African Journal of Biotechnology Vol. 9(24), pp. 3481-3488, 14 June, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Review

Recent progress on technologies and applications of transgenic poultry

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Accepted 31 May, 2010

Manipulation of the poultry genome has the potential to improve poultry production and offer a powerful bioreactor for the production of pharmaceutical and industrial proteins in eggs. However, before realizing this, the methods for producing transgenic poultry must become routine. The direct modification of an embryo with DNA or viral vectors is possible, but this approach does not have the ability to make locus-specific modifications to the genome. To overcome this problem, several cell-based protocols, which use mainly blastodermal cells (BDCs), embryonic stem cells (ESCs), primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) have been developed to generate transgenic chickens. At present, the complete system for isolating, expanding, transfecting, selecting and re-expanding embryonic stem cell cultures and the subsequent production of high-grade somatic chimeras has been reported, but germline chimeras with transgenic progeny have not yet been achieved. Although the use of viral systems can achieve highly efficient gene transfer, the potential safety issues may limit their practical application. Among the many possible permutations and combinations of target cell and gene transfer methods described in this review, targeting SSCs *in vitro* using non-viral-based gene transfer and re-injecting them to cock testis is the most efficient and cost-effective strategy to produce transgenic poultry.

Key words: Genetic modification, embryo, blastodermal cells, primordial germ cells, stem cells, viral vector.

INTRODUCTION

Significant progress during the last two decades has improved our understanding of the molecular basis of the genetics of growth and development. This knowledge, coupled with transgenic techniques, has the potential to accelerate conventional poultry breeding programs for improvements in desirable production traits (e.g. egg production, feed efficiency, rate of gain and body composition). Compared to mammals, avian species such as chicken and quail are easy to raise and have short reproductive cycles and high egg production; therefore, they are particularly amenable to transgenics for the production of pharmaceutical and industrial proteins in eggs (Robert, 2006). Notably, the development of techniques for producing transgenic poultry has lagged somewhat behind that for mammals. This is due primarily to the differences in the reproductive systems of avian species, the processing of the egg as it passes down the oviduct, the need for the shell in development and the difficulty of isolating germ or embryonic stem cells (Mozdziak and Petitte, 2004; van de Lavoir et al., 2006a; Fabrice and Bertrand, 2010). Nevertheless, in the past five years, some important breakthroughs have been achieved, making transgenic technology in poultry more efficient and thus, more useful for a broader spectrum of applications (Scott and Lois, 2005; van de Lavoir et al., 2006b; Kalina et al., 2007; Kwon et al., 2008; Shiue et al., 2009; Motono et al., 2010; Yu et al., 2010). The objective of this review is to present the status of technologies used to generate transgenic poultry and their recent applications.

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Abbreviations: BDCs, Blastodermal cells; **ESCs,** embryonic stem cells; **PGCs,** primordial germ cells; **SSCs,** spermatogonial stem cells.

TRANSGENIC TECHNOLOGY IN POULTRY

An efficient technology that enables the production of

transgenic birds at high frequency with reliable expression of transgenes is the prerequisite of application to both basic research and biotechnology. Two different strategies have been used to generate transgenic birds in most previous studies: (1) The direct modification of the embryo with DNA or viral vectors and (2) indirect cellular approaches, using mainly blastodermal cells (BDCs), embryonic stem cells (ESCs), primordial germ cells (PGCs) and spermatogonial stem cells (SSCs).

Direct protocols to generate transgenic poultry using DNA or viral vectors

In early studies, some investigators preferred to replicate the protocol developed in mice and applied successfully in several mammalian species, which involved pronuclear injection of newly fertilized eggs to produce transgenic poultry (Love et al., 1994). However, this protocol has some obvious drawbacks. First, the method is inconvenient and costly because every time one-cell eggs are harvested, a mature female bird must be killed, making it difficult to obtain a sufficient number of eggs to complete any procedure aimed at generating transgenic birds. Second, this protocol is technically demanding because after each injection, the ovum must be cultured *ex vivo* or transferred into a female bird to obtain a live bird. Both of these manipulations require a significant level of technical skill and an infrastructure to support the *ex vivo* culture system.

