, embryonic cells were removed by sucking with a glass micropipette with an internal diameter that was narrower than the external diameter of the embryo. Finally, 10 zona-free embryos, 10 zona-intact embryos, and 10 zonae pellucidae were prepared before use. Each group of embryos in approximately 20 μl of Whitten's medium was placed on 2 ml of 45, 22.5, 11.3, or 5.6% Percoll in a Petri dish (Falcon 1008; Becton Dickinson and Co., Franklin Lakes, N.J.). After 10 min, the status of the zona-free embryos, zona-intact embryos, and zonae pellucidae in each solution of Percoll was observed with a dissecting microscope (Leica AG, Heerbrugg, Switzerland).

Preparation of embryos by in vitro *fertilization*

Twenty-four female BDF1 mice were superovulated by treatment with eCG followed by hCG. At 14 h after hCG treatment, the mice were euthanatized by cervical dislocation, and their oviducts were removed. The oocyte-cumulus complexes were collected from the oviducts and transferred to ten 50-μl drops of TYH medium. Epididymal sperm were collected from a male BDF1 mouse and incubated for 30 min in 50 μl of TYH medium. Sperm were then added to the drops containing oocyte-cumulus complexes. The final concentration of sperm was approximately 1×10^6 sperm/ml. The drops containing sperm and oocyte-cumulus complexes were kept for 24 h at 37.5°C in an atmosphere of 5% CO_2 and 95% air.

Separation of embryos

The medium (0.4 ml) collected from eight drops containing eggs, cumulus cells, and sperm was gently layered on 4.5 ml of 22.5% Percoll in a 15-ml centrifuge tube (TPP; Midwest Scientific Inc., St. Louis, Mo.). The tube was centrifuged at 750 × g for 5 min. After centrifugation, the upper two-thirds (about 3.3 ml) of the solution, including a visible layer, was collected and transferred to another 15-ml centrifuge tube. The solution was diluted to 5 times the volume with Whitten's medium, and the tube was centrifuged at 750 × g for 5 min. The supernatant, except for the lowest 1 ml, was then discarded by using an aspirator, 4 ml of Whitten's medium was added, and the tube was again centrifuged at 750 × g for 5 min. The lowest 1 ml of supernatant was transferred to a Petri dish by Pasteur pipette (Corning Glass Works, Corning, N.Y.). The remaining layer from the lower one-third of the solution and the pellet at the bottom of the tube were also collected and transferred to another Petri dish. Several 2-cell embryos were manually collected by micropipette from the other remaining two drops, and

transferred to another Petri dish. Then, the eggs contained in the drops were separated with 22.5% Percoll, and 2-cell embryos were collected and transferred to the other Petri dish. Some embryos were transferred to a recipient female mouse, and the others were left as they were.

Culture of embryos

The embryos collected from each Petri dish were transferred to individual 50-μl drops of Whitten's medium overlaid by mineral oil. Embryos were kept for 72 h at 37.5°C in an atmosphere of 5% CO_2 and 95% air.

Number of inner cell mass and trophectoderm nuclei

Numbers of inner cell mass (ICM) and trophectoderm (TE) nuclei of blastocysts derived from manually separated 2-cell embryos and from 2-cell embryos separated with 22.5% Percoll were investigated by a modified immunosurgery method [4]. Briefly, blastocysts were treated with 0.5% pronase to remove the zona pellucida. Zona-free blastocysts were incubated in 50 μl of Whitten's medium supplemented with 10% goat anti-mouse lymphocyte antiserum at 37°C for 10 min. The embryos were incubated in 50 μl of Whitten's medium supplemented with 10% guinea pig serum as complement, 10 μg/ml bisbenzimide (Hoechst 33342), and 100 μg/ml propidium iodide. After 30 min, the embryos were washed with Whitten's medium and mounted on a glass slide. The numbers of ICM and TE nuclei in a single embryo were counted by using a fluorescence microscope (BX50, Olympus Optical Co., Ltd., Tokyo, Japan).

Embryo transfer

Embryo transfer was carried out surgically under anesthesia by sodium pentobarbital. Some 2-cell embryos were transferred into the oviducts of two ICR pregnant females that had been mated 1 day before with males of the same strain.

Statistical analysis

Data were analyzed by two-way analysis of variance.

Results

Table 1 shows the status of zona-free embryos, zona-intact embryos, and zonae pellucidae in the various solutions of Percoll. All floated in the 45% solution. In the 11.3% and 5.6% solutions, zonae pellucidae floated but zona-free and zona-intact embryos sank. In the 22.5% solution, zona-free embryos sank, but zona-intact embryos separated into the upper layer of the Percoll solution because of the buoyancy of the zona pellucida.

Table 1. The status of zona-intact embryos, zona-free embryos, and zonae pellucidae in various Percoll solutions

Samples	Percent of Percoll (vol/vol)			
	45	22.5	11.3	5.6
Zona-free embryos	floated	sank	sank	sank
Zona-intact embryos	floated	floated	sank	sank
Zonae pellucidae	floated	floated	floated	floated

When the medium containing eggs, cumulus cells, and sperm was loaded on 22.5% Percoll and subsequently centrifuged, a layer that floated in the upper part of the solution and a pellet that had sunk to the bottom of the tube were observed (Fig. 1a, b). Five hundred eighty-eight (99%) of 595 eggs (collected from 20 mice, 29.8 eggs/female) were collected from the upper two-thirds of the solution, and 7 (1%) eggs were collected from the lower one-third (Table 2). No eggs were collected from the lowermost pellets (Table 2 and Fig. 2a, b). No cumulus cells were observed in either the upper part or the lower part of the solution. Four hundred eighty-one (80%) 2-cell embryos were collected from the 595 eggs (Table 2). After culture in Whitten's medium for 72 h, 471 (98%) of these embryos developed to the blastocyst stage (Table 2 and Fig. 3).

The average numbers of ICM and TE nuclei of 19 blastocysts derived from manually separated 2-cell embryos (control) were 17.4 and 54, respectively, and those of 21 blastocysts derived from 2-cell embryos separated with 22.5% Percoll were 16.5 and 59.7, respectively (Fig. 4). There was no difference by two-way analysis of variance.

After transfer of 40 embryos into female mice, recipients gave birth to 10 offspring derived from 2-cell embryos separated with 22.5% Percoll.

Discussion

We report a simple, effective method for separating eggs from other cells using Percoll and centrifugation.