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A Dissertation

For the Degree of Doctor of Philosophy

Studies on Antimicrobial Peptides and Epigenetic Factors in Transgenic Birds

형질전환조류 생산을 위한 항균펩타이드 및 후생유전학적 인자의 영향에 대한 고찰

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The approach for the generation of disease-resistant birds; Selection of candidate gene and expected effects of epigenetic factors during generation of transgenic birds

질병저항성 조류 생산을 위한 접근법; 후보 유전자의 선발 및 형질전환조류 생산과정 중 예상되는 후생유전학적 인자의 영향

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ABSTRACT

In this study, we investigated the relative expression of the Rous sarcoma virus (RSV) promoter-driven expression of enhanced green fluorescent protein (EGFP) in fibroblasts of transgenic quails. We analyzed the direct influence of CpG methylation of the RSV promoter on the transcriptional activity of delivered transgenes. Embryonic fibroblasts collected from homozygous trans-genic quail (TQ2) were treated with 50 μM of DNA methyltransferase inhibitor followed by 5-aza-2′-deoxy-cytidine (5-azadC) for 48 h, and changes in expression were then analyzed by flow cytometry. The results show a significant increase of EGFP expression in TQ2 embryonic fibroblasts (QEFs) (2.64% to 79.84%). Subsequent methylation-specific amplification revealed that 5-azadC significantly reduced the CpG methylation status in the RSV promoters of the QEFs (86.42 to 48.41%); even after 5-azadC was withdrawn, CpG methylation remained decreased in expanded culture (16.28%). Further analysis showed that potential transcription factor binding sites existed in the CpG methylation site of the RSV promoter. These results may provide the basis for understanding the epigenetic mechanism responsible for transgenic animal production and genetic preservation.

DNA methylation reprograming of primordial germ cells (PGCs) in mammals establishes monoallelic expression of imprinting genes, maintains retrotransposons in an inactive state, inactivates one of the two X chromosomes, and suppresses gene expression. However, the roles of DNA methylation in chickens PGCs are unknown. In this study, we found a 1.5-fold or greater difference in the expression of 261 transcripts when comparing PGCs and chicken embryonic fibroblasts (CEFs) using an Affymetrix GeneChip Chicken Genome Array. In addition, we analyzed the methylation patterns of the regions ~5-kb upstream of 261 sorted genes, 51 of which were imprinting homologous loci and 49 of which were X-linked homologous loci in chicken using the MeDIP Array by Roche NimbleGen. Seven hypomethylated and five hypermethylated regions within the 5-kb upstream regions of 261 genes were found in PGCs when compared with CEFs. These differentially methylated regions were restrictively matched to differentially expressed genes in PGCs. We also detected 203 differentially methylated regions within imprinting and X-linked homologous regions between male PGCs and female PGCs. These differentially methylated regions may be directly or indirectly associated with gene expression during early embryonic development, and the epigenetic difference could be evolutionally conserved between mammals and birds.

The basic functions of DNA methylation include in gene silencing by

methylation of specific gene promoters, defense of the host genome from retrovirus, and transcriptional suppression of transgenes. In addition, genomic imprinting, by which certain genes are expressed in a parent-of-origin-specific manner, has been observed in a wide range of plants and animals and has been associated with differential methylation. However, imprinting phenomena of DNA methylation effects have not been revealed in chickens. To analyze whether genomic imprinting occurs in chickens, methyl DNA immunoprecipitation array analysis was applied across the entire genome of germ cells in early chick embryos. A differentially methylated region (DMR) was detected in the eighth intron of the L-arginine:glycine amidinotransferase (GATM) gene. When the DMR in GATM was analyzed by bisulfite sequencing, the methylation in male primordial germ cells (PGC) of 6-d-old embryos was higher than that in female PGC (57.5 vs. 35.0%). At 8 d, the DMR methylation of GATM in male PGC was 3.7-fold higher than that in female PGC (65.0 vs. 17.5%). Subsequently, to investigate mono- or biallelic expression of the GATM gene during embryo development, we found 2 indel sequences (GTTTAATGC and CAAAAA) within the GATM 3′-untranslated region in Korean Oge (KO) and White Leghorn (WL) chickens. When individual WL and KO chickens were genotyped for indel sequences, 3 allele combinations (homozygous insertion, homozygous deletion, and heterozygotes) were detected in both breeds using a gel shift assay and high resolution melt assay. The deletion allele was predominant in KO, whereas the insertion allele was predominant in WL.

Heterozygous animals were evenly distributed in both breeds ($P < 0.01$). Despite the different methylation status between male and female PGC, the GATM gene conclusively displayed biallelic expression in PGC as well as somatic embryonic, extraembryonic, and adult chicken tissues.

Cathelicidins are antimicrobial peptide components of the innate immune system. Four cathelicidins have been identified in the chicken: cathelicidin1 (CATH1), cathelicidin2 (CATH2), cathelicidin3 (CATH3), and cathelicidinB1 (CATHB1). The aim of this study was to characterize the antibacterial activities, structural conservation and expression patterns of these antimicrobial peptides. All had a highly conserved functional domain. The expression of CATH1, CATH2, and CATH3 mRNA was high in the bone marrow of adult female chickens. By contrast, CATHB1 mRNA was highly expressed in the thymus. The active domains of all four chicken cathelicidins were synthesized, and their antibacterial activities on cell viability, membrane damage, and colony formation of Escherichia coli were examined. After treatment of E. coli with 0.5–10 μM of each cathelicidin, CATH1, CATH2, and CATH3 reduced cell viability at all concentrations. When E. coli were treated with 5 μM of each cathelicidin, CATH2 and CATH3 demonstrated maximum damage to the cell membrane. To examine the effect on colony formation, an ampicillin-resistant E. coli strain was established and treated with 5 μM of each

cathelicidin. CATH1, CATH2, and CATH3 markedly arrested colony formation, whereas CATHB1 had no effect. The present results demonstrate dose-dependent antimicrobial effects of chicken cathelicidins that were mediated by membrane damage and had a mechanism independent of that of common antibiotics. Our data suggest a novel approach for controlling drug-resistant bacteria and for producing disease-resistant animals in the livestock industry.

Keywords: chicken, quail, DNA methylation, gene expression, antimicrobial peptide

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LIST OF ABBREVIATIONS

5-azadC	5-aza-2'-deoxycytidine
AMP	Antimicrobial peptide
CATH	CATHELICIDIN
CD analysis	Circular dichroism analysis
DMR	Differentially methylated region
DNMT	DNA methyltransferase
EGFP	Enhanced green fluorescent protein
GATM	L-arginine:glycine amidinotransferase
HRM assay	High resolution melt assay
Indel	Insertion-deletion
KO	Korea oge
MeDIP	Methyl-DNA immunoprecipitation
PCR	Polymerase chain reaction
PGC	Primordial germ cell
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol
QEF	Quail embryonic fibroblast
RSV	Rous sarcoma virus
SNP	Single nucleotide polymorphisms
SSEA-1	Stage specific embryonic antigen 1
WL	White leghorn

CHAPTER 1.

GENERAL INTRODUCTION

Transgenic animals could be applied commercially to the preparation of recombinant proteins, and transgenic herds could be protected against disease or have new genetic traits. The production of transgenic animals could contribute to research fields such as the in vivo study of gene function during development, organogenesis and aging and the development of therapeutics in models of human disease. However, transgene silencing has been reported during the development of transgenic animals, and the applications of transgenic animals are limited by epigenetic factors such as DNA methylation, histone modification and non-coding RNA.

In particular, DNA methylation in eukaryotes basically acts as a regulatory factor that controls transcriptional activity. Moreover, DNA methylation is important during embryonic development in mammals as it regulates genomic imprinting, X-inactivation and cell differentiation.

In chickens, the basic function of DNA methylation is similar to that in mammals. For example, the methylation of a promoter region induces gene silencing, and methylation protects against infection of the host genome by a viral genome and induces transcriptional suppression of transgenes. DNMT 1, 3a, and 3b are expressed in mammals and chickens during early embryonic development. Chicken DNMTs play a similar role as mammalian DNMTs in methylation establishment. However, chicken PGCs exhibit different migration activity in germ cell development from mammalian PGCs, and the control mechanism of DNA methylation during early embryonic development

is also different between chickens and mammals. For example, imprinted genes in mammals are instead expressed from bi-alleles in chickens. Additionally, male chickens are homogametic for sex chromosomes (ZZ), whereas females are homogametic for the sex chromosomes in mammals (XX). Furthermore, somatic Z inactivation does not occur in ZZ male chickens, whereas somatic X inactivation does take place in XX mammals. The aforementioned characteristics suggest that birds and mammals could have different control mechanisms for epigenetic regulation even though the epigenetic materials are the same.

Antimicrobial peptides serve as constitutive or inducible defense barriers against microbial infections in plants, insects, amphibians and mammals (which include humans). AMPs play important roles in killing microbes, boosting specific innate immune responses, and exerting selective immunomodulatory effects on the host. One type of AMP, CATH, forms a family of vertebrate-specific immune molecules and has been reported in fish, chickens, and mammals. From these findings, CATHs could be proposed as an appropriate gene to support antimicrobial activities.

To establish a stable strategy for disease-resistant transgenic animals, we tried to select candidate genes for transgenic birds against bacterial diseases, and we investigated the effects of epigenetic factors during the production of transgenic birds. We serially investigated the effect of DNA methylation in birds as compared to mammals. In chapter 3, we show that

silenced transgenes in quail were reactivated by 5-azadC, an inhibitor of DNA methylation, and DNA methylation was also reduced by the promotion of the silenced transgene. We also analyze genome-wide methylation patterns between male and female PGCs in chapter 4 by using a methyl ChIP array. In addition, we discuss genomic imprinting phenomena for differentially methylated regions between male and female PGCs in chapter 5. Finally, we selected functional genes to produce transgenic birds that are resistant for bacterial diseases. Chicken cathelicidins were characterized, and their antimicrobial activity is evaluated in chapter 6. The selected cathelicidins showed strong antimicrobial activity. Collectively, our results could help to induce stable germ-cell-mediated transgenesis through the controlling mechanism of transgene silencing and could lead to an understanding of the epigenetic characteristics of early avian germ cells. The antimicrobial gene introduced here, which uses stable germ-cell-mediated transgenesis, could contribute to the production of transgenic birds that are protected against bacterial diseases.

CHAPTER 2.

LITERATURE REVIEW

1. Transgenic animals

1.1. Generation of transgenic animals

The term “transgenic animal” refers to an animal that has had an “artificial transgene” introduced into its genome. Transgenic animals include a wide range of living modified organisms (LMO) beyond just genetically modified organisms (GMO), and these animals could improve human welfare while concurrently limiting dangerous side effects. A transgenic animal is produced by transplantation of an embryo or a fertilized egg with the goal of transgenesis of innate genetic traits. The production of transgenic animals is essential for the in vivo study of gene function during development, organogenesis and aging. These animals permit the evaluation of therapeutic strategies in models of human disease and the investigation of disease progression in a manner not possible in human subjects. Commercial applications of transgenesis include the preparation of recombinant proteins, protection of animals against disease, and introduction of new genetic traits into herds. Transgenic animals have been produced in a variety of species. Transgenic vertebrates have been developed in species with both scientific and commercial value including fish, amphibians, birds, and mammals.

Transgenic invertebrate species include some that are widely used in research, such as the arthropod fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, and organisms with commercial value, including eastern oysters, dwarf surf clams and the Japanese abalone (Gama Sosa *et al.* 2010). For research purposes, transgenic models in the invertebrates *D. melanogaster* and *C. elegans* and the vertebrates *Danio rerio* (zebrafish), *Xenopus laevis*, and *Xenopus tropicalis* offer the advantages of low maintenance costs and the rapid generation of large numbers of transgenic organisms. However, their distant phylogenetic relationship to humans may limit their use in modeling human pathological conditions. By contrast, the generation and maintenance of transgenic mammals that are phylogenetically closer to humans, such as sheep, cattle, and pigs, involve a large investment in time and resources that prevents their use in most research settings. As such, the use of transgenic cattle is largely limited to projects with commercial applications such as generating animals resistant to bovine spongiform encephalopathy by deleting the prion receptor (Richt et al. 2007) or producing cows resistant to staphylococcal mastitis (Donovan et al. 2005, 2006). Transgenic pigs have been generated to produce organs for xenotransplantation (MacKenzie *et al.* 2003; Sprangers *et al.* 2008; Ekser et al. 2009). Along with rabbits and cattle, pigs have also been used as protein bioreactors to produce biologically active recombinant proteins (Fan and Watanabe 2000, 2003; Houdebine 2004, 2007, 2009). Transgenesis in mice is

the most commonly used approach to generate models of human disease. Transgenic mice offer the advantages of a relatively low cost, a short gestation time (18.5–21 days depending on the strain), and, perhaps most importantly, a well-developed set of technologies by which genetic modifications can be introduced. The availability of genetically inbred strains and the relatively close evolutionary relationship of mice to humans are additional advantages. Transgenic pigs have been developed as models of retinitis pigmentosa (Li et al. 1998) and Alzheimer's disease (Kragh et al. 2009), and transgenic models of human diseases have also been generated in rabbits (Fan and Watanabe 2003) and rats (Mashimo and Serikawa 2009). However, transgenic technologies are not as widely available in these species as in mice, where the techniques for gene targeting and pronuclear injection are well-developed and widely available. Indeed, many of the methods for genetic manipulation used in the mouse are not routine or even presently possible in other mammalian species, although the recent development of rat and pig ES cells creates the potential that gene targeting may be extended to these species (Buehr et al. 2008; Wu et al. 2009). The generation of knockout, knockin and humanized rats is also being pursued using nuclear transplantation of genetically modified somatic cells (Zhou et al. 2003). Although the cost is often prohibitive and the technology is less widely available, non-rodent species may offer some advantages in modeling human disease. For example, most cholesterol in mice is contained in the form of high-density lipoprotein (HDL)

cholesterol, whereas most cholesterol in rabbits is found in low-density lipoproteins (LDL), which is more like humans. Due to the differing lipoprotein profiles between mice and rabbits, the introduction of human transgenes such as apolipoprotein A have resulted in different phenotypes in these two species (Fan and Watanabe 2000). It has also been proposed that the rat may be, in general, a better species than the mouse for modeling many human disorders (Abbott 2004). However, as the mouse remains the most widely used species for modeling human neurodegenerative diseases,

1.2. Transgenic Aves

Chicken (*Gallus g. domesticus*) and quail (*Coturnix c. japonica*) have been exploited mainly for the generation of transgenic avian lines that express fluorescent proteins. The first successful development of transgenic chickens was reported by using replication-competent reticuloendotheliosis virus (REV) (Salter *et al.* 1986). Since then, many advancements have taken place in the production of transgenic poultry. The domestic chicken is poised to become a major animal bioreactor for the production of commercial quantities of therapeutic proteins in eggs, which will move domestic fowl into the realm of protein bioprocessing. Transgenic chickens have been produced to study and

improve the efficiency of germline integration of exogenous genes. The industrial application of egg white as a bioreactor for pharmaceutical materials (e.g., antibodies or recombinant proteins) is anticipated because it is difficult to obtain large amounts of such materials from biochemical synthesis (Kamihira et al. 2005; Lillico et al. 2007; Kwon et al. 2010; Penno et al. 2010). Importantly, a recent report has demonstrated that horizontal infection with the avian influenza virus can be prevented in a transgenic chicken line carrying a shRNA construct against the influenza A virus polymerase gene; this finding suggests that applying transgenic technologies to chickens might be practical for the poultry industry (Lyall et al. 2011). Aside from transgenic chickens, quail has been exploited for the generation of tissue-specific fluorescent transgenics, which are essential for time-lapse imaging analysis (Scott & Lois 2005; Sato et al. 2010; Seidl et al. 2012). Quail is a highly effective avian species in which genetic modifications can be performed because they have shorter incubation and earlier sexual maturation periods compared to chickens (Poynter et al. 2009c). Moreover, the small body size of the quail permits the maintenance of transgenic strains in slightly modified rodent cages in the air-conditioned, pathogen-controlled environment of an animal facility (Huss et al. 2008).

1.3. Transgene silencing

The delivered transgenes can integrate into the chromosomal DNA, replicate episomally or persist as non-replicating episomal elements in non-dividing cells. Depending upon the properties of the transgene expression cassette, the particular features of specific transgene integration sites and the state of the individual recipient cells, the transgenes are expressed with varying degrees of efficiency. On some occasions, the transgenes are permanently silenced immediately after introduction; on other occasions, transgene silencing occurs only after a certain period of adequate expression. On still other occasions, transgene expression varies dramatically among the individual clones of transgene-harboring cells. Such variation is thought to be mainly due to the transgene's interaction with its immediate genetic neighborhood within the host genome. This phenomenon is similar to 'position-effect variegation' in normal development caused by spontaneous, clone-wise silencing of some resident genes (Eissenberg 1989). Typical position-effect variegation is an epigenetic instability, and it should be distinguished from variegation due to somatic mutations, e.g., due to variations in the length of polynucleotide repeat expansions (Dion *et al.* 2008) or the sorting of mitochondrial genomes in mitochondrial heteroplasmia (Zaegel *et al.* 2006). The element of randomness, which is inherently present in position-effect variegation, should not come as a surprise. In fact, stochastic

fluctuations of gene expression are typical both at the level of variation between different cells of tissue and at the level of temporal variation within one cell. Both of these modes of variation are essential for normal differentiation and tissue-patterning; the input of stochastic variation is decisive when a developmental signal is present at a near-critical level. The permanent silencing of transgene expression can occur both in post-mitotic target cells and target cells undergoing clonal expansion, whereas variegation is typically associated with clones of dividing cells. Stable long-term transgene expression in differentiating cells is particularly challenging. In fact, the introduced genes are subject to the pre-existing and developing gene expression patterns in the target cells, which can override the signals from the transgenes' own regulatory elements and thereby cause transgene expression shutdown. Indeed, at a transcriptional level, the changing scenery of transcription initiation factor pools, chromatin re-modeling and DNA methylation events during differentiation contribute to transgene expression.

2. DNA methylation

2.1. General function of DNA methylation

DNA methylation in eukaryotes plays a crucial role in the control of gene expression and the inactivation of transposable and repetitive elements. In addition, epigenetic modifications such as DNA methylation are important for the embryonic development of mammals as they regulate genomic imprinting, X-inactivation and cell differentiation (Mlynarczyk & Panning 2000; Reik & Walter 2001; Cantone & Fisher 2013).

2.1.1. Control of gene expression

DNA methylation in vertebrates refers to methylation of the 5-position of the cytosine pyrimidine ring. It occurs predominantly in regions containing a high frequency of the sequence cytosine phosphate guanine (CpG), which are termed CpG islands. Transcription repression is generally associated with methylation of CpG islands located in or near gene promoters (i.e., the 5' flanking region). Genome-wide cytosine methylation states (especially those associated with genes) differ among cell types and function as a form of memory of the identity and developmental state of a cell. In normal cells, most promoter-associated CpG islands at transcription sites are unmethylated. The absence of CpG methylation indicates either normal transcription activity or the fact that a gene can be recruited to express even if

it is basally silent. However, promoter-region CpG islands in cancer cells are more likely to become methylated, and This methylation can cooperate with other transcriptional-silencing chromatin events to strongly prevent the normal transcription of DNA, thereby “silencing” the gene (Cross & Bird 1995; Siegfried & Simon 2010).

2.1.2. Embryonic development

During mammalian development, there are two crucial developmental stages and/or cell types in which the epigenome undergoes profound reprogramming: pre-implantation embryos and primordial germ cells (PGCs), which are the precursors both for oocytes and spermatozoa (Surani *et al.* 2007). Epigenetic reprogramming in these cells involves genome-wide demethylation of 5mC; 5mC plays a crucial role in genome imprinting, X-inactivation, transposon silencing, the stability of centromeric/telomeric structures and gene expression in general (Suzuki & Bird 2008; Lister *et al.* 2009). Genome-wide chromatin changes occur in germ cells, especially during early germ cell development, and these changes contribute to the suppression of somatic cell differentiation (Sasaki & Matsui 2008). In early germ cells, termed primordial germ cells (PGCs), the genomes

obliterate most of their epigenetic markers, including DNA methylation, histone modification, and other covalent chromatin modifications that are associated with somatic gene regulation, so that germ cells can acquire the capacity to support post-fertilization development (Reik 2007; Kota & Feil 2010). As a result, the epigenetic reprogramming in PGCs establishes monoallelic expression of imprinting genes, maintains inactivated retrotransposons, inactivates one of the two X chromosomes, and suppresses gene expression (Lees-Murdock & Walsh 2008). This process also prepares the germ cells for meiosis, during which homologous chromosomes become aligned to allow synapsis and recombination. Recent research has demonstrated that histone modifications, aside from specific DNA sequence motifs, also contribute to synapsis formation and recombination and enhance recombination at preferential regions (Baudat *et al.* 2010; Myers *et al.* 2010).

2.1.3. Genomic imprinting

The genomic imprinting phenomenon was first reported in insects and has subsequently been observed in a wide range of plants and animals (de la Casa-Esperon & Sapienza 2003). The most restrictive definition of imprinting is a parent-of-origin effect that explains the mechanism by which

specific gene transcription from the allele of only one parent is regulated (Ferguson-Smith & Surani 2001; Baroux *et al.* 2002; Rand & Cedar 2003). Imprinting in mammals, plants, and many insects is necessary to important developmental processes. Although imprinting studies have focused mainly on mammals, particularly humans and mice, such parent-of-origin effects have also been observed in transcription in plants (Baroux *et al.* 2002) and *Drosophila* (Lloyd 2000). In mammals, imprinting was first reported in the mid-1980s. Embryological studies in mice demonstrated the functional nonequivalence of maternal and paternal genomes. Specifically, conceptuses derived from zygotes containing either two sets of the maternal chromosomes or two sets of the paternal chromosomes failed to develop beyond mid-gestation (Barton *et al.* 1984; McGrath & Solter 1984). These findings demonstrated that normal embryonic development requires both a maternal and a paternal genome and suggested the existence of genes whose expression depends on whether they are inherited from the mother or from the father. Germ-line imprinting is regarded as a conflict between maternal and paternal systems. Differentiation between maternal and paternal genes originates from the formation of epigenetic marks that are deposited on chromosomes in the germ line, and the sex of the parent determines the presence of these marks. Thus, imprinting creates a functional difference between the maternally and paternally derived copies of the genome.

2.2. DNA methyltransferase

The enzymes that methylate cytosine to form 5-methylcytosine (5mC) have been well characterized. DNA methyltransferase (DNMT) 1 preferentially methylates hemi-methylated cytosines in CpG sequences and thus acts as a maintenance methyltransferase to maintain genome-wide methylation patterns during replication. DNMT3A and DNMT3B can methylate unmethylated CpG sequences and, hence, function as de novo methyltransferases. Although DNMT3L does not have methyltransferase activity, it is closely related with genomic imprinting. Instead of being absent, DNMT3L links between DNMT3a and DNMT3b and reinforces them (Kinney & Pradhan 2011). DNMT1, 3a, and 3b were also expressed in chickens during early embryonic development. Chicken DNMTs play a similar role to the mammalian DNMTs in establishing methylation (Champagne 2011; Chedin 2011; Rengaraj *et al.* 2011).

