A Thesis for the Degree of Master of Science

Transcriptome Analyses and *cis*- Regulatory Element Assay of Germ Cell-Specific Genes in Chicken Primordial Germ Cells.

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SUMMARY

Primordial germ cells (PGCs), the only type of cells can transfer genetic information to next generation, are distinct from other types of cells in specialized gene expression for maintaining unique characteristics of germ cells. Successful production of functional gamete that finally generates entire new organism is largely dependent on these germ cell-specific gene expressions in PGCs. In this regards, understanding of molecular mechanism(s) related to germ cell-specific gene expression has been emphasized in germ cell biology. One of the important regulating mechanism(s) in the germ cell is the harmonious expression of germ cell-specific genes regulated by transcriptional regulatory elements. Indeed, architecture of specialized germ cell promoters can contribute to mediate distinct transcriptional signals necessary for germ cell-specific programs of appropriate gene expression. Thus, studying on *cis*-DNA elements and their unique combination of modules is important for deciphering of regulatory networks of germ cell genes that govern transcriptional control during early PGC development. Despite of the importance of transcriptional regulatory network in PGCs, very limited studies are available on *cis*- and *trans*- elements that control germ cell-specific expression in avian species.

Therefore, in this study, we investigated promoter structures of PGCs-specific genes (*CVH, cDAZL* and c*NANOG*) that includes *cis*- and *trans*-regulatory elements. To measure the promoter activity, we constructed eGFP and NanoLuc expression vectors that contain the 5' flanking region of these genes. Furthermore, we elucidated predictive transcription factors (TFs) binding to promoter region refer to RNA-seq databases, and in the case of germ cell-related genes, subsequently verified predictive TFs by knockdown analysis. Firstly, we verified the minimal promoter regions of *CVH* and *cDAZL* gene, encoding conserved RNA-binding proteins, mediated by dual luciferase

reporter assay. As results, we elucidated the minimal upstream regions (−135/+275 of *CVH* and −83/+173 of *cDAZL*) which are required for promoter activity in chicken PGCs. And our results indicated that 5' untranslated region (UTR) and intron-1 is dispensable for *cDAZL* expression, but have positive effect for *CVH* expression. Furthermore, we identified that *in silico* prediction of TFs binding to *cis*-regulatory element sequences were involved in specific expression of genes in chicken PGCs. Taken together, our results suggested that *cis*- and *trans*- regulatory factors of *cDAZL* and *CVH* genes affect germness related genes expression in chicken PGCs. Secondly, chicken *NANOG* promoter region which requires at least the 200-bp fragment (−130/+70) was verified in chicken PGCs. Through the 5' deletion assay, we identified that *cNANOG* promoter had chicken PGCs specific activity, unlike *CVH* and *cDAZL* promoter. In addition, comparing promoter activity with each of fragments gave a clue for finding putative proximal and distal enhancers, and then PGC-specific expressed TFs were predicted by referring to RNA-seq databases. Furthermore, mutation assay revealed that putative binding sites of pluripotency factors affect chicken *NANOG* promoter activity like as mammalian species.

In this study, we elucidated *cis*-transcriptional regulatory regions of chicken PGCs-specific genes, and several TFs that could bind to *cis*-regulatory regions. Collectively, our results in this study could contribute to constructing of germ cell-specific synthetic promoter for tracing germ cells as well as understanding the molecular mechanism(s) for maintaining germness in chicken PGCs.

Key words: primordial germ cells, chicken, Vasa, Dazl, Nanog, promoter, transcription factors

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LIST OF ABBERVIATION

3' UTR: 3' untranslated region

5' UTR: 5' untranslated region

AP2γ: Transcription factor AP-2 gamma

BLIMP1: B lymphocyte-induced maturation protein 1

BMP4: Bone morphogenetic protein 4

CIWI: Chicken PIWI-like protein 1

CVH: Chicken vasa homolog

DAZL: Deleted in azoospermia-like

DMEM: Dulbecco's modified eagle medium

eGFP: Enhanced green fluorescent protein

EGK: Eyal-Giladi and Kochav

EP300: E1A binding protein p300

ESC: Embryonic stem cell

FBS: Fetal bovine serum

FGF: Fibroblast growth factors

GABPA: GA binding protein transcription factor, alpha subunit 60kDa

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GSC: Gonadal stromal cell

HSF2: Heat shock transcription factor 2

KLF4: Kruppel-like factor 4

LIF: Leukemia inhibitory factor

MEF: Mouse embryonic fibroblast

NFYA: Nuclear transcription factor Y, alpha

OCT4: Octamer binding transcription factor 4

PGC: Primordial germ cell

PIWI: P-element induced wimpy testis

PRDM14: PR domain containing 14

qRT-PCR: Quantitative reverse transcription PCR

RNAi: RNA interference

SCF: Stem cell factor

siRNA: Small interacting RNA

SMAD: Sma and mad related Family

SOX2: SRY (sex determining region Y) box 2

SP3: Stimulating protein 3

TAF: TATA box binding protein associated factor

TRF: TBP-related factor

WNT3: Wingless-type MMTV integration site family, member 3

ZNF143: Zinc finger protein 143

CHAPTER 1.

GENERAL INTRODUCTION

In sexual reproducing organisms, primordial germ cells (PGCs) are the first germ line cell population set aside from other cell lineages in various species (Saitou and Yamaji, 2010). They produce offspring through fusing two functional gametes which are provided by gametogenesis. In chicken, PGCs firstly emerged in the epiblast (Eyal-Giladi et al., 1981), and begin to migrate to the area pellucida in blastoderm at EGK stage XII (Eyal-Giladi et al., 1976). As PGCs transfer from the germinal crescent to the blood stream at HH stage 10-12, a process of migration of them continues through circulatory system until settle down in the developing genital ridges (Ukeshima and Yoshinaga, 1991). PGCs minimize interaction with other cells and express germ cell specific-genes which are unique characteristics distinct from other cells through combinatorial gene expression differentially for establishment of a germline lineage while PGCs proceed migration to differentiate into germ cells (DeJong, 2006; Richardson and Lehmann, 2010). Consequently, PGCs can accomplish a task as the progenitors of germ cells.

Specially, expression of germ cell specific-genes in germ cells is the most important thing in the germ cell development aspect because is required for germ cell survival, proliferation, migration and differentiation (Wylie, 1999). Several evolutionally conserved germ cell-specific genes are expressed in germ cells such as *Vasa* and *Dazl*. Also, in chicken, *Vasa* (Tsunekawa et al., 2000) and *Dazl* (Kito et al., 2010) are expressed in germ cells. These PGCs mainly express RNA binding proteins (RBPs) by modulating tissue specific *cis*- and *trans*- regulatory elements and have the specialized genetic programs distinct from other somatic cells for maintaining their unique characteristics (Donovan, 1998; Han et al., 2006; Kim et al., 2007). PGCs also express pluripotency markers, *Nanog*, *Oct4* and *Sox2* that play a role in maintenance of PGC characteristics (Saito et al., 2003). Transcription factors act to activate a gene by binding to *cis*-regulatory elements such as enhancer, silencers and promoters

(Spitz and Furlong, 2012). Thus, it is most important evident that a study on the promoter could understand fundamental concepts of cell biology in specific cell types and transcriptional regulatory elements.

Vasa, one of well-known for an ATP-dependent RNA helicase, encode evolutionarily conserved RNA-binding protein that play critical roles in germ cell specification, supporting germ line development and RNA processes involving biosynthesis of piRNAs (Lasko, 2013). The *vasa* gene firstly isolated in *Drosophila* (Lasko and Ashburner, 1988), and *vasa* homologous genes have been characterized in germ cells across diverse organisms including chickens. Expression of the protein chicken *vasa* homologue (CVH) is restricted to chicken germ cells during early embryogenesis (Naoki Tsunekawa, 2000). And the *vasa* regulatory regions that are required for transcription have been exclusively studied in germ cells using reporter system in diverse species such as *D. melanogaster* (Sano et al., 2002), *D. rerio* (Krøvel and Olsen, 2002), *O. latipes* (Li et al., 2013). Minematsu *et al*. (2008) identified that chicken gonadal cells require a 1,555-bp sequence of 5'flanking region of *CVH* gene for germ cell specific exogenous gene expression under the *CVH* promoter (Minematsui et al., 2008). However, the intricate regulatory mechanism(s) that govern transcriptional control for *Vasa* expression during chicken germline development has yet to be investigated in detail.

Dazl (Deleted in AZoospermia-Like), a member of the *DAZ* gene family, is specifically expressed in fetal and adult gonads and is essential during gametogenesis. For researching of mechanism by which *Dazl* gene expression is restricted to germ cells, researchers have attempted on identifying its regulatory region. The regulatory sequence of *Dazl* gene has been identified to require for germline specific gene expression in human (Teng et al., 2012), mouse (Nicholas et al., 2009), pig (Linher et al., 2009) and chicken (Zhang et al., 2015). In the case of pig and human, studies on *DAZL* promoter have

reported that core promoter of *Dazl* gene and putative *trans*-acting elements are involved in expression of *Dazl* gene specifically in PGCs. Also, chicken *DAZL* core promoter was identified, but, researches on *DAZL* promoter activity in chicken PGCs and prediction of *trans*-acting elements on the promoter region have not been reported.

Nanog is a homeodomain protein, which is preferentially expressed in embryonic stem (ES), embryonic germ (EG) and embryonal carcinoma (EC) cells (Yamaguchi et al., 2005). Nanog is known as the central transcription factor for maintaining the pluripotency of cells. Indeed, in *Nanog*-null embryos show the phenomenon that E5.5 embryos fail to develop the epiblast and *Nanog*-deficient ES cells lose pluripotency (Mitsui et al., 2003). Thus, it is important that identification of upstream effectors of *Nanog* gene. Because they might govern the acquisition and maintenance of pluripotential cells such as ES and EG cells. For these researches, many studies have been performed about *cis*-regulatory elements and recognition factors of these sequences such as Octamer and Sox elements (Kuroda et al., 2005). In chicken, on the other hand, studies on the molecular mechanisms of transcriptional regulation in chicken *NANOG* gene has not been attempted. Thus, studies on identification *cis*-elements of chicken *NANOG* gene will contribute to provide new insights into the regulatory circuitry that maintains PGC fate.

Understanding the cellular and molecular mechanisms that regulate germ cell-specific gene expression during PGC development is critical for the practical use of genetic modifications and germ-cell biology. Therefore, in this study, we performed the experiments to identify the minimal promoter region for chicken *vasa* homologue (*CVH*), c*DAZL* and *cNANOG* expression and predicted *trans*-acting factors that bind to the promoter region via prediction programs. Thus, we expect that these study might offer clues on understanding the molecular mechanisms that regulate the cell fate and integrity of PGCs.

CHAPTER 2

LITERATURE REVIEW

1. Primordial germ cells

In sexual reproducing organisms, germ cells produce offspring through fusing two functional gametes which arise from primordial germ cells (PGCs), the first germ line cell population set aside from other cell lineages in various species (Saitou and Yamaji, 2010). Only genetic and epigenetic information encoded in PGCs that have it in them to connect one generation and next generation through transmitting the information.

PGCs, themselves develop in a specialized from other cell types, represent the property of pluripotency in the way that can generate an entire new organism and also are closely related to broadly studied in vitro pluripotent stem cell models (Wylie, 1999; Lesch and Page, 2012). For achievement of these their own capacity, have unique characteristics by combinational specific gene expression differentially distinct form other cells. In this sense, recent researches have been reported on the molecular mechanisms of germ cell development and specifying germ cell fate.

1.1 Origin of primordial germ cells

PGCs arise from the epiblast of early embryos at around embryonic day 6.25 (E6.25), and then migrate to the fetal gonad and differentiate into germ cells in mouse (Richardson and Lehmann, 2010). In avian germ cell development, PGCs firstly emerged in the epiblast (Eyal-Giladi et al., 1981), and begin to migrate to the area pellucida in blastoderm at EGK stage XII. As PGCs transfer from the germinal crescent to the blood stream at HH stage 10-12, a process of migration of them continues through circulatory system until settle down in the developing genital ridges (Ginsburg and Eyal-Giladi, 1987).

1.2 Specification

In diverse animal embryos, germ cells must be properly specified early in development to serve crucial purpose of generating functional gametes. Typically, there are two general modes of germ cell specification. Most is known of germ cell specification in invertebrates: the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*. In these animals, germ cell can be specified by the partitioning of maternally supplied germ plasm, is referred to cytoplasmic determinants, which is germ granules reside in specialized cytoplasm: preformation mode (Weismann, 1885). In other animal species, including mammals, PGCs must be induced at the post-implantation epiblast stage by cell signaling: induction mode (Extavour and Akam, 2003).

Formation of PGCs in preformation mode, germ plasm suppling substance comprised of RNAs, proteins and organelles are maternally loaded from oocyte to embryos. These components in germ plasm serve as transcriptional and post-transcriptional regulator for preventing activation of somatic development and establishment of germ cell property (Seydoux et al., 1996; Schaner et al., 2003). The major of germ plasm components are conserved RNA-binding proteins including the VASA-related RNA helicases, the Tudor-domain proteins, NANOS, the Arg methyltransferase PRMT5 and certain Argonaute proteins that play a crucial role for determining and maintaining germ cell fate (Strome and Updike, 2015).

In *Drosophila melanogaster*, germ granule assembly in the posterior region of the oocyte where PGCs will bud off during embryogenesis through recruiting other polar granules components (e.g., Vas and Tud) to the posterior pole by Osk protein (Breitwieser et al., 1996; Strome and Updike, 2015). To demonstrate the function of polar granules, a series of transplantation research were conducted (Illmensee and Mahowald, 1974; Okada et al., 1974). Transplantation of polar granules into anterior pole and the midventral site

caused ectopic PGC formation.

In *Caenorhabditis elegans*, segregation of 1-cell zygote (P_0) contributes to generate the germ line blastomeres, or P cells during the four asymmetric embryonic divisions, distributing P granules to the P_4 cell, and become the PGC (Strome and Wood, 1982; Strome and Lehmann, 2007).

Germ cells in mammalian embryos lack maternal germ plasm and are specified by inductive signals from neighboring extra-embryonic tissue. A small set of signaling molecules and zygotic transcription factors are critical for instructing a small number of proximal epiblast cells to become PGCs (Hayashi et al., 2007).

The expression of *Bmp4* and *Bmp8*b from the extraembryonic ectoderm, and *Bmp2* arisen from the visceral endoderm is known to be required for germ cell induction and ensure the generation of sufficient numbers of PGCs in mouse (Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). To respond BMP signals, nearly competent epiblast cells of the mouse can express key transcriptional regulators Blimp1 (Prdm1), Prdm14 and AP2γ that play a key role for PGC specification. A tripartite transcription factor network is essential for the repression of a somatic mesodermal program, activation of germ cell genes, including pluripotency genes and ensuing epigenetic reprogramme (Magnúsdóttir et al., 2013). Also, several cells in the proximal epiblast express developmental pluripotency-associated 3 (*DPP3*/*Stella*), which is required to maintain rather than specify the PGCs (Bortvin et al., 2004).

In mouse embryos, the proximal epiblast cells receiving BMP signaling from surrounding somatic tissues can induce PGCs expressing Blimp1, but not Sox17 (Kanai-Azuma et al., 2002), whereas in humans the endodermal transcription factor SOX17 is the key regulator of hPGCLCs, which promotes germ cell development through acting upstream of BLIMP1

(Irie et al., 2015). This study indicates important similarities and differences between human and mouse PGC specification.

In chicken the specification of PGCs is as yet an unsolved problem, however, a chicken *vasa* homologue (*CVH*), one of the germ plasm components, was detected in initial developmental stages. Also, CVH protein is localized to cytoplasm of germ cells and co-localized with mitochondrial cloud and spectrin in the growing oocyte. These results suggest that chicken germ cell is determined by maternally inherited factors in the germ plasm (Tsunekawa et al., 2000).

In animal's germ cell development, core germ line determinants are conserved regardless of the modes of specification such as *vasa*, *nanos* and *piwi*. *vasa*, a DEAD-box RNA helicase, widely is used as germ line specific marker and serve as regulating the translation of mRNAs (Seervai and Wessel, 2013). Although, in some organisms like mouse, germ plasm is not maternally inherited from oocyte to embryos for germ cell specification, *vasa* is found in the germ cells at some point stages of development (Toyooka et al., 2000). In particular, *D. melanogaster* and *D. rerio* require key germ plasm markers such as *dazl* and *vas* (Maegawa et al., 1999; Olsen et al., 1997). Induction of germ cells also are related to *Nanog* gene, known as a core pluripotency factor. *NANOG* can induces germ cells in primed epiblast *in vitro* by interacting enhancers of *Prdm1* and *Prdm14* in EpiLCs in mouse (Murakami et al., 2016).

1.3 Establishment of the germ cell lineage

Even after PGCs are specified, PGCs must be maintain their unique characteristics during development for the successful transmission of genetic information to next generation. In most of animals, a first step of protection from extrinsic somatic signals is achieved by the separation of PGCs from

somatic cells during early embryogenesis. During later stage of development, germ line fate requires a unique gene regulatory network in this germ line maintenance and function which accomplish at least three key events: somatic gene repression, epigenetic reprogramming and translation of only germ cell appropriate transcripts (Strome and Updike, 2015). In this review, a brief summary in aspect of intrinsic regulators (transcriptional • translational), epigenetic remodeling and extrinsic factors, for acquisition and maintenance of germness in PGCs during their development, has been given.

1.3.1 Transcriptional • translational intrinsic regulators

During PGC development, several key transcription factors (BLIMP1, PRDM14 and AP2γ) which are necessary and sufficient for the germ line competence are strongly expressed in response to WNT3 and BMP4 signaling. Combination of these factors contribute to PGC specification and maintenance of their unique properties (Magnusdottir et al., 2013).

Blimp1, a known transcriptional repressor, has a critical role in repression of the somatic programme during development. *Blimp1* is restricted to PGC precursors and appears to be critical for maintaining a PGC fate in mouse. Indeed, disruption of *Blimp1* in mouse leads to fail in migration, proliferation and consistent repression of key somatic regulators (Ohinata et al., 2005; Hayashi et al., 2007).

Prdm14, a PR domain–containing transcriptional regulator, is expressed exclusively in the germ cell lineage and conserved at least in vertebrates. *Prdm14* is essential for the reacquisition of potential pluripotency and epigenetic reprogramming. In the absence of *Prdm14*, PGCs fail to undergo these two events regardless of the presence of *Blimp1* (Yamaji et al., 2008).

In contrast, PRDM14 and AP2γ together are associated with gene

induction involved in establishing properties of PGCs (Magnusdottir et al., 2013). After BLIMP1 and PRDM14 is expressed by BMP4, AP2γ as an important transcription factor for downstream target of *Blimp1* is induced directly. Repression of AP2γ leads to upregulate of specific mesodermal differentiation markers, so AP2γ also plays a role in PGC maintenance (Weber et al., 2010). Indeed, co-expression of BLMP1, AP2γ and PRDM14 can induce PGC-like cells *in vitro*, so they are sufficient for PGC-specific fate (Magnusdottir et al., 2013).

PGCs also retain expression of several pluripotency-related transcription factors including *Nanog*, *Oct4* and *Sox2* which mediate PGCs characteristics (Clark and Reijo Pera, 2006). PGCs undergo apoptosis in *Oct4* null during embryonic development (Kehler et al., 2004). *Sox2*, is known as transcription factor that regulates Kit expression by binding to discrete regulatory regions of *Kit* gene, is required for PGCs proliferation and survival (Campolo et al., 2013). In defect of *Nanog*, PGCs fail to migrate on the genital ridge, thus *Nanog* is required for formation of germ cells (Chambers et al., 2007). These studies suggest that *Nanog*, *Oct4* and *Sox2*, known as pluripotency-markers, are necessary in maintaining viability of mammalian germline.

One of the major factors which is involved in maintenance of PGCs characteristics is germ line specific RNA-binding proteins such as VASA, DAZL and DND1. The germ line-associated RNA-binding proteins may have a role in repressing translation, thereby preventing their differentiation into somatic cells and maintaining their ability to develop into PGCs (Eddy, 2006).

The most universal of a group of conserved molecular determinants which function in germ line maintenance are *vasa* and *vasa*-like DEAD box RNA helicase genes. Vasa is a member of the DEAD box protein family that have nine conserved sequence motifs. Motif of typify all DEAD-box genes

promotes ATP-dependent RNA helicase catalytic activity. Structural data also suggest that Vasa unwinds duplex RNA in a non-processive manner (Linder, 2006; Sengoku et al., 2006). DEAD-box proteins encompass pre-mRNA splicing, ribosome biogenesis, nuclear export, translational regulation and degradation. The *vasa* gene firstly isolated in *Drosophila* (Lasko and Ashburner, 1988), and *vasa* homologous genes have been characterized in germ cells across diverse organisms.

In mammals, the expression of VASA protein also is detected specifically in germ cells at the late migrating stage (Castrillon et al., 2000; Toyooka et al., 2000). In chicken, expression of the protein chicken *vasa* homologue (CVH) also is restricted to chicken germ cells during early embryogenesis (Tsunekawa et al., 2000). Also, *Vasa* gain-of-function analyses in chicken cell lines have provided more insight into Vasa's role in development. Ectopic *VASA* expression in chicken embryonic stem cells (ESCs) induces expression of specific germ line and meiotic genes (Lavial et al., 2009). As a consequence, these ESCs exhibit improved germ line colonization and adopt a germ cell fate. This supports a fundamental role of *VASA* in germ line identity and function (Gustafson and Wessel, 2010). The molecular function of the VASA protein is estimated to interact with linking the sequences in the 3'UTR target mRNAs involve in germ cell maintenance (Gustafson and Wessel, 2010).

Dazl (Deleted in AZoospermia-Like), a member of the *DAZ* gene family, which is germ cell specific RNA-binding protein to regulate translation in various organisms (Collier et al., 2005). DAZL play a role in spermatogenesis and oogenesis (Ruggiu et al., 1997). Disruption of *Dazl* gene causes loss of germ cells in the gonads and the increase in the number of apoptotic germ cells (Lin and Page, 2005). Also in chicken, *DAZL* is detected in germ cells until the adult stage (Rengaraj et al., 2010). Unlike in mammals, *cDAZL* (chicken *DAZL*) is expressed specifically in PGCs during their migration from EGK stage X to

HH stage11. Therefore, investigation of *cDAZL* expression pattern could explains origin and central formation of PGCs in chicken (Lee et al., 2016).

Dnd1 (*dead-end*) gene, an RNA binding protein, was first characterized in zebrafish germ cells and is specifically expressed in the germ cells of vertebrates. Loss of *Dnd1* expression in germ cells results in abnormal migration and gametogenesis (Weidinger et al., 2003). DND1 regulates mitotic arrest in male germ cells by translational regulation of cell cycle genes (Cook et al., 2011) and can protect some genes expressed in germ cells against miRNA mediated translational repression by binding uridine-rich regions in the 3'untranslated region of target transcripts (Kedde et al., 2007). Chicken *dead end* homologue was isolated, and CDH protein is strongly detected in the nuclei of chicken PGCs during embryogenesis. It may work on translational regulation factor in common with other vertebrates (Aramaki et al., 2007).

Above experiments indicate that germ cell-specific RNA-binding proteins are required for germ cell development and survival.

1.3.2 Epigenetic remodeling

Proper epigenetic programming of primordial germ cells (PGCs) are major early events that have an impact on germ line development during embryogenesis and PGC migration to the gonad (Durcova-Hills et al., 2006; Nicholas et al., 2009).

Epigenetics represents a range of chromatin modifications including DNA methylation, posttranslational modifications of histone proteins, remodelling of nucleosomes and higher order chromatin reorganization, which in turn modulate chromatin structure. These epigenetic modifications are mediated in regulating gene expression allowing to constitute a unique profile in each cell. As a consequence, the preexisting epigenetic marks from the

genomes must be reset during germ-cell specification and new ones established to guarantee renewal of totipotency at each generation (Smallwood and Kelsey, 2012). Therefore, epigenetic modifiers have key roles in germ cell development in aspects of having a specific fate such as meiosis and maintaining genomic integrity through acquiring a unique gene expression programme. Indeed, germ cells go through a series of epigenetic events that are unique to this cell type (Sasaki and Matsui, 2008).

In the early germ cells, an extensive remodelling of the epigenetic landscape occurs from their specification to their colonization of the developmental gonads, so that germ cells can acquire the capacity to support post-fertilization development (Jang et al., 2013). Extensive epigenetic reprogramming play a crucial role in a mechanism to obliterate repressive modification from the genome that enable to reacquire totipotency in premigratory and migratory gem cells (Seki et al., 2005).

