SYMPOSIUM

Inserting random and site-specific changes into the genome of chickens[1]

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ABSTRACT During the past decade, modifications to the chicken genome have evolved from random insertions of small transgenes using viral vectors to site-specific deletions using homologous recombination vectors and nontargeted insertions of large transgenes using phi-31 integrase. Primordial germ cells (PGC) and gonocytes are the germline-competent cell lines in which targeted modifications and large transgenes are inserted into the genome. After extended periods of in vitro culture, PGC retain their capacity to form functional gametes when reintroduced in vivo. Rates of stable germline modification vary from 1×10^{-5} for nontargeted insertions to 1×10^{-8} for targeted insertions. Following transfection, clonally derived cell lines are expanded, injected into Stage 13–15 Hamburger and Hamilton embryos, and putative chimeras are incubated to term in surrogate shells. Green fluorescent protein (GFP) is incorporated into transgenes to reveal the presence of genetically modified PGC in culture and the extent of colonization of the gonad during the first week posthatch. If the extent of colonization is adequate, cohorts of putative chimeras are reared to sexual maturity. Semen is collected and the contribution from donor PGC is estimated by evaluating GFP expression using flow cytometry and PCR. The most promising candidates are selected for breeding to obtain G1 heterozygote offspring. To date, this protocol has been used to (1) knockout the immunoglobulin heavy and light chain genes and produce chickens lacking humoral immunity, (2) insert human V genes and arrays of pseudo V genes into the heavy and light immunoglobulin loci to produce chickens making antibodies with human V regions, (3) insert GFP into nontargeted locations within the genome to produce chickens expressing GFP, and (4) insert Cre recombinase into the genome to produce chickens that excise sequences of DNA flanked by loxP sites.

Key words: transgenic, knock-out, knock-in, homologous recombination, immnunoglobulin gene

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INTRODUCTION

Techniques for engineering the genome of plants and animals have found broad application in agriculture, biomedical research, and drug development. In chickens, the first modifications to the genome were introduced almost 30 yr ago (Salter et al., 1987), but the widespread use of genetic engineering has been thwarted by a lack of technology to introduce site-specific changes, large sequences of DNA into the genome, or both. During the past decade, our laboratory has developed routine protocols for the introduction of genetic modifications of any kind and any size. The basic elements of the technology are 1) culture of chicken primordial germ cells (**PGC**) and germline stem cells from several embryonic stages (van de Lavoir et al., 2006; Leighton et al., 2008; Song et al., 2014), 2) genetic modification of chicken primordial germ cells using homologous recombination, site-specific integrases, or transposons; and 3) prediction of the germline potential of putative chimeras by evaluation of GFP expression in gonads and semen. Here we demonstrate these principles with nontargeted insertion of GFP and Cre recombinase genes, targeted knockouts of the heavy and light-chain immunoglobulin genes, and site-specific insertion of human immunoglobulin transgenes into the knock-out immunoglobulin loci.

Culture of PGC

The conditions for culturing PGC used in our laboratory have been described by van de Lavoir et al. (2006, 2012) and Song et al. (2014). Briefly, the cells are obtained by dissecting them from the germinal crescent of Stage 4–5 Hamburger and Hamilton embryos, by collection of blood from Stage 13–15 embryos, or from gonads at embryonic d 6 to 8, and placed into cell cul-

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Table 1. Typical timetable for growth of chicken primordial germ cells in vitro

Event	Days postcollection	Days posttransfection	Number of cells
Collection from embryo	0		50–200
Transfer to 24-well plate	14–21		
Transfer to 12-well plate	21–42		100,000
Transfer to 6-well plate			
Transfer to T75[1]	40–50		2×10^6
Dilution into 48-well plates and application of selection		3–6	One per well in a few wells
Appearance of colonies		10–20	100–1,000 per colony
Transfer to 24-well plate			
Transfer to 12-well plate			100,000
Transfer to 6-well plate			
Injection into recipients			10^5
Transfer to T75			2×10^6

[1]T75 tissue culture flask.

ture medium consisting of KO-DMEM supplemented with 40% buffalo rat liver cell-conditioned medium, 4 ng/mL of fibroblast growth factor (**FGF**), 6 ng/mL of stem cell factor (FGF), 10% FBS, and 2.5% chicken serum. A feeder layer of buffalo rat liver cells inactivated by exposure to radiation is also required. The advantage of using PGC from the germinal crescent is their relative abundance, whereas blood-derived PGC are in a vast minority relative to erythrocytes. The greater abundance of germinal crescent PGC allows early recognition of the cells of the culture. By contrast, blood-derived PGC become visible as the erythrocytes die off during the first 2 wk of culture. After leaving the circulation and migrating into the gonad, PGC become gonocytes. These cells also retain the ability to contribute to the germline following extended periods in culture and genetic modification. Whereas the later stages of culture of gonocytes are similar to those of PGC, their isolation requires removal of somatic cells by panning (i.e., elimination of cells adhering to the culture plate) as the culture is established (Song et al., 2014).

