【総　説】

5. シアル酸転移酵素遺伝子ホモ導入マウスに見られた拡張型心筋症

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Dilated Cardiomyopathy in a Transgenic Mouse Line Homozygous for Sialyltransferase Transgenes

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Summary

Dilated cardiomyopathy (DCM) is a serious human health problem for which a murine model is needed. During our research on sialic acid metabolism, we developed multiple transgenic mouse lines homozygous for the Galβ1,3GalNAcα-2,3-sialyltransferase type II (ST3Gal II) transgenes. The mice in one of these lines consistently developed DCM. The mice were born healthy and grew normally until approximately seven months of age. At that point, they manifested dyspnea, deformation of the thorax, and rapid weight loss, dying about a week after the onset of symptoms. The average lifespan was 227±55 days (mean±SD, n=30). At necropsy, the hearts were enlarged, with dilated chambers and thin, low tensile strength walls. Evident peritoneal and pleural effusion indicated circulatory failure. Pathological examination revealed fibrosis with inflammatory infiltrates in the heart, and hyaline degeneration and regeneration of muscle fibers in the skeletal muscles. To determine the mechanism underlying the symptoms, we considered two potential contributing factors: the effect of genome disruption by transgene insertion, and the effect of transgene overexpression. The transgenes were mapped to approximately 200 kb upstream of δ-sarcoglycan (SGCD) on chromosome 11 by genomic walking and BLAST search of the Ensembl genome database. However, no mRNA expression of the gene found at the insertion site (LOC214380) was detected by RT-PCR in either non-transgenic or homozygous transgenic mice. In addition, the level of SGCD expression in homozygous transgenic mice was not different from that of non-transgenic mice. Despite the substrate preference of the ST3Gal II enzyme for glycolipids, no difference in ganglioside composition was found in the brain or liver of the homozygous transgenic mice, relative to non-transgenic mice. However, alteration of heart glycoproteins in the homozygous transgenic mice was detected by a combination of two-dimensional polyacrylamide gel electrophoresis and wheat germ agglutinin staining. These results suggest that perturbation of protein glycosylation by overexpressed ST3Gal II could be the principal cause of DCM in the transgenic mice; this may represent a new form of degenerative muscular disease induced by abnormal glycosylation. Still, the possible involvement of unexpected disruption of the endogenous genome cannot be ruled out, as the absence of symptoms in hemizygous mice could not be explained solely on the

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249 BOVINE GRANULOSA CELLS mRNA EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-α AND THE PROTO-ONCOGENE c-Fos

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PPARα and c-Fos are involved in regulation of gene expression and are known to be dependent on retinoic acid (RA), which in turn influences oocyte growth and developmental competence (Duque *et al.*, 2002 Hum. Reprod. 17, 2706–2714; Hidalgo *et al.*, 2003. Reproduction 125, 409–416), probably acting in part through granulosa cells. Peroxisome proliferator-activated receptor-α (PPARα) heterodimerizes with the retinoid receptor X (RXR), while c-Jun/c-Fos heterodimerizes with liganded retinoic acid receptors (RARs), then preventing formation of transcription factor activator protein 1 (AP-1) complexes capable of DNA binding. Cellular retinoic acid binding protein (CRABP) limits RA excess and regulates the transcriptional potential of RA; CRABPII has been detected in rat granulosa cells from mature follicles and luteal cells. The aim of this study was to investigate PPARα, c-Fos and CRABPII mRNA expression in bovine granulosa cells. In parallel, other genes whose expression can be influenced by RA were analyzed: luteinizing hormone receptor (LHr), follicle stimulating hormone receptor (FSHr), aromatase and growth hormone (GH). Ovaries were collected at a local abattoir and kept in saline at 30–35°C. Granulosa cells were obtained by aspirating 2- to 7-mm antral follicle contents, pelleted at 700g for 4 min and resuspended in RNA-later (Ambion®). Total RNA was isolated with a NucleoSpin® RNAII kit (Macherey-Nagel), and mRNA was reverse transcribed into single-stranded cDNA using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). A PCR standard method was made using 1 μL of the cDNA as a template. All PCR primer couples were designed on the basis of the bovine sequence, but c-Fos and CRABPII primers were designed based on the human-murine sequences. Primers within the couple were located in different exons to distinguish DNA from RNA amplification. CRABPII was further investigated in bovine whole ovary, corpus luteum (CL) and liver, in a search for positive controls. Bovine β-actin, 18S and 28S were examined in each sample as positive controls for RNA isolation and cDNA synthesis efficiency. Ten μL of product were loaded into an agarose 2% gel in TBE buffer containing ethidium bromide, and were separated by horizontal electrophoresis. Gels were visualized with ultraviolet light and photographed using a digital camera. Gene expression in granulosa was demonstrated for PPARα, c-Fos, LHr, FSHr, aromatase, GH and controls (β-actin, 18S and 28S) but CRABPII gene did not express in granulosa cells, whole ovary, CL or liver under our experimental conditions. While lacking CRABPII expression remains intriguing, the expressed genes support a role of retinoid pathway within granulosa cells under both in vivo and in vitro conditions, because granulosa cells used in the present experiments were derived from follicles providing oocytes for IVM-IVF. Grant support: Spanish Ministry of Science and Technology (AGL-2002-01175).

250 SPECIFIC GENE KNOCK DOWN OF OCT-4 IN MOUSE PREIMPLANTATION EMBRYOS USING SHORT INTERFERENCE RNA

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RNA interference is used to specifically and effectively inhibit the expression of cognate genes. In the present study we investigated the inhibitory effect of gene expression in mouse embryos developing in vitro by injecting short interference RNA (siRNA). Fertilized mouse zygotes were obtained from mated females 20–24 h after hCG injection. Chemically synthesized 21-nt siRNA was commercially obtained and injected into mouse zygotes. The zygotes were then cultured in KSOM medium supplemented with 4% BSA at 37°C. Semi-quantitative RT-PCR was used to examine Octamer-binding transcription factor (Oct-4) gene expression in a single mouse embryo developing in vitro following siRNA-injection. In order to determine the expression and distribution of Oct-4 in mouse embryos, the mouse embryos were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% triton x-100 for 10 min. Embryos were then incubated with rabbit Oct-4 polyclonal antibody for 1 h and with FITC-labeled goat anti-rabbit antibody. Propidium iodide was used for DNA staining. siRNA injection did not retard the development of mouse zygotes. The number of blastocyst cells and the ICM/TE ratio did not differ in the siRNA injected blastocysts and the non-injected control group. Semi-quantitative RT-PCR revealed that Oct-4 expression was decreased at the 4-cell embryo stage and was significantly high at the morula and blastocyst stages. Injection of siRNA into oocytes inhibited RNA expression of Oct-4 and Nanog, but not of E-cadherin and Heat shock protein 70.1. Immunocytochemical staining showed inhibition of Oct-4 synthesis of the morulae and blastocysts following injection of siRNA. After culture of the embryos in the ES cell-derived conditioned medium, the embryos were stained for alkaline phosphatase (AP), a marker specific to pluripotent cells. AP was not detected in the inner cell mass of blastocysts following siRNA injection. These results suggest that siRNA injection into a mouse zygote specifically inactivates Oct-4 in mouse embryos developing in vitro.

251 SEARCH FOR GENES INVOLVED IN DEVELOPMENTAL COMPETENCE IN MOUSE OOCYTES USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

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During the first month after birth, synchronous follicular growth occurs in the ovary of immature mice (first wave). Previously, we showed that mouse oocytes during the first wave were more competent developmentally in older females (Suzuki O *et al.*, 2002 Theriogenology 57, 628 abst), although the numbers of mature oocytes did not differ with female age (17, 18, and 24 days old). In this study, we examined the genetic factors that affect the developmental competence of mouse oocytes during the first wave using suppression subtractive hybridization (SSH). Oocytes collected from 17- and 24-day-old B6D2F1 females (D17 and D24, respectively) without hormonal treatment were matured in Waymouth medium supplemented

with pyruvate (0.23 mM), antibiotics, bovine fetuin (1 mg mL^{-1}), and polyvinylpyrrolidone (3 mg mL^{-1}). After 17-h culture at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, total RNA was isolated from oocytes whose germinal vesicles had broken down (mature oocytes), separately, in three independent culture groups per age (each group contained oocytes from four animals) using Cell-to-cDNA Cell Lysis Buffer (Ambion, Austin, TX, USA). Some of the total RNA from each independent group was pooled by age (total of RNA from approximately 100 oocytes per age) and used for SSH. A SMART cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) was used to reverse-transcribe total RNA to cDNA. SSH was performed with a PCR-Select cDNA Subtraction Kit (Clontech). The subtracted PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Clones from the subtracted library (D24−D17) were sequenced and their identities were examined using the NCBI BLAST search. The differential expression of candidate genes preferentially expressed in mature D24 oocytes suggested by SSH was confirmed with cDNA transcribed separately in the three independent culture groups per age using real-time quantitative PCR with an ABI Prism 7900HT with TaqMan technology (Applied Biosystems, Foster City, CA, USA). Of 513 clones sequenced, the top six preferentially-expressed candidate genes in more developmentally-competent D24 oocytes were spindlin (20 clones), bmi-1 (4 clones), cyclin B1 (4 clones), E330034G19Rik (4 clones), Jagged1 (4 clones), and Ndfip2 (4 clones). The expression of spindlin in mature D24 oocytes (relative threshold cycle: -3.8 ± 0.7, mean ± SD) was confirmed to be approximately 11-fold higher than in D17 oocytes (-0.3 ± 1.5) when GAPDH was used as an internal control ($P < 0.05$, *t*-test). Quantitative analyses of mRNA expression of the remaining genes are now under way. Our results suggest that spindlin is one of the key factors leading to the acquisition of developmental competence in mouse oocytes during folliculogenesis. Supported by JSPS KAKENHI (No.145716000) and MHLW.