To avoid these disadvantages, other researchers have developed several efficient approaches through the direct manipulation of the embryo at two key stages of development: (1) the embryo in newly laid fertilized eggs at the blastodermal stage (stage X) and (2) embryos that have reached ~72 h of incubation, when the primordial germ cells (PGCs) and the precursors of the gametes, can be accessed. In 2001, Mizuarai et al. (2001) generated transgenic quail with highly efficient germline transmission (80%) by directly injecting a replication-defective pantropic retroviral vector into newly laid eggs at the blastodermal stage. Later, Kwon et al. (2004) also produced transgenic chickens using similar methods. Moreover, subsequent studies on detection showed that the EGFP gene was expressed in various body parts, including head, limb, eye, toe and several internal organs. Several years later, based on this system, Kwon and colleagues (2008) once again generated transgenic chickens that produced human granulocyte colony-stimulating factor (hG-CSF), and the biological activity of this recombinant hG-CSF was significantly higher than that of its commercial counterpart derived from *Escherichia coli*. Although the successful germline transmission of the transgene was confirmed in G1 progeny, most of them died within 1 month of hatching.

To improve both the efficiency of gene expression and germline transmission, some researchers have investigated the optimal developmental stage for viral vector injection. Masamichi et al. (2005) have reported that the maximal level of transgene expression could be obtained by injecting a replication-defective retroviral vector into the heart of embryos after 55 h of incubation. Likewise, Kawabe et al. (2008) also found that both the transduction efficiency and transgene expression were the highest when the viral solution was injected into embryos at a similar stage (stages 14 – 15). Both studies suggested that PGCs migrating into the gonads from the bloodstream are effective viral targets for generating transgenic chickens using retroviral vectors. Based on previous reports, in short, we could conclude that the appropriate development stage of embryo in combination with replication-incompetent retroviral or lentiviral gene delivery systems can greatly improve the efficiency of bird transgenesis.

Indirect protocols to generate transgenic poultry via genetically modified cells *in vitro*

Although the direct modification of an embryo with DNA or viral vectors can produce transgenic poultry, this technique does not have the ability to make locus-specific modifi- cations (gene targeting) to the genome because the transgene is integrated randomly into the host chromosomes (van de Lavoir et al., 2006). One obvious drawback of this strategy is that it probably causes variable expression of the transgenes in the host due to positional effects related to the chromosomal sites of integration. For this reason, the use of gene targeting is particularly attractive for the production of transgenic poultry because the possibility of obtaining a predictable phenotype that is of commercial importance is better than having to evaluate several lines of birds produced through random integration of the transgene (Dodgson, 2007). In view of this, a cell-based strategy may provide an alternative method for creating genetically modified poultry. Due to the culture of cells, the use of this approach not only provides sufficient materials for transgenic manipulation, but also allows the selection of cells that have integrated the transgene and cells that undergo targeted changes to the genome through the selection of homologous recombination events (Jung et al., 2007; Fabrice and Bertrand, 2010). From a practical perspective, clearly, this strategy can reduce the number of birds required to isolate transgenic birds and reduce the time required for assessment of the transgenic phenotype by one generation. Several viable protocols via cell-based strategy for the production of transgenic chicken are illustrated in Figure 1.

To date, several kinds of cells have been cultured *in vitro* for the production of transgenic poultry: (1) BDCs or ESCs isolated from the blastodisk immediately after oviposition (Pain et al., 1996; Park et al., 2006; Wang et al., 2006), (2) PGCs isolated from developing embryos (Zhao and Kuwana, 2003; Mozdziak et al., 2005; Shiue et

