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비바이러스 *In Ovo* 직접주입법에 의한 메추리 형질전환 시스템

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Non-Viral Transgenesis via Direct *In Ovo* Lipofection in Quail

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ABSTRACT Transgenic animals have been widely used for developmental biology studies, as disease models, and even in industry such as transgenic bioreactor animals. For transgenic birds, quail has the great advantages of small body size, short generation time, and frequent egg production. To date, retroviral or lentiviral transduction has been used to generate transgenic quail for various purposes. However, the efficiency of transgenic offspring production with these methods is relatively low and viral vector usage has safety issues. Unfortunately, non-viral transgenesis has not been established in quail due to a deficiency of stem cell and germ cell culture systems. In this study, we established a direct in ovo lipofection method that could be used to create transgenic quail without germline-competent cells or viruses. To optimize the injection stage during embryo development, the liposome complex (containing piggyBacCMV-GFP and transposase plasmids) was introduced into an embryonic blood vessel at 50 hr, 55 hr or 60 hr. GFP expression was detected in various tissues (heart, kidney, liver and stomach) on day 12 of incubation under a fluorescence microscope. Additionally, GFP-positive cells were detected in the recipient embryonic gonads. In conclusion, the direct in ovo lipofection method with the piggyBac transposon could be an efficient and useful tool for generating transgenic quail.
(Key words : transgenic quail, *in ovo* lipofection, *piggyback* transposon, transgene expression)

INTRODUCTION

Genetically modified transgenic birds have the great potentials for studying embryo development, functional genomics, and animal models for human diseases. In addition, the transgenic lines would be also applied to efficiently improve poultry production and massively produce industrial proteins.

To generate transgenic chicken, DNA microinjection into zygote was first adopted but the efficiency of transgenic chicken production was too low and furthermore, lots of hens should be sacrificed for collecting the fertilized eggs (Love et al., 1990). At the early stage of avian transgenesis, the blastodermal cells of freshly laid eggs were used as a vehicle of transgene into the offspring genome because the germline chimeras have been produced with the undifferentiated cells at stage X (Petitte et al., 1990; Carsience et al., 1993). However, eventually, the generation of transgenic offspring via blastodermal cells and embryonic stem cells derived from stage X blastoderm has been failed. Zhu et al. (2005) reported the production of human monoclonal antibody in eggs of chimeric founder chickens after transfer of chicken embryonic stem cells (cES cells) comprising human antibody gene. However, the transgenic progeny was also not produced from the founder chimeric chickens.

The most promising protocol for transgenic birds is a virus-mediated transgene transfer. Since Salter et al. (1987) first demonstrated the production of transgenic chickens by injecting avian leukosis viruses into blastoderm, many types

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of virus such as retrovirus and lentivirus were attempted to generate the transgenic birds. Using a replication-deficient retroviral vector based on the avian leukosis virus (ALV), Harvey et al. (2002) reported that the biofunational β-lactamase was secreted and deposited into egg white of four generations of transgenic chickens which were transferred the transgene controlled by the ubiquitous cytomegalovirus (CMV) promoter. Subsequently, for the massive production of recombinant therapeutic proteins, Lillico et al. (2007) advanced the transgenic chicken bioreactor system using lentiviral vector controlled by oviduct-specific promoter. Recently, GFP-expressing transgenic zebra finches were also generated by lentivirus and so, these transgenic birds can provide a useful tool for studying vocal learning as a model animal (Agate et al., 2009).

In despite of a versatile viral transduction for generating transgenic birds, there are a lot of obstacles for the practical applications due to relatively low and variable rates of germline transmission and transgenic offspring production as well as safety issues of viral vector usages (Park & Han, 2012). Thus, an alternative transgenic strategy was attempted by using germline-competent cells, primordial germ cells (PGCs) to improve the efficiency of germline transmission and develop non-viral transgenic techniques. In chicken, two types of PGCs at different stages can be retrieved and manipulated from embryonic blood vessel and embryonic gonads because they migrate through the blood vessel and finally, settle down into the developing genital ridges. Both of the circulating and gonadal PGCs were reintroduced into the blood vessel of recipient embryos at the PGC migration stages and the germline transmittable capability was verified during the last two decades (Tajima et al., 1993; Naito et al., 1994; Park et al., 2003). More recently, *in vitro* culture system for chicken PGC expansion was established (van de Lavoir et al., 2006; Choi et al., 2010; McGrew et al., 2010). Based on PGC culturing technique, avian transgenesis is rapidly advanced and would be potentially applied to the pharmaceutical protein production as well as the poultry industry. Additionally, non-viral transposon elements that can relocate between different genomic loci were successfully adopted to generate transgenic chickens (Macdonald et al., 2012; Park and Han, 2012).

