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Germline Replacement by Transfer of Primordial Germ Cells into Partially Sterilized Embryos in the Chicken[1]

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ABSTRACT

We report a novel technique for almost complete replacement of the recipient germline with donor germ cells in the chicken. Busulfan solubilized in a sustained-release emulsion was injected into the yolk of fertile eggs before incubation. A dose of 100 μg was found to provide the best outcome in terms of reducing the number of endogenous primordial germ cells (PGCs) in embryonic gonads (0.6% of control numbers) and hatchability (36.4%). This was applied for preparing partially sterilized embryos to serve as recipients for the transfer of exogenous PGCs. Immunohistochemical analysis showed that the proportion of donor PGCs in busulfan-treated embryos was significantly higher than in controls (98.6% vs. 6.4%). Genetic cross-test analysis revealed that the germline transmission rate in busulfan-treated chickens was significantly higher than in controls (99.5% vs. 6.0%). Of 11 chimeras, 7 produced only donor-derived progenies, suggesting that these produced only donor-derived gametes in the recipient's gonads. This novel germline replacement technique provides a powerful tool for studying germline differentiation, for generating transgenic individuals, and for conserving genetic resources in birds.

chicken, germline replacement, primordial germ cells, sterilization

INTRODUCTION

Primordial germ cells (PGCs) are the progenitor cells of gametes. Recently, Tsunekawa et al. [1] clarified that the chicken PGC precursors comprise just six cells expressing the chicken VASA homolog (CVH) protein among approximately 300 residing in the center of the blastodisc at stage IX (Roman numerals refer to the staging system of Eyal-Giladi and Kochav [2]). Thereafter, they increase to about 30–150 cells in the central zone of the area pellucida of stage X embryos, which contain about $3–6 \times 10^4$ cells [1, 3]. With the formation of the primitive streak, PGCs move anteriorly to the edge of the extraembryonic region, the so-called germinal crescent region [4]. They enter the developing vascular system from stage 11 (Arabic numerals refer to the staging system of Hamburger and Hamilton [5]) and then circulate temporarily throughout the embryo by stage 18. Finally, PGCs leave the blood vessels and migrate to the intermediate mesoderm from stage 15 [3] and then differentiate into gametes.

Since the clarification of the timing and migration pathway of avian PGCs, production of germline chimeric birds by the transfer of foreign PGCs has been a topic of interest. The transfer of PGCs provides a means to study avian germ cell development and differentiation. Establishment of embryo manipulation techniques has made it possible to produce somatic as well as germline chimeric chickens by transferring blastodermal cells containing PGCs from stage X embryos into the subgerminal cavity of recipient embryos at stage X [6]. In addition, the unique migration pattern of avian PGCs via the vascular network enables researchers to produce germline chimeric chickens by transferring PGCs retrieved from the germinal crescent region [7], from blood [8], and from gonads [9] into the vascular system of recipient embryos. Avian germline chimeras using blastodermal cells or PGCs have potential application in generating transgenic birds [10, 11] and preserving endangered species [12, 13]. However, there is no definitive method to incorporate donor PGCs into avian host germlines. Therefore, development of a germline replacement technique is needed.

To produce only donor-derived sperm or eggs from germline chimeric animals, the use of sterile recipients is essential. Transplantation of donor spermatogonia of rainbow trout into sterile triploid salmon has resulted in the successful production of only donor-derived sperm or eggs [14]. In normal chickens, as in other birds, the male is the homogametic sex (ZZ) and the female is heterogametic (ZW). At present, three genotypes of triploid embryos have been identified: ZWW, ZZW, and ZZZ. Embryos with ZWW sex chromosomes cannot survive to hatching [15]. On the other hand, some ZZW and ZZZ triploid embryos are viable and can survive to maturity, although neither genotype is fertile [16]. In ZZW chicken, the right gonad develops into a testis and the left gonad develops into an ovotestis at hatching. However, leukocytes infiltrate both gonads at approximately 9 mo of age, and the seminiferous epithelium degenerates in most chicken aged over 1 yr [17]. In ZZZ chicken, gonadal and excurrent duct development is normal [17]. ZZZ triploid chicken embryos can be used as recipients for germline chimera production, but this strategy is only available for males. Genetic mutants lacking germ cells are also unavailable in the chicken. Alternatively, several methods to eliminate the endogenous PGCs or spermatogonia of recipients have been applied for germline replacement in zebrafish [18] and mice [19, 20], respectively. Therefore, partial, if not complete, sterilization of recipient embryos prior to the transfer of donor

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PGCs would be an ideal approach for germline replacement in chicken.

We have developed an efficient method for delivering busulfan (1,4-butanediol dimethanesulfonate), an alkylating agent with cytotoxic effect on chicken germ cells [21, 22], to the chicken embryo [23]. This method enables an efficient depletion of the endogenous PGCs and repopulation with exogenous PGCs in early chicken embryos [24]. Here we have exploited these advances to produce chicken chimeras in which recipient germlines were almost totally replaced by donor cells.

MATERIALS AND METHODS

Fertilized Eggs and Animal Care

All animal care and use in this study was conducted in accordance with the animal experimentation guidelines of our institute (NILGS Animal Care Committee). White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens were maintained at the NILGS farm.

