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Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells

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BACKGROUND: The development of a spermatogonial transplantation technique has provided new possibilities for the treatment of male infertility. Previous studies have shown that spermatogonial stem cells could reinitiate spermatogenesis after cryopreservation and reintroduction into the seminiferous tubules of infertile recipient males, and this raised the possibility of banking frozen stem cells for male infertility treatment. It remains unknown, however, whether germ cells from freeze–thawed stem cells are fertile, leaving the possibility that the procedure compromises the integrity of the stem cells. METHODS AND RESULTS: Dissociated mouse testis cells were cryopreserved and transplanted into infertile recipient testes. The freeze–thawed testis cell populations contained higher concentrations of stem cells than fresh testis cell populations. Offspring were obtained from freeze–thawed stem cells transplanted into infertile males, and fertility restoration was more efficient in immature (5–10 days old) than in mature (6–12 weeks old) recipients. However, offspring were also obtained from infertile adult recipients using in-vitro microinsemination. CONCLUSIONS: This first successful application of frozen stem cell technology in the production of offspring by spermatogonial transplantation suggests the superiority of immature recipients for clinical applications. Thus, the combination of cryopreservation and transplantation of stem cells is a promising approach to overcome male infertility.

Key words: cryopreservation/infertility/stem cell/testis/transplantation

Introduction

Sperm cryopreservation has proven to be a valuable procedure for the management of infertility, for the protection of fertility in oncology patients, and for couples undergoing IVF (Bunge and Sherman, 1953; Fossa *et al.*, 1989; Royére *et al.*, 1996; Lass *et al.*, 2001). However, there is a lack of methods by which the germlines of patients with few sperm may be preserved. Examples include prepubertal patients or those with clinical conditions such as severe spermatogenic maturation arrest or who lack sperm. The infertility of these patients cannot be overcome by the use of traditional assisted-reproduction technology.

The development of a spermatogonial transplantation technique has provided a new treatment strategy for male infertility (Brinster and Zimmermann, 1994). Following the transplantation of dissociated testis cells into a seminiferous tubule microenvironment, the spermatogonial stem cells colonize and initiate spermatogenesis (Nagano *et al.*, 1999). Since spermatogonial stem cells self-renew and differentiate into proliferating spermatogonia, they provide a limitless supply of mature spermatozoa. Thus, spermatogonial stem cell transplantation may be useful for the treatment of different types of male infertility; for example, it was used to treat a mouse model of Sertoli cell-only syndrome (Ogawa *et al.*, 2000; Shinohara *et al.*, 2001). The mice that underwent transplantation recovered normal fertility and produced progeny for the rest of their lives. In addition to congenital infertility, this technique should also benefit oncology patients who are undergoing stem cell-destroying irradiation or chemotherapy, by prior isolation of stem cells and autotransplantation (Aslam *et al.*, 2000). Transplantation of stem cells should rescue fertility in a manner similar to that of bone marrow stem cell transplantation. Therefore, the establishment of methods to preserve stem cells and restore fertility has important clinical implications.

Brinster and colleagues first demonstrated that frozen stem cells retain the ability to carry out spermatogenesis and to produce spermatozoa (Avarbock *et al.*, 1996). Surprisingly, in contrast to the difficulties associated with the freezing of sperm, the protocol used for freezing germline cells was very simple, and similar to those generally employed for somatic cells. Further studies indicated that the same procedure could be applied to the freezing of spermatogonial stem cells from several other species, such as rats, hamsters, cattle, primates and humans (Dobrinski *et al.*, 1999; 2000; Ogawa *et al.*, 1999;

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Allogeneic Offspring Produced by Male Germ Line Stem Cell Transplantation into Infertile Mouse Testis[1]

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ABSTRACT

The testis is one of several immune-privileged organs and is known for its unique ability to support allogeneic or xenogeneic tissue transplants. We investigated the possibility of deriving offspring from mice that underwent transplantation with allogeneic male germ line stem cells in the testis. Although mature adult mice rejected allogeneic germ cells and were infertile, offspring were obtained by intracytoplasmic germ cell injection using partially differentiated donor cells. In contrast, complete spermatogenesis occurred when allogeneic germ cells were transplanted into immature pup testes. Tolerance induction by monoclonal antibody administration allowed the pup transplant recipients to produce allogeneic offspring by natural mating, whereas no spermatozoa were found in the epididymis of untreated recipients. Thus, these results indicate that a histoincompatible recipient can serve as a "surrogate father" to propagate the genetic information of heterologous male donors.

immunology, in vitro fertilization, Sertoli cells, spermatogenesis, testis

INTRODUCTION

The testis is one of several immune-privileged organs in the body [1]. Despite the high immunogenicity of male germ cells, much of the development of spermatogenesis occurs after maturation of the immune system's "self"-recognition, and spermatogenic cells persist within the male reproductive tract throughout life without eliciting an immune response [2]. In addition, allografts as well as xenografts survive for a considerably longer period in the testis than in most other tissues [1, 3].

Immune privilege in the testis is thought to result from the unique anatomical structure of the seminiferous tubules and local immunosuppression by Sertoli cells, a major somatic cell in the seminiferous tubule [2]. In mice, Sertoli cells form the blood-testis barrier approximately 2 wk after birth [4] and separate the immunogenic haploid cells from the immune system [2]. In addition, Sertoli cells have local immunosuppressive activity, because cotransplantation of Sertoli cells with adrenal chromaffin cells [5], islet cells [6], or dopamine-secreting neurons [7] leads to acceptance of the transplants in allogeneic or xenogeneic hosts. Although the site of expression remains controversial [8], Fas ligand expressed on Sertoli cells is believed to play a key role in the induction of transplantation tolerance in the testis as well as in other immune-privileged organs [9, 10].

The body contains several self-renewing systems, including hematopoietic tissue, intestinal epithelium, and epidermis [11]. Spermatogenesis originates from spermatogonial stem cells, and the continuous production of progenitor cells supports male reproduction throughout the life of adult animals [4]. Because stem cells are considered to proliferate indefinitely, allogeneic stem cell transplantation has been a subject of intense investigation, mostly for clinical purposes. Particularly regarding the hematopoietic system, several decades of active investigation have made transplantation of allogeneic hematopoietic stem cells an accepted therapy for numerous hematological malignancies, metabolic disorders, and solid tumors [12]. However, little is known about the immunological response to transplantation of other types of allogeneic stem cells, particularly how they are affected by histoincompatibility [12, 13].

Recently, a technique to transfer spermatogonial stem cells was developed in which the stem cells are transplanted into the seminiferous tubules of infertile recipient animals [14]. The transplanted stem cells proliferate on the basement membrane and establish colonies of spermatogenesis [15]. Spermatogonial stem cells are unique among the many types of stem cells in that they reside in an immune-privileged microenvironment. In the present study, we investigated if male germ cells that develop in a histoincompatible environment are fertile, whether used for intracytoplasmic germ cell injection or for natural mating in immunosuppressed recipients.

