cells (spermatozoa and spermatids) has been successful in several mammalian species. Furthermore, in mice, the nuclei of secondary and primary spermatocytes can complete their meiotic divisions in immature or mature oocytes and participate in full-term embryo development. The nuclei of mouse PGCs at day 10.5 of pregnancy are fully reprogrammed following nuclear transfer and support embryo development to term. The developmental ability of embryos reconstructed from sperm/spermatogenic cells (by microinsemination) depends on the formation of a normal set of haploid chromosomes, while that of embryos from PGCs (by nuclear transfer) depends on the genomic imprinting status of the donor genome (Fig. 9).

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MOLECULAR REPRODUCTION AND DEVELOPMENT

Differential Development of Rabbit Embryos Following Microinsemination With Sperm and Spermatids

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ABSTRACT Microinsemination is the technique of delivering male germ cells directly into oocytes. The efficiency of fertilization after microinsemination and subsequent embryo development may vary with the animal species and male germ cells used. The present study was undertaken to observe the in vitro and in vivo developmental ability of rabbit embryos following microinsemination with male germ cells at different stages. First, we assessed their oocyte-activating capacity by injecting them into mouse and rabbit oocytes. The majority of mouse oocytes were activated irrespective of the type of rabbit male germ cell injected (61–77%), whereas rabbit oocytes were activated differently according to the type of male germ cells (89%, 75%, and 29% were activated by spermatozoa, elongated spermatids, and round spermatids, respectively; $P < 0.05$). After 120 hr in culture, 66%, 45%, and 13%, respectively, of these activated rabbit oocytes (pronuclear eggs) developed into blastocysts ($P < 0.05$). Additional electric pulse stimulation of round spermatid-injected oocytes increased the blastocyst rate to 43%. After 24 hr in culture, some four to eight cell embryos were transferred into the oviducts of pseudopregnant females. Normal pups were born from spermatozoa and elongated spermatids, but not from round spermatids. Karyotypic analysis at the morula/blastocyst stage revealed that the majority of round spermatid-derived embryos had abnormal ploidy (8 out of 12 embryos). Our study indicates that rabbit male germ cells acquire the ability to activate oocytes and to support subsequent embryo development as they undergo spermiogenesis. As these differential developmental patterns are similar to those reported for humans in vitro and in vivo, rabbits may provide an alternative small animal model for studying the biological nature and molecular basis of human microinsemination techniques, especially those using immature male germ cells. *Mol. Reprod. Dev.*
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INTRODUCTION

Microinsemination, also referred to as ICSI (intracytoplasmic sperm injection), is a technique that delivers male germ cells directly into the ooplasm. Due to the advent of techniques and apparatuses for micromanipulating gametes and embryos, microinsemination with normal mature spermatozoa, and with male germ cells that are unable to fertilize oocytes under conventional in vivo and in vitro conditions, is now possible. These male germ cells include immotile or misshapen spermatozoa, DNA-conjugated spermatozoa, freeze-dried spermatozoa, and immature spermatozoa; that is, spermatogenic cells. Thus, there are great variations in the types of male germ cells used for microinsemination studies and each provides valuable information on reproductive and developmental biology and genetics. However, as these microinsemination experiments have been undertaken predominantly in laboratory mice (Ogura et al., 2001; Yanagimachi, 2005), one may wonder if the information accumulated in one species can be broadly applied to other species. There are features in the process of fertilization and embryo development that are specific to mouse. Microtubule-organizing centers (MTOCs) at fertilization, for example, are of maternal origin in mice, but are from the centrioles of penetrating spermatozoa

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in most other species (Navara et al., 1994). Sperm with a very long tail and oocytes with a very small volume characterize mouse gametes. Major embryonic gene activation occurs at the early two-cell stage in mouse, but at the four-cell stage or later in other 65 species (Telford et al., 1990). Therefore, experimental models for microinsemination using species other than mouse would provide information that may be more applicable to other mammalian species.

The laboratory rabbit is used extensively in biomedical research and has played a leading role in the development of reproductive engineering techniques, including in vitro fertilization (Chang, 1959), embryo culture and transfer (Al-Hasani et al., 1986), and artificial insemination (Adams, 1962). In addition, the first ICSI-derived offspring were born to the laboratory rabbit (Hosoi et al., 1988). Later this success was followed by the birth of pups from round spermatid injection, (ROSI) but the efficiency was very low (Sofikitis et al., 1994). In contrast, murine offspring can be obtained by ROSI at an effective rate. Thus developmental efficiency of rabbit embryos following microinsemination seems to depend on the maturity of male germ cells, as it does in humans (Aslam et al., 1998) and monkeys (Hewitson et al., 2000; 2002). However, no systematic experiments have been undertaken to see whether male germ cells at each maturational step have a different ability to support embryonic development in rabbits, or in other species. We undertook rabbit microinsemination experiments using mature spermatozoa (ICSI), elongated spermatids (elongated spermatid injection; ELSI), and round spermatids (round spermatid injection; ROSI), and identical conditions for oocyte collection, embryo culture, and embryo transfer. We expected that rabbits might provide an alternative experimental model for microinsemination studies, especially those using immature male germ cells.