In contrast to chicken, the non-viral transgenic techniques as well as quail PGC culture system have not been established yet to create transgenic quail. By injecting a replication-defective pantropic retrovirus into quail blastoderms, Mizuarai et al. (2001) reported the production of transgenic quail through germline transmission. Thereafter, the virus-mediated transgenic technique at stage X blastoderm demonstrated that transgenic quail expressed a biofunctional protein on the oviduct-specific manner and was used for studying vascular development and morphogenesis as an avian model (Kwon et al., 2010; Sato et al., 2010). The lentiviral transduced PGC-mediated transgenesis successfully produced transgenic quail although the efficiency was still too low (Shin et al., 2008). Thus, the present study was conducted to develop the alternative strategy for generating transgenic quail through direct *in ovo* lipofection (DiL) of *piggyBac* transposon transgene into circulating PGCs at early developmental stage.

MATERIALS AND METHODS

1. Experimental Animals

The care and experimental use of quail were approved by the Institute of Laboratory Animal Resources, Seoul National University (Approval no. SNU-070823-5). Japanese quail were maintained according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory. Birds were individually caged under a photoperiod of 14 L : 10 D (lights on at 05:00 am and off at 7:00 pm) with free access to food and water.

2. Construction of a *piggyBac* Expression Vectors

The basic plasmids of CAGG-PBase (pCyL43) and *piggyBac* transposon (pCyL50) were donated from Sanger Institute (http://www.sanger.ac.uk). Total 3.9 kb of GFP gene controlled by cytomegalovirus (CMV) immediate-early enhancer/promoter, and neomycin-resistance (Neo^R) gene with Simian vacuolating virus 40 (SV40) promoter was cloned between 5' and 3' *piggyBac* transposon elements by *Pac* I digestion and ligation.

3. Lipofection of Quail Embryonic Blood Cells

To determine the optimal lipofection for quail cells, total blood cells were retrieved from quail embryos at 50 hr and

seeded onto 96 well plate with 10% fetal bovine serum (FBS) and DMEM culture media. After an hour, the different DNA amounts of *piggyBac*CMVGFP and transposase were transfected with Lipofectamine® reagent (Invitrogen, Carlsbad, CA). Basically, the plasmid ratio of *piggyBac*CMVGFP and transposase was 1 : 1 (w : w) in all combinations. Four conditions for DNA amounts of *piggyBac*CMVGFP and transposase were used as 1 μg + 1 μg, 2 μg + 2 μg, 4 μg + 4 μg, and 5 μg + 5 μg with 4 μL Lipofectamine® reagent. For flow cytometry at one day after transfection, the transfected quail blood cells were resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and strained through a cell strainer (40 μm, BD Falcon). The transfection efficiency was measured with a FACSAriaIII (Becton Dickinson) and the subsequent analysis was performed using FlowJo software (Tree Star Inc.).

4. Detection of GFP Expression after Direct *In Ovo* Lipofection into Embryonic Blood Vessel

For direct *in ovo* lipofection (DiL), 2 μL of the liposome complex with 1 μg + 1 μg plasmids of *piggyBac*CMVGFP and transposase was injected into blood vessel of embryos at 50 hr, 55 hr, and 60 hr. At 12 days, each tissue (heart, liver, gizzard, kidney, and gonads) was dissected from the recipient embryos and then GFP-expressing cells were detected using an AZ100 multipurpose zoom confocal microscope (Nikon Corporation, Tokyo, Japan).

5. Statistical Analysis

Statistical analysis was performed using the Student *t* test in SAS version 9.3 software (SAS Institute, Cary, NC). The significance differences between the different groups were analyzed using the general linear model (PROC-GLM) in SAS software. Differences between treatments were deemed to be significant when P was less than 0.05.