2.3. DNA methylation in birds

In chickens, the basic function of DNA methylation is similar to that in mammals. For example, the methylation of a promoter region induces gene silencing (Luo *et al.* 2011), and methylation protects infection of the host genome by the viral genome and induces a transcriptional suppression of transgenes (Park *et al.* 2010; Jang *et al.* 2011). Conversely, chicken PGCs exhibit a unique migration activity. Mammalian PGCs are originally derived from the proximal epiblast and move into embryonic gonads through the hindgut by amoeboid movement. By contrast, chicken PGCs appear within the epiblast in the blastoderm and move to the hypoblast of the area pellucida (Petitte *et al.* 1997). During gastrulation, PGCs move to the germinal crescent before circulating through the blood vessels and finally settling in the gonadal ridge (Nieuwkoop. & Sutasurya 1979; Tsunekawa *et al.* 2000; Nakamura *et al.* 2007). The control mechanism of DNA methylation during early embryonic development is also different between chickens and mammals. For example, imprinted genes (*mpr*/*igf2r*, *igf2*, *ascl2*/*mash2*, *ins2*, *dlk1*, and *ube3a*) in mammals are expressed from bi-alleles in chickens (Yokomine *et al.* 2005; Colosi *et al.* 2006; Shin *et al.* 2010). Additionally, male chickens are homogametic for sex chromosomes (ZZ), whereas females are homogametic for the sex chromosomes in mammals (XX). Furthermore, somatic X inactivation takes place in XX mammals, whereas somatic Z inactivation does not occur in ZZ male chickens (Wu & Xu 2003).

A variety of genome-wide methylation analyses have been conducted to understand DNA methylation in chickens (Li *et al.* 2011; Xu *et al.* 2011). These studies have provided insight into the characteristics and roles of DNA methylation in some tissues and somatic cell lines (Itoh *et al.* 2011; Luo *et al.* 2011). However, our understanding of the epigenetic regulation of chicken PGCs remains poor despite great interest since imprinting was first proposed in epigenetic changes that occur during germ cell development. Progress in this field has been hindered by technical difficulties due to laborious germ cell isolation and questionable sample purity; however, new and highly sensitive methods have been developed that enable the analysis of very low numbers of cell samples.

3. Antimicrobial peptides

3.1. General introduction of antimicrobial peptides

Antimicrobial peptides (AMPs) serve as constitutive or inducible defense barriers against microbial infections in plants, insects, amphibians and mammals including humans (Brogden 2005; Harris *et al.* 2009; Rahnamaeian

et al. 2009). AMPs play important roles in killing microbes, boosting specific innate immune responses, and exerting selective immunomodulatory effects on the host (Hancock & Sahl 2006; Yeaman & Yount 2007; Holzl *et al.* 2008; Muller *et al.* 2008; Lai & Gallo 2009). AMPs have been known to cause differentiation, activation and chemotaxis of multiple types of leukocytes; to inhibit LPS-induced effects; and to enhance phagocytosis, DNA uptake and wound healing (Semple & Dorin 2012). It is clear that these peptides form a vital but often overlooked component of the innate immune system. The first AMPs were discovered in the 1970s, when they were extracted from tissue. The recent rise of bioinformatics and available sequence data has generated a steep increase in the number of AMPs that have been described.

3.2. Structures of antimicrobial peptides

Several classification schemes have been proposed for AMPs; however, most AMPs are generally categorized into four clusters based on their secondary structures: peptides with a linear α-helical structure (Boman 1995; Mangoni *et al.* 2000; He & Lemasters 2002), cyclic peptides with a β-sheet structure (Epand & Vogel 1999; Matsuzaki 1999; Bu *et al.* 2002; Ovchinnikova *et al.* 2004; Ostberg & Kaznessis 2005), peptides with a β-

hairpin structure (Imamura *et al.* 2010), and peptides with a linear structure (Wu & Hancock 1999; Rahnamaeian *et al.* 2009).

3.3. Activities of antimicrobial peptides

The antimicrobial activity of AMPs as membrane-agents possessing a secondary α-helical peptide structure depends on the presence of an ionic milieu that is comparable to the conditions found in mammalian body fluids (Dorschner et al. 2006; Johansson et al. 1998; Oren et al. 1999). The AMPs target the weakest spot of the microbial membrane (for example, the absence of cholesterol and negatively charged phospholipids on the outer leaflet of the cytoplasmic membrane) (Zasloff 2002). The positive net charge (+2 to +7 due to an excess of basic versus acidic amino acids) (Scott and Hancock 2000) facilitates binding of an increasing number of AMPs to the phospholipids on the bacteria's surface until the bacterial membrane collapses completely (Boman 2003; Hale and Hancock 2007; Sallum and Chen 2008; Steiner et al. 1988). Cholesterol prevents membrane damage; normal concentrations of AMPs do not cause damage to the host because this lipid is an essential part of eukaryotic membranes (Boman 2003). The membrane potential of eukaryotic cells (−15 mV) is also low compared to the bacterial

transmembrane potential (−140 mV), which also minimizes interaction (Scott and Hancock 2000). Resistance to AMPs is rare as it is particularly difficult for any microorganism to change the structural organization of its surface phospholipids (Zasloff 2002). Some AMPs target intracellular sites in addition to the bacterial membrane (Jenssen et al. 2006; Xiong et al. 1999).

3.4. Immunomodulation of antimicrobial peptides

Although many AMPs, for example, defensins, demonstrate direct antimicrobial activity against bacteria, fungi, eukaryotic parasites and/or viruses (Steinstraesser et al. 2005, 2008; Hirsch et al. 2008; Larrick et al. 1995), it has also been established that many AMPs play a key modulatory role in the innate immune response and present an important link between the innate and adaptive immune responses (Zasloff 2002). Various tissues and cell types in the body contain gene-encoded pattern recognition receptors (PRRs) and can recruit a number of different signaling pathways in response to stress, which ultimately ensures the production of all necessary signaling and effector molecules required for an appropriate and immediate host defense. Host PRRs are generally surface proteins that immediately identify conserved molecular structures associated with microbial pathogens or other impending

dangers. The repertoire of PRRs capable of regulating gene expression encompasses the TLRs and the virus-sensing RIG-I and Mda5 helicases (Onomoto et al. 2007; Yoneyama et al. 2005; Zou et al. 2009; Robinson et al. 2006). Other non-TLR recognition molecules, however, have also been described. The structures identified by a given PRR are classified either as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Classical PAMPs include LPS and lipoteichoic acid (LTA) from Gram-negative and Gram-positive bacteria, respectively, viral double-stranded RNA (dsRNA), and fungal β-glucans (Robinson et al. 2006; Jo 2008). The term DAMPs is used here as a common name to refer to PAMPs and to pathways arising from endogenous alarm signals released by dying or injured cells (Matzinger 2007; Seong and Matzinger 2004). Matzinger's Danger Model defines "dangers" as anything (exogenous or endogenous) that has the potential to cause tissue stress or destruction (Matzinger 1994, 2007). Also in the category of innate sensors are the intracellular nod-like receptors (NLRs), which present a powerful combined defense at the plasma membrane (for example, TLRs) and from within the cell (for example, NLRs) (Benko et al. 2008; Fritz et al. 2006). Both TLRs and Nodg proteins can trigger the nuclear factor-kappaB (NF-kB) transcription factor; thus, they activate a highly stereotypical signaling pathway responsible for a range of different cellular responses including the production of AMPs (Fritz et al. 2006). The NLRs have been linked to recognition of bacterial

components and endogenous danger signals (Fritz et al. 2006). TLRs initially received considerable research interest; consequently, this group of PRRs is well known. Almost 20 different members have been reported in six major families, with each member recognizing different PAMPs. LPS is the classical ligand for TLR-4, whereas LTA and CpG oligodeoxynucleotides are recognized by TLR-2 and TLR-9, respectively (Dalpke et al. 2005). NF-kB signaling is one of the main downstream pathways responsible for HDP production, although other signaling routes (including MAPKh and JAK/STATi signaling) have been implicated in their synthesis (Ji et al. 2009; Krisanaprakornkit et al. 2002). NF-kB is a transcription factor involved in the integration of numerous parallel signaling pathways and a variety of cellular responses that are central to an immediate and functional immune response, including the production of cytokines and cell-adhesion molecules (Scott and Hancock 2000). Signaling through these pathways leads to transcriptional activation and the subsequent production of AMPs. The TLRs and NLRs also result in the activation of the inflammatory caspases, which comprise a field of research beyond the scope of this manuscript (Martinon and Tschopp 2007; Scott and Saleh 2007; Steinstrasser et al. 2007). Thus, AMPs may act directly by causing physical damage to microbes or indirectly by inhibiting physiological functions of microbes, and they may function to recruit host immune systems.

3.5. Antimicrobial peptides in birds

The first avian AMPs were described in the mid-1990s: five defensins were isolated from chicken and turkey leukocytes (Evans *et al.* 1994). To date, the complete defensin and cathelicidin clusters have been described for chickens, and increasing information about AMPs in other avian species is becoming available. In recent years, interest in AMPs has increased, and these peptides are being investigated as templates for novel anti-infectives. With the rising antibiotic resistance of the last decade, an intense search is ongoing to search for alternatives to fight infectious diseases. AMPs or derivatives thereof are believed to be one of these alternatives (Cuperus *et al.* 2013). AMPs are conserved throughout a wide variety of organisms including mammals, birds, insects and plants. For avian species, three main classes of AMPs have been described: β-defensins, cathelicidins (CATH) and liver-expressed antimicrobial peptide-2 (LEAP-2).

3.5.1. Defensin

Defensins are cysteine-rich, cationic peptides characterized by three conserved disulfide bridges, a β-sheet structure (Ganz, 2003; Selsted and Ouellette, 2005) and both hydrophobic and cationic amino acids (Ganz, 2004). The defensin family can be subdivided into three main groups: the α-, β- and θ-defensins (Selsted and Ouellette, 2005; Yang et al., 2004). α-defensins are only present in mammalian species and form disulfide bridges (Lehrer and Ganz, 2002; Yang et al., 2004). β-defensins can be found in all vertebrate species and form disulfide bridges (Klotman and Chang, 2006; Lehrer and Ganz, 2002). The α- and θ-defensins are thought to have evolved from an ancient β-defensin (Xiao et al., 2004). The θ-defensins are cyclic defensins that form cystine bridges and are found in rhesus monkeys and baboons, whereas only pseudogenes for θ-defensins are present in the human genome (Lehrer et al., 2012).

The first β-defensin ever described was found in bovine epithelial cells (Diamond et al., 1991). β-defensins are the only defensins that have been observed thus far in avian species despite extensive searches for other defensins in the chicken genome (Higgs et al., 2005; Lynn et al., 2007). To date, over 25 avian β-defensins (AvBDs) have been detected (Hellgren and Ekblom, 2010; Lynn et al., 2007; van Dijk et al., 2008). β-defensins contain a signal peptide for secretion but have no, or only a short, propiece, in contrast to α-defensins, which have anionic propieces that neutralize the positive

charge of the peptide and thus prevent interaction of the cationic peptide with intracellular membranes (Ganz, 2004; Kaiser and Diamond, 2000). The first NMR structure analysis for AvBDs was performed for AvBD103b, which was isolated from the stomach contents of the King penguin (Landon et al., 2004). The structure obtained for AvBD103b contained a three-stranded β-sheet and a short α-helical structure at the N-terminus that is present in almost all β-defensins but is lacking in α-defensins. This N-terminal α-helical region also contains the first cysteine residue for the formation of a disulfide bridge. In contrast to AvBD103b, the short N-terminal region of AvBD2 does not adopt an α-helical conformation (Derache et al., 2012). However, the three-stranded β-sheet and disulfide bridges are conserved. A bulge in the second β-strand of both structures suggests that this conformation might also be conserved among avian defensins and is of importance for correct folding or flexibility of the peptide. Despite their structural similarities, AvBD103b and AvBD2 exhibit different physical properties (Derache et al., 2009b; Landon et al., 2004). Whereas AvBD103b is highly cationic and has a hydrophobic patch, AvBD2 does not have an amphipathic structure, and its hydrophobic and cationic residues are more randomly distributed. Other structural differences among AvBDs are found in post-translational modifications and dimerization. C-terminal amidation is known to occur frequently and has been found in AvBD1 and AvBD7 but not in AvBD2 (Derache et al., 2009b). C-terminal amidation is thought to provide stability against degradation and to increase

cationicity for better antibacterial activity (Bulet et al., 2004; Derache et al., 2009b). Dimerization of β-defensins was shown for human β-defensin 2 (HBD2) and some homologs thereof (Suresh and Verma, 2006), and it was also found in duck AvBD2 (Soman et al., 2010). However, not all HDB2 homologs formed dimers, which suggests that some AvBDs may also act as monomers or form larger oligomers. A number of AvBDs, e.g., 3, 11 and 13, contain a large post-piece at the C-terminal end of the mature peptide (Herve-Grepinet et al., 2010; Higgs et al., 2005; Xiao et al., 2004; Zhao et al., 2001). In one study, AvBD13 was reported to have a post-piece (Xiao et al., 2004), whereas a different study claimed that the peptide lacks this feature (Higgs et al., 2005). This disagreement suggests the possibility of strain-specific splice variants or isoforms. AvBD11 contains a post-piece with a defensin-like motif that results in six cystine bridges instead of three, which might be the result of gene duplication (Herve-Grepinet et al., 2010). The presence of a post-piece is not uncommon among defensins; for example, it was also found in β-defensin sequences in a cluster positioned on chromosome 20 in the human genome (Schutte et al., 2002).

3.5.2. LEAP-2

Liver-expressed antimicrobial peptide-2 (LEAP-2) was found during a systematic analysis of human blood and is highly expressed in the liver (Krause et al., 2003). LEAP-2 is a 40-amino-acid, cationic peptide(Hocquellet et al., 2010). LEAP-2 is also expressed in chickens (Lynn et al., 2003), and its antimicrobial activity has only been tested against *Salmonella* strains; it showed some activity against a *Salmonella typhimurium* strain (Townes et al., 2004). The observation that the antimicrobial activity of LEAP-2 increased upon reduction of its disulfide bridges (Hocquellet et al., 2010) suggests that there may be additional biological functions for this peptide. A large number of AMPs have been described originating from a wide variety of species (Wang et al., 2009). Despite the low sequence similarity, the majority of mature peptides in all classes and species are characterized by overall cationicity and amphipathicity, which appear to be essential for their biological activities.

3.5.3. Cathelicidins

Cathelicidins (CATHs) form a family of vertebrate-specific immune molecules present in fish, chickens, and mammals. CATHs exhibit unique bipartite features: a substantially heterogeneous C-terminal antimicrobial

domain of 12–100 residues and an evolutionarily conserved N-terminal cathelin-like domain of 99–114 residues (Zanetti 2005; Chang *et al.* 2006). The sequence heterogeneity of AMPs is reflected in their structural diversity, which includes all three major folding types: cysteine-free linear peptides with an α-helical and amphipathic structure; cysteine-containing peptides with a flat β-sheet structure; and peptides rich in certain amino acids such as proline, arginine, and tryptophan (Zaiou & Gallo 2002). The chicken genome encodes cathelicidin1 (*CATH1*), cathelicidin2 (*CATH2*), cathelicidin3 (*CATH3*), and cathelicidinB1 (*CATHB1*), which are clustered within a 7.5-kb region at the proximal end of chromosome 2 (Goitsuka *et al.* 2007). These closely linked genes are aligned in the order *CATH1*, *CATHB1*, *CATH2*, and *CATH3*. *CATH3* is potentially transcribed in the inverted orientation compared with the orientation of the other members. All cathelicidin genes have four exons except *CATH2*, which has five exons (Xiao *et al.* 2006a; Goitsuka *et al.* 2007). A wide variety of bacteria, both Gram-positive and Gram-negative, have been shown to be terminally susceptible to avian cathelicidins. Quail CATH2 and 3 and pheasant CATH1 show MIC values in the range of 1–10 lM for most Gram-positive and Gram-negative bacteria, which is lower compared to LL-37 (Feng et al., 2011; Wang et al., 2011). MIC values for all four chicken cathelicidins are also of the same order of magnitude as the other avian cathelicidins (Bommineni et al., 2007; Goitsuka et al., 2007; van Dijk et al., 2009b; Xiao et al., 2006a). In addition, fungi such as *Candida albicans* are

susceptible to avian cathelicidins with MIC values in the range of 1–5 lM (Feng et al., 2011; van Dijk et al., 2009b; Wang et al., 2011), and CATH2b even inhibits biofilm formation (Molhoek et al., 2011). Bacterial death appears to be very fast, occurring in 10–30 min for *S. enteritidis* (van Dijk et al., 2009a) and in 30–60 min for *E. coli* (Bommineni et al., 2007; Xiao et al., 2009). The mechanisms responsible for bacterial death are still a matter of discussion. Nevertheless, much is known about the peptide properties that are necessary to kill bacteria. Several studies have shown that the presence of an α-helical region is important in killing bacteria. Removal of the N-terminal α-helix of CATH2a and 2b results in the loss of antimicrobial activity (van Dijk et al., 2009a; Xiao et al., 2009). This finding was also observed with an α-helical synthetic peptide, where remodeling of the N-terminal α-helix by disrupting helix formation resulted in the loss of antibacterial activity (Oh et al., 2000). Removal of the C-terminal α-helix of CATH2a reduced cell death compared to the full-length peptides, but the truncated peptide still exhibited some antibacterial activity (Xiao et al., 2009). One report of a truncated CATH2b where only the N-terminal α-helix is present showed an increase in antibacterial activity (Molhoek et al., 2010; van Dijk et al., 2009a). However, the effect was mostly lost in the presence of 100 mM NaCl, whereas the full-length CATH2a and 2b were largely unaffected (Molhoek et al., 2010; Xiao et al., 2009). Other full-length avian cathelicidins also retain antimicrobial activity in the presence of 100 mM NaCl (Feng et al., 2011; Wang et al.,

2011), which indicates the importance of α-helical regions for proper functionality. Nevertheless, a higher percentage of α-helicity does not guarantee better antibacterial effects. Amino acid substitutions that increased the total α-helicity of CATH1 and LL-37 did not increase the antibacterial activity (Nan et al., 2012; Xiao et al., 2006b). Thus, although α-helicity is important for high efficacy in bacterial killing, increasing the percentage of α-helicity throughout the peptide does not necessarily lead to better activity. A second important region in many α-helical AMPs is the kink or hinge region formed around the center of the peptide (Oh et al., 2000; Tossi et al., 2000), which induces flexibility and is thought to be important during insertion into the bacterial membrane to form pores (Oh et al., 2000; Tossi et al., 2000). Removal or substitution of these glycines or prolines at the center of an α-helical peptide, including CATH2b, can indeed greatly reduce antibacterial activity (Oh et al., 2000; Shin et al., 2000; van Dijk et al., 2009a). Interestingly, a CATH2a truncation containing only the first 14 amino acids, which contains the entire N-terminal α-helix, showed a great reduction in antibacterial activity (Xiao et al., 2009). If the peptide was lengthened by one amino acid, some of the antibacterial activity was restored, and a peptide composed of the first 18 amino acids (N-terminal α-helix including the hinge region) showed comparable activities to the full-length peptide. A C-terminal truncation of CATHCATH2b that leaves the hinge region and the N-terminal α-helix intact also showed very good antibacterial activity (van Dijk et al.,

2009a). Moreover, the C-terminal α-helix of CATH2a and 2b without the hinge region showed no antibacterial activity, whereas the C-terminal α-helix of CATH2a with the hinge region showed better antibacterial activity (in the range of 1–20 lM). Thus, the hinge region is important to produce antibacterial effects in these peptides (Xiao et al., 2009). Hydrophobicity is thought to be important for the interaction of cathelicidins with bacterial membranes (Nicolas, 2009; Oren and Shai, 1998). Membrane interaction of CATH1 has been investigated by determining its structure in a DPC-micelle (Saravanan and Bhattacharjya, 2011). This model showed that the α-helical and hydrophobic centers of the peptide formed an oligomeric structure in the lipid bilayer (most likely by interacting with the acyl groups of the lipids), while the polar residues of the peptide could interact with the phosphate groups on the outside of the lipid bilayer. This structure indicates that pores are formed by these oligomeric structures through which water and ions can freely enter and exit the cell, which disrupts the osmotic balance and hampers bacterial survival (Saravanan and Bhattacharjya, 2011). Interestingly, it was shown that the hydrophobic residues of LL-37 also interact with the membrane surface but would not form a pore by aligning itself through the membrane (Porcelli et al., 2008); although different cathelicidins are alike in structure, their mechanisms of action can differ. Loss of the first tryptophan of CATH1 reduces antibacterial activity, which indicates the importance of the hydrophobic residue in this peptide (Bommineni et al., 2007). Loss of the

more hydrophobic C-terminal in CATH2a analogues also results in reduced bacterial killing ability (Xiao et al., 2009), although the C-terminal truncation of CATH2b (C1-15) resulted in greater antibacterial activity (van Dijk et al., 2009a). When using an N-terminal truncation of CATH2 that only leaves the hydrophobic C-terminal α-helix, the antibacterial activity was almost completely lost, most likely because the first interaction with the bacteria was through the polar parts of CATH2a and 2b (van Dijk et al., 2009a; Xiao et al., 2009). Substitution of phenylalanines with more hydrophobic tryptophans in the C1-15 peptide (Molhoek et al., 2010) resulted in enhanced bacterial killing ability and also better resistance to high ionic strength. It is interesting to note that substitution of a tyrosine with an alanine in a Cecropin A-Magainin-2 fusion peptide greatly reduces antibacterial activity (Oh et al., 2000). The cationic nature of most cathelicidins is most likely important for the initial interaction with the bacterial surface (Nicolas, 2009). The highly cationic CATH2a and 2b and N-terminal analogues show good antimicrobial killing ability, whereas N-terminal truncations remove most of the cationic charges and reduce the antibacterial activity (van Dijk et al., 2009a; Xiao et al., 2009). Increasing the charge of CATH2a did not result in large changes in bacterial killing ability, which is in line with results found for other α-helical peptides, where increased charge did not directly correlate with increased antimicrobial activity (Dathe et al., 2001; Nan et al., 2012).

CHAPTER 3.

Reactivation of Transgene Expression by Alleviation of CpG Methylation in Rous sarcoma virus Promoter in Transgenic Quail Cells

1. Introduction

Transgenic birds have been an model for conducting various studies such as those focusing on the production of pharmaceutical proteins in eggs, the development of animal disease models, and agricultural applications (Han 2009). Successful production of transgenic birds was achieved by injecting retroviral vectors into undifferentiated stage X embryos (Mizuarai *et al.* 2001) and by germ cell-mediated germline transmission with a lentiviral vector (Shin *et al.* 2008; Kim *et al.* 2010; Park *et al.* 2010).

An early study in the 1980s reported that long terminal repeats (LTRs) of *Rous sarcoma viruses* (RSVs) have strong promoter activity in various types of cells (Gorman *et al.* 1982). Since then, RSV promoters have been successfully used for producing transgenic animals with a high induction of transgene expression (Overbeek *et al.* 1986; Zhang *et al.* 1990). However, variations in the transcriptional activity of the promoter among tissues and organs in transgenic animals has been a major concern (Overbeek *et al.* 1986).

