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Ⅳ．研究成果の刊行物・印刷

MOLECULAR REPRODUCTION AND DEVELOPMENT 70:464–470 (2005)

Birth of Offspring After Transfer of Mongolian Gerbil (*Meriones Unguiculatus*) Embryos Cryopreserved by Vitrification

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ABSTRACT The Mongolian gerbil (*Meriones unguiculatus*) has been used as a laboratory species in many fields of research, including neurology, oncology, and parasitology. Although the cryopreservation of embryos has become a useful means to protect valuable genetic resources, its application to the Mongolian gerbil has not yet been reported. In this study, we investigated the in vitro and in vivo developmental competence of Mongolian gerbil embryos cryopreserved by vitrification. In vivo-fertilized embryos were vitrified on the day of collection using the ethylene glycol (EG)-based solutions EFS20 and EFS40, which contained 20% and 40% EG, respectively, in PB1 containing 30% (w/v) Ficoll 70 and 0.5M sucrose. First, we compared one-step and two-step vitrification protocols. In the one-step method, the embryos were directly transferred into the vitrification solution (EFS40), whereas in the two-step method, the embryos were exposed serially to EFS20 and EFS40 and then vitrified. After liquefying (thawing), late two-cell embryos (collected on day 3) vitrified by the two-step method showed significantly better rates of in vitro development to the morula stage compared to those vitrified by the one-step method (65% vs. 5%, $P < 0.0001$). We then examined whether the same two-step method could be applied to early two-cell embryos (collected on day 2), four-cell embryos (day 4), morulae (day 5), and blastocysts (day 6). After liquefying, 87%–100% of the embryos were morphologically normal in all groups, and 23% and 96% developed to the compacted morula stage from early two- and four-cell embryos, respectively. After transfer into recipient females, 3% (4/123), 1% (1/102), 5% (4/73), and 10% (15/155) developed to full-term offspring from vitrified and liquefied early two-cell embryos, late two-cell embryos, morulae, and blastocysts, respectively. This demonstrates that Mongolian gerbil embryos can be safely cryopreserved using EG-based vitrification solutions. *Mol. Reprod. Dev. 70: 464–470, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: cryopreservation; ethylene glycol; embryo transfer

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*), also called the "laboratory gerbil", is a myomorph rodent that is native to China and Mongolia. It has been widely used as a laboratory animal in biomedical research, including the study of epilepsy (Jobe et al., 1991), tumor (Meckley and Zwicker, 1979), hypercholesterolemia (Dictenberg et al., 1995), and cerebral ischemia (Levine and Payan, 1966). This species has also been used to develop good animal models for a variety of infectious diseases caused by bacteria, viruses, and parasites; for example, *Helicobacter pylori* (Yokota et al., 1991; Sugiyama et al., 1998), Borna disease virus (Nakamura et al., 1999), *Echinococcus multilocularis* (Williams and Oriol, 1976), *Cryptospordium muris* (Koudela et al., 1998), *Brugia-pahangi* (Klei et al., 1981), *Giardia duodenalis* (Buret et al., 1991), and *Entamoeba histolytica* (Chadee and Meerovitch, 1984). Although gerbils were randomly bred in closed laboratory colonies for the first decades of their use, selective breeding has recently been conducted to establish laboratory strains that are suited for each research purpose. The best-characterized strains include seizure-sensitive and -resistant strains (Loskota et al., 1974; Robbins, 1976; Seto-Ohshima et al., 1997) and mutant strains with different coat colors (Robinson, 1973; Shimizu et al., 1990).

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For many domestic and laboratory species, assisted reproductive technologies have been developed to enhance live animal production and safe cryopreservation of genetic materials. However, the major assisted reproductive technologies—including in vitro fertilization, embryo culture, embryo transfer, and embryo cryopreservation—have not yet been established for Mongolian gerbils. This is a major drawback of working with Mongolian gerbils and it impedes their broad exploitation in biomedical research. The present study was undertaken to develop a reliable experimental protocol for cryopreservation of gerbil embryos. For this purpose, we employed embryo vitrification methods, which have been successfully used for mice (Kasai et al., 1990), rabbits (Kasai et al., 1992), cattle (Tachikawa et al., 1993), horses (Hochi et al., 1994), humans (Mukaida et al., 1998), mastomys (Mochida et al., 2001), and rats (Han et al., 2003). As little information is available concerning techniques related to embryo manipulation in gerbils, we also examined whether the protocols for superovulation and embryo transfer conventionally used for mice and rats could be applied to gerbils.

MATERIALS AND METHODS

Animals

Mongolian gerbils (*Meriones unguiculatus*) from inbred strains MGS/Sea (agouti coat color, Seac Yoshitomi, Ltd., Fukuoka, Japan) and MGB (black coat color, from the Nippon Medical School, Tokyo, Japan; Shimizu et al., 1990) were maintained under specific-pathogen-free conditions at the National Institute of Infectious Diseases, Japan. They were kept under controlled lighting conditions (light: 05:00–19:00) and provided with water and commercial laboratory mouse chow *ad libitum*. All animals were maintained and handled in accordance with the guidelines of the National Institute of Infectious Diseases, Japan. As the results obtained from different strains of gerbil were not significantly different, they were combined in this study.

Collection of Embryos

Mature females (7–18 weeks of age) were induced to superovulate by intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (PMSG) between 3 and 5 pm, followed by injection of 10 IU human chorionic gonadotrophin (hCG) 44–46 hr later. The superovulated females were mated with mature males in cages with wire net floors. The next morning (designated day 1 of pregnancy), the presence of a copulation plug was confirmed. The early two-cell embryos, late two-cell embryos, four-cell embryos, and morulae were collected by flushing the oviducts (at 48, 72, 96, 120 hr post-hCG) with modified phosphate buffered saline (PB1, Whittingham, 1971a); blastocysts were also collected by flushing the uteri (144 hr post-hCG). The collected embryos were placed in culture dishes containing droplets of M16 medium (Whittingham, 1971b) covered with paraffin oil and cultured at 37°C under 5% CO_2 in air until cryopreservation or embryo transfer.

Cryopreservation of Embryos

In the first series of experiments, we assessed the toxicity of different cryoprotectants to optimize the cryopreservation solution suitable for gerbil embryos. Freshly collected late two-cell (day 3) embryos were immediately suspended in 2M solutions of ethylene glycol (EG), glycerol, dimethyl sulfoxide (DMSO), propylene glycol (PG), or acetamide in PB1 at room temperature (22°C) for 10 min. After washing by serial transfers into three drops of PB1 at room temperature, the rate of in vitro development to the compacted morula stage was assessed using the culture conditions described above. Preliminary experiments showed that the morula stage was the most advanced stage to which fresh two-cell embryos developed under our in vitro culture conditions.

