Cryopreservation of specialized chicken lines using
cultured primordial germ cells

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ABSTRACT Biosecurity and sustainability in poultry production requires reliable germplasm conservation. Germplasm conservation in poultry is more challenging in comparison to other livestock species. Embryo cryopreservation is not feasible for egg-laying animals, and chicken semen conservation has variable success for different chicken breeds. A potential solution is the cryopreservation of the committed diploid stem cell precursors to the gametes, the primordial germ cells (PGCs). Primordial germ cells are the lineage-restricted cells found at early embryonic stages in birds and form the sperm and eggs. We demonstrate here, using flocks of partially inbred, lower-fertility, major histocompatibility complex- (MHC-) restricted lines of chicken, that we can easily derive and cryopreserve a sufficient number of independent lines of male and female PGCs that would be sufficient to reconstitute a poultry breed. We demonstrate that germ-line transmission can be attained from these PGCs using a commercial layer line of chickens as a surrogate host. This research is a major step in developing and demonstrating that cryopreserved PGCs could be used for the biobanking of specialized flocks of birds used in research settings. The prospective application of this technology to poultry production will further increase sustainability to meet current and future production needs.

Key words: primordial germ cell, chicken, cryopreservation, biobank, stem cell

INTRODUCTION

Preservation of genetic diversity of extant populations that can be reintroduced at later times to avoid population bottlenecks is central to controlled flock management. This is especially important for the biosecurity of poultry production systems, which are at risk of emerging disease pandemics such as avian influenza (Whyte et al., in press). There is also a recognized need to preserve and safeguard the genetic diversity of traditional breeds of chicken (Wilkinson et al., 2012). Many of these breeds are maintained in regionally restricted populations and are vulnerable to both disease outbreaks and losses in genetic diversity due to fluctuations in population sizes. Similarly, poultry genetic resources used in research are being lost, as experimental lines of chickens developed to investigate a multitude of traits are being eliminated by research and government institutes (Fulton and Delany, 2003). Smaller-scale efforts to cryopreserve rare breeds could be combined with efforts being made in commercial breeding in order to safeguard genetic resources on both a national and global scale (Blesbois et al., 2007). It is noteworthy that cryopreservation programs could be financially viable for poultry flocks after as little as 3 years and using conventional backcrosses to re-establish the genome of the poultry breed (Silversides et al., 2012).

As it remains experimentally unachievable to cryopreserve avian oocytes or early-stage embryos, an entire chicken breed cannot be reconstituted using conventional cryopreservation technologies (Petitte, 2006). The cryopreservation of chicken male gametes can be achieved using traditional methods of semen preservation. There are inherent problems with using semen for reconstitution of chicken breeds (Blesbois et al., 2007). Semen viability after cryopreservation has proven variable between poultry breeds, and as the female chicken is the heterogametic sex containing the W sex chromosome, the entire avian genome cannot be conserved using semen preservation. Therefore, frozen semen collections can only be effectively used to safeguard and increase the genetic diversity of extant chicken breeds. An alternative method for the cryopreservation of avian gametes is the cryopreservation of gonadal tissue followed by organ transplantation into host chickens. The frozen
gonad (testicular or ovarian tissue) is transplanted into immunocompromized hosts and can produce functional semen and oocytes (Song and Silversides, 2007a,b). However, this procedure has not yet produced pure-bred offspring from the direct mating of a male host carrying donor testis tissue with a female host carrying donor ovary tissue.

The use of early germ cell precursors, the PGCs in avian species offers an innovative platform to reconstitute chicken breeds from frozen materials. The PGCs are formed very precociously during avian development. It has been demonstrated that these cells can be isolated from the embryonic circulatory system. The cells can be reintroduced into the circulatory system of host embryos and will colonize the host gonad and produce viable male and female gametes in hosts of the same sex (Naito et al., 1994; Song et al., 2005; Nakamura et al., 2010a; Nakamura et al., 2012). The difficulties associated with this method are that only 100 to 200 PGCs in total are present in the circulatory system of the embryo at this stage and the exogenous introduced PGCs must compete with the endogenous PGCs present in the surrogate host embryo. Nevertheless, using this technique, a pure rare-breed chicken was reconstituted from frozen PGCs using surrogate hosts of common chicken variety (Nakamura et al., 2010b).

