A Thesis for the Degree of Master of Science

A study of expression of avidin and cystatin in chicken cells mediated by *piggyBac* transposition system

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SUMMARY

Transgenic chicken is an irreplaceable model animal as a bioreactor because of the unique physiological characteristics which differs from mammals. Modern layer hens produce more than 330 eggs per years, which contains 6.5g of proteins. More significant advantage of the use of chicken as bioreactors, in comparison with other mammalian species, is the relatively short generation time of approximately 20 weeks. Thus, the transgenic chicken line can be built up within a comparatively shorter time. Moreover, simple composition of the egg white proteins offers additional merits on the purification process. To take advantage of using chicken as a bioreactor, development of efficient genetic modification technology in chicken system should be preceded for further improvement on avian bioreactor. As an emerging safety issues, non-viral system to produce transgenic chicken has become important.

Biologically active substances contained in egg white have drawn much attention of researchers. Avidin and cystatin are trace components of egg white, in which each makes up 0.05% of the total egg white protein. Both proteins are widely used in the fields of biotechnology and clinical researches; avidin mainly

for avidin-biotin technology and cystatin mainly for disease treatment and diagnostics, however, the low contents of avidin and cystatin in natural resources limit their applications. For commercial use, two proteins have been produced from diverse bioreactor systems, including *E.coli, Pichia Pastoris* and human cell lines. However, the safety issues and cost-effectiveness hinder practical applications of avidin and cystatin. Thus, in this study, we generate founders of transgenic chicken to produce more avidin and cystatin C with the non-viral transposon method.

For efficient gene insertion into the chicken genome, *piggyBac* transposon vector was modified to overexpress chicken avidin, human cystatin C and chicken cystatin. For higher expression, self-cleaving 2A peptide were inserted between two coding sequences (CDS) of each genes. These expression vectors were expected to produce more protein than expression vectors with one copy of genes. Particularly, codon of human cystatin with 2A peptide gene was optimized for expression in the hen. To confirm the working of constructed vectors, we transfected DF-1 chicken fibroblast cell lines with 6 types of vectors, respectively. After transfection, DF-1 cells were selected with G418 for up to 4 weeks, and RT-PCR for each cell lines were followed. As a result, we verified that the *piggyBac*

transposon system is able to introduce transgenes into chicken genome effectively.

After confirming successful transgene integration into chicken genome using *piggyBac* system, transgenes were introduced into chicken primordial germ cell (PGC), which is the precursor of functional gamete. Selection with G418 and RT-PCR were performed in the same manner as in DF-1 cell line to demonstrate integration and expression of chicken avidin, human cystatin, and chicken cystatin, respectively. Additionally, RT-PCR analysis was conducted to examine whether G418-selected PGCs preserved the germ-cell properties. The result indicated that G418-selected chicken PGCs expressed transgene without losing germ cell characteristics.

For generating transgenic chicken, transgene-expressing PGCs were transplanted into Korean Oge chicken (KO) recipient embryonic blood vessels. After hatching and sexual maturation, we collected sperm from male founders, and conducted PCR to detect donor PGCs-derived sperm in male founders. From the result of PCR, we detected a total of 17 founders. However, until now, none of founders was confirmed as a germline chimera, which can produce transgenic offspring, via testcross analysis.

In our research, founders for transgenic chicken expressing avidin and cystatin were generated using non-viral *piggyBac* transposon system. This research can contribute to the studies on avian transgenesis, and provide efficient production system for various biomaterials to overcome the limitations of the conventional system.

Key words: *piggyBac*, transgenic chicken, avidin, cystatin

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LIST OF ABBRIVIATION

chAVD : Chicken avidin

chCST : Chicken cystatin

hCST : Human cystatin C

CDS : Coding sequence

CMV : Cytomegalovirus

TK : Thymidine kinase

PGC : Primordial germ cell

WL : White Leghorn

KO : Korean Oge chicken

DMEM : Dulbecco's modified eagle medium

FBS : Fetal bovine serum

PBTR : *piggyBac* terminal DNA repeat

FRT : flippase recognition target

DAZL : Deleted in azoospermia-like

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

RT-PCR : Reverse transcription PCR

INTRODUCTION

Transgenic animal bioreactors have held great promise for revolutionizing the manufacture of human biopharmaceuticals (Houdebine, 2000; Kues and Niemann, 2004; Rudolph, 1999). To date, transgenic livestock bioreactors such as the mammary glands of goat, sheep and cattle have been developed for producing biopharmaceuticals in milk (Melo et al., 2007), and this system is now considered for practical production of pharmaceuticals (Houdebine, 2009). Nonetheless, mammalian bioreactors have several drawbacks, including long generation time, large area requirements, and protein purification difficulties (Dyck et al., 2003; Whitelaw et al., 2004). Compared with mammalian systems, bioreactors using avian species may offer several advantages, such as short generation time, high yield of protein in eggs and similar protein glycosylation to humans (Lillico et al., 2007; Raju et al., 2000; Rapp et al., 2003).

In avian species, however, it is hard to manipulate embryos due to their unique physiological characteristics which differs from the mammalian species. The first report on the production of transgenic chicken adapted technique developed from mammals (Love et al., 1994), but it showed very low efficiency. To generate transgenic birds, manipulation of the blastoderm of fertilized eggs has been attempted in efforts to improve the efficacy of transgenesis in avian species (McGrew

et al., 2004; Salter et al., 1986; Zhu et al., 2005). Recently, a number of groups have focused on primordial germ cells (PGCs) as the vehicle for producing germline chimeras and transgenesis, instead of blastodermal cells. Most recently, using advanced culture systems for chicken PGCs, non-viral methods for transgene insertion employing *piggyBac* and Tol2 transposon elements have been developed (Macdonald et al., 2012; Park and Han, 2012). These non-viral strategies are highly essential for the safety issues in industrial applications, and have additional advantages including high efficiency, multiple-insertion, and stable integration of large transgenes (Ding et al., 2005). With the development of techniques for avian transgenesis, a number of groups have reported production of various recombinant proteins using transgenic chickens as bioreactors, including human monoclonal antibody (Zhu et al., 2005), hEPO (Penno et al., 2010), and most recently hEGF (Park et al., 2015)

In the last decade, biologically active substances in eggs have been extensively conducted to seek novel substances. As the hen's egg is the largest biological cell originating from one cell division, it contains all important source of nutrient required by the developing embryo, as well as defense factors against bacterial and viral infection. The role of these biologically active substances in human health has been studied, and diverse applications of these components were extensively developed

(Mine and Kovacs-Nolan, 2004). Avidin and cystatin are one of the biologically active components, which are naturally found in the egg white with extremely low concentration, and they have been widely applied in the fields of biotechnology and clinical researches. For large-scale production of avidin and cystatin, several systems have been developed, including E. coli (Airenne et al., 1994), Pichia (Zocchi et al., 2003), and human cell lines (Chen et al., 2012). However, because of the safety issues and cost-effectiveness of conventional systems, a novel production system for avidin and cystatin is highly required.

In this study, we used non-viral *piggyBac* transposon system to introduce chicken avidin (chAVD), human cystatin C (hCST) and chicken cystatin (chCST) gene into chicken genome. Consequently, we generated male founders, which contain sperm overexpressing chicken avidin (chAVD), human cystatin C (hCST) and chicken cystatin (chCST). Our results suggest that the non-viral *piggyBac* transposon is adaptable to generate transgenic chicken and indicate the feasibility of transgenic chicken as a bioreactor for producing diverse biomaterials.

LITERATURE RIVEW

1. Recent technology for transgenic chicken production

The technology to create transgenic animals has been improved in the fields of life science, pharmacology, biomedical engineering, and agricultural science. Transgenic animals have great deal of advantages for producing model animals, bioreactors, and xenotransplantation donors. To produce transgenic animals, the first attempt focused on pronucleus injection method in mammals. During the last decade, avian species have become a major target for animal transgenesis due to their significant industrial merits.

In avian species, however, it is hard to introduce transgenes into the pronucleus because of their unique physiological characteristics which are much different from mammals. Therefore, specialized methods for the chicken have been developed. Blastodermal cells at Stage X (EG&K) had been used to produce genetically modified chicken (Petitte et al., 1990). However, it showed relatively low germline transmission rate, and also very low production efficiency of transgenic progenies.

