Production of Chick Germline Chimeras from Fluorescence-Activated Cell-Sorted Gonocytes

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ABSTRACT Modification of the chicken germline has been difficult, because it has been challenging to fractionate sufficient numbers of primordial germ cells for manipulation and implantation into developing embryos. A technique to enrich cell suspensions for primordial germ cells, using fluorescence-activated cell sorting (FACS), has recently been developed. The objective of the current study was to demonstrate that the FACS-enriched early embryonic gonocytes could fully participate in development of the germline. Therefore, cells were disassociated from stage 27 gonads, incubated with mouse anti-stage-specific embryonic antigen-1, which was detected with goat-antimouse IgM-fluorescein isothiocyanate, and the fluorescently labeled cells were sorted from the unlabeled cells using FACS. The isolated gonocyte population was injected into the blastoderm of unincubated stage X embryos, the germinal crescent of 3-d embryos, and into the circulation of stage 17 embryos that were pretreated with busulfan. Barred Plymouth Rock gonocytes were implanted exclusively into recipient White Leghorn embryos, and White Leghorn gonocytes were implanted exclusively into Barred Plymouth Rock recipient embryos. Embryos were cultured until hatch, and male putative chimeras were reared to sexual maturity. Germline chimerism was evaluated by observing feather color of the progeny. All injection methods resulted in germline chimeras demonstrating that FACS-sorted gonocytes can fully participate in development. Moreover, it was demonstrated that gonocytes isolated from stage 27 embryonic gonads can be introduced into embryos at an earlier stage of development, and the introduced gonocytes can fully participate in germline development.

Key words: gonad, stage-specific embryonic antigen-1, testes, fluorescence-activated cell sorting, primordial germ cell

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INTRODUCTION

Primordial germ cells (**PGC**) are the embryonic precursors to the ova and the spermatozoa found in the adult animal (Wentworth and Wentworth, 2000). Presently, PGC can first be recognized in the stage X blastoderm (Karagenc et al., 1996), and, after gastrulation, they can be identified in the extraembryonic region called the germinal crescent (Swift, 1914). Consequently, the PGC migrate from the germinal crescent into the embryonic gonadal ridge, which develops into the mature gonad. During the first phase of this migratory process, PGC are passively carried to the vicinity of the gonadal ridge through the extra- and intraembryonic circulation (Meyer, 1964; Fujimoto, 1976 a,b). During the second phase, PGC leave the blood vessels and actively migrate to the gonadal ridge. This second phase of migration involves chemotactic signals from the gonad (Dubois and Croisill, 1970; Kuwana et al., 1986) and extracellular matrix (Urven et al., 1989), and the vascular system surrounding the germinal epithelium may direct the PGC to the embryonic gonad. Once the PGC arrive in the gonadal anlage, they are called gonocytes.

The production of germline chimeras is an important tool for studies aimed at understanding germ cell development and germ cell specification. Germline chimeras have important practical applications in the conservation of species and existing genetic stock, because it may be possible to store frozen PGC to maintain genetic material rather than maintaining living stocks of birds. Furthermore, PGC are the best target cell type to stably insert a gene into the germline to secrete therapeutic proteins in eggs (Lillico et al., 2005). Success in generating germline transgenic chickens has mostly arisen through injecting blastoderms with lentiviral or retroviral vectors to insert the transgene into the germline (Mozdziak et al., 2003; McGrew et al., 2004; Chapman et al., 2005). The rate of germline transmission using viral gene insertion has been extremely variable, making it an enticing option to directly target isolated PGC for transgene insertion. However, a challenge to creating germline chimeras using PGC is the ability to isolate PGC in sufficient numbers and culture PGC to serve as donor cells.

