The Embryonated Egg: A Practical Target for Genetic Based Advances to Improve Poultry Production

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ABSTRACT The embryonated avian egg is an attractive target for applying technology-based advances to improve poultry production. There are a number of reasons for this. First, the egg is immobile and can be easily accessed by high-speed automated equipment such as the commercial egg injection system used for vaccination of broilers worldwide. Second, due to successful breeding techniques, the embryonic period now composes 30 to 40% of a broiler's total lifespan, underscoring the importance of this window in the bird production life cycle. Third, the period of incubation involves rapid development from a ball of 40,000 to 60,000 undifferentiated blastodermal cells to a fully formed chick and associated extra-embryonic compartments in 21 d, allowing development of novel approaches for improving poultry production. Some of these novel approaches will be discussed in this paper and include gender discrimination of embryos and the possibility of changing the breeding paradigm through introduction of undifferentiated cells such as avian blastodermal or embryonic stem cells.

(*Key words*: avian embryo, poultry production automation)

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INTRODUCTION

Unlike mammals, chick embryonic development takes place within the egg (in ovo) but outside its mother's body, making the embryo readily accessible to intervention. The development process is extremely rapid, taking only 21 d of incubation compared to the months of embryonic development required by other important agricultural amniotes such as cattle, sheep, and pigs. Successful breeding has resulted in dramatic reductions in chick growout times. In the 1950s it took 84 d to reach half the weight of today's broiler. Today a chick may reach marketable weight in just 32 to 49 d posthatch (Haverstein et al., 1994). Thus the chick embryonic development period composes from 30 to 40% of the chick's total lifespan, making it a very important component of the chick production cycle (Figure 1).

Incubation technology developed in the early twentieth century has been adopted worldwide. Hatcheries capable of producing in excess of one million chicks per week are not uncommon. Until the early 1990s, very few further technological advances had been made at the hatchery level. This changed in the late twentieth century when a high-throughput automated egg injection system was introduced worldwide. The initial application was for Marek's vaccination of poultry embryos at transfer from incubation to hatching setters; i.e., d 18 to 19 of incubation (Sharma and Burmester, 1982). Today over 85% of all U.S. broiler hatcheries employ these in ovo vaccination systems and the technology is gaining acceptance in many other countries. Available in ovo vaccines include various forms of Marek's disease, infectious bursal disease vaccines (Haddad et al., 1997), and fowlpox (used in Japan and Brazil). In ovo vaccination technology has been reviewed by Ricks et al. (1999). Benefits of the automated egg injection system include reduced labor costs, precise delivery, healthier chicks due to earlier development of immunity, and reduced stress associated with the elimination of manual handling and injection.

In ovo vaccination technology is just one example of the benefits of targeting the embryonated egg for improved poultry production. The purpose of this paper is to introduce the reader to other opportunities for improving poultry production by targeting the embryonated egg, particularly those areas in which high-throughput or automated systems could facilitate practical applications of advancing technology. Special emphasis on those interventions that build on advances in genetic- or molecular-based techniques will be made. It is not intended that these examples be all-encompassing, but that they educate the reader as to the possibilities available for embryonic targeting, including delivery and sampling (diagnosis) at various stages of incubation as a means to improve poultry production practices.

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Abbreviation Key: PGC = primordial germs cells.

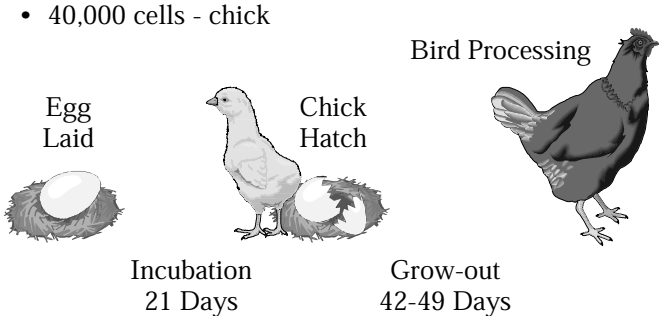

FIGURE 1. Chick lifespan.

