

◀Research Note▶

Production of Inter-Genus Somatic Nuclear Transferred Gonadal Germ Cells (snt-GGCs) in Avian Species

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Interspecies or even inter-genus somatic nuclear transfer is considered to be an effective means for conserving a wide variety of avian genetic resources. However, somatic nuclear transfer offspring production is currently limited to mammals. Therefore, the present experiment was designed in an attempt to produce inter-genus somatic nuclear transferred gonadal germ cells (snt-GGCs) between chicken and quail. Electrofusion was carried out between embryonic blood cells (EBCs) collected from 4-day-old embryos (donor cells) and gonadal germ cells (GGCs) from 7-day-old chick embryos or 6-day-old quail embryos (recipient cells). GGCs were labeled with PKH26 fluorescent dye as a marker. Electrofusion was carried out according to previously described methods. The combinations of donor-recipient cells were designed to contain all four possible combinations: E(c)-G(c), E(q)-G(q), E(q)-G(c) and E(c)-G(q), where E, G, c and q are abbreviations for EBCs, GGCs, chick and quail, respectively. Following electrofusion, the fusion solution containing cells were stained with 1 μ g/mL Hoechst 33342. PKH26-labeled cells with two or more nuclei of different sizes were determined to be snt-GGCs. The experiment was replicated ten times and snt-GGCs were observed in five (50%), three (30%), four (40%) and five (50%) replicates. The average number of snt-GGCs produced per replicate were 0.6 (1.2%), 0.3 (0.6%), 0.5 (1.0%) and 0.5 (1.0%) for E(c)-G(c), E(q)-G(q), E(q)-G(c) and E(c)-G(q), respectively. The present results demonstrate that inter-genus snt-GGCs can be produced by electrofusion using EBCs and GGCs in avian species.

Key words: chicken, electrofusion, gonadal germ cells, quail, somatic nuclear transfer

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Introduction

The conservation of animal genetic resources is critical for the successful breeding programs that seek to increase disease resistance and productivity. The common methods by which genetic resources are conserved in many animal species are cryopreservation of spermatozoa and embryos. In avian species, however, methods to freeze spermatozoa were established by Lake and Stewart (1978), but the cryopreservation of avian embryos is considered to be difficult due to their large size as well as the presence of abundant yolk materials. Alternatively, methods using cryopreserved primordial germ cells (Naito *et al.*, 1994) as well as gonadal germ cells (Tajima *et al.*, 1998) have been developed in an attempt to conserve avian genetic re-

sources.

Recent developments in somatic nuclear transfer techniques in mammals as reported by Wilmut *et al.* (1997) provide a new option for conserving genetic resources using somatic cells. However, the somatic nuclear transfer technique developed for mammals cannot be directly applied to avian species due to the aforementioned anatomical and physiological differences of avian embryos.

The basic strategy for producing nuclear transferred avian offspring using somatic nuclear transferred primordial germ cells (snt-PGCs) was described by Tajima (2002). Under this scenario, the production of somatic nuclear offspring could occur by injecting snt-PGCs into the bloodstream of the early embryo. This method assumes that snt-PGCs injected into the bloodstream of the early embryo are capable of migrating toward the developing gonad and are able to differentiate into spermatozoa in the testes, and oocytes in the ovary. Recently, the production of snt-PGCs was reported by Minematsu *et al.* (2004) and Naito *et al.* (2005) using circulating blood cells collected from early chick embryos as the somatic nuclear donor.

To apply this technique to the conservation of avian genetic resources, the development of methods to produce

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interspecies or even inter-genus nuclear transfer techniques are necessary. The present experiment was therefore designed to produce inter-genus somatic nuclear transferred gonadal germ cells (snt-GGCs) between chicken and quail as a model for inter-genus somatic nuclear transfer in avian species.

Materials and Methods

Fertilized Chicken and Quail Eggs

Gonadal germ cell (GGC) and embryonic blood cell (EBC) samples used in the present experiment were collected from fertilized chicken and quail eggs. Chicken eggs used in this study were produced by Rhode Island Reds (*Gallus gallus domesticus*) maintained at the Agricultural and Forestry Research Center, University of Tsukuba, Japan. Japanese quail (*Coturnix japonica*) eggs were purchased from Tokai-Yuki (Toyohashi, Japan).

GGC Sample Preparation

Fertilized eggs were incubated at 37.8°C for either 7 days (chicken) or 6 days (quail). Following incubation, the left gonad was collected from five fertilized embryos under a stereomicroscope for each experiment (L2; Leica, Tokyo, Japan). The collected gonads were sliced into small pieces using the tip of a 30-G needle and dissociated in 0.05% trypsin in phosphate buffered saline [PBS (-)] at 37.0°C for 5 min. The dissociated samples were filtered through a 20- μ m nylon filter (CHN-20D Small Parts Inc., Miami Lakes, FL, USA) to remove cell clusters. The morphological criterion used to identify PGCs was adopted for the identification of GGCs, i.e., a large granulated round cell with a large nucleus. Nuclear diameter of GGCs was measured after staining cells using Hoechst 33342 fluorescent dye.