The first wave of epigenetic remodelling including histone modifications and DNA methylation marks occurs during implantation of the blastocyst. Pre-existing DNA methylation patterns are erased at CpG islands to refuse undergoing somatic fate, however, some regions escape epigenetic remodelling, for example, evolutionarily young and potentially hazardous retroelements, like SVA, remain highly methylated (Tang et al., 2015; Ramathal et al., 2011).

When PGCs migrate to the genital ridge, the second wave of epigenetic remodelling which is observed only in germ cells has started including their paternal or maternal imprinted loci. H3K9 dimethylation (H3K9me2) that is associated with transcriptional repression and DNA methylation decreases in migrating PGCs. Subsequently, the H3K27 tirmethylation (H3K27me3), another repressive mark, becomes more prominent to maintain a proper repressive chromatin state of the PGC genome.

It will be important to repress many somatic genes in PGCs. It is likely that these changes of repressive marks contribute to acquire totipotency in migrating PGCs (Sasaki and Matsui, 2008). Following sex-determination of the embryo, these phase enable to obtain distinct sex-specific DNA remethylation profiles of mature oocytes and sperm during germ cell development (Hajkova et al., 2002; Yamazaki et al., 2003; Durcova-Hills et al., 2006).

In the mouse, *de novo* methylation including the re-establishment of imprints takes place before meiosis in mitotically arrested cells in male prospermatigonia and is completed prior to birth. In female PGCs, *de novo* methylation takes place during the postnatal oocyte growth phase arrested in meiotic prophase I, and continues even after birth (Lucifero et al., 2002). Recent genome-wide analysis identified the greatest difference in methylation pattern between mature oocytes and sperm. These germ line differentially methylated regions contribute to control the parent-of-origin specific monoallelic expression of imprinted genes (Smallwood and Kelsey, 2012).

Recent studies have reported that some epigenetic factors, involved in resetting of the epigenome and preventing activation of transposons, are specifically expressed in germ line cells and interact with some pluripotency genes. Indeed, in hPGCs, expression of some pluripotency genes, including TFCP2L1, KLF4, NANOG and OCT4 may contribute to epigenome resetting and global hypomethylation (Tang et al., 2015). In the germline, PRMT5, an arginine methyltransferase, interacts with BLIMP1 resulting in high levels of H2A/H4R3 (arginine 3 of the histones H2A and H4) methylation which is detected on the LINE1 and LAP in PGCs. Conditional loss of PRMT5 in early PGCs leads to male and female sterility, precede by transposable elements-induced DNA damage response (Kim et al., 2014). It will be important to investigate understanding the epigenetic profiles of germ cells to clarify comprehensive insights on germline biology and inheritance.

1.3.3 Extrinsic factors for signaling

In mammalian, BMP signals (BMP2, BMP4 and BMP8b) are important for PGC specification. Especially, BMP4 functions as an activator directly either indirectly of germ cell specific genes, *Prdm1* and *Prdm14* (Ohinata et al., 2009). Several researches regarding the successful maintenance of PGCs *in vitro* have been reported. Based on these information, lots of extrinsic factors which stimulate signal transduction were identified for the expression key transcriptional regulators for PGC development (Saitou and Yamaji, 2010). The growth factors BMP4, LIF, SCF, retinoic acid and FGF are required for early survival and proliferation of PGCs in the short-term culture experiments (Hong et al., 2012; Dolci et al., 1991; Matsui et al., 1991; Dolci et al., 1993; Resnick et al., 1998). In the absence of somatic cell during mammalian PGCs in culture, a cocktail of soluble growth factors, KLF4, LIF, BMP4, SDF-1, bFGF and compounds (N-acetyl-l-cysteine, forskolin, retinoic acid) enable to support the survival and self-renewal of mouse PGCs (Farini et al., 2005). In chicken, long-term *in vitro* conditions are established for maintaining lineage specificity and germline competency of PGCs. FGF, insulin and SMAD signaling are the minimal signaling pathways to cooperating for avian germ cell self-renewal (Whyte et al., 2015).

2. Transcriptional regulatory elements in germ cells

Gene expression is controlled, spatially and temporally, at the level of transcription. As the result of signaling pathways, transcriptional activation is governed by altering the interactions of multisubunit transcription factor complexes with their cognate *cis*-regulatory elements, including enhancers, silencers, insulators and promoters (Novina and Roy, 1996). In the general mechanism(s) of transcription initiation, production of transcript is controlled

at the promoter level. (Goodrich and Tjian, 2010). Proper expression of specific sets of genes are mediated by enhancers and promoters which provide recognition sequence to bind one or more transcription factors. Thus, studies on *cis*-DNA elements and their unique combination of modules is important for deciphering of regulatory networks of global genes that govern transcriptional control.

2.1. Core promoter

Core promoter, which is located between approximately −35 and +35 relative to the transcription start of a metazoan gene, is recognized by the general transcription factor TFIID that promote the process of forming pre-initiation complexes (Goodrich and Tjian, 2010). Generally core promoter contain an A/T-rich sequence, with the consensus TATAAA, was called the TATA box. Based on these reasons, it was considered that a similar core promoter structure would be found in every cellular genes and core promoter cannot be an active contributor to combinatorial regulation. So, it was expected that only distal promoters and enhancers are involved in transcriptional regulation by interacting cell-type specific trans-acting factors. However, recent evidence now indicates that a number of 'non-prototypical' core promoter recognition complexes have existed and required for regulating cell-specific programmes of transcription during development. Therefore, revealing the function of 'non-prototypical' subunits in core promoter of genes may clue in on comprehending transcriptional regulation and the maintenance of gene expression states (Smale, 2001). Indeed, recent studies have observed that germ cells express core promoter-associated regulatory factors and core promoter architecture is various according to genes and these diversity of core promoter contribute to mediate distinct transcriptional signals necessary for germ-cell-specific programs of appropriate gene expression. In addition, specialized core

promoters are used in germ cell transcription.

Architecture of germ cell-specific promoters used in producing transgenic animals reveals several notable features, including small size (~100bp) and relatively high GC content (DeJong, 2006). Recently, cell-type-specific TAFs and TRFs seem to have unique functions during development, differentiation and cell proliferation. In the case of TRF2 and TRF3, these proteins with sequence similarity to TBP (Rabenstein et al., 1999; Persengiev et al., 2003) are highly expressed in testes and ovary respectively. In *Trf2* knockout mice, defects in spermatogenesis and transcription of multiple post-meiotic, testes-specific genes is severely decreased (Zhang et al., 2001). Also, TRF3 also is required for normal levels of transcription in developing oocytes, in *Trf3* knockout mice defects in oocyte growth and follicular development result in sterility (Gazdag et al., 2009). Several non-prototypical TAFs also have roles in germ cell differentiation such as TAF5, TAF4, TAF6, TAF8 and TAF12 which are selectively expressed in spermatocytes in *Drosophila* and required for proper spermatocyte differentiation (Hiller et al., 2004). Based on these results, core promoter recognition components can be related in cell-type-specific gene regulation especially germ cell development. In this respect, investigation of core promoter and their recognition factors may help to understanding transcriptional mechanisms controlling cell fate and identity.

2.2 Enhancer

One of the *cis*-regulatory elements, is involved in tissue-specific gene expression, is enhancer. Enhancers are DNA sequences that have activation ability of transcription independent of their location, distance or orientation, so they accomplish regulation of gene expression patterns by interacting with a variety of transcription factors (Banerji et al., 1981). The distinct chromatin

features of enhancers provide the cell-specific gene expression programmes. Indeed, *Oct-4* expression is regulated by stage/tissue-specific enhancer in mouse. Proximal enhancer and distal enhancer is active in the epiblast of mouse embryos and in the germline, respectively (Yeom et al., 1996). In the *Drosophila* embryonic gonad, a 40-bp enhancer identified that is involved in germline specific expression of *vasa* gene (Sano et al., 2002). Also, in *Oryzias latipes*, the first 35-bp of exon 1 acts as a transcriptional enhancer driving germ cell-specific expression of *vasa* gene (Li et al., 2015).

2.3 Transcription factor

The central mechanism of promoter-specific regulation is achieved by site-specific binding regulatory factors. Variants of the core promoter recognition factors TFIIA, TBP and TAFs have discovered that have roles in spermatocytes, oocyte and the early embryo expressing specifically in germline cells. Apart from these core promoter factors, a number of transcription factors, including Sp1, NF-κB, CREMτ, BORIS, SREBP2, FIGα, and may others, have been proposed to regulate germ cell-specific gene expression (Foulkes et al., 1992; Lilienbaum et al., 2000; Loukinov et al., 2002; Thomas et al., 2005).

A recent approach of studying promoter of germ cell-specific genes contribute to predict putative transcription factors and their binding sites that control germ cell-specific gene expression. A zinc finger protein, Zfp143, as an important regulator of mammalian embryonic stem cell renewal for regulating transcription of *Nanog* gene in mouse binds to *Nanog* promoter interact with Oct4 (Chen et al., 2008). Another germ cell associated transcription factor is microphthalmia-associated transcription factor (*Mitf*) which has been reported to express in male mouse germ cells of the adult testis (Saito et al., 2003). In medaka, *Mitf* binding motif was found in the promoters of germ cell-specific

genes encoding RNA-binding proteins such as *dazl*, *dnd* and *vasa.* Indeed, *Mitf* expression in medaka cell culture mediated the transcriptional activity of the promoters of *dazl*, *dnd* and *vasa*. These results suggested that Mitf is a transcriptional activator of medaka germ genes *in vitro.*

3. Studies on the regulatory mechanisms of gene expression in germ cells

Regulation of gene transcription is the main control point in regulation of a tissue/cell-specific gene expression pattern. The mechanism of transcriptional regulation is orchestrated by transcription factors and their binding sites including core promoter and enhancer (Pereyra, 2010). Each gene has its own promoter, and some promoters can only be activated in a specific cell type. So, identification of promoters and their regulatory elements is one of the answer to questions of how germ cell-specific programs of gene expression. Many research have attempted to analyze the promoter of germ cell-specific genes to understanding germline specific regulatory mechanisms. In fact, recent studies in various species such as mouse, frogs and flies have revealed that germ cells have unique mechanisms of transcription initiation using alternate forms of core promoter transcription (DeJong, 2006).

3.1. The regulatory elements of *Vasa* gene

The vasa regulatory sequence has been shown to be able to drive GFP expression exclusively in germ cells of transgenic animals. The 5.1-kb, 4.7-kb, 5.6-kb. 8-kb, 4.3-kb and 2.4-kb of *vasa* promoter have been used for germ cell specific expression of reporter genes in medaka (Tanaka et al., 2001), rainbow trout (Yoshizaki et al., 2002), mice (Gallardo et al., 2007), cows (Luo et al., 2013), pig (Song et al., 2016) and zebrafish (Krøvel and Olsen, 2002) respectively.

Identification of *vasa* regulatory regions through reporter assay have been studied in invertebrates including fruit flies, malaria mosquito and vertebrates including medaka and chicken.

In *Drosophila*, once initiated, VAS protein is present in germline cells and is crucial role in germline development (Hay et al., 1988). Therefore, studies on the regulatory mechanism of *vas* gene could provide insight into the aspects of germline development. It is reported that germline specific *vas* gene expression in oogenesis is required for a 40-bp genomic region of the *vas* gene though interacting specifically with certain ovarian protein (Sano et al., 2002).

In malaria mosquito, another invertebrate animal, vasa-like gene is specifically expressed in both the male and female gonads in adult mosquitoes and is characterized the regulatory regions that are the entire 5'UTR and only 380bp of upstream sequence for the specific germline expression in the GSCs of both sexes (Papathanos et al., 2009).

In the case of medaka, several regulatory regions of *vasa* promoter and enhancer for germ cell specific expression have been found. To identify regulatory element of vasa promoter, they conducted 5'-deletion and 3'-deletion assay from the 5.1-kb sequence. By introducing 34 external and internal deletions, a total of 11 regions have been identified within the 5.1-kb vasa promoter and also found that the first intron plays an important role in the VAS activity (Li et al., 2013). Later, based on these results, enhancer controlling germline expression is have identified. The first 35-bp of exon 1 of the medaka *vasa* gene is sufficient to increase transcriptional activity of a heterologous promoter and contain E box or E box like motifs (Li et al., 2015).

In chicken, using hrGFP expression vector derived by *CVH* promoter, 2,718-bp, 1,555-bp and 827-bp promoter activity are compared in gonadal cells *in vitro.* As a result, germ cell-specific gene expression required at least a 1,555-

bp sequence of the 5' flanking region of *CVH* gene and 1,555-bp sequence could induced germ cell-specific exogenous gene expression during embryonic development (Minematsu et al., 2008). However, a standard approach of studying of regulatory regions of *CVH* gene and the prediction of putative binding sites has remained rare in chicken.

3.2. The regulatory elements of *Dazl* gene

Researchers have attempted on identifying its regulatory region of *Dazl*. The regulatory sequence of *Dazl* gene has been identified to require for germline specific gene expression in human, mouse, pig and chicken.

In mouse, a 1.7-kb sequence in the 5' flanking region of *Dazl* gene is sufficient to the germ cell specificity for driving the expression of GFP in transgenic mice. Following these transgenic mice, they can used to isolate putative germ cells from mouse ESCs (Nicholas et al., 2009). However, this reporter did not recapitulate early *Dazl* expression, so lacks essential regulatory elements for the early expression of *Dazl* in developing PGCs. In the case of containing of all 5' and 3' regulatory elements, reliably express endogenous *Dazl* gene, even during early PGC development (Chen et al., 2014).

By reporter assays, core promoter of *Dazl* gene and putative trans-acting elements are found in pig and human. According to reporter assay, they identified core promoter which germ cell-specific activity and these results are explained by the pattern of DNA methylation on the promoter region that is putative transcription factors binding sites.

In the case of pig, a 1,981-bp region corresponding to the putative *DAZL* promoter including its 5' flanking region was cloned and 5' deletion assay was performed. As a result, the minimal 149-bp promoter region was sufficient to activate transcription as basal levels of *DAZL* promoter activity in

both PGCs and fibroblasts. Furthermore, they identified that the sp1-2 binding element at position -83/-71 is essential for *DAZL* promoter activity and these sites are methylated in fibroblasts, so core promoter can specifically regulate expression of *Dazl* gene in PGCs (Linher et al., 2009).

Also, core promoter was identified in human *DAZL* gene based on in-silicon searches. 5' deletion assay of putative promoter region and comparing promoter activity between HeLa and GC 1 cells, Teng *et al*. have suggested that the *DAZL* core promoter is located in the region from -808 to -473 from the TSS. They also demonstrated that regulatory SNPs in -792 position which is located within the CpG islands contribute to susceptibility to spermatogenic failure in Taiwanese men. It may be explained that the G to A substitution can disrupt binding NRF-1 transcription factor on core promoter region and the consequent reduce DAZL expression (Teng et al., 2012).

In chicken, the 5' flanking region of the *DAZL* gene promoter was cloned and transfected into DF-1 cells, and then quantification of the activity of the chicken *DAZL* promoter fragment was measured by reporter assay for investigating core promoter. To obtain the core promoter activity, -383 to -39-bp region of the *DAZL* promoter is sufficient (Zhang et al., 2015). Although core promoter has identified in chicken, researches on *DAZL* promoter activity in chicken PGCs and prediction of trans-acting elements on the promoter region have not been found.

3.3. The regulatory elements of *Nanog* gene

In mammals, maintenance of pluripotency in ESCs is under the control of the core transcription factors Nanog (Mitsui et al., 2003; Chambers et al., 2003), Oct4 (Nichols et al., 1998) and Sox2 (Avilion et al., 2003). The homeodomain transcription factor Nanog is expressed in pluripotential cells,

embryonic stem cells and embryonic germ cells (Yamaguchi et al., 2005). *Nanog* expression in PGCs is maintained in proliferating germ cells during migration of PGCs, in which nuclear reprograming is processing (Yamaguchi et al., 2005).

Recent studied have identified an evolutionary functional conservation among vertebrate *Nanog* orthologs from chick (Lavial et al., 2007), zebrafish (Theunissen et al., 2011) and axolotl salamander (Dixon et al., 2010). In 2006, chicken *NANOG* was identified and expressed exclusively in PGCs during early embryo development (Canon et al., 2006). Also, it was found that chicken *NANOG* regulate pluripotency and self-renewal in chick ESC, like mammals (Lavial et al., 2007).

Regulation of *Nanog* gene have been studied in aspect of transcription factors, epigenetic factors and autoregulation. Major regulators of *Nanog* expression in mammals are Oct4 and Sox2. Through luciferase assays, it was found that transcriptional regulatory region of *Nanog* gene exist within 332-bp upstream of the transcriptional start sites and this region contains Octamer and Sox elements (Kuroda et al., 2005). Oct4 and Sox2 bind to the *Nanog* promoter in living mouse and promote upregulation of *Nanog* gene expression (Rodda et al., 2005). In 2008, Oct4 binding to the *Nanog* promoter mechanism that is regulated by Zfp143 which direct bind to *Nanog* proximal promoter region has identified (Chen et al., 2008).

To better understand how the *Nanog* gene is regulated, several studies have started. Besides the importance of HMG/POU cassette which means Sox2 and Oct-3/4 binding sites (Boer et al., 2007), lots of transcription factors binding cis-regulatory elements were identified including a STAT3 (Pan et al., 2006), FoxD3 (Suzuki et al., 2006), Sp1/Sp3 (Wu and Yao, 2006), Sall4 (Wu et al., 2006), Tcf/Lef (Kim et al., 2011), Bmi1 (Paranjape et al., 2014), Brd4 (Liu et al., 2014; Horne et al., 2015) and Znf143 (Chen et al., 2008) binding sites

which are involve in controlling transcription positively. In fact, several signaling transduction are required such as the JAK/STAT3 pathway via Klf4 and the PI3K/AKT pathway via Tbx3 (Niwa et al., 2009). In fact, STAT3 bind to an enhancer region upstream of the *Nanog* promoter and direct activate *Nanog* transcription (Do et al., 2013). And transcription of *Nanog* gene is regulated by negative *cis*-regulatory elements such as p53 (Lin et al., 2005), Klf4 and Pbx1 (Chan et al., 2009).

Even though Oct4 and Sox2 protein are important role in control transcription of *Nanog* gene, Nanog itself can bind to its own promoter and regulate its own transcription either positively or negatively (Saunders et al., 2013; Das et al., 2012). In the case of Nanog autorepression, an endogenous negative feedback loop occurs independently of Oct4 and Sox2 (Navarro et al., 2012) and by interacting with the transcriptional regulator Zfp281 (Fidalgo et al., 2012).

To understand the molecular mechanisms that regulate *Nanog* gene expression, in mouse and human, promoter assays of *Nanog* gene have been performed. In 2005, mouse *Nanog* promoter was characterized to identify the regulatory region for transcriptional activity of the gene. Through primer extension analysis and dual luciferase assay, it was identified that *Nanog* gene has two transcription start sites, separated by 190-bp and two promoter regions. Also comparing with promoter activity, positive and negative regulatory regions were verified. In addition, it was confirmed that the Oct-1 binding sites located in -190 are important for the activity of the major promoter (WU and Zhen, 2005). And the *Nanog* proximal promoter (-258/+34) has highly activity in mouse ESCs (Kuroda et al., 2005).

In human, the proximal (P1) and the distal (P2) promoters as an alternative promoter were identified using promoter reporter in ESCs. P2 is located at 1.8kb upstream region from the known TSS of *NANOG*. On the

contrary to mouse Nanog promoter, the minimal of distal promoter region (-1788/-1737) is much stronger than proximal promoter in ESCs. In chicken, on the other hand, studies on the molecular mechanisms of transcriptional regulation in chicken *NANOG* gene has not been attempted.

CHAPTER 3

Regulation of Chicken *VASA* and *DAZL* Gene by Several Regulatory Elements

1. Introduction

Primordial germ cells (PGCs) that emerge during early embryogenesis undergo a series of developmental events, such as specification, migration, and differentiation, to produce a new organism in the next generation (Han, 2009; Park and Han, 2012). They express RNA binding proteins (RBPs) by modulating tissue-specific *cis*- and *trans*-regulatory elements and have specialized genetic programs distinct from those of other somatic cells for maintaining their unique characteristics (Donovan, 1998; Han et al., 2006; Kim et al., 2007). Significant efforts have been made to elucidate the detailed molecular mechanisms regulating transcriptional control in germ cells (Zheng et al., 2009; Rengaraj et al., 2010; Kim et al., 2012). At the transcriptional level, certain genes are effectively silenced, whereas other genes are exclusively expressed to maintain the levels of germline-expressed gene products (Reinke, 2006; Seydoux and Braun, 2006).

In chicken, PGCs separate from the epiblast in the blastoderm at Eyal-Giladi and Kochav stage X, which consist of 40,000 to 60,000 undifferentiated embryonic cells, and translocate into the hypoblast area of the pellucida (Hamburger and Hamilton, 1951; Ginsburg and Eyal-Giladi, 1986). During gastrulation, they circulate through the vascular system and finally settle in the gonadal anlagen. After the arrival of PGCs, these cells continue to proliferate until they enter meiosis. This development of the PGC lineage is a highly complex process that is controlled by the coordinated action of many key factors, such as the expression and regulation of germline-specific genes (Lee et al., 2007; Lee et al., 2011).

Evolutionarily conserved germ cell-specific *Vasa* has been characterized in germ cells in several organisms, including chicken (Tsunekawa et al., 2000; Minematsu et al., 2008; Lavial et al., 2009), zebrafish (Yoon et al., 1997), mouse (Fujiwara et al., 1994), and human (Castrillon et al., 2000).

Several studies have demonstrated that *Vasa* plays critical roles in germ cell specification, supporting germ line development, translational control of transcribed genes, and RNA processes involving the biosynthesis of Piwi-interacting RNAs in germ cells at the post-transcriptional level (Styhler et al., 1998; Carrera et al., 2000; Raz, 2000; Noce et al., 2001; Liu et al., 2009; Xiol et al., 2014). However, the intricate regulatory mechanism(s) that governs transcriptional control of *Vasa* expression during chicken germline development has yet to be investigated in detail.

Also, in *Dazl* gene, for researching of mechanism by which *Dazl* gene expression is restricted to germ cells, researcher have attempted on identifying its regulatory region. The regulatory sequence of *Dazl* gene has been identified to require for germline specific gene expression in human (Teng et al., 2012), mouse (Nicholas et al., 2009), pig (Linher et al., 2009) and chicken (Zhang et al., 2015). In chicken, *DAZL* core promoter was identified (-383 to -39 bp), but, researches on *DAZL* promoter activity in chicken PGCs have not been reported.

Understanding the cellular and molecular mechanisms that regulate germ cell-specific gene expression during PGC development is critical for the practical use of genetic modifications and germ-cell biology. In the current study, to characterize the promoter of chicken *vasa* homologue (*CVH*) for inducing germ cell-specific gene expression, we conducted 5′ deletion and fragment assays using both enhanced green fluorescent protein (eGFP) and NanoLuc luciferase expression vector. Furthermore, we investigated the predicted putative binding of transcription factors (TFs) on the promoter for *CVH*. Finally, we demonstrated that the transcriptional control of *CVH* expression through *cis*-elements and TFs is important for germ cell-specific gene expression in chicken PGCs.

2. Materials and Methods

Experimental animals and animal care

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

Construction of eGFP and NanoLuc luciferase expression vectors controlled by the *CVH* and *cDAZL* promoter of different sizes

For construction of eGFP expression vector, the 5' flanking regions of *CVH* (NM_204708.2) and *cDAZL* (NM_204218.1) gene were amplified using the genomic DNA extracted from adult chicken blood, and subsequently into the pGEM T easy vector (Promega, USA). Primer sets were used to clone fragments of the *CVH* and *cDAZL* promoter of different sizes (Table 1 and 2). The eGFP coding sequence and polyadenylation (Poly-A) tail were inserted into the clone vectors including *CVH* and *cDAZL* promoter using restriction enzymes *SpeI* and *NdeI*. For construction of NanoLuc luciferase expression vectors, different lengths of the 5'-upstream region of *CVH* gene were inserted between the *KpnI* and *XhoI* sites of the pNL1.2 vectors (Promega, USA). In the case of *cDAZL* gene, fragments of promoter were used restriction enzyme sites by *KpnI* and *SacI*.

Culture of chicken PGC and DF-1

Chicken PGCs were cultured according to our standard procedure (Park and Han, 2012). Briefly, chicken PGCs from White Leghorn embryonic gonads at 6-day-old (HH stage 28) were maintained in knockout Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 20%

fetal bovine serum (FBS) (Hyclone, USA), 2% chicken serum (Sigma-Aldrich, USA), 1x nucleosides (Millipore, USA), 2mM L-glutamine (Gibco, USA), 1x nonessential amino acids (Gibco, USA), β-mercaptoethanol (Gibco, USA), 1mM sodium pyruvate (Gibco, USA), and 1x antibiotic-antimycotic (Gibco, USA). Human basic fibroblast growth factor (bFGF) (KomaBiotech, Korea) at 10 ng/ml was used for PGC self-renewal. The cultured PGCs were subcultured onto mitomycin-inactivated mouse embryonic fibroblasts at 5 to 6-day intervals by gentle pipetting without any enzyme treatment. For DF-1, the cells were maintained in DMEM with high glucose (Hyclone, USA), 10% FBS, and 1x antibiotic-antimycotic. Cultured cells were grown at 37°C in a 5% CO2 incubator.