MATERIALS AND METHODS

Collection of Oocytes

For collection of rabbit mature metaphase II (M II) oocytes, 4–6-month-old Japanese White (JW) or F1 hybrid (JW × Dutch-belted) females were superovulated by subcutaneous injection of 1,000 IU follicle-stimulating hormone (FSH; Fertinorm P, Serono, Tokyo, Japan) dissolved in saline containing 50% glycerol, followed 48–50 hr later by intravenous injection of 100 IU human chorionic gonadotropin (hCG; Gonatropin, Teikoku Zoki, Tokyo, Japan) in saline. Fifteen to sixteen hours after hCG injection, the oocytes were collected by flushing the oviducts with RD (RPMI-DMEM) medium (Carney and Foote, 1991). Cumulus cells were removed from oocytes by gentle pipetting in RD medium containing 0.1% bovine testicular hyaluronidase (Calbiochem, EMD Biosciences, San Diego, CA). Cumulus-free oocytes were incubated in RD medium at 38.5°C under 5% CO_2 in air until microinjection.

B6D2F1 female mice (7–10 weeks old) were induced to superovulate by consecutive injections of 7.5 IU pregnant mare's serum gonadotropin (PMSG; Peamex, Sankyo, Tokyo, Japan) and 7.5 IU hCG 48–52 hr apart. M II stage oocytes were collected from the oviducts 15–17 hr after hCG injection and were freed from cumulus cells by 0.1% hyaluronidase treatment in CZB medium (Chatot et al., 1989). Oocytes were then washed with fresh CZB medium and cultured at 37°C under 5% CO_2 in air until microinjection.

Collection of Male Germ Cells

Rabbit semen was collected from mature males (JW and Dutch-belted) using an artificial vagina and washed three times with Dulbecco's phosphate-buffered saline (PBS) by centrifugation at 500*g* for 4 min. The supernatant was discarded and the pellet was resuspended in PBS. Spermatogenic cell suspensions were prepared according to a mechanical method previously described for the hamster (Ogura and Yanagimachi, 1993). In brief, testes were isolated from mature males and placed in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA, pH 7.2). The tunica albuginea were removed, and the seminiferous tubule masses were transferred into cold (4°C) PBS supplemented with 5.6 mM glucose, 5.4 mM sodium lactate, and 0.1 mg/ml polyvinyl alcohol (GL-PBS). The seminiferous tubules were cut into small pieces and gently pipetted to allow spermatogenic cells to disperse. The cell suspension was filtered through a 38 µm nylon mesh and washed three times by centrifugation (200*g* for 4 min).

Microinjection of Sperm and Spermatogenic Cells

As the ability of male germ cells to activate oocytes significantly affects subsequent embryo development, we first assessed the oocyte-activating capacity of rabbit sperm and spermatogenic cells by injecting them into mouse oocytes. Because mouse oocytes are rarely activated by the injection stimuli alone, they provide a good heterologous microinsemination model to assess the oocyte-activation capacity of male germ cells from different species (Rybouchkin et al., 1995; Yazawa et al., 2000; Ogonuki et al., 2001). Microinjection was carried out using a piezo-driven micromanipulation system (Primetech, Ibaraki, Japan). Rabbit spermatozoa and spermatogenic cells were suspended in a 12% polyvinylpyrrolidone (PVP) drop. A single spermatozoon was picked up tail first into the injection pipette and injected into a mouse oocyte. When an elongated spermatid or round spermatid was injected, their plasma membrane was broken in a relatively narrow injection pipette before injection. After 4–5 hr incubation, oocytes were fixed and stained with aceto-orcein to allow cytological examination by phase-contrast microscopy. Oocytes were considered to be activated when they possessed the second polar body and two (or three in a very few cases) pronuclei.

The method of homologous microinsemination using rabbit oocytes was the same as described above, except

that some round spermatids were injected into pre-activated oocytes to see whether artificial activation of oocytes would improve development of the resultant embryos. Oocyte activation was carried out in a solution containing 300 mM mannitol and 0.3 mM Ca^{2+} by applying electrical stimulation twice with a 20 min interval. Each pulse consisted of a DC pulse (1,500 V/cm, 99 μsec) with pre- and post-pulse AC (100 V/cm, 10–30 sec, 2 MHz). Ten minutes after the second pulse, oocytes were injected with round spermatids. The injected oocytes were incubated in RD medium.