EMBRYONATED EGG STRUCTURE AND TARGETING

The avian egg and embryonic development have been described in detail (Romanoff, 1949; Romanoff and Romanoff, 1960). Nomenclature used to describe the various stages of embryonic development are those used by Eyal-Giladi and Kochav (1975) and Hamburger and Hamilton (1951). Eyal-Giladi and Kochav describe Stages I to XIV, a narrow window of incubation that starts just before the egg is laid and concludes at about 6 to 7 h of incubation. At Stage I of development the primitive streak is about half formed (about 0 to 1 h in shell gland before egg expulsion from the hen). Stage X corresponds to the stage of embryonic development in the freshly laid egg. Stage XIV corresponds to about 6 to 7 h of incubation. Hamburger and Hamilton describe Stages 1 to 45, a broader period of embryonic development beginning when the egg is laid and concluding at about 19 to 20 d of incubation, shortly before hatch. Stages 1 and 2 of Hamburger and Hamilton probably correspond roughly with stages XII to XIV of Eyal-Giladi and Kochav. For the purposes of this discussion, the focus will be on targeting embryonated eggs at different stages for specific applications as shown in Table 1.

TARGETING AT DAYS 18 TO 19 OF INCUBATION FOR DISEASE PREVENTION

As shown in Table 1 and described above, the primary application at this point of incubation is vaccination. This is well understood but, for the sake of completeness, will be described briefly here. The embryo is well developed, has expanded to fill most of the space within the eggshell (except air cell), and is enclosed in the amniotic fluid filled sac, which in turn is surrounded by the allantoic membranes (Figure 2). Access is achieved by punching through the shell and associated shell membranes into the air cell and extending a needle into the amniotic or embryonic compartment. The importance of site of delivery for effective Marek's vaccination has been described (Avakian et al., 2002; Wakenell et al., 2002). Vaccines in development include an in ovo coccidiosis vaccine (Poston et al., 2001) and a novel Newcastle vaccine (Haddad et al., 2002). Efforts also include development of molecular-based vaccines reviewed by Sharma and Ricks (2002). Use of competitive exclusion agents in ovo have reduced salmonella colonization (Cox et al., 1992). In ovo feeding for improvement of chick quality and posthatch performance is under investigation by Ferket and Uni (2002).

Automated candling devices that allow discrimination of clear and early dead from other embryonated eggs are commercially available. For example, vaccine administration to eggs identified by such methods can be "shut off," preventing vaccine waste. Alternatively, such eggs can be removed from the process allowing a better quality hatch.

TARGETING AT DAY 13 TO 17 OF INCUBATION—GENDER DETERMINATION

Sorting of chicks or poults by sex is important to the efficiency of the global poultry industry. All egg layers,

TABLE 1. Applications for embryonic targeting

Targeting application	Eyal-Giladi and Kochav stage	Hamburger and Hamilton stage (H&H)	Day of incubation	Methods
Vaccines	NA	44	18	
Sex discrimination	NA	39 to 43	13 to 17	Allantoic fluid extraction for estrogen detection
Poultry breeding and transgenesis	NA	4 to 20	Unincubated to d 3	PGC[1] delivery Blood migration stage 10–16 (H&H)
Poultry breeding and transgenesis	Stage X	NA	Unincubated	Embryonic stem cell delivery

[1]Primordial germ cell.