In Vitro transfection

In vitro transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). For expression analysis of eGFP, constructed *CVH* promoter vector (1 μg) and 2 ul of Lipofectamine 2000 were separately diluted with 50 ul of Opti-MEM I reduced serum medium (Invitrogen) 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. Liposome-DNA complex solution was added to $2.5x10^5$ cultured PGCs in 500 ul of PGC culture medium. Transfected cells were incubated for 24hr without feeders. After incubation, cells were analyzed using a fluorescence microscope.

Luciferase reporter assay

Nano-Glo Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure the *CVH* and *cDAZL* promoter activities. The prepared cells were seeded in 96-well plate and co-transfected with pGL4.53 firefly luciferase (Fluc) and pNL1.2 (NlucP/CVH RE or cDAZL

RE) NanoLuc luciferase (Nluc) plasmid using Lipofectamine 2000 (Invitrogen). Transfected cells were lysed with lysis buffer with Fluc substrate and incubated on an orbital shaker for 3 min. Fluc signals were then quenched and following by reaction with Nluc substrate. The signals in arbitrary unit (AU) from both Nluc and Fluc were measured by a luminometer (Glomax-Multi-Detection System, USA). The promoter activities were calculated by the ratio of respective AU values of Nluc/Fluc.

Prediction of putative transcriptional binding elements *in Silico* sequence analysis

The 591-bp fragment (−316/+275) of CVH and the 1,045-bp fragment (−872/+173)of *cDAZL* promoter that had the highest activity was analyzed for transcription factor binding sites. Transcription factor binding sites were predicted by the MatInspector, a Genomatix program (http://www.genomatix.de) using TRANSFAC matrices (vertebrate matrix; core similarity 1.0 and matrix similarity 0.8), the PROMO that uses version 8.3 TRANSFAC (http://alggen.lsi.upc.es) and the TFBIND which uses weight matrix in database TRANSFAC R.3.4 (http://tfbind.hgc.jp).

siRNA transfection in chicken PGCs

Cells were seeded at a density of 2.5 x 10^5 per well of a 12-well plate in a volume of 1 ml medium. Then, cells were transfected with each siRNAs (50 pmole) with RNAiMAX (Invitrogen, USA). Negative control siRNA that has no complementary sequence in the chicken genome was used as a control. Sequences of each siRNAs are listed in Table 3. After transfection for 48 hr, total RNA was extracted using TRIzol reagent (Invitrogen, USA). The knockdown efficiency of predictive transcription factors and their effects on the expression of germ cell-related genes including *CVH*, *cDAZL*, *CIWI* and *cDND1* were measured using quantitative RT-PCR.

RNA isolation and quantitative RT-PCR

Total RNA of siRNA-treated PGCs was extracted by TRIzol reagent (Life Technologies, USA) according to the manufacturer's protocol. 1ug of each RNA was reverse-transcribed with the Superscript III First-strand Synthesis System (Invitrogen, USA). The cDNA was diluted 4-fold and used as a template for quantitative real-time PCR which was performed using StepOnePlus real time PCR system (Applied Biosystems, USA) with EvaGreen (Biotium, USA). Each test sample was performed in triplicate. Then, the relative gene expression of individual sample was calculated after normalization with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression as an endogenous control (Livak and Schmittgen, 2001; Lee et al., 2007). The primer pairs, which were designed using the NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), used for detection of cDNAs are listed in Table 4.

Statistical analysis

All data were expressed as mean± S.D. from three independent experiments. One-way ANOVA with Bonferroni compare all pairs of columns was used to calculate the difference between experimental groups. GraphPad Prism v.5 (GraphPad Software, USA) was used to evaluate the data. $P < 0.05$ was considered statistically significant.

3. Results

Identification of *CVH* and *cDAZL* promoter for inducing germ cell-specific gene expression

To investigate the gene promoter for the expression of *CVH* mRNA, we constructed eGFP expression vectors containing different sizes of the *CVH* promoter by 5′ deletion, spanning a 1,850-bp region from the 5′ flanking region to the 5′ untranslated region (UTR) (Figure 1A). Subsequently, we tested whether the differently sized *CVH* promoters can induce the expression of eGFP in cultured chicken PGCs. As shown in Figure 1B, the following eGFP reporters were associated with the expression of green fluorescence in chicken PGCs: 1,850-bp fragment (−1,575/+275), 1,506-bp fragment (−1,231/+275), 900-bp fragment (−625/+275), 591-bp fragment (−316/+275), and 410-bp fragment (−135/+275); however, the smallest fragment (+26/+275) did not induce this expression. To evaluate the promoter activity further, we performed the dual luciferase reporter assay using the same fragments of the *CVH* promoter in chicken PGCs and DF-1. Consistent with the findings for eGFP expression, the luciferase reporters containing the promoter region of *CVH* presented strong enzyme activity, but we could not detect enzyme activity from the smallest 250-bp fragment and pNL1.2-basic, an empty vector (Figure 1C). Notably, compared with DF-1 fibroblast cells, chicken PGCs generally presented at least 10 times higher luciferase activities (Figure 1C and D). Collectively, these results suggest that the minimal promoter region of the *CVH* gene is located at −135 to +275 bp, which includes the 5′ UTR, and plays an important role in the transcription of this gene in chicken PGCs.

cDAZL promoter region also was identified with the same procedure in the case of finding *CVH* promoters. Figure 2A shows that constructs including *cDAZL* promoter with 5'UTR that can express eGFP; 1,221-bp

fragment (−1,408/+173), 1,045-bp fragment (−872/+173), 590-bp fragment (−417/+173), 519-bp fragment (−346/+173) and 256-bp fragment (−83/+173), and the results of transfection into chicken cultured PGCs of these vectors were observed through fluorescence microscopy (Figure 2B). All five constructs were expressed in PGCs. To measuring promoter activity, dual luciferase assay was performed in PGCs (Figure 2C) and DF-1 (Figure 2D). Collectively, these results suggested that the minimal part of promoter region of *cDAZL* gene is located at −83 bp to +173 bp which can cause specifically expression of exogenous genes in chicken cultured PGCs.

Investigation of the *cis*–regulatory element of *CVH* and *cDAZL* gene

For further investigation of the potential transcriptional *cis*-elements in the *CVH* promoter, we performed 5′ and 3′ fragmentation assays using the 591-bp fragment (−316/+275) that presented the highest luciferase reporter activity, as well as a 410-bp fragment (−135/+275) (Figure 3A). First, we confirmed the eGFP expression with the designed fragments of the *CVH* promoter in chicken PGCs. Among six fragment constructs, the 591-bp fragment (−316 /+275), 502-bp fragment (−227/+275), and 410-bp fragment (−135/+275) were associated with the strong expression of green fluorescence in chicken PGCs compared with the 357-bp fragment (−135/+222) and the 297-bp fragment (−135/+162). These latter two fragments (357-bp and 297-bp fragments) still showed minimal promoter activity, while the 250-bp fragment (+26/+275) showed none (Figure 3B). We also conducted a dual luciferase reporter assay using NanoLuc luciferase expression vectors to compare the *CVH* promoter activity in chicken PGCs and DF-1. As shown in Figure 3C, deletion of the 92-bp fragment between −227/+275 bp and −135/+275 bp resulted in a dramatic decrease in luciferase activity. These results suggest that a positive transcriptional *cis*-element is located in this region. Furthermore,

partial deletion of the 5′ UTR including intron 1 (−135/+222 bp and −135/+162 bp) also produced a dramatic change in promoter activity (Figure 3C). Interestingly, all tested fragments showed higher luciferase activity in PGCs than in DF-1 fibroblasts (Figure 3D). Collectively, these results indicate that the PGC-specific gene expression requires at least a 410-bp sequence of the 5′ upstream region of the *CVH* gene along with the 5′ UTR including intron 1.

To verify that whether *cDAZL* promoter is required for 5' UTR and the potential *cis*-element in *cDAZL* promoter, we performed 5' and 3' fragmentation assay using 1,045-bp fragment (−872/+173) that present the highest luciferase reporter activity and 590-bp fragment (−417/+173) (Figure 4A). The results of transfection into PGCs of these vectors were shown in figure 4B. NanoLuc luciferase expression vectors driven by *cDAZL* promoters were transfected into PGCs and DF-1 and transfected cells were analyzed via dual luciferase assay (Figure 4C and D). As shown in Figure 4C, deletion of 195-bp fragment between 912-bp fragment (−739/+173) and 717-bp fragment (−555/+173) presents dramatic decrease in luciferase activity. These results suggested that the positive transcriptional *cis*-element locate in this region. Also Figure 4C shows that fragments without 5' UTR of *cDAZL* gene can act as promoter in PGCs. In these results indicate that *CVH* promoter is required for 5' UTR containing intron-1, but *cDAZL* promoter activity has no concern with 5' UTR of *cDAZL* gene.

Prediction of transcription factors highly expressed in chicken PGCs.

Based on the findings of the *CVH* promoter activity mentioned above, we predicted common TFs that have binding sites in the 591-bp fragment (−316/+275) of the *CVH* promoter and 1,045-bp fragment (−872/+173) of the *cDAZL* promoter using three software programs (PROMO, TFBIND, and MatInspector). Additionally, we attempted to clarify the TFs that were more

highly expressed in chicken PGCs than in other cell types, such as Stage X blastodermal cells, gonadal stromal cells (GSCs), and chicken embryonic fibroblasts (CEFs) using previously obtained transcriptome data (Figure 5A) (Han et al., 2006; Kim et al., 2007; Lee et al., 2011). From these analyses, we identified six TFs (*EP300*, *GABPA*, *HSF2*, *NFYA*, *SP3*, and *ZNF143*) that were expressed at significantly higher levels in PGCs and have putative binding sites in the 591-bp fragment (−316/+275) of the *CVH* and 1,045-bp fragment (−872/+173) of the *cDAZL* promoter. To summarize our findings, we marked the consensus sequences and positions of the predicted TFs in sequences of the *CVH* and *cDAZL* promoter including TATA-box sequence and transcription start codon in Figure 5B and C.

Predictive transcription factors affect transcriptional activity of germ cell-specific RBPs

To confirm the expression of the selected TFs in chicken PGCs, we conducted quantitative RT-PCR using the RNA samples prepared from various cells/tissues (PGCs, Stage X, CEFs, DF-1, and GSCs). The results showed that the expression of five TFs is highly PGC-specific, with the exception being *GABPA*, which is expressed in both PGCs and Stage X equally (Figure 6). These results indicate that these TFs may be involved in transcriptional control of the *CVH* and *cDAZL* promoter by directly interacting with it in chicken PGCs. We further examined whether these TFs affect the transcription of germ cell-specific RBPs (*CVH*, *cDAZL*, *CIWI*, and *cDND1*) in chicken PGCs using an siRNA-mediated knockdown assay. As shown in Figure 7, in the samples with the highest knockdown efficiency of *HSF2*, *NFYA*, *SP3*, and *ZNF143* mRNA expression in chicken PGCs, the expression of RBP mRNA was significantly reduced, suggesting that these TFs function in regulating transcriptional control of the *CVH* and *cDAZL* promoter, and other PGC-specific RBPs, such as *CIWI*,

and c*DND1*, while *EP300* and *GABPA* remain unaffected. Taken together, these results suggest that these TFs (*HSF2*, *NFYA*, *SP3*, and *ZNF143*) play a role in the transcription of PGC-specific RBPs through direct binding to 5′ upstream promoter regions.

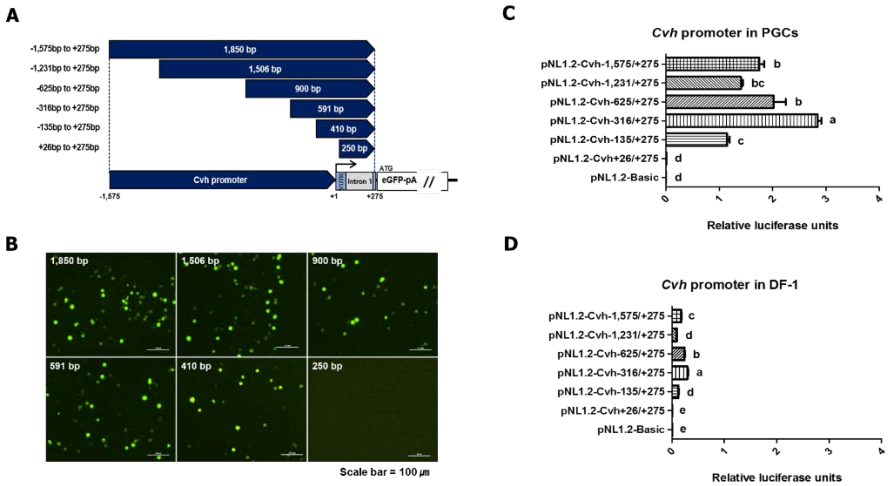

Figure 1. Identification of *CVH* promoter regions through 5' deletion assay. (A) Schematic diagram of constructed *CVH* promoter vectors which are designed for expression analysis using an eGFP vector. By 5' deletion assay, six constructs including different lengths of 5' flanking sequences including 5' UTR were randomly designed. In the case of 250-bp size vector contain only part of 5' UTR. (B) 24 hr after transfection, expression of enhanced green fluorescent protein (eGFP) under control of different constructs in cultured chicken PGCs was monitored by microscopy. Each fragments used as driving eGFP expression were ligated NanoLuc luciferase expression vector (pNL1.2-Basic) for measuring promoter activity. Dual luciferase assay of *CVH* promoter activity in PGCs (C) and DF-1 (D). NanoLuc luciferase expression levels were normalized to the luciferase activity of internal Firefly control and expressed as relative luciferase units. Scale bar = 100 μm. Different letters (a-e) indicate significant differences ($P < 0.05$).

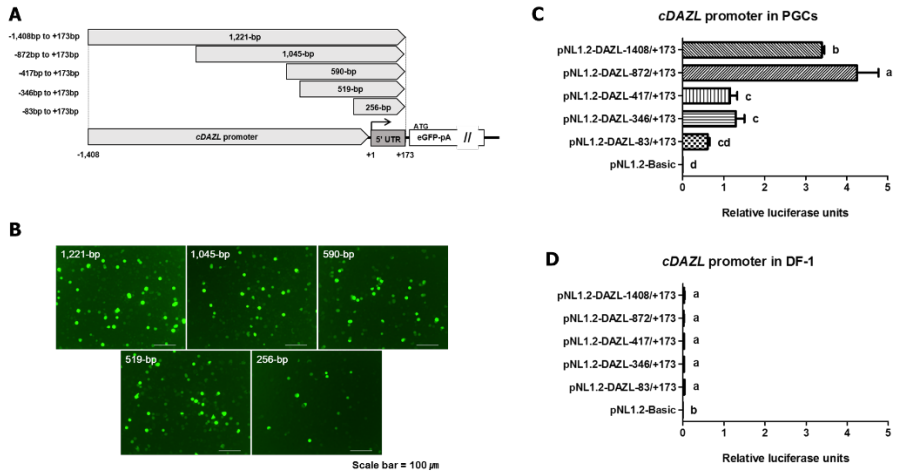

Figure 2. Identification of *cDAZL* promoter regions by 5' deletion assay. (A) Schematic diagram of constructed *cDAZL* promoter vectors which are designed for expression analysis using an eGFP vector. By 5' deletion assay, five constructs including different lengths of 5' flanking sequences including 5' UTR were randomly designed. (B) 24 hr after transfection, expression of enhanced green fluorescent protein (eGFP) under control of different constructs in cultured chicken PGCs was monitored by microscopy. Each fragments used as driving eGFP expression were ligated NanoLuc luciferase expression vector (pNL1.2-Basic) for measuring promoter activity. Dual luciferase assay of *cDAZL* promoter activity in PGCs (C) and DF-1 (D). NanoLuc luciferase expression levels were normalized to the luciferase activity of internal Firefly control and expressed as relative luciferase units. Scale bar = 100 μm. Different letters (a-d) indicate significant differences ($P < 0.05$).

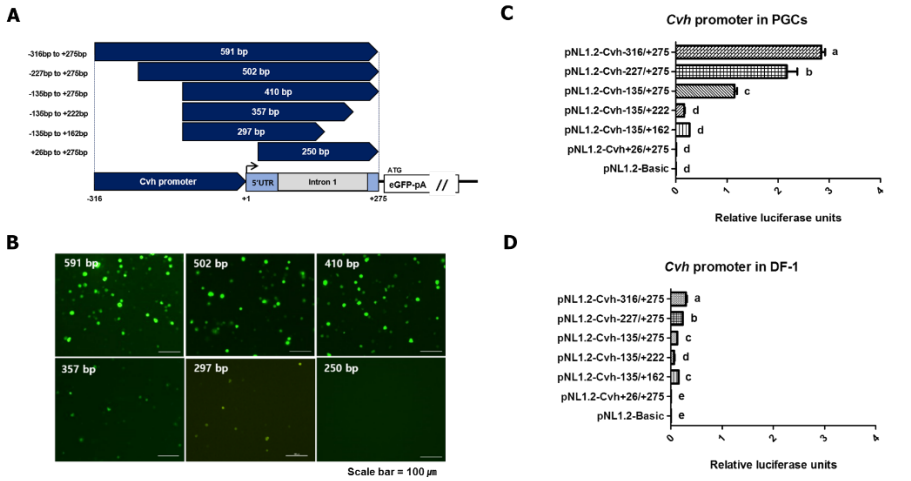

Figure 3. Identification of *CVH* promoter regions through 5' fragmentation. (A) Schematic diagram of fragmented six constructs of *CVH* promoter. Constructs were designed for expression analysis using an eGFP vector. 5' and 3' randomly deletion assay were conducted from the fragments −316/+275 and −135/+275, respectively. In the case of 3' deletion from the fragment −135/+275, two constructs (−135/+222 and −135/+162) included partial intron region. (B) Expression of enhanced green fluorescent protein (eGFP) under control of different constructs in cultured chicken PGCs transfected in vitro. 24-hr after transfection, expression of eGFP was observed by fluorescence microscopy. Each fragments used as driving eGFP expression were ligated NanoLuc luciferase expression vector (pNL1.2-Basic) for measuring promoter activity. Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs (C) and DF-1 (D). Promoter activity was measured as the ratio of NanoLuc luciferase expression levels to internal Firefly control and expressed as relative luciferase units. Different letters (a-e) indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

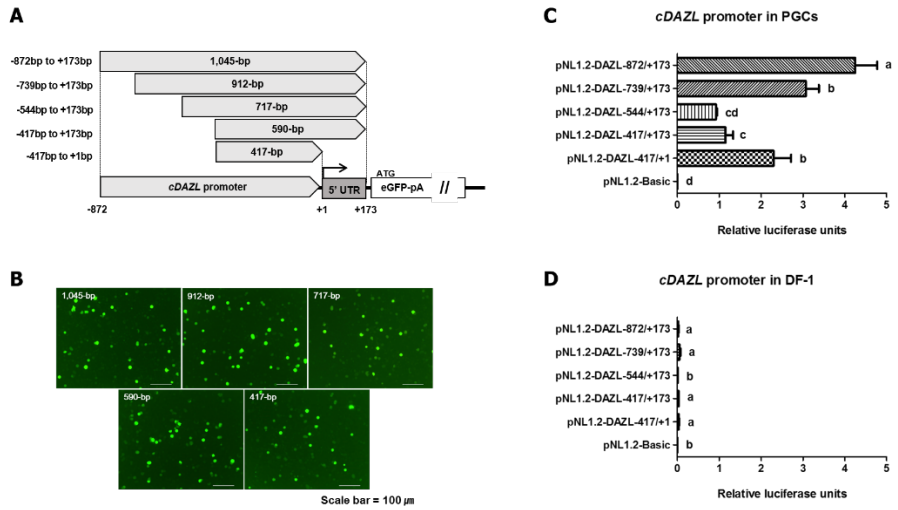

Figure 4. Analysis of *cis*-element in chicken *DAZL* promoter. (A) Schematic diagram of fragmented four constructs of *cDAZL* promoter. Constructs were designed for expression analysis using an eGFP vector. 5' randomly deletion assay were conducted from the fragments −872/+178 and −417/+178, respectively (B) Expression of enhanced green fluorescent protein (eGFP) under control of different constructs in cultured chicken PGCs transfected in vitro. 24 hr after transfection, expression of eGFP was observed by fluorescence microscopy. Each fragments used as driving eGFP expression were ligated NanoLuc luciferase expression vector (pNL1.2-Basic) for measuring promoter activity. Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs (C) and DF-1 (D). Promoter activity was measured as the ratio of NanoLuc luciferase expression levels to internal Firefly control and expressed as relative luciferase units. Different letters (a-d) indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

