Analysis of chicken primordial germ cells

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Abstract Primordial germ cells (PGCs) are precursors of germline cells. Although avian PGCs have been used to produce transgenic birds, their characteristics largely remain unknown. In this study, we isolated PGCs from chicken embryos at various developmental stages and analyzed the gene expression. Using the expression of stage-specific embryonic antigen-1 (SSEA-1) as a marker of chicken PGCs, we purified PGCs from embryos by fluorescence-activated cell sorting after incubation for 2.5–8.5 days. The number of SSEA-1⁺ cells was almost unchanged during days 2.5–8.5 of incubation in females but continuously increased in male. Expression of several genes, including *Blimp1*, *SOX2*, and *CXCR4*, was observed in SSEA-1⁺ cells but not in SSEA-1[−] cells in both female and male embryos. Quantitative reverse-transcription PCR analysis revealed that the expression of *CXCR4*, a chemokine receptor essential for migration of PGCs from the bloodstream to the gonads, was reduced after the circulating PGC stage (day 2.5).

Keywords Cell sorting · Chicken ·
CXCR4 · Gene expression · Primordial germ cells

Introduction

Transgenic techniques have been rapidly developed using various livestock species as an alternate method of producing biologically active substances (Houdebine 2000; Kues and Niemann 2004; Rudolph 1999). Transgenic chickens have various advantages, such as a relatively short maturation period and ease of breeding (Ivarie 2003; Sang 2004). Retrovirus vectors, including avian leukosis virus (Harvey et al. 2002), Moloney murine leukemia virus (Kamihira et al. 2005), and equine infectious anemia virus (McGrew et al. 2004), have been used to establish transgenic chickens. On the other hand, there are few reports describing the successful use of non-viral methods (Love et al. 1994; Zhu et al. 2005). In most of these studies, transgenes have been directly introduced into embryos in eggs and portions of DNA have been introduced into primordial germ cells (PGCs) in embryos. In general, the frequency with which G1 transgenic descendants are obtained is very low. Recently, transgenic birds have been produced by transferring genes to cultured PGCs and transplantation of the transfected PGCs to the anterior portion of the sinus terminalis of the embryo (van de Lavoie et al. 2006).

PGCs originate from the epiblast (Eyal-Giladi et al. 1981), are located at the center of the area pellucida at stage X (Eyal-Giladi and Kochav 1976), and are translocated anteriorly to the germinal crescent (Tagami and Kagami 1998). They then

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migrate through the developing blood vascular system to the germinal ridges (future gonads), where they accumulate as gonadal germ cells (GGCs). GGCs differentiate into spermatogonia after 13 days of incubation in male chickens and into oogonia in females after 8 days of incubation (Etches 1995; Howarth 1995); however, the details of germ cell development in birds remain unclear.

Germline chimeric chickens have been generated by transplantation of donor PGCs and GGCs from various developmental stages (blastoderm to day 20 embryo) to recipient embryos as described previously (Kagami et al. 1997; Naito et al. 1994, 2007; Park et al. 2003). The results indicate that isolated PGCs and GGCs partly retain the characteristics of the blood-circulating stage of PGCs, settle in the developing gonads, and undergo normal differentiation to gametes. However, the efficiency with which descendants are obtained from transplanted cells decreases with advancing developmental stage of the donor PGCs, suggesting stage-dependent changes in the characters of these cells. In the present study, we isolated PGCs by fluorescence-activated cell sorting (FACS) from embryos at various developmental stages and determined their basic characters.

