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Development of a Novel Method for Isolating Gonadal Germ Cells from Early Chick Embryos

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We conducted the present study to develop a simplified method of isolating gonadal germ cells (GGCs) from embryonic gonads to facilitate the production of germline chimeras in avians. Developing gonads recovered from 7-day-old chick embryos were incubated for 30 minutes to 24 hours at 37.8℃ in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS[−]). GGCs began to be released from gonads after 30 minutes of incubation; the number of GGCs release increased until 12 hours of incubation. The purity of GGCs (the number of GGCs released divided by the total number of cells released) was approximately 50% for the initial 1.5 hours of incubation and decreased thereafter. We stained 50 GGCs in PBS[−] with PHK26 fluorescent dye and injected them into the bloodstream of a 2-day-old chick embryo. GGCs exhibiting fluorescence were detected in the gonads of recipient embryos at 5 days after transfer. These results indicate that high-purity, highly viable GGCs can be isolated from chick embryos simply by incubating the developing gonads in PBS[−].

Key words: 7-day-old chick embryos, gonadal germ cells, isolation, PBS[−]

Introduction

Many animal species are currently under the threat of extinction. For example, 1,240 of 10,064 known avian species are listed as endangered (IUCN, 2012). Therefore, the development of a method to conserve genetic resources is crucial for the maintenance of avian genetic diversity.

Mammalian genetic resources can be conserved by cryopreservation and transfer of both spermatozoa and ova. However, the method of conserving the genetic resources for mammals cannot be applied to avian species primarily because of the technical difficulties surrounding the cryopreservation of avian eggs. To overcome this problem, attempts have been made to conserve avian genetic resources by collecting and transferring primordial germ cells or gonadal germ cells (GGCs) collected from developing embryos. It has been reported that circulating primordial germ cells and GGCs can be recovered from the blood of embryos at developmental stage 13−15 (Hamburger and Hamilton, 1951) or from embryonic gonads (Tajima *et al*., 1998), and germline chimeras can be produced in domestic chickens by transferring primordial germ cells or GGCs into the bloodstream of 2-day-old recipient embryos (Tajima *et al*., 1993; Tajima *et al*., 1998; Naito *et al*., 1999; Tajima *et al*., 2004). Germ cells can be recovered from embryonic gonads by means of long-term culture in medium (Pain *et al*., 1996), using a proteinase such as trypsin (Tajima *et al*., 1998), filtration (Tajima *et al*., 2004), and fluorescence-activated cell sorting (Mozdziak *et al*., 2005). However, it has been difficult to isolate high-purity GGCs from among the overwhelming number of somatic cells in the source tissues. We therefore performed the present study to develop a simple method of isolating high-purity GGCs without using proteinase or expensive equipment.

Materials and Methods

Isolation of gonadal germ cells

Fertilized White Leghorn eggs were incubated for 7

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days in a forced-air incubator maintained at 37.8℃. After incubation, both gonads were isolated from each embryo. Isolated left and right gonad were placed in a 1.5-mL centrifuge tube containing 500 μL phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS[−]) and returned to a 37.8℃ incubator for 0.5 to 24 hours. The number, survival rate, and purity (i.e., number of GGCs/total number of cells released) of recovered GGCs were recorded. The survival rate of isolated GGCs was determined by using Trypan blue staining. For a control, these same parameters were measured after embryonic gonads were treated with 0.1% trypsin for 20 minutes in an incubator maintained at 37.8℃. GGCs were stained with Periodic acid–Schiff (PAS) stain and anti-chicken *vasa* homologue (CVH) antibody according to the methods described by Tajima *et al*. (1999) and Tsunekawa *et al*. (2000), respectively. Each experiment was repeated more than five times.

Migratory ability of GGCs

Developing left and right gonad from 7-day-old chick embryo were incubated at 37.8℃ in 100 μL PBS [−] for about 1 hour. We then labeled 50 of the GGCs released with the fluorescent dye PKH26 and injected them into the dorsal aorta of Rhode Island Red embryos at stage 13−16 of development by using a fine glass pipette under a dissection microscope. After culturing embryos at 37.8℃ for five days, both right and left gonads were collected and incubated in PBS [−] containing 0.05% trypsin at 37.8℃ for 20 minutes. After incubation, fluorescence-labeled cells were counted under a fluorescence microscope by using a 546-nm excitation filter. This experiment was repeated 38 times.

Results and Discussion

GGCs were released from embryonic chick gonads after their incubation in PBS[−] (Fig. 1). GGCs were first observed in the PBS[−] at 30 minutes after the introduction of embryonic gonads into this medium and continued to be released for 12 hours. The gonads of 7-day-old chick embryos appeared to contain few (if any) somatic cells and sparse to nonexistent connective tissue between GGCs. The purity of the GGCs recovered remained at approximately 50% during the first 1.5 hours of incubation in PBS[−] and decreased thereafter ($P<0.05$). The survival rate of released GGCs remained at 98% until 5 hours after incubation and decreased thereafter ($P<0.05$). These results show that GGCs can be isolated by incubating embryonic chick gonads in PBS[−] at 37.8℃ for about 1 hour.

