Localization of primordial germ cells or their precursors in stage X blastoderm of chickens and their ability to differentiate into functional gametes in opposite-sex recipient gonads

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This study was performed to determine the distribution of primordial germ cells and their precursors in stage X blastoderm of chickens. The blastoderm (Barred Plymouth Rock chickens) isolated from the yolk was separated into three portions: the central disc, the marginal zone and the area opaca. The dissociated blastodermal cells derived from the central disc, marginal zone and area opaca were transferred into a recipient blastoderm (White Leghorn chicken) from which a cell cluster was removed from the centre of the central disc. The manipulated embryos were cultured in host eggshells until hatching. The chicks were raised until sexual maturity and test mated with Barred Plymouth Rock chickens to assess the donor cell contribution to the recipient germline. Germline chimaeric chickens were produced efficiently (46.7%, 7/15) when the blasto-

dermal cells derived from the central disc were transferred into recipient embryos of the same sex, whereas no germline chimaeric chickens were produced when the blastodermal cells derived from the marginal zone or area opaca were transferred into recipient embryos of the same sex (0/12). Germline chimaeric chickens were also produced by transfer of blastodermal cells derived from the central disc (6.7%, 1/15), marginal zone (10.0%, 1/10) or area opaca (11.1%, 1/9) into recipient embryos of the opposite sex. It is concluded that primordial germ cells are induced during or shortly after stage X and that the cells derived from the central disc have the highest potential to give rise to germ cells. Cells derived from the marginal zone and area opaca can also give rise to germ cells, although the frequency is low.

Introduction

Germline segregation in chickens starts at a very early embryonic stage and they first appear as primordial germ cells. Manipulation of primordial germ cells is very useful for producing transgenic chickens and also for preserving genetic material in avian species (Naito, 1997, 1998; Naito *et al.*, 1991a, 1994a,b, 1998a, 2000a,b; Tajima *et al.*, 1998; Wentworth *et al.*, 1996; Wong *et al.*, 1999), as primordial germ cells are progenitor cells of ova and spermatozoa, and manipulated primordial germ cells can give rise to viable offspring via germline chimaeric chickens (Tajima *et al.*, 1993; Naito *et al.*, 1994c, 1998b). Avian primordial germ cells originate from the epiblast (Eyal-Giladi *et al.*, 1981) and translocate to the hypoblast at stages XI–XIV (Eyal-Giladi and Kochav, 1976). The primordial germ cells are then carried anteriorly to the germinal crescent region. Finally, the cells migrate to the germinal ridges (the future gonads) via the blood vascular system and

differentiate into oogonia in female embryos and spermatogonia in male embryos (Nieuwkoop and Sutasurya, 1979; Kuwana, 1993).

An unincubated chicken blastoderm at stage X is composed of a single layered area pellucida and a peripheral area opaca. The area pellucida is subdivided into a central disc and a peripheral marginal zone. Ginsburg and Eyal-Giladi (1987, 1989) and Ginsburg (1994, 1997) analysed the distribution of primordial germ cells and their precursors in stage X blastoderm. They isolated and cultured various fragments of blastoderm *in vitro* and then determined the presence of primordial germ cells by periodic acid Schiff staining. These studies showed that most of the primordial germ cells originated from the central disc and not from the periphery of the area pellucida, and also indicated that primordial germ cells may be determined as early as stage X. Karagenc *et al.* (1996) and Petite *et al.* (1997, 1999) investigated the origin of primordial germ cells in the prestreak stage of chicken embryos using monoclonal antibodies SSEA-1 and EMA-1, which recognize murine and chicken primordial germ cells, respectively (Hahnel and Eddy, 1986; Urven *et al.*, 1988). They found

