A Dissertation

For the Degree of Doctor of Philosophy

Studies on application of primordial germ cells for interspecies chimera and molecular characterization of NANOG in aves

이종간 조류 키메라 생산을 위한 생식세포의 활용 및 NANOG 분석을 통한 생식세포의 특성 규명

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SUMMARY

Avian is one of the excellent models for basic and applied research such as reproductive system and developmental biology. In particular, avian primordial germ cells (PGCs) have unique characteristics and accessibility compared to mammals during early embryo development. Thus, the production of germline chimera has been regarded as the most powerful tool via PGCs-mediated technology. In addition, a detailed understanding of germ cells has been emphasized for maintaining distinct properties in germ cell biology. In this regards, we achieved the combination of the unique migratory route of avian germ cells with an embryo replacement method could be applied to produce interspecies germline chimeras and developed potential knowledge for the conservation of wild and endangered birds through surrogate system. Furthermore, we investigated the structural characteristics of chicken NANOG (cNANOG) protein and its biological significance using biochemical methods and cell assay in PGCs. Also, we revealed the oviduct specific genes regulated by estrogen for potential therapeutic peptides or regulation of egg white protein secretion.

The first study was performed to investigate novel germ cell transfer system to produce interspecific germline chimeras without germ cell isolation, such as PGC culture or separation using magnetic activated cell sorting with specific antibodies. A highly efficient interspecies germline chimera production system was developed via the establishment of PGCs in an *in vitro* culture system or cell sorting with specific antibodies but, only chicken PGCs have been cultured successfully *in vitro* without loss of germ cell properties and separated cell sorting with specific antibodies. Thus, Alternative

methods to produce interspecies germline chimeras that are applicable to various avian species need to developed. We therefore the original chick embryo proper was replaced with a quail embryo onto the chick yolk to produce an interspecific germline chimera. Using this method, the chick PGCs were successfully transferred into the quail gonads after replacement the 5.5-day-old embryonic gonads of quail-chick chimeras. Furthermore, the genomic DNA from the gonads of quail-chick chimeras was positive for both the chicken-specific and quail-specific markers. These results suggest that this technique is also potentially applicable to the restoration and conservation of endangered bird species.

Using surrogate system, we also applied the conservation of wild and endangered birds. To conserve the soft-shelled egg of endangered wild birds, surrogate system has been applied. However, optimal culture conditions such as effectiveness of developmental timing of donor embryos on hatchability are still deficient. Therefore, in this study, we investigated optimal system for culture Korean Oge (KO) chicken embryos in White Leghorn (WL) surrogate chicken eggshells by transfer at different periods. Donor KO embryos at 3-day and 4-day-old were transferred into recipient WL eggshells, the hatchability of KO embryos transferred into surrogate eggshells at 3-day-old was 23.1%, whereas embryos transferred at 4-day-old was 36.0%. Furthermore, the development of all viable embryos from 3-day group and 4-day group were normal. These results suggest that this method can be useful for the restoration and conservation of endangered bird species.

For the next study, we investigated function of chicken NANOG (cNANOG) in PGCs and blastoderm cells. Functional

NANOG has been reported that dimerization of NANOG is mediated by tryptophan repeat (WR) motif in C-terminal domain and required for maintaining pluripotency. However, chicken NANOG lacks the WR motifs that are conserved in the CTD of mammalian NANOG. In this study, we investigated cNANOG self-associates via the disordered CTD that switches into a helix. We also discovered novel hydrophobic interaction motif for cNANOG self-association. In addition, the self-association of cNANOG is highly important to maintain the expression of pluripotency related *POUV* that enables the proliferation of cPGCs and blastoderm cells. These results indicate that cNANOG self-associates through its CTD via a novel folding-upon-binding mechanism is important to maintenance of PGCs and blastoderm cells.

Next, we investigated oviduct specific novel gene stimulated by estrogen to discover the potential therapeutic peptides or regulation of secreted egg white protein. Egg white protein secretion pathway has not been described yet. Using DES treatment, we discovered oviduct specific gene and this gene observed tubular gland cells and the epithelium cells in adults of oviductal magnum. Finally, to validate novel gene ORF translation, we inserted a N-terminal Flag-tag and confirmed the ORF translation in primary oviductal epithelial cells (OECs). Our results suggest that the oviduct specific novel gene can be related to regulate the egg-white protein secreted pathway.

Based on the researches, the system for interspecies germline chimera will be more optimized and applied various wild birds to

restore and conservation. Also, molecular mechanism of cNANOG will contribute to understand maintaining PGCs. Furthermore, oviduct specific novel gene could be applied to investigate the new biological compound.

Keywords: chicken, interspecies germline chimera, primordial germ cells, NANOG, oviduct

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LIST OF ABBREVIATIONS

AAA	AAA triple mutation
actRIIB	Activing receptor IIB
CASP1	Caspase-1
CASP2	Caspase-1
CD	Circular dichroism
cNANOG	Chicken NANOG protein
***cNANOG*-KD**	*cNANOG* knockdown
CTD	C-terminal domain
CVH	Chicken *vasa* homologue
DAZL	Deleted in azoospermia-like
DES	Diethylstilbestrol
E2	17beta-estradiol
ESCs	Embryonic stem cells
ER a	Estrogen receptor a
ER β	Estrogen receptor β
EGK	Eyal-Giladi and Kochav
FACS	Fluorescence-activated cell sorting
HD	Homeodomain
HH	Hamburger and Hamilton
HDAC	Histone deacetylase
IDPs	Intrinsically disordered protein
KO	Korean oge

MALS	Multi-angle light scattering
MACS	Magnetic-activated cell sorting
NANOG	Nanog homeobox
NTD	N-terminal domain
OCE	Oviductal epithelial cell
PGC	Primordial germ cells
PAS	Periodic acid-Schiff -1
RNP	Ribonucleoprotein
SDF-1/CXCR4	Chemokine stromal cell-derived factor 1
SDI	Size dependent isolation
SEC	Size exclusion chromatography
SSCs	Spermatogonial stem cells
WL	White Leghorn
WR	Tryptophan repeat

CHAPTER 1

GENERAL INTRODUCTION

Avian is one of the excellent models for basic and applied research such as reproductive system and developmental biology. In particular, avian primordial germ cells (PGCs) have unique characteristics and accessibility compared to mammals during early embryo development. Thus, the production of germline chimera has been regarded as the most powerful tool via PGCs-mediated technology. In addition, a detailed understanding of germ cells has been emphasized for maintaining distinct properties in germ cell biology.

The technique for producing germline chimera has been developed to facilitate a basic and applied research in birds, such as developmental biology, avian transgenesis and endangered species conservation. Avian germline chimeras can be produced by transplanting blastodermal cells (Petitte et al., 1990), primordial germ cells (PGCs) (Han et al., 2002) or testicular cells (Han et al., 2002). Among these, PGC-mediated system has been considered as a major technique for germline chimera production because of their high efficiency of germline transmission (Park et al., 2003). The unique circulatory of avian PGCs through the blood vessels has been widely attempted to manipulate germ cell-transfer systems as well as production of transgenic birds (Park et al., 2003, Naito et al., 2004, Kim et al., 2005, Kang et al., 2008, Park et al., 2008). Therefore, germline chimeras have been successfully produced by transfer of PGCs, which are collected from the donor embryonic blood vessels during HH stages 13 to 17 (Tajima et al., 1993, Chang et al., 1995) or donor embryonic gonads (Chang et al., 1995, Han et al., 2002), into the blood vessels of recipient embryos. In addition, interspecies germline chimeras can be used for conservation of endangered birds by germ-cell transfer system (Kang et al., 2008, Wernery et al., 2010). Endangered bird species are restricted in their populations and their low ability of egg production so, collecting and manipulating PGCs has been a difficulty in endangered embryos. Thus, it is necessary to develop alternative methods to produce germline chimeras that can be applied to various species of birds. The embryo transplantation technique in which a quail whole embryo is grafted onto the host chick embryonic yolk was

originally devised by Martin (Martin, 1972). Quail-chick chimeras could then be established by grafting a quail embryo body onto the chick extraembryonic area (Dieterlen-Lievre, 1975). We postulated that the combination of the unique migratory route of avian germ cells with an embryo replacement method could be applied to produce interspecies germline chimeras.

The Surrogate eggshell culture system has been investigated to study development and hatchability of avian species. However, there are several limitations such as viability and hatchability. In previous studies, quail embryos were successfully cultured until hatch in recipient chicken eggshells, but the percentage of hatchability was only 3% (Ono and Wakasugi, 1983). Similarly, chicken embryos were cultured in recipient turkey eggshells or chicken egg-shells, and the percentage of hatchability was still low (Rowlett and Simkiss, 1985). Also, the knowledge regarding the development and hatchability of avian species in different surrogate eggshell system and culture conditions are still deficient such as effectiveness of the developmental timing of donor embryos on hatchability. Therefore, we investigated the optimal system for culture Korean Oge (KO) chicken embryos in White Leghorn (WL) surrogate chicken eggshells by transfer at different periods to improved culture conditions.

The homeobox protein NANOG is a transcription factor that plays a crucial role in maintaining the pluripotency and promoting the self-renewal of pluripotent stem cells (Chambers et al., 2003, Mitsui et al., 2003, Hart et al., 2004, Niwa et al., 2009). In addition, the predominance of NANOG expression in early germ cell development plays an important role in the proliferation and survival of migrating PGCs (Yamaguchi et al., 2005, Yamaguchi et al., 2009, Sanchez-Sanchez et al., 2010). Also, PGC specification, PGC-specific epigenetic modifications, and global enrichment of histone methylation are regulated via NANOG (Murakami et al., 2016). Tryptophan-repeat (WR) region in the C-terminal domain (CTD), where a pentapeptide W-x-x-x-x (W, tryptophan; x, polar amino acid) repeats eight to ten times WR region is critical for the

formation of a functional NANOG dimer (Mullin et al., 2008, Wang et al., 2008, Chang et al., 2009). Mammalian NANOGs share WR region in the CTD but, avian NANOG lacks the WR motifs that are conserved in the CTD of mammalian NANOG for dimerization (Saunders et al., 2013). It is thus not clear whether avian NANOG forms a dimer to function as a transcription factor, and what governs the molecular association of avian NANOG to achieve a functionally active state. We evaluated the chicken CTD belongs to a novel intrinsically disordered protein that switches into a helical oligomer via self-association, enabling the maintenance of PGCs and blastoderm cells.

To elucidate the potential interspecies germline chimeric system and understanding molecular mechanism of germ cell specific genes in chicken, a series of experiments were conducted. In CHAPTER 2, we review the history of application for germline chimera production and PGCs-specific genes and their regulation. Furthermore, we will be also discussed estrogen effects in chicken oviduct. In CHAPTER 3, we demonstrate the investigation of novel germ cell-transfer system to produce interspecific germline chimeras without germ cell isolation. In CHAPTER 4, we applied the conservation of wild and endangered birds using surrogate system. In CHAPTER 5, we report cNANOG self-associates through its CTD via a novel folding-upon-binding mechanism is important to maintenance of PGCs and blastoderm cells. Finally, in CHAPTER 6, we investigated oviduct-specific novel gene stimulated by estrogen to discover the potential therapeutic peptides or regulation of secreted egg white protein.

CHAPTER 2

LITERATURE REVIEW

1. Strategies for producing germline chimera in Avian

Chimera is composed the different cell populations that are originated from the fusion of as different zygotes. It is distinguished from genetic mosaics which contains genetically different population of cells from zygote. Thus, gemline chimera is carried the different types of germ cells in gonads.

Various technique for generating germline chimera, including embryonic cells, adult or fetal tissues, or embryonic stem cells have been attempted. in mammals. However, mammalians cloning system such as nuclear transfer are not applicable to birds because of physiological differences including embryonic position of the yolk, different cleaving stages. In avian, due to the developmental differences compared to mammals, methods of production of germline chimera are not similar with other species. Thus, unique migratory route of PGCs has been developed to produce germline chimera in birds. Also, blastodermal cells, spermatogonial stem cells (SSCs) and embryonic stem cells (ESCs) has been suggested as a method to generated germline chimera.

1.1 Blastoderm cells mediated germline chimera

In many researchers have been attempted to produce germline chimera through the blastodermal cells, which consist of 4000-6000 undifferentiated cells at Eyal-Giladi and Kochav (EGK) stage X (Eyal-Giladi et al., 1981). Blastodermal cells can easily be isolated and manipulated from the freshly laid eggs. In addition, somatic and germline chimera were produced by transplantation of blastoderm cells into germinal cavity of EGK stage X (Petitte et al., 1990, Carsience et al., 1993). Also, retroviral or lentiviral system have been approached to produce transgenic avian species through the EGK stage X

embryos (Bosselman et al., 1989, McGrew et al., 2004). However, efficiency of germline chimera was too low. To overcome this limitation, several studies reported that blastoderms were exposure to gamma rays and X-ray irradiation to deplete endogenous germ cells at EGK stage X embryo (Carsience et al., 1993, Aigegil and Simkiss, 1991, Park et al., 2010). Removal blastodermal cells from recipient embryos has been also attempted to produce chimera (Kagami et al., 1997)

In other approach, in 1996, Pain et al., established that long term in vitro culture of chicken ES cells, which are separated from blastoderm at EGK stage X, and these cells were contributed to generate somatic and germline chimera (Pain et al., 1996). Despite several efforts, the production of chimera system using ES cells was low rate of germline transmission and not repeated experimentally (Lavial and Pain, 2010). One possible reason is that chicken ESCs were restricted the germline potency during the culture (Lavial et al., 2009). In addition, germ cell and somatic cells have already been segregated at EGK stage X, according to the presence of the chicken *vasa* homologue (CVH) in chicken oocyte and early embryos (Tsunekawa et al., 2000).

