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Review

Primordial germ cells (PGCs) as a tool for creating transgenic chickens

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Abstract

The transgenic chicken has great potential as a bioreactor for the production of valuable pharmaceutical proteins, notably in the oviduct/egg. Whereas conventional transgenic approaches have significant limitations in this species, an alternative approach employing primordial germ cells (PGCs), the progenitor cells to ova and spermatozoa, has now been successfully applied to the insertion of exogenous genes into birds. Recent developments in manipulating avian embryos make it possible to produce germline chimeras derived from transferred PGCs. In this review we describe the migration pathway of chicken PGCs during early development. We then summarize different methods for the isolation of PGCs and the diversity of techniques used to introduce genes into these cells. Finally, we describe an *in vitro* assay for testing tissue-specific vectors designed to express heterologous proteins in transgenic chickens.

Key words: primordial germ cells, transgene expression, transgenic chickens, bioreactor, ovalbumin

Introduction

Many years of research into avian transgenesis have culminated in the production of transgenic or chimeric chickens expressing important therapeutic proteins in the oviduct. The use of transgenic hens as bioreactors has several advantages over other approaches: the pattern of protein glycosylation is very similar in chickens and humans, birds have short generation times and large numbers of progeny, and eggs can provide a cost-effective and efficient source of therapeutic proteins. The low biochemical complexity of native egg proteins is also likely to facilitate purification of expressed proteins. Moreover, the use of avian species permits to production of recombinant proteins that are toxic for mammals (Lilico et al. 2007).

Attempts to direct the expression of recombinant proteins to the oviduct of genetically manipulated chickens have focused on the use of powerful regulatory sequences, such as those derived from the ovalbumin gene, whose expression is restricted to the tubular gland cells of the oviduct. Ovalbumin constitutes 54% of the protein in chicken egg white, and modern hens produce more than 300 eggs per year.

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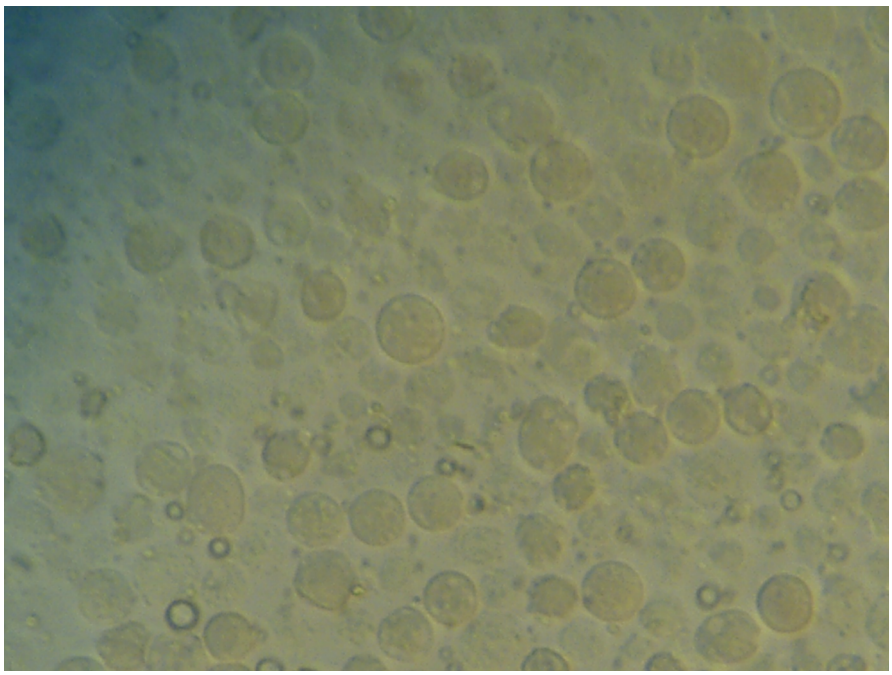

Fig. 1. Primordial germ cells isolated from gonads of 5-6 day old chick embryos. (magnification 400x)

Potentially, a single manipulated individual could produce several grams per year of a recombinant protein in the naturally sterile environment of the egg.