Administration of Busulfan

A sustained-release emulsion [23] was used as the vehicle for busulfan delivery. Busulfan (Sigma-Aldrich, St. Louis, MO) was dissolved in N,N-dimethyl formamide (DMF; Nacalai Tesque, Kyoto, Japan), diluted 10-fold in Ca^{2+}- and Mg^{2+}-free PBS (PBS[−]). A volume of 1 ml busulfan solubilized solution and the same volume of sesame oil (Nacalai Tesque) were separately added in each of the two 2.5-ml syringes (Terumo, Tokyo, Japan). These syringes were connected with a Shirasu porous glass (SPG) membrane filter (SPG pumping connector, pore diameter 10 μm; SPG Technology, Miyazaki, Japan) and the busulfan solubilized solution and sesame oil were emulsified by passing through the membrane 100 times. The sustained-release emulsion was stored for a few minutes for cooling to room temperature and then used for injection. In fresh egg, the position of blastodisc is unsettled because a high amount of thick albumen covering with the yolk blocks the yolk rotation. To position the blastodisc at the top of the yolk, the thick albumen was detached from the yolk. It is well known that the proportional amount of thick albumen decreases gradually, whereas that of thin albumen increases gradually during the storage of chicken eggs [25]. Fertilized WL eggs were obtained freshly from our farm and then stored for 7 days at 14°C in an incubator to reduce the proportional amount of thick albumen. Subsequently, detachment of thick albumen from the yolk was carried out by vortexing the egg for 10 sec at the lowest speed using a mixer with a pop-off cup (Vortex-Genie 2; Scientific Industries, Bohemia, NY). A small hole was made at the blunt end of each egg without damaging the inner shell membrane. Doses of 0, 25, 50, 75, 100, or 125 μg of busulfan in 50 μl sustained-release emulsion were injected horizontally into the yolk through a small hole using a sharp needle (32 gauge × 3.2 cm; Hamilton, Reno, NV) attached to a 50-μl syringe (Hamilton) and then the hole was sealed with wax.

Embryo Culture

Treated or untreated control eggs were incubated for 48–52 h until they reached stage 14 at 39.0°C and relative humidity of 50%–60%, with tilting at a 90° angle once an hour, in a forced-air incubator (P-008B Biotype; Showa Furankli, Saitama, Japan). A window with diameter ≈20 mm was opened in the narrow end and a portion (4–8 ml) of albumen was discarded. After the window was sealed with clingfilm wrap, the embryos were incubated for ≈6 days until they reached stage 29 or until hatching in the same forced-air incubator conditions with tilting at a 30° angle once every 2 h.

Immunohistochemistry and Counting PGCs

Whole gonads attached with the adjacent mesonephros were collected from stage 29 embryos, fixed overnight in 4% paraformaldehyde (PFA) in PBS(−) and washed three times in PBS(−) containing 0.1% Tween-20 (PBS-T) for 15 min each. Fixed gonads were treated with methanol containing 2% H_2O_2 for 30 min to inactivate endogenous peroxidase activity and rinsed twice with PBS-T for 20 min each. Immunohistochemical staining was performed using the biotin/avidin-conjugated horseradish peroxidase system (Vectorstain Elite ABC Rabbit IgG Kit; Vector Laboratories, Burlingame, CA) and NovaRed substrate (NovaRed substrate kit for peroxidase; Vector Laboratories). After blocking in 4.5% normal goat serum in PBS-T for 6 h, gonads were incubated overnight in a 1:10 000 dilution of rabbit anti-CVH antibody [3] and then washed three times in PBS-T for 25 min each. Gonads were incubated with biotinylated secondary antibody (1:200) overnight and then rinsed three times with PBS-T for 25 min each. After incubation with Vectorstain Elite ABC Reagent for 30 min, gonads were washed four times in PBS(−) for 5 min each. Finally, gonads were stained using NovaRed solution according to the manufacturer's instructions. All steps were carried out at 4°C. Both left and right gonads were detached from mesonephros and then the labeled cells in the stained gonads were counted using a microscope (DFC480-Note OY; Leica Microsystems, Tokyo, Japan).

Preparation of Donor PGCs

Fertilized BPR eggs were incubated in the same conditions as described above for ≈55 h to obtain embryo stages 14–16. Blood was collected from the dorsal aorta of the embryos with a fine glass micropipette under a microscope (MS5; Leica Microsystems). Each collected blood sample was added to 100 μl of PBS(−). After sexing of each sample, male and female blood samples were pooled separately in PBS(−). PGCs were concentrated from embryonic blood using Nycodenz density gradient centrifugation [26] with some modification. Briefly, PBS(−) containing 10% fetal bovine serum (FBS) was used as a buffer instead of KAv-1 medium [27]. Some concentrated PGCs were labeled with the fluorescent lipophilic carbocyanine dye PKH-26 (Zynaxis, Malvern, PA) according to the protocol of Yamamoto et al. [28]. Chicken PGCs are easily distinguished from erythrocytes by their morphological characteristics, such as large size and the presence of many refractive granules in the cytoplasm observed using phase-contrast microscopy [29]. Collected PGCs were stored in 1 ml of PBS(−) containing 10% FBS for 4 h until transfer at room temperature.

Repopulation of Recipient Germline with Donor PGCs

WL embryos treated with 100 μg busulfan or untreated controls were prepared as recipients and then incubated for 48–52 h until they reached stage 14 in the same conditions as described above. Approximately 0.5 μl of blood was collected from the dorsal aorta of recipient embryos through a window (diameter ≈20 mm) in the narrow end of the egg. Blood collection from recipient embryos was finished within 60 min. Each collected blood sample was diluted to 100 μl with PBS(−) and used for sex determination. Sets of 200 PKH-26-labeled PGCs from the BPR strain were microinjected into the dorsal aorta of recipient embryos at stages 15–16 with the same sexes of donors and recipients. Manipulated embryos were incubated further and whole gonads attached with the adjacent mesonephros were collected at stage 29. After fixing in 4% PFA in PBS(−), gonads were rinsed three times with PBS-T for 15 min each. Fluorescent immunostaining was done as described elsewhere [23]. After blocking in 4.5% normal goat serum in PBS-T for 6 h, gonads were incubated overnight with rabbit anti-CVH antibody (1:10 000) and were then rinsed three times with PBS-T for 25 min each. Gonads were incubated overnight in a 1:400 dilution of Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and washed three times in PBS-T for 25 min each. After detachment of both left and right gonads from mesonephros, fluorescent PKH-26-labeled cells (donor PGCs) and CVH-positive cells (donor and recipient PGCs) in the immunostained gonads were monitored by fluorescence microscopy (DFC480-Note OY, Leica Microsystems). When the immunostained samples were obtained from male embryos, the numbers of PKH-26-labeled cells and CVH-positive cells in both right and left gonads were counted. The numbers of PKH-26-labeled cells and CVH-positive cells were counted in the left gonad in female embryos because only this one develops into a functional ovary in birds.