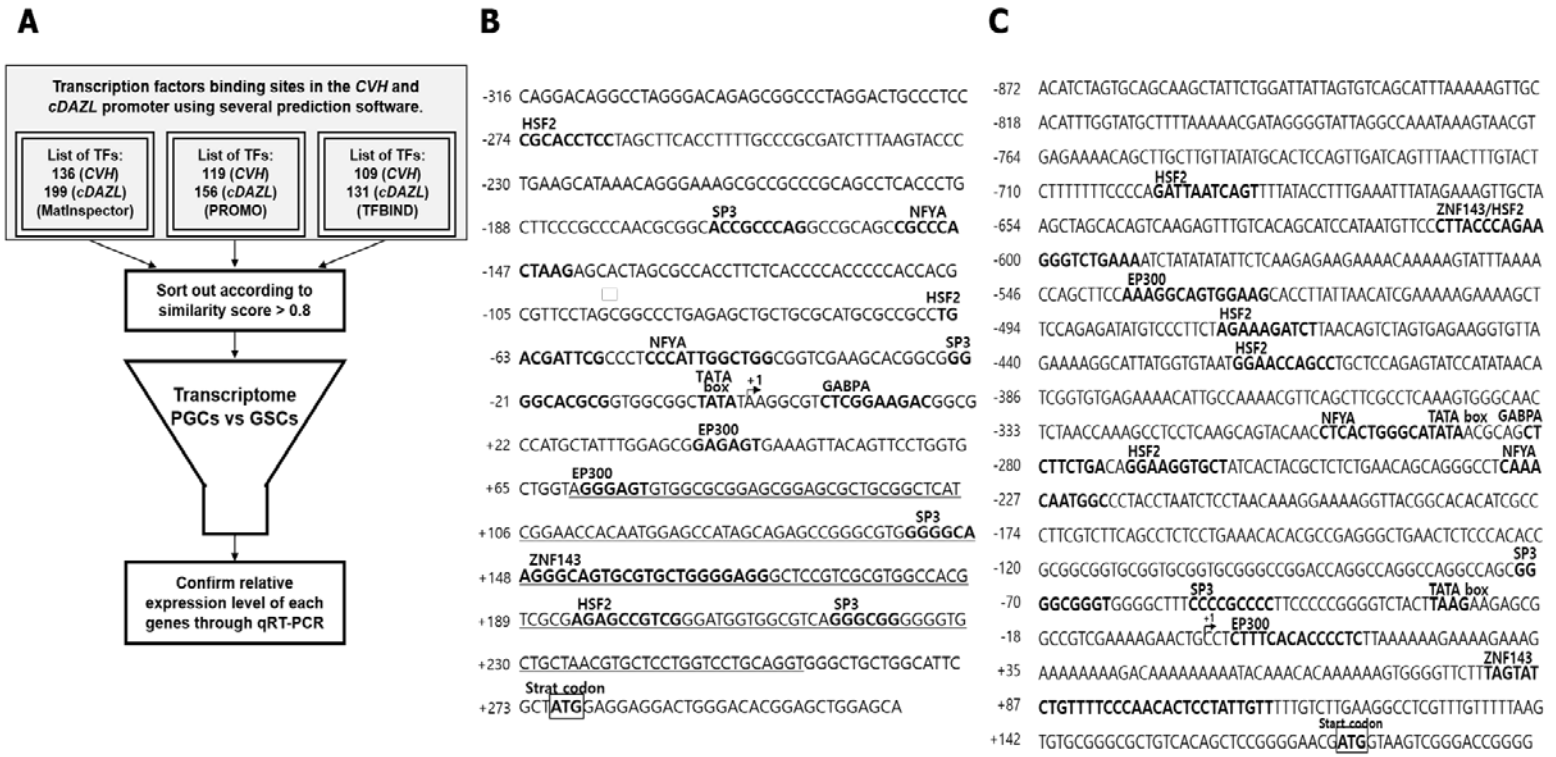
A
Transcription factors binding sites in the CVH and cDAZL promoter using several prediction software.
List of TFs: 136 (CVH) 199 (cDAZL) (MatInspector)
List of TFs: 119 (CVH) 156 (cDAZL) (PROMO)
List of TFs: 109 (CVH) 131 (cDAZL) (TFBIND)
Sort out according to similarity score > 0.8
Transcriptome PGCs vs GSCs
Confirm relative expression level of each genes through qRT-PCR
B
-316 CAGGACAGGCCTAGGGACAGAGCGGCCCTAGGACTGCCCTCC
HSF2
-274 CGCACCTCCTAGCTTCACCTTTTGCCCGCGATCTTTAAGTACCC
-230 TGAAGCATAAACAGGGAAAGCGCCGCCCGCAGCCTCACCCTG
SP3 NFYA
-188 CTTCCCGCCCAACGCGGCACCGCCCAGGCCGCAGCCGCCCA
-147 CTAAGAGCACTAGCGCCACCTTCTCACCCCACCCCCACCACG
HSF2
-105 CGTTCCTAGCGGCCCTGAGAGCTGCTGCGCATGCGCCGCCTG
NFYA SP3
-63 ACGATTCGCCCTCCCATTGGCTGGCGGTCGAAGCACGGCGGG
TATA box +1 GABPA
-21 GGCACGCGGTGGCGGCTATATAAGGCGTCTCGGAAGACGGCG
EP300
+22 CCATGCTATTTGGAGCGGAGAGTGAAAGTTACAGTTCCTGGTG
EP300
+65 CTGGTAGGGAGTGTGGCGCGGAGCGGAGCGCTGCGGCTCAT
SP3
+106 CGGAACCACAATGGAGCCATAGCAGAGCCGGGCGTGGGGGCA
ZNF143
+148 AGGGCAGTGCGTGCTGGGGAGGGCTCCGTCGCGTGGCCACG
HSF2 SP3
+189 TCGCGAGAGCCGTCGGGATGGTGGCGTCAGGGCGGGGGGTG
+230 CTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTC
Strat codon
+273 GCTATGGAGGAGGACTGGGACACGGAGCTGGAGCA
C
-872 ACATCTAGTGCAGCAAGCTATTCTGGATTATTAGTGTCAGCATTTAAAAAGTTGC
-818 ACATTTGGTATGCTTTTAAAAACGATAGGGGTATTAGGCCAAATAAAGTAACGT
-764 GAGAAAACAGCTTGCTTGTTATATGCACTCCAGTTGATCAGTTTAACTTTGTACT
HSF2
-710 CTTTTTTTCCCCAGATTAATCAGTTTTATACCTTTGAAATTTATAGAAAGTTGCTA
ZNF143/HSF2
-654 AGCTAGCACAGTCAAGAGTTTGTCACAGCATCCATAATGTTCCCTTACCCAGAA
-600 GGGTCTGAAAATCTATATATATTCTCAAGAGAAGAAAACAAAAAGTATTTAAAA
EP300
-546 CCAGCTTCCAAAGGCAGTGGAAGCACCTTATTAACATCGAAAAAGAAAAGCT
HSF2
-494 TCCAGAGATATGTCCCTTCTAGAAAGATCTTAACAGTCTAGTGAGAAGGTGTTA
HSF2
-440 GAAAAGGCATTATGGTGTAATGGAACCAGCCTGCTCCAGAGTATCCATATAACA
-386 TCGGTGTGAGAAAACATTGCCAAAACGTTCAGCTTCGCCTCAAAGTGGGCAAC
NFYA TATA box GABPA
-333 TCTAACCAAAGCCTCCTCAAGCAGTACAACCTCACTGGGCATATAACGCAGCT
HSF2 NFYA
-280 CTTCTGACAGGAAGGTGCTATCACTACGCTCTCTGAACAGCAGGGCCTCAAA
-227 CAATGGCCCTACCTAATCTCCTAACAAAGGAAAAGGTTACGGCACACATCGCC
-174 CTTCGTCTTCAGCCTCTCCTGAAACACACGCCGAGGGCTGAACTCTCCCACACC
SP3
-120 GCGGCGGTGCGGTGCGGTGCGGGCCGGACCAGGCCAGGCCAGGCCAGCGG
SP3 TATA box
-70 GGCGGGTGGGGCTTTCCCCGCCCCTTCCCCCGGGGTCTACTTAAGAAGAGCG
+1 EP300
-18 GCCGTCGAAAAGAACTGCCTCTTTCACACCCCTCTTAAAAAAGAAAAGAAAG
ZNF143
+35 AAAAAAAAGACAAAAAAAAATACAAACACAAAAAAGTGGGGTTCTTTAGTAT
+87 CTGTTTTCCCAACACTCCTATTGTTTTTGTCTTGAAGGCCTCGTTTGTTTTTAAG
Start codon
+142 TGTGCGGGCGCTGTCACAGCTCCGGGGAACGATGGTAAGTCGGGACCGGGG

Figure 5. Location of predicted transcription factors binding sites on the *CVH* and *cDAZL* promoter. (A) A flowchart of selection processing transcription factors which have the putative binding sites in the 591-bp fragment (−316 bp to +275 bp) of *CVH* and 1,045-bp fragment (−872 bp to +173 bp) of *cDAZL* promoter. The input is sets of transcription factors which are predicted by several prediction software (MatInspector, PROMO and TFBIND). Then, TFs which are similarity score below 0.8 are removed. The putative transcription factors are extracted depending on significant expression in chicken PGCs from transcriptome data. Nucleotide sequences of the 5' flanking region of the 591-bp fragment (−316 bp to +275 bp) of *CVH* (B) and 1,045-bp fragment (−872 bp to +173 bp) of *cDAZL* promoter (C). Selected transcription factors were marked on the sequences of *CVH* and *cDAZL* promoter region. The +1 indicates the transcriptional initiation site and boxed ATG indicates the translational start site. The bold sequences show predicted binding sites of the transcription factors. Underline indicates the intron region of *CVH* gene.

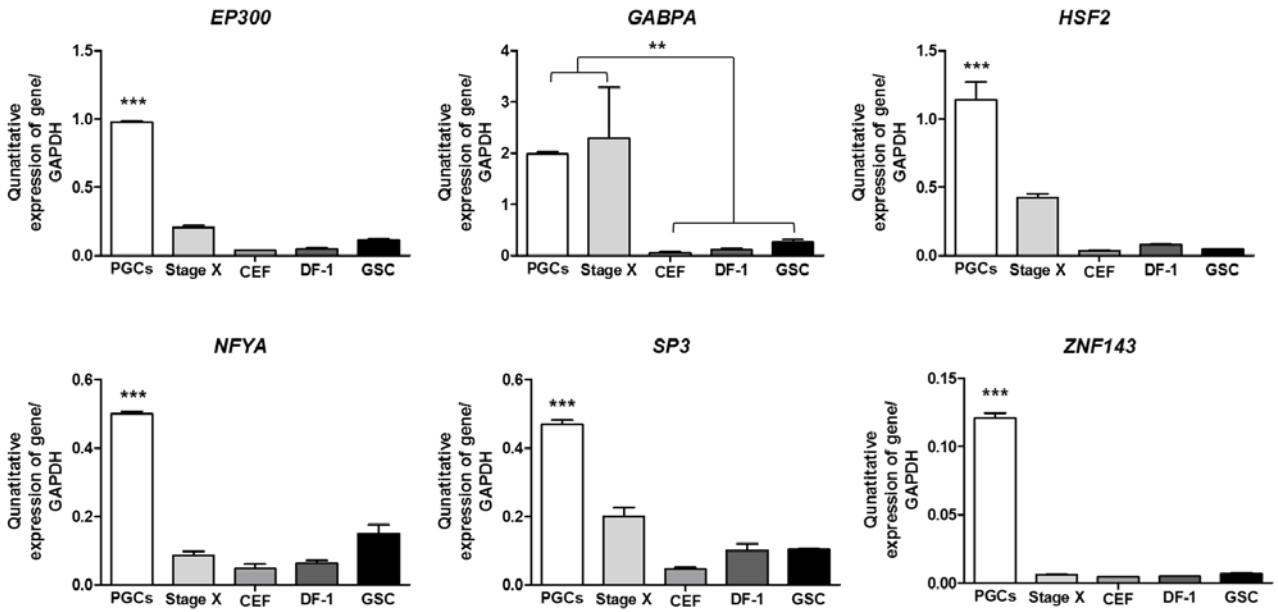

Figure 6. Quantitative expression analysis of predicted transcription factors in various cell types. By quantitative RT-PCR analysis, predicted transcription factors was analyzed with the prepared PGCs, stage X blastoderm, CEFs (chicken embryonic fibroblast), DF-1 and GSCs (gonadal stromal cells). Error bars indicate the standard deviation of triplicate analysis. Significant differences are indicated as ***$P < 0.001$ and **$P < 0.01$.

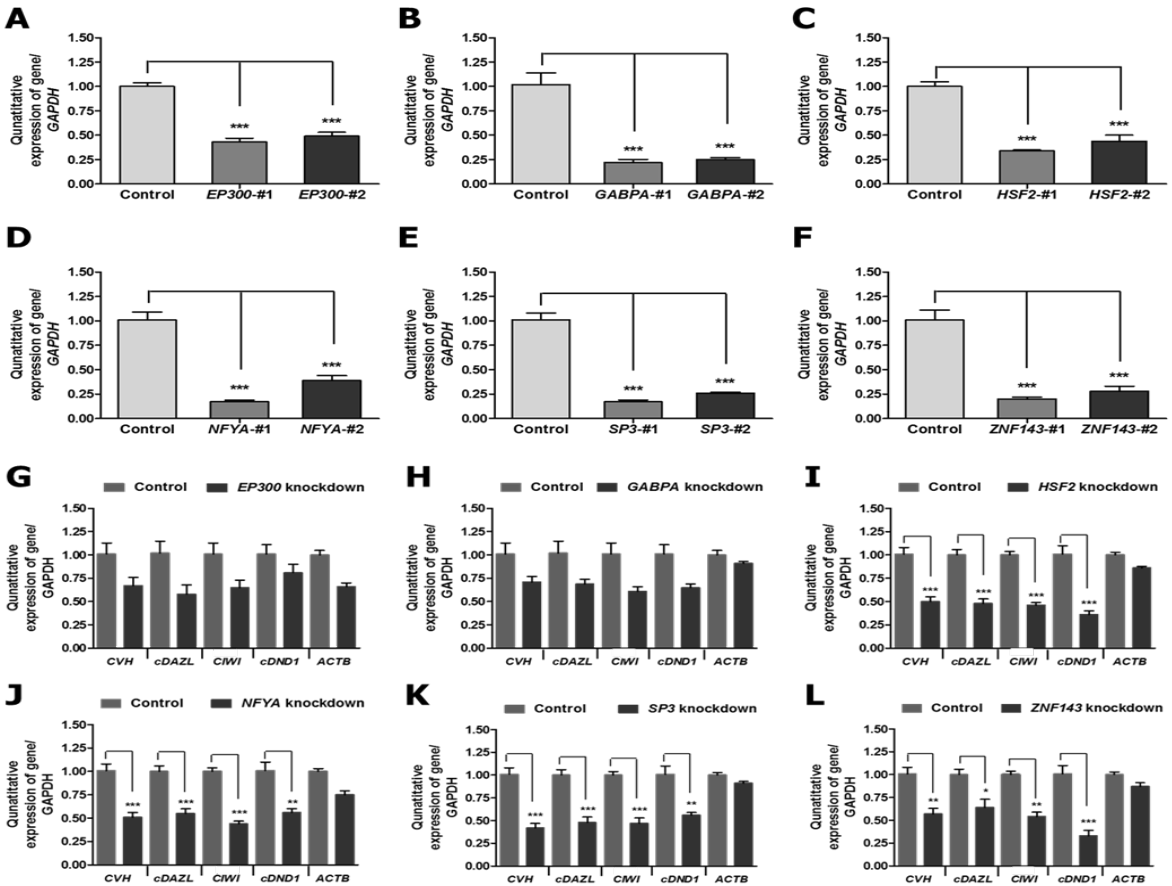
A
B
C
D
E
F
G
H
I
J
K
L
Qunatitative expression of gene/ GAPDH
Control
EP300-#1
EP300-#2
GABPA-#1
GABPA-#2
HSF2-#1
HSF2-#2
NFYA-#1
NFYA-#2
SP3-#1
SP3-#2
ZNF143-#1
ZNF143-#2
EP300 knockdown
GABPA knockdown
HSF2 knockdown
NFYA knockdown
SP3 knockdown
ZNF143 knockdown
CVH
cDAZL
CIWI
cDND1
ACTB

Figure 7. Relative gene expression analysis after knockdown of predicted transcription factors in cultured PGCs. (A-F) Efficiency of siRNA-mediated knockdown *in vitro* for predicted transcription factors was analyzed by quantitative reverse-transcriptase PCR. (G-L) The relative expression analysis of germ cell-related RBPs (*CVH*, c*DAZL*, *CIWI* and *cDND1*) in cultured PGCs after treatment of each siRNA. *ACTB* (*β-actin*) was used as a control for silencing specificity of the knockdown probes. qPCR was conducted in triplicated, normalizing data to control expression of *GAPDH*. Significant differences between control and treatment groups are indicated as ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. Error bars indicate the standard error of triplicate analyses.

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

Primer set	Primer sequence (5'→3')
CVH -1,575 bp_F	GACACAGCTTTCCCACGTGAG
CVH -1,231 bp_F	TGGCCACGTGCTATCATATTAGT
CVH -625 bp_F	CTCTGATCATGCCTGCAGCC
CVH -316 bp_F	CAGGACAGGCCTAGGGACAGA
CVH -227 bp_F	AGCATAAACAGGGAAAGCGC
CVH -135 bp_F	GCGCCACCTTCTCACCCC
CVH +25 bp_F	GCTATTTGGAGCGGAGAGTGAAA
CVH promoter_R	AGCGAATGCCAGCAGCC
CVH -135/+222_R	CGCCCTGACGCCACCAT
CVH -135/+162_R	AGCACGCACTGCCCTTGC
eGFP poly A _F	ACTAGTCCGCGGATGGTGAGCAAG
eGFP poly A_R	CATATGGACGTCTCCCCAGCATGCC

Table 2. List of primer sequences for cloning of the *cDAZL* promoter using genomic PCR

Primer set	Primer sequence (5'→3')
cDAZL -1,422 bp_F	CTGGTACTGATGTGTTGCC
cDAZL -872 bp_F	ACATCTAGTGCAGCAAGCTATTCTGG
cDAZL -752 bp_F	GCACTCCAGTTGATCAGTTTAACTT
cDAZL -555 bp_F	CCAGCTTCCAAAGGCAG
cDAZL -428 bp_F	GGAACCAGCCTGCTCCAGA
cDAZL -357 bp_F	CTCAAAGTGGGCAACTCTAACCAA
cDAZL -83 bp_F	GGCCAGGCCAGCGGGGCGG
cDAZL promoter_R	CGTTCCCCGGAGCTGTG
cDAZL promoter_R2	CAGTTCTTTTCGACGGCC
eGFP poly A _F	ACTAGTCCGCGGATGGTGAGCAAG
eGFP poly A_R	CATATGGACGTCTCCCCAGCATGCC

Table 3. List of siRNA sequences of each transcription factor for knockdown analysis

Target gene#	siRNA sequence (5'→3')	
	Sense	**Antisense**
EP300#1	GAGUUCUCCUCACUACGAA	UUCGUAGUGAGGAGAACUC
EP300#2	GAUGAAUGCUGGCAUGAAU	AUUCAUGCCAGCAUUCAUC
GABPA#1	GAGCAAGGUAUGUGUCUGU	ACAGACACAUACCUUGCUC
GABPA#2	CACAAGAAGUCAACCAUCA	UGAUGGUUGACUUCUUGUG
HSF2#1	GUGUUGGAUGAACAGAGAU	AUCUCUGUUCAUCCAACAC
HSF2#2	CAGAACUGAGAGCAAAACA	UGUUUUGCUCUCAGUUCUG
NFYA#1	UCAGACAGCUUACAGACUA	UAGUCUGUAAGCUGUCUGA
NFYA#2	CAGUACACAGCCAACAGUA	UACUGUUGGCUGUGUACUG
SP3#1	CAGUACAGUGCUGGCAUCA	UGAUGCCAGCACUGUACUG
SP3#2	CUGGUAAUAUAGUACAGA	AUCUGUACUAUAUUACCAG
ZNF143#1	GCAGCGUUUCAUAGCACCU	AGGUGCUAUGAAACGCUGC
ZNF143#2	GAGCUUGAAAACUCCUAGU	ACUAGGAGUUUUCAAGCUC

Table 4. List of primer sequences for quantitative real-time PCR

				Primer sequence (5'→3')		
No.	**Gene Symbol**	**Description**	**Accession No.**	**Forward**	**Reverse**	**Product Size**
1	***EP300***	E1A binding protein p300	XM_004937710.1	AGCTGCAGATGGAGGAGAAATC	ACGGTAAAGTGCCTCCAGTG	242
2	***GABPA***	GA binding protein transcription factor, alpha subunit 60kDa	NM_001007858.1	TGAACAGGTGACACGATGGG	GGGACTCGCTGGAAGAAGTC	225
3	***HSF2***	heat shock transcription factor 2	NM_001167764.1	CCAGTTATCACCTGGAGCC	CCAACAAGTCCTCTCGACCC	242
4	***NFYA***	nuclear transcription factor Y, alpha	NM_001006325.1	TCAGCCACCTGTGAAGACAC	TCGAACTGGGCTTTCACCTC	231
5	***SP3***	Sp3 transcription factor	NM_204603.1	GGCAAAAGGTTCACTCGCAG	GTGTTGTTCCTCCCGCAGTA	229
6	***ZNF143***	zinc finger protein 143	XM_004941377.1	GAAGCGGCACATCCTTACCT	CCCTGACTTCCACAGCGATT	216

4. Discussion

The results of the current study suggest that the minimal promoter region of the *CVH* and *cDAZL* gene, which extends from −135 to +275 bp containing the 5′ UTR and intron 1 and from −83 to +173 bp respectively, can control the transcription of the *CVH* and *cDAZL* gene in chicken PGCs. They also suggest that significantly upregulated TFs such as *HSF2*, *NFYA*, *SP3*, and *ZNF143* in chicken PGCs play a role in expression of the *CVH* and *cDAZL* gene by directly interacting with putative binding sites of the *CVH* and *cDAZL* gene promoter.

VASA, an evolutionarily conserved RBP that promotes translational control of germ cell-specific genes, is expressed specifically in germ cells during germline development (Gustafson and Wessel, 2010). Several reports have shown that *Vasa* plays a critical role in the formation of the germplasm and gametogenesis in invertebrates such as *Caenorhabditis elegans* and *Drosophila* (Illmensee and Mahowald, 1974; Spike et al., 2008). In addition, VASA expression in germ cells is essential for their survival and proliferation (Parvinen, 2005; Medrano et al., 2012). Although studies on the transcriptional control of *CVH* for temporal and spatial regulation hold great promise for practical applications, regarding using a germ cell-specific promoter for tracing germ cells as well as understanding the molecular network of transcriptional regulation behind their unique characteristics, very limited information is available on the regulatory elements involved in transcriptional control of the *CVH* gene in chicken.

DAZL is also associated with germ cell specification and development. In mammalian germ cell, DAZL is involve in germ cell survival and meiotic progression (Medrano et al., 2012; Saunders et al., 2003). In the chicken, chicken DAZL (cDAZL) protein was found that conserved expression pattern in germ line cell (Rengaraj et al., 2010). Knockdown of *cDAZL* cause defective

PGC development such as reduced proliferation, aberrant gene expression profiles and PGC apoptosis (Lee et al., 2016). These facts suggested that VASA and DAZL expressed specifically in germ line cells are essential for germ cell survival and proliferation, supporting germ cell development.

A previous study showed that the *CVH* gene requires a 5′ flanking region of 1,555-bp for higher induction of specific expression in germ cells at the transcriptional level (Minematsu et al., 2008). However, as shown in Figure 1, we described that the highest promoter activity region of the *CVH* gene, which is a 591-bp fragment (−316/+275) containing the 5′ UTR, is sufficient for the induction of specific expression in chicken PGCs, as determined by a 5′ deletion assay. Additionally, our findings demonstrate for the first time that the 5′ UTR containing intron 1 is required for promoter activity of the *CVH* gene in chicken PGCs, as determined through 5′ and 3′ fragmentation assays (Figure 3). With regard to the roles of introns in transcriptional control in diverse organisms, several reports have shown that introns play a pivotal role in controlling transcription, including that of germline-specific genes, and act as enhancers to control gene expression (Kawamoto et al., 1988; Liu et al., 1990; Henkel et al., 1992; Tomaras et al., 1999; Li et al., 2013; Wong et al., 2013; Mohapatra and Barman, 2014). Therefore, it seems likely that the 5′ UTR containing intron 1 of the *CVH* gene would be valuable for constructing a germ cell-specific *CVH* promoter vector for the practical utilization of genetic resources.

Transcriptional control is required for regulatory elements such as specialized promoter sequences and promoter recognition *trans*-acting factors (DeJong, 2006). Therefore, we investigated whether predicted TFs that have putative binding sites in the 591-bp fragment (−316/+275) of the *CVH* promoter and 1,045-bp fragment (−872/+173) of the *cDAZL* promoter can directly regulate the expression of chicken *CVH* and *cDAZL*. Using our previous

transcriptome analysis, we identified TFs that were more highly expressed in chicken PGCs than in other cell types. The integrated approaches used in this study were complementary for finding novel TFs with putative binding sites in the *CVH* and *cDAZL* promoter. Finally, we selected six TFs (*EP300*, *GABPA*, *HSF2*, *NFYA*, *SP3*, and *ZNF143*) that have putative binding sites in the 591-bp fragment and 1,045-bp fragment of the *CVH* and *cDAZL* promoter respectively, through a series of experiments. In Figure 5, all TFs are marked for consensus sequences and positions in the sequence of the *CVH* and *cDAZL* promoter, including the TATA-box sequence for transcriptional initiation and the start codon. We also validated their expression levels using quantitative RT-PCR. Based on the results, five TFs (*EP300*, *HSF2*, *NFYA*, *SP3*, and *ZNF143*) were significantly expressed in PGCs compared with their levels in other samples, while *GABPA* were significantly expressed in PGCs compared with CEF, DF-1, and GSC, but showed no significant difference in expression compared with that at Stage X (Figure 6). We further examined whether these TFs affect the transcription of germ cell-specific RBPs (*CVH*, *cDAZL*, *CIWI*, and *cDND1*) in chicken PGCs through siRNA-mediated knockdown.

With regard to significant expression and functions in germ cells, it has been reported that heat shock factor 2 (HSF2) plays a role during embryonic development and under stress conditions, prevents the formation of damaged gametes, and ensures the integrity of the reproductive process (Abane and Mezger, 2010). In addition, knockout mouse models have shown that HSF2 is involved in oogenesis and spermatogenesis (KEVIN D. SARGE, 1994; Kallio M, 2002). As a general transcription activator, NF-Y binds strongly at CCAAT motifs and consists of NF-YA, -YB, and -YC subunits (Mantovani, 1999). In *C. elegans*, mutations in *nfya-1* affect the development of germ cells and also reduce the number of sperm (Deng et al., 2007). Moreover, NF-Y is greatly affected by the CCAAT motif in terms of its transcriptional activity regarding *Miwi* and *CIWI* gene (Yu Hou, 2012; Sohn et al., 2014). Additionally, SP3 is

one of the Sp family of TFs, which is characterized by three conserved zinc fingers (Philipsen and Suske, 1999), and positively or negatively controls the transcriptional activity of numerous genes through binding to the GC box in *cis*-regulatory elements (Suske, 1999). Importantly, it has been reported that regulation of *Nanog* gene expression is required for Sp1 and Sp3 expression, besides Oct4 and Sox2, in mouse (Wu and Yao, 2006). Zinc finger protein 143 (ZNF143) was first identified in *Xenopus* (Myslinski et al., 1998), and most ZNF143 binding sites are disturbed in promoters associated with CpG islands near the transcription start site in the mammalian genome (Myslinski et al., 2006). *Znf143* is particularly expressed in the mouse ICM (Yoshikawa et al., 2006) and its expression has been implicated in the regulation of mammalian embryonic stem cell survival and renewal (Chia et al., 2010). It was also proven that ZNF143, interacting with Oct4, governs Nanog expression through direct binding to the *Nanog* proximal promoter (Chen et al., 2008). In addition, ZNF143 has recently been identified as a new factor connecting promoters and distal regulatory elements as an insulator function for lineage-specific gene expression (Bailey et al., 2015). As mentioned above, *HSF2*, *NFYA*, *SP3*, and *ZNF143* would be expected to function as transcriptional regulators in chicken PGCs. Collectively, our results demonstrate for the first time that these TFs are involved in promoter activity of germ cell-specific RBPs in chicken PGCs; however, it remains to be determined whether these TFs directly act on each gene promoter during chicken PGC development.

