A Dissertation

For the Degree of Doctor of Philosophy

Studies on avian germline competent stem cells and practical application for bioreactor system

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SUMMARY

Avian species have become valuable models for biotechnological purposes including production of functional proteins, disease resistance model, and industrial traits as well as developmental studies of vertebrates. To produce genome modified aves, germline-competent stem cells will be required to achieve robust, expedite, and precise genetic modification. Therefore, here, we demonstrated the derivation and manipulation of germline-competent stem cells for various purposes.

Firstly, we attempted the derivation of induced pluripotent stem cells-like cells (iPSLCs) from avian feather follicle cells (FFCs). The induced pluripotent stem cell (iPSC) as a novel class of pluripotent stem cells contribute to all three germ layers including germ line. Therefore, we attempted the derivation of induced pluripotent stem cells-like cells (iPSLCs) from avian feather follicle cells (FFCs) as a novel approach to stem cell production. The FFC-iPSLCs can proliferate with the pluripotent property and differentiate into all three germ layers in vitro. This experimental strategy should be useful for conservation and restoration of endangered or high-value avian species without sacrificing embryos.

The primordial germ cell (PGC) is the most well established germline-competent stem cells in avian species especially in chicken. In second part, we generated the production of anti-cancer monoclonal antibody against the CD20 protein from egg whites of transgenic hens, and validated the bio-functional activity of the protein in B lymphoma and B lymphoblast cells. This experiment well represent as an efficient system for producing anti-cancer therapeutic antibodies from

transgenic chicken with consistent expression and highly enhanced Fc effector functions. Meanwhile, to produce efficient genome modification in quail PGCs, we optimize the transfection and drug-selection system into quail genome of primary PGCs. Applying the CRISPR/Cas9 system on quail genome of primary PGCs using electroporation and puromycin drug-selection system, we confirmed that the efficient genome editing ratio compared to non-selected cells.

Apart from the PGCs, the spermatogonial stem cells (SSCs) also regarded a reliable way to produce genome-edited animals. In terms of adopting genome modification techniques on quail genome, we produced the germline chimeric quail using testicular cells (TCs) and SSCs transplanted into busulfan-treated recipient testis. The transplantation of male germ cells including spermatogonia and SSCs is an efficient method to study spermatogenesis, control male fertility and transgenesis. Compared with the embryo-mediated method, this strategy is simple and leads to rapid generation of quail germline chimeras. This will lead to production of transgenic models using adult germ cells and, through the production of germline chimeras, also help in efforts to conserve avian species.

In conclusion, these experimental strategies for derivation of avian stem cells, manipulation of them and its application using genome editing tools can be used to efficient way for produce birds of economic traits or non-sacrificible avian conservation system.

Keywords: bioreactor, chicken, CRISPR/Cas9, genome editing, germline chimera, iPSLCs, PGCs, quail, SSCs

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
ALV	avian leucosis virus
AP	Alkaline Phosphatase
A_{pr}	A pair spermatogonia
A_s	A single spermatogonia
bFGF	Basic fibroblast growth factor
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CDC	complement-dependent cytotoxicity
CHO	chinese hamster ovary
CLL	chronic lymphocytic leukemia
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
crRNA	CRISPR RNA
CVH	chicken vasa homologue
CXCR4	C-X-C chemokine receptor type 4
Cyp26b1	cytochrome P450 family 26 subfamily b member 1
D	Dominant black
DAZL	deleted in azoospermia-like
DLL1	delta-like 1
DSA	datura stramonium agglutinin
EAA	essential amino acids

EB	embryoid body
EDTA	ethylenediaminetetraacetic acid
EGC	embryonic germ cell
EGF	epidermal growth factor
EGK	Eyal-Giladi stage
EMA	Equi merozoite antigen
ESC	embryonic stem cell
EW	egg white
ExE	extra-embryonic ectoderm
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Fc	fraction crystallisable
FFC	Feather Follicular cell
FGF9	fibroblast growth factor 9
FITC	fluorescein isothiocyanate
GDNF	glial cell line-derived neurotrophic factor
H3K27me3	trimethylation of histone H3 on lysine 27
H3K9me3	trimethylation of histone H3 on lysine 9
HCAM	homing cell adhesion molecule
HH	Hamburger and Hamilton
iPSC	induced pluripotent stem cell
ITGA6	integrin alpha 6
ITGB1	Integrin Subunit Beta 1

JAD1	jadomycin polyketide synthase cyclase
KO	Korean Oge
LIF	leukemia inhibitory factor
LRCs	label-retaining cells
mAb	monoclonal antibodies
MACS	magnetic-activated cell sorting
MC1R	melanocortin 1 receptor
MEF	mouse embryonic fibroblast
miRNAs	micro RNAs
MoA	mechanism of action
MSC	mesenchymal stromal cell
MSTN	myostatin
NEAA	non-essential amino acids
NFAT	nuclear factor of activated T-cells
NHEJ	non-homologous end-joining
NR6A1	nuclear receptor subfamily 6, group A, member 1
OV	ovalbumin
PAS	Periodic acid-Schiff
PAS	Periodic acid–Schiff
PFA	paraformaldehyde
PGC	primordial germ cell
PI	using target-specific primers
piRNAs	piwi-interacting RNAs

PRC1	polycomb repressive complex 1
PTM	post-translational modification
QEF	quail embryonic fibroblast
RA	retinoic acid
RALDH2	retinaldehyde dehydrogenase 2
RT-PCR	polymerase chain reaction
SDF-1	stromal cell-derived factor 1
shRNA	short hairpin RNA
SNP	single nucleotide polymorphism
SSC	spermatogonial stem cell
SSEA1	stage-specific embryonic antigen 1
STO	SIM mouse embryo-derived thioguanine- and ouabain-resistant
Stra8	stimulated by retinoic acid 8
TALEN	transcription activator-like effector nuclease
TC	testicular cells
TF	ovotransferrin
tracrRNA	trans-activating CRISPR RNA
TSP	target-specific primers
WG	whole gonadal cells
WL	White Leghorn
WP	wild plumage

CHAPTER 1

GENERAL INTRODUCTION

Avian species have become valuable models for biotechnological purposes including production of functional proteins, disease resistance model, and industrial traits as well as developmental studies of vertebrates. Moreover, avian models have been used as excellent bioreactor systems due to their numerous merits including relatively short generation time and plentiful reproductive capacity, and also proper for using them as a human disease model including cancer and inherited disease (Ivarie, 2003; Han, 2009; Johnson and Giles, 2013). To produce genome modified aves, germline-competent stem cells will be required to achieve robust, expedite, and precise genetic modification.

In 2006, induced pluripotent stem cells (iPSCs or iPS cells) technique was introduced as a one of prominent system for acquisition of pluripotency from somatic cells which have potential to develop all lineages of cells and tissues (Takahashi and Yamanaka, 2006). For some highly endangered species iPSCs offer the possibilities for animal restoration and prevent extinction through somatic cell reprogramming. This strategy has also been applied to various endangered species (Verma *et al.*, 2012; Ben-Nun *et al.*, 2015; Ramaswamy *et al.*, 2015). Therefore, generation of iPSCs from avian somatic cells could be an alternative for avian restoration and conservation.

The primordial germ cell (PGC) is the most well established germline-competent stem cells in avian species especially in chicken. Avian PGCs are initially localized to the central zone of the area pellucida in stage X embryos (Ginsburg and Eyal-Giladi, 1987; Ginsburg *et al.*, 1989), and they migrate to the germinal crescent at stage 4 (18–19 h after incubation) and, between stages 10 and 12, move into blood vessels and begin circulating in the bloodstream, finally settle in genital ridge (Nieuwkoop and Sutasurya, 1979). Using these unique characteristics, the avian PGCs were efficiently isolated, and it can be maintaining for long-term with stable transfection of exogenous DNA and drug selection for purposed cells. According to previous reports, the recombinant proteins from transgenic chicken bioreactor is known to produce *N*-glycan species terminated by

high mannose and core afucosylated form (Pinkert, 2014), which increasing antibody-dependent cellular cytotoxicity (ADCC) activity of anti-cancer antibodies. Therefore, it could be an efficient system for producing anti-cancer therapeutic antibodies from transgenic chicken with consistent expression and highly enhanced Fc effector functions.

Spermatogonial stem cell (SSC), type of adult stem cell, located on the basement membrane of seminiferous tubule and act as foundation for spermatogenesis throughout life in males by balancing self-renewal and differentiation (Phillips *et al.*, 2010). SSC is a reliable germline competent stem cell that could deliver their genetic information to successive generations, it has been used continuously for transgenic studies. To produce of desirable offspring after testicular transplantation, endogenous germ cell depletion by irradiation and anti-spermatogonial alkylating reagent treatment are effective. The combination of novel genome editing tools such as CRISPR/Cas9 and the SSC transplantation system may produce various kind of animal model including avian species.

Genome editing platforms are developing rapidly in recent years, and broadly adopting on genome modification in living organisms. Especially, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) regarded as a most convenience and efficient tool for genome modification (Cong *et al.*, 2013; Sander and Joung, 2014). These are successfully applied in somatic lines and stem cell of chicken and quail (Abu-Bonsrah *et al.*, 2016; Ahn *et al.*, 2017). However, despite several works for isolation and manipulation of PGCs in quails (Ono *et al.*, 1998; Kim *et al.*, 2005b; Park *et al.*, 2008) the long-term *in vitro* culture methods of quail PGC and efficient delivering exogenous gene are still limited. Therefore, highly efficient gene transfer technology and gene targeting system for primary quail PGCs should be required during short period for genome edited quail production.

To demonstrate the derivation and manipulation of germline-competent stem cells for various purposes, we evaluate the capacity of the cells and genome modification in avian genome. In CHAPTER 2, we review the germline competent stem cells and its application in avian species and its future applications. In CHAPTER 3, we established the iPSCs from chicken FFCs for alternative way of avian conservation. In CHAPTER 4, we demonstrate the production of transgenic chicken expressing human CD20 mAb, and evaluate the functionalities focused on its Fc effector functions. In CHAPTER 5, we demonstrated the production of quail germline chimera after transplantation of testicular cells (TCs) and SSCs into busulfan treated recipients' testes. Finally in CHAPTER 6, we optimized the genome editing systems on quail primary PGCs using CRISPR/Cas9 system.

CHAPTER 2

LITERATURE REVIEW

1. Induced pluripotent stem cells (iPSCs) for avian conservation

1.1 Pluripotent stem cells

Stem cells are undifferentiated cells that can self-renew but also possess an ability to differentiate into more specialized cell types. The classification of a stem cell has also broadened to include ones that are pluripotent, multipotent, oligopotent, and unipotent, depending on the number of cell types into which they can differentiate from an initial self-renewing state (Ezashi *et al.*, 2016). Pluripotent stem cells are maintaining their undifferentiated state and having differentiation potentials for all three germ layer which can be forming other tissues and organs in both of *in vitro* and *in vivo*. In mammals, pluripotent stem cells derived from various origins were well established such as embryonic stem cells (ESC) which originate from inner cell mass (ICM) of blastocyst and embryonic germ cells (EGC) which established from primordial germ cell (PGC) in embryonic gonads (Evans and Kaufman, 1981; Resnick *et al.*, 1992; Thomson *et al.*, 1998). In human, ESC were first created from human blastocysts (Thomson, Itskovitz-Eldor et al., 1998), and these stem cells were phenotypically distinct from those obtained from mice (Hanna *et al.*, 2010). The morphology of mouse ESCs which forms dome-shaped colonies with the naive or inner cell mass (ICM)-like state, but and ESCs derived from the primed or epiblast-like state, which exhibits a more flattened morphology (Ezashi, Yuan et al., 2016). The flattened colonies can grown on a Matrigel substratum or mitotically-arrested mouse embryonic fibrablast and require FGF2 and growth factors that operate through the ACTIVIN/TGFB receptor signaling pathway and activation of the transcription factors SMAD2 and SMAD3 (Amit *et al.*, 2004; Ludwig *et al.*, 2006). Although, the long history of attempts at ESC derivation from domesticated mammalian species, authentic ESC cells like rodents and human have not been established conclusively in any of other species (Ezashi, Yuan et al., 2016).

In avian, ESCs were firstly established from EG&K stage X blastoderm and maintained *in vitro* for long period (Pain *et al.*, 1996), and also reported EGCs from embryonic gonads (Park and Han, 2000). These cells showed that *in vitro* propagation on somatic cell feeder layers differentiated into all three germ layer lineages and produced somatic chimeras. Moreover, when the cells were transplanted into the subgerminal cavity of recipient embryos at EGK stage X, somatic and germline chimeras were produced successfully, demonstrating their stem cell characteristics

1.2. iPSCs as a novel pluripotent stem cells

In 2006, induced pluripotent stem cells (iPSCs or iPS cells) technique was introduced as a one of prominent system for acquisition of pluripotency from somatic cells which have potential to develop all lineages of cells and tissues (Takahashi and Yamanaka, 2006). By the reason that homeodomains of reprogramming factors such as *Nanog*, *Oct3/4*, *Sox2*, *cMyc* and *Klf4* are functionally conserved in most of animal (Theunissen *et al.*, 2011; Rossello *et al.*, 2013). To date, many studies regarding iPSCs from many kinds of animals including avian species were reported (Esteban *et al.*, 2009; Li *et al.*, 2009; Lu *et al.*, 2014; Mo *et al.*, 2014).

Most general interest of iPSCs is focused on clinical application. Generating patient-specific stem cells has been a long-standing goal in the field of regenerative medicine. Therefore, there are considerable challenges were attempt to generating disease-specific and patient-specific iPSCs establishment (Robinton and Daley, 2012). For example, iPS cells to demonstrate disease modelling and drug screening for familial dysautonomia, a rare genetic disorder of the peripheral nervous system (Lee *et al.*, 2009).

Another interest of iPSCs for clinical application, tissue engineering

and regenerative medicine. These issue mainly focused on generating progenitor cells such as dental pulp, neural spheres, hematopoietic stem cells, cartilage, and cardiogenic cells from iPSCs and its transplantation (Amimoto *et al.*, 2011; Inui *et al.*, 2012; Kerkis and Caplan, 2012; Uemura *et al.*, 2012; Uto *et al.*, 2013). These studies have high potential for medicinal value and are expected to have unlimited outcomes in the future.

1.3. iPSCs for species conservation

More recently, functional germ cells were capably reprogramming from iPSCs under specific conditions (Hayashi *et al.*, 2011; Hayashi *et al.*, 2012). For some highly endangered species iPSCs offer the possibilities for animal restoration and prevent extinction through somatic cell reprogramming. Ben-Nun et al., firstly reported the generation of iPSCs from the endangered primate, the drill *Mandrillus leucophaeus*, and from the nearly extinct northern white rhinoceros, *Ceratotherium simum cottoni* (Ben-Nun *et al.*, 2011). Because they are self-renewing and pluripotent, iPSCs from endangered species have several possible applications. This strategy has also been applied to other endangered species such as snow leopard (*Panthera uncia*), Somali wild ass (*Equus africanus somaliensis)*, Javan banteng (*Bos javanicus javanicus)* and orangutan (*Pongo tapanuliensis*) (Verma, Holland et al., 2012; Ben-Nun, Montague et al., 2015; Ramaswamy, Yik et al., 2015). Although, there have been no reports on the substantial species restoration using iPSCs and will be necessary to more accurate reprogramming technology to conserve endangered species, but these success in generating iPSCs from endangered species may offer significant possibilities to rescue species on the verge of extinction.

1.4. A strategy for avian conservation using iPSCs

In avian species, pluripotent stem cells such as ESCs and EGCs has been already reported (Pain, Clark et al., 1996; Park and Han, 2000). However, all kind of these cells have limitation that cell sources should be obtained from embryonic tissues that accompany with the sacrifice of embryos. Thus, traditional pluripotent stem cells are inadequate for the conservation of rare and highly endangered avian species.

There are few cases of iPSCs has been reported using both chicken and quail embryonic fibroblast cells. The previous results suggest that human and mouse reprogramming factors can induce the somatic cell reprogramming of avian cells (Lu *et al.*, 2012; Rossello, Chen et al., 2013; Choi *et al.*, 2016). Although, the previous reports were showed that the successful reprogramming on avian cells, it regarded to the origin of reprogrammed cells were inevitably isolate from embryonic tissues.

Avian feathers provide the most easily accessible somatic cell sources and have a great potential for regenerative ability due to the various differentiation capabilities (Xi *et al.*, 2003). Feather follicle has been known to contain stem cell source which called label-retaining cells (LRCs) in collar bulge of avian epidermis, controlled the epidermis development and homeostasis (Yue *et al.*, 2005). This particular cell presents progenitor or stem cell properties that control the epidermis development and homeostasis during physiological molting cycles. With these reasons, FFCs are regraded to one of the adult stem cell sources for epidermal tissue regeneration and somatic cell reprogramming in birds (Xu *et al.*, 2011). Avian FFCs have been exhibited to have the characteristics of mesenchymal stromal cells (MSC)-like cells (Xu, Sun et al., 2011). Because of reprogramming of human hair follicle cells into iPSCs are very efficient and have high differentiation potential (Shi *et al.*, 2016), avian FFCs may suitable for iPSC induction. Therefore, generation of iPSCs from FFCs could be an alternative for avian restoration and conservation even without sacrificing embryos.

2. The primordial germ cells (PGCs) in avian biotechnology

Germ cells are the only cells that can transfer the entirety of an organism's genetic information to the next generation. In many multicellular organisms, mature gametes, including sperm or eggs, originate from primordial germ cells (PGCs). PGCs occupy a small proportion of the early embryo and are clearly distinguishable from somatic cells. In the fields of developmental and evolutionary biology, gaining a comprehensive understanding of the underlying mechanisms for germ cell specification is a major goal. Germ cells are important not only for basic biological research, but also as a means of preserving genetic resources. Thus, how and when germ cells are initially originated and specified is a crucial question. There are two general models of germ cell specification: preformation and induction (Extavour and Akam, 2003). The preformation model involves germ cell determinants, which are unique cytoplasmic organelles generically termed 'germ granules' or 'germ plasm.' These organelles contain maternally inherited mRNAs, proteins, and small RNAs, and play a crucial role in germ cell specification in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, and *Xenopus laevis* (Strome and Wood, 1983; Hay *et al.*, 1988; Ephrussi *et al.*, 1991; Tada *et al.*, 2012; Strome and Updike, 2015). On the other hand, in the induction model (which applies to taxa including *Homo sapiens* and *Mus musculus*) germ cells arise from proximal-posterior epiblasts, a process induced by bone morphogenetic protein (BMP) signalling from neighbouring extra-embryonic tissue during gastrulation (Lawson *et al.*, 1999; Tang *et al.*, 2016).

In avian species, PGCs are initially detected as a scattered pattern in the area pellucida, the central region of Eyal-Giladi (EGK) stage X embryos (Eyal-Giladi and Kochav, 1976; Ginsburg and Eyal-Giladi, 1987; Tsunekawa *et al.*, 2000). Between EGK stage X and Hamburger and Hamilton (HH) stage 2 (Hamburger and Hamilton, 1951), PGCs are moved passively by the overall

movement of embryonic cells; they actively move to the germinal crescent at HH stage 4 (Swift, 1914; Hamburger and Hamilton, 1951; Eyal-Giladi *et al.*, 1981). Subsequently, between HH stages 9 and 12, PGCs move into blood vessels and circulate through the bloodstream (Fujimoto *et al.*, 1976b; Ukeshima *et al.*, 1991), finally settling in the genital ridge (Meyer, 1964; Fujimoto, Ukeshima et al., 1976b). To date, although numerous studies have been performed to elucidate the origin and specification of avian PGCs, they still remain incompletely defined.

2.1. Derivation and cultivation of PGCs in chicken

Since the migratory route for avian PGCs differ from mammalian species (Niewkoop and Sutasurya, 1979), they can be isolated at various stages of embryonic development. This characteristic provides them with a huge advantage in germ cell research. Various methods have been used to isolate PGCs from embryonic tissue or blood vessels, including fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS) using PGC-specific antibodies, and density gradient centrifugation, and size-dependent isolation without using antibodies (Chang *et al.*, 1992; Ono and Machida, 1999; Zhao and Kuwana, 2003; Mozdziak *et al.*, 2005; Jung *et al.*, 2017a).

PGC isolation have been attempted not only in chicken, but also in other avian species such as pheasant, quail, turkey, duck and guinea fowl, and have been produced using PGCs for the purpose of restoring endangered birds (Reynaud, 1969; Ono *et al.*, 1996; Kang *et al.*, 2008; Wernery *et al.*, 2010; Liu *et al.*, 2012; van de Lavoir *et al.*, 2012). However, historically the germline transmission rate of donor-derived progeny has been prohibitively low, owing to differences between species in traits such as reproductive cycle. PGC culture is therefore required for endangered bird restoration efforts, but the culture process has not yet been fully optimised.

Among aves, the long-term culture system of chicken PGC has been successfully established (van de Lavoir *et al.*, 2006). It has been shown that bFGF plays an essential role in the proliferation and survival of chicken PGCs via the mitogen-activated protein kinase kinase (MEK) / extracellular signal–regulated kinases (ERK) signaling pathway, and helps to maintain telomerase activity, migratory activity, and germline contribution in PGCs cultured for extended periods (Choi *et al.*, 2010; Macdonald *et al.*, 2010). More recent studies have shown that MEK1, AKT (also known as protein kinase B, PKB), and SMAD family member 3 (SMAD-3) signalling is required for PGCs to maintain germline transmission capacity, and that Wnt/β-catenin signalling is required for PGC proliferation *in vitro* (Whyte *et al.*, 2015; Lee *et al.*, 2016b). Furthermore, it has been reported that stem cell factor 2 (SCF2) derived from chickens has potent and prolonged effects on PGC proliferation via FGF2- and c-KIT-mediated growth signals, and is thus crucial for the maintenance of germ cell characteristics and germ line transmission (Daichi *et al.*, 2016).

2.2. PGC-mediated transgenic technology in chicken

Gene transfer into the avian genome was first achieved using avian leucosis virus (ALV) by transduction of stage X blastoderm (Salter *et al.*, 1986). However, this way for produced transgenic aves proved to be inefficient with very low germline chimera production efficiency and transgene expression. In the first study that established transgenic chickens using PGCs, were produced by viral infection on germinal crescent PGC by avian leucosis virus or spleen necrosis virus after replication-defective manners (Vick *et al.*, 1993). However, this system showed very low efficiency, mainly due to the non-purified germinal crescent cells and blood cells containing small population of PGCs. Therefore, the method of direct lentiviral infection to purified PGCs were suggested in quail and chicken (Shin *et al.*, 2008; Kwon *et al.*, 2010; Motono

et al., 2010).

In recent, long-term cultivation and establishment of PGC cell lines have been reported. Cultured PGCs have been utilised not only for research on germ cell signalling pathways (Choi, Kim et al., 2010; Whyte, Glover et al., 2015; Lee, Lim et al., 2016b), but also for transgenic studies (van de Lavoir, Diamond et al., 2006). Because of the PGC *in vitro* culture allowed the stable transfection of exogenous DNA and drug selection for purposed cells. Thus, established transgenic PGCs were showed highly efficient of offspring production rate after transplanted into dorsal aorta of HH13-15 embryos (Park and Han, 2012). Moreover, combined to transposable element piggyback and Tol2 were enhanced efficiency of the transgene integration (Macdonald *et al.*, 2012; Park and Han, 2012; Lee *et al.*, 2015a).

2.3. PGC-mediated genome editing technology in chicken

PGC-mediated genome editing technology including targeted gene deletion and insertion were rapidly developed as well as PGC-mediated genome editing. Recently, novel genome editing tools such as transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) have been developed (Boch, 2011; Jinek *et al.*, 2012). Both systems are originally adopted in bacterial host immune reaction. The TALE motif from the pathogenic bacterium *Xanthomonas* can be readily engineered to bind virtually any DNA sequence. The DNA binding domain of TALE motif contains a repeated highly conserved 33–34 amino acid sequence and the FokI nuclease that are actively excise the host genome of yeast (Christian *et al.*, 2010; Li *et al.*, 2011). Using the highly affinity of target genome sequence, TALEN adapted

as avian genome editing tools in several works adapted as promising methods for developing avian model (Park *et al.*, 2014; Taylor *et al.*, 2017).

More recently, CRISPR/Cas9 system as a cutting-edge genome editing tools were developed. The CRISPR/Cas9 system composed to two small RNA molecules, CRISPR RNA (crRNA) which later guides the Cas nuclease, trans-activating CRISPR RNA (tracrRNA) and Cas protein an RNA-guided DNA endonuclease (Jinek, Chylinski et al., 2012). Because of the synthesis of crRNA and tracrRNA is much simpler and its high accuracy on targeted sequence, CRISPR/Cas9 rapidly adapted in various organisms and purposes (Reardon, 2016). Likewise applied on other living organisms, the CRISPR/Cas9 system has also been obviously adopted in avian genome including chicken and quail (Veron *et al.*, 2015; Abu-Bonsrah, Zhang et al., 2016; Ahn, Lee et al., 2017). In subsequently, the combinatory technology of CRISPR/Cas9 system and cultured PGC lines, successfully generated genome-edited chicken with high knockout efficiency (Dimitrov *et al.*, 2016; Oishi *et al.*, 2016). Applying such germline modification techniques combined with PGC culture to a variety of valuable avian species will permit expansion of this research area and have considerable benefits for the poultry industry.

2.4. Characteristics and purification of quail PGCs

In their morphological aspect, the ultrastructural characteristics of quail PGCs were exhibited differences to chicken PGCs in terms of no glycogen particles, electron-dense and membrane bounded granules, and prominent nucleolus associated with condensed chromatin (Yoshinaga *et al.*, 1993). For the detection of quail PGCs, specific marker QH1 antibody have been suggested during early embryonic development (Pardanaud *et al.*, 1987). The number PGCs increases significantly until the primitive streak stage (HH stage 4), but until 7-

somite stages (HH stage 9) there were no significant increase of PGC-number (Pardanaud, Buck et al., 1987). As in both the duck and the chick, the colonization in the quail PGCs are rapid and regular increase, at stages from HH stage 13 to 18 and from HH stage 24 to 30 (Didier and Fargeix, 1976). In subsequent, immunomagnetic purification of viable quail PGCs from embryonic blood were introduced by monoclonal antibodies (MAb) specific to the cells (Aoyama *et al.*, 1992b; Ono and Machida, 1999). The purified PGCs by MACS separation were more efficiently enriched compared with the ficoll density-gradient centrifugation system (FICS) which the previously used enrichment system (Ono and Machida, 1999).

For using this system, producing quail germline chimeras by the transfer of gonadal primordial germ cells (gPGCs) were successfully introduced and the percentage of germline transmission to the donor-derived gametes in the chimeras that received cultured and noncultured gPGCs were 1.9 and 2.2-4.7%, respectively (Kim, Park et al., 2005b). Apart from the efficiencies of germline transmission, the property of primary PGCs was not adversely affected by culture for up to 3 days (Kim, Park et al., 2005b). Moreover, this experimental methods have been proved that germline competency can be maintained through *in vitro* maintenance up to 20 days (Park, Kim et al., 2008). Effective isolation of quail PGC and germline transmission have increased the possibility of quail PGC-mediated transgenesis as much as chickens.

2.5. PGC-mediated transgenesis in quail

The study on gene transfer into quail PGC were initially performed by directly introduced of PGCs were circulatin in the blood vessels with liposomes consisting of LacZ expressing plasmid DNA (Ono *et al.*, 1995). Since then, several case of production of transgenic quails has been reported through direct

gene transfer into PGC of blood vessels (Sun *et al.*, 2012; Zhang *et al.*, 2012). In contrast, until now, transgenic quail production is mainly through the direct gene transfer subgerminal cavity of blastoderm using viral particles (Kamihira *et al.*, 2004; Poynter *et al.*, 2009a; Kwon, Choi et al., 2010). Although transgenic quail production using gonadal PGC has only been reported so far (Shin, Kim et al., 2008), it has been shown to be a more promising method because number of cells and a relatively high production efficiency of transgenic progenies. In addition, advanced gene delivery technology and genome editing tools are enhance the potentiality of gPGC-mediated transgenic quail production.

3. Production of recombinant proteins in therapeutic use

3.1. Therapeutic recombinant proteins

Proteins are now well established as a clinically and commercially important class of therapeutics. The production of therapeutic recombinant proteins allowed for revolutionary advances in the treatment of many previously unmet medical demands (Li and d'Anjou, 2009). Since the early 1980s, proteins have regareded as a novel class new class of pharmaceuticals and with currently about 200 marketed therapeutic products are commercially available with a small number of diagnostics reagent and vaccines (Walsh, 2010). The classification of therapeutic proteins into molecular types that include: antibody-based drugs, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, Fc fusion proteins, growth factors, hormones, interferons, interleukins, and thrombolytics (Walsh, 2010).

To make recombinant proteins, the gene is isolated and cloned into an expression vector whose code is carried by a recombinant DNA. Most recombinant proteins in therapeutic use such as anti-cancer antibodies are from humans but are expressed in other organisms such as bacteria, yeast, animal cells in culture and tissues of plant and animal (Celik and Calik, 2012; Overton, 2014; Picanco-Castro *et al.*, 2014; Sheshukova *et al.*, 2016; Gifre *et al.*, 2017). Many issues affect the expression of recombinant proteins including expression level, consistence yield, posttranslational processing such as glycosylation that are available only in eukaryotic cells and also yeast, insect cells, and mammalian cell culture systems (Brooks, 2004). And another posttranslational regulatory factors for expression of recombinant proteins are myristoylation, palmitoylation, isoprenylation, phosphorylation, sulfation, *C*-terminal amidation, β-hydroxylation, and methylation, because organisms differ in which codons are preferred for each amino acid, the level of corresponding tRNAs varies between organisms (Palomares *et al.*, 2004). The gene for a recombinant protein can also be altered to make the protein more stable. Changing the *N*-terminal amino acid, removing the PEST region (sequences enriched with Pro, Glu, Ser, and Thr), or co-expressing a molecular chaperone all increase protein stability (Palomares, Estrada-Mondaca et al., 2004). In case much recombinant protein is produced too fast, it may aggregate into inclusion bodies (Singh and Panda, 2005). Therefore, using regulated expression vectors can control the rate at which recombinant protein production. The production of therapeutic recombinant proteins allowed for revolutionary advances in the treatment of many previously unmet medical demands (Li and d'Anjou, 2009).

3.2. Bioreactor systems for recombinant protein production

The production of therapeutic recombinant proteins allowed for

revolutionary advances in the treatment of many previously unmet medical demands (Li and d'Anjou, 2009). The conventional concept of bioreactor refer to a device system to grow engineered cells, to harvest cell extracts, or enzymes carried out a biological reaction for the production of recombinant proteins especially for production of monoclonal antibodies (mAbs) (Nelson and Geyer, 1991). To date, the manufacturing process of these therapeutic proteins has undergone major changes and has now achieved high volume productivity (approximately, 10-15 g / L) (Wurm, 2004; Huang *et al.*, 2010). However, the strategies used for these progresses could not guaranteed quality, therapeutic efficacy and immune response of products (Smalling *et al.*, 2004). This concerns mainly caused by their glycosylation, the most prevalent and complex post-translational modification naturally occurring in proteins, which influences important protein properties such as conformation, stability, solubility, pharmacokinetics, *in vivo* activity and immunogenicity (Helenius and Aebi, 2001; Li and d'Anjou, 2009). Especially in therapeutic antibody, since the glycosylation is highly variable and is affected by several parameters of the process of efficacy, understanding of glycosylation and effects to control and manipulation is essential to favor of product quality. For the optimal production of therapeutic mAbs are needed containing the delicate disulfide bond structures and posttranslational modification including glycosylation (Chames *et al.*, 2009). In this regards, the stirred-tank system is the most suitable way for producing mAbs majorly in mammalian cells especially in Chinese hamster ovary (CHO) cells for last decades (Nelson and Geyer, 1991; Kim *et al.*, 2012).

