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Production of Interspecific Germline Chimeras via Embryo Replacement[1]

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ABSTRACT

In avian species, primordial germ cells (PGCs) use the vascular system to reach their destination, the genital ridge. Because of this unique migratory route of avian germ cells, germline chimera production can be achieved via germ cell transfer into a blood vessel. This study was performed to establish an alternative germ cell-transfer system for producing germline chimeras by replacing an original host embryo with a donor embryo, while retaining the host extraembryonic tissue and yolk, before circulation. First, to test the migratory capacity of PGCs after embryo replacement, Korean Oge (KO) chick embryos were used to replace GFP transgenic chick embryos. Four days after replacement, GFP-positive cells were detected in the replaced KO embryonic gonads, and genomic DNA PCR analysis with the embryonic gonads demonstrated the presence of the GFP transgene. To produce an interspecific germline chimera, the original chick embryo proper was replaced with a quail embryo onto the chick yolk. To detect the gonadal PGCs in the 5.5-day-old embryonic gonads, immunohistochemistry was performed with monoclonal antibodies specific to either quail or chick PGCs, i.e., QCR1 and anti-stage-specific embryonic antigen-1 (SSEA-1), respectively. Both the QCR1-positive and SSEA-1-positive cells were detected in the gonads of replaced quail embryos. Forty percent of the PGC population in the quail embryos was occupied by chick extraembryonically derived PGCs. In conclusion, replacement of an embryo onto the host yolk before circulation can be applied to produce interspecies germline chimeras, and this germ cell-transfer technology is potentially applicable for reproduction of wild or endangered bird species.

chicken, embryo replacement, interspecies germline chimera, primordial germ cell, quail

INTRODUCTION

The germline chimera production technique has been a major tool for basic and applied research in birds, such as developmental biology, animal transgenesis, and endangered species conservation. Avian germline chimeras can be produced by transplanting blastodermal cells, primordial germ cells (PGCs), or testicular cells [1–3]. Among these, PGCs have been used as a major source for germline chimera production because of their high efficiency of germline transmission [4]. PGCs, which are found in the central region of the area pellucida [5, 6] at Eyal-Giladi and Kochav stage X [7], gradually translocate anteriorly to an extraembryonic region called the germinal crescent at Hamburger and Hamilton (HH) stage 4 [8]. The PGCs remain in the extraembryonic region until around HH stage 10, when the blood vascular system starts to form; soon afterward, the PGCs move to the future gonad region through blood vessels [6, 9, 10]. This unique migratory route through the bloodstream has been widely applied as a powerful tool for germ cell-transfer systems as well as production of transgenic (TG) birds [4, 11–14]. Germline chimeras have been successfully produced by transfer of PGCs, which are collected from the donor embryonic blood vessels during HH stages 13 to 17 [15, 16] or donor embryonic gonads [2, 17] into the blood vessels of recipient embryos. In addition, with germ cell-mediated techniques, interspecies germline chimeras can be used for conservation of endangered birds [14, 18]. Endangered birds are greatly restricted in their populations because of their limited capacity for egg production and seasonal breeding. On the other hand, in the case of the chicken, hens can produce at least 330 eggs nonseasonally throughout the year. Therefore, production of interspecies germline chimeras with the fecundity of chickens can be helpful for conservation of endangered birds. However, the efficiency of interspecies germline chimera production is still low.

A highly efficient germline-chimera production system was developed via the establishment of PGCs in an in vitro culture system [19–22], and targeted gene knockout chickens were produced [23, 24]. However, only chicken PGCs have been cultured successfully in vitro without loss of germ cell properties [21, 22]. Quail PGCs have also been cultured to produce germline chimeras, but these cells could not be cultured for longer than 20 days [11, 12]. Alternative methods to produce germline chimeras that are applicable to various avian species have yet to be developed.