In conclusion, we have identified the promoter region of the *CVH* and *cDAZL* gene for PGC-specific gene expression and found TFs such as *HSF2*, *NFYA*, *SP3*, and *ZNF143* associated with transcriptional control of the *CVH* and *cDAZL* gene in chicken PGCs. This information should aid a wide range of studies in constructing germ cell-specific synthetic promoters for tracing germ cells using transgenesis, as well as our understanding of the transcriptional regulation that maintains germness in PGCs.

CHAPTER 4

Characterization of Chicken *NANOG* Gene Promoter

1. Introduction

Primordial germ cells (PGCs) are the progenitors of germ cells, have the potential to differentiate into sperm and egg. They also carry the property of pluripotency because germ cells can generate an entire new organism (Wylie, 1999). The molecular mechanisms for germ cell specification diverge on species, but the several researches have been reported that major regulators associated with maintenance of germ cell characteristics during development are largely conserved (Extavour and Akam, 2003). For example, *Vasa* and *Dazl* which encodes evolutionally conserved RNA-binding proteins, are expressed in germ line cells of various organisms (Lesch and Page, 2012). They support the germ cell development through promoting translation of other germ line genes (Takeda et al., 2009; Gustafson and Wessel, 2010). PGCs also express many pluripotency genes such as *Nanog*, *Oct4* and *Sox2*, as well as germ cell markers in various organisms. And the mis-regulation of these genes cause apoptosis in PGCs, but little is known about how they the function in germ cells (Saito et al., 2003; Kehler et al., 2004; Chambers et al., 2007). Recently, many studies have been reported about relation between the germline and pluripotency. Some of researchers assumed that pluripotency factors are required for controlling a derestricted epigenome in germ cells (Leitch and Smith, 2013).

In the case of *Nanog*, known as a core pluripotency factor in mammals, it is important for acquisition of pluripotency during embryonic development and its protein is exclusively expressed in pluripotent cells and developing germ cells (Chambers et al., 2003). Primordial germ cells lacking *Nanog* fail to mature on reaching the genital ridge, indicating that *Nanog* is specifically required for formation of germ cells (Chambers et al., 2007). Especially, *Nanog* play a role of key transcription factor for inducing mouse PGC-like cells (mPGCLCs) in mouse epiblast-like cells (mEpiLCs) while it directly binds and

activates enhancers of *Prdm1* and *Prdm14* that are important for PGC specification (Murakami et al., 2016). Recent studied have found an evolutionary functional conservation among vertebrate Nanog orthologs from chick (Lavial et al., 2007). In chicken, *NANOG* was identified, and expression pattern of NANOG protein was verified during early embryonic development. Chicken *NANOG* is expressed in exclusively PGCs, but is not detected throughout the epiblast at stage HH1 or HH3 (Canon et al., 2006). These data suggest that *Nanog* is an important regulator of pluripotency and self-renewal of ES cells and PGCs. So, many studies have been tried to identify *cis*-regulatory elements of *Nanog* gene to understand the molecular mechanisms that how the pluripotential cell-specific expression of *Nanog* is controlled.

From these studies, it has developed that Nanog gene expression require transcriptional *cis* –elements which are adopted by Oct4 and Sox2 in mouse (Kuroda et al., 2005). Also, in other vertebrates including cow, pig, goat and human, DNA consensus sequences which recognized by Oct4 and Sox2 highly conserved at the proximal region of upstream of *Nanog* gene (Guo et al., 2014), demonstrating that Oct4 and Sox2 are major regulators of *Nanog* expression in ESCs. Besides, lots of transcription factors are involved in transcriptional regulation of *Nanog* gene as an activator or repressor (Saunders et al., 2013). On the other hand, studies on the molecular mechanisms of transcriptional regulation in chicken *NANOG* gene has not been attempted. Thus, in this study, we tried to elucidate the sequences which act as a promoter and enhancers of chicken *NANOG* gene in PGCs. And we confirmed the effect of pluripotency factors in chicken *NANOG* promoter activity, comparing with various mammalian species.

2. Materials and Methods

Experimental animals and animal care

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

5’ rapid amplification of cDNA ends (5’RACE)

5’ RACE PCR were performed using GeneRacer Kit (Invitrogen, USA) following the manufacturer’s instructions. The GeneRacer RNA Oligo ligated mRNA is reverse transcribed into cDNA and the single-stranded cDNA serves as template in nested 5’ RACE PCR using GeneRacer 5′ Primer and reverse gene specific primers (Reverse GSP) designed from exon 2 of c*NANOG* gene. The sequences of GSP primers are as follow: GSP (antisense exon 2), 5’-GTCTGCAGTAGGGCTAGTGGCAGAGTCT-3’.

Construction of eGFP and NanoLuc luciferase expression vectors derived from the c*NANOG* Promoter

For construction of eGFP expression vector, amplified the 5’ flanking region of the c*NANOG* gene from genomic DNA of adult chicken were cloned into the pGEM T easy vector (Promega, USA) then, ligated eGFP coding sequence and polyadenylation (Poly-A) tail using restriction enzymes *Spe I* and *Nde I*. As different sizes of the c*NANOG* promoter sequences were cloned, each of PCR products amplified though primer sets (Table 1). The fragment −3,550 bp to +70 bp of chicken *NANOG* gene was cloned from the National Center for Biotechnology Information (NM_001146142.1) for the promoter 5’ deletion and fragment assay. In the case of cloning NanoLuc luciferase expression

vectors, different lengths of the 5'-upstream region of the *cNANOG* gene were inserted between the *Kpn I* and *Xho I* sites of the pNL1.2 vectors (Promega, USA).

Culture of chicken PGC and DF-1

Chicken PGCs were cultured according to our standard procedure (Park and Han, 2012). Briefly, chicken PGCs from White Leghorn embryonic gonads at 6-day-old (HH stage 28) were maintained in knockout Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, USA), 2% chicken serum (Sigma-Aldrich, USA), 1x nucleosides (Millipore, USA), 2mM L-glutamine (Gibco, USA), 1x nonessential amino acids (Gibco, USA), β-mercaptoethanol (Gibco, USA), 1mM sodium pyruvate (Gibco, USA), and 1x antibiotic-antimycotic (Gibco, USA). Human basic fibroblast growth factor (bFGF) (KomaBiotech, Korea) at 10 ng/ml was used for PGC self-renewal. The cultured PGCs were subcultured onto mitomycin-inactivated mouse embryonic fibroblasts at 5 to 6-day intervals by gentle pipetting without any enzyme treatment. For DF-1, the cells were maintained in DMEM with high glucose (Hyclone, USA), 10% FBS, and 1x antibiotic-antimycotic. Cultured cells were grown at 37°C in a 5% CO2 incubator.

***In Vitro* transfection**

In vitro transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). For expression analysis of eGFP, constructed *cNANOG* promoter vector (1 μg) and 2 ul of Lipofectamine 2000 were separately diluted with 50 ul of Opti-MEM I reduced serum medium (Invitrogen) 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. Liposome-DNA complex solution was added to $2.5x10^5$ cultured PGCs in 500 ul of PGC culture medium.

Transfected cells were incubated for 24 hr without feeders. After incubation, cells were analyzed using a fluorescence microscope.

Luciferase assays

Nano-Glo Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure the *cNANOG* promoter activities. The prepared cells were seeded in 96-well plate and co-transfected with pGL4.53 firefly luciferase (Fluc) and pNL1.2 (NlucP/cNANOG RE) NanoLuc luciferase (Nluc) plasmid using Lipofectamine 2000 (Invitrogen). Transfected cells were lysed with lysis buffer with Fluc substrate and incubated on an orbital shaker for 3 min. Fluc signals were then quenched and following by reaction with Nluc substrate. The signals in arbitrary unit (AU) from both Nluc and Fluc were measured by a luminometer (Glomax-Multi-Detection System, USA). The promoter activities were calculated by the ratio of respective AU values of Nluc/Fluc.

Construction of mutation vector in OSNT site

Mutation sequences of predictive OSNT site containing restriction enzyme sites (*Kpn I* at 5' overhang and *Xho I* at 3' overhang) was synthesized by Bioneer Corp. (Daejeon, Korea). NanoLuc luciferase expression vector was cloned by means of *Kpn I* and *Xho I* digestion and ligation.

Prediction of putative transcriptional binding elements

Transcription factor binding sites were predicted by the MatInspector, a Genomatix program (http://www.genomatix.de/) using TRANSFAC matrices (vertebrate matrix; core similarity 1.0 and matrix similarity 0.8)

3. Results

Analysis of 5’ UTR of chicken *NANOG* gene

In order to identify the promoter region of chicken *NANOG* gene, we firstly verified the 5’ end sequence through 5’ RACE PCR. In figure 1A, lane 2 shows that a 470-bp DNA fragment was amplified from GeneRacer 5′ Primer and gene-specific reverse primer (GSP) which located in exon 2 of chicken *NANOG* gene. To confirm the quality of adapter-ligated RNA, we also loaded 5’ RACE PCR product of β-actin gene which is a 872-bp size band containing 828-bp of *β-actin* gene and 44-bp of the GeneRacer RNA Oligo at lane 1. From the sequence analysis of amplified product, we identified the transcription start site of chicken *NANOG* gene and upstream 70-bp from the *NANOG* translation start site is 5’ UTR region (Figure 1B).

Identification of chicken *NANOG* promoter in PGCs

To investigate transcriptional regulatory region of chicken *NANOG* gene, we were cloned eGFP and NanoLuc luciferase expression vectors controlled by different size fragments of 5’ flanking region which are randomly designed from the sequence of −3,550/+70 using primers located in different positions (Table 1). As shown in Figure 2A, the following the 5’ deletion assay, six constructs containing *NANOG* promoter ligated eGFP coding sequences were represented: 3,620-bp fragment (−3,550/+70), 2,058-bp fragment (−1,988/+70), 1,015-bp fragment (−945/+70), 697-bp fragment (−627/+70), 512-bp fragment (−442/+70) and 320-bp fragment (−250/+70). Each these constructs were transfected into chicken cultured PGCs, and then eGFP were observed through fluorescence microscopy (Figure 2B). All constructs expressed eGFP in PGCs. Comparing the promoter activity between the different fragments, we conducted dual luciferase assay using NanoLuc

luciferase expression vectors including different sizes of 5' flanking region, as on eGFP expression vectors. The results of dual luciferase reporter assay in PGCs revealed that only 320-bp of upstream sequence of *cNANOG* gene retained promoter activity and pNL1.2-NANOG−3,550/+70 had activity about 3 times that of pNL1.2-NANOG−250/+70 (Figure 2C). Interestingly, *cNANOG* promoters does not worked in DF-1 (Figure 2D). It seems likely that upstream regions of *cNANOG* gene we've cloned had promoter activity only in PGCs. It would be explained that we haven't found the region which has the basal promoter activity. To exclude a possibility of this case, we examined the basal promoter region by 5' deletion assay.

Investigation of putative proximal and distal enhancer in *cNANOG* promoter

In the 5' deletion series, the fragment −3,550/+70 induced luminescence signal a 3 fold increase than other fragments and only 250-bp sequence of *cNANOG* promoter (−250/+70) was required for having promoter activity exclusively in PGCs, suggesting that the transcription of *cNANOG* gene is regulated by PGC-specific proximal and distal enhancers. In order to investigate this points in detail, we designed a series of 4 and 3 constructs through deletion of 5' upstream region at regular intervals from the fragment −250/+70 vector and −3,550/+70 respectively. From dual luciferase assay, we found that it was required for at least 200-bp sequence of *cNANOG* promoter (−130/+70) to induce gene expression in PGCs (Figure 3A). In the case of luciferase assay in DF-1, all of constructs still didn't work (data not shown). These results revealed that the minimal promoter region to active gene expression in PGCs is the 200-bp fragment (−130/+70) and *cNANOG* promoter functions restricted in PGCs. Comparing the promoter activity between fragments in proximal region from the TSS, pNL1.2-NANOG−210/+70 had

higher activity, so we predicted transcription factors binding to −210/+70 region (Figure 3B). We assumed that these transcription factors play critical role in regard to modulate PGC-specific promoter activity. In addition, previous data showed pNL1.2-NANOG−3,550/+70 had three times more activity than other vectors. Following 5' deletion assay from the 3,620-bp fragment (−3,550/+70), we confirmed the region which induce significant promoter activity difference between other vectors (Figure 3C). The region is located between −3,154 and −2,928 sequence, might serve as a distal enhancer. Then, we also examined PGC-specific expressed putative transcription factors binding to distal enhancer (Figure 3D). These results suggested that the transcriptional regulation of chicken *NANOG* gene in PGCs is involved in proximal and distal enhancer interacting with PGC-specific trans-acting factors.

Putative pluripotency factor binding sites affect *cNANOG* promoter activity

In previous study, we found that several putative transcription factors that might be involved in PGC-specific promoter activity, binding to proximal enhancer. To examine what it functions to be critical regulator modulating PGC-specific promoter activity, we performed mutation assay of OSNT site which composes binding site for Oct4, Sox2, Nanog, Tcf3 (Tcf7l1) and Sall4b. Following an alignment of upstream region of *cNANOG* gene among cow, goat, human, mouse, pig and chicken (refer to Guo et al., 2014), it revealed that DNA consensus sequences recognized by core pluripotency factors such as Oct4, Sox2 and Nanog shown overall similarities in chicken *NANOG* promoter region (−88/-70) (Figure 4A). We employed a random mutagenesis procedure on OSNT sites from ACTTTTAACATGATAATGG to GTCCGCGTTCATCGCCATT following dual luciferase assay to confirm the contribution of putative ESC three core transcription factors (Oct4, Sox2 and

Nanog) in chicken *NANOG* promoter activity. And then, we compared with another NanoLuc luciferase expression vector (pNL1.2-NANOG−130/+70) to verify effect of the disruption on OSNT site. The result of mutation assay was shown in figure 4B. There was significant difference between pNL1.2-mNANOGm−130/+70 and pNL1.2-NANOG−130/+70 which is confirmed region for having promoter activity. These results indicated that putative core pluripotency factors binding sites had effect on luciferase expression like as other mammalian species. Therefore, we suggested that the Oct4-Sox2 and Nanog binding *cis*-regulatory elements, which are perfectly conserved throughout mammalian evolution, affect the regulation of chicken *NANOG* promoter.

The region from position −130 to −89 of the chicken *NANOG* gene is required for germline-specific expression in chicken PGCs

In order to locate the regulatory element(s) responsible for PGC-specific expression of *cNANOG*, as shown in Figure 5A, we introduced a series of deletions into the pNL1.2-NANOG−130/+70 constructs: 200-bp fragment (−130/+70), 178-bp fragment (−108/+70), 158-bp fragment (−88/+70) and 139-bp fragment (−69/+70). To evaluate the promoter activity, we performed the dual luciferase reporter assay using the four constructs and empty luciferase vector (pNL1.2-Basic) in chicken PGCs (Figure 5B). The luciferase reporters containing 200-bp fragment (−130/+70) promoter region of *cNANOG* presented strong enzyme activity, but we could not detect enzyme activity from the smallest 139-bp fragment and pNL1.2-basic, an empty vector. Notably, compared with 200-bp fragment, 178-bp fragment and 158-bp fragment presented at least 18 times and 75 times higher luciferase activities despite 178-bp and 158-bp fragment contained OSNT biding site that plays a critical role in *cNANOG* promoter activity mentioned above (Figure 5B). Collectively, these

results indicate that the chicken NANOG promoter requires a 42-bp sequence from −130 to -89 of the 5’ upstream region of the chicken *NANOG* gene as well as OSNT binding site.

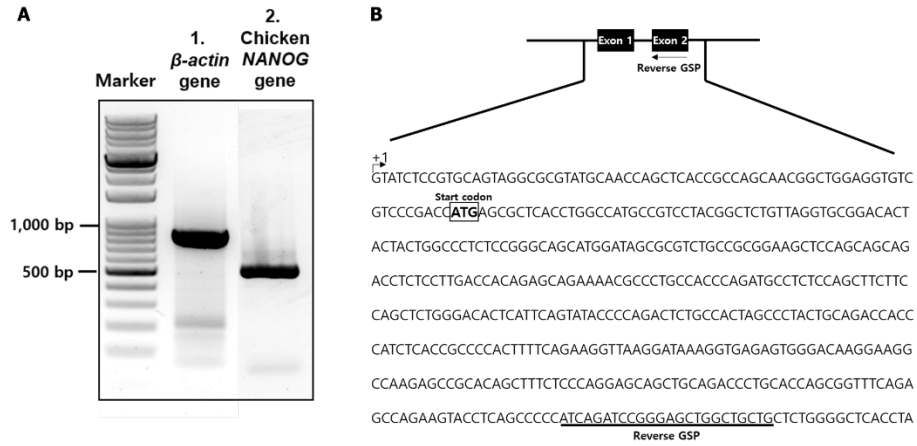

Figure 1. RACE analysis of chicken *NANOG* gene. 5’ RACE-PCR was performed to identify full length 5’ end sequence of chicken *NANOG* gene from an RNA transcript according to the manufacturer's protocol. (A) The results of 5’RACE PCR were confirmed via electrophoresis analysis. Lane1 and 2 were loaded of β-actin gene and *NANOG* gene respectively. (B) The 5’RACE PCR product of *NANOG* gene was confirmed by DNA sequencing. +1 indicates the potential TSS of *NANOG* gene.

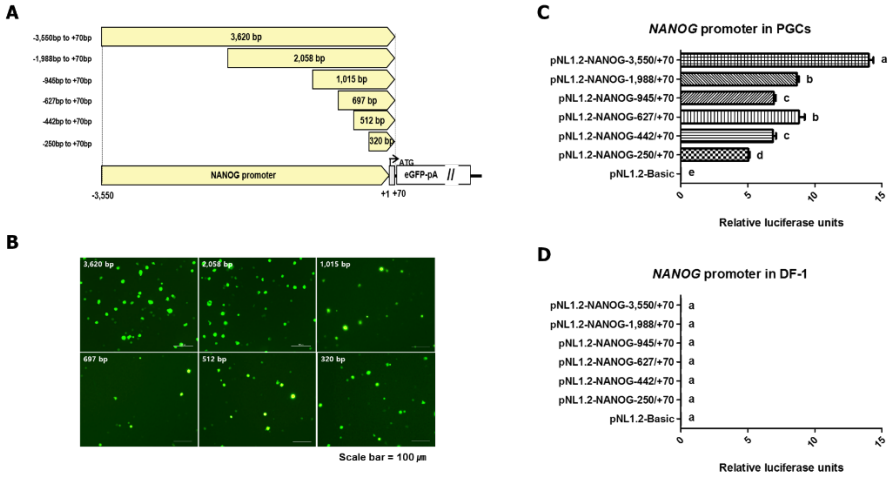

Figure 2. Identification of *cNANOG* promoter regions using 5' deletion assay. (A) Schematic diagram of constructed eGFP expression vectors under the 5' flanking region of *NANOG* gene. Constructs of each promoters containing the 5' UTR. (B) Expression of enhanced green fluorescent protein (eGFP) under control of different constructs in cultured chicken PGCs was monitored by microscopy. Each fragment used as driving eGFP expression were ligated NanoLuc luciferase expression vector (pNL1.2-Basic) for measuring promoter activity. Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs (C) and DF-1 (D). Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. Scale bar = 100 μm. Different letters (a-e) indicate significant differences ($P < 0.05$).

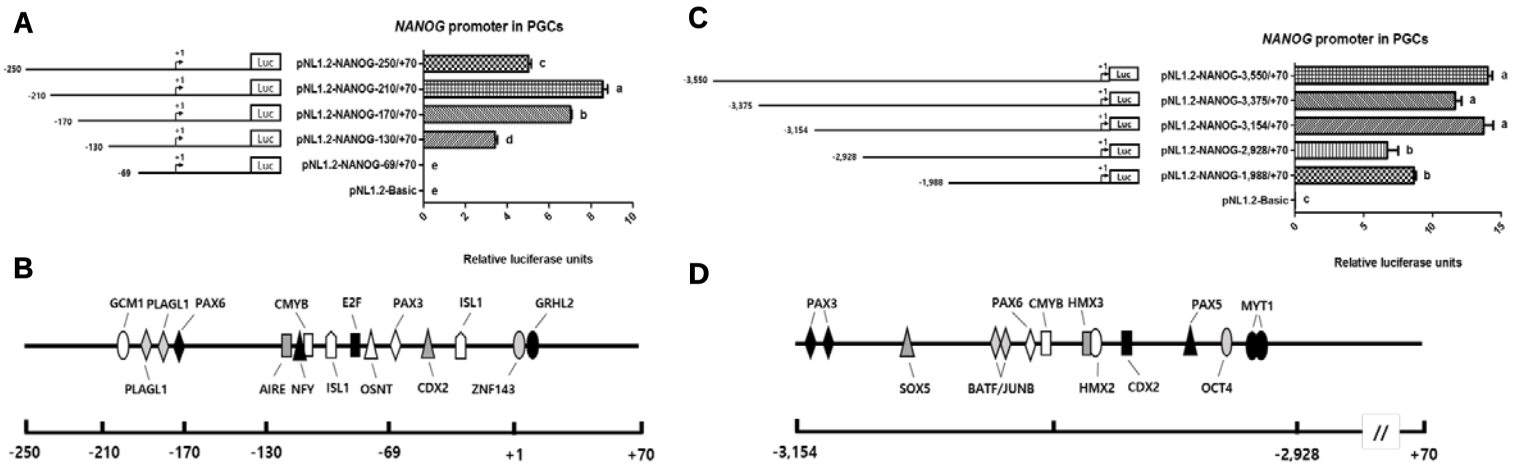

Figure 3. Verification of proximal and distal enhancer of *cNANOG* gene. (A), (C) Schematic diagram of constructed *NANOG* promoter vectors. Constructs of each promoters containing the 5' UTR. Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs. Following the results of 5' deletion assays, prediction of transcription factors was performed using MatInspector software in putative proximal enhancer (B) and distal enhancer (D). Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. Scale bar = 100 μm. Different letters (a-e) indicate significant differences ($P < 0.05$).

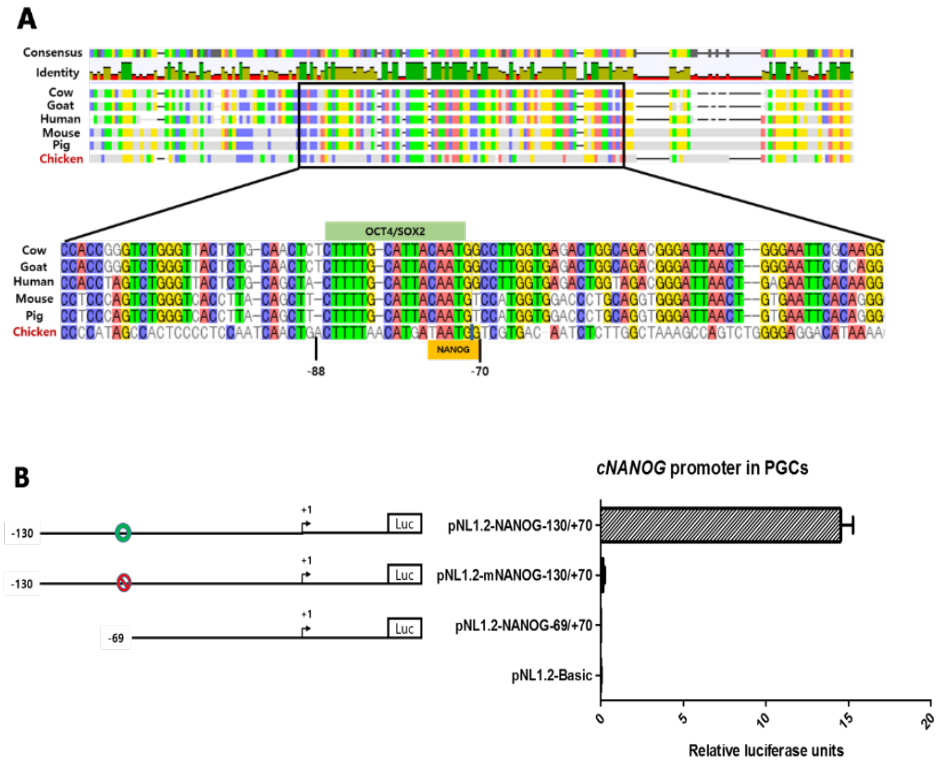

Figure 4. Mutation analysis in putative pluripotency factors binding site. (A) Multiple alignment of putative chicken *NANOG* proximal enhancer with *cis*-regulatory elements of *Nanog* gene from five vertebrates. Predictive OCT4, SOX2 and NANOG binding sites mostly conserved in chicken. (B) Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs. NANOG binding check vector contain the OSNT site, and OSNT site was mutagenized from ACTTTTAACATGATAATGG to GTCCGCGTTCATCGCCATT in pNL1.2-mNANOG−130/+70 vector. Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression.

