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Migratory Ability of Gonadal Germ Cells (GGCs) Collected from 5, 7, and 10-day-old Chick Embryos

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Abstract

In order to evaluate the migratory ability of the gonadal germ cells, embryonic gonads were collected from 5-, 7- and 10-day-old White Leghorn (WL) embryos and dispersed in medium containing 0.25% trypsin. Gonadal germ cells (GGCs) were collected and labeled with fluorescent dye (PKH-26). Approximately 150 GGCs were injected into the dorsal aorta of 2-day-old recipient embryos, which were then incubated for 5 days in a forced-air incubator. After incubation, gonads from recipient embryos were recovered, dispersed using 0.25% trypsin, and observed under a fluorescence microscope. Fluorescence-labeled GGCs were detected in the gonads of recipient embryos injected with GGCs from 5- and 7-day-old chick embryos, but not from 10-day-old chick embryos. These results indicate that GGCs retain the migratory ability for at least 7 days of incubation *in vivo*.

Key words : Chicken, Gonadal germ cells, Germ-line chimeras, Migration

Introduction

Recent advancements in the technique that produces germ-line chimeras in domestic chickens provide the means to answer some fundamental biological questions related to germ-line development in avian species. Primordial germ cells (PGCs), predecessors of spermatogonia or oogonia, arise from the central zone of the area pellucida (Ginsburg and Eyal-Giladi, 1987; Kagami *et al.*, 1997) and gradually translocate to the lower layer during the early stages of primitive streak formation (Sutasurya *et al.*, 1983; Ginsburg and Eyal-Giladi, 1986). The PGCs then migrate anteriorly via the hypoblast and translocate in the germinal crescent (Swift, 1914; Ginsburg and Eyal-Giladi, 1986).

Upon development of the vascular system, by Day 2 of incubation, the PGCs circulate temporarily through the blood stream along with blood cells. The concentration of PGCs is reported to be high at stages 14 to 15, decreasing rapidly thereafter (Tajima *et al.*, 1999).

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Circulating PGCs then escape from the vascular system and migrate into the germinal ridge (Swift, 1914; Fujimoto *et al.*, 1976; Ando and Fujimoto, 1983). After this migration, gonadal germ cells (GGCs) are known to proliferate and finally differentiate to either spermatogonia in the testis (Swift, 1916) or oogonia in the ovary (Swift, 1915).

The migrating nature of the PGCs has been harnessed to produce germ-line chimeras in domestic chicken by transferring PGCs (Yasuda *et al.*, 1992; Tajima *et al.*, 1993, 2003; Naito *et al.*, 1994a,b). Various strategies for conserving avian genetic resources have also been reviewed (Tajima, 2002).

Chang *et al.* (1995) reported that GGCs recovered from the gonadal ridge of embryos cultured *in vitro* for 5 days (stage 27) can still migrate into the germinal ridge of 2-day-old chick embryos when injected into their blood stream. Furthermore, the production of germ-line chimeras was confirmed by a progeny test using chickens injected with GGCs collected from embryos that were cultured for 5 days (Chang *et al.*, 1997). Germ-line chimeras have also been produced by transferring frozen-thawed GGCs collected from gonads of 5-day-old chick embryos (Tajima *et al.*, 1998) or incubated for 53 hours in Japanese quail (Chang *et al.*, 1998). The results of these experiments suggest the need to evaluate the migrating ability of GGCs recovered from embryo gonads that have been incubated for more than 5 days.

Therefore, the present experiment was conducted to build on previous experiments by evaluating the migrating ability of GGCs collected from the gonads of 5-, 7-, and 10-day-old chick embryos.

Materials and Methods

Fertilized White Leghorn (WL) eggs produced at the Agricultural and Forestry Research Center, University of Tsukuba, Japan, were used in the experiment. The eggs were incubated at 37.8℃ for 5-, 7-, or 10-days in a forced-air incubator (P-008, Showa Incubator Laboratory, Japan) and used as germ-cell donors.