MATERIALS AND METHODS

Animals and Transplantation Procedure

In the first set of experiments using adult recipients, donor cells were recovered from the testes of a transgenic mouse line B6-TgR(ROSA26)26Sor (ROSA26; H-2^b haplotype) purchased from The Jackson Laboratory (Bar Harbor, ME) [16]. This mouse expresses the *Escherichia coli LacZ* transgene in all cells of the seminiferous tubules [15]. Donor cells used for intracytoplasmic germ cell injection experiments were isolated from the testes of a transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 (Green; H-2^b haplotype) provided by Dr. M. Okabe (Osaka University, Osaka, Japan). The spermatogonia and spermatocytes of these mice express the enhanced green fluorescent protein (EGFP) gene, which gradually decreases after meiosis [17].

Donor cells were transplanted into the testes of C57BL/6J (B6; H-2^b haplotype) and C3H/HeJ (C3H; H-2^k haplotype) adult males that had been treated with busulfan (44 mg/kg) at 6 wk of age [14]. To protect from the

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Pregnancy by the tubal transfer of embryos developed after injection of round spermatids into oocyte cytoplasm of the cynomolgus monkey (*Macaca fascicularis*)

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BACKGROUND: Round spermatids have been used as substitute gametes in basic reproductive research and in infertility clinics. In humans, however, the efficiency of fertilization and pregnancy is generally much lower after round spermatid injection (ROSI) than after injection with mature sperm. We examined the ability of round spermatids to support embryonic development using a non-human primate as a model. We chose cynomolgus monkeys because, as in humans, their round spermatids have the oocyte-activating capacity of mature sperm. METHODS: We examined fertilization and subsequent development of embryos after ROSI and then transferred the embryos into the oviducts of female monkeys. RESULTS: Seventy-seven per cent of survived oocytes were activated and had formed pronuclei or the second polar body; 79% of the oocytes cultured developed to the 2-cell stage, and 23% developed to the blastocyst stage. Ultrasonography showed a normal-sized fetus in the uterus of a recipient, but the fetus spontaneously aborted at day 103. CONCLUSIONS: The round spermatids of cynomolgus monkeys can be used as substitute gametes to support embryonic development at least to mid-gestation. This non-human primate is a suitable animal model for round spermatid conception in mammals, especially humans, and for biological and genetic characterization of events following ROSI.

Key words: cynomolgus monkey/embryo transfer/oocyte activation/ROSI/round spermatid

Introduction

Since the first success of ICSI in humans, it has been used for treatment of male factor infertility. However, idiopathic azoospermia caused by spermatogenic arrest cannot be remedied by this technique, and therefore many attempts have been made to develop new techniques of assisted fertilization using immature male germ cells. It is now accepted that elongated spermatids can be efficiently used (Antinori *et al.*, 1997; Sofikitis *et al.*, 1998a) as substitute gametes because of their high fertilizing ability comparable with that of mature sperm. In contrast, many controversial issues have been raised concerning the use of round spermatids. While several normal pregnancies following round spermatid injection (ROSI) have been reported (Fishel *et al.*, 1995; 1996; Tesarik *et al.*, 1995; 1996; Antinori *et al.*, 1997), disappointingly poor fertilization and subsequent embryonic development are the common outcome in some clinics (Yamanaka *et al.*, 1997; Levran *et al.*, 2000; Vicdan *et al.*, 2001). Round spermatids differ biologically from mature sperm in some important respects, such as immaturity of certain cytoplasmic and nuclear proteins (Ziyyat and Lefevre, 2001). Therefore, it is very likely that this immaturity is one of the causes of the poor pregnancy rates following ROSI in humans (Ghazzawi *et al.*, 1999). Consistent with this assumption is the finding from mouse experiments that the overall efficiency of ROSI is relatively poor compared with that of ICSI. However, the circumstances in human ROSI are more complex because the fertilizing ability of patients' round spermatids might be congenitally impaired, or reduced to some extent by an inadequate testicular environment (Vanderzwalmen *et al.*, 1998). Thus, to assess the fertilizing ability of round spermatids, it is essential to recover them from fertile individuals. Non-human primates are best suited for this end. To our knowledge, however, there are only a few reports of successful ICSI in non-human primates and no reports of successful ROSI.

We have previously confirmed the technical basis for embryo culture, embryo transfer (Sankai *et al.*, 1994; Sankai,

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New microinsemination techniques for laboratory animals

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Abstract

Since the development of a reliable mouse intracytoplasmic sperm injection (ICSI) technique in 1995, microinsemination techniques have been widely applied in several laboratory species. As gametes and embryos have specific biological and biochemical features according to the species, technical improvements are necessary for successful microinsemination that subsequently leads to normal fetal development in several species. Recent advanced reproductive research involving genetic engineering often depends on microinsemination techniques that require a high degree of skill, and new human assisted reproductive technology (ART) requires experimental models using laboratory animals. The accumulation of technical improvements in these fields should accelerate the development of microinsemination techniques in mammals, including humans.

Keywords: Microinsemination; Spermatozoon; Spermatid

1. Introduction

Microinsemination techniques in laboratory animals have been developed independently from those used for humans because ease and efficiency are most important in the former, but biological and genetical safety are the greatest concerns in the latter. As a result, the microinsemination technique first developed for laboratory animals was direct microsurgical injection of sperm heads into the ooplasm (ICSI) [1], although those developed for humans were partial zona dissection (PZD) and subzonal insemination (SUZI), which are less invasive techniques than ICSI [2].

In 1976, microinsemination was first used to study mammalian fertilization using a golden hamster model, because hamster oocytes tolerate injection and because abundant information on mammalian fertilization had accumulated from hamster in vitro fertilization (IVF) [1]. Following injection with isolated sperm heads, hamster oocytes undergo a

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Do cloned mammals skip a reprogramming step?

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It is widely accepted that at least some populations of cloned animals have an attenuated lifespan compared with their conventionally bred counterparts. This has been attributed both to premature aging or senescence and to accumulation of abnormalities in gene expression in their tissues. Here, we argue that these problems arise because the process of nuclear transfer used to create cloned animals skips one of the two essential, independent steps involved in the reprogramming of cell nuclei.

Senescence

Since the birth of Dolly the sheep in 1996, the 'real biological age' of cloned animals has been a matter of much debate[1]. It has been argued that Dolly was either 6 years old (on the basis of her date of birth) or 12 years old (on the basis of the age of the donor mammary gland cell used in her creation) when she was euthanased because of serious progressive lung disease.

One proposed means of assessing a clone's age is by measuring the length of its telomeres and the speed of their erosion. Simple measurements of telomere length suggest that cloned animals have telomeres that are similar in length to, or even longer than, telomeres from naturally bred animals[2–5]. Telomeres from Dolly[6] and from bovine clones[7] are shorter than those of age-matched controls, however. In addition, certain cloned animals have even shorter telomeres than those in the somatic donor cells from which they were actually derived. As many cloned large animals reach 4–6 years of age with no signs of premature aging, these variations and errors in telomere restoration do not necessarily seem to lead to premature aging.

A comparison by Clark *et al.*[8] of *in vitro* culture parameters and characteristics of sheep fibroblast cells used as nuclear donors in cloning and cells derived from corresponding cloned fetuses showed that complete telomere restoration is not necessarily achieved after nuclear transfer; in fact, the proliferation and lifespan of the cloned cells from the fetus are the same as those of the donor cell line. The authors thus concluded that the lifespan of a clone is influenced by the genetically determined speed of telomere erosion.

Because of their short life cycle, mice are an ideal system for studying the longevity of cloned animals. A study by Ogonuki *et al.*[9] showed that cloned mice die significantly earlier than controls. As many of the cloned mice suffer from serious pathologies (*e.g.*, pneumonia and hepatic failure), however, premature aging might not be the primary cause of death.

