GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Generation of transgenic chicks using an oviduct-specific expression system

H.A. Kaleri[1,2]*, S.Y. Xu[1,3]* and H.L. Lin[1]

[1]College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, China
[2]Department of Animal Breeding and Genetics,
Faculty of Animal Husbandry and Veterinary Sciences,
Sindh Agriculture University, Tando Jam, Pakistan
[3]Department of Animal Sciences, Jinlin Institute of Nanjing, Nanjing, China

*These authors contributed equally to this study.
Corresponding authors: H.L. Liu / H.A. Kaleri
E-mail: liuhonglin@njau.edu.cn / hkaleri@hotmail.com

Genet. Mol. Res. 10 (4): 3046-3055 (2011)
Received February 16, 2011
Accepted July 27, 2011
Published December 8, 2011
DOI http://dx.doi.org/10.4238/2011.December.8.1

ABSTRACT. We successfully replaced the ovalbumin gene of a magnum region in chickens with a human plasminogen activator. We constructed pL-eGFP, pL-tPAGFP and pL-2.8OVtPAGFP vectors and cultured 293FT chicken embryo fibroblasts, chicken primordial germ cells, Hela C127 cells, and oviduct epithelial cells. All vectors were expressed in the transfected cells, except pL-2.8OVtPAGFP vector, which was only expressed in oviduct epithelial cells. A lentivirus with pL-2.8OVtPAGFP was injected in fertilized eggs; 11 chicks hatched in the G_0 generation, four of them carried the tPAGFP. Two cockerels from the G_0 generation were crossed with four wild-type hens. Three chicks in G_1 carried the tPAGFP. We concluded that by using an oviduct-specific vector for transfection, human recombinant plasminogen activator protein can be expressed in the oviducts of laying hens. This character is inherited and can be reproduced with a need for repeated transfection.

Key words: Ovalbumin gene; GFP; Tissue plasminogen activator; Laying hen

INTRODUCTION

In recent years, the most advanced research in the world has been the genetically modified technology. The manipulation of animal genome has offered a powerful bioreactor for the production of pharmaceutical and industrial proteins (Li and Lu, 2010). Transgenic insects, mammals and birds are among the major candidates for the production of biopharmaceutical proteins (Harvey et al., 2002; Tomita et al., 2003; Zhu et al., 2005; Lillico et al., 2007; Houdebine, 2009). The use of transgenic chicken as a bioreactor to synthesize therapeutic proteins, as a component of egg yolk or white may have several advantages over a mammalian expression system. This includes short-time scale for setup and easy to raise, lower costs associated with husbandry, a short reproductive cycle and high production, the potential to produce proteins that are toxic to mammalian cells, beneficial glycosylation profile, and reduced immunogenicity of the purified product (Robert, 2006; Lillico et al., 2005). Chicken ovalbumin promoter is considered to be the strongest major tissue-specific promoters that yield more than 50% of the total egg white protein content (Yamamoto, 1997). The lentiviral vector has been successfully used to create transgenic mice with a high rate of incorporation of foreign gene (Lois et al., 2002). Lentiviral vectors permit high levels of transgene expression in quails and chickens (Scott and Lois, 2005). Tissue plasminogen activator (tPA), a major protein involved in the breakdown of blood clots to treat major life threaten diseases, including heart attacks, strokes, clots in the lungs, and cancer, catalyzes the conversion of plasminogen to plasmin that is also used to dissolve thrombi associated with ischemic strokes and brain injury (Ichinose et al., 1986; Bruen et al., 2007). Taking into account the importance of tissue plasminogen-activator protein, the present study was designed to replace ovalbumin gene using lentiviral vector containing oviduct-specific promoter and human tissue plasminogen activator protein. The pL-eGFP, pL-tPAGFP and pL-2.8OVtPAGFP vectors were constructed and 293FT, Hela, C127, chicken embryo fibroblasts (CEF), chicken primordial germ cells (cPGC), and oviduct epithelial cells were cultured. Lentivirus was produced using pL-2.8OVtPAGFP vectors and injected into fertilized eggs.

