—Research Note—

Conditioning of Karyoplasts for Producing Somatic Nuclear Transferred Gonadal Germ Cells in Domestic Chickens

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Abstract. The objective of this study was to establish a protocol for generating karyoplasts that can be used to produce somatic nuclear transferred gonadal germ cells (snt-GGCs) in domestic chickens. Karyoplasts were produced by centrifuging cultured fibroblasts from 10-day-old chick embryos at $10,000 \times g$ in the presence of $1.0 \mu\text{g/ml}$ cytochalasin B. The number of karyoplasts was significantly ($P < 0.05$) higher and the diameters of the karyoplasts were significantly ($P < 0.05$) smaller when fibroblasts were centrifuged for 60 min than for 10 or 30 min. It was possible to generate snt-GGCs by electrofusion of GGCs with karyoplasts produced from cryopreserved or serum-starved fibroblasts. These results indicate that karyoplasts generated from 10-day-old chick embryos can be used to produce snt-GGCs even after cryopreservation and serum starvation of the fibroblasts.

Key words: Chicken, Cryopreservation, Gonadal germ cell, Karyoplast, Nuclear transfer, Serum starvation

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Since the birth of Dolly the sheep [1], the first mammal derived from the transfer of a somatic nucleus, somatic nuclear transfer technology has become key in reproductive and developmental biology. However, somatic nuclear transfer technology developed for mammals cannot be applied directly to avian species owing to major differences in the basic reproductive physiology between these two classes. An alternative strategy of producing somatic nuclear transferred offspring in avian species by using avian primordial germ cells (PGCs) or gonadal germ cells (GGCs) has been described by Tajima [2].

PGCs are undifferentiated germ cells that subsequently differentiate into spermatogonia in the testis or into oogonia in the ovary. In avian, PGCs originate from the epiblast [3] and appear in the area pellucida [4]. After development of the vascular system, PGCs circulate along with embryonic blood cells and finally migrate into the embryonic gonads [5]. After migration, PGCs are called GGCs. Avian germline chimeras have been produced by transfer of cryopreserved PGCs [6] or GGCs [7] into recipient embryos. When a somatic nucleus is introduced into an enucleated PGC or GGC, which is then transferred into the bloodstream of a recipient embryo, the recipient theoretically produces sperm or eggs originating from the somatic nucleus.

Somatic nuclear transferred PGCs (snt-PGCs) have been produced from PGCs and embryonic blood cells (EBCs) by electrofusion [8, 9] and virus-stimulated fusion [9]. Ishiguro *et al.* [10] described a method for producing inter-genus somatic nuclear transferred GGCs (snt-GGCs) from chickens and quails. In all the reports mentioned here, EBCs were used as nuclear donors, but limited information is available as to whether cells other than EBCs can be used for producing snt-PGCs or snt-GGCs.

Fibroblasts can be recovered readily from adult chickens and embryos, cultured *in vitro* and cryopreserved. These characteristics make them suitable as nuclear donors. In fact, the production of nuclear transferred offspring using fibroblasts from a female fetus as donor has been described for cattle [11]. In chickens, however, our preliminary study failed to yield snt-GGCs from intact fibroblasts cultured *in vitro* because of an inability to form a 'pearl chain' in an alternating electrical field during electrofusion (data not shown).

Karyoplasts, defined as nuclei surrounded by thin rings of cytoplasm and enclosed by a plasma membrane [12], have been considered as alternative nuclear donors. Karyoplasts can be produced by centrifuging fibroblasts on glass disks in the presence of cytochalasin B [12], but limited data are available on their production from domestic chickens. The present study was thus conducted to establish a protocol for generating karyoplasts from embryonic fibroblasts to be used in generating snt-GGCs in domestic chickens.

Materials and Methods

Animals

Fertilized eggs were obtained from White Leghorn chickens maintained at the Agricultural and Forestry Research Center (University of Tsukuba, Japan). The animals were treated in accordance with institutional standards for the humane treatment of animals.

Experiment 1. Effect of duration of centrifugation on diameter and recovery of karyoplasts

Karyoplast production: Skin samples were collected from the lower abdominal region of 10-day-old chick embryos. Each sample was minced and dispersed in 2 ml of minimum essential

medium (MEM; M0769; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; 26140, Gibco); cells were cultured in 35-mm plastic dishes (627161, Greiner, Graz, Austria) coated with type I collagen (Cell Matrix Type I-P; Nitta Gelatin, Tokyo, Japan) at 37.8 C in an atmosphere of 5% CO₂. After two or three passages, fibroblasts were cultured on 22-mm-diameter glass disks (22GDs; Komatsuya, Saitama, Japan) coated with type I collagen. When fibroblasts on the 22GDs reached subconfluency, the disks were immersed in 10 ml MEM-10% FBS supplemented with 1 µg/ml cytochalasin B (C6762; Sigma-Aldrich Chemical, St. Louis, MO, USA). Karyoplasts were produced by centrifuging the glass disks at 10,000 × g for 10, 30 or 60 min at 37.8 C.