Embryo Culture and Transfer

Injected oocytes with two distinct pronuclei and the second polar body were considered to be normally fertilized and were cultured in RD medium at 38.5°C under 5% CO_2 and 5% O_2 in air. They were examined daily to determine their developmental stage. Some embryos that reached the two to four cell stage 24 hr after injection, were transferred into the oviducts of day one pseudopregnant females, which had been treated with 100 IU hCG and finger stimulation 18 hr before. Embryos that reached the morula/blastocyst stage in culture were examined for their total cell number. They were whole-mounted on a glass slide, fixed with acetone, and stained with propidium iodide (Sigma-Aldrich, St Louis, MO). The total cell number was counted under a fluorescent microscope.

Chromosomal Analysis of Embryos

Embryos that reached the blastocyst stage were incubated in RD medium containing 100 ng/ml vinblastine for 10 hr. After treatment with hypotonic solution (1% sodium citrate) for 20 min, embryos were fixed with methyl alcohol–acetic acid (1:1) solution for a few seconds and spread on a clean glass slide. They were fixed again on the glass slide with methyl alcohol–acetic acid (3:1). Cells at metaphase were examined for their ploidy. The chromosomes of three to eight cells were counted for each embryo.

Statistical Analysis

Data were analyzed using Fisher's exact probability test or one-way ANOVA analysis as appropriate. A P-value less than 0.05 was considered statistically significant.

RESULTS

We did not observe any strain-dependent differences in the efficiencies of oocyte activation and embryo development; therefore, we combined the data from the JW and Dutch-belted rabbits, and their F1 hybrids, unless otherwise indicated.

Oocyte-Activating Capacity of Rabbit Sperm and Spermatogenic Cells

To assess the ability of rabbit spermatogenic cells to activate oocytes, we first injected rabbit spermatozoa, elongated spermatids, and round spermatids into mouse oocytes. Following microinjection, the majority (61–77%) of mouse oocytes were activated irrespective of the stage of rabbit male germ cells used, forming two well-developed pronuclei and the second polar body within 5 hr (Fig. 1). We then injected rabbit male germ cells into rabbit oocytes using the same technique. Most rabbit (89%) oocytes were activated by mature spermatozoa (Fig. 1); however, the rate of oocyte activation significantly decreased when immature male germ cells were injected. Those oocytes, which were not successfully activated were at either M II or anaphase II with decondensed sperm heads or prematurely condensed spermatid chromosomes. When rabbit oocytes were activated prior to ROSI, 81% were activated and formed male and female pronuclei.

Development of Embryos Following Microinsemination

To assess the ability of male germ cells to support embryo development, we examined development of embryos in vitro and in vivo following microinsemination. The pattern of in vitro embryo development is shown in Figure 2. Once oocytes were activated following microinsemination, the majority cleaved and developed into four-cell embryos within 24 hr, irrespective of the male germ cells used. However, the viability of those derived from round spermatids apparently decreased with time. After 120 hr in culture, 66%, 45%, and 13% of the activated rabbit oocytes (pronuclear eggs) from mature spermatozoa, elongated spermatids, and round spermatids, respectively, developed into blastocysts. The developmental ability of round spermatid-derived embryos significantly improved (43%) when oocytes were activated by an electric pulse shortly before microinsemination (Fig. 2).

Another batch of embryos at the four to eight cell stage was transferred into oviducts. Normal pups were born from embryos derived from sperm and elongated spermatids, but none from round spermatids (Table 1). All pups were born alive and showed active movement (Fig. 3).

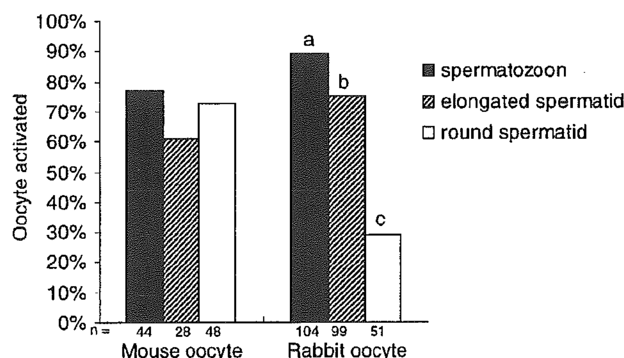

Fig. 1. Activation of mouse and rabbit oocytes following microinjection with rabbit male germ cells. Differential activation rates were observed when rabbit male germ cells were injected into homologous oocytes. Values with different letters differ significantly ($P < 0.05$; Fisher's exact probability test). The number below each bar indicates the number of embryos examined.