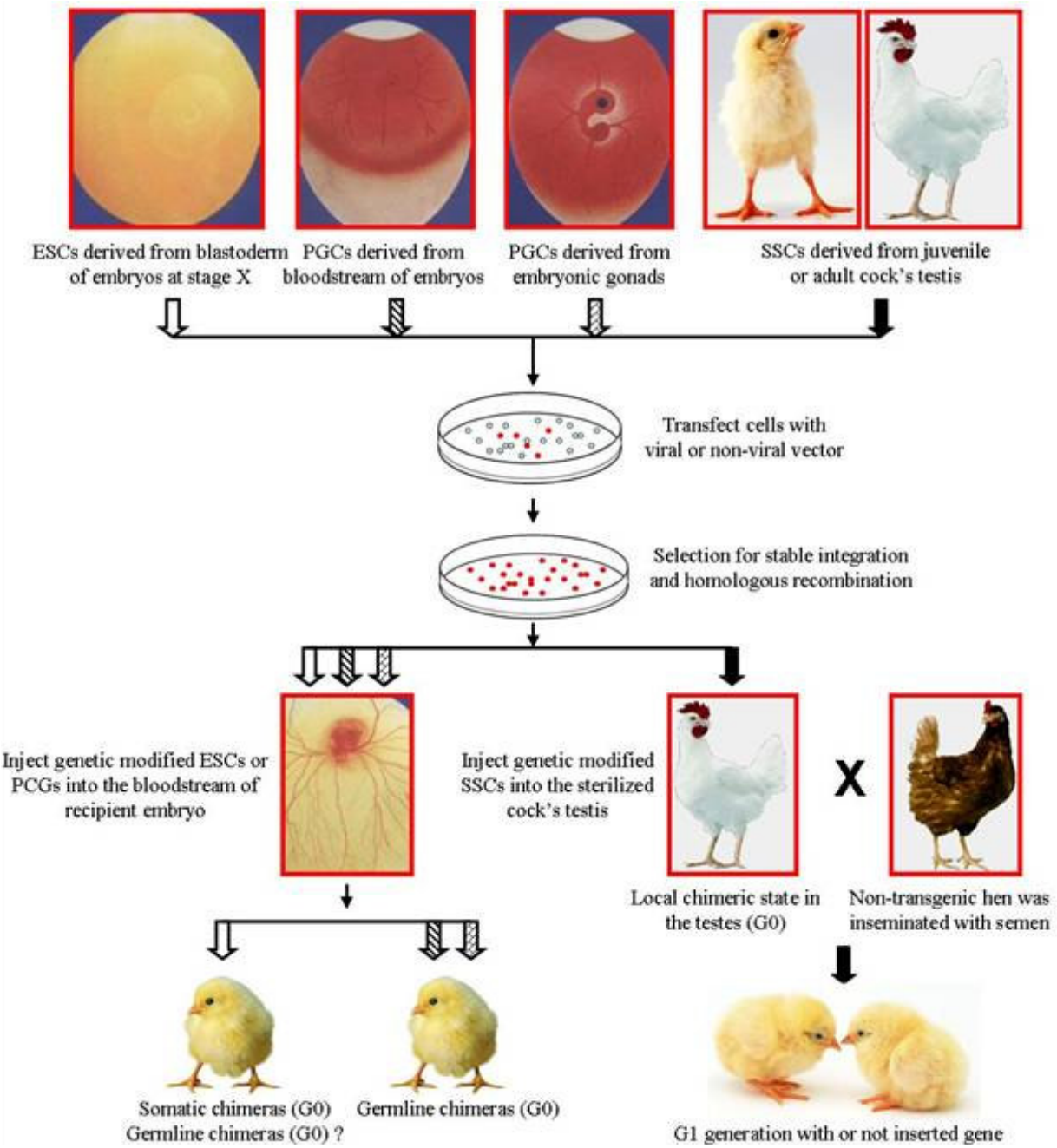

Figure 1. Diagram of methods that have been used for the production of transgenic chicken via cell-based strategy. These methods involve several major steps for targeted modifications to the genome. (1) To obtain cells that will contribute to the germline from the embryo or cock's testis and culture them in conditions that keep the cells in a relatively undifferentiated status; (2) these cells are transfected with viral or non-viral vectors designed to allow homologous recombination, and cells in which the correct recombination event has occurred are selected and expanded *in vitro*; (3) injecting genetic modified cells into the bloodstream of embryo or cock's testis, where they integrate and become part of the cellular makeup of the recipient.

al., 2009) and (3) SSCs isolated from the testes of cocks (Wu et al., 2006; Jung et al., 2007).