The embryo transplantation technique in which a quail whole embryo is grafted onto the host chick embryonic yolk was originally devised by Martin [25]. Quail-chick chimeras could then be established by grafting a quail embryo body onto the chick extraembryonic area [26]. These techniques have

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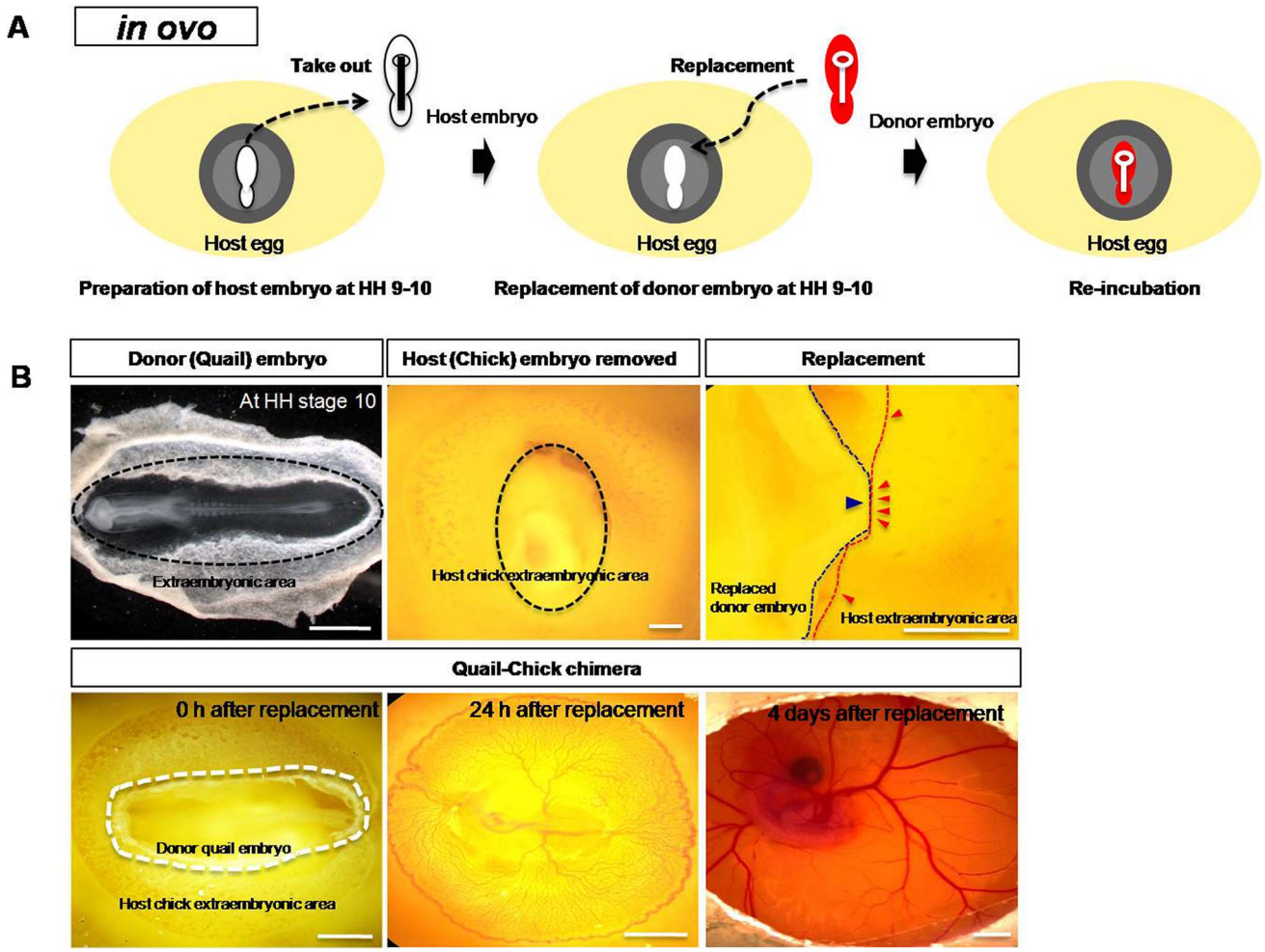

FIG. 1. Experimental strategy for chimera generation. **A**) Schematic diagram of embryo replacement. **B**) The operation was performed with HH stage 9–10 embryos. The donor embryo was cut along the black dotted lines and removed. The host embryo was also cut in an identical manner along the black dotted lines and removed. Both the host and donor embryo membranes were pinched with forceps to attach. After embryo replacement, the embryo was incubated for 24 h or 4 days. Blue arrowhead and blue dotted line, donor membrane boundary. Red arrowheads and red dotted line, host yolk sac membrane boundary. White dotted line, suture points. Bar = 1 mm.

been used in studies to investigate the development of the embryonic vascular system and to trace the cell origin of the hematopoietic system, which initiated an important paradigm shift in research fields pertaining to the vascular system [27–29]. Recent studies have indicated an increased survival rate after embryo replacement with manipulations such as electroporation and ex ovo culture, which enables the further application of embryo replacement [30, 31].