1.2 Spermatogonial stem cells mediated germline chimera

Spermatogonial stem cells (SSCs), which are maintained the spermatogenesis, are derived from PGCs during early embryo development and settled at the basement membrane of the seminiferous tubules. SSCs are the only adult stem cells that have the ability to transmit the genetic information (Oatley and Brinster, 2006). Thus, transplanted SSCs into recipient testes could be generated functional sperm (Brinster and Zimmermann, 1994, Ogawa et al., 1999). Recently, SSCs-mediated system has successfully proven the generation

of genome modified progenies (Hamra et al., 2002, Ryu et al., 2007).

In vitro culture system of SSCs have been developed to approach for study the biology of SSCs (Kanatsu-Shinohara et al., 2003, Nagano et al., 2003). Currently, *in vitro* cultured SSCs or freshly isolated SSCs have been reported in mammals (Nagano et al., 2003, Kossack et al., 2009, Nasiri et al., 2012). In avian species, SSCs have been freshly isolated and successfully cultured (Jung, Lee et al. 2010). In addition, transplated SSCs into germinal cavity or vascular system can be produced donor-derived progeny. However, germline transmission efficiency is quite low. Despite of limitation, transgenic efficiency is high and the SSCs-mediated system can be utilized for time-efficiency because production of germline chimera using blastoderm cells or PGCs is need time for the generation. Therefore, it is required to study maintenance of their properties during in vitro culture or enrichment of exogenous SSCs in recipient.

1.3 Primordial germ cells mediated germline chimera

1.3.1 Biological characteristics of PGCs

Germ cells transmit genetic and epigenetic information to connect next generation through gametogenesis. During the first established germ cell population, PGCs are common origins of functional gametes, sperm or ova. Regulation of germ cells are differed from somatic cells due to the unique function in early development.

In most species, germ cells emerge from the first germline cell population known as PGCs. In mouse, PGCs are originated from the proximalepiblast cells and identified during early gastrulation (Saitou and

Yamaji, 2010). In chicken, PGCs firstly arise from epiblast and normally localized to central region of the area pellucida at EGK stage X (Eyal-Giladi et al., 1981). PGCs migrate toward the germinal crescent (Hamburger and Hamilton, 1951, Ginsburg and Eyal-Giladi, 1986) and then enter the blood vessels during Hamburger and HH 12-15 (Fujimoto et al., 1976). Finally, they use the blood stream to settle down in future gonadal regions at HH stage (Fujimoto et al., 1976).

In the early developmental stages, the origin of PGCs was identified by assessing morphological characteristics. In 1914, Swift reported that chicken PGCs are large cells (14-22 μm diameter) with abundant glycogen granules within the cytoplasm (Swift, 1914). PGCs arise from the margin region between area pellucida and opaca, which is known as germinal crescent, followed by their morphological features. Also, PGCs easily confirmed the periodic acid-Schiff (PAS) staining because of cytoplasmic glycogen (Meyer, 1960, Clawson and Domm, 1969). Using electron microscopy and PAS staining, the origin of PGCs described fine morphological characteristics and PGCs in germinal crescent were observed (Fujimoto et al., 1976a, Fujimoto et al., 1977). Following these methods, PGCs are induced their specification like mammals. However, using the tracing germ cell specific markers, it is discovered that avian germ cells is determined by maternally inherited factors (Tsunekawa et al., 2000, Lee et al., 2016).

The *vasa* homologues are highly conserved germline marker among all species. The germ plasm component Vasa protein is essential role in germ cell development (Hay et al., 1990, Lasko and Ashburner, 1990, Gruidl et al., 1996, Ikenishi and Tanaka, 1997). In 2000, Tsunekawa et al., reported that a chicken *vasa* homologue (CVH), which is known as germ cell specific marker, expressed in first cleavage of fertilized eggs (Tsunekawa et al., 2000). The CVH was localized in the mitochondrial clouds so, CVH-containing materials are required

for the formation of germ plasm in chicken. Following this research, chicken specification might be formed by preformation mode. In 2016, Lee et al., reported expression of *Deleted in azoospermia-like (DAZL)* in intrauterine stage embryos (Lee et al., 2016). In addition, DAZL was asymmetric localized in oocyte and translocated subcellular region after zygotic genome activation. This finding supports that avian PGCs specified maternally inherited germ plasm. However, function of germ plasm components is poorly understood.

After specification, in most animals, PGCs are generally acquired motility to migrate toward the somatic gonadal precursors. The basic feature of PGCs migratory pathway is well characterized in various species (Richardson and Lehmann, 2010, Nakamura et al., 2013, Kang et al., 2015). In mammalian species, PGCs translocate from the proximal epiblast region to the extraembryonic ectoderm (Lawson and Hage, 1994, Hayashi et al., 2007) and then, migrate to genital ridges through endodermal hindgut (Richardson and Lehmann, 2010). Chicken PGCs are localized in the central one of the area pellucida before primitive streak formation and starts their translocation from the epiblast to hypoblast (Ginsburg and Eyal-Giladi, 1987). Following the formation of the primitive streak, they migrate from the central zone to the germinal crescent (Hamburger and Hamilton, 1951, Ginsburg and Eyal-Giladi, 1986). After formation of blood vessels, they undergo circulation in the blood stream during HH stages 12-15 (Swift, 1914, Fujimoto et al., 1976b). Finally, they reach to the genital ridge at HH stage 17 by amoeboid movement (Firket, 1920, Fujimoto et al., 1976a). During the PGCs migration, they are regulated by distinct mechanism which involves signaling pathway and adhesion molecules (Richardson and Lehmann, 2010). First, PGC migrate from the central zone to germinal crescent by passive regulation and attach to the basement membrane of epiblast (Wakely and England, 1979). In 2015, Kang et al., reported PGCs passively regulated from central zone to anterior region and then actively moved toward the germinal crescent during formation of primitive streak (Kang et al.,

2015). However, the mechanism of attraction and repulsion is still unknown. The migrating or cultured PGCs form the pseudopodia so, initial migration could be mediated by amoeboid movement (Fujimoto et al., 1976a, Kuwana et al., 1987, Choi et al., 2010). PGCs subsequently move into the blood vascular system at HH stage 9-12 (Fujimoto et al., 1976b, Lee et al., 1978) and circulating PGCs settle in genital ridge (Ukeshima, 1987). PGCs from HH stage 13 embryos isolated and transplanted genital ridge or other embryonic tissues. Only genital ridge groups moved by attraction factors (Kuwana et al., 1987). In 2004, Stebler et al., investigated that the chemokine stromal cell-derived factor 1 (SDF-1/CXCR4) is required for guiding chicken migration (Stebler et al., 2004). Recently, Bernardo et al., investigated anterior vitelline veins have important role for directly accumulation of migrating PGCs into the intraembryonic vasculature (Bernardo, 2012).

PGCs initiate the proliferation before differentiation into the germ cells at EGK stage X. In 1972, Swartz and Domm reported that number of germ cells increased during the PGCs migration and germ cells were undergoing division (Swartz and Domm, 1972). Thus, these results suggest that PGCs occur the rapid proliferation during the migration. Approximately 30 CVH-expressing cells were observed in central zone of area pellucida and then 45-60 cells are existed in the hypoblast layer. 200-250 PGCs population are subsequently increased during migration to anterior region which is known as germinal crescent (Tsunekawa et al., 2000). After settle down to genital ridge, PGCs also rapidly proliferate in both male and female (Zaccanti et al., 1990). From embryonic day 6.5, the morphological differentiation between male and female begin to appear (Hudson et al., 2005). The differentiation of PGCs into functional gametes in oogonia or spermatogonia at embryonic day 8 (Nakamura et al., 2007). In males, the medullary cord differentiates into seminiferous tubules enclosing germ cells. In female, the cortex of left ovary becomes occupied with proliferating oogonia and the right gonad of female regresses.

1.3.2 PGCs-mediated germline chimera in avian

Avian PGCs are more commonly used to produce the germline chimera. Since unique migratory route of avian PGCs (Niewkoop and Sutasurya 1979), they can usually be isolated ate three different embryonic developmental stages including germinal crescent, blood vessels or gonad tissues and generated donor-derived progenies from isolated PGCs. To isolated the PGCs, various methods have been investigated. Density gradient centrifugation and size dependent isolation (SDI) have been attempted to isolate PGCs without cell surface marker (Zhao and Kuwana, 2003, Jung et al., 2017). PGCs also collected by Ficoll density gradient isolation method (Yasuda et al., 1992, Park et al., 2003). After identification of PGCs-specific antibody, it is applied to collect high efficient rate of avian PGCs using magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) (Chang et al., 1992, Ono and Machida, 1999, Mozdziak et al., 2005). In addition, isolation methods of PGCs have been investigated not only in chicken but also other avian species including quail, pheasant, guinea fowl, duck and turkey. However, these systems are still limited to isolate small number of PGCs and applicate in wild or endangered birds. Thus, in vitro culture system was required for application to generate wild or endangered birds but, it has not been done yet (Chang et al., 1992, Ono and Machida, 1999, Mozdziak et al., 2005, Wernery et al., 2010, Liu et al., 2012a).

To overcome these limitation, among the avian species, chicken PGCs have been successfully established *in vitro* culture system (van de Lavoir et al., 2006). However, it is need to develop an optimization of culture condition due to limitation of number of cells. In 2010, Choi et al., reported that PGCs signaling pathway involved in MEK/ERK signal is crucial role in proliferation and survival of chicken PGCs (Choi et al., 2010). This result contributes to in vitro

long-term culture system of PGCs, germ cell characteristics, and migration activity. Moreover, MEK1, AKT, SMAD-3 signaling and Wnt/β-catenin signaling is important for maintaining the germline transmission capacity and proliferation during PGCs culture (Whyte et al., 2015, Lee et al., 2016b). Stem cell factor 2 (SCF2) with FGF2 signaling is also crucial factor for PGCs proliferation (Miyahara et al., 2016). Cultured PGCs have been applied not only the germ cell study, but also producing germline chimera. Thus, after developed in vitro culture PGCs system (van de Lavoir et al., 2006), germline chimera can be advanced production of germline transmission efficiency (Choi et al., 2010, Macdonald et al., 2010, Song et al., 2014). Although, germline transmission efficiency was dependent on the PGCs cell lines, the PGCs-mediated system is regarded as the most powerful tool for producing germline chimera.

2. Production of interspecies germline chimera

Conservation of endangered birds is addressed an important issue. In mammals, cloning technique using nuclear transfer has been developed but, it is not applicable to avian species. Thus, an interspecies germ line chimera has been regarded as the powerful tool for the restoration of avian genetic resource. Interspecies germline chimeras have been generated by PGCs, blastoderm cells and SSCs-mediated system.

2.1. Quail to chicken

Production of interspecies quail-to-chick germline chimeras have been attempted to produce by blood PGCs, blastoderm cells and SSCs. The production

of interspecies germline chimeric chickens by transfer the circulating quail PGCs has been reported (Ono, 1996). Blastoderm cells have also conducted on the production of qual-to-chick chimera. The results shown that doner-derived cells contributed to a 6-day old recipient embryonic gonads as well as somatic tissues (Watanabe et al., 1992). Adutl quail SSCs-mediated system are firstly employed the production of interspecies germline chimera to apply for reproduction of endangered birds (Roe et al., 2013). However, the frequency of donor-derived offspring from interspecific chimera was too low due to the different physiological properties.

2.2. Turkey to chicken

The transfer of turkey PGCs isolated from the germinal crescent has produced interspecies germline chimera (Reynaud, 1969, Reynaud, 1976). After transferred PGCs, both turkey and chick PGCs obtained repopulation in gonad but, it was difficult to distinguish the donor PGCs. In addition, no donor-derived offspring has been produced from turkey-to-chicken germline chimera even though chimeric females had fertilization.

2.3. Duck to chicken and chicken to duck

In other research related to duct-to-chicken or chicken-to-duck chimeras, germline chimeras were successfully produced by transferring blastoderm cells. In 2002, Li et al., reported germ line chimeras were produced between duck and chicken even somatic chimera were induced (Li et al., 2002). Production of chimeric ducks were introduced by transferring chicken PGCs

isolated from gonad into duck embryos (Liu et al., 2012a). Female chimeric ducks were induced infertility but, male could develop functional sperm. However, generation of donor-derived offspring from male chimeric ducks was still low.

2.4. Others

In different avian species, using guinea fowl as hosts, interspecific germline chimera was attempted to produce by cultured PGCs transplantation (van de Lavoir et al., 2012). Also, pheasant-to-chicken germline chimeras were conducted to apply germ cell transfer system to restore the endangered birds (Kang et al., 2008). It has been reported that hatchability was significantly affected in the sexes of PGCs and the production of donor-derived offspring from female chimeric pheasant was absence because of collecting semen from wild male pheasant. In 2010, houbara bustard, which is known as wild seasonal breeding bird, has been employed by gonadal PGCs transplantation into chick embryo (Wernery et al., 2010). Only one male progeny could reach sexual maturity.