Production of Chimeric Chickens

Recipient WL embryos treated with 100 μg busulfan or untreated controls were incubated for 48–52 h until they reached stage 14 in the same conditions as described above. About 0.5 μl of blood was drawn from the dorsal aorta of recipient embryos through a window (diameter ≈20 mm) in the narrow end of the egg. Blood collection from recipient embryos was completed within 60 min. Each collected blood sample was added to 100 μl of PBS(−) and used for sexing. Sets of 200 PGCs from BPR embryos were transferred to the dorsal aorta of recipient embryos at stages 15–16 with the same sex combinations of donors and recipients. Manipulated embryos were incubated until hatching.

Sexing

The tissues obtained from donor BPR embryos were digested in 70 μl of buffer composed of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% Tween 20, and 50 μg/ml Proteinase K (pH 7.5) using vigorous vortexing for 20 min at room temperature. Blood samples from recipient WL embryos were digested in 10 μl of the same buffer using vigorous vortexing for 20 min at room temperature. Molecular sexing was conducted by detecting conserved regions of the CHD-W and CHD-Z genes using primers 2550 F and 2718 R and

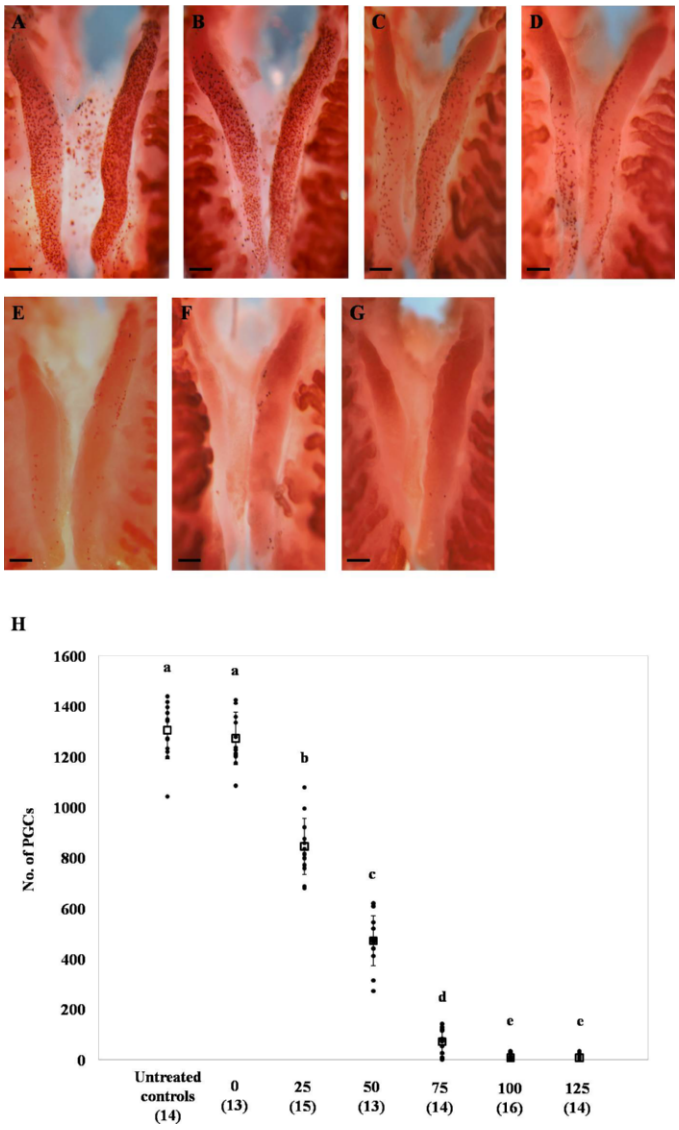

FIG. 1. The number of endogenous PGCs in embryonic gonads after 6 days incubation. Endogenous PGCs were detected by immunohistochemical staining using a specific antibody raised against chicken VASA homolog protein. **A**) Untreated control embryo. Embryos treated with doses of 0 (**B**), 25 (**C**), 50 (**D**), 75 (**E**), 100 (**F**), and 125 μg (**G**) busulfan solubilized in 50 μl sustained-release emulsion. Bar = 100 μm. **H**) The numbers of endogenous PGCs in whole gonads at embryo stage 29 were reduced by busulfan treatment dose-dependently. Means with different superscripts are significantly different ($P < 0.01$). The number of embryos evaluated is shown below each treatment.

polymerase chain reaction amplification, following the protocol of Fridolfsson and Ellegren [30]. Sex determination of donors and recipients was completed within 3 h.

Progeny Testing

Putative germline chimeric chickens derived from PGC transfer that survived to sexual maturity were crossed with BPR (i/i) chickens by artificial insemination and the feather color of their offspring was examined. Black offspring (i/i) indicated that the offspring was derived from BPR donor PGCs, whereas white offspring with small patches of black pigmentation (I/i) indicated that the offspring was derived from recipient WL PGCs.

Regeneration of BPR Individuals from Germline Chimeras

When complete germline replacement has been identified by test mating, the chimeric chickens were crossed using artificial insemination for regenerating BPR chickens as a result of fertilizing eggs with spermatozoa, both derived from donor PGCs. The hatchlings were classified as black (BPR × BPR), spotted white (BPR × WL), or pure white (WL × WL).

Statistical Analysis

All data are expressed as the mean ± SD. The survival rate of the embryos in each group was analyzed using the chi-square test. The numbers of PGCs in the gonads in each of the seven groups was analyzed by one-way ANOVA. If model effect was significant, differences between mean values for each treatment were then evaluated using the Bonferroni test. Differences in the numbers of PKH-26-labeled PGCs and CVH-positive cells, and in the proportions of donor PGCs and donor-derived offspring, were compared between busulfan-treated and untreated control groups using unpaired Student *t*-tests. Differences in survivability and the other factors were considered significant at $P < 0.05$ and $P < 0.01$, respectively.

