A Thesis for the Degree of Master of Science

Sex Dimorphic Proliferation Profiles and Its Regulation in Chicken Germ Cells during Embryonic Development

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SUMMARY

Germ cell is the only cells undergoing two types of cell cycle changes during lifetime. This cell cycle changes is the process that is necessary to halve the number of chromosomes for formation of haploid germ cells. The cell cycle change of primordial germ cells (PGCs) indicates the initiation of germ cell differentiation leading to the highly specialized gametes, oocyte or sperm.

Usually, mammals and bird undergo the first cell cycle changes by sex is occurred during embryonic development. Female initiate the first meiotic division in embryonic day, while male pause the mitotic division and enter the G0/G1 arrest. Through this differentiation pathway, strict controls of cell cycle event both proliferation and meiosis induction is required at proper timing to establish the formation of functional gametes. Under aberrant regulations, germ cells undergo excessive apoptosis, formation of TGCT (Testicular Germ Cell Tumor), infertility (Jorgensen et al., 2013). Despite the importance of regulation of cell cycle in germ cells during sex differentiation, the clear definition and mechanisms remained as questions in chicken. In this study, we try to definite the proliferation and cell cycle profiles in chicken germ cells. And also, we focused on the mitotic arrest in male germ cells and try to elucidate the biological meaning of this mechanisms involved in sex differentiation.

We try to examine the proliferation profiles in chicken germ cells

during sex differentiation. To examine the dynamics, we performed the comparison study of germ cells based on DNA replication cell cycle and mitosis dynamics during embryonic development. From these studies, we showed that chicken germ cells undergo proliferation and cell cycle changes in sex dimorphic pattern prior to germ cell differentiation. It have been showed that female germ cells progress the cell cycle changes with high proliferation than male germ cells. And also, from embryonic day 16, the population of G2 phase were increased drastically. On the other hand, male germ cells continues the cell cycle changes with low proliferation phase during overall embryonic development. In addition, at embryonic day 14, almost germ cells enter the mitotic arrest. However, some germ cells lasting DNA synthesizing are existed in both embryonic testis and ovary after onset of sexual differentiation. Collectively, chicken germ cells trigger the sex dimorphic proliferation and differentiation in asynchronous pattern in their gonad.

In our research, the proliferation profiles based on DNA contents was investigated for the first time in chicken germ cells and it was revealed that chicken germ cell differentiation has the distinguished mechanism compared with mammal. This study can contribute to the studies on relationship of cell cycle changes and sex differentiation in chicken germ cells.

Keyword : germ cell, proliferation, sex differentiation, meiosis, mitotic arrest

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CONTENTS

LIST OF FIGURES

CHAPTER 3

LIST OF ABBEREVIATION

PGC : Primordial Germ Cell

CVH : Chicken Vasa Homolog

cDNA : Complementary DNA

GAPDH : Glyceraldehyde – 3 – phosphatate dehydrogenase

DMSO : Dimethyl sulfoxid

RA : Retinoic Acid

RAR : Retinoic Acid Receptor

STRA8 : *Stimulated by retinoic acid gene 8*

CYP26B1 : Cytochrome p450 (CYP)-mediated RA metabolism

SRY : Sex-determining region Y

FGF9 : Fibroblast growth factor 9

SOX9 : Sex determining region Y- box 9

DMRT1 : Drosophila Doublesex and C. elegans Mab-3 Related Transcription factor, #1

AMH : Anti Mullerian Hormone

RALDH2 : Retinaldehyde dehydrogenase type 2

EdU : 5-ethynyl-2'-deoxyuridine

PFA : Paraformaldehyde

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

ABAM : Antibiotic Antimycotic

MACS : Margnetic activated cell sorting

FACS : Fluorescence-activated cell sorting

SSEA-1 : Stage Specific embryonic antigen - 1

CHAPTER 1.

GENERAL INTRODUCTION

Germ cell plays a uniquely important role in biology, providing a mechanism for sexual reproduction and for passing genetic legacy from one generation to the next. The pioneer type of germ cell is called primordial germ cells (PGCs). During gastrulation, PGCs continue the proliferation and migrate into their gonad where they gain capability forming functional germ cells; oocyte or sperm. Before sex determination, PGCs display similar patterns of morphological, physical dynamics both male and female germ cells. In this timing, PGCs express pluripotency related markers. The sex determination of germ cells is dictated by cues from both the somatic environment and germ cell autonomous decision (Adams and McLaren, 2002; McLaren, 1981; McLaren and Southee, 1997; Palmer and Burgoyne, 1991). In mammal and bird, the sex dimorphic pathway of germ cells occurs during embryonic development. Female germ cells initiate the first meiotic division in embryonic day, thereby committing to oogenesis. On the other hand, male germ cells do not commit into meiosis and enter the mitotic arrest of G0/G1.

Retinoic acid (RA) is a crucial molecule in developmental biology. It has been revealed that RA regulates the cell growth, differentiation (Duester, 2008). RA gains the capability to action by binding the retinoic acid receptor (RAR) and regulates genes expression by the activation or repression of transcription. *Stra8(Stimulated by retinoic acid gene 8*, pre-meiotic marker of germ cells, is the target of RA receptor-dependent transcription. Recent studies have been reported that meiosis is induced by retinoic acid signaling (Bowles et al., 2006; Koubova et al., 2006). In female, germ cells begin the meiosis under the RA activation during embryogenesis. However, male germ cells do

not enter the meiosis and undergo G0/G1 mitotic cell cycle arrest. Because the cytochrome p450 (CYP)-mediated RA metabolism prevents premature Stra8 expression in embryonic testis (Koubova et al., 2006). The meiosis mediated mechanisms are conserved in a lot of perspectives in mammals and birds (Smith et al., 2008; Yu et al., 2013).

In this context, chicken female germ cells commit the meiotic events in coincidence at similar developmental timeline and molecular changes (Smith et al., 2008). n contrast, there are also several distinctive characteristics in chicken compared to mouse. Mouse female germ cells initiate meiosis at simultaneous timing of the onset of gonadal differentiation. In contrast to the mouse, the gonadal sex determination occurs at embryonic day 6.5 and appearance of meiotic germ cells is at E.15.5. From this perspective, chicken is considered as a delayed model while mouse takes the simultaneous model. In sex determination of chicken, hormonal regulation has crucial roles in female sex determination through estrogen, estrogen synthetase and aromatase. In spite of these interesting perspectives, a few studies about the proliferation and differentiation have been reported. So, we concentrated in elucidation of distinguished mechanisms of chicken germ cell proliferation during embryonic development. We analyze proliferation profiles based on the general cell cycle status, DNA synthesizing germ cell visualization and estimation of germ cell numbers during embryonic development. In addition, unique proliferation profiles distinguished from mammals are discussed.

CHAPTER 2.

LITERATURE REVIEW

1. Germ Cell Development

Germ cell is the only cells transferring their genetic information from one generation to next generation. And also, germ cell undergoes two types of cell cycle changes, mitosis and meiosis. Germ cells possess the distinguished mechanisms to specify, maintain and protect their own during its life cycle. Through these changes, germ cells finally become forming gametes as functional germ cells. The appropriate conversions have to take place during germ cell development; to leave the mitotic cell cycle and begin progression through meiotic cell cycle. The mitosis/meiosis decisions are regulated by various molecular collaborations.

1.1 Primordial germ cell

1.1.1 Specification

In many animals, germ cell is originated as primordial germ cells (PGCs). However, some specification mechanisms are not conserved in animals. To categorize the mechanism of germ cell specification, there are two models suggestions; pre-formation and induction model.

In pre-formation model, PGCs are separated from the somatic lineage firstly. In this species, maternally synthesized germ plasm is deposited into the egg during oogenesis. The ‘germ plasm’ within cytoplasm indicates the organelles containing mRNA, protein, smallRNA and others and functions determinant the germ cell fate. Flies, worms, fish and frogs belong in pre-

formation model.

The germ cell specification in induction model occurs in the proximal epiblast after the segregation of embryonic and extraembryonic tissue, just prior to gasturulation. In this timing, a subpopulation of embryonic pluripotent epiblast cells deposited to inductive signals come from the extraembryonic tissue. Finally, The deposited cells induce into PGCs. A BMP4/8 signal from the extra embryonic ectoderm initiates the germ-cell-specific program by activating the repressor protein Blimp1 (also known as Prdm1) (Lawson et al., 1999, Ohinata et al., 2005 and Ying et al., 2001). Mammals and many other species are in induction model.

Chicken PGCs were first identified in the germinal crescent region and thought to originate from hypoblast (Swift, 1914). In this study, PGCs were identified based on morphological characteristics such as a large amount of glycogen granules and large size of cells compared to surrounding somatic cells. In specification, the type of specification mode of chicken is not categorized clearly. Fabian et al. suggested that chicken is the induction model, based on the absence of the germ granule in cytoplasm within germ cells (Fabian et al., 1981). However, a chicken *vasa* homologue (*CVH*) was isolated and chicken PGCs was traced by this protein (Tsunekawa et al., 2000). In this study, CVH protein is localized surrounding spectrin and mitochondrial clouds in growing oocyte. From this study, chicken PGCs are considered in specification by pre-formation. However, it need to more studies to determine the PGCs specification model of chicken.

1.1.2 Migration

The PGCs arise far from the somatic cells and migrate into their gonad, where they can gain the capability to formation of functional gametes, by various migration process. During this progression, PGCs can establish the epigenetic marks, active proliferation and other events (Kunwar et al., 2006; Raz, 2004; Santos et al., 2004).