Germline transmission of PGC cultured under similar conditions has been reported by Macdonald et al. (2010, 2012), Choi et al. (2010), and Park and Han (2012). More recently, a defined medium that does not require a feeder layer has been developed by McGrew (2013).

Genetic Modification of PGC

In most cases, transgene design includes incorporation of GFP under the control of a ubiquitous promoter to assist in downstream evaluations of the cells in vitro and in vivo (see below). The selection cassette also includes a gene encoding antibiotic resistance under the control of a ubiquitous promoter to allow selection of the transfected cells. Both genes are flanked by loxP sites to facilitate their removal in vivo (see below). Homologous recombination vectors are designed with isogenic DNA in the regions of homology. Although the frequency of targeted clones per transfected cell is low, on the order of 1×10^{-8}, the proportion of correctly targeted clones out of the total number of clones is high. Targeting efficiencies at the 2 loci we have targeted, the immunoglobulin heavy and light chain loci, have been 10% for a vector with 4 kb total homology and up to 30% for vectors with 8 kb of homology. We speculate that nontargeted (random) insertion of the targeting vectors leads to silencing of the selectable markers most of the time, raising the apparent frequency of targeting. However, targeted loci do not need to be expressed in order for successful targeting because the immunoglobulin loci are not expressed in PGC. The genetic modifications are introduced into PGC by electroporation. Following transfection, the emergence of stable transfectants is facilitated by expression of GFP incorporated into the selectable marker cassette.

A typical timeline from collection of PGC or gonocytes from embryos to injection of a transfected cell line is shown in Table 1. Although not included in the table, a typical timeline also includes aliquots of 10^6 cells using standard protocols for freezing cells that have been maintained in vitro (Robertson, 1987).

It is preferable to use cells at an early passage, although in some cases, we have obtained germline transmission of cells that have been in culture for approximately 1 yr and serially transfected. Although we have not formally examined the relationship between duration in culture and germline transmission, our empirical observations suggest that the rate of germline transmission declines as the duration in culture increases.

Although both male and female PGC colonize the ovary and testis in ovo, functional gametes are formed only when female cells are introduced into female recipients or male cells are introduced into male recipients. Because males mature several weeks earlier than females and males can be mated to many females, we use only male cell lines for the introduction of genetic modifications.

Injection of PGC into Recipient Embryos

Injection of PGC into recipient embryos is scheduled when the culture contains at least 5×10^5 cells. Typically, 40 embryos are injected per cell line to produce about 20 hatched chicks. The cells are injected using

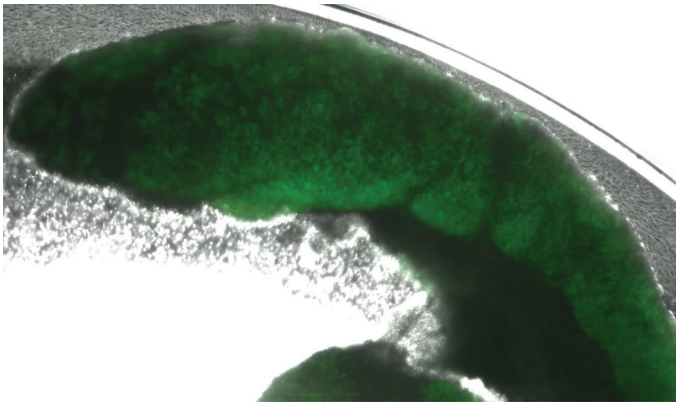

Figure 1. A typical ovary colonized by male primordial germ cells (PGC) expressing green fluorescent protein (GFP). The ovarian cortex contains GFP expressing male PGC whereas they are absent in the medulla. The chick was approximately 1 wk of age when the ovary was collected.

a mouth pipette with a 40-μm glass needle inserted into the peripheral veins or aortic artery of embryos at Stage 13–15 Hamburger and Hamilton. The embryos are transferred to surrogate shells and incubated to term as described by van de Lavoir and Mather-Love (2006).