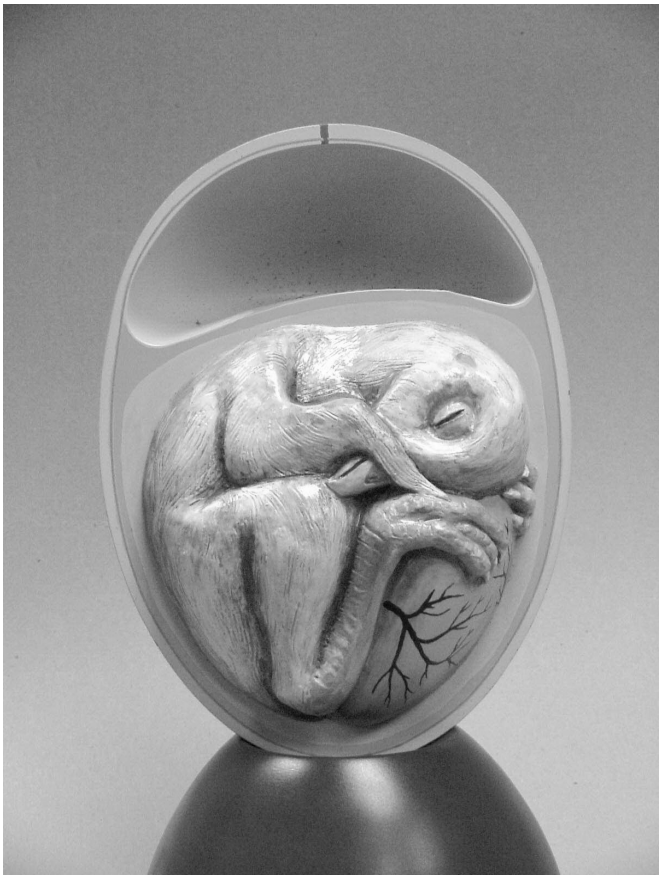

FIGURE 2. Embryonated egg at transfer.

breeders of all types, meat-producing turkeys, and 27% of broilers are sexed at hatch. Reasons for sexing include the use of all females for table egg production, use of male and female bird lines during the breeding amplification process with the need for disposal of the "off-sex," and sex-separate rearing of meat producing turkeys and broilers. Current procedures for sex segregation are manual procedures practiced by highly skilled workers at hatch. They include vent sexing or feather sexing of chicks that carry a slow-feathering gene. A brief review of current practice is provided by Phelps et al. (2001, 2002). Such methods have significant drawbacks including a dwindling supply of highly trained manual labor, biosecurity risks encountered when contract sexers travel from hatchery to hatchery, increased processing time, and handling stress to chicks at hatch. Vent sexing involves identifying distinctive characteristics and folds in the vent of a chick. Successful practice of vent sexing requires excellent manual dexterity and Zen-like concentration. Feather sexing, although less expensive than vent sexing, is not applicable to turkeys, most broiler breeds, or some layer breeds. There have been reports that the K gene for slow feathering is closely linked to an endogenous virus (Bacon et al., 1988). This can cause immunological tolerance to lymphoid leukosis (Crittendon et al., 1987) and is therefore a disadvantage in breeding stock. An automated method to sex segregate chicks prior to hatch would alleviate many problems associated with current practice. A rapid, inexpensive method would further facilitate adoption by the broiler meat production industry.

Potential sex specific markers include differences in physiological parameters such as heart rate (Laughlin et al., 1976; Glahn et al., 1987), PCR identification of the W-specific chromosome in females (Clinton, 1994; Uryu et al., 1989; Petitte et al., 1992), presence of anatomical differences such as gonads (Vandenabeele et al., 1993), or sex-specific compounds present in the blood or extra-embryonic fluid (Gill et al., 1983; Tanabe et al., 1979). The challenge has been to identify a method that is inexpensive, fast, and compatible with high-throughput automation. Recently a method that appears to meet these requirements has been reported by Phelps (2001). Embryos can be distinguished on the basis of their sex by the level of estrogen conjugates present in allantoic fluid. Typically, allantoic fluid derived from female embryos contains higher levels of estrogen than that derived from corresponding males. Estrogen levels in female samples determined using a commercially available RIA Kit[2] range from 113 to 830 pg/mL, whereas levels in males are not detectable or are less than 42 pg/mL. Avian embryos can be sorted by gender based on the presence of estrogenic compounds in the allantoic fluid of female embryos from d 13 to 17 of incubation in broiler, broiler breeder, turkey, and layer embryos regardless of flock age or strain.

Detection Method

A variety of methods may be used to detect estrogen. One example is a yeast biosensor (LiveSensors)[3] that has been genetically modified to respond to estrogen present in allantoic fluid and can discriminate males from females. LiveSensors is composed of *Saccharomyces cerevisiae* transformed with yeast expression vector for the human estrogen receptor, the reporter gene that contains promoter with estrogen response elements coupled with *Escherichia coli* β-galactosidase (Butt and Chen, 1999). In the presence of estrogens released by action of exogenously added β-glucuronidase, the estrogen receptor binds to the estrogen response elements and initiates transcription of the reporter gene. The concentration of estrogens in the allantoic fluid is correlative with the level of induction of the reporter gene. Activity of the reporter gene is measured using ONPG substrates, which yield a yellow colorimetric signal that can be monitored by a CCD camera. As little as 4 μL of allantoic fluid need be used for effective gender determination.

