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—Original Article—

X-irradiation Removes Endogenous Primordial Germ Cells (PGCs) and Increases Germline Transmission of Donor PGCs in Chimeric Chickens

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Abstract. Primordial germ cells (PGCs) are embryonic precursors of germline cells with potential applications in genetic conservation, transgenic animal production and germline stem cell research. These lines of research would benefit from improved germline transmission of transplanted PGCs in chimeric chickens. We therefore evaluated the effects of pretransplant X-irradiation of recipient embryos on the efficacy of germline transmission of donor PGCs in chimeric chickens. Intact chicken eggs were exposed to X-ray doses of 3, 6 and 9 Gy (dose rate = 0.12 Gy/min) after 52 h of incubation. There was no significant difference in hatching rate between the 3-Gy-irradiated group and the nonirradiated control group (40.0 *vs.* 69.6%), but the hatching rate in the 6-Gy-irradiated group (28.6%) was significantly lower than in the control group ($P<0.05$). No embryos irradiated with 9 Gy of X-rays survived to hatching. X-irradiation significantly reduced the number of endogenous PGCs in the embryonic gonads at stage 27 in a dose-dependent manner compared with nonirradiated controls. The numbers of endogenous PGCs in the 3-, 6- and 9-Gy-irradiated groups were 21.0, 9.6 and 4.6% of the nonirradiated control numbers, respectively. Sets of 100 donor PGCs were subsequently transferred intravascularly into embryos irradiated with 3 Gy X-rays and nonirradiated control embryos. Genetic cross-test analysis revealed that the germline transmission rate in the 3-Gy-irradiated group was significantly higher than in the control group (27.5 *vs.* 5.6%; $P<0.05$). In conclusion, X-irradiation reduced the number of endogenous PGCs and increased the germline transmission of transferred PGCs in chimeric chickens.
Key words: Chicken, Germline chimera, Primordial germ cell, X-ray

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Primordial germ cells (PGCs) are the founder germline cells. In chickens, PGCs are scattered in the center of the blastodisc of freshly oviposited eggs (stage X: Roman numerals refer to the staging system of Eyal-Giladi and Kochav [1]). Following the formation of the primitive streak, PGCs move passively to the anterior border of the extraembryonic region, the so-called germinal crescent region [2]. They then enter the developing vascular network in the germinal crescent region and are transported by the embryonic circulation to the intermediate mesoderm, where they leave the blood vessels and migrate to the genital ridge [3, 4]. After settling in the gonads, the PGCs proliferate and then differentiate into functional gametes.

A technique for producing live offspring from isolated PGCs following their intravascular transplantation into developing embryos was initially established in chickens in 1993 [5]. Chicken PGCs can be stored at –196 C using a simple protocol, without losing their ability to transmit to the germline [6–8]. In addition, a novel method for long-term culture of PGCs that maintains their commitment to the germ cell lineage has recently been developed in chickens [9]. PGCs have therefore received considerable attention as a potentially valuable resource for genetic conservation, genetic modification and germline stem cell research in chickens. However, further development of such applied research using PGCs requires the efficiency of obtaining donor-derived offspring to be improved. The proportion of donor-derived gametes seems to be determined by the ratio between the numbers of donor- and recipient-derived germ cells in the recipient gonads. Accordingly, two approaches can be used to improve germline chimerism: one approach is increasing the number of PGCs transferred, and the other is decreasing the number of endogenous PGCs in the recipient embryos. Increasing the number of PGCs transferred increases the number of exogenous PGCs migrating into the gonads of recipient embryos [10], and the germline transmission rate would thus be expected to increase using this approach. In contrast, endogenous PGCs can be removed following administration of the alkylating agent busulfan (1,4-butanediol dimethanesulfonate) [11–13]. In regard to the latter, our group has developed an efficient method for delivering busulfan to developing chicken embryos using a sustained-release emulsion [14]. This method enables the efficient and stable depletion of endogenous PGCs. When

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donor PGCs were transferred into recipient embryos treated with busulfan, the germline transmission rate was increased 16.6-fold compared with control, untreated embryos [15]. The production of germline chimeric chickens usually involves the transfer of donor PGCs into the bloodstream of recipient embryos between 48 and 64 h of incubation (stages 13–17: Arabic numerals refer to the staging system of Hamburger and Hamilton [16]). Because the maximal interval after administration is 64 h, the sterilizing effect of the drug used to destroy the endogenous PGCs interferes with the transferred PGCs [17], and relatively more donor PGCs are therefore needed for germline chimera production using this method. Thus although this method seems to be the most efficient currently available method for increasing germline chimerism, it is not appropriate if the number of donor PGCs is limited. Alternative methods for removing endogenous PGCs without damaging the transferred PGCs are needed in such situations.