The development of in vitro culture conditions for expanding the population of PGCs before cryopreservation potentiates the ability to safely store cells before transplantation and reduces the technical skill needed to isolate and purify the cells before transplantation. A schematic showing the experimental steps in this protocol is shown in Figure 1. Germ line transmission has been shown from many laboratories using high serum medium conditions (van de Lavoir et al., 2006; Choi et al., 2010; Macdonald et al., 2010; Macdonald et al., 2012; Miyahara et al., 2014; Song et al., 2014; Naito et al., 2015). Recently, we developed a defined medium for the culture of chicken PGCs (Whyte et al., 2015). Defined serum conditions should allow for the standardized culture of PGCs that can subsequently be transferred between research institutes.

As a first step in demonstrating the biobanking of chicken breeds using in vitro cultured and cryopreserved PGCs we have used 5 highly inbred White Leghorn lines of chicken currently maintained at the National Avian Research Facility (NARF), UK. These White Leghorn lines were back crossed and selected to express a single MHC haplotype and vary in their susceptibility to many avian viral and bacterial pathogens. These lines used in this study are the inbred line 6 (varying in their susceptibility to Marek’s virus) the partially inbred line O lacking endogenous avian leukosis viruses (ALVs), Cornell partially inbred lines N and P (differing in resistance to MD), and the Wellcome inbred line W; (lines 6, O, N, P, W) (Cole, 1968; Bacon et al., 2000). These chicken lines have been maintained for several decades as breeding populations in several countries and have been used in vaccine development and the identification of genes involved in disease resistance to several pathogens. Although partially inbred, these lines still contain some genetic variability which is much reduced compared to outbred lines (Gheyas et al., 2015). The use of inbred lines also demonstrates the capacity to biobank low-fertility, specialty breeds using cultured PGCs.

Here we describe the establishment of a biobank of frozen primordial germ cells from 5 chicken lines of research interest. Each archived line comprises between 15 to 32 individuals in total, and at least 5 individuals of each sex.

MATERIALS AND METHODS

PGC Culture Medium

Avian PGC culture medium contained 1 × B-27 supplement, 2.0 mM GlutaMax, 1 × NEAA, 0.1 mM β-mercaptoethanol, 1 × nucleosides, 1.2 mM pyruvate, 0.2% ovalbumin (Sigma), 0.2% sodium heparin (Sigma) 5 μg/mL in avian DMEM, a custom basal medium (a modification of knockout DMEM [250 mosmol/L, 12.0 mM glucose, and CaCl-free; ThermoFisher Scientific]). The following growth factors were added.
before use: human Activin A, 25 ng/mL (Peprotech);
human FGF2, 4 ng/mL (R&D Biosystems); 0.2% 
chicken serum (Biosera). All reagents were purchased 
from ThermoFisher Scientific unless otherwise speci-

**Chicken Lines, PGC Line Derivations and Embryo Manipulations**

Chicken flocks of several partially inbred White 
Leghorn lines were produced and maintained at IAH, 
Compton and are now housed at the NARF, UK. Line 
0, 6 and N birds originate from the Poultry Research 
Laboratory, East Lansing, MI. Lines N and P line birds 
from Cornell University, USA and Wellcome B14 line 
(W line) from Wellcome Research Laboratories, Beck-

Inbred PGC lines were derived by placing ~1.0 µL 
of blood isolated from stage 15 to 16 (H&H) embryos 
in 300 µL medium in a 48-well plate. One-third of the 
medium was changed every 2 d. When total cell number 
reached 1.0 × 10^5, total volume of medium was changed 
every 2 d and cells were propagated at 2 to 4 × 10^5 
cells/mL medium. Cells were frozen in Avian DMEM 
containing 4% DMSO/5% chicken serum and stored at 
−150°C. The donor embryo was isolated and sex was 
determined as published (Macdonald et al., 2010).

To investigate the efficiency of germline transmis-
sion, one male and one female line 6 cell line were ex-
panded in culture in FAcs medium and cryopreserved 
for 1 wk. Cells were thawed, cultured for several wk 
and counted. Eggs were incubated until stage 16 HH 
and windowed through the pointy end. 5,000 to 6,000 
cells total of male or female cells were injected into the 
dorsal aorta, the egg was resealed with parafilm and in-
cluded in the sample lysed and PGCs present in the well pro-
liferated as single dispersed cells (Figure 2). At the end 
of 3 wk, the cultures containing more than 100,000 cells 
were scored as a positive derivation of a cell line, and 
these cells were cultured for an additional wk in increas-
ing volume (100,000 cells per 0.5 mL medium) and then 
frozen in aliquots of 50,000 to 100,000 cells per vial.