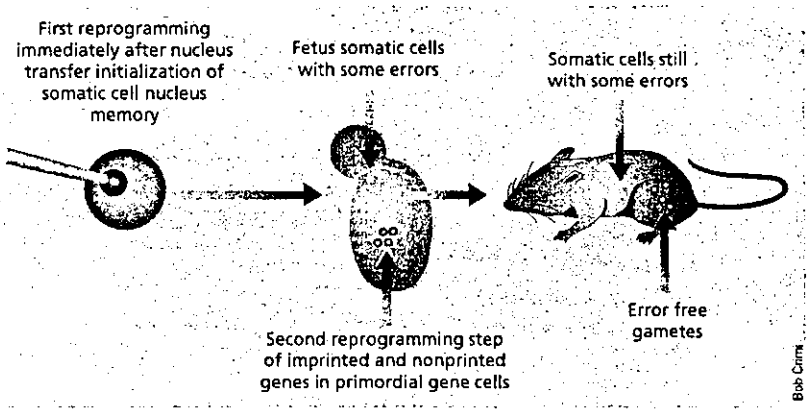

Figure 1 Reprogramming in two steps. The first step occurs after the nucleus is transferred into the enucleated oocyte and probably during the next few divisions, depending on the species. In about 1–5% of reconstructed oocytes, this reprogramming, even with some errors, permits further development and results in viable offspring. In the remaining cases, the reprogramming is incomplete and embryos die or offspring are not viable. During the second reprogramming step, imprinted and nonimprinted genes are reprogrammed, and errors that were not corrected during the first step are repaired. As this step occurs only in the germline cells, cloned animals contain somatic cells with some abnormalities, but their spermatozoa or oocytes are error-free.

What is normal?

This raises the question of whether any clones are completely 'normal'[10]? The expression of several (imprinted and nonimprinted) genes differs substantially in cloned animals compared with conventionally bred counterparts. Of about 10,000 genes analyzed in mouse clones, approximately 400 show abnormal expression patterns, especially in placentas[11]. Notably, aberrant expression seems to be somewhat tissue-specific, with nonplacental organs having a lesser extent of abnormal gene expression.

A similar analysis of expression of genes in the *Oct4* group in mice showed that embryos derived from embryonic stem cells have a normal expression pattern[12,13], whereas blastocysts produced by somatic cell transfer have abnormal expression (additional factors, *e.g.*, culture conditions, may also influence the expression of certain genes[14,15]). Thus, we conclude that the premature aging of clones is not the only (or the main) reason why cloned animals die earlier than naturally bred counterparts.

Reprogramming by steps

To elucidate this phenomenon, we must look more closely at the reprogramming of the nucleus after its transfer to the recipient cell. There are essentially two independent natural periods when cell nuclei can be reprogrammed. The first period begins immediately after fertilization when, for example, the

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LETTER

Tissue-Specific Distribution of Donor Mitochondrial DNA in Cloned Mice Produced by Somatic Cell Nuclear Transfer

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Summary: Highly diverse results have been reported for mitochondrial DNA (mtDNA) hetero-plasmy in nuclear-transferred farm animals. In this study, we cloned genetically defined mice and investigated donor mtDNA inheritance following somatic cell cloning. Polymerase chain reaction (PCR) analysis with primers that were specific for either the recipient oocytes or donor cells revealed that the donor mtDNA coexisted with the recipient mtDNA in the brain, liver, kidney, and tail tissues of 96% (24/25) of the adult clones. When the proportion of donor mtDNA in each tissue was measured by allele-specific quantitative PCR and subjected to ANOVA analysis, a tissue-specific mtDNA segregation pattern ($P < 0.05$) was observed, with the liver containing the highest proportion of donor mtDNA. Therefore, the donor mtDNA was inherited consistently by the cloned offspring, whereas donor mtDNA segregation was not neutral, which is in accordance with previous notions about tissue-specific nuclear control of mtDNA segregation. genesis 39:79–83, 2004. © 2004 Wiley-Liss, Inc.

Key words: mitochondrial DNA; cloning; mouse; embryo; somatic cell

The production of animals by somatic cell cloning is now possible in several mammalian species, including sheep, mice, cattle, goats, pigs, cats, rabbits, mules, and horses (Wilmut *et al.*, 2002; Galli *et al.*, 2003; Woods *et al.*, 2003). The derived clones are considered genetic duplicates of the donor nuclear genome that was used for the nuclear transfer. However, since the recipient oocytes contain 10^2 to 10^3 times higher copy numbers of mtDNA, as compared to the donor cells, the resultant cloned animals are assumed to be transmitochondrial, i.e., having nuclear and mitochondrial genomes of different origins. Evans *et al.* (1999) analyzed mtDNA inheritance in Dolly the sheep, derived from an adult somatic cell line, and in nine sheep that were derived from fetal cells, and found that the mtDNAs in these clones were exclusively oocytic in origin. This result indicates that the donor mtDNA is eliminated by unknown mechanisms after nuclear transfer, and that only the mtDNA from oocytes proliferates in cloned animals. However, contrasting results were obtained when more sensitive polymerase chain reaction (PCR) methods were employed for the detection of mtDNA. Steinborn *et al.* (2000) revealed that the donor mtDNA could be detected in seven out of ten somatically cloned cattle produced by electrofusion, with the donor mtDNA comprising 0.4–4% of the total number of mtDNA copies, which indicates that mtDNA heteroplasmy occurs in the majority of cloned animals. They estimated that the ratio of donor cells to recipient cytoplast mtDNAs before nuclear transfer was in the range of 0.4–0.8%. Thus, it appears that the levels of donor mtDNAs are maintained throughout clone development, whereas they may increase or decrease at certain times during development. However, more recent analyses by other researchers using bovines have added further layers of complexity to this topic by describing a variety of potential donor mtDNA fates, e.g., neutral segregation (Steinborn *et al.*, 2002; Hiendleder *et al.*, 2003), significant reductions (Meirelles *et al.*, 2001), and significant increases (Takeda *et al.*, 2003). These discrepancies may be due, at least in part, to the preexisting heteroplasmy in recipient oocytes (Takeda *et al.*, 2003), the types of donor cell used, and the tissues examined.