Two approaches are available for reducing the number of endogenous PGCs in chickens without interfering with transferred PGCs: one is drawing blood, and the other is irradiation. However, drawing blood from recipient embryos prior to the transfer of donor PGCs resulted in only a slight improvement in germline chimerism because only about a third of the total blood was removed, leaving endogenous PGCs intact [18]. Irradiation with soft (low-energy) X-rays can effectively reduce the number of endogenous PGCs in chickens [19–21] and quail [22]. Because the sterilizing effects on PGCs are restricted to the irradiation period, donor PGCs would proliferate in irradiated recipient embryos after transplantation. However, no studies have been reported that have assessed the feasibility of X-irradiation as a means of increasing germline transmission of transferred PGCs.

In this study, we therefore developed a simple method using X-irradiation to reduce endogenous PGCs in chicken embryos. The utility of applying X-irradiation to the recipient embryos proper to enhance the germline transmission of exogenous PGCs was demonstrated.

Materials and Methods

Animal care and use

All animal care and use in this study was conducted in accordance with the animal experimentation guidelines issued by the Animal Care and Use Committee of Shinshu University. White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens were maintained at the Faculty of Agriculture farm, Shinshu University.

Experimental design

The use of X-irradiation for preparing recipient embryos for germline chimera production requires that the irradiation conditions be optimized to efficiently deplete the endogenous PGCs and maintain high hatchability. Irradiation can be performed up until transfer of donor PGCs, but the timing is important. Lim *et al*. [21] reported that although exposure of chicken embryos to soft X-rays at stages X, 9, and 14 resulted in similar decreases in the numbers of endogenous PGCs, the hatching rate increased with the age of the embryos at the time of irradiation. In the present study, chicken embryos were therefore exposed to various doses of X-rays at stages 13–15. In the first experiment, WL embryos at stages 13–15 were exposed to X-rays at selected doses (0, 3, 6 and 9 Gy), and embryonic development, teratogenicity and hatchability were monitored. In the second experiment, the numbers of endogenous PGCs in the whole gonads in stage-27 embryos irradiated with selected doses of X-rays (0, 3, 6 and 9 Gy) were monitored by immunohistochemical analysis. In the third experiment, sets of 100 PGCs isolated from BPR chickens were transferred into irradiated (3 Gy) and nonirradiated recipients, and germline transmission rates of donor PGCs were compared by test-cross analysis.

Embryo culture and X-irradiation

In the present study, chicken embryos were therefore exposed to various doses of X-rays at stages 13–15. Intact WL embryos were incubated normally for 52 h to obtain stage 13–15 embryos at 39.0 C and a relative humidity of 50–60%, with tilting at 90° once an hour, in a forced-air incubator (P-008B Biotype; Showa Furankli, Saitama, Japan). The eggs were then placed with the narrow end upwards on a turntable (6 rpm) installed in the chamber of an X-ray apparatus (MBR-1505R2; Hitachi Medical, Tokyo, Japan). To expose 3, 6 and 9 Gy at rate of 0.12 Gy/min, embryos were X-irradiated for 25, 50 and 75 min, respectively. It is difficult to keep the temperature at 39.0 C during irradiation due to lack of a heating feature of this apparatus. To ignore the effects of temperature change during irradiation, embryos in each group were temporarily incubated at room temperature (approximately 26 C) for 75 min. A window with a diameter of 10–15 mm was opened in the narrow end, and a portion (approximately 4–8 ml) of albumen was discarded. The window was sealed with cling film wrap, and the embryos were incubated until they reached stage 27 or until hatching under the same forced-air incubator conditions with tilting at 30° once every 2 h.