252 A COMPARATIVE EXPRESSION ANALYSIS OF GENES IN PREIMPLANTATION DEVELOPMENTAL STAGES OF BOVINE EMBRYOS PRODUCED IN VITRO OR IN VIVO

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A comparative analysis of mRNA expression patterns between embryos produced under different in vitro and in vivo culture systems allows the isolation of genes associated with embryo quality and investigation of the effect of culture environment on the embryonic gene expression. In this study, expression analysis of four known (PSCD2, TCF7L2, NADH-subunit and PAIP1) genes and one novel transcript, derived from differential display PCR, was performed in in vitro (Ponsuksili *et al.*, 2002, Theriogenology 57, 1611–1624) or in vivo- (Moesslacher *et al.*, 2001 Reprod. Dom. Anim. 32, 37) produced bovine 2-, 4-, 8-, 16-cell, morula and blastocyst stage embryos using real time PCR technology. Poly(A) RNA was isolated from four separate individual embryos from each developmental stage and embryo group (in vitro or in vivo) using Dynabeads mRNA kit (Dynal, Oslo, Norway). After reverse transcription, quantitative PCR was performed with sequence specific primers in an ABI PRISM® 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA, USA) using SYBR® Green as a double-strand DNA-specific fluorescent dye. Standard curves were generated for target and endogenous genes using serial dilutions of plasmid DNA. Final quantification was done using the relative standard curve method, and results were reported as relative expression or *n*-fold difference to the calibrator cDNA (i.e., the blastocyst stage) after normalization with the endogenous control (Histone2a). Data were analyzed using SAS version 8.0 (SAS Institute Inc., NC, USA) software package. Analysis of variance was performed with the main effects being the developmental stage and embryo source (in vitro or in vivo) and their interactions followed by multiple pairwise comparisons using Tukey's test. No significant difference was observed in the relative abundance of the PSCD2 gene between the two embryo groups. However, its expression was higher (20-fold) ($P < 0.05$) at the 8-cell stage than the other developmental stages among in vitro embryos. Higher expression ($P < 0.05$) of NADH-subunit mRNA was detected in vivo than in vitro at the 2-cell stage of development. The TCF7L2 mRNA was expressed in the in vitro embryos but not in the in vivo ones. PAIP1 mRNA was higher ($P < 0.05$) in in vitro (1500-fold) than in the in vivo embryos (500-fold) at the 2-cell developmental stage compared to the calibrator. The novel transcript was also detected at higher level ($P < 0.05$) in the in vitro than in the in vivo embryos at the 2-cell stage of development. However, the PAIP1 and the novel transcript showed no significant difference in their expression between the two embryo groups beyond the 2-cell developmental stage. Both PAIP1 and the novel transcript were detected only up to 8-cell stage in both embryo groups, suggesting their maternal origin. In conclusion, the variations in the expression of studied genes between in vitro and in vivo may reflect the effect of the two culture systems on the transcriptional activity of early embryos.

253 BOVINE OOCYTE CYCLIN B1 mRNA UNDERGOES CYTOPLASMIC POLYADENYLATION BEFORE THE BEGINNING OF IN VITRO MATURATION

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Maternal oocyte Cyclin B1 mRNA is known to be stored in the cytoplasm with a short poly(A) tail and be translationally dormant at GV stage. During maturation, Cyclin B1 poly(A) tail is elongated by a process called cytoplasmic polyadenylation and driven by A/U-rich cis-acting elements in its 3′ untranslated region (UTR) known as cytoplasmic polyadenylation elements (CPEs). The objective of this study was to elucidate whether GV-stage bovine oocytes possess a stockpile of Cyclin B1 mRNA stored with a short a poly(A) tail that is elongated during maturation by CPE regulation. The mRNA poly(A) tail length was measured by Rapid Amplification of cDNA Ends Polyadenylation test (Race-PAT) on oocytes ($n = 100$) at the GV stage and 3, 5, 8, 10, 15, 20, and 25 h of in vitro maturation. The mRNA poly(A) tail length was also measured in triplicate ($n = 20$) on cold oocytes in GV (all manipulations on ice), warm oocytes in GV (ovaries transported in warm saline and manipulations on ice) and warm + 2 h 30 min oocytes in GV (oocytes left for an additional 2 h and 30 min at room temperature). To assess for variation in mRNA quantity, Cyclin B1 mRNA level was quantified by real-time PCR (Lightcycler, Roche, Indianapolis, IN, USA) in cold, warm or warm + 2 h

Restoration of spermatogenesis by lentiviral gene transfer: Offspring from infertile mice

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Disruption of spermatogenesis found in azoospermia and oligozoospermia is thought to be of primarily genetic origin. *Sl/Sl^d* mutant mice offer a model system in which lack of transmembrane type c-*kit* ligand (KL2) expression on the somatic Sertoli cell surface results in disruption of spermatogenesis. We investigated the ability of adeno-, adeno-associated-, retro-, and lentiviral vectors to transduce Sertoli cells and found that transduction with either adeno- or lentiviral vectors led to reporter gene expression for more than 2 mo after testicular tubule injection. Because adenoviral vectors showed toxicity, lentiviral vectors were used to express the c-*kit* ligand in *Sl/Sl^d* Sertoli cells. Restoration of spermatogenesis was observed in all recipient testes. Furthermore, the sperm collected from recipient testes were able to generate normal pups after intracytoplasmic sperm injection. None of the offspring carried the transgene, suggesting the inability of lentiviral vectors to infect spermatogenic cells *in vivo*. We propose that lentiviral vectors can be used for gene therapy of male infertility without the risk of germ-line transmission.

Worldwide, up to 20% of couples are infertile. Approximately 30–50% of human infertility is attributable to male infertility, 70–90% of which arises from disrupted or impaired spermatogenesis with a clinical outcome of azoo- or oligospermia (1, 2). Spermatogenesis takes place within the testicular seminiferous tubules that are composed of germ cells, Sertoli cells, and peritubular cells lining the tubule. This process involves a mitotic germ cell proliferation, meiosis, and morphological changes of haploid germ cells to mature spermatozoa. Successful spermatogenesis, however, requires participation outside tubules, interstitial cells such as Lydig cells that produce androgen, macrophages, mast cells, and lymphoid cells. This intricate network of interactions is regulated by many growth factors and hormones and depends on intimate contact between germ cells and somatic Sertoli cells (3). Sertoli cells play a seminal role in normal spermatogenesis by providing not only structural support but also a variety of growth factors required for differentiation and proliferation of germ cells. In addition, inter-Sertoli cellular tight junctions confer the "blood–testis barrier" and partition the testis into an intratubular compartment (4, 5). Therefore, Sertoli cell dysfunction will impair spermatogenesis and result in male infertility. Currently there are no effective modalities to correct such genetic defects in animals or humans (6, 7).

To investigate whether viral-vector mediated gene transfer can be used for correcting Sertoli cell dysfunction, we used *Sl/Sl^d* mutant male mice that have Sertoli cell dysfunction and are consequently infertile. The *Sl* locus (steel) encodes both the soluble and membrane-bound forms of c-*kit* ligand (KL) that bind to the c-*kit* tyrosine kinase receptor synthesized by the *W* locus (dominant white spotting). In the testis, the c-*kit* receptor is expressed on the germ cells from spermatogonia, whereas the c-*kit* ligand is produced by the somatic Sertoli cells, and interaction between these two factors is essential for spermatogonial cell proliferation. The *Sl* mutation deletes the entire *Sl* gene, whereas the *Sl^d* mutation deletes the transmembrane and intracellular domain, thereby generating only soluble forms of the c-*kit* ligand (KL1) and leading to azoospermia. Additionally, seminiferous tubules of these mice are virtually devoid of germ cells, a clinical condition known as Sertoli cell only syndrome (8). We therefore asked whether spermatogenesis can be restored in *Sl/Sl^d* mice by transducing Sertoli cells with lentiviral vectors generating functional the c-*kit* ligand, KL2. We report that not only is spermatogenesis restored in all recipient testes, but also spermatozoa collected from transduced testes were able to generate normal pups by microinsemination.

Materials and Methods

Preparation of Viral Vector Plasmids and Viral Vector Production. The AdV-CMV-nls*lacZ* (nls, nuclear localization signal; AdV, adenoviral vector; CMV, cytomegalovirus) vector and the AAV-CMV-*lacZ* (AAV, adeno-associated virus type 2) vector were prepared as described (9, 10). In this study, we constructed the pRV-CMV-nls*lacZ* plasmid in the murine leukemia virus-based retroviral vector, pCLNCX (11) by replacing a fragment containing the neo resistant cassette and the CMV promoter with CMV-nls*lacZ*-WPRE (WPRE, woodchuck hepatitis virus posttranscriptional regulatory element). We constructed the pLV-CMV-nls*lacZ* and pLV-CMV-*lacZ* (LV, lentiviral vector) plasmids in the HIV-based self-inactivating lentiviral vector, pRRLsin-hPGK-EGFP (EGFP, enhanced green fluorescent protein) by replacing the hPGK-EGFP fragment with the CMV-nls*lacZ* or CMV-*lacZ* fragment, respectively (12). To construct pLV-CMV-KL2, we replaced the nls*lacZ* fragment of pLV-CMV-nls*lacZ* with the KL2 fragment amplified by PCR from the testicular cDNA library with primers (KL2for: 5′-ctggatccgccaccatgaagaagacacaaacttgg-3′) and (KL2rev: 5′-ctgtcgactattacacctcttgaaattctctc-3′). Vesicular stomatitis virus G envelope protein-pseudotyped retroviral and lentiviral vectors were generated as described (12).