Triage of Putative Chimeras from Hatch to Sexual Maturity

During the first 7 d posthatch, colonization of ovaries by the GFP-expressing cell line is evaluated under a dissecting microscope. Only the germline is GFP-positive because PGC do not contribute to somatic lineages. Because male cell lines are capable of initially colonizing the female gonad but do not develop into oocytes in chimeras, we use the gonads from female chicks to evaluate the level of colonization produced by each cell line. Although the evaluation of the extent of GFP fluorescence in the whole gonad is a subjective measure, we try to be consistent by having the same investigator score the gonads each time. Usually, but not always, the level of colonization is sufficient and the males are reared to sexual maturity. We define sufficient as at least half of the chimeras displaying over 50% colonization. A typical ovary colonized by male PGC expressing GFP is shown in Figure 1.

The first semen samples from male chimeras are analyzed by flow cytometry and PCR to obtain a qualitative assessment of the contribution of the donor PGC to the ejaculate. Examples of these evaluations are shown in Figure 2.

Several examples following the fate of transfected cell lines from injection to germline transmission are listed in Table 2. Examination of these data reveals that not all clonal cell lines derived from a transfection will yield offspring carrying the transgene. A few clones are eliminated because their ability to colonize the gonad is poor. Others are eliminated because their contribution to the ejaculate is judged inadequate after flow cytometry analysis, PCR of the transgenes in semen, or both. Approximately 60% of the chimeras carrying clones that are deemed to be good candidates for germline transmission after evaluation are bred to hens. For reasons that are not obvious at the present time, however, chimeras carrying clones that are deemed to be good candidates do not always yield transgenic offspring (for example, 229–46 in Table 2). In the examples presented in Table 2, about 75% of the chimeras carrying selected clones transmitted the transgene to their offspring. It should also be noted that the rate of germline transmission within cohorts of chimeras from a given clone is variable and unpredictable. For example, a chimera injected with PGC from clone #3 from the HG-193 cell line transmitted the transgene to 2% of its offspring, whereas another member of this cohort transmitted the transgene to 62% of its offspring. Although this is an extreme example, it highlights the differences in the ability of chimeras made with the same cells on the

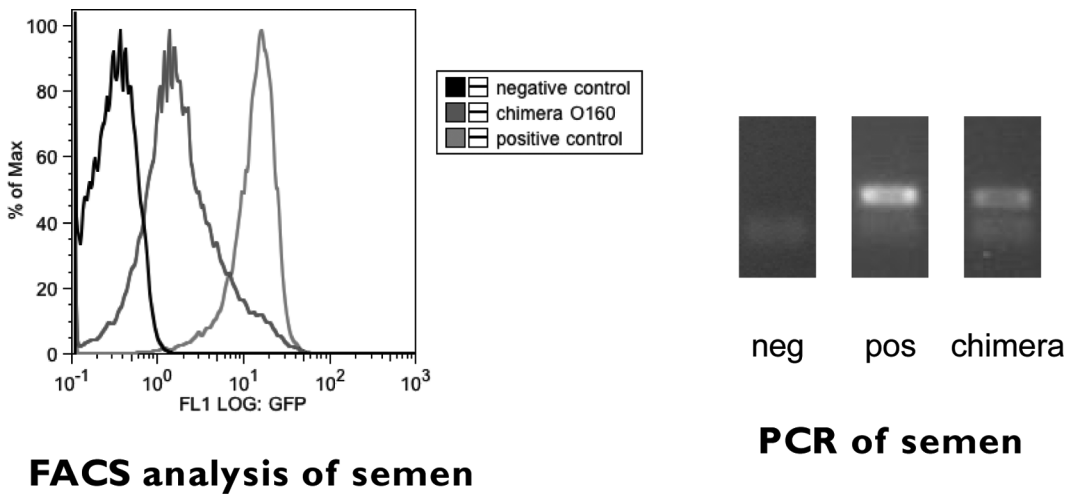

Figure 2. An example of detection of green fluorescent protein (GFP) in semen of chimeras by fluorescence-activated cell sorting (FACS; left panel) and by PCR (right panel). Color version available online.