Immunohistochemistry and counting of PGCs

Whole gonads with the attached adjacent mesonephros were collected from stage 27 embryos, fixed overnight in 4% paraformaldehyde (PFA) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(–)) and, then 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 then rinsed twice with PBS-T for 20 min each. Immunostaining was performed using the biotin/avidin-conjugated horseradish peroxidase system (VECTASTAIN Elite ABC Rabbit IgG Kit; Vector Laboratories, Burlingame, CA, USA) 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-chicken Vasa homolog antibody [4] and then rinsed three times in PBS-T for 25 min each. Gonads were incubated with biotinylated secondary antibody (1:200) overnight and then washed three times with PBS-T for 25 min each. After incubation with VECTASTAIN 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 conducted at 4 C. Both left and right gonads were detached from the mesonephros, and labeled cells in the stained gonads were counted under a microscope (DFC480-Note OY; Leica Microsystems, Tokyo, Japan).

Table 1. Effects of X-irradiation on teratogenicity and the development of chicken embryos

Dose (Gy)	No. of embryos	Percentage of embryos showing abnormalities	Incubation period until stage 27	Developmental delay* (compared with controls)
Nonirradiated controls	50	0[a]	5 d	NA
3	46	6.5[ab]	5 d	0 d
6	38	15.8[ab]	5.5 d	0.5 d
9	17	35.3[b]	6 d	1 d

* NA, not available. [a, b] $P<0.05$.

Table 2. Survival and hatching rates of chicken embryos in windowed eggs with and without X-irradiation

Dose (Gy)	No. of embryos	Survival of embryos on incubation day (%)						Hatched (%)
		3	6	10	14	17	20	
Nonirradiated controls	92	98.9	96.7[a]	94.6[a]	90.2[a]	89.1[a]	87.0[a]	69.6[a]
3*	20	100.0	95.0[ab]	90.0[ab]	90.0[ab]	90.0[a]	80.0[ab]	40.0[ab]
6*	42	95.2	78.6[b]	69.0[b]	57.1[b]	50.0[b]	42.9[b]	28.6[b]
9*	104	93.3	28.8[c]	17.3[c]	11.5[c]	5.8[c]	19.2[c]	0[c]

* Embryos incubated for 52 h were irradiated. [a–d] Means with different superscripts in the same column are significantly different (χ^2 test, $P<0.05$).

Preparation and transfer of donor PGCs

Fertilized BPR eggs were normally incubated in the same conditions as described above for 55 h to obtain embryos at stages 14–16. Blood (3–7 µl) was collected from the dorsal aorta and peripheral vein of the embryos using a fine glass micropipette under a microscope (MS5; Leica Microsystems). Each collected blood sample was added to 100 µl of PBS(–). PGCs were concentrated from embryonic blood by Nycodenz density gradient centrifugation [23] with minor modifications. Briefly, PBS(–) containing 10% fetal bovine serum was used as a buffer instead of KAv-1 medium [24]. Chicken PGCs are easily distinguished from erythrocytes by their large size and the presence of a considerable number of refractive granules in the cytoplasm when observed under phase contrast microscopy (IX71; Olympus, Tokyo, Japan) [25]. One hundred PGCs were picked up from the concentrated cell suspensions using fine glass micropipettes and microinjected intravascularly into irradiated (3 Gy) and nonirradiated recipient WL embryos.

Genetic cross-testing

Donor PGCs were obtained from BPR chickens, which have pigmented feathers and are homozygous recessive (*i*/*i*) for dominant white. The recipient WL embryos have white feathers and are homozygous dominant (*I*/*I*) for dominant white. Presumptive germline chimeric chickens derived from PGC transfers that survived to sexual maturity were mated with BPR (*i*/*i*) chickens by artificial insemination, and the feather color of their offspring was examined. Black offspring (*i*/*i*) were derived from BPR donor PGCs, whereas white offspring with small patches of black pigmentation (*I*/*i*) were derived from recipient WL PGCs.

Production of BPR progenies by crossing germline chimeras

To produce BPR offspring using sperm and eggs derived from donor PGCs, male and female germline chimeras in the irradiated group were mated using artificial insemination, and the feather color of the offspring was examined.

Statistical analysis

All data are presented as means ± SEM. The survival and hatching rates of the embryos in each of the four groups were analyzed using χ^2 tests. The numbers of endogenous PGCs in the whole gonads in each of the four groups were analyzed by one-way ANOVA. If the model effect was significant, differences between mean values for each treatment were then evaluated using the Bonferroni test. Differences in the proportions of donor-derived offspring were compared between the irradiated- and the nonirradiated control groups using unpaired Student's *t*-tests. Statistical significance was set at $P<0.05$.