Meanwhile, in order to efficiently produce mAbs with shortening of production period and cost reduction, alternative way are also developed using transgenic plants (De Muynck *et al.*, 2010), insects (Xu, 2014) and animals (Houdebine, 2009; Bertolini *et al.*, 2016; Monzani *et al.*, 2016). The silkworm, an invertebrate bioreactor has been applied to the production of recombinant proteins. Through the transient expression systems with baculovirus-infection,

has been widely used in silkworm for recombinant protein production (Usami *et al.*, 2010; Usami *et al.*, 2011). In more recent work, CD20 mAb were successfully produced in transgenic silkworm which a highly domesticated insect that has been widely used in invertebrate bioreactor (Tada *et al.*, 2015). However, they have unique glycosylation patterns and the baculovirus system is more appropriate for laboratory scale production (Dyck *et al.*, 2003).

In this regard, transgenic animal bioreactor system has been suggested an alternative way for producing recombinant proteins (Wang *et al.*, 2013b). In comparison of other bioreactor system, transgenic animal could overcome complex, biologicals active proteins with efficient growth and economic manner (Dyck, Lacroix et al., 2003). The mammary gland of has generally been regarded the tissue of choice to express valuable recombinant proteins in transgenic animal bioreactors because animal milk such as sheep, goats and cows is easily collected in large volumes (Eyestone, 1999). For example, a recombinant human antithrombin alfa (ATLyn) produced in transgenic goats has been approved in the US and EU for the treatment of patients with hereditary antithrombin deficiency (Adiguzel *et al.*, 2009; Kling, 2009). However, since the long generation time, discontinuous characteristic of the lactation cycle and the complexity of the milk composition, the production of proteins in milk from the species is very limited (Wall *et al.*, 1997).

3.3. Transgenic avian bioreactor system for production of recombinant proteins

Transgenic poultry has the potential to be used for the production of pharmaceutical and industrial proteins in eggs (Ivarie, 2003; Sang, 2004; Han, 2009) and several successfully generated transgenic chickens as a bioreactor

have been reported indeed (Zhu *et al.*, 2005a; Lillico *et al.*, 2007a; Cao *et al.*, 2015). Especially, among the animal bioreactors, chicken have become remarkably expanded during the recent decades because of their numerous merits including relatively short generation time, plentiful reproductive capacity and proper for using them as a human disease model including cancer and inherited disease (Ivarie, 2003; Han, 2009; Johnson and Giles, 2013). Regarding recombinant protein production, chicken egg white (EW) contains ~6.5g of proteins in single egg, composed of only10 major proteins and approximately more than half of term comes from a single gene, ovalbumin which means easily purified the functional proteins compared to other animal system (Harvey *et al.*, 2002; Abeyrathne *et al.*, 2013)**.** More importantly, it is considered to be an ideal model for animal bioreactors for the production of therapeutic antibodies, because it has optimal genetic stability among generations in transgenic lines, and can maintain livestock in virus or pathogen free system (Chadd and Chamow, 2001; Harvey, Speksnijder et al., 2002; Lillico *et al.*, 2007b).

For the development of transgenic birds producing recombinant proteins, virus-mediated methods which injection of viral particles into subgerminal cavity of embryos were regarded a most promising strategy (Harvey, Speksnijder et al., 2002; Lillico, Sherman et al., 2007b; Kyogoku *et al.*, 2008; Kwon, Choi et al., 2010). Although the low efficiencies for transgenic birds production (approximately 1% ~ 4%) and this way difficult to select the desired genotype after transgene integration, this technique have been a useful way for the produce transgenic birds due to its effective introduction efficiency of foreign genes into recipients' genome (Harvey, Speksnijder et al., 2002; Lillico, Sherman et al., 2007b; Kyogoku, Yoshida et al., 2008; Kwon, Choi et al., 2010). Establishment of somatic chimeras by chicken embryonic stem cells (ESCs)-mediated system is successfully demonstrated that producing monoclonal antibody (mAb) from chicken oviduct and egg white (Zhu *et al.*,

2005b). Not only the extensibility with effective selection during cultivation but the high quantity of mAb productivity (maximally over 3mg per egg), this strategy were regarded an alternative production way for chicken bioreactor (Pain *et al.*, 1999; Zhu, van de Lavoir et al., 2005b). However, despite the high efficiency of therapeutic antibody production, this approach is impractical because of the inability to produce germline transgenic chicken. For the production of germline transgenic chicken, transgene deliver to germ cell is essential for successive transmit next generation. The usage of primordial germ cells (PGCs) which the precursor of functional gametes, are well-defined in chicken including culture system and PGC mediated transgenesis (van de Lavoir, Diamond et al., 2006; Lee, Lee et al., 2015a). Due to this technological advancement, chickens' bioreactor system is increasingly available.

Antibodies composed of two light chains and two heavy chains containing three domains, CH1, CH2 and CH3. CH2 domains of two glycan chains contribute to the asymmetric binding of the fraction crystallisable (Fc) to the Fc receptor and its glycosylation stabilizes the Fc structure (Bowden *et al.*, 2012; Reusch and Tejada, 2015). *N*-glycosylation of CH2 domain is one the most important post-translational modifications in therapeutic antibodies due to activation of immune effector functions including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Hodoniczky *et al.*, 2005; Houde *et al.*, 2010). The lack of α1,6-core fucose on Fc region increase ADCC effects by improvement of binding affinity of human IgG1 for the Fcγ receptors IIIa (FcγRIIIa) expressed on immune cells (Umana *et al.*, 1999; Niwa *et al.*, 2004a; Chung *et al.*, 2012). In some cases, produce glycoengineered antibodies lacking core fucose, which results 50-fold increase in ADCC by containing highly afucosylated Fc glycans of IgG1 (Shinkawa *et al.*, 2003). Other glycan structure that high mannose *N*-glycosylation also enhances ADCC activity through higher FcγRIIIa binding affinity, but tends to decreased C1q binding and CDC activity (Yu *et al.*, 2012). The efficacy of CDC

enhanced by high level terminal galactosylation providing increasing binding affinity to C1q and induced activating the complement system, but does not significantly affect ADCC activity. (Hodoniczky, Zheng et al., 2005; Jefferis, 2009). Therefore, *N*-glycosylation including levels of afucosylation, high mannose formation and galactosylation in CH2 domian, is a critical factor in the mechanism of action (MoA) of therapeutic antibodies.

In this regard, chicken believes that *N*-glycan can be terminated by high mannose and rare fucosylated form (Raju *et al.*, 2000). Production of therapeutic mAb from transgenic somatic chimeric chicken EW of previous reports, showed that high mannose and afucose *N*-glycan features (Zhu, van de Lavoir et al., 2005b). And in more recent, the US Food and Drug Administration (FDA) approval on recombinant protein drug (Kanuma; sebelipase alfa) produced by transgenic hens which believes that *N*-glycan can be terminated by high mannose form, it may use this *N*-glycan feature to transport therapeutic enzymes efficiently into diseased cells (Pinkert, 2014; Sheridan, 2016). In this aspect the development of transgenic chicken as a valuable animal bioreactor system are continuously effort (Sheridan, 2016).

4. Transplantation of spermatogonial stem cells (SSCs) for avian transgenesis

4.1. Characteristics of SSCs

Spermatogonial stem cell (SSC), type of adult stem cell, located on the basement membrane of seminiferous tubule and act as foundation for spermatogenesis throughout life in males by balancing self-renewal and differentiation (Phillips, Gassei et al., 2010). In general model of self-renewal and differentiation of SSCs, A_s (A single) spermatogonia were recognized as stem cells in spermatogenesis process (Oakberg, 1971). When dividing A_s cells, the daughter cells divide into two independent stem cells (self-renewal), or they are linked by intercellular bridges each cells that can differentiate into A_{pr} (pair) cells. A_{al} spermatogonial cells divide and differentiate into A-type mature cells (A1-A4), and later develop into intermediate mature type (In) spermatogonial cells and spermatogonia type B (type B spermatogonia) and finally differentiate into primary spermatocytes (Phillips, Gassei et al., 2010). The SSCs self-renewal mechanisms are firmly controlled and regulated by Sertoli cells present in the definite testis microenvironment (Jegou, 1993).

Recent studies demonstrate the importance of transplantation of SSCs in transgenesis and cryopreservation for clinical application (Phillips, Gassei et al., 2010; Goossens *et al.*, 2013). SSC is an important foundation for infertility therapy in cancer survival patients, therefore, its isolation, purification and long-term culture are valuable for clinical application (Singh *et al.*, 2011). However, SSCs are estimated to include only ~ 0.03% of germs cells in mammalian testis (Tegelenbosch and de Rooij, 1993). The scarce availability of SSCs in the testes demand for the efficient and newer methods of isolation and subsequent enrichment of these cells.

Isolation of SSCs with high purity and viability from testis is necessary

for both basic research and clinical applications of SSCs. Isolation of SSCs involves a multi-step enzymatic digestion of the testicular tissues followed by purification using differential plating method or magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) (Shinohara *et al.*, 2000; Izadyar *et al.*, 2002). The SSC culture presents a novel approach for understanding the molecular mechanisms and cell-signaling pathways that control SSC function. Several studies have been tried long and short-term cultures of SSCs (Kanatsu-Shinohara *et al.*, 2003; Nagano *et al.*, 2003). In the past few decades, methods for long-term *in vitro* culture of SSCs have been significantly improved by co-culturing on feeder cells and/or in defined medium including different growth factors, such as glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) (Kanatsu-Shinohara, Ogonuki et al., 2003; Kubota *et al.*, 2004). The feeder system consists of co-culturing of SSCs with a Sertoli cells (Aponte *et al.*, 2006), SIM mouse embryo-derived thioguanine- and ouabain-resistant (STO) monolayer (Kubota, Avarbock et al., 2004) and mouse embryonic fibroblast cells (MEF) (Aoshima *et al.*, 2013).

Numerous studies have been performed for the recognition of specific markers for SSC as it would be helpful for the characterization and isolation of these cells (Klisch et al., 2011). The SSC markers would further assist in preservation of germplasm (Avarbock et al., 2011), germ cell transplantation (Honaramooz et al., 2011) and transdifferentiation process (Simon et al., 2009). However, markers engaged in the regulation of self-renewal and maintenance of SSC in mice and human have only gained special attention.

4.2. Transplantation of SSCs and transgenesis in animals

Since, Brinster & Zimmermann (1994) established germ cell transplantation system, this approach extremely contribute the study of SSCs and spermatogenesis (Brinster and Zimmermann, 1994). This have been verified the reconstitution of spermatogenesis and generate functional sperm after SSCs transplantation into recipient testes in mice and rats (Brinster and Zimmermann, 1994; Ogawa *et al.*, 1999). Later, transplantation of SSCs was tested in boars, bulls, goats, sheep, dogs and chicken and the development of the donor germ cells was observed in those recipients (Honaramooz *et al.*, 2003; Izadyar *et al.*, 2003; Lee *et al.*, 2006; Kim *et al.*, 2008; Herrid *et al.*, 2009a; Herrid *et al.*, 2009b).

To produce of desirable offspring after testicular transplantation, endogenous germ cell depletion by irradiation and anti-spermatogonial alkylating reagent treatment are effective. In 1972, Jones and Jackson were revealed that the busulfan (Myleran) an anti-spermatogonial chemical successfully reduced the spermatogenesis and fertility of male quails (Jones and Jackson, 1972; Jones *et al.*, 1972). This chemical treatment has been widely used to date for spermatogenesis and xenoplantation studies in other animals (Krawczyk and Szymik, 1989; Boujrad *et al.*, 1995; Jahnukainen *et al.*, 2006; Tagirov and Golovan, 2012; Lin *et al.*, 2017). Irradiation of gamma-rays or X-rays can be another effective strategy for spermatogenesis of receptors. Despite lethality risk, this strategy is effective in depletion of recipient spermatogenic cells for spermatogenesis and spermatogonial cell transplantation in many species (Izadyar *et al.*, 2000; Izadyar, Den Ouden et al., 2003; Trefil *et al.*, 2006; Herrid, Olejnik et al., 2009b). These strategies have been used in SSC-mediated spermatogenesis, male sterility and transgenic models.

SSC is a reliable germline competent stem cell that could deliver their genetic information to successive generations, it has been used continuously for

transgenic studies. In rats, with infection of lentiviral vectors, transfected SSCs were successfully generated the genome modified progenies after xenograft into heterologous testes (Hamra *et al.,* 2002; Ryu *et al.,* 2007; Kanatsu-Shinohara *et al.,* 2008). Recently, this strategy has proven effective in species such as tree shrew (Li *et al.,* 2017). In addition, using SSC, transposable element mediated gene knockout and genome editing through homologous recombination have also been successfully proven (Izsvak *et al.,* 2010; Kanatsu-Shinohara *et al.,* 2011). The combination of novel genome editing tools such as CRISPR/Cas9 and the SSC transplantation system may produce various kind of animal model including avian species.

4.3. Application of SSCs in avian species

Currently, SSCs have been successfully isolated and cultured from many species, including human, mice, cattle and Pig (Nagano, Ryu et al., 2003; Kossack *et al.*, 2009; Kuijk *et al.*, 2009; Nasiri *et al.*, 2012). Also in avian species the SSCs were isolated and short termly maintained *in vitro* in chicken and quail (Jung *et al.*, 2007; Pramod *et al.*, 2017). However, till now, reports on studies on SSCs and its application are limited in poultry. Because of the avian models especially quail, considered as an ideal models with small size, easy to grow in laboratory, quick development and early sexual maturity. Therefore, quail SSCs could be suitable germline competent stem cell for transgenesis and conservation studies than any other poultry species.

CHAPTER 3

Induction of Pluripotent Stem Cell-like Cells from Chicken Feather Follicle Cells

1. Abstract

Pluripotent stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are regarded as representative tools for conservation of animal genetic resources. Although ESCs have been established from chicken, it is very difficult to obtain enough embryos for isolation of stem cells for avian conservation in most wild birds. Therefore, the high feasibility of obtaining the pluripotent cell is most important in avian conservation studies. In this study, we generated induced pluripotent stem cell-like cells (iPSLCs) from avian feather follicle cells (FFCs). Avian FFCs are one of the most easily accessible cell sources in most avian species, and their reprogramming into pluripotltient stem cells can be an alternative system for preservation of avian species. Intriguingly, FFCs had mesenchymal stromal cells (MSC)-like characteristics with regard to gene expression, protein expression, and adipocyte differentiation. Subsequently, we attempted to generate iPSLCs from FFCs using retroviral vectors. The FFC-iPSLCs can proliferate with the stem pluripotent property and differentiate into several types of cells *in vitro*. Our results suggest that chicken FFCs are an alternative cell source for avian cell reprogramming into pluripotent stem cells. This experimental strategy should be useful for conservation and restoration of endangered or high-value avian species without sacrificing embryos

2. Introduction

Pluripotent stem cells maintain their undifferentiated state and have the potential to differentiate into all three germ layers, which can form other tissues and organs both *in vitro* and *in vivo*. In mammals, pluripotent stem cells derived from various origins are well established such as embryonic stem cells (ESCs), which originate from the inner cell mass of blastocysts, and embryonic germ cells, which are established from primordial germ cells (PGCs) in embryonic gonads (Evans and Kaufman, 1981; Resnick, Bixler et al., 1992; Thomson, Itskovitz-Eldor et al., 1998). In avian species, ESCs were first established from Eyal-Giladi and *Kochav* (EG&K) stage X blastoderm and maintained *in vitro* for a long period (Pain, Clark et al., 1996). These cells differentiate into all three germ layers and produce somatic chimeras upon *in vitro* propagation on somatic cell feeder layers. However, all these cell types have a limitation in that they are obtained from embryonic tissues, which involves the sacrifice of embryos.

In 2006, the induced pluripotent stem cell (iPSC) technique was introduced as a prominent system with which to convert somatic cells into pluripotent cells, which have the potential to develop into all cell lineages (Takahashi and Yamanaka, 2006). The homeodomains of reprogramming factors such as *Nanog*, *Oct3/4*, *Sox2*, *cMyc*, and *Klf4* are functionally conserved in most animals (Theunissen, Costa et al., 2011; Rossello, Chen et al., 2013). Many studies regarding iPSCs from many kinds of animals including avian species have been reported (Esteban, Xu et al., 2009; Li, Wei et al., 2009; Lu, West et al., 2014; Mo, Li et al., 2014). These techniques can be applied to restoration of endangered animals (Ben-Nun, Montague et al., 2011; Verma, Holland et al., 2012) and acquisition of required cells from somatic cells. More recently, functional germ cells were reprogrammed from iPSCs under specific conditions (Hayashi, Ohta et al., 2011; Hayashi, Ogushi et al., 2012). These findings offer the possibility of animal restoration through somatic cell reprogramming.

In avian species, a few studies have reported the generation of iPSCs using chicken and quail embryonic fibroblasts. Previous results suggest that human and mouse reprogramming factors can induce the somatic cell reprogramming of avian cells (Lu, West et al., 2012; Rossello, Chen et al., 2013; Choi, Kim et al., 2016). Although previous reports demonstrated the successful reprogramming of avian cells, the cell sources used to generate reprogrammed cells were isolated from embryonic tissues. Therefore, it is necessary to identify alternative cell sources from which to establish iPSCs for avian restoration and conservation, without sacrificing embryos.

Avian feathers provide the most easily accessible somatic cell source and have a great potential for regeneration due to various differentiation capabilities (Xi, Nada et al., 2003). Feather follicles contain a source of stem cells, called label-retaining cells, in the collar bulge of avian epidermis (Yue, Jiang et al., 2005). These cells have progenitor or stem cell properties that control epidermis development and homeostasis during physiological molting cycles. For these reasons, FFCs are regarded as a source of adult stem cells for epidermal tissue regeneration and somatic cell reprogramming in birds (Xu, Sun et al., 2011).

In this study, we characterize chicken FFCs as an easily accessible source of adult mesenchymal stromal cells (MSCs) as well as their reprogramming into pluripotent stem cells as an alternative strategy for avian conservation.

3. Materials and methods

Experimental animals and animal care

The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Chickens 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.

Isolation, cultivation and adipogenic differentiation of FFCs

Feather Follicular cells (FFCs) were isolated from feather sheaths of an adult (older than 24 weeks) male GFP-expressing TG chicken (Park and Han, 2012) using angled forceps and washed with PBS containing 1% antibiotic-antimycotic (GIBCO, Grand Island, NY, USA) solution. Isolated FFCs clumps were minced with a sharp blade and dissociated by gentle pipetting in 0.05% (v/v) trypsin solution supplemented with 0.53 mM EDTA (GIBCO). FFCs were then cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Hyclone) and 1% antibiotic-antimycotic in a 5% CO2 atmosphere at 37°C. For adipogenic differentiation potential analysis of FFCs, FFCs (at passage 5) were differentiated into adipocytes by incubation for 2 weeks in adipogenic differentiation medium consisting of high-glucose DMEM (Welgene Inc., Daegu, Korea) supplemented with 10% (v/v) heat-inactivated FBS, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, Mom USA), 200 µM indomethacin (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 10 µg/ml human insulin (GIBCO), and 1% (v/v) antibiotic¬-antimycotic. The adipogenic differentiated cells were stained for 10 min at room temperature in

an Oil Red O (Sigma-Aldrich) solution consisting of distilled water containing 60% (v/v) isopropyl alcohol and 0.3% (wt/v) Oil Red O. After rinsing four times with distilled water, images of Oil Red O-stained cells were obtained under an inverted microscope (CKX-41; Olympus, Tokyo, Japan).

Viral transduction and induced pluripotent stem cell-like cell (iPSLC) induction

Retroviral vector particles were produced by the calcium phosphate co-precipitation method. Briefly, 3×10^6 GP293 cells were plated on a 100 mm culture dish in DMEM (Hyclone) supplemented with 10% (v/v) FBS on the day before transfection. On the day of transfection, 10 μg of pVSV-G (Invitrogen, Carlsbad, CA, USA) and 10 μg each of *pMXs-Oct3/4*, *pMXs-Sox2*, *pMXs-Klf4*, *pMXs-cMyc*, and *pMXs-Nanog* (Kitamura *et al.*, 2003) (Addgene, Cambridge, MA, USA) were added to a final volume of 400 μl of distilled water. After the addition of 80 μl of 2.5 M $CaCl_2$, 500 μl of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, and 1.5 mM Na_2HPO_4, pH 7.0) was added and gently mixed by bubbling. The DNA mixture was then incubated at room temperature for 10 min and subsequently added to the GP293 cells. After 16 h of transfection, the medium was replaced with 4 ml of DMEM containing 10% FBS. The supernatant was harvested after 48 h, filtered through cellulose acetate filters (pore size, 0.22 μm), and subsequently used to infect 3×10^5 FFCs in a 6-well cell culture plate for a further 48 h.

Culture of FFC-iPSLCs

After viral cocktail infection, FFCs-iPSLCs were maintained and sub-cultured with knockout DMEM (Invitrogen) supplemented with 20% (v/v) FBS, 2% (v/v) chicken serum (Sigma-Aldrich), 1× nucleosides (Millipore, Temecula, CA, USA), 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, and 1% antibiotic-antimycotic.

Moreover, 10 units/ml human leukemia inhibitory factor (LIF; Sigma-Aldrich) and 10 ng/ml human basic fibroblast growth factor (bFGF; Sigma-Aldrich) were used for proliferation. FFC-iPSLCs were cultured in an incubator at 37°C with an atmosphere of 5% CO2 and 60–70% relative humidity. FFC-iPSLCs were sub-cultured onto mitomycin-inactivated mouse embryonic fibroblasts (MEFs) at an interval of 3–4 days by gentle pipetting with trypsin-EDTA treatment.

RNA isolation and RT-PCR analysis

Total RNA samples of FFCs and FFC-iPSLCs were prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen). RT-PCR was performed using specific primer sets (Table 3-1). PCR conditions were 94˚C for 5 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min. PCR products were analyzed using a 1% agarose gel.

Cytochemistry and immunocytochemistry of FFC-iPSLC

FFC-iPSLCs were fixed with 3.7% paraformaldehyde for 10 min and rinsed with PBS three times. For PAS (Periodic acid–Schiff) staining, the cells then were immersed in periodic acid solution (Sigma-Aldrich) for 5 min and subsequently incubated in Schiff solution (Sigma-Aldrich) for 5–10 min. For AP (Alkaline Phosphatase) staining, fixed cells were immersed in AP staining solution (Millipore) for 30 min and then rinsed three times with PBS. All procedures were performed at room temperature. Stained cells were observed under an inverted microscope (TE2000-U; Nikon, Tokyo, Japan).

For FFCs immunostaining, cultured cells were fixed in 3.7% paraformaldehyde solution for 10 min, washed three times with PBS, blocked

with blocking buffer consisting of PBS containing 5% (v/v) goat serum and 1% bovine serum albumin for 30 min, and then incubated with primary antibodies diluted 1:200 in blocking buffer at 4°C overnight. Primary antibodies raised against CD105 (Bioss, MA, USA), CD34 (AbD Serotec, UK), CD29 (BD Bioscience, NJ, USA), and melanoma cell adhesion molecule (MCAM) (Southern Biotech, AL, USA) were used for FFCs and stage-specific embryonic antigen 1 (SSEA1) (Santa Cruz Biotechnology, CA, USA) was used for FFC-iPSLCs. Following three washes with PBS, cells were incubated with secondary antibodies labeled with phycoerythrin (Santa Cruz Biotechnology) for 1 h at room temperature. Cells were finally mounted with ProLong Gold antifade reagent (with 4',6-diamidino-2-phenylindole) (Invitrogen) and analyzed under a fluorescence microscope.

Karyotype analysis

FFCs and FFC-iPSLCs were collected for karotyping. Whole blood cells were cultivated in RPMI-1640 medium (Invitrogen, Carlsbad, CA, 90%) supplemented with penicillin and streptomycin (Invitrogen, 0.1%), phytohemagglutinin (Invitrogen, 2%) and fetal bovine serum (Hyclone, Logan, UT, 10%) for 72 h; 0.1 mL colcemid (Sigma-Aldrich, St. Louis, MO, 10 g/mL) was added and cells were incubated for 45 to 50 min. Cultured cells were harvested, treated with 0.06M KCl (hypotonic solution) for 15 min at room temperature, and fixed with acetic acid and methanol (1:3). The fixed cells were dropped onto a slide, dried on a plate warmer for 1 to 2 h, and stained with 4% Giemsa solution for 5 min.

In vitro differentiation

To examine whether FFC-iPSLCs could form embryoid bodies (EBs), colonies were gently agitated and harvested by centrifugation. FFC-iPSLCs

were resuspended in cell culture media lacking growth factors (LIF and bFGF) and placed in a non-adhesive culture plate. The medium was changed every other day and morphology was monitored daily for EB formation. After 7 days, floating masses of cells were collected and plated into a 24-well plate to attach and differentiate. These cells were stained for VIMENTIN (Abcam, UK), FOXA2 (Abcam), and α smooth muscle actin (αSMA; Abcam), mounted with ProLong Gold antifade reagent (with 4',6-diamidino-2-phenylindole), and analyzed under a fluorescence microscope.

Production of Chimera

Stage X White Leghorn chicken embryos from freshly laid eggs were used as recipients for the injection of FFC-iPSLCs. A small window was made on the lateral part of the egg and the shell membrane was removed. Approximately 2 ml of cell suspension at 103 cells/μl was injected into the subgerminal cavity using a micropipette. The egg window of recipient embryo was sealed twice with parafilm and then the sealed eggs were laid down with the pointed end at the bottom. After 7 days of incubation, intestine, liver, skin, brain, heart and gonads were collected from embryos. GFP positive cells of each tissues were observed under a fluorescence microscope. To confirm the somatic chimerism of the embryos, DNA from each tissues was isolated by phenol extraction and CMV-EGFP specific primers were used (Table 3-1). PCR conditions were 94˚C for 5 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min. PCR products were analyzed using a 1% agarose gel.

Statistical analysis

Differences of cell numbers between P0 FFCs and each passage cells (P1-P4) were statistically analyzed by Tukey’s test using GraphPad Prism

statistical software (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered significant.

4. Results

Derivation of FFCs from a GFP-expressing TG chicken

Avian feathers contain dermal papilla cells (Yu et al., 2002) and are thought to be the most easily accessible cell source in poultry, wild birds, and highly endangered birds. Therefore, we reasoned that avian FFCs are a promising cell source for pluripotent stem cell induction. We first attempted to determine the characteristics of FFCs based on their morphology, gene expression, and surface protein expression. To obtain cells from feather follicular tissue, feather cell clumps were collected from a 24-week-old WL chicken. The GFP-expressing TG chicken was established in our laboratory (Figure 3-1a). After removing the shaft, feather cell clumps were dissociated and seeded on a culture plate and fibroblast-like cells grew over a few days of cultivation (Figure 3-1b and 3-1c). After 7 days of continuous culture, most cells had a spindle-shaped fibroblast morphology (Figure 3-1d).

Chicken FFCs exhibit characteristics of MSCs

To examine the expression of MSC markers in FFCs, we performed RT-PCR analysis of MSC markers such as CD29 (Integrin Subunit Beta 1, ITGB1), CD44 (homing cell adhesion molecule, HCAM), CD90 (Thy-1), and CD105 (Endoglin). Selected MSC marker expression was detected in both of bone marrow and FFCs (Figure 3-1e). We confirmed their protein expression by performing immunocytochemistry using specific antibodies (Figure 3-1f). In addition, the proliferation capacity of FFCs was measured by cumulative cell counting over 20 days of continuous cultivation. FFCs maintained their stable proliferation rate up to passage 5, in our culture condition (Figure 3-1g). We next evaluated the *in vitro* differentiation potential of FFCs. After adipogenic induction, there was a marked increase in lipid-containing Oil Red O-positive cells compared with the control group (Figure 3-1h). However, the FFCs did

not differentiate into chondrocytes and osteoblasts (data not shown). These results suggest that chicken FFCs exhibit similar characteristics of bone marrow-derived MSCs described in previous studies (Keating, 2006; Khatri et al., 2009; Bai et al., 2013) and can be a cell source for somatic cell reprogramming.

Reprogramming of FFCs

Subsequently, we tried to reprogram chicken FFCs into iPSCs using the mouse transcription factors which could convert somatic cells into pluripotent stem cells in many species (Takahashi and Yamanaka, 2006; Sumer et al., 2011; Rossello et al., 2013). FFCs were infected with retroviruses that express mouse reprogramming factors (*Oct3/4, Sox2, cMyc, Klf4,* and *Nanog*) to generate chicken reprogrammed pluripotent stem cells (iPSCs), as shown in the schematic diagram (Figure 3-2a). After 3–4 days of viral infection, we observed ESC-like colony formation and could pick up isolated colonies after 7 days of culture (Figure 3-2b and 3-2c). A total of 21 colonies were collected and plated on mitogen-inactivated MEFs in ESC culture medium containing bFGF and LIF. Finally, we obtained three clones with further clonal expansion. The clones were similar to ESCs in terms of their morphology; however, the genes which related on maintained the pluripotency characteristics were silenced in reprogrammed iPSLCs and they could not be maintained for more than ten passages. Because previous studies reported varied characteristics of chicken iPSCs maintained in vitro (Rossello et al., 2013; Dai et al., 2014), we cautiously assumed that the chicken iPSCs were not fully reprogrammed using known transcription factors such as *Oct4, Sox2, Nanog, cMyc, Klf4,* and *Lin28*.

Characterization of GFP-expressing FFC-iPSLCs

To examine the expression of pluripotency marker genes in FFC-

iPSLCs, we performed RT-PCR analysis using primer sets specifically designed for the chicken reprogramming factors *NANOG, POUV, LIN28, KLF4,* and *cMYC* (Table 3-1). All three clones (#7, #19, and #20) were expressed the pluripotency marker genes *NANOG, POUV,* and *LIN28* while these genes were not detect in control FFCs (Figure 3-3a). Other pluripotency marker genes (*KLF4* and *cMYC*) were expressed in FFC-iPSLCs and GFP-expressing FFCs (GFP-FFCs) at the same level. The expression of exogenic reprogramming factors (*Oct3/4, Sox2, Nanog, cMyc,* and *Klf4*) was not silenced (Figure 3-3b). This observation demonstrates that FFC-iPSLCs are generated through partial reprogramming. FFC-iPSLCs were positively stained for AP, a stem cell functional enzyme marker, and with PAS (Figure 3-3c). In immunocytochemical analysis confirmed membrane-specific SSEA1 protein expression in FFC-iPSLCs (Figure 3-3d). Chromosome karyotyping was conducted in FFCs and FFC-iPSLCs. In both of cells were showed normal diploid chromosome (Figure 3-3e).

In vitro differentiation of FFC-iPSCs

The differentiation potential of FFC-iPSLCs was evaluated by investigating EB formation and spontaneous formation of the three germ layers in vitro. For this, FFC-iPSLCs were maintained in suspension culture in the absence of LIF and bFGF. After 7 days in suspension, we observed EBs of various sizes. After transfer to a gelatin-coated plate for further differentiation, we observed expression of all three germ layer-specific markers, FOXA2 (endoderm), VIMENTIN (mesoderm), and αSMA (ectoderm) (Figure 3-4a).

