PHYSIOLOGY, ENDOCRINOLOGY, AND REPRODUCTION

Isolation of Chicken Primordial Germ Cells Using Fluorescence-Activated Cell Sorting

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ABSTRACT Presently, it is difficult to undertake germ line modification of the chicken with primordial germ cells (PGC) because it has been difficult to efficiently fractionate the PGC from the total somatic cell population. The objective of this study was to develop a method that allows isolation of an enriched population of viable PGC from embryonic blood and embryonic gonadal tissue. Blood was harvested from early chick embryos (stages 13 to 15), and cells were liberated from the gonads of stage 27 chick embryos. Subsequently, viable PGC were labeled with anti-stage–specific embryonic antigen-1 (SSEA-1), which was detected with goat-anti-mouse IgM-fluorescein isothiocyanate. Fluorescently labeled cells were sorted from the unlabeled cells using fluorescence-activated cell sorting (FACS), and the identities of the PGC were confirmed using periodic acid-Schiff (PAS) staining or anti-embryonic mouse antigen-1 (EMA-1) staining followed by microscopic evaluation. Finally, PGC were sorted from somatic cells of sex-identified embryos. Less than 0.1% of the blood cell population was collected as SSEA-1–positive cells. Similarly, approximately 2% of the gonadal cell population were collected as SSEA-1–positive cells. Therefore, fewer (~1,000 to 9,000) PGC were recovered from each isolate. Placing the sorted SSEA-1–positive cells on a glass slide from a microcentrifuge tube resulted in a recovery rate of 53 to 73% relative to the number detected by FACS. Furthermore, the proportions of sorted cells that stained with PAS or anti-EMA-1 following sorting were 92 ± 4% PAS positive and 94 ± 1% anti-EMA-1 positive. Finally, the sorted SSEA-1–positive cells were maintained in vitro to demonstrate their viability after sorting. It was demonstrated that it is possible to label blood and gonadal chicken PGC with SSEA-1 and subsequently to sort viable SSEA-1–positive PGC from somatic cells.

(*Key words*: stage-specific embryonic antigen-1, gonad, periodic acid-Schiff, embryonic mouse antigen-1)

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INTRODUCTION

Primordial germ cells (PGC) are embryonic precursor cells to the ova and spermatazoa found in adult animals (D'Costa et al., 2001; Wentworth and Wentworth, 2000). PGC originate outside the gonad, and they can be readily identified in the extraembryonic region called the germinal crescent (Swift, 1914). The subsequent movement of PGC from the germinal crescent to the gonadal ridge, which develops into the mature gonad, occurs in 2 phases. First, the PGC are passively carried to the vicinity of the gonadal ridge through the extra- and intraembryonic circulation (Swift, 1914; Meyer, 1964; Fujimoto et al., 1976a,b). Second, PGC leave the blood vessels and actively migrate into the gonadal ridge. In the second phase of migration, chemotactic signals from the gonad (Dubois and Croisill, 1970; Kuwana et al., 1986), extracellular matrix (Urven et al., 1988a), and the vascular system surrounding the germinal epithelium are thought to be important factors governing PGC fate.

The classic histological stain for differentiating PGC from somatic cells is periodic acid-Schiff (PAS; Meyer, 1960). PAS is a generally accepted method to identify PGC because these cells have a high glycoprotein content compared with surrounding somatic cells, making germ cells PAS positive and somatic cells PAS negative (Meyer, 1964; Fujimoto et al., 1976a,b). However, there are also immunological markers against cell-surface glycoproteins that are found in PGC but not somatic cells. The stage-specific embryonic antigen-1 (SSEA-1) is likely one of the best-characterized antigens on PGC in mammalian and avian embryos. Anti-SSEA-1 was directed against a carbohydrate epitope with a galactose (β1-4) N-acetylglucosamine (α1-3) fucose linkage similar to the Lewis × antigen (Gooi et al., 1981). Undifferentiated multipotential mouse cells, chick embryonic stem cells, and murine

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Abbreviation Key: EMA-1 = embryonic mouse antigen; FACS = fluorescence-activated cell sorting; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; PAS = periodic acid-Schiff; PGC = primordial germ cells; SSEA-1 = stage-specific embryonic antigen-1.