Recipient Mice and Viral Vector Injection Procedure. In the first experiment, we injected recombinant adenoviral, AAV, retroviral, and lentiviral vectors into male C57BL/6 × DBA2 F1 hybrid mice (B6D2F1) at 6 wk of age. Approximately 10 μl of viral vector solution containing 0.04% trypan blue was injected into seminiferous tubules via the efferent ductules. Viral vector was injected into the right testis and the left testis remained as control. In the second experiment, B6D2F1 male mice were injected with the LV-CMV-nls*lacZ* vector into both testes at 6 wk of age and housed with 3 B6D2F1 females for 3 mo. In the third experiment, ~3 μl of LV-CMV-KL2 vector was injected into *Sl/Sl^d* male mice at 3–6 wk of age. In all experiments, recipient mice were anesthetized by i.p. injection of Avertin before the operation. The seminiferous tubule injection was performed according to the method described (13), and more than 70% of seminiferous tubules were filled with solution as determined by trypan blue.

Abbreviations: AdV, adenovirus; CMV, cytomegalovirus; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; nls, nuclear localization signal; IU, infectious unit.

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METHODS

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Production of mitochondrial DNA transgenic mice using zygotes

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Abstract

Several animal models of human disease, which have been developed by random or targeted modifications of genomic DNA sequences, have furthered our understanding of pathogenesis and the development of therapeutics. However, these models have not facilitated studies on mitochondrial diseases, since modifications to mitochondrial DNA (mtDNA) sequences are not possible using current recombination techniques. Consequently, information on human mitochondrial diseases is relatively sparse, and issues related to mitochondrial pathogenesis and inheritance remain unresolved. Recently, we reported the development of a new technique to generate mice carrying mutant mtDNA from a mouse cell line. In this report, we describe our techniques in detail, with emphasis on the preparation of donor cytoplasts and the micromanipulative procedures for electrofusion of cytoplasts and recipient zygotes. These steps are critically important for the successful introduction of exogenous mtDNA into embryos, and thereby into animals, so that the mutant mtDNA is efficiently propagated in subsequent generations.

Keywords: Mitochondrial DNA; Mitochondrial disease; Mouse model; Electrofusion; Cytoplast; Zygote

1. Introduction

Point mutations or deletions in mitochondrial DNA (mtDNA) may result in defective oxidative phosphorylation [1] and thus affect a variety of tissues, particularly those that require high-level energy sources for normal function, such as the brain or skeletal muscles [2]. Moreover, it has been discovered that mutated mtDNA participates in aging [3–5], diabetes mellitus [6,7], and renal [8–10] and neurodegenerative [11–13] diseases. Genetic studies on patients suffering from these diseases have provided important clues as to the inheritance and genetics of mitochondrial disorders. Recently, mtDNA-depleted ρ^0 cells have been used in vitro to examine the relationship between mutated mtDNA sequences and the pathogenicity of mitochondrial diseases [14–16]. However, the pathogenesis and genetics of mitochondrial disorders remain largely unresolved due to the lack of suitable animal models for mitochondrial disorders.

Several technical difficulties have been encountered in generating animal models for mitochondrial diseases. First, unlike nuclear DNA, it has not been possible to introduce mutations into mtDNA by conventional transgenic techniques, because mtDNA recombination is poor [17]. Second, it is extremely difficult to introduce exogenous mtDNA into the intact mitochondria of most animal cells. Third, a large proportion of the mtDNA in each cell must be replaced with mutated mtDNA for clinical symptoms to appear [15]. Fourth, no efficient method has been established for the introduction of exogenous mtDNA into living animals. We previously established a mouse model for mitochondrial disease (Fig. 1) that overcomes these difficulties [18,19]. The model was developed by: (1) the establishment of a cell line with a consistent proportion of mtDNA that had a 4696-bp deletion; (2) the preparation (enucleation) of donor cells; (3) the introduction of mtDNA into mouse zygotes by electrofusion with the donor cytoplasts; (4) embryo transfer of reconstructed embryos into recipient females; and (5) analysis of the mice into which the 4696-bp deletion had been introduced. A relatively small amount of the exogenous mtDNA was detected in the founder mice,

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with increasing amounts of DRAP1 protein inhibited FoxH1 binding, whereas incubation with nonspecific proteins or with Dr1 had no effect (fig. S3). Finally, we assessed binding of a FoxH1-Smad2-Smad4 transcription factor complex (activin-responsive factor, ARF) (*25, 26*) to an activin/Nodal-response element in nuclear extracts from activin-treated cells. We found that addition of DRAP1 alone, or DRAP1 together with Dr1, could effectively inhibit ARF binding, whereas Dr1 alone had no detectable effect (Fig. 4F) (fig. S3). These results indicate that DRAP1, independently of Dr1, can effectively compete for FoxH1 binding to DNA.

Our loss-of-function analysis has revealed the earliest essential role for *Drap1* in embryogenesis, in repressing the activity of the Nodal signaling pathway. Although these findings do not preclude multiple subsequent functions for *Drap1*, they show that *Drap1* is not essential for numerous patterning and differentiation events at pregastrulation stages. On the basis of our protein interaction data, we propose that DRAP1 regulates Nodal signaling in vivo through an interaction between DRAP1 and FoxH1 that precludes FoxH1-Smad2-Smad4 complex binding to its cognate DNA targets. Notably, this model implies that DRAP1-mediated repression is not universally exerted by forming a complex with Dr1 and TBP, in agreement with earlier suggestions (*11, 13*).

These findings suggest that a normal function of DRAP1 is to down-modulate the transcriptional response to Nodal signaling, particularly by attenuation of its positive feedback loop. Such a mechanism is likely to be essential for Nodal, which can function as a long-range morphogenetic signal (*27*). First, *Drap1* might function in nascent mesoderm to allow specification of distinct mesoderm fates in response to differing levels of inducing signal. Second, *Drap1* might function in epiblast cells to buffer the response to mesoderm-inducing signals and maintain prospective ectoderm unresponsive to low levels of mesoderm-inducing signals. Thus, *Drap1* may represent a key component of a mechanism for limiting the spatial or temporal extent of the response to a potent morphogenetic signal.

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www.sciencemag.org/cgi/content/full/298/5600/1996/DC1
Materials and Methods
Figs. S1 to S3

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Regulation of Spermatogenesis by Testis-Specific, Cytoplasmic Poly(A) Polymerase TPAP

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Spermatogenesis is a highly specialized process of cellular differentiation to produce spermatozoa. This differentiation process accompanies morphological changes that are controlled by a number of genes expressed in a stage-specific manner during spermatogenesis. Here we show that in mice, the absence of a testis-specific, cytoplasmic polyadenylate [poly(A)] polymerase, TPAP, results in the arrest of spermiogenesis. TPAP-deficient mice display impaired expression of haploid-specific genes that are required for the morphogenesis of germ cells. The TPAP deficiency also causes incomplete elongation of poly(A) tails of particular transcription factor messenger RNAs. Although the overall cellular level of the transcription factor TAF10 is unaffected, TAF10 is insufficiently transported into the nucleus of germ cells. We propose that TPAP governs germ cell morphogenesis by modulating specific transcription factors at posttranscriptional and posttranslational levels.

Poly(A) tails of eukaryotic mRNAs are implicated in various aspects of mRNA metabolism, including transport into the cytoplasm, stability, and translational control (*1, 2*). Thus, the control of poly(A) tail length is one of the posttranscriptional regulators of gene expression. Spermatogenesis—differentiation of male germ cells—is a specialized developmental process, which is precisely regulated at the transcriptional, posttranscriptional, and translational levels (*3, 4*). In previous work, we identified a testis-specific, cytoplasmic poly(A) polymerase, TPAP (PAPβ), as a candidate molecule involved in the additional extension of poly(A) tails of preexisting mRNAs in haploid germ cells, because this gene is expressed predominantly in round spermatids (*5*).