Since the initial success of mouse somatic cell cloning in 1998 (Wakayama *et al.*, 1998), this experimental system has provided valuable information on the phenotypes and gene expression profiles that are specific to cloned embryos/animals (Ogura *et al.*, 2001). The biological advantages of laboratory mice over domestic species, which include the availability of well-characterized

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The Novel Dominant Mutation *Dspd* Leads to a Severe Spermiogenesis Defect in Mice[1]

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ABSTRACT

Spermiogenesis is a complex process that is regulated by a plethora of genes and interactions between germ and somatic cells. Here we report a novel mutant mouse strain that carries a transgene insertional/translocational mutation and exhibits dominant male sterility. We named the mutation dominant spermiogenesis defect (*Dspd*). In the testes of *Dspd* mutant mice, spermatids detached from the seminiferous epithelium at different steps of the differentiation process before the completion of spermiogenesis. Microinsemination using spermatids collected from the mutant testes resulted in the birth of normal offspring. These observations indicate that the major cause of *Dspd* infertility is (are) a defect(s) in the Sertoli cell-spermatid interaction or communication in the seminiferous tubules. Fluorescent in situ hybridization (FISH) analysis revealed a translocation between chromosomes 7F and 14C at the transgene insertion site. The deletion of a genomic region of chromosome 7F greater than 1 megabase and containing at least six genes (*Cttn, Fadd, Fgf3, Fgf4, Fgf15,* and *Ccnd1*) was associated with the translocation. *Cttn* encodes the actin-binding protein cortactin. Immunohistochemical analysis revealed localization of cortactin beside elongated spermatids in wild-type testes; abnormality of cortactin localization was found in mutant testes. These data suggest an important role of cortactin in Sertoli cell-spermatid interactions and in the *Dspd* phenotype.

sertoli cells, spermatid, spermatogenesis

INTRODUCTION

Human infertility is observed in 10–15% of couples and the contributing factors are attributed to males and females equally. Spermatogenic arrest is the major cause of male infertility [1, 2]. The most common cause of spermatogenic arrest identified genetically is a microdeletion of the Y chromosome; however, this deletion accounts for less than 5% of male infertility [3]. There are likely many other autosomal genes and loci that affect spermatogenesis, but only a limited number have been identified.

The random integration of foreign DNA during the production of transgenic mice often leads to genomic rearrangements. Nakanishi et al. reported that chromosomal translocation was observed in about 5% of their EGFP transgenic lines [4]. Translocation is often accompanied by a genomic deletion that can vary enormously in size. It has not been easy to determine the size of deletions, but recently improved mouse genome databases that contain information on single nucleotide polymorphisms (SNPs) provide platforms for the quick size determination of even massive deletions.

A number of mutant mice showing abnormalities in spermatogenesis, including some insertional mutants [5–7], have provided important models for male infertility [3]. However, much remains to be understood. One of the obstacles to the analysis of spermatogenesis is that the spermatogenic process has not been reproduced in vitro [8]. The interaction between somatic Sertoli cells and germ cells is an important factor in spermatogenesis, making it difficult to reconstitute spermatogenesis in vitro. Sertoli cells are multifunctional nurse cells that provide nutritional and physical support for spermatogenic cells throughout sperm development. The Sertoli cell-spermatid interaction in particular is thought essential for the formation of a normal sperm head and for the release of sperm at the correct time. Filamentous (F-) actin plays an important role in Sertoli cell-spermatid interactions. The ectoplasmic specialization (ES) [9], a specialized junctional structure between Sertoli cells and elongated spermatids after step 8, contains an actin layer [10, 11]. The ES is damaged by treatment with the actin-disrupting drug cytochalasin D [12]. Mice treated with bisphenol-A or β-estradiol 3-benzoate exhibit an ab-

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—Original—

Microinsemination with First-Wave Round Spermatids from Immature Male Mice

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Abstract. In several mammalian species, including mice, round spermatids have been used to produce normal offspring by means of microinsemination techniques. In this study, we examined whether mouse round spermatids retrieved from immature testes undergoing the first wave of spermatogenesis had acquired fertilizing ability comparable to cells from mature adults. Microinsemination with round spermatids was performed by direct injection into preactivated oocytes, as previously reported. About 60–85% of the successfully injected oocytes developed to the morula/blastocyst stage after 72 h in culture, irrespective of the age of the males (17–25 days old). After embryo transfer, normal pups were obtained from all age groups, including the day-17 group, the stage at which the first round spermatids appeared. A high correlation ($r=0.90$) was found between the birth rate and male age ($P<0.01$, Spearman rank correlation), indicating that the efficiency of producing offspring was dependent on the age of the donor males. Imprinted genes (*H19*, *Igf2*, *Meg3*, and *Igf2r*) were expressed from the correct parental alleles (maternal, paternal, maternal, and maternal, respectively) in all (n=12) day-9.5 fetuses derived from day-20 spermatids. These results clearly indicate that at least some first-wave spermatogenic cells have a normal haploid genome with the correct paternal imprint and are capable of supporting full-term embryo development, as do mature spermatozoa from adults. The use of male germ cells from immature animals may save time in the production of inbred/congenic strains and rescue male-factor infertility of early onset.
Key words: Microinsemination, Spermatogenesis, Spermatid, Genomic imprinting, Mouse

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Microinsemination, also called intracytoplasmic sperm injection (ICSI), is a technique that is used to deliver spermatozoa directly into oocytes with micromanipulation devices. This technique has provided invaluable information on several biological and molecular aspects of mammalian fertilization that could never be achieved by conventional *in vitro* fertilization (IVF). With ICSI, it was first demonstrated that sperm-egg membrane fusion can be bypassed for normal fertilization and subsequent embryo development [1–3]. With recent technical advances, immature sperm cells (spermatogenic cells) at certain stages in the testes have also been used to construct

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Oligo-astheno-teratozoospermia in mice lacking Cnot7, a regulator of retinoid X receptor beta

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Spermatogenesis is a complex process that involves cooperation of germ cells and testicular somatic cells. Various genetic disorders lead to impaired spermatogenesis, defective sperm function and male infertility[1]. Here we show that *Cnot7*$^{-/-}$ males are sterile owing to oligo-astheno-teratozoospermia, suggesting that Cnot7, a CCR4-associated transcriptional cofactor[2], is essential for spermatogenesis. Maturation of spermatids is unsynchronized and impaired in seminiferous tubules of *Cnot7*$^{-/-}$ mice. Transplantation of spermatogonial stem cells from male *Cnot7*$^{-/-}$ mice to seminiferous tubules of *Kit* mutant mice (*Kit*$^{W/W\text{-}v}$) restores spermatogenesis, suggesting that the function of testicular somatic cells is damaged in the *Cnot7*$^{-/-}$ condition. The testicular phenotypes of *Cnot7*$^{-/-}$ mice are similar to those of mice deficient in retinoid X receptor beta (*Rxrb*)[3]. We further show that Cnot7 binds the AF-1 domain of Rxrb and that Rxrb malfunctions in the absence of Cnot7. Therefore, Cnot7 seems to function as a coregulator of Rxrb in testicular somatic cells and is thus involved in spermatogenesis.

Genetic analyses in yeast suggest that CAF1, a component of the CCR4-NOT complex, has multiple roles in regulating transcription[4]. In addition, proteins in the CCR4-NOT complex are involved in mRNA metabolism in yeast[5,6]. Two mammalian homologs of yeast CAF1 are known: Cnot7 (also called CAF1) and Cnot8 (also called CALIF)[7,8]. Both are expressed in a variety of tissues, with relatively high expression of Cnot7 in lung, liver and thyroid gland[7,8]. Cnot7 interacts with members of the TOB-BTG antiproliferative family, which comprises Tob1, Tob2, Btg1, Btg2 (also called PC3 and TIS21), Btg3 (also called ANA) and Btg4 (also called PC3B; refs. 7,9,10). The proteins of this family are implicated in regulation of transcription[11–14].

To elucidate the physiological role of mammalian Cnot7, we produced mutant mice lacking the gene *Cnot7* by means of homologous recombination (**Fig. 1a–d**). Homozygous *Cnot7* knockout (*Cnot7*$^{-/-}$) mice were alive at birth and born at the predicted mendelian frequency. Adult *Cnot7*$^{-/-}$ mice had normal health, size and behavior, except that male *Cnot7*$^{-/-}$ mice were sterile. *Cnot7*$^{+/-}$ males had normal fertility and *Cnot7*$^{-/-}$ females produced average-size litters (6.3 ± 2.1 offspring per litter; $n = 14$ litters).