Vitrification was performed according to the method developed for mouse embryos by Kasai et al. (1990), with slight modifications. We prepared two vitrification solutions, EFS20 and EFS40, which consisted of 20% and 40% (v/v) EG, respectively, in PB1 solution containing 30% (w/v) Ficoll (average molecular weight 70,000), and 0.5M sucrose. In this study, we employed both one- and two-step vitrification protocols. For the one-step method, 13–20 embryos, together with a minimal amount of culture medium, were directly introduced, using a fine glass pipette, into approximately 40 μl EFS40 solution in a 0.25-ml plastic straw held horizontally. The plug ends were sealed with polyvinyl alcohol powder. After exposure of embryos to the EFS solution for 2 min at room temperature, the straw was immersed in liquid nitrogen. For the two-step method, 13–20 embryos were suspended in EFS20 solution for 2 min at room temperature. They were then directly transferred to approximately 40 μl EFS40 solution in a straw, as described above. After exposure of the embryos to EFS40 solution at room temperature for 30 sec, the sealed straw was immersed in liquid nitrogen.

To liquefy, (thaw; for terminology, see Shaw and Jones, 2003) the embryos for further evaluation, after storage in liquid nitrogen for at least 2 days, a straw was warmed rapidly in 22°C water for about 8 sec. Immediately after warming, the EFS solution containing the embryos was expelled from the straw onto a watch glass using a metal rod. The solution was diluted by addition of 0.8 ml PB1 medium containing 0.25M or 0.5M sucrose (S-PB1) and the embryos were retrieved into fresh S-PB1 medium. At 5 min after liquefying, the embryos were transferred to PB1 medium.

Embryo Transfer

Two types of pseudopregnant recipient females were prepared for embryo transfer: hormone-treated recipients and nontreated recipients. For the former, females were pre-treated with hormones for superovulation, as described above. Only virgin females were used for both groups. The vasectomized males for induction of pseudopregnancy were prepared at least 4 weeks before the experiments and infertility was confirmed by the

absence of sperm in the vagina of females after mating. Each recipient female was mated with a mature vasectomized male in a cage with a wire net floor. On the following morning (designated day 1 of pseudopregnancy), the females were examined for the presence of a vaginal plug and used for embryo transfer. Shortly before embryo transfer, the recipient females were anesthetized with an intraperitoneal injection of sodium pentobarbital (57.5 mg/kg).

Statistical Analysis

The results were evaluated using Fisher's exact probability test. Values of P less than 0.05 were considered statistically significant.

RESULTS

Effects of Cryoprotectants on the Development of Gerbil Embryos

Gerbil embryos were exposed to one of five different cryoprotectants and their subsequent development was assessed. As shown in Figure 1, embryos exposed to EG and PG showed rates of development to the compacted morula stage similar to those of nonexposed control embryos ($P > 0.05$). In contrast, those exposed to glycerol or acetamide had significantly decreased developmental competence (Fig. 1). Exposure to DMSO had an intermediate effect on embryo development. Therefore, we selected EG-based solutions for use in our cryopreservation experiments for gerbil embryos, as has been done for mouse embryos (Kasai et al., 1990).

Comparison of One- and Two-step Methods

Late two-cell (day 3) gerbil embryos that had been vitrified using the one- or two-step methods were liquefied and examined for subsequent development in vitro. More embryos developed to the compacted morula stage in vitro when they had been vitrified by the two-step method using EFS20 and EFS40, and liquefied in 0.25M sucrose solution (36/55, 69%) as compared to the other vitrification and liquefying procedures (Fig. 2). Only 5% of embryos (2/43) developed to the morula stage after the one-step vitrification method using EFS40 alone (Fig. 2).

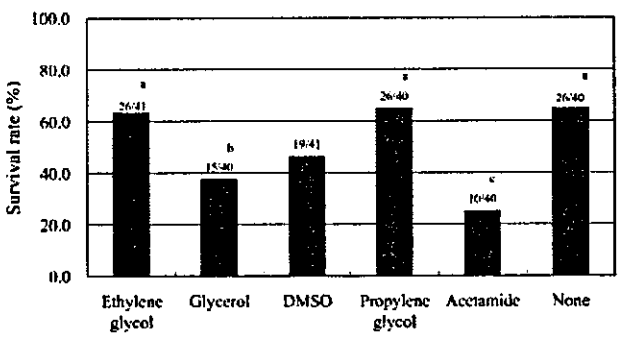

Fig. 1. Survival of late two-cell (day 3) gerbil embryos after exposure to cryoprotectants in PB1 for 10 min at 22°C. The numbers on the bars indicate surviving embryos per exposed embryos. The values with different letters (a, b, and c) are statistically different ($P < 0.05$).

In light of these results, we employed the two-step method for the subsequent vitrification experiments.

Effect of the Stage of Development Upon Cryopreservation on Subsequent In Vitro Embryo Development

Using the two-step method described above, we vitrified embryos at different stages of development and later liquefied and examined them to determine their survival and subsequent development in vitro and in vivo. When recovered into normal culture medium, most embryos (>87%) appeared to be morphologically normal, regardless of the stage at which the embryos were vitrified (Table 1). When cultured in vitro, embryos vitrified at the late two-cell (day 3) and four-cell (day 4) stages showed in vitro development comparable to that of nonvitrified control embryos at the same stage (Table 1). When embryos were vitrified at the early two-cell stage (day 2), in vitro developmental competence was significantly lower than that of controls (Table 1).

Development of Vitrified Gerbil Embryos After Embryo Transfer

We found that hormonal treatment of the recipient females made the efficiency of mating with males more consistent compared with natural mating, which occurs at random. However, as hormonal treatment may compromise the oviductal and uterine environments for transferred embryos, we first assessed whether hormonally treated recipient females conceived after embryo transfer. Early two-cell embryos (day 2) and blastocysts (day 6) transferred into the recipient oviducts and uteri, respectively, developed to term, regardless of whether the recipient females had been treated with hormones (Table 2). For further embryo transfer experiments, therefore, we used recipient females prepared by natural mating without hormone treatment, to optimize the conditions for efficient embryo transfer.

Early two-cell embryos (day 2), late two-cell embryos (day 3), morulae (day 5), and blastocysts (day 6) were vitrified using the two-step method, liquefied, and assessed for their developmental competence after transfer into recipient females. Four-cell embryos (day 4) were not transferred because day 3 oviduct (ampullar) is not an appropriate transfer site in gerbils owing to the distance from the position of the native oocytes, which at this stage have descended to a point near the uterine–oviductal junction.

