An Attempt at Fusing Primordial Germ Cell with Embryonic Blood Cell in Chickens Using Inactivated Sendai Virus or Electrical Stimulation

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In order to produce nuclear transferred PGCs, fusion of primordial germ cell (PGC) with embryonic blood cell (EBC) was attempted. PGCs and EBCs were isolated from the blood of stages 13–15 embryos. The collected PGCs and EBCs were mixed, and fusion of these cells was carried out using inactivated Sendai virus (HVJ) or electrical stimulation. PGC-EBC fused cells were identified by the morphology and difference in size of the two nuclei. The mean fusion rate of PGC with EBC was 0.96% using inactivated HVJ and 5.2% using electrical stimulation. The present results provide useful information for nuclear transfer in PGCs.

Key words: cell fusion, electrical stimulation, embryonic blood cell, HVJ, primordial germ cell

Introduction

Production of cloned animals by somatic cell nuclear transfer (Wilmut et al., 1997) provides useful techniques in basic science as well as applied science, such as the preservation of genetic resources, the multiplication of endangered species, and the production of transgenic animals. Although many mammals cloned by somatic cell nuclear transfer have been produced (Wilmut et al., 2002), no cloned chickens have been successfully produced due to the distinctive structure of the ovum. Recently, a chicken embryo culture system for newly ovulated fertilised ova through hatching has been developed (Naito et al., 2005). Thus, the application of nuclear transfer techniques developed in mammals to chickens has now become possible. But, in this system, only a limited number of ova could be manipulated because only one ovum can be obtained from a hen, and the hatching rate of the cultured embryos is still low. Thus, the nuclear transfer techniques developed in mammals still remain difficult to apply in chickens.

Manipulation of primordial germ cells (PGCs) provides an alternative method for
nuclear transfer in chickens (Tajima, 2002; Naito, 2003). The system for producing germline chimaeric chickens by the transfer of PGCs has already been established (Tajima et al., 1993; Naito et al., 1994). If a nucleus of PGC can be replaced by a somatic cell nucleus, the nuclear transferred PGC could give rise to viable offspring via germline chimaeric chicken after being transferred to recipient embryo. Enucleation of PGCs could be done by UV irradiation (Minematsu et al., 2004a) or some other methods, and somatic cell nuclear transfer could be achieved by fusing the enucleated PGC with a somatic cell (Minematsu et al., 2004b). Through these manipulations, it is expected that the nuclear transferred PGCs are produced and the manipulated PGCs migrate to the germinal ridges after being transferred to the bloodstream of recipient embryos and successfully differentiate into germ cells in the gonads of the chimaeric chickens. By mating these chimaeric chickens, somatic cell-derived offspring are expected to be produced. In order to apply this strategy for nuclear transfer in chickens, the techniques for each step involved in the nuclear transfer in PGCs should be developed.

The present study was conducted to produce nuclear transferred PGCs, and here we report the preliminary results of fusing PGC with embryonic blood cell (EBC) using inactivated Sendai virus (Hemagglutinating Virus of Japan; HVJ) or electrical stimulation.

Materials and Methods

Fertilised Eggs and Animal Care

Fertilised eggs of Barred Plymouth Rock (BPR) chickens were obtained by artificial insemination. BPR population is maintained at the National Institute of Livestock and Grassland Science. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences Animal Care Committee).

Preparation of PGCs and EBCs

Fertilised eggs of BPR were incubated at 38°C, with a relative humidity of 50–60%, for about 53 h in a forced-air incubator (P-008B Bio-type; Showa Furanki, Saitama, Japan). Blood was collected from the dorsal aorta of embryos at stages 13–15 (Hamburger and Hamilton, 1951) using a fine glass micropipette. PGCs were concentrated by the Nycodenz density gradient centrifugation method (Zhao and Kuwana, 2003; Naito et al., 2004). Briefly, the collected blood was pooled, washed and dispersed in a 400μl KAv-1 medium (Kuwana et al., 1996). Five millilitres of 11% Nycodenz solution was placed in a 50ml tube (Cat. No. 2070, Becton Dickerson, Franklin Lakes, NJ, USA), and 5ml of 5.5% Nycodenz solution and subsequently 400 μl of blood solution were overlaid. The tube was centrifuged at 400 g for 20 min, and then 10 ml of the PGC-rich solution (about 1,000 PGCs) was recovered from the top. The residual EBC solution was used for preparing EBCs.

Fusion of PGC and EBC Using Inactivated HVJ

The recovered PGC-rich solution was washed with KAv-1 medium and dispersed in 50μl fresh KAv-1 medium. The residual EBC solution was also washed with KAv-1
medium and then the cell concentration was adjusted to $5 \times 10^4$ cells/µl. Ten microlitres of EBC solution was added in the PGC solution and mixed gently. Sixty microlitres of inactivated HVJ-E solution (GenomONE-Neo, Ishihara Sangyo, Shiga, Japan) was added to the mixture and placed in a 5 ml polystyrene culture tube (Cat. No. 2003, Becton Dickerson, Franklin Lakes, NJ, USA) and incubated for 5 hours at 37°C. Then, 1 ml of KA-1 medium was added in the mixed solution together with 0.25 µg of Hoechst 33342 (Code 04929-82, Nakarai Tesque, Kyoto, Japan), placed in a well of a 4-well culture plate (Cat. No. 176740, Nunc, Roskilde, Denmark) and incubated for 2–18 hours at 37°C. The cells were observed under an inverted fluorescence microscope (DMIRE2, Leica Microsystems, Tokyo, Japan).

