A Thesis for the Degree of Master of Science

Studies on Expression Regulatory Mechanisms of *CIWI* gene in Chicken Primordial Germ Cells

닭 원시 생식세포에서 *CIWI* 유전자의 발현조절기전에 대한 연구

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SUMMARY

Primordial germ cells (PGCs) are the progenitors of germ cells, which produce functional gametes and transmit genetic information to the next generation. Understanding the molecular mechanisms that regulate cell-fate decisions during early PGC development is important for fundamental research in germ cell biology. The P-element-induced wimpy testis (PIWI) protein, which associates with small non-coding RNAs, is responsible for maintaining the integrity of germ cells and stem cells. Thus, transcriptional regulation of *PIWI* is critical for effective functional modulation.

In this study, we identified the promoter region of the *PIWI* homolog in chicken (*CIWI*) and investigated the transcriptional regulatory elements that control expression of *CIWI* in chicken primordial germ cells (PGCs).

We constructed a vector that included the enhanced green fluorescent protein (eGFP) gene controlled by the 4-kb *CIWI* promoter. The vector was expressed in chicken PGCs, but not in chicken embryonic fibroblasts (CEFs). Based on promoter deletion and fragmentation assays, we found that a 252-bp fragment of the *CIWI* promoter (-577 to -326 bp) was crucial for *CIWI* expression in PGCs.

A transcriptional regulatory element CCAAT (−498 to −494 bp) was detected in the proximal region from the transcription initiation site. Mutational analysis further confirmed that the CCAAT element in the *CIWI* promoter regulates transcriptional initiation in chicken PGCs. Based on multiple sequence alignment analysis, the 4−kb promoter of *CIWI* was not conserved with the human and mouse promoters of *PIWI*. Interestingly, the flanking regions of the CCAAT element, which are positioned differently in *HIWI* (human), *Miwi* (mouse) and *CIWI* were highly conserved.

In addition, the specificity protein 1 (Sp1) motifs were predicted to modulate the transcriptional initiation of *CIWI* by binding to the 5' −flanking regions of the CCAAT box. We also identify several transcription factors which might affect the transcriptional activity of germ cell−specific genes. Thus, a short promoter sequence and a single transcription factor, such as the CCAAT box, NF−Y and SP1, in chicken germ cells could control transcriptional initiation of genes specific for cellular proliferation and cell−lineage−specific expression.

In the present study, we analyzed the transcriptional regulatory elements in the promoter region of *CIWI* to examine functional modulation of avian germ cell−specific gene expression. Identifying short promoter sequences or regulatory transcription

factor in germ cell-specific promoter like *CIWI* gene may be valuable not only for an efficient production of transgenic chickens having the reporter gene specifically only in germ cell lineage, but also for the basic study of reproductive biology of germ cell development and differentiation in the early embryonic stages.

Key words: chicken, primordial germ cell, *CIWI* promoter, CCAAT element.

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CONTENTS

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATION

PIWI : P-element-induced wimpy testis

CIWI : Chicken PIWI-like protein 1

CILI : Chicken PIWI-like protein 2

PGC : Primordial germ cell

CEF : Chicken embryonic fibroblast

GSC : Gonadal stromal cell

MEF : Mouse embryonic fibroblast

eGFP : Enhanced green fluorescent protein

5' UTR : 5' untranslated region

3' UTR : 3' untranslated region

HIWI : Human PIWI-like protein 1

MIWI : Mouse PIWI-like protein 1

MILI : Mouse PIWI-like protein 2

ZIWI : Zebrafish PIWI-like protein 1

ZILI : Zebrafish PIWI-like protein 2

ncRNA : Non coding RNA

piRNA : PIWI interacting RNA

siRNA : small interfering RNA

miRNA : micro RNA

DAZL : Deleted in azoospermia-like

CR1 : Chicken repetitive element 1

NF-Y : Nuclear transcription factor Y

SP1 : Specificity protein 1

TFAP2α : Transcription factor activating enhancer binding protein 2 alpha

ZIC3 : Zic family member 3

PAX2 : Paired box 2

PAX5 : Paired box 5

Blimp1 : B lymphocyte-induced maturation protein 1

PRDM14 : PR domain containing 14

CBF : CCAAT-binding factor

USF1 : Upstream stimulatory factor

WL : White Leghorn

SSEA-1 : Stage-specific embryonic antigen 1

DAPI : 4',6-diamidino-2-phenylindole

qRT-PCR : Quantitative reverse transcription PCR

TE : Transposable elements

RNAi : RNA interference

MACS : Magnetic activated cell sorting

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

DMEM : Dulbecco's modified eagle medium

FBS : Fetal bovine serum

CHAPTER 1.

GENERAL INTRODUCTION

Primordial germ cells (PGCs) play a role in transferring genetic information to the next generation, which is essential for maintaining a species. PGCs differentiate into germ cells, which can undergo meiosis as well as mitosis, and consequently, produce gametes. Understanding the molecular mechanisms of germ-line specification and development is important not only for the fundamental studies of germ cell biology, but also for the practical utilization of genetic resources.

The P-element induced wimpy testis (PIWI) subfamily of protein functions specifically within the germ cells and stem cells. PIWI proteins play a role in maintaining fertility. Mutation of PIWI showed sterile in diverse organisms including mouse, fruit flies and zebrafish. PIWI proteins also play a role in maintaining genomic integrity. Loss of PIWI proteins leads to germline-specific apoptosis, which may be triggered by DNA damage. In addition, the PIWI-piRNA pathway induces silencing of retrotransposon, which is a DNA fragments that integrate into the genome and interrupt the translation of genes. PIWI proteins direct retrotransposon silencing both transcriptionally and post-transcriptionally. Recent studies have suggested that the PIWI-piRNA complex may also regulate epigenetic states via a similar mechanism in multicellular organisms (Juliano et al. 2011). PIWI proteins are implicated in directing heterochromatin formation and transcriptional silencing by promoting euchromatic

histone modifications (Yin and Lin 2007). PIWI-piRNA pathway mediates transcriptional and post-transcriptional regulation which may be responsible for its function in germline specification, gametogenesis and differentiation in diverse organisms.

In chicken, *Gallus gallus* PIWI-like protein 1 (CIWI) and 2 (CILI) were identified from adult chicken testis. mRNA levels of both *CIWI* and *CILI* were highly expressed in germ cells in both adult testis and ovary. The expression levels of *CIWI* and *CILI* in the embryonic stages also showed similar pattern with adult tissues. Furthermore *CIWI* and *CILI* were more highly expressed in PGCs than embryonic gonad. siRNA-mediated knockdown analysis of *CIWI* and *CILI* in chicken PGCs showed up-regulated expression of the chicken repetitive element (CR1) retrotransposon and induced DNA double strand breakage(Kim et al. 2012). It suggests that *CIWI* and *CILI* play a critical role in maintaining the genome integrity of germ cells in chickens.

Transcription is an initiation step in gene expression and a primary control process used in living cells. Tissue/cell-specific promoters are a promoter that has specific activity in certain cell types. (Zheng and Baum 2008) Regulation of cell type-specific gene transcription may be a critical for the cellular development. Germ cell-specific promoters can be used for programmed gene expression in germ cells. We expected that element for the germ

cell-specific promoter can be identified in promoter of *CIWI.* Studying promoter region of *CIWI* is required for comprehending expression pattern of not only for *CIWI,* but also for other germ cell-specific genes. Most *cis*-regulatory sequence has more than one type of transcription factor binding site, and most transcription factors bind to the several genes of *cis*-regulatory sequence. Different complexes of transcription factors can alter the gene regulation in different genes (Wittkopp 2010). In addition, one transcription factor can serve both as a positive regulator and as a negative regulator for the expression of genes (Li and Ou 2001).

In the present study, we analyzed the transcriptional regulatory elements in the promoter region of *CIWI* to examine functional modulation of avian germ cell-specific gene expression. The main hypothesis of this study is that cis-element which is crucial for germ cell-specific transcription is exists in *CIWI* promoter. In addition, germ cell-specific transcription factors which bind to the promoter region of *CIWI* plays a important role in germ cell-specific regulation. In chapter 3, we cloned the promoter of *CIWI* gene and estimated the cultured chicken PGC-specific expression using fluorescent protein. We perform deletion analysis and fragmentation analysis in order to identify proximal promoter of *CIWI.* In addition, we performed mutation analysis to identify the *cis* element in the proximal promoter of

CIWI. Multiple alignment of *CIWI* promoter with mouse *PIWI* and human *PIWI* promoter was performed. In chapter 4, we predicted several transcription factors which bind to the proximal promoter of *CIWI*. Predicted transcription factors were analysed by qRT-PCR in order to identify the specificity in PGCs. Each synthetic siRNAs were used for knockdown analysis of transcription factors. Expression level of *CIWI* was examined in transcription factor knockdown-PGCs by qRT-PCR .

The *CIWI* promoter could be used to study reproductive biology, especially during the development and differentiation of germ cells in the early embryonic stages.

CHAPTER 2.

LITERATURE REVIEW

1. PRIMORDIAL GERM CELLS IN CHICKEN

Primordial germ cells (PGCs) are the progenitors of germ cells, which produce functional gametes and transmit genetic information to the next generation. PGCs are capable of specializing themselves from other somatic cells and undergoing extensive regulation during development. Understanding the molecular mechanisms that regulate cell-fate decisions during early PGC development is important for fundamental research in germ cell biology.

In most of the vertebrate species, germ cells are developed from PGCs. PGC arise from the epiblast of early embryos, migrate to reach the bilateral embryonic gonads. PGC undergo meiosis followed by rapid proliferation by mitosis. PGCs develop as haploid sperm and eggs that generate a new organism upon fertilization (Magnusdottir and Surani 2014). In birds, PGCs localize initially to the central zone of the zona pellucida in undifferentiated embryos at stage X and then migrate into the developing gonads through embryonic blood vessels. This migration route differs from that in mammals (Park and Han 2012).

1.1 Specification

The specification, migration and differentiation events of PGCs are similar in many vertebrate. However the mechanism of germ cell specification is not conserved among animals. Typically, specification of germ cells occurs either through the inheritance of preformed germ plasm (Weissmann, 1885), or is induced among equipotent cells by instructive signals. For example, germ cell specification in *Xenopus* and *C. elegans* occurs via the inheritance of germ plasm, whereas, fruit flies, zebrafish and mice can be specified by inductive signals from surrounding somatic tissues (Extavour and Akam 2003).

Specification of germ cell through the preformed germ plasm is called 'preformation mode'. In the preformation mode, maternally inherited germ-plasm containing mRNAs, proteins and small RNAs play a crucial role for germ cell specification in initial developmental stages. Preformation is the method of germ cell specification in fruit flies (Illmensee and Mahowald 1974) and zebrafish (Olsen et al. 1997). In these organisms, PGCs can be detected very early in embryonic development, and germ cell differentiation is determined by the localization of inherited maternal determinants, the germ plasm, prior to, or immediately after, fertilization. In particular, fruit flies and zebrafish require germ plasm ; a specialized cytoplasm contains maternal RNAs

and proteins like *Vasa* and *Dazl*. In induction mode, germ cells are induced by signals from neighboring somatic cells during gastrulation. The induction mode of specification is conserved in most mammalian species. In mice, when blastocysts were implanted, specification of PGCs occurred. One of the major signal molecules for germ cell specification is the bone morphogenetic proteins (BMPs). The expression of Bmp4, Bmp8b and Bmp2 is known to be required for germ cell induction in the mouse (Lawson et al. 1999; Ying et al. 2000; Ying and Zhao 2001).

In chicken, VASA homologue protein, one of the germ plasm components, was detected at very early stage in blastoderm. At present, such cells containing Vasa protein are considered to be presumptive PGCs in chickens (Tsunekawa et al. 2000). These results support that chicken germline is determined by maternally inherited determinants of germ plasm, drawing a conclusion that germline specification in chickens is based on the preformation model.

The role of PIWI proteins in germ cell specification during early embryogenesis has been demonstrated in fruit flies. PIWI proteins are required for patterning and pole cell formation. In embryos lacking maternal PIWI, there is a severe decrease in germ cell formation (Megosh et al. 2006).

1.2 Differentiation

After arrive to the genital ridge, PGCs differentiate into germ cells which then undergoes mitosis followed by meiosis to produce spermatozoa in the testis or ova in the ovaries after sexual differentiation. The differentiation of PGCs into male or female germ cells is determined by the sex of the gonadal somatic cells (Burgoyne 1988; Hajji et al. 1988). In mammals, the expression of the *SRY*, the Y-linked testis-determining gene, located in Y chromosome, influences sex determination. Expression of *SRY* gene induce gonads to develop into testes. Without expression of SRY, the gonads develop into ovaries, but *SRY* gene is sufficient to induce testis differentiation in XX transgenic mice embryos (Hawkins et al. 1991; Koopman et al. 1991).

The sex chromosome system is different in avian. The male bird has a homogametic pair of sex chromosome, ZZ, whereas the female has a heterogametic pair of sex chromosome, ZW. The genetic trigger for sex determination in birds remains unknown. However, some promising candidate genes have recently emerged. The Z-linked gene, *DMRT1*, which is the best candidate for male determining gene identified in birds, supports the Z-dosage model of avian sex determination. The expression of *DMRT1* was highly detected in males during gonadal

differentiation, compared to female (Raymond et al. 1999; Smith et al. 2009). Two novel W-linked female determinants, *ASW* and *FET1* were considered to be important candidates (Smith and Sinclair 2004).

Despite the different sex chromosome patterns and suspected difference in sex-determining mechanisms, mammals and birds have essentially similar patterns of sexual differentiation (Mittwoch 1998). After sex determination, PGCs continue to divide mitotically and enter the first mitotic prophase to become oocyte in the ovary, and PGCs are mitotically arrested until birth in the male gonad (Hilscher et al. 1974).

The differentiation of male and female gonads begin around E6.5 in chickens (Hudson et al. 2005). In ZZ embryos, bilateral testis differentiation occurs. In ZW embryos, the left and right sides of gonads develop asymmetrically. During the gonadal development, the right gonad represses and the left gonad develops into a functional ovary (Ukeshima 1996). After morphological differentiation of the embryonic gonads, the PGCs differentiate into oogonia at E8.0 in females or differentiate into prospermatogonia at E13.0 in males (Nakamura et al. 2007).

In mouse, there are three mouse PIWI homolog, MIWI, MILI and MIWI2, which display distinct expression patterns and

functions during male germ cell differentiation and development. Each PIWI mutant displays unique defects in spermatogenesis. *Miwi* mutants are arrested at the round spermatid phase while *Miwi2* mutant mice display defects during meiosis, with arrest in leptotene spermatocyte and mutant spermatogonia undergoing massive apoptosis. In addition, *Mili* mutant mice was arrested at the mid-pachytene stage during spermatogenesis.

1.3 Migration & Proliferation

Since the germ cells are specified in positions that are distinct from the location where the gonad develops, in most organisms, PGCs have to migrate through the developing embryo. Therefore, PGCs undergo variable processes of morphogenesis and differentiation and become incorporated in the developing gonads (Doitsidou et al. 2002; Starz-Gaiano and Lehmann 2001).