Although the rates of development to full-term offspring were not high, normal pups were born from embryos vitrified at all stages, indicating that at least a portion of the embryos were completely viable and competent after vitrification and liquefying (Table 3; Fig. 3). The implantation rates were also low (Table 3), indicating that most embryos died before implantation, or simply failed to attach to the uterine epithelium. The weaning rates varied according to the experiment, but all of weaned pups developed into adults with normal appearance, to the extent examined.

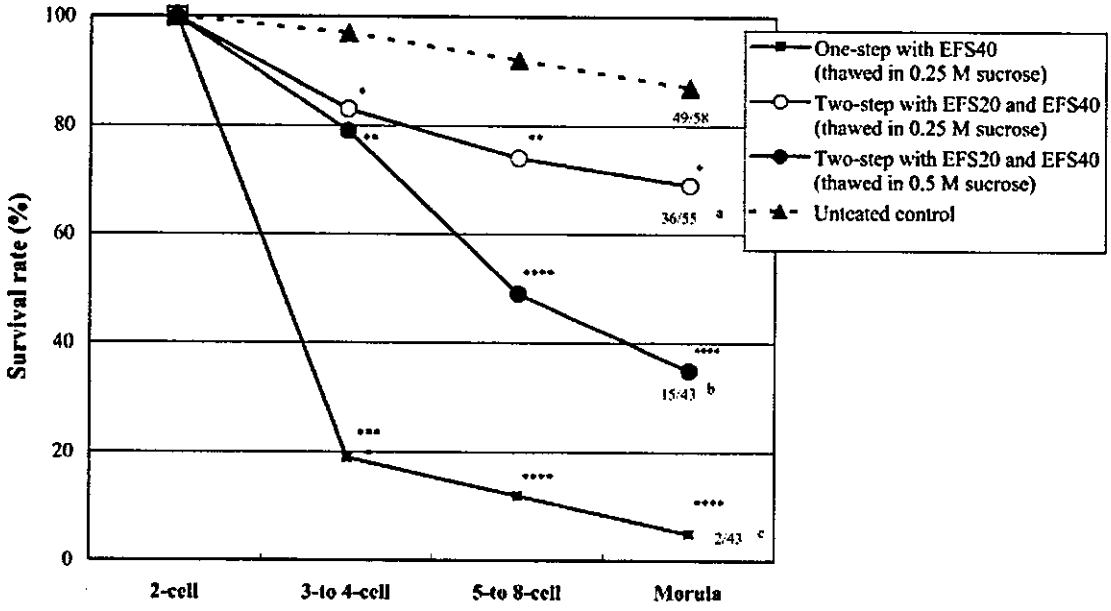

Fig. 2. In vitro development of late two-cell (day 3) gerbil embryos vitrified in EFS solution by the one-step or two-step method. Numbers indicate surviving embryos per cultured embryos. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$, as compared with the nonvitrified control at the same stage. $^{a,b}P < 0.001$, $^{b,c}P < 0.005$, and $^{a,c}P < 0.0001$.

DISCUSSION

The present study clearly demonstrated that Mongolian gerbil embryos could survive freezing and liquefying procedures at high rates and that some of them develop into normal full-term offspring. We employed a vitrification method using EG-based cryoprotectant solutions. Embryo cryopreservation by vitrification was first developed by Rall and Fahy (1985) for mouse embryos. It has potential advantages over conventional slow-freezing methods because of its very rapid cooling time and minimal cell injury caused by extracellular crystallization (Rall, 1987). However, the original vitrification solution consisted of four cryoprotectants, including acetamide, which is known to be very toxic to embryos; therefore, its application eventually was limited to embryos of certain strains of mice. Later, this complication was overcome by development of less toxic vitrification solutions using EG or glycerol (Kasai et al., 1990; Zhu et al., 1994; Rall et al., 2000). The use of EG also increased the flexibility of the conditions for vitrification protocols (e.g., exposure time to cryoprotectant) and thus increased the reproducibility of the vitrification experiments (Kuleshova et al., 1999; Nowshari and Brem, 2001). In this study, we confirmed the low toxicity of EG for gerbil embryos, but glycerol showed moderate toxic effects for gerbil embryos. To date, successful vitrification using EG has been reported for

TABLE 1. In Vitro Development of Gerbil Embryos After Vitrification by the Two-Step Method With EFS20 and EFS40

		No. (%) of embryos				
Stage of embryos (day)	Treatment	Vitrified	Recovered	Morphologically normal	Cultured	Developed to morula
Early two-cell (2)	Vitrified	40	39 (98)	39 (100)	39	9 (23)*
	Control	—	—	—	36	31 (86)*
Late two-cell (3)	Vitrified	176	169 (96)	147 (87)	55	38 (69)
	Control	—	—	—	49	39 (80)
Four-cell (4)	Vitrified	54	50 (93)	50 (100)	50	48 (96)
	Control	—	—	—	60	51 (85)
Morula (5)	Vitrified	94	92 (98)	87 (95)	—	—
Blastocyst (6)	Vitrified	50	46 (92)	46 (100)	—	—

*$P < 0.001$.

TABLE 2. In Vivo Development of Gerbil Embryos Transferred Into Pseudopregnant Recipients With or Without Treatment for Induction of Sterile Mating

Stage of embryos (day)	Treatment before sterile mating	No. (%) of recipients that became pregnant	No. of embryos transferred	No. (%)		
				Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	No treatment	3/7 (43)	80	12 (15)	9 (11)	4 (5)
	Hormone treatment	4/8 (50)	124	23 (19)	10 (8)	7 (6)
Blastocyst (6)	No treatment	5/5 (100)	77	40 (52)	37 (48)*	31 (40)*
	Hormone treatment	5/7 (71)	111	34 (31)	20 (18)*	15 (14)*

Early two-cell and blastocyst were transferred into the oviducts (day 1) and uteri (day 5) of recipient females, respectively.
*$P < 0.001$.

many mammalian species (see Introduction). In mice, its applicability for embryos at each developmental stage has been assessed in detail (Miyake et al., 1993).

As vitrification solutions contain very high concentrations of cryoprotectants and sucrose to circumvent intracellular ice formation, the embryos are exposed to extremely high osmolality before they are vitrified. It had been reported that this osmotic shock may compromise the viability of the embryos, but can be reduced by the step-wise exposure of embryos to solutions with increasing osmolalities. We found that this was also the case with gerbil embryos. When late two-cell gerbil embryos were vitrified using the two-step method with EFS20 and EFS40, their developmental competence was significantly improved; as many as 65% of the frozen and liquefied embryos reached the morula stage in vitro. The osmolalities of EFS20 and EFS40 were about 4.5 Osmol/kg and 9 Osmol/kg, respectively, as measured by an automated osmometer. In mice, we found that better survival rates could be achieved for embryos from certain strains (e.g., DBA/2, ddY) using the two-step method, as compared to a one-step method with EFS30. Thus far, mouse embryos from 248 strains, including genetically modified mice, have been safely cryopreserved using the two-step method and their viability confirmed by full-term development after liquefying and embryo transfer (unpublished).