Materials and methods

Isolation of PGCs

Fertilized eggs (line M; Nisseiken, Yamanashi, Japan) were incubated at 38 °C and 60% humidity, with rocking at an angle of 90° every 15 min. After incubation for 53 h, the sharp end of the eggs was cut horizontally with a diamond cutter (Minitor, Tokyo, Japan). After checking that embryos had developed to Hamburger–Hamilton stages 13–16 (Hamburger and Hamilton 1951), blood was drawn with a glass micropipette from 20 embryos and resuspended in DMEM containing 10% fetal bovine serum (FBS). Embryos were isolated from fertilized eggs at days 4.5, 5.5, 6.5, 7.5, and 8.5 (stages 26–36) of incubation and rinsed with phosphate-buffered saline (PBS); germinal ridges or gonads were then isolated and cells were dispersed in 0.1% trypsin and 0.02% EDTA.

Cells from blood and gonads were reacted with diluted (100-fold) anti-stage-specific embryonic antigen-1 (SSEA-1) antibody (Santa Cruz Biotechnology,

Santa Cruz, CA, USA) for 30 min on ice. After centrifugation (300g, 5 min), cells were reacted with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM (Chemicon, Millipore, Bedford, MA, USA) for 30 min on ice followed by washing with PBS. Stained cells were suspended in PBS containing 0.5% FBS and separated by FACS (EPICS ALTRA; Beckman-Coulter, Fullerton, CA, USA).

The sex of embryos at days 4.5–8.5 was determined by detecting *Xho* I repeats in the W chromosome, as described (Clinton 1994). For this purpose, organs other than gonads were collected simultaneously and DNA was isolated with MagExtractor-Genome (Toyobo, Osaka, Japan). PCR was conducted in standard PCR buffer containing 0.2 mM dNTPs, 1 mM MgSO₄, 0.2 μM primers, and 1 U of DNA polymerase (KOD Plus; Toyobo) (94 °C, 2 min followed by 20 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). Primers used were 5'-CCCAAATATAACACGCTTCACT-3' and 5'-GAAATGAATTATTTTCTGGCGAC-3'. PCR products were analyzed by agarose gel electrophoresis.

Anti-VASA antibody

The peptide cGPAGVLKGRSEEIDSGRGPVKVTVY (c, cytosine, needed for binding to a carrier protein), corresponding to the N-terminal portion of chicken VASA, was synthesized and conjugated to carrier (keyhole limpet hemocyanin) (Toray Research Center, Mishima, Japan). BALB/c mice were immunized with 100 μg (first and second immunizations) and 50 μg (third and fourth immunizations) of the peptide conjugate. After checking the antibody titer in the blood sample by enzyme-linked immunosorbent assay using a BSA-conjugated synthetic peptide as the antigen, blood was collected and antiserum was obtained by centrifugation. This antiserum and anti-mouse IgG-peroxidase (Santa Cruz Biotechnology) were used for Western blotting.

For immunostaining, the sorted cells were dispersed on a slide glass and fixed with 4% paraformaldehyde for 10 min. After washing with PBS, cells were permeabilized with 0.1% Triton X100 for 5 min. They were stained with 100-fold-diluted anti-VASA antiserum and rhodamine-labeled anti-mouse IgG (Protos Immunoresearch, Burlingame, CA, USA) and analyzed under a microscope (BZ-8000, Keyence, Osaka, Japan).

Reverse transcription (RT)-PCR

mRNA was purified from sorted cells using an RNeasy Microkit (Qiagen, Hilden, Germany) and reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). cDNA was amplified by PCR with the following primers:

actin, forward: 5'-GGATTTTCGAGCAGGAGATGG-3' and reverse: 5'-ACCCAAGAAAGATGGCTGGA-3';

CXCR4, forward: 5'-GCCATTCTGGTCTGTGGA TG-3' and reverse: 5'-GGCATGGACTATTGC CAGGT-3';

VASA, forward: 5'-TGACTTATGTCCCCCTC CT-3' and reverse: 5'-GTAATGGTGCTGGAGG GTCA-3';

SOX2, forward: 5'-ATAAATACCGACCCCGGA GG-3' and reverse: 5'-CGGTCGTCATGGTATT GGTG-3';

Blimp1, forward: 5'-AGCAACTGGATGCGCTA TGT-3' and reverse: 5'-AGGGATGGGCTTAATG GTGT-3';

integrin $\alpha 6$, forward: 5'-GAAACCCGGGATATC ATTGG-3' and reverse: 5'-CAGCAACACCTTGC TGACAG-3'; and

integrin $\beta 1$, forward: 5'-TGTTTGTGGGGAC CAGATTG-3' and reverse: 5'-CCAGGTGACAC TTCCCATCA-3'.

