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An overview of the basics of generate transgenic hen 'bioreactors'

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ABSTRACT

The hen has long held promise as a low-cost, high-yield bioreactor for the production of human biopharmaceuticals in egg whites using genetic engineering. Recently finding appeared indicating the production of substantial levels of human monoclonal and single chain antibodies (O 3mg and O 150 mg, respectively) in eggs of transgenic hens. These promising findings indicate that the hen is close to becoming a competitive manufacturing platform for the production of human biopharmaceuticals. The most successful method of gene transfer in birds is to use PGC and retroviral vectors injected into the embryos following oviposition. The germline transmission rate of the founder birds is very low. Nevertheless, the transgenic lines demonstrate the stable integration and inheritance of the transgene to subsequent generations. In this review, general avian reproduction and establishment of germ cells will be described for possible routes to gene transfer. Later, the current gene transfer methodologies will be summarized with a focus on PGC gene transfer. Finally, the application of transgenics to poultry will be discussed.

Keyword: transgenic chickens, bioreactors, PGC.

INTRODUCTION

Primordial germ cells (PGCs) are the precursors of sperm and eggs (1). In most animals, segregation of the germ line from the somatic lineages is one of the earliest events in development (2); in avian embryos, PGCs are first identified in an extra-embryonic region, the germinal crescent, after approximately 18 h of incubation. After 50–55 h of development, PGCs migrate to the gonad and subsequently produce functional sperm and oocytes (3, 4). So far, cultures of PGCs that remain restricted to the germ line have not been reported in any species (5, 6). Van de Lavoie et al showed that chicken PGCs can be isolated, cultured and genetically modified while maintaining their commitment to the germ line. Furthermore, they showed that chicken PGCs can be induced in vitro to differentiate into embryonic germ cells that contribute to somatic tissues. Retention of the commitment of PGCs to the germ line after extended periods

in culture and after genetic modification combined with their capacity to acquire somatic competence *in vitro* provides a new model for developmental biology (7). The utility of the model is enhanced by the accessibility of the avian embryo, which facilitates access to the earliest stages of development and supplies a facile route for the reintroduction of PGCs into the embryonic vasculature. In addition, these attributes create new opportunities to manipulate the genome of chickens for agricultural and pharmaceutical applications.

An ovum, a female sex cell, in the domestic hen's ovary gradually accumulates yolk, which provides nutritive materials for the embryo and ovulation occurs approximately every 24 h. The nucleus and cytoplasm of the ovum is located at the surface of the yolk mass within a small white area called the germinal disc (8). After ovulation, the ovum is immediately engulfed by the funnel shaped infundibulum, the first portion of hen's oviduct where the spermatozoa, the male sex cells, fertilize the ovum. Fertilization in birds is polyspermic (9). The forming zygote remains in this section approximately 15 min (10). Subsequently the egg enters the magnum which secretes albumen that encases the yolk. The egg spends about 3 h in the magnum. After the magnum, the yolk together with the albumen capsule enters the isthmus where the inner and outer shell membranes are laid down and the first cleavage division of the fertilized egg occurs (8). Leaving the isthmus, the developing egg moves to the uterus, or the shell gland, where the eggshell forms. The egg spends the longest time in uterus than in any other portion of the hen's reproductive tract, about 18 to 21 h (11). For the period before oviposition, Eyal-Giladi and Kochav (1976) classified a series of normal stages describing the sequence of developmental events from the first cleavage up to primitive streak into 14 stages using Roman numerals (12). These events can be divided into three developmental periods: cleavage, formation of the area pellucida, and hypoblast formation. During 10 to 12 h in the uterus, the cytoplasmic mass of germinal disc is cleaving very rapidly to form an epithelial sheet 5 – 6 cells thick (E.G&K stage VI). The germ is now known as the blastoderm). The blastoderm is separated from the underlying yolk mass by the subgerminal cavity. About 6 to 8 h before the egg is laid, the lower layers of the blastoderm start to detach and fall into the subgerminal cavity to form a transparent thinned-out area that marks the future posterior end of the embryo (12). This process marks the beginning of area pellucida formation. At about the time of oviposition the developing blastoderm appears to the naked eye as a whitish circular structure approximately 2 mm in diameter that is composed of two regions. The outer edge is opaque, and is called area opaca; and the inner layer is translucent from a shedding process, and is referred to as the area pellucida (13). The chick embryo at the time of oviposition is known as a stage X embryo (12) and is composed of about 50,000 to 60,000 cells (Figure 1).