Allantoic Fluid Sampling

Allantoic fluid is the waste reservoir of an embryo (Romanoff, 1960). This fluid begins to form around d 5

[2]Diagnostic Products, Los Angeles, CA; www.dpcweb.com.
[3]LifeSensors, Inc., Malvern, PA; www.lifesensors.com.

of incubation, attains a maximum volume of 6.1 mL on d 13 of incubation, and wanes in volume as incubation continues due to moisture loss and fluid resorbtion but 1 to 2 mL is still present at d 18 of incubation. It is most easily sampled from d 13 to 17 of incubation (Phelps, 2001). In an egg in the typical upright position (blunt end up), allantoic fluid is dispersed as a thin layer under the inner shell membrane making it difficult to sample. When eggs are tilted from the typical upright position, it has been found that the allantoic fluid pools at the uppermost surface as the embryo displaces the fluid by gravity (Figure 3). This facilitates consistent and accurate sampling.

Prototype System

A prototype system has been developed and is currently being optimized (Phelps et al., 2002). The process requires three main steps or modules: fluid sampling, assay of sampled fluid, and egg sorting. In the first step, conducted at d 16 of incubation, eggs are removed from the setter; viable eggs are separated from non-viable eggs, and the viable eggs are transferred to a sampling module in which the eggs are brought into a suitable position for sampling. A needle samples fluid from each egg and dispenses it into a well in a bar-coded template. Templates contain multiple wells in the same array as the egg flat. Eggs are replaced in bar-coded egg flats and are manually replaced into the setting incubator. A computer matches the bar codes of the egg flat and the template containing allantoic fluid samples. In the second step, templates are transferred into an assay module where the assay using LiveSensors takes place. At transfer, typically d 18 of incubation, eggs are removed from setters. The computer identifies which eggs are male and female based on the bar code present on the egg flat. Eggs are then sorted by gender, vaccinated, and transferred to hatching baskets.

TARGETING AT DAYS 0 TO 2 OF INCUBATION—CHANGING THE BREEDING PARADIGM

Current breeding techniques involve complex strategies employed by the broiler and layer industry in which valuable traits are maintained as separate "elite" or "pedigree" poultry lines. From these lines are produced the great-grandparent lines. These lines are then crossed and multiplied to produce birds for meat or eggs (Figure 4). The process of amplification from great-grandparents to commercial broilers and egg layers takes 3 to 4 yr, and in broilers results in dilution of the genetic merit of the elite lines. For example, growth and feed efficiency of elite birds is greater than their descendant commercial broilers. Inclusion of new traits at the elite level can take several years. Advances in biotechnology suggest several novel approaches that may be used in the hatchery of the future to produce birds via embryonic targeting prior to and during the early incubation process.