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that SSEA-1 and EMA-1 positive cells first appeared at stage X on the ventral surface of the epiblast and later on the dorsal surface of the hypoblast. These SSEA-1 positive cells were proven to give rise to primordial germ cells by culturing them on feeder cells *in vitro* (Karagenc and Petite, 2000). Kagami *et al.* (1997) also investigated the localization of the developmental origin of primordial germ cells in stage X blastoderm. These authors removed a cell cluster from various parts of the recipient blastoderm, injected donor blastodermal cells derived from whole blastoderm and examined the donor contribution to the recipient germline. When a cell cluster was removed from the centre of the central disc, donor-derived offspring were produced efficiently from the germline chimaeras. Kagami *et al.* (2000) performed a similar experiment by transferring donor cells obtained from various parts of stage X blastoderm into recipient blastoderm and examined the number of primordial germ cells in the bloodstream of early recipient embryos. The number of primordial germ cells in the bloodstream was reduced by removing a cell cluster from the centre of the central disc of the stage X blastoderm, and the number recovered only when the blastodermal cells derived from the central disc were transferred into the stage X recipient blastoderm. These authors concluded that primordial germ cells originate in the central part of the area pellucida and that the development of cells into primordial germ cells is determined at the stage X blastoderm.

Germline chimaeric chickens were produced with high frequency by transfer of stage X blastodermal cells derived from the area pellucida (Carsience *et al.*, 1993; Kagami *et al.*, 1995), which indicates that most germ cells arise from the area pellucida of the stage X blastoderm. However, it has not been determined whether blastodermal cells derived from the marginal zone or area opaca can enter the germline of recipients and give rise to viable offspring.

The present study was performed to determine the distribution of primordial germ cells and their precursors in stage X blastoderm. Germline chimaeric chickens were produced by transfer of blastodermal cells derived from central disc, marginal zone or area opaca and the efficiency of production of donor-derived offspring from germline chimaeric chickens was examined. The ability of primordial germ cells and their precursors at stage X to differentiate into functional gametes in the gonads of recipient embryos and chickens of the opposite sex was also examined.

Materials and Methods

Fertilized eggs and animal care

Fertilized eggs of White Leghorn and Barred Plymouth Rock chickens were obtained by artificial insemination from the genetic stocks maintained at the National Institute of Animal Industry. All animals received humane care as outlined in the *Guide for the Care and Use of Experimental Animals* (National Institute of Animal Industry Animal Care Committee).

Collection of stage X blastoderm and separation into central disc, marginal zone and area opaca

Unincubated fertilized Barred Plymouth Rock eggs were broken. The blastoderm was isolated from the yolk using a filter paper ring, placed in Dulbecco's PBS without Ca^{2+} or Mg^{2+} (No. 21300-025; Gibco BRL, Grand Island, NY) and separated into three portions (central disc and marginal zone of the area pellucida, and the area opaca) using plastic straws by pressing from above. Each portion of the blastoderm was dissociated by trypsin treatment (0.25 g trypsin l^{-1} , 0.2 g EDTA l^{-1} ; T4049, Sigma, St Louis, MO) at 37°C for 5 min and then dispersed in KAv-1 medium (Minimum Essential Medium Alpha Medium (α -MEM No. 11900-024; Gibco BRL) supplemented with 1 mmol D-glucose l^{-1} , 5×10^{-5} mol 2-mercaptoethanol l^{-1} , 10 mmol N-2-hydroxyethylpiperazine-N'-3-propanesulphonic acid (EPPS) l^{-1} , 50 ml fetal bovine serum l^{-1} , 50 ml chicken serum l^{-1} , pH 8.0 (Kuwana *et al.*, 1996).

Preparation of recipient embryos and injection of donor blastodermal cells

Unincubated fertilized White Leghorn eggs were broken and the contents were put into glass vessels. The thick albumen was removed from the yolk and the blastoderm was placed on top of the yolk by rotating the yolk. A needle (27G $\times$ 3/4; Terumo, Tokyo) connected to a micropipette was inserted underneath the blastoderm layer and a cell cluster containing about 700 cells was sucked from the centre of the central disc (Kagami *et al.*, 1997; Naito *et al.*, 1999). The cells collected were used for DNA extraction and sexing of recipient embryos. Donor blastodermal cells (500–1000 cells) derived from the central disc, marginal zone and area opaca were injected into the subgerminal cavity of the recipient embryos using a fine glass micropipette (Naito *et al.*, 1991b). After injection, the residual cells of each blastoderm were used for DNA extraction and sexing of donor embryos. The manipulated embryos were transferred into host eggshells filled with thin albumen and sealed with cling film, plastic rings and elastic bands (Perry, 1988; Naito *et al.*, 1990). The reconstituted eggs were incubated at 38°C and 60% relative humidity for 3 days in a forced air incubator (P-008B; Showa Incubator Laboratory, Yono-shi, Saitama). The embryos were then transferred into large host eggshells and incubated at 38°C and 60% relative humidity until hatching (Rowlett and Simkiss, 1987; Perry, 1988; Naito *et al.*, 1990).