Fluorescence-labeled GGCs transferred into recipient embryos migrated into the embryonic gonads (Fig. 2). The numbers of fluorescence-labeled GGCs that recovered from the left and right gonads of recipient embryos were 45.18±5.26 and 33.55±7.28, respectively.

Germline chimeras can be produced by transferring GGCs collected from 7-day-old chick embryonic gonads incubated in PBS[−] (Nakajima *et al*., 2012).

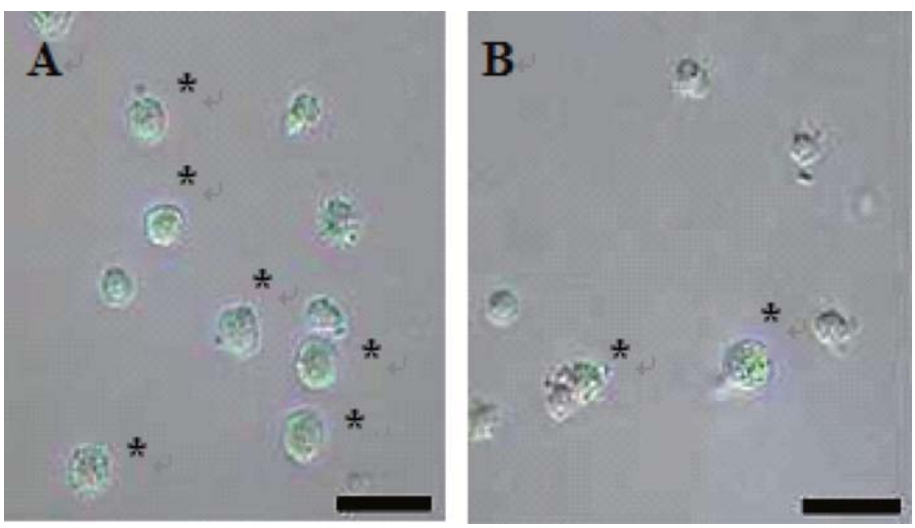

Fig. 1. Gonadal germ cells (*) from embryonic chick gonads after (A) incubating gonad in PBS[−] for 1 hour or (B) using trypsin treatment.

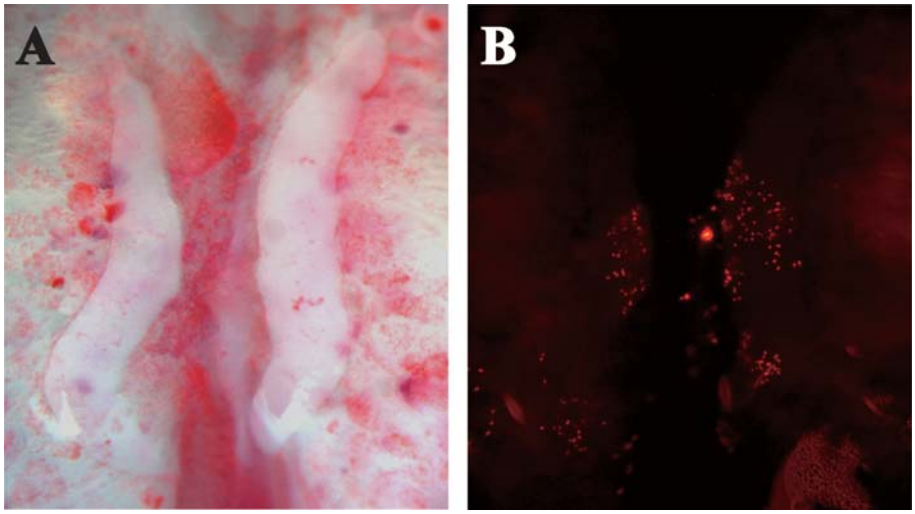

Fig. 2. Migration of harvested gonadal germ cells after their injection into recipient embryos. (A) Bright field microscopy. (B) Fluorescence microscopy of PKH26-stained gonadal germ cells in gonad of recipient embryo.

This result shows that GGCs collected from chick embryonic gonads by using PBS[−] can differentiate into normal gametes in the gonads of recipient chickens. It has been reported that GGCs are used to generate interspecies germline chimeras yielding chicken-pheasant (Kang *et al*., 2008) and chicken-duck (Liu *et al*., 2012). The GGCs harvesting methods we describe here might be used in the establishment of species-specific reproductive management systems for the successful conservation of avian genetic resources.

These new technologies can help reduce the cost of maintaining foundation stocks in poultry and secure wild birds and poultry from unexpected events, such as a highly pathogenic avian influenza outbreak or even a tsunami. This benefit is particularly realized when germline chimera technology is combined with the cryopreservation of germ cells, thus enabling long-term banking of avian genetic resources. Furthermore, germline chimera technology likely reduces the potentially negative environmental effects of conventional *ex situ* strategies for conserving avian genetic resources.

Conclusion

Viable GGCs can recovered efficiently and simply by incubating embryonic chick gonads in PBS[−] at 37.8℃ for about 1 hour. Even though the mechanism underlying the release of GGCs from developing gonads and into PBS[−] is not understood. The rapid and simple method that we describe here will greatly facilitate the production of germline chimeras. Future studies likely will focus on extending the present method to the entire period of embryonic development.

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