Chicken PGC first arise from the epiblast and migrate to the hypoblast of the area pellucida in the blastoderm. In stage 10 to 12, PGCs are carried from the germinal crescent into the blood stream and migrate through the circulatory system. (Ando and Fujimoto 1983; Ginsburg and Eyal-Giladi 1986; Ukeshima et al. 1991) Subsequently, PGC actively leaves capillary vessels, and finally settle in the developing genital ridges (Fujimoto et al.

1976a; Hamburger and Hamilton 1951; Meyer 1964). Distinct from avian PGC, mammal PGCs migrate through the embryonic tissues to reach their destination (Han 2009).

Before PGCs differentiate into germ cells, PGC undergo mitotic proliferation at stage X. Approximately 30 PGCs, which express Chicken *VASA* homolog (*CVH*), are scattered in the central zone of the area pellucida in the stage X embryo. 200-250 *CVH* expressing PGCs exist in the germinal crescent, which is the anterior part of the embryo (Muniesa and Dominguez 1990; Tsunekawa et al. 2000). After migration, the proliferation of PGCs rapidly increases until they differentiate into germ cells. PGC division from stage 3 to 27 (H&H) shows that rapid proliferation during migration cause increase in PGC number (Swartz and Domm 1972). More than 1,000 of PGCs exist at stage 31 (7 days of incubation) of both male and female gonads (Zaccanti et al. 1990).

The *Caenorhabditis elegans* genome encodes two PIWI orthologs, which is *prg-1* and *prg-2.* Prg-1 is expressed in perinuclear P-granules of the male germ cells. RNAi knockdown of both prg-1 and prg-2 in *C. elegans* leads to decreased germ-cell proliferation (Cox 1998).

1.4 Regulation of germness

PGCs highly express several key genes during their development. Several key genes are expressing in order to regulate the germline competence. Transcription factors BLIMP1. PRDM14, TCFAP2C and POUV are strongly expressed in PGC during specification. Four transcription factors are expressed strongly to repress somatic program and for their survival (Kehler et al. 2004; Yamaji et al. 2008). PGC undergo apoptosis or show impaired specification when these genes are disturbed.

Expression of pluripotency-related genes are also important in PGCs. Genes are expressed in early pluripotent embryonic cells at the first time, and the expression become restricted to PGCs after their specification. OCT4 might have a role in establishing competence for PGC fate (Kehler et al. 2004). Genes such as Nanog are necessary for the maintenance of early germ cells because mutant cells undergo apoptosis during migration (Chambers et al. 2007; Yamaguchi et al. 2009). PGCs express several more pluripotency-related genes including *Sox2* and *Pouv* during their development (Chambers et al. 2007; Graham et al. 2003). Mis-regulation of these genes cause apoptosis in PGCs.

During specification of primordial germ cells, several germ cell-specific genes are expressed and play a role for maintaining germ cell-competency among various species. They contain *Nanos, Vasa, Piwi, Dazl* and etc.

Deleted in azoospermia-like (*DAZL*) is known to play a critical role during spermatogenesis and oogenesis. Several studies have been performed to evaluate the function of DAZL from the perspectives of germ cell development. Mice deficient for *Dazl* are infertile, lacking any formation of spermatozoa or oocyte (Ruggiu et al. 1997). Also in chicken, cDAZL is expressed specifically in germ cells from embryonic stages to adult stages (Rengaraj et al. 2010). VASA protein is one of the key molecules in the differentiation and development of germ cells in several animals (Hay et al. 1998). VASA protein is specifically localized in the germ plasm and is exclusively expressed in germline cells throughout subsequent stages of development. Tsunekawa et al. found that the germline-specific *CVH* gene is expressed from the first cleavage of fertilized eggs (Tsunekawa et al. 2000).

The P-element-induced wimpy testis (PIWI) protein family, one of two subfamily members of Argonautes that regulate gene expression by associating with small noncoding RNAs, plays a role in maintaining the integrity of germ cells and stem cells. In

fruit flies, loss of maternal *piwi* transcript results in loss of PGC formation. Furthermore, overexpression of increasing amounts of maternal *piwi* leads to a linear increase in the number of new PGCs formed (Megosh et al. 2006). In chicken, *CIWI* and *CILI* genes were identified not only in chicken testis and ovary, but also in PGCs.

2. THE FUNCTIONS OF PIWI PROTEINS IN DIVERSE ORGANISMS

In early embryo development, PGC lineage is governed by action of many factors including key regulators in transcription and post-transcription. Recent evidence suggests that the PIWI-piRNA complex may regulate epigenetic states via a similar mechanism to the multicellular organisms. Studies in animals from diverse taxa demonstrate that PIWI proteins have a conserved germ cell and stem cell function (Juliano et al. 2011).

2.1 Discovery of piRNA and PIWI family proteins

Distinct population of PIWI associated small RNAs were discovered in studies about RNA silencing in fruit flies. They investigated the silencing of testis-expressed stellate genes, which are known to be involved in the maintenance of male fertility (Aravin et al. 2001). They suggested the possibility of the existence of piRNA. About 25 to 27 nucleotide RNA was forming complexes with PIWI clade and the silencing stellate protein. These RNAs were identified in the testes and ovaries of zebrafish, *C. elegans*, and some unicellular eukaryotes. Small RNA partners of PIWI proteins were identified in mammalian

testes and termed PIWI interacting RNAs (piRNAs) (Tomari et al. 2007).

During early embryonic development, the PGC lineage is governed through the orchestrated actions of many transcriptional and post-transcriptional regulators. Several recent studies demonstrated that a large number of small non-coding RNAs (ncRNAs) act as transcriptional and post-transcriptional regulators. ncRNA is a functional RNA molecule that is not translated into a protein. Among ncRNAs, there are three major types of small ncRNAs ; microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and piwi-interacting RNAs (piRNAs). These small RNAs range in size from 18 to 32 nucleotides (nts), and they differ in both their biogenesis and function. Small RNAs have proven to be robust regulators of pluripotency and cell fate in many contexts, including germ cells. Common characteristic of these ncRNAs is that they bind to proteins of the Piwi/Argonaute family. These proteins are evolutionarily conserved, and characterized by the presence of the PAZ (Piwi-Argonaute-Zwille) and PIWI domains that are essential for their function (Saxe and Lin 2011). siRNAs and miRNAs, which bind to Argonaute (Ago) subfamily, is ubiquitously present in somatic and germline cells. Otherwise, piRNAs bind to Piwi subfamily, highly enriched in the germline.

2.2 Characterization of PIWI proteins in diverse organisms

PIWI proteins have been identified and characterized in many species. PIWI family is expressed in specialized germ cells and stem cells in all animals examined so far. However PIWI proteins have been detected in different subcellular locations with different expression patterns among animals. It suggests that PIWI protein display distinct function among species. The PIWI protein was first cloned and characterized in *D. melanogaster*, and its expression was found to be necessary to maintain germ line stem cells (Lin and Spradling 1997).

PIWI proteins bind to piRNAs and function in germ cells and stem cells throughout animal phylogeny. There are three PIWI proteins in *D. melanogaster melanogaster*; *PIWI*, *Aubergine*, and *AGO3*. They are expressed in both male and female germ lines (Aravin et al. 2001). *PIWI* can be detected in both the nucleus of somatic and germ cells in fruit flies ovaries. *PIWI* is expressed in germ cells of testes, but is also strongly expressed in somatic niche cells, which supports the maintaining of germline stem cells (Saito and Siomi 2010). In fruit flies, the PIWI/AGO family was discovered in a p-element insertion screen for genes that disrupt GSC function (Lin and Spradling 1997). The gonads of *piwi* mutant flies contained fewer egg chambers and sperm bundles, and showed abolished proliferation

of germline stem cells. *piwi* mutant flies are sterile due to the defects in self-renewal of GSCs (Cox et al. 1998; Cox et al. 2000).

Three PIWI homolog proteins are present in mouse; MIWI (PIWI), MILI (PIWIL2) and MIWI2 (PIWIL4), which show distinct expression patterns and functions in germ cell lineages (Carmell et al. 2007; Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004). In detail, a murine *PIWI* gene (*Miwi*) encodes a cytoplasmic protein specifically expressed in spermatocytes and spermatids. MIWI is first detected at 14 dpp (days postpartum), which is a stage that corresponds to the appearance of pachytene stage spermatocytes. MIWI is present in midpachytene stage spermatocytes and round spermatids in adult testis, and it becomes much more abundant in diplotene stage spermatocytes. In *Miwi* mutant mice, spermatogenesis was arrested at the early stages and it suggests that MIWI is a key regulator of spermiogenesis (Bak et al. 2011; Deng and Lin 2002). *Mili* is expressed at an earlier stage than *Miwi*. The expression of *Mili* was detected up to the pachytene spermatocytes, whereas that of *Miwi* was detected from the mid-pachytene stage to the elongated spermatids. In *Mili* mutant mice, spermatogenesis was arrested at the mid-pachytene stage (Kuramochi-Miyagawa et al. 2004). Whereas MILI and MIWI are cytoplasmic proteins, MIWI2 is found in both the nucleus and perinuclear nuage.

MIWI2 has a relatively narrow expression window during spermatogenesis, which is detected from 18 dpc (days post coitum) in the gonocytes to three days dpp in spermatogonia stem cells. *Miwi*2 mutant mice showed significantly increased levels of apoptosis in the seminiferous tubules compared to the wild-type (Carmell et al. 2007; Kuramochi-Miyagawa et al. 2001). These results indicated that the various members of PIWI in mice have functions required for stage-specific differentiation of germ cells.

Zebrafish has two homologs of PIWI called Ziwi and Zili. Ziwi protein localizes to granules that cluster along the cleavage planes in two and four cell embryos, which is strikingly similar to the characteristic localization of Vasa mRNA at these stages (Yoon et al. 1997). Ziwi has the highest expression during the mitotic and early meiotic stages of germ cell differentiation (Kane and Kimmel 1993). Zili protein can be detected in primordial germ cells (PGCs) from 3 days post-fertilization (dpf) (Houwing et al. 2008). Ziwi affects germ cells during earlier development, triggering apoptosis in premeiotic cells, most likely in a cell-autonomous manner. Loss of *ziwi* induces progressive loss of germ cells due to apoptosis not only during larval development, but also in adult germ cells (Houwing et al. 2007).

The human HIWI protein showed significant homology with other members of the PIWI family in mouse and fruit flies (Qiao et al. 2002). HIWI (PIWI), HILI (PIWI2), HIWI3 (PIWI3) and HIWI2 (PIWI4) have been identified. In the case of HIWI, the highest level of expression in fetal tissues was found in the kidney. Analysis of adult samples showed that the highest level of expression was seen in the testis followed by the kidney. *HIWI* is expressed in spermatocytes and round spermatids during spermatogenesis. Also, *HIWI* is expressed in hematopoietic stem and progenitor cells, but not in the differentiated products of these cells, thus suggesting a role for PIWI proteins in at least one type of human somatic stem cell (Sharma et al. 2001). HILI and HIWI3 are mainly expressed in the testes, but interestingly, HIWI2 was ubiquitously observed in all tissues (Sugimoto et al. 2007).

2.3 Germline functions of PIWI proteins

Interestingly, expression of PIWI protein is mainly restricted to germ cells and stem cells, and is required to maintain fertility. Also repressing transposons in the germline and regulating gene expression at the epigenetic, post-transcriptional and translational levels are the main function of PIWI protein in germ cells (Juliano et al. 2011).

2.3.1 PIWI proteins are required for fertility.

The crucial role of PIWI proteins can be found from the earliest stage of germline development (germline fate specification) to late stages of gametogenesis. PIWI proteins maintain fertility in *D. melanogaster*. *C. elegans* and mouse. For example, fruit flies *piwi* mutant flies become sterile both in male and female. *Miwi* mutant mice also become sterile in male.

PIWI proteins have a conserved role in maintaining GSCs during gametogenesis in diverse animals. In fruit flies, Piwi is required for the self-renewal of GSCs and gonadal development. In *piwi* mutant flies, gonads contain fewer number of egg chambers or sperm bundles respectively, and *piwi* mutation abolish the proliferation ability of both male and female germline stem cells (Cox et al. 1998; Lin and Spradling 1997). In *ago3* mutant flies, GSCs are not properly maintained either, and it also reduced fertility in male (Li et al. 2009). In *Miwi* mutant males, spermatogenesis is arrested at the early stages of spermiogenesis (Deng and Lin 2002). *Miwi2* mutant mice displayed an interruption of spermatogonia and the complete loss of male germ cells at six months, strongly suggesting a function for these proteins in GSC maintenance (Carmell et al. 2007).

Function of the role of PIWI proteins is demonstrated during meiosis. *Miwi2* mutants mice show predominant arrest at the leptotene stage of meiosis. Mutant spermatogenic cells displayed defects in DSB (Double strand breakage) repair system, suggesting that *Miwi2* has role in proper meiotic recombination (Carmell et al. 2007; Kuramochi-Miyagawa et al. 2004). In *Mili* mutants, spermatogenesis is blocked at the mid-pachytene stage (Kuramochi-Miyagawa et al. 2004). In zebrafish, while *ziwi* mutant male displayed fertile and normal, *ziwi* mutant female showed sterile, due to an oogenic block in meiosis I (Houwing et al. 2007).

PIWI proteins are also involved in embryogenesis and germline specification. *D. melanogaster* PIWI protein and AUB protein are essential for the formation of pole cells (Harris and Macdonald 2001; Megosh et al. 2006). In fact, Piwi, Aub, Ago3, all three PIWI proteins are required for normal body-axis patterning during early embryogenesis (Li et al. 2009; Schupbach and Wieschaus 1991). In conclusion, PIWI proteins are required early in embryogenesis for patterning and pole cell formation in fruit flies.

2.3.2 PIWI proteins repress transposon activation in the germline

Transposable elements (TE) are sequences of DNA that can transpose themselves to new positions in the genome. Two class of transposons, Class I and Class II exists, and they have different mechanism of transposition. Class I transposon, also called retrotransposons, copy themselves through transcription, and reverse transcribe RNA into DNA, which may integrate back to the genome. There are 2 types of retrotransposons, which are LTR and non-LTR. LTR represents long terminal repeats. Non-LTR retrotransposons can divide into LINE (long interspersed nuclear elements) and SINE (short interspersed nuclear elements). Class II transposons are only DNA fragments and integrate into the genome through a cut and paste mechanism.

PIWI proteins are required to repress transposons in the germline. In fruit flies, mutations in *piwi, aub* and a*go3*, the three members of *PIWI* family, cause transposon derepression in the germ line (Klenov et al. 2007; Lehmann and Nusslein-Volhard 1991; Vagin et al. 2006). MILI and MIWI2, which are the members of PIWI family in mouse, are needed for the methylation of transposon which encodes genomic regions. Transposon activity was increased when methylation of *Mili* and *Miwi2* were absent. Mutations in *Mili* and *Miwi2* caused

increased expression of LINE and LTR retrotransposons such as IAP. They also erased DNA methylation of retrotransposons (Aravin et al. 2007; Kuramochi-Miyagawa et al. 2008).