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The first chicken germline chimera was produced by transferring isolated blastodermal cells from a Barred Plymouth Rock (**BPR**) embryo into a recipient White Leghorn (**WL**) embryo. The resulting somatic chimera transferred the BPR genotype to its offspring (Petitte et al., 1990). Subsequently, other investigators have successfully isolated PGC from embryonic chick blood or gonads, enriched the initial cell populations for PGC using a Ficoll density gradient (Park et al., 2003) or magnetic cell sorting (Kim et al., 2004, 2005), and they have demonstrated germline transmission. Similarly, it has been recently shown that blood-derived PGC can migrate from a stage X embryo to the germinal ridge, but germline transmission of PGC was not reported for the technique, and the ability of gonadal PGC to participate in the development of a stage X embryo was also not evaluated (Naito et al., 2004). Recently, fluorescence-activated cell sorting (**FACS**) has been used to enrich isolated embryonic gonadal and blood cell populations for PGC, but germline transmission of these cells was not evaluated (Mozdziak et al., 2005). Cells reactive for generally accepted PGC markers, such as mouse anti-stage-specific embryonic antigen-1 (**SSEA-1**; Resnick et al., 1992; Karagenc et al., 1996; D'Costa and Petitte, 1999), harvested from the embryonic gonad will be called gonocytes, herein. The objective of the present study was to generate germline chimeras from cell populations that were enriched for gonocytes using FACS and to evaluate the ability of gonocytes to participate in germ cell development when injected into earlier stages of development.

MATERIALS AND METHODS

Isolation of Donor Germ Cells

Male gonads were removed from 10 to 14 embryos (stage 27) and pooled in 200 μL of 0.25% trypsin (room temperature) and were subsequently incubated at 37°C for approximately 15 min for cell liberation. The trypsin was inactivated with 200 μL of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum. The suspension was filtered with a 35-μm filter (Mozdziak et al., 2005).

PCR Sexing of Embryos

Samples of extraembryonic membranes with blood vessels surrounding embryos were dissected and placed on ice in a microcentrifuge tube containing 50 μL of 2:1 lysis solution (10 m*M* Tris pH 8.0, 10 m*M* EDTA, 6 *M* NaCl, and 0.6 *M* NH_3OH) to liberate DNA. The sample was heated to 100°C for 13 min and then placed on ice. Sterile water (350 μL) was added to the DNA solution. After centrifugation, 2 μL of the supernatant was used as a DNA template in the PCR reaction to sex the embryo. Polymerase chain reaction components were 2.5 m*M* each W-chromosome primer [5'gprimerWxho-1:5'-CCCAAA TATAACACGCTTCACT-3',3'gprimerWxho-2:5'-GAA ATGAATTATTTTCTGGCGAC-3'],b 0.5 m*M* each 18S ribosomal RNA primer (5'gprimer Ribo-1: 5'-AGCTCTTT CTCGATTCCGTG-3',3'primerRibo-2:5'-GGGTAGACA CAAGCTGAGCC-3'), 0.2 m*M* each of deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Promega, Madison, WI), 1× MasterAmp PCR Enhancer (Epicenter, Madison, WI), 2 μg of DNA template in a buffer of 3.0 m*M* $MgCl_2$, 20 m*M* KCl, 50 m*M* Tris-HCl, 1 m*M* Ficoll (Idaho Technology, Idaho Falls), 1 m*M* tartrazine, and 500 μg of BSA/mL (Clinton et al., 2001; Mozdziak et al., 2005). Rapid amplification was performed in a 10-μL volume loaded into a glass capillary tube (1-mm outside diameter, 0.5-mm inside diameter). Rapid cycling was accomplished in a 1605 AirThermocycler (Idaho Technology) with initial denaturation at 95°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. The DNA was separated by electrophoresis, and the gel was stained with ethidium bromide before ultraviolet visualization. All procedures were performed as previously reported (Petitte and Kegelmeyer, 1995; Mozdziak et al., 2005). After evaluation, all female embryos were discarded from further analysis.

FACS

The disassociated male gonadal cells were suspended in Media 199 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% embryonic stem cell-qualified fetal bovine serum (Invitrogen Corp.). Subsequently, the gonadal cells were incubated with a mouse monoclonal antibody against SSEA-1 (Developmental Studies Hybridoma Bank, Iowa City, IA) for 30 to 60 min on ice. Stage-specific embryonic antigen-1 is a generally accepted cell surface marker for mouse and avian PGC (Resnick et al., 1992; Karagenc et al., 1996; D'Costa and Petitte, 1999). After the cell suspension was washed with PBS, goat-antimouse IgG conjugated to fluorescein isothiocyanate was added to the cell suspension at a 1:1,000 dilution and incubated for 30 min on ice. The cell suspension was washed with Media 199 and 10% embryonic stem cell-qualified fetal bovine serum. Fluorescence-activated cell sorting was performed using MoFlo high-speed FACS (Cytomation, Fort Collins, CO) to collect a suspension of SSEA-1–positive germ cells. All flow cytometry procedures were previously detailed in Mozdziak et al. (2005). The enriched embryonic gonocyte suspensions were subsequently injected into developing embryos.