Real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) in 20 μ L reaction mixture containing 10 μ L Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 500 nM of each primer, 1 μ L of 20 $\times$ bovine serum albumin (1 mg/mL), and 2 μ L of sample DNA. LightCycler amplification involved a first denaturation at 95 $^{\circ}$ C for 60 s followed by amplification of the target DNA for 45 cycles (94 $^{\circ}$ C for 5 s, 53 $^{\circ}$ C for 10 s, and 72 $^{\circ}$ C for 10 s). The amount of each gene was determined with LightCycler Software version 3.5 (Roche Diagnostics). The expression level was normalized by that of actin.

Results

Flow cytometric analysis of PGCs

We separated and analyzed PGCs and GGCs from male and female embryos of various developmental

stages (day 2.5, stages 13–16, to day 8.5, stages 35–36). Figure 1a shows typical cell sorting patterns obtained with day 2.5 embryos (blood-circulating PGCs) and day 5.5 embryos (gonadal PGCs). Since the sex difference is not obvious by microscopy until day 7.5 even though gonadal organogenesis begins around day 4, each embryo was analyzed individually by FACS and genetic sex was determined by PCR of genomic DNA from other parts of body, except for day 2.5 embryos, from which it was difficult to obtain sufficient PGCs for individual analysis. On day 2.5, the region shown in Fig. 1a (left, region A), substantially represented SSEA-1⁺ cells. The sorted SSEA-1⁺ cells were approximately 9–20 μ m in diameter, as reported for PGCs (Fujimoto et al. 1976). Thus, cells were collected with this FACS gate setting at several developmental stages (days 2.5–8.5). However, in later developmental stages, relatively small cells with low-fluorescence intensity appeared (region B, Fig. 1a). For instance, on day 5.5, the number of cells of this type was less than 10% of that in region A in male embryos. However, the number of cells in region B (Fig. 1a) was almost equal to that in region A (Fig. 1a) in female embryos. In general, the population of the cells expressing a low amount of SSEA-1 increased with advancing development, especially in female embryos, although the number of these cells as a percentage of the total cell population varied among individuals. In this study, cells in region A (Fig. 1a) with a relatively high level of expression of SSEA-1 were analyzed as PGCs. As shown in Fig. 1b, the number of PGCs in females was constant (around 300 cells in the gonads) despite the increase in total cell number in the gonads (data not shown). On the other hand, the number of PGCs increased in males with an increase in total cell number in the gonads. By day 7.5, the number of PGCs had increased to 2.5×10^3 in the gonads. In addition, mean SSEA-1 fluorescence intensity declined on day 4.5 in both males and females compared with that in circulating stage (day 2.5), and their expression level was almost constant until day 8.5 (Fig. 1c). On average, fluorescence intensity in males was found to be higher than that in females. Although we analyzed SSEA-1 expression as the mixture of male and female on day 2.5, it is likely that sex difference in SSEA-1 expression is not evident at this stage because we observe just single population by FACS analysis. These results agree with the