In the last series of experiments, we assessed the viability and competence of vitrified gerbil embryos by transfer into recipient females. Because there have been very few studies on embryo transfer in gerbils, the best combinations of the embryo stage and the day of pseudopregnancy of the recipient females are not known. In preliminary experiments, late two-cell (day 3) embryos did not survive when transferred into 1-day pseudopregnant oviducts. In this study, therefore, the recipient females were implanted with embryos 1 day older than the female's pseudopregnancy. The oviducts and uteri of gerbils accepted embryos at different stages of development as long as a 1-day difference existed between the recipients and embryos. Embryos at every developmental stage developed into normal offspring, regardless of whether the embryos had been vitrified. However, the rates of normal birth per transfer were very low, being less than 10% in most cases. We also found that the implantation rates of both vitrified and fresh embryos were low, except in the case of fresh 6-day embryos. This indicates that embryo transfer techniques for gerbils can still be improved, probably by optimizing the transfer timing. The in vitro culture medium for gerbil embryos should also be improved, because no two-cell or four-cell embryos reached the blastocyst stage in M16 medium, which was originally developed for mouse embryos. We have previously reported that embryos of

TABLE 3. In Vivo Development of Vitrified Gerbil Embryos After Transferred Into Pseudopregnant Females

Stage of embryos (day)	Recipient female			No. of embryos transferred	No. (%) of		
	Stage (day)	Transfer site	No. (%) that became pregnant		Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	1	Oviduct	2/8 (25)	123	6 (5)*	4 (3)**	2 (2)**
Late two-cell (3)	2	Oviduct	1/6 (17)	102	6 (6)*	1 (1)*	1 (1)**
Morula (5)	4	Uterus	2/6 (33)	73	9 (12)	4 (5)	2 (3)
Blastocyst (6)	5	Uterus	3/10 (33)	155	30 (19)*	15 (10)*,**	13 (8)**
Blastocyst (6)	5[a]	Uterus	3/7 (43)	110	25 (23)	17 (15)	15 (14)

Embryos were transferred into recipient females on the day of thawing.
[a]Without hormone treatment (natural cycle).
*$P < 0.005$.
**$P < 0.05$.

Fig. 3. Gerbil pups born after transfer of vitrified blastocysts (black). They looked normal and showed active movement.

mastomys, a laboratory rodent native to Africa, developed into blastocysts in glutamine-containing medium, but not in a medium lacking glutamine (Ogura et al., 1997). Future experiments should examine whether this is also the case with gerbil embryos.

No conventional methods yet exist for embryo transfer in the Mongolian gerbil, in part because, unlike other rodents such as mice and rats, induction of pseudopregnancy in female gerbils is difficult due to the unique character of the reproductive biology of this species. First, females and males caged together after reaching sexual maturity often show very aggressive behavior because of their monogamous nature. It has been reported that the incidence of fighting and mortality can be decreased to some extent by combining an elder male and a virgin female (Norris and Adams, 1972). We employed this combination for our embryo transfer experiments. Second, unlike in mice and rats, it is difficult to identify the estrous cycle in gerbils by vaginal smears due to their irregular patterns. The estrous cycle of the Mongolian gerbil generally lasts for 4 to 7 days, varying by individual animal (Marston and Chang, 1965). In a preliminary experiment, we examined whether the cycle could be synchronized by the hormone treatment used for superovulation. On the day following hCG administration and mating, 61% (n = 41) of females had a copulation plug. As these hormone-treated females were proven to conceive after embryo transfer, we conventionally employed this method to conduct transfer experiments using vitrified embryos. Successful embryo transfer in Mongolian gerbils was first reported by Norris and Rall (1983), who used lactating pregnant females as recipients after ligating their single oviduct during early pregnancy. This method gave excellent results by exploiting lactation-induced delayed implantation for embryo transfer.

In conclusion, Mongolian gerbil embryos can be cryopreserved safely using a two-step vitrification method with EG-based cryoprotectant solutions. The offspring derived from vitrified embryos appeared normal and grew into fertile adults. This strategy will enable efficient maintenance of gerbil breeding colonies and avoid microbiological and genetic contamination that may occur during natural breeding.

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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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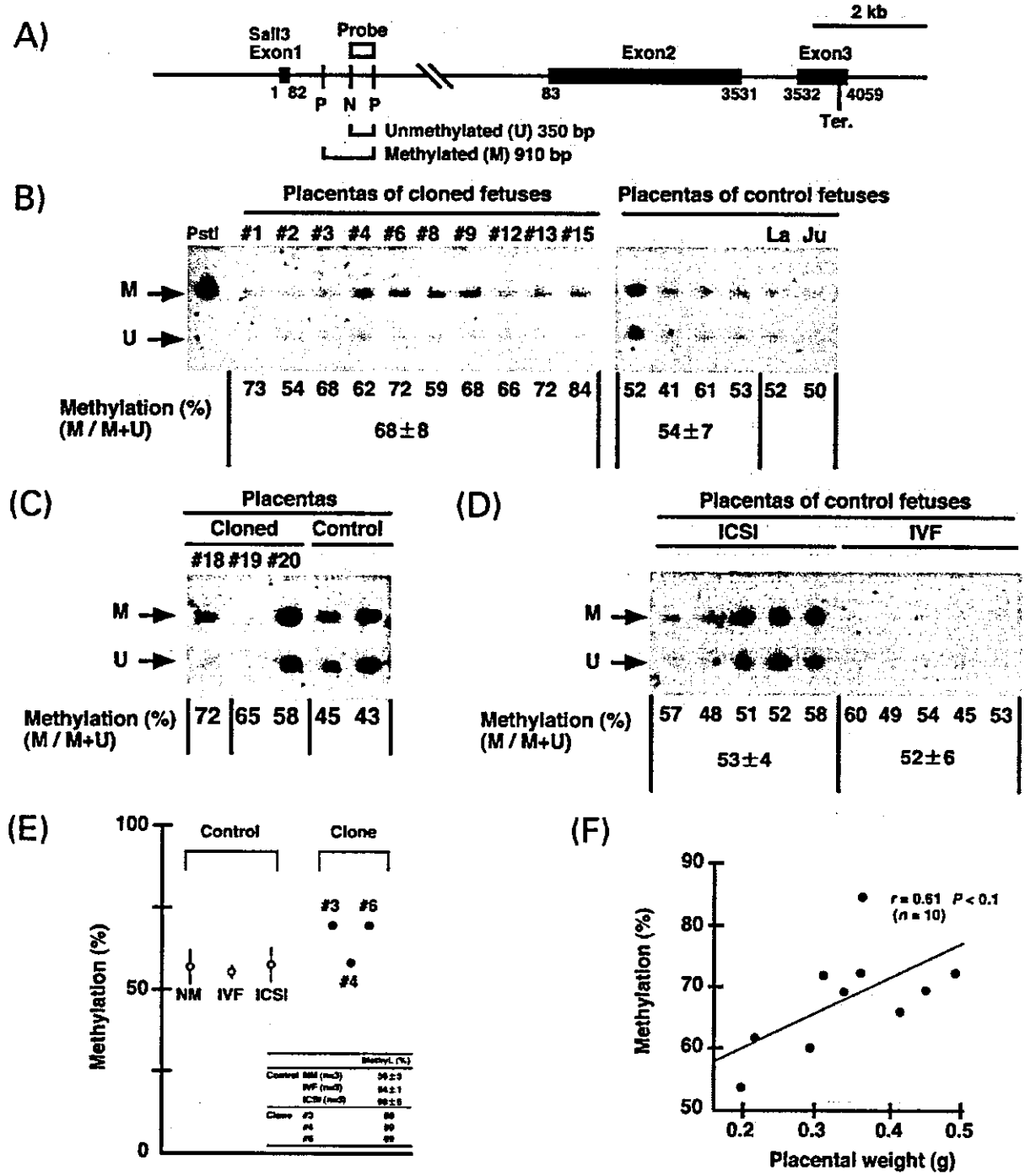

