Effects of Recloning on the Efficiency of Production of α1,3-Galactosyltransferase Knockout Pigs

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Abstract. Obtaining sufficient transgenic cells via selective cultivation of genetically manipulated somatic cells is difficult due to the limited number of cell divisions. Additionally, if irreversible mutations in a cell’s chromosomes occur during selective cultivation and the cell is used as the nuclear donor, somatic cell nuclear transfer (SCNT) embryos often exhibit abnormal development. On the other hand, a SCNT method in which fetal cells derived from SCNT embryos are used as the nuclear donor (recloning method) is an effective technique for obtaining large quantities of transgenic cells. In this study, we compared the in vivo development rate of SCNT embryos produced from porcine α1-3 galactosyltransferase gene knockout (GTKO) cells by a recloning method with that of SCNT embryos produced without recloning from porcine GTKO cells (direct method). In the direct method, 557 and 462 cloned embryos were produced using two types of activation methods, the two-step activation (TA) method and the delayed activation (DA) method, and then transferred into 6 and 4 recipients, respectively, but no piglets were born from these recipients. In the recloning method, 956 and 1038 cloned embryos were produced using the TA and DA methods, respectively, and then transferred to 8 and 7 recipients, respectively. Two piglets were born from one recipient in the TA group and 6 piglets were born from 3 recipients in the DA group. This report indicates that the recloning method improved the developmental capacity of SCNT embryos reconstructed with gene-targeted somatic cells.

Key words: α1,3-galactosyltransferase, Gene knockout, Nuclear transfer, Pig, Recloning

The lack of transplantable organs is a major problem in transplantation medicine. Xenotransplantation of porcine organs may be a solution to this problem, and studies to this end are currently being conducted. However, to perform pig-to-human transplantation successfully, it is necessary to overcome the problem of xenotransplantation-specific rejection. Porcine cells contain αGal epitopes (carbohydrate antigens), but these epitopes are not present in human and Old World monkey cells. On the other hand, humans and Old World monkeys possess anti-αGal antibodies as natural antibodies. If a porcine organ is transplanted into a human, the natural human antibodies (anti-αGal antibodies) react with αGal epitopes in the porcine organ, resulting in hyperacute vascular rejection due to complement reaction [1, 2]. The αGal epitopes are carbohydrates biosynthesized by α1,3-galactosyltransferase (GT). In humans and Old World monkeys, the GT-encoding gene has mutated, and as such, GT is not expressed [3, 4]. To prevent this type of rejection, several research groups have produced GT knockout (GTKO) pigs by means of somatic cell nuclear transfer (SCNT) [5–11]. When organs derived from homozygous GTKO pigs were transplanted into baboons, the organs survived for 3 to 6 months [12, 13], demonstrating that development of GTKO pigs is essential and that further multiplex genetic modification is necessary to extend the survival period for clinical use.

Recently, our team successfully knocked out the GT gene in cells obtained from transgenic (Tg) pigs expressing both decay accelerating factor (DAF, a complement regulatory protein) and N-acetylglucosaminyltransferase-III (GnT-III, a carbohydrate-remodeling enzyme) [14] and subsequently produced GTKO pigs by SCNT [15]. In that study, we first produced cloned embryos from GTKO cells by SCNT and then transferred the embryos into recipient gilts. Next, we recovered the fetuses from the gilts on Day 29 of gestation and performed SCNT using cells from these fetuses (recloning), ultimately producing GTKO pigs. The reason for recloning is as follows. To create GTKO cells, gene transfection must first be carried out using a knockout vector, successful homologous recombinant cells must be selected and then these cells must be cultured (selective culture). However, the lifespan of somatic cells is limited, and therefore the cells become senescent if they are cultured for a long time to obtain enough cells for SCNT [16]. Because of this, it is often the case that only a limited number of homologous recombinant cells are available for SCNT. Moreover, it is thought that culturing somatic cells for a long period increases the percentage of cells with chromosomal aberrations [17]. This is the major obstacle in producing gene-knockout pigs using SCNT following homologous recombination. The advantage of the recloning method lies in its ability to selectively extend the lifespan of transgenic donor cells, which usually consist of a heterogeneous cell population that includes cells that are close to senescence or have an increased percentage of chromosomal aberrations caused by long culturing periods [18]. Furthermore, it is possible to check for chromosomal aberration at this time. So far, Cibelli et al. [18] and Kuroiwa et al. [19], in addition to our team, have successfully