We postulated that the combination of the unique migratory route of avian germ cells with an embryo replacement method could be applied to produce interspecies germline chimeras. We detected chick PGCs in 5.5-day-old embryonic gonads of quail-chick chimeras. We confirmed the potential application of this novel germ cell-transfer system to produce germline chimeras without germ cell isolation, such as PGC culture or separation using magnetic activated cell sorting with specific antibodies. Thus, this germ cell transfer technique may be applicable to conservation of wild or endangered avian species.

MATERIALS AND METHODS

Experimental Animals and Animal Care

The care and experimental use of animals were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). A broiler-type quail strain, ''Jumbo'' (*Coturnix japonica*), and Korean Oge chicken (KO; *Gallus gallus*) were used as donor and host embryos, respectively. The experimental animals were maintained according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Embryo Replacement

Fertilized host and donor eggs were incubated horizontally for 33 h to reach HH stage 9–10. The flatness of the embryo is important to prevent leaking of the host yolk during embryo replacement; it also facilitates dissection, allowing replacement with the donor embryos in ovo. The host and donor size must be matched to prevent technical difficulties during the replacement steps. Next, 5 ml of albumen was removed from the host egg with a 21-gauge syringe by making a tiny hole on the narrow end of the egg. After cutting and removing of the host and donor embryos with a pair of microscissors, resulting in smooth cut edges, the donor embryo was replaced in the original position of the host embryo. Both the donor embryo and host membranes were gently pinched together with a pair of fine forceps to attach the donor embryo to the host membrane. During suturing, the host yolk sac margin was carefully pulled out from underneath the vitelline membrane, and the margins of the donor and host were gently held and pressed together for a few seconds and then released. After suturing was complete, the extra tissue along the suture line was trimmed to expand the donor embryo over the suture boundary. The operation window was sealed with clear tape and the eggs were further incubated at 38.5°C in an incubator until Embryonic Day (E) 5.5 (Fig. 1).

TABLE 1. Information of the primer sets used for genomic DNA PCR analysis.

Primer ID	Sequence	Size (bp)
CSP#1[a]	GAGTGTAGACAGTAGTGTATC CTCAGGGCACCATTTTCACTG	363
QSP#7[b]	AATTGGTGACGGGTACACAGATTC AGGATCACGATATTGAACCATCAC	667
GFP[c]	CAAGGACGACGGCAACTACA CGGTCACGAACTCCAGCAG	377

[a] Chicken-specific marker.
[b] Quail-specific marker.
[c] GFP primers.

Immunohistochemistry

For identification of gonadal PGCs (gPGCs) in quail-chick chimeras, monoclonal antibody specific to quail PGCs (QCR1) [32–35] and stage-specific embryonic antigen-1 (SSEA-1; Santa Cruz Biotechnology, Santa Cruz, CA) specific to chicken PGCs were used. Whole gonads were retrieved from 5.5-day-old embryos and fixed with 4% paraformaldehyde for 1 day at room temperature, then rinsed three times with 1× PBS. The tissue was then permeabilized with 1% Tween-20 and rinsed with 1× PBS. The gonads were incubated with blocking solution (10% BSA and 5% goat serum in 1× PBS) for 3 h, and then incubated with 1:200 diluted QCR1 (mouse immunoglobulin [Ig] G) or anti-SSEA-1 (mouse IgM) antibody at 4°C overnight. After washing with 1× PBS, gonads were incubated with fluorescein isothiocyanate (Santa Cruz Biotechnology)-conjugated goat anti-mouse IgG (diluted 1:200) for QCR1 or phycoerythrin (Santa Cruz Biotechnology)-conjugated goat anti-mouse IgM (1:200) for anti-SSEA-1 for 1 h at room temperature. Immunostained gonads were examined under a fluorescence microscope (Eclipse Ti; Nikon, Tokyo, Japan), and the SSEA-1 or QCR1-positive cells were counted.