Production of Chimera

To determine the in vivo differentiation potential of FFC-iPSLCs, about

1,000 cells were injected into the subgerminal cavity of White Leghorn chicken embryos at stage X. After 7 days incubation, Of 30 manipulated embryos we collected the embryonic tissues from 4 putative chimeric chicken embryos including intestine (endoderm), liver (endoderm), skin (ectoderm), brain (ectoderm), heart (mesoderm) and gonads (germline). Dissect tissues were analyzed under a fluorescence microscope whether determine by GFP positive cells. As a results, we observed GFP positive cells in all tissues except in intestine (Figure 3-4b). We also confirm the incorporation of FFC-iPSLCs in recipient embryos, 1 (E4) of 4 putative chimeras were positive for EGFP specific primers in all three germ lineage tissues and also in gonad. Therefore, FFC-iPSLCs are regarded to have a sufficient differentiation potential to differentiate into all three germ layers also in germline.

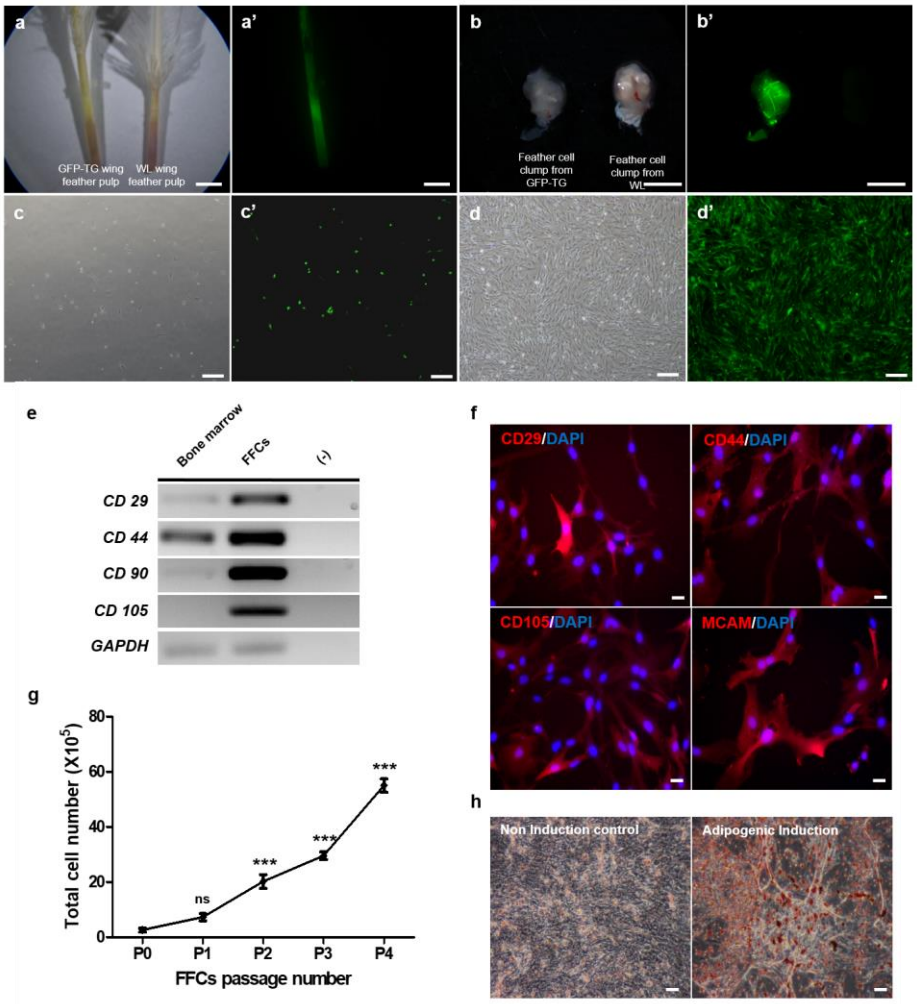
a
a'
b
b'
GFP-TG wing feather pulp
WL wing feather pulp
Feather cell clump from GFP-TG
Feather cell clump from WL
c
c'
d
d'
e
Bone marrow
FFCs
(-)
CD 29
CD 44
CD 90
CD 105
GAPDH
f
CD29/DAPI
CD44/DAPI
CD105/DAPI
MCAM/DAPI
g
Total cell number (X10^5)
80
60
40
20
0
ns
***
***
***
P0
P1
P2
P3
P4
FFCs passage number
h
Non Induction control
Adipogenic Induction

Figure 3-1. Derivation of GFP-FFCs and its characterization. (a, a') Images of wing feather pulp from an adult GFP-expressing TG chicken and an adult WL chicken. (b, b') Morphology of feather follicle cell clumps from feather pulp of a GFP-expressing TG chicken and a WL chicken. (c, c') P0 GFP-FFCs at 1 day after seeding. (d, d') Morphology of *in vitro*-expanded P3 FFCs obtained from feather pulp. Culture-expanded FFCs have a spindle-shaped fibroblastic morphology. (e) RT-PCR analysis of CD29, CD44, CD90, and CD105 in cultured GFP-FFCs (P3). Chicken bone marrow cDNA was used as a positive control of all the markers [–: negative control (no template)]. (f) Immunocytochemical analysis of GFP-FFCs. FFCs were immunostained for the MSC markers CD29, CD44, CD105, and MCAM. (g) Total number of GFP-FFCs in the primary culture (P0, Day 0: initial cell seeding) and four successive sub-passages, P1 (Day 5), P2 (Day 10), P3 (Day 15), and P4 (Day 20). The data points represent mean ± SD. ***Significantly different from passage 1; $P < 0.05$. (h) Adipogenic differentiation of GFP-FFCs *in vitro*. FFCs were cultured in adipogenic induction medium for 2 weeks. Oil Red O-stained accumulated lipids (right panel). Scale bars = 1 cm (A, B), 200 μm (C, D and F) and 50 μm (H).

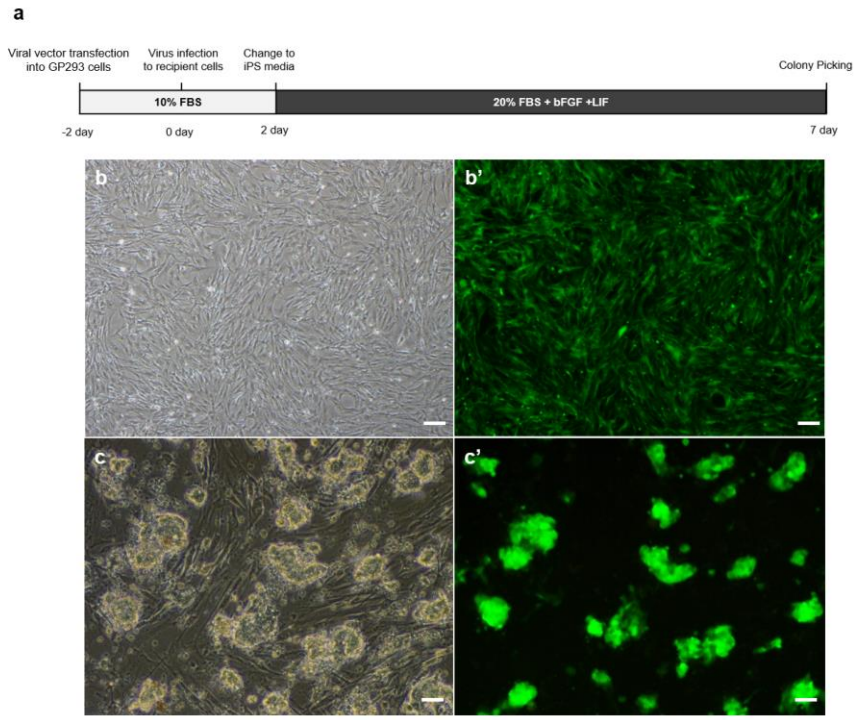

Figure 3-2. Generation of FFC-iPSLCs. (a, a') Schematic diagram of the experimental procedures used to generate chicken iPSLCs from FFCs. (b, b') Morphology of FFCs at 1 day after infection. (c, c') Morphology of P5 FFC-iPSLCs on a MEF feeder layer. Scale bars = 50 μm.

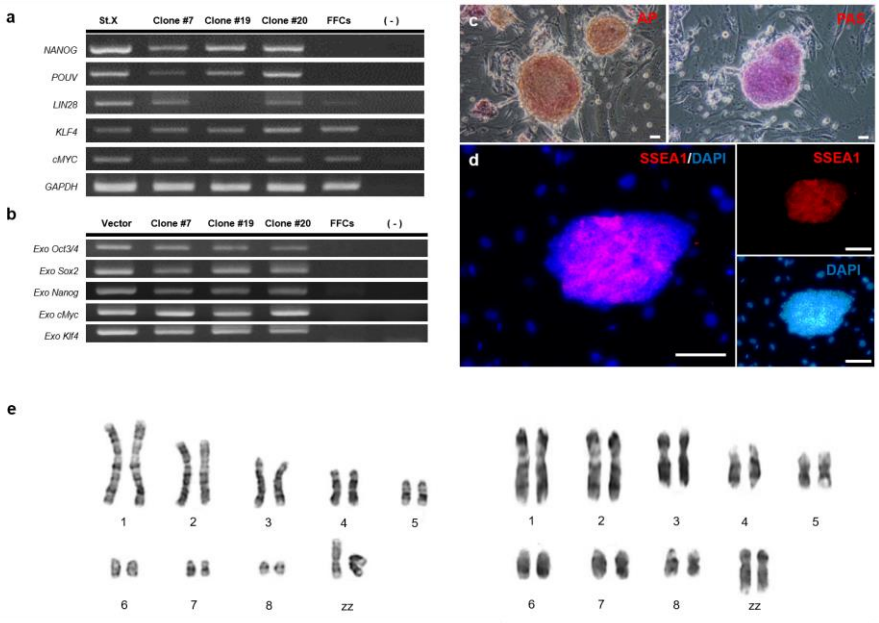

Figure 3-3. Characterization of FFC-iPSLCs. (a) RT-PCR analysis of pluripotency-related gene expression in EG&K stage X blastodermal cells, FFC-iPSLCs (clones #7, #19, and #20), and FFCs. (b) Gene expression of exogenous Oct3/4, Sox2, Nanog, cMyc, and Klf4 in FFC-iPSLCs. pMXs vectors were used for each inserted gene. [–: negative control (no template)]. (c) Cytochemical analysis of FFC-iPSLCs (P5 of clone #20). The cells were characterized by AP (left panel) and PAS (right panel) staining. (d) Immunostaining of FFC-iPSLCs for SSEA1. (e) Chromosomal karyotyping and analysis of male FFCs (left panel) and FFC-IPSLCs (right panel). Chromosomes for sex chromosomes (Z and Z) and macrochromosomes (chromosomes 1 to 8) were banded by Giemsa staining. Scale bar = 20 μm (c and d).

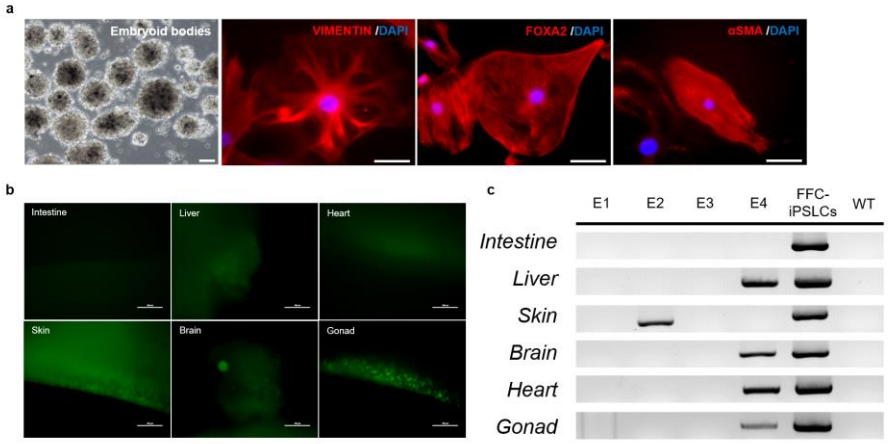

Figure 3-4. Differentiation of FFC-iPSLCs. (a) Embryoid bodies of FFC-iPSLCs formed after 7 days of suspension culture and immunocytochemical analysis of FFC-iPSLC-derived EBs with three germ layer-specific antibodies after attachment and differentiation. Ectoderm (VIMENTIN), endoderm (FOXA2), and mesoderm (αSMA) markers were used to examine in vitro differentiation. (b) Production of day 7 chimeric chicken embryos derived from FFC-iPSLCs. GFP positive cells were detected in various tissues including intestine (endoderm), liver (endoderm), skin (ectoderm), brain (ectoderm), heart (mesoderm) and gonad (germline tissue) of chimeric embryos. (c) PCR analysis of chimeric chicken embryos using EGFP-specific primers for somatic contribution of FFC-iPSLCs cells from 4-chimeric embryos. DNA of FFC-iPSLCs was used as a positive control and WT (Wild type chicken gDNA) was used as a negative control. Scale bar = 100 μm.

Table 3-1. Primer sets for characterization of FFCs and FFC-iPSLCs

Gene	Forward primer	Reverse primer	Product size (base pair)
CD29	GAACGGACAGATATGCAACGG	TAGAACCAGCAGTCACCAACG	300
CD44	GGTTTTATAGTGGGGCATATTGTTATCCC	TTAACCGCGATGCACACGGC	700
CD90	GGTCTACATGTGCGAGCTGA	AAAGCTAAGGGGTGGGAGAA	471
CD105	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	704
NANOG	CAGCAGACCTCTCCTTGACC	AAGCCCTCATCCTCCACAGC	586
POUV	GCCAAGGACCTCAAGCACAA	ATGTCACTGGGATGGGCAGA	511
LIN28	TGCGAAGCCAGGCGAGGAGC	GGACCGGTCACCCGGATGGA	285
Klf4	CCGCTCCCTTCAACCTGGCG	GGCAGTCCCTGCTGCTCAGC	441
MYC	CTTCTACCTGGCGGCGCAGC	AGCTTGGCGGCGGCGGAGAA	297
Oct3/4	GTGGGGGTGAGAAGGCGAAGT	GACGGCATCGCAGCTTGGATACAC	204
Sox2	CGACCGGCGGCAACCAGAAG	GACGGCATCGCAGCTTGGATACAC	260
c-Myc	GGGCTGTACGGAGTCGTAGTC	GACGGCATCGCAGCTTGGATACAC	233
Klf4	CGGTTAGTCGGGGCACCTGC	GACGGCATCGCAGCTTGGATACAC	271
GAPDH	CACAGCCACACAGAAGACGG	CCATCAAGTCCACAACACGG	443
EGFP	TGATGCCGCATAGTTAAGCC	TCCACGCCCATTGATGTACT	595

5. Discussion

In an attempt to develop an alternative cell source for avian conservation, we established a strategy by which to acquire pluripotent cells from adult chicken FFCs. Three individual lines of chicken iPSLCs were readily generated from adult chicken FFCs using mouse reprogramming factors (*Oct3/4*, *Sox2*, *cMyc*, *Klf4*, and *Nanog*). Established chicken iPSLCs expressed ESC markers and pluripotency-associated genes at comparable levels to those in stage X blastodermal cells (Pain, Clark et al., 1996; Lavial *et al.*, 2007) and were morphologically indistinguishable from ESCs/iPSCs from other species. Furthermore, chicken iPSLCs, although partially reprogrammed, had a capacity to differentiate into all three germ layers *in vitro*.

Although more than 1,200 species of birds (13% of living bird species) in the world are considered to be threatened or endangered (Hoffmann *et al.*, 2010), ways to conserve their germplasm are extremely limited, except for some poultry species whose germ cells can be isolated and expanded (Karagenc *et al.*, 1996; Ono and Machida, 1999; Kim *et al.*, 2004; van de Lavoir, Diamond et al., 2006; Park, Kim et al., 2008). A number of previous studies demonstrated induction of iPSCs from embryonic fibroblasts in avian species (Lu, West et al., 2012; Rossello, Chen et al., 2013; Lu, West et al., 2014; Choi, Kim et al., 2016). However, this embryonic cell source is inappropriate for avian restoration, mainly due to limited accessibility to PGCs by sacrificing embryos at early development stages.

In human, iPSCs are formed significantly more efficiently (20–1400-fold) from hair follicle dermal papilla-derived cells than from fibroblasts (Tsai *et al.*, 2010). Similar to human hair follicles, avian feathers contain dermal papilla cells (Yu *et al.*, 2002) and are thought to be the most easily accessible cell source in poultry, wild birds, and highly endangered birds. Therefore, we reasoned that avian FFCs are a promising cell source for pluripotent stem cell induction. We first attempted to determine the characteristics of FFCs based on

their morphology, gene expression, and surface protein expression. Chicken FFCs, similar to human hair follicle cells, had MSC characteristics (Wang *et al.*, 2013a). The characteristics of chicken FFCs were similar to those of chicken MSCs described in previous studies (Khatri *et al.*, 2009; Bai *et al.*, 2013). However, the differentiation potential of FFCs differed from that of bone marrow-derived MSCs, especially with regard to osteogenic differentiation (data not shown). This differentiation bias of FFCs might be explained by an origin-dependent differentiation potential, similar to that of mouse dermal papilla cells (Myllyla *et al.*, 2014). Bone morphogenetic proteins might be indispensable for osteogenic lineage induction of FFCs, in addition to normal induction medium.

Subsequently, we tried to reprogram chicken FFCs into iPSCs using the mouse transcription factors *Oct4*, *Sox2*, *Nanog*, *cMyc*, and *Klf4*. Although chicken and mouse transcription factors have relatively low homology, the sequences containing the transcription factor-binding motif of each factor exhibit higher homology in each species (Theunissen, Costa et al., 2011; Rossello, Chen et al., 2013). Our results showed that mouse transcription factors can induce pluripotent stem cells from chicken FFCs. However, the pluripotency-related genes were silenced in reprogrammed iPSLCs expanded for more than ten passages (data not shown). Because previous studies reported varied characteristics of chicken iPSCs maintained *in vitro* (Rossello, Chen et al., 2013; Dai *et al.*, 2014; Choi, Kim et al., 2016), we cautiously assumed that the chicken iPSCs were not fully reprogrammed using known transcription factors such as *Oct4*, *Sox2*, *Nanog*, *cMyc*, *Klf4*, and *Lin28*.

Despite the potential of FFC-iPSLCs, the germline competency or germline differentiation of these cells was not demonstrated in this study. In the field of avian cloning, PGC-mediated germline transmission is regarded as the most efficient way to conserve wild birds (Kang, Choi et al., 2008; Wernery, Liu et al., 2010; van de Lavoir, Collarini et al., 2012). However, ways to isolate and expand germ cells are extremely limited in most avian species, except some

poultry species (Kim *et al.*, 2005a; Park, Kim et al., 2008; Choi, Kim et al., 2010; Song *et al.*, 2014). Therefore, it might be ideal to derive germ line-competent cells from avian iPSCs or iPSLCs in future studies. In mammalian systems, germ cell induction from iPSCs has been reported (Imamura *et al.*, 2010; Hayashi, Ohta et al., 2011; Hayashi, Ogushi et al., 2012). One group reported the expression of germ cell markers such VASA and DAZL in chicken iPSCs (Lu, West et al., 2014). Although these results do not fully explain the mechanism by which to induce germ cell properties in chicken iPSCs, they may provide clues to generate germ cells from avian somatic cell sources.

In summary, we successfully generated iPSLCs from FFCs in the first case of avian adult cell reprogramming into a pluripotent state. Chicken FFCs had MSC-like characteristics, which indicates that they have a high potential to give rise to iPSCs, similar to MSCs, and are easily isolated (Tsai, Clavel et al., 2010). This was supported by the demonstration of iPSLC induction and their *in vitro* characterization. This system may provide an alternative way to conserve wild and endangered birds and provide a substitute for traditional embryo-dependent cell sources.

CHAPTER 4

Evaluation of a germline transgenic chicken system as an efficient bioreactor for the production of therapeutic antibody with enhanced Fc effector functions

1. Abstract

With the modern genetic techniques, various animal bioreactor systems play crucial role in the production and functional enhancement of anti-cancer antibodies. Chicken is considered as the most efficient animal bioreactor for the production of anti-cancer antibodies because of its relatively short generation time, plentiful reproductive capacity, and daily deposition in the egg white. Although several studies have focused on the production of anti-cancer antibodies in egg white, in-depth studies about biological activity with physiological characteristics of transgenic chicken derived anti-cancer antibodies have not been fully carried out. Here, we reported the production of anti-cancer monoclonal antibody against the CD20 protein from egg whites of transgenic hens, and validated the bio-functional activity of the protein in B lymphoma and B lymphoblast cells. Quantitative analysis showed that deposition of the chickenised CD20 monoclonal antibody (cCD20 mAb) from transgenic chickens was accelerated even in successive generations as copy number accumulation. As a result of ultra performance liquid chromatography (UPLC) tandem mass spectrometry (LC/MS/MS) analysis showed that the cCD20 mAb exhibited 14 kinds of N-glycan patterns with high mannose, afucosylation and terminal galactosylation forms. The cCD20 mAb did not show the significant improvement on Fab binding affinity, but remarkable increases of Fc-related functions including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) compared to commercial rituximab, a chimeric mAb against CD20. Our results suggest that the transgenic chicken bioreactor is an efficient system for producing anti-cancer therapeutic antibodies in terms of consistent expression and highly enhanced Fc effector functions.

2. Introduction

The application of recombinant monoclonal antibodies (mAbs) widely used in therapeutic applications such as anti-cancer therapy and, inflammatory diseases (Nelson *et al.*, 2010; Scott *et al.*, 2012). As one of the therapeutic mAbs, rituximab, targeting CD20 antigen in B cells for treating non-Hodgkin lymphoma, chronic lymphocytic leukemia (CLL), and rheumatoid arthritis (Hainsworth *et al.*, 2000; Taylor, 2007). The therapeutic mechanisms of rituximab in the treatment of these diseases based on several actions in including induced apoptosis, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Cartron *et al.*, 2004; Glennie *et al.*, 2007). However, although rituximab has been widely used in the treatment of lymphoma and other autoimmune disorder, its efficacy remains variable and often modest (Boross and Leusen, 2012). Therefore, it is still needed to improve the efficacy of CD20 mAbs.

Meanwhile, in order to efficiently produce mAbs with high efficacy in bioreactor systems, alternative way are developed using living organisms including transgenic plants (Wang *et al.*, 2015) and, insects (Tada, Tatematsu et al., 2015). The transgenic animal bioreactor system has been suggested an another way for producing recombinant proteins (Wang, Zhao et al., 2013b). In comparison of other bioreactor system using living organism, transgenic animal could overcome complex, biologically active proteins with efficient growth and economic manner (Dyck, Lacroix et al., 2003). The use of chicken as an animal model has been increased in recent decades due to its relatively short generation time; plentiful reproductive capacity; and suitability for use as a human disease model, including in studies of cancer and inherited disease (Ivarie, 2003; Han, 2009; Johnson and Giles, 2013). Regarding the production of recombinant protein, a single egg white of chicken contains ~6.5 g protein and it composed of only 10 major proteins (Harvey,

Speksnijder et al., 2002). In addition, more than half of egg white proteins come from a single gene, ovalbumin (OV), which means that chicken bioreactor system is more competitive for mass production and following purification of exogenous proteins compared to other animals (Harvey, Speksnijder et al., 2002; Lillico *et al.*, 2005). More important, its genetic stability among generations enhances the merit in the uses of the chicken system for the production of therapeutic antibodies (Chadd and Chamow, 2001; Harvey, Speksnijder et al., 2002; Lillico, Sherman et al., 2007b).

Previous researches about development of transgenic birds producing recombinant proteins were largely relied on virus-mediated methods which injection of viral particles into the subgerminal cavity of embryos (Harvey, Speksnijder et al., 2002; Lillico, Sherman et al., 2007b; Kwon, Choi et al., 2010). However, it is considered to be difficult to select of the desired genotype among the progenies due to low germline transmission efficiency (approximately 1–4%), although the viral infection to embryonic stem cell (ESC) showed possibilities on mass production of recombinant protein from chicken oviduct and egg white(Pain, Chenevier et al., 1999; Zhu, van de Lavoir et al., 2005b). Worse, it has fundamental limitation on safety issues of the produced recombinant proteins for human uses due to its viral origin (Thomas *et al.*, 2003; Park, 2007). These limitations have been overcome with development of primordial germ cells (PGCs)-mediated transgenic technology and non-viral vector system such as *piggyBac* transposition. The use of transgenic PGCs much more enhanced the efficiency for producing germline transgenic chicken (van de Lavoir, Diamond et al., 2006; Lee, Lee et al., 2015a), and recent studies have shown this technological advancement could be used as an efficient system for development of chicken bioreactors (Park and Han, 2012; Park *et al.*, 2015).

The one of another distinctive advantages on chicken bioreactor system is post-translational modification (PTM) of the produced recombinant protein. The recombinant proteins from transgenic chicken bioreactor is known to produce *N*-

glycan species terminated by high mannose and core afucosylated form (Pinkert, 2014). The similar *N*-glycan profiles are reported in therapeutic monoclonal antibody (mAb) produced from egg white of somatic chimeric transgenic chicken (Zhu, van de Lavoir et al., 2005b), and more recent studies have noted that the U.S. Food and Drug Administration's approval of recombinant protein drugs (Kanuma; sebelipase alfa) produced by transgenic hens states that *N*-glycan can be terminated by the high mannose form, and this *N*-glycan feature is used to transport therapeutic enzymes efficiently into targeted cells (Sheridan, 2016). In this context, the *N*-glycan feature of recombinant proteins from chickens can be used to enhance the efficacy of human therapeutic proteins, especially, anti-cancer antibody's function. Indeed, *N*-glycosylation of the CH2 domain of antibody is one of the most important PTM in therapeutic antibodies, because it has a role in activation of the immune effector functions, including ADCC and CDC by stabilisation of the Fc structure (Hodoniczky, Zheng et al., 2005; Houde, Peng et al., 2010; Pinkert, 2014; Park, Lee et al., 2015). Previous researches reported that the lack of α1,6-core fucose *N*-glycan of Fc region dramatically increases its ADCC activity through the improvement of binding affinity of human IgG1 for the Fcγ receptors IIIa (FcγRIIIa) expressed on immune cells (Umana, Jean-Mairet et al., 1999; Shinkawa, Nakamura et al., 2003; Niwa, Hatanaka et al., 2004a; Chung, Quarmby et al., 2012). And the antibody bearing high level of *N*-linked high mannose species also shows enhanced ADCC activity and increased FcγRIIIa binding affinity (Yu, Brown et al., 2012). Moreover, the increase of terminal galactose in *N*-glycan content is correlated with higher CDC activity and C1q binding affinity but does not significantly affect ADCC activity (Hodoniczky, Zheng et al., 2005; Jefferis, 2009). Therefore, the characterization of *N*-glycan species on Fc region is one of the critical steps for the production of therapeutic antibodies relying on ADCC and CDC for their mode of action. In this regards, we anticipated that the anti-cancer antibody produced in transgenic chickens would show high levels of Fc functional effects on ADCC and CDC.

In this study, the germline transgenic chicken was applied in order to strengthen the function of therapeutic antibodies using the unique feature of chicken bioreactor system in term of *N*-glycan profile. We produced an anti-CD20 human-mouse chimeric mAb rituximab as a representative anti-cancer antibody from transgenic chickens. Then, we purified the chickenised CD20 mAb (cCD20 mAb) from different genotyped, transgenic chicken egg whites, and performed the quantitative and qualitative analysis including the *N*-glycan structures. We further explored the biofunctional activity of cCD20 mAb from transgenic chickens with commercial rituximab.

3. Materials and methods

Experimental animals and animal care

The care and experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Chickens 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.

Construction of cCD20 mAb expression vector

Codons of the anti-CD20 mAb gene were optimised for expression in the hen using the *Gallus gallus* codon database (https://www.kazusa.or.jp/codon). A codon-optimised cCD20 mAb gene consisting of a chicken lysozyme signal peptide sequence with a VL + hIg kappa region and VH hIgG constant region, consecutively conjugated with internal ribosome entry site sequences (Figure 4-1a), was synthesised by Bioneer Company (Daejeon, Korea). This cassette was ligated into the *piggyBac*, which contained 3.5kb of chicken OV promoter and 1.6 kb of the 3'-UTR, including the poly A tail sequences. This vector based on the *piggyBac* OVcEGF vector from our previous study (Park, Lee et al., 2015).

Transfection and G418-selection of PGCs and transplanted donor germ cells in recipient embryos

A male PGC line was established using day-6 (stage 28) WL embryonic

gonads and was maintained in knockout Dulbecco Modified Eagle Medium (KO-DMEM) (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 20% (v/v) foetal bovine serum (Invitrogen, Life Technologies), 2% (v/v) chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1× nucleoside mix (EMD Millipore, Temecula, CA, USA), 2 mM L-glutamine, 1× nonessential amino acid mix, 2-ME, 10 mM sodium pyruvate, and 1× antibiotic-antimycotic mix (Invitrogen, Life Technologies). Human basic fibroblast growth factor (10 ng/mL; Koma Biotech, Seoul, Korea) was used to activate PGC proliferation. PGCs were cultured at 37°C in an atmosphere of 5% (v/v) CO_2 and at 60–70% relative humidity. Cultured PGCs were subcultured onto mitomycin-inactivated layers of mouse embryonic fibroblasts at 5- to 6-day intervals via gentle pipetting (in the absence of any enzyme treatment). The cCD20 mAb expression vector and the CAGG-PBase (pCyL43) were co-transfected into the PGC line via lipofection using the Lipofectamine 2000 reagent (Invitrogen, Life Technologies). At a day after transfection, G418 (to 300 mg/mL) was added to culture media to allow the selection of transfected PGCs. The complete selection period required up to 3 weeks. All transfection and selection procedures followed those established in our previous reports (Park and Han, 2012; Park, Lee et al., 2015).

To inject selected transfected PGCs into recipient embryos, we made a small window on the pointed end of each recipient KO egg at Hamburger and Hamilton (HH) stages 14–17, and microinjected via micropipette a 2 μL aliquot containing at least 3000 PGCs into the dorsal aorta of the recipient embryo. Each egg window was sealed with paraffin film, and the eggs were incubated with the pointed end down prior to further screening and eventual hatching. Testcrossing and detection of transgenic WL chickens with a dominant pigmentation inhibitor gene (*I/I*) and black KOs with a recessive pigmentation inhibitor gene (*i/i*) was performed to obtain donor PGCs and recipient embryos, respectively. Via testcrossing analysis by mating with KO females (*i/i*), putative germline chimeras were identified by their offspring.

Sperm from KO recipient male chickens (*i/i*) can father only black KOs because of the presence of only the recessive pigmentation inhibitor gene (*i/i*), whereas WL donor-derived sperm (*I/I*) can father white hybrids with the *I/i* genotype pigmentation genotype.

Identification of the transgene integration site by DNA walking

Transgene insertion sites were identified using a DNA Walking SpeedUp Premix Kit-II (Seegene, Seoul, Korea) according to the manufacturer's protocol. PCR products after the third round of DNA walking were excised from the agarose gel, purified using a Power Gel Extraction Kit (Promega, Madison, WI, USA), and then cloned into the pGEM-T Easy Vector (Promega). The cloned PCR products were sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences of the 5'-flanking regions were analysed using the Basic Local Alignment Search Tool (BLAST) Assembled Genome database (http://blast.ncbi.nlm.nih.gov/BLAST.cgi) and the UCSC Genome Bioinformatics browser (http://www.genome.ucsc.edu) to identify transgene insertion sites in the genomes of transgenic chickens.