3. PGCs-specific genes and their regulation

To obtain biological functions of germ cells, specialized programs of regulation of gene must be established. In somatic cells, The RNA transcription regulated by various distinct ways (Eddy and O'Brien, 1998, Hecht, 1998, Grimes, 2004). In contrast, biochemical mechanisms of transcription in germ cells have unique regulation of gene expression. In addition, tissue-specific core

promotors are initiated their transcription so, identification of their regulatory genes is important to understand for germ cell-specific gene.

In most animals, they have unique PGCs features of migration to somatic gonads and proliferate into germ cells in early embryogenesis. Despite developmental differences, germ cell-specific factors are conserved among the species. One of the major factors such as germline-associated RNA-binding proteins, VASA, DAZL, and DND1 is well characterized in germline. These genes play an important role in maintenance of PGCs which is involved in repressing translation and preventing differentiation into somatic cells (Houston and King, 2000). PGCs also express the several pluripotency-related transcription factors including NANOG, OCT4 and SOX2 (Clark and Reijo Pera, 2006).

3.1 Regulatory mechanism of NANOG gene

3.1.1 Molecular characteristic of NANOG

Transcription factor, NANOG, is essential role for maintaining the pluripotency in ESCs in mammals (Chambers et al., 2003, Mitsui et al., 2003). Generally, the unique homeodomain transcription factor, NANOG, is detected in embryonic germ cells (Yamaguchi et al., 2005) as well as pluripotent cells and ESCs (Mitsui et al., 2003, Chambers et al., 2003, Hart et al., 2004, Niwa et al., 2009). Moreover, NANOG is required for proliferating germ cells during PGCs migration. Recent study reported NANOG are regulated PGC specification, PGC-specific epigenetic modifications, and global enrichment of histone methylation (Murakami et al., 2016).

In mammals, NANOG is expressed in the compact morula and disappeared in the blastocyst stage. detected in PGCs during the early embryo development (Chambers et al., 2003, Hart et al., 2004, Hatano et al., 2005). NANOG is enriched in pluripotent embryo cells and lacking NANOG is failed to develop the blastocyst because of absence of epiblasts (Mitsui et al., 2003). In addition, overexpression of NANOG is maintained the pluripotency upon LIF withdrawal (Darr et al., 2006). In contrast, downregulation of NANOG is induced differentiation to extraembryonic tissue (Hough et al., 2006, Hyslop et al., 2005). Thus, NANOG is required for maintaining pluripotency.

In 2007, Lavial et al., identified chicken NANOG is conserved an evolutionary function among the vertebrate NANOG orthologs (Lavial et al., 2007). Chicken NANOG was exclusively expressed in PGCs during early embryo development (Canon et al., 2006). Like mammals, chicken NANOG was also existed in ESCs to regulated pluripotency and self-renewal (Lavial et al., 2007). Modulation of chromatin structure is also important for stemness. The Satb1, the adenine/thymine (AT)-rich sequence binding protein, is negatively regulated the NANOG expression level whereas the Satb2 is positively regulated NANOG expression (Savarese et al., 2009).

3.1.2 Regulation of NANOG

The regulation mechanism of NANOG gene have been investigated in aspect of transcription factors, epigenetic factors, and autoregulation. In mammals, major regulators of NANOG are OCT4 and SOX2. The NANOG promoter has biding site of OCT4 and SOX2 and these gene promote the NANOG gene level (Rodda et al., 2005). Also, NANOG itself can regulate its transcription positively or negatively (Boyer et al., 2005). Zfp143 is directly bind

to NANOG proximal promoter region to OCT4 binding of OCT4 (Chen et al., 2008).

Many researchers have been attempted to understand transcription factors biding mechanism. NANOG regulation by cis-regulatory elements, including STAT3, FoxD3, Sp1/Sp3, Sall4, Tcf/Lef, Bmi1, Brd4, and Xnf143, was identified (Pan et al., 2006, Suzuki et al., 2006, Wu and Yao, 2006, Kim et al., 2011, Paranjape et al., 2014, Liu et al., 2014, Chen et al., 2008b). Expression of NANOG is activated by transcription factors in specific cues. Tcf3 can bind in NANOG locus to downregulate NANOG expression via Wnt signalling (Pereira et al., 2006). Esrrb is also directly modulated the NANOG expression with Oct4 and interaction between Esrrb and Ncoa3 is upregulated NANOG expression level (Percharde et al., 2012, van den Berg et al., 2008). Furthermore, NANOG is activated by JAK/STAT3 pathway and PI3K/AKT pathway in response to LIF (Niwa et al., 2009). NANOG is regulated own transcription either positively or negatively. NANOG itself binds to its promoter by cooperating with OCT4 and SOX2 and binds to the NuRD complex by interacting with Zfp281 (Fidalgo et al., 2012; Liang et al., 2008). In addition, NANOG transcription is regulated independently of OCT4 and SOX2 by autorepressive loop (Navarro et al., 2012).

NANOG transcription can modulate the chromatin modifiers in ESCs. Sin3a/histone deacetylase (HDAC) complex was directly repressed NANOG expression through binding P53 to NANOG promoter upon differentiation (Lin et al., 2005). In 2009, Sin3a/HDAC complex can upregulate through association with SOX2 (Baltus et al., 2009).

3.1.3 Function of NANOG protein

NANOG interacted with core transcription factors in ESCs and it is important for maintaining pluripotency and early development (Wang et al., 2006). NANOG protein is composed N-terminal domain, homeodomain and C-terminal domain. Homeodomain as DNA-binding domain bound to an OCT4 promoter DNA (Hayashi et al., 2015). NANOG protein forms homodimerization through tryptophan repeat (WR) region within the C-terminal domain (Mullin et al., 2008, Wang et al., 2008). Mutation of WR region was unable to dimerize and loss the mouse ESCs self-renewal and pluripotency. Dimerization of NANOG was also important to interact core pluripotency factors such as Sall4, Zfp198, Zfp281, Dax1, Nac1 and Oct4. Thus, NANOG dimerization is sufficient to support self-renewal in ESCs.

Chicken and zebrafish express NANOG but, these proteins exhibit low conservation of sequence due to lack of WR region. Despite this feature, zebrafish NANOG is able to form dimerization through N-terminal domain and homeodomain (Schuff et al., 2012). In chicken, functional NANOG form has not yet been investigated. However, chick NANOG is functionally conserved among the other species because chick NANOG can replaced with mouse NANOG during somatic reprogramming (Theunissen et al., 2011).

3.2 Regulatory mechanism of VASA gene

VASA gene is most common gene expressing germ cells in various animals. VASA is a member of the DEAD-box protein family that have nine conserved sequence motifs. These motifs confer ATP-dependent RNA helicase catalytic activity. Secondary structure of DEAD-box protein facilitates the RNA folding and interactions with other proteins (Lorsch, 2002, Mohr et al., 2002). Due to these features, DEAD-box proteins encompass pre-mRNA splicing,

ribosome biogenesis, nuclear export, translational regulation and degradation (Linder, 2006). In mammals, VASA protein detected specifically during early embryogenesis and germ cells development.

In *Drosophila melanogaster,* VASA gene has a crucial role in several aspects of germ cell development. Especially, the germ plasm component of VASA protein is important for germ cell determination (Saffman and Lasko 1999). To investigate the function in female, germline development, mutation of VASA coding region was required for female sterility including abnormal differentiation (Styhler et al., 1998). Thus, VASA is not only essential for the specification but also, required for the gametogenesis in adults. The posterior of the oocyte is concentrated the VASA protein and its expression is only observed in germ cells during embryogenesis in both male and female (Lasko and Ashburner, 1990).

The VASA gene is conserved in most animals including *Caenorhabditis elegans, Xenopus laevis, Danio rerio* and *Mus musculus* (Fujiwara et al., 1994, Gruidl et al., 1996, Ikenishi and Tanaka, 1997, Yoon et al., 1997). In *Caenorhabditis elegans*, three VASA-like genes have been identified (Gruidl et al., 1996). The VASA subfamily is specifically detected in germ cells during embryo development in all species, and required for germ cell specification in *Caenorhabditis elegans* and *Xenopus laevis.* In *Danio rerio,* VASA is utilized for germ cell-specific marker at early embryo development (Yoon et al., 1997). Also, mouse VASA gene is specifically detected in germ cells lineage (Fujiwara et al., 1994). Mutation of this gene could not produce sperm in male mice and the proliferation of PGCs is restreicted (Tanaka et al., 2000). In chicken VASA hoomologue is traced the origin of PGCs and specifically expressed in germ cells (Tsunekawa et al., 2000).

3.3 Regulatory mechanism of DAZL gene

Deleted in azoospermia (DAZ) family proteins are composed two conserved domains, the ribonucleoprotein (RNP)-type RNA recognition motif (RRM) and DAZ motif (Cooke et al., 1996, Eberhart et al., 1996). Deleted in azoospermia-like (DAZL), which is conserved gene family members including BOULE and DAZ, is critical for germ cell development. However, DAZL shows variable and distinct expression in different tissues. In addition, the DAZ family is applied infertility animal model because of their function in male fertility (Xu et al., 2009).

BOULE is only family members in *Drosophila melanogaster* and *Caenorhabditis elegans*. A homologue of DAZ in *Drosophila melanogaster*, named boule, is essential in spermatogenesis not in oogenesis (Eberhart et al., 1996). In contrast, in *Caenorhabditis elegans*, DAZ-1 is an important factor for female meiosis (Karashima et al., 2000). Mutation of BOUL in *Drosophila melanogaster*, males are sterile and spermatocyte undergoes at the G2/M phase arresting (Eberhart et al., 1996, Cheng et al., 1998).

In vertebrate, deletion of DAZL in *Xenopus laevis*, are diffected PGCs development (Houston and King, 2000a). In mouse, inhibition of DAZL are induced sterility in both male and female (Ruggiu et al., 1997). Failure of spermatogonial differentiation in testis was also observed. Disruption of DAZL gene lead to loss population of gem cells and induced apoptotic germ cells (Lin and Page, 2005). In chicken, DAZL gene is specifically expressed in PGCs during the migration. In addition, it is in asymmetrically localized in oocyte during intrauterine embryo stages (Lee et al., 2016). Therefore, DAZL may play an important role in germ cell lineage in chicken.

4. Estrogen regulation of gene in chicken oviduct

4.1. Molecular mechanisms of estrogen action

Estrogen plays important role in the regulation of reproductive organs and differentiation and maintenance of several tissues in the development. This hormone is regulated the transcription of specific genes in reproductive organs (Pearce and Jordan, 2004, Hewitt et al., 2005). The biological functions of estrogen are mediated by two receptors; estrogen receptor a (ER a) and β (ER β). These two receptors are evolutionary conserved (Hewitt et al., 2005) and rapidly response to estrogen (Razandi et al., 2004).

Estrogen receptors are nuclear transcription factors, these receptors interact with associated factors to activate regulation of genes expression through directly binding to the estrogen response elements within the promoters or other transcription factors such as AP-1 and Sp1 (Hall et al., 2001). The activated estrogen receptors are simulated by cytoplasmic signaling through its phosphorylation or phosphorylation of others (Wu et al., 2005).

Multiple co-regulators involve in activation of gene expression by Estrogen receptors to precisely regulate gene (Smith and O'Malley, 2004). The activated estrogen receptors are inhibited by biding to antagonist. In contrast, estrogen receptor agonist is play important role in chromatin remodeling and transcriptional initiation through stimulation to co-activators (Smith and O'Malley, 2004).

4.2. Effect of estrogen in the chicken oviduct

Chicken is excellent model for research on development of female reproductive system because endocrine system is regulated by estrogen. In particular, the dynamic morphological changes occur in chicken oviduct by estrogen (Dougherty and Sanders, 2005, Hrabia et al., 2014). In various species, development of the female reproductive organs is regulated by both endogenous and exogenous estrogens (Palmiter and Wrenn, 1971, Song et al., 2011). Specifically, diethylstilbestrol (DES) as and exogenous estrogen induced differentiation a d development of the immature chicks by activating expression of estrogen-responsive genes.

The immature oviduct of chick induces cytodifferentiation by estrogen. In 1969, Kohler et al., reported that glandular structures were presented invagination into subepithelial stroma and epithelial cells were differentiated (Kohler et al., 1969). In addition, apoptosis induced by estrogen in the chick oviduct. Expression of BMP-7 gene is reduced by treatment of exogenous estrogen to prevent apoptosis whereas expression of BMP-7 gene is upregulated by withdrawal exogenous estrogen to trigger apoptosis (Monroe et al., 2000). In human, 17beta-estradiol (E2) is inhibited BMP-7 gene and activing receptor IIB (actRIIB) and apoptosis in mouse uterus 134 (Kusumegi et al., 2004). In addition, estrogen treatment stimulates the caspase activity including the expression of caspase-1 (CASP1) and -2 (CASP2) genes thus, estrogen plays an important role in protection of apoptosis (Monroe et al., 2002).

Estrogen treatment controls major egg-white proteins synthesis, including ovalbumin, ovotransferrin, ovomucoid and avidin. These proteins were synthesized and secreted from tubular glands cells (Kohler et al., 1969). Interestingly, short time progesterone treatment with estrogen can be not induced initial tissue growth into tubular gland cells and can specifically produce lysozyme and ovalbumin (Oka and Schimke, 1969a). This finding suggests that progesterone inhibits initial proliferation.

Although many researchers have been attempted to clarify the effect of estrogen in the female chick reproductive system. However, the molecular mechanism of their target gene and regulation mechanism of egg-white secretion by hormones.