The embryo proper was separated from the egg yolk and placed in a plastic dish filled with phosphate buffered saline (PBS) solution without Ca^{2+} or Mg^{2+} (PBS(-), cat #05913, Nissui, Japan). The embryo was rinsed thoroughly in PBS(-) to remove egg yolk. Embryonic gonads were excised under a dissecting microscope (SZH, Olympus, Japan) using a 30G needle (Terumo, Japan). The gonads were placed in 0.25% trypsin solution in PBS(-) for 5 minutes at 37℃ and gently suspended using a pipette. Bovine serum (cat #10082-139, GIBCO-BRL, USA) was then added to terminate the enzyme reaction. The gonadal cell suspension was transferred to a 1.5-ml centrifuge tube and centrifuged at 800 *g* for 15 minutes. After discarding the supernatant, the gonadal cells were resuspended in 500 μl Minimum Essential Medium (MEM; 12370-037, GIBCO BRL, USA) at 37℃.

Gonadal germ cells collected from 5-, 7-, and 10-day embryos were labeled with fluorescent dye (PKH-26, Dainippon-Seiyaku Corp, Japan). The half-life of PKH-26 is more than 100 days, according to the manufacturer. Labeling of the GGCs was performed according

to the manufacturer's instructions, except that the concentration of the fluorescent dye was increased tenfold. Fluorescence-labeled GGCs were kept in an incubator at 37℃ until they were injected into recipient embryos. Care was taken to avoid exposing the samples to ultraviolet light during incubation. Embryos that reached stages 14 to 15 were used as recipients of GGCs.

The surface of the eggshell was swabbed with 70% ethanol before use. A hole of approximately 1.5 cm diameter was opened at the sharp end of the egg. Approximately 150 GGCs (as judged by morphology) were collected in a fine glass pipette under an inverted microscope (IMT-2, Olympus, Japan), and were then injected into the dorsal aorta of the embryo under a dissection microscope. Care was taken to avoid hemorrhage during injection. After injection, the hole was sealed with plastic tape (Scotch mailing tape, 3M, USA), and the recipient embryo was incubated for 5 days in a forced-air incubator until it was 7 days old.

Gonads were isolated from the 7-day-old recipient embryos, and GGCs were in turn isolated from the gonads using the same methods described previously. The GGCs were observed under an inverted fluorescence microscope (IMT-2-RTF; excitation 490 nm, emission 515 nm). Cells emitting fluorescence and displaying typical GGC morphology were judged to be injected GGCs and their descendent cells. The number of GGCs emitting fluorescence was counted using a hemocytometer (Thoma) under an inverted florescence microscope.

Results

The GGCs collected from 5-, 7-, and 10-day-old chick embryos are referred to as GGC(5), GGC(7), and GGC(10), respectively. The proportions of live embryos and embryos with fluorescence-labeled GGCs, 5 days after GGC transfer, are shown in Table 1. The proportions of live embryos for GGC(5), GGC(7), and GGC(10) were 82.8%(24/29), 72.0% (36/50) and 65.6%(21/32), respectively. No significant differences were observed among treatments ($P>0.05$). The proportions of live embryos with fluorescence-labeled GGCs for

Table 1 Migration of Gonadal Germ Cells (GGCs) collected from 5-, 7- and 10-day-old chick embryos.

	Age of GGC-donor embryo		
	5 days	7 days	9 days
Number of Eggs Received GGCs	29	50	32
Number of Live Embryos 5 Days After GGC Transfer	24 (♀9 ♂15) 82.8%	36 (♀16 ♂20) 72.0%	21 (♀12 ♂9) 65.6%
Number of Live Embryos with Fluorescence-Labeled GGCs	10 (♀5 ♂5) 41.7%[a]	14 (♀5 ♂9) 38.9%[a]	0 (♀0 ♂0) 0.0%[b]

* Different letters in the same row differ significantly ($P<0.05$).

GGC(5), GGC(7) and GGC(10) were 41.7%(10/24), 38.9%(14/36), and 0%(0/21), respectively. The proportion was significantly lower in GGC(10) than in GGC(5) and GGC(7) ($P<0.05$), whereas no significant difference was observed between the proportions for GGC (5) and GGC(7).