There were no gross anatomical differences in the seminal vesicles and prostates among *Cnot7*$^{+/+}$, *Cnot7*$^{+/-}$ and *Cnot7*$^{-/-}$ males, but the testes of *Cnot7*$^{-/-}$ mice were smaller than those of *Cnot7*$^{+/+}$ or *Cnot7*$^{+/-}$ mice (**Fig. 1e**). *Cnot7*$^{-/-}$ mice produced only 7% as much sperm as *Cnot7*$^{+/+}$ or *Cnot7*$^{+/-}$ mice (**Fig. 1f**), and their spermatozoa beat less vigorously and generated less forward momentum (**Fig. 1g**). Almost all spermatozoa from *Cnot7*$^{-/-}$ mice had irregularly shaped heads, abnormally arranged mitochondria and erroneously attached flagella (**Fig. 1h,i**). Electron microscopic analysis detected ultrastructural components, such as condensed chromatin, acrosomes and flagella, including axoneme, mitochondrial sheath, outer dense fibers and fibrous sheath, in spermatozoa from *Cnot7*$^{-/-}$ mice. But their arrangements were irregular and maturation of sperm was abnormal (**Fig. 1i**). The degree of morphological irregularity varied: spermatozoa of *Cnot7*$^{-/-}$ mice were round-headed (73%), tapered-headed (20%), symplast (5%) or nearly normal (2%). We also observed malformation of spermatids in the seminiferous tubules (see **Fig. 3d**). Taken together, these data indicate that oligo-astheno-teratozoospermia (low sperm number and motility and abnormal sperm morphology) underlies the sterility of *Cnot7*$^{-/-}$ males.

To further analyze sperm competence for fertilization, we carried out *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) experiments. For the IVF experiment, we freed oocytes from cumulus cells and the zona pellucida, because epididymal spermatozoa of *Cnot7*$^{-/-}$ mice had poor motility and were unable to penetrate these egg layers. Even under these experimental conditions, spermatozoa of *Cnot7*$^{-/-}$ mice hardly fertilized oocytes, whereas *Cnot7*$^{+/-}$ spermatozoa had normal fertilizing ability (**Table 1**). Direct injection of

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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.

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Generation of Pluripotent Stem Cells from Neonatal Mouse Testis

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Summary

Although germline cells can form multipotential embryonic stem (ES)/embryonic germ (EG) cells, these cells can be derived only from embryonic tissues, and such multipotent cells have not been available from neonatal gonads. Here we report the successful establishment of ES-like cells from neonatal mouse testis. These ES-like cells were phenotypically similar to ES/EG cells except in their genomic imprinting pattern. They differentiated into various types of somatic cells in vitro under conditions used to induce the differentiation of ES cells and produced teratomas after inoculation into mice. Furthermore, these ES-like cells formed germline chimeras when injected into blastocysts. Thus, the capacity to form multipotent cells persists in neonatal testis. The ability to derive multipotential stem cells from the neonatal testis has important implications for germ cell biology and opens the possibility of using these cells for biotechnology and medicine.

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Introduction

Germ cells are unique in that they have the capacity to contribute genes to offspring. Although germ cells are highly specialized cells for the generation of gametes, several lines of evidence suggest their multipotentiality. For example, teratomas are tumors containing many kinds of cells and tissues at various stages of maturation, which occur almost exclusively in the gonads (Stevens, 1984). Furthermore, primordial germ cells (PGCs) from embryos between 8.5 and 12.5 days postcoitum (dpc) give rise to pluripotent cells when cultured under appropriate conditions (Resnick et al., 1992; Matsui et al., 1992). These EG cells have differentiation properties similar to ES cells isolated from inner cell mass (Martin, 1981; Evans and Kaufman, 1981). While these observations suggest that the germline lineage retains the ability to generate pluripotent cells, it has not been possible to establish pluripotent cells from normal neonatal gonads (Labosky et al., 1994).

We recently reported the in vitro culture of mouse spermatogonial stem cells (Kanatsu-Shinohara et al., 2003a), the only type of stem cell in the body that transmits genetic information to offspring (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). When neonatal testis cells were cultured in the presence of glial cell line-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF), the germ cells developed uniquely shaped colonies, and the stem cells proliferated logarithmically over a 5 month period. Upon transplantation into the seminiferous tubules of infertile mice, the cultured cells produced normal sperm and offspring, and neither somatic differentiation nor teratoma formation was observed, indicating that the cultured cells were fully committed to the germ cell lineage (Kanatsu-Shinohara et al., 2003a). This was in contrast to ES cells, which produced teratoma after being transferred into seminiferous tubules (Brinster and Avarbock, 1994). Based on these results, we named these cells germline stem (GS) cells to distinguish them from ES or EG cells. Thus, GS cells represent a third method of expanding germline cells, but they are clearly distinct from ES/EG cells in their differentiation capacity.

In this manuscript, we describe the derivation of pluripotent stem cells from the neonatal mouse testis. Neonatal testis cells were cultured in conditions similar to those used for GS cell culture. In addition to the GS cell colonies, colonies indistinguishable from ES cell colonies appeared. This second cell type could be expanded selectively under culture conditions used for ES cells. Although they produced teratomas when transplanted subcutaneously or into the seminiferous tubules of the testis, they participated in normal embryonic development following injection into blastocysts.

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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.

DNA RESEARCH 11, 371–379 (2004)

A Mutation in the Serum and Glucocorticoid-Inducible Kinase-Like Kinase (*Sgkl*) Gene is Associated with Defective Hair Growth in Mice

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Abstract

YPC is a mutant mouse strain with defective hair growth characterized by thin, short hairs and poorly developed hair bulbs and dermal papillae. To identify the gene associated with the phenotype, we performed genome-wide linkage analysis using 1010 backcross progeny and 123 microsatellite markers covering all chromosomes. The mutant locus (*ypc*) was mapped to a 0.2-cM region in the proximal part of mouse chromosome 1. This 0.2-cM region corresponds to a 450-kb region of genome sequence that contains two genes with known functions and five ESTs or predicted genes with unknown functions. Sequence analysis revealed a single C-to-A nucleotide substitution at nucleotide 1382 in the *Sgkl* gene, causing a nonsense mutation at codon 461. *Sgkl* encodes serum and glucocorticoid-inducible kinase-like kinase (SGKL), which belongs to a subfamily of serine/threonine protein kinases and has been suggested to have a role downstream of lipid signals produced by activation of phosphoinositide 3-kinase (PI3K). In the mutant SGKL, a serine residue in the C-terminal end of the protein (Ser486), which is indispensable for activation of SGKL upon phosphorylation, is abolished by premature termination. Specific expression of the *Sgkl* gene in the inner root sheath of growing hair follicles was also identified by *in situ* hybridization. Therefore, we concluded that the nucleotide substitution in the *Sgkl* gene is the causative mutation for defective hair growth in the *ypc* mutant mouse and that the signaling pathway involving SGKL plays an essential role in mammalian hair development.