Testcross for generation of homozygous chickens with multi copies of transgene

To generate homozygous chickens with two copies of the cCD20 mAb transgene at the same locus, we testcrossed G1 transgenic males with the cCD20 mAb transgene on chromosome 4; Tg4(he) and G1 transgenic females with the transgene on chromosome 33; Tg33(he) with opposite-sex wild-type chickens. Subsequently, we produced transgenic chicken containing a transgene at a different locus [Tg33(he)4(he); containing a heterozygous transgene on both chromosomes 4 and

33]. To generate four-copy homozygous chickens [Tg33(ho)4(ho); containing a homozygous transgene on both chromosomes 4 and 33], we mated Tg33(he)4(he) heterozygous roosters (G2) with Tg33(he)4(he) heterozygous transgenic hens (G2). Pedigrees of the transgenic progeny of rooster Tg33(he) and hen Tg4(he) and successive progeny are shown in Figure 4-1b. We used specific primer sets (Table 4-1) to distinguish between the wild-type and transgenic loci. In the presence of the transgenic locus, the forward primer binds to the chicken genome and the reverse primer binds to the chicken genome or transgene. To distinguish the genotype of progeny of Tg4, a 315 bp PCR product was derived from non-transgenic (Non-TG) wild-type chickens, whereas a 480 bp PCR product was amplified from transgenic chickens. In progeny of Tg33, a 431 bp PCR product was derived from Non-TG wild-type chickens, whereas a 610 bp PCR product was amplified from transgenic chickens. In heterozygous transgenic chickens both the wild-type and transgenic loci were amplified, yielding two PCR amplicons in both wild-type loci and transgenic loci, whereas in the homozygous transgenic chickens only transgenic loci were amplified (Figure 4-1c and 4-1d). PCR was performed via initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All reactions were terminated by final incubation at 72°C for 7 min.

Extraction and purification of the cCD20 mAb in transgenic hens

Egg white was sheared for 30 min at 25°C and homogenised. The egg white suspension was added to three volumes of reverse osmosis water and stirred for 30 min. The egg white suspension was adjusted to pH 6.0 using 0.5 M phosphoric acid and centrifuged for 20 min at 12,100 × g, which removed most egg white proteins, including ovomucin. The supernatant was adjusted to pH 7.4 using 0.5 M dibasic sodium phosphate. Then the sample was filtered through a 0.2 μm syringe filter. The

human IgG was purified on a Protein A column (5 mL; GE Healthcare) at a 4 mL/min flow rate with a pH gradient. The Protein A column was washed with 5 column volumes (CV) of loading buffer (20 mM sodium phosphate, pH 7.4) for equilibration. The column was loaded with a 200 mL sample at a flow rate of 4 mL/min and then washed with 16 CV loading buffer. Bound human IgG proteins were eluted using 3 CV elution buffer (100 mM sodium citrate, pH 3.0). Eluted fractions were examined using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Quantification of crude egg white and purified cCD20 mAb using ELISA

Purified cCD2 mAb were measured using human IgG ELISA kits (Koma Biotech, Seoul, Korea) according to the manufacturer's instructions. This kit used the double-antibody sandwich method. In this method, the optical density is proportional to the amount of monoclonal antibodies. We measured the concentration of monoclonal antibodies by comparing the optical density of the standard protein. The human reference serum was used for the calibration curve containing various diluted concentrations of IgG.

Western blot analysis of anti-CD20 mAb

Purified cCD20 mAbs were loaded in each lane for separation on 10% SDS-polyacrylamide gels. Resolved proteins were transferred onto a Hybond 0.45 PVDF membrane (GE Healthcare Life Sciences, Little Chalfont, UK) and blocked with 3% skim milk for 1 h at room temperature (Sigma-Aldrich). The blocked membrane was incubated overnight at 4°C in the presence of goat anti-human IgG (H+L) primary antibody (Alpha Diagnostic) diluted with blocking buffer (1:1000) and then incubated with horseradish peroxidase–conjugated secondary antibody (Thermo Fisher

Scientific) at a dilution of 1:10,000 for 1 h. Immunoreactive proteins were visualised using the ECL Western blot detection system (GE Healthcare Life Sciences).

N-glycan profiling of cCD20 mAbs from G3 Tg33(ho)4(ho) transgenic chickens

N-glycan analysis of cCD20 mAbs from G3 Tg33(ho)4(ho) transgenic chickens was performed by LC/MS/MS. Briefly, purified cCD20 mAb (200 μg) was incubated with 10 mM dithiothreitol in 50 mM ammonium bicarbonate buffer for 30 min at 56°C. PNGase F (500 units) was added and incubated at 37°C for 16 h. To precipitate deglycosylated mAbs, we added 2 mL cold ethanol to the reacted sample and then incubated it at –20°C for 4 h. After incubation, the sample was centrifuged at 10,000 × g for 10 min and the supernatant, having released *N*-glycan, was transferred to a new tube and dried completely using a Speed-Vac concentrator. The dried sample was further labelled with procainamide for fluorescence analysis. For the labelling, 350 μL dimethyl sulfoxide and 150 μl glacial acetic acid were added to the glass vial. Subsequently this mixture (100 μL) was added to 13 mg procainamide, and the solution was completely dissolved. The mixture was completely dissolved after the addition of 6 mg sodium cyanoborohydride ($NaBH_3CN$). 5 μL of the mixture was added to a completely dried *N*-glycan sample and reacted for 16 h at 37°C. To remove any excess procainamide reagent, we performed solid phase extraction using an S-cartridge. We activated and equilibrated the S-cartridge by mixing 1 mL HPLC-grade H_2O, 1 mL 30% acetic acid (five times), and 1 mL 100% acetonitrile (four times). The procainamide-labelled sample was mixed with 100 μL 100% acetonitrile, loaded on an S-cartridge, and washed with excess procainamide reagent not a part of the fluorescent labelling with 1 mL acetonitrile (five times). Then we eluted procainamide-labelled *N*-glycan by adding 1.5 mL H_2O. The procainamide-labelled *N*-glycan sample was analysed and quantified by UPLC/FLD coupled with mass

spectrometry. An ACQUITY UPLC BEH Glycan column (2.1×150 mm, 1.7 μm; Waters, New Castle, DE, USA) was used for the separation and detection of *N*-glycan on the fluorescence detector (Waters iClass UPLC). LC conditions were as follows: flow rate (0.5 mL/min), column temperature (60°C), mobile phase buffer A (100 mM ammonium formate, pH 4.5), buffer B (100% acetonitrile), injection volume (8 μL), linear gradient (75–60% B for 46.5 min, 60–0% B for 1.5 min, 0% B for 1 min, 0–75% B for 1 min, and 75% B for 13 min). A high-resolution mass spectrometry, Triple-TOF MS (AB SCIEX, Concord, Ontario, Canada), was used for *N*-glycan identification. The percentage distribution of the *N*-glycan was analysed with Empower (Waters).

CD20 binding assay

The cellular reactivity of the CD20 mAbs from transgenic chickens and rituximab (MabThera, Roche) was assessed using the CD20-expressing B-cell lymphoma cell line Raji. Cells were incubated with monoclonal antibodies at various concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, or 10 μg/mL) for 30 min at room temperature. Bound monoclonal antibodies were detected using FITC-conjugated goat anti-human IgG (Invitrogen). Cells were analysed with FACSCalibur, and data were analysed with FlowJo (ver. 7.6.5; Tree Star, Ashland, OR, USA).

Annexin V assay for cCD20 mAb-induced apoptotic cells

Raji cells were plated in 24 well plates (2×10^5 cells per well) with 0.1, 1, or 5 μg/mL of monoclonal antibodies. After incubation for 24 h, cells were transferred to centrifuge tubes for the apoptosis assay. To examine apoptotic cell death, we performed Annexin V (Thermo Fisher Scientific) staining according to the

manufacturer's protocol and analysed cells by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). Annexin V negative cells were considered viable, and Annexin V–positive cells were considered apoptotic. Cells were analysed with FACSCalibur, and data were analysed with FlowJo (ver. 7.6.5; Tree Star).

CDC assay

We performed complement-dependent cytotoxicity (CDC) assays by modifying the technique used in a previous study(Natsume *et al.*, 2009). Briefly, Raji cells were incubated at 50,000 cells per well in an opaque 96 well tissue culture plate with various concentrations of anti-CD20 antibodies (rituximab and cCD20 mAbs) including 16% human serum (Sigma, St. Louis, MO, USA) in supplemented RPMI-1640 for 2 h at 37°C, 5% CO_2. After incubation, the cell proliferation reagent WST-1 (Roche Diagnostic, Basel, Switzerland) was added (15 μL/well) and the plates were further incubated for 4 h to detect the live cells. The absorbance (A_{450}–A_{650}) of the formazan dye produced by metabolically active cells of each well was detected on an epoch microplate reader (BioTek, Winooski, VT, USA). Cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = 100 \times (E–S)/(M–S),$$

where E is the absorbance of the experimental well, S is that in the absence of mAb (cells were incubated with medium and complement alone), and M is that of medium and complement in the absence of target cells and antibody.

ADCC assay

The ADCC assay was performed using an ADCC Reporter Bioassay

complete kit (Promega) according to the manufacturer's protocol. All components came from the ADCC Reporter Bioassay complete kit. Briefly, CD20-positive WIL2-S cells were centrifuged and resuspended in RPMI 1640 + low IgG serum and seeded at 10,000 cells per well in an opaque 96 well tissue culture plate with effector cells. Control anti-CD20 antibody, rituximab, and chicken cCD20 mAbs were serially diluted and incubated with the WIL2-S cells for approximately 6 h at 37°C, 5% CO_2. Cell death was determined using the Bio-Glo Luciferase assay, and measurements were taken on a microplate reader. Data were fitted to a 4PL curve using GraphPad Prism.

Statistical analyses

Significant differences between groups were examined statistically using Student's *t* test and one-way ANOVA. A p value < 0.05 was considered statistically significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).

4. Results

Production of cCD20 mAb expressing transgenic chickens and generation of homozygous chickens with multi copies of transgene

To produce transgenic chickens expressing cCD20 mAb, the gonadal PGC line from White Leghorn (WL) containing the *piggyBac* transposon-based cCD20 mAb expression cassette (Figure 4-1a) was established and transplanted into recipient. After sexual maturation of the recipients, the founder chickens (G0) were testcrossed with Korean Oge (KO) female chickens and the feather colours of the progenies were evaluated for identifying germline transmission. Three male germline chimeric chickens were used for testcross analyses to generate transgenic chickens. The efficiency of the generation of transgenic chickens was 69.6%, 80.0%, and 91.7%, respectively, and transgene integration locus of the transgenic chickens were identified by DNA walking analyses using target-specific primers (TSP) (Table 4-1). Among the G1 transgenic chickens, one transgenic male (Tg4; transgene integrated at chromosome 4) and one transgenic female (Tg33; transgene integrated at chromosome 33) were selected and confirmed as having the transgene integration in their genome sequence (Figure 4-2) for further study.

To generate homozygous chickens with multi copies of transgene, we mated two G1 transgenic chickens Tg33 and Tg4 with a non-transgenic chicken, respectively (Figure 4-1b). To evaluate the genotype of transgenic progenies, we designed two primer sets to detect the wild-type or transgene locus by amplicon size (Table 4-1 and Figure 4-1c). The progenies bearing one copy of the transgene locus at chromosome 33 were designated as the Tg33(he), the progenies bearing one copy of the transgene locus at chromosome 4 were designated as Tg4(he) and/or two copies of both loci were designated as Tg33(he)4(he). The representative results of the genomic PCR-based genotyping on transgenic progenies showed that Tg33(he)4(he)

contained the 480 bp and 610 bp amplicons which means transgene integrated on chromosome 4 and 33, but also exhibit 315 bp and 431 bp amplicons indicating the progeny has wild-type locus in other allele chromosome 4 and 33 (heterozygous). PCR analysis of Tg33(ho)4(ho) showed only 480 bp and 610 bp amplicons, which means homozygous transgenic chicken bearing transgene in each allele on different chromosomes (Figure 4-1d).

Detection and purification of cCD20 mAb from egg whites of transgenic chickens

The chickenised anti-CD monoclonal antibodies (cCD20 mAbs) from two G2 egg-laying transgenic hens' egg whites that have different transgene integration site G2 Tg4(he) and G2 Tg33(he) were used to measure the concentration after purification. The concentrations of mAbs was 2.04 ± 0.93 μg/mL and 2.21 ± 0.82 μg/mL in G2 Tg4(he), G2 Tg33(he), respectively, and there was no significant difference in expression levels between the transgenic chickens (Figure 4-3a). Western blot analysis showed that both heavy (H; about 50 kDa) and light (L; about 25 kDa) chain–specific bands for anti-human IgG antibody were clearly detected in purified egg white samples from both transgenic hens (Figure 4-3b). To confirm expression of the heavy chain and light chain of cCD20 mAb from Tg33(he)4(he) and Tg33(ho)4(ho), we further analysed the eluted mAbs by SDS-PAGE under non-reducing (N) and reducing (R) conditions (Figure 4-3c). Under non-reducing conditions, the full-length assembled major band was detected at 150 kDa (2 sets of heavy chains and of light chains), whereas 50 kDa and 25 kDa bands were abundantly detected in both of Tg33(he)4(he) and Tg33(ho)4(ho) under reducing conditions (Figure 4-3c). These results indicate that cCD20 mAbs are successfully deposited in the chicken body, are secreted into the egg white, and possibly form H_2L_2 mature antibody subunit structures in different transgene integration types.

We then compared concentrations of cCD20 mAbs from G2 Tg4(he) (one-copy), G2 Tg33(he)4(he) (two-copy), and G3 Tg33(ho)4(ho) (four-copy) transgenic hen egg whites. Compared to chickens bearing one copy of the transgene [1.96 ± 0.22 μg/mL in G2 Tg4(he) transgenic chickens], concentrations of cCD20 mAbs from multi-copy transgene-integrated chickens, 12.88 ± 0.84 μg/mL in G2 Tg33(he)4(he) and 18.17 ± 1.4 μg/mL in G3 Tg33(ho)4(ho), respectively, were significantly higher (Figure 4-3d). Comparing the concentrations between G2 Tg33(he)4(he) and G3 Tg33(ho)4(ho), concentration of mAbs from the later one was significantly higher. These results confirmed the improved yield of cCD20 mAbs from the transgenic chicken as increase of transgene copy number. Collectively, these results indicate that the cCD20 mAb produced from transgenic chickens showed stable expression regardless of the integration site and improvement of the protein deposition in successive generations with combinatory transgene quantities.

Glycosylation analysis of cCD20 mAbs derived from transgenic chickens

The effective biological activity of therapeutic antibodies depends on *N*-glycosylation profiles (Raju and Jordan, 2012). *N*-glycan profiles of cCD20 mAbs produced in G3 Tg33(ho)4(ho) were analysed by ultra performance liquid chromatography (UPLC) tandem mass spectrometry (LC/MS/MS). Analytical sample was treated with PNGase F, and then the charged *N*-glycan was separated from the protein and labeled with a fluorescent labeling substance. The charged *N*-glycan was analysed by LC/MS/MS, and the content of the charged *N*-glycan was confirmed. As a result of the analysis, 14 *N*-glycans were identified from the purified cCD20 mAb (Figure 4-4 and Figure 4-5). The fluorescence chromatogram and extracted ion chromatogram obtained from mass spectrometry were confirmed at the same time, and the structure of the glycan of each peak was also confirmed by

assignments of the following tandem mass spectra (Figure 4-4a). cCD20 mAb produced from Tg33(ho)4(ho) egg white exhibited all non-fucosylated species and 8 of 14 major *N*-glycans contain the terminal galactose residues (74.1% of total monosaccharides) (Figure 4-4b). As previously reported (Pinkert, 2014; Sheridan, 2016), the cCD20 mAb showed that high mannose residues accounts for approximately 35.7% of total monosaccharides and there were no fucosylated *N*-glycans, and only one species contained sialic acid (Figure 4-4b). These results show that the cCD20 mAb from transgenic chicken eggs exhibited a typical chickenised antibody *N*-glycosylation pattern with high mannose and afucosylation.

Fab related functions of cCD20 mAbs derived from transgenic chickens

Stable Fab binding affinity and consistency from different batches of therapeutic antibodies are prerequisites for product quality, stability, and subsequent evaluation of biological activity. To characterise the binding affinity of cCD20 mAbs to the CD20 antigen, we performed a flow cytometry assay after treating cCD20 mAbs from G2 Tg33(he)4(he) and G3 Tg33(ho)4(ho) to CD20-expressing Raji cells. All three groups showed very similar binding efficiencies to Raji cells at all concentration (0.01, 0.05, 0.1, 0.5, 1, 5 or 10 µg/mL) (Figure 4-6a). Similarly, the median fluorescent intensity (MFI) values of all three groups were no significantly different at all concentrations (Figure 4-6b). The results indicate that binding affinity of cCD20 mAbs from two transgenic chickens for Raji cells was no significant difference with rituximab in dose-dependent treatment.

Cell apoptosis due to binding of the antibody itself is one of the important features of therapeutic antibodies. To further evaluate the induction of apoptosis by cCD20 mAbs, a series of dose response studies were performed with an Annexin V staining assay on CD20-expressing Raji cells (Figure 4-6c and 4-6d). The number of

apoptotic cells after the mAb treatment was significantly increased the G2 Tg33(he)4(he) mAb-treated group (60.7%), and the G2 Tg33(ho)4(ho) mAb-treated group (55.3%) at 5 μg/mL concentration compared to the rituximab treated group (42.8%) (Figure 4-6c). Furthermore, there were significant differences between rituximab treated group and cCD20 mAbs from two-transgenic chickens in induced apoptosis by dose-dependent manner (0.1, 1 and 5 μg/mL) (Figure 4-6d). Collectively, these results indicated that cCD20 mAb obtained from transgenic chickens had similar Fab properties in terms of direct binding affinity and higher efficiencies in induced apoptosis compared to rituximab. The results also revealed that the Fab related function of the cCD20 mAbs obtained from each transgenic chicken line showed no significant difference, indicating consistent production of the cCD20 mAb regardless of generations and strains of the transgenic chickens.

Fc effector activities of cCD20 mAbs derived from transgenic chickens

Fc function is the most important evaluation index of the efficacy of anti-cancer therapeutics such as rituximab. CDC is an important effector mechanism in the eradication of B lymphoma cells by anti-CD20 mAb. To evaluate CDC of the cCD20 mAbs, Raji cells were co-incubated with serially diluted rituximab and two types of cCD20 mAbs from G2 Tg33(he)4(he) and G3 Tg33(ho)4(ho)) from 10^{-4}–10^{2} μg/mL in human serum–containing medium. When the cell lysis efficiencies were observed in a dose-dependent manner, the half-maximal effective concentration (EC_{50}) values of cCD20 mAbs from each line showed significantly higher CDC activity than rituximab (Figure 4-7a). The EC_{50} values were 7.317 μg/mL for rituximab, 3.202 μg/mL for Tg33(he)49(he), and 3.27 μg/mL for Tg33(ho)49(ho), representing 2.3-fold and 2.25-fold higher efficacy for B lymphoma cells than rituximab (Figure 4-7a). However, the maximal efficacies of CDC activity of cCD20

mAbs from transgenic chickens was similar to rituximab (38.81 μg/mL for rituximab, 41.62 μg/mL for Tg33(he)4(he), and 40.94 μg/mL for Tg33(ho)4(ho), respectively).

To determine the ADCC activity of cCD20 mAbs, we examined immune-mediated effector functions of cCD20 mAbs to B lymphoblast, WIL2-S, compared to rituximab and commercially available CD20 mAb. We co-cultured FcγRIIIa-expressing Jurkat cells and WIL2-S lymphoblast cells with the control anti-CD20 mAb, rituximab and cCD20 mAb, respectively. When appropriate binding antibodies FcγRIIIa of Jurkat cells activate gene transcription through the nuclear factor of activated T-cells (NFAT) pathway, firefly luciferase is produced in the effector cell. As a result, luciferase expression was induced by the activation of FcγRIIIa after incubation of WIL2-S and Jurkat/FcgRIIIa/NFAT-Luc reporter cells under the existing control anti-CD20 mAb, rituximab and two types of cCD20 mAbs, and the intensity of luminescence increased in a dose-dependent manner. The intensity of luminescence was significantly higher in cCD20 mAb groups than in the control anti-CD20 mAb and rituximab groups (Figure 4-7b). More specifically, control anti-CD20 mAb and rituximab showed ADCC activity in EC_{50} of 2.63 ng/mL and 1.05 ng/mL, respectively, but in two types of cCD20 mAbs from transgenic chickens, the EC_{50} values were 0.126 ng/mL in Tg33(he)4(he), and 0.0677 ng/mL in Tg33(ho)4(ho); this is 8.3-fold and 15.5-fold higher efficacy on B lymphoblast cells than rituximab, respectively (Figure 4-7b). In addition, the maximal ADCC activity of cCD20 mAbs from transgenic chickens was 50% higher than rituximab. These results suggest that the cCD20 mAb antibody has greater efficacy as an anti-cancer therapeutic than rituximab in Fc effector functions, including CDC and ADCC, and the Fc effector functions of cCD20 mAb from transgenic chickens is also maintained consistently despite of differences between generations and integration patterns.

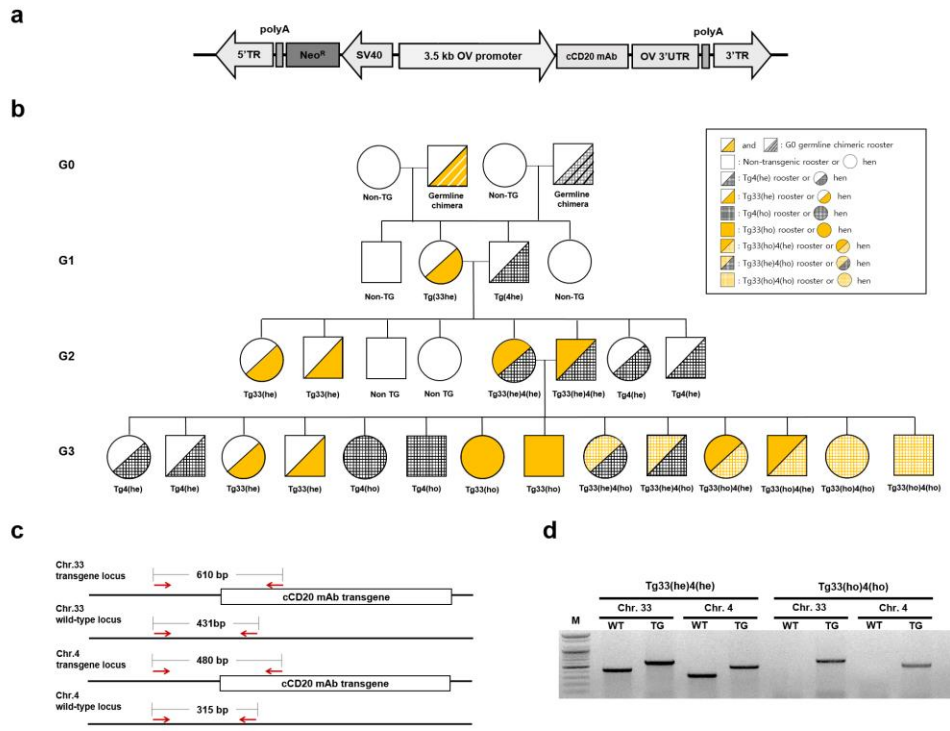

Figure 4-1. cCD20 mAb expression vector and production of transgenic chickens. (a) Schematic representation of the cCD20 mAb expression vector. The vector is based on *piggyBac* transposon and contains 3.5 kb ovalbumin (OV) promoter with 5' UTR of the chicken OV gene, chickenized anti-CD20 monoclonal antibody (cCD20 mAb) expression coding sequence, and 3'UTR of the chicken OV gene. (b) Schematic representation of producing multi-copy transgene bearing transgenic (TG) chickens. The founder germline chimeric roosters were mated with Non-TG hens. In G1 progenies, G1 Tg4(he) transgenic rooster that contains one-copy of transgene in chromosome 4 was mated with G1 Tg33(he) transgenic hen that contains one-copy of transgene in chromosome 33, and finally produced homozygous chickens containing 4-copies of transgene in their chromosome 4 and 33. (c) Detection of transgenic locus by PCR analysis. The specific primer sets used to detect each

transgenic loci (610 bp PCR product for transgenic loci in chromosome 33 and 480 bp PCR product for transgenic loci in chromosome 4) and wild-type alleles (431 bp PCR product for wild-type loci in chromosome 33 and 315 bp PCR product for wild-type loci in chromosome 4). (d) In Tg33(he)4(he) transgenic chickens, both the transgenic loci and wild-type loci were amplified, yielding 4-PCR amplicons, while in Tg33(ho)4(ho) chickens, only transgenic loci were amplified. he : heterozygous transgenic, ho : homozygous transgenic.

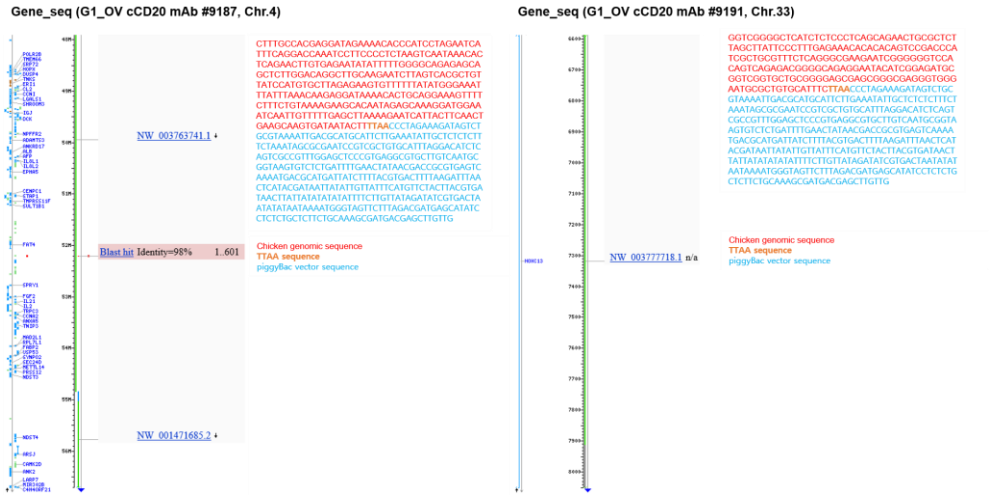

Figure 4-2. Identification of transgene insertion sites in the genomes of G1 transgenic chickens via DNA walking. The transgene locus map of G1 transgenic chicken #9187 and #9191 was detected using the BLAST and UCSC genome databases in which the transgene was localized to chromosome 4 (Tg4) and chromosome 33 (Tg33), respectively. In #9187 transgenic chicken, no functional gene or transcript was found within ~0.1 Mb of the transgene integration site. In #9191 transgenic chicken, the transgene integrated exogenic locus of HOXC13 gene (XM_001235165.3).

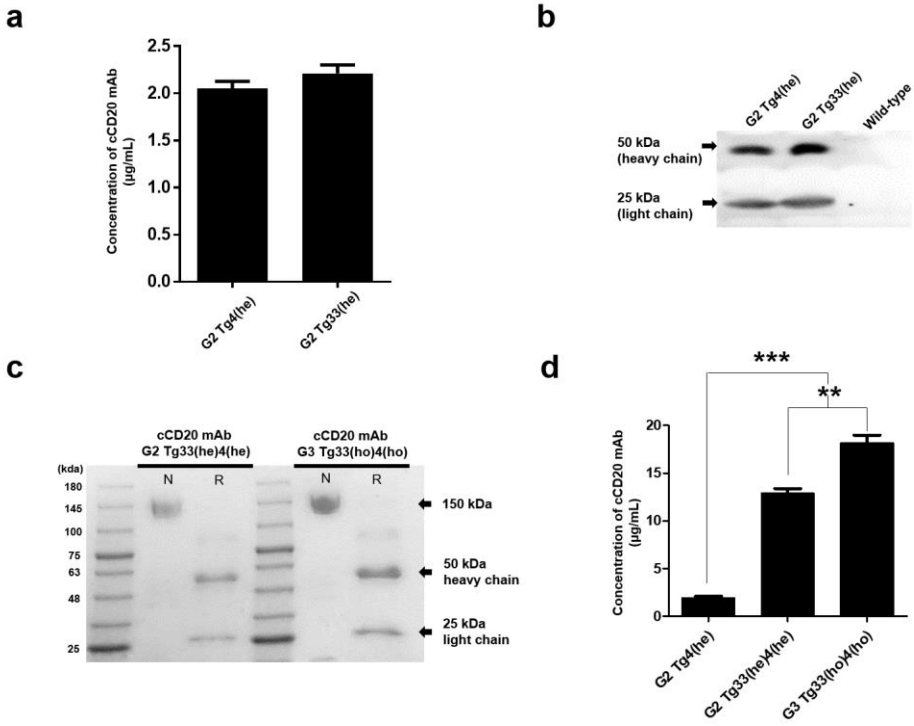

Figure 4-3. Quantification and consistency of cCD20 mAbs from transgenic chickens. (a) Quantification of purified cCD20 mAb from G2 transgenic chickens' egg white by ELISA. There is no significant difference in the cCD20 mAb from either type of heterozygous G2 transgenic chickens' egg white. (b) Western blot analysis for anti-human IgG of egg white from G2 transgenic chickens. Heavy (about 50 kDa) and light (about 25 kDa) chains of cCD20 mAb were detected in both groups. Egg white protein of wild-type (Non-TG) White Leghorn (WL) hen was used as the negative control. (c) Coomassie blue staining of cCD20 mAbs from G2 Tg33(he)4(he) and G3 Tg33(ho)4(ho) transgenic chickens after SDS-PAGE under non-reducing (N) and reducing (R) conditions. (d) Expression of cCD20 mAb from transgenic chickens of various genotypes. Concentrations of mAbs from transgenic chickens that contains two-copy of transgene, G2 Tg33(he)4(he) or four-copy of transgene, G3

Tg33(ho)4(ho) were significantly higher compared to that of G2 transgenic chicken that contains one-copy of transgene, G2 Tg4(he). Significant differences in concentration of mAb are indicated as *** $p < 0.001$, **$p < 0.01$. Bars indicate the SD of triplicate analyses.

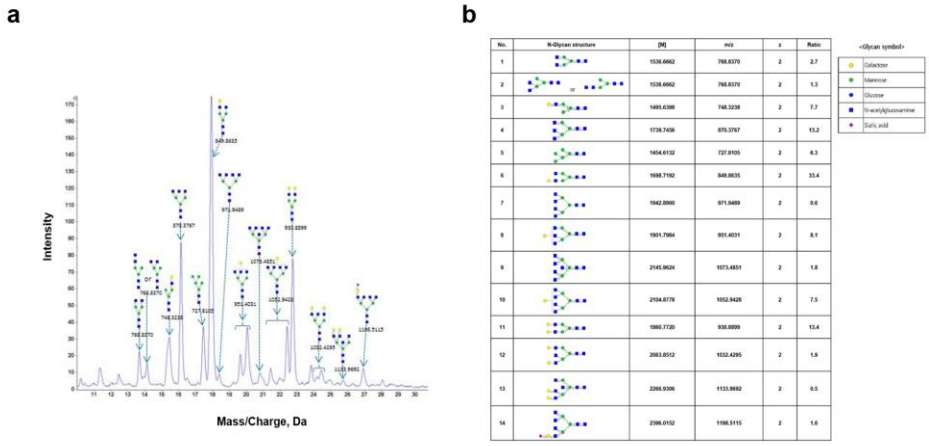

Figure 4-4. Charged *N*-glycan profile of cCD20 mAb from transgenic chicken egg. (a) The individual sugar structure content of the cCD20 mAb from Tg33(ho)4(ho) chicken. (b) Fourteen *N*-glycan profiles of cCD20 mAb through fluorescence and extracted-ion chromatogram (XIC).