CHAPTER 3

Production of Interspecific Germline Chimeras via Embryo Replacement

1. Introduction

The germline chimera production technique has been a major tool for basic and applied research in birds, such as developmental biology, animal transgenesis and endangered species conservation. Avian germline chimeras can be produced by transplanting blastodermal cells (Petitte et al., 1990), primordial germ cells (PGCs) (Han et al., 2002), or testicular cells (Lee et al., 2006). Among these, PGCs have been used as a major source for germline chimera production because of their high efficiency of germline transmission (Park et al., 2003). PGCs, which are found in the central region of the area pellucida (Ginsburg, 1994, Nakamura et al., 2007) at Eyal-Giladi and Kochav (EGK) stage X (Eyal-Giladi et al., 1981), gradually translocate anteriorly to an extraembryonic region called the germinal crescent at Hamburger and Hamilton (HH) stage (Hamburger and Hamilton, 1951). The PGCs remain in the extraembryonic region until around HH stage 10 when the blood vascular system starts to form; soon afterward, the PGCs move to the future gonad region through blood vessels (Fujimoto et al., 1976, Nakamura et al., 2007, De Melo Bernardo et al., 2012). This unique migratory route through the bloodstream has been widely applied as a powerful tool for germ cell-transfer systems as well as production of transgenic birds (Park et al., 2003, Naito et al., 2004, Kim et al., 2005, Kang et al., 2008, Park et al., 2008). Germline chimeras have been successfully produced by transfer of PGCs, which are collected from the donor embryonic blood vessels during HH stages 13 to 17 (Tajima et al., 1993, Naito et al., 1994) or donor embryonic gonads (Han et al., 2002, Chang et al., 1995), into the blood vessels of recipient embryos. In addition, with germ cell-mediated techniques, interspecies germline chimeras can be used for conservation of endangered birds (Kang et al., 2008, Wernery et al., 2010). Endangered birds are greatly restricted in their populations because of their limited capacity for egg production and seasonal breeding. On the other hand, in the case of the chicken, hens can produce at least 330 eggs non-seasonally throughout the year.

Therefore, production of interspecies germline chimeras with the fecundity of chickens can be helpful for conservation of endangered birds. However, the efficiency of interspecies germline chimera production is still low.

A highly efficient germline-chimera production system was developed via the establishment of PGCs in an in vitro culture system (van de Lavoir et al., 2006, Park and Han, 2012, Choi et al., 2010, Macdonald et al., 2010), and targeted gene knockout chickens were produced (Park et al., 2014, Park et al., 2013). However, only chicken PGCs have been cultured successfully in vitro without loss of germ cell properties (Choi et al., 2010, Macdonald et al., 2010). Quail PGCs have also been cultured to produce germline chimeras, but these cells could not be cultured for longer than 20 days (Kim et al., 2005, Park et al., 2008). Alternative methods to produce germline chimeras that are applicable to various avian species have yet to be developed.

The embryo transplantation technique in which a quail whole embryo is grafted onto the host chick embryonic yolk was originally devised by Martin (Martin, 1972). Quail-chick chimeras could then be established by grafting a quail embryo body onto the chick extraembryonic area (Dieterlen-Lievre, 1975). These techniques have been used in studies to investigate the development of the embryonic vascular system and to trace the cell origin of the hematopoietic system, which initiated an important paradigm shift in research fields pertaining to the vascular system (Francoise-Lievre, 2005, Dzierzak and Medvinsky, 2008, Martin et al., 1978). Recent studies have indicated an increased survival rate after embryo replacement with manipulations such as electroporation and ex ovo culture, which enables the further application of embryo replacement (Tanaka, Harada et al. 2010, Nagai, Sezaki et al. 2014).

We postulated that the combination of the unique migratory route of avian germ cells with an embryo replacement method could be applied to

produce interspecies germline chimeras. We detected chick PGCs in 5.5-day-old embryonic gonads of quail-chick chimeras. We confirmed the potential application of this novel germ cell-transfer system to produce germline chimeras without germ cell isolation, such as PGC culture or separation using magnetic activated cell sorting with specific antibodies. Thus, this germ cell transfer technique may be applicable to conservation of wild or endangered avian species.

2. Materials and methods

Experimental animals and animal care

The care and experimental use of animals were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). A broiler-type quail strain, "Jumbo" (*Coturnix japonica*), and Korean Oge chicken (KO; *Gallus gallus*) were used as donor and host embryos, respectively. The experimental animals 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.

Embryo replacement

Fertilized host and donor eggs were incubated horizontally for 33 h to reach HH stage 9–10. The flatness of the embryo is important to prevent leaking of the host yolk during embryo replacement; it also facilitates dissection, allowing replacement with the donor embryos *in ovo*. The host and donor size must be matched to prevent technical difficulties during the replacement steps. Next, 5 ml of albumen was removed from the host egg with a 21-gauge syringe by making a tiny hole on the narrow end of the egg. After cutting and removing of the host and donor embryos with a pair of microscissors, resulting in smooth cut edges, the donor embryo was replaced in the original position of the host embryo. Both the donor embryo and host membranes were gently pinched together with a pair of fine forceps to attach the donor embryo to the host membrane. During suturing, the host yolk sac margin was carefully pulled out from underneath the vitelline membrane, and the margins of the donor and host were gently held and pressed together for a few seconds and then released. After suturing was complete, the extra tissue along the suture line was trimmed to

expand the donor embryo over the suture boundary. The operation window was sealed with clear tape and the eggs were further incubated at 38.58°C in an incubator until Embryonic Day (E) 5.5 (Figure 3-1).

Immunohistochemistry

For identification of gonadal PGCs (gPGCs) in quail-chick chimeras, monoclonal antibody specific to quail PGCs (QCR1) (Ono et al, 1998, Aoyama et al., 1992, Ono et al., 1996) and stage specific embryonic antigen-1 (SSEA-1; Santa Cruz Biotechnology, Santa Cruz, CA) specific to chicken PGCs were used. Whole gonads were retrieved from 5.5-day-old embryos and fixed with 4% paraformaldehyde for 1 day at room temperature, then rinsed three times with 1x PBS. The tissue was then permeabilized with 1% Tween-20 and rinsed with 1x PBS. The gonads were incubated with blocking solution (10% BSA and 5% goat serum in 1x PBS) for 3 h, and then incubated with 1:200 diluted QCR1 (mouse immunoglobulin [Ig] G) or anti-SSEA-1 (mouse IgM) antibody at 48°C overnight. After washing with 1x PBS, gonads were incubated with fluorescein isothiocyanate (Santa Cruz Biotechnology)-conjugated goat anti-mouse IgG (diluted 1:200) for QCR1 or phycoerythrin (Santa Cruz Biotechnology)-conjugated goat anti-mouse IgM (1:200) for anti-SSEA-1 for 1 h at room temperature. Immunostained gonads were examined under a fluorescence microscope (Eclipse Ti; Nikon, Tokyo, Japan), and the SSEA-1 or QCR1-positive cells were counted.

Analysis of chimeric status after replacement

To evaluate the chimeric status and monitor the migration of PGCs from the host yolk sac to the donor embryo, embryonic gonads were collected from 5.5-day-old chimeric embryos. Other parts of the collected chimeric embryos were used for genomic DNA PCR with GFP-specific, chicken-specific (CSP#1),

and quail-specific (QSP#7) primers (Kang et al., 2008, Li et al., 2001). The sequences and product sizes of these primers are shown in Table 3-1. Genomic DNA PCR was performed in a total volume of 20 μl containing 200 ng of collected tissue genomic DNA, 1.6 μl of 2.5 mM dNTP mixture (Core BioSystems, Seoul, Korea), 2 μl of 10 reaction buffer (Core BioSystems), 2 pmol of each primer, and 2.5 units of Taq polymerase (Core BioSystems) under the following conditions: 10 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 63°C, and 30 sec at 72°C, with a final extension for 5 min at 72°C.

Statistical analysis

Data obtained from at least three replicates were subjected to statistical analysis by a generalized linear model (PROC-GLM) using Statistical Analysis System software (SAS Institute, Cary, NC). When a significant effect was detected, the treatment effect was compared using the least-squares method. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

Establishment of embryo replacement for production of germline chimeras

The germ cell-transfer system was attempted to establish whether the PGCs derived from an extraembryonic region (i.e., the area opaca in the host yolk) can be incorporated into replaced donor embryos. First, we performed replacement of GFP-TG chick embryos (Park and Han, 2012) with KO chick embryos (Figure 3-2, A and B). As shown in Table 3-2, among the 37 KO-TG chimeric embryos, 25 embryos survived until 24 h after the operation. Of these 25 embryos, 17 survived until HH stage 28 (4 days after replacement). GFP-expressing cells, which were observed in GFP-TG chick embryonic gonads, were also detected in the embryonic gonads of KO-TG chimeras (Figure 3-2, C and E), whereas they were not detected in KO chick embryonic gonads (Figure 3-2D). Genomic DNA PCR analysis of 5.5-day-old embryonic gonad confirmed the presence of GFP-positive cells in KO-TG chimeras (Figure 3-3A).

Production of quail-chick germline chimeras by embryo replacement

To produce quail-chick germline chimeras, the quail embryo proper was used to replace the host embryo on its yolk (Figure 3-1B). Of 55 quail-chick yolk sac chimeric embryos, 42 embryos survived until 24 h after replacement. Of these 42 embryos, 25 survived until HH stage 28 (Table 3-2). We also confirmed the viability of the transplanted embryos (n = 30). The transplanted embryos survived until a maximum of E16.5, and quail-chick chimeras showed normal development while they were alive (Figure 3-4). Of 30 quail-chick yolk sac chimeric embryos, 12 embryos (40%) survived until E5.5 after embryo replacement. Of these 12 embryos, 2 (6.7%) survived until E16.5.

Migration of chick PGCs toward the embryonic gonads of replaced quail

embryos

To monitor the migration of PGCs from the host extraembryonic region of the yolk to the embryonic gonads after replacement, the 5.5-day-old embryonic gonads of quail- chick chimeras were immunostained with QCR1 and anti-SSEA-1 antibodies (Figure 3-5). QCR1 was specific to PGCs in quail gonads (Figure 3-5, A and B), and SSEA-1 was specific to PGCs in chick gonads (Figure 3-5, C and D). When the gonads of the quail-chick chimeras were immunostained with QCR1 and SSEA-1, the PGCs positive for each of the antibodies were detected, indicating that the chick PGCs were successfully transferred into the quail gonads (Figure 3-5, E–G).

To confirm the genotype of the gonads of quail-chick chimeras, genomic DNA was extracted from the 5.5-day-old embryonic gonads of intact quail embryos, KO chick embryos, and quail-chick chimeras. PCR was then performed with these genomic DNAs using species-specific markers. The genomic DNA from the gonads of quail-chick chimeras was positive for both the chicken-specific and quail-specific markers (Figure 3-3B).

Contribution of chick PGCs in the gonads of chimeras

To investigate the contribution of chick PGCs in the gonads of the quail-chick chimeras, QCR1 and SSEA-1 positive PGCs were counted. As shown in Table 3, the average numbers of QCR1-positive and SSEA-1-positive PGCs were 242.3 ± 66.9 and 200.5 ± 43.9, respectively (n = 8).

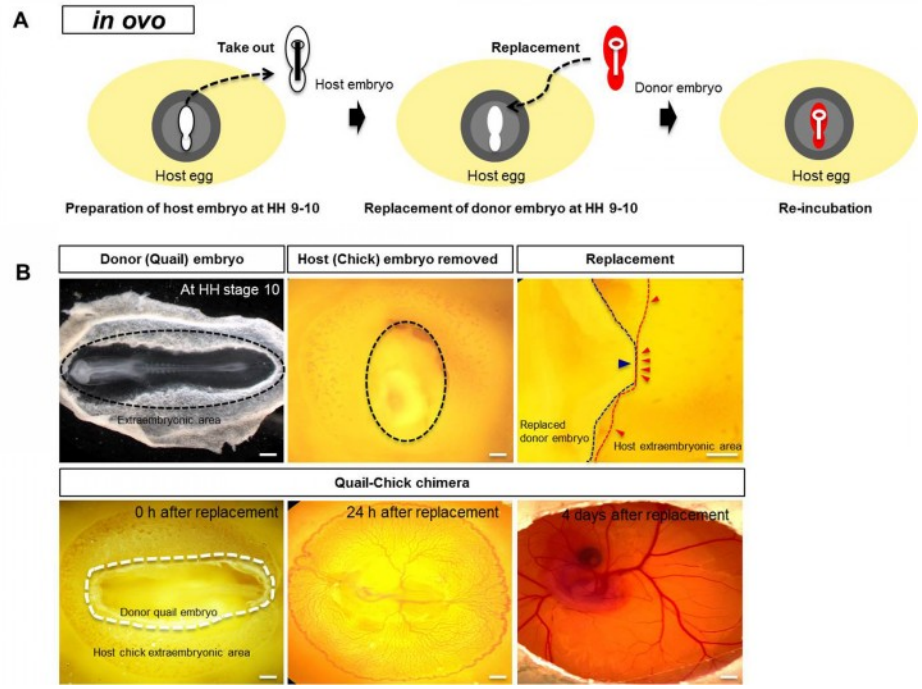


Figure 3-1. Experimental strategy for chimera generation. (A) Schematic diagram of embryo replacement. (B) The operation was performed with HH stage 9–10 embryos. The donor embryo was cut along the black dotted lines and removed. The host embryo was also cut in an identical manner along the black dotted lines and removed. Both the host and donor embryo membranes were pinched with forceps to attach. After embryo replacement, the embryo was incubated for 24 h or 4 days. Blue arrowhead and blue dotted line, donor membrane boundary. Red arrowheads and red dotted line, host yolk sac membrane boundary. White dotted line, suture points. Bar = 1 mm.