Key words: Hair follicle; SGKL/SGK3/CISK; WNT signaling; Mutant mouse; IRS

1. Introduction

Hair follicle morphogenesis and the hair growth cycle are complex processes dependent on a series of mesenchymal-epithelial interactions in skin.[1] Reciprocal exchange of signals between dermal and epidermal cells of skin regulates the formation of hair placodes during embryonic development, and it also regulates cyclic transformation of the growth (anagen), regression (catagen), and quiescent (telogen) phases in the hair cycle in adult skin. As these processes show a high degree of organization and self-renewal, hair follicle development and hair cycling are thought to be excellent models for investigating the molecular mechanisms of mesenchymal-epithelial interactions.

Numerous growth factors and cytokines have been shown to be involved in morphogenesis and cycling of hair follicles. WNT,[2,3] TGFα,[4,5] BMPs,[6,7] and FGFs[8] in particular, as well as their signal transduction molecules,[9,10] play essential roles in these processes. Experiments with transgenic mice or those with knockout mutations in these genes have demonstrated a number of abnormalities in morphogenesis and cycling of hair follicles, including a short-hair phenotype and cyclical balding in transgenic mice overexpressing the *Wnt3* gene in skin[3] and abnormally long hair in *Fgf5* knockout mice, which is caused by defective regulation of the hair cycle.[8] On the other hand, spontaneous mutant mouse strains showing abnormalities in hair morphogenesis have also provided useful information on the molecular mechanisms of these processes. For example, the hairless (*hr*) mutant, which

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Cytoplasmic Asters Are Required for Progression Past the First Cell Cycle in Cloned Mouse Embryos[1]

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ABSTRACT

Unlike the oocytes of most other animal species, unfertilized murine oocytes contain cytoplasmic asters, which act as microtubule-organizing centers following fertilization. This study examined the role of asters during the first cell cycle of mouse nuclear transfer (NT) embryos. NT was performed by intracytoplasmic injection of cumulus cells. Cytoplasmic asters were localized by staining with an anti-α-tubulin antibody. Enucleation of MII oocytes caused no significant change in the number of cytoplasmic asters. The number of asters decreased after transfer of the donor nuclei into these enucleated oocytes, probably because some of the asters participated in the formation of the spindle that anchors the donor chromosomes. The cytoplasmic asters became undetectable within 2 h of oocyte activation, irrespective of the presence or absence of the donor chromosomes. After the standard NT protocol, a spindle-like structure persisted between the pseudopronuclei of these oocytes throughout the pronuclear stage. The asters reappeared shortly before the first mitosis and formed the mitotic spindle. When the donor nucleus was transferred into preactivated oocytes (delayed NT) that were devoid of free asters, the microtubules and microfilaments were distributed irregularly in the ooplasm and formed dense bundles within the cytoplasm. Thereafter, all of the delayed NT oocytes underwent fragmentation and arrested development. Treatment of these delayed NT oocytes with Taxol, which is a microtubule-assembling agent, resulted in the formation of several aster-like structures and reduced fragmentation. Some Taxol-treated oocytes completed the first cell cycle and developed further. This study demonstrates that cytoplasmic asters play a crucial role during the first cell cycle of murine NT embryos. Therefore, in mouse NT, the use of MII oocytes as recipients is essential, not only for chromatin reprogramming as previously reported, but also for normal cytoskeletal organization in reconstructed oocytes.

developmental biology, early development, embryo, gamete biology, ovum

INTRODUCTION

Although somatic cell cloning has been performed successfully in several mammalian species, it has emerged from recent studies that the biological factors and technical issues that affect the efficiency of cloning differ from species to species. For example, the timing of nuclear transfer (NT) and oocyte activation has a major impact on the outcome of the cloning procedure. In livestock (cattle, sheep, swine, and goats) animals, this timing seems to be relatively flexible as compared with mice. In goats and sheep, preactivated oocytes have been used for NT, leading to the production of normal offspring [1, 2]. In cattle, although the use of MII oocytes as recipients is known to support the optimal in vitro development of reconstructed embryos, at least some of the embryos that are derived from preactivated oocytes undergo preimplantation development [3]. In contrast, in mice the use of MII oocytes is critical for reconstructed embryos to complete the first cell cycle [4]. Even oocytes that receive the donor nucleus 1–2 h after activation inevitably arrest their development during the S phase of the pronuclear stage and undergo severe fragmentation [5]. This is one of the major obstacles to mouse cloning, since murine oocytes may be activated accidentally during handling in vitro (e.g., during enucleation) before NT. Previously, it has been demonstrated that the use of MII oocytes in NT is critical for the transferred donor nuclei to be able to reprogram their chromatin structures and initiate zygotic gene activation (ZGA) according to the normal schedule [6]. However, the major round of ZGA occurs during the second cell cycle (two-cell stage) in the mouse [7], which makes it very unlikely that incomplete genomic reprogramming causes the severe fragmentation seen in delayed NT oocytes during the first cell cycle.

In unfertilized murine oocytes, the microtubule-organizing centers (MTOCs), which comprise the so-called cytoplasmic asters (cytoasters), play central roles in the apposition of the male and female pronuclei and in centrosomal inheritance of cleavage stage–embryos [8]. In most animals other than the mouse, the centrosomes are inherited mainly from the fertilizing spermatozoa, from which the MTOC is organized (reviewed in [9]). Therefore, it is possible that the interactions that occur between microtubules and chromosomes during the reconstruction and first cell cycle of cloned embryos differ between mice and other animals. The present study was undertaken to determine 1) the roles of cytoplasmic asters during the first cell cycle of cloned murine embryos; and 2) the effects of NT timing on aster behavior, which may be related to the embryo fragmentation that is observed specifically in delayed NT murine oocytes.

MATERIALS AND METHODS

Culture Media

The oocytes were cultured in bicarbonate-buffered potassium simplex optimized medium (KSOM) that was supplemented with 0.1 mg/ml poly-

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The *Sall3* locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice

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DNA methylation controls various developmental processes by silencing, switching and stabilizing genes as well as remodeling chromatin. Among various symptoms in cloned animals, placental hypertrophy is commonly observed. We identified the *Spalt-like gene3* (*Sall3*) locus as a hypermethylated region in the placental genome of cloned mice. The *Sall3* locus has a CpG island containing a tissue-dependent differentially methylated region (T-DMR) specific to the trophoblast cell lineage. The T-DMR sequence is also conserved in the human genome at the *SALL3* locus of chromosome 18q23, which has been suggested to be involved in the 18q deletion syndrome. Intriguingly, larger placentas were more heavily methylated at the *Sall3* locus in cloned mice. This epigenetic error was found in all cloned mice examined regardless of sex, mouse strain and the type of donor cells. In contrast, the placentas of *in vitro* fertilized (IVF) and intracytoplasmic sperm injected (ICSI) mice did not show such hypermethylation, suggesting that aberrant hypermethylation at the *Sall3* locus is associated with abnormal placental development caused by nuclear transfer of somatic cells. We concluded that the *Sall3* locus is the area with frequent epigenetic errors in cloned mice. These data suggest that there exists at least genetic locus that is highly susceptible to epigenetic error caused by nuclear transfer.

Introduction

Most cells of higher eukaryotes differentiate without changing DNA sequence. Cells differentiate into specific types by activation and inactivation of particular sets of genes. DNA methylation is involved in various biological phenomena (Bird 2002; Li 2002) such as cell differentiation (Takizawa *et al.* 2001), X chromosome inactivation (Norris *et al.* 1991), genomic imprinting (Stoger *et al.* 1993), heterochromatin formation (Jones *et al.* 1998) and tumorgenesis (Issa *et al.* 1994).