2.3.3 The PIWI-piRNA complex is required for genome integrity in germline

The piRISC (piRNA-induced silencing complex) in the germline protects the integrity of the genome from invasion of transposable elements by repressing them. Loss of PIWI proteins induces germline-specific apoptosis. It may be triggered by DNA damage and elevated transposition (Carmell et al. 2007; Houwing et al. 2007; Klattenhoff et al. 2007; Kuramochi-Miyagawa et al. 2004).

The phosphorylated histone H2AX (γ-H2AX) marks the sites of DNA double-stranded breaks that naturally occur during the leptotene stage of meiosis. However, double strand breaks are repaired spontaneously from zygotene to pachytene stages of meiosis. In *Miwi2* mutant mice, level of γ-H2AX relatively increased in zygotene-stage spermatocytes. Due to defect of *Miwi2*, double strand breaks prevent germ cells to enter the pachytene stage of spermatogenesis, and leads to apoptosis (Carmell et al. 2007). *Miwi2* mutant show increased level of apoptosis, but *Mili* mutant and wild type animals has no

significant difference in staining of γ-H2AX. It suggests that apoptosis in *Miwi2* mutant mice has an alternate pathway in inducing apoptosis (Kuramochi-Miyagawa et al. 2004).

Mutation in *PIWI* can also induce activation of transposon element. Active TEs are able to move randomly in genome, and it induces generation of double stranded DNA breakage easily during transposition. Double strand breakage by transposons induces increased levels of phosphorylated histone H2AX in the *PIWI* mutant.

2.4 Functional characteristics of the PIWI in chicken germ cell lineage

In avian species, the biological functions of PIWI family members remain unclear. In a recent study, we characterized the PIWI members CIWI and CILI in chickens (Kim et al. 2012). In another study, the complete genomic structure of the *CIWI* gene including a 161-bp fragment of the 5' UTR and 660 bp of the 3' UTR followed by a poly(A) tail was reported (Chen et al. 2013). In contrast to mouse PIWI homologs, which is detected only in testis, high-level expression of *CIWI* and *CILI* was detected in the germ cell lineage in testis and ovary. While *CIWI* transcript was detected not only in testes and ovaries but also

weakly in both male and female kidneys, *CILI* transcript was detected only in testes and ovaries. Expression pattern of *CIWI* and *CILI* observed in testis and ovary was different from each other and it suggests that each PIWI family has a different role during gametogenesis. Localization of *CIWI* and *CILI* mRNA was observed in the perinuclear cytoplasm of germ cells. *CIWI* mRNA was detected where undifferentiated spermatogonial stem cells, spermatogonia or primary spermatocytes exist and undergo division, but *CILI* mRNA was detected in the differentiating germ cells in the middle of the seminiferous tubule. In female ovaries, *CILI* mRNA was highly expressed in all kinds of follicles in the ovaries, whereas *CIWI* mRNA was detected at a low level in small prehierarchical and preovulatory hierarchical follicles. We also investigated the expression levels of *CIWI* and *CILI* throughout the embryonic stages. Furthermore we determined that *CIWI* and *CILI* were more highly expressed in PGCs than embryonic gonads (Kim et al. 2012). siRNA-mediated knockdown analysis of *CIWI* and *CILI* in chicken PGCs showed up-regulated expression of the chicken repetitive element (CR1) retrotransposons and induced DNA double strand breakage. These results indicated that *CIWI* and *CILI* play a critical role in maintaining the genome integrity of germ cells in chickens.

3. UNIQUE ASPECTS OF TRANSCRIPTION REGULATION IN GERM CELLS

Transcription is the first step of gene expression and primary control strategy used in living cells. Altered rates of protein syntheses are regulated by induction and repression of promoter initiation. Studying promoter sequences and regulations are required for comprehending regulation of global gene (Lu et al. 2004). Each gene has its own promoter, and some promoters can only be activated in a specific cell type.

3.1 Germ-cell specific programs of gene expression

A tissue/cell-specific promoter is a promoter that has activity in only certain cell types. (Zheng and Baum 2008) Regulation of cell type-specific gene transcription is central to cellular differentiation and development of the organism. (Reddi et al. 2007) Tissue/cell-specific promoters can be used for variety of applications, including programmed gene expression in cell types, tissues and organs of interest, and for developing different cell culture models. (Kuzmin et al. 2010) Questions of how germ cell-specific programs of gene expression have been studied but not yet fully understood. Part of the answer comes from the regulatory factors, which is associated with core promoter and

transcription factor which binds to the promoter. Perhaps germ cell-specific promoter and transcription may be required to separate somatic and germ cell lineage and may be related to the meiotic and mitotic cell cycles (DeJong 2006). Issues with distinctions between germ cell and somatic cell transcription system can be revealed from the germ cell-specific promoter and transcription factor. To carry out the biological functions, spermatocytes and oocytes must establish specialized gene expression programs. Many researchers suggest that biochemical mechanisms of transcription initiation may be unique in germ cells. In fact, recent studies in various species such as mouse, frogs and flies have shown that germ cells use alternate forms of core promoter transcription. In addition, initiation of transcription in germ cells is generated from tissue-specific core promoters that are silent in somatic cells (DeJong 2006).

3.2 Putative transcriptional binding elements in germ cell-specific gene

A number of regulatory factors, including Sp1, NF-κB, CREMτ, BORIS, SREBP2, FIGα, and many others, have been proposed to regulate genes in germ cells (Foulkes et al. 1992; Lilienbaum et al. 2000; Thomas et al. 2005; Wang 2004).

Cyclic AMP response element (CRE) binding protein (CREB) and cyclic AMP response element modulator (CREM) are proteins that regulate transcription in response to various stress, metabolic and developmental signals (Hummler et al. 1994; Sassone-Corsi 1995). CREM exists in multiple isoforms that act as repressors or activators (Foulkes and Sassone-Corsi 1992). CREM knockout leads to early apoptosis of haploid cells and male sterility, suggests that CREM plays an essential role in spermiogenesis (Blendy et al. 1996; Nantel et al. 1996; Nantel and Sassone-Corsi 1996).

In mouse, testis-expressed paralogs of TFIID components have been identified, which is TBP-related factor, TRF2. In mouse, TRF2 is highly expressed in male germ cells, whereas TRF3 expression is restricted to the ovary (Martianov et al. 2001; Xiao et al. 2006). TRF2 deficient mice in male showed sterile while TRF2 deficient mice in female showed fertile. Almost complete arrest of spemiogenesis due to the apoptosis of round spermatid was identified in TRF2 deficient mice in male (Martianov et al. 2002).

Recently, co-expression of BLIMP1, AP2γ and PRDM14 can induce PGC-like cells (PGCLCs) by substitution of the cytokines, which directs the induction of PGC, *in vitro*. BLIMP1, PRDM14 and AP2γ contribute to the repression of mesodermal

genes in PGCs to set them apart from their somatic neighbors. These three factors proposed tripartite genetic network for PGC specification (Magnusdottir et al. 2013).

3.3 PIWI interacting transcription factors in diverse organisms

In case of *PIWI*, promoter of mouse Miwi gene was studied. Hou et al. cloned the functional promoter of the mouse *Miwi* gene and they identified a 303-bp proximal promoter region of *Miwi* that controls gene expression from mid-pachytene spermatocytes to round spermatids during meiosis. They also found that CpG islands within the proximal promoter of the *Miwi* gene showed an inverse correlation between the methylation position and germ cell-specific expression (Hou et al. 2012). Interestingly, the *Miwi* gene promoter lacks a TATA box, while a CCAAT box was identified within the core promoter region. Thus, the mutated CCAAT box significantly decreased expression activity, indicating that it is important for transcriptional regulation of the *Miwi* gene (Hou et al. 2012). The CCAAT box is one of the most common *cis* elements found in eukaryotic promoters, and acts as a binding site for the RNA transcription factor NF-Y, which is identical to the CCAAT-binding factor (CBF). The CCAAT box-NF-Y

interaction is required for transcriptional activation of several eukaryotic genes (Dolfini et al. 2012).

In mouse, Hou et al. identified another two enhancer boxes (E-box) in 303bp proximal promoter region of *Miwi* gene (Hou et al. 2012). E-box is a DNA sequence which exists upstream of some promoter regions and acts as a transcription factor to initiate gene transcription (Massari and Murre 2000). In the *Miwi* gene promoter, methylation at the E2 box in the proximal region inhibited binding of the upstream stimulatory factor (USF), and caused a complete block of *Miwi* gene expression (Hou et al. 2012). They suggest that USF controls *Miwi* expression from mid-pachytene spermatocytes to round spermatids through methylation-mediated regulation. Another transcription factor A-MYB also binds to the *Miwi* promoter and associates with production of pachytene piRNA. Induction of A-MYB at the pachytene stage of spermatogenesis initiates production of pachytene piRNAs. A-Myb mutant testes showed the reduced level of *Miwi* and disrupted transcription of piRNA precursor.

Thus, a short promoter sequence and a single transcription factor, such as the CCAAT box and NF-Y, in germ cells could control transcriptional initiation of genes specific for cellular proliferation and cell-lineage-specific expression (Qyang et al. 1999).

CHAPTER 3.

THE CCAAT ELEMENT IN THE *CIWI* PROMOTER REGULATES TRANSCRIPTIONAL INITIATION IN CHICKEN PRIMORDIAL GERM CELLS.

1. Introduction

Primordial germ cells (PGCs) are the progenitor of germ cells, which produce functional gametes and transmit the genetic information to the next generation. PGCs are capable of specializing themselves from other somatic cells and undergoing extensive regulations during development. Understanding the molecular mechanisms that regulate cell fate decisions during early PGC development is important for fundamental research in germ cell biology.

In early embryo development, PGC lineage is governed by orchestrated actions of many factors including transcriptional and post-transcriptional regulators. The recent evidence suggests that the PIWI-piRNA complex may regulate epigenetic states via a similar mechanism in multicellular organisms. (Juliano et al. 2011). Interestingly, PIWI protein expression is mainly restricted to germ cells and stem cells, (Thomson and Lin 2009) and are required to maintain fertility. Also repressing transposons in the germline and regulating gene expression at the epigenetic, post-transcriptional, and translational levels are the main function of PIWI protein in germ cells. (Juliano et al. 2011) PIWI-piRNA complex involves in post-transcriptional regulation in diverse organisms.

We identified chicken PIWI-like protein 1 (CIWI) and 2 (CILI) from 25-wk-old adult male testis cDNA library. We have found that PIWI domain in the PIWIL1 protein showed relatively high similarities between mammals and birds. *CIWI* and *CILI* genes of the Piwi family were identified in chicken embryonic and adult stages in both males and females. Also superior expression of *CIWI* and *CILI* in PGCs suggests that *CIWI* and *CILI* were specifically restricted to germ cell lineage during development. Expression of CR1 retrotransposons were elevated in both *CIWI*- and *CILI*-knockdown PGCs compared to the control. Also we found r-H2A. X, which is a marker for double strand breakage, in *CIWI* knockdown PGCs, while we could not detect it in the control PGCs (Kim et al. 2012).

In chicken, *CVH* (Chicken *Vasa* homologue) gene has been cloned. Regulating manner of *CVH* transcription was estimated by an analysis of the promoter sequence. VASA is an RNA binding protein with an RNA dependent helicase which is essential for germ cell development (Raz 2000). Its expression is exclusively restricted to the germ cell lineage throughout the embryonic development (Hay et al. 1988; Lasko and Ashburner 1988). Homologous of VASA were identified in chickens, and it showed the germline-specific expression throughout embryogenesis and in the adult both testis and ovary (Tsunekawa et al. 2000). Minematsu et al. has found that germ cell-specific gene

expression required 1,555-bp sequence of the 5' flanking region of *CVH* gene. Germ cell specificity of GFP expression driven by the *CVH* promoter was disclosed both *in vitro* and *in vivo* (Minematsu et al. 2008).

In 2012, Hou et al. have identified the functional promoter of the mouse *Miwi.* MIWI is one of mouse PIWI homolog which expressed in cytoplasm specifically in the testis from mid-pachytene spermatocytes to round spermatids (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2001). 303-bp proximal promoter region of *Miwi* gene has been identified. CpG islands within the proximal promoter of *Miwi* gene showed an inverse correlation between the methylation position and germ cell-specific expression. Interestingly, the promoter of *Miwi* gene lacks a TATA box. Instead, CCAAT box was identified within the core promoter region. Mutated CCAAT box significantly decreased expression activity of *Miwi* gene, suggesting that CCAAT box is important for transcriptional regulation of the *Miwi* gene (Hou et al. 2012).

Eukaryotic transcription is regulated by several DNA elements. Regulation of transcription is conducted with promoter-proximal elements, and distal enhancer elements, and both are recognized by several transcription factors. TATA, GC and CCAAT boxes are the main regions of core promoter. The CCAAT box is one

of the most typical elements for transcriptional activation (Kabe et al. 2005). The CCAAT box is one of the most common *cis* elements found in eukaryotic promoters, and acts as a binding site for the RNA transcription factor NF-Y, which is identical to the CCAAT-binding factor (CBF). The CCAAT box and NF-Y interaction is required for transcriptional activation of several eukaryotic genes (Dolfini et al. 2012). Especially, NF-Y is necessary for recruiting RNA polymerase II to TATA-less gene (Kabe et al. 2005). NF-Y is ubiquitous transcription factor which is necessary for CCAAT box binding. NF-Y associates with TATA box binding protein (TBP) and several TBP-associated factors (TAFs) which are essential factors for initiation of transcription (Bellorini et al. 1997; Frontini et al. 2002). SP1, a zinc finger transcription factor that binds to GC-rich motifs of many promoters, is involved in many cellular processes, such as cell differentiation, cell growth and DNA damage. TFAP2α also functions as a sequence-specific DNA-binding transcription factor, which binds to the specific DNA sequence and recruits the transcriptional machinery (Williams and Tjian 1991).

In the present study, we cloned a promoter of *CIWI* gene and estimated the cultured chicken PGC-specific expression using fluorescent protein. *CIWI*_eGFP vector revealed that 252-bp core promoter of the *CIWI* gene control the specific expression

in germ cell. In addition, we also found that proximal promoter of *CIWI* has no TATA box sequence. Similar to *Miwi* promoter, transcriptional initiation element CCAAT was identified in the 252-bp of *CIWI* promoter. Mutation assays by substituting CCAAT with other nucleotide sequences showed complete disruption of transcriptional activity in chicken PGCs. Sequence alignment among *CIWI, Miwi* and *HIWI* (Human *PIWI* like-1) showed high similarity. Specifically, all flanking regions of the CCAAT element were present in the predicted binding sites for the same transcription factors. We are suggesting that chicken and mammals have a similar regulation mechanism of genes that are transcribed specifically in germ cells. The sequence of *CIWI* promoter will be widely applicable to research studies in gametogenesis and other areas of reproductive biology, especially in development and differentiation of primordial germ cells in the early stage embryos using transgenic chickens.