Analysis of Chimeric Status after Replacement

To evaluate the chimeric status and monitor the migration of PGCs from the host yolk sac to the donor embryo, embryonic gonads were collected from 5.5-day-old chimeric embryos. Other parts of the collected chimeric embryos were used for genomic DNA PCR with GFP-specific, chicken-specific (*CSP#1*), and quail-specific (*QSP#7*) primers [14, 36]. The sequences and product sizes of these primers are shown in Table 1. Genomic DNA PCR was performed in a total volume of 20 µl containing 200 ng of collected tissue genomic DNA, 1.6 µl of 2.5 mM dNTP mixture (Core BioSystems, Seoul, Korea), 2 µl of 10× reaction buffer (Core BioSystems), 2 pmol of each primer, and 2.5 units of Taq polymerase (Core BioSystems) under the following conditions: 10 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 63°C, and 30 sec at 72°C, with a final extension for 5 min at 72°C.

Statistical Analysis

Data obtained from at least three replicates were subjected to statistical analysis by a generalized linear model (PROC-GLM) using Statistical Analysis System software (SAS Institute, Cary, NC). When a significant effect was detected, the treatment effect was compared using the least-squares method. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Establishment of Embryo Replacement for Production of Germline Chimeras

The germ cell-transfer system was attempted to establish whether the PGCs derived from an extraembryonic region (i.e., the area opaca in the host yolk) can be incorporated into replaced donor embryos. First, we performed replacement of GFP-TG chick embryos [19] with KO chick embryos (Fig. 2, A

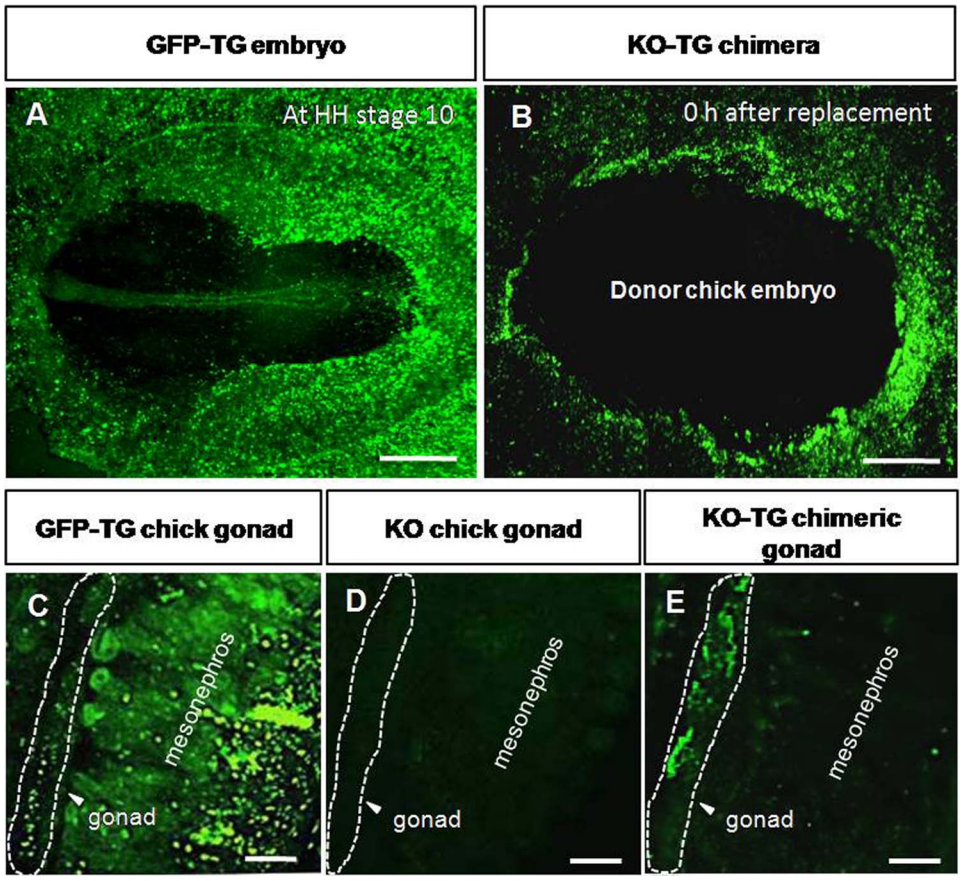

FIG. 2. Detection of the GFP transgene in embryonic gonads of a chimera. **A**) GFP-TG chick embryo showed GFP expression at stage 9–10. **B**) KO embryo was replaced onto the yolk of the recipient. GFP expression was investigated in the 5.5-day-old embryonic gonads of GFP-TG (**C**) and KO chicks (**D**) and KO-TG chimeras (**E**) using a confocal laser scanning microscope. Bars = 1 mm (**A**, **B**) and 500 µm (**C**–**E**).