Mammalian cloning using adult somatic cells has been successful in several species (Renard *et al.* 2002; Wilmut *et al.* 2002). Cloned offspring develop a variety of abnormal phenotypes such as increased body weight (large fetus syndrome), pulmonary hypertension, placental overgrowth, respiratory problems and early death (Lanza *et al.* 2000; Hill *et al.* 2000; Tamashiro *et al.* 2000; Tanaka *et al.* 2001; Ogonuki *et al.* 2002). This suggests a disruption of the normal developmental program. On this basis we expected and have identified several aberrantly methylated loci in the tissues of full-term cloned fetuses (Ohgane *et al.* 2001). Interestingly, each cloned animal has a different DNA methylation pattern and the extent of hyper- or hypo-methylation varies among the individuals. Cloned embryos at blastocyst or earlier developmental stages were reported to have unusual DNA methylation patterns at both repetitive and single copy gene regions (Santos *et al.* 2002; Bourc'his *et al.* 2001; Kang *et al.* 2001, 2002). Cloned fetuses of later developmental stages also showed aberrant DNA methylation at loci of imprinted and X-chromosomal genes compared with control fetuses (Humpherys *et al.* 2001; Xue *et al.*

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Generation of Pluripotent Stem Cells from Neonatal Mouse Testis

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Summary

Although germline cells can form multipotential embryonic stem (ES)/embryonic germ (EG) cells, these cells can be derived only from embryonic tissues, and such multipotent cells have not been available from neonatal gonads. Here we report the successful establishment of ES-like cells from neonatal mouse testis. These ES-like cells were phenotypically similar to ES/EG cells except in their genomic imprinting pattern. They differentiated into various types of somatic cells in vitro under conditions used to induce the differentiation of ES cells and produced teratomas after inoculation into mice. Furthermore, these ES-like cells formed germline chimeras when injected into blastocysts. Thus, the capacity to form multipotent cells persists in neonatal testis. The ability to derive multipotential stem cells from the neonatal testis has important implications for germ cell biology and opens the possibility of using these cells for biotechnology and medicine.

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Introduction

Germ cells are unique in that they have the capacity to contribute genes to offspring. Although germ cells are highly specialized cells for the generation of gametes, several lines of evidence suggest their multipotentiality. For example, teratomas are tumors containing many kinds of cells and tissues at various stages of maturation, which occur almost exclusively in the gonads (Stevens, 1984). Furthermore, primordial germ cells (PGCs) from embryos between 8.5 and 12.5 days postcoitum (dpc) give rise to pluripotent cells when cultured under appropriate conditions (Resnick et al., 1992; Matsui et al., 1992). These EG cells have differentiation properties similar to ES cells isolated from inner cell mass (Martin, 1981; Evans and Kaufman, 1981). While these observations suggest that the germline lineage retains the ability to generate pluripotent cells, it has not been possible to establish pluripotent cells from normal neonatal gonads (Labosky et al., 1994).

We recently reported the in vitro culture of mouse spermatogonial stem cells (Kanatsu-Shinohara et al., 2003a), the only type of stem cell in the body that transmits genetic information to offspring (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). When neonatal testis cells were cultured in the presence of glial cell line-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF), the germ cells developed uniquely shaped colonies, and the stem cells proliferated logarithmically over a 5 month period. Upon transplantation into the seminiferous tubules of infertile mice, the cultured cells produced normal sperm and offspring, and neither somatic differentiation nor teratoma formation was observed, indicating that the cultured cells were fully committed to the germ cell lineage (Kanatsu-Shinohara et al., 2003a). This was in contrast to ES cells, which produced teratoma after being transferred into seminiferous tubules (Brinster and Avarbock, 1994). Based on these results, we named these cells germline stem (GS) cells to distinguish them from ES or EG cells. Thus, GS cells represent a third method of expanding germline cells, but they are clearly distinct from ES/EG cells in their differentiation capacity.

In this manuscript, we describe the derivation of pluripotent stem cells from the neonatal mouse testis. Neonatal testis cells were cultured in conditions similar to those used for GS cell culture. In addition to the GS cell colonies, colonies indistinguishable from ES cell colonies appeared. This second cell type could be expanded selectively under culture conditions used for ES cells. Although they produced teratomas when transplanted subcutaneously or into the seminiferous tubules of the testis, they participated in normal embryonic development following injection into blastocysts.

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Birth of mice after in vitro fertilization using C57BL/6 sperm transported within epididymides at refrigerated temperatures

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Abstract

The transportation of cryopreserved spermatozoa is an economical, efficient, and safe method for the distribution of mouse strains from one facility to another. However, spermatozoa from some strains, including C57BL/6 (B6), are very sensitive to freezing and thawing and frequently fail to fertilize eggs by conventional in vitro fertilization methods at the recipient mouse facility. Since many genetically engineered mice have the B6 genetic background, this sensitivity poses a major obstacle to studies of mouse genetics. We investigated the feasibility of transporting spermatozoa within epididymides under non-freezing conditions. First, we examined the interval that B6 and B6D2F1 (BDF1) spermatozoa retained their ability to fertilize when stored within epididymides at low temperatures (5 °C or 7 °C). Fertilization rates were >50%, irrespective of the spermatozoa used, when epididymides were stored for 3 d at 7 °C. B6 spermatozoa, but not BDF1 sperm, had better retention of fertilizing ability at 7 °C versus 5 °C. We then transported freshly collected B6 and BDF1 epididymides from a sender colony to a recipient colony using a common package delivery service, during which the temperature was maintained at 5 °C or 7 °C for 2 d. Sufficiently high fertilization rates (68.0–77.5%) were obtained for all experimental groups, except for B6 spermatozoa transported at 5 °C. These spermatozoa were successfully cryopreserved at the recipient facility and, yielded post-thaw fertilization rates of 27.6–66.4%. When embryos derived from the B6 spermatozoa that

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LETTER

Birth of Mice Produced by Germ Cell Nuclear Transfer

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Summary: That mammals can be cloned by nuclear transfer indicates that it is possible to reprogram the somatic cell genome to support full development. However, the developmental plasticity of germ cells is difficult to assess because genomic imprinting, which is essential for normal fetal development, is being reset at this stage. The anomalous influence of imprinting is corroborated by the poor development of mouse clones produced from primordial germ cells (PGCs) during imprinting erasure at embryonic day 11.5 or later. However, this can also be interpreted to mean that, unlike somatic cells, the genome of differentiated germ cells cannot be fully reprogrammed. We used younger PGCs (day 10.5) and eventually obtained four full-term fetuses. DNA methylation analyses showed that only embryos exhibiting normal imprinting developed to term. Thus, germ cell differentiation is not an insurmountable barrier to cloning, and imprinting status is more important than the origin of the nucleus donor cell per se as a determinant of developmental plasticity following nuclear transfer. genesis 41:81–86, 2005. © 2005 Wiley-Liss, Inc.

