Production of cloned mice from embryonic stem cells arrested at metaphase

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In mammals, cloned individuals can be produced from somatic cells. The combined use of gene targeting in embryonic stem cells and cloning contributes to the investigation of gene function in mammals. However, one of the major limitations to cloning is the low viability of cloned embryos, leading typically to high rates of pre- and postnatal death. The present study investigated whether cloning efficiency is influenced by the procedural differences involved in using transfected embryonic stem cells arrested at M phase for cloning by both single and serial transfer. In contrast to a previous study, in which fibroblasts were used, in the present study using embryonic stem cells there was no difference in the rate of

production of cloned pups after the use of a single or serial nuclear transfer, although the proportion of blastocysts (70% versus 51%) was significantly higher ($P < 0.001$) after serial nuclear transfer. After embryo transfer of 445 blastocysts, 218 (49%) implanted and 27 (6% of blastocysts transferred) live pups were born. Of these 27 pups, 23 developed to adults of apparently normal fertility. Of these adults, 39% ($n = 9$) were derived from targeted embryonic stem cells, which is similar to the proportion of targeted embryonic stem cells in the population used for cloning. This study showed that cloning with embryonic stem cells is a viable procedure resulting in the production of transgenic cloned adults.

Introduction

After the production of cloned sheep from cultured embryonic disc cells (Campbell *et al.*, 1996), a succession of other individuals, including sheep (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997), cattle (Kato *et al.*, 1998), goats (Baguisi *et al.*, 1999) and mice (Wakayama *et al.*, 1998; Ogura *et al.*, 2000), were cloned from somatic cells. These studies revealed the capacity of unfertilized oocytes to reprogramme the DNA of differentiated cells, although the mechanism by which this occurs remains poorly understood (Wakayama *et al.*, 2000). One of the practical uses for cloning is its application as part of a gene targeting strategy, especially in mice, since functional embryonic stem (ES) cells have been established and used widely for the analysis of gene function *in vivo*. This potential use was realized by Wakayama *et al.* (1999) and Rideout *et al.* (2000), who produced cloned pups from ES cells established from a 129 sub-strain and 129XC57BL/6 hybrid strain mice. However, Wakayama *et al.* (1999) also reported disadvantages in cloning from ES cells. A large

proportion of the clones produced from 129 ES cells died postnatally for reasons as yet unknown, and it has not proved possible to produce clones from the TT2 ES cell line established from B6CBF1 hybrid strain mouse embryos. In general, fetal loss occurs throughout the gestation period in somatic cell cloning (Wakayama *et al.*, 1998; Ogura *et al.*, 2000; Ono *et al.*, 2001).

Cloned mice can be generated from fetal fibroblast cells synchronized at metaphase by serial nuclear transfer (Ono *et al.*, 2001). The donor nuclei are transferred into ovulated oocytes and, after artificial activation, the nucleus formed is again transferred to enucleated fertilized one-cell embryos. The present study used the original procedure (Ono *et al.*, 2001) to examine whether cloned mice can be produced from TT2 ES cells, which had been targeted previously at the *G9a* gene (Tachibana *et al.*, 2001), homologous to the human *G9A*, a novel mammalian lysine-preferring histone methyltransferase.

Materials and Methods

Animals

Adult female and male B6CBF1 (C57BL/6XCBA) and ICR mice were obtained from SLC Japan Inc. (Shizuoka). All

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Fig. 1. Construction of cloned embryos from embryonic stem (ES) cells arrested at metaphase. (a) ES cells were exposed to the synthetic microtubule inhibitor nocodazole at $0.4 \mu\text{g ml}^{-1}$ for 2 h before nuclear transfer. Cells going through the process of cell division are attached less firmly to the substratum. (b) Metaphase cells 18–20 μm in diameter were selected for nuclear transfer as donor cells. (c) Condensed metaphase chromosomes were observed when a donor cell (arrow) was sucked into a injection pipette. Chromosomes are viewed here by Hoffman modulation contrast microscopy. (d) Constructed oocyte formed by the nuclear transfer of the ES cell into an enucleated oocyte. Transformation of the normal metaphase spindle was observed in 80% of oocytes. (e) Constructed oocyte activated with 10 mmol strontium I^{-1} formed a single diploid pronucleus (DN) after extruding the extra polar body (PB). (f) Blastocysts derived from constructed oocytes using ES cells. Scale bars represent 100 μm (a, f) and 20 μm (b–e).

mice were kept under controlled temperature ($23 \pm 2^\circ\text{C}$) and lighting conditions of 12 h light:12 h dark during experiments and were given food and water *ad libitum*. All mice were maintained and used in accordance with the *Guide for Care and Use of Laboratory Animals* by the Japanese Association for Laboratory Animal Science.