Results

Embryonic development and hatchability

The effects of X-irradiation on teratogenicity and the development of chicken embryos are shown in Table 1. The teratogenic effects on chicken embryos observed at stage 27 increased with the X-ray dose. The most common abnormalities in the irradiated embryos were abnormal limb buds and small eyes. Apparent anemia was also observed after irradiation of embryos in the 6- and 9-Gy-irradiated groups. No developmental delay was observed in the 3-Gy-irradiated group compared with the nonirradiated control group, while embryos irradiated with 6 or 9 Gy of X-rays showed delayed development. Embryos in the nonirradiated control and 3-Gy-irradiated groups took 5 days of incubation to reach stage 27 compared with 5.5 and 6 days in the 6- and 9-Gy-irradiated groups, respectively. The effects of X-irradiation on the survivability and hatchability of chicken embryos are shown in Table 2. There were no significant differences in survival and hatching rates of chicken embryos between the 3-Gy-irradiated group and the nonirradiated control group. However, when embryos were exposed to 6 Gy or 9 Gy of X-rays, the survival rates of irradiated embryos decreased significantly between days 3 and

6 of incubation compared with nonirradiated control embryos. The hatching rate of chicken embryos in the 6-Gy-irradiated group was significantly lower than that in the nonirradiated control group. No chicken embryos irradiated with 9 Gy of X-rays survived to hatching.

Removal of endogenous PGCs in embryonic gonads

Immunohistochemical analysis confirmed that the three irradiated groups (3, 6 or 9 Gy) had fewer endogenous PGCs than the nonirradiated control group (Fig. 1, A–D). The numbers of endogenous PGCs in whole gonads of embryos at stage 27 in each group are shown in Fig. 1E. The relative number (range) of endogenous PGCs in whole mounts of stage-27 gonads in nonirradiated controls was 741.3 ± 44.8 (513–1076). The mean number of endogenous PGCs in whole mounts of gonads at stage 27 was reduced by X-irradiation in a dose-dependent manner ($P<0.05$), with the corresponding values for the 3-, 6- and 9-Gy-irradiated groups being 155.5 ± 28.4 (40–347), 71.3 ± 16.5 (7–236) and 34.2 ± 7.4 (6–69), respectively.

Germline transmission of donor PGCs

For germline chimera production, WL embryos were irradiated with 3 Gy of X-rays. These embryos and nonirradiated controls then received sets of 100 PGCs isolated from BPR embryos. In the 3-Gy-irradiated group, 18 embryos were manipulated, five (1 male and 4 female) hatched, and all hatchlings reached sexual maturity. In the nonirradiated control group, 17 embryos were manipulated, 11 hatched (4 males and 7 female), and six (2 male and 4 female) survived to sexual maturity. As shown in Table 3, sexually mature chickens in both the 3-Gy-irradiated group (3G-1, 2, 4, 13 and 14) and nonirradiated control group (C-4, 9, 10, 12, 15 and 16) were stable germline chimeras that produced black offspring (BPR) derived from donor BPR PGCs (Fig. 2). The frequency of donor-derived offspring from germline chimeras in the 3-Gy-irradiated group (27.5 ± 4.4%) was significantly higher than in the nonirradiated control group (5.6 ± 2.1%) ($P<0.05$).

Production of donor PGC-derived chickens by mating germline chimeras

A female germline chimera (3G-13) was artificially inseminated with semen from a male germline chimera (3G-4). Eighteen chickens were obtained from this combination of germline chimeras; three were black offspring (BPR) derived from donor BPRs. Further trials could not be performed due to the death of the male chimeric chicken.