Blastodermal cells and embryonic stem cells

The technique for obtaining germline chimeras by direct modification of the embryo at the blastodermal stage (stage X) has been well established in both chicken and quails (Mizuarai et al., 2001; Kwon et al., 2004; McGrew et al., 2004; Koo et al., 2006). Moreover, some reports have indicated that blastodermal cells are amenable to transfection using various methods such as liposomes

and viral vectors (Wang et al., 2006). For instance, Zhu et al. (2005) have developed a cell transplantation method in chicken, wherein human monoclonal antibody were purified from the eggs of somatic chimeras after introducing transfected blastodermal cells into stage-X embryo. However, there is no report as yet that blastodermal cells of the bird that have been cultured *in vitro* for more than a week contribute to the germline chimeras, even though they apparently make up approaching 100% of the somatic cells of the chimera. To the cause of this, we think it is possible that during the long-term *in vitro* culture, these transfected BDCs have lost the ability to contribute to germline tissues.

To facilitate the germline transmission, some research groups have attempted to isolate pluripotent embryonic stem cells from the avian species, because pluripotent embryonic stem cells are undifferentiated cells capable of proliferation in cell culture and self-renewal and have the ability to differentiate into all somatic cell types and the germ line (van de Lavoir et al., 2006b). In the past, most efforts to culture avian embryonic stem cells have focused on the stage X non-incubated blastodermal cells, because within this population exist some cells that have the ability to enter the germline. For instance, Pain et al. (1996) study showed that chicken early blastoderm contains cells characterized as putative avian embryonic stem (ES) cells that can be maintained *in vitro* for long-term culture. These cells exhibit features similar to those of murine ES cells such as typical morphology, strong reactivity toward specific antibodies, cytokine-dependent extended proliferation and high telomerase activity. These cells also present high capacities to differentiate *in vitro* into various cell types including cells from ectodermic, mesodermic and endo- dermic lineages. At present, the complete system for isolating, expanding, transfecting, selecting and re-expanding ES cell cultures and the subsequent production of high- grade chimeras has been reported (van de Lavoir et al., 2006a). Up to date, however, there are no reports that exist about using transfected avian ESCs to generate germline chimeras with transgenic progeny (Fabrice et al., 2009) (Figure 1). Previous study once demonstrated that blastodermal cells can gradually transform into ESC-like cells during *in vitro* culture, while losing large amounts of lipid vesicles from the cytoplasm (Park et al., 2006). From these studies, we can conclude that although ESC can be easily amplified *in vitro* and genetically modified but it is still unknown under what culture conditions chicken ESCs would be able to keep long-term germinal competency once maintained in long-term *in vitro* culture.

Primordial germ cells

PGCs are the progenitor cells of ova and spermatozoa and are the genetic link among generations (Mozdziak et al., 2005). At first, PGCs can be recognized at the center of the area pellucida in the stage X blastoderm (freshly laid eggs). Upon incubation and as development continues, they can be found in the extra-embryonic region called the germinal crescent. They then enter and circulate in the blood vessels of the embryo, and migrate to the gonadal ridges, which develop into the mature gonads (Nakamura et al., 2007). It is generally thought that the migration is assisted by the extracellular matrix, which acts as a substrate containing adhesion molecules along the migratory path of PGC to the gonadal ridge (D'Costa et al., 2001). This unique feature, the migration of PGCs through the bloodstream and commitment to the germline, facilitates the production of germline chimeras via the injection of allogenic or xenogenic pluripotent cells into the blood vessels of the recipient embryos. Moreover, some experi- ments have demonstrated that isolated PGCs could be transferred to other recipient embryos at the stages in which PGCs circulate in the blood, without losing the ability to contribute to the formation of germ cells (Naito et al., 1999; Kim et al., 2004; Mozdziak et al., 2006). Therefore, it is assumed that gene transfer to PGCs and then trans- plantation to recipient embryos is an alternative way to generate transgenic birds. Furthermore, it is expected that the use of PGCs may improve the efficiency of germline transmission since PGCs are progenitors of ova and spermatozoa. Due to these reasons, a number of researches have focused on PGCs, instead of BDCs or ESCs, as a way to generate transgenic birds.