Figure 1 Aberrant DNA hypermethylation at the *Sall3* locus in placentas of cloned mice. (A) The genomic structure of the *Sall3* locus with the position of the probe and expected bands in Southern blotting. The *Sall3* locus is at the telomeric E3 subregion of chromosome 18. *Not*I is a methylation-sensitive restriction enzyme. The *Not*I site, aberrantly methylated in the placenta of the cloned mouse, is located approximately 1.1 kb downstream of *Sall3* exon 1. Closed boxes represent three exons of the *Sall3* gene. Numbers below the boxes are nucleotide numbers, designating the translation-starting site as number 1. An open box indicates the position of the probe. Methylation status of the *Sall3* locus was analysed by Southern blotting using *Not*I and *Pst*I. The bands expected in Southern blotting in Figs 1 and 3

2002). These findings suggest that cloned animals produced by somatic nuclear transfer have different methylation patterns from normal animals. At present, however, it remains to be seen whether aberrant DNA methylation at certain loci is related to the phenotypes specific to cloned animals.

Overgrowth of the placenta is one of the commonly observed symptoms in all cloned mice regardless of the sex and strain of animal and the type of donor cell (Wakayama & Yanagimachi 2001; Ogura *et al.* 2000a). Abnormal gene expression has been detected in term placentas of cloned mice (Humpherys *et al.* 2002; Suemizu *et al.* 2003). Thus, there may be genomic loci associated with the abnormal placental development in cloned mice. By using restriction landmark genomic scanning (RLGS) method, we have previously investigated genome-wide DNA methylation of CpG islands in mouse embryonic stem (ES) cells, embryonic germ (EG) cells and trophoblast stem (TS) cells before and after differentiation (Shiota *et al.* 2002). We have also investigated CpG islands of terminally differentiated germ cells and several somatic tissues using the same technique. There are many CpG islands with tissue-dependent differentially methylated regions (T-DMRs) (Imamura *et al.* 2001; Shiota *et al.* 2002). Here, we report that a T-DMR within the *Sall3* locus at the telomeric E3 subregion of mouse chromosome 18 is hypermethylated in the placentas of all cloned mice examined.

Results

Hypermethylation of the Sall3 locus in placentas of cloned mice

Based on our previous studies, we prepared a T-DMR panel consisting of 247 loci detected by RLGS, one of which, locus #148, was methylated only in TS cells (Shiota *et al.* 2002). Intriguingly, locus #148 was matched to one of the aberrantly methylated loci in the placenta of cloned mice reported in the previous paper (spot 8 in Ohgane *et al.* 2001). It is also mapped to the *Sall3* locus at the telomeric E3 subregion of mouse chromosome 18 (Fig. 1A).

We first used fetuses cloned with adult cumulus cells of B6D2F1 mice (Fig. 1B). The degree of methylation at the *Sall3* locus was 54 ± 7% in the naturally mated controls (NM) as deduced from the previous report (Ohgane *et al.* 2001; Shiota *et al.* 2002). Placentas of all 10 cloned fetuses, with the exceptions of #2 and #8, showed over 60% methylation (average 68 ± 8%). Clone #15 showed the highest DNA methylation (84%). These facts indicated that the *Sall3* locus is commonly hypermethylated in these clones. In other words, cloning by somatic cell nuclear transfer may consistently result in the epigenetic error at this specific locus of the placental genome.

Mouse placenta consists of junctional and labyrinth zones (Cross *et al.* 1994). Morphological examination of placentas revealed that an expanded spongiotrophoblast layer in the junctional zone is the major cause of placentomegaly in cloned mice (Tanaka *et al.* 2001). DNA methylation of the *Sall3* locus was not different between the labyrinth and junctional zones (52% and 50%, respectively) (Fig. 1B). This implies that the hypermethylation of the *Sall3* locus is not the result of change in the proportion of certain trophoblast subtypes in the placentas of cloned mice.

Methylation status of the Sall3 locus in placentas of cloned mice with various donor cells

Since placentomegaly is observed regardless of the types of donor cell, we next investigated whether DNA