A number of group focused on PGCs as the vehicle for producing germline chimeras, instead of blastodermal cells. In

avian species, PGCs arise from the epiblast and migrate to the germinal crescent at stage 4, then move into the blood stream and finally migrate into the genital ridges (Swift, 1914). The unique migration patterns of PGCs through the circulatory system facilitates the production of germline chimeras via the injection of donor PGCs into the blood vessels of recipients (Tajima et al., 1993). Interestingly, only chicken PGCs have been maintained continuously without loss of germ-cell competency, whereas *in vitro* long-term culture system for PGCs remains to be established in mammals. The first study of culture and germ-line transmission of chicken using PGCs was published in 2006, which accelerated further researches on the transgenic chicken (Van de Lavoir et al., 2006). Germ-line chimeras are produced by collecting PGCs from the blood vessels of chick embryo at H&H stage 14~17. Collected PGCs are cultured *in vitro*, and confirmed with germ cell markers such as DAZL and VASA. However, not all PGC cell lines showed efficient germ-line transmission rates. As a further study, PGCs were transfected *in vitro* with beta-actin promoter and enhanced GFP (eGFP) gene. The GFP expression was observed in progeny embryos, but the transgene integration rate was still low.

To overcome the low efficiency of blood PGCs-mediated germline chimera production, PGCs were collected from embryonic gonads instead of the blood vessels (Park et al.,

2003). Germline chimeras have subsequently been produced by the transfer of gonadal PGCs retrieved from 5.5day-old embryos, and the generation of transgenic chicken has been followed with higher efficiency. However, the concerns about efficiency of genome transgene integration in PGCs still remain.

In 2012, two groups reported the generation of PGC-mediated transgenic chicken by non-viral integration systems (Macdonald et al., 2012; Park and Han, 2012). Both groups used transposon and transposase, and the result showed an average of 95.2% germline transmission efficiency with constant and strong GFP expression when *piggyBac* transposon and transposase system was applied (Park and Han, 2012). Subsequently, half of the donor-derived offspring were transgenic chicks because of the heterozygosity. Now with the *piggyBac* transposon and transposase system, we can efficiently overexpress transgene and additional endogenous gene to produce pharmaceuticals from eggs. To fully make use of chicken as a bioreactor system, transgenic chickens producing biologically active substances is highly required.

2. Transgenic chicken as a bioreactor

Since the first transgenic mice were produced in 1984, the demands of transgenic animals have increased steadily. Various researches have been performed to generate transgenic animals for a wide range of purposes; one of them is production of pharmaceuticals. The idea has been mainly focused on production of pharmaceuticals in the mammary gland of cattle, goats and sheep. These bioreactor systems are proposed to be more cost-effective than large-scale bacterial culture or cell culture. In 2009, antithrombin III produced from transgenic goats firstly obtained the license for application from FDA. In these days, a number of studies have been actively performed to produce high-value products using bioreactor animals.

Chicken also has been widely investigated as a bioreactor for commercial use because of its unique reproductive system which is different from mammals. The advantages of the hen as a bioreactor is clear. Chicken has shorter generation time, approximately 5 months, and high reproductive potential than that of other livestock species, which enable us to establish the transgenic chicken lines rapidly. Furthermore, the production of pharmaceuticals in the eggs of transgenic hens would be highly cost-effective because one hen can lay at least 330 eggs per year, which contains 6.5g of protein on average. Most

importantly, the composition of egg white proteins is relatively simple which offers distinct advantage on cost-effective purification. In addition, the post-translational modifications of protein in birds such as glycosylation resembles those in humans (Raju et al., 2000). For these reasons, chickens have been regarded as a fine bioreactor system for large-scale production of medical proteins.

As chicken has become a promising model animal to produce biopharmaceuticals compared to mammalian species, various attempts have been made to produce functionally active proteins in eggs. Since beta-galactosidase and beta-lactamase were successfully produced in transgenic chicken (Harvey et al., 2002), further researches have been performed to utilize chicken as a bioreactor to produce pharmaceuticals. In 2003, human interferon-alpha-2b, which is widely used for human cancer treatment has been produced in eggs (Rapp et al., 2003). In 2005, human monoclonal antibody (mAb) - to prostate-specific antigen - has been produced more than 3g in the egg white of transgenic hens using the promoter of OV gene (Zhu et al., 2005).This study has shed light on the expression elements of OV gene and their ability to drive high level of protein production in eggs. Around the same period, production of human antiprion single-chain antibody has been reported (Kamihira et al., 2005), and production of miR24, GCSF and human FSH has

followed (Harel-Markowitz et al., 2009; Kwon et al., 2008; Lillico et al., 2007). Recently, generation of transgenic chicken have been reported, which produces human EPO/Fc protein in their eggs (Penno et al., 2010). Most of the studies, however, are based on virus-mediated gene transduction systems which have safety issues. Therefore, production of transgenic chicken to produce pharmaceuticals using non-viral system is highly required.

3. Chicken Avidin

3.1 Basic characteristics of chicken avidin

Avidin is a tetrameric glycoprotein which is naturally found in the oviducts of oviparous vertebrates including birds, reptiles and amphibians, and is deposited in the egg white of them as a trace component. No analogous protein has been reported in mammalian species. In 1940, avidin was first isolated from chicken egg white (Thompson, 1940). It was demonstrated that avidin comprises four identical subunits of 15.6kDa and 128 amino acids long in 1971 (Huang and DeLange, 1971). Soon after, the cDNA of the chicken oviduct avidin gene was documented (Gope et al., 1987) and a genomic clone was isolated (Keinänen et al., 1988).

The main function of avidin is to bind biotin which is small soluble vitamin H, essential to life as it functions as an enzymatic cofactor in metabolic reactions (Green, 1990). Each subunits of avidin, a homotetramer, can bind one molecule of biotin; thus a total of four biotin molecules can bind to a single avidin molecule. In nature, avidin can function as a defense agent against microbial pathogens, which require biotin for their survival (Wallén et al., 1995). Avidin possesses antimicrobial activity against biotin-requiring bacteria and yeasts (Green, 1974). The antimicrobial activity of avidin has also inhibited various bacteria,

including *Escherichia coli, Staphylococcus aureus,* and *Staphylococcus epidermis* (Korpela et al., 1984).

As avidin binds biotin with extremely high affinity and specificity (Kd $10^{13} - 10^{15}/M$) (Green, 1963), avidin–biotin system has been established and widely used as probes and affinity matrices without compromising the activity of the target in numerous studies, including cancer treatment and pre–targeting (Weber et al., 1989). Because of its high biotin binding and signal amplification due to the tetrameric structure, avidin can used for increased persistence of biotinylated anti–cancer drugs (Hytonen et al., 2003). Moreover, radiolabelled avidin can bind to lectins of tumor cells and localized rapidly in diverse types of tumors in mice, which makes avidin a useful tool for tumor pre–targeting (Yao et al., 1998). Avidin can also be utilized for affinity purification of biotinylated molecules (Green and Toms, 1973). Moreover, avidin is an exceptionally highly stable molecule against harsh conditions including high concentrations of protease and a wide range of pH and temperature (Green, 1990). Therefore, avidin–biotin complex shows extremely strong binding affinity with high stability, and it is exploited in a wide range of biochemical assays, involving western blot, ELISA, and ELISPOT.

Streptavidin, secreted by Streptomyces, is known as functionally and structurally analogous protein of chicken avidin,

isolated from the egg white (Livnah et al., 1993; Weber et al., 1989). Avidin, however, shows different characteristics from streptavidin, including primary amino acid sequence, glycosylation pattern and immunological reactivity (Green, 1990). In case of avidin, a single oligosaccharide is found in avidin monomer, whereas no oligosaccharide is found in streptavidin, which means they differ in glycosylation patterns. Moreover, the isoelectric point (pI) of avidin and streptavidin is different, which makes avidin a basic protein (pI 10.5) and streptavidin an acidic protein (pI 6) (Rosebrough, 1993). From this differences, avidin shows relatively higher nonspecific binding property to negatively charged molecules and shorter plasma half-life period than streptavidin (SCHECHTER et al., 1990). The different glycosylation patterns of avidin and streptavidin also determine the tendency to accumulate in the liver. Despite higher binding specificity and longer half-life period of streptavidin, avidin has a much lower immunogenicity than streptavidin, thus being more relevant for biological applications (Chinol et al., 1998; Marshall et al., 1996).