EBC Sample Preparation

Fertilized chicken and quail eggs were incubated at 37.8°C for 4 days. The embryos were placed in a glass dish and blood was collected under a stereomicroscope using a fine glass pipette, prepared by pulling a 50- μ L glass pipette (Drummond Scientific, Broomall, PA, USA) with a micropipette puller (PA-81-8811; Narishige, Tokyo, Japan). The collected blood samples were transferred into 50 μ L of a 0.25 mol/L sucrose solution and EBC concentrations were adjusted to a concentration of 2.0×10^3 cells/ μ L. Nuclear diameter of EBCs was measured after staining cells using Hoechst 33342 fluorescent dye.

Electrofusion of GGCs and EBCs

The EBC samples (10 μ L) containing roughly 20,000 EBCs were transferred into the fusion chamber (FTC-12; Shimadzu, Kyoto, Japan). Fifty GGCs labeled with PKH26 fluorescent dye (Z-PKH26-GL; Zynaxis, Malvern, PA, USA) were then placed into a droplet containing the EBCs. The droplet in the fusion chamber was covered with approximately 100 μ L of liquid paraffin oil.

Electrofusion was carried out according to the methods described by Minematsu *et al.* (2004). Briefly, pearl chain formation was induced under an alternating current (AC) field (frequency, 0.5 MHz; strength, 350 V/cm; duration,

60 s) followed by three direct current (DC) pulses for cell fusion (strength, 4 kV/cm; pulse width, 0.6 ms; pulse interval, 1 s).

The combinations of donor-recipient cells for electrofusion were designed to contain all four possible combinations of treatments: E(c)-G(c), E(q)-G(q), E(q)-G(c) and E(c)-G(q), where E, G, c and q are abbreviations for EBCs, GGCs, chick and quail, respectively. Following electrofusion, the fusion solution containing cells were stained with 1 μ g/mL Hoechst 33342. The cells were examined under an inverted fluorescence microscope (IMT-2; Olympus, Tokyo, Japan) with 334- and 546-nm excitation filters. PKH26-labeled cells that displayed morphological characteristics of GGCs, that is, two or more nuclei of different sizes, were determined to be snt-GGCs. The experiment was repeated ten times.

Results

Intact GGCs and EBCs collected from chicken and quail are shown in Fig. 1. Average nuclear diameter (means \pm se) of EBC and GGC was $5.60 \pm 0.06 \mu$ m and $9.81 \pm 0.10 \mu$ m in chicken, and $5.18 \pm 0.07 \mu$ m, and $9.56 \pm 0.10 \mu$ m in quail, respectively (n=50). A significant difference in nuclear diameter was observed among four cell types ($P < 0.01$).

After electrofusion, fused cells were observed under an inverted microscope (Fig. 2). Fused cells with typical morphological characteristics of GGCs that carry two nuclei of different sizes and also display PKH26 fluorescence signal were determined as snt-GGCs.

Out of ten replications, snt-GGCs were observed in five (50%), three (30%), four (40%) and five (50%) replicates. Furthermore, the average number of snt-GGCs produced per replicate were 0.6 (1.2%), 0.3 (0.6%), 0.5 (1.0%) and 0.5 (1.0%) for E(c)-G(c), E(q)-G(q), E(q)-

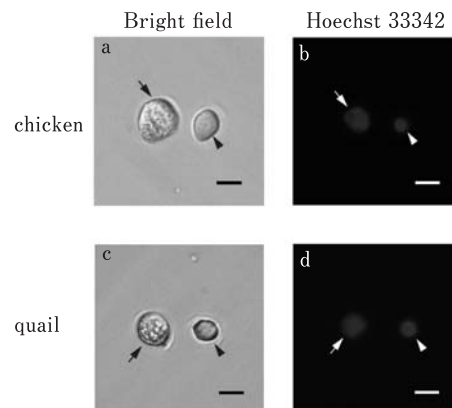


Fig. 1. Microscopic observation of EBCs and GGCs collected from chicken and quail. The arrows indicate GGC nuclei and the arrowheads denote the somatic nuclei. GGC and EBC under bright field microscopy (a: chicken, c: quail) and under fluorescent microscopy after staining with Hoechst 33342 (b: chicken, d: quail). Bar: 10 μ m.

G(c) and E(c)-G(q), respectively (Table 1).