2. Material and Methods

Experimental animals and animal care

The care and experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University, Korea (Approval No: SNU-070823-5). Chickens were maintained according to a standard management program at the University Animal Farm, Seoul National University. Procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Construction of expression vectors controlled by the CIWI promoter

For cloning of the *CIWI* promoter, genomic DNA from adult chicken blood was isolated using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) and used as PCR template. Primer sets shown in Table 1 were used to clone fragments of the *CIWI* promoter of different sizes. PCR products of the correct size were cloned into the pGEM T easy vector (Promega,

Madison, WI, USA). A 4-kb genomic fragment from -3,839 to +161 bp, including the predicted transcription start site, was cloned, but the +1 to +161-bp region includes the 5' -untranslational region (5' -UTR) and does not include the ATG start codon (Chen et al. 2013). The eGFP-coding sequence and polyadenylation (poly A) tail were inserted into the cloned vectors containing the *CIWI* promoter using restriction enzymes *Spe*I and *Nde*I. For the promoter deletion assays, another four expression vectors containing *CIWI* promoter fragments of different sizes and positions were constructed; a 2,986-bp fragment (-2,825 to +161), 1,985-bp fragment (-1,824 to +161), 1,000-bp fragment (-839 to +161), and 3,000-bp fragment (-3,839 to -839). Subsequently, the following fragmented promoters between -839 and +161 bp were designed and constructed: -839 to -567-bp fragment, -577 to -326-bp fragment, -329 to -90-bp fragment, and -89 to +161-bp fragment.

Culture of chicken primordial germ cells

Chicken PGCs were cultured according to our standard procedure (Park and Han 2012b). Briefly, chicken PGCs from White Leghorn (WL) embryonic gonads at day 6 (stage 28) were maintained and sub-passaged in knockout Dulbecco's

Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 2% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1× nucleosides (Millipore, Billerica, MA, USA), 2 mM L-glutamine (Gibco), 1× nonessential amino acids (Gibco), β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco) and 1×antibiotic-antimycotic (Gibco). Human basic fibroblast growth factor (10 ng/ml) (bFGF, Koma Biotech, Korea) was used for PGC self-renewal. Chicken PGCs were maintained in an incubator at 37° C with an atmosphere of 5% CO_2 and 60-70% relative humidity. The cultured PGCs were subcultured onto mitomycin-inactivated mouse embryonic fibroblasts (MEFs) at 5 to 6-day intervals by gentle pipetting without any enzyme treatment.

Culture of chicken embryonic fibroblasts (CEFs)

The primary culture of CEFs was prepared from the muscles of 6-day-old WL chicken embryos. Single cell populations were obtained using 0.05% trypsin-EDTA (Gibco) treatment to dissociate cells and maintained in DMEM with high glucose (Hyclone), 10% FBS and 1×antibiotic-antimycotic. The cells were seeded approximately 5×10^5 cells/well. Cultured cells were grown at 37° C in a 5% CO_2 incubator.

In vitro *transfection*

In vitro transfection was performed using the lipofection method with Lipofector EZ (Aptabio Therapeutics, Suwon, Korea) according to the manufacturer's instructions. The 4-kb *CIWI* promoter vector (10 μg) and 5 μl of Lipofector EZ were separately diluted with 100 μl of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA) and incubated at room temperature for 5 min. Liposome-DNA solutions were then mixed and incubated at room temperature for 20 min to form the lipid-DNA complex, which was added to $1x10^5$ cultured PGCs in 500μl of PGC culture medium. Transfected cells were cultured for 24 h without feeders and maintained for another 48 h on MEFs. After 3 days of incubation, cells were observed under a fluorescence microscope.

Immunocytochemistry of transfected PGCs

Cultured chicken PGCs were fixed in 4% paraformaldehyde for 1 day at room temperature, and incubated with 1:200 diluted anti-mouse stage-specific embryonic antigen 1 antibody (SSEA-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4° C overnight. After washing with PBS, PGCs were incubated with a secondary antibody labeled with phycoerythrin (Santa

Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Cells were finally mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) and visualized under a fluorescence microscope.

Flow cytometry analysis

For flow cytometry analysis, transfected PGCs were resuspended in PBS containing 1% BSA and passed through a cell strainer (40 μm, BD Falcon; Becton Dickinson, Canada). Fluorescence levels in transfected PGCs were calculated using FACSCalibur (Becton Dickinson) with excitation at 488 nm for GFP detection. All subsequent analyses were performed using the FlowJo software (Tree Star, Ashland, OR, USA).

In vivo *transfection*

About 6ul of Lipofector EZ reagent was diluted with 4ul of Opti-MEM I and 5ug of DNA plasmid was diluted with 5ul of Opti-MEM I. After incubation at room temperature for 5 min, Lipofector EZ and DNA plasmid were combined together and incubated at room temperature for 20 min in order to form the

lipid-DNA complexes. Using a well sharpen capillary tube, 2ul of lipid-DNA complex solution was injected into the bloodstream of 2.5-day incubated embryos. The manipulated embryos were incubated at 37 ℃ for another 3.5 days. Embryonic gonads dissected from 6-day-old recipient embryos were observed under a fluorescent microscope.

Construction of the CCAAT mutation vector

CCAAT sequences of the *CIWI* promoter were substituted by TTCCT sequences, and TTCCT sequences were cloned into the pUC57 vector by Bio Basic Inc. (MO, Canada). The synthesized mutant sequences were cloned into the *CIWI* promoter expression vector by means of *Spe*I and *Sac*II digestion and ligation.

Multiple sequence alignment and comparative analysis of putative transcriptional binding elements in PIWI promoters between species

Mouse PIWI like-1 (*Miwi)* and human PIWI like-1 (*HIWI)* promoter was used to predict transcription factor and multiple sequences align. About 325 bp of *Miwi* promoter was derived

from Hou et al. 2012. 200 bp of *HIWI* promoter was induced by the University of California at Santa Cruz Genome Browser (UCSC, http://www.genome.ucsc.edu). Transcriptional binding elements in these promoters were predicted using the software MatInspector (Genomatix, Munich, Germany) and PROMO (Alggen, Barcelona, Spain). Multiple sequence alignment with *HIWI*, *Miwi* and *CIWI* promoters was conducted using the Geneious software (ver. 6.0.5, Auckland, New Zealand).

3. Results

The eGFP vector containing 4-kb of *CIWI* promoter was expressed only in cultured chicken primordial germ cells (PGCs).

For the identification of promoter region in *CIWI*, the gene sequences from the National Center for Biotechnology Information (NCBI, Gene ID: 416804) and the 5′-flanking sequences from the UCSC Genome Browser were analyzed initially. To disclose transcriptional elements responsible for *CIWI* expression, about 4kb genomic fragment from -3,839bp to +161bp at which the predicted transcription start site was cloned. However, +1 to +161bp is the 5'-untranslational region (5' UTR) that does not include the ATG start codon. To construct the expression vectors, eGFP gene and bovine polyA tail was ligated to the downstream of 4kb CIWI promoter. This 4kb expression vector containing *CIWI* promoter was specifically expressed in cultured chicken primordial germ cells (PGCs), which was also positive to PGCs specific marker SSEA-1 (Figure 1A). In contrast, eGFP vector containing *CIWI* promoter was not expressed in chicken embryonic fibroblasts (CEFs)(Figure 1B).

4-kb *CIWI* promoter vector is able to activate in chicken embryonic gonads

In embryogenesis, avian PGCs circulate temporarily in the blood vessels at stage 10-15, before reaching the gonads (Fujimoto et al. 1976b). Subsequently, PGC actively leaves capillary vessels, and finally settle in the developing genital ridges (Fujimoto et al. 1976a; Hamburger and Hamilton 1951; Meyer 1964). To validate the cloned *CIWI* promoter during developmental stages, *in vivo* transfection was conducted in chicken embryos. When injecting the complex of 4-kb *CIWI* promoter vector plasmid and liposome, circulating PGCs in blood vessel were lipofected during migration into embryonic gonads. In embryonic gonads of 6 days, eGFP-expressing PGCs were detected under fluorescence microscopy (Figure 1C).

Promoter deletion assay of the *CIWI* promoter

For the promoter deletion assay, we constructed another four different expression vectors containing *CIWI* promoter fragments of different sizes and positions; these included a 2,986-bp fragment (-2,825 to +161 bp), 1,985-bp fragment (-1,824 to +161 bp), 1,000-bp fragment (-839 to +161 bp), and 3,000-bp fragment (-3,839 to -839 bp) (Figure 2A). All

vectors, except the 3,000-bp fragment (-3,839 to -839 bp) vector, were strongly expressed in chicken cultured PGCs (Figure 2B). In contrast, none of expression vectors were expressed in CEFs (Figure 2C). Based on these results, the transcriptional *cis* elements located between -839 and +161 bp may be critical for *CIWI* expression. To further verify the efficiency of expression of each construct, the percentage of GFP-expressing cells was measured using flow cytometry in PGCs and CEFs. Compared to the CMV promoter, vector containing the 2,986-, 1,985- and 1,000-bp fragments of the *CIWI* promoter showed GFP expression in over 2% of PGCs, and the *CIWI* promoter showed significant promoter activity in PGCs. The flow cytometry results suggested that expression constructs (excluding the 3,000-bp construct) were strongly expressed in chicken PGCs, while none were expressed in CEFs.

Promoter fragmentation assay of the *CIWI* promoter

To identify the transcriptional elements crucial for *CIWI* expression, four fragmented promoters were designed and constructed : -839 to -567 bp, -577 to -326 bp, -329 to -90 bp and -89 to +161 bp (Figure 3A). Expression of these four constructs was examined using fluorescence microscopy and flow cytometry. Interestingly, the fragmented promoter from -577 to

−326 was expressed in PGCs only (Figure 3B). In contrast, no construct, including the −577 to −326 fragmented promoters, were expressed in CEFs (Figure 3C). Compared to other vectors, the −577 to -326 bp fragment of the *CIWI* promoter led to 1.44% of PGCs expressing GFP. This result suggests that the elements for transcriptional initiation of *CIWI* is located between −577 and −326 bp.

Mutated CCAAT box lost transcriptional activity in chicken PGCs

To identify the essential transcriptional elements in the 252bp *CIWI* gene promoter, putative transcription elements were predicted by using the PROMO software. However, no TATA box sequence was detected, but another transcriptional initiation element, a CCAAT box was observed within the 252bp (−577 to −326 bp) of *CIWI* promoter sequences. Sequence of CCAAT box was substituted by TTCCT sequences in order to verify the transcriptional activity of the CCAAT element (Figure 1A). Vector with mutated CCAAT element (TTCCT vector) completely lost the transcriptional activity of *CIWI* gene in chicken PGCs. Either CCAAT or TTCCT element were not expressed in CEFs. The flow cytometry results suggested that *CIWI* promoter including normal CCAAT element led to 2.01% of

PGCs expressing GFP, but mutated CCAAT showed 0% of PGCs expressing GFP (Figure 1B). This result suggested that the CCAAT box within the −577 to -326 bp region of the *CIWI* promoter is important for specific transcriptional expression of the *CIWI* gene in chicken PGCs.

Comparative analysis of putative transcriptional binding elements in *PIWI* promoters

Promoter sequences of Human PIWI like−1 (*HIWI*) and mouse PIWI like−1 (*Miwi*) were used for transcription factor prediction and multiple sequences alignment. About 200 bp (−100 to +100) of *HIWI* (from the University of California at Santa Cruz Genome Browser, http://www.genome.ucsc.edu) and 325 bp (−143 to +181) of *Miwi* promoter sequences (from Hou et al. 2012) were compared with 252−bp (−577 to −326 bp) fragments of the *CIWI* promoter. Sequence alignment showed overall similarities between chicken and human or chicken and mouse of ~50% (Figure 2A). Interestingly, all flanking regions of CCAAT element which is differently positioned in −76 to −7 bp of mouse, −132 to −65 bp of human, and −537 to −467bp of chicken showed higher similarity. In addition, all *HIWI, Miwi* and *CIWI* promoters contained a binding sequence of nuclear transcription factor Y (NF−Y), which bound strongly with the

CCAAT box. All of *Miwi, HIWI* and *CIWI* promoter contains NF-Y, which defined to mainly bind to CCAAT box. Additionally, the specificity protein 1 (Sp1) and transcription factor activating enhancer binding protein 2 alpha (TFAP2α) motifs were predicted to modulate the transcriptional initiation of *HIWI*, *Miwi* and *CIWI* by binding to the 5' or 3' -flanking regions of the CCAAT box (Figure 2B).

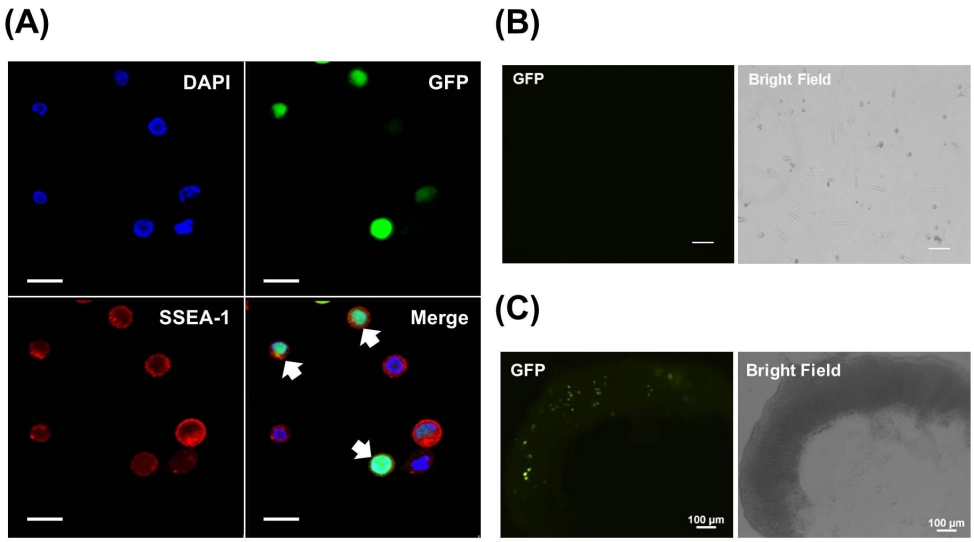

Figure 1. Expression of *CIWI* promoter vector in primordial germ cells both *in vitro* and *in vivo*. *In vitro* expression analysis of enhanced green fluorescent protein (eGFP) vector containing 4 kb of the chicken P-element induced wimpy testis-like 1 (*CIWI*) promoter in cultured chicken primordial germ cells (PGCs, A) and chicken embryonic fibroblasts (CEFs, B). After transfection of the *CIWI*-eGFP construct, PGCs were immunostained with a marker protein stage-specific embryonic antigen 1 (SSEA-1). Arrows indicate PGCs expressing eGFP vector containing the *CIWI* promoter, and SSEA-1 (scale bar = 20 μm). eGFP vector containing the *CIWI* promoter was not expressed in CEFs (scale bar = 100 μm). *In vivo* expression analysis of eGFP vector containing 4 kb of the *CIWI* promoter in gonad of 6-day embryo (C). scale bar = 100 μm.