In *Drosophila,* it has been reported that PGCs are originated as pole cells and migrate from posterior end of the embryo to the posterior midgut pocket during gastrulation (Jaglarz et al., 1995).

In mouse, PGCs originated from epiblast and migration is initiated from the posterior primitive streak to the endoderm(Anderson et al., 2000). Following subsequent migration in the hindgut during its anterior extension (E8–9.5), mouse PGCs follow a path remarkably similar to that in D. melanogaster, in which they migrate through hindgut tissue to the mesoderm, followed by bilateral migration to the gonadal ridges and gonad formation (E10.5–11.5) (Kurokawa et al., 2006).

In chicken, the PGCs arise from the epiblast at stage X and translocate to the germinal crescent region at stage 3. From germinal crescent, PGCs initiate the first migration step toward to lateral extraembryonic membranes and enter the developing vascular system around stage 10 (Ukeshima, 1996). Next, the PGCs migrate through blood vessel at stage

HH15 and settle in the genital ridges at E4.0 (England et al., 1986; Meyer, 1964).

During migration, chicken PGCs undergo mitotic division in difference patterns dependent on located region and developmental stage. Around HH stage 2-3, about 40-60 CVH positive cells are appeared at blastodisc. Also, about 200-250 CVH positive cells are localized in the anterior region of the presumptive amniocardiac vesicle at stage X to stage 4 (Tsunekawa et al., 2000). From stage 11 to 15, chicken PGCs undergo migration within vascular system together with blood cells and settle at gonadal region at stage 17-18. In comparion the proliferation rate before and after of settlement in gonad region, chicken PGCs displayed higher proliferation late after they had settled in the germinal ridge(Maeda, 1998)

1.2 Oogonia, Spermatogonia and Sex determination

Primordial germ cells(PGCs) are the pioneer cells that possess unique capability to form gamete. In vertebrates, PGCs are originated from epiblast and settled to gonad through circulation. After this event, somatic environment in gonad leads that germ cells differentiate into oogonia or spermatogonia (Bowles 2010).

In Drosophila, germ line stem cells (GSCs) are found in both ovary and testis and produce egg and sperm throughout adult life. In both sexes, GSCs divide asymmetrically and generates a daugthter GSC destined to differentiate; “cystoblast in females and a “gonialblast” in males. Cystoblast divide through

four rounds division incompletely, generating a 16-cell cyst which become functional oocyte. In ovary, multiple niches are existed. In males, 6-12 GSCs forming single cluster of somatic support cells, called hub, undergo meiotic S-phase progression and generate 64 sperm. In both males and females, asymmetrical division of GSCs generates functional germ cells, like oocyte and sperm. But symmetrical replenishment of germ stem cell pools maintains supplement of differentiated germ cells throughout whole life.

To differentiate PGCs to oogonia or spermatogonia, the proper gonadal environment is established for supplying differentiation inducing substance. In this mechanism, mammals and chicken shared both conserved and divergent elements.

Mammals and birds exhibit genetic sex determination, in which sex is determined at fertilization by the inheritance of sex chromosomes. Mammals possess the XX:XY sex chromosome system, and male is the heterogametic sex; XY. Bird possess the ZZ:ZW sex chromosome system, and female is the heterogametic sex; ZW. At the top of the sex determining cascade, the strong sex determining genes are located in sex chromosomes in sex specific patterns; sry in mammal and DMRT1 in mouse.

Sry, is found only in mammals, is the switch gene for male-sex determination (Gubbay et al., 1990; Koopman et al., 1991). During embryogenesis, Sry is expressed to start from 10.5 dpc and peaks at 11.5 dpc in somatic cells of XY genital ridges. *Sry* functions as the top regulator of positive-feedback loops including related male differentiation genes; FGF9,

SOX9, prostaglading D2. Especially, Sox9 expression is regulated by the stimulation of *Sry.* In XY mouse gonads, Sox9 expression is upregulated in pre Sertoli cells immediately after the onset of *Sry* gene epxreesion. *Sox9* upregulates other genes involved in the differentiation of Sertoli cells(Kent et al., 1996; Morais da Silva et al., 1996; Sekido et al., 2004; Wilhelm et al., 2005). In the absence of SRY, female fate related genes including *Wnt4, Rspo1* and *Foxl2,* are expressed in a female-specific manner and induce ovarian development.

By contrast to mammals, chicken have no *Sry* homology in chromosomes. But instead of Sry, *DMRT1*((Drosophila Doublesex and C. elegans Mab-3 Related Transcription factor, #1) functions as strong male-differentiation factor. *DMRT1* is expressed specifically in the gonads of chicken embryos, being more highly expressed in males compared to females prior to and during gonadal sex differentiation (Raymond et al., 2000; Smith et al., 1999). In ZW genetic females that are sex-reversed with an aromatase inhibitor, Z-linked *DMRT1* is up-regulated in parallel with testis differentiation, despite being present in a single copy (Smith et al., 2003). Transcriptional suppression of DMRT1 by RNAi leads to feminization of the testis at both morphological and molecular levels (Smith et al., 2009). In chicken, Dmrt1 is expressed prior the 2 days of expression Sox9. And also, the expression level of Sox9 is up-regulated at AMH expression. Collectively, the functions of Sox9 are speculated to decide the male fate. In mammals, sox9 activates the expression of the fgf9 gene and establish the male specific gonadal environment (Bowles et al., 2010).

Anti-Mullerian hormone (AMH) is responsible for regressing the Mullerian ducts which develop into oviducts and upper vagina of the female reproductive tracts. produced by Sertoli cells and concerned in testicular differentiation in mammal. In chicken, AMH starts to be expressed in both male and female gonad of early embryo. However, the expression level of AMH is decreased in female gonad after sex determination (Oreal et al., 2002). In knock-down the DMRT1 in testis, AMHR2, AMH receptor 2, was not expressed in the developing seminiferous cords of males (Cutting et al., 2014). The expression of *AMH* gene is regulated by the interaction of the respective binding of *Sox9* and *Sf1* (Jiang et al., 2014) .

In ovarian differentiation, crucial determinant has not been defined, but the β-catenin signaling is considered for establishing ovary microenvironment. Both the mammal and chicken, β-catenin signaling related genes including R-Spo1, Wnt4 and β-catenin are upregulated in female reproductive tracts during sex differentiation(Ayers et al., 2013; Cutting et al., 2013).

Estrogen is required for ovarian differentiation in most invertebrate. Actually, mouse ovarian differentiation is initiate prior to stimulation of estrogen. Also, the role of estrogen in mouse has not been reported clearly. However, chicken is unique because their ovarian differentiation is sensitive to the effects of estrogen although it is considered as vertebrate. Also, the regulation of meiotic initiation is required for roles of estrogen in chicken(BYSKOV, 1979).

1.3 Meiosis and Mitotic Arrest in Sex Differentiation

After onset of differentiation into testis or ovary, germ cells accompany specialized form of cell cycle events; female germ cells finish the mitosis and enter the first stage of meiosis and male germ cells cease the mitosis and arrest in G0/G1 phase(McLaren, 1984). And timing of meiosis entry dependent on sex is determined by existence of retinoic acid signaling(Koubova et al., 2006). In developing ovary of mouse and chicken, germ cells are stimulated by retinoic acid and initiates meiosis at embryonic life. However, CYP26B1, catabolic cytochrome P450, is secreted from developing testis and down-regulate levels of retinoic acid delay initiation of meiosis during embryonic life(Bowles et al., 2006; Li et al., 2009). In this timing, *Stra8* is expressed in ovarian germ cells at E.12.5, just 1 day before meiotic germ cells can be observed and meiotic markers, *Dmc1* and *Scp3* (Bullejos et al., 2004; Koubova et al., 2006; Menke et al., 2003; Yao et al., 2003). The B type cyclins regulate that ovarian embryonic germ cells are prevented on of progressing mitotic cell cycle and accumulation in meiotic prophase 1 with a G2 DNA content. ATM and CHK2, DNA damage repair proteins, are highly activated in this timing and functions as inhibitors of G2/M progression into meiotic cell cycle(Miles et al., 2010). In cell cycle status, expression levels of CyclinE1, CyclinE2 and CyclinD3 are down-regulated in mitotic arrest status at E.14.5(Western et al., 2008). And also, Retinoblastoma 1, a potent cell cycle regulator, modulates of entry into normal cell cycle status in G0/G1 arrest(Spiller et al., 2010).

During embryonic day, female germ cells develop in clusters of

interconnected cells, cyst. The cyst is need for sharing distinctive characteristics and unifying the status of germ cells (Buning et al., 1988; de Cuevas et al., 1997). The number of cysts is increased by mitosis from individual progenitor cells during development. The cells in cyst are connected by intercellular bridge. Through intercellular bridges, a lot of organelles and mRNA can transport into one of the cystocytes. In mammal, female germ cell cyst appeared from 10.5 d.p.c. to 15.5 d.p.c. The size of clusters is increased in size from 10.5 d.p.c. until meiosis at 14.5 d.p.c. embryo. At 10.5 d.p.c., almost cyst consist of 2~4 cells per one cluster. However, a lot of larger clusters are seen including 8 or 16 mitotic cells. Finally, the size of clusters are decreased about 2 or mitotic cells at 15.5 d.p.c(Pepling et al., 1998). The formed cyst are broken apart and a lot of germ cells undergo apoptosis and the remained germ cells become primordial follicles. In same stages, male germ cell forms lower number of cyst and only 2.2 cells per one cyst are remained to birth. The remainder resumes proliferation at P0 and establish the spermatogonial stem cell pools(Lei et al., 2013).