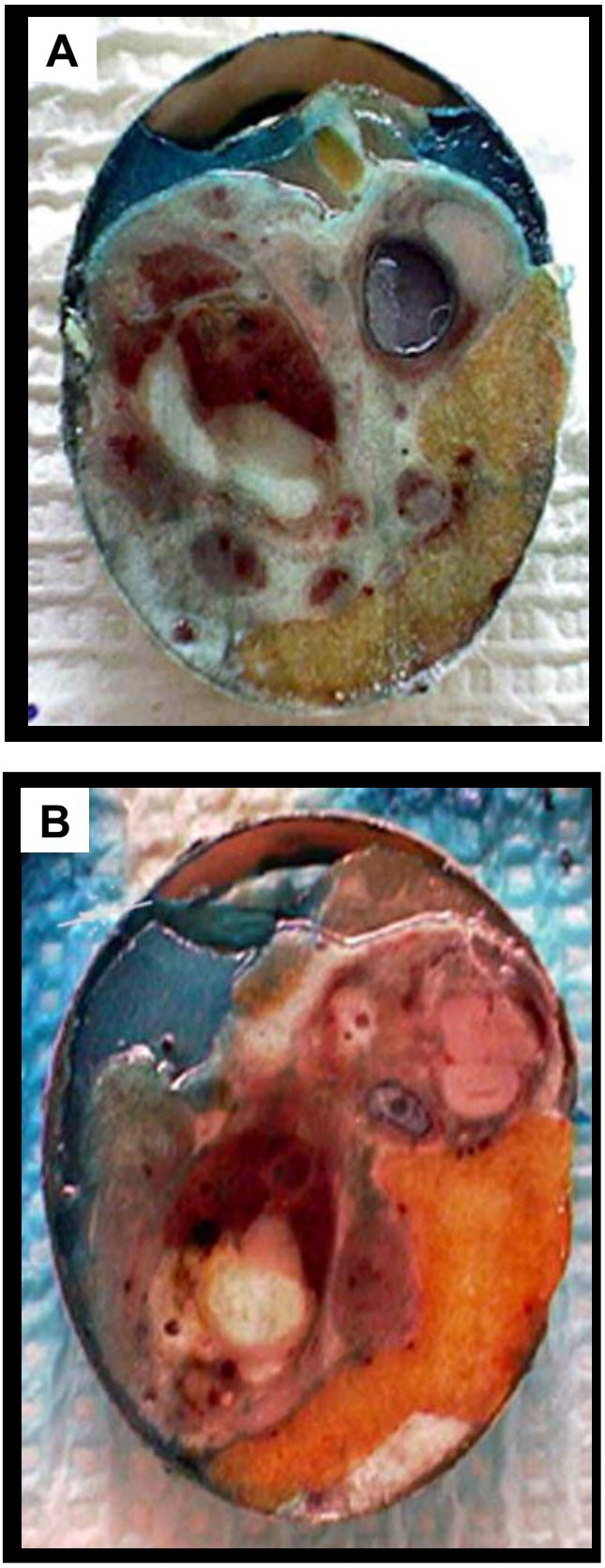

FIGURE 3. a) Egg at d 16 of incubation (nontilt); b) embryonated egg at d 16 of incubation (tilt).

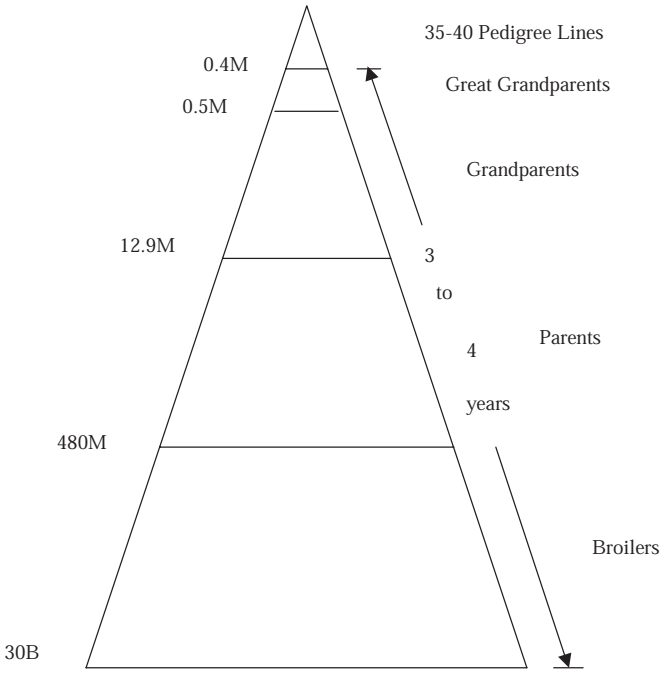

FIGURE 4. Breeding pyramid. Multiplication scheme for reproducing broilers resulting in dilution of genetic merit. M = millions; B = billions.