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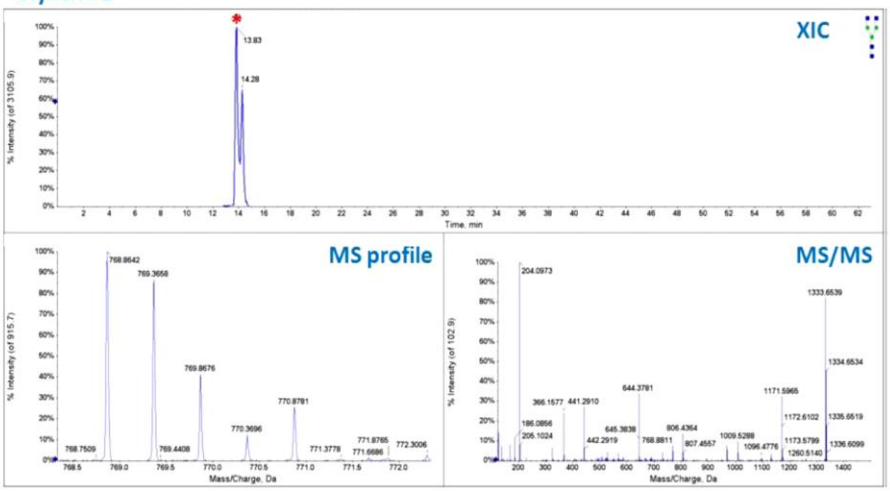

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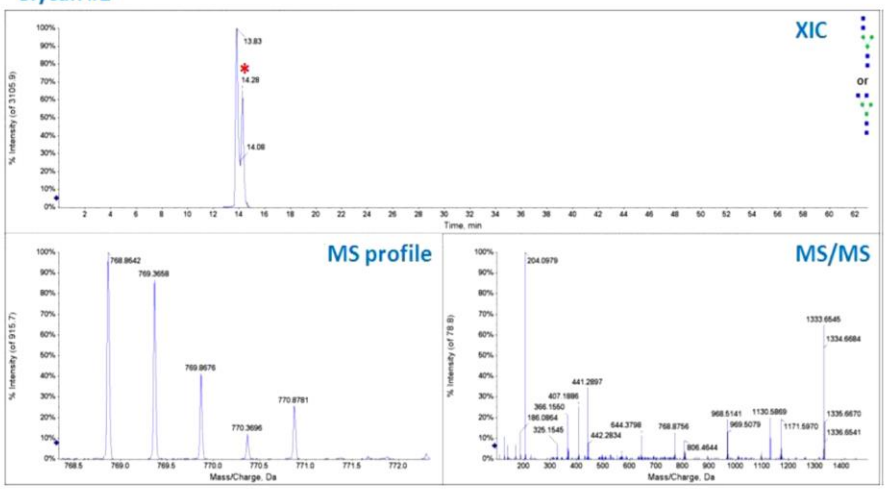

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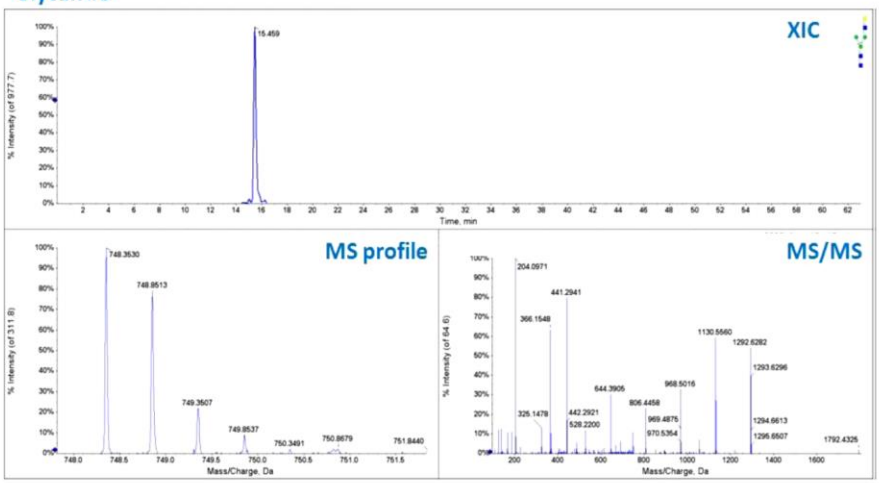

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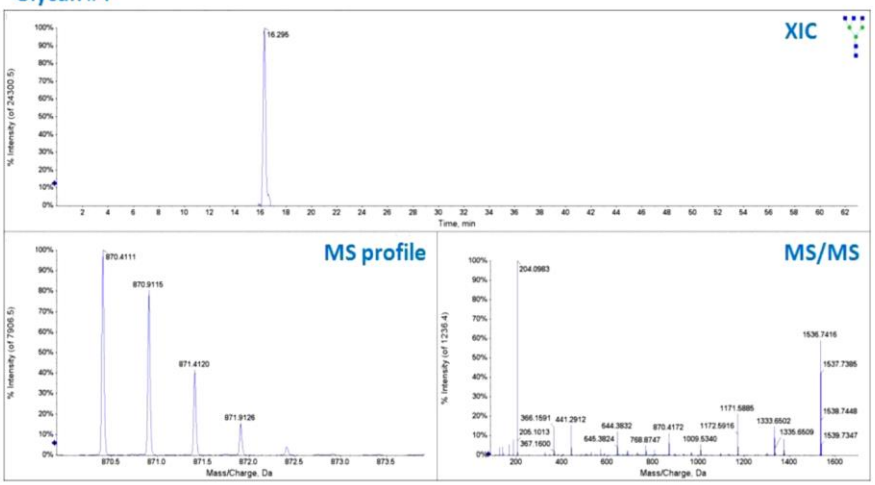

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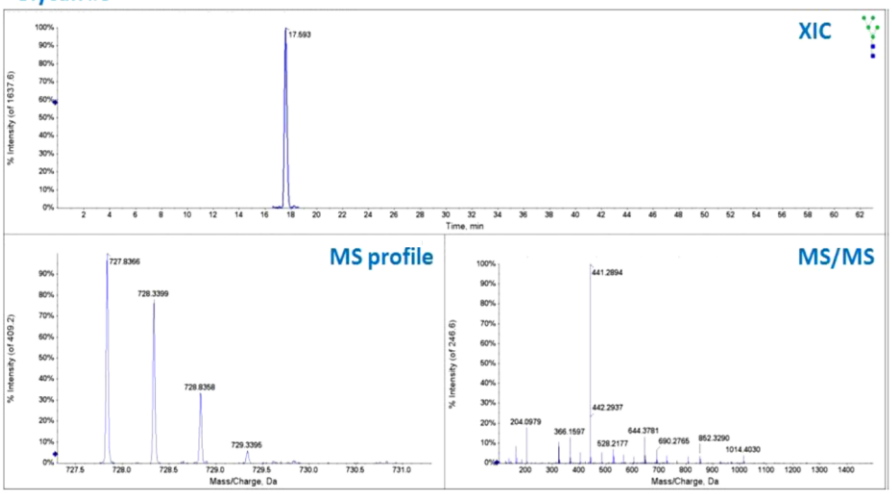

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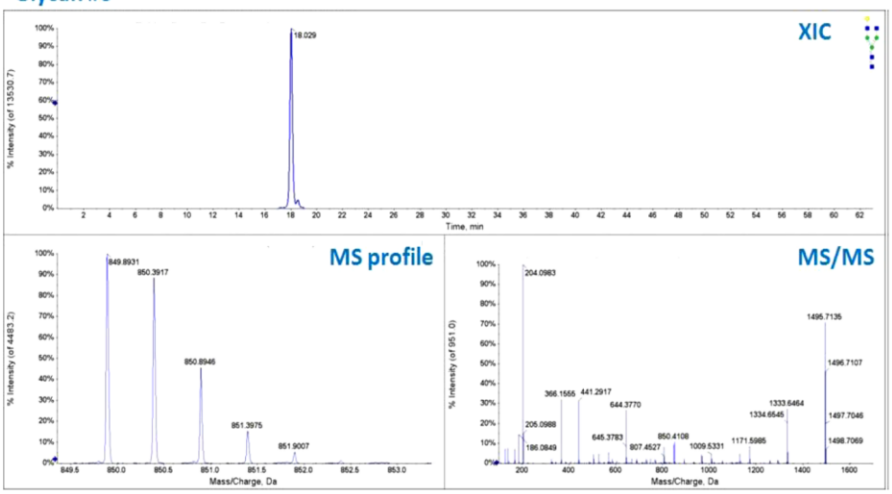

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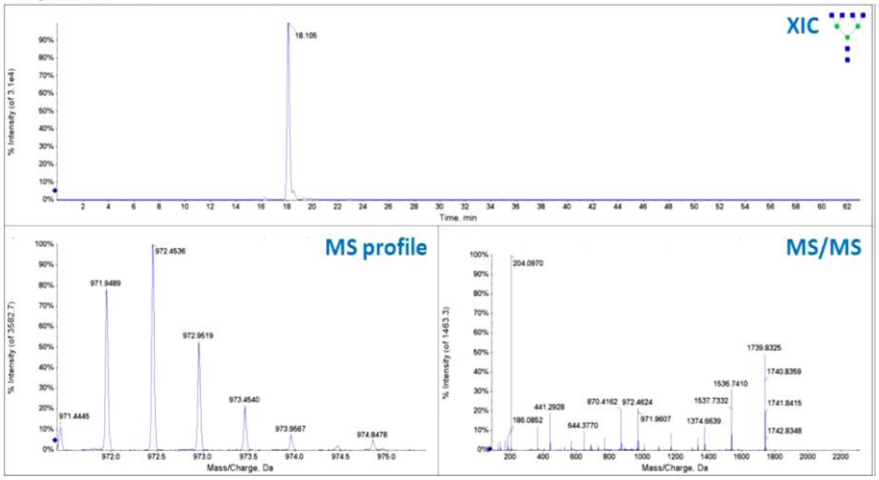

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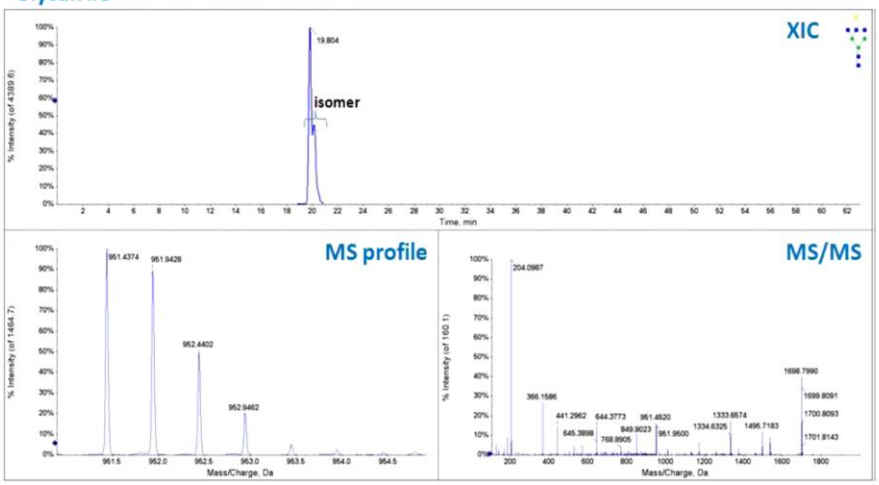

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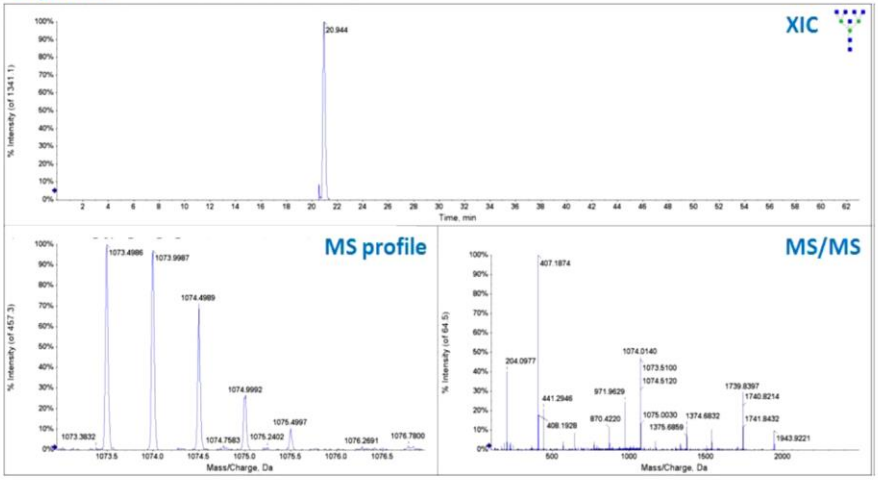

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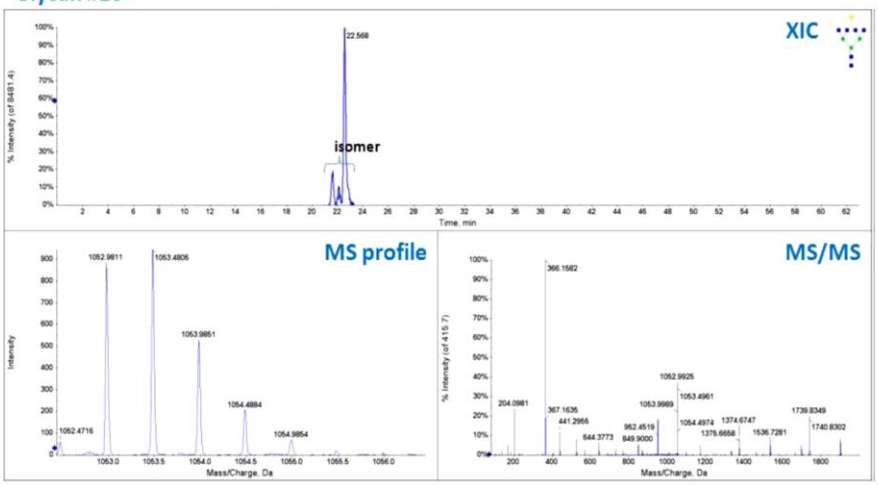

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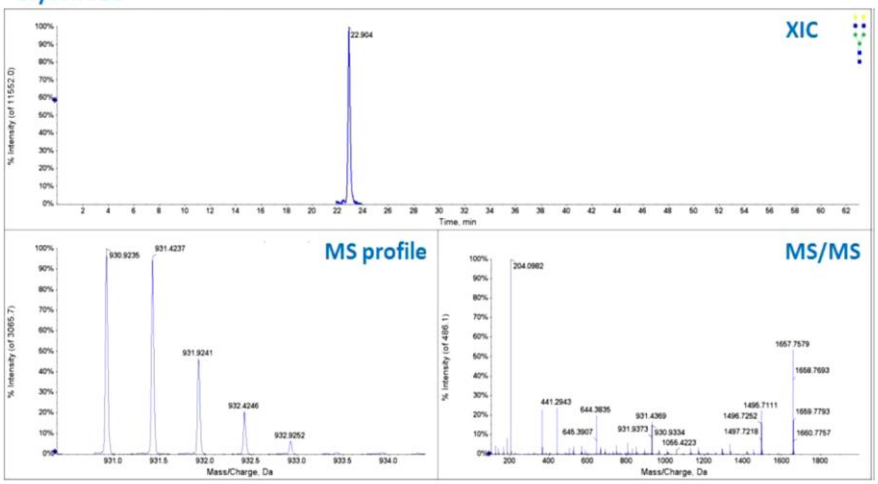

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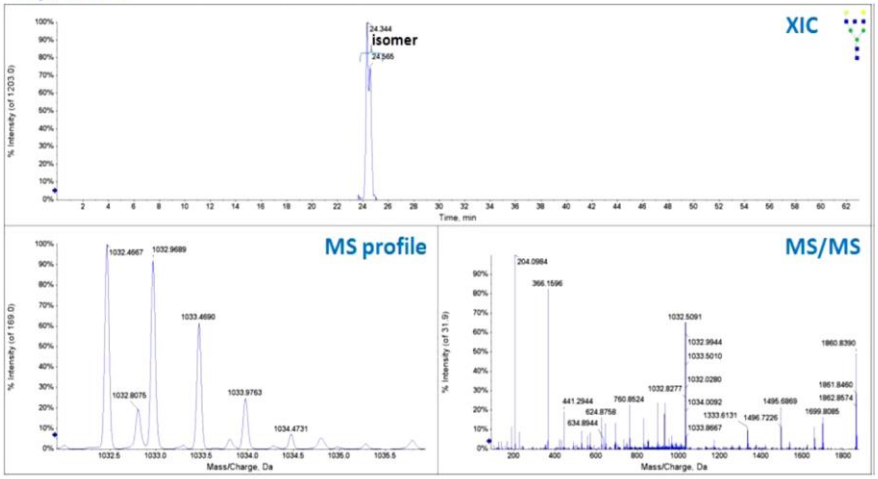

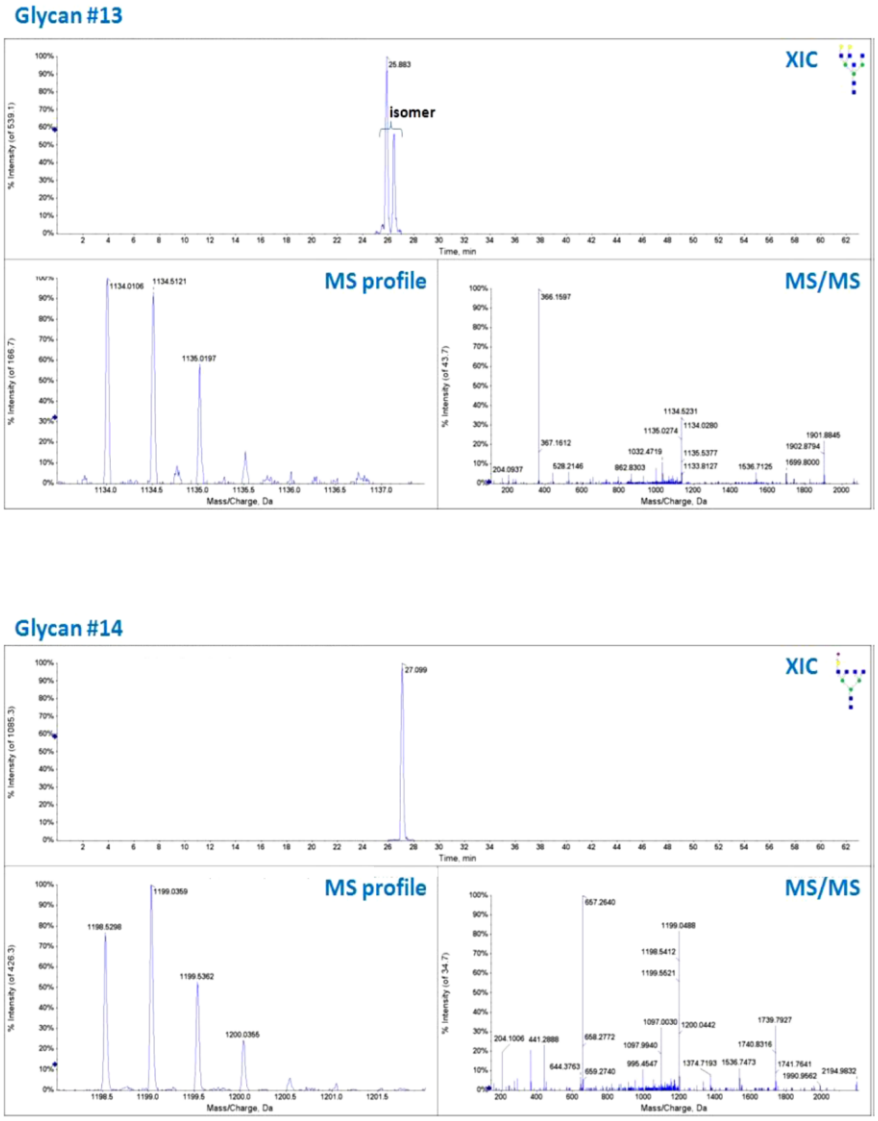

Figure 4-5. Profiling of *N*-glycan structure in the cCD20 mAb using XIC and MS/MS spectrophotometer.

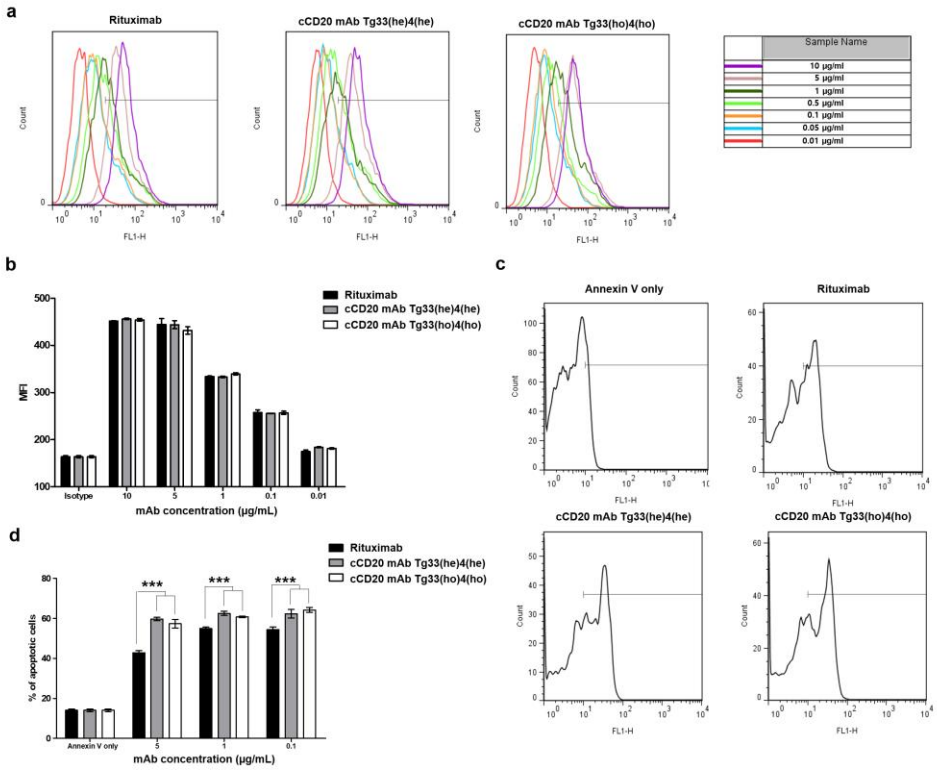

Figure 4-6. Fab binding affinity and induced apoptosis of cCD20 mAbs from transgenic chickens in a CD20-expressing B-cell lymphoma cell line. (a) Flow cytometry analysis for CD20 binding affinity after cCD20 mAb treatment (0.01, 0.05, 0.1, 0.5, 1, 5, or 10 μg/mL). (b) The median fluorescence intensity (MFI) curves for binding affinity in cCD20 mAbs from Tg33(he)4(he) and Tg33(ho)4(ho) chickens and rituximab in a dose-dependent (0.01, 0.1, 1, 5 and 10 μg/mL) manner following antibody treatment. There are no significant differences between rituximab and cCD20 mAbs at all concentration. (c) Flow cytometry analysis for anti-apoptotic activity of cCD20 mAb in CD20 expressing B-lymphoma cell line. Annexin V analysis in Raji cells by flow cytometry after 5 μg/mL cCD20 mAb treatment. Untreated group (Annexin V only) and rituximab were used as control. (d)

Quantitative analysis using annexin V for detecting induced apoptosis in the Raji cells mediated by treatment of cCD20 mAbs from Tg33(he)4(he) and Tg33(ho)4(ho) chicken and rituximab in a dose-dependent (0.1, 1, and 5 μg/mL) manner. Annexin V-positive cells indicate apoptotic cells. Untreated group (Annexin V only) and rituximab were used as control. There are significantly different between rituximab and cCD20 mAbs at all concentrations, and no significant differences between transgenic chickens (one-way ANOVA; ***$p < 0.001$, no significant differences; $p > 0.05$). Bars indicate the SD of triplicate analyses.

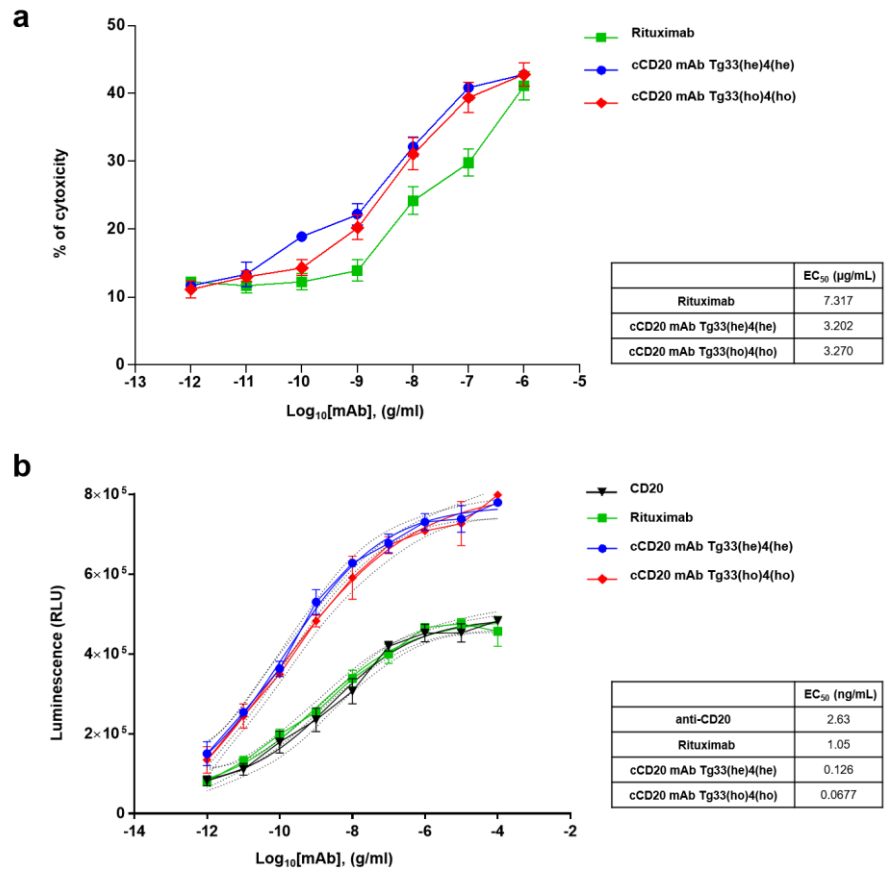

Figure 4-7. Fc effector activities of cCD20 mAbs derived from transgenic chickens. (a) Complement-dependent cytotoxicity (CDC) of cCD20 mAb from Tg33(he)4(he) and Tg33(ho)4(ho) chickens. cCD20 mAb-mediated CDC assays on Raji cell. Rituximab and cCD20 mAb were treated on Raji cells cultured in human serum containing medium in a dose-dependent manner (10^{-4}–10^{2} μg/mL). The EC_{50} values represent 2.29-fold and 2.23-fold higher efficacy for B lymphoma cells in cCD20 mAbs than rituximab. Significant differences between rituximab and cCD20 mAb are shown (one-way ANOVA; $p < 0.001$), but no significant difference was found between cCD20 mAbs. (b) Antibody-dependent cell-mediated cytotoxicity (ADCC) of cCD20 mAb from Tg33(he)4(he) and Tg33(ho)4(ho) chickens on human B lymphoblasts, WIL2-

S. The concentration is plotted against the percentage of ADCC after treatment of anti-CD20 Ab, rituximab, and cCD20 mAb purified from Tg33(he)4(he) and Tg33(ho)4(ho) chickens with Jurkat effector cells. The EC_{50} values represent 8.3-fold and 15.7-fold higher efficacy for B lymphoblast cells in cCD20 mAbs than rituximab (one-way ANOVA; $p < 0.001$). RLU, relative luminescence unit. Bars indicate the SD of triplicate analyses.

Table 4-1. List of primers used in this study

Primer	Sequence (5'→3')	Size(bp)	Detection
Ch33 F	GTC GGG GCT CAT CTC TCC CT	431	Ch33 wild-type locus
Ch33 wild-type locus R	GCG CCA AGT GAC GAT CTG GT		
Ch33 F	GTC GGG GCT CAT CTC TCC CT	610	Ch33 transgene locus
Ch33 transgene locus R	CCG CTA GCC AAC AAG CTC GT(vector seq)		
Ch4 F	AGA AGC ACA ATA GAG CAA AGG ATG GAA	315	Ch4 wild-type locus
Ch4 wild-type locus R	TCC TGT GAT GCT CCA GGC CT		
Ch4 F	AGA AGC ACA ATA GAG CAA AGG ATG GAA	480	Ch4 transgene locus
Ch4 transgene locus R	AGC TCG TCA TCG CTT TGC AGA(vector seq)		
piggyBac TSP1*	GGATCTCATGCTGGAGTTCT	-	Integration site
piggyBac TSP2	GGCGTAATCATGGTCATAGCT	-	Integration site
piggyBac TSP3	GAGCCGGAAGCATAAAGTGT	-	Integration site

*: Target specific primers (TSP)

5. Discussion

In this study, we described the production of transgenic chicken which expressing cCD20 mAb as biosimilar of rituximab a representative anti-cancer antibody, in egg white, and furthermore, we analyzed the physiological characteristics and bio-functional activities of the produced antibody for identifying practical potentials of transgenic chicken system as bioreactors.

The yield of recombinant protein from transgenic chicken is particularly determined by regulatory elements including promoters. The use of variously sized promoters from the OV gene was reported in a number of studies for tissue-specific derived recombinant proteins in birds (Zhu, van de Lavoir et al., 2005b; Lillico, Sherman et al., 2007b; Kwon, Choi et al., 2010; Cao, Wu et al., 2015). Zhu *et al*. found that the production rate of mAb driven by 7.5 kb OV promoter was ranged from 1.4 ng/mL to 10.8 ng/mL in egg whites from somatic chimeric chickens, however, the production rate of mAb driven by 15 kb OV promoter was significantly increased compared to those of the 7.5 kb OV promoter (from 1.2 mg/mL to 1.6 mg/mL on average) (Zhu, van de Lavoir et al., 2005b). These results suggest that 10-15 kb of 5' upstream region of the chicken OV gene is required for enhanced production of the transgene through tissue-specific expression (Zhu, van de Lavoir et al., 2005b). One of another studies demonstrating on productions of recombinant protein from transgenic chicken showed that 2.8 kb OV promoter combined with estrogen response elements (ERE) induced mass production of therapeutic proteins in transgenic chicken egg white hIFNβ1a (3.5-426 μg/mL) and humanised ScFv-Fc miniantibody (15-50 μg/mL), respectively (Lillico, Sherman et al., 2007b). This result also suggests that regulatory elements including promoter region is one of the critical determination for the yield of recombinant protein in transgenic chicken eggs (Pinkert, 2014). In this study, quantitative analysis of the produced antibodies from the transgenic chickens showed that the amount of antibody in the egg white of G1 heterozygous transgenic chicken and G3

homozygous transgenic chicken was approximately 2 μg/mL and 18 μg/mL, respectively, indicating stable expression of the recombinant protein through successive generations by the 3.5 kb OV promoter.