Upon incubation of the freshly laid egg a second layer of cells beneath the area pellucida starts to appear and is completed in a few hours (12). This signals the process of hypoblast formation, which is completed at stage XIII (12). When viewed ventrally, the hypoblast is smooth and has a well-defined border between area opaca and area pellucid (12, 13, and 14).

The cellular sheet of the area pellucida, the upper layer, is now known as an epiblast (12, 13, and 14). The resulting space between the two-layered blastoderm is a blastocoel, and the space between the hypoblast and the underlying yolk is the subgerminal cavity (12). This marks the beginning of gastrulation, a process which gives rise to three germ layers: ectoderm, mesoderm and endoderm (14).

The first visible sign of gastrulation is a thickening of epiblast that results in the primitive streak (15, 16), marking the anterior-posterior axis where the embryo proper develops (12, 13, and 14). From this point on, the changing appearance of the embryo is distinguished by the table of normal stages developed by Hamburger and Hamilton (1951), a system that is based on morphological characteristics. The stages are numbered from 1 to 46, from the pre-streak embryo to the newly hatched chick.(17)

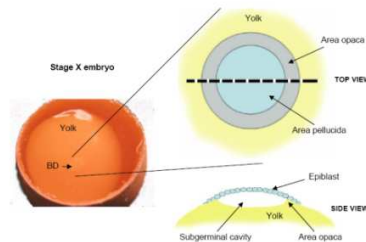


Figure 1. The basic structure of blastoderm (BD) at the time of oviposition. A freshly laid egg often contains an embryo at stage X characterized by a fully formed area pellucida (AP).

Avian germ cells develop early in embryogenesis and are the ultimate target of gene transfer for the production of transgenic chickens (Figure 2). Tsunekawa et al. (2000) has proposed that the chicken VASA protein, an RNA binding protein, could act as germplasm containing materials for predetermining the formation of germ cells similar for that observed in *Drosophophila*. The chicken VASA protein can be detected histoimmunohistochemically from early cleavage stages to the presumptive primordial germ cells (PGCs) of stage X embryos and later (18). The mechanism of the establishment of the germ line in birds is yet unknown. Primordial germ cells (PGCs), the precursors of spermatozoa and oocytes, in the early chicken embryo have been characterized by their morphological characteristics, their high glycogen content stained with periodic acid-Schiff (PAS) reaction, and the presence of cell surface antigens such as EMA-1 and SSEA-1 (19).

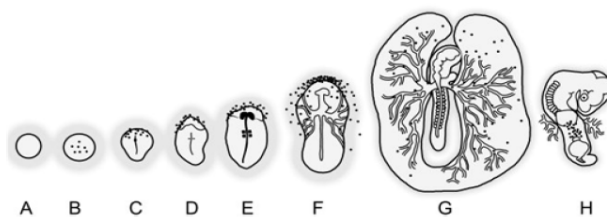


Figure 2. The developmental history of primordial germ cells from oviposition to their colonization of the genital ridge

Approximately 50 PGCs are first recognized in the stage X embryo based on immunostaining using anti-SSEA-1 and EMA-1 and later on the dorsal surface of the hypoblast. Then, PGCs migrate to an extra embryonic region anterior to the head fold, referred to as the germinal crescent that contains about 200 PGCs (4, 20). From the germinal crescent region, PGCs immigrate into the newly formed vascular system and are passively carried to the vicinity of the germinal ridge through the extra embryonic circulation (4, 21). From the blood, PGCs actively

migrate into the embryonic gonads (22, 23 and 24). These events suggest several ways to manipulate the avian genome. Many points of germ cell development can be accessed for gene delivery including mature oocytes/spermatozoa, the newly fertilized ova/zygotes, primordial germ cells during their early establishment, migration and colonization of gonad.

MATERIALS AND METHODS

Two methods are currently available to generate transgenic hens for biopharmaceutical production and both manipulate the embryo in a freshly laid fertilized egg – at lay, the embryo is at stage X, and w20 h old, consisting of a sheet of w 60000 pluripotent blastodermal cells (Figure1).