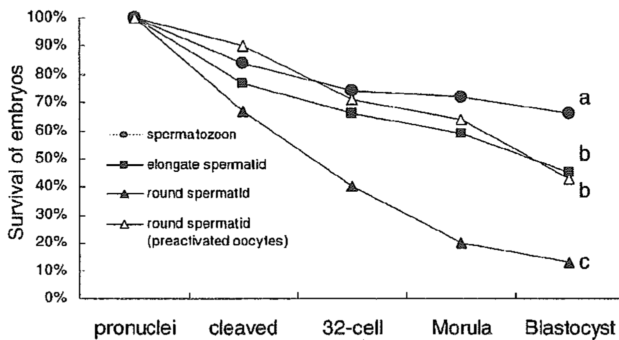

Fig. 2. Development of rabbit oocytes fertilized by microinsemination with sperm or spermatids. Embryos derived from round spermatids showed retarded development, but those subjected to preactivation stimuli showed significantly improved development. The groups of ICSI, ELSI, ROSI, and ROSI with preactivated oocytes consisted of 93, 74, 15, and 127 embryos, respectively. Values with different letters differ significantly ($P < 0.05$; Fisher's exact probability test).

Cell Number and Ploidy of Embryos

The total number of cells was counted in embryos that reached the blastocyst stage in culture (Fig. 4). The cell number of embryos derived from elongated or ROSI was significantly lower than that of ICSI and control embryos (in vivo fertilization) ($P < 0.01$). Some embryos that reached the morula/blastocyst stage were analyzed for their ploidy. In ICSI, ELSI, and ROSI (with preactivated oocytes) groups, 87%, 58%, and 33%, respectively, of morulae/blastocysts were considered diploid (Fig. 5A,B). The rate of occurrence of embryos with a diploid ($2n = 44$) set of chromosomes was significantly lower in the ROSI group than in the ICSI group ($P < 0.05$). Mixoploid embryos were observed in all groups. They were comprised of cells with two or three different ploidy; haploid (Fig. 5C), diploid, triploid, or tetraploid.

DISCUSSION

This study was undertaken to see whether development of rabbit embryos in vivo and in vitro was dependent on the maturational stage of male germ cells used for microinsemination. The results clearly demonstrated that the developmental ability of embryos was highly dependent on the stage of male germ cells. This was apparent from in vitro development into blastocysts and in vivo development into offspring. Round spermatids, in particular, showed extremely low capacity to support embryo development when compared with spermatozoa or elongated spermatids. This was unexpected, because we have routinely used round spermatids for production of offspring in mice, and this has included round spermatids from males with severe defects in spermiogenesis (Ogura et al., 2005).

TABLE 1. Birth of Pups Following Embryo Transfer of Different Microinsemination Groups

Group	No. of embryos transferred	No. (%) of pups born
ICSI	28	8 (29)
ELSI	26	3 (12)
ROSI	68	0 (0)

First we assessed the oocyte-activating capacity of rabbit male germ cells by injecting them into mouse oocytes, as we did for male germ cells from cynomolgus monkeys (Ogonuki et al., 2001) and *Mastomys* (Ogonuki et al., 2003a). The results clearly demonstrated that there was no stage-dependency in their oocyte-activating capacity. We observed 61–77% activation of mouse oocytes, which is consistent with the 71–79% reported by Yazawa et al. (2000) using the same experimental system. However, the rabbit oocyte-activating capacity following homologous microinsemination was dependent on the maturational stage of male germ cells. Subsequent embryo development was also highly dependent on the stage of male germ cells, as we demonstrated when activated oocytes with two pronuclei and the second polar body were cultured. From these results, it can be assumed that rabbit oocytes require a substantial activation stimulus, and that insufficient activation may affect the subsequent embryo development even after the second cleavage.

The requirement for substantial activation stimulus to rabbit oocytes is supported by previous studies (Ozil, 1990; Escriba and Garcia-Ximenez, 1999; Ozil and Huneau, 2001). Repetitive intracellular free Ca^{2+} rises (intracellular Ca^{2+} oscillation) occur following fertilization by sperm-borne oocyte-activation factors (SOAF), one of which is phospholipase C zeta (Swann et al., 2004). However, oocytes from many mammalian species can be artificially activated by a single Ca^{2+} rise alone and support full-term embryo development, as shown in many microinsemination and nuclear transfer studies. The rabbit seems to be the exception because repetitive stimulation is necessary to induce full oocyte activation (Ozil and Huneau, 2001; Inoue et al., 2002). Intracellular Ca^{2+} monitoring in oocytes revealed that rabbit round spermatids induce less frequent Ca^{2+} rises than rabbit mature spermatozoa or elongated spermatids (Yazawa et al., 2000). This difference in the ability to induce Ca^{2+} oscillations, which cannot be always parallel with mouse oocyte activation, might cause the stage-specific dependency on the rate of rabbit oocyte activation. Round spermatids from mice and rats do not induce even a single Ca^{2+} rise and therefore artificial oocyte activation is necessary for normal fertilization (Yazawa et al., 2000). Interestingly, hamster round spermatids induce only weak Ca^{2+} rises (Yazawa et al., 2000) as do rabbit round spermatids, but pups have been born from round spermatids without exogenous activation stimuli (Haigo et al., 2004). Thus, both male and female factors determine whether ROSI alone is enough.