Gonocyte Injections

Once the gonocytes were isolated from male embryos and enriched using FACS, they were introduced into embryos to demonstrate germline transmission using 3 different methodologies. One method was to implant 500 to 2,000 sorted germ cells into the subgerminal cavity of a stage X embryo using procedures modified from Petitte et al. (1990) and Mozdziak et al. (2003). The embryos were cultured in a surrogate chicken eggshell, followed by a

Table 1. Production of germline chimeras following injection of primordial germ cells (PGC) into stage X embryos, stage 17 embryos, or the germinal crescent

	Stage X[1]				Germinal crescent[2]					Stage 17[3]				
BirdID	PGC[4]	Prog[5]	Donor[6]	Rate[7]	Bird	PGC	Prog	Donor	Rate	Bird	PGC	Prog	Donor	Rate
1091	560	229	3	1.3	2317	1,150	214	3	1.4	3953	1,055	239	1	0.4
1095	700	177	0	0	2335	1,000	246	0	0	3955	1,055	211	1	0.4
1097	700	230	3	1.3	2337	1,000	225	0	0	3957	1,055	157	1	0.6
2301	1,033	244	0	0	2341	2,000	111	0	0	3959	1,055	403	28	6.9
2303	1,000	163	4	2.5	2343	2,000	199	0	0	B0879	670	112	1	0.8
2307	1,000	213	3	1.4						B1187	717	239	1	0.4
2309	1,000	220	0	0						2345	3,500	139	1	0.7
2323	2,000	190	11	5.7										
2327	2,000	249	0	0										
2329	2,000	282	116	41.1										
Germline transmission[8]				5.3 ± 4.0					0.3 ± 0.3					1.5 ± 0.9
Germline chimeras[9]				60					20					100

[1]Stage X embryos injected with sorted PGC.

[2]PGC were injected into the germinal crescent.

[3]Stage 17 embryos treated with busulfan at 24 h of incubation and injected with PGC into the vascular system at stage 17.

[4]Number of PGC injected.

[5]Prog = progeny examined from the chimera.

[6]Donor = donor offspring.

[7]Rate of germline transmission per male.

[8]Average germline transmission. Values represent mean ± SE ($P > 0.05$).

[9]Percentage of males generating germline chimeras.

surrogate turkey eggshell, until hatching, following procedures modified from Borwornpinyo et al. (2005).

The second method was to pretreat the eggs with an injection of 75 μg of busulfan emulsion into the yolk of embryos after 24 h of incubation, according to the methods of Song et al. (2005). The busulfan treatment has been demonstrated to improve the number and frequency of germline chimeras generated following PGC transfer (Song et al., 2005). After busulfan injection, the eggs were returned to the incubators until they reached stage 17 (Hamburger and Hamilton, 1951), when they were injected through the dorsal aorta with 600 to 3,500 sorted gonocytes. After injection, the eggshells were sealed, and the eggs were returned to the incubator and maintained until hatching.

Lastly, embryos at 3 d of incubation were injected with 1,000 to 2,000 sorted gonocytes into the germinal crescent. The injected embryos were cultured in a surrogate turkey eggshell until hatching, following the procedures of Borwornpinyo et al. (2005).

Evaluation

In all cases, BPR gonocytes were implanted exclusively into recipient WL embryos, and WL gonocytes were implanted exclusively into BPR recipient embryos. All procedures involving birds were approved by the North Carolina State University Institutional Animal Care and Use Committee. Male birds from recipient embryos were grown to sexual maturity. White Leghorn recipient roosters were mated exclusively with WL hens, and BPR feather color in the progeny was the measure of germline transmission. Similarly, BPR recipient males were mated exclusively with BPR hens, and WL feather color was the measure of germline transmission. At least 100 progeny were evaluated from each male recipient. The rate of germline transmission was calculated as the number of donor offspring relative to the total number of offspring generated by each male. The rate of germline transmission between each treatment was analyzed using the GLM procedure of the SAS Institute (1990) to perform a 1-way ANOVA, and means were separated using least significant differences.