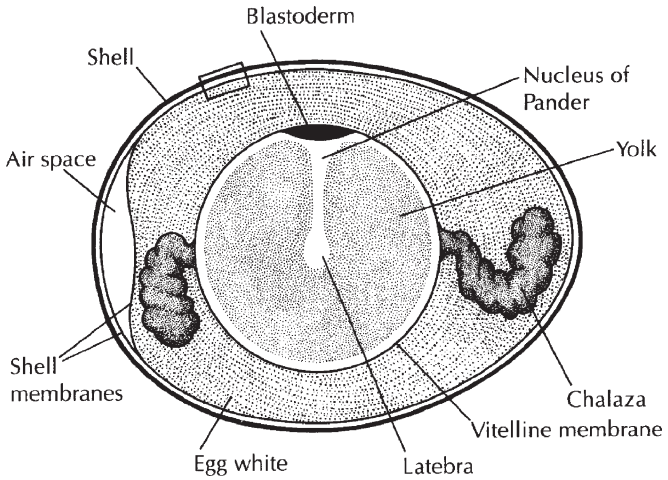

FIGURE 5. Unincubated egg of Stage X.

These approaches include strategies to 1) more effectively capture the value of elite germplasm produced through traditional quantitative selection breeding techniques, 2) select for sexual phenotype, and 3) introduce new genes into the germplasm, a process called transgenesis.

Blastodermal cells, which contain pluripotent cells capable of generating an entire organism, can be injected manually into Stage X embryos (Petitte et al., 1990) to generate somatic or germline chimeras. Likewise, primordial germs cells (PGC), which are the precursors of spermatogonia and oogonia, can be injected manually into Stage 2 through 20 embryos to generate germline chimeras (Yasuda et al., 1992; Naito et al., 1994b). Through conventional crossing techniques, donor-derived offspring can be produced (Petitte et al., 1990). Blastodermal cells and PGC can be frozen and thawed prior to use (Naito et al., 1994c; Reedy et al., 1995; and Kino et al., 1997). These properties make them useful candidates for novel breeding strategies such as reconstitution of stocks not in current use (Reedy et al., 1995) or to augment endangered populations under natural mating conditions (Tajima et al., 1999). Methods that would eliminate the time required to produce donor derived offspring through conventional breeding techniques would be highly desirable. Cloning nuclear material into PGC or blastodermal cells has been proposed (Tajima, 2002). Such a process, if achievable, would overcome difficulties encountered in use of the oocyte or newly fertilized ovum (isolation of which is constrained by the unique structure of the bird's reproductive tract, requiring surgical intervention or hen sacrifice) and difficulties in removing associated yolk-laden material. An alternative practical solution for rapid amplification of poultry has been suggested by Etches (2001) through a process of chimera production. Such chimeras would be produced through injection of donor embryonic stem cells into Stage X recipient embryonated eggs. They would be phenotypically identical but genotypically distinct from the donor embryonic stem cells.

Practical use of PGC or blastodermal cells to facilitate breeding requires advances along several dimensions. These include improvement in egg targeting methods to minimize negative effects on hatchability, improved efficiencies of donor cell incorporation typically addressed by compromising the recipient in some manner, advances in both cell culturing and cryopreservation techniques, and more efficient methods for both selection of and introduction of genes coding for improved production. A brief review of the current state of the art with respect to egg targeting and blastodermal cell and PGC targeting follows.

TARGETING THE STAGE X EMBRYO—INTRODUCING BLASTODERMAL CELLS

After oviposition, eggs may be stored for up to 7 d, typically at temperatures ranging from 15.6 to 18.0°C (60 to 65°F) and RH ranging from 75 to 80% to facilitate incubation of large numbers of eggs simultaneously. During this period the embryo, referred to as a Stage X embryo (Stepinska and Olszanska, 1983), remains as a group of about 40,000 to 60,000 undifferentiated blastodermal cells of about 2 to 4 mm in diameter. These cells float on the yolk, which is surrounded by the vitelline membrane, thick and thin albumen, inner and outer shell membranes, and the porous eggshell (Figure 5).