RESULTS

PGC Depletion in Embryonic Gonads

Embryos treated with busulfan did not show developmental delay after 6 days of incubation regardless of the dosage when compared to the untreated control embryos. Whole gonads were collected from morphologically normal embryos at stage 29. Immunohistochemical analysis confirmed that the five busulfan-treated groups (25, 50, 75, 100, or 125 μg) resulted in fewer PGCs compared with the sham-operated control group (receiving sustained-release emulsion without busulfan) and the untreated control group (Fig. 1, A–G). The numbers of PGCs in whole gonads of embryos at stage 29 in each group are shown in Figure 1H. The mean number (range) of PGCs in sham-operated controls (1272.7 ± 103.6; 1085–1426) was almost the same as in untreated controls (1304.3 ± 111.9; 1042–1442). The mean number of PGCs in whole mounts of gonads at stage 29 was reduced by administration of busulfan in a dose-dependent manner up to 100 μg ($P < 0.01$), with the values for 25-, 50-, 75-, and 100-μg groups being 844.7 ± 110.8 (681–1079), 472.1 ± 99.5 (272–620), 71.9 ± 49.0 (0–142), and 8.4 ± 12.1 (0–34), respectively. No statistically significant difference in the numbers of PGCs was observed between the 100- and 125-μg treatment groups (7.8 ± 11.5; 0–35).

Embryonic Development and Hatchability

Teratogenic effects increased with the dose of busulfan (Table 1). The most common abnormalities observed in the treated embryos were anangioplasia, abnormal limb buds, and small eyes. These embryos showing developmental abnormalities did not survive to hatching. The effects of busulfan administration on the survivability and hatchability of chicken

TABLE 1. Teratogenic effects of busulfan on chicken embryos and their hatching rate.

Dose (μg)	No. of embryos	Percentage of embryos showing abnormalities	Hatching rate of embryos showing abnormalities[a]
Untreated controls	74	0	NA
0 (Sham-operated controls)	85	0	NA
25	87	1.1	0
50	82	3.7	0
75	90	6.7	0
100	88	9.1	0
125	105	14.3	0

[a] NA, not available.

TABLE 2. Survival and hatching rates of chicken embryos in windowed eggs with and without busulfan.

Dose (μg)	No. of embryos	Survivability of embryos on incubation day (%)						Hatched (%)
		3	7	10	14	17	20	
Untreated controls	74	97.3[a]	93.2[a]	93.2[a]	89.1[a]	87.8[a]	83.8[a]	74.3[a]
0 (Sham-operated controls)	85	91.8[ab]	87.1[ab]	83.5[ab]	80.0[ab]	80.0[ab]	77.6[ab]	68.2[ab]
25	87	86.2[ab]	79.3[abc]	77.0[abc]	75.9[ab]	73.6[abc]	67.8[abc]	54.0[abc]
50	82	81.7[ab]	75.6[abc]	74.4[abc]	73.2[ab]	70.7[abc]	63.4[abc]	46.3[bcd]
75	90	81.1[ab]	74.4[abc]	70.0[bc]	65.6[b]	62.2[bc]	60.0[bc]	41.1[cd]
100	88	78.4[b]	68.2[bc]	67.0[bc]	63.6[bc]	61.3[bc]	58.0[bc]	36.4[cd]
125	105	74.3[b]	63.8[c]	61.9[c]	59.0[b]	56.2[c]	52.4[c]	29.5[d]

[a–d] Means with different superscript in the same column are significantly different (chi-square test, $P < 0.05$).

embryos are shown in Table 2. The survival and hatching rates of embryos in the sham control group was slightly lower than in the untreated control group, but the difference was not statistically significant. Survival and hatching rates were both decreased dose-dependently by the application of busulfan ($P < 0.05$).

Germline Chimerism in Embryonic Gonads

To monitor whether donor PGCs could repopulate PGC-depleted gonads after busulfan treatment, sets of 200 fluorescently labeled PGCs from BPR embryos were transferred at stages 15–16. Gonadal migration of donor PGCs was observed in all manipulated embryos, as shown in Figure 2. Embryonic germline chimerism, estimated by the number of PKH-26-labeled cells (donor PGCs) and CVH-positive cells (donor and recipient PGCs) in busulfan-treated (100 μg) and untreated control groups in males and females, is listed in Tables 3 and 4, respectively. The number of PKH-26-labeled PGCs was more than fivefold higher ($P < 0.01$) in busulfan-treated than in untreated control groups in both male (embryo numbers BME-1–13 vs. UME-1–9) and female embryos (BFE-1–11 vs. UFE-1–8). On the other hand, the number of CVH-positive cells in the busulfan-treated group was significantly lower than the number in the untreated control group ($P < 0.01$). The proportion of donor PGCs in the busulfan-treated group was 98.5% ± 2.1%, whereas in the untreated control group it was 6.4% ± 2.0% ($P < 0.01$).

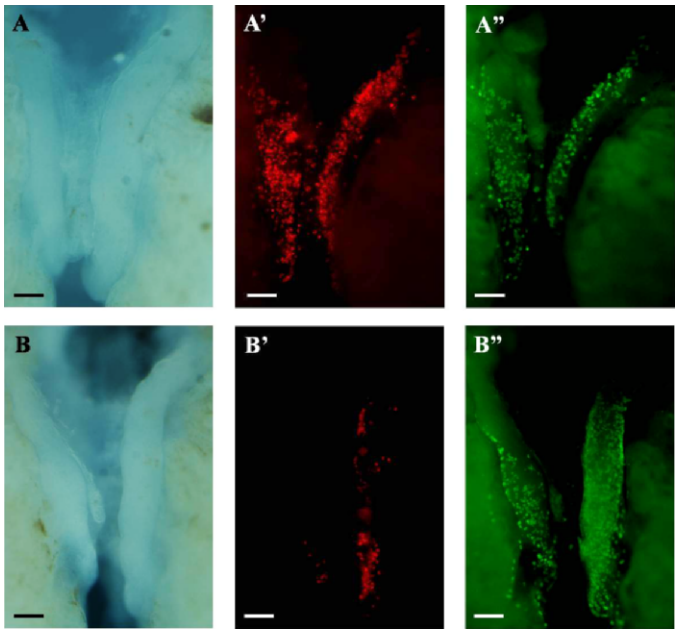

FIG. 2. Migration of donor PGCs to recipient gonads at embryo stage 29. Male gonads of busulfan-treated (**A**–**A″**) and untreated control embryos (**B**–**B″**). **A** and **B**) Bright-field images of whole mounts of gonads attached with the adjacent mesonephros. Gonadal migration of donor PGCs was tracked by staining with the fluorescent lipophilic carbocyanine dye PKH-26 (**A′**, **B′**). Chimeric gonads were immunostained with an antibody raised against CVH protein (**A″**, **B″**). Bar = 100 μm.