From these perspectives, cyst formed germ cells have begun to appear at onset of meiosis and disappeared at arrest of meiotic prophase 1. So, putative functions of cyst division are considered that synchronization the development of small groups of germline cells prior to meiosis. The synchronization by transfer the mRNA and organelles may be important that all the cells remain at the appropriate developmental stage to utilize gene products produced by their neighbor(Braun et al., 1989).

In medaka, germ cells continue the proliferation only once or twice during migration(Kurokawa et al., 2006). Before gonadal development, medaka germ cells are divided by slow division in same patterns of sex. After settlement at gonadal primordium, medaka germ cells are displayed that sex dimorphoic proliferation patterns. Female germ cells continues the proliferation actively; while male germ cells do not show active proliferation(Hamaguchi, 1982; Satoh et al., 1972). Specifically, male germ cells last the proliferation by slow intermittent division, same patterns in undifferentiated gonads, female germ cells changes the patterns to two to four rounds of continuous division, forming cysts of 4, 8, or 16 cells. These divided cells subsequently enter meiosis synchronously(Saito et al., 2007).

Chicken primordial germ cells undergo more active proliferation at upon reach the gonadal ridge (stage 17-18) than during circulation within blood vessel (stage 14-15) (Maeda, 1998). And the number of SSEA-1 positive cells in gonadal cells is drastically increased from E.4.5 to E.7.5 (Motono et al., 2008). Motono et al. reported that the number of male primordial germ cell at E.5.5~8.5 displayed drastic increase about 2.5-fold. After sex differentiation of gonad, chicken germ cells displayed dimorphic patterns by sex. The chicken embryonic gonad is indifferent at embryonic day 3.5-4.5, being morphologically indistinguishable between the sexes. From embryonic day 6.5, the gonad initiates differentiation to sex dependent pathway(Carlon et al., 1985). In female embryos, only the left gonad develops and right gonad regresses in asymmetric status. And, the cortex regions of light gonad of female embryo become thicken specifically. Male chicken embryos show symmetric gonadal development. Both left and right gonad develops their

medullar region as bilateral patterns. Byscov suggested that recognised mammals with so-called "immediate" versus "delayed" meiosis.

The immediate model is that meiosis occurs "immediately" at the time of somatic gonadal sex differentiation (such as the mouse). Sex differentiation and the appearance of the first meiotic germ cells are almost simultaneous while in others, as rabbits and ewes, the two events are far separated in time. Chicken initiate sex determination at E.6.5 and gonad is development to ovary or testis respectively and meiosis in germ cells initiate at E.15.5 in just female ovary.

2. Cell Cycle in Germ Cell Development

2.1 Germ Cell Proliferation

Mitosis is a process producing daughter cells with a chromosome complement that is identical to that of the progenitor cells. But, meiosis is a specialized type of cell division that generates gametes with a haploid set of chromosomes.

From yeast to mammalian, certain features of the mitotic and meiotic cell cycles are highly conserved. During Mitosis, the genome of diploid cells is replicated during S-phase, the duplicated chromosomes segregate from each other during M-phase, so that diploid daughter cells are produced. S-phase is preceded by G1, a stage when key developmental controls are broken out, and

it is followed by G2, a stage characterized by growth and preparation for M-phase. In animals, primordial germ cells proliferate using mitotis during embryonic life, and adult germline stem cells rely on the mitotic cycle for self renewal and amplification via trans-amplifying cells.

Upon un-matured germ cells beginning maturation, they initiate transition from the mitotic cell cycle into the meiotic cell cycle, a process that ultimately reduces the number of chromosomes from two sets to one in both sperm and egg. Although many aspects of the mitotic and meiotic cell cycles are conserved during germ cell development, the transition from one to the other often takes place in an organism-specific or sex-specific context.

In mouse, the specified primordial germ cells migrate in to genital ridge, where they gain the ability to generate functional gametes that enter the meiosis. Female germ cells initiate meiosis during embryonic life, then enter meiotic prophase 1 arrest until ovulation. A basic doctrine of reproductive biology is that all of female germ cells differentiate into meiotic germ cells and pause the mitotic division before birth in mammal. However, Tilly and his colleagues discovered that proliferative germ cells are existed in postnatal mouse ovary(Johnson et al., 2004). In 2012, they found that mitotically active germ cells are also existed in human's ovary and are isolated by fluorescence-activated cell sorting-based protocol(White et al., 2012).

On the other hand, male germ cells enter the mitotic arrest in G0/G1 phase after birth. After birth, they resume mitosis and generate the germline stem cell pool throughout adult life. Single mitotic precursor cells, called

A_{single} (A_s) spermatogonia, divide with incomplete cytokinesis to become a joined pair of cells, called A_{paired} (A_{pr}). These pairs then divide synchronously between one and three times to become linearly conjoined chains of 4–16 cells, called $A_{aligned}$ (A_{al}). Following the A_{al} stage, spermatogonia are considered to be 'differentiating' and pass through A_1, A_2, A_3, A_4, intermediate, B and preleptotene stages before entering the leptotene phase of meiosis I(Lesch et al., 2012).

Chicken germ cells are originated from extra-gonadal region as a population of about 200 dispersed cells and migrate into the gonads via the bloodvessel(Rogulska et al., 1971). Chicken primordial germ cells are possesses large amounts of glycogen and positive at periodic acid- Schiff (PAS) reaction(Meyer, 1964).

2.2 Critical Regulator for Meiosis; Retinoic acid and CYP26B1

During embryonic development, retinoic acid (RA) functions mainly in a paracrine manner, with RNA synthesis being controlled mostly by tissue–specific expression of retinaldehyde dehydrogenase-2(Raldh2), which generates RA that stimulates to RA target cells where it directly regulates genes via ubiquitous nuclear RA receptors (RARs) bound to RA response elements (RAREs)(Duester, 2008).

In mammalian embryonic gonad, ovarian germ cells initiate meiosis during embryonic day, whereas testiscular germ cells postpone meiosis until

after birth.

From 1970s, Byskov and Saxen suggested that a existence of a “meiosis inducing substances” and this substances are derived from embryonic gonad and required for initiation of meiosis(Byskov, 1974).

To initiate meiosis in both two sexes, *Stimulated by Retinoic Acid gene 8* (*Stra8*) is expressed pre-meiotic genes over 4 days starting at E.12.5, just 1 day before meiotic germ cells with characteristically condensed chromatin can be observed(Menke et al., 2003). Koubova et al., signaling by retinoic acid (RA), an active derivative of vitamin A, is required for *Stra8* expression and thereby meiotic initiation in embryonic ovaries(Koubova et al., 2006). In embryonic gonadal microenvironment, RA is produced from somatic tissue and stimulates directly on undifferentiated germ cells to induce expression of *Stra8*. And an enzyme of the CYP26 family, likely CYP26B1, degrades RNA and thereby prevents expression of Stra8 and precludes initiation of meiosis. In this study, they suggest that germ cells respond to sex differences in the RA environments offered by embryonic ovaries and testes. Bowles et al., reported that RA is produced from mesonephroi of both sexes and existences of CYP26B1 produced from Sertoli cells in fetal testis functions in retarding meiosis in male during embryonic day(Bowles et al., 2007). In testes of *Cyp26b1*-knockout mouse embryos, germ cells enter meiosis precociously, as if in a normal ovary. So, adequate initiation of meiosis is required for producing functional germ cells, as putative gamete. However, in 2011, Kumar and his colleague reported that Stra8 is expressed in the absence of physiologically detectable levels of RA(Kumar et al., 2011). They found that

in the absence of RA producing emzymes RALDH2 and RALDH3, Stra8 was found to be expressed in fetal ovaries at 13.5 dpc and they demonstrate that Stra8 was expressed in fetal testis when CYP26b1 was inhibited by chemical inhibitor, Ketoconazole. This study suggested that the critical role Cyp26b1 has in preventing onset of meiosis in the fetal testis does not involve degradation of RA, and RA-independent function of Cyp26b1 in control of meiotic initiation. This study considered a surprising report changing the classical paradigm in regulation of induction of meiosis.

Generally, CYP26B1 is secreted from Sertoli cells in embryonic testis at embryonic day 11.5, prior to mitotic arrest, and persists throughout fetal development. In Sertoli cell specific-Cyp26b1 mutant, male germ cells exit from G0 and re-enter mitosis in E16.5 mutant testes. And also, they expressed several meiotic markers, *Stra8* and SCP3, in mutant embryonic testis(Li et al., 2009). These results suggest that CYP26B1 in Sertoli cells during embryonic development maintains male germ cells undifferentiated by arresting them in mitotic quiescence and preventing them from meiotic entry until after birth, when mitosis resumes and meiosis initiates as required for spermatogenesis. In this context, this process in essential for germ cells maturation, as an RA-deficient environment is required for the entry of male germ cells into mitotic arrest and thus prevents germ cells from entry into meiotic prophase.

Craig A Smith and his colleague reported that the molecular interaction with RA and CYP26b1 and function in induction of meiosis is conserved with mouse(Smith et al., 2008). They found that meiotic germ cells were detected from embryonic day 15.5 in left gonad of female. The premeiotic marker,

STRA8, is upregulated from embryonic day 12.5 in just left gonad of female. *RALDH2 (*Retinaldehyde dehydrogenase type 2) is expressed specifically in the left gonadal cortex region at that time of *STRA8* During.this time, CYP26B1 is expressed in both sexes, however not in the outer cortex region of left gonad in female. This phenomenon suggests that conserved molecules of meiosis may also regulate the induction of meiosis in chicken as in mouse. And also, meiosis was induced by the addition of exogenous RA and inhibition of meiosis by Raldh2 shRNA interference induces retardation of expression of meiosis marker genes(Yu et al., 2013).