Table 2. Ranges in the rates of germline transmission of chimeras selected for breeding on the basis of the ability of clones to colonize the germline, fluorescence-activated cell sorting (FACS) profile of semen from chimeras and detection of green fluorescent protein (GFP) in semen by PCR

Cell line transfected	Transfected clones injected	Age of cells at injection	Clones eliminated for low colonization	Clones eliminated for low FACS/PCR	Clones tested	# clones transmitting	% transmission
*1154–9 (blood)	4	252–290	0	2	2	1	16–80
229–77 (gonadal)	3	87–107	0	2	1	1	1.5–60
229–46 (gonadal)	3	94–111	0	0	3	0	0
229–92 (gonadal)	2	109–116	0	0	2	2	38–90/58–66
HG-124 (gonad)	2	125–135	2				
HG-193 (blood)	6	113–127	0	3	3	3	8/10–12/2–62
HG-76 (gonad-parental)	N/A[1]	24	N/A	N/A	N/A	N/A	72–100
*HG-76 transfection	5	138–160	1	1	3	2	10–18/48
GC-53 (germinal crescent)	1	122	0	0	1	1	0–5
GC-65 (germinal crescent)	2	116–129	1	0	1	1	16
472–138 (germinal crescent)	2	86	0	0	2	2	4.3/100

[1]N/A = not applicable.

same day to transmit a genetic modification to their offspring. At the present time, the reasons for this variation are also unknown.

The data in Table 2 also demonstrate that the rate of germline transmission of PGC derived from the germinal crescent or blood is approximately equal to that of gonocytes derived from the developing testis.

Transgenic Chickens

During the past decade, we have used the PGC technology described above to insert transgenes into many locations within the genome. In our original publication, transmission of the HS4-β-actin-EGFP-β-actin-puro^R transgene was reported (van de Lavoir et al., 2006). Transgenic birds carrying 2 similar GFP constructs were reported by van de Lavoir et al. (2012) and transgenic birds derived from cells carrying another GFP insertion were described by Song et al. (2014). More recently, we have described birds carrying an immunoglobulin heavy chain gene that has been inactivated by replacing the J region with a selectable marker cassette composed of β-actin-GFP and CAG-puro^R (Schusser et al., 2013a). The cassette introduced into these birds also has an attP site (i.e., the docking site for phi-31 integrase) to target human-derived immunoglobulin transgenes into this location (Schusser et al., 2013b). These birds have now been made and will be described in a future publication. We have also targeted the immunoglobulin light chain gene in a similar manner to produce birds with a nonfunctional light chain gene. These genes have been further modified in vitro to produce transgenic birds with human-derived immunoglobulin light chain transgenes.

Both β-actin and CAG induce ubiquitous and high level expression of the antibiotic resistance genes and GFP. Although these and other powerful promoters have been widely used in transgenic mice, we designed our selection cassettes with 5′ and 3′ loxP sites to facilitate their removal in our transgenic birds. To accomplish this goal, we have created transgenic chickens expressing Cre recombinase, which catalyzes excision of DNA between loxP sites. As predicted, both β-actin-EGFP and CAG-puro^R are absent in the offspring of birds expressing Cre and birds carrying the floxed selection cassettes.

Conclusions

Although the desire to precisely modify the genome of chickens by adding or subtracting specific sequences of DNA was suggested more than 30 yr ago by Shuman and Shoffner (1982), the capability to execute the technology has only recently become available. Production of these birds requires technical skill to establish cultures of PGC, to introduce genetic modifications into them, to expand the genetically modified cells into populations, to analyze the genetic modifications, and to create germline chimeras by injecting the cells into embryos. Production of these birds also requires expertise in the design and assembly of transgenes to satisfy the requirement for isolation of genetically modified cells in culture and expression of the transgenes in vivo. In addition, transgene design should provide the ability to triage putative chimeras between hatch and inclusion in a breeding program. Execution also requires reasonable competence at incubation of embryos in surrogate shells, artificial insemination, general poultry husbandry, and database management.

There are parallels to be drawn between the state of the art of modifying the genome of chickens in 2014 with the state of the art of modifying the murine genome in the early 1980s. At that time, the ability to use murine embryonic stem cells to modify the murine genome was led by a few laboratories with cutting-edge expertise. During the next 3 decades, technological expertise was acquired by many academic and industrial laboratories, and a plethora of genetically modified mice were produced. The examples of genetic modifications that have been introduced by the team at Crystal Bioscience demonstrate that engineering of the avian genome is now a reality. Application of the technology

in poultry production and biomedical research is now limited only by acquisition of technological skill and imagination.

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