Germline Chimerism in Sexually Mature Birds

For chimera production, WL embryos were treated with 100 μg of busulfan, and these embryos and untreated controls received sets of 200 PGCs from BPR embryos at stages 15–16 with the same sexes of donors and recipients. In the busulfan-treated group, 50 embryos were manipulated (25 male and 25 female), 17 (8 male and 9 female) hatched, and 11 (5 male and 6 female) reached sexual maturity. In the untreated control group, 20 embryos were manipulated (9 male and 11 female), 15 (7 male and 8 female) hatched, and 12 (5 male and 7 female) survived to sexual maturity. Genetic crossing analysis with BPR chickens showed that all sexually matured chickens both in the busulfan-treated group (BMC-2–8 and BFC-1–9) and in the untreated control group (UMC-1–6 and UFC-1–8)

TABLE 3. Proportion of donor primordial germ cells (PGCs) in male gonads of 6-day-old embryos with and without busulfan treatment.

Recipient treatment/ embryo ID	No. of PKH-26-labeled cells (donor PGCs)	No. of CVH-positive cells (donor and recipient PGCs)	Proportion of donor PGCs (%)
Busulfan (100 μg)			
BME-1	378	378	100.0
BME-3	433	457	94.7
BME-4	389	401	95.5
BME-5	298	302	98.7
BME-6	308	311	99.0
BME-7	403	403	100.0
BME-9	583	583	100.0
BME-11	511	511	100.0
BME-12	347	362	95.9
BME-13	491	491	100.0
Mean ± SD	414.1 ± 91.6[a]	419.9 ± 90.0[a]	98.4 ± 2.1[a]
Untreated controls			
UME-1	76	1241	6.1
UME-2	84	1087	7.7
UME-3	72	1219	5.9
UME-4	87	1055	8.2
UME-5	62	1130	5.5
UME-6	124	1259	9.8
UME-8	78	1118	7.0
UME-9	59	1132	5.2
Mean ± SD	80.3 ± 20.2[b]	1155.1 ± 75.1[b]	6.9 ± 1.6[b]

[a,b] Different superscripts in the proportion of donor PGCs were significantly different, $P < 0.01$.

TABLE 4. Proportion of donor primordial germ cells (PGCs) in female gonads of 6-day-old embryos with and without busulfan treatment.*

Recipient treatment/ embryo ID	No. of PKH-26-labeled cells (donor PGCs)	No. of CVH-positive cells (donor and recipient PGCs)	Proportion of donor PGCs (%)
Busulfan (100 μg)			
BFE-1	408	411	99.2
BFE-2	387	387	100.0
BFE-3	283	292	96.9
BFE-4	377	377	100.0
BFE-6	260	260	100.0
BFE-7	421	438	96.1
BFE-8	296	296	100.0
BFE-9	324	324	100.0
BFE-10	284	302	94.0
BFE-11	349	349	100.0
Mean ± SD	338.9 ± 57.6[a]	343.6 ± 58.2[a]	98.6 ± 2.2[a]
Untreated controls			
UFE-1	73	1031	7.1
UFE-2	62	905	6.9
UFE-3	83	1019	8.1
UFE-4	43	1152	3.7
UFE-5	94	967	9.7
UFE-6	28	1118	2.5
UFE-7	48	1098	4.4
UFE-8	59	1215	4.9
Mean ± SD	61.3 ± 21.7[b]	1063.1 ± 101.7[b]	5.9 ± 2.4[b]

* Data were obtained from only left gonads.
[a,b] Different superscripts in the proportion of donor PGCs were significantly different, $P < 0.01$.

were germline chimeras (Table 5). The frequency of obtaining donor-derived offspring from germline chimeric chickens in the busulfan-treated group was 99.5% ± 0.9%, whereas in the untreated control group it was 6.0% ± 2.9% ($P < 0.01$). Moreover, seven chimeras (three male and four female) produced only donor-derived black progenies, suggesting that these birds produced only donor-derived gametes.

Regeneration of BPR Chickens by Crossing Germline Replacement Chimeras

Four germline-replaced hens (BFC-1, BFC-2, BFC-5, and BFC-9) were artificially inseminated with semen from each of three germline-replaced cocks (BMC-2, BMC-4, and BMC-8). Donor-derived black embryos in recipient-derived white-shelled eggs were observed by mating male and female germline chimeric birds (Fig. 3A). All of the progenies obtained from each combination of germline chimeric birds were donor-derived black chickens (Fig. 3B; Table 6).

DISCUSSION

This is the first report of a technique that allows for the almost complete replacement of a recipient germline following transplantation of donor PGCs in birds. Stable production of recipient embryos lacking endogenous PGCs was achieved following the injection of busulfan solubilized in a sustained-release emulsion into the yolk of fertile eggs before incubation. Donor PGCs transferred to partially sterilized recipient embryos were successfully incorporated into the recipient germline and gave rise to functional gametes.

Successful replacement of a recipient's germline with donor germ cells requires prior sterilization of the recipients. Up to the present, three different approaches have been applied to sterilize recipients in vertebrates. Administration of busulfan has been used for eliminating testicular cells in mice [19]. Repeated exposure to gamma radiation has also been used for sterilizing male mice [20] and roosters [31]. In these studies, subsequent transplantation of testicular cells containing spermatogonial stem cells to the sterilized testes showed

TABLE 5. Progeny test of same-sex germline chimeras produced after transfer of primordial germ cells from Barred Plymouth Rock into White Leghorn embryos with and without busulfan treatment.