Preparation of donor cells

The TT2 ES cells, established from hybrid strain B6CBF1 male mouse embryos, had been targeted previously at the *G9a* gene. However, the ES cell line had not been selected. ES cells were cultured in collagen-coated dishes without a feeder layer for 3 days in Knockout-DMEM medium (Gibco BRL, Grand Island, NY) supplemented with 15% (v/v) fetal bovine serum (Gibco) and leukaemia inhibitory factor (Wako Pure Chemical Industries, Osaka) at a concentration of 10^3 U ml^{-1} and the following reagents: 2 mmol L-glutamine (Gibco) I^{-1} , 1% (v/v) non-essential amino acid solution (Gibco) and $5.5 \times 10^{-5} \text{ mol l}^{-1}$ 2-mercaptoethanol I^{-1} solution (Wako). ES cells were cultured with medium containing $0.4 \mu\text{g ml}^{-1}$ nocodazole (Sigma Chemical Co., St Louis, MO), a microtubule polymerization inhibitor, for 2 h

so that the cells were synchronized at metaphase. Cells floating in the medium were collected (Fig. 1b). When taken into a transfer pipette, only the cells arrested at metaphase were selected and used as nuclear donors (Fig. 1c).

Nuclear transfer and embryo transfer

Cloned embryos were constructed by single or serial nuclear transfer using ES cells arrested at metaphase (Kwon and Kono, 1996; Ono *et al.*, 2001). The oocyte donors were 6–8-week-old female B6CBF1 mice. Fertilized one-cell embryos were produced by *in vitro* fertilization using 8–10-week-old B6CBF1 females and males as oocyte and sperm donors. Micromanipulations were performed in M2 medium containing $5 \mu\text{g ml}^{-1}$ cytochalasin B (Sigma) and $0.4 \mu\text{g ml}^{-1}$ nocodazole ml^{-1} in a micromanipulation chamber. After enucleation of metaphase II chromosomes, an ES cell arrested at metaphase was introduced into the perivitelline space of the enucleated oocyte with inactivated Sendai virus (HVJ, 2700 haemagglutinating activity units ml^{-1}). The oocytes that fused successfully with a donor cell were incubated for 2 h. After brief culture, the oocytes were activated artificially with 10 mmol strontium I^{-1} for 6 h and

Table 1. Construction of mouse embryos by single or serial nuclear transfer using TT2 embryonic stem cells

Nuclear transfers	Number of oocytes fused at first nuclear transfer/manipulated (%)	Number of oocytes activated normally (%)	Number of oocytes fused at second nuclear transfer	Efficiency (%)
Serial	395/519 (76)	369 (93)	311 (84)	60
Single	489/597 (82)	458 (94)	–	77

Table 2. Development of cloned mouse embryos produced from TT2 embryonic stem cells

Nuclear transfers	Number of cloned embryos cultured	Number of oocytes developed to		Number of transferred embryos	Number of pregnancies/recipients	Number of implantation sites	Number of pups at term		Number of adults
		Two-cell embryo	Blastocyst				Dead	Alive	
Serial	311	307 (99)	217 (70) ^a	213	13/17	110 (52)	1 (0.3)	10 (3.2)	9 (2.9)
Single	458	452 (99)	232 (51) ^b	232	13/19	108 (47)	0 (0)	17 (3.7)	14 (3.1)

Values in parentheses are percentages of cloned embryos cultured, except for in 'Number of implantation sites' column, in which values in parentheses are percentages per blastocysts transferred.

