-Rapid Communication-
Birth of Cloned Calves Derived from Cultured Oviductal Epithelial Cells of a Dairy Cow

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ABSTRACT The present study aims to examine the effects of the use of two kinds of somatic cells from a single adult cow on fusion rate, cleavage rate, developmental rate into blastocysts of nuclear transplant oocytes, and pregnancy potential of reconstituted blastocysts. Oviductal epithelial cells and mammary gland cells from a single adult Jersey cow with a milking record were used in the present experiment. No significant differences were observed in the in vitro criteria described above between two cell types used as a source of donor nucleus. Although pregnancies were obtained from both cell types, live calves resulted only from embryos cloned from oviductal epithelial cells. Using oviductal epithelial cells, three out of ten recipients became pregnant. Two carried to full term and delivered one female calf each at day 285 of gestation. The birth weights of two live female calves were 27.5 kg and 30.0 kg. The third aborted at day 251 of gestation. On the other hand, using mammary gland cells, one out of six recipients became pregnant but aborted at day 81 of gestation. Genomic DNA analyses confirmed that the calves are all genetically identical to the nuclear donor cell.

Key words: Somatic cell cloning, Nuclear transfer, Oviductal epithelial cell, Jersey, Reconstituted embryos

Very recently, it was demonstrated that some kinds of differentiated cells, as well as embryonic cells, can have developmental totipotency in sheep8), in mice6), and in cattle1,3,7) through reprogramming by nuclear transfer technology. It is expected that these successful productions of offspring can bring dramatic changes in the animal breeding strategy. From a viewpoint that aims to apply cloning technology to animal breeding, differentiated cell cloning, that is, adult somatic cell cloning, has some advantages when compared with embryonic cell cloning. Firstly, gender preselection for offspring is done. Secondly, traits of the offspring from nuclear donor animal's are predictable. Thirdly, uniformity of the donor cells for nuclear transfer is possible because donor cells can be maintained in culture state. Finally, even for single production of offspring it is worth comparing some traits between the nuclear donor animal and the offspring. Considering these advantages, adult somatic cell cloning should aim to be conducted using cells obtained from the animals already proven to have ideal productivities. Our goal is to evaluate the value of adult somatic cell cloning for animal breeding as effectively as possible. The objective of the present experiment was to examine the effects of the use of two kinds of somatic cells from a single adult on rates of fusion, cleavage, development to blastocysts of nuclear transplant oocytes, and pregnancy potential of reconstituted blastocysts.

Materials and Methods
Preparation of Donor Nucleus
Oviducts and mammary glands were obtained aseptically from a multiparous Jersey cow, which had been feeding in the National Livestock Breeding Center (NLBC) Iwate Station, immediately after it was slaughtered, and transported to NLBC Headquarters' laboratory. Oviductal cells were recovered by squeezing the oviducts with scissors. After washing the cells with Dulbecco's minimum essential medium (D-ME medium), supplemented with 10% fetal calf serum (FCS) by centrifugation, the cells were cultured in the same medium at 38.5°C, in 5% CO2, in air, until confluent conditions were attained (Fig. 1). These cells were passaged three to four times. Prior to using nuclear transfer, the cells were cultured in D-ME medium and the FCS concentration was reduced from 10% to 0.5% during a 5 day period8).

Preparation of Recipient Cytoplast
Ovaries with an unknown genetic background (cows slaughtered at a local abattoir), were transported to the laboratory immersing them in a 25°C physiological saline. Cumulus oocyte complexes (COCs) were aspirated from the pooled ovaries and incubated in TC199, supplemented with 5% calf serum (CS), for 20 h for in vitro-maturation. In vitro-matured COCs were treated in the M2 medium supplemented with 0.5% hyaluronidase for 5 min and the cumulus cells were removed by gentle pipetting. The zona pellucidae of the denuded metaphase II oocytes were cut near the polar body and enucleated by pushing out the cytoplasm including the nucleus with a glass needle, in PBS supplemented with 20% CS and 5 μg/ml of cytochacarin B. Enucleation was confirmed for each removed cytoplast by fluorescent dyeing with Hoechst 33342 (Fig. 2a & 2b).

Nuclear Transfer
The procedure to make reconstituted embryos was similar to the one8) published by Roslin Institute. A single donor cell either from oviductal epithelial cells or mammary gland cells, was introduced into the perivitelline space of an enucleated matured oocyte using a micro-injection glass pipette (Fig. 2c). The enucleated oocyte with the single donor cell was transferred to Zimmerman cell fusion medium (Fig. 2d). The single donor cell was electrically fused with the oocyte immediately after enucleation, with one pulse of 25 V for 50 μ sec, using a needle-type...
Fig. 1. Bovine oviductal epithelial cells in confluent condition (original magnification: x 200)

Fig. 2. Procedures of nuclear transfer using oviductal epithelial cells. (a) enucleation by pushing out the cytoplasm including the nucleus with a glass needle, (b) enucleation was confirmed for each removed cytoplasm by fluorescent dyeing with Hoechst 33342, (c) introduction of oviductal epithelial cell into perivitelline space of the enucleated oocyte using a micro-injection glass pipette, (d) the enucleated oocyte with the single donor oviductal epithelial cell, (e) the enucleated oocyte at the phase of the beginning of fusion (original magnifications of a, c, d and e: x 200, of b: x 400, of f: x 300).

Table 1. The differences between two somatic cell types from a single adult when examining fusion rate, cleavage rate, developmental rate into blastocysts of nuclear transplant oocytes, and pregnancy potential of reconstituted embryos.

<table>
<thead>
<tr>
<th>Donor cell</th>
<th>No. of metaphase II oocytes</th>
<th>No. of nuclear transplant oocytes fused (%)</th>
<th>No. of oocytes cleaved (%)</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Total (%)</th>
<th>No. of blastocysts</th>
<th>No. of pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOEC</td>
<td>101</td>
<td>70 (69.3)</td>
<td>53 (75.7)</td>
<td>11</td>
<td>19</td>
<td>30 (42.9)</td>
<td>3 / 10</td>
<td>2</td>
</tr>
<tr>
<td>BMGC</td>
<td>83</td>
<td>57 (68.7)</td>
<td>53 (66.7)</td>
<td>9</td>
<td>11</td>
<td>20 (35.1)</td>
<td>1 / 6</td>
<td>0</td>
</tr>
</tbody>
</table>

† BOEC means bovine oviductal epithelial cells and BMGC bovine mammary gland cells.
‡ Percentage of number of fused oocytes to number of metaphase II oocytes. No significant difference was observed.
# Percentage of number of cleaved oocytes to number of fused oocytes. No significant difference was observed.
§ Percentage of number of blastocysts to number of fused oocytes. No significant difference was observed.
¶ A fetus cloned from BOEC and a fetus cloned from BMGC were aborted at day 251 and at day 81 of gestation, respectively.

Fig. 3. Two normal cloned calves at 18 days of age, Future (right) and Fortune (left).
The initial important theme of adult somatic cell cloning demonstrated that the mitochondrial DNA genotype in nuclear transfer calves was a result of the dominant distribution of mitochondrial DNA from recipient oocytes. Mannen et al. (1998) suggested that cytoplasmic genetic effects are important sources of variation for carcass traits in Japanese Black cattle. Cloning using differentiated cells which can be kept in vitro may provide important answers to this question. Although oocytes of pooled ovaries from cows with an unknown genetic background were used in the present experiment, accurate comparison of traits will be needed in the next step of the experiment under the conditions, so origin of recipient oocytes can be identified. If so, the interaction between the donor cell and the recipient oocyte can be determined as well.

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References