are depicted as unmethylated (U, 350 bp) or methylated (M, 910 bp). N, *Not*I; P, *Pst*I. (B) Aberrant hypermethylation of the *Sall3* locus in placentas of fetuses cloned with female cumulus cells of B6D2F1 mice. The methylation status of placentas of cloned fetuses (n = 10) was compared with that of placentas of control B6D2F1 fetuses produced by natural mating of C57BL/6 and DBA2 mice (NM, n = 4). The value (%) under each lane denotes the methylation rate of the *Not*I site (M/M +U). The difference in methylation rate was statistically significant between the cloned and control mice (68 ± 8% and 54 ± 7%, respectively, $P < 0.01$). A NM placenta of B6D2F1 mouse was dissected into the labyrinth zone (La) and the junctional zone (Ju), and their methylation status at the *Sall3* locus was analysed. The placental labyrinth zone and the junctional zone were methylated at almost the same rates (52% and 50%, respectively). (C) Aberrant hypermethylation of the *Sall3* locus in the placentas of fetuses cloned with fibroblast cells. #18, one B6D2F1 female cloned with a foetal fibroblast. #19, one (B6 × JF1)F1 male cloned with a foetal fibroblast. #20, one (B6 × JF1)F1 female cloned with a tail tip fibroblast. Two controls are fetuses obtained by IVF of C57BL/B6 and JF1 mice. (D) The methylation status of the *Not*I site in control placentas of B6D2F1 fetuses produced by ICSI and IVF. Five placentas each of ICSI fetuses and IVF fetuses were subjected to Southern blotting under the same conditions as in Fig. 1B. (E) The methylation degree of the *Not*I site evaluated by real time genomic PCR. The methylation levels evaluated by Southern blotting in Fig. 1B,D were confirmed by real time PCR using three of the placentas of cloned (closed circle) and control fetuses (open circle). For the controls (NM, IVF and ICSI), average methylation degrees of three placentas are shown. (F) Correlation between placental weight and methylation rate of 10 B6D2F1 fetuses cloned with cumulus cells. The placental weight and methylation rate of each cloned mouse are plotted. The methylation rate and placental weight show a positive correlation (coefficient r-value of 0.61, $P < 0.1$).

hypermethylation at the *Sall3* locus occurs in the placenta of mice cloned with other types of cells (Fig. 1C). In the placenta of a female-fibroblast clone (#18, B6D2F1), the locus was hypermethylated compared with NM controls (72% vs. 54%). Similarly, in (B6xJF1)F1 mice, the placentas of a male-fibloblast clone (#19) and a female-fibroblast clone (#20) showed hypermethylation compared with controls (65%, 58% vs. 45% and 43%, respectively). Thus, the *Sall3* locus is hypermethylated regardless of the genetic background, sex and type of the nuclear donor cells.

Oocyte manipulation and *in vitro* culture do not affect the methylation status of the Sall3 locus

To control for the possibility that the *in vitro* culture of oocytes and embryos by themselves may trigger abnormal DNA methylation at gene loci (Doherty *et al.* 2000), we examined the methylation status of the placentas of fetuses produced by ICSI and IVF. We found that the degrees of methylation of the *Sall3* locus in placentas of ICSI and IVF fetuses were 53 ± 4% and 52 ± 6%, respectively (Fig. 1D). Thus, the placentas of mice produced after *in vitro* manipulation were not significantly different from those of NM controls as far as the methylation status of the *Sall3* locus is concerned.

The extent of DNA methylation was confirmed by quantitative genomic PCR (Fig. 1E). The placental genome of the NM, IVF and ICSI controls showed 56, 54, and 58% methylation, respectively, at the *Not*I site of the *Sall3* locus. Similarly, clone #4, which showed moderate hypermethylation by Southern blotting was 59% methylated. In contrast, clones #3 and #6 showed hypermethylation (69%) compared with the NM, IVF and ICSI controls.

DNA methylation level of the Sall3 locus correlates with placental weight in cloned mice

To evaluate the relationship between methylation aberration and placental phenotype, methylation rate and placental weight of each cloned placenta are plotted in Fig. 1(F). There was a positive correlation between the extent of hypermethylation of the *Sall3* locus and placental weight in cloned mice ($r = 0.61$, $P < 0.1$).

Methylation status of CpGs within the Sall3 T-DMR

We analysed the methylation status of the *Sall3* locus in ES and TS cells by bisulfite sequencing to determine the size of the *Sall3* T-DMR. The T-DMR was 904 bp in length and located in a region just 5′ of the *Not*I site extending to 3′ region that is highly homologous with human (Fig. 2A). The T-DMR containing 31 CpGs was hypermethylated in TS cells compared with ES cells, as previously reported (Shiota *et al.* 2002). Bisulfite sequencing analysis of the *Sall3* T-DMR revealed hypermethylation in the placenta of cloned mouse (#15) throughout the T-DMR (Fig. 2B). Comparison between the human and mouse *SALL3/Sall3* locus showed the sequence homology in T-DMR as well as promoter and exon 1 (Fig. 2C). Although the methylation status of the *Not*I site was about 50% in the control placentas (Fig. 1B), the methylation pattern of the whole T-DMR was unlike the pattern of typical imprinted genes. In imprinted genes, differentially methylated regions would show about 50% methylation within whole regions.

Methylation status of the Sall3 locus in the brain and liver of cloned fetuses at term

The *Sall3* gene was suggested to play important physiological roles in various tissues (Ott *et al.* 1996). To address the question whether the epigenetic error at the *Sall3* locus of cloned mice may occur in other tissues, we investigated the methylation status of the *Sall3* locus in the brain and liver of cloned fetuses at term by Southern blotting (Fig. 3). The *Sall3* locus was not methylated in the brain and liver in the control as reported previously (Shiota *et al.* 2002). The *Sall3* T-DMR was hypomethylated in these tissues of the clones as in the control, although the liver of clones was slightly methylated. This shows that the *Sall3* locus is methylated specifically in the placenta, perhaps in the trophoblast cell lineage, as previously reported (Ohgane *et al.* 2001; Shiota *et al.* 2002) and that the aberrant hypermethylation occurs uniquely in the placenta of cloned mice.

Discussion

Sall3 mRNA was barely expressed in TS cells and did not show dramatic change after differentiation (data not shown). The mouse *Sall3* T-DMR covers the region highly homologous to human genomic sequence in equivalent location (Fig. 2B,C), suggesting that this region has important functions in both species. Human chromosome 18q is one of the regions which are lost frequently in cancer cells, and its loss is often related to abnormal genome-wide hypomethylation (Schulz *et al.* 2002). The human 18q23 region containing the *SALL3* locus is likely to be responsible for the 18q deletion syndrome (OMIM #605079 and 601808) (Kohlhase *et al.* 1999). This region includes the *MBP*, *GALR1* and *NFATC* genes which could be responsible for several