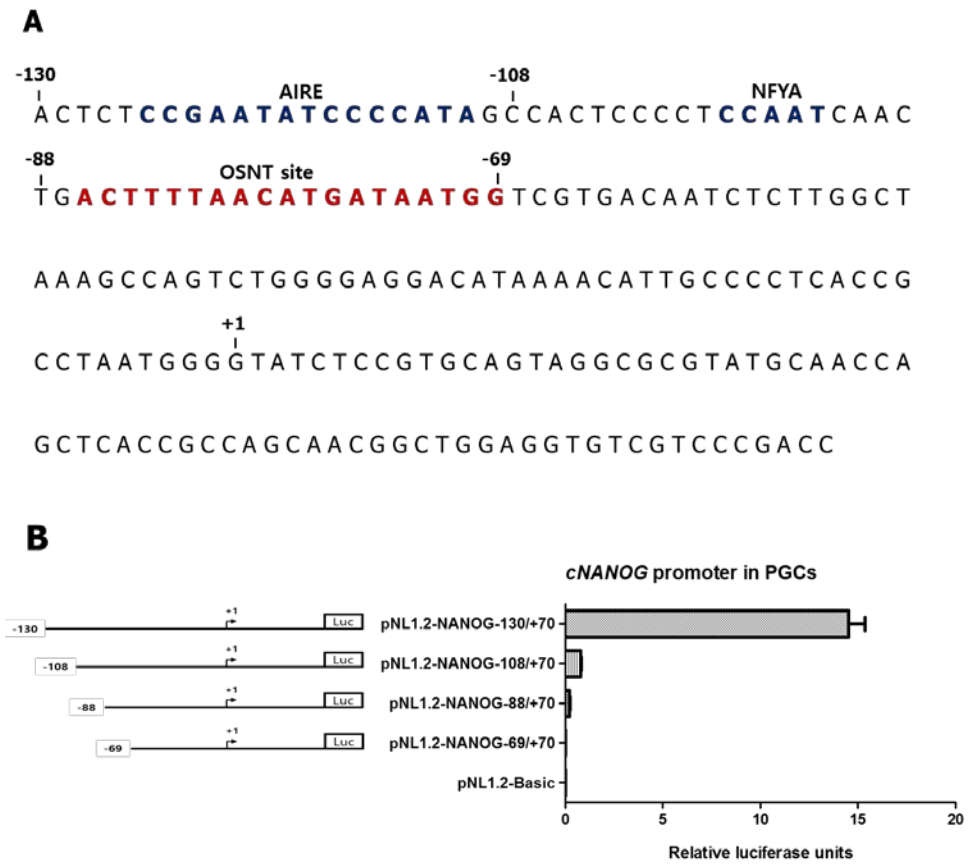

Figure 5. The 42-bp region between positions −130 and −89 is required for enhancing promoter activity. (A) Nucleic acid sequence of the 200-bp chicken *NANOG* promoter region. +1 indicates the transcriptional initiation site and the bold sequences show predicted binding sites of the transcription factors (TFs). (B) Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in PGCs. Role of sequence of (−130/−89) in the activity of pNL1.2-NANOG−130/+70. Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. AIRE, autoimmune regulator. OSNT, Oct4, Sox2, Nanog, Tcf3 (Tcf7l1) and Sall4b.

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

Primer set	Primer sequence (5'→3')
cNANOG 3,550 bp_F	AAGCTTTGTCCTTTTCTTGACC
cNANOG -3,375 bp_F	CTGGAGTCAAGGGCTGTGG
cNANOG -3,154 bp_F	TGGGCCCCTCGTTACAGCT
cNANOG -2,928 bp_F	CCAGCAGTACAAGCTCCGAA
cNANOG -1,988 bp_F	GCGACACGTGGAACA
cNANOG -945 bp_F	CATGGGGGTGTCTGCTC
cNANOG -627 bp_F	CTTCTTTGTGCTCCTCC
cNANOG -250 bp_F	CTGCAGTCTGCAATGC
NANOG -210- bp_F	CGGGTGTGCGGGCAG
NANOG -170 bp_F	CCAAAGGGGGAAGCTGG
NANOG -130 bp_F	ACTCTCCGAATATCCCCATAGC
NANOG -108 bp_F	CACTCCCCTCCAATCAACTGA
NANOG -88 bp_F	ACTTTTAACATGATAATGG
NANOG -69 bp_F	TCGTGACAATCTCTTG
NANOG promoter_R	GGTCGGGACGACACCT
eGFP poly A _F	ACTAGTCCGCGGATGGTGAGCAAG
eGFP poly A_R	CATATGGACGTCTCCCCAGCATGCC

4. Discussion

The homeodomain transcription factor Nanog is expressed in pluripotential cells, embryonic stem cells and embryonic germ cells (Yamaguchi et al., 2005). *Nanog* expression in PGCs is maintained in proliferating germ cells during migration of PGCs, in which nuclear reprograming is processing (Yamaguchi et al., 2005). In 2006, chicken *NANOG* was identified and expressed exclusively in PGCs during early embryo development (Canon et al., 2006). Recent studied have identified an evolutionary functional conservation among vertebrate Nanog orthologs from chick (Lavial et al., 2007), zebrafish (Theunissen et al., 2011) and axolotl salamander (Dixon et al., 2010).

In the present study, we characterized gene structure of chicken *NANOG* gene and confirmed the region of *NANOG* promoter in chicken cultured PGCs. Chicken *NANOG* gene is transcribed under the control of a regulatory region that lies within 130-bp upstream of the transcriptional start site. Following dual luciferase reporter assay, we identified *NANOG* promoter had not activity in DF-1. These results suggested that chicken *NANOG* promoters, which are used in our experiments, work exclusively in chicken PGCs.

Similar phenomenon has been reported in mouse *Oct4* gene (Yeom et al., 1996). There are two separate regulatory elements depend on the temporal and spatial during embryonic development. The distal element located in between -13 kb and -9 kb specifically active in embryonic stem and embryonic germ cells. The proximal enhancer, in contrast to distal enhancer, is active in the epiblast of mouse embryos. Expression patterns of mouse *Oct4* gene reveals that transcription of mouse *Oct4* gene is regulated with stage-specific manner. After that, studies on the finding trans-acting factors which recognize *cis*-regulatory elements of *Oct4* gene have been reported. Each enhancers have

several potential binding sites for transcription factors that regulate transcription of Oct4 gene as a either activator or repressor. For example, Prdm14, Klf4, Stat3, Oct4, Sox2, Nanog and Sall4 activate expression of Oct4 gene, binding to distal enhancer. On the contrary to this, Cdx2 functions as a repressor. In the case of proximal enhancer, Nanog, Nr5a2 and Esrrb are involved in promoting transcription of *Oct4* (Wu and Schöler, 2014). There are truncated isoforms of *Oct4* gene identified in human (Takeda et al., 1992) and mouse (Guo et al., 2012).

Interestingly, *Oct4* expression is reported in adult normal tissues as well as pluripotential cells. Based on this information, it was found that *Oct4* expression in various somatic cells is regulated by novel promoter region which promote production of novel alternative transcripts in somatic cells (Mizuno and Kosaka, 2008). In this report, the existence of several alternative Oct4 transcript in the eye was identified. And these isoforms are transcribed from the internal region of intron 1 using novel promoter region located in intron 1 through exon 3. Thus, we assumed that chicken *NANOG* gene has novel promoter region for producing alternative transcript forms in somatic cells like as mouse *Oct4* gene.

To determine that what are core factors to regulate transcription of *Nanog* gene, lots of studies have been conducted in mammalian. It is important for understanding the molecular networks associated with governing maintenance of pluripotency state. For obtaining these information, an approach of characterization of *NANOG* promoter, which acts as recognition sites of several transcription factors, have been performed in diverse organisms such as mouse (WU and Zhen, 2005), human (Das et al., 2012) and goat (Guo et al., 2014). Taken together, several transcription factors including Oct4, Sox2 (Rodda et al., 2005), Bmi1 (Paranjape et al., 2014), Brd4 (Liu et al., 2014), Klf4, Pbx1 (Chan et al., 2009), Sall4 (Wu et al., 2006), Nspc1 (Li et al., 2013) and

Znf143 (Chen et al., 2008) were found. They directly bind to the *Nanog* promoter regions and regulate transcription of *Nanog* gene as a either activator or repressor. Especially, Oct4 and Sox2 transcription factors play critical role in promoter activity of *Nanog* gene. Their recognition sites, which mostly located in proximal region near the transcription start site, also highly conserved among the mammal species (Guo et al., 2014). Substitution mutations on Octamer and Sox elements lead to dramatically reduce promoter activity to 17% and 15%, respectively. In addition, when both of these sites were mutated, promoter activity dropped to 6% of wild type (Rodda et al., 2005). However, in chicken, there had never been any reports of *NANOG* promoter and their recognition factors in PGCs.

Then, we performed mutation assay on putative Oct4, Sox2 and Nanog binding sites which are TTGTTATGCA, AACAAAGG and AACAAT/AG/AG, respectively to verify critical factors for having promoter activity (Salmon-Divon et al., 2010). In our results, pluripotency factors binding motifs (Oct4, Sox2 and Nanog), which are the result of an alignment sequence between other species represent similar, affected promoter activity. Following comparative genome-wide studies represent that DNA sequence elements which are recognized by the mouse core pluripotency factors such as Oct4, Sox2 and Nanog was not conserved in chicken. This information suggests that a small number of nucleotide changes on genomic sequence in chicken create novel binding sites for core factors in embryonic pluripotency networks (Fernandez-Tresguerres et al., 2010). However, our results did not support these report. Interestingly, although OSNT binding site remains intact in chicken *NANOG* promoter, deletion of the 42-bp fragment from −130 to −89 region resulted in a dramatic decrease in luciferase activity. Therefore, we proposed that OSNT as a key regulator for transcriptional regulation of chicken NANOG gene requires interaction with novel transcription factors which were located at -130 to -89. To exactly confirm the effect of putative pluripotency factors

binding sites in chicken *NANOG* promoter and collaboration with novel transcription factors, further study needs to be performed concerning the detection protein-nucleic acid interactions such as electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation sequencing (ChIP-seq).

In conclusion, in this chapter, we performed the first characterization of chicken *NANOG* gene structure including 5' UTR and proposed region of putative proximal (−130/−210) and distal enhancer (−2,928/−3,154) enhancing promoter activity of chicken *NANOG* gene. Furthermore, we identified core pluripotency factors binding site affect chicken *NANOG* promoter activity through mutation assay. And we proposed the possibility of novel transcription factors for promoter activity of chicken *NANOG* gene with OSNT site. This study will contribute to understand the molecular mechanisms of transcriptional regulation in chicken *NANOG* gene and further exploration on novel characteristics of chicken *NANOG* and other pluripotency factors through comparative study.

CHAPTER 5

GENERAL DISCUSSION

One of the fundamental question in germ cell biology is how specific-sets of genes are selected for expression in germ cells. Actually, to acquire unique characteristics of germ cells, they must establish specialized gene expression patterns distinct from somatic cells. Several evolutionally conserved germ cell-specific genes are expressed in germ cells such as *Vasa* and *Dazl*. Also, pluripotency markers including Sox2, Oct4 and Nanog are expressed in germ cells (Yamaguchi et al., 2005; Saito et al., 2003). These genes play essential role in germ cell survival, proliferation, migration and differentiation (Wylie, 1999; Chambers et al., 2007). It is revealed that PGCs have specialized transcription initiation programs to control cell type-specific gene expression patterns.

Tissue-specific gene expression is accomplished by the orchestrated actions of several transcription factors interacting with *cis*-regulatory elements such as enhancer, silencers and promoters (Spitz and Furlong, 2012). Thus, studying on *cis*-DNA elements and their unique combination of modules is important for deciphering of regulatory networks of global gene that govern transcriptional control. Indeed, small size (~100bp) of germ cell-specific promoters can contribute to specific gene expression in germ cells recruiting transcription factors which are expressed in germ cells (Han et al., 2014; Bielinska et al., 2005; Han et al., 2003). So, identification of promoters and their regulatory elements is one of the answer to questions of how germ cell-specific programs of gene expression.

In the first study, we investigated promoter regions that regulate transcription *CVH* and *cDAZL* gene known as germ cell markers. Through approach of deletion assay into the fragment −1,575/+275 of *CVH* gene and the fragment −872/+173 of *cDAZL* gene, *cis*-regulatory elements were found. And we found that at least 410-bp of *CVH* promoter (−135/+275) was required for regulating gene expression. In the case of *cDAZL* promoter, transcriptional

regulation was required at least 256-bp.

In chicken, it was reported that *CVH* gene require 1,555-bp of 5' flanking region for specific expression in germ cells. And they found that the minimal promoter region is an 827-bp of the 5' flanking region of *CVH* gene (Minematsu et al., 2008). However, in our results, 591-bp (−316/+275) of 5' upstream region is efficient for gene expression restricted in chicken PGCs. Also, we identified that minimal promoter region is 410-bp (−135/+275) regarding to an approach of 5' deletion assay more detail.

Also, chicken *DAZL* promoter activity analysis was reported that core promoter region was −383 to −39-bp in DF-1. However, they didn't confirm regarding to specificity of promoter regions in PGCs and prediction of transcription factors. In our study, we tried to investigate distal region of *cDAZL* promoter compared with previous report. Also, we found that *cis*-regulatory elements and transcription factors binding to promoter region through 5' deletion assay and transcriptome analysis. These attempt can give a clue for understanding specific transcriptional regulation in germ cells, because we compared promoter activity between chicken PGCs and DF-1 known as somatic cells.

In addition, following 3' deletion assay of *CVH* promoter region, we found that 5' UTR including intron plays critical role in promoter activity of *CVH* gene. In fact, structural features of 5' UTR containing introns is discovered in diverse organisms (Mignone et al., 2002). And studies have reported that introns play a role in controlling of transcription in many genes including human *β-actin* gene (Kawamoto et al., 1988), carp *β-actin* gene (Liu et al., 1990), mouse *TNF-α* gene (Tomaras et al., 1999) and mouse *IL-4* gene (Henkel et al., 1992). These reaches suggested that introns act as enhancer in transcription of each genes and several transcription factors which bind to these intron regions were identified. The most similar phenomenon of *CVH* gene has

been reported in medaka *vasa* (Li et al., 2013). Deletion 3' into intron 1 at 5' UTR affected promoter activity. In absence of intron-1, promoter activity in all of promoter fragments were sharply reduced by 5 fold.

CVH and c*DAZL* promoter activity mentioned above, all of elements had higher promoter activity in PGCs than DF-1, even though luciferase expression vector under the same sequences of 5' flanking regions were transfected. These results suggested that germ cell-specific transcription factors exist and they may be involved in transcriptional regulation of germ cell-related genes. To demonstrate possibility of presence of specialized transcription factors in germ cells, we predicted putative *cis*-elements recognition factors through prediction software and sorted out transcription factors which are specifically expressed in germ cells refer to RNA-seq database. Then, we verified relative expression levels of germ cell-specific genes that encode RNA-binding proteins including *CVH*, *cDAZL*, *CIWI* and *cDND1* in predictive transcription factors knockdown cells. Several transcription factors (*HSF2*, *NFYA*, *SP3* and *ZNF143*) affect transcription of germ cell-related genes. Through this study, we suggested that some of transcription factors which highly expressed in germ cells may be govern transcription of germ cell-specific genes associating with specialized promoter regions of germ cell genes either directly or indirectly.

The study in respect of germline specific transcription factors has been reported in medaka. Zhao et al. suggested that *Mitf*, which is expressed in male mouse testis (Saito et al., 2003), acts as transcriptional activator of germ cell-specific genes encoded RNA-binding proteins such as *vasa*, *dazl* and *dnd* in spermatogonial cell line (Zhao et al., 2012). However, it wasn't predicted in *CVH* promoter region by prediction software.

During embryonic development, primordial germ cells also express many pluripotency genes such as *Nanog*, *Oct4* and *Sox2*, as well as germ cell

markers (Saito et al., 2003). In the case of *Nanog*, known as a core pluripotency factor in mammals, it is important for acquisition of pluripotency during embryonic development and its protein is found in pluripotent cells and developing germ cells (Chambers et al., 2003). Primordial germ cells lacking *Nanog* fail to mature on reaching the genital ridge, indicating that *Nanog* is specifically required for formation of germ cells (Chambers et al., 2007). In chicken, *NANOG* was identified, and expression pattern of NANOG protein was verified during early embryonic development. Chicken NANOG is expressed in exclusively primordial germ cells, but not found throughout the epiblast at stage HH1 or HH3 (Canon et al., 2006).

The second study was examined to identify chicken *NANOG* promoter region. Firstly, we identified 5' UTR region of chicken *NANOG* gene by 5' RACE PCR. We confirmed that upstream 70-bp from the translation start site is the TSS of chicken *NANOG* gene through analysis of DNA sequencing. Then, through 5' deletion assay from the fragment −3,550 to +70 bp, we cloned different sizes and positions of *cNANOG* promoter and found that the fragment 3,620-bp (−3,550/+70) was higher activity that pNL1.2-NANOG−250/+70 vector about 3 times. To investigate the minimal promoter and distal enhancer, we conducted 5' deletion assay more detail from the fragment 3,620-bp (−3,550/+70) and 320-bp (−250/+70) respectively. The results of these experiments indicate that only 200-bp (−130/+70) can induce gene expression restricted in chicken PGCs and distal enhancer may be located in sequences of 226-bp (−3,154/-2,928). Furthermore, we predicted transcription factors binding to putative distal enhancer using MatInspector and sorted out based on RNA-seq database.

In addition, we conducted mutation assay in predicted OSNT site to explain what is core factors cause difference of promoter activity between 200-bp (−130/+70) and 139-bp (−69/+70). It was reported that pluripotency related

factors play critical role in promoter activity of *Nanog* gene. Their recognition sites, which mostly located in proximal region near the transcription start site, also highly conserved among the mammal species (Guo et al., 2014). In our results, pluripotency factors binding motifs (Oct4, Sox2 and Nanog) affect promoter activity even the result of an alignment sequence between other species represent similar. It seems that chicken also requires pluripotency factors as transcriptional regulators associating with *NANOG* gene expression.

To determine proximal enhancer and their recognition factors, we conducted 5' deletion assay from 200-bp fragment (−130/+70) and found that proximal enhancer and novel transcriptional regulator may be located in sequences of 20-bp (−130/−110). We need more experiments to exactly confirm that whether these sequences are involved in enhancing promoter activity of chicken *NANOG* gene.

We first characterized chicken *NANOG* promoter. Therefore, this study will contribute to understand the molecular mechanisms of transcriptional regulation in chicken *NANOG* gene and further exploration on novel characteristics of chicken *NANOG* and other pluripotency factors through comparative study. Our next studies will be focused on the mechanism of temporal and spatial regulation of germ cell-specific genes by specialized enhancers in PGCs. These studies will contribute to practical applications regarding transgenesis and constructing of germ cell-specific synthetic promoter for tracing germ cells as well as understanding the molecular network of transcriptional regulation for maintaining germness in germ cells.

REFERENCES

Abane, R. and V. Mezger (2010). "Roles of heat shock factors in gametogenesis and development." FEBS Journal 277(20): 4150-4172.

Aramaki, S., F. Sato, T. Kato, T. Soh, Y. Kato and M. A. Hattori (2007). "Molecular cloning and expression of dead end homologue in chicken primordial germ cells." Cell Tissue Res 330(1): 45-52.

Avilion, A. A., S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian and R. Lovell-Badge (2003). "Multipotent cell lineages in early mouse development depend on SOX2 function." Genes & development 17(1): 126-140.

Bailey, S. D., X. Zhang, K. Desai, M. Aid, O. Corradin, R. Cowper-Sal Lari, B. Akhtar-Zaidi, P. C. Scacheri, B. Haibe-Kains and M. Lupien (2015). "ZNF143 provides sequence specificity to secure chromatin interactions at gene promoters." Nat Commun 2: 6186.

Banerji, J., S. Rusconi and W. Schaffner (1981). "Expression of a β-globin gene is enhanced by remote SV40 DNA sequences." Cell 27(2): 299-308.

Bielinska, B., J. Lü, D. Sturgill and B. Oliver (2005). "Core promoter sequences contribute to ovo-B regulation in the Drosophila melanogaster germline." Genetics 169(1): 161-172.

Boer, B., J. Kopp, S. Mallanna, M. Desler, H. Chakravarthy, P. J. Wilder, C. Bernadt and A. Rizzino (2007). "Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2: Oct-3/4 target genes." Nucleic acids research 35(6): 1773-1786.

Bortvin, A., M. Goodheart, M. Liao and D. C. Page (2004). "Dppa3/Pgc7/stella is a maternal factor and is not required for germ cell specification in mice." BMC developmental biology 4(1): 2.

Breitwieser, W., F.-H. Markussen, H. Horstmann and A. Ephrussi (1996). "Oskar protein interaction with Vasa represents an essential step in polar granule assembly." Genes & Development 10(17): 2179-2188.

Campolo, F., M. Gori, R. Favaro, S. Nicolis, M. Pellegrini, F. Botti, P. Rossi, E. A. Jannini and S. Dolci (2013). "Essential role of Sox2 for the establishment and maintenance of the germ cell line." Stem Cells 31(7): 1408-1421.

Canon, S., C. Herranz and M. Manzanares (2006). "Germ cell restricted expression of chick Nanog." Dev Dyn 235(10): 2889-2894.

Carrera, P., O. Johnstone, A. Nakamura, J. Casanova, H. Jäckle and P. Lasko (2000). "VASA mediates translation through interaction with a Drosophila yIF2 homolog." Molecular cell 5(1): 181-187.

Castrillon, D. H., B. J. Quade, T. Wang, C. Quigley and C. P. Crum (2000). "The human VASA gene is specifically expressed in the germ cell lineage." Proceedings of the National Academy of Sciences 97(17): 9585-9590.

Castrillon, D. H., B. J. Quade, T. Y. Wang, C. Quigley and C. P. Crum (2000). "The human VASA gene is specifically expressed in the germ cell lineage." Proc Natl Acad Sci U S A 97(17): 9585-9590.

Chambers, I., D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie and A. Smith (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell 113(5): 643-655.

Chambers, I., J. Silva, D. Colby, J. Nichols, B. Nijmeijer, M. Robertson, J. Vrana, K. Jones, L. Grotewold and A. Smith (2007). "Nanog safeguards pluripotency and mediates germline development." Nature 450(7173): 1230-1234.

Chan, K. K., J. Zhang, N. Y. Chia, Y. S. Chan, H. S. Sim, K. S. Tan, S. K. Oh, H. H. Ng and A. B. Choo (2009). "KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells." Stem Cells 27(9): 2114-2125.

Chen, H. H., M. Welling, D. B. Bloch, J. Munoz, E. Mientjes, X. Chen, C. Tramp, J. Wu, A. Yabuuchi, Y. F. Chou, C. Buecker, A. Krainer, R. Willemsen, A. J. Heck and N. Geijsen (2014). "DAZL limits pluripotency, differentiation, and apoptosis in developing primordial germ cells." Stem Cell Reports 3(5): 892-904.

Chen, X., F. Fang, Y. C. Liou and H. H. Ng (2008). "Zfp143 regulates Nanog through modulation of Oct4 binding." Stem Cells 26(11): 2759-2767.

Chia, N. Y., Y. S. Chan, B. Feng, X. Lu, Y. L. Orlov, D. Moreau, P. Kumar, L. Yang, J. Jiang, M. S. Lau, M. Huss, B. S. Soh, P. Kraus, P. Li, T. Lufkin, B. Lim, N. D. Clarke, F. Bard and H. H. Ng (2010). "A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity." Nature 468(7321): 316-320.

Clark, A. T. and R. A. Reijo Pera (2006). "Modeling human germ cell development with embryonic stem cells."

Collier, B., B. Gorgoni, C. Loveridge, H. J. Cooke and N. K. Gray (2005). "The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells." The EMBO journal 24(14): 2656-2666.

Cook, M. S., S. C. Munger, J. H. Nadeau and B. Capel (2011). "Regulation of male germ cell cycle arrest and differentiation by DND1 is modulated by genetic background." Development 138(1): 23-32.