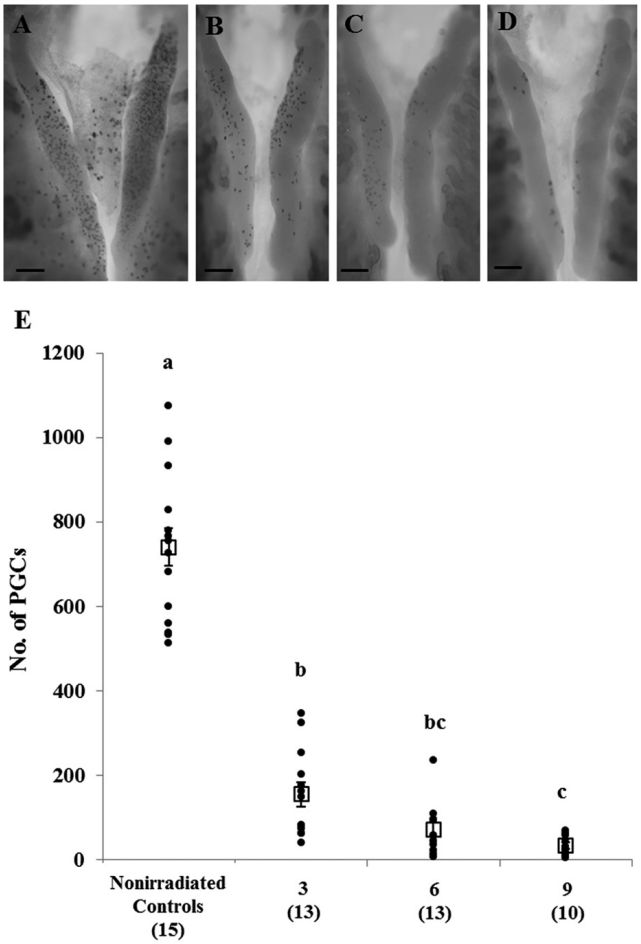

Fig. 1. The number of endogenous PGCs in embryonic gonads at stage 27. Endogenous PGCs were detected by immunostaining using a specific antibody raised against chicken Vasa homolog protein. A: Nonirradiated control embryo. Embryos irradiated with doses of 3 (B), 6 (C) and 9 (D) Gy of X-rays. Bar = 100 μm. E: The numbers of endogenous PGCs in whole gonads of stage 27 embryos were decreased by X-irradiation in a dose-dependent manner. Means with different superscripts are significantly different ($P<0.05$). The number of embryos evaluated is shown below each treatment.

Discussion

This is the first report to demonstrate the feasibility of X-irradiation of recipient embryos for increasing the germline transmission of donor PGCs. The method was very simple; intact chicken eggs were exposed to X-rays after 52 h of incubation, resulting in a significant reduction in the number of endogenous PGCs and a significant increase in germline transmission of exogenous PGCs.

Exposure of chicken embryos to X-rays at stages 13–15 in this study caused malformations such as abnormal limb buds and small eyes, and these teratogenic effects increased with increasing X-ray dose. Chicken embryos with developmental abnormalities did not survive to hatching. In addition to teratogenicity, anemia was also apparent in chicken embryos in the 6- and 9-Gy-irradiated groups. The survivability of chicken embryos irradiated with 6 or 9 Gy of X-rays decreased significantly between days 3 and 6 of incubation compared with nonirradiated control embryos as a result of teratogenic effects and anemia. Irradiation of chicken embryos with soft X-rays (around 0.7 Gy) at stage X resulted in delayed development [19, 21]. Chicken embryos exposed to 6 or 9 Gy of X-rays in this study manifested delayed development, which persisted until hatching in the 6-Gy-irradiated group. Chicken embryos in the 9-Gy-irradiated group did not survive to hatching, and this dosage was therefore considered to be lethal when embryos were irradiated at stages 13–15. The hatching rate of nonirradiated control embryos in this study was slightly lower than that in our previous study [15] (69.6 *vs.* 74.3%), probably due to the temporary drop in temperature.

Electromagnetic waves such as ultraviolet, X- and gamma-rays

Table 3. Genetic cross-test of germline chimeras produced after transfer of primordial germ cells from Barred Plymouth Rock embryos into White Leghorn embryos with and without X-irradiation

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 (%)
X-irradiation (3 Gy)				
3G-1	Female	34	83	29.1
3G-2	Female	8	18	30.8
3G-4	Male	60	114	34.5
3G-13	Female	52	107	32.7
3G-14	Female	9	77	10.5
Mean				27.5 ± 4.4[a]
Nonirradiated controls				
C-4	Female	1	83	1.2
C-9	Female	4	28	12.5
C-10	Female	4	30	11.8
C-12	Female	2	108	1.8
C-15	Male	3	123	2.4
C-16	Male	6	139	4.1
Mean				5.6 ± 2.1[b]

[a, b] $P<0.05$.