Fig. 1 Flow cytometric analysis of chicken PGCs. **(a)** Green fluorescence (stained for SSEA-1) versus forward scatter plot of blood at day 2.5 (stages 13–16 (mixture of female and male embryos)) (left) and gonadal cells at day 5.5 (stage 28) (right). The letter A indicates the region for cell sorting; B indicates the region of smaller cells expressing a lower level of SSEA-1. **(b)** Cell number of SSEA-1⁺ cells in blood and gonads of males and females in region A (Fig. 1a). M, male; F, female. Data are expressed as mean value with standard deviation of three independent experiments (day 2.5) or of 5–7 embryos analyzed individually (day 4.5–8.5). * indicates statistical significance relative to male by Student's *t* test ($p < 0.05$). ** indicates statistical significance relative to day 2.5 by Student's *t* test ($p < 0.05$). **(c)** Expression levels of SSEA-1 were indicated as mean fluorescence intensity of the cells in region A (Fig. 1a). Data are expressed as mean value with standard deviation of 5–7 embryos analyzed individually except for day 2.5 (pooled blood samples were analyzed and the mean of three independent experiments is shown)

observation that EMA-1 reactivity showed a marked sexual dimorphism in later stages (Urven et al. 1988). Thus, SSEA-1 expression by PGCs and GGCs seems to differ between males and females after residing in gonads.

Characteristics of sorted PGCs

To characterize PGCs and GGCs in detail, we then stained FACS-isolated cells (region A, Fig. 1a) with anti-VASA antibody. VASA is an RNA helicase family protein and its expression is restricted to germline cells (Tsunekawa et al. 2000). As shown in Fig. 2a, VASA was expressed in SSEA-1⁺ cells but not in SSEA-1[−] cells in both male and female embryos. Real-time RT-PCR analysis showed that the expression did not change significantly during development (days 2.5–8.5), although there was a tendency for males to express a slightly higher level of VASA compared with females in early stage (days 4.5–5.5) (Fig. 2b). Figure 2c shows immunostaining of SSEA-1⁺ cells with anti-VASA antibody on days 2.5 and 6.5. Cytoplasm seemed to be stained at high intensity. Table 1 shows the percentage of VASA⁺ cells among the sorted SSEA-1⁺ cells (region A, Fig. 1a). On day 2.5, when almost all PGCs are circulating in the bloodstream, the percentage of VASA⁺ cells was 95%, but after day 6.5, when PGCs had reached the gonads, the percentage was almost 100%. In these experiments, PGCs and GGCs were analyzed as mixtures of cells from male and female embryos, but the results suggests that almost all SSEA-1⁺ cells express VASA independently of sex. Furthermore, roughly 70–80% of cells that expressed SSEA-1 only weakly (region B, Fig. 1a) were VASA⁺ (data not shown).

We then studied the expression of several genes that have been reported to be important in the migration, development, and specification of PGCs in other species. SOX2 prevents progenitor cells from initiating differentiation and maintains the pluripotency of ES cells (Boyer et al. 2005). Integrins are postulated to be involved in PGC migration (Kunwar et al. 2006). Blimp1, a transcriptional repressor, has a crucial role in the specification of PGCs through their involvement in a novel transcriptional regulatory complex in the mouse germ cell lineage (Ancelin et al. 2006). CXCR4 is the suggested receptor of the chemokine stromal cell-derived factor-1 (SDF-1), which is reported to be involved in the migration of PGCs into the gonads in the mouse and chicken (Stebler et al. 2004).

As shown in Fig. 3a, Blimp1, CXCR4, and SOX2 were expressed only in SSEA-1⁺ cells. On the other hand, integrins $\alpha 6$ and $\beta 1$ were expressed in both

Fig. 2 Expression of VASA by SSEA-1⁺ cells. **(a)** Both SSEA-1⁺ and SSEA-1⁻ cells were sorted from embryos after various incubation times, and the expression of VASA was examined by RT-PCR. PGCs from blood (day 2.5) were analyzed as mixtures of cells from males and females. **(b)** Quantitative RT-PCR analysis of VASA in SSEA-1⁺ cells in region A of Fig. 1a. M, male; F, female. Data are expressed as mean value with standard deviation of three embryos analyzed

individually except for day 2.5 (pooled blood samples were analyzed and the mean of three independent experiments is shown). ** indicates statistical significance relative to day 2.5 by Student's *t* test ($p < 0.05$). Sex difference was not statistically significant by Student's *t* test. **(c)** Immunostaining of sorted SSEA-1⁺ cells with anti-VASA antibody. Anti-SSEA-1 (left), anti-VASA (middle), and phase-contrast (right)