Das, S., S. Jena, E. M. Kim, N. Zavazava and D. N. Levasseur (2012). "Transcriptional Regulation of Human by Alternate Promoters in

Embryonic Stem Cells." J Stem Cell Res Ther Suppl 10: 009.

DeJong, J. (2006). "Basic mechanisms for the control of germ cell gene expression." Gene 366(1): 39-50.

Deng, H., Y. Sun, Y. Zhang, X. Luo, W. Hou, L. Yan, Y. Chen, E. Tian, J. Han and H. Zhang (2007). "Transcription factor NFY globally represses the expression of the C. elegans Hox gene Abdominal-B homolog egl-5." Dev Biol 308(2): 583-592.

Dixon, J. E., C. Allegrucci, C. Redwood, K. Kump, Y. Bian, J. Chatfield, Y.-H. Chen, V. Sottile, S. R. Voss and R. Alberio (2010). "Axolotl Nanog activity in mouse embryonic stem cells demonstrates that ground state pluripotency is conserved from urodele amphibians to mammals." Development 137(18): 2973-2980.

Do, D. V., J. Ueda, D. M. Messerschmidt, C. Lorthongpanich, Y. Zhou, B. Feng, G. Guo, P. J. Lin, M. Z. Hossain, W. Zhang, A. Moh, Q. Wu, P. Robson, H. H. Ng, L. Poellinger, B. B. Knowles, D. Solter and X. Y. Fu (2013). "A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo." Genes Dev 27(12): 1378-1390.

Dolci, S., M. Pesce and M. De Felici (1993). "Combined action of stem cell factor, leukemia inhibitory factor, and cAMP on in vitro proliferation of mouse primordial germ cells." Molecular reproduction and development 35(2): 134-139.

Dolci, S., D. E. Williams, M. K. Ernst, J. L. Resnick, C. I. Brannan, L. F. Lock, S. D. Lyman, H. S. Boswell and P. J. Donovan (1991). "Requirement for mast cell growth factor for primordial germ cell survival in culture." Nature 352(6338): 809-811.

Donovan, P. J. (1998). "The germ cell-the mother of all stem cells." International Journal of Developmental Biology 42: 1043-1050.

Durcova-Hills, G., P. Hajkova, S. Sullivan, S. Barton, M. A. Surani and A. McLaren (2006). "Influence of sex chromosome constitution on the genomic imprinting of germ cells." Proceedings of the National Academy of Sciences 103(30): 11184-11188.

Eddy, E. M. (2006). "Germ plasm and the molecular determinants of germ cell fate." eLS.

Extavour, C. G. and M. Akam (2003). "Mechanisms of germ cell specification across the metazoans: epigenesis and preformation." Development 130(24): 5869-5884.

Eyal-Giladi, H., M. Ginsburg and A. Farbarov (1981). "Avian primordial germ cells are of epiblastic origin." Development 65(1): 139-147.

EYAL-GILADI, H. and S. KOCHAV (1976). "From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick: I. General morphology." DEVELOPMENTAL BIOLOGY 49, 321-337.

Farini, D., M. L. Scaldaferri, S. Iona, G. La Sala and M. De Felici (2005). "Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells." Dev Biol 285(1): 49-56.

Fernandez-Tresguerres, B., S. Canon, T. Rayon, B. Pernaute, M. Crespo, C. Torroja and M. Manzanares (2010). "Evolution of the mammalian embryonic pluripotency gene regulatory network." Proc Natl Acad Sci U S A 107(46): 19955-19960.

Fidalgo, M., F. Faiola, C.-F. Pereira, J. Ding, A. Saunders, J. Gingold, C. Schaniel, I. R. Lemischka, J. C. Silva and J. Wang (2012). "Zfp281 mediates Nanog autorepression through recruitment of the NuRD complex and inhibits somatic cell reprogramming." Proceedings of the National Academy of Sciences 109(40): 16202-16207.

Foulkes, N. S., B. Mellström, E. Benusiglio and P. Sassone-Corsi (1992). "Developmental switch of CREM function during spermatogenesis: from antagonist to activator."

Fujiwara, Y., T. Komiya, H. Kawabata, M. Sato, H. Fujimoto, M. Furusawa and T. Noce (1994). "Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage." Proceedings of the National Academy of Sciences 91(25): 12258-12262.

Gallardo, T., L. Shirley, G. B. John and D. H. Castrillon (2007). "Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre." Genesis 45(6): 413-417.

Gazdag, E., A. Santenard, C. Ziegler-Birling, G. Altobelli, O. Poch, L. Tora and M.-E. Torres-Padilla (2009). "TBP2 is essential for germ cell development by regulating transcription and chromatin condensation in the oocyte." Genes & development 23(18): 2210-2223.

Ginsburg, M. and H. Eyal-Giladi (1986). "Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast into the germinal crescent in the avian embryo." Development 95(1): 53-71.

Ginsburg, M. and H. Eyal-Giladi (1987). "Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process." Development 101(2): 209-219.

Goodrich, J. A. and R. Tjian (2010). "Unexpected roles for core promoter

recognition factors in cell-type-specific transcription and gene regulation." Nat Rev Genet 11(8): 549-558.

Guo, C. L., L. Liu, Y. D. Jia, X. Y. Zhao, Q. Zhou and L. Wang (2012). "A novel variant of Oct3/4 gene in mouse embryonic stem cells." Stem Cell Res 9(2): 69-76.

Guo, Y., L. Lei, X. Ma and H. Wang (2014). "Characterization of the proximal region of the goat NANOG promoter that is used for monitoring cell reprogramming and early embryo development." Vet J 199(1): 80-87.

Gustafson, E. A. and G. M. Wessel (2010). "Vasa genes: emerging roles in the germ line and in multipotent cells." Bioessays 32(7): 626-637.

Hajkova, P., S. Erhardt, N. Lane, T. Haaf, O. El-Maarri, W. Reik, J. Walter and M. A. Surani (2002). "Epigenetic reprogramming in mouse primordial germ cells." Mechanisms of development 117(1): 15-23.

Hamburger, V. and H. L. Hamilton (1951). "A series of normal stages in the development of the chick embryo." Journal of morphology 88(1): 49-92.

Han, J. Y. (2009). "Germ cells and transgenesis in chickens." Comparative immunology, microbiology and infectious diseases 32(2): 61-80.

Han, J. Y., T. S. Park, J. N. Kim, M. A. Kim, D. Lim, J. M. Lim and H. Kim (2006). "Gene expression profiling of chicken primordial germ cell ESTs." BMC genomics 7(1): 220.

Han, S., W. Xie, S. R. Hammes and J. DeJong (2003). "Expression of the germ cell-specific transcription factor ALF in Xenopus oocytes compensates for translational inactivation of the somatic factor TFIIA." Journal of Biological Chemistry 278(46): 45586-45593.

Han, S., W. Xie, S. H. Kim, L. Yue and J. DeJong (2004). "A short core promoter drives expression of the ALF transcription factor in reproductive tissues of male and female mice." Biology of reproduction 71(3): 933-941.

Hay, B., L. Ackerman, S. Barbel, L. Y. Jan and Y. N. Jan (1988). "Identification of a component of Drosophila polar granules." Development 103(4): 625-640.

Hayashi, K., S. M. C. de Sousa Lopes and M. A. Surani (2007). "Germ cell specification in mice." Science 316(5823): 394-396.

Henkel, G., D. L. Weiss, R. McCoy, T. Deloughery, D. Tara and M. A. Brown (1992). "A DNase I-hypersensitive site in the second intron of the murine IL-4 gene defines a mast cell-specific enhancer." The Journal of Immunology 149(10): 3239-3246.

Hiller, M., X. Chen, M. J. Pringle, M. Suchorolski, Y. Sancak, S. Viswanathan,

B. Bolival, T.-Y. Lin, S. Marino and M. T. Fuller (2004). "Testis-specific TAF homologs collaborate to control a tissue-specific transcription program." Development 131(21): 5297-5308.

Hong, N., M. Li, B. He and Y. Hong "Retinoic Acid Regulates Germ Gene Transcription In Vitro and Spermatogenesis in Testicular Organ Culture." Biological Systems: Open Access.

Horne, G. A., H. J. Stewart, J. Dickson, S. Knapp, B. Ramsahoye and T. Chevassut (2015). "Nanog requires BRD4 to maintain murine embryonic stem cell pluripotency and is suppressed by bromodomain inhibitor JQ1 together with Lefty1." Stem Cells Dev 24(7): 879-891.

Illmensee, K. and A. P. Mahowald (1974). "Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg." Proceedings of the National Academy of Sciences 71(4): 1016-1020.

Irie, N., L. Weinberger, W. W. Tang, T. Kobayashi, S. Viukov, Y. S. Manor, S. Dietmann, J. H. Hanna and M. A. Surani (2015). "SOX17 is a critical specifier of human primordial germ cell fate." Cell 160(1): 253-268.

Jang, H. J., H. W. Seo, B. R. Lee, M. Yoo, J. E. Womack and J. Y. Han (2013). "Gene expression and DNA methylation status of chicken primordial germ cells." Mol Biotechnol 54(2): 177-186.

Kallio M, C. Y., Manuel M, Alastalo TP, Rallu M, Gitton Y, Pirkkala L, Loones MT, Paslaru L, Larney S, Hiard S, Morange M, Sistonen L, Mezger V. (2002). "Brain abnormalities, defective meiotic chromosome synapsis and female subfertility in HSF2 null mice." The EMBO Journal 21.

Kanai-Azuma, M., Y. Kanai, J. M. Gad, Y. Tajima, C. Taya, M. Kurohmaru, Y. Sanai, H. Yonekawa, K. Yazaki and P. P. Tam (2002). "Depletion of definitive gut endoderm in Sox17-null mutant mice." Development 129(10): 2367-2379.

Kawamoto, T., K. Makino, H. Niwa, H. Sugiyama, S. Kimura, M. Amemura, A. Nakata and T. Kakunaga (1988). "Identification of the human beta-actin enhancer and its binding factor." Molecular and cellular biology 8(1): 267-272.

Kedde, M., M. J. Strasser, B. Boldajipour, J. A. Oude Vrielink, K. Slanchev, C. le Sage, R. Nagel, P. M. Voorhoeve, J. van Duijse, U. A. Orom, A. H. Lund, A. Perrakis, E. Raz and R. Agami (2007). "RNA-binding protein Dnd1 inhibits microRNA access to target mRNA." Cell 131(7): 1273-1286.

Kehler, J., E. Tolkunova, B. Koschorz, M. Pesce, L. Gentile, M. Boiani, H. Lomelí, A. Nagy, K. J. McLaughlin and H. R. Schöler (2004). "Oct4 is required for primordial germ cell survival." EMBO reports 5(11): 1078-1083.

Kehler, J., E. Tolkunova, B. Koschorz, M. Pesce, L. Gentile, M. Boiani, H. Lomeli, A. Nagy, K. J. McLaughlin, H. R. Scholer and A. Tomilin (2004). "Oct4 is required for primordial germ cell survival." EMBO Rep 5(11): 1078-1083.

KEVIN D. SARGE, O.-K. P.-S., JOHN D. KIRBY, KELLY E. MAYO, and RICHARD I. MORIMOTO (1994). "Expression of heat shock factor 2 in mouse testis potential role as a regulator of heat-shock protein gene expression during spermatogenesis." BIOLOGY OF REPRODUCTION.

Kim, C. G., I. Y. Chung, Y. Lim, Y. H. Lee and S. Y. Shin (2011). "A Tcf/Lef element within the enhancer region of the human NANOG gene plays a role in promoter activation." Biochem Biophys Res Commun 410(3): 637-642.

Kim, H., T. S. Park, W. K. Lee, S. Moon, J. N. Kim, J. H. Shin, J. G. Jung, S. D. Lee, S. H. Park and K. J. Park (2007). "MPSS profiling of embryonic gonad and primordial germ cells in chicken." Physiological genomics 29(3): 253-259.

Kim, S., U. Gunesdogan, J. J. Zylicz, J. A. Hackett, D. Cougot, S. Bao, C. Lee, S. Dietmann, G. E. Allen, R. Sengupta and M. A. Surani (2014). "PRMT5 protects genomic integrity during global DNA demethylation in primordial germ cells and preimplantation embryos." Mol Cell 56(4): 564-579.

Kim, T., T. Yun, D. Rengaraj, S. Lee, S. Lim, H. Seo, T. Park and J. Han (2012). "Conserved functional characteristics of the PIWI family members in chicken germ cell lineage." Theriogenology 78(9): 1948-1959.

Kito, G., S. Aramaki, K. Tanaka, T. Soh, N. Yamauchi and M.-a. Hattori (2010). "Temporal and spatial differential expression of chicken germline-specific proteins cDAZL, CDH and CVH during gametogenesis." Journal of Reproduction and Development 56(3): 341-346.

Krøvel, A. V. and L. C. Olsen (2002). "Expression of a vas:: EGFP transgene in primordial germ cells of the zebrafish." Mechanisms of development 116(1): 141-150.

Krøvel, A. V. and L. C. Olsen (2002). "Expression of a vasEGFP transgene in primordial germ cells of the zebrafish." Mechanisms of Development.

Kuroda, T., M. Tada, H. Kubota, H. Kimura, S. Y. Hatano, H. Suemori, N. Nakatsuji and T. Tada (2005). "Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression." Mol Cell Biol 25(6): 2475-2485.

Lasko, P. (2013). "The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology." Biochim Biophys Acta 1829(8): 810-816.

Lasko, P. F. and M. Ashburner (1988). "The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A." Nature 333.13.

Lasko, P. F. and M. Ashburner (1988). "The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A."

Lavial, F., H. Acloque, E. Bachelard, M. A. Nieto, J. Samarut and B. Pain (2009). "Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate." Developmental biology 330(1): 73-82.

Lavial, F., H. Acloque, F. Bertocchini, D. J. Macleod, S. Boast, E. Bachelard, G. Montillet, S. Thenot, H. M. Sang, C. D. Stern, J. Samarut and B. Pain (2007). "The Oct4 homologue PouV and Nanog regulate pluripotency in chicken embryonic stem cells." Development 134(19): 3549-3563.

Lawson, K. A., N. R. Dunn, B. A. Roelen, L. M. Zeinstra, A. M. Davis, C. V. Wright, J. P. Korving and B. L. Hogan (1999). "Bmp4 is required for the generation of primordial germ cells in the mouse embryo." Genes & development 13(4): 424-436.

Lee, B. R., H. Kim, T. S. Park, S. Moon, S. Cho, T. Park, J. Lim and J. Y. Han (2007). "A set of stage-specific gene transcripts identified in EK stage X and HH stage 3 chick embryos." BMC developmental biology 7(1): 1.

Lee, H. C., H. J. Choi, H. G. Lee, J. M. Lim, T. Ono and J. Y. Han (2016). "DAZL Expression Explains Origin and Central Formation of Primordial Germ Cells in Chickens." Stem Cells Dev 25(1): 68-79.

Lee, S. I., B. R. Lee, Y. S. Hwang, H. C. Lee, D. Rengaraj, G. Song, T. S. Park and J. Y. Han (2011). "MicroRNA-mediated posttranscriptional regulation is required for maintaining undifferentiated properties of blastoderm and primordial germ cells in chickens." Proceedings of the National Academy of Sciences 108(26): 10426-10431.

Leitch, H. G. and A. Smith (2013). "The mammalian germline as a pluripotency cycle." Development 140(12): 2495-2501.

Lesch, B. J. and D. C. Page (2012). "Genetics of germ cell development." Nat Rev Genet 13(11): 781-794.

Li, H., R. Fan, M. Sun, T. Jiang and Y. Gong (2013). "Nspc1 regulates the key pluripotent Oct4-Nanog-Sox2 axis in P19 embryonal carcinoma cells via directly activating Oct4." Biochem Biophys Res Commun 440(4): 527-532.

Li, M., G. Guan, N. Hong and Y. Hong (2013). "Multiple regulatory regions control the transcription of medaka germ gene vasa." Biochimie 95(4): 850-857.

Li, M., H. Zhao, J. Wei, J. Zhang and Y. Hong (2015). "Medaka vasa gene has an exonic enhancer for germline expression." Gene 555(2): 403-408.

Lilienbaum, A., J. Sage, S. Mémet, M. Rassoulzadegan, F. Cuzin and A. Israël (2000). "NF-κB is developmentally regulated during spermatogenesis in mice." Developmental Dynamics 219(3): 333-340.

Lin, T., C. Chao, S. i. Saito, S. J. Mazur, M. E. Murphy, E. Appella and Y. Xu (2005). "p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression." Nature cell biology 7(2): 165-171.

Lin, Y. and D. C. Page (2005). "Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice." Developmental biology 288(2): 309-316.

Linder, P. (2006). "Dead-box proteins: a family affair—active and passive players in RNP-remodeling." Nucleic acids research 34(15): 4168-4180.

Linher, K., Q. Cheung, P. Baker, G. Bedecarrats, K. Shiota and J. Li (2009). "An epigenetic mechanism regulates germ cell-specific expression of the porcine Deleted in Azoospermia-Like (DAZL) gene." Differentiation 77(4): 335-349.

Liu, N., H. Han and P. Lasko (2009). "Vasa promotes Drosophila germline stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3′ UTR." Genes & development 23(23): 2742-2752.

Liu, W., P. Stein, X. Cheng, W. Yang, N. Y. Shao, E. E. Morrisey, R. M. Schultz and J. You (2014). "BRD4 regulates Nanog expression in mouse embryonic stem cells and preimplantation embryos." Cell Death Differ 21(12): 1950-1960.

Liu, Z., B. Moav, A. Faras, K. Guise, A. Kapuscinski and P. Hackett (1990). "Functional analysis of elements affecting expression of the beta-actin gene of carp." Molecular and cellular biology 10(7): 3432-3440.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method." methods 25(4): 402-408.

Loukinov, D. I., E. Pugacheva, S. Vatolin, S. D. Pack, H. Moon, I. Chernukhin, P. Mannan, E. Larsson, C. Kanduri and A. A. Vostrov (2002). "BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma." Proceedings of the National Academy of Sciences 99(10): 6806-6811.

Lucifero, D., C. Mertineit, H. J. Clarke, T. H. Bestor and J. M. Trasler (2002). "Methylation dynamics of imprinted genes in mouse germ cells."

Genomics 79(4): 530-538.

Luo, H., Y. Zhou, Y. Li and Q. Li (2013). "Splice variants and promoter methylation status of the Bovine Vasa Homology (Bvh) gene may be involved in bull spermatogenesis." BMC genetics 14(1): 1.

Maegawa, S., K. Yasuda and K. Inoue (1999). "Maternal mRNA localization of zebrafish DAZ-like gene." Mechanisms of development 81(1): 223-226.

Magnúsdóttir, E., S. Dietmann, K. Murakami, U. Günesdogan, F. Tang, S. Bao, E. Diamanti, K. Lao, B. Gottgens and M. A. Surani (2013). "A tripartite transcription factor network regulates primordial germ cell specification in mice." Nature cell biology 15(8): 905-915.

Magnusdottir, E., S. Dietmann, K. Murakami, U. Gunesdogan, F. Tang, S. Bao, E. Diamanti, K. Lao, B. Gottgens and M. Azim Surani (2013). "A tripartite transcription factor network regulates primordial germ cell specification in mice." Nat Cell Biol 15(8): 905-915.

Mantovani, R. (1999). "The molecular biology of the CCAAT-binding factor NF-Y." Gene.

Matsui, Y., D. Toksoz, S. Nishikawa, S.-I. Nishikawa, D. Williams, K. Zsebo and B. L. Hogan (1991). "Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture."

Medrano, J. V., C. Ramathal, H. N. Nguyen, C. Simon and R. A. Reijo Pera (2012). "Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro." Stem Cells 30(3): 441-451.

Medrano, J. V., C. Ramathal, H. N. Nguyen, C. Simon and R. A. Reijo Pera (2012). "Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro." Stem Cells 30(3): 441-451.

Mignone, F., C. Gissi, S. Liuni and G. Pesole (2002). "Untranslated regions of mRNAs." Genome Biol 3(3): 1-10.

Minematsu, T., T. Harumi and M. Naito (2008). "Germ cell-specific expression of GFP gene induced by chicken vasa homologue (Cvh) promoter in early chicken embryos." Mol Reprod Dev 75(10): 1515-1522.

Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda and S. Yamanaka (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." cell 113(5): 631-642.

Mizuno, N. and M. Kosaka (2008). "Novel variants of Oct-3/4 gene expressed in mouse somatic cells." Journal of Biological Chemistry 283(45): 30997-

31004.

Mohapatra, C. and H. K. Barman (2014). "Identification of promoter within the first intron of Plzf gene expressed in carp spermatogonial stem cells." Mol Biol Rep 41(10): 6433-6440.

Murakami, K., U. Günesdogan, J. J. Zylicz, W. W. Tang, R. Sengupta, T. Kobayashi, S. Kim, R. Butler, S. Dietmann and M. A. Surani (2016). "NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers." Nature.

Murakami, K., U. Gunesdogan, J. J. Zylicz, W. W. Tang, R. Sengupta, T. Kobayashi, S. Kim, R. Butler, S. Dietmann and M. A. Surani (2016). "NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers." Nature 529(7586): 403-407.

Myslinski, E., M. A. Gerard, A. Krol and P. Carbon (2006). "A genome scale location analysis of human Staf/ZNF143-binding sites suggests a widespread role for human Staf/ZNF143 in mammalian promoters." J Biol Chem 281(52): 39953-39962.

Myslinski, E., A. Krol and P. Carbon (1998). "ZNF76 and ZNF143 are two human homologs of the transcriptional activator Staf." Journal of Biological Chemistry 273(34): 21998-22006.

Naoki Tsunekawa, M. N., Yasuhiro Sakai, Takao Nishida, Toshiaki Noce (2000). "Isolation of chicken vasa homolog gene and tracing the origin of primordial." Development.

Navarro, P., N. Festuccia, D. Colby, A. Gagliardi, N. P. Mullin, W. Zhang, V. Karwacki-Neisius, R. Osorno, D. Kelly, M. Robertson and I. Chambers (2012). "OCT4/SOX2-independent Nanog autorepression modulates heterogeneous Nanog gene expression in mouse ES cells." EMBO J 31(24): 4547-4562.

Nicholas, C. R., K. M. Haston, A. K. Grewall, T. A. Longacre and R. A. R. Pera (2009). "Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility." Human molecular genetics 18(22): 4376-4389.

Nicholas, C. R., E. Y. Xu, S. F. Banani, R. E. Hammer, F. K. Hamra and R. A. Reijo Pera (2009). "Characterization of a Dazl-GFP germ cell-specific reporter." Genesis 47(2): 74-84.

Nichols, J., B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Schöler and A. Smith (1998). "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." Cell 95(3): 379-391.

Niwa, H., K. Ogawa, D. Shimosato and K. Adachi (2009). "A parallel circuit of

LIF signalling pathways maintains pluripotency of mouse ES cells." Nature 460(7251): 118-122.

Noce, T., S. Okamoto-Ito and N. Tsunekawa (2001). "Vasa homolog genes in mammalian germ cell development." Cell structure and function 26(3): 131-136.

Novina, C. D. and A. L. Roy (1996). "Core promoters and transcriptional control." Trends in Genetics 12(9): 351-355.

Ohinata, Y., H. Ohta, M. Shigeta, K. Yamanaka, T. Wakayama and M. Saitou (2009). "A signaling principle for the specification of the germ cell lineage in mice." Cell 137(3): 571-584.

Ohinata, Y., B. Payer, D. O'Carroll, K. Ancelin, Y. Ono, M. Sano, S. C. Barton, T. Obukhanych, M. Nussenzweig, A. Tarakhovsky, M. Saitou and M. A. Surani (2005). "Blimp1 is a critical determinant of the germ cell lineage in mice." Nature 436(7048): 207-213.

Okada, M., I. Kleinman and H. Schneiderman (1974). "Restoration of fertility in sterilized Drosophila eggs by transplantation of polar cytoplasm." Developmental biology 37(1): 43-54.

Olsen, L. C., R. Aasland and A. Fjose (1997). "A vasa-like gene in zebrafish identifies putative primordial germ cells." Mechanisms of development 66(1): 95-105.

Pan, G., J. Li, Y. Zhou, H. Zheng and D. Pei (2006). "A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal." The FASEB Journal 20(10): 1730-1732.

Papathanos, P. A., N. Windbichler, M. Menichelli, A. Burt and A. Crisanti (2009). "The vasa regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito Anopheles gambiae: a versatile tool for genetic control strategies." BMC Mol Biol 10: 65.

Paranjape, A. N., S. A. Balaji, T. Mandal, E. V. Krushik, P. Nagaraj, G. Mukherjee and A. Rangarajan (2014). "Bmi1 regulates self-renewal and epithelial to mesenchymal transition in breast cancer cells through Nanog." BMC cancer 14(1): 785.