In birds, several reports showed that the RSV promoter drives transgene expression in various tissues (Mizuarai *et al.* 2001; Kwon *et al.* 2004). We also produced transgenic quail and chickens that expressed green fluorescent protein (GFP) driven by the RSV promoter (Shin *et al.* 2008; Park

et al. 2010). However, we observed that expression of GFP in the transgenic birds varied among tissues, and even among the same types of cells in the same tissues. This variation in transgene expression encouraged us to examine the CpG methylation status of the RSV promoter in each tissue, and the result suggested possible tissue-specific transgene expression by CpG methylation (Park *et al.* 2010). Studies have also suggested that CpG methylation is involved in inhibition of transgene expression driven by the RSV promoter (Guntaka *et al.* 1987; JirÏõÂ Hejnar 1999).

In this study, we hypothesized that removal of CpG methylation in the RSV promoter may reactivate transgene expression in transgenic quail. We herein describe elevated GFP expression secondary to the treatment of 5-aza-2'-deoxycytidine (5-azadC), a methyltransferase inhibitor in embryonic fibroblasts of transgenic quail.

2. Materials and methods

Animal cares and general experimental procedure

The care and experimental use of Japanese quails (*Coturnix japonica*) was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). Japanese quails were maintained in a standard management program at the University Animal Farm, Seoul National University, Republic of Korea. Procedures involved animal management, reproduction, and embryo manipulation. Eggs were brought to the laboratory within 1 to 3 h of oviposition for stage X embryos. Developing embryos under a relative humidity of 60% to 70% at 37.8°C were staged according to the Hamburger and Hamilton (HH) classification system. The production of transgenic quail has been described in previous reports (Shin *et al.* 2008). Each homozygous transgenic quail line (TQ2) was used throughout this study.

Cell culture

Quail embryos were retrieved from 6-day-old TQ2 quails. Quail embryonic fibroblasts (QEFs) were retrieved from the bodies of 6-day-old Japanese quail embryos using our standard procedure. Embryos were freed from the yolk by rinsing with calcium- and magnesium-free phosphate-buffered saline (PBS), and the bodies were retrieved by removal of embryo heads, arms, legs, tails, and internal organs with sharp tweezers under a

stereomicroscope. The embryonic bodies were collected from a total of five embryos. The bodies were dissociated by gentle pipetting in 0.05% (v:v) trypsin solution supplemented with 0.53 mM EDTA. The QEFs were then cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.1 mM MEM nonessential amino acids (Invitrogen), and 1% antibiotic–antimycotic (Invitrogen) in a 5% CO_2 atmosphere at 37°C. The expression of enhanced green fluorescent protein (EGFP) was visualized under an LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

5-Aza-2'-deoxycytidine treatment

Primary cultured QEFs were seeded at a density of 5.2×10^4 cells/cm². The cells were treated with various concentrations ranging from 0.1 μM to 100 μM of 5-aza-2'-deoxycytidine (5-azadC) (Sigma-Aldrich, St. Louis, MO) for 48 h; 5-azadC was then withdrawn, and the QEFs were continuously cultured under normal culture conditions for 20 days.

Flow cytometry

Cells were fixed in 4% paraformaldehyde in PBS. After re-suspension in 700 μL 1% bovine serum albumin in PBS, the cells were analyzed using BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA).

Bisulfite sequencing

DNA samples were prepared using an AccuPrep Genomic DNA Extraction Kit (Bioneer) and converted using MethylEasy Xceed (Human Genetic Signatures, North Ryde, NSW, Australia) according to the manufacturer's instructions. For amplifying the converted DNA, PCRs were performed with forward (5-GGG GAA ATG TAG TTT TAT GTA ATA T-3) and reverse (5-TAG TGT AGG GGA AAG AAT AGT AGA T-3) primers, which included the RSV promoter, as follows: 95°C for 1 min and 50 s, 35 cycles at 94°C for 20 s, 52°C for 20 s, 72°C for 30 s, and 72°C for 10 min for the final synthesis. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA).

Prediction of transcription factor-binding cis-elements

The presence of transcription factor-binding *cis*-elements within the RSV promoter was predicted using a bioinformatics tool for orthologous sequences (TFSEARCH ver. 1.3; http://www.cbrc.jp /research/db/TFSEARCH.html).

3. Results

Suppressed EGFP expression in TQ2 QEFs was reactivated by 5-azadC treatment

The TQ2 quail is a transgenic quail with a single copy of the pLTReGW vector, which is a lentiviral vector that drives EGFP expression driven by the RSV promoter (Shin *et al.* 2008). Embryonic fibroblasts from the 6-day-old TQ2 embryos were primary-cultured and observed under

fluorescence microscopy. However, a very small number of EGFP-expressing cells were observed (Figure 1A). Therefore, we tested whether 5-azadC, which causes DNA demethylation by inhibiting DNA methyltransferase, could reactivate transgene expression in TQ2 QEFs. The cells were treated with various concentrations of 5-azadC (0.1 to 100 μM) and observed under a fluorescence microscope at 8 days after 5-azadC withdrawal (Figure 1A to F). As a result, EGFP expression was reactivated in 5-azadC-treated cells and the green fluorescence was elevated in a dose-dependent manner.

For a more detailed analysis, flow cytometry was performed to compare EGFP expression between non-treated and 5-azadC-treated cells. Through flow cytometry, TQ2 QEFs were divided into three groups: low ($<10^1$), middle (10^1–10^3), and high ($>10^3$) levels. When 5-azadC from 0.1 to 100 μM was administered, the highly EGFP-expressing cells increased by 41.05%, 75.52%, 80.56%, 81.78%, and 80.63%, respectively, compared to the non-treated cells (Figure 1G), suggesting that 5-azadC increased EGFP expression of TQ2 in a dose-dependent manner with a plateau of EGFP expression at 50 μM of 5-azadC. At 8 days of culture, 2.64% of the control (vehicle only) cells expressed EGFP at high levels, while 79.84% of the 5-azadC-treated cells expressed EGFP at high levels (Figure 2A). In addition, at 20 days of culture, 10.41% of the control (vehicle only) cells expressed EGFP at high levels, while 55.08% of the 5-azadC-treated cells expressed EGFP at

high levels (Figure 2B). These results suggest that EGFP suppression is related to DNA methylation.

CpG methylation of the RSV promoter in 5-azadC-treated QEFs

Treatment with 5-azadC, a DNA methylation inhibitor, induced dramatic EGFP expression in TQ2 QEFs, which led us to hypothesize that demethylation of the RSV promoter by 5-azadC could be responsible for enhanced expression of the transgene EGFP. Therefore, we examined the methylation status of 5-azadC-treated cells at 13 CpG sites in the RSV promoter (Figure 3A). In non-treated cells, the CpG sites were highly methylated (86.42%). However, when treated for 48 h, 48.41% of the CpG sites maintained methylation, and most of the 6^{th} and 11^{th} CpG sites on day 8 showed demethylation (11.76%). After 5-azadC withdrawal, methylation of the CpG sites also decreased (16.28%) in the expanded culture and most sites were demethylated, except for the 4^{th} CpG site. We further analyzed transcription factor-binding sites in the RSV promoter using TSSEARCH, which estimates the location of protein-binding motifs in the DNA sequence. Consequently, 19 binding motifs were predicted (Table 1). CAP and Yin Yang-1 (YY1)-binding motifs include the first CpG site; whereas, GATA-

1/GATA-2 and S8 motifs interact with sites 3 and 11, respectively. GATA-2 covers both sites 12 and 13 (Figure 3B). These results suggest that CpG methylation of protein-binding motifs in the RSV promoter can potentially suppress EGFP expression.

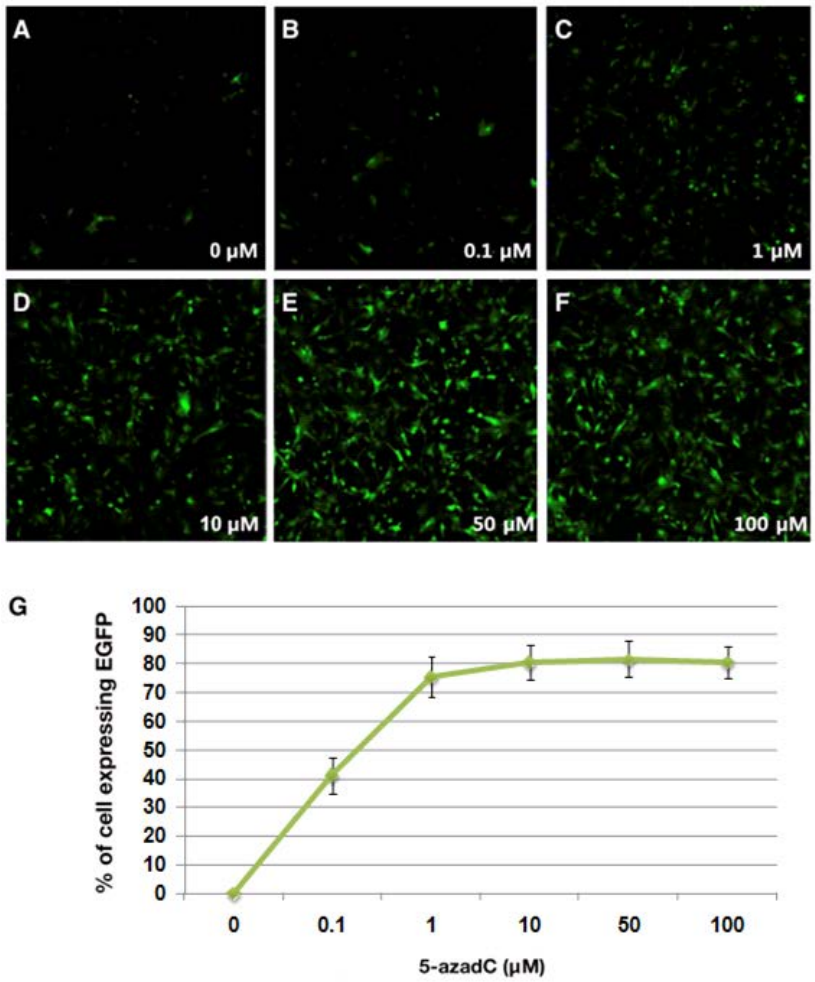

Figure 1. Effect of 5-azadC on EGFP expression in TQ2 QEFs. (A through F) Elevated EGFP fluorescence in TQ2 QEFs treated with 5-azadC in a dose-dependent manner. (G) The percentage of highly EGFP-expressing TQ2 QEFs after 5-azadC treatment. The EGFP-expressing cells in each treatment were analyzed by flow cytometry (mean ± SEM; n = 3).

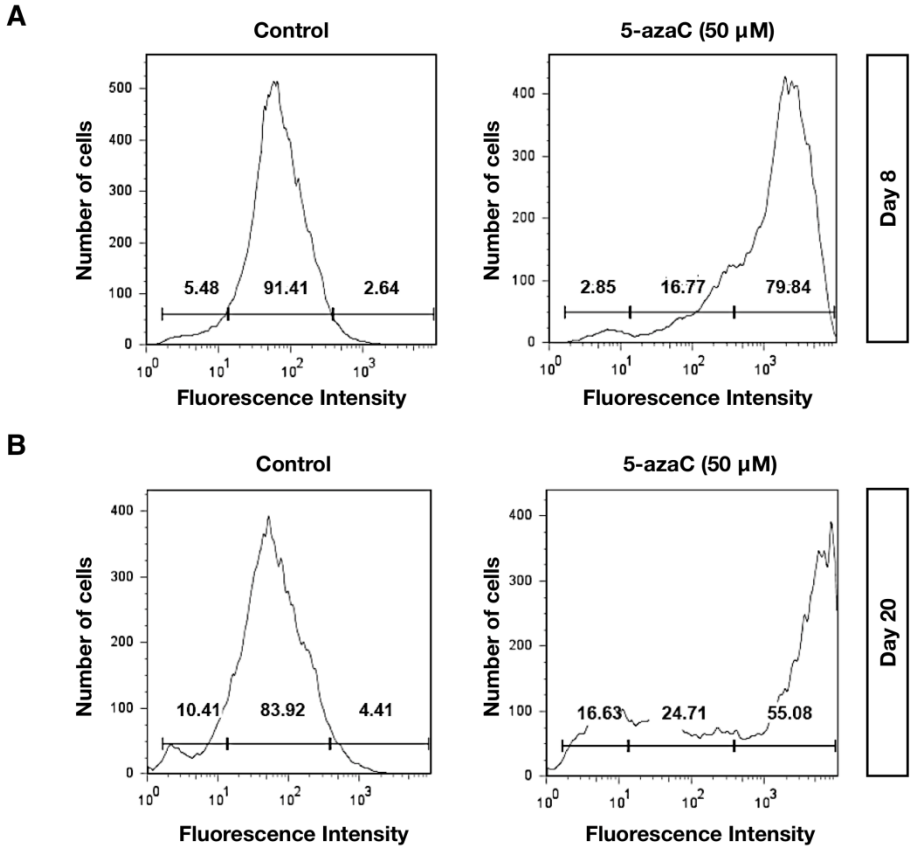

Figure 2. EGFP expression pattern in TQ2 QEFs after 5-azadC treatment. Flow cytometry analysis was performed in control (vehicle only) and 50 μM 5-azadC-treated TQ2 QEFs after 8 days of treatment (A) and 20 days of treatment (B).

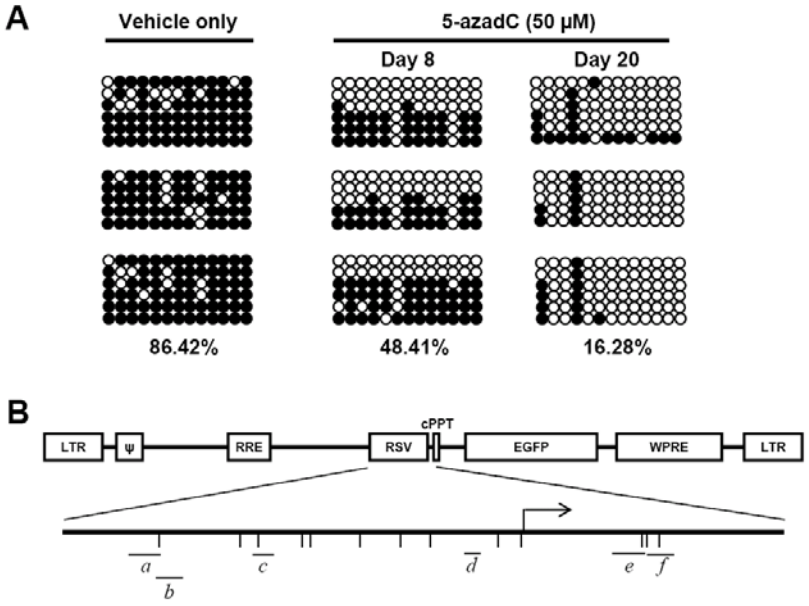

Figure 3. CpG methylation analysis and schematic of the CpG site in the RSV promoter and structure of the proviral construct. (A) The 'y-axes' indicate the numbers of the sequenced PCR products after bisulfate sequencing. Thirteen CpG methylations of the RSV promoter were analyzed in control (vehicle only) QEFs and TQ2 QEFs treated with 5-azadC for 8 and 20 days by bisulfate sequencing. Closed and open circles are methylated and unmethylated CpGs, respectively. (B) Schematic illustration of the transgene construct and CpG site of the RSV promoter. LTR, long terminal repeat; Ψ, packaging signal; RRE, Rev-response element; RSV, RSV promoter; cPPT, central polypurine tract of HIV-1; EGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. a, YY1; b, CAP; c, GATA1/2; d, TATA box; e, S8; f, GATA-2; ⏐, CpG site.

Table 1. Putative protein-binding sites within the RSV promoter

Sequence	Position	Binding TF	Species
GTCTTATGCAATAC	–210, –223	C/EBPbeta	human, mouse, rat (Akira *et al.* 1990)
TTATGCAATACTC	–208, –220	CHOP-C/EBPalpha	human, mouse, rat, chicken, frog (Ubeda *et al.* 1996)
AGTCTTATGCAATACTCT	–207, –224	C/EBPalpha	human, mouse, rat (Jenkins *et al.* 1995)
TAGTCTTGCAACAT	–193, –204	C/EBPbeta	human, mouse, rat (Jenkins *et al.* 1995)
AACGATGAGTTAG	–174, –187	cap*	eukaryotic RNA polymerase II promoter elements (Bucher 1990)
CTTGCAACATGGTAACG	–184, –200	YY1*	human (Shrivastava & Calame 1994), mouse
ATGAGTTAG	–175, –183	AP-1 binding site*	human, mouse, rat, chicken (Gallarda *et al.* 1989), frog, fruit fly
ATGAGTTAGCAA	–172, –183	NF-E2*	human, mouse, chicken (Andrews *et al.* 1993)
GCCGATTGGT	–128, –137	GATA-1/2	human, mouse (Merika & Orkin 1993)
GTAAGGTGGTA	–113, –123	deltaEF1*	chicken (Sekido *et al.* 1994)
GCCTTATTAGGAA	–93, –105	Oct-1*	human, monkey, mouse, hamster, chicken, frog (Verrijzer *et al.* 1992)
GAGAT	–35, –39	GATA-3	human (Merika & Orkin 1993)
ACCACA	15, 20	AML1a	human (Meyers *et al.* 1993)
GATAAAT	42, 48	CdxA*	chicken (Margalit *et al.* 1993)
TAAATTTAATTAATCTCG	44, 61	S8*	mouse (de Jong *et al.* 1993)
TTAATTAAT	49, 57	Pbx-1	human (Van Dijk *et al.*

			1993)
AATTAAT	51, 57	CdxA*	chicken (Margalit *et al.* 1993)
CGGTATCGGT	63, 72	GATA-2	human (Merika & Orkin 1993)
AGGGGGGA	89, 96	MZF1	human (Morris *et al.* 1994)
AGGGGGGATTGGGG	89, 102	RREB-1	human (Thiagalingam *et al.* 1996)
GGGGATTGGG	92, 101	GATA-1/2	human (Merika & Orkin 1993)
GGGATTGGG	93, 101	GATA-3	human (Merika & Orkin 1993)
GCAGGGGA	111, 118	MZF1	human (Morris *et al.* 1994)

TF, transcription factor; *, marked in Figure 3B.

4. Discussion

During the last decade, retroviral and lentiviral vector systems have continuously evolved and contributed tremendously to implementing gene therapy and transgenic animal production. HIV-1-based lentiviral vectors were once acknowledged to be not easily silenced (Escors & Breckpot 2010), but it has become apparent that genes delivered by lentiviral vectors can be silenced (Hotta & Ellis 2008; Pearson *et al.* 2008). In the current study, the status of methylation in a transgenic promoter was examined and modified to determine the relationship between different methylation statuses with EGFP expression.

Unlike 5-azacytidine, which is incorporated into both DNA and RNA and causes complex inhibitory effects of DNA, RNA, and protein synthesis (Christman 2002), 5-azadC can be incorporated into DNA during the S-phase, thus inhibiting methylation in DNA only. As a result, we observed that the effect of 5-azadC treatment for 48 h was maintained over 20 days.

The bioinformatic analysis revealed that the first CpG site was included in the cap signal element that is required for transcription initiation.

Studies have reported that the cap signal element with the TATA-box, CAAT-box, and GC-box is the most general element of the POL II promoter and exists in major protein-encoding genes. Its function is to position the RNA polymerase precisely onto the target gene, rather than influence transcription activity (Bucher 1990). Similarly, the activity of the viral promoter, which originated from the adenovirus, was inhibited by methylation of its cap signal element (Doerfler 1990). These studies suggest that the first CpG site is related to fidelity of transcription. The first CpG site was also included in the YY1-binding motif. YY1 is a multifunctional transcriptional regulator that has been reported to activate, repress, or initiate transcription (Shrivastava & Calame 1994). However, the detailed mechanism remains unknown. Recently, a study reported that the DNA-binding activity of YY1 in normal blood cells was strictly associated with an absence of *de novo* methylation in cancer (Gebhard *et al.* 2010), suggesting that the binding site of YY1 may affect the status of DNA methylation. In our data, approximately 52.94% of the first CpG methylation in 5-azadC-treated cells was maintained in the expended culture. The 11^{th} CpG site was included in the 11-bp consensus binding site, which is recognized by the murine S8 homeobox gene and was expressed in a mesenchyme-specific pattern in embryos and mesodermal cell lines (de Jong *et al.* 1993). Although transcriptional activity related to methylation of the binding site has not been reported, we propose that this binding site is related to embryonic fibroblast proliferation.

In this study, the GATA family interacted with CpG sites 3, 12, and 13. Among the vertebrates, the GATA family exhibits a distinctive or overlapping pattern of expression in tissues and cell lines. GATA-1 is found in cells of the erythroid lineage, including hematopoietic and hematopoietic progenitor cells. GATA-2 is also expressed in progenitor cells, mast cells, megakaryocytes, embryonic brain cells, primitive erythroblasts, endothelial cells, and embryonic stem cells, as well as other cells and tissues (Merika & Orkin 1993). In addition, a study reported that methylation of the U3-LTR in the murine retrovirus interfered with the binding between GATA-1 and GATA-2 and their binding sites (Barat & Rassart 1998). Based on these previous studies, we could propose that the transcriptional activity was controlled by CpG methylation of the promoter region.

We conclude that transgene expression was affected by CpG methylation of the RSV promoter in transgenic quail. Our results provide the basis for understanding the epigenetic mechanism responsible for transgenic animal production and genetic preservation.

CHAPTER 4.

Gene Expression and DNA Methylation Status of Chicken Primordial Germ Cells

1. Introduction

In vertebrates, DNA methylation occurs almost exclusively on the CpG dinucleotides. Such methylation can be inherited through cell division and transmitted from one generation to the next via germ cells. CpG dinucleotides are often clustered in particular genomic regions referred to as CpG islands. DNA hypermethylation of CpG islands is largely related to gene suppression, while their hypomethylation is associated with gene expression (Rajender *et al.* 2011). CpG methylation plays a role in the maintenance of heterochromatin as well as the inhibition of promoter activity by inhibiting interaction between transcription factors and their promoters or by changing the chromatin structure. CpG methylation is essential for embryonic development and has been implicated in genomic imprinting and X chromosome inactivation. However, DNA methylation appears to play different roles in different organisms, and it is missing entirely from many eukaryotes (Siegfried & Simon 2010).

During early mammalian germ cell differentiation, genome-wide chromatin changes occur in germ cells, contributing to the suppression of somatic cell differentiation (Sasaki & Matsui 2008). In the early germ cells, termed primordial germ cells (PGCs), the genomes obliterate most of their epigenetic markers including DNA methylation, histone modification, and

other covalent chromatin modifications that are associated with somatic gene regulation, so that germ cells can acquire the capacity to support post fertilization development (Reik 2007; Kota & Feil 2010). As a result, the epigenetic reprogramming in PGCs establishes monoallelic expression of imprinting genes, maintains inactivated retrotransposons, inactivates one of the two X chromosomes, and suppresses gene expression (Lees-Murdock & Walsh 2008). This process also prepares the germ cells for meiosis, during which homologous chromosomes become aligned to allow synapsis and recombination. Recent research demonstrated that histone modifications, aside from specific DNA sequence motifs, also contribute to synapsis formation and recombination, and enhance recombination at preferential regions (Baudat *et al.* 2010; Myers *et al.* 2010).