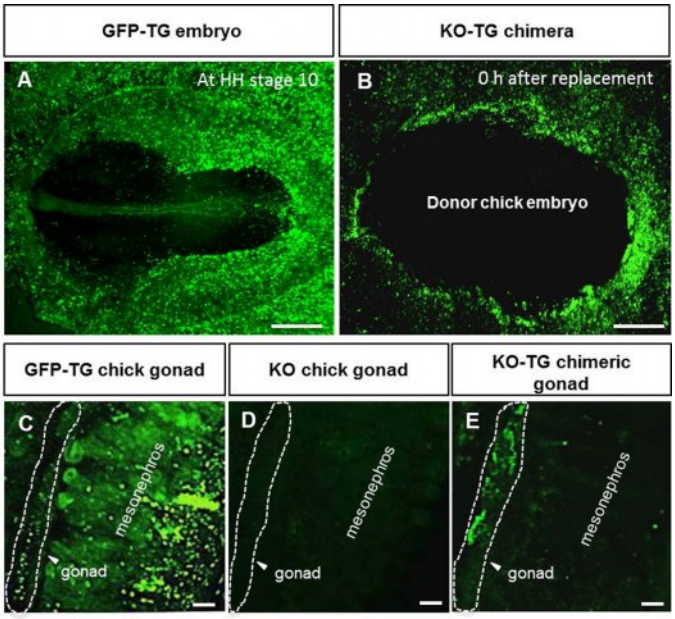


Figure 3-2. Detection of the GFP transgene in embryonic gonads of a chimera. (A) GFP-TG chick embryo showed GFP expression at stage 9–10. (B) KO embryo was replaced onto the yolk of the recipient. GFP expression was investigated in the 5.5-day-old embryonic gonads of GFP-TG (C) and KO chicks (D) and KO-TG chimeras (E) using a confocal laser scanning microscope. Bars = 1 mm (A, B) and 500 μm (C–E).

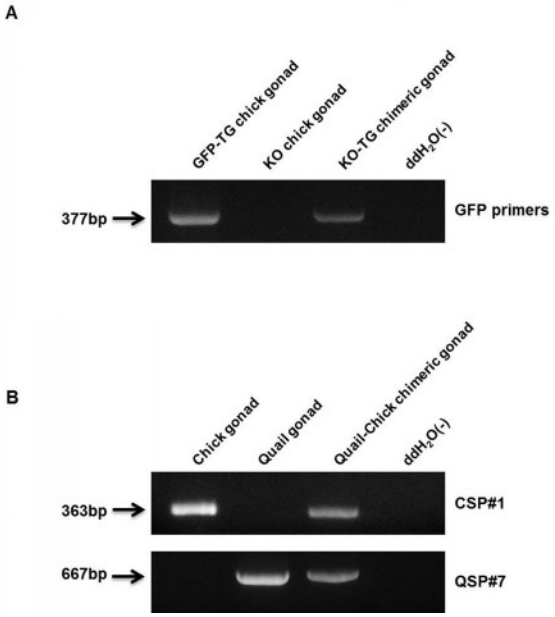


Figure 3-3. Detection of host-derived PGCs in donor embryonic gonad by PCT analysis. (A) Genomic DNA PCR analysis of 5.5-day-old embryonic gonads with GFP-specific primers showing GFP transgene detection in the GFP-TG chick and chimera. (B) The embryonic gonads of quail-chick chimeras were positive for both the chicken-specific (CSP#1) and quail-specific (QSP#7) markers.

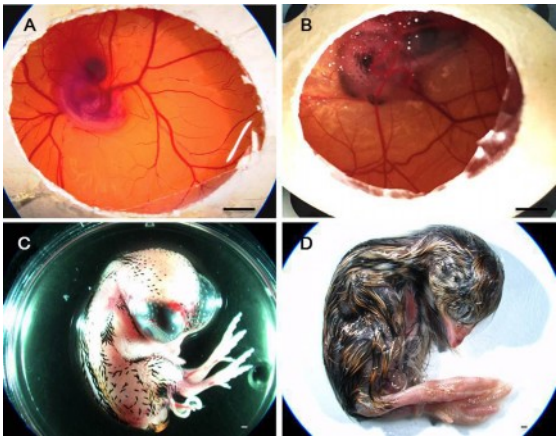


Figure 3-4. Quail-chick chimera at various stages of development. (A) Five-day-old quail embryo developed in connection with the chick yolk. An E10.5 quail-chick chimera (B, C). (D) An E16.5 quail-chick chimera was isolated from the chicken egg. Bars = 1 mm (A, B) and 2 mm (C, D).

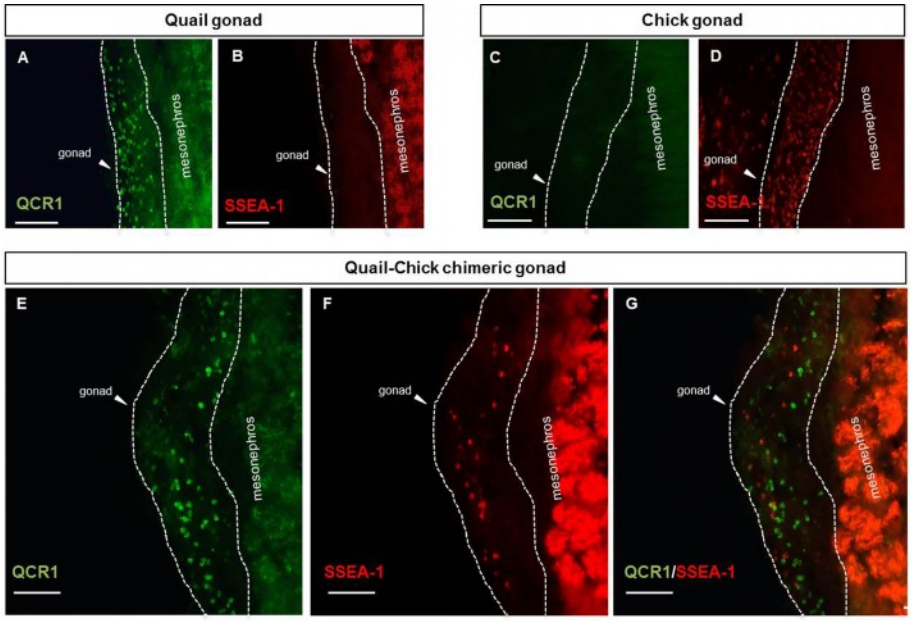


Figure 3-5. Interspecies germline chimerism in quail-chick chimeras. As controls, QCR1 and SSEA-1 showed specificity to quail PGCs (A, B) and chick PGCs (C, D), respectively. Migration of PGCs derived from the chick extraembryonic area was detected in the gonads of quail-chick chimeras by immunostaining using QCR1 (E) and SSEA-1 antibodies (F). (G) The merged image shows that QCR1 (green) and SSEA-1 (red) did not overlap in the gonads of quail-chick chimeras. Bars = 200 μm.

Table 3-1. Information of the primer sets used for genomic DNA PCR analysis

Primer ID	Sequence	Size(bp)
CSP #1[a]	GAGTGTAGACAGTAGTGTATC CTCAGGGCACCATTTTCACTG	363
QSP #7[b]	AATTGGTGACGGGTACACAGATTC AGGATCACGATATTGAACCATCAC	667
GFP[c]	CAAGGACGACGGCAACTACA CGGTCACGAACTCCAGCAG	377

[a]Chicken-specific marker.

[b]Quail-specific marker.

[c]GFP primers.

Table 3-2. Viability of replaced donor embryos onto the host yolk until 5.5 days

	Days after embryo replacement			
	1 d	2d	3 d	4 d
Quail-chick chimera No. of embryos/Total incubated eggs (%) [a]	42/55 (76.4) [c]	37/55 (67.4) [cd]	29/55 (52.7) [de]	25/55 (45.5) [e]
KO-TG chimera No. of embryos/Total incubated eggs (%) [b]	25/37 (67.6) [f]	23/37 (62.2) [fg]	18/37 (48.6) [fg]	17/37 (45.9) [g]

[a]Survival rate of replaced quail embryos on a chick.

[b]Survival rate of replaced chick embryos on a GFP transgenic chick.

[c-e]Different superscripts in the number of produced quail-chick chimeras were significantly different, ($P < 0.05$).

[f-g]Model effect for the viability of KO-TG chimera was P = 0.1811.

Table 3-3. Survival rate of quail-chick chimeras after embryo replacement

	Age reached after embryo replacement			
	5.5 d	10.5 d	12.5 d	16.5 d
Quail-chick chimera No. of embryos/Total incubated eggs (%)[a]	12/30 (40)[b]	7/30 (23.3)[bc]	4/30 (13.3)[c]	2/30 (6.7)[c]

[a]Survival rate of replaced quail embryos on chick.

[b-c]Different superscripts in the number of produced quail-chick chimeras indicate significant differences, ($P < 0.05$).

Table 3-4. Number of PGCs in 5.5-day-old quail-chick chimeric gonads

Trials	Number of gPGCs[a]	
	QCR1 (+)	SSEA-1 (+)
1	337	126
2	166	220
3	213	208
4	253	256
5	246	248
6	235	185
7	228	204
8	260	157
Average[b]	242.25 (± 66.9)	200.5 (± 43.9)

[a]The number of gPGCs was determined by counting the QCR1-positive or SSEA-1-positive cells under fluorescence microscopy.

[b]Values represent means ± SD

4. Discussion

In birds, unlike mammals, PGC-mediated system can be used to produce germline chimeras or TG birds. In mammals, germline chimeras have been produced using germline- competent stem cells (Thomas and Capecchi, 1987, Li et al., 2008). On the other hand, chicken embryonic stem cells (ESCs) derived from pre-streak-stage embryos are not suitable for producing germline chimeras because this approach exhibits significantly lower germline transmission efficiency (Petitte et al., 1990, Pain et al., 1996). Therefore, PGCs, which are precursor cells of functional gametes, have been recognized as an alternative source of germline transmission in avian species. Of the various avian species, only chicken PGCs have shown germline competency. This is due to higher germline transmission efficiency compared to ESC and stable culture of PGCs. Several groups have attempted to produce germline chimeras using PGC-mediated systems in wild/endangered birds, but the efficiency of production by interspecies PGC transfer is still very low (Kang et al., 2008, Wernery et al., 2010). In this regard, we explored an alternative method to produce germline chimeras that can be applied to most avian species. In the present study, we produced interspecies germline chimeras using the embryo replacement method. The results showed that the chicken PGCs derived from the host extraembryonic region in the yolk were incorporated into the donor quail embryonic gonads after replacement. The combination of the unique migratory route of avian germ cells with the embryo replacement technique demonstrated the possibility of producing interspecies germline chimeras.

We expected that donor embryonic gonads would be mostly occupied by the host PGCs; however, donor quail PGCs accounted for more than half of those in the quail-chick chimeric gonad. This may be explained by the fact that PGCs were already distributed at the area pellucida region at the time of transplantation, so that the donor quail PGCs could not be removed completely

from the donor embryo during the operations. In this study, embryo replacement was performed at HH stage 9 to 10 before the formation of blood vessels as described previously (Dieterlen-Lievre, 1975, Lassila et al., 1982). Nakamura et al. (Nakamura et al., 2007) reported that PGCs were observed at the area pellucida as well as the anterior part of the extraembryonic region in the area opaca at HH stage 9–10. This strongly suggests that the embryo stage at the operating time is an important factor for improving the efficiency of germline chimerism in the gonads. Thus, optimization of the embryo stage at the time of operation should be investigated in further studies. Alternatively, to eliminate endogenous PGCs in donor embryos, busulfan treatment before embryo replacement can be applied to increase the efficiency of germline transmission (Lee et al., 2013, Nakamura et al., 2010).

To evaluate germline chimerism in the offspring of interspecies germline chimeras after embryo replacement, it would be necessary to hatch the manipulated embryos. In this study, the transplanted embryos survived until a maximum of E16.5, but no hatchlings were available. We also found that quail embryos were not able to enclose the yolk after E16.5 because the chick yolk was larger than that of the quail. Thus, the large yolk size may be a major factor preventing hatching. It was further suggested that the yolk could be surgically removed to allow hatching after embryo replacement (Kenny and Cambre, 1992). Further studies are needed to improve the survival rate and hatchability for actual application. We will focus on optimization of the embryo replacement system with high viability as well as hatchability of replaced embryos in future studies.

In the present study, we confirmed that PGCs derived from the host extraembryonic region in the yolk can migrate toward the donor embryonic gonadal tissues after production of quail- chick chimeras. Our technique can be performed reciprocally and has a high success rate of chimeric embryo

development to the gonadogenesis stage. The number of transferred PGCs was also attributable to greater migration activity. Therefore, we believe that interspecific germ cell transplantation using embryo replacement will be one means of overcoming the low efficiency of interspecies germline chimera production in avian species. This technique is also potentially applicable to the restoration and conservation of endangered bird species. Through improved transplantation and culture methods, mature germ cells of endangered species can potentially be harvested from more abundant host species such as the chick or quail, thus facilitating their restoration/conservation.