TABLE 2. Viability of replaced donor embryos onto the host yolk until 5.5 days.

Parameter	Days after embryo replacement			
	1	2	3	4
Quail-chick chimera				
No. of embryos/total incubated eggs (%)[a]	42/55 (76.4)[c]	37/55 (67.4)[cd]	29/55 (52.7)[de]	25/55 (45.5)[e]
KO-TG chimera				
No. of embryos/total incubated eggs (%)[b]	25/37 (67.6)[f]	23/37 (62.2)[fg]	18/37 (48.6)[fg]	17/37 (45.9)[g]

[a] Survival rate of replaced quail embryos on a chick.
[b] Survival rate of replaced chick embryos on a GFP-TG chick.
[c–e] Different superscripts in the number of produced quail-chick chimeras were significantly different ($P < 0.05$).
[f,g] Model effect for the viability of KO-TG chimera was $P = 0.1811$.

and B). As shown in Table 2, among the 37 KO-TG chimeric embryos, 25 embryos survived until 24 h after the operation. Of these 25 embryos, 17 survived until HH stage 28 (4 days after replacement). GFP-expressing cells, which were observed in GFP-TG chick embryonic gonads, were also detected in the embryonic gonads of KO-TG chimeras (Fig. 2, C and E), whereas they were not detected in KO chick embryonic gonads (Fig. 2D). Genomic DNA PCR analysis of 5.5-day-old embryonic gonad confirmed the presence of GFP-positive cells in KO-TG chimeras (Fig. 3A).

Production of Quail-Chick Germline Chimeras by Embryo Replacement

To produce quail-chick germline chimeras, the quail embryo proper was used to replace the host embryo on its yolk (Fig. 1B). Of 55 quail-chick yolk sac chimeric embryos, 42 embryos survived until 24 h after replacement. Of these 42 embryos, 25 survived until HH stage 28 (Table 2). We also confirmed the viability of the transplanted embryos (n = 30). The transplanted embryos survived until a maximum of E16.5, and quail-chick chimeras showed normal development while they were alive (Fig. 4). Of 30 quail-chick yolk sac chimeric embryos, 12 embryos (40%) survived until E5.5 after embryo replacement. Of these 12 embryos, 2 (6.7%) survived until E16.5.

Migration of Chick PGCs Toward the Embryonic Gonads of Replaced Quail Embryos

To monitor the migration of PGCs from the host extraembryonic region of the yolk to the embryonic gonads after replacement, the 5.5-day-old embryonic gonads of quail-chick chimeras were immunostained with QCR1 and anti-SSEA-1 antibodies (Fig. 5). QCR1 was specific to PGCs in quail gonads (Fig. 5, A and B), and SSEA-1 was specific to PGCs in chick gonads (Fig. 5, C and D). When the gonads of the quail-chick chimeras were immunostained with QCR1 and SSEA-1, the PGCs positive for each of the antibodies were detected, indicating that the chick PGCs were successfully transferred into the quail gonads (Fig. 5, E–G).

To confirm the genotype of the gonads of quail-chick chimeras, genomic DNA was extracted from the 5.5-day-old embryonic gonads of intact quail embryos, KO chick embryos, and quail-chick chimeras. PCR was then performed with these genomic DNAs using species-specific markers. The genomic DNA from the gonads of quail-chick chimeras was positive for both the chicken-specific and quail-specific markers (Fig. 3B).

Contribution of Chick PGCs in the Gonads of Chimeras

To investigate the contribution of chick PGCs in the gonads of the quail-chick chimeras, QCR1-positive and SSEA-1-positive PGCs were counted. As shown in Table 3, the average numbers of QCR1-positive and SSEA-1-positive PGCs were 242.3 ± 66.9 and 200.5 ± 43.9, respectively ($n = 8$).