^a^bValues with different superscripts within the same column are significantly different, $P < 0.001$.

then placed in CZB medium (Chatot *et al.*, 1990). At second nuclear transfer, the nucleus of the constituted one-cell embryo was again transferred to previously enucleated fertilized one-cell embryos. On day 4 of culture *in vitro*, blastocysts were selected and transferred to the uterine horns of 8–10-week-old ICR female mice on day 3 of pseudopregnancy (2.5 days after mating).

Genetic analysis

Mutations of the *G9a* gene in the donor TT2 ES cells and cloned mice derived from the ES cells were analysed by PCR. DNA from the ES cell colonies and tails of the clones were obtained by extraction with a buffer containing 1 mg protease K ml⁻¹. Amplification of sequences from the wild-type *G9a* allele was carried out using a primer set forward: EX14F; 5'-CAGGACAGTGGGGGCTGGACGCCCATCATC and reverse: EX16R; 5'-CCGGCAGATGATCTTCTCGGTGCGGACAGC, and the targeted allele, including the neo-resistance gene, were amplified using a primer set forward: G3; 5'-GGGCCAGCTCATTCCTCCACTC and mG9a127; 5'-GCAGATGTGATGGCTTGGGGTAG. Twenty microsatellite markers were analysed by PCR using the specific primers described by Love *et al.* (1990) to confirm that the cloned mice were derived from the TT2 ES cells.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed on metaphase chromosomes derived from cultured lymphocytes prepared using the standard procedure described by Watanabe *et al.* (1996). Biotin-labelled mouse

whole X chromosome-specific probe was used for hybridization, and the bound probes were detected with Biotin–fluorescein isothiocyanate (FITC) painting kit (TAKARA, Tokyo; catalogue number 1187-XMB).

Statistical analysis

Data were analysed by chi-squared analysis.

Results

Donor TT2 ES cells, which had been established from a hybrid strain B6CBF1 male mouse embryo arrested at metaphase (Fig. 1a–c), were fused with enucleated oocytes using Sendai virus. After fusion, the single metaphase plate was observed in the oocyte cortex within 2 h (Fig. 1d). After artificial activation, 94% of the embryos formed a single diploid pronucleus after extruding the extra polar body (Table 1, Fig. 1e). The pronucleus was again transferred into an enucleated fertilized one-cell embryo in some cases to test the proposed superiority for development of using cytoplasm from a fertilized egg. When oocytes were produced by serial nuclear transfer, 70% of the embryos developed to the blastocyst stage (Table 2, Fig. 1f). The proportion of blastocysts was significantly lower in the group of oocytes produced from a single nuclear transfer (51%).

The ability of blastocysts to develop after implantation was assessed by transferring 445 blastocysts into 36 pseudopregnant females (Table 2). The implantation rate, which was calculated on the basis of the number of embryos transferred, was not significantly different between

Fig. 2. Cloned mice produced from gene-targeted embryonic stem cells arrested at metaphase.

oocytes produced by serial and single nuclear transfer (52% versus 47%, respectively). Twenty-seven live pups, of normal phenotype with agouti coat colour, were recovered by Caesarean operation at day 19.5 of gestation. However, the proportion of pups produced was similar in both groups (3.2% for oocytes produced by serial nuclear transfer versus 3.7% for oocytes produced by single nuclear transfer). Of these 27, 23 pups (85%) were successfully nursed by foster mothers and grew into normal adults (Fig. 2). None of the pups from embryos produced by serial nuclear transfer died postnatally, except one pup for which the foster mother refused nursing. All three cloned pups (11%) that died within a few hours after recovery were derived from embryos constructed by single nuclear transfer. The analysis of 20 microsatellite genetic markers by PCR confirmed that all cloned mice were genetically identical to the donor ES cells of TT2 line.

PCR analysis using the specific primers for the neo-resistance gene was used to detect mice that were derived from ES cells targeted at the *C9a* gene (Fig. 3). The proportions of the gene-targeted ES cells and the gene-targeted cloned mice were 40% (16 of 40 cells, data not shown), and 39% (9 of 23), respectively. The proportion of cloned mice carrying the mutation was not significantly different from that of the ES cells, indicating that gene targeting does not affect the developmental capacity of ES cells when they are reprogrammed.