First attempts to produce transgenic chickens involved the introduction of naked DNA or replication-defective viral vectors into the early embryo. Love et al. (1994) injected a fusion gene into pronuclei of fertilized eggs; more than half the embryos emerging contained plasmid DNA and a single transgenic cockerel was obtained. This transgenic bird generated more than 130 G2 offspring in which the presence of the transgene was confirmed by PCR. Kwon et al. (2004) used a replication-defective virus vector to generate transgenic chickens expressing enhanced green fluorescence protein (EGFP). The EGFP coding sequence was placed under the control of the Rous sarcoma virus promoter (RSV), and defective virus particles were injected into embryos at the blastodermal stage X (Eyal-Giladi and Kochav 1976). From 129 injected eggs, 13 hatched after 21 days of incubation; EGFP sequences were detected in several different tissues. More recently, Kwon (2008) used a similar technique to generate transgenic chickens producing recombinant human granulocyte colony-stimulating factor (hG-CSF); the biological activity of the recombinant protein was reported to be higher than that of hG-CSF produced commercially in *E. coli*.

However, transgenesis via the direct introduction of DNA sequences into the early embryo is constrained by the difficulty of pronuclei identification, and relative difficulty of culturing the treated embryo to hatching. In an alternative approach, several laboratories are developing methods of manipulating the avian genome using pluripotent cells genetically modified *in vitro*. Both blastodermal cells (BCs) of the newly laid egg and embryonic stem cells (ESCs) isolated from the blastodisc immediately after oviposition have been used to generate transgenic birds (Wang et al. 2006). Wu et al. (2006) and Jung et al. (2007) have also explored the use of spermatogonia stem cells (SSCs). However, to maximize transmission to progeny, attention is now focusing on primordial germ cells (PGCs) in view of their potential to generate germline chimeras. In this review we describe recent developments in embryo manipulation techniques and in the use of PGCs as a vehicle for the efficient production of transgenic chickens.

Localization of PGCs

In avian species, different stages of embryonic development, from first cleavage to primitive streak formation, are generally designated by Roman numerals (Eyal-Giladi and Kochav 1976). From the prestreak stage to hatching different stages are conventionally designated by Arabic numerals (Hamburger and Hamilton 1951). PGCs first arise from the epiblast and are initially localized in the central disc of the pellucida area of stage X embryos. PGCs are located

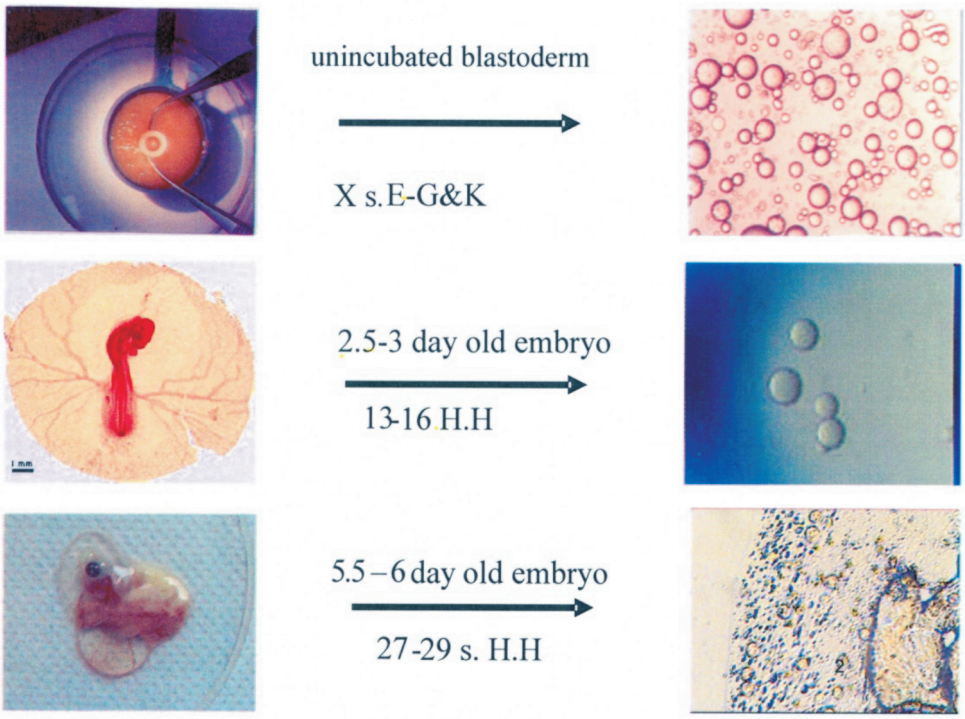

Fig. 2. Isolation of PGCs from embryos at different stages of development. E-G&K, developmental stage (s.) according to Eyal-Giladi and Kochav (1976); H.H, developmental stage according to Hamburger and Hamilton (1951).

