Table 1
Body and Placental Weights (g ± SD) at Birth in the Siblings From B16 (1 Pair) and #36 (2 Pairs) Cloned Parents

		Parents		Siblings			
		♂ (BW[a], PW[b])	♀ (BW, PW)	Sex	No.	BW	PW
Control	3 pairs	(1.71), (0.12)[c]	(1.68), (0.12)[c]	♂	13	1.30 ± 0.09	0.09 ± 0.01
				♀	8	1.29 ± 0.08	0.08 ± 0.01
				Total	21	1.30 ± 0.09	0.08 ± 0.01
Cloned	Pair 1 (B16)	(2.00), (0.52)	(1.69), (0.32)	♂	8	1.35 ± 0.08	0.09 ± 0.02
				♀	5	1.28 ± 0.09	0.09 ± 0.02
				Total	13	1.33 ± 0.09	0.09 ± 0.02
	Pair 2 (#36)	(1.69), (0.31)	(1.55), (0.41)	♂	4	1.29 ± 0.12	0.10 ± 0.02
				♀	4	1.31 ± 0.06	0.11 ± 0.01
				Total	8	1.30 ± 0.09	0.10 ± 0.01
	Pair 3 (#36)	(1.78), (0.35)	(1.58), (0.40)	♂	2	1.16 ± 0.09	0.10 ± 0.03
				♀	6	1.21 ± 0.11	0.10 ± 0.02
				Total	8	1.20 ± 0.10	0.10 ± 0.02

[a]Body weight (g).
[b]Placental weight (g).
[c]Average of BW and PW in three pairs of controls are shown. The statistical significances in BW and PW were not observed between males and females of each sibling, and among siblings from each parent.

and Pair 3 (1.78 g and 0.35 g, male) × (1.58 g and 0.40 g, female), from the #36 subline.

They were normally mated within 1 week after paring. At day 19.5 of gestation, 13, 8, and 8 live pups (F1) were recovered from the three pairs of cloned parents. All pups were successfully resuscitated and were nursed by foster mothers. No signs of hypertrophy of the placenta were observed. No retroplasic conceptuses or prenatal death were observed. The results clearly showed that the abnormalities seen in the parental cloned mice at birth were not seen in their siblings (Fig. 1A,B). The weights of the body and placenta in the siblings were within the same range as those of the controls: B16 × B16 (1.33 ± 0.09 g and 0.09 ± 0.02 g, n = 13), #36 × #36 (1.25 ± 0.10 g and 0.10 ± 0.02 g, n = 16), and control × control (1.30 ± 0.09 g and 0.08 ± 0.01 g, n = 21) (Fig. 1A,B, Table 1). The open eyelids seen in the B16 cloned mice were not observed in their siblings (Fig. 1A).

The question remained as to whether levels of recessive mutations are elevated in clones. To address this, a total of 31 pups (body weight, 1.25 ± 0.10 g and placenta weight, 0.09 ± 0.02 g) of the F3 generation were recovered by Cs at 19.5 dpc. The results showed that any anomalies, including those seen in the ES clones, were not observed in the progeny and this supports an idea that levels of recessive mutations in clones are similar to that of the wild types.

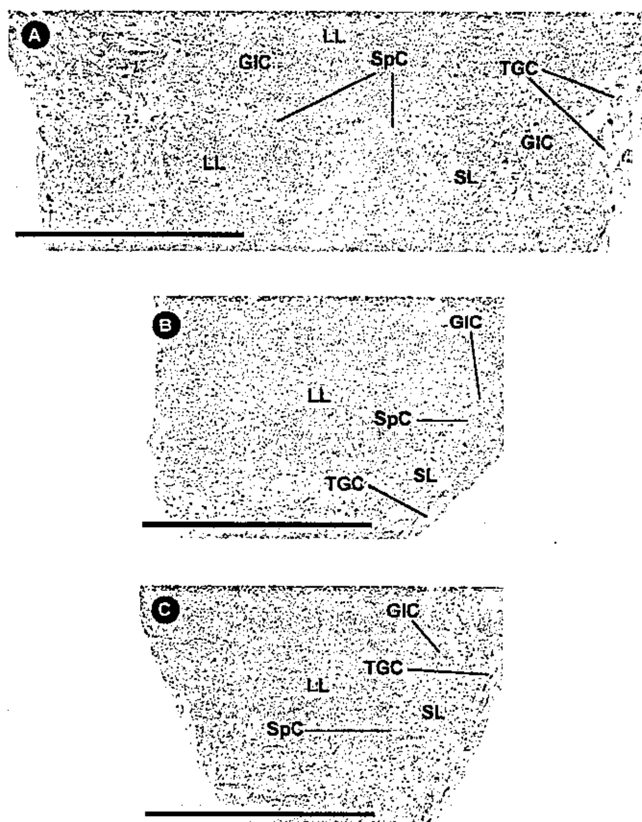

FIG. 2. Histological sections of placentas from cloned, cloned × cloned and control live pups at birth. **A:** The placentas of cloned mice showed hypertrophy caused mainly by extensive proliferation of spongiotrophoblast and glycogen cells in the spongiotrophoblast layer. But **(B)** the placentas of cloned × cloned mice were as normal as **(C)** those of control mice. LL, labyrinthine layer; SL, spongiotrophoblast layer, GIC, glycogen cells, TGC, trophoblast giant cells, SpC, spongiotrophoblast cells. Scale bar = 1 mm.