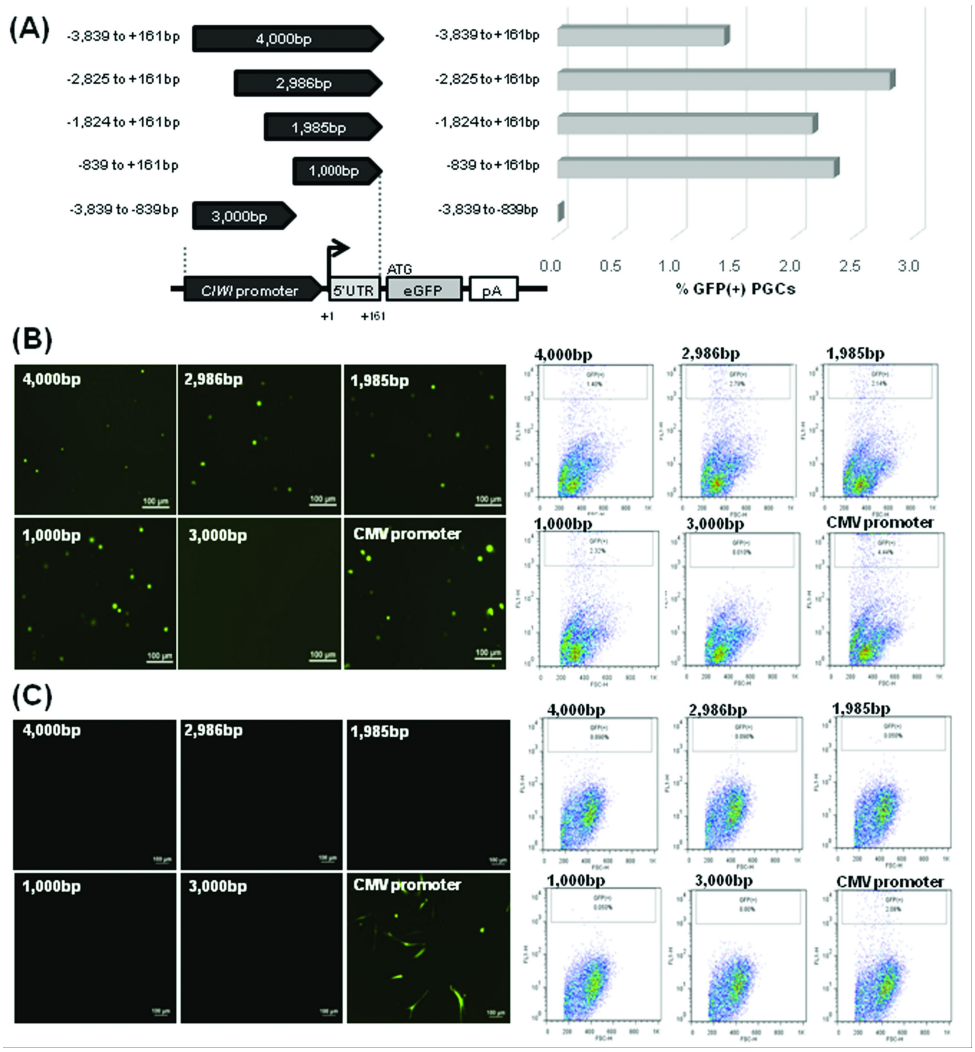

Figure 2. Deletion analysis of 4-kb *CIWI* promoter in PGCs and CEFs. (A) Schematic diagram of cloned *CIWI* promoters. About 4 kb of the *CIWI* promoter containing 161 bp of the 5' -UTR was cloned, and five constructs containing different lengths and promoter elements were designed for expression analysis using an enhanced green

fluorescent protein (eGFP) vector. The right panel indicates percentages of eGFP-expressing PGCs 3 days after transfection of each construct. Expression of eGFP vectors containing different constructs in cultured chicken primordial germ cells (PGCs, B) and chicken embryonic fibroblasts (CEFs, C) were analyzed by fluorescence microscopy and flow cytometry. eGFP construct with cytomegalovirus (CMV) promoter was used as a positive control. Scale bar = 100 μm.

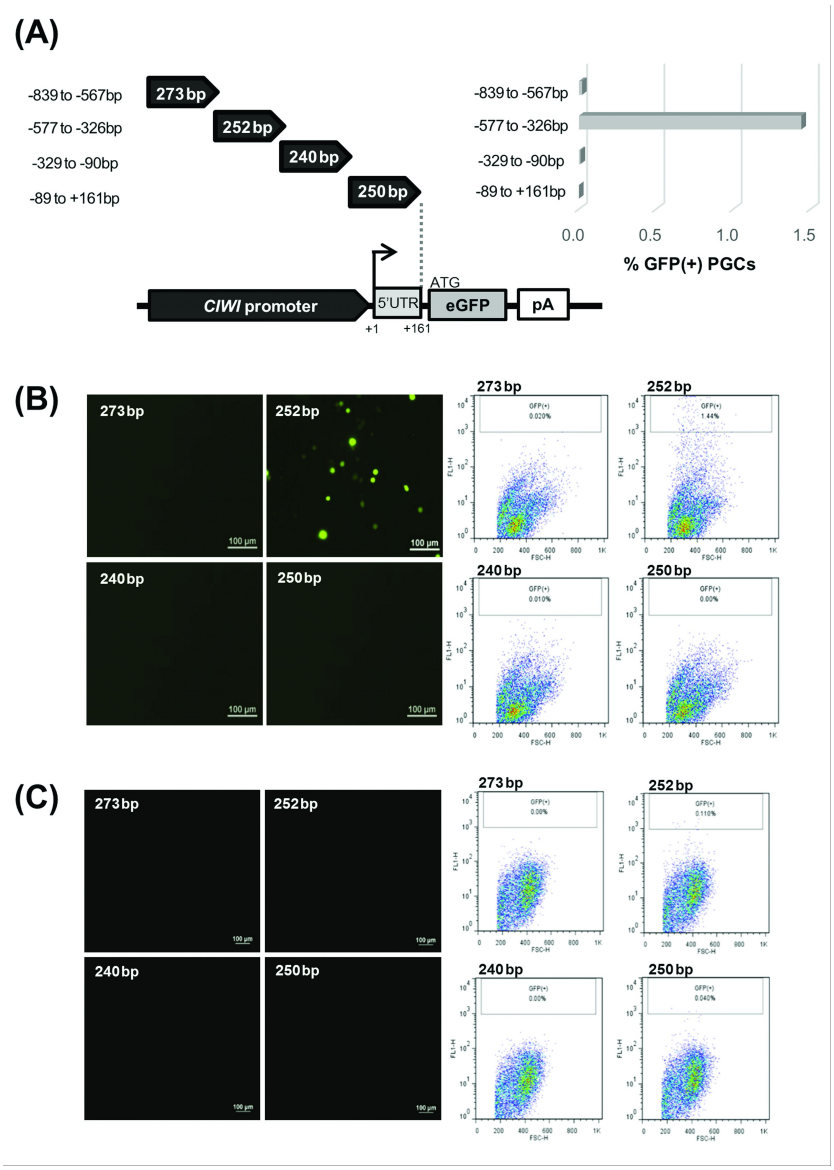

Figure 3. Identification of transcription regulatory elements in the −839−bp *CIWI* promoter containing 161 bp of the 5’ UTR.

(A) Schematic diagram of fragmented *CIWI* promoter constructs. Four constructs of the *CIWI* promoter of 250- 273 bp in size were designed

for identification of transcription regulatory elements using an enhanced green fluorescent protein (eGFP) vector. The right panel indicates percentages of eGFP-expressing PGCs 3 days after transfection of each construct. Expression of eGFP vectors containing different constructs in cultured chicken primordial germ cells (PGCs, B) and chicken embryonic fibroblasts (CEFs, C) were analyzed by fluorescence microscopy and flow cytometry. Scale bar = 100 μm.

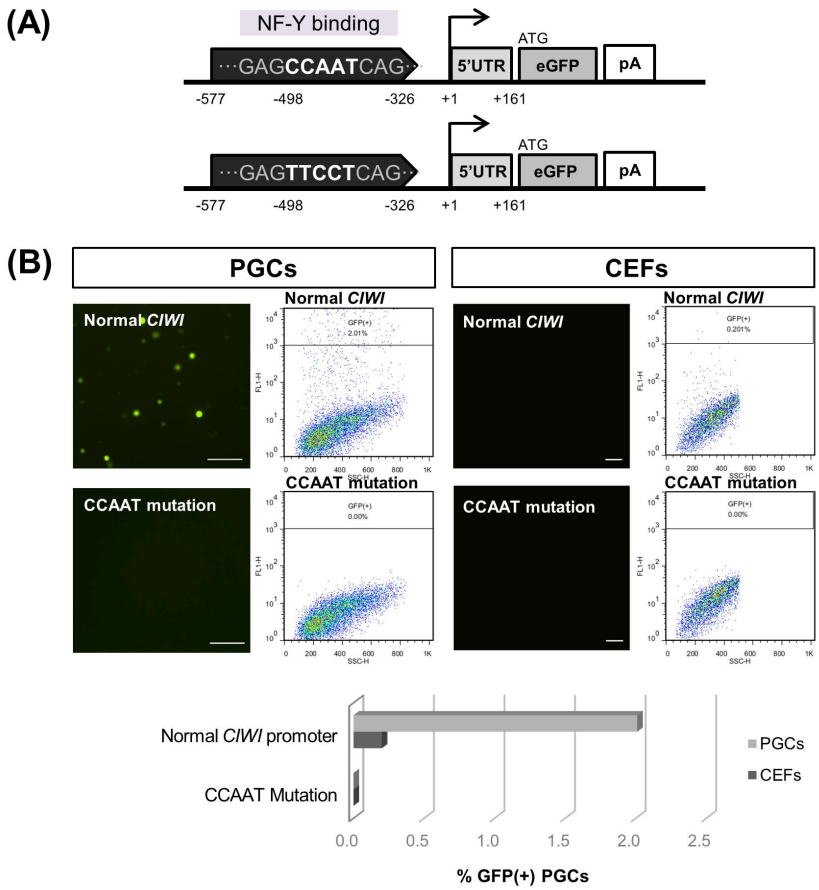

Figure 4. Mutation analysis of CCAAT box sequences in the transcriptional initiation element of *CIWI* promoter. (A) Schematic diagram shows the construct with substitution of the CCAAT box (at −498 bp) into TTCCT sequences in the RNA polymerase binding site. (B) Expression of enhanced green fluorescent protein (eGFP) vectors containing CCAAT or TTCCT sequence constructs in cultured chicken primordial germ cells (PGCs) and chicken embryonic fibroblasts (CEFs) were analyzed by fluorescence microscopy and flow cytometry. Scale bar = 100 μm.

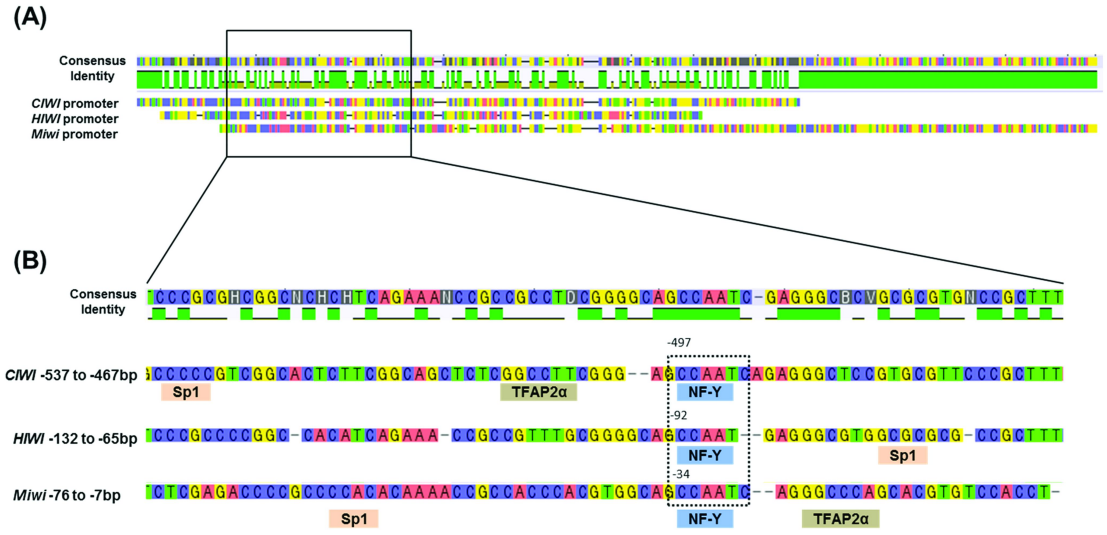

Figure 5 Multiple alignment of *CIWI* promoter with human and mouse *PIWI* promoters. (A) About 200 bp (−100 to +100 bp) of the *HIWI* promoter and 325 bp (−143 to +181bp) of the *Miwi* promoter were aligned with a 252−bp (−577 to −326 bp) fragment of the *CIWI*. (B) Alignment shows the proximal regions of the CCAAT element that were differently positioned in *HIWI*, *Miwi* and *CIWI*. The transcription factors NF−Y, SP1 and TFAP2α were predicted to bind to the 5' and 3' flanking regions of CCAAT in *HIWI*, *Miwi* and *CIWI*.

Table 1 List of primer sequences for cloning of the *CIWI* promoter using genomic PCR.

Primer Set	Primer Sequence (5' to 3')
CIWI −3,839bp_F	TGTGGTCAGGTACTCTGCTAAA
CIWI −2,825bp_F	CAGGATCCCAAAGTGCTGCTAACT
CIWI −1,824bp_F	CCTGCAGGCTACTGAGCAAGG
CIWI −839bp_F	ATGTCTGGAACAGAGCAACAAGTT
CIWI promoter_R	TTCCCTTTGAAGACAAAGCA
CIWI −3,839/−839_R	GCACAGAATGTGGACAAAAATAGAC
CIWI −839/−367_F	ATGTCTGGAACAGAGCAACAAGT
CIWI −839/−367_R	AGAGCCGAGGGCTGTGT
CIWI −577/−326_F	CCCTCGGCTCTGCCCGCT
CIWI −577/−326_R	GACCGCCGTCTCCACAACGC
CIWI −329/−90_F	GGTCTCCCGTTTGTCGCT
CIWI−329/−90_R	ACACTTACCAACACAATCCCGAC
CIWI−89/+161_F	GTCACTTCAAAGTGGCGTTG
CIWI−89/+161_R	TTCCCTTTGAAGACAAAGCAC
EGFP poly A _F	ACTAGTCCGCGGATGGTGAGCAAG
EGFP poly A_R	CATATGGACGTCTCCCCAGCATGCC

4. Discussion

PIWI protein is strictly expressed in germ cell and stem cell (Thomson and Lin 2009), and required to maintain germ cell fertility. Concurrently, epigenetic processing such as transposon repression, post-transcriptional and translational modifications is a main function of PIWI protein in germline development and maintenance of germ cell DNA integrity in variant species (Juliano et al. 2011; Klattenhoff and Theurkauf 2008).