embryonic germ cells also express the SSEA-1 epitope (Resnick et al., 1992; Pain et al., 1996). Upon differentiation, these cells are no longer SSEA-1 positive. Therefore, SSEA-1 is an appropriate marker for germ cells in chicken embryos older than stage 10 (Hamburger and Hamilton, 1951) because the only SSEA-1–reactive cells in avian embryos beyond stage 10 are PGC. Another germ cell marker is anti-embryonic mouse antigen-1 (EMA-1; Urven et al., 1988a,b), which reacts with a glycoprotein cell surface antigen against mouse embryonal carcinoma (Nulli SCC1) cells. Specifically, anti-EMA-1 reacts with a fucosylated polylactosamine carbohydrate group that also labels murine PGC (Hahnel and Eddy, 1986). SSEA-1 and EMA-1 both have been demonstrated to be expressed by chicken PGC from their appearance in the germinal crescent until their residence in the gonad (Karagenc et al., 1996).

Preceding the generation of the first chicken germ line chimera (Petitte et al., 1990), there were and subsequently have been many efforts to enrich mixed-cell populations for germ cells, which are the target for germ line gene manipulation. A pure population of germ cells will improve the potential to make genetic modifications. PGC can be implanted into an embryo to create germ line chimeras, and the transgene will be transferred to the subsequent generation through the spermatazoa or the ova. However, inserting a transgene into isolated PGC and implanting the manipulated PGC into a recipient chick embryo is not currently a practical method to generate transgenic chickens because a transgenic chicken expressing a protein has not yet been generated following PGC manipulation (Vick et al., 1993). Furthermore, the *lacZ* gene introduced into the gonads of chimeric embryos and chickens through the transfer of PGC that had been previously transfected in vitro resulted in detection of the *lacZ* gene in embryonic gonads only 3 d after PGC transfer, and the *lacZ* gene was not detected in sexually mature gonads (Naito et al., 1998). Alternatively, germ cells from superior genetic stock can be placed into inferior stock, and the superior genetics may be transferred from parent to offspring. Even though there have been recent reports of PGC enrichment with a density gradient (Zhao and Kuwana, 2003), methods to modify the germ line in chickens are presently inadequate because density gradient centrifugation has not been successful for blood- and gonad-derived PGC. Enrichment of gonadally derived PGC is more problematic than blood-derived PGC because gonadal preparations contain more cellular debris and a more complex mixture of cell types compared with blood cell preparations. The present study was undertaken to develop a reliable method to isolate PGC from embryonic chick blood and gonads that will allow for the efficient development of germ line chimeras and germ line manipulation.

MATERIALS AND METHODS

Embryonic Blood Collection

Chicken embryos between stages 13 and 15 (Hamburger and Hamilton, 1951) were removed from the yolk using a filter paper ring. The embryos were placed into petri dishes and observed under a dissecting microscope. Blood was collected from the embryos by inserting a microinjection needle into the vitelline vessels, and 3 to 8 μL of blood was collected through capillary action. Microinjection needles were made on a vertical pipette puller using 50-μL (1.47 mm o.d., 1.12 mm i.d.) glass capillary tubes.[2]

Embryonic Gonad Isolation

Stage 27 embryos (Hamburger and Hamilton, 1951) were collected by making a hole in the blunt end of the egg and removing the embryo from the yolk sac with curved forceps. The embryo was placed in a petri dish containing PBS, and the gonads were removed from the embryo with the assistance of very fine straight forceps and a dissection microscope. Gonad pairs (10 to 14) were pooled in 200 μL of room temperature 0.25% trypsin-EDTA[3] and were subsequently incubated at 37°C for 15 min. The trypsin was inactivated by adding 200 μL of Dulbecco's modified Eagle's medium with 20% fetal bovine serum (FBS[3]), and the suspension was passed through a 35-μm filter.