The phenotype of the cloned mice was male (Fig. 4a,b), except for two cases that were apparently female (Fig. 4c,d). The 'female' cloned mice mated with the wild-type males, resulting in pregnancy and the birth of normal pups. Karyotype analysis revealed that the cloned mice were of XO genotype (Fig. 4e), indicating that the ES cell line contained cells with a deleted Y chromosome.

Discussion

The ability to induce reprogramming of donor nuclei is specific in ooplasm and degrades before pronuclear

formation. Reports on embryo cloning from embryonic (Kwon and Kono, 1996) and somatic cell nuclei (Ono *et al.*, 2001) showed the advantages of serial nuclear transfer. In cloned pigs, Polejaeva *et al.* (2000) noted the advantage of serial nuclear transfer. The results of the present study show that the proportion of blastocysts produced was significantly higher after serial transfer than it was after single nuclear transfer, and that postnatal death did not occur when serial transfer was used. However, further study is required to confirm this finding and to clarify its effects on reprogramming and development.

The survival rate of cloned pups in the present study was high: 85% (23 of 27) live pups developed normally with reproductive ability (Table 2). This finding is in contrast to the results obtained by Wakayama *et al.* (1999), who failed to produce clones from TT2 ES cells. The reason for this difference is unclear, but may be the result of the nature of the ES cell lines used rather than the procedures. When J1, CCE and E14 ES cell lines derived from 129 inbred strains of mice were used as donor cells, the proportions of cloned embryos developing to the blastocyst stage were not significantly different from those of TT2 ES lines. Post-implantation development was tested by transferring 332 blastocysts to recipient females. However, only one live pup, which later died, was obtained from J1 ES cells. This finding may support the contention that ES cells derived from inbred strains of mice are difficult to reprogramme sufficiently to support development to term (Rideout *et al.*, 2000). It appears that only particular ES cells can be used as nuclear donors, although the underlying mechanisms ensuring success remain unclear.

Unexpectedly, two of the cloned mice were XO females with normal reproductive ability. As TT2 ES cell lines contain cells with a deleted Y chromosome (Uchida *et al.*, 1994), the female clones may have been produced from XO ES cells. However, it is not known whether the frequency is consistent with that of ES cells.

Hypertrophy of the placenta was observed in all cases. The mean placental mass of cloned mice was 341 ± 87 mg ($n = 27$), which is significantly higher ($P < 0.001$) than that of the controls (136 ± 31 mg; $n = 20$, data were used when litter size was < 5). Somatic cloned mice are known to exhibit hypertrophic placentae (Wakayama and Yanagimachi, 1999; Ogura *et al.*, 2000; Ono *et al.*, 2001), indicating that defective placental function contributes to the high rate of embryonic loss. Our previous study showed that the hypertrophy was caused mainly by extensive proliferation of trophoblastic and endometrial glycogen cells in the spongiotrophoblast tissues (Ono *et al.*, 2001). Genes controlling cell differentiation in the labyrinth (Basyuk *et al.*, 1999), spongiotrophoblasts (Guillemot *et al.*, 1995) and giant trophoblast cells (Riley *et al.*, 1998; Hunter *et al.*, 1999) of the placenta may be involved in this phenomenon. Imprinting genes may also be involved in the abnormal development of cloned embryos and extra-embryonic tissue including the placenta.

Fig. 3. Analysis of targeted mutation of *G9a* gene in the donor TT2 embryonic stem (ES) cells (a) and cloned mice derived from the ES cells (b). The 0.6 kb DNA fragment amplified from the wild-type *G9a* allele and the 1.1 kb fragment amplified from the targeted allele, including the neo-resistance gene.

Fig. 4. Female cloned mice have XO karyotype. Phenotypes of external genitalia in male (XY) (a,b) and female (XO) (c,d) mice. (a,c: newborn pups; b,d: adults). (e) Fluorescence *in situ* hybridization (FISH) for an X chromosome (arrow) at $\times 400$ magnification in a female cloned mouse. Scale bars represent 10 mm.

In conclusion, we have demonstrated that gene-targeted mice can be produced from TT2 ES cells by embryo cloning and that only a few postnatal deaths occur. This finding supports the contention that ES cell cloning technology can be incorporated into gene manipulation strategies, while

overcoming some of the disadvantages seen in germ line transmission using chimaeras.

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