in the ventral surface of the pellucida. These then translocate to the dorsal side of the hypoblast at stages XI-XIV, and subsequently migrate to the germinal crescent region at stage 4 via the lower layer. In general, PGCs have a larger circumference than most somatic cells and have a glycogen- and yolk-rich cytoplasm. Once blood vessels form, PGCs enter the blood vessels between stages 10 and 12 and begin to circulate in the bloodstream. PGCs migrate into the gonad primordium at the stages 20-24, where they begin to differentiate into male or female gametes. The blastoderm at stage X contains about 30 PGCs or their precursors together with pluripotent somatic cells. The numbers of PGCs vary according to developmental stage. PGCs proliferate during their migration throughout the embryo and up to 250 PGCs are found in the germinal crescent. By stage 31 there are more than 1000 PGCs (Fig. 1). In 1914, Fricket observed that the left avian sexual gland holds more PGCs than the right one (reviewed by Chang Guo-bin et al. 2010).

PGCs were originally identified using morphological criteria: their cytoplasm contains many glycogen particles (Ginsburg and Eyal-Giladi 1986). PAS (periodic acid-Schiff) is a histochemical marker first described by Meyer (1960) and PAS staining is a generally accepted method for differentiating PGC from surrounding somatic cells. PAS staining produces a diffuse staining pattern throughout the cytoplasm indicating the presence of glycogen vesicles (Macdonald et al. 2010).

A second PGC marker is the stage-specific embryonic antigen (SSEA-1). This cell-surface glycoprotein is expressed in PGCs but not in somatic cells. A specific anti-SSEA-1 antibody was developed by Solter and Knowles in 1978 (Developmental Studies Hybridoma Bank, University of Iowa, USA), and targets an epitope expressed on chick embryonic stem cells as well as on mouse primordial germ cells (Resnick et al. 1992). In chicken, PGCs are the only cells in the embryo that are SSEA-1 positive beyond stage 10.

Two other markers also have potential. Expression of the embryonic mouse antigen-1 (EMA-1) is restricted to pluripotent embryonic cells in this species (Urven et al. 1988), and antibody to EMA-1 has also been shown to react with chicken PGCs present in the gonadal crescent (Karagenc et al. 1996). Expression of another specific marker of germ cells, *vasa*, was first detected in germ cells at oviposition and expression continues until spermatid and oocyte formation in adults (Tsunekawa et al. 2000).The *vasa* gene encodes an RNA-helicase and selective germline expression is conserved across different species (reviewed by D'Costa et al. 2001).

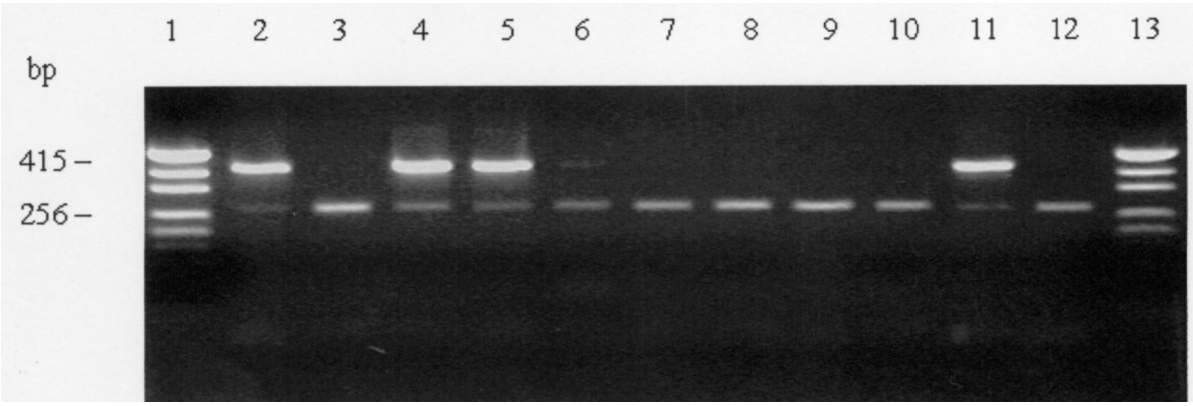

Fig. 3. Sex determination of one day old chicken embryos. Results of DNA amplification: the band at 415 bp derives from the repetitive *Xho*I fragment located on the W chromosome. The lower band (positive control) at 256 bp is sequence of the 18S rDNA gene. Lanes 1 and 13, DNA size marker (pUC19/*MspI*); lanes 2, 4, 5, 11, ♀; lanes: 3, 6, 7, 8, 9, 10, 12, ♂.