MATERIAL AND METHODS

The peGFP plasmid (preserved at the Key Laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Sciences and Technology, Nanjing Agriculture University, China) and the pLenti-lacZ plasmid (Invitrogen, Shanghai, China) containing the lentiviral vector sequence were double-digested by *Nde*I, *Xba*I enzymes and the newly constructed plasmid was subsequently named pL-eGFP. The pGEM-tPA plasmid was obtained from China Pharmaceutical University (Nanjing), tPA primers were designed P1: 5'-GCGGGAATTCTC GAGATGGATGCAA-3' and P2: 5'-GATTATCACGGATCGATGTTGT-3'.

CAC3' with *Xho*I and *Bam*HI restriction sites

PCR was carried out in a 20-μL reaction system including 50 ng genomic DNA, 2 μL PCR buffer, 0.2 mM dNTPs, 10 μM primer, 0.5 U Taq DNA polymerase (Promega, Beijing, China). PCR conditions were denaturation at 94°C for 2 min; 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min, in a total of 32 cycles, and a final extension at 72°C for 7 min. The pL-eGFP

plasmid was double-digested with *Xho*I and *Bam*HI, the PCR product of tPA was subcloned and the resultant vector was named pL-tPAGFP plasmid. The ovalbumin PCR product was subcloned into pL-tPAGFP plasmid and the resultant vector was named pL-2.8OVtPAGFP (Kaleri et al., 2011).

Preparation of plasmid DNA

Single colony of *Escherichia coli* transformants of each plasmid vector were grown overnight in 500 mL Luria-Bertani (LB) broth containing 100 µg ampicillin/mL. The plasmid DNA was prepared using the Omega kit E.Z.N.A.™ Endo-Free Plasmid Kmini Kit II.

Cell culture

293FT, C127, CEF, cPGC, and oviduct cultured cells were obtained from Key Laboratory of Animal Genetics, Breeding and Reproduction (College of Animal Sciences and Technology, Nanjing Agriculture University, China).

In vitro DNA transfection

293FT cells were transfected with pL-eGFP vector; CEF and cPGC with pL-tPAGFP vector; Hela, C127 and primary oviduct cells with oviduct-specific vector. For transfection, 4 µg of each vector was used per well of cells. Lipofectamine™ 2000 Reagent (Invitrogen) was used as the transfection reagent in a ratio of 1 µg DNA:1.5 µg Lipofectamine according to manufacturer instructions. The cells were examined at 48 h after transfection. Green fluorescent protein was checked using fluorescence microscopy.

Lentivirus production

To prepare lentiviral stock, 293FT cells were plated on 10-cm dishes with 3.5 µg 2.8OVtPAGFP plasmid, 5 µg gag/pol plasmid, 2.5 µg rev plasmid, and 3 µg VSV-G plasmid by Lipofectamine™ 2000. The culture medium was changed after 6 h. Forty-eight hours post-transfection, the culture medium was harvested and centrifuged at 3000 rpm, for 15 min, at 4°C, filtered through a 0.45-µm filter (Millipore), followed by an ultracentrifugation at 50,500 *g*, for 120 min, at 4°C. The pellet was resuspended in PBS. Aliquots of vector were stored at 80°C. For generation of lentivirus, 293FT cells were transfected with 9 µg ViraPower Packaging mix (Invitrogen) along with 5 µg pL-2.8OVtPAGFP plasmid by Lipofectamine. Twenty-four hours post-transfection, the transfection mix was replaced with fresh culture medium (without antibiotics). Virus-containing supernatant was harvested 72 h post-transfection and cleared by centrifugation at 3000 rpm/min, for 15 min, at 4°C before use in transductions.

Concentration of lentivirus via ultracentrifugation

Lentiviral supernatants were concentrated via ultracentrifugation. Initially, the supernatant was centrifuged (3000 rpm, for 15 min, at 4°C) and filtered through a 0.45-µm filter. Thereafter, the virus was collected via ultracentrifugation in ultraclear centrifugation tubes for

2 h at 25,000 rpm and 4°C. The pellet was allowed to dissolve overnight at 4°C in a volume of sterilized PBS that was 1% of the original medium volume. The next day, aliquots were prepared and stored at -80°C until use. Virus generation and infection of target cells were carried out in class-II biological safety cabinets in the class-II containment laboratories, with access restricted to authorized staff.