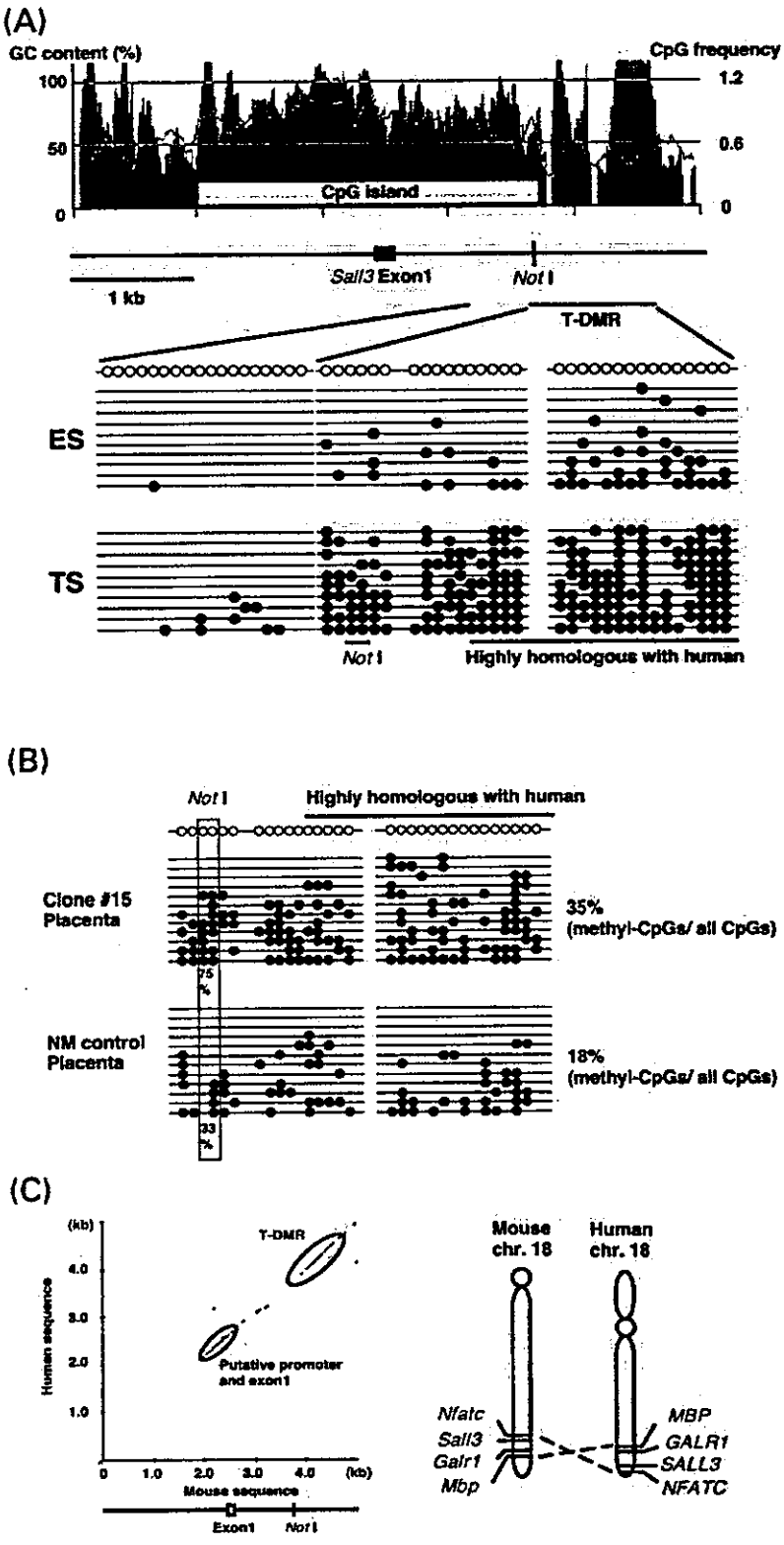

Figure 2 DNA methylation status of T-DMR at the *Sall3* locus in the placenta of cloned mouse. (A) Genomic structure of a 5 kb region that includes *Sall3* exon1, the CpG island, T-DMR and the trophoblast-specifically methylated *Not*I site. Moving averages of GC content (jagged line) and CpG frequency (black bar) are plotted on the graph. Below the graph are marked the *Sall3* exon1 (closed box) and the differentially methylated *Not*I site. The CpG island was formulated by an average GC content greater than 50% and that of CpG frequency greater than 0.6 (Gardiner-Garden & Frommer 1987). Both *Sall3* exon 1 and the trophoblast-specifically methylated *Not*I site are within the CpG island. Methylation status around the differentially methylated *Not*I site in ES and TS cells is shown below the genomic structure of the *Sall3* locus. The CpG sites analysed by sodium bisulfite sequencing are shown as open circles at the very top. Comparison of the methylation status between ES and TS cells indicated that the *Sall3* T-DMR included the differentially methylated *Not*I site and the region highly homologous with human sequence. Each line represents one DNA fragment sequenced. Only methylated CpGs are shown as closed circles. (B) Aberrant hypermethylation throughout the T-DMR in the placenta of a cloned mouse. There are 31 CpGs within the *Sall3* T-DMR, and the CpG sites are shown as open circles at the very top. The positions of two CpGs affecting *Not*I digestion are marked with a box, and their methylation rates are shown by percentage. The overall percentage of methylated CpGs is shown in the right side (methyl-CpGs/all CpGs). In cloned mouse #15, aberrant hypermethylation occurred in the region highly homologous with human sequence. (C) Genomic sequence conservation at and around the *Sall3* locus in human and mouse. (Left panel) Comparison of the nucleotide sequences of the mouse and human *Sall3/SALL3* 5′ region. Within the 5 kb orthologous regions, nucleotide sequences of a putative promoter with exon1 and T-DMR are conserved. (Right panel) Gene map of the telomeric region of mouse and human chromosome 18. The order of genes in this region is conserved in the mouse and in the human but in the reversed order.

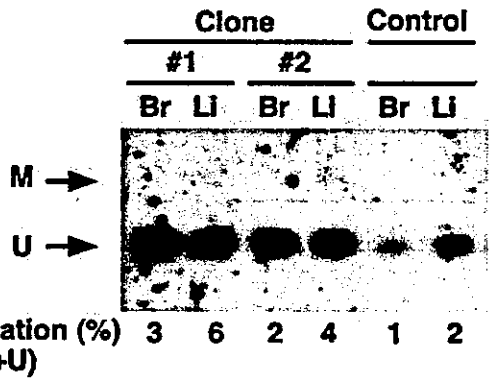

Figure 3 Methylation status at the *Sall3* locus in the brain and liver of cloned fetuses. Methylation status of the *Not*I site in foetal brain (Br) and liver (Li) of cloned and control fetuses were analysed under the same conditions as in Figure 1. Cloned mouse fetuses #1 and #2 are from the same B6D2F1 conceptuses analysed in Figure 1. M and U indicate methylated and unmethylated bands, respectively. The *Sall3* locus is barely methylated both in cloned and control fetuses.

inherited human diseases (OMIM #159430, 600377 and 600489) (Fig. 2C). Among these genes, *NFATC* is suggested to play a role in human placenta (Xia *et al.* 2002). In the case of the *Igf2* gene, the differentially methylated regulatory region is about 90 kb from the transcription start site (Wolffe 2000). Future study is necessary to know the role of the DNA methylation of the *Sall3* T-DMR in the regulation of *MBP*, *CALR1* and *NFATC*. Based on the present study, however, it is clear that the epigenetic abnormality always associates with cloned mice. Taken together, human and mouse orthologous locus including the *SALL3/Sall3* gene may be a region that is genetically and epigenetically instable. Careful examination of epigenetic errors in cloned embryos and fetuses will be needed before somatic nuclear transfer technology is applied to human therapeutics including the production of human embryonic stem cells.