Recipient treatment/ chimera ID	Sexuality of chimeras	No. of black chicks (donor-derived progenies)	No. of white chicks (recipient-derived progenies)	Proportion of donor-derived progenies (%)
Busulfan (100 μg)				
BMC-2	Male	172	0	100.0
BMC-4	Male	210	0	100.0
BMC-6	Male	197	1	99.5
BMC-7	Male	197	5	97.5
BMC-8	Male	106	0	100.0
BFC-1	Female	70	0	100.0
BFC-2	Female	99	0	100.0
BFC-4	Female	111	1	99.1
BFC-5	Female	113	0	100.0
BFC-7	Female	101	2	98.1
BFC-9	Female	74	0	100.0
Mean ± SD				99.5 ± 0.9[a]
Untreated controls				
UMC-1	Male	11	165	6.3
UMC-2	Male	10	136	6.8
UMC-3	Male	7	146	4.6
UMC-4	Male	9	113	7.4
UMC-6	Male	7	157	4.3
UFC-1	Female	5	107	4.5
UFC-2	Female	9	102	8.1
UFC-3	Female	16	123	11.5
UFC-4	Female	3	87	3.3
UFC-5	Female	2	88	2.2
UFC-7	Female	3	108	2.7
UFC-8	Female	11	102	9.7
Mean ± SD				6.0 ± 2.9[b]

[a,b] Different superscripts in the proportion of donor-derived progenies were significantly different, $P < 0.01$.

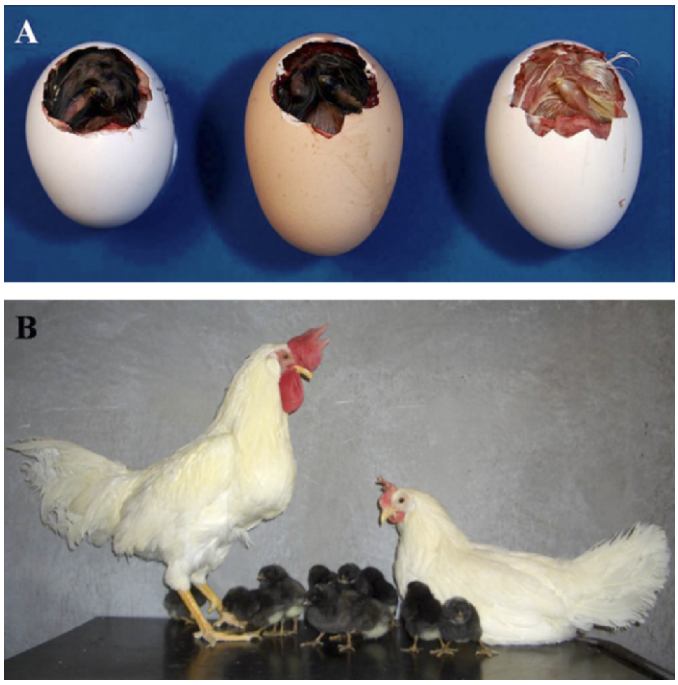

FIG. 3. Regeneration of donor-derived individuals by crossing germline-replaced chimeras. **A**) A black embryo in a white-shelled egg (left) was obtained by mating germline-replaced chimeras with transplanted PGCs from BPR into WL embryos. A colored egg (center) and a white egg (right) were laid by BPR and WL hens, respectively. **B**) Donor-derived BPR hatchlings and a male (left, BMC-1) and female (right, BFC-2) germline replacement chimera.

successful colonization of recipient testes by the exogenous germlines. In zebrafish, microinjection of a morpholino antisense oligonucleotide directed against the *dead end* mRNA in 1- to 2-cell stage embryos effectively ablated the recipient germline, and germline replacement was achieved successfully by transferring donor PGCs [18]. In practice, the use of gamma irradiation is limited because of the high cost and special equipment needed. A morpholino oligonucleotide approach is also restricted in avian species because of the technical difficulties in microinjecting the ovum immediately after fertilization and subsequent culture of the manipulated embryo. Therefore, busulfan treatment seems to be a better option for germline replacement in birds. However, the efficacy of this approach has been highly variable because of the difficulty in delivering a constant amount of busulfan to the target embryo [21]. Furthermore, busulfan treatment is feasible only when the residual busulfan does not interfere with the transferred PGCs [22]. To overcome these problems, we developed an efficient technique to deliver consistent amounts of busulfan using a sustained-release emulsion as a vehicle [23]. To maintain a consistent level of busulfan in a vehicle, the sustained-release emulsion is made by emulsifying busulfan solubilized in PBS(−) containing 10% DMF and an equal amount of sesame oil. Because the sustained-release emulsion had a lower density than the yolk, this vehicle rapidly rose and contacted the developing embryo that was lying at the top of the yolk when it was injected into the yolk. According to these advantages, the injection of busulfan solubilized in the sustained-release emulsion into the yolk resulted in a consistent and extensive depletion of endogenous PGCs. Subsequently, we showed that early application of busulfan to the recipient embryos allowed reduction of the sterilizing effect of the residual busulfan on donor PGCs [24]. In the present study, germline chimera

TABLE 6. Regeneration of Barred Plymouth Rock (BPR) progenies by crossing White Leghorn (WL) chickens that have germlines replaced by BPR.

Male chimera ID	Female chimera ID	Phenotypes of offspring*		
		Black	Spotted white	Pure white
BMC-2	BFC-1	10	0	0
BMC-2	BFC-2	10	0	0
BMC-2	BFC-5	8	0	0
BMC-2	BFC-9	6	0	0
BMC-4	BFC-1	10	0	0
BMC-4	BFC-2	10	0	0
BMC-4	BFC-5	9	0	0
BMC-4	BFC-9	8	0	0
BMC-8	BFC-5	12	0	0
BMC-8	BFC-9	14	0	0

* Black progenies are typical BPR × BPR; spotted white progenies are BPR × WL; white progenies are WL.

production and subsequent genetic cross testing were performed to validate our germline replacement technique.