In one method, retroviral particles bearing a transgene are injected to the subgerminal cavity beneath the blastoderm through a window made in the egg. Infection leads to integration of the transgene into some of the recipient cells (25). When hatched and raised to sexual maturity, G0 sperm from chimeric roosters is screened for the transgene and positive male sperm is inseminated into non-transgenic females to generate hemizygous G1 birds. In the second method, developed and used by Etches and colleagues (26) and the main subject of this article, chicken primordial germ cells (cPGC), derived from long term culture of stage X blastodermal cells, are transfected with, and selected for, transgene integration and expression and then injected into the subgerminal cavity of a recipient stage X embryo. Cells invade the blastoderm and, after hatch, populate most tissues of the bird.

Efforts to develop methods for the genetic modification of chickens have been driven not only by the importance of the chick as a model for studying vertebrate development but also by the intriguing possibility of producing human protein therapeutics in the eggs of transgenic hens.

In a report in Nature, van de Lavoie et al. genetically modified chicken primordial germ cells (PGCs), reintroduced these into chick embryos and demonstrated transmission of the transgene through the germ line of modified birds. This novel technology supplements current approaches to transgenesis in poultry and also suggests that manipulation of PGCs may allow a novel approach to genetic modification of other vertebrates (7).

Several factors hamper stable modification of the chicken genome. Not only are hens' eggs large and yolky, which makes manipulation difficult, but by the time a fertile egg is laid, development has advanced to a stage where the embryo comprises ~60,000 cells. Efforts to access the genome have involved manipulation of the embryo shortly after fertilization, modification of newly laid eggs and manipulation of PGCs (27).

Early approaches to genetically modify chickens involved injection of avian retrovirus vectors into the egg shortly after lay. The utility of these vectors was limited both by the high incidence of transgene silencing, particularly after germline transmission, and by potential safety issues associated with the risk of recombination with retroviruses that are widespread in commercial poultry populations. Developments in the use of lentiviruses as gene transfer vectors, particularly for gene therapy in humans, encouraged researchers to revisit the use of viral vectors for transgenesis. Lentiviral vector transduction from newly laid eggs can produce founder

transgenic chickens at frequencies of up to 100%, with rates of germline transmission to the G1 generation between 4% and 45% and no evidence of transgene silencing after germline transmission (28).

A related approach has been to attempt to modify the germ line directly by manipulation of PGCs. In the developing chick, as in many species, PGCs are first found outside the embryo proper (Fig. 3). They enter the circulatory system of the embryo after about two days of incubation and circulate in the blood before moving to the developing gonads. Despite the availability of effective methods to isolate PGCs and transfer them to recipient embryos, where they form functional gametes in host birds, genetic modification of avian PGCs had not been successful (19).

Van de Lavoie *et al.* first developed a method for culturing PGCs isolated from chick embryonic blood during the developmental stage when they are beginning to migrate to the developing gonads. The PGCs are grown on feeder cells in medium conditioned on buffalo rat liver cells and supplemented with stem cell factor and human recombinant fibroblast growth factor. The cultured PGCs may be transfected and after injection of the modified cells into recipient embryos at the appropriate developmental stage, the transgenic PGCs migrate to the gonads and form functional gametes, as shown by breeding experiments (Fig. 3). Male PGCs form functional sperm in male recipients and female PGCs form oocytes in female recipients, but no evidence was found that PGCs of one sex would contribute to the germline of the opposite sex (7).

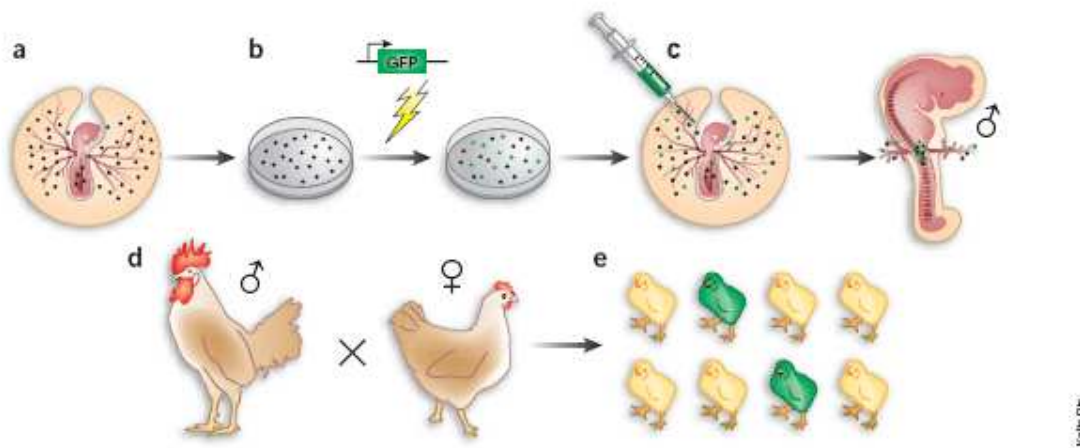


Figure 3. Production of transgenic chickens using cultured PGCs.