Histological Analysis

The hypertrophic placenta derived from the cloned pups at birth was caused mainly by extensive proliferation of trophoblast and glycogen cells in the spongiotrophoblast layer and enlargement trophoblast giant cells as compared to the placentas of the siblings from cloned parents and controls (Fig. 2A-C). The border of the

spongiotrophoblast layer and the labyrinthine layer in the placenta of cloned mice was ambiguous. However, histological analysis revealed that the placentas of the siblings of cloned parents were consistent with normally differentiated and developed placental tissues (Fig. 2B,C).

DISCUSSION

Recent arguments regarding the abnormalities of cloned animals have focused on the unregulated expressions of some imprinted/nonimprinted genes (Kono *et al.*, 1997; Kang *et al.*, 2001; Humpherys *et al.*, 2001; Inoue *et al.*, 2002). However, whether these unregulated expressions or genetic mutations are related to phenotypic abnormalities has not been clarified. In our previous study, we obtained XO female cloned mice from XY ES cell sublines (Ono *et al.*, 2001b; Shimozawa *et al.*, 2002). This provided an opportunity to address the question of whether the abnormalities seen in cloned mice are a result of epigenetic errors or of consecutive DNA mutations.

The cloned mice that we produced from two ES sublines also exhibited, as a property of somatic cloned mice, hypertrophic placentas characterized by severe defective differentiation of placental tissues with abnormal proliferation of glycogen cells and trophoblastic cells (Ono *et al.*, 2001a). Cloned mice from #36 ES cell line had only hypertrophic placentas, but B16 cloned mice showed additional phenotypic abnormalities, namely, increased body weights and open eyelids at birth. When gene-targeted mice derived from chimeric mice using B16 ES cells were produced, the same abnormalities were not observed. This finding suggests that the abnormalities seen in cloned mice were natural only for somatic/ES cell cloned mice.

If abnormalities in cloned mice are caused by accumulative DNA mutations, the deficiencies may be compensated for by normal gene expression from wild-type alleles when siblings are produced by mating with cloned and normal control wild-type mice. Therefore, the examination of siblings from XO and XY cloned mice that were derived from the same ES sublines was the most valuable procedure for addressing the question of whether abnormalities in the cloned mice were transmitted to the siblings. After mating with XY cloned mice, XO females were successfully impregnated and a normal number of normally sized pups was recovered by Cs at day 19.5 of gestation. The body and placental weights in the siblings (F1) of cloned parents were within the ranges of the controls, which were derived from pronuclear transfer and in vitro cultured embryos. As expected, histological analysis revealed that the placentas in the siblings of both cloned mice were also normal. These results suggest that cloned mice have a normal set of genomes with no genetic mutations.

Data from the F1 progeny strongly suggested that anomalies seen in the cloned mice resulted from inappropriate reprogramming of the epigenetic modifications. To confirm this further, we produced the progeny from F3 generations and confirmed that levels of recessive mutations could not be elevated in the cloned mice relative to wild types.

With some exceptions, the expression of observed imprinted genes such as H19 and Igf2 varied widely in neonates cloned from ES cells and in their placentas (Humpherys *et al.*, 2001). On the other hand, the expression of both imprinted genes was within the same range as those of the controls, but the expression of four nonimprinted genes, Igfbp2, Igfbp6, Vegfr2/Flk1, and Esx1, was reduced in neonates and their placentas cloned from immature Sertoli and cumulus cells (Inoue *et al.*, 2002). These reports show that some imprinted/nonimprinted genes are unregulated in mice cloned from somatic/ES cells and in their placentas. Telomere length and X-chromosome inactivation are reprogrammed (Wakayama *et al.*, 2000; Eggan *et al.*, 2000) while, as Humpherys *et al.* (2001) and Inoue *et al.* (2002) reported, the expression of some imprinted/nonimprinted genes in cloned mice is apparently different from that in control mice, which suggests that insufficient reprogramming occurs in embryo cloning. It is supposed that the phenotypic abnormalities were not the result of simple gene expression.