Discussion

In the present experiment, we tested the migrating ability of GGCs collected from the gonadal ridge of 5-, 7-, and 10-day-old embryos *in vivo*. Results showed that the GGCs isolated from 5- and 7-day-old, but not 10-day-old, chick embryos can migrate toward the gonadal ridge. Although inconclusive, this result may indicate that the germ cells complete differentiation to spermatogonia in the testis or oogonia in the ovary by day 10 of incubation *in vivo*. The mechanism of germ cell differentiation needs to be studied critically in the future.

From the practical point of view, there are two major advantages of using GGCs instead of circulating PGCs to produce germ-line chimeras: higher numbers of germ cells in the gonad, and flexibility in the timing of germ cell collection. Circulating PGCs are reported to be at their highest concentration in blood during developmental stages 14 and 15 (Tajima *et al.*, 1999). However, the number of circulating PGCs that can be collected from each egg is unpredictable, due to considerable variation in the number of circulating PGCs among embryos (Tajima *et al.*, 1999). Furthermore, considerable fluctuation in developmental stage among eggs, even those at the same point in incubation, has been observed (data not shown).

In contrast, the gonads of embryos can be recovered at any predetermined time and hundreds of GGCs can be collected from each gonad provided that the embryo grows normally. In fact, $1,750\pm363$ GGCs were isolated from a 5-day-old chick embryo with trypsin (Allioli *et al.*, 1994). The temporal flexibility of germ cell collection is especially important when conserving genetic resources in endangered avian species, in which eggs are valuable and the exact timetable of embryonic growth is often unknown.

The biggest problem with the use of GGCs is their identification and isolation from large numbers of somatic cells originating from the gonadal tissue, as visual identification of GGCs can be difficult. Injected GGCs are similar in morphology to circulating PGCs isolated from the blood of stage 14 chick embryos: both are large, round cells with large nuclei. A marker needs to be developed that can be used to identify live GGCs without impairing their physiological function.

The efficiency of germ-line chimera production is influenced by the male-female combination between germ cell donor and recipient (Naito *et al.*, 1999). In this study we used pooled GGCs without confirming the sex of the embryos. Future studies should take into account the male-female combination of embryos.

In conclusion, our results indicate the possibility of producing progeny by transferring GGCs that have been isolated from the gonads of 7-day-old chick embryos. Future studies

should include progeny testing of offspring derived from GGCs collected from embryos that are 7 days old and older.

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References

Allioli, N. Thomas, J.L. Cheblounе, Y. Nigon, V.M. Verdier, G. and Legras, C. 1994. Use of retroviral vectors to introduce and express the beta-galactosidase marker gene in cultured chicken primordial germ cells. Developmental Biology 165: 30-7.

Ando, Y. and Fujimoto, T. 1983. Ultrastructural evidence that chick primordial germ cells leave the blood vascular system prior to migration to the gonadal anlagen. Development, Growth and Differentiation 25: 345-352.

Chang, I.K. Jeong D.K. Hong Y.H. Park T.S. Moon Y.K. Ohno T. and Han J.Y. 1997. Production of germline chimeric chickens by transfer of cultured primordial germ cells. Cell Biology International Reports 21: 495-9.

Chang, I.-K. Naito, M. Kuwana, T. Mizutani, M. and Sakurai, M. 1998. Production of germline chimeric quail by transfer of gonadal primordial germ cells preserved in liquid nitrogen. Japanese Poultry Science 35: 321-328.

Chang, I.K. Yoshiki A. Kusakabe, M. Tajima, A. Chikamune, T. Naito, M. and Ohno, T. 1995. Germ line chimera produced by transfer of cultured chick primordial germ cells. Cell Biology International Reports 19: 569-76.

Fujimoto, T. Ukeshima, A. and Kiyofuji, R. 1976. The origin, migration and morphology of the primordial germ cells in the chick embryo. Anatomical Record 185: 139-53.

Ginsburg, M. and Eyal-Giladi, H. 1986. Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo. Journal of Embryology and Experimental Morphology 95: 53-71.

Ginsburg, M. and Eyal-Giladi, H. 1987. Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process. Development 101: 209-19.

Kagami, H. Tagami, T. Matsubara, Y. Harumi, T. Hanada, H. Maruyama, K. Sakurai, M. Kuwana, T. and Naito, M. 1997. 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. Molecular Reproduction and Development 48: 501-10.