Mouse interspecific hybrids have been reported to show a hypertrophic placental phenotype quite similar to that of cloned placentas (Zechner *et al.* 1996). As found in cloned placentas, these interspecific placentas also exhibited a wide range of placental weight, enlarged spongiotrophoblast layer, increased incidence of glycogen cell differentiation and obscure borders at spongiotrophoblast and labyrinth layers (Zechner *et al.* 1996; Tanaka *et al.* 2001). Since the methylation rate of the *Sall3* T-DMR correlated with the typical placental phenotype (placentomegaly) in cloned mice, it will be interesting to investigate DNA methylation status of the *Sall3* locus in the interspecific hybrid mice.

The production rate of cloned animals is low. Only 2–3% or less, of all reconstituted oocytes, develop into live offspring (Solter 2000; Renard *et al.* 2002; Wilmut *et al.* 2002). Most embryos/fetuses die during development, and improper placental development may be, in part, responsible for this. Establishment of cell- and tissue-specific DNA methylation is important for normal embryonic development (Bird 2002; Li 2002; Shiota & Yanagimachi 2002). Therefore, genomic loci frequently associated with the epigenetic error have been explored in the cloned animals. The finding of the *Sall3* locus showing frequent abnormal DNA methylation is crucial. From the data, we concluded that there is a genomic locus highly susceptible to epigenetic error caused by nuclear transfer.

Experimental procedures

Animals

Placentas, brains and livers of cloned and control fetuses at 19.5 dpc were collected and analysed for DNA methylation. One term placenta of naturally mated mouse was mechanically dissected into the junctional and labyrinth zones after removal of the embryo. Donor cells for cloning were adult cumulus cells (Wakayama *et al.* 1998), adult tail tip fibroblast (Wakayama & Yanagimachi 1999) and foetal fibroblast (Ogura *et al.* 2000b). Control fetuses were obtained by natural mating, *in vitro* fertilization (Toyoda *et al.* 1971) or intracytoplasmic sperm injection (Kimura & Yanagimachi 1995). Strains of males and females used for the production of fetuses are described in figure legends.

Database search and RLGS spot cloning

DNA of an RLGS spot showing aberrant methylation in one cloned mouse (spot 8 in Ohgane *et al.* 2001) was purified by the *Not*I trapper method as described elsewhere (Ohgane *et al.* 1998, 2002). The purified DNA was initially ligated into the *Not*I and *Pst*I sites of pBluescript II (Stratagene, CA). The inserted fragments were amplified by PCR with the M4-RV primer set and cloned into the pGEM-T vector (Promega, WI). Cloned DNA was sequenced by using a Shimadzu autosequencer system (Shimadzu, Kyoto, Japan) following the manufacturer's instructions. The nucleotide sequence obtained from spot DNA cloning was compared with NCBI (http://www.ncbi.nlm.nih.gov) and Ensemble (http://www.ensembl.org) mouse and human genome sequence databases by the BLAST search program. Human genes on chromosome 18 responsible for inherited diseases were ascertained by searching the OMIM database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM).

Southern blotting

Genomic DNAs (5 μg each) from cloned and control mouse tissues (placenta, brain and liver) were double-digested with *Not*I and *Pst*I, and separated on a 1.4% agarose gel followed by blotting

on to nylon membrane. A probe specific to spot 8 (the *Sall3* locus) sequence was labelled with a DIG-dUTP using a random primer labelling kit (Roche Diagnostics, Mannheim, Germany). Hybridization and washing were performed under stringent conditions as described elsewhere (Hirosawa *et al.* 1994). Signals were detected with a DIG luminescent nucleotide detection kit (Roche Diagnostics) containing alkaline phosphatase (AP)-conjugated anti-DIG antibody and AP substrate, and visualized on X-ray film (Fuji Film, Tokyo, Japan).

Statistical analysis

The intensities of the methylated and unmethylated bands were measured by NIH image software provided by the National Institutes of Health (ftp://rsbweb.nih.gov/pub/nih-image/nih-image161_fat.hqx). Methylation rate of the *Not*I site of the *Sall3* locus was calculated by the formula: (intensity of methylated band)/(intensity of methylated band) + (intensity of unmethylated band) and presented by mean ± standard deviation. The difference in methylation rate between cloned and control mice was evaluated by the independent Student's *t*-test. The relationship between placental weight and the *Sall3* methylation rate of cloned placentas was tested with Pearson's correlation coefficient, and its *P*-value was evaluated from the coefficient *r*-value.

Bisulfite sequencing

Bisulfite sequencing was performed following previously reported procedure (Imamura *et al.* 2001). In brief, 5 μg each of *Eco*RI-digested genomic DNA were modified with sodium metabisulfite, and one-tenth of each modified DNA was amplified with AmpliTaq Gold (Perkin Elmer, Norwalk, CT, USA) and the following primer sets: BisF1, 5′-GGGAAGTAAATGTTTTTT-GGTT-3′; *Bis*R1, 5′-AACTAACTAAAAAAACTCTATATC-3′; BisF2, 5′-GTTAGGGTTTTTTTAGGGTATTAGT-3′; BisR2, 5′-CCCTAATCTACCCAACATATACAAA-3′; BisF3, 5′-GATTAAATGAATGGATTTATTTTTGT-3′; and BisR3, 5′-ATTAACTATCTAAAATTTTCAACAC-3′. Amplified fragments were cloned into pGEM-T vector (Promega), and 10 or 12 independent clones for each primer set were sequenced to determine methylation status.

Real time PCR

Real time genomic PCR was performed using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, CA) following the manufacturer's instructions. Placental genomic DNAs (40 ng), with or without *Not*I-digestion (methylated DNA or total DNA, respectively) were analysed by real time PCR using F5 (5′-TACCAGCACGGAGGCCGAGGA-3′) and R3 (5′-GATCATAAAAAGTTGGCTTTTAAGG-3′) primer pair flanking the differentially methylated *Not*I site. TaqMan Rodent GAPDH Control Reagent (Applied Biosystems) was used for normalization of the template DNA amount. Methylation degree of the *Not*I site was calculated by the formula: (quantity of methylated DNA)/(quantity of total DNA).

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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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