Isolation of PGCs and generation of PGC-derived progeny

The unique migration pathway via the blood vascular system and subsequent localization of PGCs provides a method for their isolation (Fig. 2). PGCs can be isolated from (i) X stage blastoderm, (ii) blood of 2.5-3 day embryos (stages 13-17), and (iii) gonads of 5.5-6 day (stages 26-28) embryos.

At stage X, approximately 30 vasa-positive cells are scattered in the central disc of the area pellucida. It is thought that these cells are the precursors of the chick primordial germ cells and, therefore, that PGCs are determined before the egg is laid. Later, PGCs in embryonic chick blood at stages 13-15 can be isolated from the dorsal aorta by the use of fine glass micropipette; this approach allows approximately 100 PGCs to be obtained from a single embryo.

Enrichment of the initial PGC populations generally is used by the Nycodenz density gradient centrifugation method (Zhao and Kuwana 2003) or a Ficoll density gradient (Park et al. 2003). Collected PGCs were washed and dispersed in KAv-1 medium (Kuwana et al. 1996). Naito et al. (2004) demonstrated that PGCs isolated from blood, and transferred to blastoderms in X stage, could enter in the bloodstream and migrate to the gonadal ridges of recipient embryos. Mozdziak et al. (2006) used FACS to enrich PGC populations from gonocytes. Cell preparations obtained from male gonads from 10-14 embryos at stage 27 were incubated with a monoclonal antibody against SSEA-1, and fluorescently labeled cells were separated from non-reactive cells using FACS. These were introduced into embryos at different stages of development (into the germinal crescent at stage X, or into the whole stage 17 embryo). It was reported that these PGCs maintained their ability to differentiate into gametes, demonstrating that PGCs following FACS purification can participate in normal development.

Kim et al. (2004) reported that PGCs can be isolated from chick gonads and quail embryos using magnetic-activated cell sorting (MACS). PGCs were rapidly purified using a magnetic field and magnetic microbeads conjugated to a secondary antibody, and MACS-purified PGCs had a significantly improved efficiency of gonadal migration in recipient embryos. Kim et al. (2009) have also used MACS to purify PGCs following lentiviral transfection.

One observation emerging from these experiments is that it is important to match the genetic sex of the donor and recipient embryos in order to improve embryo survival and reproductive capacity of germline chimeric chickens. In birds, the female is the heterogametic sex (ZW) and produces ova with either the W or Z chromosome. The male is the homogametic sex (ZZ) and produces spermatozoa with the Z chromosome. Chicken embryos can be sexed rapidly by PCR using primers targeting W chromosome-specific *Xho*I repetitive sequences, in conjunction with an autosomal marker to validate the integrity of the PCR reaction; the absence of a W-specific marker confirms the ZZ genotype (Fig. 3).

However, it is possible that female PGCs can differentiate to generate W spermatogonia after transfer to male embryos (Tagami et al. 1997). Furuta (2008) used histological techniques to study the morphology of homosexual and heterosexual PGCs transferred to embryos. Following heterosexual transfer, PGCs contributed to the development of gonads of sex opposite to that of the resident cells, but recipient embryos had abnormal gonads. Macdonald et al. (2010) demon-

strated that cultured male PGCs can colonize the gonads of the female recipients and enter meiosis, but these cells are lost from the female ovary.

Bednarczyk (et al. 2006) injected only female or only male donor cells into recipient male embryos, and reported that cocks injected with male cells had significantly higher reproductive performance than cocks treated with female cells.

Genetic modification of PGCs

The first methods for gene transfer in birds were based on the use of avian retroviruses. These included replication-defective vectors derived from reticuloendotheliosis virus (Bosselman et al. 1984), replication-competent vectors derived from avian leucosis virus (Salter et al. 1986), and lentiviral vectors such as equine infectious anemia virus (EIAV) (McGrew and Sang 2004).