It is considered that reprogramming in cloning changes the state of the DNA to the preimplantation embryonic type from the somatic or ES cell type (Kono, 1997). This disorder mechanism may cause unregulated gene expression. Reprogramming errors suggest that epigenetic regulation for development underlies the developmental defects in clones. On the other hand, whether the abnormalities of these gene expressions are related to phenotypic abnormalities has not been demonstrated. The possibility remains that genetic mutations are imposed by cell culture, micromanipulations, or some stress. However, in this report we clearly showed that the causes of phenotypic abnormalities were not genetic mutations, as these abnormalities disappeared in the next generation of abnormal cloned parents. These findings suggest that epigenetic modifications in the chromatin that cause the abnormalities were reprogrammed in the germ line. This constitutes the first direct evidence that abnormalities seen in embryo cloning are caused by epigenetic mechanisms.

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LITERATURE CITED

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. 1999. Production of goats by somatic cell nuclear transfer. Nat Biotech 17:456-461.

Chatot CL, Lewis JL, Torres I, Ziomek CA. 1990. Development of 1-cell

embryos from different strains of mice in CZB medium. Biol Reprod 42:432-440.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256-1258.

Eggan K, Akutsu H, Hochedlinger K, Rideout WM III, Yanagimachi R, Jaenisch R. 2000. X-Chromosome inactivation in cloned mouse embryos. Science 290:1578-1581.

Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM III, Yanagimachi R, Jaenisch R. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci USA 98: 6209-6214.

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM III, Biniszkiewicz D, Yanagimachi R, Jaenisch R. 2001. Epigenetic instability in ES cells and cloned mice. Science 293:95-97.

Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogur A. 2002. Faithful expression of imprinted genes in cloned mice. Science 295:297.

Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. 2001. Aberrant methylation of donor genome in cloned bovine embryos. Nat Genet 28:173-177.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. 1998. Eight calves cloned from somatic cells of a single adult. Science 282:2095-2098.

Kono T. 1997. Nuclear transfer and reprogramming. Rev Reprod 2:74-80.

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. 2002. Production of alpha -1,3-Galactosyltransferase Knockout Pigs by Nuclear Transfer Cloning.Science 295:1089-1092.

McCreath KJ, Howcroft J, Campbell KSH, Colman A, Schnieke AE, Kind AJ. 2000. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066-1069.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science 289:1188-1190.

Ono Y, Shimozawa N, Ito M, Kono T. 2001a. Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol Reprod 64:44-50.

Ono Y, Shimozawa N, Muguruma K, Kimoto K, Hioki K, Tachibana M, Shinkai Y, Ito M, Kono T. 2001b. Production of cloned mice from embryonic stem cells arrested at metaphase. Reproduction 122: 731-736.

Renard JP, Chastant S, Chense P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X. 1999. Lymphoid hypoplasia and somatic cloning. Lancet 353:1489-1491.

Rideout WM III, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R, Jaenisch R. 2000. Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet 24:109-110.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KSH. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130-2133.

Sendai Y, Yoshida-Komiya H, Kuramochi T, Ito M, Ogasawara R, HoshiH, Shinkai Y, Araki Y. 1999. Establishment of germ-line competent embryonic stem cells lacking mouse oviduct-specific glycoprotein gene. Biol Reprod 60 (Suppl):144.

Shimozawa N, Ono Y, Muguruma K, Kimoto K, Hioki K, Araki Y, Shinkai Y, Kono T, Ito M. 2002. Direct production of gene-targeted mice from ES cells by nuclear transfer and gene transmission to their progeny. Exp Anim 51:375-381.

Tachibana M, Sugimoto K, Fukushima T, Shinkai Y. 2001. Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. J Biol Chem 276:25309-25317.

Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369-374.

Wakayama T, Shinkai Y, Tamashiro KLK, Niida H, Blanchard DC, Blanchard RJ, Ogura A, Tanemura K, Tachibana M, Perry ACF, Colgan DF, Mombaerts P, Yanagimachi R. 2000. Cloning of mice to six generations. Nature 407:318-319.

Wells ND, Pavla MM, Tervit HR. 1999. Production of clone calves following nuclear transfer with culture adult mural granulosa cells. Biol Reprod 60:996-1005.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KSH. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385:810-813.

Exp. Anim. 51(4), 375–381, 2002

Direct Production of Gene-targeted Mice from ES Cells by Nuclear Transfer and Gene Transmission to their Progeny