By applying an oocyte-activation stimulus, the development of ROSI embryos was significantly improved. They reached the blastocyst stage at a rate of 43%, which is comparable to those from sperm or ELSI. Therefore, we speculated that oocytes were successfully activated and that embryos constructed by ROSI started embryonic gene activation necessary for normal development.

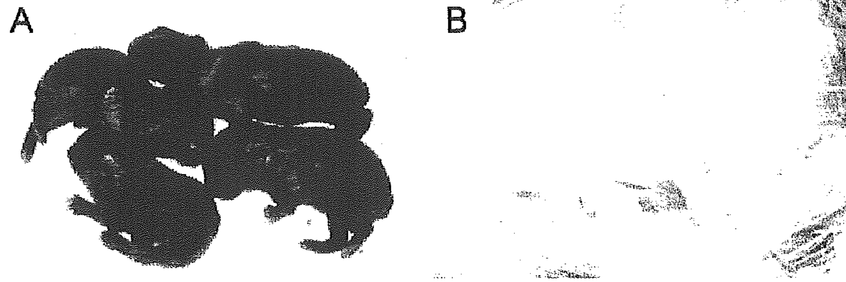

Fig. 3. Rabbit pups born after ICSI (A) and ELSI (B). All pups obtained by microinsemination were born alive in this study.

However, no pups were born following transfer of ROSI embryos. In contrast, up to 29% of ICSI embryos developed to term. To determine the cause of this differential developmental ability in vivo, we analyzed the cell number and ploidy of blastocysts obtained by microinsemination. The mean cell number of ICSI blastocysts was significantly greater than for ELSI and ROSI blastocysts, although there was no significant difference between those of ELSI and ROSI blastocysts. An apparent cell type-dependency was also found in the proportion of blastocysts with normal ploidy. In ICSI, ELSI, and ROSI groups, 87%, 58%, and 33%, respectively, of blastocysts were considered diploid. Ploidy errors do not always affect the blastocyst yield (Booth et al., 2003), but often cause embryonic loss at the peri-implantation stages (Pinto-Correia et al., 1993; Yin et al., 2002). Thus, it is very likely that the low cell number and abnormal ploidy cumulatively caused poor in vivo development of ROSI embryos in this study.

If the chromosomal composition was equally normal in the haploid male germ cells before injection, the difference in the ploidy of these blastocysts could be attributed to the difference in chromosomal behavior following microinsemination. During the process of fertilization, chromosomal behavior is directed by microtubules and their organizing center (MTOC), which is contributed by the sperm centriole in most animal species including the rabbit. The MTOC is responsible for the organization of zygote microtubules, including those of the mitotic spindles employed in subsequent cleavage divisions. As MTOC activity is acquired during spermiogenesis, round spermatids lack this activity and cannot contribute to the microtubule organization within the ooplasm. Following ROSI, therefore, the maternally synthesized MTOC, instead of the sperm-derived MTOC, drives the behavior of microtubules in fertilized oocytes and embryos (Lee et al., 1998). However, in rabbits, the maternal MTOC often functions improperly and fails to align the chromosomes or nuclei within parthenogenetically-activated rabbit oocytes, including those reconstructed with blastomeres or somatic cells (Pinto-Correia et al., 1993; Yin et al., 2002). Shi et al. (2004) reported that 83% of cloned rabbit embryos displayed aneuploidy. Although we have not investigated the chromosomal behavior during the early fertilization period, it is probable that dysregulated maternal MTOC caused abnormal ploidy in our ROSI embryos and poor ROSI conception.

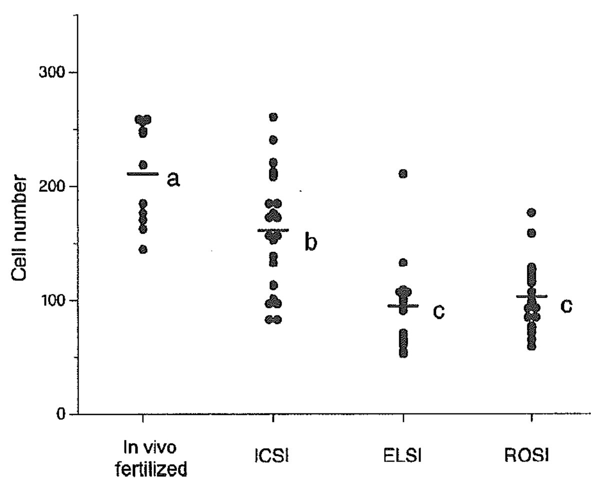

Fig. 4. The cell numbers of blastocysts obtained by microinsemination. The cell number of embryos derived from elongated or ROSI was significantly lower than that of ICSI and control embryos. Values with different letters differ significantly ($P > 0.05$; one-way ANOVA analysis followed by Scheffé's multiple comparison test).