Discussion

In the present study, electrofusion was carried out between EBCs and GGCs according to the methods described by Minematsu *et al.* (2004). These methods were originally developed for producing intra-species snt-PGCs. No significant differences were observed between fusion rates of intra-species and inter-genus combinations tested in the present study. These results indicate that the electrofusion conditions developed for intra-species nucle-

ar transfer by Minematsu *et al.* (2004) can be extrapolated for use under inter-genus somatic nuclear transfer conditions.

Fusion rates between EBCs and GGCs obtained in the present study were lower compared to previous reports by Minematsu *et al.* (2004) and Naito *et al.* (2005), who reported fusion rates between EBC and PGCs as high as 14.5% and 5.2%, respectively. A possible reason for the lower fusion rate observed in the present study could have been the use of GGCs as germ cells compared to PGCs in previous reports; the conditions for electrofusion used in the present study were originally designed for fusing circulating PGCs and EBCs in chickens as reported by Minematsu *et al.* (2004). The optimal conditions necessary for fusing EBCs and GGCs may differ from what is used for EBCs and PGCs. Fine adjustments of the fusion conditions may be necessary to improve fusion rates between EBCs and GGCs in future studies.

The primary reason for using GGCs in the present study was to collect germ cells from a limited number of eggs. The number of PGCs that can be collected from each embryo is unpredictable due to the large variation in circulating PGCs observed among embryos (Tajima *et al.*, 1999). On the other hand, significant numbers of GGCs could be collected constantly from all incubated chicken and quail embryos in the present study.

The use of 4-day-old EBCs as somatic donor cells compared to 2.5-day-old EBCs (as in previous studies) may have influenced the results as well. It is possible that structural membrane changes occurred during the course of embryo development. In future studies, the effects of the germ cell source population as well as EBC-mediated changes in fusion rates need to be examined.

Nevertheless, the results obtained in the present experiment suggest that the production of inter-genus snt-GGCs is possible in avian species. These conclusions are regarded as an important first step toward developing inter-genus somatic nuclear transfer techniques.

Inter-genus somatic nuclear transfer techniques can potentially be applied to conservation strategies of endangered avian species. In future studies, the migration ability of inter-genus snt-GGCs toward the gonadal ridge following injection into the bloodstream of 2.5-day-old embryos should be examined.

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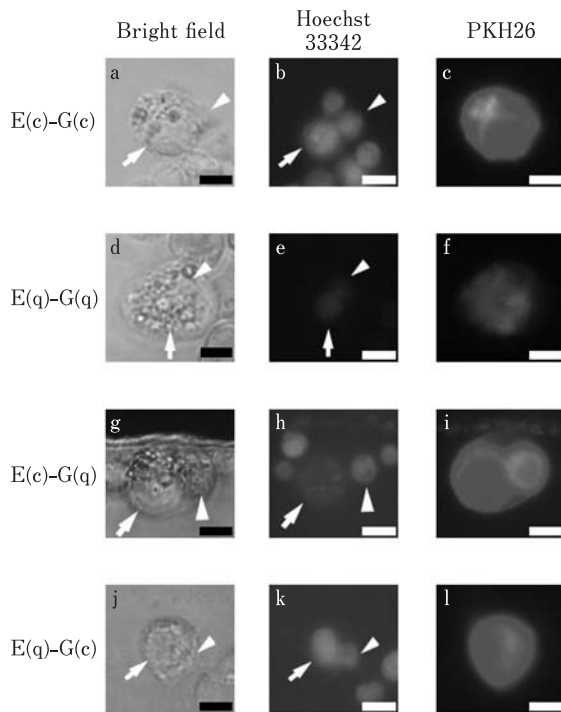


Fig. 2. Microscopic observation of intra-species or inter-genus snt-GGCs between chicken and quail. PKH26 positive cells with two nuclei of different sizes (following staining with Hoechst 33342) were identified as snt-GGCs. The white arrows indicate GGC nuclei and the white arrowheads denote the somatic nuclei. Somatic nuclear transferred GGC fused between chicken EBC and chicken GGC (a-c), quail EBC and quail GGC (d-f), chicken EBC and quail GGC (g-i), and quail EBC and chicken GGC (j-l). Bar: 10 μ m.

Table 1. Production of inter-genus somatic nuclear transferred GGCs in avian species

Donor-Recipient	Number of Replicates	Number of Replicates with snt-GGCs	Average Number of snt-GGCs/Replicate	Overall Fusion Rate
E(c)-G(c)	10	5 (50%)	0.6	1.2% (6/500)
E(q)-G(q)	10	3 (30%)	0.3	0.6% (3/500)
E(q)-G(c)	10	4 (40%)	0.5	1.0% (5/500)
E(c)-G(q)	10	5 (50%)	0.5	1.0% (5/500)

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