Accepted for publication: October 29, 2007
Published online: December 18, 2007
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produced cloned animals by means of recloning with SCNT in which transgenic bovine fetal fibroblasts were used as nuclear donor cells. However, it is not known to what extent recloning improves efficiency. Liu et al. [20] performed SCNT using bovine fetal fibroblasts as nuclear donor cells and reported that a high in vitro blastocyst development rate can be obtained by recloning. On the other hand, Hill et al. [21] conducted a similar experiment and reported that recloning is not an effective method of obtaining a high in vivo embryo development rate. However, all of the above reports used intact cells as the nuclear donor for SCNT. At present, there are no reports concerning the advantages of the recloning method using transgenic and/or homologous recombinant cells as the nucleus donor.

Therefore, in this study, we compared the in vivo development rate of SCNT embryos produced from porcine α1-3 galactosyltransferase gene knockout (GTKO) cells by a recloning method with that of SCNT embryos produced from porcine GTKO cells without recloning (direct method).

**Materials and Methods**

All the experiments were conducted in compliance with the Japanese Law Concerning the Protection and Control of Animals and the Guidelines for Animal Experimentation of the Animal Engineering Research Institute.

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

**Donor cell preparation**

Heterozygous GTKO cells were generated from human DAF and GnT-III gene expressing transgenic porcine fetal fibroblasts by homologous recombination, as described previously [15]. Briefly, porcine fetal fibroblasts were isolated from transgenic porcine fetal carcasses (Day 29 of gestation) expressing human DAF and GnT-III gene and passed three times to remove epithelial and endothelial contaminants. Then, 106 µg of the GTKO vector was introduced by means of electroporation (300 V, 975 µFD) into 2 × 106 fibroblasts. The fibroblasts were cultured for 2 days in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS; Hyclone, Logan, UT, USA), non-essential amino acid and 2 ng/ml bFGF. The karyotype of the cells was checked by the standard Giemsa staining method. The fibroblasts were passaged twice and stored frozen in cryoprotectant solution until use for nuclear transfer. Two cell lines (K2H-8 and K2H-23) were used for nuclear transfer. Once the cells were thawed, they were cultured for 2 days and then separated with trypsin-EDTA (Invitrogen) before being used as donor cells.

**Nuclear transfer**

The nuclear transfer procedure was carried out as described previously [22]. Briefly, porcine ovaries were collected from a local abattoir. Cumulus-oocyte complexes (COC) were collected from ovarian antral follicles (3–6 mm in diameter). These COCs were matured in vitro for 22 h in NCSU-23 medium [23] supplemented with 0.6 mM cysteine, 10 IU/ml eCG (Serotropin; Teikoku Zouki, Tokyo, Japan), 10 IU/ml hCG (Puberogen; Sankyo, Tokyo, Japan) and 10% (v/v) porcine follicular fluid. This process was followed by another 20 h of cultivation in fresh maturation medium without hormonal supplementation. The oocytes with expanded cumulus cells were briefly treated with hyaluronidase (1 mg/ml) and then denuded of cumulus cells using a finely drawn glass capillary pipette. Oocytes with extruded first polar bodies were used for the experiments.

As soon as the cumulus cells were removed, the oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm (about 10% of the ooplasm) using a bevelled pipette (35 µm in diameter) [24] in 10 mM Hepes-buffered Tyrode’s lactose medium (Hepes-TL) containing 0.3% (w/v) polyvinylpyrrolidone (PVP), 10% (v/v) FCS and 7.5 µg/ml cytochalasin B (CB). Enucleation was confirmed by staining the cytoplasts with 5 µg/ml Hoechst 33342.

A single donor cell was inserted into the perivitelline space of an enucleated oocyte, and cytoplasts were placed in a 10 µl droplet of fusion medium.

Fusion and activation were performed by two different methods: a two-step activation (TA) method using a calcium-containing fusion medium (0.27 M mannitol containing 0.1 mM MgSO4 and 0.1 mM CaCl2) and a delayed activation (DA) method using a calcium-free fusion medium (0.27 M mannitol containing 0.1 mM MgSO4) [22]. The donor cells and recipient oocytes were fused by applying an electric pulse (DC 180 V/mm, 10 µsec) using an electrical cell fusion instrument (SSH-1; Shimadzu, Kyoto, Japan or LF101; Bex, Tokyo, Japan).