RESULTS AND DISCUSSION

1. Optimization of Quail Embryonic Blood Cell Lipofection

To optimize lipofection of transgene, the transfection efficiency into quail blood cells from 50-hr-old embryos was compared between the different amounts of transgene plasmids at 1 : 1 (w : w) ratio of *piggyBac*CMVGFP vector and transposase plasmid. From flow analysis one day after transfection, the efficiency of GFP expressing cells in 1 μg + 1 μg of *piggyBac*CMVGFP vector and transposase plasmid was significantly higher than those of other combinations ($p<0.005$) (Fig. 1). The lipofection efficiency in 1 μg + 1 μg of *piggyBac*CMVGFP vector and transposase was 19.4% but the efficiencies in other combinations were less than half of 1 μg + 1 μg combination (10.1%, 2.2% and 0.9% in 1 μg + 1 μg, 4 μg + 4 μg and 5 μg + 5 μg, respectively) (Fig. 1B). The transfection efficiency showed the significantly negative correlation with concentrations of plasmid DNAs suggesting that the higher amounts of plasmid DNAs might hamper the formation of DNA-liposome complex.

2. Detection of GFP Expression after Direct *In Ovo* Lipofection into Embryonic Blood Vessel

Based on the result of the optimization for quail embryonic blood cell lipofection, direct *in ovo* lipofection (DiL) was conducted at the different embryonic stages of 50 hr, 55 hr, and 60 hr. After 2 μL of the liposome mixture with 1 μg + 1 μg of *piggyBac*CMVGFP and transposase plasmid was injected into blood vessel of embryos, GFP-expressing cells in five tissues including heart, liver, gizzard, kidney, and gonads were detected at 12 days (Fig. 2). The cellular toxicity induced by lipofectamine was not observed and the majority of recipient embryos survived to 12 days of incubation. The GFP expression was significantly higher in heart than other tissues regardless of injection time points (Fig. 2). This was because DNA-liposome complex was injected into blood stream and so the heart was the first contacting tissue and constantly transfected during circulation of DNA complex. In other tissues (kidney, liver and stomach), GFP expression levels were similar to each other. Interestingly, injection at 50 hr showed the higher expression of GFP transgene in all tissues compared to those of injection at 55 hr and 60 hr. Further study should be necessary to investigate the lipofection process during the blood circulation and embryo development. In embryonic gonads, the stable GFP expression was detected in all treatments (Fig. 3). Similar to other somatic tissues, GFP expression was also stronger in 50 hrs injection than those in 55 hr and 60