Chicken PIWI-like protein 1 (CIWI) and 2 (CILI) remain unclear but recently not only we characterized the PIWI members in chicken, but also other groups have tried to figure out the mechanism and regulation of *PIWI* gene in chicken. Especially, Chen et al. identified the genomic structure of the *CIWI* gene includes a 161-bp fragment of the 5' UTR and 660-bp of the 3' UTR followed by a poly (A) tail (Chen et al. 2013).

In this study, to evaluate regulatory elements of the *CIWI* promoter, we first identified 4-kb of the *CIWI* promoter including +161 bp of the 5' UTR. Subsequently, the enhanced green fluorescent protein (eGFP) as a reporter gene under the control of the cloned 4-kb *CIWI* promoter was specifically

expressed in chicken PGCs, but not in CEFs. Promoter deletion assay assists to narrow down the 5' upstream of *CIWI* sequence. 2,986-bp, 1,985-bp fragment, 1,000-bp fragment, and 3,000-bp fragment of *CIWI* promoter vector was constructed and all vector, except the 3,000-bp fragment vector, were strongly expressed in chicken cultured PGCs. Finally, promoter fragmentation assay shows that -577 to -326 bp (252-bp) of *CIWI* is the essential region for cPGCs-specific transcriptional activity. In addition, we identified a 252-bp *CIWI* promoter region containing the CCAAT box that controlled germline-specific expression of the *CIWI* gene in chickens. 252-bp of *CIWI* promoter lacks TATA box sequences, but another transcriptional initiation element CCAAT was identified in the -498 to -494 bp fragment of the *CIWI* promoter. Mutation assays by substituting CCAAT with TTCCT sequences showed complete disruption of transcriptional activity in chicken PGCs. It suggests that CCAAT specifically affects transcriptional activity of *CIWI* in cPGCs.

The CCAAT box is one of the most typical elements for transcriptional activation. Especially, CCAAT box plays important role in promoter of TATA-less gene. TATA box is necessary for recruiting RNA polymerase II to initiate transcription. In TATA-less gene, CCAAT take place for recruiting RNA polymerase II and assists initiation of transcription. NF-Y is a

ubiquitous transcription factor which binds to CCAAT box, and associates with TBP and TAFs, the essential transcription factors for initiating transcription. Since NF-Y associates with TBP and TAFs, NF-Y is able to recruit RNA polymerase II and general transcription factors onto CCAAT box-containing promoters. It suggests that NF-Y and CCAAT box is necessary in TATA-less gene.

Interestingly, similar to *CIWI*, the *Miwi* gene promoter lacks a TATA box, while a CCAAT box was identified within the core promoter region. Thus, the mutated CCAAT box significantly decreased expression activity, indicating that its importance for transcriptional regulation of the *Miwi* gene (Hou et al. 2012). These results indicated that chickens and mammals have a similar mechanism of regulation of regulatory processes that are transcribed specifically in germ cells.

In conclusion, we discovered that 252-bp sequence of the 5' flanking region of *CIWI* gene is necessary for the cultured chicken PGC expression, and that 252-bp is a fairly small sequence for regulating cell-specific gene expression. Also, we found that 252-bp of *CIWI* containing the transcriptional regulatory element CCAAT box is crucial for regulating *CIWI* expression in chicken PGCs. Thus, a short promoter sequence, such as the CCAAT box, in chicken germ cells could control

transcriptional initiation of genes specific for cellular proliferation and cell-lineage-specific expression (Qyang et al. 1999). Finally, the study about *CIWI* promoter could be one of the alternative methods for understanding the chicken PGCs specific expression induction, and also be widely applicable to research studies in development and differentiation of primordial germ cells.

CHAPTER 4.

PUTATIVE TRANSCRIPTION FACTOR AFFECTS EXPRESSION OF *CIWI* IN CHICKEN PRIMORDIAL GERM CELLS

1. Introduction

Transcription factor is a protein that binds to specific DNA sequences, controlling the transcription of genetic information from DNA to mRNA (Latchman 1997). Transcription factor such as Sp1, NF-κB, CREMτ, BORIS, SREBP2, FIGα and many other factors are proposed to regulate genes in germ cells (Foulkes et al. 1992; Lilienbaum et al. 2000; Thomas et al. 2005; Wang 2004).

In case of Mouse, Hou et al identified a 303-bp proximal promoter region of mouse Piwi-like 1 (*Miwi*) gene. They found two enhancer boxes (E-box) and one CCAAT box in a proximal promoter region (Hou et al. 2012). E-box is a DNA sequence which exists upstream of some promoter regions, and acts as a protein, especially transcription factor, binding sites. Sequences of E-box are bound by transcription factors to initiate gene transcription (Massari and Murre 2000). In the promoter of *Miwi* gene, methylation at the one of E-box inhibited binding of the upstream stimulatory factor (USF). USF is a transcription factor which activates transcription through binding of E-box motifs (Shieh et al. 1993). Since methylated E-box can't interact with USF, expression of *Miwi* gene was blocked completely. Also transcription factor A-MYB binds to the *Miwi* promoter and associated with production of pachytene piRNA. A-Myb mutant

testes showed reduced level of *Miwi* and disrupted transcription of piRNA precursor. It suggests that single transcription factor in germ cells could control transcription initiation of genes.

In Chapter 4, we verified that transcription factor NF-Y, SP1 and TFAP2α were predicted to bind the promoter of *CIWI, Miwi* and *HIWI.* NF-Y, SP1 and TFAP2α motifs were predicted to modulate the transcriptional initiation of *PIWI* family by binding to the 5' flanking region of genes. Prediction of transcription factor, which binds to the -577 to -326 bp of *CIWI*, revealed that several transcription factors, such as ZIC3, PAX2 and PAX5 might influence the CIWI expression. In this chapter, we verified that several transcription factors affects transcriptional activity of *CIWI* and other germ cell-specific gene in cPGCs.

In future study, transgenic lines having reporter gene specifically regulated by *CIWI* transcriptional elements could be utilized for the understanding of germ cell specification, differentiation and development in avian species.

2. Material and Methods

Construction of mutation vector of SP1-transcription factor binding site

Vector with normal sequences of 252-bp *CIWI* promoter were substituted by C to T point mutation of predicted SP1 transcription factor binding sites. Mutated sequences were cloned into the pUC57 vector by Bio Basic Inc. (MO, Canada). The synthesized mutant sequences were cloned into the *CIWI* promoter expression vector by means of *Spe*I and *Sac*II digestion and ligation.

In vitro *transfection*

In vitro transfection was performed using the lipofection method with Lipofector EZ (Aptabio Therapeutics, Suwon, Korea) according to the manufacturer's instructions. The 4-kb *CIWI* promoter vector (10 μg) and 5 μl of Lipofector EZ were separately diluted with 100 μl of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA) and incubated at room temperature for 5 min. Liposome-DNA solutions were then

mixed and incubated at room temperature for 20 min to form the lipid-DNA complex, which was added to $1x10^5$ cultured PGCs in 500㎕ of PGC culture medium. Transfected cells were cultured for 24 h without feeders and maintained for another 48 h on MEFs. After 3 days of incubation, cells were observed under a fluorescence microscope.

Flow cytometry analysis

For flow cytometry analysis, transfected PGCs were resuspended in PBS containing 1% BSA and passed through a cell strainer (40 ㎛, BD Falcon; Becton Dickinson, Canada). Fluorescence levels in transfected PGCs were calculated using FACSCalibur (Becton Dickinson) with excitation at 488 nm for GFP detection. All subsequent analyses were performed using the FlowJo software (Tree Star, Ashland, OR, USA).

Knockdown analysis through siRNA transfection

Chicken PGCs were cultured according to our previous report. 100 pmole of each siRNAs (ST Pharm, Seoul, Korea; Table 2) were transfected to 1 x 10^5 PGCs with Lipofectamine RNAiMax transfection reagent (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer' s protocol and the transfected PGCs were seeded to each well of 24-well culture plates. siRNA-treated PGCs were analyzed after 96h.

Bioinformatics prediction of transcription factor binding site

Transcription factor binding sites were predicted in the genomic DNA sequence of chicken *CIWI* using the software MatInspector (Genomatix, Munich, Germany) and PROMO software (Alggen, Barcelona, Spain) with a threshold score of 90 and animal matrices.

Sample collection & Quantitative RT-PCR analysis

We collected gonadal PGCs (gPGCs) and gonadal stromal cells (GSCs) on E6.5 by magnetic-activated cell sorting (MACS). Total RNA was extracted from each sample with Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the included protocol. The RNA quantity was determined by spectrophotometry at 260nm, and 0.8 μg of each RNA was reverse-transcribed with the Superscript III First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The cDNA was diluted 4-fold and quantitatively equalized for PCR

amplification using specific primer sets (Table 1). Primers were designed using the website of bioneer (http://web.bioneer.co.kr/cgi-bin/primer/primer3.cgi). Quantitative RT-PCR was performed using EvaGreen (Biotium, Hayward, CA, USA). 20 μl PCR reaction mix contained 2 μl of cDNA, 2μl of PCR buffer, 1.6 μl of 2.5mM dNTP mixture, 10pmol of each forward and reverse primer, 1 μl of 20x EvaGreen and 1 unit of taq DNA polymerase. The reaction was performed in optical 96 well standard plates (Applied Biosystems Inc. Foster City, CA, USA). Each test sample was performed in triplicate. The PCR conditions were 94℃ for 3 min, followed by 40 cycles at 94℃ for 30 sec, 59℃ for 30 sec and 72℃ for 30sec, using a melting curve program and continuous fluorescence measurement. The results are reported as the relative expression after normalization of the transcript to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control, with the nonspecific control as a calibrator, using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

3. Results

Point mutation analysis of SP1-binding site showed decreased expression of *CIWI*

The putative binding site of transcription factor SP1 was predicted in −577 to −326bp of *CIWI* promoter, Similarly, SP1 binding site was also detected in −100 to +100bp of *HIWI* promoter and −143 to +181bp of *Miwi* promoter. To verify transcription activity of SP1 transcription factor, point mutation analysis was performed on the predicted SP1 binding sequences (Figure 1A). Vector harboring the mutated SP1 binding site was expressed in cPGCs but not in CEFs (Figure 1B). The expression vector with point mutated sequence of SP1-binding site showed 0.546% while the vector with original *CIWI* sequence showed 2.01% of GFP-positive PGCs. This result indicated that the transcription factor SP1 is implicated in transcription regulatory activity of *CIWI* gene.

Transcription factor SP1, NF-Y and TFAP2α affects transcription activity of *CIWI*

The putative binding sites for transcription factor SP1, NF-Y and TFAP2α were predicted in 252bps of *CIWI* promoter. To examine whether these three transcription factors affect transcriptional activity of *CIWI* and germ cell-specific genes such as *DAZL* and *VASA*, we synthesized knockdown siRNAs for each transcription factor (Table 2) and then transfected into cPGCs. Each siRNA efficiently decreased the expression of each target gene (Figure 2A). Subsequently, the germ cell-specific gene expression was validated in the knockdown PGCs by each siRNA (Figure 6B-6D). Expression of *CIWI* was decreased in all of transcription factor-knockdown PGCs. In case of SP1-knockdown PGCs, the expression level of *CIWI* was decreased by 41% compared with control (Figure 2B) and also down-regulated with 29.7% and 24% reduction in NF-Y and TFAP2α-knockdown PGCs, respectively. *DAZL* and *VASA* were also down-regulated in all of transcription factor -knockdown PGCs (Figure 2C and 2D). These results showed that these transcription factors might affect the regulation of germ cell-specific genes including *CIWI*, *DAZL* and *VASA*, and so transcription factors of SP1, NF-Y and TFAP2α assist the transcriptional regulation of germ cell-specific genes in chicken PGCs.

Predicted transcription factors were strongly expressed in chicken PGC

Several transcription factor binding sites were predicted in −577 to -326 bp of *CIWI*. We used MatInspector and PROMO software to detect predicted transcription factor (Figure 3). Transcription factor such as NF−1, NF−Y, CDX1, ZIC2, ZIC3, PAX2, PAX5, PAX6, STAT4, MSX1, MyoD1, TFAP2α, EP300, CTCF, XBP1, NRF1, E2F3, MYT1L, GCM1, C/EBPα, SP1, GLIS3 and several other factors were detected. Using quantitative RT−PCR, expression of all transcription factor was analyzed in gPGCs (MACS (+) cells), GSCs (MACS (−) cells), CEFs and Snuhp26 (cultured chicken PGCs) (Figure 4). Several genes of transcription factor were expressed strongly in PGC but not in GSC or CEF. On the basis of the qRT−PCR data, six putative transcription factors were selected. Transcription factor ZIC3, NRF1, E2F3 and STAT4 were expressed strongly in gPGCs and cPGCs rather than GSCs and CEFs. PAX2 and PAX5 were also selected due to its original role and expression pattern.

ZIC3, PAX2 and PAX5 affect transcription activity of *CIWI, DAZL* and *VASA*, the germ cell-specific gene

Due to the lack of antibodies, we could not identify the direct binding between transcription factors and promoter of *CIWI*. Instead, we chose an indirect way to identify the binding factors through treating siRNAs of transcription factor gene into the cultured chicken PGCs and identified the gene level of *CIWI*. cDNA of siRNA treated cPGCs were used as template DNA. Quantitative RT-PCR was used to verify each rate of knockdown (Figure 5A). We used siRNA to identify the expression of *CIWI*. Compared to the cDNA of negative control cells (siRNA non-treated cells), cDNAs of each ZIC3, PAX2 and PAX5 siRNA treated cells showed relatively low level of *CIWI* expression. In addition, cDNAs of each ZIC3, PAX2 and PAX5 siRNA treated cells showed relatively low level of *DAZL* and *VASA* which are well known to be germ cell-specific genes. cDNA of ZIC3 siRNA-treated cells showed 55% decrease level of *CIWI* and 51.7%, 42.5% decreased level of each *DAZL* and *VASA* gene compared to control (Figure 5B). Expression of *CIWI, DAZL* and *VASA* were decreased by 25.6%, 30.4% and 45.3% in PAX2-knockdown PGCs compared with control (Figure 5C). Expression of *CIWI, DAZL* and *VASA* were also decreased by 21.4%, 21.9% and 14.3% in PAX5-knockdown PGCs compared with control (Figure 5D). Expression level of *CIWI, DAZL* and

VASA was not changed in NRF1, E2F3 and STAT4-knockdown PGCs (data not shown). It suggests that siRNA treated knockdown analysis showed that some of transcription factor may affect the activity of germ cell-specific gene including *CIWI.*