2.3 Cyclin and CDKs

The cell cycle progression consists of four phases responsible for the replication and transmission of genetic material to daughter cells. S phase and M phase are the main periods where chromosome replication and chromosome transmission occur, respectively. On the other hand, G1 and G2 phase are gap phases, which temporally separate S from M phase.

To maintain genetic stability during the process of generating new cells, the key cellular machinery controlling progression of cell cycle have to guarantee that chromosomal DNA is precisely duplicated, repaired and segregated to the new cell production. The critical regulator of cell cycle, Cyclins, functions as regulators the activity of their Cyclin dependent kinases (Cdks) partners and also modulate their substrate specificity. Cdks contain a serine/threonine-specific catalytic core and correlate with cyclins, which

control kinase activity and substrate specificity.

D-type Cyclins

The three mammalian D type cyclins, D1, D2 and D3, govern the progression of cells from G1 to S-phase. Besides, in addition to their role in proliferation, the D type cyclins have been implicated including differentiation and apoptosis(Satyanarayana et al., 2009). The D-type cyclins bind either Cdk4 or Cdk6 and regulate the transition from G0 to the G1 phase. Cyclin D-Cdk complexes phosphorylate pRb bound to E2F, leading to the release of active E2F which activates transcrtipion of genes needed for passage into s phase, including genes encoding cyclin E and A, Cdk2, and itself(Wolgemuth et al., 2013).

E-type Cyclins

There are also two members of the E-type cyclin family in mammals, cyclun E1 and Cyclin E2. After activation of D-type cyclins during G0 to G1 progression, the cyclin D-Cdk4/6 complexes phosphorylate the retinoblastoma protein (pRb) and the pRb-related proteins p107 and p130. When pRb is phosphorylated, its interaction with the transcription factor E2F is abolished. The free E2F stimulates cyclin E synthesis. Cyclin E then binds to Cdk2 to form cyclin E-Cdk2 complex and together with cyclin D-Cdk4/6, increase pRb phosphorylation, liberating additional amounts of E2F sufficient to induce entry into S phase of the cycle.

A-type Cyclins

The mitotic A-type cyclin is synthesized at the onset of S-phase and binds and activates Cdk2, like E-type cyclin, thus inducing progression through S-phase via phosphorylation of proteins involved in DNA replication. During the G2/M transition, cyclun A2 complexes with Cdk1, prior to B-type cyclin expression.

Cyclin A2 is ubiquitously expressed in a broad variety of tissue and cells in the adult mouse and during embryogenesis(Sweeney et al., 1996). However, cyclin A1 is restricted in only male germline(Ravnik et al., 1996) and expressed specifically in pachytene and diplotene spermatocytes at both the mRNA and protein levels(Liu et al., 1998). In female, cyclin A2 being expressed in both granulose cells and oocytes in a developmentally regulated manner (Ravnik et al., 1999).

B-type Cyclins

B-type cyclins have been identified in broad of species and organisms. In mouse, about nine B1-related sequences in their genome sequence(Hanley-Hyde et al., 1992). The functions are not understood clearly, but in general, the B-type cyclins appear during G2/M phase transition of the cell cycle and are critical for activating the major M-phase kinase Cdk1(Satyanarayana et al., 2009). They form part of Mitosis Promoting Factor, MPF, which regulate processes such as nuclear envelope fragmentation and spindle assembly. In the adult testis, *Ccnb2* was present at highest leels in the meiotically dividing

spermatocytes. On the other hand, *Ccnb1* transcripts were most abundant in the post meiotic spermatids. (Chapman et al., 1992). These study suggests that a functions for cyclin B1 which does not involve in mitosis; rather, cyclin B1 could be involved in germ cell differentiation.

CHAPTER 3.

Sex-Dimorphic Proliferation Profile of Germ Cells during Embryonic Development in Chicken.

1. Introduction

Germ cell development and differentiation pathway during embryogenesis is crucial mechanisms for producing the gametes from its precursors, and proper regulations mediated by intrinsic and external factors guide the gametes function normally (Richardson & Lehmann, 2010). Early germ cell arrest during embryogenesis, one of the regulation phenomenon, has been considered for reducing mutation on genome, allowing recombination to repair genetic damage and alleviating conflicts between gametes (Mira, 1998). Not only germ cell arrest in embryogenesis, but also specification, proliferation, arrest and resumption of cell cycle are tightly linked and coordinated each other for early zygotic process (Lesch & Page, 2012).

The origin, proliferation, arrest and resumption of cell division of early germ cells vary among the animal species. In mice, about 10 PGC precursors from proximal epiblast at 6.25 dpc migrate through tissues and colonize the gonadal ridge from 10.5 to 11.5 dpc (Byskov, 1986; Lawson et al, 1999). After settle in gonadal ridge, male germ cells last short proliferation up to 27,000 cells and enter G1/G0 arrest from 12.5 dpc to 14.5 dpc (Western et al, 2008). Subsequently, part of PGCs resume cell proliferation around day 5 postpartum (P5) and some part of PGCs recruited for spermatogonial stem cells (SSCs) (Yoshida, 2010). While female germ cells continue to proliferate up to about 25,000 cells and enter meiotic arrest from 13.5 dpc in an ovary (Borum, 1961). Meiosis of female germ cells arrested in diplotene stage of prophase I is completed subsequent hormonal stimulation and fertilization

(Hilscher et al, 1974). However, some scientists provided some experimental proofs that female mouse possess mitotically active germ cells in juvenile and adult ovary, oogonial stem cells(OSCs) since 2004 (Johnson et al, 2004). From this perspective, OSCs is considered as the capacity for differentiate into functional oocyte and generating the offspring (Zou et al, 2009). In drosophila, early germ cell development is distinguished from mammals. Initially, the pole cells containing "maternally inherited germ determinants" divide several times to about 40 cells at the posterior tip of the preblastoderm embryo (Sonnenblick, 1941). Subsequently, the pole cells migrate to dorsal mesoderm and form embryonic gonad by aggregating with somatic gonadal precursors at stage 12-14 (Boyle et al, 1997). The cells enter G2 arrest at stage 7-15 due to repression of Cyclin B production and the cells undergo mitosis in 16 h after egg-laying (AEL) (Williamson & Lehmann, 1996). In male, PGC division is resumed at the early stage of gonad development (Wawersik et al, 2005). And in female, about 12 PGCs are located in gonad of the first larva and proliferate to about 100 at the late third larval instar (Gilboa & Lehmann, 2006). About 30 PGCs associated with precursors of cap cells is going to differentiated to GSCs (Zhu & Xie, 2003)

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Compared to mammals and insects, the physiology and development of germ cells is unique in avian species. In the case of chicken, about 30 PGCs exist in area pellucida at stage X, and migrate to the germinal crescent at stage 4 (E0.5) in which 200~250 cells are present (Tsunekawa et al, 2000). Between stages 10 and 12 (around E2.0), PGCs start to move into blood stream (Ando & Fujimoto, 1983; Ukeshima et al, 1991), migrate through vascular system and finally settle in the genital ridges (Hamburger &

Hamilton, 1951; Meyer, 1964). After finished migration, more than 1,000 PGCs were found at stage 31 (E7.0) (Zaccanti et al, 1990). From E6.0, gonadal sex differentiation occurs based on genetic determination of sex chromosomes, Z and W. Also, PGCs differentiate into spermatogonia in male and oogonia in female and after E13.0 and E8.0, respectively (Aramaki et al, 2007; Nakamura et al, 2007). Male germ cells undergo mitotic arrest that means stopping proliferation and remain at the G1 stage of the cell cycle until hatching (Smith et al, 2008). By contrast, in female chicken, oogonia proliferate dramatically at E9.0, but after E15.5, oogonia start entering into meiosis and arrest at prophase I, so called meiotic arrest, upon E16.5 (Hughes, 1963; Smith et al, 2008).

However, still, direct evidences of mitotic arrest and meiotic arrest as well as proliferation profiling after sexual differentiation of chicken germ cells have not been reported yet. Therefore in this study, we investigated the sex-dimorphic proliferation profile of germ cells during chicken embryonic development using germ cell countings and flow cytometry analysis. And furthermore, we performed the immunohistochemistry analysis for identifying the existence of proliferating germ cell in developing embryonic gonads

2. Material & Method

Experimental Animal

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

5-Ethynyl-2'-deoxyuridine incorporation

To examine proliferative the proliferation status of germ cells, we injected 10ul of 10mM EdU in Dimethylsulfoxide(DMSO)(Sigma) at E.10 - E.18 into the extra-embryonic blood vessels below the air cell after removal of egg shell and shell membrane carefully. In hatched chick, we injected 10 ul of 0.4mg EdU in DMSO into bloods vessel using insulin syringe. After injection, the eggs were sealed with Parafilm and incubated at 37.5℃ for 4 hours.