PCR Sexing of Embryos

Extraembryonic membranes with blood vessels surrounding embryos designated for sexing were dissected and placed in a microcentrifuge tube on ice containing 50 μL of 2:1 lysis solutions (10 m*M* Tris pH 8.0, 10 m*M* EDTA; 6 *M* NaCl and 0.6 *M* NH_4OH) to liberate DNA. The sample was heated at 100°C for 13 min and then placed on ice, and then 350 μL of sterile H_2O was added. After centrifugation, 2 μL of the supernatant was used as a DNA template in the PCR reaction. The PCR reaction mixture consisted of 2.5 m*M* each W chromosome primer [5′ primer Wxho-1: 5′-CCCAAATATAACACGCTTCACT-3′, 3′ primer Wxho-2: 5′-GAAATGAATTATTTTCTGGCGAC-3′], 0.5 m*M* each 18s ribosomal RNA primer [5′ primer RiBo-1: 5′-AGCTCTTTCTCGATTCCGTG-3′, 3′ primer Ribo-2: 5′-GGGTAGACACAAGCTGAGCC-3′], 0.2 m*M* each of dNTP, 1 U of Taq polymerase,[4] 1× MasterAmp PCR Enhancer,[5] 2 μg of DNA template in a 1× buffer of 3.0 m*M* $MgCl_2$, 20 m*M* KCl, 50 m*M* Tris-HCl, 1 m*M* Ficoll, 1 m*M* tartrazine, and 500 μg of BSA/mL.[2] The W chromosome primers were designed to amplify a 276-bp fragment within the 717-bp *Xho*I repeat in pUGD0600 (Kodama et al., 1987), and they have been used in other studies that require embryonic sex determination (Petitte and Kegelmeyer, 1995). The 18s rRNA primers were designed to amplify a 256-bp fragment (Hedges et al., 1990), and they were originally used by

[2]Idaho Technology, Salt Lake City, UT.
[3]Invitrogen, Carlsbad, CA.
[4]Takara, Madison, WI.
[5]Epicentre, Madison, WI.

Clinton et al. (2001) as a positive control for their sex determination PCR procedures.

Rapid amplification was performed in a 10-μL volume loaded into a glass capillary tube (1 mm o.d., 0.5 mm i.d.) that was heat-sealed. Rapid cycling was accomplished in a 1605 AirThermocycler[2] with initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C (1 s), 56°C (5 s), and 70°C (20 s) with a final extension at 72°C (5 min.). After amplification, the entire reaction was loaded directly into the wells of a 1.5% agarose gel, separated by electrophoresis, and stained with ethidium bromide before visualization under ultraviolet light.

Fluorescence-Activated Cell Sorting

Blood samples from up to 33 embryos and gonadal samples from up to 14 embryos were pooled in Media 199 with 10% embryonic stem cell qualified FBS and incubated with a 1:1,000 dilution of a mouse monoclonal antibody against anti-SSEA-1[6] for 30 to 60 min on ice. After being washed with cold PBS, goat antimouse IgM-fluorescein isothiocyanate (FITC) was added to the cell suspension at a dilution of 1:1,000 and incubated for 30 min on ice. The suspension was washed with Media 199 and 10% embryonic stem cell qualified FBS, and resuspended in 500 μL of PBS. Fluorescence-activated cell sorting (FACS) was performed using a MoFlo high-speed FACS.[7] The SSEA-1–positive cells were resuspended at 400 cells/μL, based upon the flow cytometry data.

Staining of Blood-Derived PGC

Sorted blood samples were smeared onto ProbeOn Plus glass slides[8] and air-dried overnight. Similarly, 3 μL of blood was removed from a stage 14 embryo and smeared on glass slides as controls. The air-dried cells were fixed to the slides using 10% formalin in 95% ethanol for 1 min at room temperature. The slides were stained with PAS reagent and subsequently counterstained with hematoxylin. At least 408 cells were analyzed with a microscope for each trial.

Staining of Gonadally Derived PGC

Sorted gonadal samples were smeared on ProbeOn Plus glass slides and air-dried overnight. For a pre-FACS control comparison, 5 μL of gonadal single cell suspension was dried to glass slides. The air-dried cells were fixed to the slides using 10% formalin in 95% ethanol. The fixed cells were stained with anti-EMA-1,[6] which was labeled with goat-anti-mouse IgG conjugated to biotin. The secondary antibody was detected using a Vectastain ABC-AP kit[9] and BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium) substrate.[10] At least 1,259 cells were analyzed with a microscope for each trial.