Egg injection

Fifty fertilized eggs were positioned with their sides facing upwards for 8 h at room temperature in order to fix the blastoderm position. After swabbing the shell with 70% alcohol, 4.5 x 4.5-mm^2 windows were made in the equatorial plane of the egg shell using a fine drill, followed by removal of the small shell membrane inside the window with a thin forceps and a surgical blade. Five microliters of DMEM containing concentrated virus with pL-2.8OVtPAGFP was injected into the central part of the subgerminal cavity using a microinjection pipette. To increase infectivity, polybrene (10 µg/mL) was added to the virus medium. The injection pipette was drawn from a Pyrex glass tube with inner diameter at the tip of 80 µm. After injection, the window was sealed with egg shell and parafilm.

Egg incubation

After microinjection, the sealed eggs were incubated at 37.5°C and 60% relative humidity with a rocking motion every 2 h, through a 90° angle, for 18 days. Then, they were incubated at 37°C and 70% relative humidity without rocking until hatching. The age of an egg was based on days post-incubation (e.g., the day of microinjection is referred to as 0 day).

G_0 generation growth

Hatched chicks were fed until maturity according to NRC (1994). Two cockerels from the G_0 generation were mated with 4 wild-type hens and their eggs were collected for 4 days. The eggs were incubated at 37.5°C and 60% relative humidity with a rocking motion every 2 h, through a 90° angle, for 18 days. Then, they were incubated at 37°C and 70% relative humidity without rocking until hatching.

G_1 generation growth

G_1 generation chicks were grown until maturity and a cockerel was mated with two hens of the G_1 generation and wild-type hens. Their eggs were collected and checked for tPA expression.

Genomic analysis

Genomic DNA was extracted from chicken using a genomic DNA purification kit (Promega). PCR was carried out in a 20-µL reaction system including 50 ng genomic DNA, 2 µL PCR buffer, 0.2 mM dNTPs, 10 µM primer, 0.5 U Taq DNA polymerase (Promega). PCR conditions were denaturation at 94°C for 2 min; 94°C for 30 s; 58°C for 30 s, and 72°C for 2

min, in a total of 32 cycles, and a final extension at 72°C for 7 min. To achieve rapid results, the presence of transgene was screened using the DNA dot blot analysis. Briefly, 20 µg DNA was cut with ultrasound and dropped on a nylon membrane. Green fluorescence protein (GFP; 641 bp) probes were amplified with the primers 5'-ACG TAA ACG GCC ACA AGT TCA GCG-3' and 5'-GTC CAT GCC GAG AGT GAT GCC G-3', purified by agarose gel electrophoresis, and synthesized using the PCR DIG probes synthesis kit (Shanghai Roche Pharmaceuticals, Shanghai, China). A dig color detection kit (Roche) was used to detect labeled gene. GFP-specific primers (sense primer: 5'-TGG TGA GCA AGG GCG AGG AG-3' and antisense primer: 5'-CAG GGC GGA CTG GGT GCT CA-3') were designed to amplify a 674-bp fragment. PCR was carried out in a 20-µL reaction system including 50 ng genomic DNA, 2 µL PCR buffer, 10 µL of each primer, 0.2 mM dNTP, and 0.5 U Pfu DNA polymerase (Promega). PCR conditions were an initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 30 s; 64°C for 30 s, and 72°C 30 s, and a final extension at 72°C for 7 min.

RESULTS

pL-eGFP vector

The construction map of the pL-eGFP vector (7679 bp) is shown in Figure 1. Figure 1A and B show the digestion results. Figure 1C shows the diagram of plasmid digestion with a Gene Construction kit. Transfection of 293FT cells with pL-eGFP vector resulted in GFP expression in cells. Figure 2 shows the expression of GFP in 293FT cells. Figure 2A shows the image for GFP and Figure 2B shows the bright field image of A.

pL-tPAGFP vector

The amplified PCR product of tPA, construction map of pL-tPAGFP vector (9347 bp) with agarose gel of size and digestion is shown in Figure 3. Figure 3A shows the PCR product tPA in the size of approximately 1700 bp. Figure 3B shows digestion results of 1901, 3760 and 3656 bp. Transfection of CEF and cPGC with pL-tPAGFP vector revealed GFP expression. Figure 4 shows the expression of GFP in CEF and cPGC. Figure 4A and C show green images of CEF and cPGC whereas Figure 4B and D show bright photographs corresponding to the A and C fields.