The data in Table 1 show the results from these ex-
periments using 5 lines of inbred chicken. A total of 
203 cultures were started from single embryos. Approx-
imately 630 eggs were incubated to obtain this num-
ber. This 33% initiation rate was due to 1) infertility of 
the incubated eggs, 2) loss of fertility during shipment 
and storage of the eggs, 3) developmental abnormalities 
from the inbred lines, and 4) variation in development-
mental stages between lines which resulted in embryos that 
were too young (less than stage 15^+) or too old (older 
than stage 17 HH) for blood sampling.

For 203 cultures initiated from single embryos, 133 
independent cell lines (genotypes) were expanded and 
frozen in a total of 478 vials. The overall derivation rate 
was 67%, which is consistent with past cell line deriva-
tion results (Whyte et al., 2015). The derivation rates 
for male and female PGC cultures were similar (70% 
versus 65%, respectively) demonstrating that both male 
and female genotypes can be captured.

To avoid inbreeding depression, it has been estimated 
that a minimum population containing 13 breeding 
pairs is needed (FAO, 1998). We have obtained this 
number of genotypes for line 6 (27 genotypes), line N 
(29 genotypes), line O (30 genotypes) and line P (32 
genotypes). Line W comprises 15 genotypes and would 
need further cryopreserved cultures to regenerate an 
outbred population. Since these chicken lines were par-
tially inbred during the selection for single MHC hap-
lotypes, there is less of a requirement for retaining a 
higher level of genetic diversity in the stored germ cell 
lines.
Figure 2. PGCs cultured from inbred chicken lines. An example of derived PGCs culture from single embryos of Line 0 and line 6 chicken lines. Bar, 50 μm.

Table 1. Generation of frozen single-genotype, sexed PGC lines from lines of specialized inbred chicken.

| In-bred line | No. of expts | Male PGC cultures initiated | Male PGC geno-types frozen | Male PGC deri-vation rates (%) | Total Male PGC cultures initiated | Female PGC geno-types frozen | Female PGC deri-vation rates (%) | Total Female PGC cultures initiated | Total frozen geno-types | Total deri-vation rates (%) | Total vials |
|--------------|--------------|-----------------------------|---------------------------|-------------------------------|---------------------------------|-----------------------------|-------------------------------|---------------------------------|----------------------|------------------------|------------|
| 6            | 4            | 15                          | 12                        | 80                            | 45                              | 18                          | 15                           | 83                              | 52                    | 27                     | 97         |
| N            | 5            | 32                          | 13                        | 41                            | 52                              | 27                          | 16                           | 59                              | 62                    | 59                     | 114        |
| O            | 3            | 17                          | 13                        | 76                            | 52                              | 25                          | 17                           | 68                              | 68                    | 42                     | 120        |
| P            | 3            | 19                          | 18                        | 95                            | 58                              | 24                          | 14                           | 58                              | 44                    | 43                     | 102        |
| W            | 2            | 9                           | 5                         | 56                            | 15                              | 17                          | 10                           | 59                              | 30                    | 15                     | 45         |
| All lines    | 92           | 61                          | 222                       | 111                           |                                 | 72                          |                                 |                                 | 256                   | 203                    | 478        |

Summary of PGC line derivation results from multiple culture experiments and the total number of cryopreserved samples are indicated.

Germline Transmission of Inbred PGCs through Outbred Surrogate Hosts

To test that the cryopreserved PGC lines were germline competent and as a first step to demonstrate the reconstitution of a chicken breed from frozen propagated PGC lines, we wanted to verify that the cryopreserved PGCs could produce viable gametes when transplanted into an outbred commercial layer line. A male and a female line 6 PGC cryovial was thawed and expanded in culture for several wk. Five thousand to 6,000 male or female PGCs were injected into the dorsal aorta of stage 16 host ISA brown embryos. The embryos were incubated until hatching, hatched, and the hatched chicks were sexed by PCR. The hosts containing correctly sex-matched donor PGCs were raised to sexual maturity. Three hens and 3 cockerels containing donor line 6 PGCs were produced. An initial examination of the cockerel semen using microsatellite analysis tentatively identified the male containing the highest contribution of donor PGCs in the semen (used in Table 3 below). This cockerel and 2 females were crossed to wild-type ISA brown chickens. The mating data indicates that fertility was normal for these surrogate hosts (Table 2). The offspring from these matings were genotyped using microsatellite analysis to determine if they derived from a Line 6 gamete (Table 3). Three individual microsatellite primer pairs were used for this analysis. Primer pair MCW 145 produces a PCR product of 190 bp for line 6 genomic DNA. Primer pair Lei 258 produces a PCR product of 265 bp for line 6 genomic DNA. Finally, primer pair Lei 221 produces a PCR product of either 207/208 bp or 235 bp as the line 6 birds contain 2 different alleles at this locus. Table 3 presents the results of this breeding experiment. Line 6 control genomic DNA from 2 male and 2 female PGC lines (M1, M2, F1 and F2, respectively) clearly produced the predicted PCR products for all 3 primer sets. Genomic DNA from the ISA brown host line did not produce a similar product for primer set Lei221 or Lei 258 but several offspring did contain a 190 bp product for the MCW145 primer set, indicating that this primer set is not conclusive for identifying line 6 offspring from this mating. This is apparent for the offspring from hen 11-12 which contained a band of 190 bp but none of