Preparation of embryos and germ cells

The egg was incubated at 37.5 ℃ in an air atmosphere with 60 – 70 % humidity. The germ cells were collected after sex determination on E.10, 12, 14, 16, 18 and at 1 day(hatching). We dissected the gonad from embryonic

body with sharp twizzers under microscopy. At E.10, 12 and 14, gonadal tissues were chopped with small scissors and dissociated by gentle pippeting in 0.05% (v:v) trypsin solution supplemented with 0.53 mM EDTA. In E. 16, 18 and hatching testis, tissues were chopped with small scissors and incubated with 0.5 % Collagenase Ⅳ (Sigma) at 37 ℃ in 250 rpm shaking incubator during 10 min. After incubation, dissociation procedure perfomed in 0.05% (v:v) trypsin solution supplemented with 0.53 mM EDTA. The dissociated gonadal cells were washed once in PBS and fixed with 70 % EtOH for O/N at -20 ℃. The fixed gonadal cells were washed once with PBS and incubated with blocking solution for 1 hr at 4℃. Then gonadal cells were incubated with chicken germ cell specific primary antibody, rabbit Chicken VASA Homolog(CVH) IgG, for 1 hr at 4 ℃. After 3 times washing with 0.05 % Tween 20 in PBS, goat anti rabbit Alexa Fluor 488(Life Technologies) treated with germ cells as secondary antibody diluted 1:500 in 0.05 % Tween 20 in PBS for 1 hr at room temperature. After incubation, the treated cells were washed three times in 0.05 % Tween 20 in PBS and dissociated in 1 % Bovine Albumen Serum(Sigma) in PBS.

Immunohistochemistry

Embryonic gonads were dissected from E.10 – E.18 embryos and hatched chicks. Then, dissected gonad samples were washed once in PBS and fixed in 4% PFA for overnight and washed in PBS for 5 min. Then the gonads immersed in 5% sucrose in PBS, 30% sucrose in PBS and 30 % sucrose in

TBS for overnight at 4℃ to avoid crystal formation when freezing tissues. The immersed gonads were embedded in O.C.T. compound and frozen on Liquid Nitrogen. Frozen tissues were cut at 5 ㎛ under -28℃. Cutting sections dried on 55 ℃ slide warmer for 20 min, and rinsed in PBS for 20 min at room temperature. To detect incorporated EdU, sections were stained for Click-iT EdU detection kit according to the manufacturer's protocols. Then sections were incubated in blocking solution, which was consist of 2 % Normal Goat Serum and 0.2 % Triton X – 100 and 1 % DMSO in PBS, for 1 hour at room temperature. After blocking, the sections were incubated with rabbit CVH IgG antibody to detect germ cells overnight at 4 ℃. The slides were then treated with secondary antibody, goat anti rabbit Alexa Fluor 488(Life Technologies), for 1 hour at RT. The cells were kept in the dark condition to protect light during incubation. Sections were then mounted with Prolong Gold anti-fade reagent with 4',6- diamidino-2-phenylindole (Invitrogen) and visualized using fluorescence microscopy.

Flow cytometry

For analysis of cell cycle, chicken germ cells from total gonadal cells were isolated by immuno reactivity with rabbit CVH IgG detected goat goat anti rabbit Alexa Fluor 488(Life Technologies) by FACSAria Ⅲ. Auto fluorescence was removed by gating using by just secondary antibody treatment cells. Isolated germ cells was sorted and collected in PBS and were treated with 10ug/mL RNase A(Invitrogen) for 30 min at 37 ℃ and 50ug/mL

propidium iodide(Sigma) for 30 min at 4 ℃. Cell cycle status analyzed by FACSCalibur and data were analyzed using FACSDiva and Modfit LT cell cycle analysis software.

Germ cell number counting

The whole gonadal cells in a embryo were dissociated into single cells following above protocol and counted on the hematocytometer. Next, the whole gonadal cells were immunostained with anti-CVH antibody. The stained images were captured on microscope, and 10 photographs were taken randomly. The germ cell population percentages per whole gonadal cells were estimated by counting the CVH positive cells per DAPI positive cells. After estimation of germ cell population percentages in a embryo, the germ cell numbers in a embryo were estimated. The three replications were performed and statistical analysis was performed byANOVA.

3. Results

Distinctive increasing patterns of germ cell number between sexes during embryonic development

To identity proliferation profiles, the number of germ cells in both sexes during embryonic development was calculated. In male, the total number of whole gonadal cells in an embryo gradually increased until hatch (Fig. 1A and 1B). Especially, drastic increasing of cell number at E.16 and hatch were observed. On the other hand, the proportions of germ cells labeled with CVH antibody were maintained similarly from E.10 to E.14 and decreased from E.16 to hatch (Fig. 1A). According to estimated data, the number of germ cells slightly increased from E.10 to E.12 and displayed no more significant increasing from E.14 (Fig. 1A and 1C), indicating that male germ cells enter the mitotic arrest at this timing. In female, the total number of whole gonadal cells in an embryo also gradually increased until hatch (Fig. 2A and 2B). Also, the number of germ cells increased drastically about 2.5 folds from E.10 to 14. Meiosis in female chicken has been known to occur in the cortex of left ovary from E. 15.5. However, consistent increasing of female germ cells from embryonic day 16 to hatch was observed, indicating that embryonic ovary possesses mitotic cells as well as meiotic cells after induction of meiotic signals. Taken together, above results indicate that male germ cells enter the mitotic arrest from E. 14 and female germ cells last the proliferation even after onset of meiosis.

At E.6 and 8, the number of male germ cells was upper compared with female germ cells. However, female germ cells were increased their numbers excessively from E.10 and displayed gradual increasing patterns during embryonic development. On the other hand, male germ cells last very low proliferation from E.10. Subsequently, female germ cells showed higher number than male germ cells at hatch (Fig. 2A and 2C).

Sex-dependent proliferation status in chicken germ cells during embryonic development

To identify whether chicken germ cells possess the difference in cell cycle status between two sexes, cell cycle analysis by flow cytometry. As a result, germ cells of different two sexes showed different pattern of cell cycle changes (Fig. 3 and 4). Male germ cells had high proportion in G0/G1 phase and low S/G2 phase continuously during overall developmental stages (Fig. 3A). At E.10 and 12, percentage of male germ cells that were accumulated in G0/G1 phase was 79.76 ± 3.16 and 80.08 ± 3.35, respectively (Fig. 3B). The proportion of germ cells in S phase was 13.92 ± 1.63 and 12.54 ± 1.46, and G2 phase was 6.63 ± 2.93 and 7.15 ± 2.22, respectively. At E.14, 16, and 18, proportion of male germ cells in G0/G1 phase showed a slight increase than those at E.10 and 12 (88.40±1.492, 89.10±5.35 and 88.21±1.36). The proportion of germ cells in proliferative phase (S/G2) at these stages showed a decrease of approximately 10 % than E.10 and 12 (11.6±1.50, 10.89±5.35 and 11.13±1.76). Upon hatch, most of the germ cells were accumulated in G0/G1 phase (96.05±3.11) (Fig. 3B). Subsequently, the G1 phase of chicken

male germ cells is increased in gradual patterns from E.10 to hatch.

In female, E.16 is known as entry timing into meiosis showing various meiotic characteristics such as morphological differences and expression of *STRA8, cSYCP3* (Hughes, 1963; Smith et al, 2008; Zheng et al, 2009). Before onset of meiosis, a small number of germ cells were accumulated in G0/G1 phase compared to males (Fig. 4A and 4B). At E.10, 12, and 14, the female embryonic germ cells showed the proportion of 57.05 ± 9.59, 67.73 ± 1.74, and 71.59 ± 3.02 in G1 phase, respectively (Fig. 4B). From E.16, the timing of onset of meiosis in female germ cells, the proportion in G1 phase decreased drastically. At E.16 and 18, the population of G0/G1 phase was decreased from 63.17±8.67 to 45.8±10.37 and G2 phase were increased from 23.56±8.74 to 48.51±9.38. At hatch, 80.19±4.90 % of germ cells was accumulated in G2 phase (Fig. 4B). This data indicates that some female germ cells possessing 2N contents are existed in ovary since appearing the meiotic wave at hatch. Above results revealed that chicken germ cells display distinguished patterns between sexes from E.10 to hatch while differentiating into spermatogonia in male or oogonia and oocyte in female.

Chicken germ cells continue DNA replication in dimorphic patterns by sex during embryonic development

Above data demonstrate that chicken germ cells have distinctive differences in proliferation and cell cycle status and between two sexes. Next, further define DNA replication rate in germ cells, EdU incorporation analysis was performed. EdU has been known as a molecule that

incorporates into cells during S Phase. EdU was injected into embryonic blood vessels 4 hour before sampling, and gonads were sectioned and immunostained to visualize the S phase germ cells. In males, small number of germ cells continued DNA replication from E.10 and further decreasing of DNA replication of germ cells were showed from E.14, the timing of most male germ cells entering G0 arrest (Fig. 5). In female, more than number of germ cells continues DNA replication compared with male during overall embryonic development. Especially, we observed that quite a few female germ cells last DNA replication from E.16 to hatch, the timing of meiotic progression.

Based of morphological dynamics, we showed that DNA-replicating male germ cells were present singly in embryonic gonads (Fig. 5).. Also, we showed that female germ cells showed formation of cysts during DNA replication (Fig. 6). The cyst is considered as grouping germ cells and sharing some organells and same cell cycle prior to meiotic progression(de Cuevas et al, 1997; Pepling & Spradling, 1998). The DNA-replicating female germ cells resided in the cysts were detected from embryonic day 11 to hatch. Approximately 3 to 7 germ cells sharing same cell cycle were gathered in forming cyst (Fig. 6). Collectively, chicken germ cells last DNA replication asynchronously in sex-dimorphic patterns showing morphological differences during differentiation.