Key words: nuclear transfer; cloning; mouse; primordial germ cell; genomic imprinting

Since the birth of the sheep Dolly, the first eutherian mammal cloned from an adult cell, successful cloning using somatic cell nuclear transfer has been reported for the mouse, bovine, goat, pig, cat, rabbit, mule, horse, and rat (for review, see Tamada and Kikyo, 2004). Although the efficiency of cloning is low, it appears that at least some somatic cell genomes can acquire totipotency following transfer into enucleated oocytes. Somatic cell cloning is now being applied extensively to the production of clones of individuals, and to the generation of animals with genetic modifications for agricultural and pharmaceutical purposes. However, little is known about the developmental plasticity of the genome of the germ cell lineage. Only midgestation fetuses have been obtained from mouse primordial germ cells (PGCs), leading some to invoke "hemipotency" of the genome in the germ cell lineage (Kato *et al.*, 1999; Lee *et al.*, 2002; Yamazaki *et al.*, 2003). This limited efficiency of development of embryos cloned from PGCs may be attributed to the low plasticity of the germ cell genome attained during germ cell differentiation, or to erasure of the genomic imprinting "memory," known to occur in PGCs at embryo gestational day 11.5 in the mouse (Lee *et al.*, 2002).

Genomic imprinting in eutherian mammals is an epigenetic mechanism that ensures parent-allele-specific expression in some genes (imprinted genes) and plays essential roles in development and adult behavior (Reik and Walter, 2001; Li *et al.*, 1999). Imprinting "memories" are erased during early germ cell development and then reestablished in a parent-specific manner, depending on the sex of the individual. We previously examined the expression pattern of imprinted genes in fetuses cloned from fetal PGCs at embryo gestational days 11.5–13.5 (assuming the morning of the postcopulatory plug as day 0.5), and found that loss of their monoallelic expression proceeded stepwise from day 11.5 and was completed by day 12.5 (Lee *et al.*, 2002). Fetuses cloned from PGCs at days 12.5–13.5 arrested their development around day 8.5 in recipient foster mothers, as has been reported for PGCs at days 14.5–16.5 (Kato *et al.*, 1999). Those from nuclei of PGCs at day 11.5 developed furthest (to day 11.5), although they exhibited marked variation in their developmental potential, presumably reflecting the dynamic process of imprinting erasure in donor nuclei (Lee *et al.*, 2002). To extend this work, we created clones using PGCs from embryos at days 10.5 and 11.5 and examined whether such differentiated germ cell genomes could be fully reprogrammed by nuclear transfer. To ensure the accurate identification of PGCs, we used transgenic mice expressing green fluorescent protein (GFP) driven by the PGC-restricted *Oct-4* promoter (Yeom *et al.*,

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遺伝的モニタリング

Genetic monitoring of animal models for human diseases

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Hideki KATOH

◎われわれの身のまわりにあるすべての商品には品質規格が設けられている．実験動物といえども商品であるかぎり品質が問われる．実験動物の生産販売会社から市販されているマウスやラットに対して，消費者であるユーザー，研究者が高い品質を要求することは当然のことであろう．市販されている実験動物は個々の個体が評価されるのではなく，実験に使用され，得られるデータをもって評価されるのである．結果が出てから使われた個体の不適切さを知ってはすでに遅い．

Key word　マウス，遺伝的汚染，プロファイル，遺伝子操作

遺伝的汚染が過去に起こっている

1982年，アメリカの科学者があるブリーダーから購入していたBALB/c系統を使った実験に再現性がなくなったとし，その系統を調べたところ，ほかの系統との間で起こった遺伝的汚染によることを科学雑誌『*Science*』で明らかにした[1,2]．近交系が遺伝的に汚染するということは，その系統が本来もつ遺伝子型（ゲノム構成）が大きく変化するということであり，その系統が本来示すべき種々の特性（免疫応答性，自然発癌率，薬物に対する反応性など）を失うことを意味する．遺伝的要因によって強く規定される系統の特性は系統の品質とほぼ同義であるといってよく，遺伝的汚染によって系統の品質は損なわれると考える必要がある．

遺伝学的モニタリングの本来の目的は，マウス，ラットの近交系が上記のような遺伝的汚染もなく，正しく維持・生産されていることを定期的に確認し，その系統の遺伝的品質を保証することである．実験動物もいまや製品（商品）と認識すべきで，品質および品質検査のあり方が本格的に問われる時代になってきたことを本稿で知っていただきたい．

遺伝子改変マウスは遺伝的汚染と同じ状況にある

今日，遺伝子改変マウス系統が多数作出されており，それらに遺伝的汚染に匹敵する現象が観察される．たとえば，BDF1はC57BL/6とDBA/2の雑種第一代（F1）であるが，交配を続けて得られる2～3代目の個体では2系統のゲノムが混じった状態になる[3]．129，B6CBF1由来のES細胞を使用するノックアウト，ジーントラップについても同様で，まさに遺伝的汚染時にみられる現象と同じである．たとえば，*H2*遺伝子に注目した場合，ある個体ではC57BL/6のハプロタイプ*b*をホモ型にもち，他の個体ではDBA/2のハプロタイプ*d*をホモ型にもち，別の個体ではヘテロ型であるなど，個体間の遺伝的不均一性が観察される．異なる遺伝子型の発現環境の下で遺伝子の発現に個体差が現れることはよく知られており[4]，場合によっては実験結果に不都合を生じる．これを解決する一般的な方法は，操作した遺伝子を適切な近交系に入れ直し，コンジェニック系統として確立することである[5]．

標識遺伝子が遺伝的品質を保証する

C57BL/6をはじめとして多数の近交系が実験動

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A Genetic Quality Testing System for Early Stage Embryos in the Mouse

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Abstract: *We have established a genetic quality testing system for early stage embryos of the mouse. A method of preparation of template DNA for PCR was established using the lysis buffer (1 × PCR reaction buffer supplemented with proteinase K at a concentration of 40 μg/ml) developed by the authors. We demonstrated that two 8-cell embryos of an inbred strain provide sufficient volumes of template DNA for PCR to identify the strain of embryos using four microsatellite markers (*D3Mit54, D5Mit18, D6Mit15 *and* D8Mit50*) differentiating 13 inbred strains of mice. This system will be useful in embryo banks that have recently been established worldwide for demonstrating the genetic accuracy of a given strain prior to recovery of live animals.*

Key words: *early embryos, embryo bank, genetic quality, microsatellite markers, mouse*

Introduction

Embryo banks have recently been established in Japan [1], the USA [8] and Europe [5]. Cryopreservation is only the method of preserving for long periods laboratory mouse strains developed by ordinary crosses and genetic engineering techniques. Also, transport of the frozen embryos and gametes (ova and sperm) has become common, because transport of live animals is more expensive and may lead to microbial contamination.

We must pay special attention to genetic contamination that will occur even in frozen embryos, no matter what preservation and transportation methods are used. Two possible causes of genetic contamination in embryo banks are: (1) mistakes related to labels on cryotubes, and (2) unexpected mixing of embryos of two unrelated strains by errors in experimental procedures. Based on experience to date [2, 3], measures against genetic contamination are required even after recovery of live animals. We need to establish genetic testing systems assuring genetic quality of embryos preserved in embryo banks.

Frozen embryos are usually recovered on demand. It takes about three weeks to obtain live animals from frozen embryos and another one month is required to certify genotypes of recovered animals, when we use ordinary methods of genetic monitoring on live animals [6]. However, it is preferable to perform genetic tests as soon as possible after the start of recovery procedures for live animals. The tests should be mainly

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