The number of endogenous PGCs in the whole gonads of chicken embryos incubated for 6 days was reduced following busulfan administration in a dose-dependent manner. This suggests that a constant amount of drug was delivered successfully to the blastodisc when using the sustained-release emulsion as a vehicle. Doses of 100 or 125 μg resulted in nearly total depletion of the endogenous PGCs. The hatchability of the embryos was also decreased in a dose-dependent manner. A dose of 100 μg was found to be optimal for preparing partially sterilized recipient embryos for the germline replacement study, considering both the number of endogenous PGCs remaining (0.6% of control value) and hatchability (36.4%). The average proportion of chicken PGCs that migrated into the gonadal area of quail embryos within 24 h after PGC transfer was 50.7% regardless of the number of PGCs transferred [32]. In the present study, the mean number of donor PGCs observed in the gonads of untreated control embryos within 4 days of transfer was appreciably lower than the number transferred. In contrast, the number of donor PGCs in the gonads of busulfan-treated embryos within 4 days of transplantation was far higher than the number transplanted. This indicates that exogenous PGCs proliferated to repopulate the recipient's gonads without any marked sterilizing effect of the residual busulfan. Thus, the proliferation profile of exogenous PGCs after gonadal migration seems to be closely associated with the number of residual PGCs in the recipient's gonads.

We were able to produce germline chimeras by transferring sets of 200 isolated PGCs from BPR embryos into the vascular systems of recipient WL embryos treated with 100 μg of busulfan and into untreated controls. It has been reported that female PGCs in male gonads are capable of passing through the first and second meiotic divisions by adapting themselves to a male environment; however, they can barely complete spermatogenesis [33]. Accordingly, in this study sex-matched germline chimeras were produced by prior sex identification of the donor PGCs and recipient embryos to overcome this sexual mismatch problem. Crossbreeding analysis showed that the frequency of germline transmission of produced chimeric chickens in the busulfan-treated group was 99.5% whereas the rate in the untreated control group was only 6.0%. Moreover, 7 of the 11 chimeric chickens (64%; 3 male and 4 female) in the busulfan-treated group produced only donor-derived chickens. This suggests that the recipient's germline had been completely replaced by the donor germ cells and produced only donor-

derived gametes. This established method has dramatically enhanced the germline transmission rate when compared with the previously reported busulfan approach [34] (97.5%–100.0% vs. 0%–13.9%). This difference of germline transmission rate would be caused by the drug delivery system and the timing of busulfan treatment. Our established method resulted in a stable removal of endogenous PGCs, as shown in Figure 1, because of the steady delivery of consistent amounts of drug to the blastodisc. By contrast, constant PGC depletion could not be achieved using the method of Song et al. [34] because the vehicle, which was made by mixing DMF with an equal amount of sesame oil, quickly separated into DMF and oil immediately just after injection [23]. Additionally, we have elucidated that application of busulfan before incubation leads the reduction of the sterilizing effect of the residual busulfan at the time of donor PGC transfer when compared to application of busulfan after 24 h of incubation. Thus, we treated recipient embryos with busulfan before incubation, although Song et al. [34] treated recipient embryos after 24 h of incubation. Apart from the busulfan approach, several techniques have been attempted to improve the germline transmission rate of donor-derived gametes in chicken by reducing the endogenous PGCs of recipient embryos prior to donor PGC transfer. The frequencies of obtaining donor-derived offspring using gamma ray sterilization [35], surgical removal of the central zone of the area pellucida [36], or surgical removal of early embryonic blood [37] were 6.0% (0%–100%), 32.5% (0%–100%) and 33.5% (0%–95.8%), respectively. Our established method seems to be the most practical and efficient method presently available for chimera production because the frequency of germline transmission can be stabilized at a high rate.

Cryopreservation of PGCs as genetic material seems to be an effective conservation strategy because techniques for freezing ova and producing cloned individuals from somatic cells are not available in birds. Here we demonstrate the ability to produce both male and female chimeric chickens that have germlines almost completely reconstituted by donor germ cells. Moreover, purely donor-derived individuals were successfully regenerated by mating between germline-replaced chimeras. Thus, this technique will promote not only the banking of PGCs from existing chicken breeds, but also the augmentation of individual genetic stock using germline replacement chimeras. Although studies in zebrafish have demonstrated that a single PGC has the ability to repopulate the germline and give rise to functional gametes [38], little is known about the number of PGCs required for normal germline development in the chicken. We aim to determine this using our established system. Such an approach will create better conditions for enhancing PGC-mediated genetic resource conservation systems. Additionally, combining this germline replacement technique with PGC-mediated transgenesis will allow us to generate homozygous mutants efficiently in the F1 generation. Therefore, our novel technique offers a new means to study germline differentiation, conserve genetic resources, and produce transgenic individuals in birds.