DISCUSSION

In birds, unlike mammals, PGC-mediated system can be used to produce germline chimeras or TG birds. In mammals, germline chimeras have been produced using germline-competent stem cells [37, 38]. On the other hand, chicken embryonic stem cells (ESCs) derived from pre-streak-stage embryos are not suitable for producing germline chimeras because this approach exhibits significantly lower germline

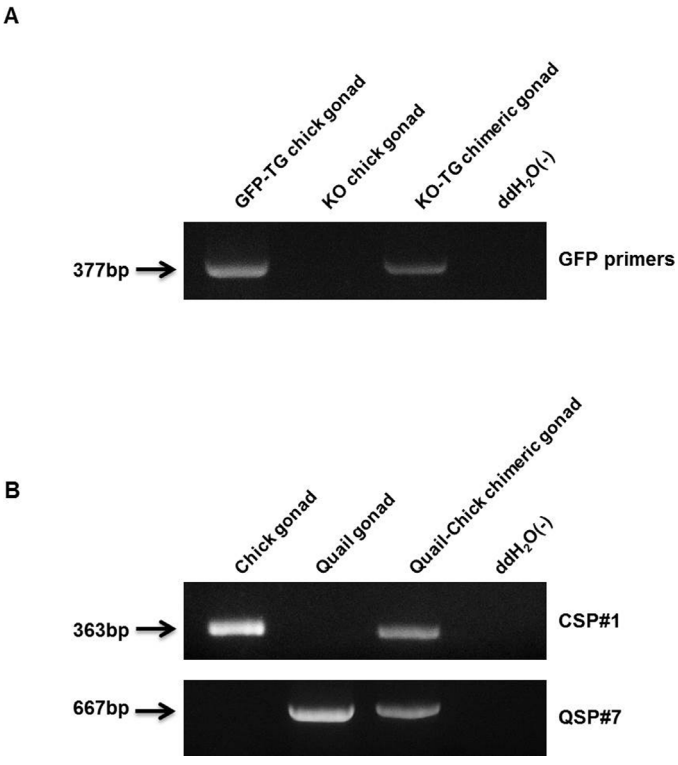

FIG. 3. Detection of host-derived PGCs in donor embryonic gonad by PCR analysis. **A**) Genomic DNA PCR analysis of 5.5-day-old embryonic gonads with GFP-specific primers showing GFP transgene detection in the GFP-TG chick and chimera. **B**) The embryonic gonads of quail-chick chimeras were positive for both the chicken-specific (*CSP#1*) and quail-specific (*QSP#7*) markers.

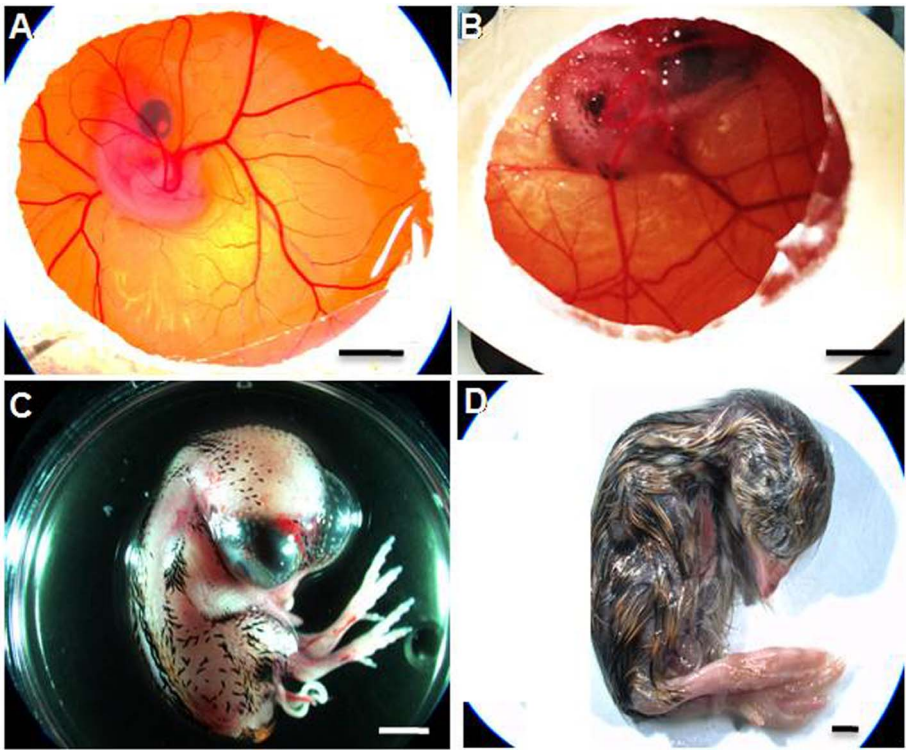

FIG. 4. Quail-chick chimera at various stages of development. **A**) Five-day-old quail embryo developed in connection with the chick yolk. An E10.5 quail-chick chimera (**B**, **C**). **D**) An E16.5 quail-chick chimera was isolated from the chicken egg. Bars = 1 mm (**A**, **B**) and 2 mm (**C**, **D**).