3.2 Production systems of chicken avidin

Chicken egg white, where the average concentration of avidin is 1.5 mg is the primary source for commercial production of avidin. Alternative expression systems including cytoplasmic

expression in *E. coli* were developed, but they showed low yield of the soluble protein (Airenne et al., 1994). Recently, eukaryotic production expression systems, which produce biologically active recombinant avidin have been developed, involving *Baculovirus*–infected insect cells (Airenne et al., 1997), *Pichia* (Zocchi et al., 2003), and maize (Kusnadi et al., 1998). Despite the diverse development of production systems, most of the previously developed systems have limitation for commercial applications. To culture insect cells, relatively expensive and special culture medium is constantly required. Meanwhile, the generation and establishment of recombinant corn are time consuming and laborious, which has limitation in practical use. More recently, improved production system using a bacterial signal peptide to produce active chicken avidin in *E. Coli* has been demonstrated (Hytonen et al., 2004). The use of *E. coli* for protein production, however, may cause aggregation in inclusion body and chronic toxicity. Hence, a safe, efficient, and modifiable eukaryotic expression system for production of avidin is highly desirable.

4. Human cystatin C and chicken cystatin

4.1 Basic characteristics of cystatin

Chicken cystatin is a protein size of 13kDa and is a well-known inhibitor of cysteine proteases, which is part of a superfamily of cystatins and belongs to the Type 2 cystatin (Abrahamson et al., 1988). It has been firstly isolated and characterized from chicken egg white (Fossum and Whitaker, 1968). From a series of studies, chicken cystatin has shown high affinity to various papain-like proteinases, including papain (Fossum and Whitaker, 1968), ficin (Sen and Whitaker, 1973) and cathepsin B, L, H (Anastasi et al., 1983) and cathepsin C (Turk et al., 1996). The following study has elucidated the mechanism of interaction between chicken cystatin and papain through N-terminally truncated forms and cyanogen bromide fragments of chicken cystatin (Machleidt et al., 1989).

On the other hand, human cystatin C, which is human analogue of chicken cystatin, has been isolated and characterized in 1961 when Jorgen Clausen described the occurrence in human cerebrospinal fluid (CSF) of a "cerebrospinal fluid-specific" protein, which he named γ-CSF (Clausen, 1961). Later, the complete amino acid sequence was determined (Grubb and Löfberg, 1982). Soon after, structural differences between human and chicken cystatin were confirmed, showing that two

proteins had a sequence identity of 44% (MACHLEIDT et al., 1983). Further determination of the structure of the human cystatin C gene and its promoter has demonstrated that the gene is of the house-keeping type, which indicates a stable production rate of cystatin C by most nucleated cell types at a constant rate (Abrahamson et al., 1990). Its main function is to regulate the activity of lysosomal cysteine proteases secreted from either lysozymes of damaged cells or invading microbes (Heidtmann et al., 1997).

4.2 Pathophysiology and applications of Cystatin

Several researches have focused on the biological functions of cystatin and pointed to multiple functions of cystatin in controlling extracellular proteolysis. Chicken cystatin and human cystatin C mainly inhibit cathepsin B, H, L and S, so that they play a key function in protection against tissue damage which is caused by cysteine peptidases. Further studies have proposed the role of chicken cystatin and human cystatin C in a number of diseases associated with alterations of the proteolytic system, such as cancer (Kyhse-Andersen et al., 1994), inflammatory diseases (Buttle et al., 1991), osteoporosis, diabetes, periodontal disease (Skalerič et al., 1989), multiple sclerosis , renal failure (Brzin et al., 1984), autoimmune diseases

and HIV infection (Cattaneo et al., 1986). Collectively, the pathophysiological roles of chicken cystatin and human cystatin C can be generally categorized into five types: 1) inhibition tumorigenesis; 2) control of the immune system; 3) bone formation; 4) antibacterial/antiviral activities; 5) and response to injury in the brain

Several proteases, including cysteine cathepsins, play role in degradation of extracellular matrix, facilitating the growth, invasion, metastasis and angiogenesis of tumor cells (Gocheva and Joyce, 2007; Joyce et al., 2004; Mohamed and Sloane, 2006). Recently, increased expression of cystatins down-regulate the activity of cathepsin, which inhibit the growth of tumor cells (Sokol and Schiemann, 2004). In case of viral infection for overexpression of cystatin C, it has been found to reduce lung metastasis (Kopitz et al., 2005) and inhibit TGF-β signaling in two tumorigenic human breast cancer cell lines (Sokol et al., 2005). Moreover, the overexpression of cystatin in glioblastoma cells also has reported significant decrease in tumor growth in nude mice (Konduri et al., 2002). Additionally, the level of cystatin C from patients suffering from colorectal cancer correlates with high levels of extracellular inhibitors (Kos et al., 2000). One of the most promising advantages of cystatin is that

it has less intensive side effects than other synthetic protease inhibitors currently used in medical treatments (Nakai, 2000).

Cystatin C also takes part in the immune system, which is thought to interfere with the phagocytic function and oxidative burst of neutrophils and monocytes (Leung-Tack et al., 1990). Although diverse researches have been conducted to determine the role of cystatin C in the immune system, it has not been fully understood yet. One of the results has shown that cystatin C plays role in MHC class II-mediated antigen presentation by controlling the degradation of the invariant chain, in which cathepsin S is involved (Pierre and Mellman, 1998). It has also been demonstrated that cystatin induced the synthesis of various cytokines and reduced the number of parasites in a mouse model of visceral leishmaniasis (Das et al., 2001).

Furthermore, cystatin C has been revealed to significantly inhibit release of calcium from bone, whereas it stimulates bone cell proliferation and matrix protein biosynthesis (Lerner et al., 1997). Recent study has demonstrated that cystatin C promotes osteoblast differentiation, mineralization, and bone formation via affecting the BMP signaling cascades in osteoblastic cells (Danjo et al., 2007).

In case of viral or bacterial attack, Cystatin C inhibits intracellular cysteine proteases of the damaged cells. Since

cysteine proteases are necessary for viral replication such as capsid formation, cystatin C plays a critical role in antibacterial and antiviral activities. The inhibitory function of chicken cystatin and human cystatin C have been demonstrated to effectively inhibit the replication of *Poliovirus* (Korant et al., 1986) and *Coronavirus* (Jormsjö et al., 2002), respectively.

The last pathophysiological role of cystatin is that it responds to injury in the brain, though its functional mechanism is not fully determined. Alzheimer's disease and many other neurodegenerative diseases are well known to be associated with the accumulation of abnormal protein in the neuronal cells. Human cystatin C is deposited in the amyloid plaque of Alzheimer's' disease or Down's syndrome. The recent study of human cystatin C correlates with soluble amyloid-beta peptide and inhibits the deposition of amyloid in amyloid-beta precursor protein, thus considerably reducing amyloid-beta deposition (Kaeser et al., 2007; Mi et al., 2007). Therefore, cystatin C has been proposed as a protectant for the pathogenesis neurodegenerative diseases, including Alzheimer's disease.

For these reasons, it is reasonable that cystatin C is used as a therapeutic protein for disease treatment in clinical practice,

including allergic diseases, and neurodegenerative disease and bone diseases.

Cystatin C is also available as a biomarker for early diagnosis and prognosis as well as a therapeutic protein. As cystatin C is freely filtered by the glomerulus and largely resorbed in proximal tubules, its concentration in serum is a good measure of glomerulus filtration rate (GFR) (Tenstad et al., 1996). GFR is the most important measure of the renal activity for clearing body wastes from the blood. Therefore, cystatin C is widely used as a biomarker for systematic evaluation of various drugs and their dosage adjustment. Moreover, the decrease in GFR indicates progressive kidney disease, therefore GFR is critical in the prevention and management of chronic kidney diseases. Cystatin C has been proposed as a more sensitive estimator of GFR than serum creatinine which is a common marker of GFR, due to its capability to detect early renal failure (Newman et al., 1995).

4.3 Production systems of cystatin C

The greatest problem in utilizing egg cystatins for medical treatments is their high cost about 140 $ USA dollar for 1 mg pure cystatin (Trziszka et al., 2004). Because the

extremely low contents of cystatins in natural resources such as eggs may limit their applications (Nakai, 2000), alternative production system has been developed. As a first step, recombinant cystatin C has been expressed in *E. coli* expression system. However, relatively high product loss during purification is expected because of inclusion body formation and intracellular expression in *E. coli* (Abrahamson et al., 1988). Recently, eukaryotic production systems, which produce biologically active recombinant human cystatin have been developed, involving *Pichia Pastoris* (Files et al., 2001) and human cell lines (Chen et al., 2012). However, these systems also have some drawbacks as poor secretion which results in high costs.