To elucidate the role of TPAP in spermatogenesis, we produced mutant mice lacking the functional TPAP gene (*Tpap*$^{-/-}$), using homologous recombination in embryonic stem cells (*6*) (fig. S1). Analysis of testicular RNA from *Tpap*$^{-/-}$ mice revealed the absence of TPAP mRNA, and protein extracts of the mutant mouse testis completely lacked 70-kD TPAP (*6*) (fig. S1). *Tpap*$^{-/-}$ male and female mice were normal in health condition, size, and behavior. However, *Tpap*$^{-/-}$ males were

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Analysis of the Mechanism for Chromatin Remodeling in Embryos Reconstructed by Somatic Nuclear Transfer[1]

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ABSTRACT

The objective of the present study was to understand the molecular/biochemical nature of chromatin remodeling that occurs in the somatic nuclei transferred into oocytes. We produced the reconstructed mouse embryos by two different protocols of nuclear transfer. The nucleus of a cumulus cell was transferred into enucleated unfertilized oocytes (transferred before activation, TA protocol) or activated oocytes (activated before transfer, AT protocol). More than half (56.1%) of the embryos reconstructed using the TA protocol developed to the morula/blastocyst stage, whereas very few (1.0%) of the embryos reconstructed using the AT protocol reached the morula/blastocyst stage. These embryos were analyzed for the events associated with transcriptional regulation. Changes in transcriptional activity, nuclear accumulation of TATA box binding protein (TBP), and DNase I sensitivity were examined after nuclear transfer. In the embryos reconstructed by TA protocol, all of these events occurred in a manner similar to that in the control diploid parthenogenetic embryos. The transcriptional activity was silenced after nuclear transfer and resumed at the late 1-cell stage. TBP was displaced and subsequently accumulated at the early and the late 1-cell stage, respectively. DNase I sensitivity was increased and then decreased at the early and late 1-cell stage, respectively. In contrast, embryos reconstructed using the AT protocol did not show such changes in transcriptional activity, TBP accumulation, and DNase I sensitivity. These events would be necessary for differentiated nuclei to restore totipotency and are useful indices to evaluate successful chromatin remodeling.

developmental biology, early development, embryo

INTRODUCTION

Successful generation of cloned animals by nuclear transfer of somatic cells has been reported in several mammalian species [1–5]. These reports proved that somatic nuclei could reverse their developmental clock to recover totipotency when introduced into appropriate cytoplasmic environments. In the initial stage of embryogenesis, the embryos are under the control of maternally derived proteins and transcripts accumulated in the oocyte until activation of the zygotic genome (zygotic gene activation). After that, the development is controlled by the zygotic nucleus. Thus, the transferred nuclei should change their gene expression pattern to that of the early embryo nuclei for further successful development. This change, which may affect the subsequent gene expression pattern, is generally termed reprogramming and is preceded by altering the configuration of chromatin, i.e., chromatin remodeling [6–8]. In spite of its biological importance, little is known about the molecular nature of the reprogramming event.

The methods used for cloning procedures fall into two groups in terms of the cell cycle stage of the recipient cytoplasm. One method is transplanting the nuclei into metaphase II (MII)-arrested unfertilized oocytes. This approach leads to nuclear envelope breakdown (NEBD) and subsequent premature chromosome condensation (PCC) due to the high level of maturation/M-phase promoting factor (MPF) in the oocyte cytoplasm [9–12]. The other method is to use activated ova, which have been relieved from MII arrest and have resumed the cell cycle, as the recipient cytoplasm. In this case, NEBD and PCC of the donor nuclei do not occur because the activity of MPF has already declined [9, 13–15]. Earlier studies demonstrated that MII-arrested nonactivated oocytes are far more effective for supporting development of embryos reconstructed with differentiated nuclei than are activated oocytes [16, 17]. Thus, success in chromatin remodeling, from differentiated nuclei to totipotent ones, is dependent on the cell cycle stage of the recipient's cytoplasm. The ability to allow remodeling of chromatin apparently exists in MII-arrested nonactivated oocytes and disappears after activation.

The purpose of this study was to investigate the molecular/biochemical basis of chromatin remodeling that occurs in the somatic nuclei transferred into oocytes. We reconstructed the embryos by two different nuclear transfer protocols, transfer of nuclei into the oocytes before and after activation, to investigate the difference in the molecular/biochemical events involved in chromatin remodeling when the nuclei are successfully remodeled and when they are not. This comparison would provide us with an important key to understanding the mechanism of chromatin remodeling. The embryos reconstructed by two different protocols were compared with each other using molecular/biochemical criteria such as transcriptional activity, distribution of basal transcription factor, and DNase I sensitivity of the chromosomes.

MATERIALS AND METHODS

Collection of Oocytes and Preparation of Donor Cells

Female B6D2F$_1$ (C57BL/6 × DBA/2 hybrid; SLC, Shizuoka, Japan) mice 8–9 wk of age were superovulated with 7.5 IU eCG followed by 7.5

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Epigenetic regulation in mammalian development and dysfunction: the effects of somatic cloning and genomic imprinting

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Abstract

Although somatic cell cloning has been accomplished in several mammalian species, its efficiency remains considerably low due to fetal mortality during the pre- and perinatal periods, which suggests incomplete initialization of epigenetic memories during the somatic cloning procedure. Genomic imprinting is an epigenetic mechanism that produces functional differences between the paternal and maternal genomes, and plays an essential role in mammalian development and growth. Therefore, it is very important to examine the genomic imprinting status of somatic clones. The placenta is one of the most commonly affected organs in the somatic clones. We confirmed that parental-origin-specific monoallelic expression of imprinted genes was maintained faithfully in cloned embryos and abnormal placentas. However, reduced expression was observed for several genes, including certain imprinted genes in both day 12.5 and term placentas. These results suggest that the development process in cloned mice is not identical to that in normal mice. We analyzed mouse clone embryos, which were produced from primordial germ cells (PGCs), and

Abbreviations: Peg, paternally expressed gene; Meg, maternally expressed gene; PGC, primordial germ cell; DMR, differentially methylated region.

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Phenotypic Effects of Somatic Cell Cloning in the Mouse

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ABSTRACT

Although a variety of phenotypes and epigenetic alterations have been reported in animals cloned from somatic cells, the exact nature and consequences of cloning remain unclear. We cloned mice using fresh or short-term cultures of donor cells (cumulus cells, immature Sertoli cells, and fetal or adult fibroblast cells) with defined genetic backgrounds, and then compared the phenotypic and epigenetic characteristics of the cloned mice with those of fertilization-derived control mice. Irrespective of the nucleus-donor cell type, about 50% of the reconstructed embryos developed to the morula/blastocyst stage, but about 90% of these clones showed arrested development between days 5 and 8, shortly after implantation. Most of the clones were alive at term, readily recovered respiration, and did not show any malformations or overgrowths. However, their placentas were two- to threefold larger than those of the controls, due to hyperplasia of the basal (or spongiotrophoblast) layer. Although there was significant suppression of a subset of both imprinted and non-imprinted placental genes, fetal gene suppression was minimal. The seven imprinted genes that we examined were all expressed correctly from the parental alleles. These findings were consistent for every cell type from the midgestation through term stages. Therefore, cloning by nuclear transfer does not perturb the parent-specific imprinting memory that is established during gametogenesis, and the phenotypic and epigenetic effects of cloning are restricted to placental development at the midgestation and term stages. Twelve male mice that were born in a normal manner following nuclear transfer with immature Sertoli cells (B6D2F1 genetic background) were subjected to long-term observation. They died earlier than the genotype-matched controls (50% survival point: 550 days vs. 1028 days, respectively), most probably due to severe pneumonia, which indicates that unexpected phenotypes can appear as a result of the long-term effects of somatic cell cloning.

INTRODUCTION

THE EFFICIENCY WITH WHICH CLONED ANIMALS are produced from somatic cells has improved consistently over the years due to optimization of the individual cloning steps, which include the enucleation of oocytes, oocyte activation, and embryo culture. However, unpredictable cloning-associated phenotypes still arise, some of which are fatal, especially in domesticated species. Al-

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Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination

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BACKGROUND: Fertility protection is an urgent clinical problem for prepubertal male oncology patients who undergo either chemotherapy or radiotherapy. As these patients do not have mature sperm to be frozen, there is as yet no effective method to preserve their fertility. METHODS AND RESULTS: Single pieces of immature mouse (1.5 × 1.5 × 1.5 mm) or rabbit (2.0 × 2.0 × ~3.0 mm) testis were cryopreserved, thawed and transplanted into mouse testes. Histological techniques were used to determine the presence of spermatogenesis, which was restored in both mouse and rabbit testicular pieces, and led to the production of mature sperm after both cryopreservation and syngeneic or xenogeneic transplantation into mouse testes. Using sperm developed in the frozen–thawed transplants, mouse offspring were born after in-vitro microinsemination. Furthermore, rabbit offspring were obtained using rabbit sperm that developed in fresh transplants in a xenogeneic surrogate mouse. CONCLUSIONS: This approach of 'testicular tissue banking' is a promising technique for the preservation of fertility in prepubertal male oncology patients. Xenogeneic transplantation into immunodeficient mice may provide a system for studying spermatogenic failure in infertile men.

Keywords: cancer/infertility/in-vitro microinsemination/ testis/transplantation

Introduction

Recent advances in chemotherapy and radiotherapy have significantly improved remission and complete recovery rates in cancer patients. However, because germ cells are highly susceptible to cytotoxic treatments, iatrogenic loss of fertility has emerged as a major side effect of successful treatment (Aslam *et al.*, 2000). In males, sperm freezing is an established method to preserve germline cells (Fossa *et al.*, 1989; Royére *et al.*, 1996; Lass *et al.*, 2001), and is performed routinely in clinics for those patients who wish to preserve their fertility before undergoing treatment for malignancy (Lass *et al.*, 2001). Despite the small amount and poor quality of semen specimens from oncology patients (Fossa *et al.*, 1989; Hovatta, 2001), pregnancies have been reported using frozen–thawed sperm (Royére *et al.*, 1996; Lass *et al.*, 2001). Unfortunately, the technique cannot be applied to prepubertal patients, who do not have sperm, although their testes contain gonocytes or immature spermatogonia. Approximately 1 in 650 children develop malignancies during childhood and it is estimated that, by 2010, one in 250 young adults (aged 20–29 years) will be long-term survivors of childhood cancer (Aslam *et al.*, 2000). Thus, the preservation of male germline cells in prepubertal boys is an urgent clinical problem (Aslam *et al.*, 2000; Hovatta, 2001).