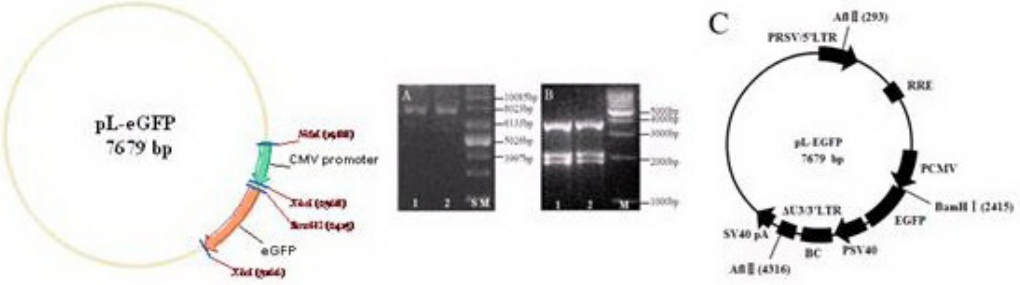

Figure 1. Map of the pL-eGFP vector (7679 bp). **A.** *Lanes 1* and *2* = PCR products; *lane SM* = super-coiled marker TaKaRa. **B.** *Lanes 1* and *2* = plasmid digestion into 1901 bp, and 2122 and 3656 bp, respectively; *lane M* = molecular marker TOYOBO. **C.** Diagram of the analysis of Gene Construction Kit 2.0.

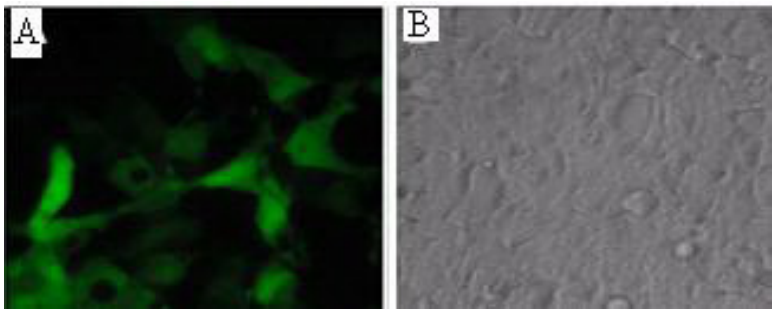

Figure 2. 293FT cells transfected with pL-eGFP plasmid. **A.** Green fluorescence image. **B.** Bright field image of A.

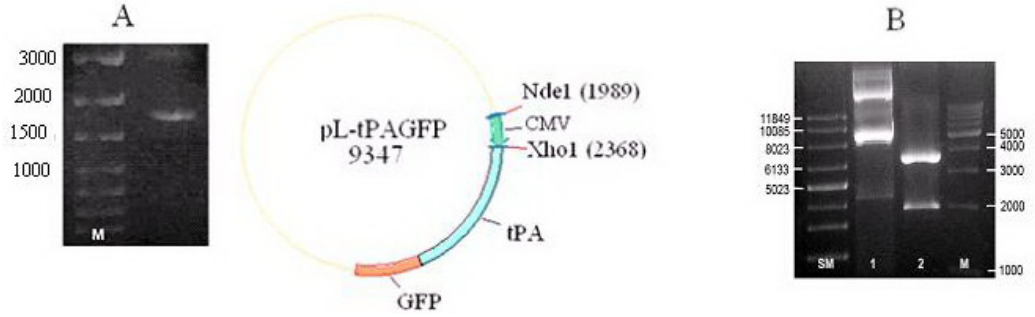

Figure 3. pL-tPAGFP vector (9347 bp). **A.** PCR product of tissue plasminogen activator (1700 bp); *lane M* = molecular marker. **B.** *Lanes 1* and *2* = plasmid digestion into 1931 bp, and 3760 and 3656 bp, respectively; *lane SM* = super-coiled marker; *lane M* = molecular marker.