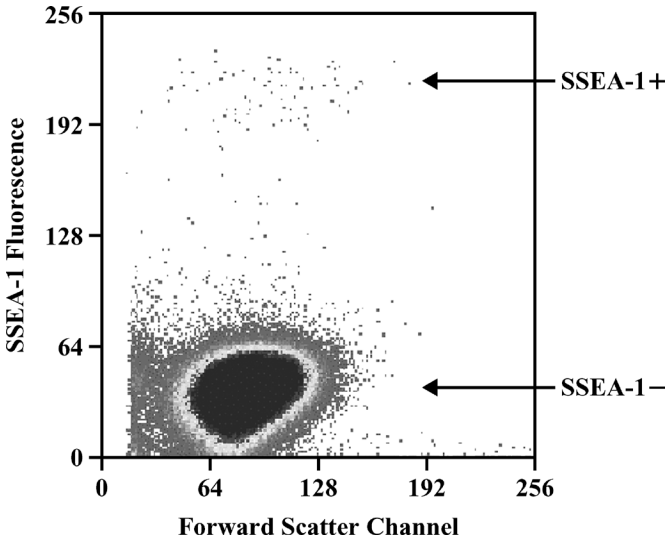

FIGURE 1. Forward scatter plotted versus anti-stage specific embryonic antigen (SSEA)-1–fluorescein isothiocyanate (FITC) fluorescence for blood cells harvested from embryos from stages 13 to 15 (Hamburger and Hamilton, 1951). The figure indicates that there is a small but identifiable SSEA-1–positive primordial germ cell population.

Data Analysis

For each cell suspension, the number of PGC in each cell suspension was determined based upon the number of SSEA-1–positive cells. The number of PGC per sample was calculated by dividing the number of PGC observed by FACS by the number of embryos used to generate each sample. Cell recovery data were collected by comparing the FACS data with microscropic observation of the sorted cells. The purity of the sorted gonadal cell population was assessed by comparing the number of PAS (PGC marker) positive cells from the blood isolates to the total number of cells. Similarly, the number of EMA-1–positive cells (PGC marker) was compared with the total number of cells as a measure of gonadally derived PGC purity. Finally, the rapid amplification PCR procedures (40 cycles of 26 s per cycle) were sufficiently rapid to allow sex determination before cell sorting. Two operators made it possible to accomplish gonadal sex determination while the gonads were being processed for cell isolation.

RESULTS AND DISCUSSION

FACS of PGC

First, PGC were fractionated from blood cells by staining with anti-SSEA-1 followed by FACS sorting of SSEA-1–positive cells from SSEA-1–negative cells. SSEA-1–positive cells exhibited a higher level of fluorescence than SSEA-1–negative cells (Figure 1). Similarly SSEA-1–positve cells from the gonad exhibited a higher level of flu-

[6]Developmental Studies Hybridoma Bank, Iowa City, IA.
[7]Cytomation, Fort Collins, CO.
[8]Fisher Scientific, Chicago, IL.
[9]Vector Labs, Burlington CA.
[10]Amresco, Vernon Hills, IL.

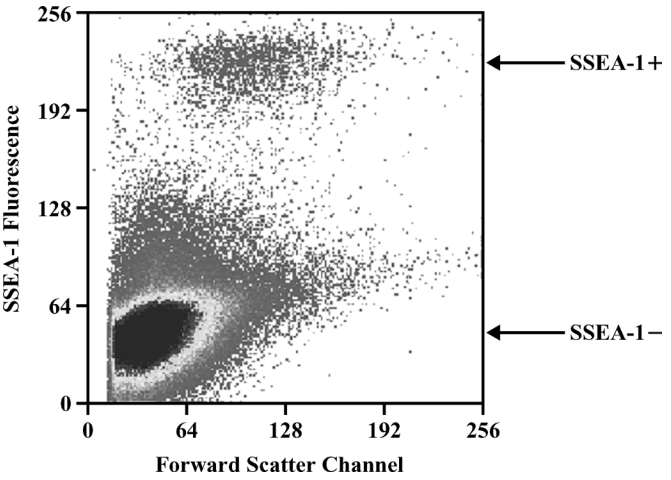

FIGURE 2. Forward scatter plotted versus anti-stage specific embryonic antigen (SSEA)-1–fluorescein isothiocyanate (FITC) fluorescence for gonadal cells harvested from embryos at stage 27 (Hamburger and Hamilton, 1951). The figure indicates that there is an identifiable SSEA-1–positive primordial germ cell population.