Other factors that affect the yield of recombinant protein produced in transgenic chicken are the transgene integration site and copy number. We identified that the mAb production yield from heterozygous or homozygous chickens containing the transgene in different loci (chromosomes 4 and 33, respectively) was significantly increased compared to heterozygous transgenic chickens that bore the transgene only on chromosome 4. The result is a dramatic increase in yield compared to the previous researches about 43% increase in yield in G3 homozygous transgenic chickens using the same vector strategy (Park, Lee et al., 2015), and 47% higher concentration of target protein from homozygous transgenic chicken eggs compared to heterozygous (Harvey, Speksnijder et al., 2002). Therefore, we assumed that placement of the transgene in different positions in the genome might allow a synergistic effect in expression of recombinant protein in chicken. Thus, we concluded that the yield of recombinant proteins from chicken bioreactors has been affected by copy number and its synergistic effects between integrated transgenes as well as length of the regulatory elements.

The *N*-glycosylation of therapeutic antibodies is an important parameter from the perspective of functionality and efficacy because of its roles on activation of immune Fc effector functions (Hodoniczky, Zheng et al., 2005; Abes and Teillaud, 2010; Houde, Peng et al., 2010). In therapeutic antibodies for cancer treatment such as rituximab, trastuzumab and cetuximab, Fc effector functions such as ADCC and CDC are especially considered as a critical mechanism of action (Clynes *et al.*, 2000; Niwa *et al.*, 2004b; Gasdaska *et al.*, 2012; Holubec *et al.*, 2016), and the PTM features are the important determinants of Fc effector functionality of anti-cancer therapeutic antibody. Rituximab is an anti-cancer therapeutic antibody for the treatment of B-

lymphoma, and its mechanism of action involves the induction of direct cell death *via* Fab-mediated specific binding to CD20 (Beers *et al.*, 2010). Furthermore, rituximab-opsonized B cell lymphoma is also killed by immune system. CD20-specific Fab region and Fc region of antibody facilitate the specific recognition and killing of cancer cells by NK cells (ADCC) and/or complement complex (CDC) (Boross and Leusen, 2012). Therefore, rituximab is one of representative therapeutic antibodies to show the importance of Fc effector function in disease treatment. The general *N*-glycosylation pattern of recombinant protein produced in transgenic chicken showed high mannose, core afucosylated form and terminal galactosylation which is expected to higher Fc effector functionalities (Zhu, van de Lavoir et al., 2005b; Pinkert, 2014), therefore, we tried demonstrating to identify the efficacy of the recombinant proteins from transgenic chicken as anticancer therapeutic antibody.

The Fab binding affinity of cCD20 mAb from transgenic chicken was not significantly different at all concentration compared to rituximab, indicating its suitability for antigen recognition. In addition, the binding affinity between Tg33(he)4(he) and Tg33(ho)4(ho) did not show any significant difference, confirming the homogeneity and consistency of the cCD20 mAb between transgenic chicken lines. In contrast, induction of apoptosis by Fab binding shows significantly different between rituximab and cCD20 mAb, and there was also no significant difference between Tg33(he)4(he) and Tg33(ho)4(ho). Consequently, the Fab quality of transgenic chicken-derived cCD20 mAb is maintained constantly across generation or genotype, and competitive with rituximab. Meanwhile, the Fc function of cCD20 mAb, including CDC and ADCC, showed a much more dramatic enhancement than the Fab function compared to rituximab. The results may come from the *N*-glycan feature of the cCD20 mAb from transgenic chicken. Commercial rituximab contains galactose residues in nine of 16 major glycans, but CD20 mAbs from transgenic silkworms (*Bombyx mori*) lacks galactose at the nonreducing terminal, which results in low CDC activity compared to rituximab (Tada, Tatematsu et al.,

2015). Different from previous research about recombinant protein production from silkworm and transgenic chicken (Kamihira *et al.*, 2009; Tada, Tatematsu et al., 2015), our results showed that cCD20 mAb produced from transgenic chicken egg white contains galactose residues in eight of 14 kinds of major *N*-glycans. Residues of rituximab involves on binding of rituximab to the complement C1q resulting in CDC activity and terminal galactosylation plays a major role in the conformation of the Fc region, galactosylation at the non-reducing terminals is correlated with the binding to complement C1q and CDC activity (Hodoniczky, Zheng et al., 2005). Therefore, the enhanced CDC activity of cCD20 mAb when compared to rituximab may be due to high level of terminal galactose (74.1%) in *N*-glycan.

In the case of therapeutic antibodies evaluation inducing ADCC, it is particularly important to measure *N*-glycan fucosylation of the Fc domain, which strongly affects ADCC activity (Shields *et al.*, 2002; Abes and Teillaud, 2010). To induce ADCC, the therapeutic antibody first binds to a cell-surface target antigen and recruits immune effector cells that lyse the target cell. Meanwhile, fucosylation of the Fc region of the antibody significantly hampers binding affinity to the cell-surface target, causing a decrease in ADCC activity (Chung, Quarmby et al., 2012). Previous researches reported that the depletion of αl-6 fucose from human IgG1 oligosaccharide significantly increases ADCC functionality (Shinkawa, Nakamura et al., 2003; Okazaki *et al.*, 2004). Therefore, lowering fucosylation is important for improving the efficacy of the antibody produced, including Rituximab and Trastuzumab (Clynes, Towers et al., 2000; Niwa, Hatanaka et al., 2004a; Niwa, Shoji-Hosaka et al., 2004b; Chung, Quarmby et al., 2012; Gasdaska, Sherwood et al., 2012). The most abundant *N*-glycan forms of rituximab are G0F, G1F, and G2F, which contain fucose residues, and six more glycans were found in the end of the GlcNAc of the Fc domain (Shang *et al.*, 2014). On the other hand, there is no fucose linkage in the egg white proteins, although B-cells produce immunoglobulin molecules that possess αl-6 fucose in chicken (An, 2011). Previous results showed that the

mAbF1 produced from somatic chimeric chicken's tubular gland and egg white exhibited afucosylated glycan structures with a significant increase in ADCC (Zhu, van de Lavoir et al., 2005b). In our study, we also found that the cCD20 mAbs produced from transgenic chicken egg white exhibited 14 *N*-glycans patterns with afucosylation. The superior ADCC activity (8- to 15-fold higher EC_{50} values) of the cCD20 mAb from transgenic chicken egg white compared to CD20 control antibody and rituximab might also cause by the distinctive *N*-glycan features.

Rituximab also exhibit the structures of high mannose *N*-glycans characteristic which prominently found in IgG antibodies (da Silva *et al.*, 2014; Shang, Saati et al., 2014; Montacir *et al.*, 2017). The portion of high-mannose glycans contribute to increased clearance of therapeutic antibodies which determined by the inherent half-life of the antibody (Goetze *et al.*, 2011). The ADCC activity were increased under the condition of both Man8/9 and Man5 high mannose glycoforms in core fucose-lacking human IgG1 compared to typical complex-fucosylated glycoform (absence of core fucose) (Kanda *et al.*, 2007). In addition, both of the Man5 and Ma6 glycoforms accelerated clearance rate in pharmacokinetic study (Yu, Brown et al., 2012) which means maintenance of high mannose glycoform also has a significant effect on ADCC activity as well as afucosylation. Therefore, the higher ADCC activity of cCD20 mAb from transgenic chicken could be explained by the high mannose *N*-glycoform.

In this study, we identified that the consistent expression of cCD20 mAbs from transgenic chicken system. We further identified the proteins contained unique *N*-glycan profiles and exhibited stronger Fc effector functionality including CDC and ADCC than commercial rituximab, which may be the result of a lack of fucose, high mannose and terminal galactose. Our results suggest that the germline-competent transgenic chicken bioreactor is a promising expression system because of its optimal PTM characteristics

causing superior Fc effector function. It also has been proved that these characteristics have maintained constantly in successive generations, which is expected to increase the value of chicken as a bioreactor. Therefore, this germline transgenic chicken bioreactor system can be a very suitable and effective system for the production of anti-cancer antibodies.

CHAPTER 5

Production of germline chimeric quails following spermatogonial cell transplantation in busulfan treated testis

1. Abstract

The effective transgenic systems in quail are limited because of the absence of long-term in vitro systems to culture stem cells or germ cells and the lack of efficient germline transmission methods. In this study, we produced germline chimeric quail using testicular cells (TCs) and spermatogonial stem cells (SSCs) transplanted into busulfan-treated recipient testis. The spermatogonial cells, derived from adult testes of the wild type plumage strain, were cultivated in vitro for 2 weeks and found to express several markers of SSCs. Meanwhile, the testes from recipient adult quail (Black quail, D strain) were sterilized using busulfan. Two weeks after treatment, the weight of the busulfan-treated testes was significantly lower than that of non-treated D strain adult testes, which express lower levels of VASA, a germ cell-specific marker. Furthermore, PKH26-labelled spermatogonial cells were detected in recipient testis, indicating successful transplantation. After transplantation of TCs and SSCs, respectively, the recipients produced donor-derived progenies with 11.5% and 16.7% germline transmission efficiency by testcross analysis. This was confirmed by breed-specific PCR analysis and determination of the feather phenotype. This is a feasible and practical method to produce transgenic quail and will help in the conservation of avian species.

2. Introduction

Quail embryos are useful materials in embryological studies, because they are easy to obtain and manipulate during all developmental stages (Nakao *et al.*, 2008; Mizushima *et al.*, 2014). In addition, quail is a suitable model for transgenic research because of its low maintenance cost, small body size, and relatively short generation time compared with other avian species. For this reason, numerous attempts have been made to produce germline chimeras, which are useful tools for transgenic model development, from primordial germ cells (PGCs) (Kim, Park et al., 2005b; Park, Kim et al., 2008; Zhang, Sun et al., 2012). Despite its practical potential, the PGC-mediated quail transgenic system is still incomplete because of the lack of long-term *in vitro* culture methods of PGC and efficient exogenous gene insertion techniques in quail.

The transplantation of male germ cells including spermatogonia and spermatogonial stem cells (SSCs) is an efficient method to study spermatogenesis and control male fertility (Brinster and Zimmermann, 1994). This technique has been thoroughly evaluated in several vertebrates such as fish, rodent, bovine and rhesus, which are used for research on fertility and transgenesis (Herrid *et al.*, 2006; Izsvak, Frohlich et al., 2010; Kanatsu-Shinohara, Kato-Itoh et al., 2011; Lee *et al.*, 2015b). Some successful cases of spermatogonial transplantation have also been demonstrated in birds (Lee, Jung et al., 2006; Trefil, Micakova et al., 2006). In quail, xenogenic transplantation of SSCs in a chicken host demonstrated the possibility of inter-species chimera production (Roe *et al.*, 2013). More recently, we reported successful cultivation of quail SSCs for certain periods by optimizing the culture conditions (Pramod, Lee et al., 2017). These results suggest male germ cell transplantation as another method to produce quail germline chimeras.

Sterilization of endogenous germ cells is often used for effective germline chimera production. Sterilization techniques include gamma ray

irradiation, X-ray irradiation and chemical methods to eliminate germ cells (Song *et al.*, 2005; Trefil, Micakova et al., 2006; Nakamura *et al.*, 2010; Park *et al.*, 2010; Lee *et al.*, 2013). Busulfan is an antispermatogonial alkylating agent that induces relatively high cytotoxicity in germ cells and therefore causes cell death in mouse and rat testes (Moisan *et al.*, 2003; Vasiliausha *et al.*, 2016). Busulfan is also considered to be a very effective antispermatogonial agent in avian testis (Jones and Jackson, 1972; Tagirov and Golovan, 2012), and it showed very high efficiency in germline chimera production when used to treat unhatched embryos (Nakamura, Usui et al., 2010). Moreover, despite that busulfan causes testicular germ cell apoptosis, a small number of SSCs survive and restore the germ cell population (Zohni *et al.*, 2012). This suggests that normal spermatogenesis can be maintained after endogenous germ cells are removed by busulfan. Therefore, busulfan treatment and spermatogonial germ cell transplantation are thought to be efficient methods to induce germ cell depletion in recipient quails and to produce germline chimeras, respectively.

In this report, we demonstrate successful production of quail germline chimeras by transplanting spermatogonial cells with and without cultivation. As was the case with chickens (Song, D'Costa et al., 2005), recipient black quails (*D/D*) were treated with busulfan to produce highly efficient germline chimeras. Donor-derived progenies, which were obtained from germline chimeras produced from spermatogonial cell transplantation, were distinguished using newly designed breed-specific markers that detect single nucleotide polymorphisms (SNPs) in the quail melanocortin 1 receptor (MC1R) gene. This technique contributed to the development of an efficient transgenic production system in quail.

3. Materials and methods

Experimental animals

Japanese quails (*Coturnix japonica*) were used as experimental animals. The Institute of Laboratory Animal Resources at Seoul National University (SNU-150827–1) approved the care and experimental use of quails. Quails were maintained according to the standard management program at the University Animal Farm at Seoul National University (Seoul, Korea). All procedures for animal management, reproduction, and embryo manipulation were performed using standard operating protocols.

Isolation of quail testicular cells (TCs) and SSCs for culture

TC and SSC isolation and culture were performed as described previously with slight modifications (Pramod, Lee et al., 2017). Briefly, whole TCs were obtained from the testes of adult (4 months of age) wild type plumage (WP: d^+/d^+) (Somes RG, 1998) using a blade to dissociate the testes into small pieces. The pieces were then treated with 1 mg/mL collagenase type IV (Gibco, Grand Island, NY, USA) in Dulbecco's Modified Eagle's Medium (DMEM) for 30 min, 7 mg/mL DNase I in DMEM for 5 min, and 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) (Invitrogen) in phosphate buffered saline (PBS) for 20 min in a shaking water bath at 37°C. The cell suspension resulting from each stage was washed with pre-warmed (37°C) PBS and then centrifuged (800 rpm, 5 min). The final cell suspension was filtered through a 100-μm and then 40-μm nylon cell strainer (BD Falcon, San Jose, California, USA). The cell pellet was finally resuspended in DMEM containing 5% fetal bovine serum (FBS). To isolate SSCs, TCs in DMEM were added to datura stramonium agglutinin (DSA; 10 μg/mL)-coated 12-well plates containing DMEM/F12 (Invitrogen) supplemented with 10% FBS, 10 ng/μL glial cell line-

derived neurotrophic factor (GDNF), 1× essential amino acids (EAAs), 1× non-essential amino acids (NEAAs) and 1× antibiotic/antimycotic solution and incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2 in air. One day after seeding, ~3 × 10^6 non-adherent TCs (putative SSCs) were separately suspended in DMEM/F12 with 10% FBS and 1× antibiotic/antimycotic solution overnight in uncoated six-well plates to enrich for SSCs. Then the suspended cells were collected, washed and co-cultured (1 × 10^5 per well) with mitotically inactivated mouse embryonic fibroblasts (MEFs) in SSC medium [DMEM/F12 supplemented with 1% FBS, 1× EAAs, 1× NEAAs, 15 ng/mL GDNF (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL basic fibroblast growth factor (Peprotech), 10 ng/mL leukemia inhibitory factor (Sigma, St Louis, MO, USA), 0.55 mM β-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), 1 μM sodium pyruvate (Gibco), and 1× antibiotic/antimycotic solution] at 37°C in a humidified CO2 incubator with 5% CO2. The medium was replaced every other day, and quail SSC clusters were passaged every 4–5 days.

Reverse transcription-polymerase chain reaction

RT-PCR was used to analyze gene expression in quail TCs, cultured SSCs, adherent Sertoli cells, and quail embryonic fibroblasts. Total RNA was prepared using TRIzol reagent (Thermo Fisher Scientific–Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific–Invitrogen). The following genes, using the respective primers, were amplified by RT-PCR: *POUV* (F: 5′-GCA GGA GAT GTG CAA TGC AGA GCA A-3′, R: 5′-GTG GCT GCT GTT GTT CAT GGA GAT C-3′), *NANOG* (F: 5′-TAG GTG CGG CCA CTA CTA CTG GCC C-3′, R: 5′-TCC ACC CAC TGA CTC TCC TTC TGG C C-3′), *VASA* (F: 5′-TTC AGT AGC AGC AAG AGG CC-3′, R: 5′-CTC CTG GGT TCA CTC TGC TG-3′), *DAZL* (F: 5′-GGC AAA AGG CTG AAA CTG GG-3′, R: 5′-TTC

TTT GCT CCC CAG GAA CC-3′), and *GAPDH* (F: 5′-TTC ACC ACC ATG GAG AAG GC-3′, R: 5′-CCA TCC CTC CAC AAC TTC CC-3′). The PCR reaction contained 2 μL 10× PCR buffer, 0.4 μL dNTPs (40 mM each), 2 pmol each primer, and 0.5 U Taq polymerase (Biofact, Daejeon, Korea). The thermocycling parameters were as follows: 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C, and finally 10 min at 72°C.

Periodic acid-Schiff (PAS) and immunocytochemical staining in quail SSCs

For PAS staining, the cultured SSCs were immersed in periodic acid solution (Sigma-Aldrich Corporation, St. Louis, MO) for 5 min, incubated in Schiff solution (Sigma-Aldrich) for 5–10 min, and then rinsed three times with PBS. All procedures were performed at room temperature, and stained cells were observed under an inverted microscope (TE2000-U; Nikon Corporation, Tokyo, Japan). For SSC immunostaining, cultured cells were fixed in 3.7% paraformaldehyde solution for 10 min, washed three times with PBS, blocked with blocking buffer (PBS containing 5% goat serum and 1% bovine serum albumin) for 30 min, and then incubated with primary antibodies diluted 1:200 in blocking buffer overnight at 4°C. Antibodies against chicken integrin alpha 6 (ITGA6, Millipore, Bedford, MA, USA) and the N-terminal peptides of chicken DAZL and CVH were used. Polyclonal antibodies against the N-terminal peptides of chicken DAZL (amino acids 2–17, SANAEAQCGSISEDNTH) and CVH (amino acids 42–57, SRPSSPLSGFPGRPNS) were raised in rabbit and purified from antisera (Lee *et al.*, 2016a; Jung *et al.*, 2017b). The samples were then incubated for 1 h at room temperature with secondary antibodies labeled with phycoerythrin or fluorescein isothiocyanate. The washed samples were mounted on slides using ProLong Gold antifade reagent and 4′,6-diamidino-2-phenylindole (Thermo Fisher–Invitrogen) and visualized under a fluorescence microscope.

Transplantation of spermatogonial cells and testcross analysis

TCs or SSCs were injected into the seminiferous tubules of D strain (*D/D*) recipients treated with 40 mg/kg busulfan at 8 weeks of age. Briefly, 40 mg busulfan (Sigma-Aldrich) dissolved in 1 mL *N*,*N*-dimethyl formamide (Merck, Darmstadt, Germany) were injected intraperitoneally into black quail (*D/D*) recipients. Busulfan-treated strain D black quails were used for TC/SSC transplantation at 2 weeks after busulfan treatment. The cell suspension (3×10^6 TCs or SSCs cultured *in vitro* for 20 days in 1 mL trypsin-EDTA-free DMEM supplemented with 10% FBS or labeled with PKH26) was surgically injected after anesthetizing the recipients with 10 mg/kg Zoletil (Virvac, Carros, France). The recipient quails were sacrificed to evaluate the localization of PKH26-labeled TCs 24 h after injection. PKH26-labeled cells were detected in 20-μm-thick cryosections stained with DAPI (1:10,000 dilution) under a fluorescent stereomicroscope. Approximately 1 month later, germline chimeric recipient quails (*D/D* with *D* and *d*+ germ cells) were subjected to testcross analysis by mating with WP (d^+/d^+) quails of the opposite sex. Germline chimerism was confirmed by hatching WP progeny, and the efficiency of germline transmission was evaluated as the ratio of the WP (d^+/d^+) to hybrid (D/d^+) progeny (Figure 5-1).

Primers designed for breed-specific PCR

Multiple sequence alignments were performed to find DNA polymorphisms of quail melanocortin 1 receptor (qMC1R) between two breeds (WP and D strains). Breed-specific primers for qMC1R (qMC1R WQ F: 5'-CGT CAG CAA CCT GGC CG-3', qMC1R BQ F: 5'-GCG TCA GCA ACC TGG CCA-3' and qMC1R R: 5'-GTC TGT GCT GCT GCC TAC CA-3') were designed with confirmed SNP at the 3' end. For PCR, these primers were paired with the reverse primers that were previously designed for DNA sequencing.

All PCR was performed in a 20μl total reaction containing 100ng of genomic DNA, 2 μl of 10× PCR buffer, 0.4 μl of dNTPs (40 mM each), 2 pmol of each primer, and 0.5 units of Taq polymerase (Biofact). The thermocycling conditions were as follows: 10 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C, and finally 10 min at 72°C. q*MC1R* WQ F and q*MC1R* BQ F were designed to detect WQ and BQ, respectively, based on the sequence variation A/G in exon 1 of q*MC1R.*

Statistical analysis

Differences of the weight between control and busulfan treated testes were analyzed by Student's t-test using GraphPad Prism statistical software (GraphPad Software, La Jolla, CA). $P < 0.01$ was considered significant.

4. Results

Isolation and culture of quail spermatogonial cells

Whole TCs isolated from WP strain quail (d^+/d^+) were seeded on DSA-coated plates using a sequential enzymatic digestion method used in previous studies (Figure 5-2a) (Pramod, Lee et al., 2017). One day after seeding, the adherent cells were mechanically removed and transferred to a culture containing mitotically inactivated MEFs as the feeder cells. The remaining attached cells had a flattened epithelioid morphology with an irregular polygon appearance. These cells are Sertoli cells, as confirmed in a previous study (Figure 5-2b) (Pramod, Lee et al., 2017). When co-cultured with the MEFs, the Sertoli cells began to cluster within 48 h and formed colonies after 6 days (Figure 5-2c). The cells were stably maintained and grew for 20 days (until the fourth passage) in the presence of the MEFs (Figure 5-2d) but then gradually decreased from the fifth passage onward. These results demonstrate that stable cultures can be achieved for a certain period; however, long-term culture of quail spermatogonial cells has not been demonstrated.

Characterization of quail SSCs

In subsequent experiments, we examined the characteristics of cultured SSCs grown on MEF feeder layers by gene expression analysis of SSC markers. The expression of representative pluripotent markers *POUV* and *NANOG* was detected in TCs and cultured SSCs, but not in Sertoli cells or quail embryonic fibroblasts (QEFs). Germ cell markers *DAZL* and *VASA* were also strongly expressed in TCs and SSCs, but not in Sertoli cells or QEFs (Figure 5-3a). Cytochemical analysis showed that the cultured SSCs stained positively for PAS, a stem cell functional enzyme marker (Figure 5-3b). Immuno-cytochemical analysis demonstrated expression of the germ cell-specific

markers DAZL and VASA, as well as the SSC-specific marker ITGA6, in cultured SSCs (Figure 5-3c). Overall, the SSCs co-cultured with MEFs maintained a stable growth pattern without a loss of their characteristics.

Germ cell depletion using busulfan and repopulation of recipient quail testes

For efficient production of germline chimeric quails, endogenous germ cells were depleted using busulfan, before TC and SSC transplantation. Eight *D* strain male quails received a single intraperitoneal injection of 40 mg/kg busulfan. After 2 weeks of busulfan treatment, the size of the testes was greatly reduced in the recipients (approximately 1 cm in diameter) compared with the non-treated controls (approximately 1.8 cm in diameter) (Figure 5-4a). The testicular weight of busulfan-treated recipients (0.408 ± 0.09 g) was also significantly lower than that of the wild type control (3.0 ± 0.39 g) (Figure 5-4b). Compared with the control group, both testicular size and weight were decreased by 84% by busulfan; these decreases were greater than those achieved in chickens treated with the same concentration of busulfan (Tagirov and Golovan, 2012). Immunohistochemical staining for the germ cell-specific marker VASA showed that the germ cell population in the seminiferous tubules was also considerably reduced in busulfan-treated testes. Furthermore, the size of the tubule itself was more compact than that in wild type controls (Figure 5-4c). This suggests that endogenous germ cell depletion was achieved by a single intraperitoneal injection of busulfan.

Quail germline chimera production by transfer of spermatogonial cells

To produce germline chimeric quails using spermatogonial cells, WP quail TCs and cultured SSCs (at P2) labeled with PKH26 red fluorescence dye were transplanted into four D strain quail testes (two quails for TC

transplantation and two quails for SSC transplantation) 2 weeks after busulfan treatment (Table 5-1). To confirm the localization of spermatogonial cells in transplanted testes, 20-μm-thick cryosections from D strain recipient testes were examined under a fluorescent microscope (Figure 5-5a). Transplanted TCs were identified in the inner spaces of the seminiferous tubules of the recipient testes, confirming the localization of the implanted cells. Subsequently, testcross analysis showed that germline transmission occurred in two of three recipients. Regarding the phenotypic characteristics, the hybrids (D/d^+) had dark brown feathers, whereas the donor (d^+/d^+)-derived progenies had yellow and black stripes (Figure 5-5b). As shown in Table 5-1, 61 progenies from 100 incubated eggs hatched from one recipient transplanted with whole TCs, and 7 donor-derived progenies were identified among them (11.5% germline transmission efficiency). Meanwhile, between the two recipients of SSCs, 64 progenies were produced from 82 incubated eggs from recipient quail #1, but no donor-derived progenies were identified. On the other hand, in recipient quail #2, 42 progenies were produced from 54 incubated eggs, 7 of which were identified as donor-derived progenies (16.7% germline transmission efficiency) (Table 5-1). To confirm the identity of the donor-derived progenies, we used genotyping to identify the breed-specific MC1R gene, which regulates hair color (Rees, 2003). Through DNA sequencing, we found a SNP at nucleotide 289 in the MC1R gene. This base is "G" in the WP strain and "A" in the D strain; thus, we designed a primer to target this simple breed-specific marker. The primer sets used, which have different annealing affinities, effectively distinguished between the different quail breeds. As shown in Figure 5-5c, WP-specific amplicons were amplified by PCR in all four progenies (SSCs-derived progeny; SP1, SP2, SP3 and SP4, respectively), but donor-derived progenies did not express D-specific amplicons (SP2 and SP3) (Figure 5-5c). This suggests that progenies expressing both amplicons are hybrids (D/d^+, SP1 and Sp4), while donor-derived progenies ($d^{+/}$ d^+) express only the WP amplicon (SP2 and SP3) (Figure 5-5c). Collectively, we successfully produced germline

chimeras by transplantation of TCs and SSCs into busulfan-treated testes and effectively confirmed the presence of WP donor-derived progenies using phenotypic and genotypic criteria.

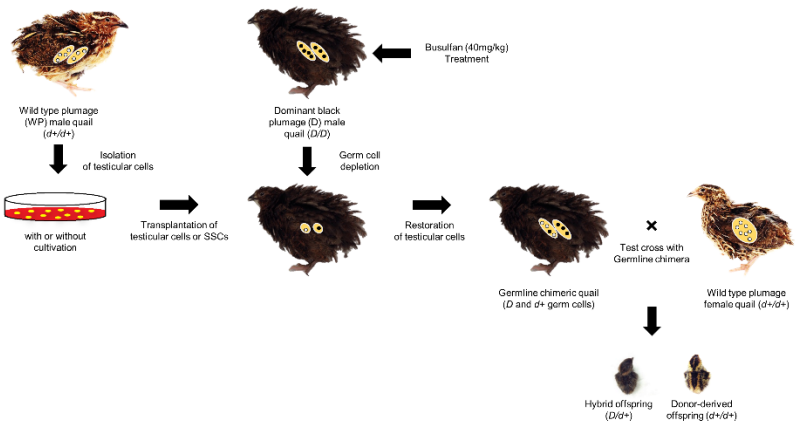

Figure 5-1. General procedure for production of germline chimeric quail and donor-derived offspring by TC/SSC transplantation.

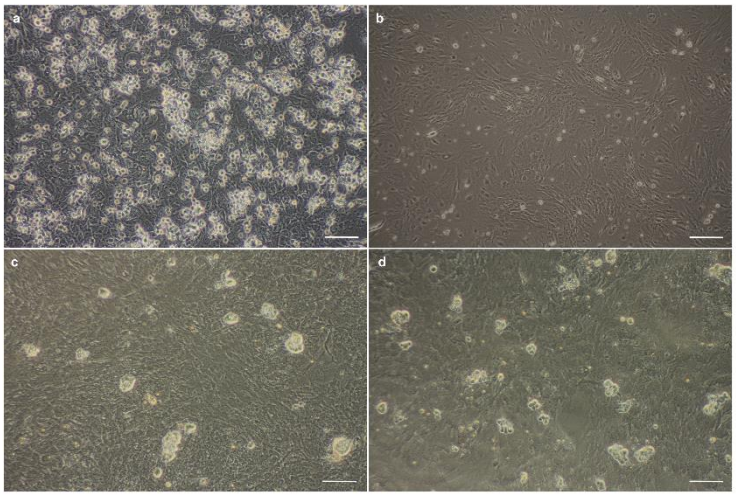

Figure 5-2. Culture of quail SSCs. (a) Morphology of TCs one day after seeding. (b) Morphology of Sertoli cells derived from whole TCs. (c) Colonized quail spermatogonial cells (6 days after culture, passage 1) and (d) SSCs (20 days after culture, passage 4) on MEF feeder layers. Scale bar = 200 μm.

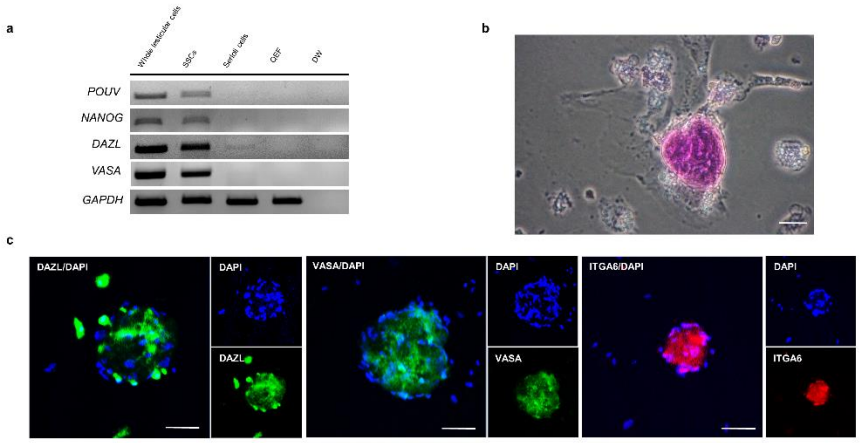

Figure 5-3. Characterization of quail SSC after 20 days of culture. (a) RT-PCR analysis of *POUV, NANOG, DAZL,* and *VASA* in quail SSCs cultured for 20 days. Quail TCs were used as the positive control, and Sertoli cells and QEFs were used as the negative controls. (b) Cytochemical analysis of SSCs by PAS staining. (c) Immunocytochemical staining of SSCs for DAZL, VASA, and ITGA6. Scale bar = 20 μm.