The reconstructed embryos were activated by electric stimulation (DC 150 V/mm, 100 µsec) 1 to 1.5 h later and cultured for 3 h with 5 µg/mL CB followed by culture in NCSU23.
Crossbred prepubertal gilts (Large White/Landrace × Duroc) were used as recipients. Estrus was induced in the recipients by intramuscular injection of 1,000 IU eCG followed by 1,500 IU hCG 71 h later. The reconstructed embryos were transferred 2 to 3 days after the hCG injection. Following a mid-ventral incision under general anesthesia, the reproductive tracts were exposed and the embryos were transferred into the ampullary-isthmic junctions of the oviducts.

Pregnancies were monitored by weekly ultrasound scanning until delivery.

Analysis of piglets

The tails were harvested from the newborn piglets and their recipient pigs and then placed in an elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS and 100 µg/ml Proteinase K) overnight at 55°C. Phenol-chloroform extraction and ethanol precipitation were then carried out.

Amplification by PCR was used to confirm gene knockout in the genomic DNA of the cloned piglets. The primer sets used for this check were the same as those described in a previous report [15]. In the case of a targeted allele in which the GT gene has been replaced with a marker gene (hygromycin-resistance gene), a DNA fragment of approximately 10 kb was amplified; however, in the case of the wild-type allele, a DNA fragment of approximately 7.6 kb was amplified. Amplification was performed on genomic DNA using the Expand Long Template PCR System (Roche; the forward primer was 5’-AGAGGTCGTGACCATAACCCGAT-3’, and the reverse primer was 5’-AGCCCATCGTGCTGAACATCAAGTC-3’). After first denaturation step of 2 min for 94°C, PCR amplification were performed for 30 cycles of 94°C for 15 sec, 65°C for 30 sec and 68°C for 10 min, with a 20-sec extension per cycle, and this was followed by 68°C for 7 min. Amplification products were analyzed by 1% agarose gel electrophoresis.

Statistical analysis

The differences between the direct and recloning methods with respect to pregnancy rate and number of piglets born were compared using the chi-square test. Differences were regarded as significant when P<0.05.

Results

In the direct method, 557 and 462 cloned embryos were produced using the TA and DA methods, respectively, and then transferred to 6 and 4 recipients, respectively. One recipient from each group aborted in the early stage of gestation (< Day 30 of gestation), and no piglets were born from these recipients (Table 1). In the recloning method, 956 and 1,038 cloned embryos were produced using the TA and DA methods, respectively, and then transferred to 8 and 7 recipients, respectively. Four recipients in the TA group became pregnant; three aborted and one delivered 2 live piglets. Four recipients in the DA group also became pregnant; one aborted and three delivered a total of 6 live and 2 stillborn piglets (Table 1). Necropsy of the stillborn piglets did not reveal any particular abnormalities. The piglets that were born alive are currently growing well. The pregnancy rates for the recloning methods were approximately 50% (50% for TA and 57% for DA), whereas those of the direct methods were approximately 20% (17% for TA and 25% for DA) (Table 1). As a whole, live piglets were only obtained from embryos produced using the recloning method.

PCR analysis was conducted to confirm the gene knockout event in the genomic DNA of live piglets (Fig. 1). In all cases of recipient genomic DNA, only a DNA fragment of approximately 7.6 kb (indicating the wild-type allele) was amplified (lanes 3, 4 and 9). By contrast, DNA fragments of approximately 7.6 and 10 kb (indicating the wild-type allele and targeted allele) were amplified in the

Table 1. Comparison of the direct method and a recloning method with respect to the in vivo development of embryos produced by SCNT

<table>
<thead>
<tr>
<th>Method</th>
<th>Activation method</th>
<th>No. of embryos transferred</th>
<th>No. of recipients</th>
<th>No. of pregnancies (%)</th>
<th>No. of abortions</th>
<th>No. of pregnancies brought to term (%)</th>
<th>No. of piglets born (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td>TA</td>
<td>557</td>
<td>6</td>
<td>1 (17)</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>462</td>
<td>4</td>
<td>1 (25)</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Recloning</td>
<td>TA</td>
<td>956</td>
<td>8</td>
<td>4 (50)</td>
<td>3</td>
<td>1 (12.5)</td>
<td>2 (20)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>1,038</td>
<td>7</td>
<td>4 (57)</td>
<td>1</td>
<td>3 (43)</td>
<td>6 (5.8)</td>
</tr>
</tbody>
</table>

*TA; two-step activation, DA; delayed activation.
cases of the genomic DNA from K2H-8 (lane 1), K2H-23 fetal cells (lane 2) and live piglets that were produced using the recloning method (lanes 5 to 8 and 10 and 11), indicating that the GT gene had been knocked out.