In females, transplantation of frozen–thawed ovarian pieces has been successful in both laboratory and domestic animals. Live births or pregnancies have been reported after orthotopic transplantation of frozen–thawed ovarian tissues or whole ovary in the mouse (Parrot, 1960), rat (Wang *et al.*, 2002) and sheep (Gosden *et al.*, 1994). Based on the success in animal experiments, the first ovarian transplantation trial was recently initiated in humans, and the results demonstrated the promise of possibly 'frozen banking' of an ovarian tissue piece for female cancer patients (Oktay, 2001; Radford *et al.*, 2001).

Given the successful outcome of tissue cryopreservation in females, a valuable strategy to preserve male fertility would be to develop spermatogenesis from immature spermatogonia in pieces of frozen–thawed testicle. Early attempts to freeze testicular pieces met with limited success (Nugent *et al.*, 1997);

クローン動物の頻発異常とエピジェネティクス

体細胞クローンマウスの異常

井上貴美子・越後貫成美・持田慶司・小倉淳郎

体細胞核移植技術の開発当初より，クローン動物に生じるさまざまな異常が深刻な問題として表面化しており，それはクローンマウスにおいても例外ではない．クローン家畜と異なり出生時の外貌奇形や死産は少ないが，それでも大多数の胚の着床前後における脱落と，ほぼ必ずといえる胎盤過形成が観察される．また遺伝的背景に依存して成体の体重増加，血清生化学値異常，免疫機能低下なども報告されている．一方では，マウスを用いることにより初めて，核移植後もドナー体細胞ゲノムの刷込み記憶が維持されていることが明らかにされた．今後もクローンマウスの解析を通じて，クローン動物の異常につながる機序が解明されることが期待される．

Key words クローンマウス，再プログラム化，刷込み遺伝子

はじめに

この数年の体細胞を用いた核移植技術の改良に伴い，ヒツジ，マウス，ウシ，ヤギ，ブタ，ネコ，ウサギとつぎつぎに体細胞クローン動物作製の成功例が報告されてきた[1~7]．このなかでもマウスはクローン作出の成功が報告されている唯一の小型実験動物種であり，これまでにクローンにかかわるさまざまな基礎的研究に利用されてきている．マウスをクローン作出に使用することは他の動物種と比較して以下の利点があげられる．①遺伝的背景の明確な多くの近交系が樹立され，またそのF_1交雑系も容易に利用できる．②寿命が短く，一生を通じた経時的変化を観察しやすい．③採卵，胚移植，飼育が簡便なため，実験個体数を多く揃えることができる．④妊娠期間が短く，次世代への影響が観察しやすい．とくに①は，クローン動物の体系的なデータ収集にはきわめて重要であるが，体細胞クローンに成功している動物のうち近交系が利用できるのはマウスのみである．これらの近交系あるいはそのF_1マウスをクローン実験に利用することにより，遺伝的背景が同一のコントロールとの比較が可能となり，クローンに特有の現象を浮き彫りにすることができる．すなわち，クローンマウスの表現型あるいは遺伝子発現における異常を科学的に特定できるわけである（実際には個体作製まで成功するマウスクローン実験のほとんどは，F_1交雑系をドナー細胞および卵子に用いている）．本稿では，これまでにマウスを用いて明らかにされたクローン動物の異常について，着床前，着床後，出生時，出生後のそれぞれの時期に分けて述べてみたいと思う．

I. マウス体細胞クローン技術の現状

筆者らはおもに細胞質内注入法によりドナー核を除核未受精卵に移植している（図1）．マウスドナー核の移植には，そのほかに電気パルスや不活化センダイウイルスを用いた膜融合の方法も用いることが可能である[8~10]．注入法による核移植は，多数の卵子に短時間で連続的に核注入ができ，そして核移植手技が1ステップで終了する（電気融合はドナーの囲卵腔への挿入と電気穿孔という2ステップになる）という利点がある．一方では，注入により卵子が死にやすい，注入に適した小さい細胞しかドナー核として使用できない，などの欠点がある．いずれの方法を用いた場合も重要なのは，そのレシピエント卵子細胞質中で，ドナー核ゲノムが効率よく受精卵のゲノムに再プログラム化（初期化）[→今月のKey Words

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Abnormalities of mice cloned from somatic cells

伝子の総数が3～4万とすると，センチュウのわずか2倍です．体の構造の複雑さには100倍からの差がありそうですが……．

発生の制御にかかわる繊維芽細胞増殖因子は，ショウジョウバエ（以下，ハエ）とセンチュウではいずれも2種類なのに，ヒトでは30種類とわかりました．細胞骨格にかかわる遺伝子の数も，ヒトでは大きく増えています．ヒトでは免疫など生体の防御にかかわる遺伝子群の発達も目につきました．他方，細胞内の情報伝達にかかわる遺伝子については，センチュウ，ハエ，ヒトで，ほとんど差がありませんでした．細胞の生存に欠かせない遺伝子は共通で，体の構造や特殊な機能にかかわる遺伝子は違う，という構図がありそうです．ヒトで見つかった1262グループのタンパク質のうち，脊椎動物に限るものは94種類だけだったことも，これを裏づけます．ヒトのもつ複雑なタンパク質も，基本単位となる構造（ドメイン）は，センチュウやハエと差がなく，ヒトではドメインを複雑に組み合わせているのです．

ヒトには，嗅覚にかかわる遺伝子が900ほどもあるうち，実に60％が壊れていました．構造からみて，ここ1000万年ほどの間に機能が失われたというのです．猿人の時代から，ヒトは嗅覚に頼らない生活を続けてきたのでしょう．生存に欠かせない遺伝子に突然変異が起きると，その個体は子孫を残せないので変異遺伝子は集団から消え，正常な遺伝子が残ります．使わない遺伝子は変化しても実害がないので，壊れた遺伝子が残るのです．

「チンパンジーとヒトとでは1％強しか塩基配列の差がない」，という話を聞きますが，これはゲノムのほんの一部について比較を行なった結果で，チンパンジーの解析がもっと進まないと本当のところはわかりません．また解析が先行している遺伝子は，ヘモグロビンや酵素など，ある程度の量のタンパク質が得られるものです．変化したら健康が大きく損なわれるのですから，ヒトとチンパンジーで差がなくても不思議はありません．問題は脳や精神にかかわる遺伝子ですが，これからの進展が楽しみという段階です．

ここで紹介した以外の動物としては，ある種のフグが注目されました．ゲノムがずいぶん小さいのですが，哺乳類に比べて反復配列が少ないことが原因でした．遺伝子の数は，あまり変わらないのです．マウスについても解析が進行中ですが，ヒトで見つかった本態不明の遺伝子と似たものをマウスで探し，それを壊して（ノックアウトマウスの作製）症状を観察し，機能を推定するといったアプローチが，広く使えるようになります．たとえば，記憶力もマウスで調べることができるのです．

なお『遺伝』では，別冊15号「遺伝学はゲノム情報でどう変わるか」（仮題；今秋刊行の予定）の出版準備を進めています．さまざまな動植物のゲノム解析の現状のほか，進化，寿命，医療など関連する話題を広く取りあげます．ゲノムに興味をおもちの方はぜひご覧下さい．

（中込弥男，順天堂大学 医学部客員教授）

クローンは正常か？

多くの細胞の分化は不可逆であるが，その分化の過程にはゲノムDNAの塩基配列の変化は伴わない（リンパ球など一部の例外を除く）．すなわち分化した細胞のゲノムDNAの塩基配列は，個体発生の出発点である受精卵のそれとまったく同じであるにもかかわらず，ゲノムの修飾（DNAシトシンのメチル化など）の状態が異なるために分化した細胞として機能するのである．それを実験的に証明したのが，

体細胞クローン動物の誕生である．現在までに，ヒツジ，マウス，ウシ，ヤギ，ブタ，ネコ，そしてウサギで成功が報告されている．

それではなぜ，体細胞の核を未受精卵子の細胞質(卵子の染色体はあらかじめ除く）へ移植すると，クローンが誕生するのであろうか．これは，ゲノムの修飾の状態を受精卵と同じ状態にする（ゲノムの初期化という）機構が，卵子の細胞質中に存在するために，体細胞のゲノムが受精卵のゲノムに戻るからである．もちろんこの初期化の機構は，クローンを作るために存在するのではない．受精する前の卵子も精子も，それぞれ分化した細胞であり，そのゲノムの修飾の状態は受精卵とは大きく異なる．この卵子と精子が合一し，受精が成立した際，両者のゲノムが受精卵のゲノムになるために初期化機構が存在するのである．

初期化させる因子の実態はつきとめられていないが，これを利用したのが核移植クローンである．この場合，体細胞のゲノムも核移植後に受精卵のゲノムとまったく同等になるであろうか．状況証拠からすると，「ノー」と言わざるをえない．マウスでも家畜でも，クローン胚が新生児まで発生する率はきわめて低く，またクローン特有の遺伝子発現パターンや発現型が生じることが知られている[1]．