Park, T. S. and J. Y. Han (2012). "piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens." Proceedings of the National Academy of Sciences 109(24): 9337-9341.

Parvinen, M. (2005). "The chromatoid body in spermatogenesis." International journal of andrology 28(4): 189-201.

Pereyra, N. B. (2010). In silico analysis of regulatory motifs in gene promoters,

Universitat Pompeu Fabra.

Persengiev, S. P., X. Zhu, B. L. Dixit, G. A. Maston, E. L. Kittler and M. R. Green (2003). "TRF3, a TATA-box-binding protein-related factor, is vertebrate-specific and widely expressed." Proceedings of the National Academy of Sciences 100(25): 14887-14891.

Philipsen, S. and G. Suske (1999). "A tale of three fingers: the family of mammalian Sp/XKLF transcription factors." Nucleic acids research 27(15): 2991-3000.

Rabenstein, M. D., S. Zhou, J. T. Lis and R. Tjian (1999). "TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family." Proceedings of the National Academy of Sciences 96(9): 4791-4796.

Ramathal, C., P. Turek and R. R. Pera (2011). Embryonic stem cells and the germ cell lineage, INTECH Open Access Publisher.

Raz, E. (2000). "The function and regulation of vasa-like genes in germ-cell development." Genome Biol 1(3): 1017.

Reinke, V. (2006). "Germline genomics."

Rengaraj, D., Y. Zheng, K. Kang, K. Park, B. Lee, S. Lee, J. Choi and J. Han (2010). "Conserved expression pattern of chicken DAZL in primordial germ cells and germ-line cells." Theriogenology 74(5): 765-776.

Rengaraj, D., Y. H. Zheng, K. S. Kang, K. J. Park, B. R. Lee, S. I. Lee, J. W. Choi and J. Y. Han (2010). "Conserved expression pattern of chicken DAZL in primordial germ cells and germ-line cells." Theriogenology 74(5): 765-776.

Resnick, J. L., M. Ortiz, J. R. Keller and P. J. Donovan (1998). "Role of fibroblast growth factors and their receptors in mouse primordial germ cell growth." Biology of reproduction 59(5): 1224-1229.

Richardson, B. E. and R. Lehmann (2010). "Mechanisms guiding primordial germ cell migration: strategies from different organisms." Nature Reviews Molecular Cell Biology 11(1): 37-49.

Rodda, D. J., J. L. Chew, L. H. Lim, Y. H. Loh, B. Wang, H. H. Ng and P. Robson (2005). "Transcriptional regulation of nanog by OCT4 and SOX2." J Biol Chem 280(26): 24731-24737.

Ruggiu, M., R. Speed, M. Taggart, S. J. McKay, F. Kilanowski, P. Saunders, J. Dorin and H. J. Cooke (1997). "The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis." Nature 389(6646): 73-77.

Saito, H., K. Takeda, K.-i. Yasumoto, H. Ohtani, K.-i. Watanabe, K. Takahashi,

A. Fukuzaki, Y. Arai, H. Yamamoto and S. Shibahara (2003). "Germ cell-specific expression of microphthalmia-associated transcription factor mRNA in mouse testis." Journal of biochemistry 134(1): 143-150.

Saitou, M. and M. Yamaji (2010). "Germ cell specification in mice: signaling, transcription regulation, and epigenetic consequences." Reproduction 139(6): 931-942.

Salmon-Divon, M., H. Dvinge, K. Tammoja and P. Bertone (2010). "PeakAnalyzer: genome-wide annotation of chromatin binding and modification loci." BMC bioinformatics 11(1): 1.

Sano, H., A. Nakamura and S. Kobayashi (2002). "Identification of a transcriptional regulatory region for germline-specific expression of vasa gene in Drosophila melanogaster." Mechanisms of development 112(1): 129-139.

Sano, H., A. Nakamura and S. Kobayashi (2002). "Identification of a transcriptional regulatory region for germline-specific expression of vasa gene in Drosophila melanogaster." Mechanisms of Development.

Sasaki, H. and Y. Matsui (2008). "Epigenetic events in mammalian germ-cell development: reprogramming and beyond." Nat Rev Genet 9(2): 129-140.

Saunders, A., F. Faiola and J. Wang (2013). "Concise review: pursuing self-renewal and pluripotency with the stem cell factor Nanog." Stem Cells 31(7): 1227-1236.

Saunders, P., J. Turner, M. Ruggiu, M. Taggart, P. Burgoyne, D. Elliott and H. Cooke (2003). "Absence of mDazl produces a final block on germ cell development at meiosis." Reproduction 126(5): 589-597.

Schaner, C. E., G. Deshpande, P. D. Schedl and W. G. Kelly (2003). "A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies." Developmental cell 5(5): 747-757.

Seervai, R. N. and G. M. Wessel (2013). "Lessons for inductive germline determination." Molecular reproduction and development 80(8): 590-609.

Seki, Y., K. Hayashi, K. Itoh, M. Mizugaki, M. Saitou and Y. Matsui (2005). "Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice." Dev Biol 278(2): 440-458.

Sengoku, T., O. Nureki, A. Nakamura, S. Kobayashi and S. Yokoyama (2006). "Structural basis for RNA unwinding by the DEAD-box protein Drosophila Vasa." Cell 125(2): 287-300.

Seydoux, G. and R. E. Braun (2006). "Pathway to totipotency: lessons from germ cells." Cell 127(5): 891-904.

Seydoux, G., C. C. Mello, J. Pettitt, W. B. Wood, J. R. Priess and A. Fire (1996). "Repression of gene expression in the embryonic germ lineage of C. elegans." Nature 382(6593): 713-716.

Smale, S. T. (2001). "Core promoters: active contributors to combinatorial gene regulation." Genes & development 15(19): 2503-2508.

Smallwood, S. A. and G. Kelsey (2012). "De novo DNA methylation: a germ cell perspective." Trends Genet 28(1): 33-42.

Sohn, Y. A., S. I. Lee, H. J. Choi, H. J. Kim, K. H. Kim, T. S. Park and J. Y. Han (2014). "The CCAAT element in the CIWI promoter regulates transcriptional initiation in chicken primordial germ cells." Mol Reprod Dev 81(9): 871-882.

Song, Y., L. Lai, L. Li, Y. Huang, A. Wang, X. Tang, D. Pang, Z. Li and H. Ouyang (2016). "Germ cell-specific expression of Cre recombinase using the VASA promoter in the pig." FEBS Open Bio 6(1): 50-55.

Spike, C., N. Meyer, E. Racen, A. Orsborn, J. Kirchner, K. Kuznicki, C. Yee, K. Bennett and S. Strome (2008). "Genetic analysis of the Caenorhabditis elegans GLH family of P-granule proteins." Genetics 178(4): 1973-1987.

Spitz, F. and E. E. Furlong (2012). "Transcription factors: from enhancer binding to developmental control." Nat Rev Genet 13(9): 613-626.

Strome, S. and R. Lehmann (2007). "Germ versus soma decisions: lessons from flies and worms." Science 316(5823): 392-393.

Strome, S. and D. Updike (2015). "Specifying and protecting germ cell fate." Nature Reviews Molecular Cell Biology 16(7): 406-416.

Strome, S. and W. B. Wood (1982). "Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans." Proceedings of the National Academy of Sciences 79(5): 1558-1562.

Styhler, S., A. Nakamura, A. Swan, B. Suter and P. Lasko (1998). "vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development." Development 125(9): 1569-1578.

Suske, G. (1999). "The Sp-family of transcription factors." Gene 238(2): 291-300.

Suzuki, A., Á. Raya, Y. Kawakami, M. Morita, T. Matsui, K. Nakashima, F. H. Gage, C. Rodríguez-Esteban and J. C. I. Belmonte (2006). "Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells." Proceedings of the National Academy of Sciences 103(27): 10294-10299.

Takeda, J., S. Seino and G. I. Bell (1992). "Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissues." Nucleic acids research 20(17): 4613-4620.

Takeda, Y., Y. Mishima, T. Fujiwara, H. Sakamoto and K. Inoue (2009). "DAZL relieves miRNA-mediated repression of germline mRNAs by controlling poly (A) tail length in zebrafish." PLoS One 4(10): e7513.

Tanaka, M., M. Kinoshita, D. Kobayashi and Y. Nagahama (2001). "Establishment of medaka (Oryzias latipes) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate." Proceedings of the National Academy of Sciences 98(5): 2544-2549.

Tang, W. W., S. Dietmann, N. Irie, H. G. Leitch, V. I. Floros, C. R. Bradshaw, J. A. Hackett, P. F. Chinnery and M. A. Surani (2015). "A Unique Gene Regulatory Network Resets the Human Germline Epigenome for Development." Cell 161(6): 1453-1467.

Teng, Y. N., Y. P. Chang, J. T. Tseng, P. H. Kuo, I. W. Lee, M. S. Lee and P. L. Kuo (2012). "A single-nucleotide polymorphism of the DAZL gene promoter confers susceptibility to spermatogenic failure in the Taiwanese Han." Hum Reprod 27(9): 2857-2865.

Theunissen, T. W., Y. Costa, A. Radzisheuskaya, A. L. van Oosten, F. Lavial, B. Pain, L. F. C. Castro and J. C. Silva (2011). "Reprogramming capacity of Nanog is functionally conserved in vertebrates and resides in a unique homeodomain." Development 138(22): 4853-4865.

Thomas, K., D.-Y. Sung, J. Yang, K. Johnson, W. Thompson, C. Millette, J. McCarrey, A. Breitberg, R. Gibbs and W. Walker (2005). "Identification, characterization, and functional analysis of sp1 transcript variants expressed in germ cells during mouse spermatogenesis." Biology of reproduction 72(4): 898-907.

Tomaras, G. D., D. A. Foster, C. M. Burrer and S. M. Taffet (1999). "ETS transcription factors regulate an enhancer activity in the third intron of TNF-alpha." Journal of leukocyte biology 66(1): 183-193.

Toyooka, Y., N. Tsunekawa, Y. Takahashi, Y. Matsui, M. Satoh and T. Noce (2000). "Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development." Mechanisms of development 93(1): 139-149.

Tsunekawa, N., M. Naito, Y. Sakai, T. Nishida and T. Noce (2000). "Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells." Development 127(12): 2741-2750.

Ukeshima, A., K. Yoshinaga and T. Fujimoto (1991). "Scanning and

transmission electron microscopic observations of chick primordial germ cells with special reference to the extravasation in their migration course." Journal of electron microscopy 40(2): 124-128.

Weber, S., D. Eckert, D. Nettersheim, A. J. Gillis, S. Schafer, P. Kuckenberg, J. Ehlermann, U. Werling, K. Biermann, L. H. Looijenga and H. Schorle (2010). "Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance." Biol Reprod 82(1): 214-223.

Weidinger, G., J. Stebler, K. Slanchev, K. Dumstrei, C. Wise, R. Lovell-Badge, C. Thisse, B. Thisse and E. Raz (2003). "dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival." Current Biology 13(16): 1429-1434.

Weismann, A. (1885). "The continuity of the germ-plasm as the foundation of a theory of heredity." Essays upon heredity and kindred biological problems 1: 163-254.

Whyte, J., J. D. Glover, M. Woodcock, J. Brzeszczynska, L. Taylor, A. Sherman, P. Kaiser and M. J. McGrew (2015). "FGF, Insulin, and SMAD Signaling Cooperate for Avian Primordial Germ Cell Self-Renewal." Stem Cell Reports 5(6): 1171-1182.

Wong, T. T., A. Tesfamichael and P. Collodi (2013). "Identification of promoter elements responsible for gonad-specific expression of zebrafish Deadend and its application to ovarian germ cell derivation." Int J Dev Biol 57(9-10): 767-772.

Wu, D. Y. and Z. Yao (2006). "Functional analysis of two Sp1/Sp3 binding sites in murine Nanog gene promoter." Cell Res 16(3): 319-322.

Wu, D. Y. and Z. Yao (2006). "Functional analysis of two Sp1/Sp3 binding sites in murine Nanog gene promoter." Cell research 16(3): 319-322.

WU, D. Y. and Y. Zhen (2005). "Isolation and characterization of the murine Nanog gene promoter." Cell research 15(5): 317-324.

Wu, G. and H. R. Schöler (2014). "Role of Oct4 in the early embryo development." Cell Regeneration 3(1): 1.

Wu, Q., X. Chen, J. Zhang, Y. H. Loh, T. Y. Low, W. Zhang, W. Zhang, S. K. Sze, B. Lim and H. H. Ng (2006). "Sall4 interacts with Nanog and co-occupies Nanog genomic sites in embryonic stem cells." J Biol Chem 281(34): 24090-24094.

Wylie, C. (1999). "Germ cells." Cell 96(2): 165-174.

Xiol, J., P. Spinelli, M. A. Laussmann, D. Homolka, Z. Yang, E. Cora, Y. Couté, S. Conn, J. Kadlec and R. Sachidanandam (2014). "RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts." Cell

157(7): 1698-1711.

Yamaguchi, S., H. Kimura, M. Tada, N. Nakatsuji and T. Tada (2005). "Nanog expression in mouse germ cell development." Gene Expr Patterns 5(5): 639-646.

Yamaji, M., Y. Seki, K. Kurimoto, Y. Yabuta, M. Yuasa, M. Shigeta, K. Yamanaka, Y. Ohinata and M. Saitou (2008). "Critical function of Prdm14 for the establishment of the germ cell lineage in mice." Nat Genet 40(8): 1016-1022.

Yamazaki, Y., M. R. Mann, S. S. Lee, J. Marh, J. R. McCarrey, R. Yanagimachi and M. S. Bartolomei (2003). "Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning." Proceedings of the National Academy of Sciences 100(21): 12207-12212.

Yeom, Y. I., G. Fuhrmann, C. E. Ovitt, A. Brehm, K. Ohbo, M. Gross, K. Hubner and H. Scholer (1996). "Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells." Development 122(3): 881-894.

Ying, Y., X.-M. Liu, A. Marble, K. A. Lawson and G.-Q. Zhao (2000). "Requirement of Bmp8b for the generation of primordial germ cells in the mouse." Molecular Endocrinology 14(7): 1053-1063.

Ying, Y. and G.-Q. Zhao (2001). "Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse." Developmental biology 232(2): 484-492.

Yoon, C., K. Kawakami and N. Hopkins (1997). "Zebrafish vasa homologue RNA is localized to the cleavage planes of 2-and 4-cell-stage embryos and is expressed in the primordial germ cells." Development 124(16): 3157-3165.

Yoshikawa, T., Y. Piao, J. Zhong, R. Matoba, M. G. Carter, Y. Wang, I. Goldberg and M. S. Ko (2006). "High-throughput screen for genes predominantly expressed in the ICM of mouse blastocysts by whole mount in situ hybridization." Gene Expression Patterns 6(2): 213-224.

Yoshizaki, G., Y. Takeuchi, H. Tominaga, T. KOBAYASHI and T. TAKEUCHI (2002). "Visualization of primordial germ cells in transgenic rainbow trout carrying green fluorescent protein gene driven by vasa promoter." Fisheries science 68(sup2): 1067-1070.

Yu Hou, J. Y., Xiang Zhou, Xiazhou Fu, Hanhua Cheng, Rongjia Zhou (2012). "DNA Demethylation and USF Regulate the Meiosis-Specific Expression of the Mouse *Miwi*." PLoS Gene Expression Patterns.

Zhang, D., T.-L. Penttila, P. L. Morris, M. Teichmann and R. G. Roeder (2001).

"Spermiogenesis deficiency in mice lacking the Trf2 gene." Science 292(5519): 1153-1155.

Zhang, L., R. Zhu, Q. Zuo, D. Li, C. Lian, B. Tang, T. Xiao, Y. Zhang and B. Li (2015). "Activity analysis and preliminary inducer screening of the chicken DAZL gene promoter." Int J Mol Sci 16(3): 6595-6605.

Zhao, H., M. Li, Y. I. Purwanti, R. Liu, T. Chen, Z. Li, N. Hong, G. Guan, A. Yin, L. Xiao, R. Ge, J. Song and Y. Hong (2012). "Mitf is a transcriptional activator of medaka germ genes in culture." Biochimie 94(3): 759-767.

Zheng, Y. H., D. Rengaraj, J. W. Choi, K. J. Park, S. I. Lee and J. Y. Han (2009). "Expression pattern of meiosis associated SYCP family members during germline development in chickens." Reproduction 138(3): 483-492.

SUMMARY IN KOREAN

생식세포는 유전정보를 다음세대로 전달해주는 유일한 세포로서, 체세포와 다른 독특한 유전자 발현 패턴을 지니며 그들만의 특성을 유지한다. 이는 생식세포가 기능을 가진 배우자로 발달하여 궁극적으로 새로운 개체를 만들기 위해 중요하다. 따라서 어떻게 생식세포가 다른 세포와 구별되는 유전자 발현 패턴을 지니게 되는지에 대한 연구는 생식세포 자체의 분자생물학적 이해에 중요하다. 이를 수행하기 위해 생식세포 특이적으로 발현하는 유전자들의 조절 메커니즘을 밝히는 연구가 많이 진행되어 왔지만, 닭에서는 생식세포 특이적 유전자 발현을 조절하는 인자에 대한 연구가 부족하다. 따라서 본 연구에서는 닭의 원시생식세포에 특이적으로 발현하는 것으로 알려진 *VASA*, *DAZL* 그리고 *NANOG*의 프로모터 부위를 동정하여 이들 유전자의 전사를 조절하는 조절 인자를 밝히고자 하였다.

첫 번째 연구에선 생식세포에서 특이적으로 발현하는 RNA 결합 단백질을 암호화하고 있는 *CVH*와 *cDAZL* 유전자의 프로모터 부위를 동정하였다. 이를 위해 각 유전자들의 5’ 상위 지역을 유전자 증폭과정을 통해 획득한 후, 녹색 형광 단백질 유전자가 발현할 수 있도록 벡터를 구축하였다. 또한 프로모터 활성도를 측정하기 위해 발광효소가 발현할 수 있는 벡터도 동시에 구축하였다. 그 결과, *CVH* 프로모터의 경우 +25 bp에서 +275 bp를 포함한 벡터를 제외하고 모든 벡터가 녹색 형광 단백질을 발현하였으며, *cDAZL*의 경우는 모든 벡터가 형광을 띠는 것을 현미경을 통해 확인할 수 있었다. 발광효소 세기를 측정하여 프로모터의 활성도를 측정한 결과에서는 *CVH* 프로모터 −316

bp에서 +275 bp 부위가 제일 높은 활성도를 가졌고, *cDAZL*은 −872 bp에서 +173 bp 부위가 제일 높은 활성도를 보였다. 흥미롭게도 모든 벡터는 닭 원시생식세포에서 프로모터 활성도가 닭 배아섬유아세포에서보다 월등히 높은 수치를 보였다. 이를 바탕으로 우리는 원시생식세포가 체세포에서는 작용하지 않는 특이적인 전사인자를 발현하고 있기 때문이라고 추정하였다. 이를 증명하기 위해 각각의 유전자 프로모터 부위에 어떤 전사인자가 결합하는지 여러 가지 예측 프로그램을 사용하여 알아내고, 닭 원시생식세포 전사체 분석을 바탕으로 하여 추정된 전사인자들 중 닭 원시생식세포에서 특이적으로 높게 발현하는 인자들만을 추려내었다. 총 6가지 전사인자들 (*EP300*, *GAPBA*, *HSF2*, *NFYA*, *SP3* 그리고 *ZNF143*) 중 *HSF2*, *NFYA*, *SP3* 그리고 *ZNF143*의 경우, 원시생식세포 내에서 이들 전사인자의 발현이 감소하면 생식세포 특이적 유전자들 (*CVH*, *cDAZL*, *CIWI* 그리고 *cDND1*)의 발현도 감소하는 것을 확인하였다. 이는 생식세포에서 특이적으로 발현하는 전사인자들에 의해 여러 생식세포 유전자의 전사가 조절될 가능성을 보여준다.

추가적으로 *CVH* 유전자의 프로모터 3’ 말단부위를 제거한 실험에서 인트론을 포함하고 있는 5’ UTR 부위가 절단되면 프로모터의 활성도가 급격히 낮아짐을 확인할 수 있었다. 이는 송사리의 *Vasa* 프로모터와 비슷한 현상을 보이며, *CVH* 프로모터의 경우도 활성을 갖기 위해 인트론 부위를 필요로 한다고 예측된다.

*Nanog*는 닭을 포함한 많은 종에서 다능성을 가진 세포에서 발현하여 세포의 특성을 유지하는데 중추적인 역할을 한다고

알려져 있다. 때문에 포유류에서 *Nanog*의 발현을 조절하는 상위 인자가 무엇인지 밝히기 위해 프로모터 부위를 동정하고 그에 결합하는 전사인자를 밝히기 위한 연구가 많이 진행되어 있다. 포유류들간 *Nanog*의 전사를 조절하는 주요 인자로 밝혀진 OCT4와 SOX2 전사인자가 결합하는 프로모터 지역이 상당히 높게 보존되어 있는 것으로 밝혀져 있다. 반면, 닭에서는 *NANOG* 프로모터 연구가 진행되어 있지 않아 전사 조절 인자에 대한 정보가 부족하다. 따라서 두 번째 연구에서는 닭의 *NANOG* 유전자 전사를 조절하는 시퀀스와 그에 결합하는 전사인자를 밝히기 위해 *cNANOG* 유전자 프로모터 부위를 동정하였다.

프로모터 부위를 동정하기에 앞서, 5’ RACE PCR을 통해 아직 밝혀지지 않은 닭의 *NANOG* 유전자 5’ UTR 지역이 전사시작부위로부터 상위 70-bp 라는 것을 찾았다. 이를 기반으로 하여 −3,550 bp에서 +70 bp까지 지역을 임의적 결실 (deletion)을 통한 벡터 구축을 하였다. 320-bp만으로도 닭 원시생식세포에서 프로모터의 활성을 나타내었고, 3,620-bp의 경우 다른 벡터에 비해 활성도가 더 높은 것으로 나타났다. 반면, 닭 배아섬유아세포에서는 프로모터 활성이 전혀 없었다. 이는 마우스의 *Oct4* 유전자가 세포에 따른 선택적 프로모터 (alternative promoter)의 사용을 보이는 것과 비슷한 양상을 보인다. 따라서 닭의 *NANOG* 유전자도 마우스 *Oct4*와 같이 세포에 따라 프로모터 활용이 다를 것이라고 예상된다.

추가적으로 닭의 *NANOG* 프로모터 인핸서를 찾기 위해 320-bp와 3,620-bp의 상위 부위를 결실한 실험에서 각각 −130 bp에서 −210 bp 그리고 −3,154 bp에서 −2,928 bp까지가 각각

인접 인핸서 (proximal enhancer)와 원거리 인핸서 (distal enhancer) 지역이라고 예상할 수 있었다. 예측 프로그램과 리보핵산 시퀀싱 (RNA-seq) 데이터를 기반으로 인핸서 부위에 결합하며 원시생식세포와 포배엽 단계의 세포에서 특이적으로 발현하는 잠정적인 전사인자들을 확인하였다. 또한 인접 인핸서를 찾기 위한 과정에서 −130 bp에서 +70 bp는 프로모터 활성을 지니지만 −69 bp에서 +70 bp는 활성이 없는 것을 확인했다. 프로모터가 활성을 갖기 위해 필요한 주요 인자가 포유류에서와 마찬가지로 다능성 관련 인자들이 관여하는지 확인하기 위한 실험을 진행하였다. 이들 인자들이 인지할 것이라고 예상되는 시퀀스에 돌연변이 (mutation)를 시켜 프로모터 활성 차이를 비교한 결과, 포유류의 *Nanog* 유전자의 전사 조절과 같이 추정된 다능성 관련 전사인자 결합 시퀀스가 닭의 *NANOG* 유전자 발현에 영향을 주는 것으로 확인되었다. 하지만 −130에서 −89까지 시퀀스가 제거가 될 경우, 다능성 관련 인자들이 변형되지 않아도 *NANOG* 프로모터의 활성이 급격히 떨어지는 것을 확인하였다.

위의 결과들을 볼 때, 닭의 원시생식세포의 특이적으로 발현하는 유전자는 생식세포에 특화된 프로모터 지역과 이를 인지하는 전사인자들의 복합체로 이루어진다는 것을 알 수 있다. 예측된 전사인자들이 직접적으로 예상된 부위에 결합하여 기능한다는 것을 알기 위해선, 단백질 결합위치 동정과 겔 이동분석과 같은 추가적 실험이 수행되어야 할 것이다. 결과적으로 본 연구에서 우리의 결과는 닭 원시생식세포에서의 기초 분자생물학적 연구뿐만 아니라 발현 조절 부위를 활용한 모델 동물 개발 등의 응용생명과학 분야에도 큰 도움을 줄 것으로 기대된다.