More recently, the generation of transgenic chickens has been attempted through chimeric intermediates produced by the transfer of blastodermal cells (precursors of PGCs) or PGCs. Microinjection of *in vitro* modified PGCs into recipient embryos generates germline transgenic chimeras. PGCs isolated from blood can be transfected *in vitro* and injected into the bloodstream of embryos at the same developmental stage, and it has been demonstrated that genetically modified PGCs do not lose their ability to contribute to germ cells (Kuwana 2004) and germline chimeric chicken were obtained. Using a similar method, transgenic chickens (G1) were created by Motono et al. (2009). Here, embryos were infected with a HIV-based replication-defective lentiviral vector plasmid in which EGFP expression was driven from the chicken β-actin promoter. Following infection, PGCs were purified by FACS and approximately 30% of PGCs expressed EGFP. Cells were transferred into the bloodstream of 2.5 or 5.5 day recipient embryos. It was reported that 10% of transferred PGCs migrated to the gonads of the recipients, although the percentage of donor-derived PGCs in the gonad tended to be higher in female recipients than in males. Germline transmission of the transgene from mature birds (G0) was reported, and 3-6.6% of progeny (G1) chickens were transgenic for the EGFP expression construct.

More commonly, however, electroporation and lipofection techniques have been used to transfer DNA constructs directly into PGCs. Van de Lavoir et al. (2004) transfected PGCs obtained from stage 14-17 embryonic blood with linearized DNA using electroporation (one decay pulse of -200V, with 900-1.100 μF; or eight square-wave pulses of -250 to -350V, 100 μs). To minimize potential silencing of transgene expression, the construct was flanked at both ends by HS4 insulator sequences. PGCs were cultured *in vitro* for more than 1 month on buffalo rat liver (BRL) feeder cells to select for cells harboring integrated exogenous DNA. These were then introduced into recipient White Leghorn embryos, generating chimeric transgenic birds. Long-term culture was not incompatible with germline transmission of the transgene: after *in vitro* culture for 35-100 days, germline transmission of birds derived from cultured cells ranged from <1% to 86%, whereas up to 69% germline transmission was obtained after 47-66 days in culture (Van de Lavoir et al. 2004).

Despite this success, long-term *in vitro* culture is generally held to be associated with reduced ability to colonize the gonad, although other researchers have successfully applied this technique (Park et al. 2008, Shine et al. 2009).

Hong et al. (1998) compared two methods for PGC transfection. Electroporation was reported to have an 80% efficiency of DNA transfer whereas transfection with DNA:liposome complexes was only 17% efficient. Nevertheless, lipofection has been successfully used for DNA transfer into both BCs (Bednarczyk et al. 2003) and PGCs (Naito 2007). In this latter study lipofection of PGCs with linearized plasmid DNA (β-actin-GFP) was performed *in vivo*; 4.3% of embryo gonads were found to be positive for the expression construct.

This group also presented results from an *in vitro* or *in vivo* electroporation-based technique termed nucleofection, employing either circular or linearized plasmid DNA, and here the GFP gene was detected in less than 1% of recipient embryos. Further work will be required to optimize the nucleofection method for PGCs transfection. More recently, Naito et al. (2010) reported the production of chimeric chickens via a cell-based method in which blood-derived PGCs were cultured for an extended period *in vitro* using gonadal chicken cells as feeder cells. A male germline chimeric chicken was obtained following transfer of 58-day cultured PGCs; this chicken produced one donor-derived offspring from 270 examined.

Transgene expression constructs

To improve the production of recombinant protein in eggs, studies have addressed potential methods to increase the activity of oviduct-specific promoters. Park and Muramatsu (1999) examined *in vitro* steroid induction of gene expression from different steroid-responsive promoters appropriate for transgene expression in the oviduct of laying hens. Two steroid-

responsive promoters, Ov 100 and Ov 900, were found to reliably express a reporter gene in a cell-specific manner. The Ov 900 contains the ovalbumin negative regulatory element and the steroid-dependent response element from the 5' flanking region of the gene. Gao Bo et al. (2005) developed a vector in which 3 kb of the 5' and 3' flanking sequences of the chicken ovalbumin gene were used to direct the expression of EGFP and LacZ. Following gene transfer by the polyethyleneimine procedure into the oviduct epithelial and fibroblast cells (*in vitro*), and via the wing vein into egg-laying hens (*in vivo*), a PCR technique detected LacZ mRNA expression in the oviduct of injected hens, whereas the kidney, liver and heart were negative. High levels of β-galactosidase (LacZ) enzyme activity were detected in the oviduct magnum (116.7 mU/ml) and eggs (16.47 mU/ml), demonstrating that cloned regulatory regions of ovalbumin gene can be used to drive efficient expression of recombinant proteins in the oviduct.