Evaluation of karyoplasts: Karyoplasts were double-stained with two fluorescent dyes according to the manufacturer's instructions: 100 µg/ml of PKH26 (Z-PKH26-GL; Zynaxis, Malvern, PA, USA), which was incorporated into the plasma membrane, and 20 µg/ml of Hoechst 33342 (B-2261, Sigma), a nuclear stain. Karyoplasts and fibroblasts were counted and assessed under an inverted fluorescence microscope (IMT-2-RFC; Olympus, Tokyo, Japan) equipped with 334- and 546-nm excitation filters. The diameters of karyoplasts were measured with an attached micrometer, and the viability of cells was evaluated by trypan blue staining.

Experiment 2. Production of snt-GGCs

Preparation of fibroblasts: Fibroblasts were cultured in 35-mm plastic dishes to subconfluency as described for Experiment 1 and then underwent one of the following four treatments.

1) Control: Fibroblasts were passaged on 22GDs.

2) Cryopreservation: Cultured fibroblasts were treated with trypsin and centrifuged at 200 × g for 5 min. The supernatant was removed, and 100 µl of MEM-10% FBS containing 10% dimethyl sulfoxide was added. The fibroblast suspension was transferred into a Cryoware (5000-0012, Nalgene, New York, NY, USA), and the tube was placed in a freezing vessel (Bicell; Nihon Freezer, Tokyo, Japan). The cell sample was frozen by placing the Bicell into a deep freezer maintained at -80 C for at least 2 h, followed by immersion in liquid nitrogen (-196 C). The cryopreserved sample was stored for more than a week before being used. After thawing at 37 C, fibroblasts were cultured in MEM-10% FBS until the cells became subconfluent, and then they were passaged on 22GDs. The survival rate of the cryopreserved fibroblasts was evaluated by trypan blue staining.

3) Serum starvation: Fibroblasts were cultured on collagen-coated 22GDs to subconfluency. The culture medium was then replaced with medium containing 0.5% fetal bovine serum (MEM-0.5% FBS), and the cells were cultured for another 3 to 4 days.

4) Cryopreservation and serum starvation: Frozen-thawed fibroblasts were passaged on 22GDs as described above until the cells reached subconfluency. The cells were then cultured in MEM-0.5% FBS for an additional 3 to 4 days before karyoplast production.

Karyoplasts were produced from fibroblasts as in Experiment 1. Karyoplasts were stained with PKH26 fluorescent dye and then centrifuged at 500 × g for 5 min. The supernatant was replaced with 50 µl of a 0.25 M sucrose solution containing 10% fetal

bovine serum (fusion fluid). The cell density was adjusted to 2,000 karyoplasts/µl, and a 10 µl sample was placed in a fusion chamber (FTC-12; Shimadzu, Kyoto, Japan) with an inter-electrode gap of 1 mm.

Preparation of GGCs: The left gonads were collected from five 7-day-old White Leghorn embryos, minced in phosphate-buffered saline that did not contain Mg²⁺ and Ca²⁺ [PBS(-)] and placed into 300 µl of PBS(-) in a 1.5-ml microcentrifuge tube. After incubation at 37.8 C for 30 min, tissue fragments were removed from the cell suspension by filtration through nylon mesh with 20 µm pores (Small Parts, Miami Lakes, FL, USA). The cell suspension was centrifuged (200 × g, 5 min), and the precipitate was dispersed in 100 µl of fusion fluid. Morphological criteria were adopted to identify GGCs (i.e., a large, round, granulated cell with a large nucleus) for identification of chicken GGCs [13]. Using fine glass needles, we collected 500 GGCs into a minimal volume of fusion fluid and dispersed them into a fusion chamber containing 20,000 karyoplasts in fusion fluid. The sample in the chamber was covered with 100 µl of liquid paraffin oil, and the fusion chamber was incubated at 37.8 C for 10 min.

Cell fusion of GGCs and karyoplasts: Electrofusion was performed, as described previously [8], using a DC and AC power supply (LF101; BEX, Tokyo, Japan). Pearl chain formation was induced by exposing cells to 350 V/cm in a 0.5 MHz AC field. Pearl-chained cells were electrofused by three DC pulses adjusted to 4 kV/cm. The pulse width and interval were fixed at 60 µs and 1 sec, respectively. After electrofusion, the cells were exposed to an AC field for 60 sec to promote cell fusion, and then the nuclei were stained with Hoechst 33342 dye and incubated at 37.8 C for 30 min.

Evaluation of snt-GGCs: Fused cells (potential snt-GGCs) were stained with PKH26 and Hoechst 33342 and evaluated under an inverted fluorescence microscope (IMT-2-RFC) with 334- and 546-nm excitation.

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance followed by Duncan's multiple range test.

Results and Discussion

In the present study, karyoplasts were used as nuclear donors to produce snt-GGCs. Karyoplasts can contain ribosomes, occasional mitochondria and a few fragments of the endoplasmic reticulum [14], but lack centrioles, microtubules and Golgi apparatuses [15]. These characteristics need to be monitored carefully when karyoplasts are generated for the production of nuclear transferred offspring.