Fig. 2. Phenotypes of offspring from a female germline chimera. Black offspring were derived from donor BPR PGCs; white offspring with small patches were derived from recipient WL PGCs. The white hen is a germline chimera (3G-13).

are known to induce DNA damage, cell cycle arrest and apoptosis. Susceptibility to irradiation varies depending on the type of cells and tissues, and germ cells are much more sensitive than somatic cells [26]. Irradiation of chicken embryos with X-rays at stages 13–15 in the present study resulted in a significant dose-dependent reduction in the number of endogenous PGCs compared with the numbers in nonirradiated controls. The relative numbers of the 3-, 6- and 9-Gy-irradiated groups were 21.0, 9.6 and 4.6% of the nonirradiated control numbers, respectively. These results suggest that X-irradiation inhibits the mitotic activity of chicken PGCs, possibly by inducing apoptosis. Similarly, exposure to soft X-rays has previously been found to reduce the number of endogenous PGCs in early chicken embryos [18–21]. When chicken embryos were irradiated with 2 Gy of soft X-rays at stage X, the number of endogenous PGCs decreased to 43.9% of the nonirradiated control numbers [19]. Apart from X-rays, ultraviolet and gamma rays have also been used to decrease the number of endogenous PGCs in chicken embryos. Exposure of chicken embryos to ultraviolet radiation at stages 4–10 (18–38 h of incubation) resulted in a reduction in endogenous PGCs to less than 30% of the nonirradiated control number, though this method also caused a number of abnormalities and mortalities [27]. Gamma irradiation of stage X chicken embryos resulted in gradual dose-dependent decreases in the number of endogenous PGCs and hatchability [28]. In that study, exposure to 5 Gy of gamma radiation significantly decreased the number endogenous PGCs to 32.3% of the nonirradiated control number. Electromagnetic waves thus appear to have similar effects on removing PGCs in early chicken embryos, though the effects vary slightly depending on the radiation form, dosage and embryo age. X-ray irradiation in the present study appeared to be as effective at removing endogenous PGCs in chicken embryos as irradiation with soft X-rays, ultraviolet light or gamma radiation in previous studies [19, 21, 27, 28].

No direct evidence for an increase in germline transmission of donor PGCs following exposure of recipient chicken embryos to micromagnetic waves has been provided. Carsience *et al*. [29] succeeded in producing a somatic chimeric chicken, as well as a germline chimeric chicken, which yielded only donor-derived offspring, by injecting 200–400 blastodermal cells into a stage X embryo that had been exposed to a gamma source (5–7 Gy) prior to injection. However, the frequency of donor-derived offspring in their study was only 6.3%, with considerable variation. Moreover, this method of producing chimeras is not appropriate for evaluating the effects of irradiation because unknown numbers of PGCs were introduced. The present study therefore adopted a comparative approach to evaluate the feasibility of X-irradiation as a means of

increasing the germline transmission of donor PGCs. A dose of 3 Gy was found to be optimal for preparing recipient embryos, taking into consideration the depletion of endogenous PGCs, teratogenicity and hatchability. Hence, sets of 100 PGCs isolated from BPR embryos were transferred into the vascular systems of recipient WL embryos irradiated with 3 Gy of X-rays and nonirradiated controls. Cross-breeding analysis revealed that the germline transmission of donor PGCs into 3-Gy-irradiated chickens was increased 4.9-fold compared with nonirradiated controls (27.5 *vs.* 5.6%). When blood (containing about 250 PGCs) from quail embryos at stages 13–14 was intravascularly injected into chicken embryos irradiated with 7.2 Gy of soft X-rays, the number of donor-derived quail germ cells in the left gonads of chicken embryos at stage 30 increased more than two-fold compared with nonirradiated control embryos [20]. Our previous results, therefore, suggest that the proliferative activity of donor PGCs is promoted after transplantation into irradiated recipient embryos when the proliferation of endogenous PGCs is inhibited. This will increase the proportion of donor-derived germ cells in the recipient gonads. These advantages of the radiation-based approach make it suitable for the production of germline chimeric chickens using a limited number of PGCs.

In conclusion, the exposure of recipient chicken embryos to 3 Gy of X-rays significantly reduced the number of endogenous PGCs and increased the germline transmission of donor PGCs after transplantation, with no significant reduction in hatchability. This simple method for producing germline chimeras using X-irradiation provides an important insight into PGC-mediated reproductive technology in chickens.