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Abstract: *In order to evaluate the usefulness of a cloning technique to produce gene-manipulated mice for the field of laboratory animal science, we produced mice cloned from gene-targeted embryonic stem (ES) cells and examined the vertical transmission of a targeted gene to their progeny. Of 1257 eggs constructed by nuclear transfer using M-phase ES donor cells targeted with an oviduct-specific glycoprotein (OGP) gene, 990 formed a pseudo-pronucleus and a polar body after activation. Of 504 cloned embryos transferred into recipients, 20 live cloned pups (2%) were recovered by Caesarean section at 19.5 days of gestation. Fourteen of these cloned mice were studied. Genotyping of the OGP locus and 20 microsatellite loci showed that they were genetically identical to the OGP gene-targeted TT2 cells. Eight cloned pups grew into adults, of which 7 were male and 1 was female (missing the Y chromosome). Mating experiments using the cloned mice were carried out. Of 89 F1 mice produced from the mating of cloned and C57BL/6J mice, 50 had the targeted OGP gene heterozygously. Thirty-seven F2 mice from 4 pairs of the $OGP^{-/+}$ mice were composed of 9 $OGP^{-/-}$, 18 $OGP^{-/+}$, and 10 $OGP^{+/+}$. Moreover, 26 offspring of one pair of the cloned mice were composed of 10 $OGP^{-/-}$, 12 $OGP^{-/+}$, and 4 $OGP^{+/+}$. These offspring were fertile and transmitted the mutant OGP gene to the next generation. Comparison of these results with those of germline chimeric mice indicates that gene-targeted mice can be produced at least one generation earlier by nuclear transfer than by the conventional methods. In addition, the targeted OGP gene was constantly transmitted to the progeny of the gene-targeted mice. Cloning techniques are potentially a more efficient way to generate gene-manipulated mice for laboratory animal science, although such techniques include many unresolved problems, such as low production efficiency, and selection of a cell source for gene manipulation among others.*

Key words: *cloned mice, ES cells, gene-targeting, nuclear transfer, gene transmission*

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Introduction

The techniques that are generally used to produce gene-manipulated mice have several disadvantages. In one method, for example, DNA is microinjected into the pronucleus of a fertilized egg, and the injected DNA is randomly integrated into the mouse genome. Often, the injected DNA is not expressed in the desired tissue or at the desired level, since it is inserted randomly. In another method, chimeric mice are produced from gene-replaced embryonic stem (ES) cells by homologous recombination. But the gene-manipulated ES cells do not always contribute to germ cells, even though mutations can be introduced into specific gene sites in mouse ES cells. In addition, the gene-manipulated mice are usually seen in the next generation of chimeric mice.

Cloning techniques to produce mice directly from cultured cells may be able to overcome these disadvantages. Following the report by Wakayama *et al.* [17] who successfully cloned mice from non-targeted ES cells, Rideout *et al.* [12] and Ono *et al.* [10] have reported cloning mice using gene-targeted ES cells. However, the ability of gene-targeted cloned mice to reproduce and vertically transmit targeted genes has not yet been fully examined. If vertical transmission of the targeted gene to the progeny of gene-targeted cloned mice can be proven clearly, the use of gene-targeted cloned mice would have several advantages over the chimeric mating of gene-targeted mice in laboratory animal science: no chimeric mice would be needed to confirm the transmission to germ cells by mating, generations would be shorter, and so on.

In this study, we describe the direct production of gene-targeted mice, without chimera, from gene-targeted ES cells, as well as the transmission of the targeted gene to their progeny, in order to clarify the potential of cloning technology to generate gene-targeted mice for the field of laboratory animal science.

Materials and Methods

Gene-targeted ES cells: ES cells of the TT2 line [19], derived from a B6CBF1 (C57BL/6N Crj × CBA/JN Crj) embryo, were targeted with the mouse oviduct-specific glycoprotein (OGP) gene coding region identified by Sendai *et al.* [15] and Takahashi *et al.* [16]. OGP$^{-/-}$ and OGP$^{-/+}$ mice derived from chimeric mice showed normal fertilizing ability *in vivo* (Araki *et al.*, unpublished data). Frozen-thawed OGP$^{-/+}$ ES cells were cultured in gelatin-coated dishes without a feeder layer for 3 or 4 days in Dulbecco's Modified Eagle's Medium (Life Technologies, Grand Island, NY, USA) containing 20% fetal bovine serum (Life Technologies), 10^3 U/m*l* leukemia inhibitory factor (ESGRO, Chemicon, Temecula, CA, USA), 2 mM L-glutamine, 1% non-essential amino acid (× 100 solution, Life Technologies) and 5.5×10^{-5} M β-mercaptoethanol (ES medium). Before nuclear transfer, ES cells were cultured with ES medium containing 0.4 μg/ml nocodazole (Sigma, St. Louis, MO, USA), a microtubule polymerization inhibitor, for 2 h in order to synchronize the cells at the metaphase [9]. Cells floating in the medium were collected and used as donors for nuclear transfer.

Preparation of oocytes and embryos: Oocytes were collected from female Slc: B6CBF1 mice (C57BL/6Cr Slc × CBA/N Slc; Japan SLC Inc., Shizuoka, Japan) superovulated with injections of 5 IU pregnant mare's serum gonadotropin (PMSG; Serotropin, Teikokuzoki Co., Tokyo, Japan) and 5 IU human chorionic gonadotropin (hCG; Gonatropin, Teikokuzoki) given 48 h apart. Oocytes were collected from oviducts 14 h after hCG injection, and cumulus cells were removed by brief incubation in 300 units/ml hyaluronidase in M2 medium [11]. One-cell embryos were produced by *in vitro* fertilization using Slc: B6CBF1 females and males.