In chickens, the basic function of DNA methylation is similar to that in mammals. For example, the methylation of a promoter region induces gene silencing (Luo *et al.* 2011), and methylation protects infection of the viral genome into the host genome as well as induces a transcriptional suppression of transgenes (Park *et al.* 2010; Jang *et al.* 2011). Conversely, unlike mammals, chicken PGCs exhibit unique migration activity. Mammalian PGCs are originally derived from the proximal epiblast and move into embryonic gonads through the hindgut by amoeboid movement. On the other hand, chicken PGCs appear within the epiblast in the blastoderm and move to the hypoblast of the area pellucida (Petitte *et al.* 1997). During gastrulation, PGCs

move to the germinal crescent then circulate through the blood vessels, finally settling in the gonadal ridge (Nieuwkoop. & Sutasurya 1979; Tsunekawa *et al.* 2000; Nakamura *et al.* 2007). The control mechanism of DNA methylation during early embryonic development is also different between chickens and mammals. For example, imprinted genes (*mpr*/*igf2r*, *igf2*, *ascl2*/*mash2*, *ins2*, *dlk1*, and *ube3a*) in mammals are expressed from bi-alleles in chickens (Yokomine *et al.* 2005; Colosi *et al.* 2006; Shin *et al.* 2010). Additionally, in chickens, the males are homogametic for sex chromosomes (ZZ), whereas females are homogametic for the sex chromosomes in mammals (XX). Additionally, whereas somatic X inactivation takes place in XX mammals, somatic Z inactivation does not occur in ZZ male chickens (Wu & Xu 2003).

A variety of genome-wide methylation analyses have been conducted to understand DNA methylation in chickens (Li *et al.* 2011; Xu *et al.* 2011), which have provided insight into the characteristics and roles of DNA methylation in some tissues and somatic cell lines (Itoh *et al.* 2011; Luo *et al.* 2011). However, our understanding of the epigenetic regulation of chicken PGCs remains poor despite the great interest shown in epigenetic changes that occur during germ cell development since imprinting was first proposed. Progress in this field has been hindered by technical difficulties caused by laborious germ cell isolation and questionable sample purity; however, new, highly sensitive methods have been developed that enable the analysis of very few cell samples. Here, we attempted to identify genes specifically expressed

in chicken PGCs as compared with CEFs and analyzed the methylation patterns of differentially expressed PGC genes as well as X-linked and imprinting homologous loci among male PGCs, female PGCs, and CEFs.

2. Materials and methods

Experimental animals and cell samples

The Institute of Laboratory Animal Resources of Seoul National University (SNU-070823-5) approved the care and experimental use of the animals. White Leghorn (WL) chickens were maintained according to a standard management program at the University Animal Farm. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Preparation of PGCs and CEFs for microarray data generation

For preparing PGCs, gonadal cells were retrieved from the gonads of 6-day-old (HH stage 29) WL embryos using our standard procedure (Park *et al.* 2003). Embryos were freed from the yolk by rinsing with calcium- and magnesium-free phosphate buffered saline (PBS) and the gonads were

retrieved by dissection of the embryo abdomen with sharp tweezers under a stereomicroscope. Embryonic gonads were collected from a total of 1,080 embryos. Gonadal tissues were dissociated by gentle pipetting in 0.05% (v:v) trypsin solution supplemented with 0.53 mM EDTA. After centrifugation at 200 x g for 5 min, total gonadal cells were loaded into MACS (Miltenyi Biotech, Germany), and the separated primordial germ cells (PGCs) were immediately stored in liquid nitrogen (-190ºC) until further processing. For the CEF culture, all internal organs and limbs were removed from WL embryos at E6.5. The remaining embryonic body was then dissociated using 0.25% (v/v) trypsin-EDTA at 37°C for 20 min. Next, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (Invitrogen, CA, USA) in a 5% CO_2 atmosphere at 37°C until passage 2.

Microarray data generation

Total RNA was extracted from PGCs and CEFs with Trizol reagent (Invitrogen, CA, USA). DNA traces from total RNA were degraded with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) before gene-expression analysis on an Affymetrix GeneChip Chicken Genome Array (Affymetrix, CA, USA). The Seoulin Bioscience Corporation (Korea) generated the GeneChip data from PGCs and CEFs in triplicate. Approximately 5 μg total RNA from

each sample was used for labeling. Probe synthesis, hybridization, detection, and scanning were performed according to standard Affymetrix protocols (Lee *et al.* 2007). cDNA was synthesized using the One-Cycle cDNA Synthesis Kit (Affymetrix, CA, USA). Single-stranded cDNA (ss-cDNA) was synthesized using Superscript II reverse transcriptase and T7-oligo(dT)-primers at 42°C for 1 h. Double-stranded cDNA (ds-cDNA) was obtained using DNA ligase, DNA polymerase I, and RNase H at 16°C for 2 h, followed by T4DNA polymerase at 16°C for 5 min. After cleanup using the Sample Cleanup Module, ds-cDNA was used for *in vitro* transcription. cDNA was transcribed using the GeneChip *in vitro* transcription Labeling Kit (Affymetrix, CA, USA) in the presence of biotin-labeled CTP and UTP. The biotin-labeled, *in vitro*-transcribed RNA was then fragmented and hybridized to the chicken genome GeneChip array at 45°C for 16 h. After hybridization, the arrays were washed in a GeneChip Fluidics Station 450 with a nonstringent wash buffer. The arrays were stained with a streptavidin–phycoerythrin complex. After staining, intensities were determined with a GeneChip scanner controlled by GeneChip Operating Software (GCOS) (Affymetrix, CA, USA)).

Microarray data analysis

Gene expression data analysis was conducted using the Microarray

Suite 5.0 software (Affymetrix, CA, USA) and the GenPlex software v1.8 (ISTECH, Inc., Goyang, Korea). The MAS5 algorithm was employed for expression summary and signal calculation (Hubbell *et al.* 2002). Global saline normalization was performed using a GCOS algorithm, after which the normalized data were log2-transformed. Fold changes were determined and Welch's t-test was applied for the selection of significant transcripts. The fold-change cut-off maintained 1.5-fold, and the minimum significance level was 0.05. For better visualization and comparison of significantly expressed transcripts in PGCs as compared with CEFs, significantly upregulated and downregulated transcripts in each test sample were clustered via hierarchical clustering with Pearson's correlation.

All upregulated and downregulated transcript-matched genes in the PGCs were identified by microarray analysis, and gene-matched proteins were identified using proteomics. These genes were then subjected to searches for relative signaling and metabolic pathways mostly through the KEGG database (http://www.genome.jp/kegg/pathway.html) using over-representation analysis.

PCR-sexing of chicken embryos and preparation of male and female PGCs and CEFs for the DNA methylation chip

The sex of each donor embryo was determined before PGC transfer via PCR using a nonrepetitive DNA sequence on the W chromosome.

Embryonic blood cells (1 μl) were collected once from the dorsal aorta of 3-day-old embryos through the egg shell window. Each blood sample was diluted 100-fold in 1× PBS and then boiled for 5 min at 99°C prior to the PCR. The Psex primer pair (forward primer 5'-CTA TGC CTA CCA CAT TCC TAT TTG C-3' and reverse primer 3'-AGC TGG ACT TCA GAC CAT CTT CT-5') designed for sex determination in birds was used to amplify the 396-bp fragment (Ogawa *et al.* 1997). After collection, the window of the egg was sealed with parafilm. The egg was subsequently incubated before PGC collection at 37.5°C in an air atmosphere with 50–60% humidity according to our standard protocol (Kang *et al.* 2008). We then obtained gonads from the sex-confirmed chicken embryos at E6 using our standard procedure. To collect purified chicken male and female PGCs, FACS was performed using a chicken PGC-specific marker, anti-stage-specific embryonic antigen 1 (anti–SSEA-1) antibody, and confirmed by staining with anti-SSEA-1 antibody and the periodic acid-Schiff (PAS) reaction, which are specific to chicken PGCs (Park *et al.* 2003; Kim *et al.* 2004). We also retrieved chicken female embryonic fibroblasts (CEFs) from 6-day-old chicken embryos using our standard procedure. Embryos were freed from the yolk by rinsing with calcium- and magnesium-free PBS, and the embryonic bodies were retrieved after removal of embryo heads, arms, legs, tails, and all internal organs with sharp tweezers under a stereomicroscope. The bodies were dissociated by gentle pipetting in 0.05% (v/v) trypsin solution supplemented with 0.53 mM

EDTA. CEFs were collected from the dissociated bodies (Jang *et al.* 2011).

Immunoprecipitation of methylated genomic DNA

Each 4 ug/200 ul of genomic DNA from PGCs and CEFs was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, CA, USA) per the manufacturer's instructions and the genomic DNA was sheared into 100–500 bp (mean ~300 bp) fragments by sonication. Methyl-CpG binding-domain protein affinity capture was performed using MethylMiner™ Methylated DNA Enrichment kits (Invitrogen, CA, USA) following the manufacturer's protocol. For salt-gradient elution of the fragmented genomic DNA, successive fractions were obtained by elution using buffer containing the following NaCl concentrations: 200 mM, 350 mM, 450 mM, 600 mM, and 2 M NaCl.

DNA methylation chip analysis

Each feature on the array has a corresponding scaled log2 ratio, which is the ratio of the input signals for the experimental (IP) and control (input) samples co-hybridized to the array. The log2 ratio is computed and scaled to center the ratio data around zero. Centering is performed by subtracting the bi-weight mean for the log2 ratio values for all features on the

array from each log2 ratio value. From the scaled log2 ratio data, a fixed-length window (default = 750 bp) is placed around each consecutive probe and the one-sided Kolmogorov-Smirnov (KS) test is applied to determine whether the probes are drawn from a significantly more positive distribution of intensity log ratios than those in the rest of the array. The resulting score for each probe is the -log10 p-value from the windowed KS test around that probe. Using NimbleScan software, peak data files (.gff) are generated from the p-value data files (Genocheck, Ansan, Korea). NimbleScan software detects peaks by searching for the user-specified number of probes (default = 2) above a user-specified p-value minimum cutoff (-log10, default = 2) and merges peaks within a user-specified distance (default = 500 bp) of each other.

3. Results and discussion

PGC-upregulated and downregulated genes

To isolate PGCs from six day-old gonads, we sorted SSEA1-positive cells using FACS analysis. The percentage of SSEA-1+ and PAS+ cells after the FACS analysis were 93 ± 1.4% and 96 ± 0.8, respectively, and the viability of the sorted PGCs was 95.0 ± 0.8%. A total of 261 genes met the fold-change and p-value cut-off values of 1.5-fold and 0.05, respectively, 168

of which were upregulated in the PGCs compared to the CEFs (control) (Figure 1A).

DNA chip construction

We constructed a DNA chip comprising the 5kb-upstream regions of differentially PGC-expressed genes, X-linked homologous loci and imprinting homologous loci in the chicken genome. Most imprinting homologous genes were clustered around particular loci of chromosomes 1–5 (Table 2 and Figure 2) and X-linked homologous genes exclusively existed on chromosomes 1 and 4 with the exception of *hccs*, *opn1lw*, *hsd17b10*, and *plp1* (Table 3 and Figure 2), while differentially PGC-expressed genes spanned chromosomes 1–24, 26-–28, and Z.

Methylation status of imprinting homologous genes in chicken

Clustering of imprinting genes is typical in mammals. For example, approximately 300 kb of the distal portion of mouse chromosome 7 contains four imprinted genes (*mash2*, *ins2*, *igf2*, and *H19*) (Leighton *et al.* 1996). While in the male germ line resetting of methylation occurs before meiosis, maternal imprinting control regions (ICRs) are hypomethylated until after the pachytene stage of meiosis I, which occurs in the postnatal growing oocyte. It

is now clear from studies performed on PGCs isolated without culture and directly analyzed by bisulfite sequencing following isolation, that the differentially methylated regions (DMRs) of imprinted genes, including the maternally methylated Snrpn *DMR1*, *Peg3*, *Lit1,* and *Igf2* and the paternally methylated *H19* and *Rasgrf1,* are synchronously demethylated between E11.5 and E12.5. These genes are maintained in a partially methylated state until they become fully methylated again in the male germ line. They continue to slowly lose methylation in the female germ line until the fully unmethylated pattern seen in the mature oocyte is achieved (Hajkova *et al.* 2002; Li *et al.* 2004; Yamazaki *et al.* 2005). Our DNA methylation chip data showed partial imprinting homologous genes in chicken PGCs at E6 that were differentially methylated (Figure 2).

Methylation status of X-linked homologous genes in chicken

X-linked homologous genes were only found on chromosomes 1 and 4, with the exception of *hccs*, *opn1lw*, *hsd17b10,* and *plp1* (Table 3 and Figure 2). Such clustering phenomena of X-linked homologous genes in chicken were reported previously (Deakin *et al.* 2008). The X chromosome was inactive in migrating PGCs, but becomes reactivated upon arrival at the gonadal ridge, presumably reflecting both demethylation and alteration of any other epigenetic marks that may be associated with inactivation, such as

histone changes, although no methylation analyses of the transgenes in germ cells were performed (Tam *et al.* 1994; Boumil *et al.* 2006). Interestingly, most X-linked homologous loci were differentially methylated in both PGCs and somatic cells (CEFs) (Figure 2).

Differentially expressed PGC genes and methylation of their promoters

DNA methylation in mammals is generally linked to imprinting genes and X-linked genes during embryo development (Wu & Xu 2003; Lees-Murdock & Walsh 2008; Kota & Feil 2010). Additionally, DNA methylation can directly inhibit the transcriptional activities of promoters. Similarly, promoters in chickens can be suppressed by methylation (Weber & Schubeler 2007; Siegfried & Simon 2010). We examined the DNA methylation status of the regulatory regions 5-kb upstream of 261 differentially expressed PGC genes to study the epigenetic regulation between PGC-specific genes and their promoters. Differences in the methylation status between PGCs and CEFs were only detected in 12 of the analyzed regions (Table 1). When the regulatory regions of *nanog*, *lgals2*, *ambp*, *rcjmb04_8j10,* and *col4a4* were demethylated in PGCs, the expression levels of those genes were increased, whereas *rbm33* and *gng11* exhibited decreased expression when their respective promoters were methylated. Therefore, we suggest that transcription of these genes is controlled by DNA methylation of their

regulatory regions (Figure 1B).

In addition, three genes (*tnnc1*, *rft1*, and *gpd2*) of those differentially expressed in the PGCs were insensitive to DNA methylation, while the expressions of Gga.14707.1.S1_at (unknown gene) and *eif2ak3* were decreased in PGCs compared to CEFs despite hypomethylated regulatory regions (Figure 1B). Therefore, we hypothesize that the transcriptional regulation of *tnnc1*, *rft1*, *gpd2*, Gga.14707.1.S1_at, and *eif2ak3* in PGCs is controlled in a DNA methylation-independent manner.

Direct correlation between DNA methylation and gene expression

To examine the epigenetic correlation between the expression of a gene and its regulatory region in PGCs, we focused on the inverse correlation between a gene's expression and the methylation of its regulatory region. When the expression of *nanog* was increased 4.43-fold in PGCs compared to CEFs, the methylation of its regulatory region was decreased 2.25-fold (Table 1), and a ~300 bp methylated region was detected within the region ~3 kb upstream of *nanog* (Figure3A). *Nanog* encodes one of the major transcription factors associated with pluripotent maintenance in mammals (Do & Scholer 2009). In mammals, *Nanog* is important for embryonic development and acts as a guide gene during the epiblast/primitive endoderm lineage decision (Mitsui *et al.* 2003; Ralston & Rossant 2005). A hypomethylated state of the

promoter is necessary for the normal expression of *nanog* in pluripotent cells (Farthing *et al.* 2008), such as the inner cell mass. During development, DNA demethylation of the upstream region occurs (Farthing *et al.* 2008) to induce the erasure portion of the parental mark acquired during gamete formation (Western *et al.* 2010). Similarly, specific features of the promoter participate in the regulation of the pluripotency state in mammals (Rodda *et al.* 2005). In mice, *Nanog* also mediates germline development such as cell state transition during germ cell development (Chambers *et al.* 2007). Furthermore, in human cells, apoptosis of migrating PGCs was induced by *nanog* knockdown (Yamaguchi *et al.* 2009) as well as controlled by the methylation of the *nanog* promoter region (Nettersheim *et al.* 2011). Thus, we suggest that the methylated region of *nanog* is closely associated with its expression in PGCs, and that its expression is related to PGC maintenance or germ cell differentiation.

Other genes (*lgals2*, *ambp*, *col4a4*, and *rcjmb04_8j10*) and their promoters exhibit similar epigenetic regulation to *nanog*. When the gene expression levels of *lgals2*, *ambp*, *col4a4*, and *rcjmb04_8j10* were increased 3.44-, 2.93-, 1.10-, and 1.20-fold, respectively, in PGCs compared to CEFs, methylation was also decreased by 2.56-, 2.15-, 2.360, and 2.64-fold, respectively (Table 1), and approximately 100–300 bp of differentially methylated sequence was found within the regions 5kb-upstream of these genes (Figure 3B, 3C, 3D and 3E). In addition, the gene expression levels of

rmb33 and *gng11* were decreased 0.86- and 1.09-fold, while DNA methylation was increased 2.34- and 2.25-fold (Table 1), respectively, and approximately 100–400 bp of differentially methylated regions existed within 5kb-upstream of these genes (Figure 3F and 3G). Therefore, we suggest that DNA methylation controls these genes via an inverse correlation between gene expression and the methylation of their regulatory regions.

Different methylation statuses between male and female PGCs

When we compared the male and female PGCs, 289 genes exhibited significantly different methylation (< 0.05) patterns in the 5 kb-upstream regions of the PGC-expressed genes or the X-linked and imprinting homologous loci (including promoters, exons, introns, intergenic sequences, and even neighboring genes). A total of 144 of the differentially methylated regions were hypermethylated in male PGCs and 145 regions were hypermethylated in female PGCs. When we analyzed the methylated regions, 38 and 56 of the 5 kb-upstream regions were hypermethylated, and 106 and 89 of the genes and promoters were hypermethylated in male and female PGCs, respectively. The methylation ratio between male and female PGCs was similar despite the fact that the methylation chip did not cover the whole genome of the PGCs. These results suggest that DNA methylation is evenly distributed among male and female PGCs, but that the methylated loci differ

(Figure 4).

Here, we identified differentially expressed genes in chicken PGCs compared to CEFs and analyzed the methylation patterns of the 5 kb-upstream regions of the differentially PGC-expressed genes, X-linked and imprinting homologous loci among male PGCs, female PGCs, and CEFs. We determined that epigenetic variation mainly occurs in imprinting homologous and X-linked homologous loci in PGCs and CEFs. Differential methylation was detected in the differentially expressed genes between PGCs and CEFs. Thus, we propose that epigenetic characters of imprinting and X-linked homologous genes are evolutionally conserved in birds, although the epigenetic mechanisms in birds are different from those in mice after DNA methylation. Additionally, DNA methylation during early embryonic development mainly affected X-linked and imprinting-related loci, whereas normal genes are affected by DNA methylation in birds. These results provide information for the epigenetic characterization and regulation of chicken PGCs.

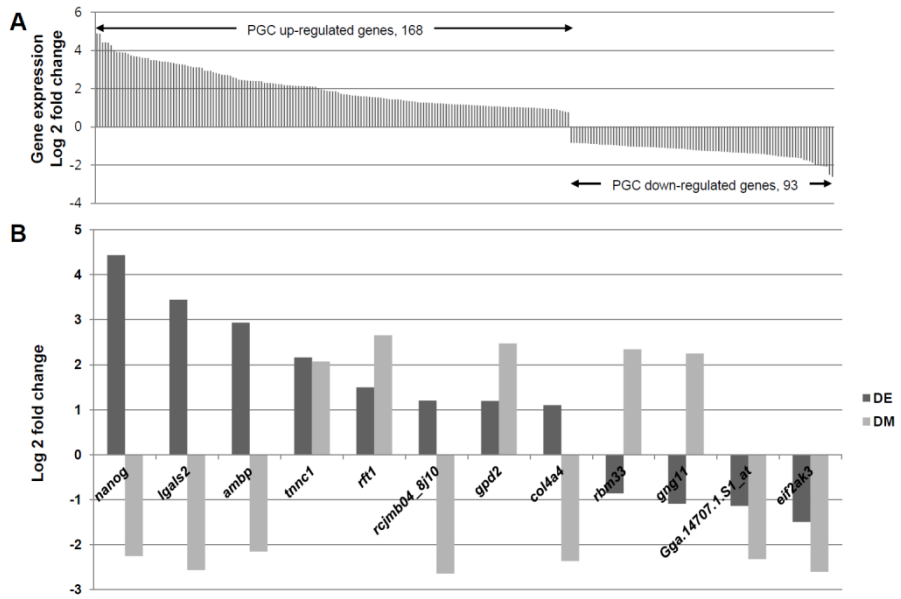

Figure 1. Differentially expressed PGC genes. (A) Profiles of PGC-upregulated and downregulated genes. The listed genes exhibited a minimum of 1.5-fold differential expression at a significance level of ≤ 0.05. (B) Comparison between gene expression and DNA methylation. DE, differentially expressed; DM, differentially methylated.