5. Conclusion

In avian species, PGCs use the vascular system to reach their destination, the genital ridge. Because of this unique migratory route of avian germ cells, germline chimera production ca be achieved via germ cell transfer into a blood vessel. This study was performed to establish an alternative germ cell-transfer system for producing germline chimeras by replacing an original host embryo with a donor embryo, while retaining the host extraembryonic tissue and yolk, before circulation. First, to test the migratory capacity of PGCs after embryo replacement, KO chick embryos were used to replace GFP transgenic chick embryos. Four days after replacement, GFP-positive cells were detected in the replaced KO embryonic gonads, and genomic DNA PCR analysis with the embryonic gonads demonstrated the presence of the GFP transgene. To produce an interspecific germline chimera, the original chick embryo proper was replaced with a quail embryo onto the chick yolk. To detect the gonadal PGCs in the 5.5-day-old embryonic gonad, immunohistochemistry was performed with monoclonal antibodies specific to either quail or chick PGCs, i.e, QCR1 and SSEA-1, respectively. Both the QCR1-positive and SSEA-1-positive cells were detected in the gonads of replaced quail embryos. Forty percent of the PGC population in the quail embryos was occupied by chick extraembryonically derived PGCs. Thus, replacement of an embryo onto the host yolk before circulation can be applied to produce interspecies germline chimeras, and this germ cell-transfer technology is potentially applicable for reproduction of wild or endangered bird species.

CHAPTER 4

Normal Development and Hatchability of Korean Oge Chickens in White Leghorn Surrogate Eggshells

1. Introduction

Culture of avian embryos in the surrogate eggshell is an interesting investigation to study the development and hatchability of avian species in this condition. In previous studies, quail embryos were successfully cultured until hatch in recipient chicken eggshells, but the percentage of hatchability was only 3% (Ono et al., 1994). Similarly, chicken embryos were cultured in recipient turkey eggshells or chicken egg-shells, and the percentage of hatchability was 20% or 23%, respectively (Rowlett and Simkiss, 1985).

Perry has developed three kinds of surrogate eggshell system for the culture of chicken embryos from single cell stage to hatching (Perry, 1988). In that experiment, the percentage of hatchability was only 7%. Later, many researchers were attempted to increase the percentage of hatchability in surrogate eggshell system. For instances, the culture condition of Perry's method was slightly modified by Naito group to increase the hatchability up to 34% (Naito et al., 1990). Kamihira group modified Perry's system by the addition of calcium and eggshell powder (Kamihira et al., 1998). Surrogate eggshell system was also improved by making optimum size of window, and sealing the window after embryo transfer which gave 42~59% of hatchability (Borwornpinyo et al., 2005; Andatch et al., 2004). Furthermore, Lui et al investigated surrogate system by using different eggshells or albumens from other species that produced 7.7~60% of hatchability (Liu et al., 2012, Liu et al., 2013). However, the knowledge regarding the development and hatchability of avian species in different surrogate eggshell system and culture conditions are still deficient such as effectiveness of the developmental timing of donor embryos on hatchability.

The aim of this study was to investigate the optimal system for culture Korean Oge (KO) chicken embryos in White Leghorn (WL) surrogate chicken

eggshells by transfer at different periods.

2. Materials and methods

Experimental animals

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

Preparation of Surrogate Eggshells

The recipient surrogate eggshells were prepared from freshly laid and normal-sized WL chicken eggs (65~70 g). Using an electric drill, we cut the narrow ends of WL eggshells and all egg components were removed. The approximate diameter of surrogate eggshell window was 32 to 35 mm.

Culture of KO Chicken Embryos in WL Surrogate System

Perry has developed three surrogate systems for the culture of chicken embryos (Perry, 1988). According to Perry's method, fertilized ova were cultured until the formation of blastoderm stage (1 day of incubation) in system I. The blastoderm stage embryos were cultured until early embryogenesis (1~4 days of incubation) in system II. The 4-day-old embryos were cultured until hatch in system III (4~22 days of incubation). The present study was designed mainly based on Perry's surrogate system III (Figure 4-1A). Freshly laid KO eggs (38~40 g) were incubated for 3 or 4 days. The 3-day and 4-day-old KO embryos

along with albumin and yolk were carefully transferred into WL surrogate eggshells. Then, the edges of surrogate eggshell windows were sealed with cling film and tied up with a rubber band (Figure 4-1B). Figure 4-2 shows the original images of KO chicken culture in WL surrogate eggshells. After transferring the KO donor components, the embryos projected with blood vessels were gently re-located to the top without damaging the airspace of the surrogate eggshells (Figure 4-2B). After sealing, the eggs were incubated at 37.5°C with 70% of humidity, and the eggs were rotated 30 degree angle at every hour (Figure 4-2D). The number of live embryos was counted everyday until embryonic day 18. At embryonic day 19, the sealing film was removed and the eggs were transferred to an incubator with 37.5°C and 60% of humidity until hatching (Figure 4-2, E and F).

3. Results

Culture of Korea Oge (KO) chicken embryos in White Leghorn (WL) surrogate eggshells

Initially, KO embryos at 3-day and 4-day-old (HH stage 20~24) (Hamburger and Hamilton, 1951) were transferred into WL surrogate eggshells and incubated until hatching of chicks. In this surrogate culture, we monitored the development and viability of embryos, and the percentage of hatching. Most of the KO embryo development was normal in WL surrogate eggshells (Figure 4-3). For instances, feather germs were visible in the edges of wings, and eyelids were extended to eyeball at embryonic day 8. At the time close to hatching, yolk was enclosed in body cavity and chorioallantoic membrane contained less blood (Figure 4-3).

Viability and hatchability of chicken embryos

The viability and hatchability of KO chicken embryos in surrogate system III are shown in Table 4-1 and 4-2, respectively. Two days of incubation after transferring the 3-day-old and 4-day-old embryos into surrogate system, their viability was 92.2% and 92.5%, respectively (Table 4-1). Then, a few embryos were died in the surrogate system during different developmental stages. Therefore, at embryonic day 14 to 21, the viability of 3-day and 4-day group was decreased from 43.8% to 23.1% and from 55.6% to 36.0%, respectively. All viable embryos in both 3-day and 4-day group were hatched normally at embryonic day 22. However, the percentage of hatchability rom 3-day group was 23.1% and 4-day group was 36.0% (Table 4-2).

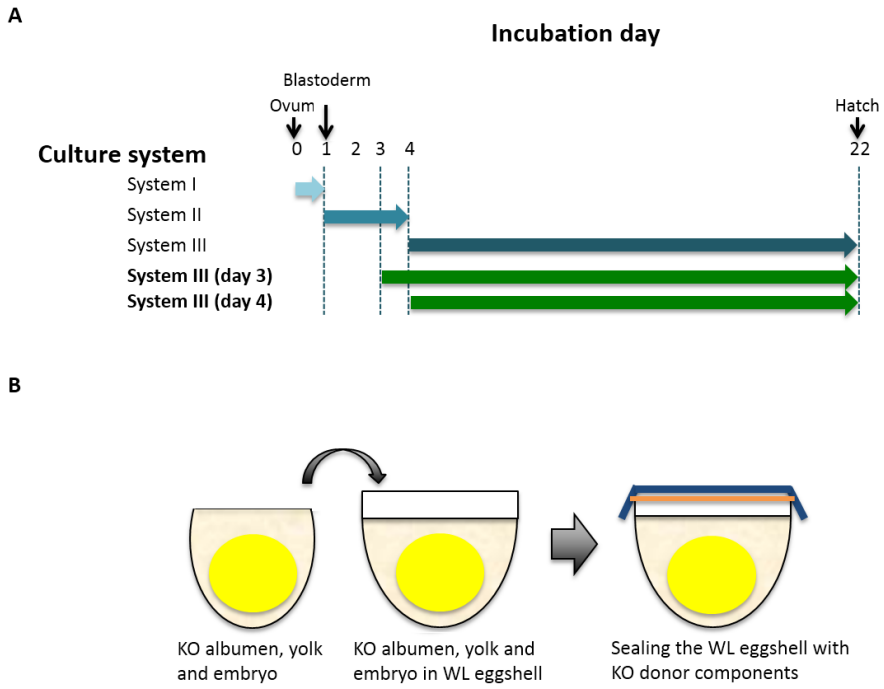


Figure 4-1. Schematic representation of the study. (A) Schematic diagram of Perry's surrogate systems and surrogate system III (3-day-old and 4-day-old) used in this study. (B) Schematic diagram of preparation of surrogate system for the culture of Korean Oge (KO) chickens in White Leghorn (WL) eggshells.

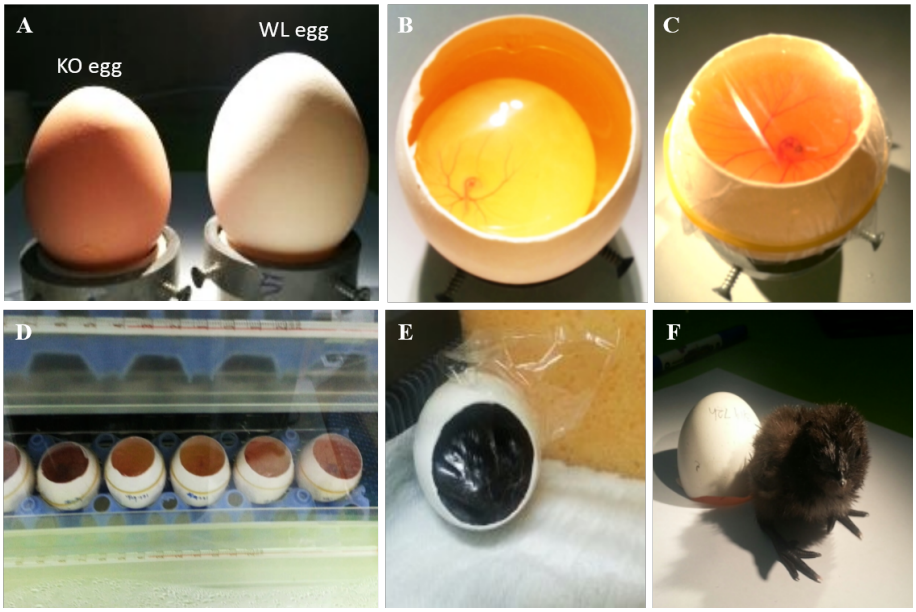


Figure 4-2. Culture of Korea Oge (KO) chicken embryos in White Leghorn (WL) surrogate eggshells. (A) Eggs of KO and WL chickens. (B) KO albumin, yolk and embryo projected with blood vessels in WL surrogate eggshell. (C) Sealing of WL surrogate eggshell with cling film. (D) Incubation of WL surrogate eggshells containing KO embryos 37.5°C with 70% of humidity, and rotated 30° every hour. (E) Removing the cling film before hatching of the KO chick. (F) KO chick hatched from WL surrogate eggshell.

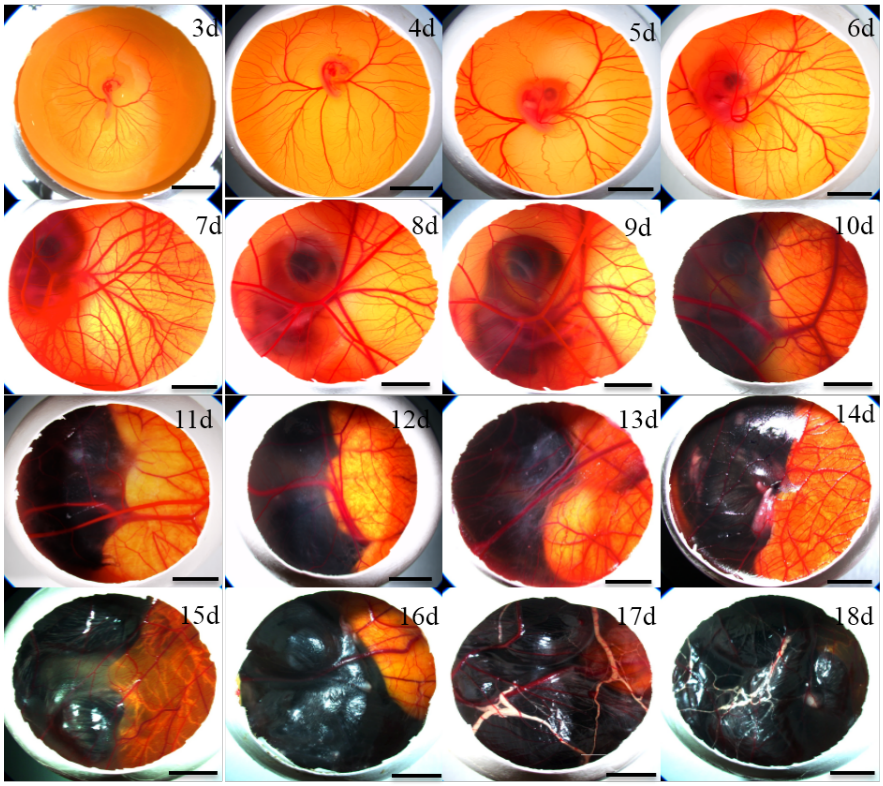


Figure 4-3. Normal development of Korean Oge (KO) chicken embryos in White Leghorn (WL) surrogate eggshells. The KO embryos at 3-day and/or 4-day were transferred into WL surrogate eggshells, and the embryo development was monitored until hatching the chicks.