受精卵クローン（受精後に分割を始めた着床前胚の割球をドナー細胞として使う）と体細胞クローン(体細胞をドナー細胞として使う）を比べると，明らかに後者のほうが異常を生じていることから，やはり体細胞ゲノムが初期化される過程でエラーが生じている可能性が高い．そのエラーがどうして起こるのかは明らかになっていないが，少なくともそのエラーは短期的（出産まで）にも長期的（出産後）にも影響を与えるようである．短期的な影響としては，着床前の胚の発生停止，妊娠中および出産前後の胎児死亡，奇形，胎盤異常（口絵7頁下）などがある．ウシやヒツジでは，これらの異常は頻繁であり，またその形は非常に多様である．長期的な影響としては，免疫能の低下（ウシ，ヤギ，マウス），肥満（マウス），肺炎（マウス，ウシ），心臓肥大(ウシ)，早期死亡（マウス）などの表現型が知られている．

遺伝学的に純系あるいはその交雑系の実験用マウスを用いた結果から，これらクローン特有の影響が遺伝的背景により左右されることがわかってきている．筆者らは，クローンマウスが短命であったと報告した[2]が，それは特定のマウス系統に限ったことであり，別のマウス系統を用いればまた違った影響が現れるのである．まったく同じ遺伝子型に固定された近交系マウスとは異なり，遺伝的に不均質である家畜におけるクローン個体の表現型のばらつきも，これで説明がつく．そうなると，家畜よりもさらに遺伝学的に多様性に富むヒトでは，クローンの影響として何が起こるかは予期不能と考えられ，ヒトクローン出産計画などとんでもないということがわかる．

では，こんなに何年も核移植クローン研究が行われていながら，科学的に説明できないことばかりなのであろうか．じつは徐々にではあるが，分子遺伝学レベルでの解析が進んでいる．たとえば，ゲノム刷込みと核移植クローンの関係がある．ゲノム刷込みとは，哺乳類特有の現象であり，全遺伝子のごく一部（刷込み遺伝子という）が両親からくる二つの遺伝子座のうち一方からだけ発現する機構のことである．配偶子の発生過程において，それぞれの遺伝子座に配偶子発生過程で付けられた刷込み記憶が存在し，これらの刷込み遺伝子は，父方あるいは母方の遺伝子座からのみ発現するように制御されている．これらの遺伝子（刷込み遺伝子）の多くは，個体発生の段階で重要な役割を担っている．哺乳類の場合，単為発生胚（卵子のゲノムのみをもつ）が子どもにまで発生しないのはそのためである．この刷込み記憶も，DNAの塩基配列の変更を伴わないゲノム修

疾患モデルとしてのクローンマウス

Cloned mice as experimental models for epigenetic diseases

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Atsuo Ogura

◎哺乳類の体細胞クローン産子はきわめて多様な異常表現型を示すことが知られている．著者らは，マウスのクローン胎仔および産子を解析することにより，核移植クローン特有の遺伝子発現や表現型の異常パターンを明らかにした．まだ研究ははじまったばかりであるが，遺伝的背景の明確なマウスをクローンの実験系に用いることにより，近い将来に，もっとも根本にあるクローン異常の原因が明らかにされることが期待される．

Key word 核移植クローン，マウス，胚性幹細胞，体細胞，胎盤

この数年間の哺乳類における体細胞核移植クローン技術の進展は著しい．1996年の胎仔細胞由来ヒツジクローンにはじまり[1]，1997年の成体細胞ヒツジクローン（ドリー）[2]，そして現在までにマウス[3]，ウシ，ヤギ，ブタ，ネコ，ウサギで体細胞核移植クローンが誕生している．それらクローン個体の表現形はドナー個体と比較していくつかの相違点があり，とくに家畜ではクローン個体間におけるばらつきがめだつ傾向にある．遺伝子型（塩基配列）に変異がないとすれば，これらの表現形の多様性はおもにゲノム修飾や遺伝子発現系の変異に起因すると推察される．しかし，一連の核移植クローン個体作出手技には，ドナー細胞の処理や体外胚培養など核移植とは直接関係なくエピジェネティックな変異を生じさせる要因が含まれており，核移植による影響そのものを特定することは困難である．

クローン個体の表現型を詳細にみると，①個体差の範疇と考えられる表現型（ぶちのパターンなど），②体外操作時や胚培養時の人為的影響（ウシのlarge calf syndrome[4]など）や用いた細胞の性質など周辺技術に依存した表現型，そして，③体細胞核移植に特有の表現型，に分類できると思われる．同様の顕微操作を行う技術として顕微授精などがあるが，顕微授精がいかに高度になってもあくまで“受精”の域を脱しないのに対し，体細胞核移植クローン技術は配偶子形成などの過程をショートカットして個体を作出するという不自然さがある．この無理に由来するクローン個体の表現型，すなわち上の③にあたる表現型あるいは遺伝子発現パターンが，核移植クローン技術を生物学的に理解するうえでもっとも重要であると考えられる．そのうちいくつかは明らかに病理学的な表現型を伴い，エピジェネティクな変異による疾患であるといえる．

これらを解析するうえでマウスは最適な動物種である．すなわち，遺伝学的情報が豊富で遺伝学的に均質な集団の作出が可能であるので，正確な対照を用いた実験系の確立が可能である．もちろん家畜に比較するとコストも時間も桁違いに節約できる．しかし，マウスでの研究成果の多くがほかの動物種にあてはまらない可能性もある．

本稿ではこれらの利点を生かして，これまでに明らかにした核移植クローン技術に由来する異常な表現型を解説する．なお，これまでに報告されたマウスクローンの表現型異常を表1にまとめた．

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Short Communication

Improved Postimplantation Development of Rabbit Nuclear Transfer Embryos by Activation with Inositol 1,4,5-Trisphosphate

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ABSTRACT

Cloned rabbit embryos are characterized by their extremely poor postimplantation development, despite their high survivability until the blastocyst stage *in vitro*. This study examined whether the developmental failure of cloned rabbit embryos *in vivo* can be overcome by technical improvements to the activation protocol. Freshly collected cumulus cells were transferred into enucleated oocytes by intracytoplasmic injection. One to two hours later, the oocytes were activated by electroporation with Ca^{2+} or inositol 1,4,5–trisphosphate (IP3), which is known to induce repeated rises in intracellular Ca^{2+}, as in normal fertilization. After transfer of embryos at the two- to four-cell stages, well-defined implantation sites with remnant fetal tissue were observed at term (day 28) only in the IP3-stimulation groups (0.9% and 5.8% per transferred embryo for single and triple stimulation groups, respectively). When some recipients in the same group were examined at days 16–20, a viable cloned fetus (day 19) with normal organogenesis was obtained. These findings clearly demonstrate that the oocyte activation protocol using IP3 enhances the postimplantation development of nuclear-transferred rabbit embryos.

INTRODUCTION

FOR ABOUT 50 YEARS, rabbits have provided good experimental models for the study of reproductive biology. In 1988, the first intracytoplasmic sperm injection (ICSI)–derived offspring in mammals was reported in the rabbit (Hosoi et al., 1988). Successful rabbit blastomere cloning was also reported as early as 1988 (Stice and Robl, 1988), following success in sheep (Willadsen, 1986) and cows (Prather et al., 1987). Despite much effort, however, rabbit somatic cell cloning has been hampered because of the inability of the reconstructed embryos to undergo the implanta-

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Paternal Expression of a Novel Imprinted Gene, *Peg12/Frat3*, in the Mouse 7C Region Homologous to the Prader–Willi Syndrome Region

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Paternally expressed imprinted genes (*Pegs*) were systematically screened by comparing gene expression profiles of parthenogenetic and normal fertilized embryos using an oligonucleotide array. A novel imprinted gene, *Peg12/Frat3*, was identified along with 10 previously known *Pegs*. *Peg12/Frat3* is expressed primarily in embryonic stages and might be a positive regulator of the Wnt signaling pathway. It locates next to the *Zfp127* imprinted gene in the mouse 7C region, which has syntenic homology to the human Prader–Willi syndrome region on chromosome 15q11-q13, indicating that this imprinted region extends to the telomeric side in the mouse. © 2002 Elsevier Science

Key Words: **genomic imprinting; Prader–Willi syndrome; *Peg12/Frat3*; paternally expressed gene.**

Among vertebrates, genomic imprinting is a mammalian-specific phenomenon in which functional differences between paternal and maternal genomes produce several parental-origin-specific phenotypes in development, growth, behavior, and some human genetic diseases and cancers (1–5). It is explained by the existence of two kinds of imprinted genes: paternally expressed genes (*Pegs*) and maternally expressed genes (*Megs*). To systematically isolate imprinted genes, a number of candidate genes were collected that showed differential expression in uniparental (parthenogenetic or androgenetic) and normal fertilized embryos using subtraction-hybridization and oligonucleotide arrays. So far, nine *Pegs* (*Peg1/Mest, Igf2, Peg3, Snrpn, Peg5/Nnat, Peg9/Dlk1, Ndn, Sgce, Impact*) have been identified by these methods (6–10). Using an improved version of GeneChip (Murine Genome U74 probe array), we obtained additional candidates and proved that *Peg12/Frat3* is a novel paternally expressed imprinted gene.

Peg12/Frat3 locates on the mouse 7C chromosomal region syntenic to human 15q11-q13, which is involved in Prader–Willi (PWS, MIM 176270) and Angelman (AS, MIM 105830) syndromes. PWS is a neurogenic disorder that is associated with significant developmental, behavioral, and mental problems, which result from a deficiency of the expression of paternally expressed imprinted genes in this region (5, 11). It is a well-conserved region in human and mouse and, so far, twelve *Pegs—Zfp127, Zfp127as, Magel2, Ndn, Sunrf, Snrpn, Pwcr1, MBII-85, MBII-52, MBII-13, Ipw,* Ube3aas—are known to be clustered in the mouse 7C region (http://www.mgu.har.mrc.ac.uk/research/imprinted/imprin.html).