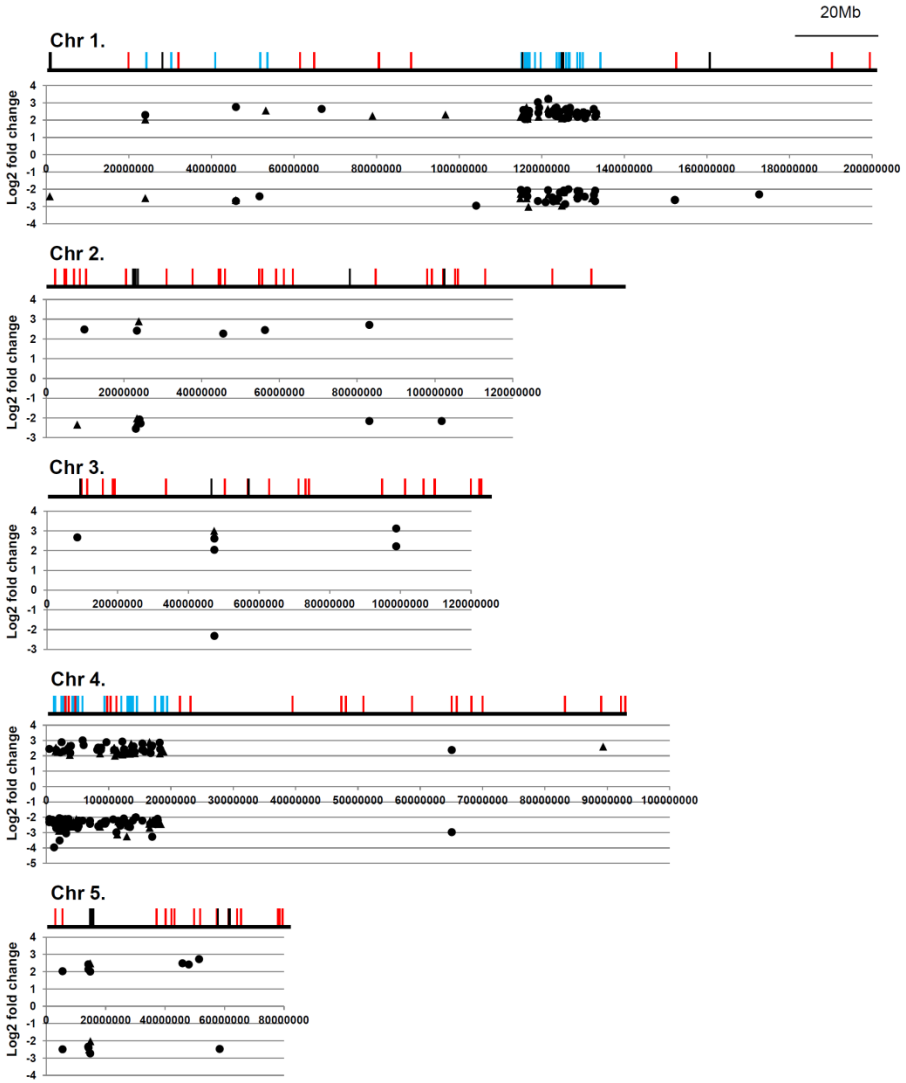

Figure 2. Methylated positions and values of 1 to 5 chromosomes among male PGCs, female PGCs, and CEF. Bar position (blue, X-linked; black, imprinting; red, differentially PGC-expressed) indicates the probe-targeted region; ●, differentially methylated ratio in CEFs as compared with PGCs; ▲, differentially methylated ratio in male PGCs as compared with female PGCs.

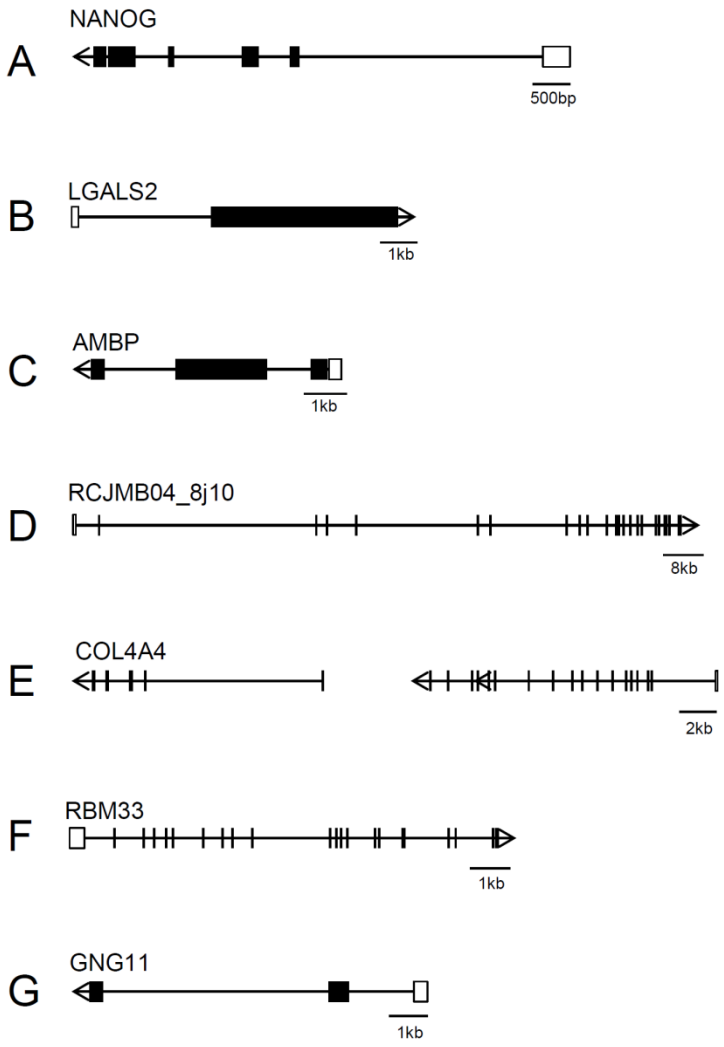

Figure 3. Gene structure and DNA methylation status of genes controlled in a DNA methylation-dependent manner. Gene structure and DNA methylation status of *nanog* (A), *lgals2* (B), *ambp* (C), *rcjmb04_8j10* (D), *col4a4* (E), *rbm33* (F), and *gng11* (G). Arrows indicate transcriptional direction; ■, coding region; □, methylated region.

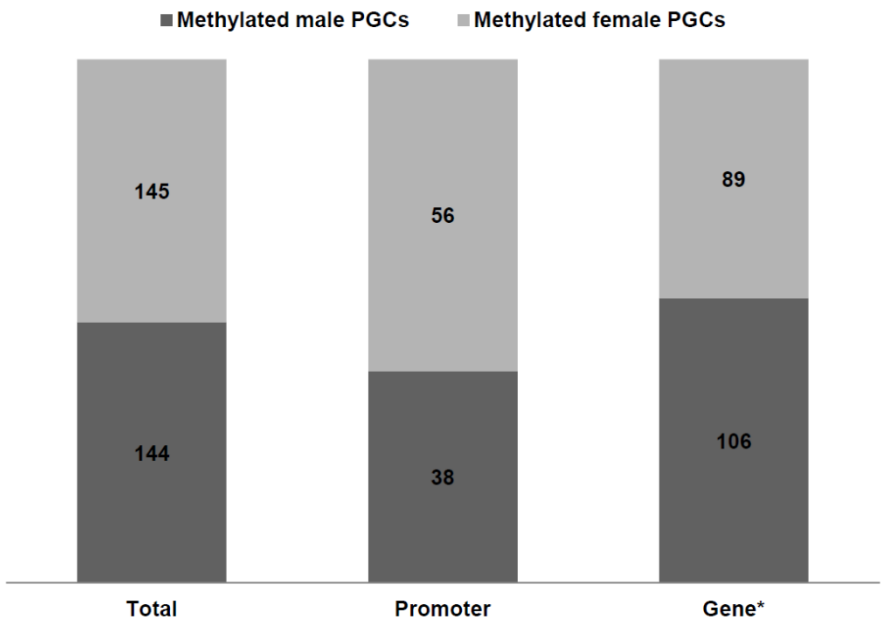

Figure 4. Comparison of methylated regions between male and female PGCs. Gene* includes exons, introns, and intergenic sequences.

Table 1. Differentially methylated promoter regions of genes differentially expressed between PGCs and CEFs. DE, differentially expressed; DM, differentially methylated.

Probe Set ID	Gene Symbol	DE (Log2 fold change)	DM (Log2 fold change)	p-value	Gene Title
GgaAffx.24398.1.S1_at	NANOG	4.43	-2.25	0.007002	nanog homeobox
Gga.6146.2.S1_a_at	LGALS2	3.44	-2.56	0.003988	lectin, galactoside-binding, soluble, 2
Gga.11647.1.S1_at	AMBP	2.93	-2.15	<0.0001	alpha-1-microglobulin/bikunin precursor
Gga.3041.1.S1_at	TNNC1	2.16	2.07	0.002294	troponin C type 1 (slow)
GgaAffx.1286.1.S1_at	RFT1	1.50	2.65	0.001444	RFT1 homolog (*S. cerevisiae*)
GgaAffx.12040.1.S1_at	RCJMB04_8j10	1.20	-2.64	0.002142	transducin (beta)-like 1 X-linked receptor 1
Gga.11036.1.S1_s_at	GPD2	1.19	2.47	0.002492	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
GgaAffx.3084.1.S1_s_at	COL4A4	1.10	-2.36	0.007684	collagen, type IV, alpha 4
GgaAffx.20263.1.S1_s_at	RBM33	-0.86	2.34	0.005369	RNA binding motif protein 33
Gga.15320.1.S1_at	GNG11	-1.09	2.25	0.007447	guanine nucleotide binding protein (G protein), gamma 11
Gga.14707.1.S1_at		-1.14	-2.32	0.006875	
GgaAffx.10171.1.S1_at	EIF2AK3	-1.49	-2.6	0.00185	eukaryotic translation initiation factor 2-alpha kinase 3

Table 2. Chicken homologues of X-linked genes

Chicken chromosome	Gene* (chromosome position, Mb)
No homologue	ABCD1, ALAS2, AMELX, CACNA1F, EMD, FGD1, FLNA, FOXP3 ,G6PD, IKBKG, L1CAM, MECP2, MED12, OPN1MW, PORCN, POU3F4, SERPINA7, SMC1A, TAZ, UBA1
1	AVPR2 (30.28), BCOR (115.95), CDKL5 (123.97), DMD (118.07), GPR143 (128.97), KAL1 (129.62), MID1 (128.31), NDP (114.76), NR0B1 (119.4), NYX (115.58), OFD1 (126.36), OTC (116.46), PIGA (125.51), RPS6KA3 (123.2), RS1 (123.94), SHOX (133.84), TRAPPC2 (126.39)
4	ABCB7 (12.61), AR (0.42), ARX (16.9), ATP7A (13.01), ATRX (12.9), BTK (2.01), CD40LG (4.38), CHM (8.62), COL4A5 (14.01), DCX (13.33), EDA (0.59), F8 (2.13), F9 (5.03), FMR1 (18.82), FRMD7 (3.45), GJB1 (2.3), GLA (2), GPC3 (3.85), HPRT1 (4.03), IDS (18.23), IL2RG (2.37), MTM1 (17.88), NSDHL (11.37), OCRL (1.64), PRPS1 (1.95), SLC16A2 (12.41), TAF1 (2.2), TIMM8A (2.02)
8	HCCS (14.02)
19	OPN1LW (7.00)
Not determined	HSD17B10, PLP1

* X-linked genes were extracted from the official website of National Library of Medicine , USA (http://ghr.nlm.nih.gov/chromosome=X/show/Genes).

Table 3. Candidate imprinting genes

Referred chicken chromosome	Gene* (chromosome position, Mb)
No homologue	ARHI, NAP1L5, PEG10, PON1, PON3, ASCL2, CDKN1C, ZNF215, ZIM2, ZIM3
1	U2AF1-RS1 (115.16), SLC38A4 (28.02), DCN (40.83), HTR2A (160.49), MKRN32 (53.46), UBE3A (124.93), ATP10A (124.71), GABRB3 (124.45), GABRA5 (124.29), GABRG3 (123.96)
2	GRB10 (80.79), CALCR (22.36), SGCE (22.95), PPP1R9A (23.02), PON2 (23.15), ASB4 (23.18), DLX5 (23.74), IMPACT (102.57)
3	COMMD1 (8.31), PLAGL1 (51.22), IGF2R (41.59), SLC22A2 (41.57), SLC22A3 (41.53), WT1[1] (5, 43.05)
5	IGF2 (11.33), INS (11.3), PHEMX (11.21), CD81 (11.18), TSSC4 (11.17), TRPM5, (11.13), KCNQ1 (10.78), SLC22A18 (10.71), PHLDA2 (10.7), NAP1L4 (10.67), TNFRSF23 (10.61), OSBPL5 (10.55), DLK1 (45.75), DIO3 (46.1)
10	GATM (11.49), RASGRF1 (20.54)
14	CPA4[2], MEST[1] (1, 14.85), COPG2[1] (1, 14.61), RTL1[2],
18	Zim1[2]
20	SNRPN (10.26), L3MBTL[1] (1, 24.9kb), GNAS (10.43)
21	TP73 (849kb)
23	TCEB3C[2]
24	SDHD (5.81)

[1] chromosome position is different between the reference and NCBI database. (NCBI chromosome number, position [Mb])

[2] not found within the NCBI chicken genome database

* genes were referred from the previous study (Dunzinger *et al.* 2007)

CHAPTER 5.

Biallelic Expression of the L-Arginine:Glycine Amidinotransferase Gene with Different Methylation Status between Male and Female Primordial Germ Cells in Chickens

1. Introduction

The genomic imprinting phenomenon was first reported in insects and has subsequently been observed in a wide range of plants and animals (de la Casa-Esperon & Sapienza 2003). It has been described as a parent of origin effect, explaining the mechanism that regulates specific gene transcription from the allele of only one parent, which is the most restrictive definition of imprinting (Ferguson-Smith & Surani 2001; Baroux *et al.* 2002; Rand & Cedar 2003). Imprinting in mammals, plants, and many insects is necessary for important developmental processes. Although imprinting studies have focused mainly on mammals, particularly humans and mice, such parents of origin effects have also been observed on transcription in plants (Baroux *et al.* 2002) and *Drosophila* (Lloyd 2000). In mammals, imprinting was first reported in the mid-1980s. Embryological studies in mice demonstrated the functional nonequivalence of maternal and paternal genomes. Specifically, conceptuses derived from zygotes containing either two sets of the maternal chromosomes or two sets of the paternal chromosomes failed to develop beyond mid-gestation (Barton *et al.* 1984; McGrath & Solter 1984). These findings demonstrated that normal embryonic development requires both a maternal and a paternal genome and suggested the existence of genes whose expression depends on whether they are inherited from the mother or from the father. Germ line imprinting is regarded as a conflict between maternal and

paternal systems. Differentiation between maternal and paternal genes originates from the formation of epigenetic marks that are deposited on chromosomes in the germ line and the sex of the parent determines the presence of these marks. Thus, imprinting creates a functional difference between the maternally and paternally derived copies of the genome.

In chickens, the basic function of DNA methylation is similar to that in mammals. Methylation at a specific gene promoter causes gene silencing (Luo *et al.* 2011) and DNA methylation defends against infection of the viral genome into the host genome, and induces the transcriptional suppression of transgenes (Park *et al.* 2010; Jang *et al.* 2011). However, differences exist in the control mechanism during early embryonic development between chickens and mammals. The genes *mpr/igf2r*, *igf2*, *ascl2/mash2*, *ins2*, *dlk1*, and *ube3a*, imprinted in mammals, are expressed from both alleles in chickens (Yokomine *et al.* 2005; Colosi *et al.* 2006; Shin *et al.* 2010). Thus, gene regulation by imprinting is not considered to be conserved in avian species.

Our laboratory used methyl-DNA immunoprecipitation array data to identify differentially methylated regions in the genome of chicken germ cells. The *L-arginine:glycine amidinotransferase* (*GATM*) gene was differentially methylated in the eighth intron, leading us to the hypothesis that differential expression might occur within the *GATM* genes between male and female germ cells during embryonic development. Therefore, results of this study

provide novel insights into the *GATM* gene with respect to its biallelic expression in primordial germ cells (PGCs) as well as somatic embryonic, extraembryonic, and adult chicken tissues. Moreover, our current data contribute to a more comprehensive understanding and ongoing comparative studies of epigenetic reprogramming across birds and mammals.

2. Materials and methods

Animal care and general experimental procedures

The care and experimental use of White Leghorn (WL) and Korean Oge (KO) chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). WL and KO chickens were maintained within a standard management program at the University Animal Farm, Seoul National University, Korea. Procedures involved with animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory. Developing embryos under a relative humidity of 60–70% at 37.5°C were staged according to the Hamburger and Hamilton (HH) classification.

Sex determination, PGC isolation, and embryonic tissue sampling

The sex of each donor embryo was determined before PGC transfer via PCR using a non-repetitive DNA sequence on the W chromosome. Embryonic blood (1 μl) was collected from the dorsal aorta of 3-day-old embryos through the egg shell window. Blood sample was diluted 100-folds in 1× PBS and then boiled for 5 min at 99 ℃ prior to the PCR. Sex of each embryo was determined using the chicken W chromosome specific primer pair (USP1 5′-CTA TGC CTA CCA CMT TCC TAT TTG C-3′ and USP3 3′-AGC TGG AYT TCA GWS CAT CTT CT-5′) and Z chromosome specific primer pair (CPE15 5'-AAG CAT AGA AAC AAT GTG GGA C-3' and CPE15 3'-AAC TCT GTC TGG AAG GAC TT-5') (Itoh *et al.* 2001). After blood collection, the window of the egg was sealed with parafilm. The egg was subsequently incubated at 37.5 ℃ in an air atmosphere with 60 – 70 % humidity. We then obtained gonads from the sex-determined chicken embryos at day 6 and day 8 using our standard procedure. To prepare PGCs, the total gonadal cells were retrieved from the gonads of WL and KO embryos (Park *et al.* 2003). Embryos were freed from the yolk by rinsing with calcium- and magnesium-free phosphate-buffered saline (PBS) and the gonads were

retrieved by dissection of the embryo abdomen with sharp tweezers under a stereomicroscope. Gonadal tissues were dissociated by gentle pipetting in 0.05% (v:v) trypsin solution supplemented with 0.53 mM EDTA. After centrifugation at 200 × *g* for 5 min, total gonadal cells were loaded into MACS (Miltenyi Biotech, Bergisch Gladbach, Germany), and the separated PGCs and remaining gonadal stromal cells (GSCs) were stored immediately in liquid nitrogen (–190ºC) until further processing. Embryos were freed from the yolk by rinsing with calcium- and magnesium-free PBS, and brain, heart, liver, kidney, gonad, and fibroblasts from 8-day-old embryos were sampled with sharp tweezers under a stereomicroscope.

Methyl-DNA immunoprecipitation (MeDIP) array

MeDIP was performed according to the NimbleGen Custom DNA Methylation Arrays of Methylated DNA Immunoprecipitation protocol (Roche, Indianapolis, IN) with modifications. Each 4 μg/200 μl of genomic DNA from PGCs and CEFs was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions and the genomic DNA was sheared into 100–500-bp (mean ~300 bp) fragments by sonication. Methyl-CpG-binding domain protein affinity capture

was performed using MethylMine Methylated DNA Enrichment kits (Invitrogen) according to the manufacturer's protocol. For salt-gradient elution of the fragmented genomic DNA, successive fractions were obtained by elution using buffer containing the following NaCl concentrations: 200 mM, 350 mM, 450 mM, 600 mM, and 2 M (Jang *et al.* 2012).

Bisulfite sequencing

DNA samples were prepared using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and converted using MethylEasy Xceed (Zymo Research, Irvine, CA) according to the manufacturer's instructions. For amplifying the converted DNA, PCR was performed with forward (5'-AAT TGT TGT AGG TTA AGT TTA TGG TGA T-3') and reverse (5'-ATT CAC CCC TAT AAA CTC TCT CAA AT-3') primers, which included the eighth intron of the *GATM* gene, as follows: 95°C for 1 min, 35 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The PCR products were cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) (Jang *et al.* 2011).

Reverse transcription PCR and quantitative real-time PCR

RNA extraction, cDNA synthesis, reverse transcription PCR (RT-PCR), and quantitative real-time PCR (real-time qPCR) were performed according to our previous reports (Seo *et al.* 2011). To estimate the gene expression level, total RNA samples were extracted from 6-day and 8-day embryonic tissues, 6-, 8- and 20-day extraembryonic tissues including the yolk sac, and 42-week chicken tissues. Next, 1 μg of total RNA from the samples was used to create single-stranded cDNA using the Superscript III First-Strand Synthesis System (Invitrogen). Sequence-specific primers based on chicken *GATM* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Table 1) were designed using the Primer3 program (http://frodo.wi.mit.edu/). Real-time PCR was performed using the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and EvaGreen (Biotium, Hayward, CA, USA). Non-template wells without cDNA were included as negative controls. Each sample was tested in triplicate. The PCR conditions were 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, using a melting curve program (increasing temperature from 55°C to 95°C at a rate of 0.5°C per 10 s) and continuous fluorescence measurement. The results are reported as the relative expression after normalization of the transcript to *GAPDH* (endogenous control) and the nonspecific control as a

calibrator using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Analysis of chicken GATM genotype by indel region

To perform genotype selection, gatm5_1D (indel-GTTTAATGC) and gatm5_2D (indel-CAAAAA) primer pairs were designed for the high-resolution melt assay (HRM) and gel shift assay (Table 1). Genotypes of individual WL and KO were confirmed by HRM and gel shift assay. For the gel shift assay, genomic indels of GTTTAATGC and CAAAAA were amplified by PCR. The PCR conditions were 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 58°C for 15 s, and 72°C for 30 s. The PCR products were separated in a 5.0% agarose gel stained with 0.5 μg/ml ethidium bromide. Genomic DNA was also amplified using SsoFast EvaGreen Supermix (Bio-Rad) and gatm5_1D primer. The amplicon covering indel GTTTAATGC was amplified using the CFX96 real-time PCR detection system, and results were analyzed using CFX Manager and Precision Melt Analysis software (Bio-Rad). A chi-square test on the allele frequencies was performed using SAS version 9.2 (SAS Institute, Cary, NC).

Analysis of allelic expression by indel regions

To analyze allelic expression of the *GATM* gene, a GTTTAATGC and CAAAAA homozygous insertion line and GTTTAATGC and CAAAAA homozygous deletion line were test-crossed. cDNAs of the heterozygous embryonic tissues at 8-days, extraembryonic tissues at 6-, 8- and 20-day, and 42-week chicken tissues were amplified by PCR using gatm5_1D and gatm5_2D primer pairs. The PCR conditions were 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 58°C for 15 s, and 72°C for 30 s. The PCR products were separated in a 5.0% agarose gel and stained with 0.5 μg/ml ethidium bromide.

3. Results

Methyl-DNA immunoprecipitation (MeDIP) array and methylation status of the GATM gene

In a previous study, we compared the methylation pattern between

male and female PGCs using the methyl-DNA immunoprecipitation (MeDIP) array, and we found about 80 genes that differentially methylated between male PGCs and female PGCs (Jang *et al.* 2012). *GATM* was one of the identified genes which shows the highest value of different methylation. In this study, DNA methylation of the eighth intron region within the *GATM* gene was 2.22-fold higher in male PGCs than in female PGCs ($P < 0.05$) (Figure 1A). To confirm the methylation difference between male and female PGCs, we performed bisulfite-treated sequencing for the differentially methylated region (DMR). At 6 days, the methylation of *GATM* in male PGCs was higher than that in female PGCs (57.5% vs. 35.0%), whereas methylation of female fibroblasts was a little higher than that of male fibroblasts (32.5% vs. 25.0%). At 8 days, the methylation of *GATM* DMR in male PGCs was 3.7-fold higher than in female PGCs (65.0% vs. 17.5%), whereas almost no difference was observed between male and female fibroblasts (55.0% vs. 50.0%) (Figure 1B).

Conservation of GATM in vertebrates

The nucleotide and amino acid sequences of *GATM* are conserved among vertebrates (humans, mice, cattle, pigs, zebrafish, and chickens)

(Figure 2A and Table 2). The genomic structure of the *GATM* gene, which has nine exons and eight introns, is also similar among pigs, mice, humans, and chickens (Figure 2B).