Table 4-1. Viability percentage of Korean Oge chicken embryos cultured in White Leghorn surrogate eggshells

Incubation day[a]	Surrogate system III	
	3-day-old embryos(%)[b] (n=84)[c]	4-day-old embryos(%)[b] (n= 76)[c]
3	100	-
4	95.0	100
5	92.2	94.1
6	72.8	92.5
7	70.0	87.1
8	59.3	73.7
9	59.3	70.1
10	59.3	66.3
11	55.5	58.2
12	53.0	55.6
13	48.0	55.6
14	43.8	55.6
15	39.6	53.7
16	39.6	52.1
17	34.8	52.1
18	33.4	51.1
19	33.4	48.3
20	23.1	36.0
21	23.1	36.0
22 (Hatch)[d]	23.1(±17.5)[d]	36.0(±22.6)[d]

[a]Also represent the days of embryo development (Hamburger and Hamilton, 1951).

[b] Percentage of viable KO embryos in WL surrogate eggshells.

[c] Total number of KO embryos transferred into WL eggshell.

[d] Values in brackets represent mean±S.D.

Table 4-2. Hatchability percentage of Korean Oge chicken embryos cultured in White Leghorn surrogate eggshells[1]

Trials	Incubation period			
	3-day to hatch		4-day to hatch	
	n	(%)	n	(%)
1	17	11.8	6	16.7
2	7	14.3	5	40.0
3	9	33.3	7	14.3
4	10	10.0	8	25.0
5	10	10.0	11	36.4
6	9	44.4	9	44.4
7	7	57.1	10	90.0
8	6	16.7	9	30.0
9	9	11.1	11	27.3
Average[a]	23.1(±17.5)		36.0(±22.6)	

[a]Values represent mean±S.D.

[1]Hatchability percentage was evaluated on the basis of viable embryos at 22-day of incubation.

Table 4-3. Characteristics and results of different surrogate system conducted in earlier studies

Culture systems	Characteristics	Hatchability (%)	References
System I (Day 0~1) System II (Day 1~3) System III (Day 3~22/23)	System I: Sealing jar with liquid albumen in CO_2 incubator System II: Transfer into recipient eggshell with liquid albumen in incubator angle 90° at 38℃, 40-50% RH System III: Transfer into more large size eggshell in incubator angle 30° at 38℃, 40-60% RH	7%	Perry, 1988
System I (Day 0~1) System II (Day 1~3) System III (Day 3~22/23)	System I: Sealing jar with liquid albumen in CO_2 incubator System II: Transfer into recipient eggshell and replacement of thick albumen by thin albumen or not, and incubation angle 90° at 38℃, 40-50% RH System III: Transfer into more large size eggshell in incubator angle 30° at 38℃, 40-60% RH	34.40%	Naito et al., 1990
System I (Day 0~1) System II (Day 1~3) System III (Day 3~22/23)	System I: Cultured for 24 h at 41.5℃ in a tightly sealed 20ml plastic cup with chicken thin albumen System II: Cultured for 52 h at 37.5℃ while being rocked at an angle of 90° at 30min intervals System III: Cultured at 37.5℃ with rocking at an angle of 30°	25%	Ono et al., 1994
System I (Day 0~1) System II (Day 1~3) System III (Day 3~22/23)	System I: Cultured for 26 h at 41℃ in a tightly sealed 20ml plastic cup with chicken thin albumen System II: Transferred into small recipient eggshells filled with thin albumen System III: Transferred into large recipient eggshells	19.50%	Naito et al., 1995
System II (Day 1~3) System III (Day 3~Hatch)	Embryos cultured with oxygen and calcium environment	30%, 80%	Kanihira et al., 1998

System II (Day 1~3) System III (Day 3~Hatch)	Used turkey eggshells and different sealing films	45%, 75%	Borwornpinyo et al., 2005
System II (Day 1~3) System III (Day 3~22)	Interspecific egg white on the development of chicken embryos	47%, 19%	Liu et al., 2012

4. Discussion

The present study investigated the optimal system for development and hatchability of KO chicken embryos in WL surrogate eggshells. For the fitness of development of donor embryos, WL eggshells which is larger than those of donor embryos (KO) were used as a recipient for the surrogate system (Naito and Perry, 1989). We examined the viability, normal development and hatchability of KO chicken embryos in WL surrogate eggshells. Donor KO embryos at 3-day and 4-day-old were transferred into recipient WL eggshells, incubated for further 18 days at 37.5°C with 70% of humidity until hatching. The viability of 3-day-old KO embryos at 7, 14 and 21 day in surrogate eggshell were 70.0%, 43.8% and 23.1%, respectively. In contrast, the viability of 4-day-old KO embryos at 7, 14 and 21 day in surrogate eggshells were 87.1%, 55.6% and 36.0%, respectively. The hatchability of KO embryos transferred into surrogate eggshells at 3-day-old was 23.1%, whereas embryos transferred at 4-day-old was 36.0%. Furthermore, the development of all viable embryos from 3-day group and 4-day group were normal. These results indicate that KO embryos of different age (3-day or 4-day) could survive and develop normally in WL surrogate eggshells, but the percentage of hatchability from 3-day group and 4-day group suggests that the embryos transferred at 4-day are more stable in the age for transferring into surrogate system, because these embryos are still in the phase of early embryogenesis.

Several previous studies were also attempted to optimize the surrogate system by applying different culture conditions such as eggshell sizes, temperature of incubator, angle of rotor, albumin in the eggshell, humidity etc. In comparison with different condition of surrogate systems, also, shown that the percentage of hatchability varied in all these trials (Table 4-3). In this study, we wanted to identify the optimum starting point for embryo transfer that produce higher viability and hatchability. Previous studies showed that 3-day-old

embryos were easily damaged when they were transferred (Perry, 1988). In this regard, we compared the viability from the surrogate culture system with different developmental timings of donor embryos. As a result, the higher percentage of viability (36.0±22.6) was received from the 4-day group when compared with 3-day group (23.1±17.5) (Table 4-2). The percentage of hatchability was raised over 30% in certain surrogate system used the interspecific egg white as culture medium (Liu et al., 2012) or oxygen with calcium supplement (Kanihira et al., 1998). Also, the replacement of thick albumin by thin albumin in the surrogate eggshells produced over 30% of hatchability (Naito et al., 1990). In this study, KO embryos transferred at 4-day-old were produced over 30% of viability and hatchability. Therefore, we strongly recommend 4-day-old embryos as a stable condition for surrogate eggshell culture.

4. Conclusion

The avian embryos have been used as a good model to study embryonic development. Due to its unique development in the eggshell, avian embryos can be cultured and hatch in the surrogated eggshell system. In this study, we examined the viability, normal development and hatchability of KO chicken embryos in WL surrogate eggshells. Donor KO embryo at 3-day and 4-day-old were transferred into recipient WL eggshell, incubated for further 18 days at 37.5°C with 70% of humidity until hatching. The viability of 3-day-dold KO embryos at 7, 14 and 21 day in surrogate eggshell were 70.0%, 43.8% and 23.1%, respectively. In contrast, the viability of 4-day-old KO embryos at 7, 14 and 21 day in surrogate eggshells were 87.1%, 55.6% and 36.0%, respectively. The hatchability of KO embryos transferred into surrogate eggshells at 3-day-old was 23.1%, whereas embryos transferred at 4-day-old was 36.0%. Furthermore, the development of all viable embryos from 3-day group and 4-day group were normal. Our results suggested that culture of KO embryos in WL surrogate eggshells is highly possible, and transfer of donor embryos at 4-day-old may yield higher percentage of hatchability. This study may provide potential knowledge for the conservation of wild and endangered birds through surrogate system.

CHAPTER 5

Chicken NANOG self-associates via a novel folding-upon-binding mechanism

1. Introduction

The homeobox protein NANOG is a transcription factor that plays a crucial role in maintaining the pluripotency and promoting the self-renewal of pluripotent stem cells (Mitsui et al., 2003, Chambers et al., 2003, Hart et al., 2004, Niwa et al., 2009). In mouse, NANOG is expressed in the compact morula (Mitsui et al., 2003, Chambers et al., 2003, Hart et al., 2004, Niwa et al., 2009) and the primordial germ cells (PGCs) during early development (Yamaguchi et al., 2005). NANOG expression is essential for regulating cell differentiation in the early embryo, and for maintaining the pluripotency of the epiblast shortly after implantation (Mitsui et al., 2003, Siu et al., 2013, Torres-Padilla and Chambers, 2014). In addition, the predominance of NANOG expression in early germ cell development plays an important role in the proliferation and survival of migrating PGCs (Yamaguchi et al., 2005, Yamaguchi et al., 2009, Sanchez-Sanchez et al., 2010). Also, PGC specification, PGC-specific epigenetic modifications, and global enrichment of histone methylation are regulated via NANOG (Murakami et al., 2016).

The NANOG protein consists of an N-terminal domain (NTD), a central homeodomain (HD). The sequence identity is generally low between mammalian, avian, and reptile NANOGs except for the HD. Mammalian NANOGs, however, share tryptophan-repeat (WR) region in the CTD, where a pentapeptide W-x-x-x-x (W, tryptophan; x, polar amino acid) repeats eight to ten times. Mutational analysis revealed that the conserved WR region is critical for the formation of a functional NANOG dimer (Mullin, Yates et al. 2008, Wang, Levasseur et al. 2008, Chang, Tsai et al. 2009). The tryptophan-to-alanine mutations in the WR region disrupted the dimerization, abolishing the pluripotent properties of mouse embryonic stem (ES) cells in the absence of leukaemia inhibitory factor (Mullin et al., 2017, Wang et al., 2008, Mullin et al., 2008).

In avian species, NANOG also has an important role in maintaining the pluripotency and proliferation of chicken ES cells (Lavial et al., 2007), and its expression is restricted to PGCs during early embryo development, similar to mammals (Canon et al., 2006). Avian NANOG is comprised of an NTD, a HD, and a CTD, similar to mammalian NANOG. Avian NANOG, however, lacks the WR motifs that are conserved in the CTD of mammalian NANOG for dimerization (Saunders et al., 2013). It is thus not clear whether avian NANOG forms a dimer to function as a transcription factor, and what governs the molecular association of avian NANOG to achieve a functionally active state. Here, we address the structural characteristics and molecular interaction of chicken NANOG protein (cNANOG) and its biological significance using spectroscopic and biochemical methods as well as cell-based assays. We demonstrate that cNANOG self-associates to form a multimer via a disorder-to-order conformational transition of its CTD. Remarkably, a single mutation can perturb the self-association of cNANOG and abolish the maintenance of PGCs and blastoderm cells.

2. Materials and methods

Cloning, expression, and purification of cNANOG

cNANOG cNTD (residues 1–97) and cCTD (residues 158–309) were cloned into a pET32a vector (Merck Millipore, Darmstadt, Germany) with an N-terminal His_6 and thioredoxin tag and verified by DNA sequencing. The plasmids were introduced into *Escherichia coli* strain BL21star (DE3) (Invitrogen, Carlsbad, CA, USA) cells for expression. Transformed cells were grown in Luria Bertani or minimal medium with $^{15}NH_4Cl$ as the sole nitrogen sources. Protein expression was induced by 1 mM isopropyl-D-thiogalactopyranoside at an OD_{600} of 0.6 ~ 0.8, and the cells were harvested by centrifugation after 16 h of induction at 17°C.

For cNTD purification, the pellets were resuspended in 50 mL (per litre of culture) of 20 mM Tris, pH 7.4, 200 mM NaCl, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonylfloride, lysed using Emulsiflex C3 (Avestin, Ottawa, Canada), and centrifuged at 25,000 × *g* for 30 min. The supernatant fraction was loaded onto a HisTrap HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and the fusion protein was eluted with a 100 mL gradient of imidazole (0–500 mM). The fusion protein was then dialysed against 50 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM β-mercaptoethanol, and the His_6 tag was cleaved by TEV protease. The digestion reaction was loaded onto the HisTrap column and the cleaved protein was fractionated. The protein was further purified and fractionated by size exclusion chromatography using a HiLoad Superdex 75 column (GE Healthcare Bio-Sciences AB) pre-equilibrated with 20 mM Tris, pH 7.4, 200 mM NaCl, and 5 mM β-mercaptoethanol.

cCTD and its truncated derivatives and mutants were lysed using the same lysis buffer, centrifuged, and the pellet was collected and stored at –80°C for 30 min. The pellet was re-suspended in 100 mL of 6 M Urea, 50 mM Tris, pH 8.0, 1 M NaCl, and 5 mM β-mercaptoethanol onto a HisTrap HP column (GE Healthcare Bio-Sciences AB) and refolded on the column using 200 mL of (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM β-mercaptoethanol). After the fusion protein was eluted with a 100 mL gradient of imidazole (0–500 mM), the target protein was further purified using the same procedure of the cNTD purification.

SEC and MALS Analysis

Size exclusion chromatography (SEC) and multi-angle light scattering (MALS) analysis were performed on a Superdex 75 10/300 GL column (GE Healthcare Bio-Sciences AB) at 25°C. The column was calibrated with conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa). The sample was loaded onto a pre-equilibrated column with 50 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM β-mercaptoethanol buffer. Eluted samples were confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. SEC-MALS experiments were performed using an Agilent 1200 high performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) combined with a Wyatt DAWN HELEOS II MALS instrument and a Wyatt OPtilab rEX differential refractometer (Wyatt, Santa Barbara, CA, USA). For chromatographic separation, samples were loaded and eluted in the same column and buffer as used in the SEC analysis. The equipment was pre-calibrated with 1 mg/mL bovine serum albumin (dimer, 133 kDa; monomer, 66 kDa) and the results were normalised and analysed using ASTRA software (Wyatt).