orsecence than SSEA-1–negative cells (Figure 2). The cells exhibiting the elevated level of fluorescence were sorted from the cells with low fluorescence signal. The number of PGC identified per sample by FACS was 41 ± 1 for blood PGC (Table 1), which was slightly lower than the number of PGC per embryo identified for blood PGC by other investigators who used PAS (Tajima et al., 1999) or cell size (Zhao and Kuwana, 2003) to identify PGC, but was within the same order of magnitude. Variation in PGC number during passive migration in the blood could be related to the short time PGC are found in the blood, the amount of blood harvested from an embryo, and variation in embryo stages within any batch of similarly incubated embryos. Given the goal of collecting a highly enriched population of PGC, it is likely that our methods were stringent to minimize inclusion of any non-PGC in our sorted cell populations. Similarly, the number of PGC per sample identified for gonadally derived PGC was 706 ± 41 mixed sex donors (Table 1), 500 ± 115 male donors, and 624 ± 177 female donors (Table 2), which was less than other investigators who used trypsin:EDTA for cell isolation (~1,700 PGC per sample) but was approximately the same as results from investigators who only used EDTA (~800 PGC per sample) (Allioli et al., 1994). The lower yield could be related to the goal of collecting a highly enriched population of PGC because it is likely that our methods were stringent to minimize inclusion of any non-PGC in our sorted cell populations.

Another difference between the present study and previous studies (Allioli et al., 1994; Tajima et al., 1999; Zhao and Kuwana, 2003) was that the other investigators did not perform immunostaining on the cell populations followed by cell sorting; they simply observed the cells microscopically after isolation. A goal of PGC isolation is to eventually inject PGC into developing embryos, and given the relatively low numbers of PGC in a cell suspension from several embryos, it is important to examine the number of PGC available to inject after sorting. Therefore, SSEA-1–positive cells were sorted directly in a microcentrifuge tube, the fraction was placed on a slide, and the number of cells was directly counted under a microscope. It was determined that approximately 51 to 73% of the cells collected after FACS were successfully placed on a microscope slide (Tables 1 and 2). All cells appeared to be PGC based upon morphology. Similarly, sorted SSEA-1–positive gonadal PGC were cultured in 20–μL drops of medium for 24 h, and the sorted cells appeared viable based upon microscopic examination of the cultures (Figure 3). Although propidium iodide exclusion was not used as a measure of viability for the present data, blood and gonadal PGC preparations were stained with propidium iodide prior to cell sorting, and 95 to 99% of the SSEA-1–positive cells were propidium iodide negative (i.e., viable).

TABLE 1. Flow cytomety, recovery, and purity data for primordial germ cells (PGC) isolated from the blood and gonad

Cell source	Trial	Total cells	SSEA-1+[1]	Recovery[2] (%)	Purity[3] (%)	Donors[4] (n)	PGC per sample
Blood	1	6,169,753	1,621	52	96	33	49
	2	5,270,832	1,229	52	95	31	36
	3	4,045,914	1,010	54	85	27	37
Average[5]		5,162,166 ± 615,502	1,287 ± 178	53 ± 1	92 ± 4	30 ± 2	41 ± 4
Gonad	1	210,116	9,070	50	93	14	647
	2	605,212	6,853	47	93	10	685
	3	282,387	7,864	56	ND[6]	10	786
	4	250,979	ND[6]	ND[6]	95	10	ND[6]
Average		337,174 ± 90,562	7,929 ± 641	51 ± 3	94 ± 1	11 ± 1	706 ± 41

[1]Number of stage-specific embryonic antigen-1 (SSEA-1)–positive cells determined by fluorescence-activated cell sorting (FACS).

[2]Percentage of cells enumerated on a microscope slide compared with FACS data.