Sex identification of donor blastodermal cells and recipient embryos

DNA was extracted from the residual cells after injection of the donor blastodermal cells and from the blastodermal cells collected from the recipient embryos using a DNA extraction kit (SepaGeneTM; Sanko Junyaku, Tokyo) according to the manufacturer's instructions. The extracted DNA was dissolved in 5 μl Tris-EDTA (TE) buffer (10 mmol

Table 1. Survival and hatching rates of manipulated chicken embryos

Portion of donor blastoderm	Number of eggs manipulated	Number (%) of embryos surviving at day 3 of incubation	Number (%) of chicks hatched
Central disc	724	395 (54.6)	58 (8.0)
Marginal zone	435	231 (53.1)	29 (6.7)
Area opaca	435	193 (51.4)	18 (4.1)
Total	1594	819 (51.4)*	105 (6.6)

*Twin embryos (178/819, 21.7%) were included.

Tris-HCl I⁻¹, 0.25 mmol EDTA I⁻¹, pH 8.0). PCR analysis for sexing was carried out on the 1 µl DNA samples (Naito *et al.*, 1999). The reaction conditions and female specific DNA primers (5'-CCCAAATATAACACGCTTCACT-3' and 5'-GAAATGAATTATTTCTGGCGAC-3') for the PCR were performed according to the method of Clinton (1994) using a programmable thermal controller (Model 9700; Perkin Elmer, Foster City, CA). Control PCR reactions were also carried out to ensure the presence of genomic DNA using primers (5'-CAGATCAGTTTCTATCAGC-3' and 5'-TGTGACTTCAATGGTGACA-3') to amplify the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Clinton, 1994). After amplification, 5 µl aliquots of each reaction were combined, separated on a 2% (w/v) agarose gel and visualized under UV irradiation after ethidium bromide staining. A band of 315 bp was detected in females but not males.

Progeny test of putative chimaeric chickens

For the autosomal pigment inhibitor gene, White Leghorn is homozygous dominant (*II*) and Barred Plymouth Rock is homozygous recessive (*ii*). Germline chimaerism of the putative chimaeric chickens produced by transfer of blastodermal cells (central disc, marginal zone or area opaca portion) from Barred Plymouth Rock to White Leghorn chickens was tested by mating with Barred Plymouth Rock chickens using artificial insemination, and the feather colour of their offspring was examined. Black offspring (*ii*) were derived from the donor blastodermal cells (Barred Plymouth Rock) and white offspring (*II*) were derived from the recipient embryos (White Leghorn).

Analysis for the presence of the W chromosome-specific repeating sequences in sperm samples

Sperm samples were obtained from the male chimaeric chickens (*n* = 15 birds) produced by transfer of female blastodermal cells. DNA was extracted and dissolved in TE buffer. PCR analysis was carried out on 500 ng DNA samples to examine the presence of the W chromosome-specific repeating sequences according to the method of Clinton (1994).

Results

Survival and hatching rates of the manipulated embryos

The survival rates of the manipulated embryos at day 3 of incubation were about 50% in any portion of the donor blastoderm transferred (Table 1). The average hatching rate was 6.6% (105/1594). The hatching rate was highest when blastodermal cells derived from the central disc were transferred and it was lowest when blastodermal cells derived from the area opaca were transferred. Twin embryos were sometimes observed (21.7%, 178/819). Only one twin embryo developed to day 20 of incubation (just before hatching), but the others died by day 10 of incubation.