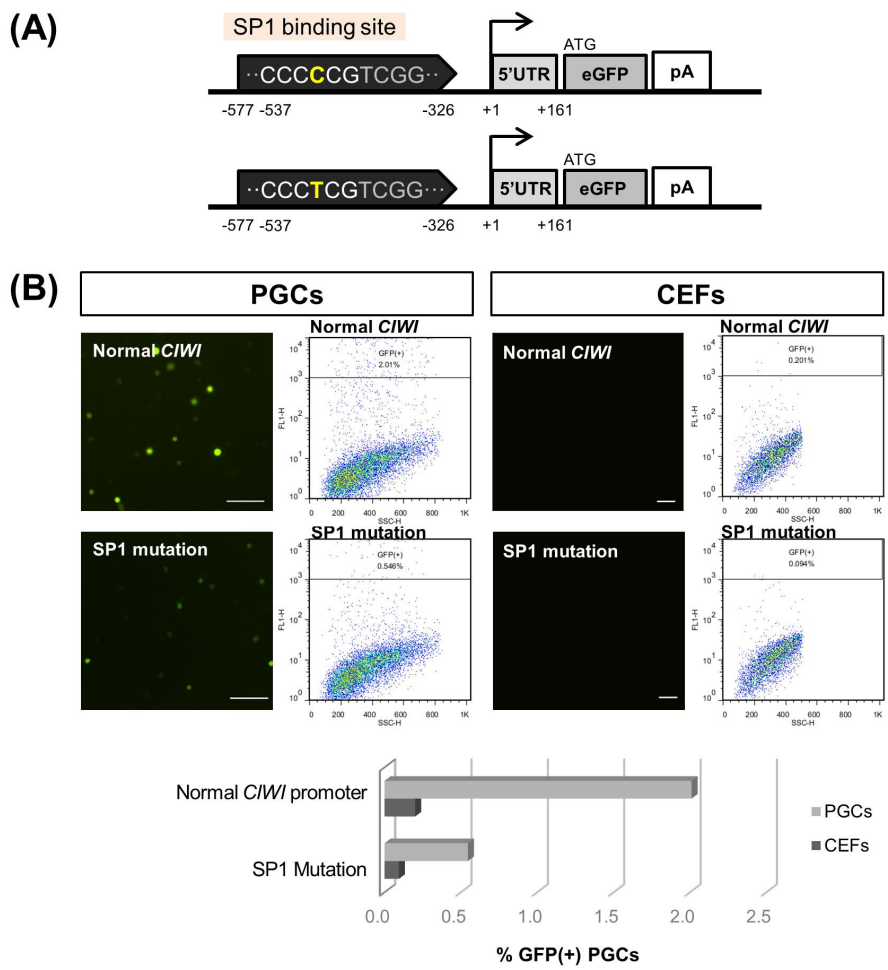

Figure 1. Point mutation analysis of binding site of SP1 transcriptional factor in the *CIWI* promoter. (A) Schematic diagram shows the construct with point mutation of the SP1 binding site. (B) Expression of enhanced green fluorescent protein (eGFP) vectors containing normal or point mutated sequence constructs in cultured chicken primordial germ cells (PGCs) and chicken embryonic fibroblasts (CEFs) were analyzed by fluorescence microscopy and flow cytometry. Scale bar = 100 μm.

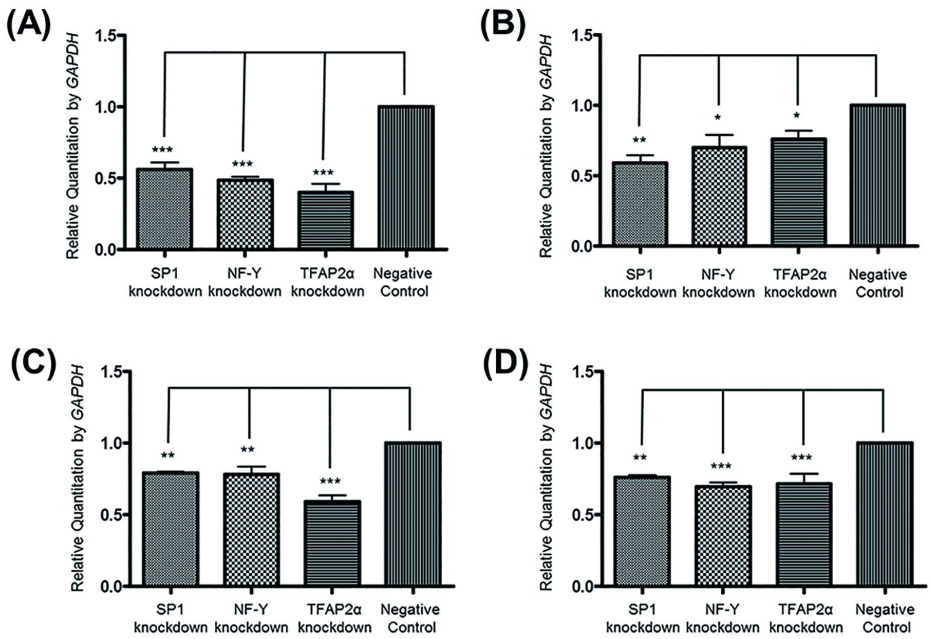

Figure 2. Knockdown analysis of transcription factors-treated cultured chicken primordial germ cells (PGCs). siRNA for each transcription factor SP1, NF-Y and TFAP2α was transfected into cPGCs. Knockdown rate was examined by qRT-PCR (A). Expression of *CIWI* (B), *DAZL* (C) and *VASA* (D) was examined by qRT-PCR after knockdown of each transcription factor in chicken PGCs. Expression level of each gene was compared to negative control. Significant differences between control and treatment groups are indicated as ***P < 0.001, **P < 0.01, and *P < 0.05. Error bars indicate the SE of triplicate analyses.

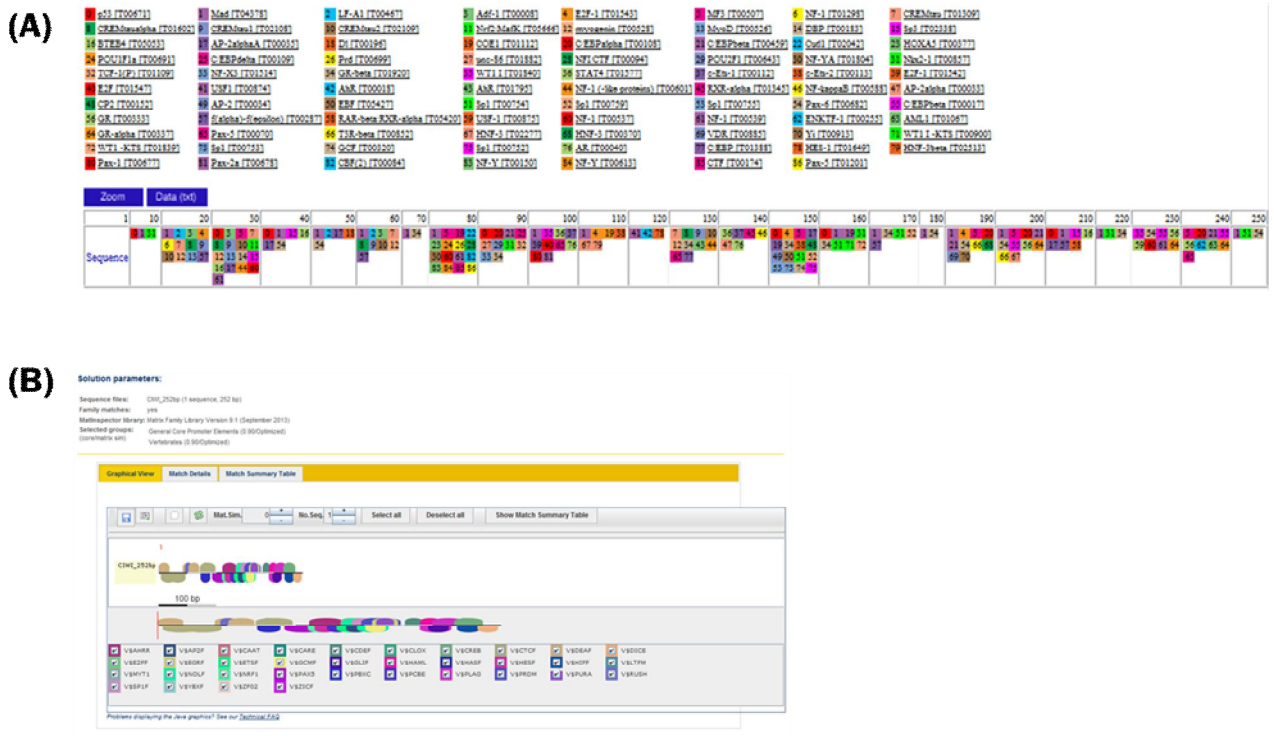

Figure 3. Several transcription factor binding sites were predicted in −577 to -326 bp of *CIWI*. PROMO (A) and MatInspector (B) software were used to detect predicted transcription factor.

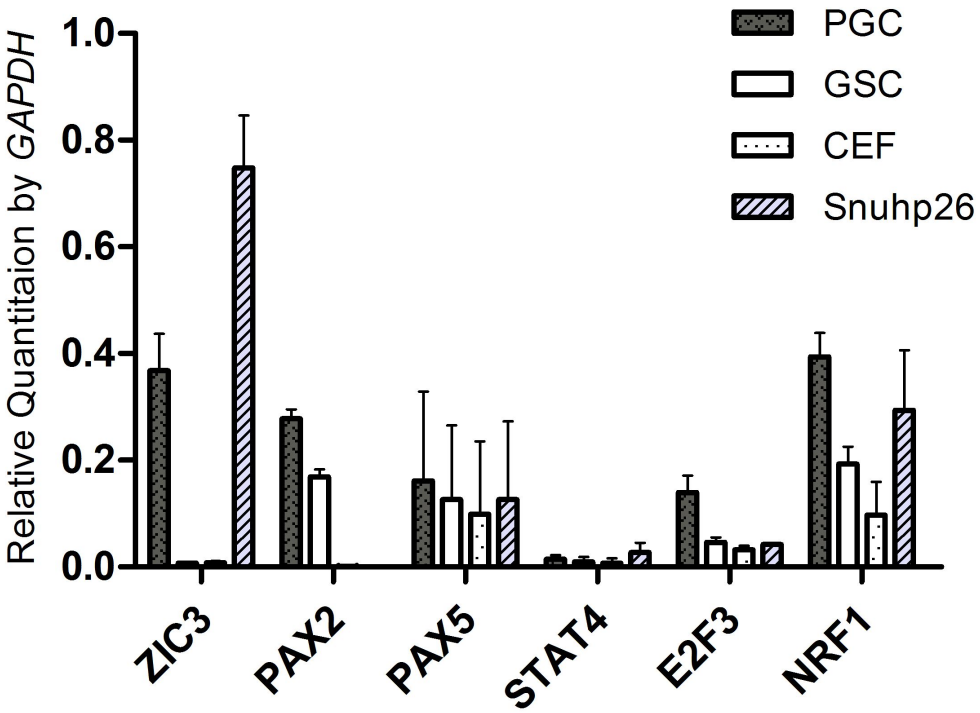

Figure 4. Six Putative transcription factor which binds to 252-bp of *CIWI* promoter. Using quantitative RT-PCR, expression of all transcription factors detected by MatInspector and PROMO were analyzed in gPGCs (MACS (+) cells), GSCs (MACS (-) cells), CEFs and Snuhp26 (cultured chicken PGCs). Several transcription factors were expressed strongly in PGC but not in GSC or CEF. Six putative transcription factors were selected including ZIC3, PAX2, PAX5, STAT4, E2F3, and NRF1.

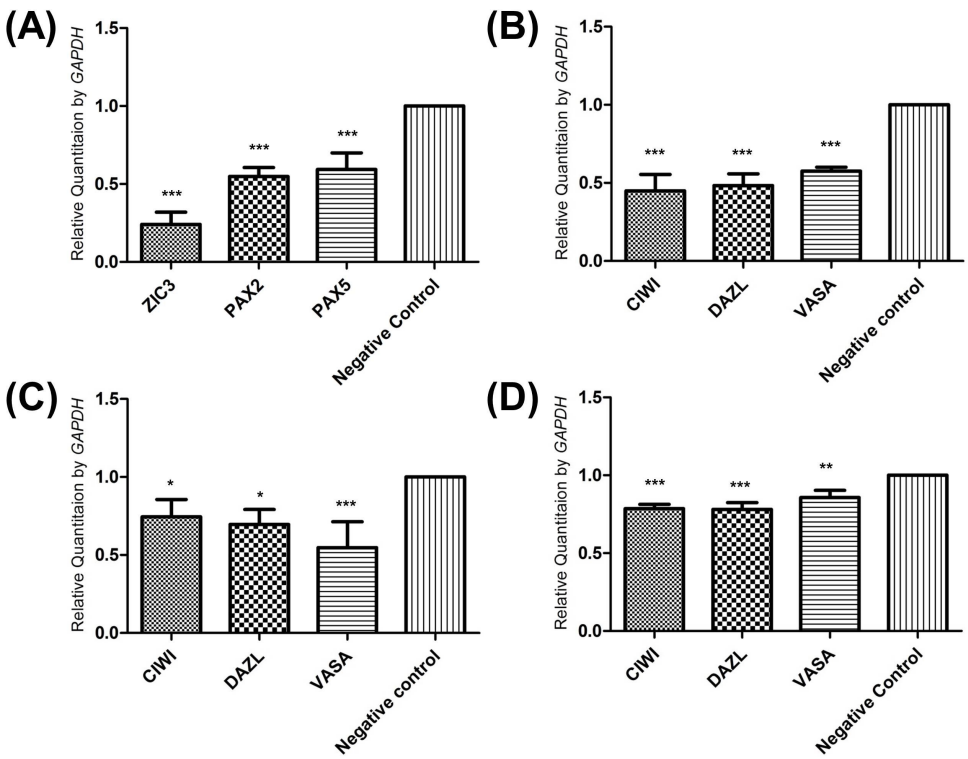

Figure 5. Knockdown analysis of ZIC3, PAX2 and PAX5-treated cultured chicken primordial germ cells (PGCs). siRNA for each transcription factor ZIC3, PAX2 and PAX5 was transfected into cPGCs. Knockdown rate was examined by qRT-PCR (A). Expression of *CIWI, DAZL* and *VASA* was examined by qRT-PCR after knockdown of ZIC3 (B), PAX2 (C) and PAX5 (D) transcription factor in chicken PGCs. Expression level of each gene was compared to negative control. Significant differences between control and treatment groups are indicated as ***P < 0.001, **P < 0.01, and *P < 0.05. Error bars indicate the SE of triplicate analyses.

Table 1 List of gene specific PCR primer sequences for qRT-PCR.