[3]Percentage of cells on the microscope slide that were periodic acid-Schiff (PAS; blood PGC) or anti-embryonic mouse antigen-1 (EMA-1; gonadal PGC) positive.

[4]Number of donor embryos for blood and number of embryos that donated gonadal pairs.

[5]Values are means ± SE.

[6]ND = not determined.

TABLE 2. Flow cytomety and recovery data for primordial germ cells (PGC) isolated from gonads of sexed embryos

Sex	Trial	Total cells	SSEA-1+[1]	Recovery[2] (%)	Donors[3] (n)	PGC per sample
Male	1	443,204	6,847	64	10	685
	2	146,864	2,890	62	10	289
	3	248,985	5,249	78	10	525
Average[4]		279,685 ± 86,912	4,995 ± 1,150	68 ± 5	10	500 ± 115
Female	1	245,324	6,832	81	10	683
	2	115,995	2,929	64	10	293
	3	242,859	8,957	ND[5]	10	896
Average[4]		201,393 ± 42,705	6,239 ± 1,765	73 ± 9	10	624 ± 177

[1]Number of stage-specific embryonic antigen-1 (SSEA-1)–positive cells determined by fluorescence-activated cell sorting (FACS).

[2]Percentage of cells enumerated on a microscope slide compared with FACS data.

[3]Number of donor embryos for blood and number of embryos that donated gonadal pairs.

[4]Values are means ± SE.

[5]ND = not determined.

PGC Purity

An average of 5,162,166 blood cells were subjected to cell sorting based upon SSEA-1 fluorescence. Less than 0.1% of the blood population was SSEA-1 positive, which is consistent with the results of other investigators (Chang et al., 1992; Yasuda et al., 1992). Sorted cell preparations from the blood were examined microscopically to confirm that SSEA-1–positive cells were PGC based upon their morphology and purity using PAS staining, which is a generally accepted marker for blood PGC (Meyer, 1964; Fujimoto et al., 1976a,b). It has been demonstrated that all SSEA-1–positive PGC give rise to PAS-positive PGC (Karagenc et al., 1996). The sorted SSEA-1 FITC-positive cells appeared qualitatively large and round with a luminescent granular cytoplasm characteristic of PGC (Figure 4). The PAS staining confirmed that the purity of the sorted cells was 92 ± 4% (Table 1), which is equal or greater than the purity reported by Zhao and Kuwana (2003). Furthermore, the number of PGC per sample reported by Zhao and Kuwana (2003) is approximately the same order of magnitude as the present study. An advantage of the FACS methodology compared with the methods of Zhao and Kuwana (2003) is that it is applicable to blood-derived PGC and gonadally derived PGC.

An average of 279,193 cells from gonads harvested from 10 to 14 embryos were sorted based upon SSEA-1 FITC fluorescence. Approximately 2% of the total cell population was SSEA-1 positive based upon FACS analysis yielding between 706 ± 41 PGC per sample (Table 1), which is consistent with others' estimates (Trefil et al., 1997). Sorted cell preparations were examined microscopically to confirm that the SSEA-1 FITC-labeled cells were PGC based upon their morphology and also based upon anti-EMA-1 staining (Figure 4), which is another PGC marker (Urven et al., 1988a,b; Karagenc et al., 1996). The sorted SSEA-1–positive cells were greater than 94 ± 1% EMA-1 positive. Therefore, it appears that the majority of cells sorted based upon SSEA-1 fluorescence were PGC because they also expressed the second PGC marker EMA-1.

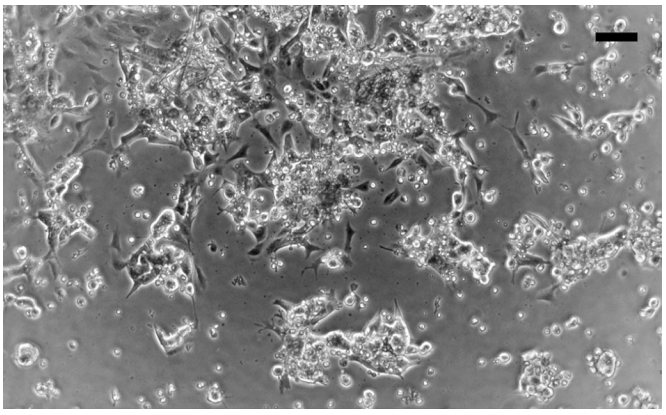

FIGURE 3. Phase contrast view of gonadal primordial germ cells (PGC) cultured for 24 h after sorting. The PGC have attached to the dish in small clusters. Scale bar = 100 μm.