GATM expression pattern during chick embryo development

To analyze *GATM* expression in 6-day and 8-day embryos, we performed real-time qPCR from brain, heart, liver, kidney, fibroblasts, GSCs, and PGCs from 6-day and 8-day-old embryos. *GATM* was specifically expressed in liver, kidney and PGCs in both 6-day-old and 8-day-old embryos, while *GATM* were rarely expressed in other tissues (Figure 3). Especially after embryonic gonad was separated into PGCs and GSCs, PGCs were only expressing *GATM* when compared to GSCs (Figure 3). When *GATM* expression was compare between 6-day-old and 8-day-old embryos, the expression in 8-day female PGCs was 5.49-fold lower than in 6 day female PGCs, and the expression in 8-day male PGCs was 1.44-fold lower than in 6 day male PGCs. ($p < 0.05$). The expression of *GATM* in 8-day male and female kidneys was also decreased than in 6-day male and female kidneys. However, no significantly different expression was observed in the other tested samples between male and female embryos.

Indel analysis of the chicken GATM gene for examining allele expression patterns

To reveal the allelic expression pattern of *GATM*, we analyzed single nucleotide polymorphisms (SNPs) within the chicken *GATM* from 29 WL and 26 KO chickens. We found in total 7 SNPs within *GATM* transcripts: five of them are non-referred (T12594291C, G12596992A, T12600530C, C12600843T and T12601107G) and two of them are referred (C12600843T and A12600641G). We also found two indel sequences (GTTTAATGC and CAAAAA) within *GATM* transcripts. The two referred SNPs (C12600843T and A12600641G) and two indels (GTTTAATGC and CAAAAA) were in total linkage disequilibrium in the 55 animals we observed. SNP C12600843T and insertion GTTTAATGC were in phase in one haplotype and SNP T12601107G and insertion CAAAAA were in phase in the other (Figure 4A). When individual WL and KO chickens were genotyped across GTTTAATGC of the indel sequences, only the 100bp amplicon was produced from homozygous insertion animals, and only the 91bp amplicon from homozygous deletion animals. Heterozygous animals produced both the 91bp and 100bp amplicons from 3' UTR region of *GATM* by genomic PCR (Figure 4B). The

three genotypes were also categorized in both breeds using HRM assay (Figure 4C). The insertion alleles were predominant in WL chickens, whereas deletion alleles were predominant among KO chickens ($p < 0.01$) (Table 3).

Subsequently, we analyzed the allelic expression of *GATM* using two indel sequences (GTTTAATGC and CAAAAA) within *GATM* transcripts. The *GATM* transcripts from PGCs of heterozygous male and female embryos indicated biallelic expression (Figure 5A). We also investigated the allelic expression of *GATM* using GTTTAATGC in the brain, heart, liver, kidney, and fibroblasts of 8-day embryos (Figure 5B), 6-, 8- and 20-day of extraembryonic tissues including the yolk sac, which functions as mammalian placenta (Figure 5C), and adult chickens (Figure 5D). The results showed biallelic expression in all tissues.

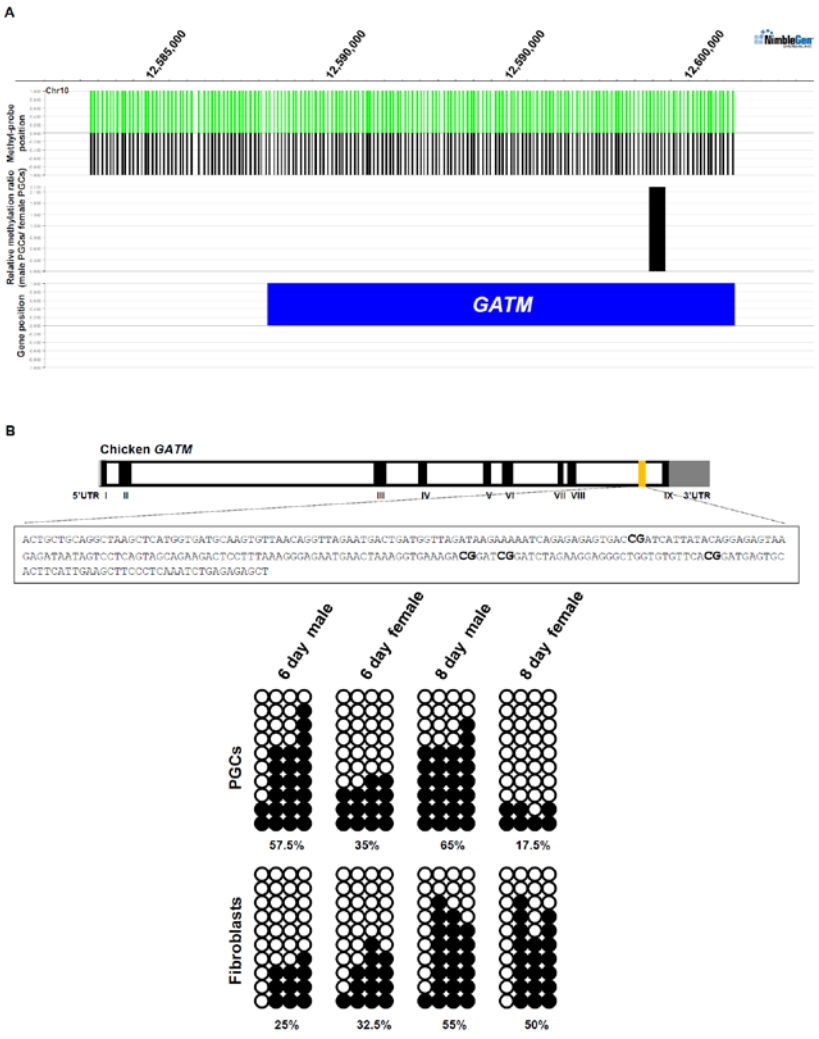

Figure 1. (A) Methylation status of L-arginine:glycine amidinotransferase (*GATM*) and the differentially methylated region (DMR) of *GATM* between male and female PGCs from the MeDIP array. (B) Methylation status of *GATM* in primordial germ cells (PGCs) and fibroblasts by bisulfite-treated sequencing.

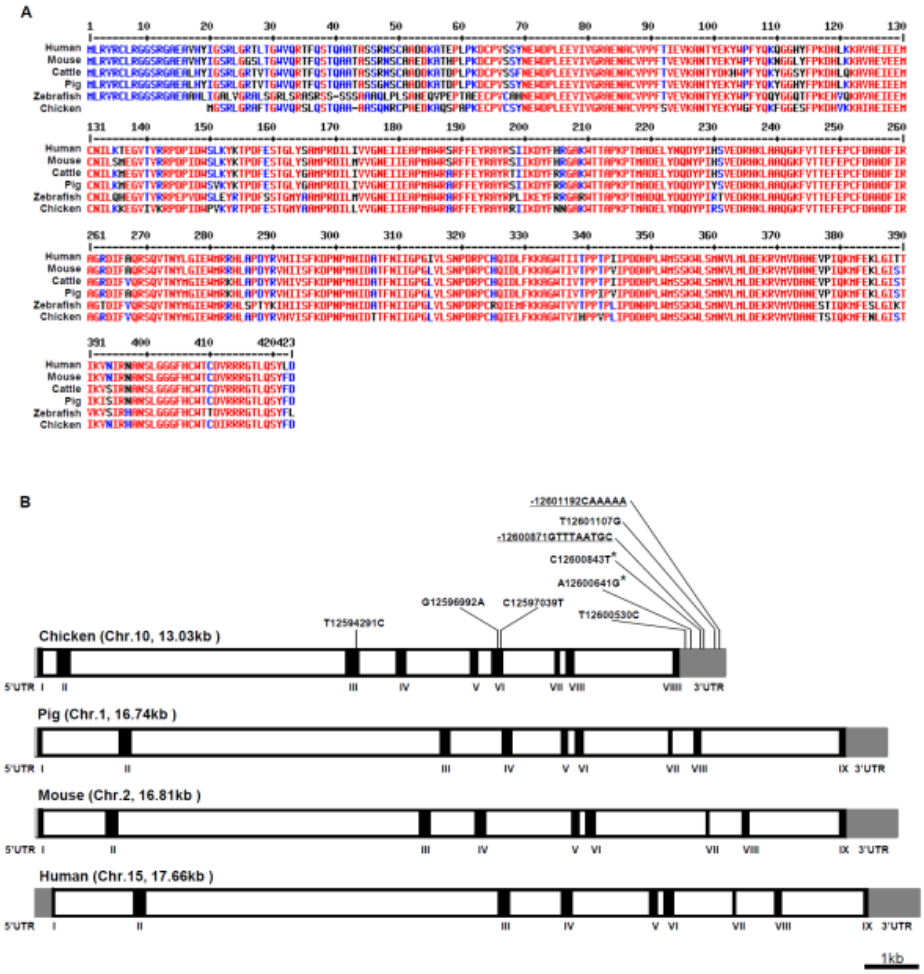

Figure 2. (A) Alignment of L-arginine:glycine amidinotransferase (*GATM*) between various species. Amino acid sequences alignment among humans, mice, cattle, pigs, zebrafish, and chickens. (B) Comparison of genomic structures among pigs, mice, humans, and chickens. *; referred SNPs in NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp/).

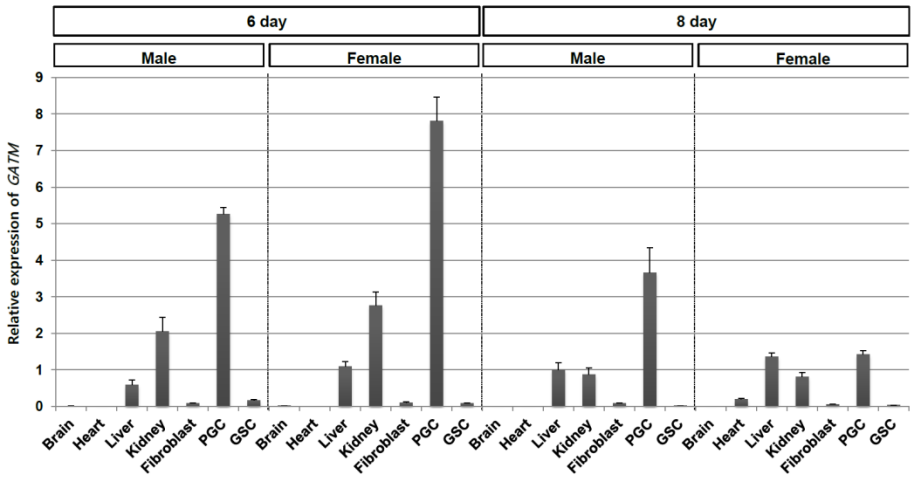

Figure 3. L-arginine:glycine amidinotransferase (*GATM*) expression in 6-day-old and 8-day-old embryos. Primordial germ cells (PGCs) and gonadal stroma cells (GSCs) were isolated from 6-day-old and 8-day-old embryonic gonads by MACS using an anti-SSEA1 antibody. Expression levels of *GATM* in PGCs, GSCs, and the embryonic tissues were determined by quantitative PCR. Relative quantification of *GATM* was normalized with *GAPDH*.

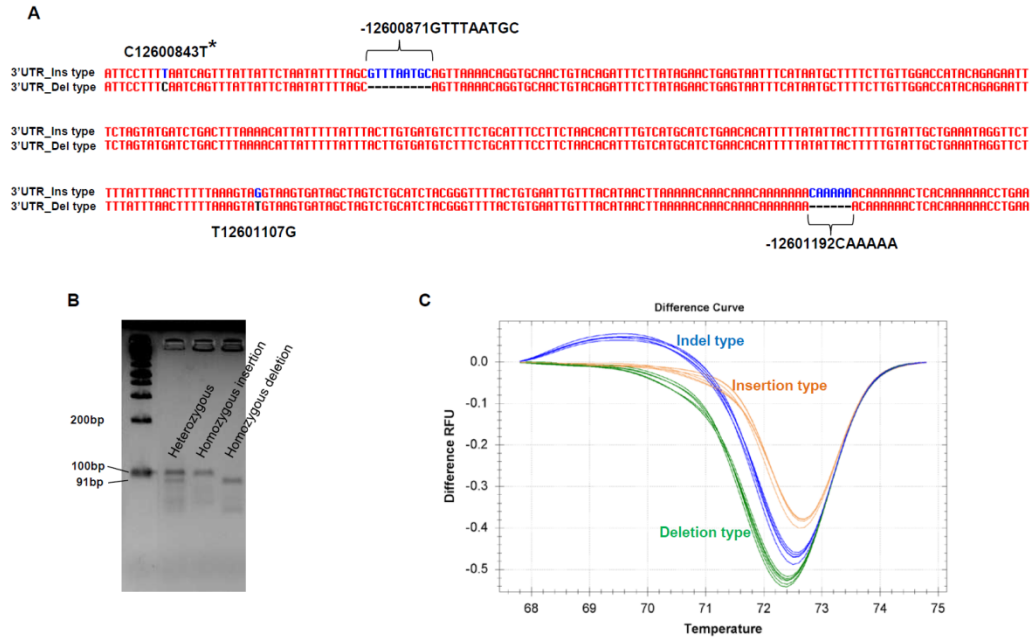

Figure 4. Insertion-deletion (indel) polymorphism of GTTTAATGC. (A) Comparison of the indel sequence within the 3'-untranslated region (UTR) of L-arginine:glycine amidinotransferase (*GATM*). Gel shift assay of insertion-deletion genomic polymorphism (B) and its HRM analysis (C).

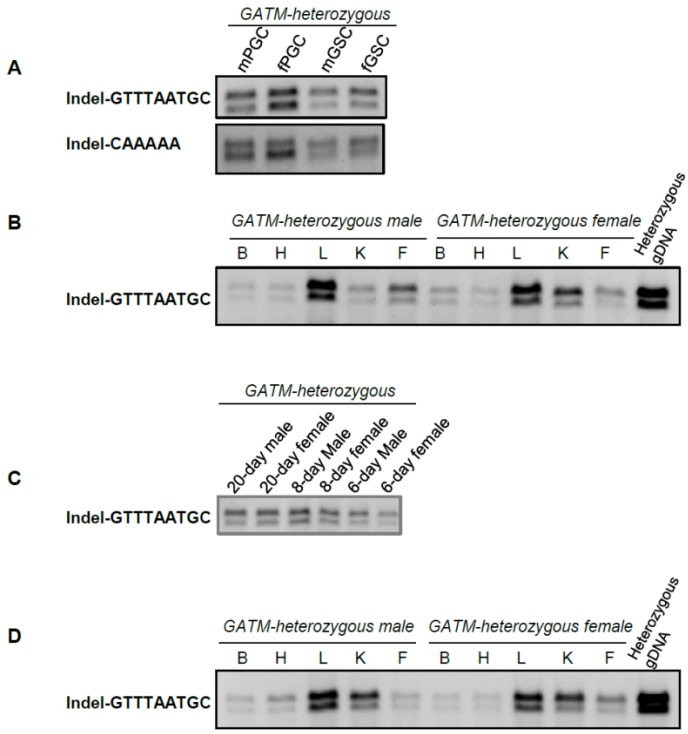

Figure 5. Allelic expression of the chicken L-arginine:glycine amidinotransferase (*GATM*) gene. Allelic expression pattern of *GATM* in primordial germ cells (PGCs) and gonadal stroma cells (GSCs) of 8-day-old chickens in PGCs and GSCs (A) and embryonic somatic tissues (B), 6-day-old, 8-day-old and 20-day-old extraembryonic yolk sac (C), and 42-week chicken tissues (D) using a gel shift assay. B, brain; H, heart; L, liver; K, kidney; F, fibroblast; mPGCs, male PGCs; fPGCs, female PGCs; mGSCs, male GSCs; fGSCs, female GSCs.

Table 1. Primers used for the identification of SNPs, indel sequences, and genotyping of the chicken L-arginine:glycine amidinotransferase (*GATM*) gene

Primer	Sequence (5'-3')	Size (bp)	Annealing Temperature (°C)
gatm1	F: 5’-ggcgaatttcatgggctc	514	58
	R: 5’-gccataggcgcttcaatgat		58
gatm2	F: 5’-cccctggaagaggtcattgt	511	58
	R: 5’-attttccctgagcagccagt		58
gatm3	F: 5’-ccatgccaagagacatcctg	618	58
	R: 5’-ccatcacacgcttctcatcc		58
gatm4	F: 5’-ggacagtgattcacccacca	532	58
	R: 5’-gccaatgtgaaaaggcttca		58
gatm5	F: 5’-tgaagccttttcacattggc	598	58
	R: 5’-ggaggtttcagctgggagat		58
gatm5_1D	F: 5’-gcagcttgaaatgttaaacttgaatta	100/91 (Ins/Del)	58
	R: 5’-gttgcacctgttttaact		58
gatm5_2D	F: 5’-tctgcatctacgggttttactgtga	97/91 (Ins/Del)	58
	R: 5’-ctttcaggtttttgtgagtttttt		58

Table 2. Comparisons of amino acid sequences and nucleotide sequences among the L-arginine:glycine amidinotransferases (GATMs) of various species

Gene		Identity (%)	
Species	Symbol	Protein	DNA
Chicken	*GATM*		
vs. Human	*GATM*	86.4	79.8
vs. Cattle	*GATM*	86.4	80
vs. Mouse	*Gatm*	86.6	79
vs. Zebrafish	*gatm*	79.9	70.6
vs. Pig	*GATM*	87	No similarity

Table 3. Genotype and frequencies of the L-arginine:glycine amidinotransferase (*GATM*) gene in White Leghorn (WL) and Korea Oge (KO) chickens

Breed	No. of birds used	Genotype			Allele frequency	
		Homozygous Insertion	Heterozygous Indel	Homozygous Deletion	Insertion	Deletion
KO	26	3	9	13	0.33	0.67
WL	29	15	10	4	0.69	0.31

4. Discussion

The imprinting phenomenon was originally discovered in insects (Crouse 1960) and later in plants (Kermicle 1970) and zebrafish, although an understanding of the mechanisms controlling imprinting in nonmammalian species has lagged behind studies in mammals. It remains unclear whether imprinting in mammals and other species is a conserved biological process with the same underlying molecular mechanisms. Since the mammalian imprinting phenomenon was first reported in mice, more than 100 imprinted mouse genes have been identified, and most of them are conserved in humans as well (Arnaud 2010). However, no evidence has demonstrated imprinting phenomena in chicken. Moreover, many studies have also shown that birds do not exhibit imprinting mechanisms and account for this with a variety of reasons (Bourc'his *et al.* 2001; Yokomine *et al.* 2005; Colosi *et al.* 2006; Itoh *et al.* 2007; Shin *et al.* 2010).

In this study, we compared the methylation status between male and female PGCs at day 6, which is an initiation stage of differentiating germ cells, by DNA methyl-binding chip analysis. Subsequently, the *GATM* gene was identified as a DMR between male and female PGCs. Additionally, bisulfite sequencing showed that the differentially methylated ratio became more

pronounced between male and female PGCs at 8 days compared those at 6 days, whereas the methylation ratios between male and female fibroblasts were not significantly different through 6-day and 8-day embryos. Such DMRs between males and females have been found frequently in intron regions in imprinted genes (Peters & Williamson 2007; Kaaks *et al.* 2009; van Dijk *et al.* 2010). Moreover, the DMR of *GATM* seemed to undergo sex-dependent epigenetic modification during germ cell specification. Thus, we hypothesized that the DMR of *GATM* could result in imprinting in chicken PGCs. However, when we analyzed the allelic expression pattern of *GATM* in chicken PGCs, we found that it was biallelically expressed.

GATM encodes an arginine:glycine amidinotransferase protein/enzyme (GATM, alternative name is AGAT). GATM controls the first step of creatine biosynthesis, whereby GATM transfers a guanidino group from arginine to glycine to produce L-ornithine and guanidinoacetic acid. This enzyme activity is found in most vertebrates including humans, mice, cattle, pigs, zebrafish, and chickens (Wyss & Kaddurah-Daouk 2000). In this study, *GATM* was highly expressed in PGCs, as well as in the liver and kidney of chickens (Figure 3A). In mammals, *GATM* is expressed in the brain, heart, liver, kidney, lung, and skeletal muscle (Cullen *et al.* 2006; McMinn *et al.* 2006; Monk *et al.* 2006). *GATM* is highly expressed in the liver, kidney, and muscle, which are involved in creatine biosynthesis (Wyss & Kaddurah-

Daouk 2000; Nasrallah *et al.* 2010). However, the role of *GATM* in early germ cells is unclear although a function of creatine has been reported in testis including Sertoli cells and male germ cells (Moore *et al.* 1992; Moore *et al.* 1998; Rolf *et al.* 1998; Sidhu *et al.* 1998; Timbrell 2000).

The imprinting of *GATM* has been studied in mammals and was reported to be expressed exclusively from the maternal allele in extraembryonic tissues of mice (Sandell *et al.* 2003). Human *GATM* is biallelically expressed in first-trimester fetal and placenta tissues, and immune-selected trophoblast cells (Monk *et al.* 2006). Biallelic expression of the porcine *GATM* gene was also found in placentas on days 75 and 90 of gestation (Zhou *et al.* 2007). Thus, imprinting of *GATM* remains controversial.

We demonstrated conclusively that the *GATM* gene displays biallelic expression in chicken PGCs, although males and females show different methylation status in early embryos. Thus, the differential methylation of *GATM* is not correlated with its allelic expression pattern in chickens. These findings can contribute to studies of epigenetic reprogramming during early embryonic development and evolutionary studies on imprinting phenomena across birds and mammals.

CHAPTER 6.

Structure, expression and antibacterial analysis of chicken cathelicidin

1. Introduction

Antimicrobial peptides (AMPs) serve as constitutive or inducible defense barriers against microbial infections in plants, insects, amphibians, and mammals, including humans (Brogden 2005; Harris *et al.* 2009; Rahnamaeian *et al.* 2009). AMPs play important roles in killing microbes, boosting specific innate immune responses, and exerting selective immunomodulatory effects on the host (Hancock & Sahl 2006; Yeaman & Yount 2007; Holzl *et al.* 2008; Muller *et al.* 2008; Lai & Gallo 2009). Several classification schemes have been proposed for AMPs; however, most AMPs are generally categorized into four clusters based on their secondary structures: peptides with a linear α-helical structure (Boman 1995; Mangoni *et al.* 2000; He & Lemasters 2002), cyclic peptides with a β-sheet structure (Epand & Vogel 1999; Matsuzaki 1999; Bu *et al.* 2002; Ovchinnikova *et al.* 2004; Ostberg & Kaznessis 2005), peptides with a β-hairpin structure (Imamura *et al.* 2010), and peptides with a linear structure (Wu & Hancock 1999; Rahnamaeian *et al.* 2009).

AMPs demonstrate functional activities through various processes. One representative function of AMPs is an interaction with microbial membranes to form a pore; pore-forming mechanisms are mainly associated with the

effects of α-helical membrane peptides (Brogden 2005). The pores act as non-selective channels for ions, toxins, and metabolites, preventing the microbe from maintaining homeostasis (Boland & Separovic 2006). Another notable function of AMPs is the inhibition of DNA and protein functions. After entering host cells, certain AMPs attack ribosomes to inhibit protein synthesis or DnaK protein (a heat shock 70 kDa protein) to inhibit protein folding (Otvos *et al.* 2000; Kragol *et al.* 2001). In addition, AMPs have been shown to possess several immunomodulatory functions such as modification of host gene expression (Bowdish *et al.* 2004; Yu *et al.* 2007). Thus, AMPs may act directly by causing physical damage to microbes or indirectly by inhibiting physiological functions of microbes, or may function to recruit host immune systems.