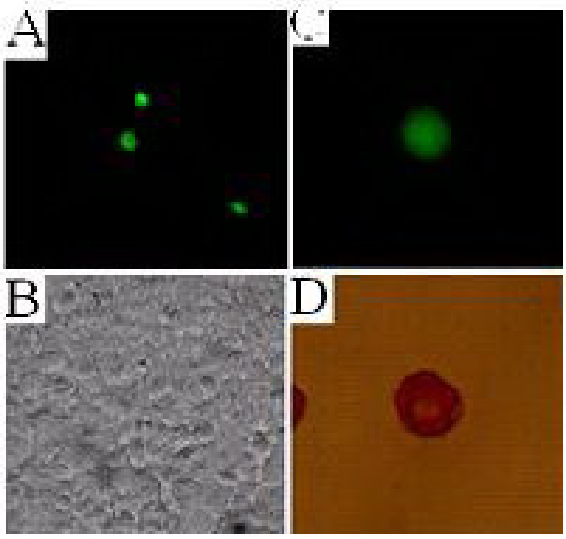

Figure 4. A. C. Green florescence image of chicken embryo fibroblasts and chicken primordial germ cells. **B. D.** Bright-field photographs corresponding to A and C.

Oviduct-specific vector

The oviduct-specific vector was transfected with oviduct epithelial cells, Hela, and C127 cells; this revealed that the oviduct-specific vector was only expressed in the oviduct cells, but was a control vector in oviduct epithelial, Hela and C127 cells. Figure 5 shows expression of GFP in oviduct epithelial cells, Hela and C17 cells.

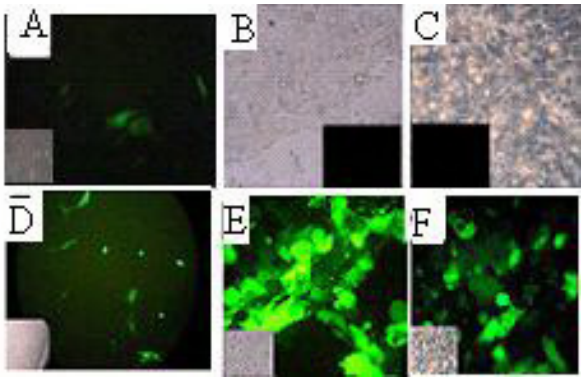

Figure 5. A. Green fluorescence image of oviduct epithelial cells transfected with oviduct-specific vector pL-2.8OVtPAGFP. **B. C.** Bright field photograph of Hela and C127 cells; small black photos show that no GFP was expressed in Hela and C127 cells. **D. E. F.** Green fluorescence image for oviduct epithelial, Hela and C127 cells transfected with control vector.

Transgenic detection

G_0 generation

The 11 (22%) chicks hatched and four (36%) of them contained exogenous gene tPAGFP marked by DNA dot blot analysis, which was also confirmed by PCR analysis. Figure 6A,B show DNA dot blot and PCR analysis result of chicks in the G_0 generation.

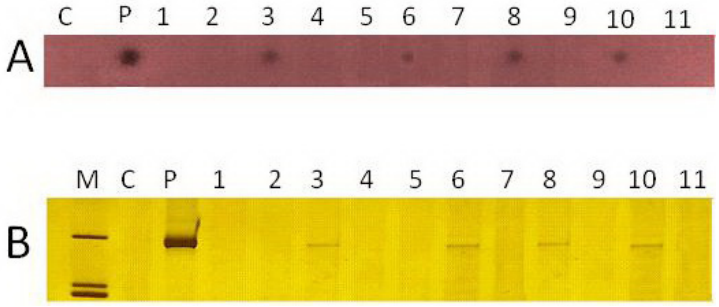

Figure 6. A. B. DNA dot blot and PCR analysis of the G_0 generation. **A.** *Lane C* = negative control; *lane P* = positive control or DNA from oviduct cells transfected with oviduct-specific promoter plasmid pL- 2.8OVtPAGFP; *lanes 1-11* = transgenic chickens of the G_0 generation. **B.** *Lane M* = molecular marker; *lane C* = negative control or genome DNA from non-transgenic chicken; *lane P* = positive control, genomic DNA from oviduct cells transfected with plasmid pL-2.8OVtPAGFP; *lanes 1-11* = hatched chickens.

G_1 generation

Nine (28%) chicks hatched and three (33%) of them were carriers of the tPA marked with GFP by DNA dot blot analysis, which was also confirmed by PCR analysis. Figure 7A,B show the DNA dot blot analysis and PCR analysis of G_1 generation chicks.