Mice that fail to express all the *Pegs* in this region show phenotypes similar to PWS and serve as potential mouse models of PWS. These include mice with (i) maternal duplication/paternal deficiency of the 7C chromosomal region, (ii) a large deletion of the PWS/AS homologous region associated with a transgenic insertion, and (iii) a 42-kb deletion of the putative PWS imprinting center (IC) region (12–14). All of these model mice show characteristics similar to human PWS infants, such as feeding difficulties, decreased movement, and failure to thrive. Although they show 100% lethality within approximately one week (for example, 72% of IC mutation mice died within 48 h after birth and none survived past 9 days), why the model mice showed such a severe lethality remains unknown.

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Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells

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SUMMARY

Genomic imprinting is an epigenetic mechanism that causes functional differences between paternal and maternal genomes, and plays an essential role in mammalian development. Stage-specific changes in the DNA methylation patterns of imprinted genes suggest that their imprints are erased some time during the primordial germ cell (PGC) stage, before their gametic patterns are re-established during gametogenesis according to the sex of individuals. To define the exact timing and pattern of the erasure process, we have analyzed parental-origin-specific expression of imprinted genes and DNA methylation patterns of differentially methylated regions (DMRs) in embryos, each derived from a single day 11.5 to day 13.5 PGC by nuclear transfer. Cloned embryos produced from day 12.5 to day 13.5 PGCs showed growth retardation and early embryonic lethality around day 9.5. Imprinted genes lost their parental-origin-specific expression patterns completely and became biallelic or silenced. We confirmed that clones derived from both male and female PGCs gave the same result, demonstrating the existence of a common default state of genomic imprinting to male and female germlines. When we produced clone embryos from day 11.5 PGCs, their development was significantly improved, allowing them to survive until at least the day 11.5 embryonic stage. Interestingly, several intermediate states of genomic imprinting between somatic cell states and the default states were seen in these embryos. Loss of the monoallelic expression of imprinted genes proceeded in a step-wise manner coordinated specifically for each imprinted gene. DNA demethylation of the DMRs of the imprinted genes in exact accordance with the loss of their imprinted monoallelic expression was also observed. Analysis of DNA methylation in day 10.5 to day 12.5 PGCs demonstrated that PGC clones represented the DNA methylation status of donor PGCs well. These findings provide strong evidence that the erasure process of genomic imprinting memory proceeds in the day 10.5 to day 11.5 PGCs, with the timing precisely controlled for each imprinted gene. The nuclear transfer technique enabled us to analyze the imprinting status of each PGC and clearly demonstrated a close relationship between expression and DNA methylation patterns and the ability of imprinted genes to support development.

Key words: Genomic imprinting, Primordial germ cells, PGC clones, Imprinted genes, DNA methylation, Mouse

INTRODUCTION

The initialization and reprogramming processes of epigenetic information during germ cell development are not fully understood. In mammals, a parental-origin-specific gene regulation mechanism, known as genomic imprinting, plays an essential role in development, growth and behavior, by regulating the expression of two kinds of imprinted genes: paternally and maternally expressed genes (Pegs and Megs, respectively) (Surani et al., 1984; McGrath and Solter, 1984; Cattanach and Kirk, 1985; Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991; Kaneko-Ishino et al., 1995; Miyoshi et al., 1998). Parental imprinted memories persist in somatic cells after fertilization, while it is necessary for them to be erased and re-established during germ cell development to reflect the gender of the individual (Reik and Walter, 2001). The immigration of PGCs to the genital ridges starts at around day 10.5 of the embryonic stage and is completed by day 11.5 (Rugh, 1990; Yeom et al., 1996; Molyneaux et al., 2001), when differentiation of the testes and ovaries commences.

Previous studies have indicated that imprinted memories were erased from day 11.5 to day 15.5 PGCs, judging from changes in DNA methylation and the loss of the monoallelic expression of imprinted genes (Grant et al., 1992; Kafri et al., 1992; Brandeis et al., 1993; Szabo and Mann, 1995). Region 2 of the *Igf2r* gene, which shows the fully methylated pattern of maternal alleles and the unmethylated pattern of paternal alleles in somatic cells, becomes totally unmethylated in both

Adenovirus-mediated gene delivery and *in vitro* microinsemination produce offspring from infertile male mice

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Sertoli cells play a pivotal role in spermatogenesis through their interactions with germ cells. To set up a strategy for treating male infertility caused by Sertoli cell dysfunction, we developed a Sertoli cell gene transfer system by using an adenovirus vector, which maintained long-term transgene expression in the testes of infertile mice. Introduction of an adenovirus carrying the mouse Steel (*Sl*) gene into Sertoli cells restored partial spermatogenesis in infertile Steel/Steel^{dickie} (*Sl*/*Sl*^{d}) mutant mouse testes. Although these males remained infertile, round spermatids and spermatozoa from the testes produced normal fertile offspring after intracytoplasmic injection into oocytes. None of the offspring showed evidence of germ line transmission of adenoviral DNA. Thus, we demonstrate a successful treatment for infertility by using a gene therapy vector. Therefore, adenovirus-mediated gene delivery into Sertoli cells not only provides an efficient and convenient means for studying germ cell–Sertoli cell interactions through manipulation of the germ cell microenvironment *in vivo*, but also is a useful method to treat male infertility resulting from a Sertoli cell defect.

Infertility affects ≈20% of couples, and severe spermatogenic defects are present in ≈5% of these cases, representing ≈1% of the male population (1). Spermatogenesis depends on an intimate interaction between germ cells and Sertoli cells (2). Sertoli cells are the only somatic cells in the seminiferous tubules that have direct contact with germ cells. A defect in Sertoli cells may result in abnormal spermatogenesis and male infertility. It is possible that the germ cells in some infertile testes might be functionally competent but deficiencies in Sertoli cells could possibly inhibit the normal differentiation of germ cells. There are currently no effective methods to correct such genetic defects in animals or humans (3).

The *Sl* mouse is used as a murine model of infertility (4). The *Sl* locus encodes soluble and membrane-bound forms of *Sl* factor that binds to the c-*kit* tyrosine kinase receptor produced by the *W* locus (5–7). In the testis, c-*kit* and *Sl* are expressed in spermatogonia and Sertoli cells, respectively, and the interaction between these two factors play an important role in the regulation of spermatogonial cell proliferation (8). A mutation in *Sl* deletes the entire *Sl* gene, whereas the *Sl*^{d} mutation deletes the transmembrane and intracellular domains (9). Thus, mice with the *Sl*/*Sl*^{d} mutation do not have the membrane-bound *Sl* factor and spermatogenesis does not occur in the male. In addition, the seminiferous tubules of these mice are virtually devoid of germ cells, a histological outcome that is similar to the clinical condition called Sertoli cell-only syndrome (10), although Sertoli cell-only is often associated with very small numbers of sperm (11, 12). Nonetheless, studies have shown the presence of healthy spermatogonia in the *Sl*/*Sl*^{d} testis. Aggregation chimeras between *Sl* and wild-type embryos produced progeny with the *Sl* phenotype, indicating the presence of functional *Sl* germ cells (13). Furthermore, transplantation of germ cells from *Sl*/*Sl*^{d} mice into infertile *W* mutant mice restored fertility to the latter, and the donor haplotype was transmitted to the offspring of *W* mutant mice (14). Thus, the *Sl* mutant mouse is an example of male infertility arising from defective Sertoli cells. The evidence suggests that primitive spermatogonia in the *Sl* testis may undergo spermatogenesis if provided with healthy Sertoli cells, despite long-term exposure to a defective environment.

Bearing in mind the close interaction between germ cells and Sertoli cells, a valuable approach to better understanding of spermatogenesis would be to introduce foreign genes into Sertoli cells and then monitor the outcome. Furthermore, genetic modification of Sertoli cells could be used to correct defective Sertoli cells, thus providing a method to treat those cases of male infertility that might be the results of defects in Sertoli cell function. However, although germ cells can be transfected by using several methods (15–17), there is currently no technique that allows long-term, widespread transgene expression in Sertoli cells (16, 18). Adenoviruses have potential as gene therapy vectors in human patients because of their relatively high cloning capacity and amenability to production in high titers (19). Sertoli cells represent an ideal target for adenoviral infection for two reasons. First, Sertoli cells may have the ability to express the DNA from infecting adenoviruses over long periods of time because they are mitotically quiescent after puberty (2). Although an adenovirus can transduce both dividing and nondividing cells (19), the division of infected cells results in viral DNA loss because an adenovirus cannot integrate into the host genome (19). Second, Sertoli cells have immunosuppressive activity. Because testis may be an immune-privileged organ and Sertoli cells can protect immunogenic tissues by Fas ligand expression (20, 21), an adenovirus infection may not trigger an immune reaction, a major drawback in this technology (19). The absence of an immune response could thus be beneficial.