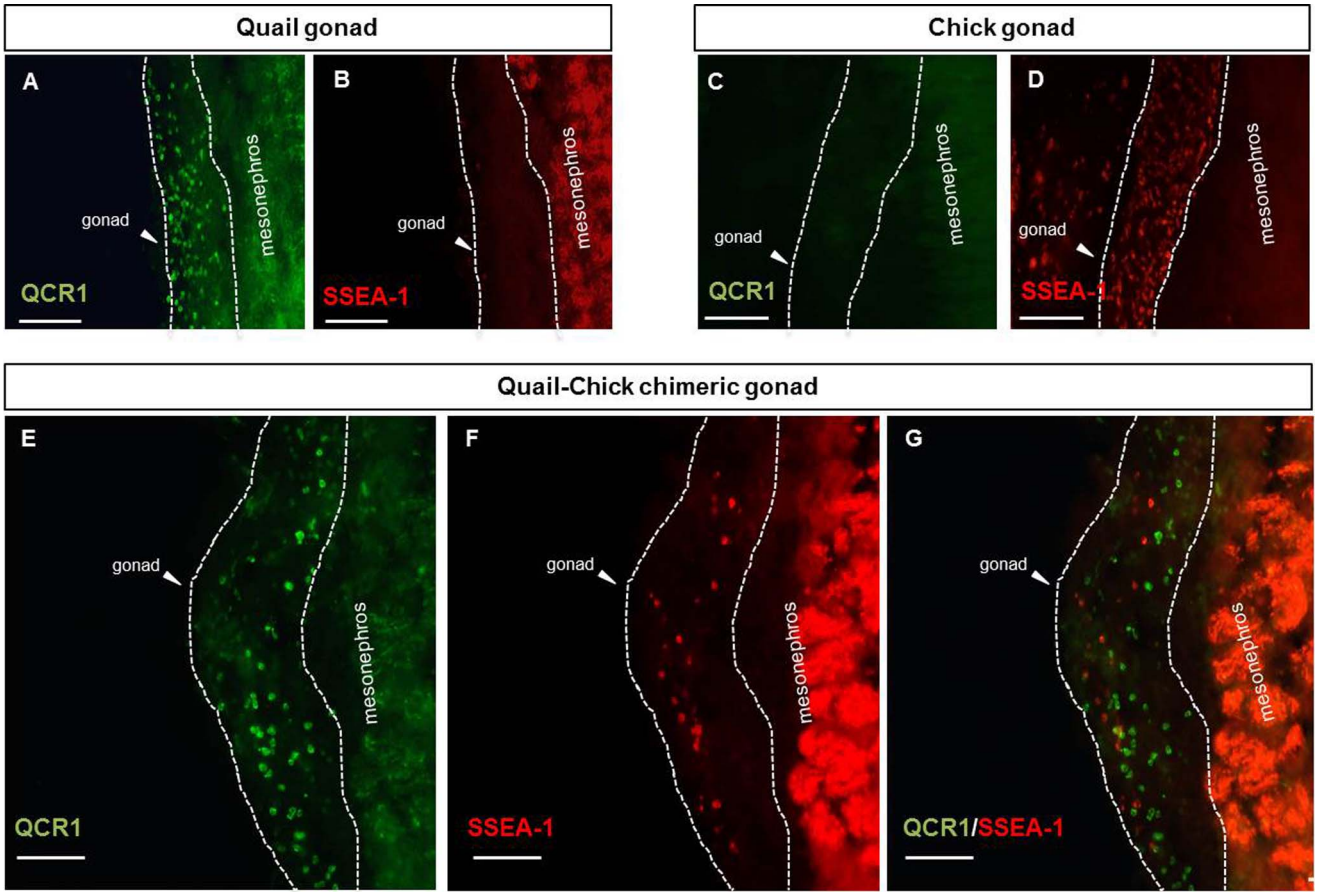

FIG. 5. Interspecies germline chimerism in quail-chick chimeras. As controls, QCR1 and SSEA-1 showed specificity to quail PGCs (**A**, **B**) and chick PGCs (**C**, **D**), respectively. Migration of PGCs derived from the chick extraembryonic area was detected in the gonads of quail-chick chimeras by immunostaining using QCR1 (**E**) and SSEA-1 antibodies (**F**). **G**) The merged image shows that QCR1 (green) and SSEA-1 (red) did not overlap in the gonads of quail-chick chimeras. Bars = 200 μm.