MATERIALS AND METHODS

Experimental animals and animal care

The experimental use of chickens was approved by Seoul National University Institutional Animal Care and Use Committees. Chickens were maintained according to a standard management program of the Seoul National University Animal Farm, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Construction of the expression vector

To construct 1XchAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST expression vectors, previously reported *piggyBac* transposon vector were modified (Park and Han, 2012). The 5′ FRT sequence was inserted between the 5'(PBTR) and TK promoter of the *piggyBac* transposon vector, and the 3′ FRT sequence was inserted between poly-A tail sequence of CMV-GFP and 3'(PBTR).

CDS of 1XchAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST, and 2XchCST gene were synthesized by Bioneer Company (Daejeon, Korea). Especially, codons of the 2XhCST gene were optimized for expression in chicken. To allow doubled

production of each proteins in 2XchAVD, 2XhCST, and 2XchCST expression vectors, self-cleaving 2A peptide sequence (5′-AACGTGAGAAGAAAGAGATACGCCGGCTACTTCGCCGATCTGCTGATCCACGATATCGAGACCAACCCCGGCCCC-3′) was placed between two CDS of each gene (Figure 1B). Subsequently, the synthesized 1XchAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST genes were ligated into *piggyBac* transposon vector, replacing the CDS of enhanced green fluorescent protein (eGFP) in original *piggyBac* transposon vector (Figure 1).

Culture of DF-1 cell line

Chicken DF-1 fibroblast cell line was maintained and subcultured with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) (Hyclone, Utah, USA), and 1X Antibiotic-Antimycotic (Gibco, CA, USA). DF-1 cells were cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60-70% relative humidity.

Culture of the chicken gonadal PGC line

Primordial germ cells from White Leghorn chicken embryonic gonads at day 6 (stage 28) were maintained and

subcultured with knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 20% (vol/vol) Fetal Bovine Serum (FBS, Hyclone, Utah, USA), 2% (vol/vol) chicken serum (Sigma-Aldrich, St.Louise, MO, USA), 1×nucleosides (Millipore, Bedford, MA, USA), 2mM L-glutamine (Gibco), 1× non-essential amino acids (Gibco), β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco), and 1× Antibiotic-Antimycotic (Gibco, CA, USA). Human bFGF (10 ng/mL; Koma Biotech, Seoul, Korea) was used for PGC self-renewal. Chicken PGCs were cultured in an incubator at 37℃ with an atmosphere of 5% CO2 and 60-70% relative humidity. The cultured PGCs were subcultured onto mitomycin-inactivated MEFs in 5- to 6-day intervals by gentle pipetting without any enzyme treatment (Park and Han, 2012).

In vitro Transfection and G418-Selection

All transfection and selection procedures followed those established in our previous report. Each *piggyBac* 1XAVD, 2XAVD, 1XchCST, 2XchCST, 1XhCST and 2XhCST vector and GAGG-PBase (pCyL43B) were co-introduced with the ratio of 7:3 (7 μg : 3 μg = *piggyBac* transposon vector : pCyL43B) into the DF-1 cell line and SNUhp26 PGC cell line, respectively, by lipofection with Lipofectamine 2000 reagent (Invitrogen, Life Technologies). At one day after transfection, 300 μg/mL G418

was added to the culture media for selection, and cell lines were selected for up to 4 weeks.

Detection and Characterization Following G418-Selection

The expression of chAVD, hCST, and chCST genes driven by CMV promoter in G418-selected DF-1 cell line and PGCs was detected by RT-PCR analysis. RNA of cultured cells was isolated using TRIZOL (Invitrogen, Life Technologies). Subsequently, we used Superscript III® First Strand Kit (Invitrogen, Life Technologies) to synthesize cDNA of each RNA. 1XAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST expression vectors were used as a positive control, respectively, and wild-type DF-1 cells and PGCs genomic DNA were used as a negative control. Primers are listed in Table 1.

In addition, RT-PCR analysis was conducted to examine whether G418-selected PGCs harbor the germ-cell properties. The information of transgene-specific, germness related genes (*VASA* and *DAZL*) and stemness related genes (*POUV* and *NANOG*) primers are listed in Table 1.

Microinjection of G418-selected PGCs into Recipients.

To inject selected transfected PGCs into recipient embryos, a small window was made on the pointed end of the

recipient Korean Oge chicken (KO) egg and a 2-μL aliquot containing more than 3,000 PGCs was microinjected via a micropipette into the dorsal aorta of the stage 14-17 recipient embryo. Each egg window of the recipient embryo was sealed with paraffin film, and the eggs were incubated with the pointed end down until further screening and hatching.

Detection of donor-PGC derived sperm in the sperm of founder roosters.

After sexual maturation, the sperm of each founder were collected for genomic DNA analysis. Genomic DNA was isolated from the sperm, and analyzed to detect donor-PGC (White Leghorn, WL) derived sperm, using specific primers listed in Table 1. 1XAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST expression vectors were used as a positive control, respectively, and wild-type KO genomic DNA was used as a negative control. In addition, we estimated germ cell chimerism in sperm of transgenic founder chickens by qRT-PCR analysis with WL and KO specific primers.

Testcross analysis and detection of mutant chickens

WLs with dominant pigmentation inhibitor gene (*I/I*) and black KOs with a recessive pigmentation inhibitor gene (*i/i*) were used to obtain donor PGCs and recipient embryos, respectively.

Through testcross analysis by mating with WL hens (*I/I*), the germ-line chimeras were identified by offspring phenotype. Endogenous germ cells in the KO recipient males (*i/i*) produced hybrid chicks (*I/i*) with black spots, whereas WL donor-derived germ cells (*I/I*) produced white chicks with (*I/I*). Offspring derived from the transplanted mutant PGCs were screened by PCR analysis. 1XAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST expression vectors were used as a positive control, respectively, and wild-type Korean WL genomic DNA was used as a negative control.

Table 1. Primer sequences for RT-PCR and qRT-PCR.

Target	Primer sequence (5` → 3`)
chAVD F	AAGCTTGCCACCATGGTGCACGCAACCTCC
chAVD R	GCGGCCGCTCACTCCTTCTGTGTGCGCA
hCST F	AAGCTTGCCACCATGGCCGGACCCCTGAGA
hCST R	GCGGCCGCCTAGGCATCCTGGCAGGTGC
chCST F	AAGCTTGCCACCATGGCGGCAGCGGCGTGT
chCST R	GCGGCCGCTTACTGGCACTTGCTTTCCAGCA
hCST F	AAGCTTGCCACCATGGCCGGACCCCTGAGA
hCST R	GCGGCCGCCTAGGCATCCTGGCAGGTGC
CMV F	CAAATGGGCGGTAGGCGTGT
2A R	GTGGATCAGCAGATCGGCGA
VASA F	GCAGACCAAACAGCCCCTTC
VASA R	TTAAATCCCGCCCTGCTTGT
DAZL F	TCGTCAACAACCTGCCAAGG
DAZL R	AACCCACTGTGGTGGAGCCT
POUV F	TGAAGGGAACGCTGGAGAGC
POUV R	ATGTCACTGGGATGGGCAGAC
NANOG F	AACTCTGCGGGGCTGTCTTG
NANOG R	AAAAGTGGGGCGGTGAGATG
GAPDH F	TCAAATGGGCAGATGCAGGT
GAPDH R	CGGCAGGTCAGGTCAACAAC
WL-specific F	AGCAGCGGCGATGAGCGGTG
WL-specific R	CTGCCTCAACGTCTCGTTGG
KO-specific F	AGCAGCGGCGATGAGCAGCA
KO-specific R	CTGCCTCAACGTCTCGTTGG

III. RESULTS

piggyBac transposon system is able to introduce transgene into chicken genome

To explore the expression of 1XchAVD, 2XchAVD, 1XhCST, 2XhCST, 1XchCST and 2XchCST *piggyBac* vectors, driven by CMV promoter, each expression vector was co-transfected into the DF-1 cell line with pCyL43B (Figure 1). 1XchAVD and 2XchAVD transfected DF-1 cells were completely selected with 300ug/mL G418 for up to 4 weeks, respectively (Figure 2). Subsequently, the RT-PCR analysis of G418-selected DF-1 cell line was performed to confirm the expression of transgenes, indicating that transgenes were properly introduced into chicken genome and were expressed well (Figure 2). To confirm whether transgene detection was originated from introduced plasmids or integrated transgene in chicken genome, we performed RT-PCR with vector-specific primers, indicating that transgene was expressed by integrated gene in chicken genome (Figure 2). Likewise, 1XhCST and 2XhCST transfected DF-1 cells were selected with 300ug/mL G418 for up to 4 weeks, respectively. 1XhCST and codon-optimized 2XhCST-transfected DF1 cell

line confirmed that codon-optimized two genes are normally expressed in chicken DF-1 cell line through RT-PCR analysis (Figure 3). 1XchCST and 2XchCST were also efficiently introduced into chicken genome, followed by G418 selection, and G418-selected DF-1 cells were characterized by RT-PCR analysis that those cells expressed each transgene, respectively (Figure 4).

