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, 28 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<sup>+</sup> and OGP<sup>−</sup> mice derived from chimeric mice showed normal fertilizing ability in vivo (Araki et al., unpublished data). Frozen-thawed OGP<sup>−</sup> 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<sup>3</sup> U/ml 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<sup>−5</sup> M β-mercaptoethanol (ES medium). Before nuclear transfer, ES cells were cultured with ES medium containing 0.4 μg/ml nucodazole (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 SIC × CBA/N SIC; 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 SIC: 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 nucodazole. 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<sup>2+</sup>-free M16 medium [18], containing 10 mM Sr<sup>2+</sup> [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% CO₂ 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: GTTCTTCCTGATGAACACAGTGG and GCACACCGTTFAGTACCG, respectively. To identify the mutant allele, the following sequences were used for the 5’-primer and 3’-primer: ACCCTGACAAACATTTAGGCTCC and CATAACGGTTCCTGACTCC, 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 MgCl₂, 0.2 mM dNTP, and Takara LA Taq polymerase (TakaRa Shuzo Co., Shiga, Japan). Multiplexed 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: B6C3F1), 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<sup>−/−</sup> embryonic stem cells

<table>
<thead>
<tr>
<th>Nuclear transfer (NT) method</th>
<th>No. of oocytes activated normally&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of embryos developed to M+B&lt;sup&gt;b&lt;/sup&gt; cultured (%)</th>
<th>No. of M+B transferred (%)</th>
<th>No. of pregnant/ recipients (%)</th>
<th>No. of live pups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>587/745 (78.8)</td>
<td>356/587 (60.6)</td>
<td>313 (53.3)</td>
<td>17/28 (60.7)</td>
<td>11 (1.9)</td>
</tr>
<tr>
<td>Serial NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>403/512 (78.7)</td>
<td>209/396 (52.8)</td>
<td>191 (48.2)</td>
<td>12/19 (63.2)</td>
<td>9 (2.3)</td>
</tr>
<tr>
<td>Total</td>
<td>990/1257 (78.8)</td>
<td>565/983 (57.5)</td>
<td>504 (51.3)</td>
<td>29/47 (61.7)</td>
<td>20 (2.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>No significant differences (P>0.05) were observed between both methods. <sup>b</sup>Oocytes with a pronucleus and a polar body. <sup>1</sup>Morula and Blastocyst stage.

Table 2. Cloned pups obtained by Caesarian section

<table>
<thead>
<tr>
<th>No. of cloned pups</th>
<th>Died soon</th>
<th>Died before weaning</th>
<th>Weaned</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>6 (30.0)</td>
<td>6 (30.0)</td>
<td>8 (40.0)</td>
</tr>
</tbody>
</table>

Fig. 1. Cloned mice (C1 to C4), OGP<sup>−/−</sup> 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<sup>−/−</sup> F1 mice were observed to be fertile and produced 37 offspring (F2 mice). The genotypes of the F2 mice were as follows: 9 OGP<sup>−/−</sup>, 18 OGP<sup>−/+</sup> and 10 OGP<sup>+</sup> (Table 4). Furthermore, the one female cloned mouse mated with
Table 3. Profile of microsatellite markers in cloned mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Note</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
<th>D7</th>
<th>D8</th>
<th>D9</th>
<th>D10</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1c: B6CBF1</td>
<td>a strain of recipient oocytes</td>
<td>a, c</td>
<td>a, c</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>TT2 cells*</td>
<td>derived from C57BL/6J: B6CBF1</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Cloned mice</td>
<td>derived from TT2 cells*</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a, b</td>
<td>a</td>
<td></td>
</tr>
</tbody>
</table>

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 mouse

<table>
<thead>
<tr>
<th>Generation</th>
<th>Parents</th>
<th>No. of offspring</th>
<th>Genotype of offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>♀: Genotype</td>
<td>♂: Genotype</td>
<td>OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>F1</td>
<td>B6J&lt;sup&gt;+&lt;/sup&gt;: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>Clone 1&lt;sup&gt;+++&lt;/sup&gt;: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>Clone 2: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Clone 3: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>Clone 4: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Clone 5: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>Clone 6: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>B6J: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Clone 7: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>0</td>
<td>50 (56.2%)</td>
</tr>
<tr>
<td>F2</td>
<td>A&lt;sup&gt;+++&lt;/sup&gt;: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>E: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>B: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>E: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>C: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>F: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>D: OGP&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>F: OGP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>9 (24.3)</td>
<td>18 (48.6)</td>
</tr>
</tbody>
</table>

*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<sup>−/−</sup>, 12 OGP<sup>−/+</sup> and 4 OGP<sup>++/−</sup>. 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<sup>−/+</sup> genotype, and mating with C57BL/6J females yielded offspring (F1
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 germ line 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-prefering 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.
Acknowledgments

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References