To date, there have been some successful reports using PGCs for transgenic research. van de Lavoir et al., (2006b) have successfully produced transgenic chickens using embryonic blood derived PGCs. In addition, this study also presents a robust system that allows PGCs in culture for extended periods and can be genetically manipulated while retaining their commitment to the germ line. The advent of this technology provides the ability to introduce transgenes of any size and to make site-specific changes to the genome. However, the scarcity of PGCs retrieved from embryonic blood (as low as 0.05% of total blood cells) still limited technical feasibility of PGCs for more advanced manipulation for transgenesis (Park et al., 2003). To resolve this problem, some research groups have attempted to isolate PGCs from the embryonic gonads instead of the circulatory system. Kim et al. (2005) reported the successful production of quail germline chimera by transfer of gPGCs immediately after collection. Subsequently, they also found that the capacity of gPGCs to induce germline transmission was not decreased by culturing for extended periods *in vitro* prior to trans- plantation (Park et al., 2008). Similarly, Shiue et al. (2009) recently also established a robust culture system maintain chicken gPGCs for long-term *in vitro* culture without losing their capacity to express pluripotent markers and to integrate into the gonads. In order to optimize conditions for the establishment of transgenic chickens, Motono et al. (2010) have directly compared the efficiency of PGCs

from 2.5- and 5.5-day embryos in migrating to the recipients' gonads and succeeded in establishing transgenic progeny using gPGCs infected with a lentiviral vector.

To further enhance the efficiency of germline transmission and genetic modification, some effective methods have also been widely employed in recent years. One approach is to purify PGCs by nycodenz density gradient centrifugation (Zhao and Kuwana, 2003) or magnetic cell sorting (Kim et al., 2004, 2005). The other is to sterilize the recipient embryos using busulfan emulsion (Furuta and Fujihara, 1999) or gamma-ray irradiation device (Lim et al., 2006) since the percentage of the transferred PGCs to the whole PGC population (transferred plus endogenous) is one important factor determining the efficiency of obtaining transgenic progeny.

Spermatogonial stem cells

In previous studies, most transgenic protocols were using BDCs, ESCs or PGCs as the mediated cells to produce transgenic birds. These protocols, however, are time-consuming and laborious, because they all need to massively produce founder stocks and to maintain the founders until sexual maturation for testcross analysis of the progenies derived from manipulated embryo (Li et al., 2008). To facilitate the production of transgenic poultry, many investigators have directed their attention toward developing an alternative germline chimera production system that operates via SSCs rather than through BDCs, ESCs or PGCs. Since early study in mice have indicated that testicular cells contain a small proportion of SSCs, which are the precursor cells for spermatogenesis and can be genetically modified *in vitro* and transplanted into seminiferous tubules of recipients (Kanatsu-Shinohara et al., 2003; Hamra et al., 2005). Furthermore, the work done by Jung et al. (2007) also identified SSCs in chicken testicular cells. This system could create a local chimeric state in the testes, and eliminate the background of mosaic animals and produce hemizygously transgenic animals in G1 generation. Pavel et al. (2006) for the first time in birds validated that the transfer of dispersed testicular cells from fertile donor cocks results in the recolonization of recipient testes previously sterilized by repeated radiation treat- ments. These recipient cocks ultimately produced viable and fertilization-competent spermatozoa. Similar results were also obtained by Lee et al. (2006) but with a low chimeric efficiency (7.8%). These studies suggest a possibility that efficient gene delivery into the crude mix of testicular cells might target the SSCs and pave way for bird transgenesis. Recently, Kalina et al. (2007) demonstrated that chicken testicular cells could be efficiently infected *in vitro* with a replication-defective reporter retrovirus vector pantropized by vesicular stomatitis virus envelope glycol-protein (VSV-G). Moreover, all cell types present in the mix of testicular cells express the transduced reporter enhanced green fluorescent protein (EGFP) after *in vitro* cultivation and freshly explanted infected testicular cells restore spermatogenesis in sterilized recipient cockerels.