G418-selected chicken PGCs expressed transgene without losing germ cell characteristics

As in case of DF1 cells, each expression vectors were transfected into chicken PGCs and transgene-expressing PGCs were completely selected after 4 weeks with 300ug/mL G418. G418-selected PGCs with 1XchAVD and 2XchAVD were characterized by the RT-PCR analysis, indicating that G418-selected PGCs expressed chAVD, driven by CMV promoter (Figure 2). Both 1XhCST and 2XhCST transfected chicken PGCs showed expression of 1XhCST and 2XhCST, respectively, after selection for 4 weeks with G418 (Figure 3). In case of 1XchCST and 2XchCST, transfected and G418-selected PGCs expressed 1XchCST and 2XchCST, respectively, as in other cases (Figure 4). Interestingly, the

expression of chicken cystatin was detected in chicken primordial germ cells (Figure 4.A). The possibility of remaining vector plasmids in each G418-selected PGCs was dispelled through the RT-PCR analysis. In addition, these six types of PGC lines continuously exhibited germ-cell properties with the expression of germness related and stemness related genes (*VASA, DAZL, POUV,* and *NANOG*), indicating that G418-selected chicken PGCs expressed transgene without losing germ cell characteristics (Figure 5).

G418-selected chicken PGCs developed into sperm in male founder

After lipofection of each of the six expression vectors into the SNUhp26 PGC line, stably transgene-expressing PGCs were selected using G418. Subsequently, G418-selected PGCs were transplanted into recipient embryonic blood vessels. After hatching and sexual maturation, sperm of each founder roosters was collected and analyzed to detect donor-PGCs derived sperm, which contains CMV-1XchAVD, CMV-2XchAVD, CMV-1XhCST, CMV-2XhCST, CMV-1XchCST and CMV-2XchCST sequences, respectively. A total of 17 founders were detected and the rates of founders among the donor-PGC transplanted chicken were 25% (7/28),

16% (4/25) and 3.8% (1/26) for 1XchAVD, 1XhCST and 1XchCST, respectively. On the other hand, founder roosters containing 2XchAVD and 2XchCST integrated sperms were detected at the rate of 18.8% (3/16) and 15.4% (2/13), respectively (Figure 6, Table 2). The founder of 2XhCST was not established yet.

Germ cell chimerism of transgenic founder chickens

The germ cell chimerism of transgenic founder chickens was estimated by qRT-PCR analysis with WL and KO specific primers, indicating that transgenic founder chickens retain both endogenous germ cells and donor germ cells in their testis in varying ratio (Figure 7). 14 founders were estimated, and seven founders (#1502, 1514, 1515, 1517, 1523, 1524, 1529) showed higher ratio of donor germ cells than endogenous germ cells, whereas, another seven founders (#1526, 1533, 1550, 1563, 1571, 1577, 1580) showed higher ratio of endogenous germ cells than donor germ cells. The donor germ cells account for up to 74% and at least 21%. #1514 founder rooster showed the highest proportion of donor germ cells and #1577 founder rooster showed the lowest proportion of donor germ cells.

Germline chimeras were identified through testcross analysis by mating

As the founders produced donor PGC-derived WL sperm as well as KO sperm, germline chimeras can be identified by their offspring genotype, and transgenic chicks only can be produced from germline chimeras. Through testcross analysis, one donor germ cell-derived chick was produced from #1549 founder rooster which is 1XchAVD. On the other hand, three donor germ cell-derived chicks were produced from #1514 and #1515 founder roosters which are 1XhCST. Following PCR analysis, however, those chicks were not transgenic chicks (Figure 8, Table 3).

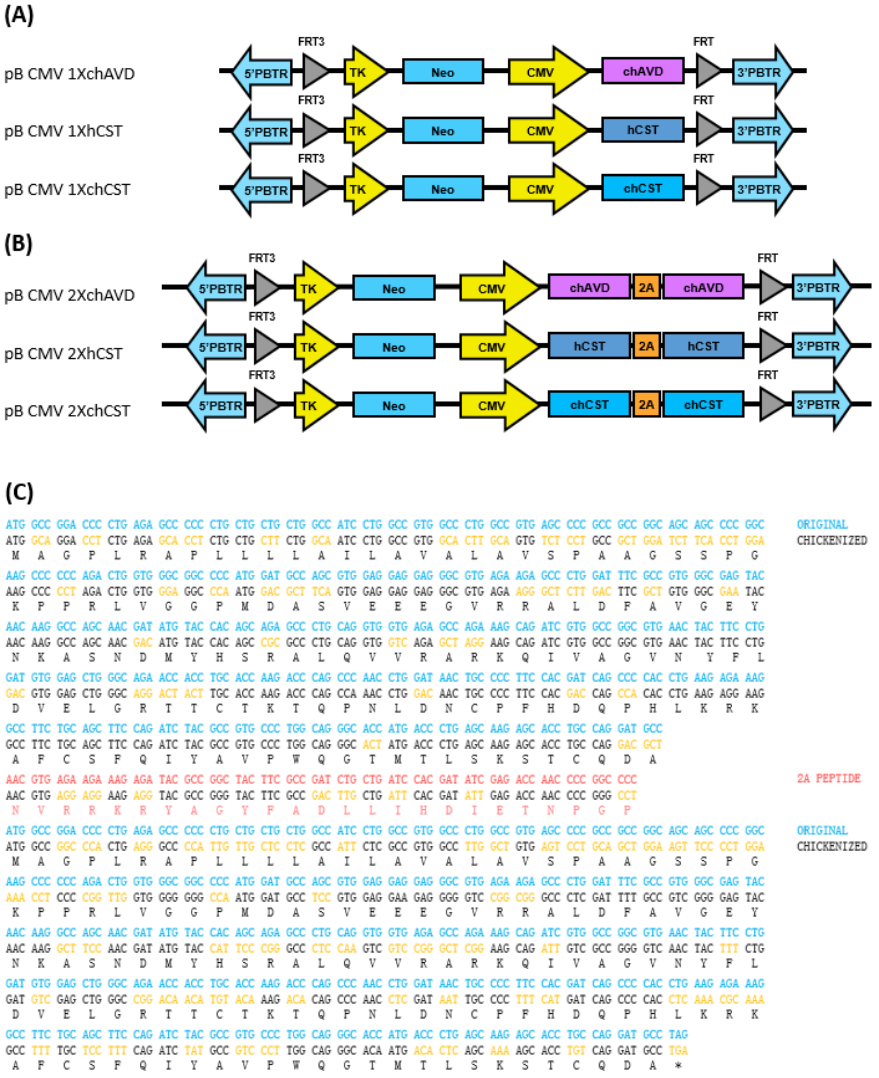

Figure 1. Construction of expression vectors. (A) The schematic representation of 1XchAVD, 1XhCST, 1XchCST expression vectors. The 1X expression vectors carry single copy of AVD, chCST and hCST gene driven by the CMV promoter, respectively, and neomycin resistant gene driven by the TK

promoter and flipase recognition target sequences (*FRT3*–*FRT*) between *piggyBac* transposon terminal repeat (PBTR) **(B)** The 2XchAVD, 2XhCST, 2XchCST expression vectors. The 2X expression vectors carry two copies of CDS, which are linked with 2A peptide cleavage sequence. **(C)** The codons of the 2XhCST gene were optimized to reflect *G. gallus* codon usage. The hCST sequence and the optimized codons are indicated in blue and yellow, respectively. The self–cleaving 2A peptide sequence is indicated in red. PBTR; piggyback terminal repeat, FRT; flipase recognition target, TK; thymidine kinase, CMV; cytomegalovirus,