Primer Set	Primer Sequence (5' to 3')
*ZIC2*_F	ACGTCCTCAACGGGCAGATG
*ZIC2*_R	CTGCCGCATGTAGCGGAAA
*ZIC3*_F	GGCAGCAAGGACTCCACGAA
*ZIC3*_R	CGGTCGTCGGTGTGGAAACT
*PAX2*_F	CGGTTCAGCAACCCTGCATT
*PAX2*_R	AGGCACGGCAAGGCTCTCTT
*PAX5*_F	CCTGCGCTGACAGGAATGGT
*PAX5*_R	TTTGGGTCCGAGGTCAGTGG
*STAT4*_F	CAGTGCCCAGATGACTGACCAA
*STAT4*_R	TTTCGTGGATGACACGGCCT
*E2F3*_F	GCCACCAGTATCTTGCTGAAGGA
*E2F3*_R	TCCGCCGCTCTGTTCAAATC
*NRF1*_F	AGAAGCAGCGGGTGTCATGG
*NRF1*_R	GCCAACCTGGATGAGGGACA
*GAPDH*_F	GGTGGTGCTAAGCGTGTTAT
*GAPDH*_R	ACCTCTGTCATCTCTCCACA

Table 2 List of gene specific siRNA sequences for knockdown analysis.

siRNA	Sense (5' to 3')	Antisense (5' to 3')
NF-Y	GCAAGUCUGUUCUACCUUA	UAAGGUAGAACAGACUUGC
SP1	CCAUCAGCUCGUCCAACAU	AUGUUGGACGAGCUGAUGG
TFAP2α	CCCUCUCCAAGUCUAACAA	UUGUUAGACUUGGAGAGGG
ZIC3	GCUUAGGAAACACAUGAAG	CUUCAUGUGUUUCCUAAGC
PAX2	GCUGCACCAGGACAUACUA	UAGUAUGUCCUGGUGCAGC
PAX5	CCAGUACCCAAUGGCCAUU	AAUGGCCAUUGGGUACUGG
NC	GGAUGCGGUGGUUAAAGCA	GGAUGCGGUGGUUAAAGCA

*NC (Negative Control) ; Control siRNA has no complementary sequence in the chicken genome.

4. Discussion

Transcription factor binds to specific DNA sequences, controls the transcription of genetic information from DNA to mRNA (Karin 1990; Latchman 1997). Transcription factor accomplished their role alone or with other proteins in a complex. Transcription factor can promote or activate the RNA polymerase to initiate transcription, or it can block the access of RNA polymerase to interrupt transcription (Nikolov and Burley 1997; Roeder 1996).

During gametogenesis in chickens, several germ cell-specific genes such as *CIWI, CVH, DND1* and *DAZL* are expressed in germ cells. They play critical roles in differentiation of germ cells (Kito et al. 2014). *DAZL* encodes potential RNA binding proteins that are expressed in prenatal and postnatal germ cells of males and females. In chicken testis, DAZL is localized to the nucleus of spermatogonia and relocates to the cytoplasm during meiosis. *DAZL* gene is localized to the nucleus and cytoplasm of fetal germ cells and to the cytoplasm of developing oocytes. Expression of chicken *DAZL* (*cDAZL*) was detected continuously in the germ-line cells of males and females until the adult stage (Rengaraj et al. 2010). VASA protein is an RNA binding protein with ATP-dependent RNA helicase of the DEAD-box family

protein (Raz 2000). It is specifically localized in the germ plasm and expressed in germline cells throughout subsequent stages of development. Chicken VASA homolog gene could be used as a reliable molecular marker for investigating avian germ cell lineages (Tsunekawa et al. 2000).

Most *cis*-regulatory sequence has more than one type of transcription factor binding site, and most transcription factors bind to the several genes of *cis*-regulatory sequence. Different complexes of transcription factors can alter the gene regulation in different genes (Wittkopp 2010). In addition, one transcription factor can serve both as a positive regulator and as a negative regulator for the expression of genes (Li and Ou 2001). Mutations that alter the *cis*-regulatory sequence to which transcription factors bind can change gene expression as well as that alter the activity of transcription factors. Mutation in *cis*-regulatory sequence is able to affect the binding affinity of transcription factor, trigger the alteration of *cis*-regulatory activity (Wittkopp 2010).

The SP family of zinc-finger transcription factors are important mediators of selective gene activation during embryonic development and cellular differentiation. Transcripts of *Sp1* are expressed in a stage and cell type-specific manner in differentiating male mouse germ cells and SP1 proteins were

expressed mainly in the primary spermatocytes (Thomas et al. 2005). Transcription factor ZIC3 is a nuclear protein, which functions in early stages of left-right body axis formation (Ware et al. 2004). ZIC3 is required for maintenance of pluripotency in embryonic stem cells. Down-regulation of Zic3 resulted in a significant decrease in Nanog expression and loss of pluripotency in ESCs (Lim et al. 2007). The PAX proteins are important regulators in early development, they play a critical role in the formation of tissues and organs during embryonic development. The PAX gene family is also important for maintaining the normal function of certain cells after birth. The Pax-5 gene is the first member of the Pax gene family reported to be expressed in testis. PAX5 has been identified with neural development and b-cell differentiation. PAX5 is also play an important role in spermatogenesis (Adams et al. 1992; McManus et al. 2011). PAX2 can promote proliferation and cell survival, and influence differentiation (Park et al. 2006). In the chick, Pax2 expression within the lateral plate is controlled by BMP signaling (Dudley et al. 2010; Obara-Ishihara et al. 1999).

In this chapter, we identified several transcription factors which might bind to 252-bp of *CIWI* promoter and assist the transcription of not only *CIWI* gene, but also *DAZL* and *VASA* gene in cultured chicken PGCs. We found transcription factor SP1, NF-Y and TFAP2α which is predicted to bind in 252-bp

of *CIWI* promoter in last chapter. Transcription factor SP1 was predicted to bind not only to *CIWI,* but also to *Miwi* and *HIWI* promoter. Mutation of SP1-binding site showed decreased expression level of *CIWI*. SP1, NF-Y and TFAP2a were knocked down in cPGCs using siRNAs and shown decreased level of *CIWI* expression. Expression of *DAZL* and *VASA* were also decreased in each transcription factor knockdown PGCs compared with control. We additionally identified 3 more transcription factors which might affect expression level of *CIWI*, *DAZL* and *VASA*. Expression of each *CIWI*, *DAZL* and *VASA* showed decreased level in transcription factor ZIC3, PAX2 and PAX5-knockdown PGCs compared with control. We suggest that 6 transcription factors might affect transcriptional activity of germ cell-specific genes including *CIWI*, *DAZL* and *VASA*.

CHAPTER 5.

GENERAL DISCUSSION

Understanding the molecular mechanisms that regulate cell-fate decisions during early PGC development is important not only for fundamental research in germ cell biology and embryology but also for the practical utilization of genetic resources and use of genetic modifications. Studying *PIWI* family genes, which plays crucial roles during germline development and gametogenesis of many metazoan species, is necessary to understand the properties of germ cells. Since expression of PIWI is restricted germline and stem cells, PIWI can be a great tool for studying germ cell-specific expression. *Gallus gallus* PIWI-like protein 1 (CIWI) was identified both in embryo and adult chicken testis and ovary. In addition *CIWI* was more highly expressed in PGCs than embryonic gonads. Knockdown of *CIWI* resulted in double-strand breakage after higher activation of retrotransposons, such as CR1. It suggests that *CIWI* is responsible for silencing of transposable elements in germ line.

Since transcriptional regulation of *CIWI* can be critical for effective functional modulation, studying promoter sequences and regulations are required. We thought minimal sequence of chicken *PIWI* promoter may posses the regulatory *cis*-element and transcription factors, which are crucial for germ cell-specific transcription. Identifying the minimal sequence of *CIWI* promoter can be a great tool for production of transgenic chickens. In addition, it can be of help on the basic study of reproductive

biology and germ cell development.

To evaluate regulatory elements of the *CIWI* promoter, we first identified 4 kb of the *CIWI* promoter including +161 bp of the 5' UTR. Subsequently, the enhanced green fluorescent protein (eGFP) as a reporter gene under the control of the cloned 4-kb *CIWI* promoter was specifically expressed in chicken PGCs, but not in CEFs. *In vivo* transfection analysis showed that 4-kb *CIWI* promoter vector was also expressed in E 6.0 embryonic gonads. For the promoter deletion assay, four expression vectors containing *CIWI* promoter fragments of different sizes were constructed including a 2,986-bp, 1,985-bp and 1,000-bp fragment. All expression constructs were strongly expressed only in chicken PGCs. No expression constructs were expressed in CEFs. This result suggests that 1,000-bp fragment may be the critical for *CIWI* expression. Promoter fragmentation assays were performed in order to identify the minimal *CIWI* promoter. Vector with -577 to -330-bp of *CIWI* was only expressed in cPGCs among four fragmented promoters. It suggests that the elements for transcriptional initiation in *CIWI* were located between -577 and -326 bp.

The second experiment was examined to identify the transcriptional initiation elements in promoter of *CIWI*. Interestingly, there was no TATA box sequences, but another

transcriptional initiation element CCAAT that was identified in the −498 to −494−bp fragment of the *CIWI* promoter. Mutation assays by substituting CCAAT with TTCCT sequences showed complete disruption of transcriptional activity in chicken PGCs. We also found that CCAAT element was present in the predicted binding sites of mouse PIWI like−1 (*Miwi*) and human PIWI like−1 (*HIWI*). Transcription factor SP1 and NF−Y were also positioned in predicted promoter region of *HIWI, Miwi* and *CIWI*. These results indicated that chickens and mammals have a similar mechanism of regulation of regulatory processes that are transcribed specifically in germ cells (Dolfini et al. 2012).

We also identified several transcription factors which might bind to −577 to −330 bp of *CIWI* and affects the transcriptional activity of *CIWI* gene. Transcription factor SP1 was predicted to bind in *CIWI, Miwi* and *HIWI* promoter. Mutation of SP1−binding site showed decreased expression level of *CIWI*. Other transcription factors including NF−Y, TFAP2α, ZIC3, PAX2 and PAX5 were predicted to affect the transcriptional activity of *CIWI*. Knockdown analysis of transcription factors was conducted in order to identify the alteration of *CIWI, DAZL* and *VASA* expression, which are germ cell−specific genes. Expression of each *CIWI*, *DAZL* and *VASA* showed decreased level in 6 transcription factors−knockdown PGCs compared with control. It suggests that 6 transcription factors might affect transcriptional

activity of germ cell-specific genes including *CIWI*, *DAZL* and *VASA*.

In the present study, we identified the promoter region of *CIWI* gene and transcriptional elements in the *CIWI* promoter, which regulates *CIWI* expression in chicken germ cell lineages. We also identify the transcription factor which might affect the transcriptional activity of germ cell-specific genes. Thus, a short promoter sequence and a single transcription factor, such as the CCAAT box and SP1, in chicken germ cells could control transcriptional initiation of genes specific for cellular proliferation and cell-lineage-specific expression. Also single transcription factor can affect the expression level of germ cell-specific gene such as *CIWI, DAZL* and *VASA*.

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SUMMARY IN KOREAN

원시생식세포는 정자와 난자, 즉 생식세포의 전구체로써 다음 세대로 유전 정보를 전달하는 세포이다. 생체내 원시생식세포의 발달에 따른 세포 운명 조절 메커니즘 연구는 생식세포 자체의 분자생물학적 연구뿐만 아니라, 이를 활용한 응용생물학 분야 연구에도 큰 의미를 가진다. P-element-induced wimpy testis (*PIWI*) 는 small non-coding RNA의 종류 중 하나인 piRNA와 결합하여 생식세포와 줄기세포의 특성 유지에 필수적인 역할을 하는 단백질로써, *PIWI* 유전자의 전사적 조절 메커니즘은 생식세포를 효과적으로 조절하는데 중요한 역할을 한다고 밝혀져 있다. 그리하여, 본 연구는 닭 원시생식세포 특성을 유지하는데 필수적인 역할을 하는 *PIWI* 유전자의 homolog인 *CIWI* 유전자의 발현 조절 부위를 밝히고 *CIWI* 유전자 발현 조절 부위에 존재하는 특정 전사 조절 인자를 탐색하여, *CIWI* 유전자의 닭 원시생식세포 내 발현 조절 메커니즘을 이해하고자 진행되었다.

먼저, 우리는 *CIWI* 유전자의 5′ upstream 영역 4-kb를 획득한 후 이 조절 부위에 의해 녹색 형광 단백질 유전자가 발현하도록 벡터를 구축하였고, 이를 닭의 원시생식세포와 닭 배아섬유아세포에 전이시켜 그 발현을 관찰하였다. 그 결과, 녹색 형광 단백질은 *CIWI* 유전자의 4-kb 5′ upstream 영역에 의해 닭의 원시생식세포에서 성공적으로 발현하였으나 닭 배아섬유아세포에서는 발현하지 않았다. 이는 *CIWI* 유전자의 4-kb 5′ upstream 영역이 닭 원시생식세포에서 특이적으로 유전자 발현을 유도함을 시사 하였다. 추가적으로 *CIWI* 유전자 4-kb 5′ upstream 영역의 결

실 (deletion) 및 파쇄 (fragmentation) 실험을 진행한 결과, *CIWI* 유전자의 4-kb 5′ upstream 영역 중 –577 bp에서 -326 bp의 252-bp 영역이 닭 원시생식세포내의 *CIWI* 발현에 필수적이라는 것을 확인하였으며, 특히 –577 bp 에서 -326 bp 사이에 존재하는 CCAAT 염기서열이 *CIWI* 유전자의 발현에 필수적 역할을 하고 있음을 돌연변이 실험을 통해 확인하였다. 뿐만 아니라 다른 종간의 비교 연구를 통해 *CIWI* 유전자 발현 조절 부위의 CCAAT 염기서열이 사람의 *PIWI* 유전자 (*HIWI*) 발현 조절 부위, 쥐의 *PIWI* 유전자 (*Miwi*) 발현 조절 부위에서도 동일하게 존재하고 있음을 확인하였으며, CCAAT 주위의 염기 서열 역시 *CIWI* 와 높은 상보성을 나타내고 있음을 확인하였다. 이러한 결과를 통해 *CIWI* 유전자 5′ upstream 영역의 CCAAT 염기서열이 닭 원시생식세포에서 *CIWI* 유전자 발현에 필수적이며 이러한 양상은 조류 뿐 아니라 인간과 쥐에서도 상보적임을 알 수 있었다.

그 다음으로, *CIWI* 유전자 발현 조절 부위에 결합하여 유전자의 발현을 조절하는 전사 인자를 찾는 예측 프로그램을 통해 NF-Y 전사 인자가 특이적으로 CCAAT 염기서열에 결합하는 것을 확인하였으며, *CIWI* 유전자 발현 조절 부위 내 다른 영역에서도 NF-Y 전사인자 외에 SP1 전사 인자를 포함한 다양한 전사 인자가 결합하여 유전자 발현을 조절하는 것을 확인하였다. 또한 *CIWI* 외에 생식세포에 특이적으로 발현하는 유전자 역시 SP1 등의 다양한 전사 인자가 생식세포 특이적 발현에 관여하고 있음을 발견하였다. 이러한 결과는 닭 원시생식세포에서의 생식 세포 특이적 유전자 발현에 다양한 전사 인자가 관여하고 있다는 사실을 나타낸다.

본 연구에서 우리는 닭 원시생식세포에서 특이적으로 발현하여 그 특징을 유지하는데 필수적인 *CIWI* 유전자의 발현 조절 부위와 그 발현 조절 부위 내에 존재하는 전사 조절 인자를 발굴하였다. 이러한 결과는 닭 원시생식세포에서의 특이적 발현을 보이는 유전자들 간의 발현 조절 메커니즘 연구를 포함한 기초 분자생물학적 연구 뿐 아니라 발현 조절 부위를 활용한 모델 동물 개발 등의 응용생명과학 분야에서도 큰 도움을 줄 것으로 기대된다.