Intracytoplasmic injection of round or elongated spermatids has been proposed as a treatment for men with nonobstructive azoospermia, where mature spermatozoa are not detected by testicular biopsy. However, application of this technique by different groups using immature sperm cells resulted in highly variable success (Aslam et al., 1998) and therefore its clinical value and safety is still a matter of controversy. To understand the cellular biology of new microinsemination techniques, we should use appropriate preclinical animal models. Mice have been most frequently used for this purpose, but their mechanisms of fertilization, including microtubule behavior, are apparently distinct from those of other animals (Yanagimachi, 2005).

Mouse ROSI, for example, has few technical obstacles and has been practicably used for rescue of strains with poor reproductive performance or failure of spermiogenesis (Ogura et al., 2005; Yanagimachi, 2005). The

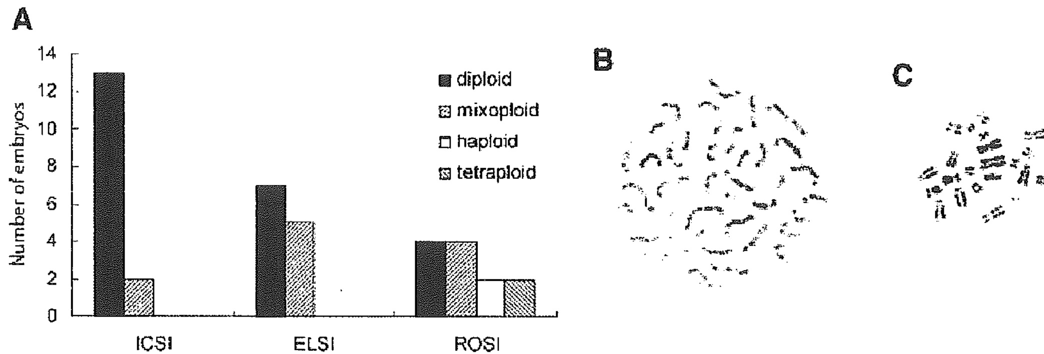

Fig. 5. Ploidy analysis of morulae and blastocysts derived from microinsemination. **A:** Number of embryos by ploidy classification. The rate of normal ploidy (diploid) in ROSI embryos was significantly lower than that of ICSI embryos. $*P < 0.05$ (Fisher's exact probability test). **B:** Diploid set of chromosomes ($2n = 44$) in an ICSI embryo. **C:** Haploid set of chromosomes ($n = 22$) in a ROSI embryo.

nonhuman primates may provide the best model for human fertilization studies (Hewitson et al., 1999; Chan et al., 2000; Ogonuki et al., 2003b), but their use is often limited due to ethical and financial reasons. The differential microinsemination efficiency in rabbits, shown here to be dependent on the maturity of male germ cells, is very similar to that in humans. Spermatid conception treatment in several human infertility clinics revealed that pregnancy was achieved in the ELSI cycles at a practicable rate, but not in the ROSI cycles (Vanderzwalmen et al., 1997; Aslam et al., 1998; Sousa et al., 1999). Our study highlights the possibility of rabbit microinsemination as a model to develop new approaches for evaluating the 300 risks and benefits of this technique and for developing new strategies to treat human male-factor infertility. The behavior of the chromosomes and MTOC following ELSI and ROSI in rabbits would be of considerable interest for further investigation.

In conclusion, our study indicates that rabbit male germ cells acquire the ability to activate oocytes and to support subsequent embryo development as they undergo spermiogenesis. Immaturity of the nuclear genome or difficulty in coordinating the behavior of the male and female chromosomes might compromise embryo development. Finally, rabbits may provide a good, alternative experimental model for studying mammalian microinsemination, which would have particular relevance to increasing the efficiency of round spermatid conception in humans.