Table 1 Percentage of VASA⁺ cells among the sorted SSEA-1⁺ cells

Incubation (day)	% VASA ⁺ cells/SSEA-1 ⁺ cells
2.5	95 (79) ^a
5.5	98 (168)
6.5	100 (48)
7.5	100 (256)

Sorted cells (region A (Fig. 1a)) were stained with anti-VASA antibody and the number of VASA⁺ and VASA⁻ cells were determined. Data from 3 to 5 independent experiments were collected and statistically analyzed by χ^2 test, which indicates difference between stages was not statistically significant ($p > 0.05$)

^a Numbers in parenthesis indicate the number of cells analyzed

SSEA-1⁺ and SSEA-1⁻ cells in both sexes. Stage- and sex-specificity were not evident under our analytical conditions. However, quantitative RT-PCR analysis revealed that the expression of CXCR4 was the greatest on day 2.5 and decreased during incubation (Fig. 3b).

Discussion

We isolated SSEA-1⁺ cells from chicken embryos on days 2.5–8.5. VASA, a germ cell-specific protein (Tsunekawa et al. 2000), was expressed in PGCs and GGCs from all stages studied. However, not all

Fig. 3 RT-PCR analysis of SSEA-1⁺ and SSEA-1⁻ cells. **(a)** Expression of several genes was analyzed by RT-PCR. **(b)** Quantitative RT-PCR analysis of CXCR4 in SSEA-1⁺ cells. M, male; F, female. Data are expressed as mean value with standard deviation of three embryos analyzed individually except for day 2.5 (pooled blood samples were analyzed and the mean of three independent experiments is shown). ** indicates statistical significance relative to day 2.5 by Student's *t* test ($p < 0.05$). Sex difference was not statistically significant by Student's *t* test

SSEA-1⁺ cells were also positive for VASA on day 2.5. In our preliminary analyses, the percentage of VASA⁺ cells in SSEA-1⁺ cells from the area pellucida of the blastoderm (unincubated eggs) was less than 30%, suggesting that SSEA-1⁺ cells are heterologous at the very early stage of embryogenesis, possibly because their final specification may not have been completed before day 2.5. Detailed analysis of this point is now being undertaken. SSEA-1⁺ cells (region A, Fig. 1a) after day 2.5 were substantially PGCs.

In the chicken, PGCs migrate via blood vessels (days 2–3) and attach to the gonadal anlage. CXCR4 and integrins are thought to be involved in the migration of PGCs (Kunwar et al. 2006). Our finding of relatively high expression of CXCR4 in day 2.5 PGCs agrees well with this notion. Previous reports suggest that the efficiency with which descendants are obtained from transplanted cells depends on the developmental stage of PGCs when the cells are transferred into the bloodstream, i.e., gonadal PGCs or GGCs have shown decreased frequency as compared with circulating PGCs (Naito et al. 1994; Park et al. 2003). This can be explained partly by the

decrease in CXCR4 expression when the cells in later stages are used instead of circulating PGCs.

In the gonads of male chickens, the number of PGCs increased and the level of expression of SSEA-1 modestly decreased when PGCs settled in the gonads (Fig. 1). On the other hand, the number of SSEA-1⁺ cells was constant in females but the SSEA-1 expression level decreased. Furthermore, the cells with a lower level of SSEA-1 expression and relatively small size appeared in females (region B, Fig. 1a). It has been reported that sex-dependent gene expression occurs from day 3.5 in the gonads and that morphological differentiation of the gonads is evident on day 6 (Smith and Sinclair 2004). Thus, the observations described in this paper may reflect the underlying sexual differentiation of PGCs. Detailed analysis will be necessary to characterize the developmental events that occur in this period and to determine which stage of PGC is suitable for the establishment of transgenic chickens.

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