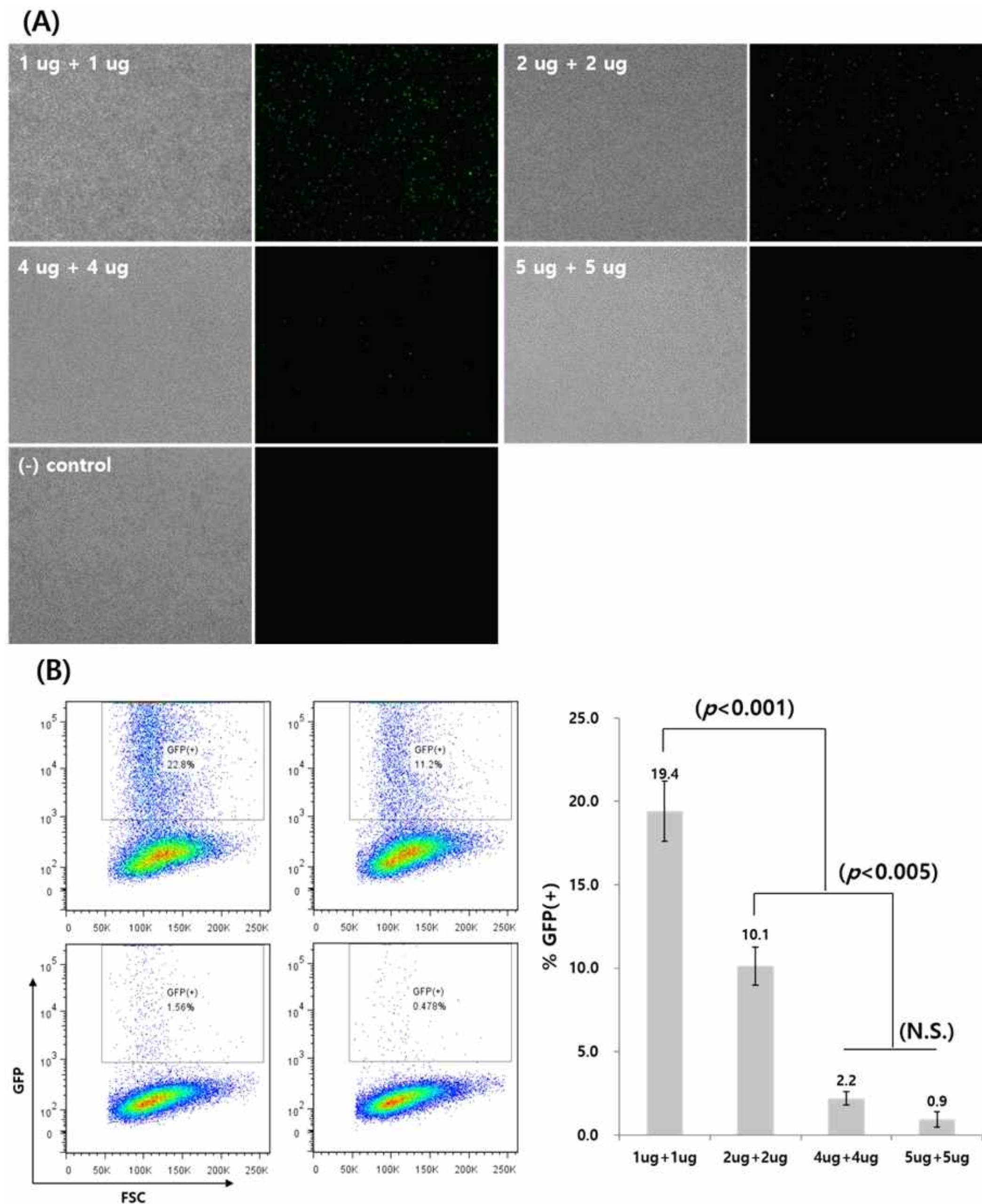

Fig. 1. (A) GFP expression in quail blood cells isolated from blood vessel of 50-hr-old embryos after lipofection with the different concentrations of *piggy*BacCMV-GFP and transposase DNAs (magnification; 10×). (B) Comparison of lipofection efficiencies in quail blood cells with the different amounts of plasmid DNAs by flow analysis. The ratio of *piggy*BacCMV-GFP and transposase plasmid was 1 : 1 (w : w) in all treatments. Three separate experiments were replicated and statistically analysed ($p<0.005$) (NS; no significant difference).

hr. However, in the absence of the transposase, *piggyBac*-CMVGFP transgene expression gradually disappeared at late developmental stages suggesting that *piggyBac* transposon and transposase played a crucial role in the constant transgene expression as well as the stable genomic integration in quail.

Transgenic animals have been used as a model animal for studying development biology and drug evaluation (Park and Han, 2012). Particularly, transgenic birds embody one of the most potent research tools in biotechnology for agriculture, medicine and animal model (Park and Han, 2012). In despite