Table 2. Fertility of founder birds containing exogenous PGCs.

| Founder birds | Days PGCs cultured before injection | Eggs | Chicks |
|---------------|-----------------------------------|------|-------|
| IBL 11–12 ♂   | 36                                | 32   | 21 (65%) |
| IBL 14–7 ♂    | 64                                | 19   | 12 (63%) |
| IBL 11–6 ♂    | 36                                | 169  | 114 (67%) |
Table 3. Microsatellite analysis of offspring from surrogate host chickens.

|                | Lei 221  | MCW145 | Lei 258 |
|----------------|----------|---------|---------|
|                | 207/208, 235 | 190     | 207     |
| Donor line 6   |          |         |         |
| Line 6 PGC♀1   | 208      | 190     | 265     |
| Line 6 PGC♂1   | 207      | 190     | 265     |
| Line 6 PGC♀2   | 207      | 190     | 265     |
| Line 6 PGC♂2   | 207      | 190     | 265     |
| Host line      |          |         |         |
| ISA brown♀     | 203      | 190     | 310     |
| ISA brown♂1    | 206      | 204     | 308     |
| ISA brown♂2    | 206      | 204     | 308     |
| Female host 11–12 |        |         |         |
| Offspring      |          |         |         |
| 9              | 207      | 190     | 265     |
| 10             | 207      | 190     | 265     |
| 14             | 207      | 190     | 265     |
| 15             | 207      | 190     | 265     |
| 18             | 207      | 190     | 265     |
| 19             | 207      | 190     | 265     |
| 20             | 207      | 190     | 265     |
| 24             | 207      | 190     | 265     |
| 25             | 207      | 190     | 265     |
| 32             | 207      | 190     | 265     |
| Female host 14–7 |        |         |         |
| Offspring      |          |         |         |
| 9              | 207      | 190     | 265     |
| 10             | 207      | 190     | 265     |
| 14             | 207      | 190     | 265     |
| 18             | 207      | 190     | 265     |
| 19             | 207      | 190     | 265     |
| Male host 11–6 |          |         |         |
| Offspring      |          |         |         |
| 12             | 207      | 190     | 265     |
| 13             | 207      | 190     | 265     |
| 19             | 207      | 190     | 265     |
| 22             | 207      | 190     | 265     |
| 25             | 207      | 190     | 265     |
| 39             | 207      | 190     | 265     |
| 41             | 207      | 190     | 265     |
| 42             | 207      | 190     | 265     |
| 43             | 207      | 190     | 265     |
| 44             | 207      | 190     | 265     |
| 45             | 207      | 190     | 265     |
| 46             | 207      | 190     | 265     |
| 47             | 207      | 190     | 265     |
| 48             | 207      | 190     | 265     |
| 49             | 207      | 190     | 265     |
| 50             | 207      | 190     | 265     |
| 51             | 207      | 190     | 265     |
| 52             | 207      | 190     | 265     |
| 53             | 207      | 190     | 265     |
| 54             | 207      | 190     | 265     |
| 66             | 207      | 190     | 265     |
| 79             | 207      | 190     | 265     |
| 94             | 207      | 190     | 265     |
| 95             | 207      | 190     | 265     |
| 96             | 207      | 190     | 265     |
| 100            | 207      | 190     | 265     |
| 102            | 207      | 190     | 265     |
| 112            | 207      | 190     | 265     |
| 113            | 207      | 190     | 265     |
| 114            | 207      | 190     | 265     |
| 117            | 207      | 190     | 265     |
| 118            | 207      | 190     | 265     |
| 120            | 207      | 190     | 265     |
| 121            | 207      | 190     | 265     |
| 122            | 207      | 190     | 265     |
| 123            | 207      | 190     | 265     |
| 124            | 207      | 190     | 265     |
| 126            | 207      | 190     | 265     |
| 127            | 207      | 190     | 265     |
| 128            | 207      | 190     | 265     |
| 130            | 207      | 190     | 265     |
| 135            | 207      | 190     | 265     |
| 136            | 207      | 190     | 265     |
| 138            | 207      | 190     | 265     |
| 140            | 207      | 190     | 265     |
| 143            | 207      | 190     | 265     |
| 146            | 207      | 190     | 265     |
| 152            | 207      | 190     | 265     |
| 153            | 207      | 190     | 265     |
| 155            | 207      | 190     | 265     |
DISCUSSION