Lilico et al. (2007) reported the use of EIAV-derived lentiviral vectors to express heterologous proteins in the oviduct of laying hens, and tissue-specific expression was obtained using estrogen response element (ERE) regulatory sequences from the ovalbumin gene. The integration site of vector and the genetic background of the hens were found to influence the expression level, nevertheless, transgenic chickens were obtained that synthesized biologically active recombinant miR24 (a chimeric ScFv-Fc miniantibody) and human interferon β1 (hIFNβ1a) in egg white. Interestingly, there was no evidence for transgene silencing over several generations (Lilico et al. 2007).

The specific elements associated with the ovalbumin gene that direct oviduct-specific expression have not yet been fully defined. Thorough analysis has identified additional oviduct-specific regulatory elements upstream of the ovalbumin coding sequences, including a tissue-specific element, the chicken oviduct-specific and enhancer-like region (COSE) (Park et al. 2006) and EREs (Lilico et al. 2007). Further studies on these and other elements, and how they interact *in vivo* to drive tissue-specific expression, will be required in order to generate reproducibly transgenic chickens with high-level tissue-specific expression for use as bioreactors.

With this objective, it will be important to develop a reliable *in vitro* assay system for testing tissue-specific vector constructs. One promising model is cultured chicken oviduct epithelial cells (COEC) that can be easily transfected with constructs based on oviduct-specific regulatory sequences, for example those derived from the ovalbumin or lysozyme genes. We have recently developed culture techniques that permit the preparation of confluent primary cultures of COEC in serum-free medium; after 5-7 days of cell culture, efficient *in vitro* transfection was obtained using commercially available transfection reagents. As shown in Fig. 4, introduction of an ovalbumin gene-base vector expressing GFP into cultured COEC gave rise to strong green fluorescence.

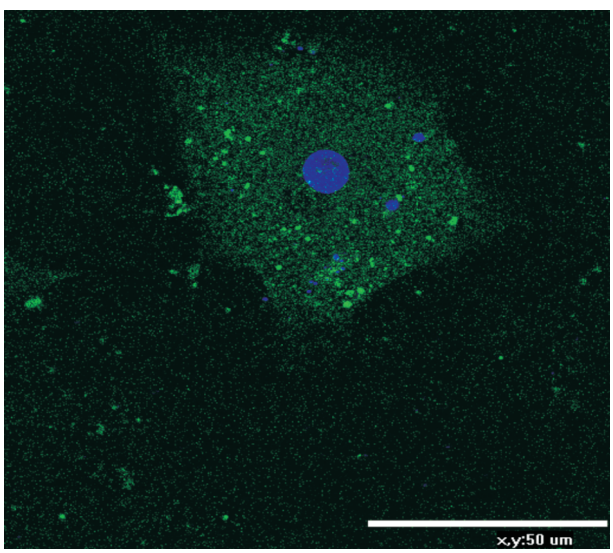

Fig. 4. Cultured oviduct epithelial cell following transfection with the GFP gene under control of the ovalbumin promoter; 48h post-transfection (Nikon Eclipse confocal microscope, original magnification 100x). Diaminobenzidine stained nucleus. Scale bar 50 µm.

In conclusion, many new methods are being explored for the generation of transgenic chickens. This is primarily in view of their enormous potential as bioreactors for the production of biopharmaceuticals; other potential applications include the modification of production traits such as disease resistance and the use of transgenesis as a tool to facilitate molecular studies on chicken development and physiology. In particular, ongoing technical advances have confirmed the enormous commercial potential of the chicken as a production vehicle (Penno et al. 2009). For the future, *in vitro* models based on chicken PGCs and COECs hold great promise; further work in this field will be required to realize the full potential of transgenic chicken technology.

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