RESULTS AND DISCUSSION

Mozdziak et al. (2005) has documented that it is possible to select a population of cells based upon SSEA-1 immunoreactivity and recover a highly enriched population of viable PGC from blood or early gonads. Most of the enriched cells were identified as germ cells based upon monoclonal antibody EMA-1 immunoreactivity and periodic acid-Schiff staining, which are generally accepted PGC markers (Meyer, 1964; Fujimoto et al., 1976 a,b; Urven et al., 1988, 1989). Therefore, the current study was undertaken to demonstrate that sorted gonadal PGC or gonocytes could fully participate in development and, ultimately, pass their genetic material through the germline to subsequent generations.

In the current study, the enriched suspensions of male gonocytes were injected into the embryos, and the resulting putative male chimeric chicks were raised to sexual maturity and mated with hens to test for germline transmission. Three different methods of delivering the gonocytes to the embryo were evaluated. First, germ cells were delivered through the vasculature to stage 17 (Hamburger and Hamilton, 1951) embryos that had been pretreated with busulfan. The current results support and

expand previous work (Mozdziak et al., 2005), because it has now been demonstrated that FACS-sorted gonocytes can generate germline chimeras. Ficoll density gradients have been used to enrich PGC and generate chimeras (Park et al., 2003), but the current work is the first to demonstrate that germline chimeras will result from sorted gonocytes introduced into busulfan-treated embryos (Table 1).

The second method to generate germline chimeras was to inject germ cells into the subgerminal cavity of the embryo from a freshly laid egg. It may be possible for the gonocytes implanted into the stage X embryo to survive, because PGC appear to be determined before stage X (Zhu et al., 2005; van de Lavoir et al., 2006), and it has been shown that blood-derived PGC implanted into the stage X embryo can migrate and populate the genital ridge (Naito et al., 2004). Therefore, it was hypothesized that the gonocytes would enter into the genital ridge and participate in germline development. Furthermore, it has been suggested that chicken embryonic stem cells are not capable of participating in germline development (Zhu et al., 2005; van de Lavoir et al., 2006), which may support the concept that PGC are predetermined in the chick embryo. Similar to the results of injecting stage 17 embryos as recipients, it is also possible to implant gonocytes into stage X embryos and generate germline chimeras (Table 1). The most novel aspect of the results is that gonocytes can fully participate in normal development when implanted into an earlier developmental stage embryo, suggesting that there is germline plasticity.

The last method to generate chimeras was to implant gonocytes into the germinal crescent. Because the germinal crescent is the first stage when PGC have been shown to be committed to the germline, it was surmised that the exogenous germ cells would participate in normal development and again generate germline chimeras. Similar to the other 2 methods, chimeras could be generated by directly implanting gonocytes into the germinal crescent, but germline chimerism was infrequent (Table 1).

Although there were no significant differences among the rates of germline chimera production among the 3 injection methods ($P > 0.05$), it appears that implanting cells directly into the germinal crescent may be the least efficient way to generate chimeras, because only 1 of 5 embryos (20%) produced any donor-derived offspring. Similarly, the rate of donor-derived offspring was numerically lower for injection into the germinal crescent compared with the other 2 methods, making it the least attractive way for an investigator to initiate germline chimera production in their laboratory. The second method to generate germline chimeras was to treat embryos with busulfan (Song et al., 2005), followed by introducing the sorted gonocytes into the embryo; it was efficient at generating germline chimeras because 7 of the 7 recipient embryos generated at least 1 donor-derived offspring. Similarly, the rate of germline transmission per individual was numerically higher compared with the germinal crescent injections. However, 6 of 10 recipient embryos generated germline chimeras using sorted gonocytes when the germ cells were implanted directly into the blastoderm of a stage X embryo. Therefore, it appears that blastodermal injection might be less reliable at generating chimeras compared with busulfan treatment. Lastly, it should be noted that the gonocytes injected into the stage X embryo never resulted in any donor feather pigmentation in the resulting chimeras. Hence, suspensions of FACS-enriched gonocytes from the early gonad are clearly committed to the germ cell lineage, even when injected into the relatively plastic stage X embryo.

Overall, the results of the current study confirm that heterologous, viable gonadal cells can be enriched for gonocytes through immuno-based FAC analysis. Furthermore, the results show that sorted gonocytes will ultimately result in germline chimeras and that to maximize the number of chimeras, it may be optimal to use busulfan treatment, followed by introduction of germ cells into the vasculature of stage 17 embryos, or to directly implant the cells into the stage X blastoderm. Lastly, the results demonstrate for the first time that gonocytes implanted into an earlier developmental stage embryo will fully participate in germline development.

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