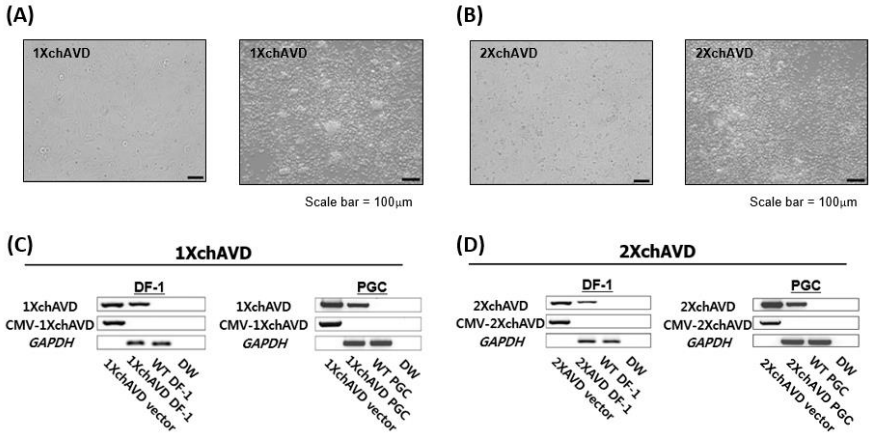

Figure 2. G418 selection and RNA expression of *piggyBac* CMV-1XchAVD and CMV-2XchAVD expression vector transfected DF-1 and PGCs. (A) 1XchAVD transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (B) 2XchAVD transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (C) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-1XchAVD vector. 1XchAVD transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 1XchAVD. (D) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-2XchAVD vector. The 2XchAVD expression vector contains two copies of chicken avidin coding sequence linked by self-cleaving 2A peptide sequence. 2XchAVD transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 2XchAVD. WT; wild type, DW; distilled water.

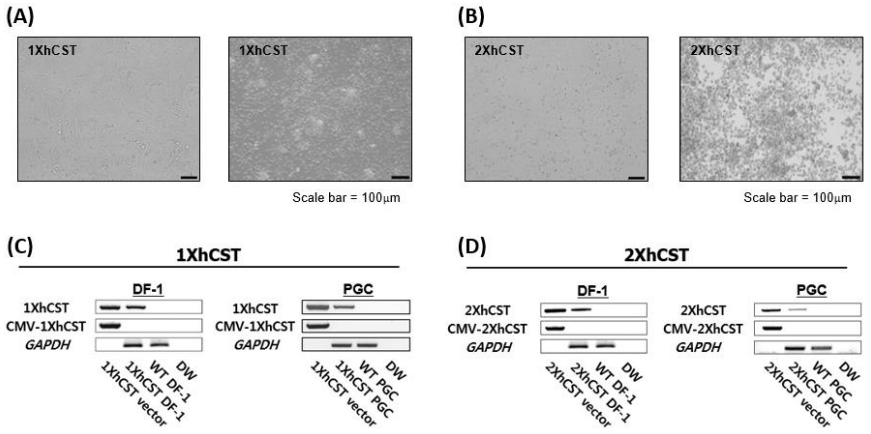

Figure 3. G418 selection and RNA expression of *piggyBac* CMV-1XhCST and CMV-2XhCST expression vector transfected DF-1 and PGCs. (A) 1XhCST transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (B) 2XhCST transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (C) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-1XhCST vector. 1XhCST transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 1XhCST. (D) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-2XhCST vector. The 2XhCST expression vector contains two copies of human cystatin C coding sequence linked by self-cleaving 2A peptide sequence. 2XhCST transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 2XhCST. WT; wild type, DW; distilled water.

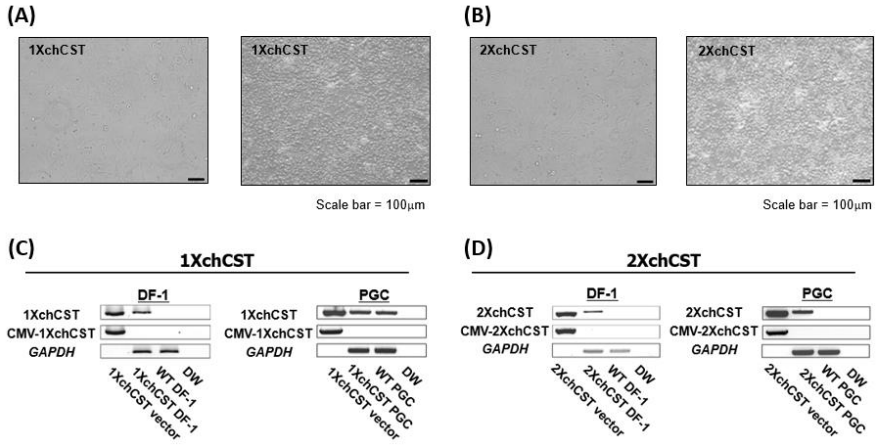

Figure 4. G418 selection and RNA expression of *piggyBac* CMV-1XchCST and CMV-2XchCST expression vector transfected DF-1 and PGCs. (A) 1XchCST transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (B) 2XchCST transfected DF-1 cells and chicken PGCs followed by G418 selection. (Bar=100 μm) (C) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-1XchCST vector. 1XchCST transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 1XchCST. (D) RT-PCR analysis of G418-selected DF-1 cells and chicken PGCs after transfection with pB-CMV-2XchCST vector. The 2XchCST expression vector contains two copies of chicken cystatin coding sequence linked by self-cleaving 2A peptide sequence. 2XchCST transfected DF-1 cell line and PGCs proliferated normally during selection with 300 μg/mL G418 for up to 4 weeks. RT-PCR analysis represented that G418-selected DF-1 cells constantly expressed 2XchCST. WT; wild type, DW; distilled water.

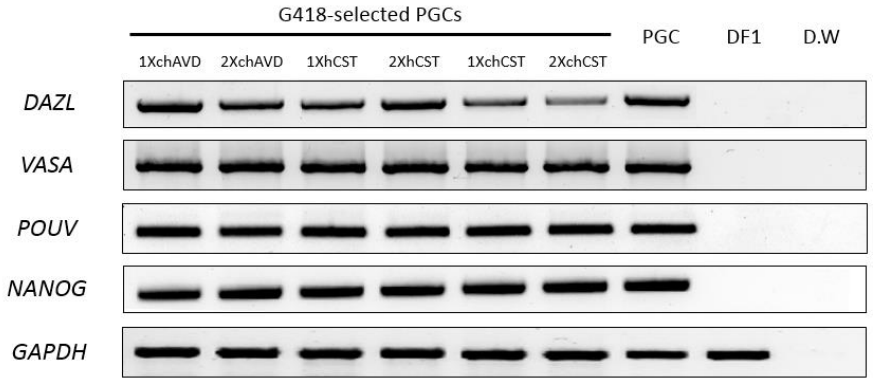

Figure 5. Expression of germness and stemness related genes in G418-selected PGCs. G418-selected PGCs expressed both germness related genes (*DAZL* and *VASA*) and stemness related genes (*POUV* and *NANOG*). SNUhp26 cell line was used as a positive control and DF-1 cell line was used as a negative control. PGC; primordial germ cell. DW; distilled water.

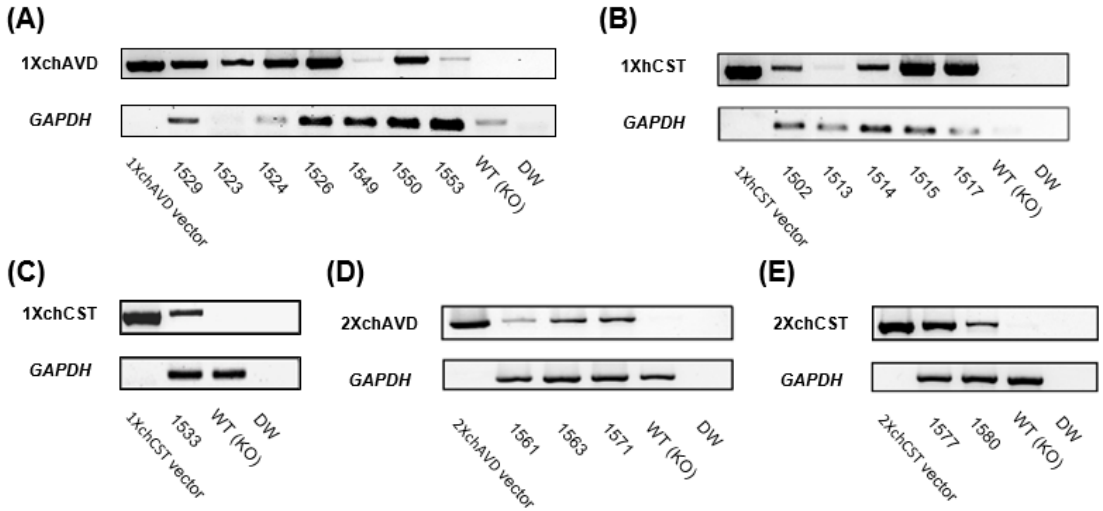

Figure 6. Genomic DNA analysis of founder chickens' sperm. **(A)** In founder KO chickens, donor PGC-derived sperm (WL) was detected with gene-specific primers. Founders of 1XchAVD produced donor PGC-derived sperm. **(B)** Founders of 1XhCST. **(C)** Founders of 1XchCST. **(D)** Founders of 2XchAVD. **(E)** Founders of 2XchCST. KO; Korean Oge, WL; White Leghorn, WT; wild type, DW; distilled water.