As shown in Table 1, the diameter of the cells after centrifugation was significantly smaller than that of the control fibroblasts ($P < 0.05$). Furthermore, staining with Hoechst 33342 and PKH26 identified nuclei within these small cells and membranes around them, respectively (Fig. 1). On the basis of these observations, we considered that the small cells generated after centrifugation were karyoplasts.

The diameter and recovery rate of karyoplasts are determined

Table 1. Effect of the duration of centrifugation on the diameter and recovery of chicken karyoplasts (Experiment 1)

Duration of centrifugation (min)	Diameter of karyoplasts after centrifugation (μm)	Diameter ratio [#]	No. of karyoplasts recovered after centrifugation ($\times 10^4$)	Survival rate after centrifugation (%)
0	13.20 ± 0.06^a	—	26.90 ± 1.19^a	96.8 ± 0.49
10	8.15 ± 0.21^{bc}	0.617	2.61 ± 0.47^c	97.2 ± 1.50
30	8.46 ± 0.19^b	0.641	6.15 ± 0.52^c	98.4 ± 0.40
60	7.62 ± 0.23^c	0.577	12.81 ± 2.04^b	96.0 ± 1.67

Data are shown as the mean $\pm$ standard error with 5 replicates. [#] Diameter ratio=(diameter of cell after centrifugation) / (diameter of fibroblast without centrifugation). ^{a, b, c} Values with different letters in the same column are significantly different ($P < 0.05$).

Fig. 1. Identification of karyoplasts produced by centrifugation of cultured chicken fibroblasts. A: bright-field microscopy. B: karyoplast stained with PKH26 (a fluorescent dye that stains the plasma membrane). C: karyoplast stained with Hoechst 33342 (a fluorescent dye that stains the nucleus). Scale bar=10 μm . **Fig. 2.** Identification of chicken somatic nuclear transferred gonadal germ cells (snt-GGCs) produced by electrofusion of GGC and karyoplasts. A: bright-field microscopy. B: PKH26-positive snt-GGC. C: nuclei in snt-GGC stained with Hoechst 33342. Scale bar=10 μm .**Table 2.** Number of chicken gonadal germ cells (GGCs) and karyoplasts fused with karyoplast by electrofusion (Experiment 2)

Treatment	No. of GGCs fused with karyoplast	No. of GGCs fused with GGC
Control	3.4 ± 0.5	2.4 ± 0.4
Cryopreservation	4.4 ± 1.1	2.8 ± 1.4
Serum starvation	3.0 ± 0.9	2.8 ± 0.6
Cryopreservation and serum starvation	2.8 ± 0.7	2.0 ± 1.0

Data are shown as the mean $\pm$ standard error of 5 replicates. No significant differences among treatments ($P > 0.05$).

primarily by 2 factors: centrifugal force and duration of centrifugation. The force and duration required to produce karyoplasts varies among reports: 15 min at $17,369 \times g$ [16], 20 to 40 min at $12,000$ to $24,000 \times g$ [17] and 60 min at $10,000 \times g$ [18]. The differences in centrifugation parameters for producing karyoplasts can be attributed to differences in experimental conditions, materials used and the objectives of the studies. In the present study, significantly smaller karyoplasts were produced in significantly higher numbers when the duration of centrifugation was 60 min instead of 10 or 30 min ($P < 0.05$). In future studies, we will further assess the effect of the karyoplast preparation method on the efficiency of producing snt-GGCs from domestic chickens.

In experiment 2, snt-GGCs were produced by fusing GGCs with karyoplasts, which were created from cultured fibroblasts after cryopreservation, serum starvation and cryopreservation and serum

starvation (Table 2). It was considered that PKH26-labeled cells that had two different-sized nuclei after staining with Hoechst 33342 were GGCs fused with karyoplasts (Fig. 2). The rate of fusion between GGCs and karyoplasts did not differ among the fibroblast treatments. These results indicate that neither cryopreservation nor serum starvation significantly interfered with production of snt-GGCs from chickens.

Somatic nuclear transferred embryos have been reconstituted using serum-starved cumulus cells in bovine [19]. In the present experiment, chicken fibroblasts were cultured under serum starvation before karyoplast production. Serum starvation suppresses cell proliferation and synchronizes cells in the quiescent (G_0) phase of the cell cycle [1]. For the purposes of mammalian nuclear transfer, cells in G_0 or G_1/S are reprogrammed more readily than are those in the M phase [20, 21].

In the present study, the nuclei of GGCs were neither eliminated nor inactivated prior to electrofusion with karyoplasts. It is considered that physical removal of the nuclei of GGCs would be difficult owing to their cellular characteristics (i.e., large nuclei and scant cytoplasm). Alternatively, Minematsu *et al.* [22] reported that the migratory ability of PGCs was maintained after nuclear inactivation by UV exposure at 254 nm and $0.9 \pm 0.1 \mu\text{W}/\text{cm}^2$ for 60 sec. Naito *et al.* [23] inactivated the nuclei of PGCs using soft X-ray irradiation. Future studies should be conducted using UV- or soft X-ray-irradiated GGCs as somatic nuclear recipients. Furthermore, the migratory ability of sntGGCs should be evaluated.

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