A

	E.6	E.8	E.10	E.12	E.14	E.16	E.18	HATCH
Total gonadal cell number	126400± 7760	366233± 30435	1243000± 33808	1540000± 14730	1634333± 47815	3160000± 206639	3880000± 60828	5796666± 295014
Germ cell portion (%)[a]	1.03±0.1298	1.596±0.611	1.241±0.304	1.038±0.315	1.067±0.159	0.557±0.127	0.457±0.104	0.303±0.099
Estimated germ cell number[b]	1301±75	5782±486	15429±420	16289±153	17438±510	17589±515	17629±276	17450±894

[a] Germ cell portion = SSEA-1 or CVH-positive cell number / whole gonadal cell number
[b] Estimated germ cell number = Whole gonadal cell number * SSEA-1 or CVH-positive cell percentage

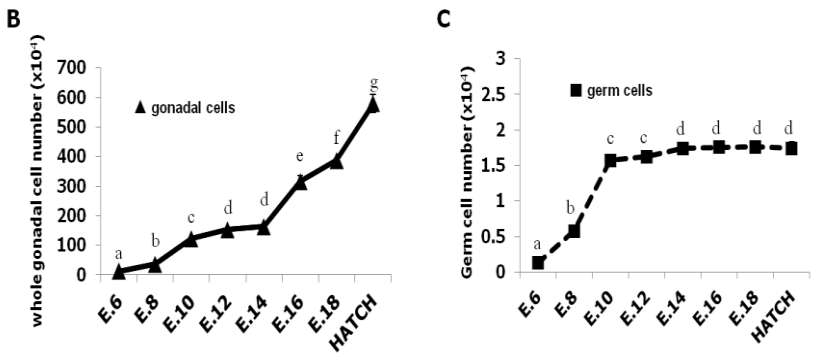


Figure 1. Changes in number of male germ cells during embryonic development. Number of SSEA-1 or CVH-positive cells among whole gonadal cells in the tissue sections was counted to estimate germ cell number during sex differentiation (**A**). Based on the estimation, number of germ cells and whole gonadal cells was represented at E.6–hatch (**B**). Whole gonadal cells increased continuously during embryo development. Male germ cells did not show drastic increase of number from E.16, indicating mitotic arrest. Data represent mean ± SD of replications. Different letters indicate significant differences at $P < 0.05$.

A

	E.6	E.8	E.10	E.12	E.14	E.16	E.18	HATCH
Total gonadal cell number	108300± 7143	311033± 3523	832666± 46715	1393333± 15275	1746667± 49328	2433333± 100166	2663333± 83266	3143333± 149778
Germ cell portion (%)[a]	1.03±0.12	1.203±0.10	2.928±0.94	4.45±0.17	4.20±0.95	3.04±0.58	3.15±1.93	2.82
Estimated germ cell number[b]	1124±83	3744±105	24388±1368	62623±686	73440±2074	74138±3051	83766±2618	88763

[a] Germ cell portion = SSEA-1 or CVH-positive cell number / whole gonadal cell number
[b] Estimated germ cell number = Whole gonadal cell number * SSEA-1 or CVH-positive cell percentage

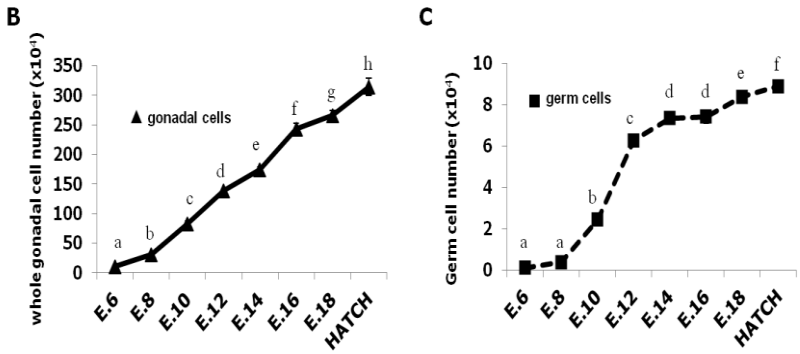
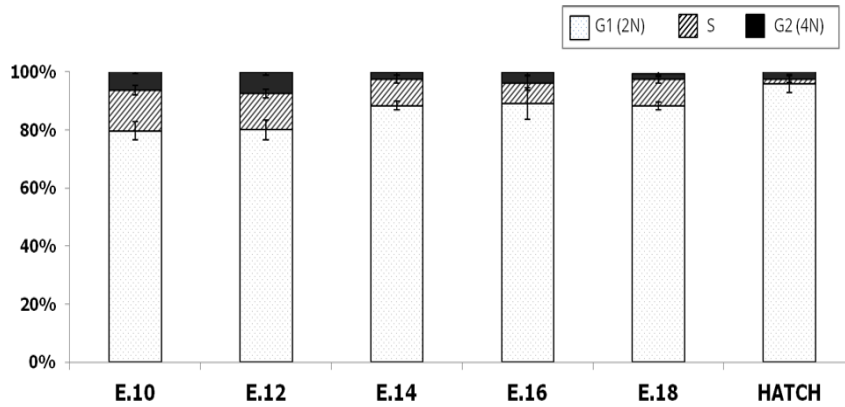


Figure 2. Changes in number of female germ cells during embryonic development. Number of SSEA-1 or CVH-positive cells among whole gonadal cells in the tissue sections was counted to estimate germ cell number during sex differentiation (**A**). Based on the estimation, number of germ cells and whole gonadal cells was represented at E.6–hatch (**B**). Whole gonadal cells increased continuously during embryo development. Female germ cells continued the mitosis after onset of meiosis (at around E.16). Data represent mean ± SD of 10 replications. Different letters indicate significant differences at $P < 0.05$.

A

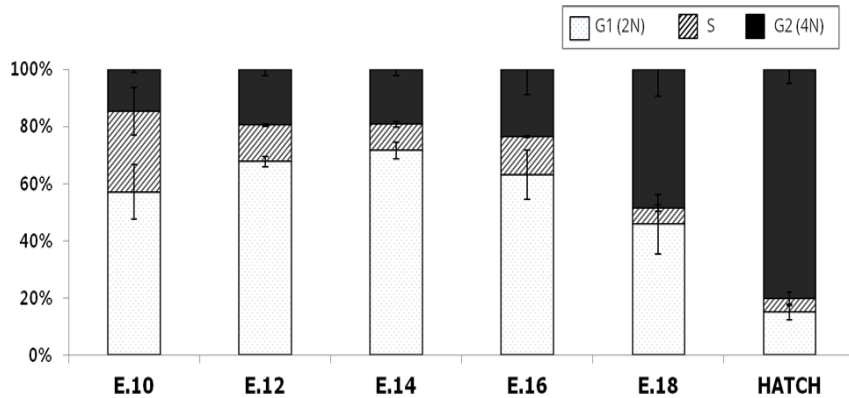


B

	E.10	E.12	E.14	E.16	E.18	HATCH
G0/G1	79.76±3.16	80.08±3.35	88.40±1.49	89.10±5.35	88.22±1.36	96.05±3.11
S	13.92±1.63	12.54±1.46	9.08±1.39	7.01±2.50	9.32±1.24	1.48±1.00
G2/M	6.63±2.93	7.15±2.22	2.52±0.11	3.88±3.31	1.81±0.95	3.70±2.85

Figure 3. Proliferation profiles of male embryonic germ cells based on cell cycle phase during embryonic development. Proliferation status was analyzed based on DNA content assessed by propidium iodide staining in CVH-positive germ cells. (**A**) Representation of cell cycle distribution of chicken male germ cells during sex differentiation. Each block indicates the percentage of cells within different cell cycle phases (G0/G1; dotted, S; striped, G2; black). Bars indicate SD of the mean of triplicate analyses. (**B**) Percentage values of cells in each cell cycle stage analysis of embryonic male germ cells at E.10–hatch. Data represent mean ± SD.