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Genetic and epigenetic properties of mouse male germline stem cells during long-term culture

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Summary

Although stem cells are believed to divide infinitely by self-renewal division, there is little evidence that demonstrates their infinite replicative potential. Spermatogonial stem cells are the founder cell population for spermatogenesis. Recently, in vitro culture of spermatogonial stem cells was described. Spermatogonial stem cells can be expanded in vitro in the presence of glial cell line-derived neurotrophic factor (GDNF), maintaining the capacity to produce spermatogenesis after transplantation into testis. Here, we examined the stability and proliferative capacity of spermatogonial stem cells using cultured cells. Spermatogonial stem cells were cultured over 2 years and achieved ~10^{85}-fold expansion. Unlike other germline cells that often acquire genetic and epigenetic changes in vitro, spermatogonial stem cells retained the euploid karyotype and androgenetic imprint during the 2-year experimental period, and produced normal spermatogenesis and fertile offspring. However, the telomeres in spermatogonial stem cells gradually shortened during culture, suggesting that they are not immortal. Nevertheless, the remarkable stability and proliferative potential of spermatogonial stem cells suggest that they have a unique machinery to prevent transmission of genetic and epigenetic damages to the offspring, and these characteristics make them an attractive target for germline modification.

Key words: Culture, Genomic imprinting, Karyotype, Spermatogenesis, Stem cell, Telomere

Introduction

An important characteristic of stem cells is their ability to self-renew (Potten, 1992). Although many stem cells are believed to divide infinitely by self-renewal division, there is little evidence that demonstrates the infinite replicative potential of stem cell populations. Numerous studies have also shown that cells with a high replicative potential often exhibit many abnormalities when they are maintained in vitro. The serial propagation or aging of cells in culture not only induces chromosomal abnormalities, but also other degenerative cellular changes, including abnormal structures in the cytoplasm, changes in metabolism, replicative efficiency or growth rate that culminates in apoptosis or tumorigenic behavior (Rubin, 2002). Although stem cells are considered to have a special machinery to maintain their replicative potential without accumulating abnormalities (Cairns, 2002), stem cells derived from germline of embryos are sensitive to such stress, as embryonic stem (ES) or embryonic germ (EG) cells very often exhibit abnormalities in chromosome structure and genomic imprinting patterns after culture (Labosky et al., 1994; Liu et al., 1997; Longo et al., 1997; Dean et al., 1998; Humpherys et al., 2001; Draper et al., 2004), which lead to loss of germline potential. However, owing to the lack of appropriate system, it remains unknown whether stem cells from postnatal tissues are similarly unstable in culture.

Spermatogonial stem cells are the founder cell population for spermatogenesis (Meistrich, 1993; de Rooij and Russell, 2002). Although spermatogonial stem cells are infrequent and divide slowly in vivo, the addition of GDNF to in vitro cultures of these cells induces self-renewing division of spermatogonial stem cells: the cells increase logarithmically in vitro without losing the capacity to produce spermatogenesis when transferred into the seminiferous tubules of infertile mouse testes (Kanatsu-Shinohara et al., 2003b; Ogawa et al., 2004; Kubota et al., 2004). Recipient mice that received grafts of transfected cultured spermatogonial stem cells sired transgenic offspring at a frequency approaching 50%, which is five- to 10-fold higher than the frequencies achieved using traditional methods based on oocytes or embryos (Kanatsu-Shinohara et al., 2005a). Owing to their unique characteristics, we

designated these cells 'germline stem' (GS) cells to distinguish them from ES cells or EG cells (Evans and Kaufman, 1981; Martin, 1981; Matsui et al., 1992; Resnick et al., 1992; Kanatsu-Shinohara et al., 2003b). Thus, GS cells created a new possibility to study spermatogonial stem cells.

In this study, we examined the replicative potential and stability of spermatogonial stem cells during long-term culture. Two independent GS cell cultures were maintained for 2 years, and the cultured cells were analyzed for their phenotypic and functional characteristics, including the germline potential.

Materials and methods

Cell culture

The GS cells were established from the testes of a newborn transgenic mouse from the C57BL6/Tg14(act-EGFP-OsbY01) line that was bred on the DBA/2 background (Kanatsu-Shinohara et al., 2003b), whose cells express enhanced green fluorescence protein (EGFP). A single-cell suspension was prepared using a two-step enzymatic digestion with collagenase and trypsin, and the cultures were initiated as previously described (Kanatsu-Shinohara et al., 2003b). The established GS cells were transferred onto a feeder cell layer of mitomycin C-treated mouse embryonic fibroblasts (MEFs). ES and multipotent germline stem (mGS) cells were cultured using standard ES cell culture conditions, as previously described (Kanatsu-Shinohara et al., 2004b). The cells were cryopreserved using Cellbanker (DIA-IATRON, Tokyo, Japan) (Kanatsu-Shinohara et al., 2003c).