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References

1. **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. [Medline] [CrossRef]
2. **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. [Medline]
3. **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. [Medline]
4. **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. [Medline]
5. **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. [Medline] [CrossRef]
6. **Naito M, Tajima A, Tagami T, Yasuda Y, Kuwana T.** Preservation of chick primordial germ cells in liquid nitrogen and subsequent production of viable offspring. *J Reprod Fertil* 1994; **102**: 321–325. [Medline] [CrossRef]
7. **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. [CrossRef]
8. **Nakamura Y, Usui F, Miyahara D, Mori T, Ono T, Takeda K, Nirasawa K, Kagami H, Tagami T.** Efficient system for preservation and regeneration of genetic resources in chicken: concurrent storage of primordial germ cells and live animals from early embryos of a rare indigenous fowl (Gifujidori). *Reprod Fertil Dev* 2010; **22**: 1237–1246. [Medline] [CrossRef]
9. **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. [Medline] [CrossRef]
10. **Kim JN, Park TS, Park SH, Park KJ, Kim TM, Lee SK, Lim JM, Han JY.** Migration and proliferation of intact and genetically modified primordial germ cells and the generation of a transgenic chicken. *Biol Reprod* 2010; **82**: 257–262. [Medline] [CrossRef]
11. **Aige-Gil V, Simkiss K.** Sterilisation of avian embryos with busulphan. *Res Vet Sci* 1991; **50**: 139–144. [Medline] [CrossRef]
12. **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. [Medline] [CrossRef]
13. **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. [Medline] [CrossRef]
14. **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. [Medline] [CrossRef]
15. **Nakamura Y, Usui F, Ono T, Takeda K, Nirasawa K, Kagami H, Tagami T.** Germline replacement by transfer of primordial germ cells into partially sterilized embryos in the chicken. *Biol Reprod* 2010; **83**: 130–137. [Medline] [CrossRef]
16. **Hamburger V, Hamilton HL.** A series of normal stages in the development of the chick embryo. *J Morphol* 1951; **88**: 49–92. [CrossRef]
17. **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. [CrossRef]
18. **Nakamichi H, Sano A, Harumi T, Matsubara Y, Tajima A, Kosugiyama M, Naito M.** Effects of soft X-ray irradiation to stage X blastoderm on restriction of proliferation of primordial germ cells in early chicken embryos. *J Poult Sci* 2006; **43**: 394–400. [CrossRef]
19. **Atsumi Y, Tagami T, Kagami H, Ono T.** Restriction of germline proliferation by soft X-ray irradiation of chicken embryos and its application to chimera production. *J Poult Sci* 2008; **45**: 292–297. [CrossRef]
20. **Atsumi Y, Yazawa S, Usui F, Nakamura Y, Yamamoto Y, Tagami T, Hiramatsu K, Kagami H, Ono T.** Depletion of primordial germ cells (PGCs) by X-irradiation to extra-embryonic region of chicken embryos and expression of xenotransplanted quail PGCs. *J Poult Sci* 2009; **46**: 136–143. [CrossRef]
21. **Lim JM, Kwon HM, Kim DK, Kim JN, Park TS, Ono T, Han JY.** Selective decrease of chick embryonic primordial germ cells *in vivo* and *in vitro* by soft X-ray irradiation. *Anim Reprod Sci* 2006; **95**: 67–74. [Medline] [CrossRef]
22. **Li HC, Kagami H, Matsui K, Ono T.** Restriction of proliferation of primordial germ cells by the irradiation of Japanese quail embryos with soft X-rays. *Comp Biochem Physiol A Mol Integr Physiol* 2001; **130**: 133–140. [Medline] [CrossRef]
23. **Zhao DF, Kuwana T.** Purification of avian circulating primordial germ cells by nycodenz density gradient centrifugation. *Br Poult Sci* 2003; **44**: 30–35. [Medline] [CrossRef]
24. **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. [Medline]
25. **Fujimoto T, Ukeshima A, Kiyofuji R.** The origin, migration and morphology of the primordial germ cells in the chick embryo. *Anat Rec* 1976; **185**: 139–153. [Medline] [CrossRef]
26. **Gladstone RJ, Colwell HA.** On some effects of X-rays on the developing chick embryo. *J Anat Lond* 1933; **68**: 87–95.
27. **Aige-Gil V, Simkiss K.** Sterilizing embryos for transgenic chimeras. *Br Poult Sci* 1991; **32**: 427–438. [Medline] [CrossRef]
28. **Park KJ, Kang SJ, Kim TM, Lee YM, Lee HC, Song G, Han JY.** Gamma-irradiation depletes endogenous germ cells and increases donor cell distribution in chimeric chickens. *In Vitro Cell Dev Biol Anim* 2010; **46**: 828–833. [Medline] [CrossRef]
29. **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. [Medline]