Cathelicidins (CATHs) form a family of vertebrate-specific immune molecules present in fish, chickens, and mammals. CATHs exhibit unique bipartite features: a substantially heterogeneous C-terminal antimicrobial domain of 12–100 residues and an evolutionarily conserved N-terminal cathelin-like domain of 99–114 residues (Zanetti 2005; Chang *et al.* 2006). The sequence heterogeneity of AMPs is reflected in their structural diversity, which includes all three major folding types: cysteine-free linear peptides with an α-helical and amphipathic structure; cysteine-containing peptides with a flat β-sheet structure; and peptides rich in certain amino acids such as proline,

arginine, and tryptophan (Zaiou & Gallo 2002). The chicken genome encodes cathelicidin1 (*CATH1*), cathelicidin2 (*CATH2*), cathelicidin3 (*CATH3*), and cathelicidinB1 (*CATHB1*), which are clustered within a 7.5-kb region at the proximal end of chromosome 2 (Goitsuka *et al.* 2007). These closely linked genes are aligned in the order *CATH1*, *CATHB1*, *CATH2*, and *CATH3*. *CATH3* is potentially transcribed in the inverted orientation compared with the orientation of the other members. All cathelicidin genes have four exons except *CATH2*, which has five exons (Xiao *et al.* 2006a; Goitsuka *et al.* 2007). Additionally, chicken cathelicidins have shown antimicrobial activities against a broad spectrum of Gram-positive and Gram-negative bacteria (Xiao *et al.* 2006a; Molhoek *et al.* 2011). In the present study, we compared the expression patterns and antimicrobial activities of four chicken cathelicidins. Our results reveal features of chicken cathelicidins that suggest they may be applicable as an alternative to antibiotics in the animal care industry.

2. Materials and methods

Animal care and general experimental procedures

The care and experimental use of White Leghorn (WL) chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). WL chickens were maintained within a standard management program at the University Animal Farm, Seoul National University, Korea. Procedures regarding animal management and reproduction adhered to the standard operating protocols of our laboratory.

Quantitative real-time polymerase chain reaction (PCR) analysis

RNA extraction, cDNA synthesis, and quantitative real-time PCR (real-time qPCR) were performed according to our previous reports (Lee *et al.* 2010). To estimate the gene expression levels, total RNA was extracted from several tissues (bone marrow, brain, cornea, esophagus, gall bladder, heart, isthmus, kidney, large intestine, liver, lung, magnum, rectum, respiratory tract, small intestine, shell gland, spleen, thymus, tongue, and vagina) of female WL chickens at 42 weeks of age. One microgram of total RNA from each sample was used to synthesize single-stranded cDNA using a Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Sequence-specific primers based on chicken *CATH1*, *CATH2*, *CATH3*, *CATHB1*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) sequences were

designed using the Primer-BLAST program of the National Center for Biotechnology Information (NCBI). Real-time qPCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and EvaGreen (Biotium, Hayward, CA, USA) reference dye. Non-template wells without cDNA were included as negative controls. Each sample was tested in triplicate. Real-time qPCR expression of *each chicken cathelicidin* mRNA was analyzed using the following specific primer pairs: forward primer 5'-TGG GCT CGT CAA GGA CTG CG-3' and reverse primer 5'-CTG ATG ACC AGC GGC CAG ACG-3' for *CATH1*; forward primer 5'-GAC AGA GTG CAC CCC GAG CG-3' and reverse primer 5'- GGC CCC GTT GGA CCA GAA CG-3' for *CATH2*; forward primer 5'-CGG GCT CGT CAA GGA CTG CG-3' and reverse primer 5'-CCC GCA GCC ACC GTG TTG AT-3' for *CATH3*; and forward primer 5'-GCC GAT CAC CTA CCT GGA TG-3' and reverse primer 5'- TGG TGA CGT TCA GAT GTC CG-3' for *CATHB1*. The PCR conditions were 95°C for 3 min, followed by 39 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s, using a melting curve program (increasing temperature from 55°C to 95°C at a rate of 0.5°C per 10 s) and continuous fluorescence measurement. The results are reported as the relative expression after normalization to *GAPDH* and the nonspecific control as a calibrator using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Synthesis of cathelicidin peptides

Variable domains (CATH1, CATH2, CATH3, and CATHB1) were synthesized based on the respective full-length cathelicidin sequences (Figure 1 and Table 1), and the peptides were purified to >95% through reversed-phase high-performance liquid chromatography (AbClon, Seoul, Korea). The lyophilized peptides were stored in a desiccant at −20°C; they were dissolved in dimethyl sulfoxide and diluted in phosphate buffer (pH 7.2) before use.

Preparation of liposomes

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). A small unilamellar vesicle (SUV) solution was prepared by dissolving the appropriate amounts of lipids in a chloroform and methanol (2:1) mixture. The lipid solution was evaporated in a 50-ml round-bottomed flask using a rotary evaporator at 25°C under vacuum for 10 min. The dried mixture was suspended in 10 mM sodium phosphate buffer (pH 7.2) and hydrated overnight at 4°C. The total lipid

concentration was 2 mg/ml. The solution was extruded through a polycarbonate membrane with a pore diameter of 100 nm at 37°C. The resulting SUVs were collected and stored at 4°C until use.

Circular dichroism spectra analysis of synthetic cathelicidin peptides

To analyze the secondary structures of synthetic cathelicidin peptides, circular dichroism (CD) spectra were measured in 10 mM sodium phosphate buffer (pH 7.4) at peptide concentrations of 0.3 mg/ml, with a JASCO J-715 ultraviolet (UV) spectropolarimeter (JASCO, Inc., Easton, MD, USA) at room temperature. The light path of the cell was 0.2 cm for wavelengths ranging from 190 to 260 nm. All data are expressed as molar ellipticity. The CD spectra were obtained by averaging three scans and are expressed as ellipticity (*h*) m, (deg·cm^2·mol) (Lee *et al.* 2012).

Cell viability analysis of E. coli after treatment with synthetic cathelicidin peptides

To test the cell viability of peptide-treated *E. coli*, 6.5×10^6 colony forming units (CFU) of *E. coli* were incubated with 0.5, 1.0, 2.5, 5.0, or 10 μM of synthetic cathelicidin peptides in 10 mM phosphate buffer (pH 7.0), respectively, at 37°C for 2 h, and 100 μl of each peptide-treated sample were equilibrated to room temperature in an opaque-walled 96-well plate. One hundred microliters of BacTiter-Glo reagent were added to each well, and the mixed contents were incubated at 25°C for 10 min in the dark. The ATP concentration of each sample was measured using a BacTiter-Glo Microbial Cell Viability Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol.

Detection of damaged E. coli membranes after treatment with synthetic cathelicidin peptides

To visualize *E. coli* membrane damage, 6.5×10^6 CFU of *E. coli* were incubated with 5 μM CATH1, CATH2, CATH3, or CATHB1 in 10 mM phosphate buffer (pH 7.0), respectively, at 37°C for 2 h, and the membranes were observed by confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany) after staining with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen) according to the manufacturer's protocol.

The damaged membranes were also observed under a field-emission scanning electron microscope (Carl Zeiss).

Establishment of an antibiotic-resistant E. coli strain and test of antimicrobial activity

An ampicillin-resistant *E. coli* DH5α strain was established by transformation with an ampicillin-resistance gene, and the strain was treated with ampicillin (100 μg/ml) and 5 μM CATH1, CATH2, CATH3, or CATHB1 in 10 mM phosphate buffer (pH 7.0) at 37°C for 2 h. Three microliters of each treated *E. coli* sample were spread on LB agar medium containing ampicillin (100 μg/ml) and incubated at 37°C for 16 h to examine colony formation. Another 3 μl of sample were added to 3 ml of LB broth medium containing ampicillin (100 μg/ml) and incubated at 37°C for 16 h with shaking at 250 rpm. The rate of cell proliferation was measured as the optical density at 600 nm.

3. Results

Sequence conservation and expression patterns of chicken cathelicidins

The amino acid sequences of the four chicken cathelicidins (CATH1, CATH2, CATH3, and CATHB1) were aligned to compare the conservation of their primary structures, including the signal peptide and cathelin-like functional domain. All sequences were highly conserved, although the sequence of CATHB1 was longer than those of the other three peptides (Figure 1). We analyzed the mRNA expression patterns of chicken *CATH1*, *CATH2*, *CATH3*, and *CATHB1* in the bone marrow, brain, cornea, esophagus, gall bladder, heart, kidney, large intestine, liver, lung, rectum, respiratory tract, small intestine, spleen, thymus, and bursa of juvenile female chickens at 1 month of age, and the in the bone marrow, brain, cornea, esophagus, gall bladder, heart, isthmus, kidney, large intestine, liver, lung, magnum, rectum, respiratory tract, small intestine, shell gland, spleen, thymus, tongue, and vagina of adult female chickens at 42 weeks of age (Figure 2). *CATH1*, *CATH2*, and *CATH3* showed higher expression in the bone marrow than in any other tissues of 1-month-old and 42-week-old chickens (Figure 2A–C, E–G). By contrast, *CATHB1* was highly expressed in the bursa of 1-month-old chickens and in the thymus of 42-week-old chickens compared with all other tissues examined (Figure 2D and H).

Effects of lipid binding on the secondary structure of functional cathelicidin domains

We synthesized the functional peptides (variable domains) of CATH1, CATH2, CATH3, and CATHB1 (Table 1 and Figure 1), and determined their secondary structures in lipid-free and lipid-bound states by the far-UV CD method. The CD spectra of CATH1 and CATH3 in aqueous buffer (pH 7.2) displayed a single negative minimum at ~200 nm, suggesting a linearized form (Figure 3A and C). The CD spectrum of CATH2 in aqueous buffer had a minimum at ~217 nm and a maximum at ~200 nm, characteristic of a β-sheet structure (Figure 3B). The CD spectrum of CATHB1 exhibited two minima at ~208 nm and ~222 nm, indicating a helical structure (Figure 3D). CATH1 and CATH3 underwent structural changes from a linearized form to a helical structure, whereas CATH2 and CATHB1 maintained their structures in aqueous buffer. Additionally, CATH1, CATH2, CATH3, and CATHB1 displayed apparent secondary structures with the addition of POPG liposomes compared with the addition of POPC liposomes. Thus, we conclude that membrane-like structures (liposomes) can change the secondary structure of the peptides, possibly via electrostatic interactions between the liposomes and

the peptides.

Antimicrobial activities of synthetic chicken cathelicidin peptides

The effects of the synthesized peptides on *E. coli* viability were measured using a BacTiter-Glo Microbial Cell Viability Assay. At 5 μM peptide, all of the cathelicidin peptides showed effective antimicrobial activity, reducing cell viability to <20%. At 0.5 μM peptide, CATH1, CATH2, and CATH3 reduced cell viability to <50%, compared with the antimicrobial activity of CATHB1 (Figure 4A). These results suggest that the functional peptides had effective antibacterial activities under a broad range of peptide concentrations.

Membrane damage by synthetic chicken cathelicidin peptides

The damage to *E. coli* cell membranes after treatment with cathelicidins was assessed using a LIVE/DEAD BacLight Bacterial Viability Kit. In the absence of peptide, most of the *E. coli* cells were stained green,

indicating an intact membrane. In the presence of 5 μM CATH1, CATH2, CATH3, or CATHB1, many *E. coli* cells were stained red, indicating membrane damage (Figure 4B). The membrane damage was greater with CATH2 and CATH3 than with CATH1 or CATHB1. By scanning electron microscopy, control *E. coli* cells showed normal shape and membrane surface (Figure 5A and B). However, after treatment with each cathelicidin, *E. coli* cell shrinkage and rupture were observed (Figure 5C–J). These findings suggest that the cathelicidin peptides have a high potential for destroying bacterial cells via membrane damage.

Effects of synthetic chicken cathelicidin peptides on antibiotic-resistant E. coli

We established an ampicillin-resistant *E. coli* strain and determined the effects of the cathelicidin peptides on colony formation and proliferation of the resistant strain. When ampicillin-resistant *E. coli* cells were treated with the cathelicidin peptides, CATH1, CATH2, and CATH3 effectively suppressed colony formation, but CATHB1 treatment showed no significant effect on colony formation of ampicillin-resistant *E. coli* compared with control treatment (Figure 5K). The inhibition of cell proliferation was highly

correlated with the effects on colony formation (Figure 5L). Thus, the antimicrobial effects of CATH1, CATH2, and CATH3 appear to occur by a mechanism different from that of ampicillin.

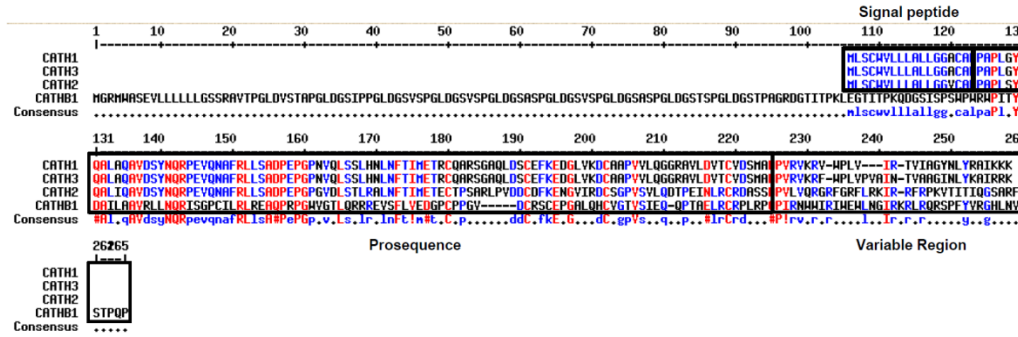

Figure 1. Amino acid sequence alignment of four chicken cathelicidin peptides: cathelicidin1 (CATH1), cathelicidin2 (CATH2), cathelicidin3 (CATH3), and cathelicidinB1 (CATHB1). Sequences that are identical or similar between peptides are indicated in different colors. Boxed areas are the conserved signal peptide pro-sequence and variable region in the cathelin-like functional domain of chicken cathelicidins.

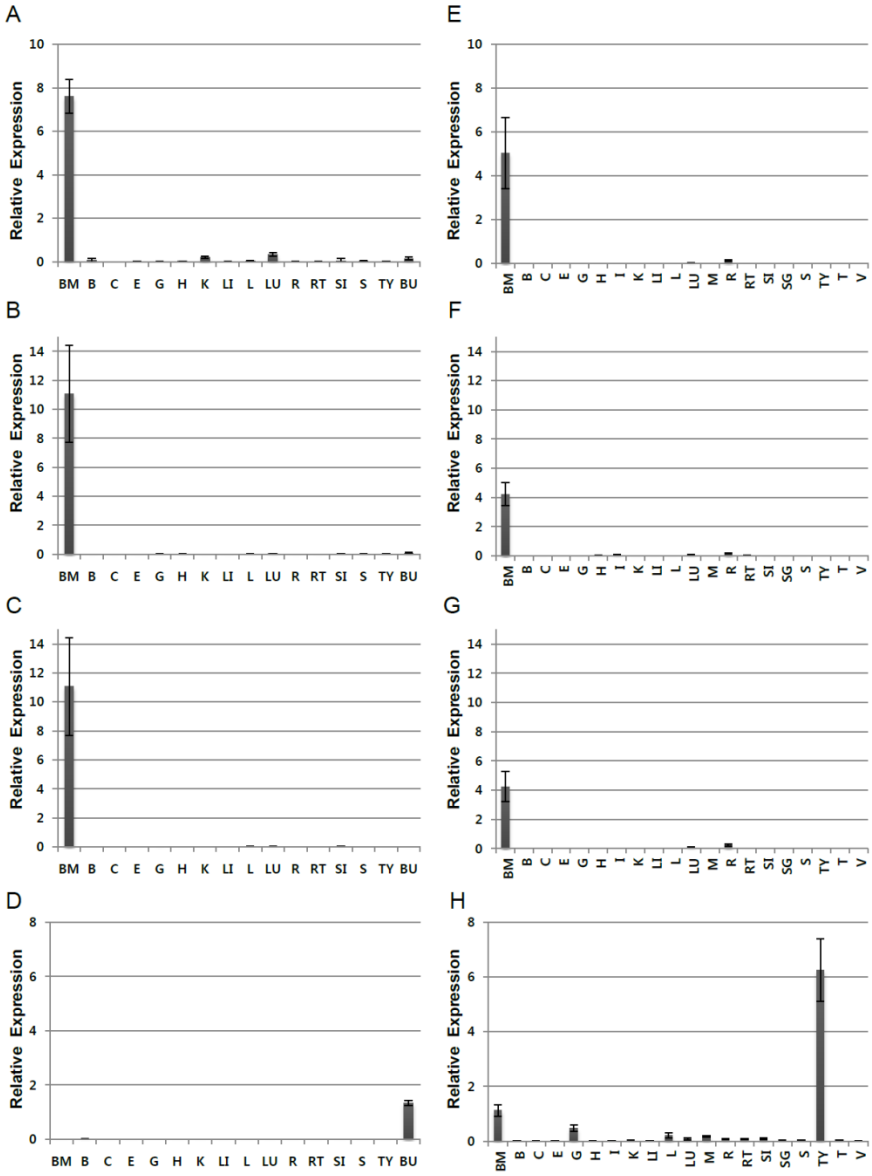

Figure 2. Expression of chicken cathelicidins in 1-month-old and 42-week-old hens. Expression patterns of cathelicidin1 (*CATH1*, A and E), cathelicidin2 (*CATH2*, B and F), cathelicidin3 (*CATH3*, C and G), and cathelicidinB1

(*CATHB1*, D and H) in tissues from 1-month-old and 42-week-old hens were analyzed by quantitative real-time polymerase chain reaction. BM, bone marrow; BU, bursa; B, brain; C, cornea; E, esophagus; G, gall bladder; H, heart; I, isthmus; K, kidney; LI, large intestine; L, liver; LU, lung; M, magnum; R, rectum; RT, respiratory tract; SI, small intestine; SG, shell gland; S, spleen; TY, thymus; T, tongue; and V, vagina.

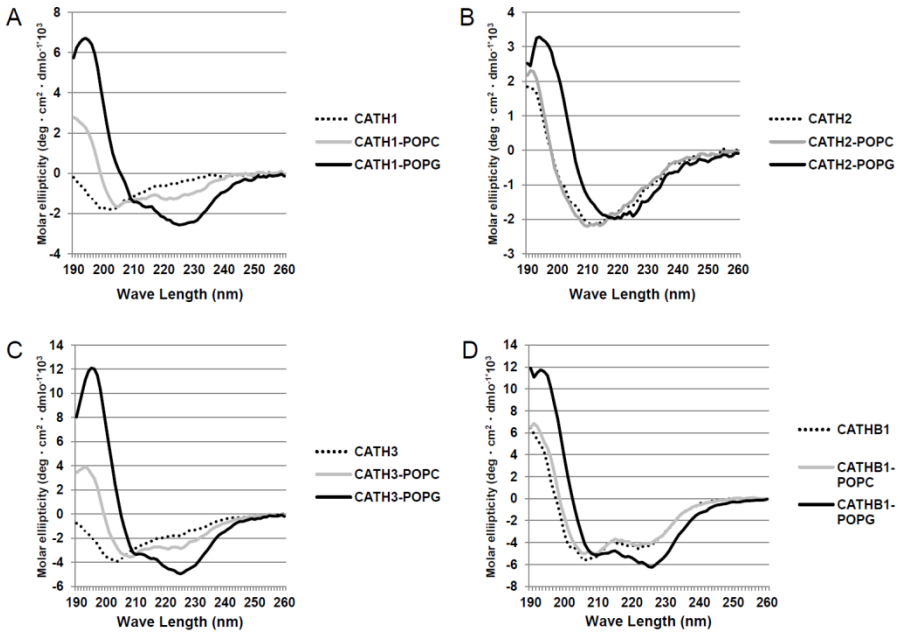

Figure 3. Secondary structures of the synthesized cathelicidin peptides in the absence and presence of liposomes, determined by using circular dichroism. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) liposomes were incubated with each cathelicidin peptide: cathelicidin1 (CATH1, A), cathelicidin2 (CATH2, B), cathelicidin3 (CATH3, C), or cathelicidinB1 (CATHB1, D).

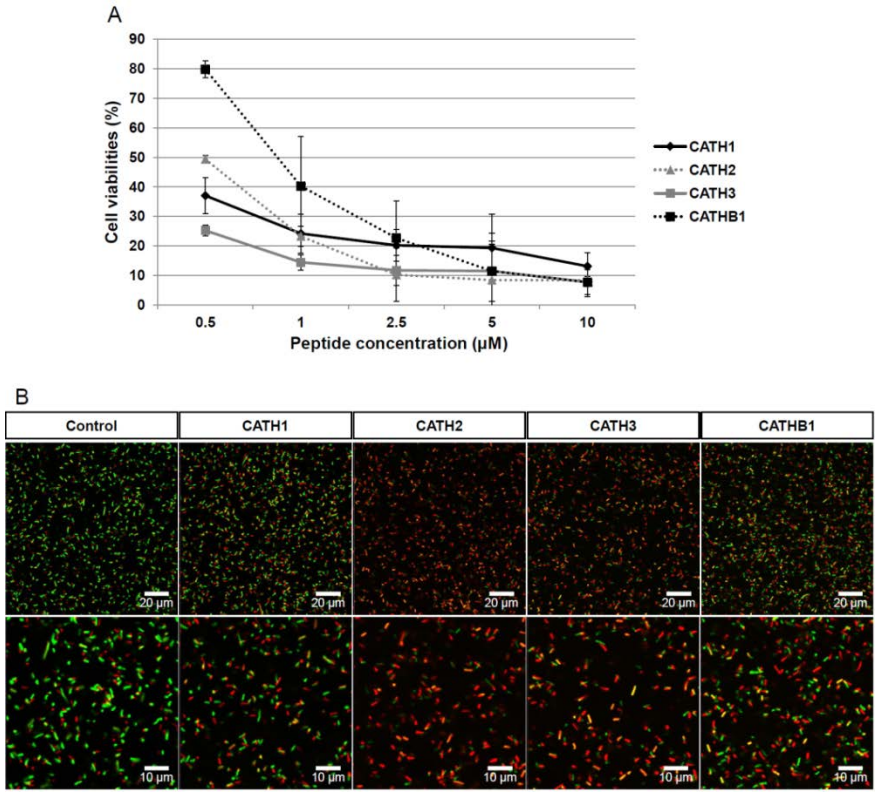

Figure 4. *Escherichia coli* viability and membrane damage following treatment with cathelicidins. The effects of different concentrations of cathelicidins on *E. coli* cell viability were analyzed by a BacTiter-Glo Microbial Cell Viability Assay (A). Membrane damage in *E. coli* treated with each synthetic cathelicidin peptide (5 μM) was detected with a LIVE/DEAD BacLight Bacterial Viability Kit. Green fluorescence indicates live bacteria with intact membranes; red fluorescence indicates dead bacteria with damaged membranes (B). CATH1, cathelicidin1; CATH2, cathelicidin2; CATH3, cathelicidin3; and CATHB1, cathelicidinB1.