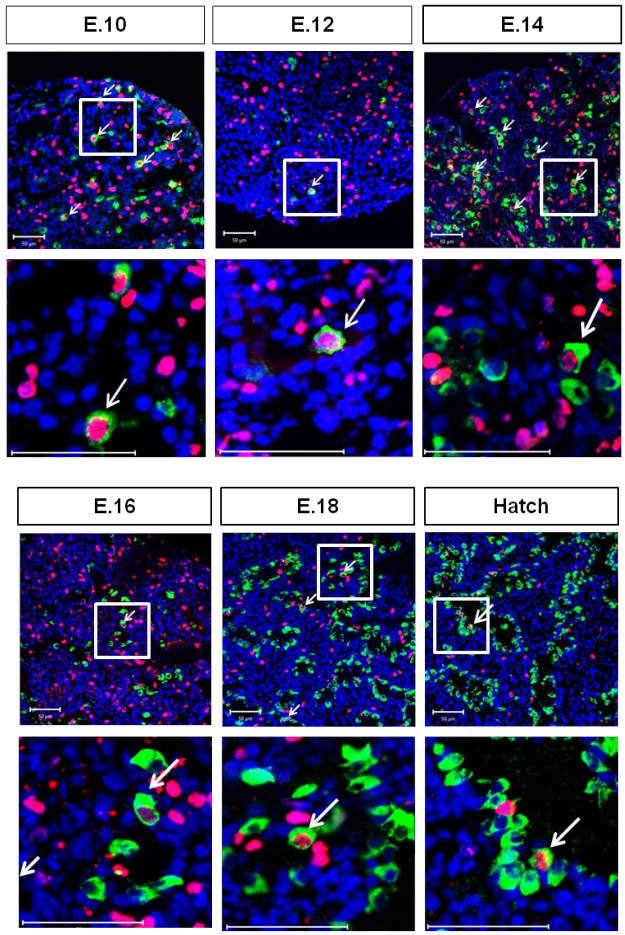
A



B

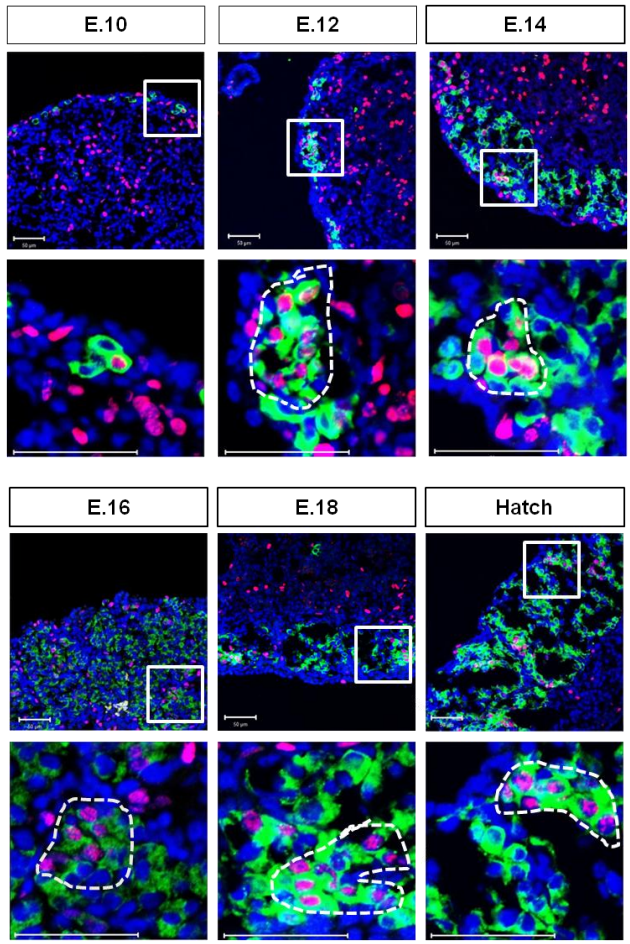
	E.10	E.12	E.14	E.16	E.18	HATCH
G0/G1	57.05±9.59	67.73±1.74	71.59±3.02	63.17±8.67	45.8±10.37	15.14±2.79
S	28.24±8.37	12.73±0.48	9.19±0.94	13.27±0.26	5.69±1.02	4.67±2.34
G2/M	14.72±1.22	19.23±2.23	19.23±2.07	23.56±8.74	48.51±9.38	80.19±4.90

Figure 4. Proliferation profiles of female embryonic germ cells based on cell cycle phase during embryonic development. Proliferation status was analyzed based on DNA content assessed by propidium iodide staining in CVH-positive germ cells. (**A**) Representation of cell cycle distribution of chicken female germ cells during sex differentiation. Each block indicates the percentage of cells within different cell cycle phases (G0/G1; dotted, S; striped, G2; black). Bars indicate SD of the mean of triplicate analyses. (**B**) Percentage values of cells in each cell cycle stage analysis of embryonic male germ cells at E.10–hatch. Data represent mean ± SD of triplicate analyses.



DAPI; CVH; EdU

Figure 5. Asynchronous entry of mitotic arrest in male germ cells during embryonic development. To identify the proliferative embryonic germ cells during sex differentiation, gonads were isolated at E.10, 12, 14, 16, 18, and hatch (4 hours after EdU incorporation) and sectioned for staining with anti-CVH and EdU. Arrows indicate proliferating male germ cells that were present singly. The boxed regions in upper panels were magnified in lower panels. Scale bars = 50 ㎛.



DAPI; CVH; EdU

Figure 6. Continued DNA synthesizing of female germ cells via cysts formation after onset of meiosis during embryonic development. To identify the proliferative embryonic germ cells during sex differentiation, gonads were isolated at E.10, 12, 14, 16, 18, and hatch (4 hours after EdU incorporation) and sectioned for staining with anti-CVH and EdU. Arrows indicate proliferating female germ cells that formed cysts. The boxed regions in upper panels were magnified in lower panels. Scale bars = 50 ㎛.

4. Discussion

Germ cells originate from PGCs and commit the differentiation pathway to play their genuine roles. During this differentiation pathway, strict controls of cell cycle events, such as proliferation and meiosis induction, are required at proper timing to establish the formation of functional gametes. Under aberrant regulations, germ cells undergo excessive apoptosis, formation of testicular germ cell tumor, or infertility (Jorgensen et al, 2013). To our knowledge, there is still no study on proliferation profile of germ cells comparing male and female in birds. In this study, we performed the studies on proliferation of germ cells from onset of gonadal differentiation to germ cell differentiation during embryonic development. From our results, it is suggested that chicken germ cells continue proliferation in sex dimorphic patterns during germ cell differentiation. Based on the results in this study, chicken germ cell possess the distinctive proliferation characteristics comparable with other species.

In the overall context, chicken germ cells displayed different proliferation patterns dependent of sex from E.10. During embryonic development from E.10 to 14, male germ cells constantly exhibited high G1 proportion, about 79.76 % - 88.40 %, while female germ cells displayed relatively low G1 proportion, 57.05 % - 71.59 %. These results reveal that there is difference in proliferation activity between sexes prior to meiosis initiation. In contrast, in murine germ cells, the cell cycle changes of each sex occurred in similar patterns prior to onset of meiosis (Western et al, 2008). In this regard, although several molecular

mechanisms are conserved between two species, it is suggested that timing of action and extent of regulation of them are quite different.

In mammal, mitotic arrest of germ cells takes place in male, at the timing of onset of meiosis in female. In mouse, male germ cells pause the mitotic division from E.13.5 (Tam & Snow, 1981), displaying different morphological characteristics compared to female germ cells displaying meiotic morphological changes (Adams & McLaren, 2002). Western and colleagues reported that significantly higher proportion of the germ cell is accumulated in G0/G1 phase during mitotic arrest. (Western et al, 2008). Also, the G1-S phase proteins are strongly up-regulated during this time. In chicken, several previous studies have reported that chicken germ cell development has conserved mechanisms with mammals regarding timeline and molecular expressions (Lee et al, 2010; Smith et al, 2008; Yu et al, 2013; Zheng et al, 2009). Although some studies have focused on the mitotic arrest of male germ cells in chick (Mendez et al, 2005; Zheng et al, 2009), it still remains controversial and is not fully studied. In this study, we found that most male germ cells enter the G0/G1 phase at around E.14 with displaying reduced proportion in S phase and without increase of germ cell number. Considering the definition of mitotic arrest as studied in mammals, this change can be considered as mitotic arrest in chicken. However, the mitotic arrest of male germ cells in chicken display several distinguished patterns with mouse. First, we found that small number of male germ cells still undergoes DNA synthesizing even after E.14. In mouse, however, germ cell differentiation of both sexes, such as meiosis in female and mitotic arrest in male, is taken place in synchronized pattern

via molecular signaling changes in gonadal environment (Bullejos & Koopman, 2001; Byskov & Saxen, 1976; Koopman). These results indicate that chicken male germ cells take mitotic arrest in an asynchronous way. Also, chicken male germ cells displayed low proliferation rate from E.10 and the proliferation status were decreased in gradual pattern. In mouse, the proliferation changes into first meiotic prophase or mitotic arrest are occurred drastically during 24 – 48 hours.(Miles et al, 2010; Western et al, 2008).

Initiation of meiosis is the most important process of female germ cells to differentiate functional gametes. In mouse, female germ cells progress differentiation with meiosis wave at E.14.5 during 24-48 hours (Bullejos & Koopman, 2004; Menke et al, 2003; Western et al, 2008). In contrast, chicken female germ cells undergo meiosis during E.14-18, relatively long time compared to mouse (Hughes, 1963). Also, it was reported that the number of female germ cells last proliferation and keeps cell number increasing until E.17 (Erickson, 1974). In our study, chicken female germ cells last their proliferation after the onset of meiosis and up to hatch. Also, DNA replicating germ cells are observed after onset of meiotic progression. In similar context, we found that the cysts are existed in ovary after the onset of meiosis and up to hatch. Cyst is the group of cells that are formed from a found cell throughout synchronous mitotic division. In this cyst, cells are linked by intercellular bridges by which the some organelles and mRNA are shared. The cysts always appeared prior to onset of meiosis, and disruption of cyst formation affects in oocyte differentiation and sex determination (Hawkins et al, 1996; Pauli & Mahowald, 1990). The cyst formation of germ cells was discovered in

various species including lower insect, high insect, *Xenopus*, mammals and etc (Buning & Sohst, 1988; Gondos & Zamboni, 1969; Klymkowsky & Karnovsky, 1994; Pepling & Spradling, 1998; Ruby et al, 1969)..