Sex identification of donor blastodermal cells and recipient embryos

The sexes of the donor blastodermal cells and recipient embryos were identified clearly by PCR. Each DNA sample was analysed twice and the same results were obtained in all samples. In all the recipient embryos, the sex identified by PCR was the same as the phenotypic sex of the hatched chickens.

Chimaeric chickens and progeny tests

Somatic and germline chimaeras were produced by transfer of Barred Plymouth Rock blastodermal cells to White Leghorn recipient embryos (Table 2). The percentages of feather pigmentation in the putative chimaeric chickens were variable (0–90%). These percentages of feather pigmentation did not correlate with the germline chimaerism.

Sixty-one chickens were matured and test mated with Barred Plymouth Rock chickens; ten chickens proved to be germline chimaeras. The efficiency of producing germline chimaeric chickens was high when the blastodermal cells derived from the central disc were transferred into recipient embryos of the same sex; 50% (5/10) for males and 40% (2/5) for females. In contrast, no germline chimaeric chickens were produced when blastodermal cells derived from the marginal zone or area opaca were transferred into recipient embryos of the same sex (0/12). Germline chimaeric chickens were also produced by transfer of blastodermal

Table 2. Progeny test of chimaeric chickens produced by transfer of stage X blastodermal cells from Barred Plymouth Rock to White Leghorn chickens

Portion of donor blastoderm	Sex		Number of birds test mated	Mean percentage of donor-derived pigmentation (range)	Number (%) of germline chimaeras (G0)	Mean number of offspring (G1) tested (range)	Percentage of donor-derived offspring (G1)
	Donor	Recipient					
Central disc	Male	Male	10	12.0 (0–70)	5 (50.0)	248 (40–583)	0.65–4.68
Marginal zone	Male	Male	5	3.0 (0–15)	0	190 (33–410)	–
Area opaca	Male	Male	4	4.0 (0–20)	0	158 (16–324)	–
Central disc	Female	Male	9	4.4 (0–20)	0	241 (10–409)	–
Marginal zone	Female	Male	3	0.0 (–)	1 (33.3)	178 (37–328)	0.58
Area opaca	Female	Male	3	6.7 (0–20)	0	300 (232–364)	–
Central disc	Male	Female	6	22.5 (0–90)	1 (16.7)	244 (114–410)	0.31
Marginal zone	Male	Female	7	0.0 (–)	0	122 (9–208)	–
Area opaca	Male	Female	6	13.3 (0–80)	1 (16.7)	172 (14–418)	0.49
Central disc	Female	Female	5	5.0 (0–10)	2 (40.0)	160 (50–233)	0.43–1.28
Marginal zone	Female	Female	1	0.0 (–)	0	320 (–)	–
Area opaca	Female	Female	2	0.0 (–)	0	135 (87–182)	–

cells derived from the central disc (6.7%, 1/15), marginal zone (10.0%, 1/10) or area opaca (11.1%, 1/9) into recipient embryos of the opposite sex.

Both male and female donor-derived offspring were produced from same sex and mixed sex germline chimaeric chickens and the reproductive performance of these donor-derived offspring was normal.

Analysis for the presence of the W chromosome-specific repeating sequences in the sperm samples

W chromosome-specific repeating sequences were not detected in any of the DNA samples by PCR analysis, probably due to the low contribution of donor-derived germ cells in recipient gonads.

Discussion

In this study, stage X blastodermal cells derived from the central disc, marginal zone or area opaca were transferred into recipient embryos of the same developmental stage from which a cell cluster was removed from the centre of the central disc. The putative chimaeric chickens produced were test mated to examine the germline chimaerism. Germline chimaeric chickens were produced efficiently (46.7%, 7/15) when blastodermal cells derived from the central disc were transferred into recipient embryos of the same sex, whereas no germline chimaeric chickens were produced when blastodermal cells derived from the marginal zone or area opaca were transferred into the recipient embryos of the same-sex (0/12). Germline chimaeric chickens were also produced by transfer of blastodermal cells derived from the central disc, marginal zone or area opaca into the recipient embryos of the opposite sex. These results indicate that the development of

the germline is not limited to the cells from the central disc of the area pellucida and that the cells from other regions (marginal zone or area opaca) can give rise to germ cells, although the frequency is low.