Nuclear transfer: Cloned embryos were constructed by single and serial nuclear transfer using the procedures described by Ono *et al.* [9, 10]. Micromanipulations were performed in M2 medium containing 5 μg/ml cytochalasin B (CB; Sigma), or 5 μg/ml CB and 0.4 μg/ml nocodazole. After enucleation of the M II chromosome [5], ES cells synchronized at the metaphase were introduced into the perivitelline spaces of the enucleated oocytes with inactivated Sendai virus (HVJ: hemagglutinating activity 2,700 units/ml). The oocytes fused with ES cells were incubated for 2 h in modified CZB medium [2], containing 5.56 mM glucose (mCZB). After the incubation, the oocytes were artificially activated with Ca^{2+}-free M16 medium [18], containing 10 mM Sr^{2+} [1], for 6 h and then placed in mCZB (single nuclear transfer). In the second nuclear transfer, the nucleus of the

constructed egg was again transferred to a previously enucleated fertilized one-cell embryo 10–12 h after activation (serial nuclear transfer) [6].

In vitro culture and embryo transfer: Embryos that had undergone nuclear transfer were cultured in mCZB at 37°C under 5% CO_2 in air. On day 4 of *in vitro* culture, morulae and blastocysts were transferred into the uterine horns of 2.5 days postcoitum pseudopregnant females. Pups were recovered at 19.5 days of gestation by Caesarean section (Cs).

Breeding of cloned mice: The animals were maintained in an air-conditioned room with controlled illumination (12 h light/12 h dark), temperature (22–25°C) and humidity (60–70%), and were given a commercial food preparation (CA-1, Japan CLEA Co., Tokyo, Japan) and tap water. Adult male cloned mice were mated with C57BL/6J females (Japan CLEA) and adult female cloned mice. The F1 mice produced from cloned mice were also mated to obtain F2 mice. The mice were maintained with the approval of the Laboratory Animal Use and Care Committee of the Central Institute for Experimental Animals.

Genotyping of the OGP gene: To distinguish between the wild-type and mutant alleles, the cloned mice and their offspring were genotyped by PCR using two sets of primers. The tails of the mice were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH: 8.0, 0.1 M NaCl, 20 mM EDTA, 100 μg/ml proteinase K, 1% SDS), and DNA was extracted from 100 μl of lysate using a MagExtractor (MFX-2000, Toyobo Co., Osaka, Japan). To identify the wild-type allele, the following sequences were used for the 5'-primer and 3'-primer: GTTCTTCTGATGAAACACAGTG and GCACACCAGTTAGTAGGCAG, respectively. To identify the mutant allele, the following sequences were used for the 5'-primer and 3'-primer: ACCCTGACAACATTGAGGCTCC and CATACACGGTGCCTGACTGCG, respectively. Both PCR reactions were carried out for 35 cycles (94°C, 1 min; 57°C, 1 min; 72°C, 1 min) in an LA PCR™ buffer containing 2 mM $MgCl_2$, 0.2 mM dNTP, and Takara *LA Taq* polymerase (TaKaRa Shuzo Co., Shiga, Japan). Multiplied fragments in both reactions were approximately 500 bp.

Genotyping of microsatellite loci: Twenty microsatellite markers, one marker for each chromosome (except for the Y chromosome) were also analyzed to determine their genetic background. Since the differences in PCR patterns in 8 of the 20 microsatellite loci are present between CBA/JN Crj (a parent strain of TT2 cells) and CBA/N Slc (a parent strain of Slc: B6CBF1), PCR results can easily determine the strain from which a cloned mouse originated. PCR amplification of microsatellite loci was performed in accordance with a previously described method [14]. The amplified products were electrophoresed on 3–4% agarose gel and visualized with ethidium bromide.

Chromosome counts: Chromosome preparations were obtained from mitogen-stimulated peripheral blood lymphocytes [3] and stained with Quinacrine mustard (Sigma) and Hoechst 33258 (Sigma). The chromosomes were then counted and the Y chromosomes were differentiated (× 1000 magnification).

Statistical analysis: The data were analyzed by chi-square analysis. Differences were considered statistically significant at $P<0.05$.

Results

In vitro and in vivo development of cloned embryos: Of 1257 eggs constructed by single and serial nuclear transfer, 990 (78.8%) formed a pseudo-pronucleus with a polar body after activation with strontium. Five hundred sixty-five (57.5%) of 983 cultured cloned embryos developed to the morula and blastocyst stage. In order to assess the ability of embryos to develop to term, 504 cloned embryos were transferred into 47 recipient females. Twenty-nine recipients became pregnant, and 20 live pups (2.0%) were recovered by Cs at 19.5 days of gestation. No significant differences ($P>0.05$) were observed with regard to *in vitro* and *in vivo* development following single and serial nuclear transfer of ES cells arrested at the metaphase for the production of cloned mice (Table 1). Of the 20 pups, 6 pups died within 1 hr after Cs, and 6 more pups died before weaning. The reasons for the neonatal deaths of the cloned pups were unclear. Finally, 8 pups (40%) grew into adults (Table 2). External genitalia and chromosome counts revealed that 1 of the 8 pups was female.