The storage and faithful recovery of breeds of poultry from stored germplasm is needed for the long-term safeguarding and management of poultry genetic resources (Whyte et al., in press). Due to the structure of the laid chicken egg, the storage of ova and early embryos has not been possible so multiple cryopreservation methods have not been possible. Cryopreservation of avian species wholly relies on the use of semen. Semen preservation in chicken is variable between breeds and much more difficult than the routine laboratory procedures used for many mammalian species (Whyte et al., in press). Recently, cryopreservation and transplantation of gonadal tissue from both males and females have been developed (Song and Silversides, 2007a,b). It remains to be seen if this will be a viable method and join semen as a preferred method for breed preservation. The requirement of surgery and immunosuppressants shows that this will be a highly technical procedure with welfare issues. The use of the early precursors to the germ cell lineage, the PGCs, offers an alternative method to safeguard valuable flocks of chickens. It has previously been demonstrated that PGCs can be isolated from the early circulatory system of the embryo or from the embryonic gonad, purified, and cryopreserved. These cells can later be introduced into the circulatory system of chicken embryos and will develop into sperm or oocytes that can produce viable offspring. Here we were able to expand and capture hundreds of genotypes over short (4 wk) culture periods. Our results demonstrate that over the period of a few months sufficient PGC lines could be generated to reconstitute a breeding population for a traditional breed of chicken. A method to increase the throughput of germ-cell line processing is needed if this system is to be applied to commercial pedigree breeds of chicken. This is a prerequisite, as commercial chicken lines comprise several hundreds of genetically diverse individuals for the preservation of genetic resources for sustainability and adaptation for future poultry demands.

The reconstitution of a chicken breed using cryopreserved chicken semen and backcrossing is predicted to take 4 crosses to re-establish 97% of the genome of the original breed (Blesbois et al., 2007). The cost of such a program is predicted to be fiscally equivalent to the costs of maintaining a chicken flock for 3 years (Silversides et al., 2012). The additional costs required for the culture and cryopreservation of PGCs to produce a cell-based biobank could be met if we are able to directly mate the surrogate host males and females to reconstitute a chicken breed in a single cross. In the germline transmission experiments reported here, the male transmission rate was low. Although, using cryopreserved semen, we could have reproduced a pure-bred line from the transmitting female surrogate host (14-7). In our previous work, we obtained 80 to 100% transmission rate from female hosts yet only 10% transmission from the male host indicating that transmission rates using male cultured PGCs are much lower than from females in our culture medium (Whyte et al., 2015). Our results reinforce the need for sterile hosts to reconstitute a pure breed especially from male PGCs. Germ cell ablation can be obtained through chemically induced sterility (Nakamura et al., 2008, 2010a) or genetically engineered sterility by mutation of a gene important for avian germ cell development. It was demonstrated that a pure rare breed of chicken could be produced from isolated cryopreserved PGCs via a surrogate host with depleted germ cells (Nakamura et al., 2010b). Future experiments will either use chemically ablated or genetically ablated host embryos to increase the germ line transmission rates.

TABLE 3. continued

|                 | Lei 221 |     |    | MCW145 |       |    | Lei 258 |     |    |
|----------------|---------|-----|-----|--------|-------|-----|---------|-----|-----|
|                | 207/208 | 235 |    | 190    |       |    |        | 265 |    |
|                | Call 1  | Call 2 |        |  Call 1 | Call 2 |       |  Call 1 | Call 2 |       |
| 158            | 214     | 214  |    | 201    | 201   |    | 308     | 310  | |
| 162            | 210     | 214  |    | 201    | 204   |    | 249     | 364  | |
| 164            | 210     | 214  |    | 201    | 201   |    | 249     | 310  | |
| 165            | 214     | 214  |    | 201    | 201   |    | 310     | 364  | |
| 166            | 214     | 218  |    | 201    | 201   |    | 310     | 364  | |
| 168            | 203     | 218  |    | 201    | 204   |    | 249     | 364  | |

The PCR product size is indicated for genomic DNA from control and offspring from the surrogate host chickens. Two allelic calls were made for each genomic DNA sample and line 6 alleles are indicated in **bold**.
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