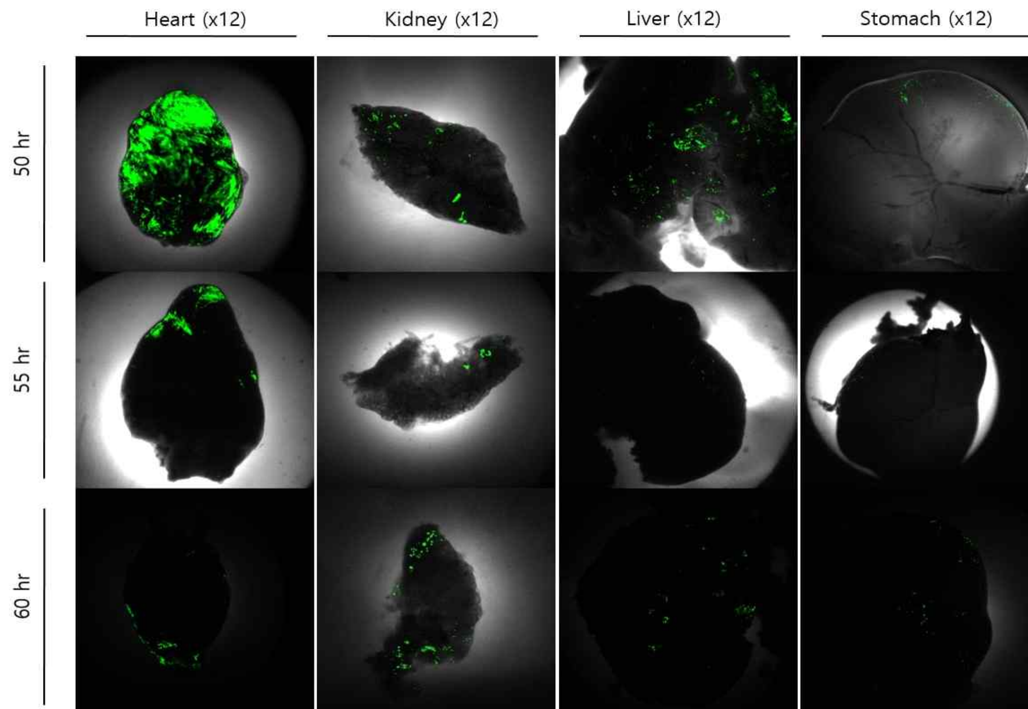

Fig. 2. GFP detection in quail tissues after direct *in ovo* lipofection (DiL). Mixture of *piggy*BacCMV-GFP and transposase plasmid with liposome was transferred into blood vessel at 50-hr-old quail embryos. After injection of DNA-liposome complex, the GFP expression was detected in heart, kidney, liver and stomach at 12 days of incubation under a confocal laser scanning microscope.

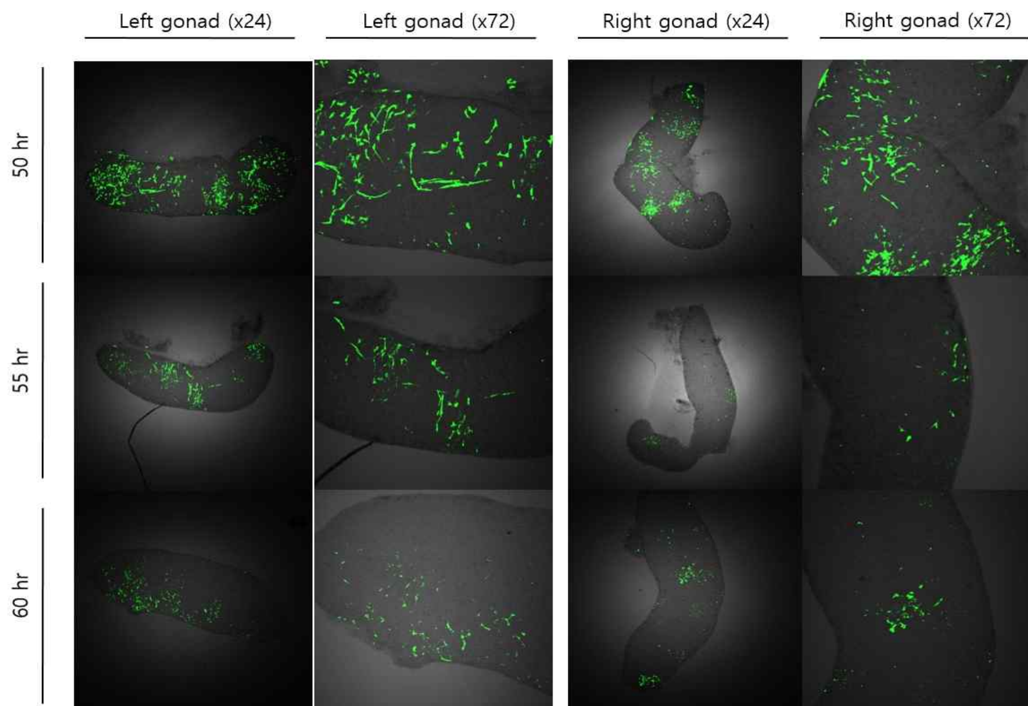

Fig. 3. GFP detection in quail tissues after direct *in ovo* lipofection (DiL). Mixture of *piggy*BacCMV-GFP and transposase plasmid with liposome was transferred into blood vessel at 50-hr-old quail embryos. After injection of DNA-liposome complex, the GFP expression was detected in recipient embryonic gonads at 12 days of incubation under a confocal laser scanning microscope.

of many advantages of transgenic quail as a model animal, the uses of transgenic quail have a lot of limitations due to the difficulty to generate transgenic lines. Recently, Scott et al. (2013) reported on production of transgenic chickens through stably transformed primordial germ cells induced by a direct *in ovo* lipofection with Tol2 transposon and transposase. In conclusion, combined with the direct *in ovo* lipofection strategy with *piggyBac* transposon could be easily applicable and an efficiently useful tool to generate transgenic quail.