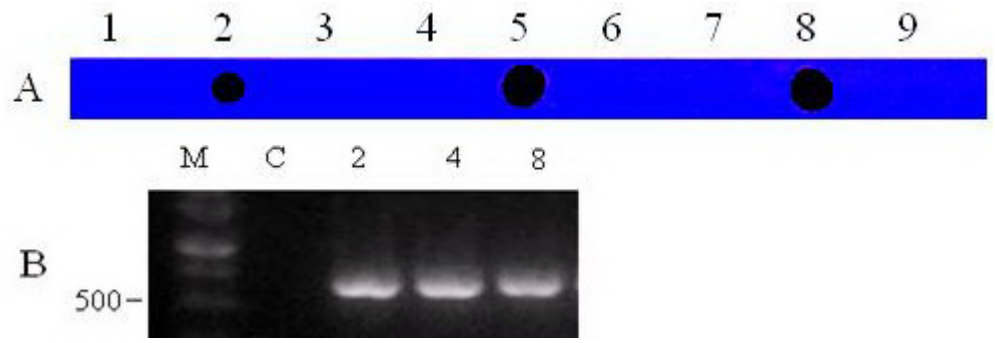

Figure 7. DNA dot blot and PCR analysis of the G_1 generation. **A.** DNA dot blot analysis of hatched chicks (*lanes 1-9*). **B.** PCR of positive chicks (*lanes 2*, *4*, *8*); *lane C* = control; *lane M* = molecular marker.

DISCUSSION

In the present study, we constructed pL-eGFP, pL-tPAGFP, and pL-2.8OVtPAGFP vectors and transfected them with 293FT, CEF, cPGC, Hela, C127, and oviduct epithelial cells for confirmation of vector. The vector digestion, agarose gel results for size and expression of GFP in cultured cells revealed that vectors were constructed successfully. The expression of oviduct-specific vector in oviduct cells alone revealed that this vector is only expressed in oviduct cells, but other vectors in a different type of cells. The result of DNA dot blotting and PCR analysis of the G_0 generation chick revealed that of 11, only four chicks in G_0 and three of nine in G_1 were positive, containing integration of tPA with GFP. The 36% integration rate in G_0 and 33% in G_1, the hatching rate 22% in G_0 and 28% in G_1 were slightly higher than other studies that reported results within the range from 4 to 27% (Chapman et al., 2005; Kwon et al., 2010). However, in accordance with other authors (Harvey et al., 2002; Mozdziak et al., 2003), hatching rates were reported to be between 23 to 36% using oncoretroviruses. The lower hatching rate in G_0 may possibly be due to results of cytotoxic effect on EGFP expression in early embryos (Perry et al., 1999) and lentiviral integration into key developmental genes, which resulted in embryonic abnormality and death. Many other researchers have attempted to generate transgenic birds (Salter et al., 1986, 1987; Bosselman et al., 1989; Vick et al., 1993; Love et al., 1994; Sherman et al., 1998; Mizuarai et al., 2001; Mozdziak and Petitte, 2004; Sang, 2004), but reasonably high production of recombinant proteins with a complicated structure has not been reported yet. We obtained positive results showing that oviduct-specific vector containing tissue plasminogen activator can be expressed in magnum of oviduct cell. This vector can be very successful for integration of tissue plasminogen activator protein on genome level in oviduct of laying hens. Our studies will continue to check tissue plasminogen

activator excluding GFP from vector and ongoing research should be fruitful for expression of tissue plasminogen activator as biopharmaceutical protein in eggs.

ACKNOWLEDGMENTS

Research supported by the National High-Technology Research and Development Program of China (883 Key program #2007AA100504 Annhui Natural Science Foundation #050410201).

REFERENCES

Bosselman RA, Hsu RY, Boggs T, Hu S, et al. (1989). Germline transmission of exogenous genes in the chicken. *Science* 243: 533-535.

Bruen KJ, Ballard JR, Morris SE, Cochran A, et al. (2007). Reduction of the incidence of amputation in frostbite injury with thrombolytic therapy. *Arch. Surg.* 142: 546-551.

Chapman SC, Lawson A, Macarthur WC, Wiese RJ, et al. (2005). Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. *Development* 132: 935-940.