Table 1. *In vitro* and *in vivo* development of cloned embryos derived from $OGP^{-/+}$ embryonic stem cells

Nuclear transfer (NT) method	No. of oocytes activated normally$/ fused oocytes (%)	No. of embryos developed to M+B#/ cultured (%)	No. of M+B transferred (%)	No. of pregnants/ recipients (%)	No. of live pups (%)
Single NT*	587/745 (78.8)	356/587 (60.6)	313 (53.3)	17/28 (60.7)	11 (1.9)
Serial NT*	403/512 (78.7)	209/396 (52.8)	191 (48.2)	12/19 (63.2)	9 (2.3)
Total	990/1257 (78.8)	565/983 (57.5)	504 (51.3)	29/47 (61.7)	20 (2.0)

*No significant differences ($P>0.05$) were observed between both methods. $Oocytes with a pronucleus and a polar body. #Morula and Blastcyst stage.

Table 2. Cloned pups obtained by Caesarian section

No. of cloned pups	No. of pups (%)		
	Died soon	Died before weaning	Weaned
20	6 (30.0)	6 (30.0)	8 (40.0)

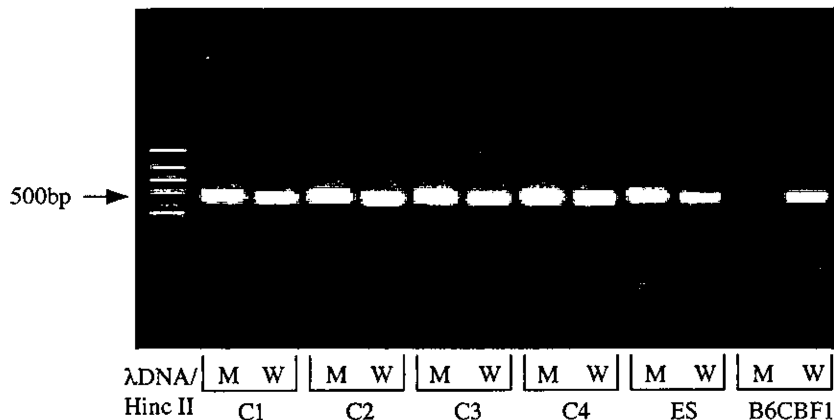

Fig. 1. Cloned mice (C1 to C4), $OGP^{-/+}$ ES cells (ES) and an Slc: B6CBF1 mouse were genotyped by PCR using two sets of primers in order to distinguish the wild-type from the mutant OGP gene, as described in the Materials and Methods section. Lanes M and W show PCR products amplified with specific primers for the mutant- and wild-type, respectively.

Genotyping of cloned mice: Mutation-specific PCR confirmed the transmission of the mutant OGP gene in all 14 cloned mice that were tested, excluding 3 of the pups that died soon after Cs and 3 of the pups that died before weaning (Fig. 1). All cloned mice tested were also identical to OGP gene-targeted TT2 cells but not to B6CBF1 from the SLC used as a source of recipient oocytes (Table 3).

Vertical transmission of the targeted OGP gene: When the 7 adult male cloned mice were paired and mated with C57BL/6J females, a total of 89 offspring (F1 mice) resulted. Genotyping the results of PCR showed that 50 (56.2%) of the F1 mice had the targeted OGP gene in one allele. Four pairs of $OGP^{-/+}$ F1 mice were observed to be fertile and produced 37 offspring (F2 mice). The genotypes of the F2 mice were as follows: 9 $OGP^{-/-}$, 18 $OGP^{-/+}$ and 10 $OGP^{+/+}$ (Table 4). Furthermore, the one female cloned mouse mated with

Table 3. Profile of microsatellite markers in cloned mice

Sample	Note	D1 Mit21	D2 Mit61	D3 Mit44	D4 Mit53	D5 Mit18	D6 Mit33	D7 Mit77	D8 Mit88	D9 Mit22	D10 Mit28
Slc: B6CBF1	a strain of recipient oocytes	a, c	a	a, c	a, b	a, b	a, b	a	a, b	a, b	a
TT2 cells*	derived from Crj: B6CBF1	a, b	a	a, b	a	a, b	a, b	a	a, b	a	a
Cloned mice	derived from TT2 cells*	a, b	a	a, b	a	a,. b	a, b	a	a, b	a	a

Sample	D11 Mit51	D12 Mit79	D13 Mit26	D14 Mit7	D15 Mit13	D16 Mit4	D17 Mit16	D18 Mit40	D19 Mit41	DX Mit19
Slc: B6CBF1	a, b	a, b	a, b	a, b	a	a, c	a	a	a, c	a
TT2 cells*	a, b	a, b	a, b	a, b	a	a, b	a, b	a, b	a, b	a
Cloned mice	a, b	a, b	a, b	a, b	a	a, b	a, b	a, b	a, b	a

PCR band patterns in 8 markers (underlined) were different between recipient oocytes and cloned mice. *OGP targeted TT2 cells.