In brief the basics stage of generate transgenic hen 'bioreactors' is as follows:

(a) PGCs (black) are isolated from the blood of chick embryos after approximately two days of incubation. (b) The embryonic blood is cultured under conditions that promote proliferation of PGCs, and the cultured cells are electroporated with a transgene construct, here encoding green fluorescent protein (GFP). (c) Transgenic PGCs (green) are injected into the circulatory system of embryos after two days of incubation and migrate to the developing gonads. The recipient embryos are incubated until they hatch. (d) Hatched males are reared to sexual maturity and

crossed with wild-type hens. (e) The offspring of the cross are screened to identify those derived from the GFP-expressing transgenic PGCs (Fig. 3).

DISCUSSION

Efforts to generate transgenic hen 'bioreactors' have focused on targeting expression of therapeutic proteins to the oviduct, using regulatory sequences of one of the major egg white proteins. Although van de Lavoie *et al.* did not evaluate expression of a transgene designed to direct protein accumulation in the eggs of the transgenic progeny, a recent report (26) from the same laboratory described production of chimeric birds from chicken ES cells transgenic for a construct in which sequences from the chicken gene encoding ovalbumin, the most abundant egg white protein, direct expression of a monoclonal antibody. Although functional monoclonal antibody was secreted into egg white, transgene expression was not tightly limited to the oviduct cells, which could be problematic.

As gene targeting (e.g., introducing the sequence encoding a therapeutic protein into an endogenous egg white protein gene) is an important goal of cell-based transgenesis in poultry, demonstration of effective gene targeting in cultured chicken PGCs is the next challenge to maximize the impact of this new method. Targeting a therapeutic protein sequence to the endogenous ovalbumin gene could yield very high levels of protein, but this may have a deleterious effect on egg white structure and egg shell formation.

One potential advantage of producing therapeutic proteins in egg white is that the proteins are likely to be glycosylated similarly to human proteins. Appropriate glycosylation is often necessary for protein function and may have other beneficial characteristics, such as increasing the circulatory half-life.

There is some evidence that therapeutic proteins synthesized as a component of egg white may be appropriately glycosylated (26,29), but further data are required from larger-scale synthesis of a range of different classes of protein.

Although there are many challenges ahead in the development of transgenic hen bioreactors, recent advances in chick transgenic technologies suggest that these challenges can now be explored and their potential evaluated.

CONCLUSION

After 25 years, the search for the avian cell that can be cultured indefinitely, genetically modified, and clonally derived while retaining its ability to enter the germline has ended. Van de Lavoie *et al.* have defined the conditions for culture and genetic modification of primordial germ cells (PGCs) and shown that these cells are transmitted at high rates through the germline. The advent of this technology provides the ability to introduce transgenes of any size and to make site-specific changes to the genome (7).

Although PGCs are committed to the germline, they can be induced into somatically committed embryonic germ (EG) cells by changing the culture conditions. EG cells resemble embryonic stem (ES) cells that are also committed to the somatic lineages. These cell-based systems

facilitate insertion of larger transgenes that provide high level, developmentally regulated and tissue-specific expression in transgenic chimeras and their offspring. Following introduction of a transgene, high-grade somatic chimeras can be made with ES and EG cells within 4 weeks and 4 months respectively, allowing quick assessment of the transgenic phenotype. Following introduction of a transgene into PGCs, high-grade germline chimeras can be made within 8–9 weeks and the high rate of germline transmission of G0 chimeras produces a large cohort of transgenic chicks in 16–17 weeks. PGC, EG and ES cells can be grown in conventional laboratory settings and small flocks of recipient birds or third-party vendors can supply recipient embryos to make somatic and/or germline chimeras. In general, animal management is routine although some specialized equipment and technical skill is required to incubate chimeras in surrogate shells.

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