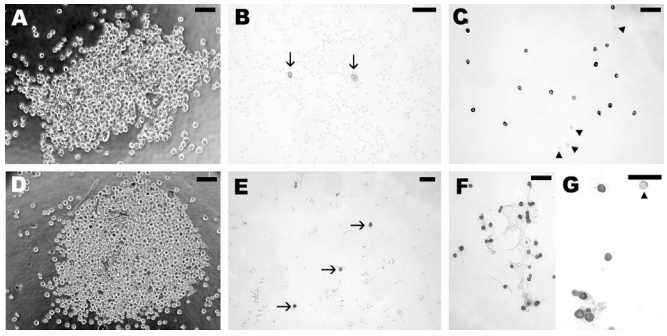

FIGURE 4. FACS of avian primordial germ cells from stage 13 to 15 embryonic blood samples (A through C) and stage 27 dispersed embryonic gonads (D through G). Phase contrast views of sorted primordial germ cells (PGC) from blood (A) and gonads (D). PGC from embryonic blood smears were stained with periodic acid-Schiff (PAS) before sorting (B) and after sorting (C). PGC were detected in dispersed embryonic gonadal cells using the antibody embryonic mouse antigen-1 (EMA-1) before sorting (E) and after sorting (F and G). Arrows indicate stained PGC before sorting in B and E. Arrow heads indicate unstained cells after sorting in C and G. Scale bar = 100 μm.

Sorting Sexed PGC

Embryos were identified as male or female using PCR. Gonadal PGC were isolated and subsequently sorted using the same procedures as PGC from unsexed embryos. It is important to sort PGC from sexed embryos because they can be used in different experimental situations. It is possible to alter sexual phenotype by treating chick embryos with an aromatase inhibitor to induce female chickens to exhibit a male phenotype (Vaillant et al., 2001, 2003), and implantation of testes into female embryos can cause genetically female chickens to take on a male phenotype (Stoll et al., 1980; Maraud et al., 1987; Stoll et al., 1993). Taken together (Stoll et al., 1980; Maraud et al., 1987; Stoll et al., 1993; Vaillant et al., 2001, 2003) these studies suggest that there is plasticity in sexual phenotype. It is unknown, however, if simply implanting cells of a different sexual phenotype will induce any systemic changes, but it is unlikely because the genotype of the gonad directs gamete development independently of PGC genotype (Hajji et al., 1988). Naito et al. (1999) placed male germ cells into female recipient embryos and female germ cells into male recipient embryos. The rates of donor-derived offspring from the chimaeric chickens were 0.4 to 0.9% for male donor into female recipient embryos and 0.1 to 0.3% for female donors into male recipient embryos, suggesting that the germ cell survival was very low. The present study provides a new method to obtain large numbers of PGC for transfer into recipient embryos of a different sex than the donor embryo. The new approach will allow investigators to more thoroughly assess the effect of sex-specific PGC implantation on sexual phenotype or the appearance of ovo-testes.

Male chickens, in contrast to male mammals, are the homogametic sex (ZZ), whereas the female chickens are the heterogametic sex (ZW). Therefore, if genetically male PGC placed into a female embryo do not alter the sexual phenotype, they may increase the number of Z-bearing ova that are produced by the chickens. Subsequently, it is possible that proportion of male progeny will increase because there will be more Z-bearing ova to meet the Z-bearing sperm. Hence, the ratio of male progeny from the manipulated female embryo should theoretically be larger than progeny from unmanipulated embryos (Kagami, 2003).

In summary, it has been demonstrated that it is possible to sort viable PGC from the embryonic blood and embryonic gonad based upon SSEA-1 fluorescence and to recover enriched PGC. Similarly, it is possible to sort PGC from embryos of a specific sex. The utility of this new technology is that it will be possible to use it in studies aimed at understanding PGC biology and sex determination.

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