Discussion

Several problems occur when transgenic cells are used as nuclear donors to produce cloned animals. One problem is that it is difficult to obtain sufficient numbers of transgenic cells that can be used for SCNT. The number of cell divisions is limited in somatic cells, and therefore when a transgenic cell is selectively cultured, it proliferates to form a colony, but soon reaches senescence, and cell division stops [16]. Published reports indicate that the efficiency rate of knockout pig production is still low, and therefore it is desirable to obtain several thousand knockout cells per colony; however, many colonies do not contain sufficient numbers of these cells. Cibelli et al. [18] reported that they generated a 40-day-old cloned fetus using a cell line at 0.8 population doublings (PD) from senescence, and the number of PDs until senescence of a fibroblast cell line isolated from the fetus increased to 31. Similarly, Kuroiwa et al. [19] demonstrated that bovine fetal fibroblasts introduced an artificial chromosome containing human immunoglobulin were rejuvenated by recloning and were able to be used as donor cells again. As such, recloning is capable of extending the lifespan of donor cells and therefore makes it possible to obtain sufficient numbers of cells for use as donor cells in nuclear transfer.

Another problem is that the development rate of cloned embryos is often low when genetically modified cells (e.g., knockout cells and Tg cells) are used as donors. Iguma et al. [25], Forsberg et al. [26] and Zakharchenko et al. [27] reported that in the case of cattle, genetic modification of donor cells influences the number of cloned calves born. An increase in chromosomal aberration, such as mutation and deletion due to a long culturing period, is considered one of the causes. DePinho et al. [17] indicated that chromosomal aberration increases as cells age or the passage number increases. An increase in the rate of chromosomal aberration in donor cells affects the development rate of cloned embryos produced using the donor cells. Liu et al. [20] and Roh et al. [28] reported that an increase in the passage number adversely affects the in vitro development rate of cloned bovine embryos. Jang et al. [29] reported that the rate of apoptosis in blastomeres is high in cloned bovine embryos produced using donor cells that have a high passage number.

In the present study, reconstructed embryos produced by the direct method were transferred to 7 recipients, and laparotomy was performed on Day 29 of gestation. Two of the recipients became pregnant, and a total of 14 fetuses were obtained. However, aneuploidy was observed in 6 of the 14 fetuses (43%). We could not obtain data to prove that a high rate of chromosomal aberration was the reason that live piglets were not obtained by the direct method. Two of the 10 recipients in the direct method and 4 of the 15 recipients in the recloning method aborted, although this difference was not significant. However, the pregnancy rate of the recloning method was approximately 50% (50% for TA and 57% for DA), whereas the pregnancy rate of the direct method was approximately 20% (17% for TA and 25% for DA), which is a low value. This difference is likely the reason why live piglets were only produced in the recloning method. This suggests that a high initial pregnancy rate is an important element in efficiently obtaining cloned live offspring. In a previous study, the birth rates (cloning efficiency) of cloned pigs produced by the TA and DA methods were compared, but it was unclear which method was more effective for cloned pig production [22]. In present study, both activation methods were also compared for the cloning efficiency of GTKO pigs. In the recloning method, viable GTKO pigs were obtained by both the TA (2.0%) and DA (5.8%) methods, which was not significant. On the other hand, viable GTKO pig production was not observed for either activation method in the direct method. Based on these results, there was no difference in cloning efficiency between the TA and DA activation methods.

It has been reported that the hearts and kidneys of GTKO pigs were transplanted into baboons and that the greatest survival periods for these organs were 179 and 83 days, respectively [12, 13]. These results suggest that GTKO porcine organs effectively avoid hyperacute rejection following pig-to-human xenotransplantation and that further effective genetic modification is necessary in addition to GTKO. In fact, Miyagawa et al. [30] reported that the H-D antigen could be xenotantigen following removal of the αGal antigen. Moreover, Sharma et al. [11] reported that knocking out only the GT gene does not completely remove the αGal antigen. It is probably necessary to produce pigs with more complex genetic modification, and the recloning method would be very useful for that purpose.

References