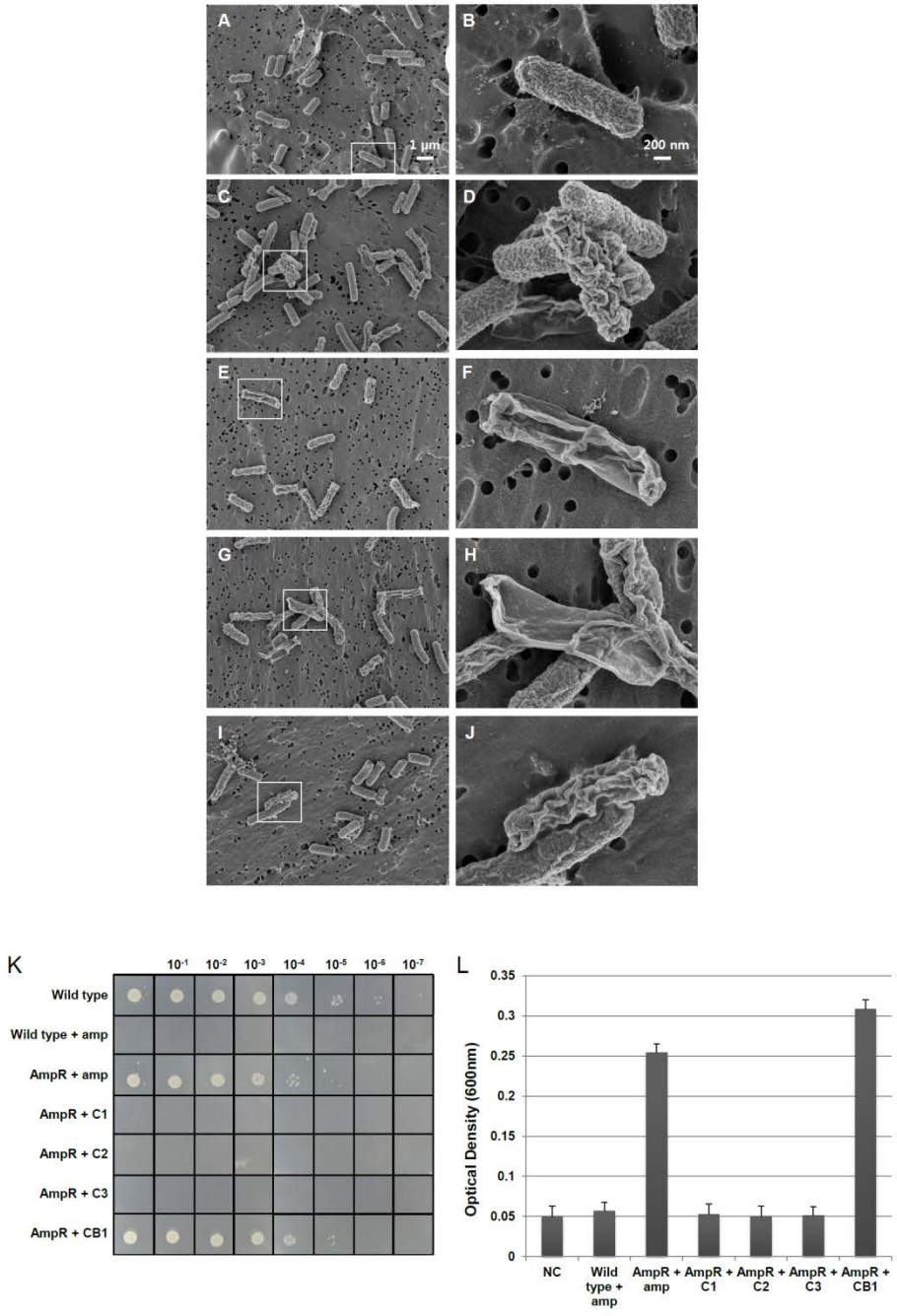

Figure 5. Scanning electron micrographs of cathelicidin-induced cell

membrane damage, and the antibacterial effects of cathelicidins on ampicillin-resistant *E. coli*. Scanning electron micrographs of *E. coli* with no treatment (control, A and B) or treated with cathelicidin1 (CATH1, C and D), cathelicidin2 (CATH2, E and F), cathelicidin3 (CATH3, G and H), or cathelicidinB1 (CATHB1, I and J). Effects of synthetic cathelicidin peptides (5 μM) on colony formation (K) and cell proliferation (L; determined by optical density) of an ampicillin-resistant *E. coli* strain. B, D, F, H, and J: Magnified views of the boxed areas in A, C, E, G, and I, respectively. Wild type, *E. coli* DH5α strain; amp, ampicillin; AmpR, ampicillin-resistant strain; NC, non-treated, culture medium only; C1, cathelicidin1; C2, cathelicidin2; C3, cathelicidin3; and CB1, cathelicidinB1.

Table 1. Design of synthetic cathelicidin peptides

Name	Size (no. of amino acids)	Synthetic peptide sequence	Molecular weight (Da)	Charge	Hydrophobic ratio	Source
CATH1	28	PVRVKRVWPLVIRTVIAGYNLYRAIKKK	3338.14	8	28	Chicken cathelicidin1
CATH2	34	PVLVQRGRFGRFLRKIRRFRPKVTITIQGSARFG	4014.84	10	44	Chicken cathelicidin2
CATH3	31	PVRVKRFWPLVPVAINTVAAGINLYKAIRRK	3547.35	7	61	Chicken cathelicidin3
CATHB1	40	PIRNWWIRIWEWLNGIRKRLRQRSPFYVRGHLNVTSTPQP	5028.86	7.1	45	Chicken cathelicidinB1

4. Discussion

The breeding of chickens represents a major portion of stockbreeding (http://www.thepoultrysite.com). Cathelicidins constitute a family of host defense peptides that have been identified in several vertebrate classes, including, fish, birds, and mammals (Zanetti 2005; Chang *et al.* 2006; Xiao *et al.* 2006a). The *cathelicidin* genes are expressed in various tissues such as those of the epithelium, mouth, tongue, esophagus, intestine, cervix, and vagina (Bals *et al.* 1998; Frohm Nilsson *et al.* 1999; Hase *et al.* 2002). All members of the cathelicidin family share a highly conserved cathelin pro-sequence at the N-terminal domain and have extremely variable C-terminal domain sequences (Zanetti 2005). The structures of cathelicidin peptides have been characterized as α-helical, disulfide bridged, tryptophan rich, proline rich, and two-disulfide bridged in various species (Zanetti 2005). Most cathelicidins form pores via interactions between lipid polar head groups of microbial membranes and the helical bundles of the peptides, and the pores act as non-selective channels that disturb the vital homeostasis of microbes (Oren *et al.* 1999; Wildman *et al.* 2003; Morgera *et al.* 2009). Some cathelicidins may also induce the host immune response by stimulating immune-related cells such as macrophages, neutrophils, T cells, B cells, and mucosal cells (Agerberth *et al.* 2000; De *et al.* 2000; Tjabringa *et al.* 2003).

Several previous studies in chickens have identified four mature peptides of the cathelicidin family: CATH1, CATH2, CATH3, and CATHB1 (Xiao *et al.* 2006a; Xiao *et al.* 2006b; Bommineni *et al.* 2007; Goitsuka *et al.* 2007; Xiao *et al.* 2009). Among these, CATH1, CATH2, and CATH3 formed an α-helical structure when incorporated with trifluoroethanol (Xiao *et al.* 2006b; Bommineni *et al.* 2007; Xiao *et al.* 2009).

In the present study, we examined the expression patterns of the cathelicidin members in three adult (42-week-old) and three juvenile (1-month-old) hens. CATH1, CATH2, and CATH3 showed similar mRNA expression patterns, with expression detected mainly in chicken bone marrow from 1-month-old and 42-week-old hens. In contrast, CATHB1 was highly expressed in the bursa of juvenile hens, as previously reported (Goitsuka *et al.* 2007; Achanta *et al.* 2012), as well as the thymus of adult hens; its expression in bone marrow was comparatively low. In chicken, the thymus and bursa are the primary lymphoid organs related to T cell and B cell differentiation and maturation (Rose 1979; McCormack *et al.* 1991; Ribatti *et al.* 2006). The bone marrow also participates in the immune system, producing B cells and macrophages (Qureshi 2003). The bursa, which is present exclusively in birds, arises from bursal epithelial cells around embryonic day 4, reaches a maximum size at 6–12 weeks after hatching, and undergoes retrogression at 18–22 weeks (McCormack *et al.* 1991). In the present study, the expression of

CATHB1 differed from that of the other members of this family, suggesting that CATHB1 may be activated by a different immune response.

To analyze the secondary structures of the four chicken cathelicidin peptides, we synthesized the peptides and mixed them with POPC and POPG, which form neutral and negatively charged liposomes, respectively (Liu *et al.* 2001). The secondary structures of CATH1 and CATH3 were changed from random structures to weakly α-helical structures in the presence of POPC liposomes, but displayed well-defined α-helical structures in the presence of POPG liposomes. CATHB1 exhibited obvious α-helical structures after the addition of either POPC or POPG liposomes. These results suggest that CATH1, CATH3, and CATHB1 can interact with liposomes, and that charged polar lipids strengthen the interaction.

An α-helical structure has been reported previously for CATH2 (Xiao *et al.* 2009). When we synthesized CATH2 in the present study, proline and valine were added at the N-terminus, and glycine was added at the C-terminus, compared with the sequences in previous studies (Xiao *et al.* 2006a; Xiao *et al.* 2009). In the present study, CATH2 displayed β-sheet structures in aqueous buffer, and the secondary structure of CATH2 maintained β-sheets in the presence of liposomes. As the addition or deletion of a single amino acid is sufficient to change the structure of a protein (Schaefer & Rost 2012), the added amino acids might have altered the secondary structure of CATH2 in

the present study, resulting in the β-sheet structure in aqueous solution and in the presence of liposomes.

In the present study, all four cathelicidins reduced *E. coli* cell viability and severely damaged *E. coli* cell membranes, with CATH2 and CATH3 showing the highest efficiency. Furthermore, CATH1, CATH2, and CATH3 markedly arrested colony formation and cell proliferation of ampicillin-resistant *E. coli*. However, CATHB1 showed no effect on colony formation or cell proliferation. In previous studies, the variable domains of CATH1, CATH2, and CATH3 were clearly discriminated, but the variable domain of CATHB1 was not identified (Xiao *et al.* 2006a; Goitsuka *et al.* 2007). Here, we predicted the variable domain sequence of CATHB1, but as no effect on colony formation or cell proliferation by CATHB1 was detectable in our assay, the antimicrobial activity of CATHB1 remains unclear.

In conclusion, the present study focused on the identification of chicken cathelicidin peptides, their expression patterns in various tissues, and their antibacterial effects on viability, membrane damage, and colony formation of *E. coli* bacteria. Chicken CATH1, CATH2, and CATH3 showed a similar pattern of expression in juvenile and adult hens, whereas CATHB1 was expressed in the bursa of juvenile chickens and in the thymus of adult chickens, after bursa degeneration. All synthetic chicken cathelicidins showed a notable dose-dependent antibacterial effect. In particular, CATH2 and

CATH3 showed highly efficient antibacterial activities in all of the assays performed in the current study. We propose that the mechanisms of chicken cathelicidins for killing bacteria are independent of the mechanisms of antibiotics such as ampicillin. Our data provide novel information that may be useful for controlling drug-resistant bacteria and for producing disease-resistant animals in the livestock industry.

CHAPTER 7.

GENERAL DISCUSSION

Although transgenic animals could be employed in a wide range of scientific fields and industries, transgene silencing has restricted those applications due to epigenetic factors such as DNA methylation, histone modification and non-coding RNA.

From previous research, it has been established that transgenic quails and chickens express EGFP driven by the RSV promoter. However, the EGFP expression in transgenic birds varied among tissues and even among the same types of cells in the same tissues (Shin *et al.* 2008; Park *et al.* 2010). This variation in transgene expression was also modulated by the CpG methylation status of the RSV promoter in chickens (Park *et al.* 2010). In this study, the status of methylation in a transgenic promoter was examined and modified to determine the relationship between different methylation statuses and EGFP expression. When DNA methylation was inhibited, we observed that the effect of 5-azadC treatment for 48 h was maintained over 20 days, and we performed a bioinformatic analysis. Finally, we concluded that transgene expression was affected by CpG methylation of the RSV promoter in transgenic quail. Our results provide the basis for understanding the epigenetic mechanism responsible for transgenic animal production and genetic preservation.

Next, we identified differentially expressed genes in chicken PGCs as compared to CEFs and analyzed the methylation patterns of the 5-kb-

upstream regions of the differentially PGC-expressed genes and X-linked and imprinting homologous loci among male PGCs, female PGCs, and CEFs. We determined that epigenetic variation mainly occurs in imprinting homologous and X-linked homologous loci in PGCs and CEFs. Differential methylation was detected in the differentially expressed genes between PGCs and CEFs. Thus, we propose that the epigenetic characters of imprinting and X-linked homologous genes are evolutionally conserved in birds and that the epigenetic mechanisms in birds are different from those in mice after DNA methylation. Additionally, DNA methylation during early embryonic development mainly affected X-linked and imprinting-related loci, whereas normal genes are affected by DNA methylation in birds. These results provide information for the epigenetic characterization and regulation of chicken PGCs.

To compare the mechanisms of epigenetic regulation between mammals and birds, we investigated imprinting phenomena in chickens for differentially methylated patterns. Since the mammalian imprinting phenomenon was first reported in mice, more than 100 imprinted mouse genes have been identified, and most of them are conserved in humans as well (Arnaud 2010). However, no evidence has arisen for imprinting phenomena in chickens. Moreover, many studies have also shown that birds do not exhibit imprinting mechanisms and account for this finding with a variety of explanations (Bourc'his *et al.* 2001; Yokomine *et al.* 2005; Colosi *et al.* 2006;

Itoh *et al.* 2007; Shin *et al.* 2010). In this study, we compared the methylation status between male and female PGCs at day 6, which is the initiation stage of germ cell differentiation, by DNA methyl-binding chip analysis. Subsequently, the *GATM* gene was identified as a DMR between male and female PGCs. Additionally, bisulfite sequencing showed that the differentially methylated ratio became more pronounced between male and female PGCs at 8 days compared to those at 6 days, whereas the methylation ratios between male and female fibroblasts were not significantly different in 6-day and 8-day embryos. Such DMRs between males and females have been found frequently in intron regions in imprinted genes (Peters & Williamson 2007; Kaaks *et al.* 2009; van Dijk *et al.* 2010). Moreover, the DMR of *GATM* seemed to undergo sex-dependent epigenetic modification during germ cell specification. Thus, we hypothesized that the DMR of *GATM* could result in imprinting in chicken PGCs. However, when we analyzed the allelic expression pattern of *GATM* in chicken PGCs, we found that it was biallelically expressed. Thus, we demonstrated conclusively that the *GATM* gene displays biallelic expression in chicken PGCs even though males and females exhibit different methylation statuses in the early embryo stage. Thus, the differential methylation of *GATM* is not correlated with its allelic expression pattern in chickens. These findings can contribute to studies of epigenetic reprogramming during early embryonic development and to evolutionary studies on imprinting phenomena across birds and mammals.

In the present study, we also focused on the identification of chicken cathelicidin peptides; their expression patterns in various tissues; and their antibacterial effects on viability, membrane damage, and colony formation of *E. coli* bacteria. Chicken CATH1, CATH2, and CATH3 showed a similar pattern of expression in juvenile and adult hens, whereas CATHB1 was expressed in the bursa of juvenile chickens and in the thymus of adult chickens after bursa degeneration. All synthetic chicken cathelicidins showed a notable dose-dependent antibacterial effect. In particular, CATH2 and CATH3 showed highly efficient antibacterial activities in all of the assays performed in the current study. We propose that the mechanisms by which chicken cathelicidins kill bacteria are independent of the mechanisms of antibiotics such as ampicillin. Our data provide novel information that may be useful to control drug-resistant bacteria and to produce disease-resistant animals in the livestock industry.

From these epigenetic studies, we can understand the mechanisms of transgene silencing in birds and investigate epigenetic status and its function in avian PGCs. Thus, these studies may contribute to improving the efficiency of PGC-mediated transgenesis. We are also able to identify genes against bacterial diseases and to analyze their functional activities. Collectively, our data suggest an appropriate approach to produce disease-resistant birds.

SUMMARY IN KOREAN

형질전환동물은 외래 유전자를 유전체에 도입한 동물로 정의되며 배아발달, 장기발생, 노화 등을 포함한 유전자 기능의 기초연구 및 인간 질병 모델, 유용성 단백질 생산과 관련된 생물반응기로써 이용될 수 있을 뿐만 아니라 축산분야에 있어서 상업형질의 도입 및 질병저항성 동물의 생산 등 다양한 분야에 이용되고 있다. 그러나 인위적으로 도입된 외래 유전자의 경우 생물체에서 DNA methylation, histone modification, non-coding RNA등 후생유전학적 조절 기작을 통하여 그 발현이 억제되는 현상을 관찰할 수 있다.

특히 그 가운데 DNA methylation은 진핵 생물에서 유전자 발현을조절하는데 중요한 역할을 하는 것으로 알려져 있다. 특히 척추동물이상에서 DNA methylation은 씨토신 피리미딘 링의 5번 위치에 메틸기가 붙고 다음 염기서열로 구아닌이 오는 형태가 기능적으로 의미를 갖는다고 알려져 있으며, 이러한 CpG가 조밀하게 배열된 부분을 CpG island라고 한다. 이러한 CpG island는 주로 유전자의 promoter 부위에 위치하게 되며, 이들 부위에 methylation이 일어나게 되면 유전자 발현이 억제되는 것으로 알려져 있다. 포유류를 비롯한 고등생물에서 DNA methylation은 histone등 다른 다른 후

생유전학적 인자들과 복합적으로 기능하면서 배아의 발달에 따른 X 염색체의 inactivation, genomic imprinting 그리고 초기 생식세포 발달에 큰 영향을 주는 것으로 밝혀졌으나, 닭을 비롯한 조류에서 DNA methylation의 대한 알려진 부분은 지극히 제한적이다.

본 연구에서는 형질전환 메추리로부터 유도한 QEFs로부터 프로모터의 methylation 패턴을 분석하고 EGFP의 발현과 상관관계를 분석하였을 때 EGFP의 발현이 낮고 DNA methylation이 높게 일어난 현상을 관찰하였으며, DNA methylation 억제제인 5-azadC를 48시간 처리하여 DNA methylation이 억제되었고 그 효과가 20일 이상 유지되는 것을 확인할 수 있었다. 그러므로 형질전환 프로모터의 DNA methylation이 외래유전자 발현에 직접 영향을 줄 수 있다는 가능성을 제시할 수 있었다.

또한 MeDIP array를 통해서 원시생식세포와 체세포 사이에 발현에 차이를 보이는 유전자의 프로모터 부위 그리고 X-linked 와 imprinting 관련 상동부위의 DNA methylation 패턴을 비교한 결과 DNA methylation의 변화는 주로 X-linked 와 imprinting 관련 상동부위에서 일어난다는 사실을 확인할 수 있었다. 그리고 원시생식세포와 체세포에서 서로 다른 발현양상을 보이는 유전자에

서 DNA methylation 또한 차이를 보인다는 것을 확인하였다. 그러므로 본 연구를 통하여 비록 DNA methylation 이후에 마우스와 닭 사이에 후생유전학적 조절 기작이 다르다 할지라도 X-linked 와 imprinting 관련 상동부위에 유전자들 후생유전학적 특징들이 진화적으로 조류에서도 보존될 수 있다는 가능성을 확인하였다.

마우스에서 imprinting 현상이 처음 보고된 뒤 현재까지 100개 이상의 유전자가 새롭게 밝혀진 가운데 닭에서는 아직까지도 imprinting 현상에 대한 결정적인 증거가 보고되지 않은 상황이다. 본 연구에서는 MeDIP array에서 암수 원시생식세포 사이에 methylation에 차이를 보였던 부위를 대상으로 각각 6일령과 8일령 암수 원시생식세포를 대상으로 allelic expression 여부를 조사하였다. 그 가운데 GATM 유전자는 bisulfite sequencing으로 methylation 패턴을 검정하였을 때 암수 원시생식세포간에 분명한 차이를 나타내었으나, 체세포에서는 차이를 보이지 않았다. 그러므로, 원시생식세포에서 GATM 유전자가 성별에 따른 후생학적 변이에 차이를 나타내는 유전자로 확인되었으나 최종적으로 biallelic expression을 확인할 수 없었다.

항균펩타이드는 다양한 종의 생물에서 발견되며 미생물을 죽이거나 면역반응을 강화시키고, 호스트에 선택적 면역조절을 유도하는 것으로 알려져 있다. 최종 펩타이드의 경우 100개 미만의 짧은 아미노산으로 구성되며, 기본적으로 α-helical 구조와 β-sheet 구조로 구성된다. 본 연구에서는 AMP의 일종인 cathelicidins에 초점을 맞추어 연구를 진행하였다. 닭에는 총 CATH1, 2, 3 그리고 B1이 존재하며, CATH 1, 2, 3가 병아리와 성축에서 유사한 발현패턴을 보이는 것과 달리 병아리에서 bursa에서 주로 발현하는 CATH B1의 경우 성축이 되어 bursa가 퇴화하게 되면 thymus에서 발현한다는 사실을 발견하였다. 항균 활성을 측정한 결과 CATH 2와 3이 가장 높은 활성을 보였으며, 전자현미경을 통하여 항균펩타이드에 의해서 미생물의 세포막이 직접 데미지를 입는다는 사실을 확인할 수 있었다. 또한 이런 항균 펩타이드를 통한 미생물의 저해 기작은 ampicillin 저항성 균주를 이용한 항균 활성 테스트 결과 ampicillin과는 독립된 메커니즘으로 미생물을 저해한다는 것을 증명하였다.

위의 연구결과를 토대로 조류에서 후생학적 요인들 특히 DNA methylation을 조절함으로써 외래 유전자의 silencing 현상을 조절할 수 있다는 가능성을 보여 주었으며, 조류에서 형질전환동물의 생산에 있어 가장 효율적인 방법으로 부각되는 원시생식세포의 후생

유전학적 특징들을 분석함으로써 향후 원시생식세포를 이용한 외래 유전자 도입 시에 미치는 후생학적 요인들을 이해하는데 기여할 수 있을 것으로 사료된다. 또한 닭에서 강력한 항균력을 갖는 cathelicidin 유전자를 선발하고 그 항균 활성을 분석함으로 질병 저항성 조류 생산에 적합한 후보 유전자를 제시하였다. 그러므로 본 연구에서 수행한 다각적 연구결과들을 토대로 향후 효율적인 질병 저항성 닭 생산을 위한 통합적 접근법을 제시할 수 있을 것으로 기대한다.

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I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

- Isaac Newton

언제가 저 진리의 바다에서 특별한 작은 조개 껍데기를 하나쯤 발견해볼 생각입니다.

도움주신 모든 분들께 감사 드립니다.