Table 4. Transmission of targeted OGP gene to the offspring produced from the male cloned mice

Generation	Parents		No. of offspring	Genotype of offspring (%)		
	♀: Genotype	♂: Genotype		$OGP^{-/-}$	$OGP^{-/+}$	$OGP^{+/+}$
F1	B6J*: $OGP^{+/+}$	Clone 1**: $OGP^{-/+}$	10	0	6	4
	B6J: $OGP^{+/+}$	Clone 2: $OGP^{-/+}$	21	0	14	7
	B6J: $OGP^{+/+}$	Clone 3: $OGP^{-/+}$	23	0	13	10
	B6J: $OGP^{+/+}$	Clone 4: $OGP^{-/+}$	6	0	4	2
	B6J: $OGP^{+/+}$	Clone 5: $OGP^{-/+}$	9	0	4	5
	B6J: $OGP^{+/+}$	Clone 6: $OGP^{-/+}$	9	0	4	5
	B6J: $OGP^{+/+}$	Clone 7: $OGP^{-/+}$	11	0	5	6
	Total		89	0	50 (56.2%)	39 (43.8%)
F2	A**: $OGP^{-/+}$	E: $OGP^{-/+}$	9	1	5	3
	B: $OGP^{-/+}$	E: $OGP^{-/+}$	11	6	5	0
	C: $OGP^{-/+}$	F: $OGP^{-/+}$	8	1	5	2
	D: $OGP^{-/+}$	F: $OGP^{-/+}$	9	1	3	5
	Total		37	9 (24.3)	18 (48.6)	10 (27.0)

*C57BL/6J. **Individual number of cloned mice and their offspring.

a male cloned mouse, producing and nursing 26 offspring. The genotypes of their 26 offspring were as follows: 10 $OGP^{-/-}$, 12 $OGP^{-/+}$ and 4 $OGP^{+/+}$. These mice derived from the female cloned mouse were fertile and their progeny had the targeted gene (Table 5).

Discussion

In this study, we demonstrated that the application of cloning technology to the production of gene-manipulated animals is comparatively effective insofar as it enables the production of gene-targeted mice one generation earlier, since chimeric mice are not needed, and the targeted gene is consistently transmitted to their progeny. The advantages and disadvantages in producing gene-manipulated animals using this cloning technique are discussed below.

All cloned mice used for mating experiments were fertile and able to normally produce their progeny in a Mendelian manner. These data confirmed the usefulness of cloning technology to produce gene-manipulated mice. As shown in Table 4, all of the cloned mice produced in this study had the $OGP^{-/+}$ genotype, and mating with C57BL/6J females yielded offspring (F1

Table 5. Transmission of targeted OGP gene to the offspring produced from the cloned parents

	Parents		No. of	Genotype of offspring		
Generation	♀: Genotype	♂: Genotype	offspring	OGP$^{-/-}$	OGP$^{-/+}$	OGP$^{+/+}$
F1	Clone 8*: OGP$^{-/+}$	Clone 2: OGP$^{-/+}$	26	10	12	4
F2	a*: OGP$^{+/+}$	f: OGP$^{-/+}$	3	0	0	3
	b: OGP$^{-/+}$	〃	8	1	6	1
	c, d, e: OGP$^{-/-}$	〃	20	9	11	0

*Individual number of cloned mice and their offspring.

mice). The ratio of OGP$^{-/+}$ to OGP$^{+/+}$ genotypes among F1 mice was near 1:1. The ratio of OGP$^{-/-}$ to OGP$^{-/+}$ to OGP$^{+/+}$ genotypes among F2 mice was approximately 1: 2: 1.

An advantage of this technique is that targeted mice can be produced one generation earlier than conventional methods, because mice cloned from the targeted ES cells were themselves targeted mice rather than chimeric mice. The conventional methods to produce gene-targeted mice cannot produce such mice directly from ES cells; rather, it relies on the production of chimeric mice with germ cells derived from ES cells [13]. Since ES cells often fail to contribute to germline transmission, the direct production of mice cloned from ES cells might overcome the disadvantages of the conventional methods.

Our experiments also demonstrated that gene-targeted mice could be produced two generations earlier by this method than by the conventional methods, by the use of both XY and XO commercially available ES clones. A female cloned mouse was accidentally produced from the TT2 line (40, XY) [10, 19] in this study. This female cloned mouse had an XO karyotype (39, XO), indicating that the Y chromosome had been somehow lost. The R1 and D3 cell lines, which originate from mouse substrain 129, have also been described as possibly containing XO-type cells [7, 8]. Accordingly, the ES clone used for the female cloned mouse might have lost the Y chromosome during the long-term culture of ES cells for drug selection. As a result, however, we showed that OGP$^{-/-}$ mice could be obtained two generations earlier than the conventional methods and with high efficiency: 10 OGP$^{-/-}$ from 26 offspring by mating between a female cloned and a male cloned mouse. These data suggest that cloning technology will achieve faster production of gene-targeted mice.

