Production of cloned mice from embryonic stem cells arrested at metaphase

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Materials and Methods

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

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mice were kept under controlled temperature (23 ± 2°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) l^{-1}, 1% (v/v) non-essential amino acid solution (Gibco) and 5.5 × 10^{-5} mol 2-mercaptoethanol l^{-1} solution (Wako). ES cells were cultured with medium containing 0.4 μg nocodazole ml^{-1} (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 μg cytochalasin B ml^{-1} (Sigma) and 0.4 μg 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 l^{-1} for 6 h and

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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 μg 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 an 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 l^{-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).
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’-GGGCCAGCTCATTCCTCCACT 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

<table>
<thead>
<tr>
<th>Nuclear transfers</th>
<th>Number of oocytes fused at first nuclear transfer/ manipulated (%)</th>
<th>Number of oocytes activated normally (%)</th>
<th>Number of oocytes fused at second nuclear transfer</th>
<th>Efficiency (%)</th>
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<tr>
<td>Serial</td>
<td>395/519 (76)</td>
<td>369 (93)</td>
<td>311 (84)</td>
<td>60</td>
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<tr>
<td>Single</td>
<td>489/597 (82)</td>
<td>458 (94)</td>
<td>–</td>
<td>77</td>
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<table>
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<tr>
<th>Nuclear transfers</th>
<th>Number of cloned embryos cultured</th>
<th>Number of oocytes developed to</th>
<th>Number of transferred embryos</th>
<th>Number of pregnancies/ recipients</th>
<th>Number of implantation sites</th>
<th>Number of pups at term</th>
<th>Number of adults</th>
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<tr>
<td>Serial</td>
<td>311</td>
<td>307 (99)</td>
<td>217 (70)</td>
<td>213</td>
<td>110 (52)</td>
<td>1 (0.3)</td>
<td>10 (3.2)</td>
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<tr>
<td>Single</td>
<td>458</td>
<td>452 (99)</td>
<td>232 (51)</td>
<td>232</td>
<td>108 (47)</td>
<td>0 (0)</td>
<td>17 (3.7)</td>
</tr>
</tbody>
</table>

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.

Values with different superscripts within the same column are significantly different, P < 0.001.

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

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

Discussion

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

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