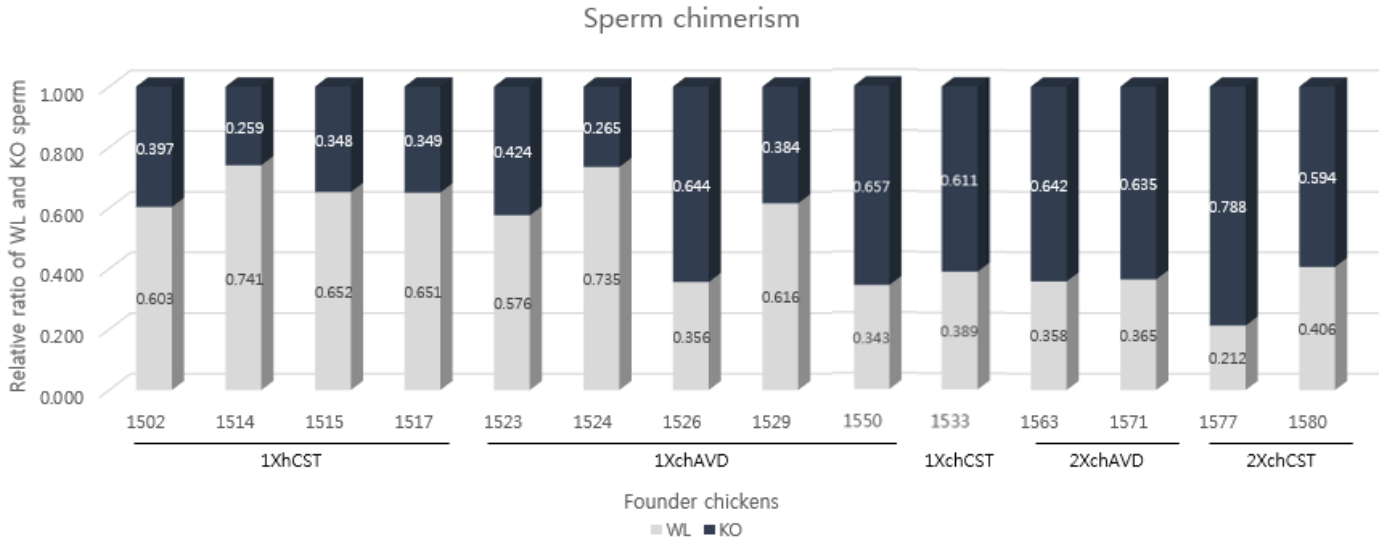

Figure 7. Sperm chimerism of founder chickens. Transgenic Founder chickens retain both endogenous germ cell-derived sperm and donor germ cell-derived sperm in varying ratio. The donor germ cell-derived sperm accounts for up to 74% and at least 21%. #1514 founder rooster showed the highest proportion of donor germ cell-derived sperm and #1577 founder rooster showed the lowest proportion of donor germ cell-derived sperm. WL; white leghorn, KO; Korean oge.

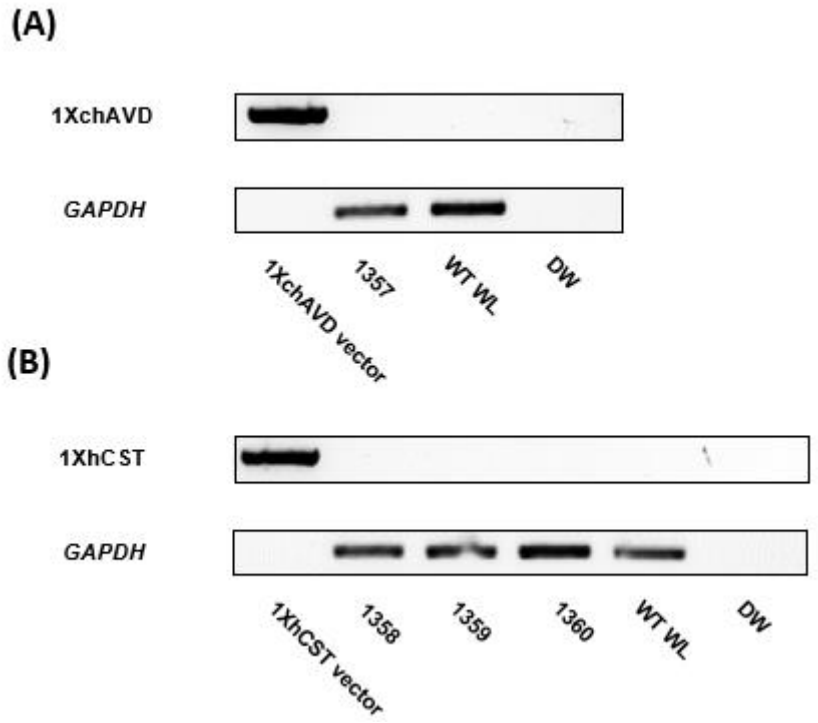

Figure 8. Genomic DNA analysis of germline chimera. (A) Genomic DNA PCR analysis of a donor germ cell derived chick from #1549 (1XhCST) founder rooster. It was revealed as a non-transgenic chick. (B) Genomic DNA PCR analysis of three donor germ cell derived chick from #1514 and #1515 (1XchAVD) founder roosters. They were revealed as non-transgenic chicks. WT; wild type, WL; White Leghorn, DW: distilled water.

Table 2. Transgene transfection and selection in chicken DF−1 cells and PGCs (SNUhp26)

Cell	No. of Cells	Vector	DNA (μg)	G418 selection (μg/mL)
DF-1	6×10^5	pCyL34B : pB-CMV-1XchAVD	3 : 7	300
	6×10^5	pCyL34B : pB-CMV-2XchAVD	3 : 7	300
	6×10^5	pCyL34B : pB-CMV-1XchCST	3 : 7	300
	6×10^5	pCyL34B : pB-CMV-2XchCST	3 : 7	300
	6×10^5	pCyL34B : pB-CMV-1XhCST	3 : 7	300
	6×10^5	pCyL34B : pB-CMV-2XhCST	3 : 7	300
SNUhp26	5×10^5	pCyL34B : pB-CMV-1XchAVD	3 : 7	300
	5×10^5	pCyL34B : pB-CMV-2XchAVD	3 : 7	300
	5×10^5	pCyL34B : pB-CMV-1XchCST	3 : 7	300
	5×10^5	pCyL34B : pB-CMV-2XchCST	3 : 7	300
	5×10^5	pCyL34B : pB-CMV-1XhCST	3 : 7	300
	5×10^5	pCyL34B : pB-CMV-2XhCST	3 : 7	300

Table 3. Production of founders via transplantation analysis.