Naito, M. Matsubara, Y. Harumi, T. Tagami, T. Kagami, H. Sakurai, M. and Kuwana, T. 1999. Differentiation of donor primordial germ cells into functional gametes in the gonads of mixed-sex germline chimaeric chickens produced by transfer of primordial germ cells isolated from embryonic blood. Journal of Reproduction and Fertility, 117: 291-298.

Naito, M. Tajima, A. Tagami, T. Yasuda, Y. and Kuwana, T. 1994a. Preservation of chick primordial germ

cells in liquid nitrogen and subsequent production of viable offspring. Journal of Reproduction and Fertility 102: 321-325.

Naito, M. Tajima, A. Yasuda, Y. and Kuwana, T. 1994b .Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Molecular Reproduction and Development 39: 153-61.

Sutasurya, L.A. Yasugi, S. and Mizuno, T. 1983. Appearance of primordial germ cells in young chick blastoderms cultured *in vitro*. Development, Growth and Differentiation 25:517-521.

Swift, C.H. 1914. Origin and early history of the primordial germ cells of the chick. American Journal of Anatomy 15: 483-516.

Swift, C.H. 1915. Origin of the definitive sex cells in the female chick and their relationship to the primordial germ cells. Amer. J. Anat. 18: 441-470,

Swift, C.H. 1916. Origin of the sex cords and the definitive spermatogonia in the male chick. American Journal of Anatomy 20: 375-410.

Tajima, A. 2002 Production of germ-line chimeras and their application in domestic chicken. Avian and Poultry Biology Review. 13: 15-30.

Tajima, A. Barbato, G.F. Kuwana, T. and Hammerstedt, R.H. 2003. Conservation of a genetically selected broiler line (42L) using cryopreserved circulating primordial germ cells (PGCs) isolated by filtration method. Journal of Poultry Science 40: 53-61.

Tajima, A. Hayashi, H. Kamizumi, A. Ogura, J. Kuwana, T. and Chikamune, T. 1999. Study on the concentration of circulating primordial germ cells (cPGCs) in early chick embryos. Journal of Experimental Zoology 284: 759-764.

Tajima, A. Naito, M. Yasuda, Y. and Kuwana, T. 1993. Production of germ line chimera by transfer of primordial germ cells in domestic chicken (*Gallus domesticus*). Theriogenology 40: 509-519.

Tajima, A. Naito, M. Yasuda, Y. and Kuwana, T. 1998. Production of germ-line chimeras by transfer of cryopreserved gonadal primordial germ cells (gPGCs) in chicken. Journal of Experimental Zoology 280, 265-267.

Yasuda, Y. Tajima, A. Fujimoto, T. and Kuwana, T. 1992. A method to obtain avian germ-line chimaeras using isolated primordial germ cells. Journal of Reproduction and Fertility 96: 521-8.

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ニワトリ５日，7日および10日胚の未分化生殖巣に存在する生殖細胞（GGCs）の移住能力に関する研究

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要　約

ニワトリ初期胚の生殖巣に存在する生殖細胞の移住能力を検討することを目的に，白色レグホーン（WL）の５日，７日および10日胚から生殖巣を摘出し，0.25%トリプシンを含む培養液で処理することにより生殖巣細胞懸濁液を調整した。生殖巣細胞をPKH-26を用いて蛍光標識した後，その中に存在する生殖細胞（GGCs）のうち150個を２日胚の大動脈内に移植し，５日間孵卵した。孵卵後，胚から生殖巣を摘出し，0.25%トリプシンを含む培養液で処理した後，蛍光顕微鏡下で観察した。その結果，生殖細胞ドナーとして５日および７日胚の生殖巣から分離されたGGCsを用いた場合，生殖細胞レシピエント胚の生殖巣内に蛍光標識された生殖細胞が認められた。これに対して，生殖細胞ドナーとして10日胚の生殖巣から分離されたGGCsを用いた場合，生殖細胞レシピエント胚の生殖巣内に蛍光標識された生殖細胞は認められなかった。以上の結果から，GGCは少なくとも孵卵７日目までは移住能力を保持していることが明らかになった。

キーワード：移住，生殖系列キメラ，生殖巣内の生殖細胞，ニワトリ

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