For improving the efficiency of germline transmission and genetic modification of SSCs *in vitro*, Wu et al. (2006) first developed a method for SSCs isolation, purification and culture *in vitro*. Based on this system, their colleagues also established a highly efficient protocol to produce transgenic chickens by directly injecting transfected SSCs into the cock's testis (Li et al., 2008) (Figure 1). Likewise, Yu et al. (2010) recently, also isolated chicken SSCs from fetal testes, and cultured them more than 2 months after purification *in vitro*. Moreover, they found that transferred SSCs could stably localize to the basal membrane, prolife- rate and differentiate into sperm. This simple and efficient method provides a new way to produce transgenic chicks on a large scale. All these studies suggest that it is a feasible approach to produce transgenic birds using SSCs.

PROTOCOLS FOR GENE TRANSDUCTION

All methods for producing transgenic poultry rely on techniques designed to transfer foreign genes into target cells. The first methods developed for gene transfer in poultry were based on the use of avian retroviruses: replication-competent vectors derived from avian leucosis virus (Salter et al., 1986) and replication-defective vectors derived from reticuloendotheliosis virus (Bosselman et al., 1989). Since then, retroviral vectors have become a common gene transfer vehicle for bird cells. Retroviruses vector are small RNA viruses that, upon infection of dividing cells, are reverse-transcribed into DNA and then integrated into the host genome and passed down from generation to gene- ration (Mozdziak and Petitte, 2004). For use in transgenics, nonessential parts of the viral genome are replaced with the gene for transfer, which is subsequently inserted into the host genome along with viral sequences. To date, many researchers have successfully produced transgenic chickens or quail using retrovirus vectors (Harvey et al., 2002; Masamichi et al., 2005; Koo et al., 2006; Kawabe et al., 2008; Kamihira et al., 2009). Nonetheless, DNA transfer by a retrovirus has some limitations, including: (1) the upper limit of the introduced DNA fragment is <8 kb; (2) the transgene only integrates into dividing cells; (3) specific target cells must be recognized; and (4) the process is characterized by high susceptibility to gene silencing. In order to overcome some of these limitations, a new group of vectors, derived from the lentivirus class of retroviruses, has been developed. These lentiviral vectors do not require dividing cells for their integration because the import of their genetic information into the host cell is independent of cellular division, and they have a wide spectrum of target hosts cells (Cockrell and Kafri, 2007). In addition, in

recent years, lentivirus systems have been improved for tissue-specific expression. In 2005, Scott and Lois were successful in producing transgenic quail expressing the GFP gene using lentiviral vectors. Furthermore, they found that the GFP expression was specific to neurons and consistent across multiple generations. This approach provides a powerful tool for tissue-specific expression of desired transgenes or directed knockout of deleterious genes in birds. Since then, several classes of lentiviral vectors have been used to produce transgenic birds; some results have resulted in estimates that the production frequency of transgenic founders using these vectors is 10- to 100-fold higher than that obtained using the previous methods (Chapman et al., 2005; Motono et al., 2010). Moreover, to date, few studies have found the incidence of gene silencing or the loss of transgene expression over time or generations.

Despite extraordinary progress, the use of viral systems still has certain drawbacks, e.g., the potential safety issues, the possible integration of retroviruses in close proximity to potential oncogenes, followed by the activation of these oncogenes, and the initiation of conversion of a normal cell into a tumor cell (Cockrell and Kafri, 2007). In this situation, several non-viral-based technologies for gene transfer have been described including microinjection, liposome transfer, electroporation and calcium acid phosphate precipitation. DNA microinjection into the pronucleus of newly fertilized chicken eggs has been reported since 1994, but so far, it has not been used widely in poultry due to its low transfer efficiency and the laboriousness of the procedure, as described above (Love et al., 1994). In contrast, the liposome transfer method can be applied widely to transfer foreign genes efficiently into cells and embryos (Wang et al., 2006; Suraeva et al., 2008). Never- theless, a recent study on *in vitro* SSC transfection showed that both the efficiency of transfection and cell survival rate were higher using electroporation rather than liposomes or calcium acid phosphate (20.52 versus 9.75 and 5.61; 69.86 versus 65.00 and 51.16%, respectively) (Yu et al., 2010).