TABLE 3. Number of PGCs in 5.5-day-old quail-chick chimeric gonads.

	Number of gPGCs[a]	
Trials	QCR1 (+)	SSEA-1 (+)
1	337	126
2	166	220
3	213	208
4	253	256
5	246	248
6	235	185
7	228	204
8	260	157
Average[b]	242.25 (±66.9)	200.5 (±43.9)

[a] The number of gPGCs was determined by counting the QCR1-positive or SSEA-1-positive cells under fluorescence microscopy.
[b] Values represent means ± SD.

transmission efficiency [1, 39]. Therefore, PGCs, which are precursor cells of functional gametes, have been recognized as an alternative source of germline transmission in avian species. Of the various avian species, only chicken PGCs have shown germline competency. This is due to higher germline transmission efficiency compared to ESC and stable culture of PGCs. Several groups have attempted to produce germline chimeras using PGC-mediated systems in wild/endangered birds, but the efficiency of production by interspecies PGC transfer is still very low [14, 18]. In this regard, we explored an alternative method to produce germline chimeras that can be applied to most avian species. In the present study, we produced interspecies germline chimeras using the embryo replacement method. The results showed that the chicken PGCs derived from the host extraembryonic region in the yolk were incorporated into the donor quail embryonic gonads after replacement. The combination of the unique migratory route of avian germ cells with the embryo replacement technique demonstrated the possibility of producing interspecies germline chimeras.

We expected that donor embryonic gonads would be mostly occupied by the host PGCs; however, donor quail PGCs accounted for more than half of those in the quail-chick chimeric gonad. This may be explained by the fact that PGCs were already distributed at the area pellucida region at the time of transplantation, so that the donor quail PGCs could not be removed completely from the donor embryo during the operations. In this study, embryo replacement was performed at HH stage 9 to 10 before the formation of blood vessels as described previously [26, 40]. Nakamura et al. [6] reported that PGCs were observed at the area pellucida as well as the anterior part of the extraembryonic region in the area opaca at HH stage 9–10. This strongly suggests that the embryo stage at the operating time is an important factor for improving the efficiency of germline chimerism in the gonads. Thus, optimization of the embryo stage at the time of operation should be investigated in further studies. Alternatively, to eliminate endogenous PGCs in donor embryos, busulfan treatment before embryo replacement can be applied to increase the efficiency of germline transmission [41, 42].

To evaluate germline chimerism in the offspring of interspecies germline chimeras after embryo replacement, it would be necessary to hatch the manipulated embryos. In this study, the transplanted embryos survived until a maximum of E16.5, but no hatchlings were available. We also found that quail embryos were not able to enclose the yolk after E16.5 because the chick yolk was larger than that of the quail. Thus, the large yolk size may be a major factor preventing hatching. It was further suggested that the yolk could be surgically removed to allow hatching after embryo replacement [43]. Further studies are needed to improve the survival rate and hatchability for actual application. We will focus on optimization of the embryo replacement system with high viability as well as hatchability of replaced embryos in future studies.

In the present study, we confirmed that PGCs derived from the host extraembryonic region in the yolk can migrate toward the donor embryonic gonadal tissues after production of quail-chick chimeras. Our technique can be performed reciprocally and has a high success rate of chimeric embryo development to the gonadogenesis stage. The number of transferred PGCs was also attributable to greater migration activity. Therefore, we believe that interspecific germ cell transplantation using embryo replacement will be one means of overcoming the low efficiency of interspecies germline chimera production in avian species. This technique is also potentially applicable to the restoration and conservation of endangered bird species. Through improved transplantation and culture methods, mature germ cells of endangered species can potentially be harvested from more abundant host species such as the chick or quail, thus facilitating their restoration/conservation.

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