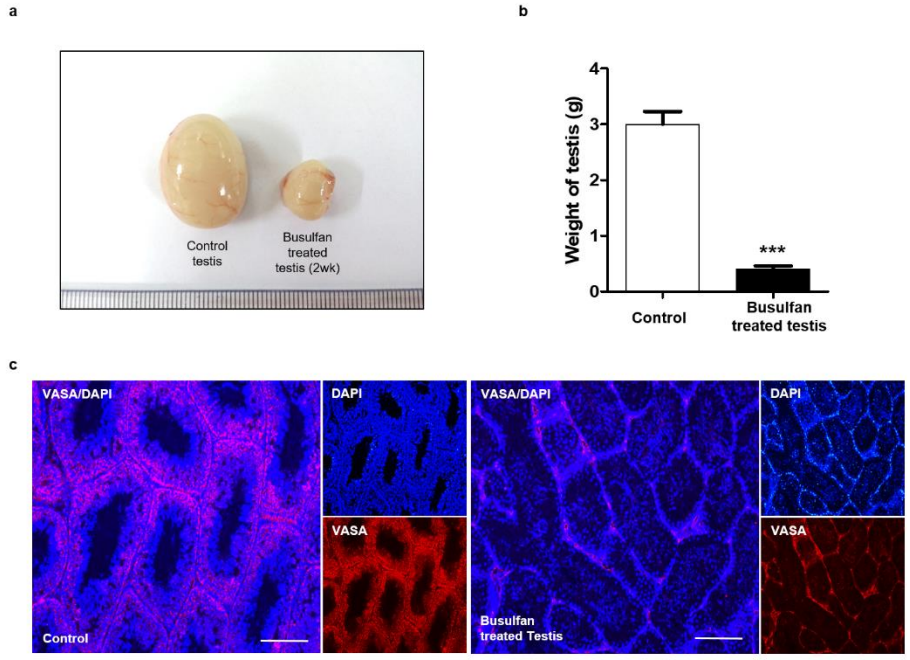

Figure 5-4. Effect of busulfan treatment on testicular weight and germ cell sterilization. (a) Effect of busulfan treatment on adult quail testicular size. Morphological comparisons between control and busulfan-treated testes (2 weeks after treatment). (b) The weight of the testes 2 weeks after busulfan treatment was significantly lower than that of control testes ($P < 0.01$). (c) The number of germ cells in adult testes decreased after busulfan treatment. Scale bar = 100 μm.

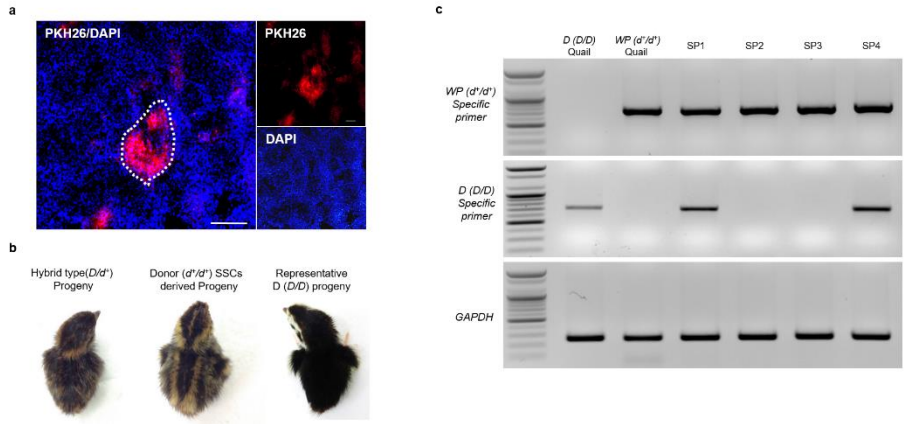

Figure 5-5. Transplantation of WP (d^+/d^+) TCs and SSCs into strain D (*D/D*) testis after busulfan treatment and production of donor (d^+/d^+)-derived progenies. (a) Localization of cultured SSCs (d^+/d^+) in the seminiferous tubule (white dotted line) after injection into busulfan-treated testis (*D/D*). Scale bar = 100 µm. (b) Donor-derived progenies (d^+/d^+) from strain D quail recipients (D/d^+) mated with WP quail (d^+/d^+). Hybrid progeny (D/d^+) and strain D progeny (*D/D*) are presented as controls. (c) Genomic DNA analysis for breed determination of germline chimeric quail. Genomic DNA was isolated from the progenies, and PCR was used to distinguish between strain D (*D/D*) and WP (d^+/d^+). In progenies SP1 and SP4, both strain D-specific and WP-specific markers were detected, indicating that these progenies are hybrids (D/d^+). In progenies SP2 and SP3, WP-specific markers were detected, indicating that these progenies are donor-derived (d^+/d^+). SP, SSCs-derived progeny.

Table 5-1. Birth of germline chimeras produced by transfer of whole testicular cells and/or quail SSCs

Type of Donor cells	No. of transplanted cells	No. of incubated eggs	No. of hatched quails	No. of donor-derived quail	Donor-derived quail Produced/Total (%)*
Whole testicular cells	3×10^6 cells	100	61	7	11.5
14-day cultured SSCs #1	3×10^6 cells	82	64	0	0
14-day cultured SSCs #2	3×10^6 cells	54	42	7	16.7

* Percentage of hatchlings shown to be of the donor-derived WP (d^+/d^+) genotype

5. Discussion

The merits of using quail as an avian experiment model include its high egg production, low maintenance cost, small body size and short generation period (approximately 6-8 weeks). Indeed, these characteristics make quail an ideal species for transgenic research and many biology models (Zhang, Sun et al., 2012; Huss *et al.*, 2015). Methods of germ cell-mediated germline chimera production are considered very reliable and are used in avian transgenesis experiments (Han, 2009; Han *et al.*, 2015). The production of germline chimeras for transgenic research mediated by PGCs is the most prominent system used to develop avian, especially chicken, models, (van de Lavoir, Diamond et al., 2006; Park and Han, 2012). Several studies have reported successful production of germline chimeric quails by transferring gonad-derived PGCs that had been cultured for 3 and 20 days (Kim, Park et al., 2005b; Park, Kim et al., 2008). Studies have also reported successful production of transgenic quails after transduction of transgenes in germ cells (Shin, Kim et al., 2008; Kwon, Choi et al., 2010). However, compared with chickens, the long-term culture and *in vitro* manipulation required with quail germ cells remain obstacles to their use.

In our previous report, we described the isolation, characterization and *in vitro* culture of chicken and quail SSCs (Lee, Jung et al., 2006; Jung, Lee et al., 2007; Pramod, Lee et al., 2017) and identified SSCs as a distinct type of germline stem cell and an alternative to PGCs. Here, we report the successful production of germline chimeric quails after transplantation of exogenous spermatogonial cells (whole TCs and SSCs) maintained *in vitro* for up to 20 days. This is the first study to report the production of donor-derived progenies via spermatogonial cell transplantation in the quail. Using germ cells from birds after hatching, this system is expected to be highly applicable to transgenic quail production and species conservation.

SSCs are self-renewing adult stem cells that contribute to male germ cells and can differentiate into sperm. Currently, SSCs have been investigated mainly in rodents, domestic animals and even in humans for male fertility preservation and transgenesis (Kossack, Meneses et al., 2009; Kanatsu-Shinohara, Kato-Itoh et al., 2011; Zheng *et al.*, 2014). Because of their high proliferative and differentiation capacities, the depletion of spermatogonial cells, including SSCs in recipient testes, significantly enhances the efficiency of germline transmission. Here, we successfully enriched spermatogonial cells under MEF feeder conditions, while maintaining their germ cell/stem cell characteristics (Figure 5-2 and 5-3) (Pramod, Lee et al., 2017). In each independent experiment, TC transplantation showed 0.4-0.9% efficiency of donor-derived progeny production in wild type testis, while recipients treated with gamma irradiation showed 20.0-22.2% efficiency of donor-derived progeny production (Lee, Jung et al., 2006; Trefil, Micakova et al., 2006).

Busulfan, a antispermatogonial alkylating agent, significantly reduced the chicken testicular weight and number of spermatozoa (Tagirov and Golovan, 2012). Similar to chicken, a single dose of busulfan (40, 30 or 20 mg/kg intraperitoneal injection) significantly induced quail sterility (Jones and Jackson, 1972). Therefore, in the present study, the depletion of endogenous germ cells was induced under similar conditions, because fertility after 2 weeks was significantly reduced by a single dose of busulfan (40 mg/kg) (Jones and Jackson, 1972). As shown in Figure 5-4c, a small population of germ cells remained in the basement membrane of the seminiferous tubules of the recipients even after treatment with busulfan, indicating that the endogenous germ cells had not been depleted completely. Even though germ cells remained under those conditions, over 40 mg/kg of busulfan treatment causing lethal (Bucci and Meistrich, 1987); therefore, we determined 40 mg/kg to be the optimal busulfan concentration for the recipient for spermatogonial transplantation. After transferring 3×10^6 non-cultured TCs and cultured SSCs, 11.5% and 16.7% of donor-derived progenies were produced, respectively.

These efficiencies are slightly lower than that achieved using gamma irradiation to produce germline chimeras in chicken testis (Trefil, Micakova et al., 2006) but higher than that achieved using spermatogonial cell transplantation in chicken testis without sterilization (Lee, Jung et al., 2006). In addition, progeny did not occur in one SSCs transplant. Because of busulfan-induced infertility was highest after approximately 20 days, but the effect lasted up to 50 days in quails (Jones and Jackson, 1972), we presumed that residual busulfan inhibited donor cell (SSCs #1) spermatogenesis.

In this study, we used two breeds of quail: one with black plumage (*D*: homozygous for the autosomal incomplete dominant gene D) and one with WP (d^+/d^+) (Somes RG, 1998). D strain quail have black feathers over the entire body, except the chest where the feathers are white, and WP quail have yellow and black striped feathers. As shown in Figure 5-5b, their phenotypic differences make it easy to distinguish between the two breeds, and thus between donor and recipient. However, the phenotype of the hybrid, a combination of the WP and D strains, is dark brown. The dark brown color of the hybrid is the result of an autosomal incomplete dominant trait inherited from the D strain, which interferes with genotype identification. Therefore, we conducted genotyping using breed-specific markers specific to the MC1R gene, which is involved in regulating hair color (Rees, 2003). WP-specific amplicons were amplified by PCR in all four progenies, but donor-derived progenies did not express D-specific amplicons. These results may be effective in the analysis of germline chimeric quails through testcross as a faster, simpler and more accurate molecular method.

Our study demonstrates that quail germline chimeras can be produced by spermatogonial cell transplantation after busulfan treatment. We are the first to use this strategy to produce donor-derived progeny using adult germ cells. Compared with the embryo-mediated method, this strategy is simple and leads to rapid generation of quail germline chimeras. This will lead to production of transgenic models using adult germ cells and, through the production of

germline chimeras, help in efforts to conserve avian species.

CHAPTER 6

Targeted gene deletion and insertion on quail genome by programmable genome editing in primary primordial germ cells

1. Abstract

Quail is one of the most advantageous avian species for producing genome edited birds because of its low maintenance cost, small body size, and relatively short generation time compared with other avian species. In recent years, the development of a cutting-edge genome engineering technology, Clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated (Cas) 9 has provided numerous opportunities for efficient genome modification in living organisms including avian species. However, the efficient genome modification tools and its deliver into germline competent cells are very limited in quail. In this study, we optimize the tranfection system into quail genome of primary primordial germ cells (PGCs) as one of reliable germline competent cells in aves. We transfected the CMV EGFP plasmid into quail whole gonadal cells using with liposome-mediated method and electroporation method. The transfection efficiencies were varied from 20.75% to 83.64% in whole gonadal cells and from 6.39% to 84.27% in MACS purified PGCs. In subsequently, we employed the CRISPR/Cas9 system to induce mutations in the quail myostatin (qMSTN), and quail ovotransferrin (qTF) genes. The results of sequencing shown that the mutations rate at four different loci of qMSTN were 30%, 10%, 33.3% and 10%, respectively and of qTF were 11.1%, 30%, 22.2% and 30%, respectively. We selected one of guide RNA of each genes, and co-transfected with CMV PuroR vector to improve the mutation efficiency. Only, one day puromycin treatment were accelerate the mutation rate in PGCs to 60% of qMSTN and to 50% of qTF genes. We also attempt same strategy to exogenous gene insertion using non-homologous end joining (NHEJ) with EGFP vector. As a result, the designed nucleases sufficiently modified the targeted locus on the chicken genome and introduced EGFP gene cassettes. The results suggest that targeted genome modification mediated by programmable genome editing tools can be applied to quail genome, and precise gene insertion on targeted locus can be introduced by enhanced

DNA cleavage event. Collectively, these results are the first report of targeted gene deletion and insertion on quail genome by CRISPR/Cas9 system in primary PGCs and it could be supply fabulous chance for producing genome-edited quails.

2. Introduction

Quail is a prominent avian species for transgenic model because of their small body size, less maintaining cost or space, short generation time for sexual maturity, and large egg production capacity compared to other species (Poynter *et al.*, 2009b). Moreover, quail embryos are useful materials in embryological studies, because they are easy to obtain and manipulate during all developmental stages (Nakao, Ono et al., 2008; Mizushima, Hiyama et al., 2014), and it allowed the advantageous for *in ovo* imaging of embryos with reporter transgenes for observing developmental process (Sato *et al.*, 2010; Huss, Benazeraf et al., 2015). For this reason, numerous attempts have been made to produce transgenic quails, which are useful tools for transgenic model development, mainly using lentiviral system (Scott and Lois, 2005; Poynter and Lansford, 2008; Shin, Kim et al., 2008; Kwon, Choi et al., 2010).

Genome editing platforms are developing rapidly in recent years, and broadly adopting on genome modification in living organisms. Especially, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) regarded as a most convenience and efficient tool for genome modification (Cong, Ran et al., 2013; Sander and Joung, 2014). These are successfully applied in somatic lines and stem cell of chicken and quail (Abu-Bonsrah, Zhang et al., 2016; Ahn, Lee et al., 2017). Additionally, CRISPR/Cas9-allowed producing of genome edited birds applied in chicken primordial germ cell (PGC) lines through germline transmission system (Dimitrov, Pedersen et al., 2016; Oishi, Yoshii et al., 2016). Because the chicken PGCs, the precursors to mature germ cells, could be stably expanded *in vitro* without loss of their nature characteristics including self-renew, migrate to genital ridge, germline competency and produce mature gametes, widely used for produce transgenic and genome edited chickens (van de Lavoir, Diamond et al., 2006; Park

and Han, 2012; Park, Lee et al., 2014; Taylor, Carlson et al., 2017). However, chicken is still the only avian species capable of PGC long term cultivation.

Although there were reported several works for isolation and manipulation of PGCs in quails (Ono, Matsumoto et al., 1998; Kim, Park et al., 2005b; Park, Kim et al., 2008) the long-term *in vitro* culture methods of quail PGC and efficient delivering exogenous gene are still limited. In contrast to cell lines, primary cells which freshly isolated from *in vivo* are highly sensitive and arduous for maintaining *in vitro* with optimal nutrients and environment. Moreover, to optimize survival and growth condition, primary cells needed in optimal media for customized for each cell type (Kaur and Dufour, 2012). Therefore, despite its practical potential and universal applicable usage over all organisms, CRISPR/Cas9 mediated genome-edited quails are not reported because of the lack of germline competent cell lines for genome modification and selection system of purposed cells.

In this study, we firstly describe an efficient way for genome edited quail germ cells overcoming aforementioned limitations via efficient transfection and selection methods within several days *in vitro.* To optimizing high efficient transfection we describe the process of optimizing electroporation conditions, and only one day drug-selection. The period that *in vitro* maintain of quail PGCs are only 10 to 20 days, we attempt to all process with non/short term-culture conditions.

3. Materials and methods

Experimental animals and animal care

Protocols for the care and experimental use of Japanese quail (C*oturnix japonica*) were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Quails were maintained according to the standard management program at the University Animal Farm, Seoul National University, Korea. The animal management, reproduction, and embryo manipulation procedures adhered to the standard operating protocols of our laboratory.

QCR1 Staining and MACS purification for quail PGCs

Whole gonadal cells from wild plumage (WP) quail embryonic gonads at day 5 (stage 28) were maintained with knockout DMEM (Invitrogen) supplemented with 20% (vol/vol) FBS (Invitrogen), 2% (vol/vol) chicken serum (Sigma-Aldrich), 1× nucleosides (Millipore), 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, 1× antibiotic–antimycotic (Invitrogen) and human bFGF (10 ng/mL, Sigma-Aldrich). To purify quail PGCs, One million gonadal cells were labeled with QCR1 of mouse immunoglobulin (Ig) G isotype for 20 min at a room temperature of 20-25 °C and washed with the buffer solution. The cells were then placed in 100 μL of buffer solution supplemented with 20 μL of goat anti-mouse IgG microbeads (Miltenyi Biotec, Germany) for 15 min at 4 °C. After the treatment, 500 μL of buffer solution were carefully added to the labeled cells, and MACS was conducted to increase the PGC population. The isolated PGCs were fixed in 4% paraformaldehyde (PFA) for 10 min. After washing in PBS, blocking was performed in PBS containing 5% (v/v) goat serum and 1% bovine serum albumin (BSA). Samples were then incubated overnight at 4 °C

with primary antibody against QCR1 monoclonal antibodies were raised in a mouse host and the resulting hybridomas were provided (Aoyama *et al.*, 1992a; Ono and Machida, 1999). After washing three times in PBS, samples were incubated in the presence of secondary antibodies labeled with fluorescein isothiocyanate (FITC) for 1 h at room temperature. After washing, samples were mounted with ProLong® Gold antifade reagent and 4′,6-diamidino-2-phenylindole (Thermo Fisher–Invitrogen) and visualized under a fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from quail whole gonadal cells and PGCs was prepared using TRIzol reagent (Thermo Fisher Scientific–Invitrogen, CA, USA) and reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific–Invitrogen). RT-PCR was performed using primer sets from a previous report (Jung, Kim et al., 2017b). The quail primers are showed in Table 6-2. RT-PCR reactions comprised 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

Migration assay

To evaluate migration to the gonads, isolated or transfected PGCs were labeled with/without PKH-26 fluorescent dye (Sigma-Aldrich, MO, USA) by a modified protocol. After centrifuged at 1,250 rpm for 5 min, the cells were suspended in 500 μL serum-free medium containing PKH-26 at the concentration of 2×10^{-6} M. Approximately 1,000 cells were injected into the dorsal aorta of HH stages 13–16 embryos of quail. Embryos were sealed with Parafilm™ and further incubated until HH stage 27. Fluorescent cells in the gonad were enumerated under a fluorescence microscope.

Construction of vectors

The *EGFP* Plasmids containing the *EGFP* gene and puromycin

resistant (*Puro*^{R}) gene have been modified our previous report of *piggyBac CMV-GFP-FRT* plasmid (Lee *et al.*, 2016c). For the construct of CRISPR/Cas9 vectors targeting quail *MSTN* and *TF*, we used pX330A all-in-one CRISPR kit used for constructing multiplex CRISPR/Cas9 vectors was a gift from Takashi Yamamoto (Addgene Kit #1000000054) (Sakuma *et al.*, 2014). To insert guide RNA sequences into CRISPR/Cas9 vectors, we synthesized sense and antisense oligonucleotides (Bionics, Seoul, Korea) and carried out annealing using the following thermocycling conditions: 30 s at 95 ˚C, 2 min at 72 ˚C, 2 min at 37 ˚C, and 2 min at 25 ˚C. The oligonucleotides used are listed in Table 6-3. Donor construct for expression of green fluorescent protein and neomycin-resistance gene was amplified from *piggyBac CMV-GFP-FRT* plasmid by forward and reverse primers containing guide RNA recognition site with PAM sequence at their 5' ends. And the amplicon was cloned into pGEM T easy vector (Promega, Madison, WI, USA), and then it was sequenced using an ABI Prism 3730 XL DNA Analyzer (Thermo Fisher–Applied Biosystems, FosterCity, CA, USA). The sequences of forward and reverse primers are listed in Table 6-2.

Transfection and puromycin-selection.

The *CMV-EGFP-CMV-Puro*^{R} vector, gRNA hCas9 expressing vector and Bait-*CMV-EGFP* donor vector were single-introduced (5μg of GFP expression vector) of co-introduced (for knockout test, 5μg of *CMV-EGFP-CMV-Puro*^{R} vector and 5μg of gRNA hCas9 expressing vector, and for knockin test, 5μg Bait-*CMV-EGFP* donor vector, 5μg of gRNA hCas9 expressing vector and 5μg of *CMV-EGFP-CMV-Puro*^{R} vector) into the 5×10^{6} of QM7 myoblast cells lines or primary gonadal cells using the Amaxa Nucleofector (V buffer and A-023, A-020, T-020 and X-001 program) or lipofection with Lipofectamine 2000 reagent (Invitrogen). For lipofection One day after transfection, 1 μg/mL puromycin was added to the culture media for selection, and GFP-expressing cells or colonies were picked under a fluorescence

microscope during puromycin selection.

Validation of viable cells and transfection efficiency

The portion of dead cells and viable cells after gene transfer were separated by Dead Cell Removal Kit using MACS isolation methods (Miltenyi Biotec). Breifly, the transfected cell pellet were resuspend in 100 μL of Dead Cell Removal MicroBeads per approximately 10^7 total cells and incubate for 15 minutes at room temperature. After the treatment, 500 μL of 1xbinding solution were carefully added to the labeled cells, and MACS was conducted to increase the live cell population. After separation, the potion of dead cells and viable cells were calculated by cell counting under the microscopy with Trypan Blue (GIBCO). After validation of viable cells, the transfection efficiency was determined by the proportion of GFP positive cells to the total number of cells.

T7E1 assay

Genomic DNA was extracted from transfected whole cells and PGCs before/after puromycin selection. Genomic regions encompassing the CRISPR/Cas9 target sites were amplified using specific primer sets (Table 6-2). PCR analysis of the targeted loci was examined in a total volume of 20 mL containing 100 ng genomic DNA, 10 × PCR buffer (BioFACT, Daejeon, Korea), 0.4 mL dNTPs (10 mM each), 10 pmol of each primer, and 0.5 U Taq polymerase (BioFACT) under the following thermocycling conditions: 5 min at 94 ˚C, followed by 35 cycles of 20 s at 94 ˚C, 40 s at 61 ˚C, and 120 s at 72 ˚C, and a final 10 min at 72 ˚C. Primers are listed in Table 6-2. The PCR amplicons were re-annealed to form a heteroduplex DNA structure after denaturation. Subsequently, the heteroduplex amplicons were treated with 5 units T7E1 endonuclease (New England Biolabs) for 20 min at 37 ˚C and then

analyzed by 1% agarose gel electrophoresis.

Statistical analysis

Differences showed in this study were analyzed by Student's *t*-test and One-way ANOVA using GraphPad Prism statistical software (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered to indicate statistical significance (***$p < 0.001$, and *$p < 0.05$).

4. Results

Isolation and characterization of quail PGCs purified by QCR1 antibody

Firstly, we isolated whole gonadal cells (WG) from 5-day old WP strain quail embryos containing unpurified PGCs and gonadal stromal cells. Through the MACS separation, we obtaining lipid granule contained floating cells apart from somatic cells (Figure 6-1). The portion of cells, 85.23 ± 6.05 % were MACS negative and 6.33 ± 2.84 % were positively isolated by MACS (Figure 6-1b). The MACS separated cells were specifically expressed germ cell specific genes (*DAZL* and *VASA*) and pluripotency markers (*NANOG* and *POUV*) (Figure 6-1c) and these cells are also expessing QCR1 itself (Figure 6-1d). To examined functionality of PGCs, we injecting 1,000 PKH-26-stained cells into the dorsal aorta of HH stages 13–16 quail recipient embryos (Figure 6-1e).

Optimizing transfection conditions in primary PGCs

To optimize the transfection efficiency, we transfected the *EGFP* expressing vector containing Puromycin resistant gene ($Puro^R$) into quail whole gonadal cells using Amaxa Nucleofector (V buffer and A-023, A-020, T-020 and X-001 program) or lipofection with Lipofectamine 2000 reagent. One day of transfection, we validated the number of GFP expressing cells of the transfected cells (Figure 6-2a). The portion of GFP positive cells were significantly higher in X-001 nucleofection group than other nucleofection groups or lipofection group (Figure 6-2c). After MACS separation, the GFP positive cells were also higher in X-001 nucleofection group than others (Figure 6-2b and 6-2d). In the validation of viable cells, A-023 nucleofection group was lower than other transfection groups. We conclude that the X-001 nucleofection group is the optimal one for delivering exogenous gene into quail PGCs

(Table 6-1).

CRISPR/Cas9 nuclease-mediated targeted mutagenesis in QM7 cell line

Subsequently, we select optimal CRISPR/Cas9 nuclease vectors for q*MSTN* gene and q*TF* gene using QM7 myoblast cells. We used all-in-one vector for expressing guide RNA for each genes and containing human Cas9 expressing cassette. We designed two of q*MSTN* exon 1 targeted gRNAs and two of q*MSTN* exon 3 targeted gRNAs (Figure 6-3a). The results showed that varied mutations efficiencies from 10% to 30% were observed for the q*MSTN* gene (Figure 6-3b). Similarly, we designed four different gRNAs for q*TF* exon7, exon8, exon9 and exon 12 (Figure 6-3c). The sequencing results showed that the efficiencies of targeted mutation of q*TF* qRNAs were 11.1% to 30% (Figure 6-3d). We selected q*MSTN* #1 gRNA and q*TF* #4 gRNA for subsequent experiments.

***CRISPR/Cas9 nuclease-mediated targeted mutagenesis* in quail PGCs**

In order to enrichment of targeted mutant cells, we co-transfected q*MSTN* #1 gRNA plasmid vector and $Puro^R$ vector containing EGFP cassette (Figure 6-4a). The mutation rate was 20% in whole gonadal cells after one day of Nucleofection (X-001 program). With the one day of puromycin treatment (1μg/ml) and MACS separation for purifying PGCs, the mutation rate was enhanced to 60% (Figure 6-4b). In a similar way, after q*TF* #4 gRNA plasmid vector and $Puro^R$ vector (Figure 6-4c) transfection, the mutation rate was 30% in whole gonadal cells and increasing the mutation rate to 50% in one-day puromycin selection PGCs (Figure 6-4d).

CRISPR/Cas9 improved targeted exogenous gene knockin

For validating targeted gene insertion to quail genome in PGCs, we constructed EGFP containing donor plasmid with Bait sequence which for gRNAs targets, have been designed for the cleavage induction of non-homologous end-joining (NHEJ) circular donor plasmids and adding *Puro*^{R} vector containing EGFP cassette for drug selection (Figure 6-5a). We targeted qMSTN exon 1 using three independent circular vectors including q*MSTN* #1 gRNA, q*MSTN Bait EGFP* donor plasmid and *CMV EGFP CMV Puro*^{R}. After one day of Nucleofection to quail whole gonadal cells, we treated puromycin (1μg/ml) for increasing cells of targeted mutagenesis (Figure 6-5b). For the confirmation of transfected cells we conducted immunocytochemistry with QCR1 antibody and DAZL as specific markers for germ cells and the stained cells with each marker were highly co-localized with transfected PGCs (Figure 6-5c).

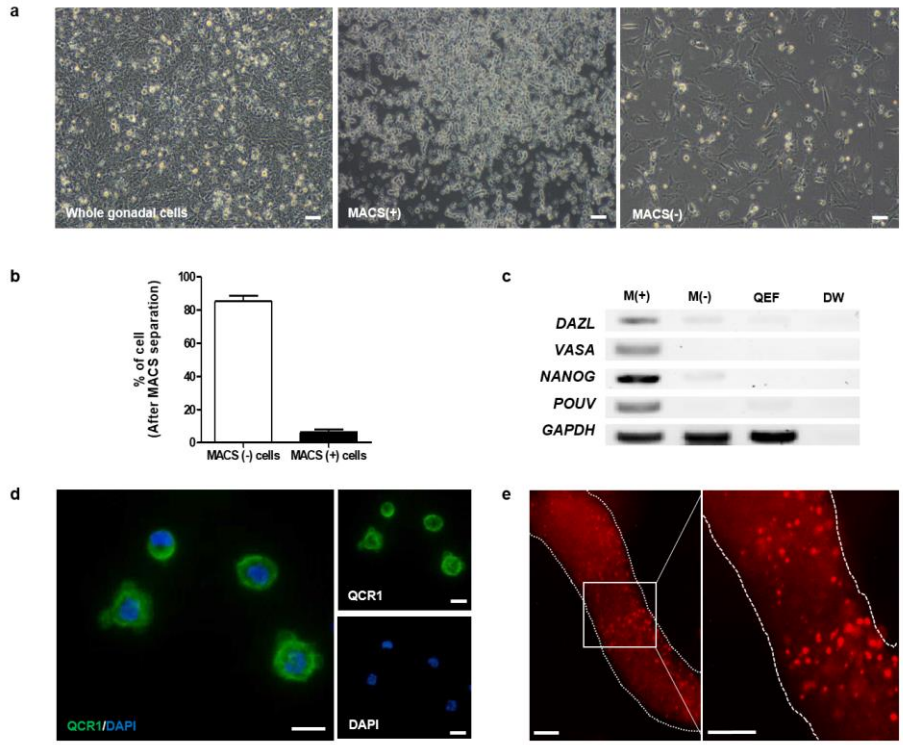

Figure 6-1. Characterization of QCR1 MACS$^+$ cells isolated from whole gonadal cells. (a) Morphology of whole gonadal cells, QCR1 MACS positive cells (MACS$^+$) and negative cells (MACS$^-$) a day after seeding. Scale bar, 100 µm. (b) Yields of QCR1 MACS negative (-) and QCR1 MACS positive (+) cells obtained after purification. (c) Expression of germ cell-marker genes (*DAZL and VASA*) and pluripotency marker genes (*NANOG* and *POUV*) and was determined by RT-PCR in MACS$^+$ cells. M(+), QCR1 MACS positive cells; M(-), QCR1 MACS negative cells; QEF, quail embryonic fibroblast; DW, distilled water. (d) Immunostained with antibody against QCR1 of MACS positive cells from quail embryonic gonads. Scale bar, 20 µm. (e) Migration of QCR1 MACS$^+$ cells. Approximately 1,000 QCR1 MACS$^+$ cells were labeled with PKH-26 fluorescent dye and injected into the dorsal aorta of HH stages 13–16 quail embryos, then incubated until HH stage 27. Scale bar, 100 µm.

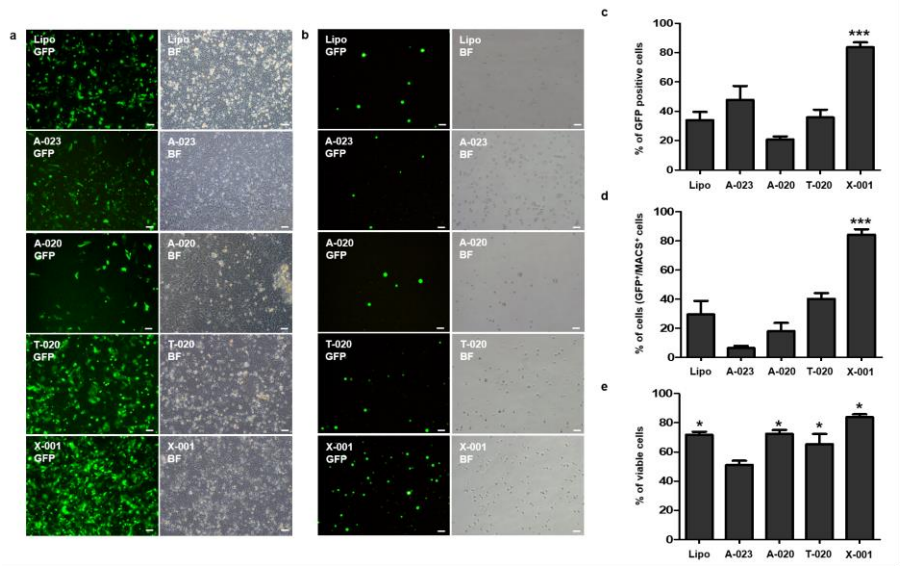

Figure 6-2. Validation of transfection efficiencies using lipofection and nucleofection to quail gonadal cells. (a) Images of whole gonadal cells a day after *CMV EGFP CMV Puro*^{R} transfection, and (b) PGCs. Scale bar, 100 μm. (c) Percentage of GFP positive cells in whole gonadal cells and (d) PGCs. Significant differences between X-001 group with other groups (one-way ANOVA; ***$p < 0.001$). (e) Percentage of viable cells after transfection. Viable cells a day after nucleofection using A-023 program were significantly lower than others (one-way ANOVA; *$p < 0.05$). Bars indicate the SD of triplicate analyses.