Cell	Vector	No. of injected PGCs	No. of injected embryos	No. of hatched embryos (%)	Founder ID
SNUhp26	pB-CMV-1XchAVD	5×10^4	60	28 (46.7)	1523, 1524, 1526, 1529, 1549, 1550, 1553
SNUhp26	pB-CMV-1XhCST	5×10^4	59	25 (42.4)	1502, 1514, 1515, 1517
SNUhp26	pB-CMV-1XchCST	5×10^4	57	26 (45.6)	1533
SNUhp26	pB-CMV-2XchAVD	5×10^4	28	16 (57.1)	1561, 1563, 1571
SNUhp26	pB-CMV-2XhCST	5×10^4	-	-	-
SNUhp26	pB-CMV-2XchCST	5×10^4	59	13 (22.0)	1577, 1580

Table 4. Detection of germline chimera and production of transgenic chicks via testcross analysis

Founder ID	Vector	Sex	No. of hatched chicks	No. of endogenous germ-cell-derived chicks (%)*	No. of donor germ-cell-derived chicks (%)**	No. of transgenic chicks (%)***
#1529	pB-CMV-1XchAVD	Male	15	15 (100)	0 (0)	0 (0)
#1523	pB-CMV-1XchAVD	Male	7	7 (100)	0 (0)	0 (0)
#1524	pB-CMV-1XchAVD	Male	0	0 (100)	0 (0)	0 (0)
#1526	pB-CMV-1XchAVD	Male	1	1 (100)	0 (0)	0 (0)
#1549	pB-CMV-1XchAVD	Male	7	6 (85.7)	1 (14.3)	0 (0)
#1550	pB-CMV-1XchAVD	Male	3	3 (100)	0 (0)	0 (0)
#1502	pB-CMV-1XhCST	Male	2	2 (100)	0 (0)	0 (0)
#1514	pB-CMV-1XhCST	Male	10	9 (90)	1 (10)	0 (0)
#1515	pB-CMV-1XhCST	Male	9	7 (77.8)	2 (22.2)	0 (0)
#1517	pB-CMV-1XhCST	Male	4	4 (100)	0 (0)	0 (0)
#1533	pB-CMV-1XchCST	Male	17	17 (100)	0 (0)	0 (0)

* Test-cross analysis was conducted by mating between KO and germ-line chimeric KO; that is, transplanted avidin, chicken cystatin, and human cystatin expressing donor PGCs of WL. The phenotype of endogenous recipient germ-cell-derived chick is black KO.
** The phenotype of offspring derived from donor PGCs of WL is white hybrid between KO and WL.
*** The percentage of transplanted avidin, chicken cystatin, and human cystatin expressing transgenic chicks in donor germ-cell-derived hybrid chicks.

DISCUSSION

The hen's egg is considered as the best model system for an animal bioreactor because genetically selected commercial hens lay more than 330 eggs per year, with 6.5 g of protein in each egg. Particularly, the purification of proteins from egg white is relatively straightforward due to the simple protein composition of egg white, and it makes eggs a cost-effective bioreactor. Over the past few decades, alternative expression systems to produce avidin and cystatin for the industrial application were developed, including *E. coli (Hytonen et al., 2004)*, *Baculovirus*-infected insect cells (Airenne et al., 1997), *Pichia* (Zocchi et al., 2003), and maize (Kusnadi et al., 1998). However, those methods still had limitation for commercial applications because of the expensive culture cost for insect cells and the requirement of relatively long time and large area for maize. Thus, chicken, which is a natural resource of avidin and cystatin, can be as a safe, efficient and cost-effective bioreactor to produce avidin and cystatin.

In this thesis, we focused on the expression of avidin and cystatin, which are naturally found in chicken

eggs at low level. To induce the expression of avidin and cystatin in chicken cells, we adopted *piggyBac* transposition system for stable integration into chicken genome. We constructed six sets of expression vectors bearing one (1X) or two copies (2X) of chicken avidin, chicken cystatin, and human cystatin, respectively. Self-cleaving 2A peptide sequence is inserted between the two CDS for doubled production of proteins compared to 1X-vectors. Using *piggyBac* transposition system, transgene was efficiently integrated into chicken genome in both chicken DF-1 cells and chicken PGCs. The avidin and cystatin expressing chicken DF-1 cells and chicken PGCs were successfully established after G418 selection for up to 4 weeks. Our results indicated that *piggyBac* transposition system can be an applicable tool for the stable genomic integration in chicken, as previously reported (Park et al., 2015). G418-selected chicken PGCs stably expressed chicken avidin, chicken cystatin, and human cystatin, respectively. Furthermore, G418-selected chicken PGCs expressed both germness related genes (*DAZL* and *VASA*) and stemness related genes (*POUV* and *NANOG*), which indicates G418-selected chicken PGCs

expressed avidin and cystatin while maintaining unique characteristics of germ cells. These unique characteristics of genetically modified chicken PGCs were thought to be a valuable parameter in transgenesis technology.

Interestingly, obvious expression of chicken cystatin was observed in chicken PGCs via RT-PCR analysis. It is known that cystatin C is secreted from Sertoli cells in mouse testis (Tavera et al., 1990). It has been suggested that cystatin C have interactive roles in the adherence of germ cells to Sertoli cells and subsequent formation of the intercellular junctions (Mruk et al., 1997). Further research has conducted that Cystatin C is expressed not only in the testis cord but also in the mesonephric region at 12.5 dpc, including the Sertoli cells and spermatocytes, in adult testis ((Kanno et al., 1999)). However, there has been no report of cystatin C expression in primordial germ cells. Our result assumed that chicken cystatin might play a potential role to protect chicken PGCs from damages.

In present study, we generated founder roosters, specifically containing transgene-integrated sperms in their testes. Through the sperm chimerism analysis,

relatively high proportion of donor PGC derived germ cells (up to 74%) was detected in sperm of founder rooster. However, no offspring was detected as a transgenic chick yet. One possible reason for this is the imperfect development of donor PGC derived germ cells to form functional sperm. As chicken primordial germ cells were cultured *in vitro* for long period, the ability of PGCs to be a functional germ cells could be partially decreased. In this study, we used chicken PGCs over passage 80, and this might result in incomplete development of PGCs into sperm in testes of founder roosters. Another possible reason is that the over-expression of avidin and cystatin might result in the abnormal development of sperm or embryonic lethality in developing chicken embryos. Avidin exhibits one of the highest known affinities in nature between a protein and their ligand, biotin. As biotin is a coenzyme required for all cells, excess avidin can cause a biotin deficiency and may interfere with enzymes that depend on enzyme-bound biotin, such as those involved in carboxylation, decarboxylation, and transcarboxylation reactions, leading to stunted growth and mortality (Kramer et al., 2000). Thus, overexpression of avidin in

chicken primordial germ cells may cause incomplete development into functional sperm or embryonic lethality in transgenic chick embryos.

Since transgenic offspring was not generated, further studies are required to generate transgenic offspring. To overcome low germline transmission efficiency, germline competent chicken PGCs, which can be differentiate and develop into functional sperm, should be used to establish avidin and cystatin expressing cell lines, respectively. For this aspect, the use of early passage of chicken PGCs or primary cultured chicken PGCs could be a way to improve the current problems. Furthermore, the treatment to chicken eggs with toxic chemicals such as busulfan, which eliminates endogenous germ cells, can be performed to improve the proportion of donor-derived offspring. Once the transgenic offspring is generated, the identification of the copy number and sites of integrated DNA should be performed, followed by the establishment of transgenic chicken lines. In addition, it is necessary to evaluate the expression of transgene in transgenic chicken tissues, especially plasma, oviduct

and eggs. For the last step, the functional activity of avidin and cystatin C from the transgenic chicken will be investigated.

In present study, we failed to generate transgenic offspring via germline transmission, but nevertheless, it should certainly find great usefulness in introducing transgenes to chicken cells. We confirmed *piggyBac* transposition system mediated genomic insertion showed high efficiency with the stable expression in chicken cells, especially chicken PGCs. In avian species, PGCs only can be used to overcome germline competency and have high germline transmission efficiency when they are compared to ESCs. In addition, chicken PGCs can be maintained *in vitro* for desired time without apparently losing their plasticity, compared with other mammalian species. Therefore, gene manipulation in chicken PGCs is the fundamental technique in avian biotechnology, which is not possible in other species. The application *piggyBac* transposition system in chicken PGCs study opens up tremendous possibilities in the field of transgenic birds. In addition, the chicken egg is the nature resource of avidin and cystatin, which are widely used in the field of biosciences. Although

recombinant avidin and cystatin are produced from various bioreactors, it is obvious that the chicken egg is the ideal bioreactor for avidin and cystatin. Therefore, our present study sheds light on the intrinsic worth of the chicken egg as a bioreactor.

In conclusion, our results suggest that the *piggyBac* transposition system-mediated gene transfer could be great use to create bioreactor chicken in the agriculture and pharmaceutical industries, and could be employed for efficient production of various biomaterials. Combined technology with novel culture system of germline-competent cell lines and highly efficient gene integration system could create economical and industrial development as well as academic and technical development.

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