In order to view and manipulate the blastoderm, investigators to date have used methods such as the windowing method of Petitte et al. (1990) or the surrogate eggshell culture technique or modifications thereof (Perry, 1988; Naito, 1990). Both of these procedures re-

quire a high level of technical skill and result in hatchabilities of 30% or less. In a typical windowing procedure, the more practical method for automation, the egg is placed in the horizontal position, the eggshell is cut with a Dremel tool[4] to expose the shell membranes, and a window is cut in the top of approximately 5 mm diameter. Thus, in this method, a 5-mm circle comprising shell and inner and outer shell membranes is removed. Eggs are turned under illumination in order to bring the blastoderm into view. Injections are made into the subgerminal cavity, and the eggs are resealed with fresh shell membrane and Duco[5] cement or other suitable adhesive. Hatchabilities can be as low as 8.2%. Speksnijder and Ivarie (2000) reported that hatch could be improved to 32% by depositing a small drop of fluid (PBS) on the shell hole prior to piercing the shell membrane. By restricting entry of air into the egg contents, these authors hypothesized that hatchability would be improved. Bednarczyk et al. (2000) have reported improved hatchabilities of 41% compared to 9.8% when eggs are stored for 5 to 7 d prior to injection and injected in the upright position versus the horizontal position. Recently a noninvasive method employing nuclear magnetic resonance imaging (MRI) has been reported to localize the germinal disc within 2 mm thick slices (Klein et al., 2002) with minimal if any effect on hatchability. Mendu and coworkers (2002, Embrex, Inc., Research Triangle Park, NC, personal communication) are working to determine whether a practical and cost-effective means for injecting Stage X embryos can be developed to support production of high numbers of high-quality chimeras.

TARGETING THE STAGE 4 THROUGH 20 EMBRYO—INTRODUCING PGC

A number of methods for introducing PGC into recipient embryos have been used based on the fact that PGC have some unique translocation and migratory properties (Figure 6; Ginsburg, 1997; Tajima, 2002). PGC migrate from the epiblast to the hypoblast followed by translocation to the extra-embryonal germinal crescent where approximately 200 cells locate. At Stages 10 through 14 PGC migrate in the blood stream and concentrate in the area of gonadal development. Transfer of PGC has been reported using transplanted germinal crescents, isolated cells, homogenates, or direct transfer into the blood stream during the PGC migratory phase (Gonzales and Wentworth, 1989; Petitte et al., 1991). More effective uptake of donor PGC occurs when recipients are compromised in some manner such as blood removal (Naito et al., 1994b). Many investigators do not report hatchabilities of manipulated eggs; of those that do, hatch appears to be poor and ranges from 11 to 40% (Naito et al., 1994b). It is clear, therefore, that substantial improvements in the efficiency of PGC transfer could be made through improvements to the delivery process.

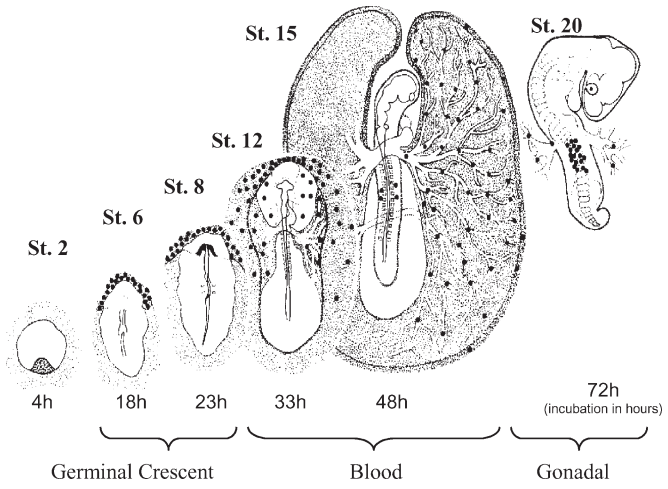

FIGURE 6. Origin and migration of primordial germ cells. From Nieuwkoop and Sutasurya, 1979. Page 119 in *Primordial Germ Cells in the Chordates*. Reprinted with the permission of Cambridge University Press. St. = Stage.

SUMMARY

In ovo technology is advancing to include not only prevention of disease through vaccination of avian embryos at transfer but also the possibility of gender discrimination of avian embryos through identification of sex-specific hormone levels in allantoic fluid samples withdrawn from broiler embryos at d 13 to 17 of incubation. Investigators are also exploring the possible use of blastodermal cells, embryonic stem cells, or PGC as vehicles to improve traditional poultry breeding schemes.

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[4]Dremel, Racine, WI; www.dremel.com.
[5]Devcon, Danvers, MA; www.devcon.com.

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