Ginsburg (1994) examined the distribution of primordial germ cells or their precursors in stage X blastoderm by counting the number of primordial germ cells after culturing various portions (central disc, marginal zone, area pellucida or dissected pieces of central disc) of the blastoderm, and estimated that 90.2% of primordial germ cells arise from the central disc, 9.7% from the marginal zone and 0.1% from the area opaca. Ginsburg (1994) also demonstrated that the main population of primordial germ cells arises from the centre of the central disc. Kagami *et al.* (1997, 2000) showed that removal of a cell cluster from the centre of the central disc of stage X blastoderm followed by injection of donor blastodermal cells derived from the central disc fully recovered the number of primordial germ cells in the bloodstream of recipient embryos. Taken together, these results indicate that primordial germ cells seem to be determined during or shortly after stage X and that while most primordial germ cells or their precursors locate in the central part of the area pellucida of the stage X blastoderm, some locate in the marginal zone or area opaca.

Many genes are involved in the determination of germline and, of these, the *vasa* gene plays an essential role in germline formation in *Drosophila* (Liang *et al.*, 1994). Expression of the *vasa* gene is not transient but persists throughout the germline; therefore, germline formation and germ cell movement can be traced by analysing the expression of the *vasa* gene. Tsunekawa *et al.* (2000) isolated the chicken homologue to the *vasa* gene and examined the germline-specific expression of the chicken *vasa* homologue (CVH) protein. CVH protein was localized in the cytoplasm of germ cells ranging from presumptive

primordial germ cells in uterine stage embryos to spermatids and oocytes in adult gonads. At stage X blastoderm, about 30 CVH expressing cells were scattered in the central part of the area pellucida. The subsequent movement of CVH-positive cells was in accordance with the cells recognized by SSEA-1 and EMA-1, or periodic acid Schiff staining. The results of the distribution of CVH-positive cells strongly support the contention that most primordial germ cells in stage X blastoderm are located in the central part of the area pellucida. Furthermore, CVH protein was predominantly localized in oocytes. The CVH-containing cytoplasmic structure seems to be the precursory germ plasm, indicating that chicken germline is determined by maternally inherited factors in the germ plasm (Tsunekawa *et al.*, 2000).

In the present study, same sex and mixed sex germline chimaeric chickens were produced by transfer of stage X blastodermal cells as described by Kagami *et al.* (1995, 1997). The production of donor-derived offspring from mixed sex germline chimaeric chickens indicates that primordial germ cells at stage X have the ability to differentiate normally into both male and female gametes irrespective of their genetic sex. However, this ability is lost rapidly during the first 2–3 days of incubation, as few donor-derived offspring were produced from the mixed-sex germline chimaeric chickens produced by transfer of primordial germ cells isolated from the blood of embryos incubated for 2.5 days (Naito *et al.*, 1999).

Sexual differentiation in chickens starts at days 5–7 of incubation (Mizuno *et al.*, 1993), at the time the aromatase gene is expressed specifically in female embryos. The primary differentiation of primordial germ cells takes place by day 3 of incubation, either actively or passively, as the number of primordial germ cells in the bloodstream of embryos incubated for 2 days is greater in females than in males (Naito *et al.*, 1999). Raymond *et al.* (1999) reported that the chicken *Dmrt1* gene (Z-linked) is expressed in the genital ridge and Wolffian duct before sexual differentiation and that expression is higher in male embryos than in female embryos. The *Dmrt1* gene is expressed in mouse primordial germ cells as well as Sertoli cells, indicating that the gene may play a role in the development or function of germline. As the expression of the *Dmrt1* gene in early chicken embryos by day 3 of incubation has not been examined, the primary differentiation of primordial germ cells before sexual differentiation and the expression of the *Dmrt1* gene in the early chicken embryos should be examined.

In conclusion, the results of the present study indicate that primordial germ cells are induced during or shortly after stage X and confirm that cells derived from the central disc have the highest potential to give rise to germ cells and that cells derived from the marginal zone and area opaca can also give rise to germ cells, although the frequency is low.

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