Harvey AJ, Speksnijder G, Baugh LR, Morris JA, et al. (2002). Expression of exogenous protein in the egg white of transgenic chickens. *Nat. Biotechnol.* 20: 396-399.

Houdebine LM (2009). Production of pharmaceutical proteins by transgenic animals. *Comp. Immunol. Microbiol. Infect. Dis.* 32: 107-121.

Ichinose A, Takio K and Fujikawa K (1986). Localization of the binding site of tissue-type plasminogen activator to fibrin. *J. Clin. Invest.* 78: 163-169.

Kaleri HA, Xiang L, Juken A and Shiyong X (2011). Oviduct-specific expression of tissue plasminogen activator in laying hens. *Genet. Mol. Biol.* 34: 231-236.

Kwon SC, Choi JW, Jang HJ, Shin SS, et al. (2010). Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. *Biol. Reprod.* 82: 1057-1064.

Li JJ and Lu LZ (2010). Recent progress on the technologies and applications of transgenic poultry. *Afr. J. Biotechnol.* 9: 3481-3488.

Lillico SG, McGrew MJ, Sherman A and Sang HM (2005). Transgenic chickens as bioreactors for protein-based drugs. *Drug Discov. Today* 10: 191-196.

Lillico SG, Sherman A, McGrew MJ, Robertson CD, et al. (2007). Oviduct-specific expression of two therapeutic proteins in transgenic hens. *Proc. Natl. Acad. Sci. U. S. A.* 104: 1771-1776.

Lois C, Hong EJ, Pease S, Brown EJ, et al. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295: 868-872.

Love J, Gribbin C, Mather C and Sang H (1994). Transgenic birds by DNA microinjection. *Biotechnology* 12: 60-63.

Mizuarai S, Ono K, Yamaguchi K, Nishijima K, et al. (2001). Production of transgenic quails with high frequency of germ-line transmission using VSV-G pseudotyped retroviral vector. *Biochem. Biophys. Res. Commun.* 286: 456-463.

Mozdziak PE and Petitte JN (2004). Status of transgenic chicken models for developmental biology. *Dev. Dyn.* 229: 414-421.

Mozdziak PE, Borwornpinyo S, McCoy DW and Petitte JN (2003). Development of transgenic chickens expressing bacterial beta-galactosidase. *Dev. Dyn.* 226: 439-445.

NRC (1994). Nutrient Requirements of Poultry. 9th Revised Edition. National Academy Press, Washington, 19-34.

Perry AC, Wakayama T, Kishikawa H, Kasai T, et al. (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284: 1180-1183.

Robert I (2006). Competitive bioreactor hens on the horizon. *Trends Biotechnol.* 24: 99-101.

Salter DW, Smith EJ, Hughes SH, Wright SE, et al. (1986). Gene insertion into the chicken germ line by retroviruses. *Poult. Sci.* 65: 1445-1458.

Salter DW, Smith EJ, Hughes SH, Wright SE, et al. (1987). Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology* 157: 236-240.

Sang H (2004). Prospects for transgenesis in the chick. *Mech. Dev.* 121: 1179-1186.

Scott BB and Lois C (2005). Generation of tissue-specific transgenic birds with lentiviral vectors. *Proc. Natl. Acad. Sci. U. S. A.* 102: 16443-16447.

Sherman A, Dawson A, Mather C, Gilhooley H, et al. (1998). Transposition of the *Drosophila* element mariner into the chicken germ line. *Nat. Biotechnol.* 16: 1050-1053.

Tomita M, Munetsuna H, Sato T, Adachi T, et al. (2003). Transgenic silkworms produce recombinant human type III procollagen in cocoons. *Nat. Biotechnol.* 21: 52-56.

Vick L, Li Y and Simkiss K (1993). Transgenic birds from transformed primordial germ cells. *Proc. Biol. Sci.* 251: 179-182.

Yamamoto T (1997). Hen Eggs: Their Basic and Applied Science. CRC Press, Boca Raton.

Zhu L, van de Lavoir MC, Albanese J, Beenhouwer DO, et al. (2005). Production of human monoclonal antibody in eggs of chimeric chickens. *Nat. Biotechnol.* 23: 1159-1169.