(색인어 : 형질전환, 메추리, *in ovo* 주입법, 피기백 트랜스포존, 외래유전자 발현)

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REFERENCES

Agate RJ, Scott BB, Haripal B, Lois C, Nottebohm F 2009 Transgenic songbirds offer an opportunity to develop a genetic model for vocal learning. Proc Natl Acad Sci. 106: 17963-17967.

Carsience RS, Clark ME, Verrinder Gibbins AM, Etches RJ 1993 Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development. 117:669-675.

Choi JW, Kim S, Kim TM, Kim YM, Seo HW, Park TS, Jeong JW, Song G, Han JY 2010 Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. PLoS One. 5:e12968.

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

Kwon SC, Choi JW, Jang HJ, Shin SS, Lee SK, Park TS, Choi IY, Lee GS, Song G, Han JY 2010 Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. Biol Reprod. 82:1057-1064.

Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM 2007 Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci. 104:1771-176.

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

Macdonald J, Glover JD, Taylor L, Sang HM, McGrew MJ 2010 Characterisation and germline transmission of cultured avian primordial germ cells. PLoS One. 5:e15518.

Macdonald J, Taylor L, Sherman A, Kawakami K, Takahashi Y, Sang HM, McGrew MJ 2012 Efficient genetic modification and germ-line transmission of primordial germ cells using *piggyBac* and Tol2 transposons. Proc Natl Acad Sci. 109:E1466–E1472.

Mizuarai S, Ono K, Yamaguchi K, Nishijima K, Kamihira M, Iijima S 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.

Naito M, Tajima A, Yasuda Y, Kuwana T 1994 Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Mol Reprod Dev. 39:153-161.

Park TS, Han JY 2012 Genetic modification of chicken germ cells. Ann NY Acad Sci. 71:104-109.

Park TS, Han JY 2012 *piggyBac* transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci. 109:9337-9341.

Park TS, Jeong DK, Kim JN, Song GH, Hong YH, Lim JM, Han JY 2002 Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. Biol Reprod. 68:1657-1662.

Petitte JN, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ 1990 Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development. 108:185-189.

Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB 1987 Transgenic chickens: Insertion of retroviral genes into the chicken germ line. Virology. 157:236-240.

Sato Y, Poynter G, Huss D, Filla MB, Czirok A, Rongish BJ,

Little CD, Fraser SE, Lansford R 2010 Dynamic analysis of vascular morphogenesis using transgenic quail embryos. PLoS One. 5:e12674.

Tyack SG, Jenkins KA, O'Neil TE, Wise TG, Morris KR, Bruce MP, McLeod S, Wade AJ, McKay J, Moore RJ, Schat KA, Lowenthal JW, Doran TJ. 2013 A new method for producing transgenic birds via direct *in vivo* transfection of primordial germ cells. Transgenic Res. 22:1257-1264.

Shin SS, Kim TM, Kim SY, Kim TW, Seo HW, Lee SK, Kwon SC, Lee GS, Kim H, Lim JM, Han JY 2008 Generation of transgenic quail through germ cell-mediated germline transmission. FASEB J. 22:2435-2444.

Tajima A, Naito M, Yasuda Y, Kuwana T 1993 Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (*Gallus domesticus*). Theriogenology. 40:509-519.

van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ 2006 van de Lavoir MC, et al. (2006) Germline transmission of genetically modified primordial germ cells. Nature. 441:766-769.

Zhu L, van de Lavoir MC, Albanese J, Beenhouwer DO, Cardarelli PM, Cuison S, Deng DF, Deshpande S, Diamond JH, Green L, Halk EL, Heyer BS, Kay RM, Kerchner A, Leighton PA, Mather CM, Morrison SL, Nikolov ZL, Passmore DB, Pradas-Monne A, Preston BT, Rangan VS, Shi M, Srinivasan M, White SG, Winters-Digiacinto P, Wong S, Zhou W, Etches RJ 2005 Production of human monoclonal antibody in eggs of chimeric chickens. Nat Biotechnol. 23:1159-1169.

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