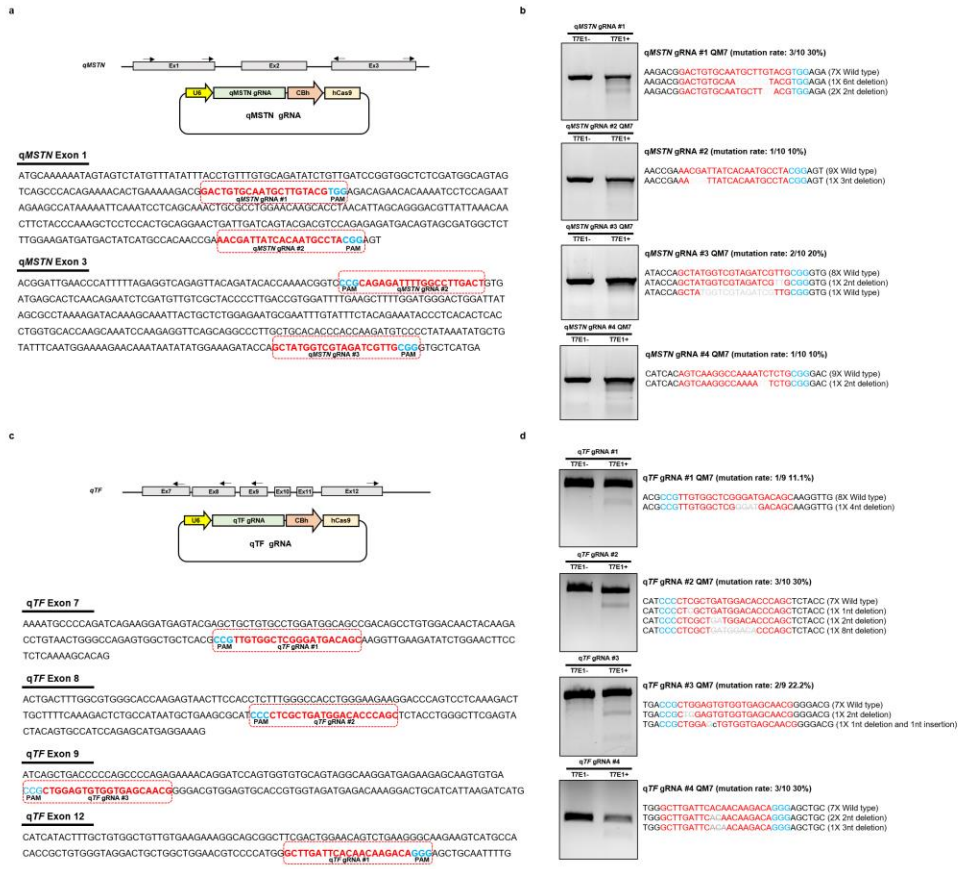

Figure 6-3. Validation of CRISPR/Cas9 expressing vectors targeted for q*MSTN* and q*TF* genes in QM7 cell line. (a) A schematic diagram of the q*MSTN* targeted CRISPR/Cas9 expression vectors. Four different the q*MSTN* targeted CRISPR/Cas9 expression all-in-one vectors were constructed. Red line boxes indicate target site for each guide RNA. (b) Results of T7E1 assay and sequencing results of q*MSTN* mutations induced by CRISPR/Cas9 in QM7 cell line. (c) A schematic diagram of the q*TF* targeted CRISPR/Cas9 expression vectors. Four different the q*TF* targeted CRISPR/Cas9 expression all-in-one vectors were constructed. Red line boxes indicate target site for each guide RNA. (d) Results of T7E1 assay and sequencing results of q*TF* mutations induced by CRISPR/Cas9 in QM7 cell line.

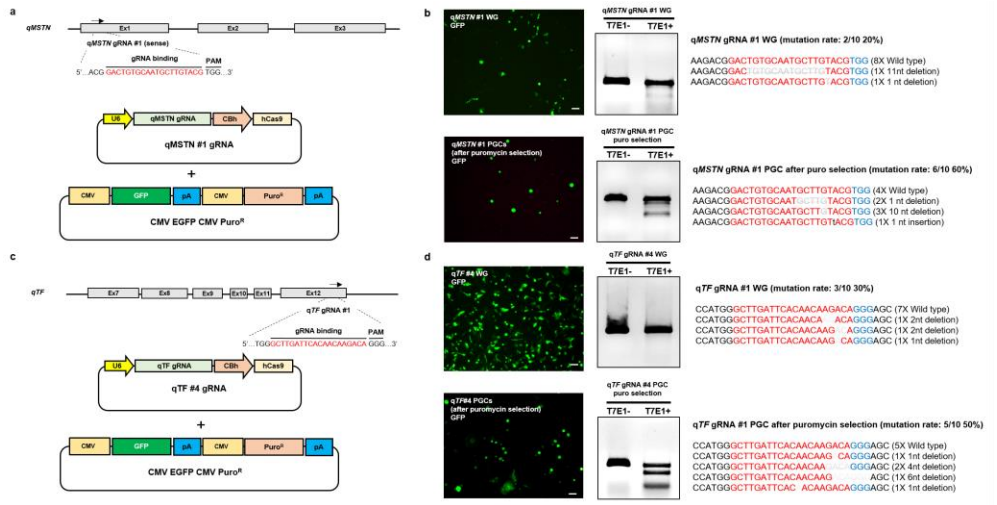

Figure 6-4. Validation and the enrichment of PGCs with targeted modification with puromycin selection. (a) A schematic diagram of the q*MSTN* #1 targeted CRISPR/Cas9 expression vector and *CMV EGFP CMV Puro*^{R} expressing vector. (b) Fluorescent image of q*MSTN* #1 targeted CRISPR/Cas9 and *CMV EGFP CMV Puro*^{R} transfected whole gonadal cells and PGCs. Results of T7E1 assay and sequencing results of q*MSTN*#1 mutations induced by CRISPR/Cas9 in whole gonadal cells and PGCs after puromycin selection for a day. (c) A schematic diagram of the q*TF* #4 targeted CRISPR/Cas9 expression vector and *CMV EGFP CMV Puro*^{R} expressing vector. (d) Fluorescent image of q*TF* #4 targeted CRISPR/Cas9 and *CMV EGFP CMV Puro*^{R} transfected whole gonadal cells and PGCs. Results of T7E1 assay and sequencing results of q*TF*#4 mutations induced by CRISPR/Cas9 in whole gonadal cells and PGCs after puromycin selection for a day. Scale bar, 100 μm.

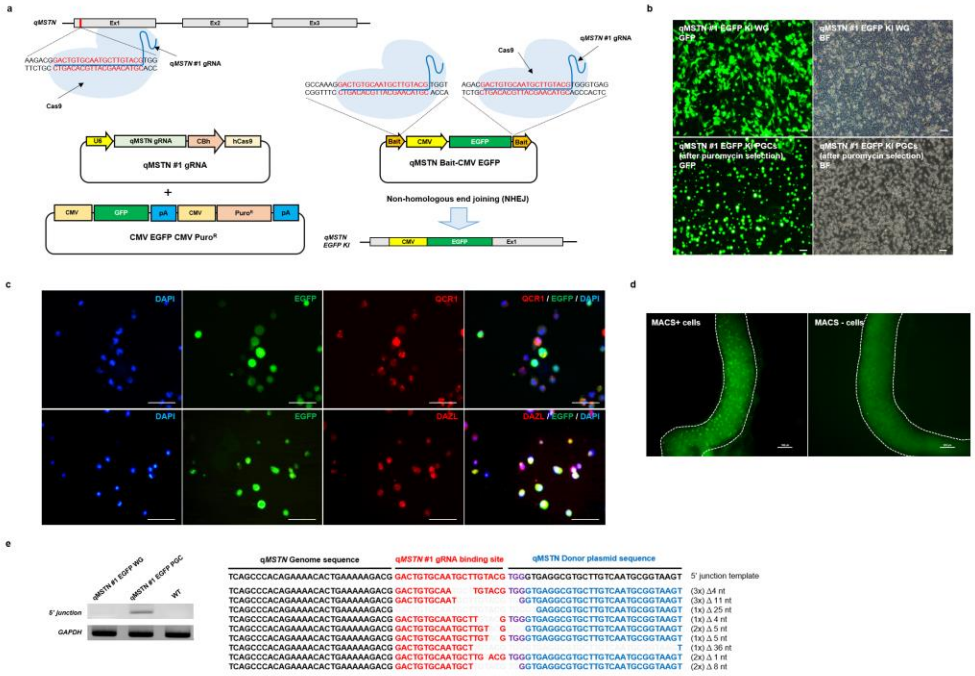

Figure 6-5. Tageted *EGFP* gene insertion into q*MSTN* gene by cleavage induction of non-homologous end joining (NHEJ). (a) A schematic diagram of the q*MSTN* #1 targeted CRISPR/Cas9 expression vector, *CMV EGFP CMV Puro*^{R} expressing vector and q*MSTN* Bait *CMV-EGFP* donor plasmid. (b) Fluorescent images of q*MSTN* #1 targeted CRISPR/Cas9, and q*MSTN* Bait *CMV-EGFP* donor plasmid were transfected to whole gonadal cells and one day puromycin selected PGCs. Scale bar, 100 μm. (c) Immunostained with antibody against QCR1 and DAZL of q*MSTN* #1 targeted CRISPR/Cas9, and q*MSTN* Bait *CMV-EGFP* donor plasmid transfected PGCs. Scale bar, 100 μm. (d) Migration of MACS separated (both of positive and negative cells) q*MSTN* #1 targeted CRISPR/Cas9, and q*MSTN* Bait *CMV-EGFP* donor plasmid transfected PGCs. Approximately 1,000 transfected cells were injected into the dorsal aorta of HH stages 13–16 quail embryos, then incubated until HH stage 27. Scale bar, 100 μm. (e) Genomic DNA analysis of EGFP knockin primary quail PGCs. 5' junctions between donor plasmid and qMSTN gene were

analysed by genomic DNA PCR analysis and sequencing analysis. Genomic DNA PCR analysis of knockin q*MSTN* #1 *EGFP* KI WG cells and puromycin selected PGCs. Positive for knockin specific PCR conditions compared to wild type. WT; wild type. Sequences of 5' junction in q*MSTN* knockin PGCs. The donor vector DNA was integrated into q*MSTN* genes with mutations including deletions and insertion.

TABLE 6-1. Transfection efficiencies in quail whole gonadal cells and purified PGCs using various conditions

Transfection methods	Transfection efficiency (%, WG[a])	Transfection efficiency (%, PGC[b])	viable cells (%, PGC[c])
Lipofection 2000	33.94 ± 9.67	29.45 ± 16.15	71.67 ± 3.8
A-023	47.75 ± 16.56	6.39 ± 2.59	51.01 ± 5.14
A-020	20.75 ± 3.69	17.99 ± 9.78	72.37 ± 4.63
T-020	35.76 ± 10.30	40.18 ± 6.77	65.29 ± 12.15
X-001	83.64 ± 6.56	84.27 ± 6.42	83.78 ± 3.42

[a]: Transfected whole gonadal cells

[b]: Transfected PGCs

[c]: Viable PGCs after transfection

Table 6-2. Primers for PCR analysis in this study

Primer name	Sequences (5'- 3')	Note
POUV F	GCAGGAGATGTGCAATGCAGAGCAA	Primers for endogenous genes
POUV R	GTGGCTGCTGTTGTTCATGGAGATC	
NANOG F	TAGGTGCGGCCACTACTACTGGCCC	
NANOG R	TCCACCCACTGACTCTCCTTCTGGC	
VASA F	TTCAGTAGCAGCAAGAGGCC	
VASA R	CTCCTGGGTTCACTCTGCTG	
DAZL F	GGCAAAAGGCTGAAACTGGG	
DAZL R	TTCTTTGCTCCCCAGGAACC	
GAPDH F	TTCACCACCATGGAGAAGGC	
GAPDH R	CCATCCCTCCACAACTTCCC	
q*MSTN* #1,2 T7E1 F	GGCACACCAGTGTGGCAAGC	Primers for T7E1 analysis
q*MSTN* #1,2 T7E1 R	TCTCAGCGGAGCAGTGCGAG	
q*MSTN* #3,4 T7E1 F	AGCAGGGAACAAACGTAGCAGACT	
q*MSTN* #3,4 T7E1 R	CGAAGTGGTGGATCTCACGATGGTC	
q*TF* #1,2 T7E1 F	TAGCAATTCTGAGCGTGCCA	
q*TF* #1,2 T7E1 R	GAGCTCATCCTAGTTGCCCC	
q*TF* #3 T7E1 F	AGCCCTGCTGGGCAGCATCA	
q*TF* #3 T7E1 R	GTCCCCATCTTGAGGCTCTG	
q*TF* #4 T7E1 F	GTTGTGAAGAAAGGCAGCGG	
q*TF* #4 T7E1 R	CTTGGGGAACGCTTTTTGGG	
q*MSTN* EGFP KI 5' R	CATTTTGACTCACGCGGTCG	Primers for EGFP KI validation.
q*MSTN* EGFP KI 3' F	GCCACTCCCACTGTCCTTTC	q*MSTN* EGFP KI 5' R paired with q*MSTN* #1,2 T7E1 F primer
q*MSTN* EGFP KI 3' R	TGCAGTGGAGGAGCTTTGGG	

Table 6-3. Primers for pX330A CRISPR/Cas9 expression vectors

aPrimer name	Sequences (5'- 3')
q*MSTN* #1 F	caccgGACTGTGCAATGCTTGTACG
q*MSTN* #1 R	aaacCGTACAAGCATTGCACAGTCc
q*MSTN* #2 F	caccgGACTGTGCAATGCTTGTACG
q*MSTN* #2 R	aaacCGTACAAGCATTGCACAGTCc
q*MSTN* #3 F	caccgGACTGTGCAATGCTTGTACG
q*MSTN* #3 R	aaacCGTACAAGCATTGCACAGTCc
q*MSTN* #4 F	caccgGACTGTGCAATGCTTGTACG
q*MSTN* #4 R	aaacCGTACAAGCATTGCACAGTCc
q*TF*#1 F	caccgGCTGTCATCCCGAGCCACAA
q*TF* #1 R	aaacTTGTGGCTCGGGATGACAGCc
q*TF* #2 F	caccgGCTGGGTGTCCATCAGCGAG
q*TF* #2 R	aaacCTCGCTGATGGACACCCAGCc
q*TF* #3 F	caccgCGTTGCTCACCACACTCCAG
q*TF* #3 R	aaacCTGGAGTGTGGTGAGCAACGc
q*TF* #4 F	caccgGCTTGATTCACAACAAGACA
q*TF* #4 R	aaacTGTCTTGTTGTGAATCAAGCc

Note: Letters with underline were target sequence

5. Discussion

The quail is an ideal organism for producing avian model for various purposes including transgenic and programmable genome edited birds because of their small size, short generation time and economical traits (Zhang, Sun et al., 2012; Huss, Benazeraf et al., 2015). In avian species, the PGC-mediated system is the most prominent way for production of germline transgenic model birds (van de Lavoir, Diamond et al., 2006; Park and Han, 2012). However, compared with chickens, the long-term culture and *in vitro* manipulation required with quail germ cells remain obstacles to their use. Several studies have reported successful production of germline chimeric quails by transferring gonad-derived PGCs that had been cultured for 3 and 20 days (Kim, Park et al., 2005b; Park, Kim et al., 2008). Studies have also reported successful production of transgenic quails after transduction of transgenes in germ cells (Shin, Kim et al., 2008; Kwon, Choi et al., 2010). However, compared with chickens, the long-term culture and *in vitro* manipulation required with quail germ cells remain obstacles to their use. Therefore, in this study, we established efficient transfection of genome modification tools, CRISPR/Cas9 into quail genome of primary PGCs to overcome the limitations of *in vitro* cultivation and long-term selection.

For the characterization of quail PGCs, we attempted to MACS cell separation using QCR1 antibody which specifically recognize the quail PGCs from the bloodstream and the gonad (Ono, Yokoi et al., 1996; Ono and Machida, 1999). The QCR1 positive cells were successfully generated the mature gametes after transplanted into recipients that demonstrated the germline cells, PGCs (Ono, Matsumoto et al., 1998). One of the representative characteristics of avian PGCs showed large cellular size compared to other neighboring cells (Yoshinaga, Nakamura et al., 1993), the MACS positive cells were resemble the large size compared to MACS negative population (Figure 6-1a). The separated cells after MACS exhibit the germ cells specific marker genes

including *DAZL* and *VASA,* and also expressed the pluripotent marker genes, *NANOG* and *POUV (*Figure 6-1c and Figure 6-5c). These genes are regarded as representative PGC marker in avian species (Choi, Kim et al., 2010; Jung, Kim et al., 2017b). Migration to the genital ridge is another key characteristic of PGCs. Especially, the PGCs from avian species are circulate in the embryonic blood vessels during the early embryonic stages (Meyer, 1964; Fujimoto *et al.*, 1976a; Urven *et al.*, 1989). We confirmed the migration activity of MACS positive cells by microinjection of the cells into the dorsal aorta of HH stages 13-16 of quail embryos, and the results showed that normal migration activity (Figure 6-1e and 6-5d). Collectively, we assumed that the cells of MACS separated by QCR1 antibody are purified quail PGCs.

We have examined the efficiency of introducing exogenous DNAs into quail PGCs using lipofectamine 2000 reagent and nucleofection. To delivering non-viral exogenous gene into chicken PGCs, lipid-DNA complex were successfully established (Macdonald, Taylor et al., 2012; Park and Han, 2012; Lee, Lee et al., 2016c). However, lipid-DNA mediated transfection has successfully been used in chicken PGCs, this way hardly applying to certain primary cell types such as mouse PGCs, zebrafish fibroblasts and human fibroblasts (Watanabe *et al.*, 1997; Zauner *et al.*, 1999; Badakov and Jazwinska, 2006). It is believed that this limitation is mainly due to intracellular barriers, including poor endocytosis, endosomal escape and nuclear localization of transformed DNA (Zabner *et al.*, 1995). The nucleofection, one of the electroporation technology facilitates transfer of exogenous gene into cytoplasm and nucleus of target cells including stem cells and primary cells (Siemen *et al.*, 2005; Aluigi *et al.*, 2006; Badakov and Jazwinska, 2006; Goffinet and Keppler, 2006). In our case, transfection efficiency and viability were very poor in both of direct lipofection or nucleofection to MACS purified PGCs (data not shown). In contrast, transfection into whole gonadal cells before MACS purification, the cell viability was higher (51.01% to 83.78% in each conditions) than direct transfection to PGCs in each conditions (Figure 6-2).

Next, we performed targeted genome deletion and insertion of quail PGCs using CRISPR/Cas9 system. We selected *MSTN* gene as a representative muscle development and increase meat productivity in various animals such as mice, goats, rabbits, sheep and pigs (Ni *et al.*, 2014; Crispo *et al.*, 2015; Qian *et al.*, 2015; Zhou *et al.*, 2015; Lv *et al.*, 2016), and *TF* as a major egg-associated allergen (Aabin *et al.*, 1996; Urisu *et al.*, 2015). To interrupt of those gene we designed four gRNAs per each gene, we selected one of them to each gene that exhibits the highest efficiency of mutation efficiency, and applied to gonadal cells. Recent works showed that the efficient selectivity of transfected cells using puromycin especially in avian cell lines. CRISPR/Cas9 mediated transfected cell selection by puromycin was performed at 2-4 days, and nearly 100% indel mutation was induced in this process (Abu-Bonsrah, Zhang et al., 2016; Oishi, Yoshii et al., 2016; Wang *et al.*, 2017). Interestingly, only one day of puromycin treatment were increasing the indel mutation rate on the quail genome purified PGCs (60% indel mutation in *MSTN* and 50% indel mutation of *TF* gene) compared to unpurified whole gonadal cells (20% indel mutation in *MSTN* and 30% indel mutation of *TF* gene)(Figure 6-4). Although the increasing genome mutation rate the viability of transfected whole gonadal cells were decreasing after two days of treatment (data not shown), one day treatment period is considered appropriate.

Additionally, we performed CRISPR/Cas9 mediated knockin in quail PGCs. Site specific double-strand beaks (DSBs) in genome induced efficient homologous recombination (HR) in cultured cells (Jasin, 1996). As the development of advanced genome-editing technologies such as transcription activator-like effector nuclease (TALEN) and CRISPR/Cas9 system, the DSB induced HR were generally used for gene insertion into specific genome locus of various organisms (Zu *et al.*, 2013; Cui *et al.*, 2015; Peng *et al.*, 2015; Taylor, Carlson et al., 2017). However, in general, the efficiency of HR relatively low in most of cases (Carroll, 2014), we used other recombination way, non-homologous end-joining (NHEJ) for CRISPR/Cas9 mediated knockin in quail

PGCs. Here, we employed the target sequences for q*MSTN* #1 gRNAs of donor plasmid, so called bait sequence which reported in zebrafish genome modification (Auer *et al.*, 2014). The homology-independent DNA integration mechanism were allowed the highly efficient targeted knockin of desirable genes for many purposes (Bae *et al.*, 2014; Kimura *et al.*, 2014; Nakade *et al.*, 2014). Here, we successfully demonstrated CRISPR/Cas9 mediated knockin of *CMV-EGFP* cassettes into quail genome of PGCs using this system, and the 5' junction specific amplicon were more intense after puromycin treatment (Figure 6-5).

In conclusion, in this study we optimizing the transfection way on primary quail PGCs. With the short term treatment of puromycin were enhancing the genome modification efficiencies of CRISPR/Cas9 mediated gene deletion and insertion on quail genome even in primary cells. This strategy enable to generation of CRISPR/Cas9 mediated knockout and knockin quails via a homology independent repair with simple and highly efficient way. It might be allow to easily establish novel avian models for industrial, genetic research and developmental biology.

CHAPTER 7

GENERAL DISCUSSION

Avian species have regarded as great models for various purposes including production of functional proteins, disease resistance model, and industrial traits as well as developmental studies of vertebrates. To produce genome modified aves, germline-competent stem cells will be required to achieve robust, expedite, and precise genetic modification combined with cutting-edge biotechnology tools. Moreover, endangered avian species could be persistently preserve using avian germline competent stem cells. In this regard, here, we demonstrated the derivation and manipulation of germline-competent stem cells for various purposes.

In the first study, we reprogrammed the feather follicle cells (FFCs) into pluripotent state cells using mammalian reprogramming factors. For some highly endangered species induced pluripotent stem cells (iPSCs) offer the possibilities for animal restoration and prevent extinction through somatic cell reprogramming. In few studies were suggested the restoration of endangered animal species using iPSCs, thus we attempted to derivation of iPS-like cells (iPSLCs) from GFP-TG chicken FFCs. Because of the avian feathers provide the most easily accessible somatic cell sources and have a great potential for regenerative ability due to the various differentiation capabilities, generation of iPSCs from FFCs could be an alternative for avian restoration and conservation even without sacrificing embryos. We successfully generated iPSLCs from FFCs in the first case of avian adult cell reprogramming into a pluripotent state. Chicken FFCs had MSC-like characteristics, which indicates that they have a high potential to give rise to iPSCs, similar to MSCs. This was supported by the demonstration of iPSLC induction and their *in vitro* characterization. We expect that the system may provide an alternative way to conserve wild and endangered birds and provide a substitute for traditional embryo-dependent cell sources.

In the second study, we identified that the consistent expression of chickenized CD20 monoclonal antibodies (cCD20 mAbs) from transgenic chicken

system. We further identified the proteins contained unique *N*-glycan profiles and exhibited stronger Fc effector functionality including complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) than commercial rituximab, which may be the result of a lack of fucose, high mannose and terminal galactose. Our results suggest that the germline-competent transgenic chicken bioreactor is a promising expression system because of its optimal post translational modification (PTM) characteristics causing superior Fc effector function. It also has been proved that these characteristics have maintained constantly in successive generations, which is expected to increase the value of chicken as a bioreactor. Therefore, this germline transgenic chicken bioreactor system can be a very suitable and effective system for the production of anti-cancer antibodies.

In the third study, we provided a simple and efficient way for generation of germline chimeric quails. Because, the effective transgenic systems in quail are limited because of the absence of long-term *in vitro* systems to culture germ cells and the lack of efficient germline transmission methods. Therefore, we produced germline chimeric quail using testicular cells (TCs) and spermatogonial stem cells (SSCs) transplanted into busulfan-treated recipient testis. The TCs and SSCs from wild type plumage quail (WP strain, d^+/d^+) were successfully isolated, cultivated *in vitro*, and transplanted into busulfan treated recipient black quail (D strain, *D/D*). After transplantation of TCs and SSCs, respectively, the recipients produced donor-derived progenies with 11.5% and 16.7% germline transmission efficiency by testcross analysis. In quail, the first to use this strategy to produce donor-derived progeny using adult germ cells. Compared with the embryo-mediated method, this strategy is simple and leads to rapid generation of quail germline chimeras. This will lead to production of transgenic models using adult germ cells and, through the production of germline chimeras, help in efforts to conserve avian

species.

In the next study, we optimized the transfection way on primary quail PGCs for targeted genome editing. Although the numerous advantageous for producing genome edited quails, the efficient genome modification tools and its deliver into germline competent cells are very limited in quail. Therefore, we optimize the tranfection system into quail genome of primary primordial germ cells (PGCs) as one of reliable germline competent cells in aves. With the combine of nucleofection and short term treatment of puromycin were enhancing the genome modification efficiency of CRISPR/Cas9 mediated gene deletion and insertion on quail genome even in primary PGCs. Collectively, these results are the first report of targeted gene deletion and insertion on quail genome by CRISPR/Cas9 system in primary PGCs and it could be supply fabulous chance for producing genome-edited quails. This strategy provide numerous opportunities for efficient genome modification in quail.

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SUMMARY IN KOREAN

조류는 난중 발달 특성을 이용한 발생학 연구뿐 만 아니라 기능성 단백질, 질병 저항성 모델 생산 및 경제 형질 강화 등을 비롯한 새로운 응용 연구 분야에 매우 중요한 생물자원이다. 모델 조류의 생산은 유전자의 삽입 및 편집을 통해 신속하고 정확하게 이루어 질 수 있으며, 이러한 조류 모델의 생산을 위해서는 생식선 전이 줄기 세포 (germline competent stem cells)가 필수적으로 요구된다. 조류에서는 배아줄기세포, 생식선 줄기세포 및 원시생식세포 등의 생식선 전이 줄기 세포가 알려져 있으며, 이러한 세포들은 생식선 키메라 시스템, 형질 전환 및 유전자 편집 기술들과 결합하여 조류 생명 공학 기술 개발 및 활용을 위한 효율적인 도구가 될 수 있다.

첫 번째 연구에서는 조류 깃털낭 세포 (feather follicle cells; FFCs)로 부터 유도만능 줄기세포 유사세포 (induced pluripotent stem cell-like cells; iPSLCs)의 형성을 시도하였다. 유도만능 줄기세포는 외래 유전자 도입을 통해 생성되는 전능성 줄기세포로서 삼배엽 분화와 생식선 전이가 가능한 세포로 알려져 있다. 본 연구에서 확립된 유도만능 줄기세포 유사세포는 삼배엽 분화와 생식선으로의 전이가 가능함을 보였으며, 이러한 실험적 접근은 체세포 유래의 생식선 전이 줄기세포의 생성을 통해 멸종위기 조류 및 고부가가치 조류 종의 보전 및 복원에 효과적으로 적용 될 수 있다.

두 번째 연구에서는 조류에서 가장 잘 확립된 생식선 전이 줄기세포인 원시생식세포 (Primordial germ cells; PGCs)를 이용하여 항체치료제의 일종인 인간 CD20 단클론 항체 (anti-human CD20 monoclonal antibody; mAb)를 발현하는 생식선 형질전환 닭의 생산과 이로부터 생산된 인간 CD20 단클론 항체의 약리적 특성을 평가하였다. 형질전환 닭으로부터 생산된 인간 CD20 단클론 항체는 14종류의 *N*-글리칸을 포함하였고, Fc 기능 향상에 효과적인 높은 수준의 만노스 (mannose) 및 퓨코스가 결여된 (afucose) 형태를 보였다. 이러한 특성을 통해 형질전환 닭 유래의 CD20 단클론 항체는 상용화된 rituximab에 비해 높은 수준의 보체 의존

성 세포 독성 (complement dependent cytoxicity; CDC) 및 항체 의존성 세포 독성(antibody dependent cell cytoxicity; ADCC) 활성을 나타내었다.

다음 연구에서는 다른 생식선 전이 줄기세포인, 정원줄기세포 (spermatogonial stem cells; SSCs)의 정소내 직접 주입을 통해 생식선 키메라 메추리를 생산하였다. 본 연구에서는 효과적인 메추리 생식선 키메라 생산을 위해 생식세포 저감 약물인 busulfan이 처리된 수용체 정소 내에 정원 세포와 정원 줄기 세포를 이식하여 생식선 키메라를 생산하였다. 배아 매개의 방법과 비교하여 이 전략은 간단하고 신속한 생식선 키메라 생성을 유도한다. 이러한 방식은 정원 줄기세포 매개의 형질 전환 모델 및 유전자 편집 조류 생산을 위한 전략으로 이용 될 수 있고, 정원 세포를 이용한 조류 종 보존에도 기여 할 수 있다.

마지막으로 우리는 유전자 편집 메추리 생산을 위해 원시생식세포 (PGCs) 내로의 유전자 도입 최적화를 진행 하였다. GFP 발현 벡터를 이용해 유전자 전이 효율의 최적화 및 CRISPR/Cas9 system 도입과 Puromycin의 처리를 통해 유전자 편집 효율을 향상시켰다. 또한 non-homologous end-joining (NHEJ) 기술을 통해 목적 유전자 편집 기술을 이용한 재조합 유전자 도입을 유도 하였다. 이러한 결과는 프로그램 가능한 유전자 편집 도구의 메추리 원시생식세포 내로의 효과적인 도입과 효율적인 유전자 편집이 가능함을 보여준다.

결과적으로, 이러한 생식선 전이 줄기세포의 생산과 응용 대한 실험적 접근들은 효과적인 유전자 조절 조류의 생산에 기여할 수 있으며, 이를 통한 유전자 연구, 목적 단백질의 대량 생산 및 질병 저항성 모델 생산 등에 이용 될 수 있다. 또한, 배아 비파괴적 방식의 생식선 전이 줄기세포를 통해 조류 복원 및 보전에 크게 기여 할 수 있을 것이다.