Transplantation

The cultured cells were collected by trypsinization and were filtered through 30 μm nylon mesh before transplantation. Approximately 3 μl of the cell suspension were microinjected into the seminiferous tubules of the testes of 4- to 8-week-old WBB6F1-W/W^v recipients (W mice; Japan SLC, Shizuoka, Japan) via the efferent duct (Ogawa et al., 1997). The injections filled 75 to 85% of the tubules in each recipient testis. The recipient W mice received anti-CD4 antibody injections to induce tolerance to the allogeneic germ cells (Kanatsu-Shinohara et al., 2003a). For the testing of tumor-forming potential, approximately 3×10^6 cells were injected into KSN nude mice (Japan SLC). The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Analysis of testis

Donor cell colonization was analyzed by observation of fluorescence under UV light (Kanatsu-Shinohara et al., 2003a). This method allows the specific identification of transplanted cells, because the host testis does not fluoresce. The recipient testes were also fixed in 10% neutral buffered formalin, and processed for paraffin sectioning. All sections were stained with Hematoxylin and Eosin for histological analysis.

Antibodies and staining

The primary antibodies used were: rat anti-mouse EpCAM (G8.8), mouse anti-mouse SSEA-1 (MC-480; Developmental Studies Hybridoma Bank, University of Iowa), rat anti-human α6-integrin (CD49f) (GoH3), biotinylated hamster anti-rat β1-integrin (CD29) (Ha2/5), biotinylated rat anti-mouse CD9 (KMC8) and allophycocyanin (APC)-conjugated rat anti-mouse Kit (2B8; all from BD Biosciences, Franklin Lakes, NJ). APC-conjugated goat anti-rat-IgG (Cedarlane Laboratories, Ontario, Canada), APC-conjugated streptavidin (BD Biosciences) and Alexa Fluor 633-conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR) were used as secondary antibodies. The cell staining technique used for flow cytometry was as previously described (Shinohara et al., 1999). The stained cells were analyzed using a FACS-Calibur system (BD Biosciences), and only EGFP-positive cells were gated for analysis. At least 10,000 events were acquired for each sample. Alkaline phosphatase staining was carried out using a Vector Blue substrate kit (Vector Laboratories, Burlingame, CA) according to manufacturer's protocol.

Analysis of marker gene expression

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). For reverse transcriptase-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized using Superscript™ II (RNase H$^-$ reverse transcriptase, Invitrogen). PCR was carried out using appropriate primer sets, as described previously (Kanatsu-Shinohara et al., 2004b; Kanatsu-Shinohara et al., 2005b; Falender et al., 2005).

Pulsed-field gel electrophoresis, terminal restriction fragments (TRF) analysis, and measurement of telomerase activity

The DNA was harvested from 2×10^6 cells by salting out method, using a buffer containing 20 mM Tris-HCl pH 8.0, 10 mM EDTA, 400 mM NaCl, 0.5% SDS and 100 μg/ml of proteinase K. The DNA was digested with *Hin*fI overnight and separated by electrophoresis on a 1.1% agarose gel at 14°C, using a pulsed-field apparatus (BioRad, Hercules, CA). Pulsed-field electrophoresis was performed with 6 V/cm constant for 12 hours and a ramped pulse from 1 to 10 seconds. To examine the TRFs containing telomeric sequences, the gel was dried at 60°C for 3 hours, soaked in denaturing solution (1.5 M NaCl-0.5 M NaOH solution for 30 minutes, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) buffer for 30 minutes, and probed with $(C_3TA_2)_4$ telomeric DNA oligonucleotides at 37°C overnight. Telomerase activity was measured using the TeloChaser detection kit (Toyobo, Osaka, Japan) as previously described (Tatematsu et al., 1996).

Microinsemination

Microinsemination was performed by intracytoplasmic injection (Kimura et al., 1995) of round spermatids from EGFP-positive spermatogenic colonies from donor testes into C57BL/6×DBA/2 F1 (BDF1) oocytes, collected from superovulated females. The embryos were transferred into the oviducts of pseudopregnant ICR females, 24 hours after in vitro culture. Live fetuses were retrieved on day 19.5 and were raised by lactating foster mothers.

Combined bisulfite restriction analysis (COBRA)

Genomic DNA was treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils but does not affect 5-methylated cytosines. PCR amplification of differentially methylated regions (DMRs) from the bisulfite-treated genomic DNA was carried out using specific primers as previously described (Xiong and Laird, 1997; Kanatsu-Shinohara et al., 2004b). The amplified PCR products were digested with the indicated restriction enzymes, which have recognition sequences containing CpG in the original unconverted DNA. The intensity of the digested DNA bands was quantified using ImageGauge software (Fuji Photo Film, Tokyo, Japan).

Results

Long-term culture of GS cells

Neonatal testis cells from EGFP-transgenic mice were used to establish GS cells. After dissociation into single cell suspensions, testicular cells were cultured in the presence of a cytokine cocktail containing epidermal growth factor, basic fibroblast growth factor, leukemia inhibitory factor and GDNF. Under these conditions, the germ cells began to proliferate and form uniquely shaped colonies. After two to three passage in tissue culture, the germ cell colonies were transferred onto mitomycin C-treated MEFs.

Development