**Fusion of PGC and EBC Using Electrical Stimulation**

The recovered PGC-rich solution was washed with a fusion solution (0.3 M Mannitol supplemented with 0.1 mM CaCl₂ and 0.1 mM MgSO₄) and dispersed in 30 µl fusion solution. The residual EBC solution was also washed with the fusion solution, and the cell concentration was adjusted to $5 \times 10^4$ cells/µl. Ten microlitres of the EBC solution was added in the PGC solution and mixed gently, then put in the fusion chamber (CUY500G1, Nepa Gene, Chiba, Japan) with an electrode gap of 1 mm. The AC and DC power was supplied for electrofusion by ECM200 (BTX, San Diego, CA, USA). The movement of the cells was observed under an inverted microscope (DMIL, Leica Microsystems, Tokyo, Japan). Cells were exposed to AC field (400 V/cm) for 20 seconds, then a DC pulse (1.5 kV/cm, 99 µ second pulse width) was applied to the cells three times at one-second intervals. The manipulated cells were recovered, dispersed in 1 ml KA-1 medium containing 0.25 µg of Hoechst 33342 (Code 04929-82, Nakarai Tesque, Kyoto, Japan), then placed in a well of a 4-well culture plate (Cat. No. 176740, Nunc, Roskilde, Denmark), incubated for 2 hours, and observed under an inverted fluorescence microscope (DMIRE2, Leica Microsystems, Tokyo, Japan).

**Results**

Fused cells of PGC and EBC were produced using inactivated HVJ as shown in Fig. 1 (A-I). PGC-EBC fused cells were identified by their morphology and the difference in size of the two nuclei after staining with Hoechst 33342. PGC has a large nucleus while EBC has a relatively small nucleus. The fused cell has thus both large and small nuclei. The fused cells were PGC-EBC, PGC-PGC, and EBC-EBC. More than three cells were occasionally fused. The mean fusion rate of PGC with EBC using inactivated HVJ was 0.96% (Table 1).

When cell fusion was carried out using electrical stimulation, pearl chains were formed within 20 seconds by exposing cells to the AC field. After applying DC pulses, adjacent cells in a pearl chain were fused in some places. Fused cells of more than three cells were also occasionally observed. The fused cells of PGC and EBC were shown in Fig. 1 (J and K). The mean fusion rate of PGC with EBC using electrical stimulation was 5.2% (Table 1).
Discussion

In the present study, fusion of PGC with EBC was attempted for the purpose of developing the techniques of somatic cell nuclear transfer into PGCs. Cell fusion was carried out using inactivated HVJ or electrical stimulation, and the fused cells of PGC and EBC were successfully obtained although the fusion efficiency was low. Fusion

Table 1. Fusion rate of PGC and EBC using inactivated HVJ or electrical stimulation

<table>
<thead>
<tr>
<th>Fusion method</th>
<th>Times</th>
<th>Number of PGCs observed</th>
<th>Number of PGC-RBC fused cells</th>
<th>Fusion rate (Mean ± S.D., %)</th>
</tr>
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<tbody>
<tr>
<td>Inactivated HVJ</td>
<td>n=17</td>
<td>8,500</td>
<td>82</td>
<td>0.96 ± 0.64</td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>n=10</td>
<td>5,000</td>
<td>260</td>
<td>5.2 ± 2.5</td>
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efficiency was higher when using electrical stimulation, but the fused cells seemed to be less damaged from using inactivated HVJ. Since cell fusions tend to occur in the same kinds of cells, that is PGC-PGC or EBC-EBC, it is necessary to improve the fusion efficiency of PGC with EBC. Since only a limited number of PGCs can be collected in one experiment, cell fusion treatment for PGCs should be carried out under the conditions of contacting PGC with EBC instead of simply mixing the cells.

Minematsu et al. (2004 b) tried to fuse PGC with EBC using electrical stimulation and obtained a fusion rate of about 16% under their optimum conditions. The difference in the fusion rate using electrical stimulation by Minematsu et al. (2004 b) and the present study seems to be caused mainly by the difference in the fusion fluid and the fusion chamber. They also reported that UV-irradiated PGCs can successfully migrate to the germinal ridges after transfer to the bloodstream of recipient embryos (Minematsu et al., 2004 a). If the fused cell of UV-irradiated PGC with EBC has characteristics of normal PGC, the fused cells can migrate to the germinal ridges after transfer to recipient embryos. As a result, it is expected that a somatic cell-derived nucleus could be introduced into the germline of recipient embryos. However, the size of the fused cell of PGC and EBC is larger than for an intact PGC, so the migration of fused cells transferred to the germinal ridges of recipient embryos might be interrupted. Also, the fused cells are fragile just after fusing compared with intact PGCs. In these cases, it would be better to culture the fused cells in vitro for some time and then transfer to recipient embryos after recovering cell structure and reducing the size of the fused cells by disrupting the PGC nucleus.

The system for producing viable offspring derived from nuclear transferred PGCs makes it possible to manipulate the germline of chickens through somatic cells, although cloned chickens cannot be produced by this system due to the recombination of chromosomes during meiosis. Further studies are necessary to develop nuclear transfer techniques in chickens.

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