REFERENCES

1. Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 2000; 127:2741–2750.
2. Eyal-Giladi H, Kochav S. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev Biol 1976; 49:321–337.
3. Nakamura Y, Yamamoto Y, Usui F, Mushika T, Ono T, Setioko AR, Takeda K, Nirasawa K, Kagami H, Tagami T. Migration and proliferation of primordial germ cells in the early chicken embryo. Poult Sci 2007; 86: 2182–2193.
4. Ginsburg M, Eyal-Giladi H. Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo. J Embryol Exp Morphol 1986; 95:53–71.
5. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol 1951; 88:49–92.
6. Petitte JN, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ. Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 1990; 108:185–189.
7. Vick L, Luke G, Simkiss K. Germ-line chimaeras can produce both strains of fowl with high efficiency after partial sterilization. J Reprod Fertil 1993; 98:637–641.
8. Tajima A, Naito M, Yasuda Y, Kuwana T. Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (*Gallus domesticus*). Theriogenology 1993; 40:509–519.
9. Tajima A, Naito M, Yasuda Y, Kuwana T. Production of germ-line chimeras by transfer of cryopreserved gonadal primordial germ cells (gPGCs) in chicken. J Exp Zool 1998; 280:265–267.
10. van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ. Germline transmission of genetically modified primordial germ cells. Nature 2006; 441:766–769.
11. Shin SS, Kim TM, Kim SY, Kim TW, Seo HW, Lee SK, Kwon SC, Lee GS, Kim H, Lim JM, Han JY. Generation of transgenic quail through germ cell-mediated germline transmission. FASEB J 2008; 22:2435–2444.
12. Kuwana T, Kawashima T, Naito M, Yamashita H, Matsuzaki M, Takano T. Conservation of a threatened indigenous fowl (Kureko Dori) using the germline chimeras transplanted from primordial germ cells. J Poult Sci 2006; 43:60–66.
13. Kang SJ, Choi JW, Kim SY, Park KJ, Kim TM, Lee YM, Kim H, Lim JM, Han JY. Reproduction of wild birds via interspecies germ cell transplantation. Biol Reprod 2008; 79:931–937.
14. Okutsu T, Shikina S, Kanno M, Takeuchi Y, Yoshizaki G. Production of trout offspring from triploid salmon parents. Science 2007; 317:1517.
15. Bloom SE. Chromosome abnormalities in chicken (*Gallus domesticus*) embryos: types, frequencies and phenotypic effects. Chromosoma 1972; 37:309–326.
16. Abdel-Hameed F, Shoffner RN. Intersexes and sex determination in chickens. Science 1971; 172:962–964.
17. Lin M, Thorne MH, Martin IC, Sheldon BL, Jones RC. Development of the gonads in the triploid (ZZW and ZZZ) fowl, *Gallus domesticus*, and comparison with normal diploid males (ZZ) and females (ZW). Reprod Fertil Dev 1995; 7:1185–1197.
18. Ciruna B, Weidinger G, Knaut H, Thisse B, Thisse C, Raz E, Schier AF. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci U S A 2002; 99:14919–14924.
19. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 1994; 91:11298–11302.
20. Creemers LB, Meng X, den Ouden K, van Pelt AM, Izadyar F, Santoro M, Sariola H, de Rooij DG. Transplantation of germ cells from glial cell line-derived neurotrophic factor-overexpressing mice to host testes depleted of endogenous spermatogenesis by fractionated irradiation. Biol Reprod 2002; 66:1579–1584.
21. Aige-Gil V, Simkiss K. Sterilisation of avian embryos with busulphan. Res Vet Sci 1991; 50:139–144.
22. Hallett JS, Wentworth BC. The effects of busulfan on gonadal differentiation and development in Japanese quail (*Coturnix coturnix japonica*). Poult Sci 1991; 70:1619–1623.
23. Nakamura Y, Yamamoto Y, Usui F, Atsumi Y, Ito Y, Ono T, Takeda K, Nirasawa K, Kagami H, Tagami T. Increased proportion of donor primordial germ cells in chimeric gonads by sterilisation of recipient embryos using busulfan sustained-release emulsion in chicken. Reprod Fertil Dev 2008; 20:900–907.
24. Nakamura Y, Usui F, Atsumi Y, Otomo A, Teshima A, Ono T, Takeda K, Nirasawa K, Kagami H, Tagami T. Effects of busulfan sustained-release emulsion on depletion and repopulation of primordial germ cells in early chicken embryos. J Poult Sci 2009; 46:127–135.
25. Romanoff AL, Romanoff AJ. The Avian Egg. New York: John Wiley and Sons Inc.; 1949:665–669.
26. Zhao DF, Kuwana T. Purification of avian circulating primordial germ cells by Nycodenz density gradient centrifugation. Br Poult Sci 2003; 44: 30–35.
27. Kuwana T, Hashimoto K, Nakanishi A, Yasuda Y, Tajima A, Naito M. Long-term culture of avian embryonic cells in vitro. Int J Dev Biol 1996; 40:1061–1064.
28. Yamamoto Y, Usui F, Nakamura Y, Ito Y, Tagami T, Nirasawa K,

Matsubara Y, Ono T, Kagami H. A novel method to isolate primordial germ cells and its use for the generation of germline chimeras in chicken. Biol Reprod 2007; 77:115–119.
29. Fujimoto T, Ukeshima A, Kiyofuji R. The origin, migration and morphology of the primordial germ cells in the chick embryo. Anat Rec 1976; 185:139–145.
30. Fridolfsson A, Ellegren H. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 1999; 30:116–121.
31. Trefil P, Micáková A, Mucksová J, Hejnar J, Poplstein M, Bakst MR, Kalina J, Brillard JP. Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. Biol Reprod 2006; 75:575–581.
32. Ishiguro S, Minematsu T, Naito M, Kanai Y, Tajima A. Migratory ability of chicken primordial germ cells transferred into quail embryos. J Reprod Dev 2009; 55:183–186.
33. Tagami T, Kagami H, Matsubara Y, Harumi T, Naito M, Takeda K, Hanada H, Nirasawa K. Differentiation of female primordial germ cells in the male testes of chicken (*Gallus gallus domesticus*). Mol Reprod Dev 2007; 74:68–75.
34. Song Y, D'Costa S, Pardue SL, Petitte JN. Production of germline chimeric chickens following the administration of a busulfan emulsion. Mol Reprod Dev 2005; 70:438–444.
35. Carsience RS, Clark ME, Verrinder Gibbins AM, Etches RJ. Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development 1993; 117:669–675.
36. Kagami H, Tagami T, Matsubara Y, Harumi T, Hanada H, Maruyama K, Sakurai M, Kuwana T, Naito M. The developmental origin of primordial germ cells and the transmission of the donor-derived gametes in mixed-sex germline chimeras to the offspring in the chicken. Mol Reprod Dev 1997; 48:501–510.
37. Naito M, Tajima A, Yasuda Y, Kuwana T. Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Mol Reprod Dev 1994; 39: 153–161.
38. Saito T, Goto-Kazeto R, Arai K, Yamaha E. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol Reprod 2008; 78:159–166.