The studies described here sought to determine whether an adenovirus vector could be used in mice to treat male infertility caused by a Sertoli cell defect. Earlier attempts to infect Sertoli cells in wild-type testes met with limited success, as transgene expression was transient and inflammation was apparent (18). Nonetheless, we hoped that adenovirus infection of infertile testes might provide a better outcome. Microinjection of adenovirus vectors into the seminiferous tubules of infertile mouse testes resulted in long-term transgene expression. By using this technique, we determined the potential of adenovirus infection system to rescue spermatogenesis in the *Sl*/*Sl*^{d} testis. This approach may be a powerful method to study germ cell–Sertoli cell interaction and might provide a novel treatment strategy for male infertility caused by defects in Sertoli cells.

DEVELOPMENTAL BIOLOGY

Abbreviations: *Sl*, Steel; *Sl*/*Sl*^{d}, Steel/Steel^{dickie}; pfu, plaque-forming unit.

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Effects of Donor Cell Type and Genotype on the Efficiency of Mouse Somatic Cell Cloning[1]

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ABSTRACT

Although it is widely assumed that the cell type and genotype of the donor cell affect the efficiency of somatic cell cloning, little systematic analysis has been done to verify this assumption. The present study was undertaken to examine whether donor cell type, donor genotype, or a combination thereof increased the efficiency of mouse cloning. Initially we assessed the developmental ability of embryos that were cloned from cumulus or immature Sertoli cells with six different genotypes (i.e., 2 × 6 factorial). Significantly better cleavage rates were obtained with cumulus cells than with Sertoli cells ($P < 0.005$, two-way ANOVA), which probably was due to the superior cell-cycle synchrony of cumulus cells at G0/G1. After embryo transfer, there was a significant effect of cell type on the birth rate, with Sertoli cells giving the better result ($P < 0.005$). Furthermore, there was a significant interaction ($P < 0.05$) between the cell type and genotype, which indicates that cloning efficiency is determined by a combination of these two factors. The highest mean birth rate (10.8 ± 2.1%) was obtained with (B6 × 129)F1 Sertoli cells. In the second series of experiments, we examined whether the developmental ability of clones with the wild-type genotype (JF1) was improved when combined with the 129 genotype. Normal pups were cloned from cumulus and immature Sertoli cells of the (129 × JF1)F1 and (JF1 × 129)F1 genotypes, whereas no pups were born from cells with the (B6 × JF1)F1 genotype. The present study clearly demonstrates that the efficiency of somatic cell cloning, and in particular fetal survival after embryo transfer, may be improved significantly by choosing the appropriate combinations of cell type and genotype.

assisted reproductive technology; cumulus cells; embryo; implantation; Sertoli cells

INTRODUCTION

Mammalian somatic cell cloning has progressed dramatically in recent years and now promises significant improvements in the generation of genetically modified animals for agricultural and biomedical purposes. In addition, cloning provides us with unique experimental models for studying the key mechanisms in mammalian development, such as genome reprogramming, genomic imprinting, DNA methylation, and telomere restoration because it generates individual copies of the donors by bypassing the normal reproduction process. The laboratory mouse is ideal for this type of research because it has defined genetic backgrounds, abundant genetic information, and a short gestation period and life span. However, mouse cloning has proven to be difficult because of the extremely poor development to term of reconstructed embryos [1, 2]; this constitutes an obstacle not only to the precise characterization of cloned mice but also to the elucidation of the molecular basis for cloning in mammals.

Since the first successful somatic cell cloning in the mouse in 1998 [3], cumulus cells with the hybrid F1 genotype (BDF1 or B6C3HF1) have become the standard donors in mouse cloning experiments. Generally, normal cloned mice are not born alive from donor cells from inbred strains, with the exception of the 129 strains (cumulus cells [4] and tail-tip cells [5]). The 129 group represents inbred strains of mice that are excellent sources of embryonic stem (ES) cell lines [6]. The underlying mechanism of this unique feature of the 129 mouse strain is not fully understood, but it is possible that the epigenetic status of the 129 genome is less stable and more easily modified in comparison with those of other strains of mice. If this is true, it may be possible to reprogram the genome of the 129 donor cells and thereby improve the subsequent development of reconstructed embryos.

The donor cell type is another factor that is generally thought to affect cloning efficiency in mammals. Precise assessment of the effects of cell type on somatic cell cloning may be performed using the mouse as a model because different donor cells with the same genetic background are readily available in this species. Previously we reported that the nuclei of immature Sertoli cells from neonatal BDF1 mice supported embryonic development at a relatively higher rate than did cumulus cells with the BDF1 background [7]. More recently it was reported that fetal neural BDF1 cells further increased the efficiency of cloning [8]. In contrast, fetal fibroblasts, which are the most commonly used donor cells in domestic species, are not necessarily good donors in the mouse [9], irrespective of their genotype [2, 5].

The present study was undertaken to examine whether donor cell type, donor genotype, or a combination thereof increased the efficiency of mouse cloning. In the first series of experiments, we used cumulus cells and immature Sertoli cells from 129/Sv-*ter* mice, their F1 hybrids, and BDF1 mice as donors in a 2 × 6 factorial analysis. In the second

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Long-Term Proliferation in Culture and Germline Transmission of Mouse Male Germline Stem Cells[1]

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ABSTRACT

Spermatogenesis is a complex process that originates in a small population of spermatogonial stem cells. Here we report the in vitro culture of spermatogonial stem cells that proliferate for long periods of time. In the presence of glial cell line-derived neurotrophic factor, epidermal growth factor, basic fibroblast growth factor, and leukemia inhibitory factor, gonocytes isolated from neonatal mouse testis proliferated over a 5-month period (>10^{14}-fold) and restored fertility to congenitally infertile recipient mice following transplantation into seminiferous tubules. Long-term spermatogonial stem cell culture will be useful for studying spermatogenesis mechanism and has important implications for developing new technology in transgenesis or medicine.

developmental biology; gametogenesis; Sertoli cells; spermatogenesis; testis

INTRODUCTION

Because spermatogonial stem cells are the only stem cells in adults that divide to contribute genes to subsequent generations, they are valuable for biological experimentation, medical research, and biotechnology [1]. However, attempts to expand and manipulate these cells in vitro have not been successful [2, 3]. Although a previous study showed that some spermatogonial stem cells could survive in vitro for longer than 3 months [4], generally only 10–20% remained after a week in culture [2]. More recently genetic materials, such as SV40 T antigen or telomerase, were used to immortalize spermatogonia, and long-term proliferation was reported [5, 6]. However, it is unclear whether these cells have the capacity to generate spermatozoa and act as true stem cells. In addition, no quantitative assessment of in vitro stem cell proliferation has been performed by transplantation assay [4, 7–10].

In the experiments described here, we report the in vitro culture of spermatogonial stem cells that proliferate for long periods of time. We used the spermatogonial transplantation technique [11] to evaluate the level of self-renewal activity of stem cells in vitro, and transplantation of the cultured cells restored fertility to congenitally infertile recipient mice.

MATERIALS AND METHODS

Animals

Testis cells were collected from a newborn transgenic mouse line C57BL6/Tg14(act-EGFP-OsbY01) that was bred into DBA/2 background (designated Green) (provided by Dr. M. Okabe, Osaka University, Osaka, Japan). The spermatogonia and spermatocytes of these mice express the enhanced green fluorescent protein (EGFP) gene, the level of expression of which decreases gradually after meiosis [12]. Therefore, donor cells can be readily identified following transplantation. Testis cells were collected by two-step enzymatic digestion and used for culture [13]. Briefly, testis cells were digested with 1 mg/ml collagenase (type IV, Sigma, St. Louis, MO) for 15 min, followed by 0.25% trypsin/1 mM EDTA digestion (both from Invitrogen, Carlsbad, CA) for 10 min. Approximately 4×10^5 cells were collected from a neonatal testis by this procedure. The number of dead cells was generally less than 5%, as assessed by trypan blue staining.

Cultured cells were transplanted into BALB/C nude or infertile WBB6F1W/W^v (designated W) pups (5–10 days old, Japan SLC, Shizuoka, Japan). To deplete endogenous spermatogenesis, nude mice were treated with busulfan (44 mg/kg) at 6 wk of age [11] and were subsequently injected with homologous bone marrow cells to reduce mortality [14]. In experiments using W recipients, 50 μg anti-CD4 antibody (GK1.5) was administered intraperitoneally on Days 0, 2, and 4 after transplantation to induce tolerance to the allogeneic donor cells [14]. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Culture Conditions

Dissociated testis cells were allocated to 0.2% (w/v) gelatin-coated tissue culture plate (2×10^5 cells/3.8 cm^2). The plates were washed twice with PBS before use. Culture medium for the testis cells was StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma), 5 mg/ml bovine albumin (ICN Biomedicals, Irvine, CA), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, minimal essential medium (MEM) vitamin solution (Invitrogen), MEM nonessential amino acid solution (Invitrogen), 10^{-4} M ascorbic acid, 10 μg/ml d-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone (Sigma), 20 ng/ml mouse epidermal growth factor (Becton Dickinson, Bedford, MA), 10 ng/ml human basic fibroblast growth factor (Becton Dickinson), 10^3 U/ml ESGRO (murine leukemia inhibitory factor; Invitrogen), 10 ng/ml recombinant rat glial cell line-derived neurotrophic factor (GDNF) (R&D Systems, Minneapolis, MN) and 1% fetal calf serum (JRH Biosciences, Lenexa, KS). The cells were maintained at 37°C in an atmosphere of 5% carbon dioxide in air.

Antibodies and Staining

Primary antibodies used were: rat anti-EpCAM (G8.8) and mouse anti-SSEA-1 (MC-480) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rat antihuman α6-integrin (CD49f) (GoH3), bio-

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