The production of cloned mice may be dependent on the characteristics of donor cells. We previously demonstrated that, when 4- and 8-cell blastomeres [6] and fetal fibroblast cells [9] were used as donor cells, serial nuclear transfer was a more efficient means to produce cloned mice than single nuclear transfer. However, no significant differences were observed between single and serial nuclear transfer of ES cells arrested at the metaphase for the production of cloned mice. The same observation was made in the production of cloned mice targeted to G9a, homologous to the human G9A, a mammalian lysine-preferring histone methyltransferase [10]. It is unclear as to why no difference was observed between the two transfer methods when TT2 cells were used as the donors of nuclei. The similarity might be due to the characteristics of TT2 cells, or it might reflect the degree of differentiation between cells.

Although this technique has several advantages over the conventional method, there are still some obstacles to using this technique to produce gene-targeted mice. These obstacles are the low efficiency of the technique: only 1–3% of the treated embryos developed into mice; the instability of the results obtained in each experiment; and the limited information available on cloning techniques. If these issues are addressed and overcome, cloning techniques may prove to be more efficient tools in generating gene-manipulated mice. For instance, the production of gene-manipulated mice by using ES cells that do not transmit to the germline will greatly contribute to the field of laboratory animal science. The application of this technique in the production of other gene-manipulated laboratory animals, besides mice, by using other lineage cells but not ES cells would also be of great value.

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References

1. Bos-Mikich, A., Swann, K., and Whittingham, D.G. 1995. Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol. Reprod. Dev.* 41: 84–90.
2. Chatot, C., Lewis, J., Torres, I., and Ziomek, C. 1990. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol. Reprod.* 42: 432–440.
3. Davisson, M.T. and Akeson, E.C. 1987. An improved method for preparing G-banded chromosomes from mouse peripheral blood. Cytogenet Cell Genet. 45: 70–74.
4. Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. U.S.A.* 77: 7380–7384.
5. Kono, T., Kwon, O.Y., and Nakahara, T. 1991. Development of enucleated mouse oocytes reconstituted with embryonic nuclei. *J. Reprod. Fertil.* 93: 165–172.
6. Kwon, O.Y. and Kono, T. 1996. Production of live young by serial nuclear transfer with mitotic stage of donor nuclei in mice. *Proc. Natl. Acad. Sci. U.S.A.* 93: 13010–13013.
7. Nagy, A., Gócza, E., Diaz, E.M., Prideaux, V.R., Iványi, E., Markkula, M., and Rossant, J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110: 815–821.
8. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell cultured-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90: 8424–8428.
9. Ono, Y., Shimozawa, N., Ito, M., and Kono, T. 2001. Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol. Reprod.* 64: 44–50.
10. Ono, Y., Shimozawa, N., Muguruma, K., Kimoto, K., Hioki, K., Tachibana, M., Shinkai, Y., Ito, M., and Kono, T. 2001. Production of cloned mice from embryonic stem cells arrested at metaphase. *Reproduction* 122: 731–736.
11. Quinn, P., Barros, C. and Whittingham, D. J. 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.* 66: 161–168.
12. Rideout, W.M. III., Wakayama, T. Wutz, A., Eggan, K. Jckson-Grusby, L., Dausman, J., Yanagimachi, R., and Jaenisch, R. 2000. Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nat. Genet.* 24: 109–110.
13. Robertson, E., Bradley, A., Kuehen, M., and Evans, M. 1986. Germ-line transmission of gene introduced into cultured pluripotential cells by retroviral vector. *Nature* 323: 445–448.
14. Routman, E.J. and Cheverud, J.M. 1995. Polymorphism for PCR- analyzed microsatellite between the inbred strains LG and SM. *Mamm Genome* 6: 401–404.
15. Sendai, Y., Yoshida-Komiya, H., Kuramochi, T., Ito, M., Ogasawara, R., Hoshi, H., Shinkai, Y., and Araki, Y. 1999. Establishment of germ-line competent embryonic stem cells lacking mouse oviduct-specific glycoprotein gene. *Biol. Reprod.* 60: Suppl. 1. 144.
16. Takahashi, K., Sendai, Y., Matsuda, Y., Hoshi, H., Hiroi, M., and Araki, Y. 2000. Mouse oviduct-specific glycoprotein gene: genomic organization and structure of the 5'-flanking regulatory region. *Biol. Reprod.* 62: 217–226.
17. Wakayama, T., Rodriguez, I., Perry, A., Yanagimachi, T., and Mombaerts, P. 1999. Mice cloned from embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 96: 14984–14989.
18. Whittingham, D. 1971. Culture of mouse ova. *J. Reprod. Fertil.* 14: 7–12.
19. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* 214: 70–76.