APPLICATIONS

Transgenic techniques have several possible applications in poultry industry, including production of valuable bioactive proteins and accelerating conventional breeding programs for improvements in desirable production traits. Owing to special physiological and developmental characteristics of the bird, in addition, these techniques have also been used to transfer foreign genetic materials into the chicken embryo to investigate the function of gene involved in vertebrate development (Brown et al., 2003; Hen et al., 2006; Raman et al., 2006). In previous studies, nevertheless, the objectives of the most transgenic works in poultry are taking chickens or quails as the bioreactor to produce some recombinant proteins. This is because transgenic poultry bioreactor system, compared with using plants, microorganisms and mammalian, has several advantages. Firstly, poultry has high protein productivity in eggs, and more than half of which comes from the expression of a single gene (ovalbumin gene), so it is easy to obtain large amounts of recombinant protein with a high purity (Robert, 2006). Secondly, the glycosylation patterns of some human proteins are more similar to those of chicken than to other bioreactor system (Raju et al., 2000). Thirdly, the presence of natural protease inhibitors in egg content and the natural sterile microenvironment of the egg system provided an ideal environment for stabilizing the biological activity of foreign proteins (Rapp et al., 2003). In addition, some previous studies indicated that the production of some proteins that are toxic for mammals could some- times be realized in transgenic birds only (Zhu et al., 2005; Lillico et al., 2007).

The first successful production of a recombinant protein in eggs was achieved using replication-defective avian leucosis virus (ALV) to deliver the transgene (Harvey et al., 2002). In this experiment, the biologically active bacterial β-lactamase was secreted into the serum and egg white of four generations of transgenic chickens, and the expression levels were similar in successive generations. This study supports the potential of the chicken as a bioreactor for the production of commercially valuable and biologically active proteins in egg white. Since then, several kinds of recombinant proteins, such as antibodies, enzymes and cytokine, were produced in the eggs using transgenic chickens or quails as the bioreactor. Recently, Lilico et al. (2007) demonstrated that transgenic chicken could produce large quantities of biologically active pharma- ceutical proteins in egg white by using oviduct-specific expression promoter, without any evidence of transgene silencing after several generations of germline transmission. Similarly, Kamihira et al. (2009) also generated transgenic chickens that can produce whole antibodies in both egg white and yolk throughout the breeding period. From previous reports, we can conclude that most of recom- binant proteins in the eggs were produced through two possible strategies. One is to introduce a transgene to oviduct cells that produce egg proteins (Harvey et al., 2002; Zhu et al., 2005). This includes the use of either tissue- specific promoters such as an ovalbumin promoter or constitutive promoters. The other possible strategy is to produce target proteins containing the Fc region of an antibody into blood, leading to the accumulation of the proteins into the yolk via IgY transfer (Kawabe et al., 2006; Lilico et al., 2007; Kyogoku et al., 2008; Kamihiraa et al., 2009). In the latter case, although target proteins may be limited to antibodies and Fc-fusion proteins, the method is effective for recovering such proteins at least without sacrificing the birds. All of these works mentioned above support the potential for efficient generation of transgenic birds and the commercial production of pharmaceutical and industrial

proteins in the egg.

CONCLUSION

Technologies for the production of transgenic poultry and protocols for the transgenic manipulation of gene expression have advanced considerably over the past decade and have had an important impact on basic research and poultry industries. Nevertheless, some major problems or drawbacks remain to be solved before the production of transgenic poultry becomes commonplace. Generally, it is considered that BDCs cultured for extended periods could transform into ESC-like cells. To date, however, there has been no report of the successful production of germline chimeras in birds using these ESC-like cells. Therefore, further research is required to conclusively establish and characterize bird ESCs to exploit their plasticity and ability to dedifferentiate in transgenic research. Apart from these problems, the increasing public concern regarding the safety of products derived from transgenic poultry may also restrict the practical application of some efficient viral-mediated transgenic technologies in the future. Under this circumstance, we believe that among the many possible permutations and combinations of target cell and gene transfer methods currently available, targeting SSCs *in vitro* using non-viral-based gene transfer and re-injecting them into cock testis is the most efficient and cost-effective strategy to produce transgenic poultry.

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