In insect, both male and female undergo meiosis during overall adult life. So, the cysts in these species are observed during whole their life (de Cuevas et al, 1997). In contrast, vertebrate female germ cells undergo meiosis during pre-adult life. And the cysts that appear during early development disappear in adult ovary. For example, mouse germ cells are connected within cysts in the ovaries from E.11.5, and the size and number of the cysts are peaked at E.12.5 to 13.5 (Pepling & Spradling, 1998). The number of meiotic germ cells and related molecules are up-regulated E.13.5 (Bowles et al, 2006; Koubova et al, 2006; Kumar et al, 2011; McLaren, 1984). After most female germ cells entering the meiosis, the cysts disappear (Pepling & Spradling, 1998). The onset of meiosis in female chick is known to start at E.15.5 with up-regulation of meiotic gene level and morphological changes. In this study, however, the cysts were found from E.12 to hatch in chicken embryonic ovary. Taken together these observations, it is supposed that chicken embryonic ovary possess two types of germ cells undergoing mitosis or meiosis. The reason of these observations could be postulated 2 hypothesis; meiotic wave in chicken occurs asynchronously similar in male or the OSCs in existed in chick's ovary. In reproductive biology, germline stem cells (GSCs) are possessed in lower vertebrates and female invertebrates, males in mammal (Spradling et al, 2011). However, Johnson and colleagues reported that some female germ cells in juvenile and adult mouse continue the mitotic division and this germ cells can differentiate into follicles in postnatal mouse ovary

(Johnson et al, 2004). Subsequently, the isolation and culture conditions of OSCs were established and offspring were produced from cultured OSC line from neonatal ovaries (Hu et al, 2012; Zou et al, 2011; Zou et al, 2009). If the mitosis undergoing female germ cells continues the mitosis after hatch, it is assumed the existence of OSCs in chicken. To elucidate this hypothesis, the experimental method for tracing the germ cell fate during oogenesis.

In mouse, before the onset of entry in mitotic arrest or meiotic progression about at 13.5 dpc, female possess about 22,000 germ cells and male possess about 27,000 germ cells (Tam & Snow, 1981). On the other hand, our results indicate that, in chicken embryo, female possess about 73,000 germ cells and male possess about 17,000 germ cells in an embryo at E.14, that shows remarkable difference between sexes compared to mouse. Taken together, the germ cell population in mouse prior to sex differentiation displays similar level in each sex, while there is a gap between male and female germ cell populations before E.14 in chicken (before the timing of mitotic arrest in male or meiotic progression in female).

During sex differentiation, mouse and chicken has many conserved mechanisms. For example, sex differentiation initiates during embryonic development while onset of occurs meiosis only in female but not in male during embryonic phase. And a lot of genes involved in gonadal differentiation are conserved between both species functionally and structurally. In this context, it is thought that the cell cycle event of chicken embryonic germ cells during sex differentiation is similar to mouse germ cells. However, several crucial features, such as increasing pattern of germ

cell number, and the timing of mitotic arrest and meiosis, make it possible to look inside the difference between two species. In mouse, germ cells enter the genital ridge area between 10.5 and 11.5 dpc, and primordial germ cells proliferate dynamically entering the genital ridge during about 2 days (Byskov, 1986; Tam & Snow, 1981). At 13.5, germ cells in the mouse initiate progression into distinctive differentiation way depend on sex (McLaren, 2001). In chicken, however, germ cells migrate and colonize into embryonic gonad at about E.3~E.4.5 (HH stage 20~26) (Hamburger & Hamilton, 1951; Meyer, 1964). Compared to mouse, chicken takes a long time to initiate cell cycle changes depend on sex after settlement of primordial germ cells. Thus, the asynchronous and delayed germ cell differentiation pathway including proliferation, mitotic arrest in male, and meiotic progression in female in chicken is distinguished from mammalian one; synchronous and speedy differentiation pathway.

In this study, we report that the chicken germ cells proliferation profiles based on germ cells number, DNA contents, and visualization of DNA synthesizing germ cells. From these results, we suggest that chicken possess the both conserved mechanisms and distinguished patterns during germ cell development. Also, the establishment of germ cell population by proliferation and differentiation is triggered in asynchronous way in both male and female gonad. To understand this unique germ cell development in chick embryos, further research will focus on discovering the molecular signaling network causing the asynchronous germ cell development in their gonad.

CHAPTER 4.

GENERAL DISCUSSION

Germ cell is the only cells undergoing two types of cell cycle changes during lifetime. This cell cycle changes is the process that is necessary to halve the number of chromosomes. The cell cycle change of PGCs indicates the initiation of germ cell differentiation leading to the highly specialized gametes, oocytes or sperm (McLaren, 1988). The first sex dimorphic cell cycle change take place in embryogenesis in mammal and bird; female enter the first meiotic division, while male germ cells enter the G0/G1 arrest(Hilscher, 1974; Hughes, 1963; Mendez et al., 2005; Speed, 1982). Throughout all stages of germ cell development, the germ cells have to possess the strict regulation of cell cycle changes. To trigger the appropriate sex-sepcific cell cycle states in germ cells, the somatic cells can induce appropriate apoptosis to maintain the DNA integrity in germ cells by somatic cell signaling(Petre-Lazar et al., 2007). In appropriate apoptosis fails in gonad, germ cells can from carcinoma in situ and testicular germ cell tumors(Bartkova et al., 2003). Despite the importance of regulation of cell cycle in germ cells during sex differentiation, the clear definition and mechanisms have not yet been investigated in chicken. So in this study, we try to elucidate the proliferation and cell cycle dynamics in chicken germ cells during sex differentiation. After that, we find that chicken possess the slow and asynchronous proliferation patterns in germ cells. Especially, we demonstrated that male germ cells continues proliferation in very low rate compared with other species and by sex. So, we try to elucidate the biological meaning of mitotic arrest based on data of part 3.

To examine the cell cycle changes in chicken germ cells during sex differentiation, we performed the comparison study of germ cells based on

DNA replication cell cycle and mitosis dynamics from onset of gonadal differentiation to germ cell differentiation. From these studies, we showed that chicken germ cells undergo cell cycle and mitosis dynamics in sex dimorphic pattern prior to germ cell differentiation. Also, it showed that chicken germ cells progress the sexual differentiation pathway slowly. It have been showed that female germ cells progress the cell cycle changes with high proliferation than male germ cells. And also, from embryonic day 16, the population of G2 phase were increased drastically. On the other hand, male germ cells continues the cell cycle changes with low proliferation phase during overall embryonic development. In addition, at embryonic day 14, almost germ cells enter the mitotic arrest.

In our research, the cell cycle changes based on DNA contents was investigated for the first time in chicken germ cells, and the clear determination of mitotic arrest was revealed. This study can contribute to the studies on relationship of cell cycle changes and sex differentiation in chicken germ cells.

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SUMMARY IN KOREAN

생식세포는 자신이 지닌 유전정보를 한세대로부터 다음세대로 전달할 수 있으며, 두번의 세포 주기 변화를 겪는 유일한 세포이다. 이런 세포 주기 변화는 염색체수를 이수체에서 반수체로 만들기 위하여 필수 적인 과정이다. 생식세포의 분화 과정 중 이 변화는 원시생식세포가 분화하면서 나타나는 최초의 성별 특이적 기작이며, 난자 혹은 정자와 같은 배우자 형성 생식세포로 필수적인 과정이다.

일반적으로 포유류와 조류에서는 성별 특이적인 세포 주기의 변화가 배아 발달 중 발생한다. 여성은 첫번째 감수분열을 배아 시기에 시작하고, 이때 남성은 세포 주기 G0/G1기에 세포 분열을 멈추게 된다. 이 변화가 적재적소에 발생하지 않을 경우, 생식세포는 세포사멸을 겪거나, 추후 암종의 발생을 야기한다.

이와 같이 세포 주기의 적절한 조절은 생식세포의 발달에 있어 중요한 메커니즘임에도 불구하고, 정확한 변화의 원인이나, 생물학적 의미에 대한 연구는 매우 미비하다. 따라서, 우리는 이 연구에서 닭 원시 생식세포 분화 과정 중에 나타나는 성특이적 세포주기의 변화를 실험적으로

첫째로 우리는, DNA contents, DNA 합성 생식세포의 시각화, 세포

수 증식 변화를 바탕으로 성 특이적인 세포 증식 및 세포 주기 변화를 확인하였다. 그 결과 전반적인 분화 과정 내내 웅성 및 자성생식세포는 구분되는 변화를 겪고 있었다. 웅성생식세포는 전반적으로 낮은 세포분열 변화를 보였고, 배아 일령 16일에 모든 세포들이 세포분열을 중지하였다. 그러나, 이후에도 몇몇 생식세포들은 DNA 합성을 계속하고 있는 것을 확인하였다. 한편, 자성생식세포는 웅성 생식세포에 비하여 전반적으로 활발하게 세포분열을 하고 있음을 확인하였으며, 감수분열이 시작되는 것으로 알려져 있는 배아일령 16일부터 4N contents를 지닌 생식세포가 급증하는 것을 알 수 있었다. 그러나, 자성생식세포 역시 감수분열이 시작된 이후에도 증식하는 세포를 확인하였다. 결과적으로, 생식세포 내 일괄적인 변화를 거쳐 분화 과정을 겪는 포유류와는 달리, 닭은 생식선 내에서 생식세포들이 비동시적으로 분화과정을 겪는 것을 알 수 있었다.

이 연구는, 닭 생식세포의 분화과정 중, 세포 증식 정도와 DNA contents를 기반하여 성특이적 세포주기의 변화를 확인함으로서 닭이 다른 종들과는 구분되는 메커니즘을 지니고 있다는 단서를 제공한다. 위와 같은 연구는 닭 생식세포의 세포 주기와 성분화에 관련된 연구에 기여 할 수 있을 것이다.