CD spectroscopy

Circular dichroism (CD) spectroscopy was conducted at 25°C using a Jasco J-815 CD spectroscopy. Wave scans were acquired by sampling data at 0.5 nm intervals between 190 and 260 nm for far UV CD measurement. Far UV CD spectroscopy was carried out with 5–10 μM of protein, 20 mM sodium phosphate, pH 7.4, and 1 mM β-mercaptoethanol using a 1 mm quartz cuvette. Each far UV CD spectrum was obtained from an average of three scans and the results were presented as the mean residue ellipticity (deg•cm^2/dmol) at each wavelength.

NMR spectroscopy

The NMR samples contained cNTD, cCTD, and cCTD derivatives in 20 mM sodium phosphate, pH 7.4, 50 mM NaCl, and 5 mM β-mercaptoethanol. NMR spectra were recorded at 25℃ on Bruker AVANCE 600 and 800 MHz spectrometers equipped with a z-shielded gradient triple resonance cyroprobe. NMR spectra were processed using the NMRPipe (Delaglio, Grzesiek et al. 1995) and analysed using the NMRView (Johnson and Blevins 1994) program.

Sequence analysis and comparison

To identify NANOG orthologues, we performed multiple alignment of protein sequences and phylogenetic analysis in the 15 species including zebrafish, mouse, monkey, human, chimpanzee, pig, bovine, cattle, bovine, goat, snake, turtle, alligator, woodpecker, chicken, and quail using the ClustalX (http://www.clustal.org).

Chicken PGC and chicken blastoderm cell culture

Chicken PGCs (cPGCs) were cultured in accordance with our standard procedure (Park and Han, 2012). Undifferentiated stage X blastoderm cells were

prepared as previously described (Lee et al., 2011, Hwang et al., 2016). Blastoderm cells collected from the White Leghorn chicken embryos at EGK.X stage and dissociated mechanically into single cells. Single blastoderm cells were centrifuged (850 rpm, 3min) and washed twice in PBS. Blastoderm cells were culture according to previous descriptions with minor modified conditions (Nakanoh et al., 2013). Cells were cultured in N2B27/2i medium containing DMEM/F-12 (GIBCO Invitrogen, Grand Island, NY, USA), Neurobasal (Gibco), 55 mM β-mercaptoethanol (Gibco), 200 mM L-glutamine (Gibco), N2-Supplement (100×, Gibco), and B27 supplement-vitamin A (50×, Gibco). The medium was supplemented with two inhibitors, 3 μM CHIR99021 and 1 μM PD0325901 (Stemgent, San Diego, CA, USA).

cNANOG knockdown and enforced cNANOG expression

To shut down the endogenous cNANOG expression, *cNANOG*-specific siRNA (5'-AAC UGU GUG AUA CUA UCA GUU-3') was designed against unique regions. Expression vectors of wild-type cNANOG and the AAA mutant were constructed using a PCR strategy. We used primers for 5' and 3' ends of *cNANOG* cDNA, or primers for the triple mutation region (Y255/Y258/Y264) in the CTD of *cNANOG* cDNA for the PCR (Table 5-1). The PCR products contained EcoRI sites at each end, and were subsequently cloned into the EcoRI sites of the pCE-hSK vector (Addgene plasmid #41814).

Sequential transfection of PGCs and blastoderm cells was performed by the transfection with *cNANOG*-specific siRNA for 24 hours, followed by the transfection with expression vectors of wild-type cNANOG or the AAA mutant for 48 hours in fresh medium. We used cPGCs and blastoderm cells transfected with non-targeting siRNA as a control. The siRNA was transfected into *in vitro* cultured PGCs or blastoderm cells using lipofectamine RNAiMAX (Thermo Fisher Science Inc., Waltham, MA, USA). The vectors of wild-type or mutant

cNANOG were transfected into *in vitro* cultured PGCs or blastoderm cells using lipofectamine 2000 (Thermo Fisher Science Inc.).

qRT-PCR of pluripotency marker genes and cell proliferation assay

After sequential transfection, total RNA was extracted using TRIzol reagent (Invitrogen) and analysed using quantitative reverse transcription PCR (qRT-PCR). Relative expression levels of pluripotency marker genes (endogenous and exogenous *cNANOG,* endogenous *cNANOG and cPOUV*) were calculated after the threshold cycle, and normalised to chicken glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The sequences of the endogenous and exogenous *cNANOG*, endogenous *cNANOG*, *cPOUV*-specific and *GAPDH* primers are shown in Table 5-2.

Effect of *cNANOG* knockdown and overexpression of wild-type or mutant cNANOG on the cell proliferation was quantified using the water-soluble tetrazolium-1 (WST-1) cell proliferation reagent (TaKaRa, Tokyo, Japan). The WST-1 assay employs water-soluble and membrane-impermeable tetrazolium salts to assess cell viability and cytotoxicity. Viable cells reduce WST-1 to a formazan using the mitochondrial dehydrogenase, which can be monitored by the optical absorption spectroscopy with high sensitivity. cPGCs and blastoderm cells were seeded into 96-well plates at a density of 1.5 x 10^4 cells per well in 100 µL medium. Cell viability was measured by differential optical absorbance at the wavelengths of 450 nm and 650 nm (A_{450}−A_{650}) at indicated time points according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism statistical software (GraphPad Software, La Jolla, CA, USA). Significant differences

between groups were evaluated by one- or two-way ANOVA against control and treatment groups. A value of $P < 0.05$ indicated statistical significance.

3. Results

Avian NANOG has a NTD and a CTD distinct from mammalian NANOG

The protein sequence alignment shows that NANOGs in chicken, mouse, and human are divided into NTD, HD, and CTD in a similar manner (Figure 5-1A). The HD is well conserved among all three species with 66–86% of pairwise sequence identity (Figure 5-1D) (Saunders, Faiola et al. 2013), whereas the NTD and CTD are divergent between avian and mammalian NANOGs (Figure 5-1, C and E). In particular, avian NANOG does not contain the conserved WR region of mammalian NANOG in the CTD (Figure 5-1B). The sequences of the NTD and CTD bear similarity among avian NANOGs and some of reptile NANOGs (Figure 5-1, C and E; Figure S5-1).

The NTD of cNANOG is a disordered monomer

Mammalian NANOG generally employs the CTD for functional dimerization (Mullin et al., 2008, Wang et al., 2008, Chang et al., 2009), whereas zebrafish NANOG employs the NTD for dimerization (Schuff et al., 2012). We first examined whether the NTD of cNANOG (cNTD; residues 1–97) is required for dimerization. Size exclusion chromatography with inline multi-angle light scattering (SEC-MALS) showed that cNTD eluted as a monomer with a molecular mass of 11.6 ± 0.7 kDa (Figure 5-2A). The circular dichroism (CD) spectra of cNTD showed a negative band around 195 nm and lacked the characteristic ellipticity originating from α-helices or β-strands, indicating the absence of regular secondary structures (Figure 5-2B). Further, the $^{1}H–^{15}N$ heteronuclear single quantum correlation NMR spectra of cNTD exhibited poorly dispersed backbone amide resonances, which are typically observed in unfolded proteins (Figure 5-2C). Taken together, cNTD was largely disordered in solution and did not show any sign of self-association.

cNANOG self-associates via the disordered CTD that switches into a helix

The CTD of cNANOG (cCTD, residues 158–309) eluted as two peaks in the SEC-MALS experiment (Figure 5-3A). The major peak eluted as a large multimer in the void volume of the Superdex 75 column, and the minor peak eluted as a monomer. The SEC-MALS data from the multimer fraction of cCTD showed a broad distribution of molecular mass ranging from 30 to 50 MDa, which was a thousand-fold larger than that of the cCTD dimer (34 kDa) (Figure 5-3A). We separated the cCTD multimer and monomer using SEC, and obtained the CD spectra to identify the secondary structures of each state. The cCTD multimer exhibited two negative bands at 222 and 208 nm, which is a signature of α-helical structures (Figure 5-3B). Unexpectedly, the cCTD monomer exhibited a CD spectrum of a disordered protein (Figure 5-3B).

We examined whether the cCTD monomer is in equilibrium with the helical multimer or remains as a permanent monomer due to misfolding. The populations of cCTD multimers and monomers exchanged in a concentration-dependent manner, such that the disordered cCTD monomers associated to form helical higher-order multimers upon concentration (Figure 5-3C). Our result demonstrates that cCTD undergoes a conformational transition from a disordered state to an α-helix upon self-association. In addition, the fact that cCTD self-associates to form such a large multimer suggests that individual cCTD subunits are likely to elongate in a head-to-tail manner. Thus, cCTD represents a novel protein–protein interaction motif that is distinct from the WR motif of mammalian NANOG.

The CD measurement employed 5 μM of cCTD to avoid the saturation of CD signals. The injection concentration of cCTD for the MALS measurement was 70 μM, but serial dilutions down to 3 μM did not change the elution volume, indicating that the molecular weight of the multimer remained

unchanged. Thus, the cCTD multimer was not disrupted at concentrations for the CD measurement. We also performed the size exclusion chromatography of the cCTD multimer sample for the CD measurement, and confirmed that the cCTD multimer fully maintained its multimeric state. It is notable that cCTD monomers progressively formed a multimer upon concentration, but the multimer, once formed, did not immediately dissociate back to the monomer upon dilution. The slow dissociation kinetics might be attributed to the unfavourable cCTD unfolding that accompanies the dissociation of cCTD multimer.

Novel hydrophobic interaction motif for cNANOG self-association

We employed a series of cCTD truncation mutants to define the minimal domain size for self-association (Figure 5-4A). Up to 42 residues of the C-terminus of cCTD could be progressively removed without affecting the self-association, and $cCTD_{158–267}$ formed a multimer similar to that produced by an untruncated cCTD (Figure 5-4B). Further C-terminal truncation of cCTD prevented the self-association, and $cCTD_{158–253}$ was exclusively a monomer from SEC (Figure 5-4B). N-terminal truncation was less tolerant, and the deletion of eight residues ($cCTD_{166–267}$) completely abolished the self-association (Figure 5-4B). The CD spectra indicated that the cCTD truncation mutants capable of self-association maintained the α-helical structure, whereas monomeric $cCTD_{158–253}$ and $cCTD_{166–267}$ were disordered (Figure 5-4C). We note that $cCTD_{158–267}$ formed both a multimer and a monomer as was observed in untruncated cCTD. In addition, the $cCTD_{158–267}$ multimer adopted an α-helical structure, whereas the $cCTD_{158–267}$ monomer was disordered (Figure 5-4D). Taken together, $cCTD_{158–267}$ fully maintained the self-associating properties of untruncated cCTD, as well as the disorder-to-helix conformational transition upon association. Hence, our finding defines the minimal length for cCTD self-association as residues 158–267.

Although cCTD does not contain the WR motif, we hypothesised that aromatic residues might provide key intermolecular interactions for cCTD self-association. Since $cCTD_{158–267}$ maintained the self-association, whereas $cCTD_{158–253}$ did not, we examined the conserved aromatic residue between residues 254–267. Tyr255 and Tyr258 were found highly conserved across all species, and Tyr264 was conserved among avian and reptile species (Fig. 1C; Figure S5-1B). We mutated three tyrosine residues to alanine, individually and in combination (Figure 5-5A). We first discovered that the AAA triple mutant (AAA) was exclusively a monomer and disordered (Figure 5-5, B and C). We then examined the single mutant of each tyrosine residue to identify the key residue for the association. A single mutation of Tyr255 (AYY mutant) completely prevented the self-association, and the AYY mutant remained disordered as a monomer. The mutation of Tyr264 (YYA mutant) also resulted in a monomer, but a small population of multimers was observed (Figure 5-5B). In addition, the CD spectra indicated the YYA mutant partially maintained the helical structure. Tyr258 was least important, and the YAY mutant was able to form a helical multimer. Double mutation of Tyr258 and Tyr264 (YAA mutant), however, completely prevented the self-association, and the YAA mutant was largely disordered. In summary, the aromatic side chain of Tyr255 was critical for the cCTD self-association, and that of Tyr264 also significantly contributed to the self-association. The single mutation of Tyr258 did not cause a noticeable change in the self-association of cCTD, but the double mutations of Tyr258 and Tyr264 completely prevented the self-association.

Self-association of cNANOG is a prerequisite for proliferation of chicken PGCs and blastoderm cells

For functional assessment of the hydrophobic interaction motif (Tyr255, Tyr258, and Tyr264) in the cNANOG protein, we examined the impact of the AAA triple mutation (AAA) on the proliferation of chicken PGCs and

blastoderm cells that represent undifferentiated embryonic stem cells (Pain et al., 1996, Lavial et al., 2009). We prepared two types of cNANOG expression vectors that induced the wild-type (WT) cNANOG protein or the AAA mutant (Figure 5-6A). Small interfering RNA (siRNA) to mediate *cNANOG* knockdown (*cNANOG*-KD) was transfected into the cultured cPGCs and blastoderm cells to achieve endogenous cNANOG depletion. *cNANOG*-KD greatly suppressed the proliferation of both cPGCs and blastoderm cells from the WST-1 assay (Figure 5-6, B and C). Overexpression of WT cNANOG rescued the proliferation of cPGCs and blastoderm cells after *cNANOG*-KD to a level comparable to the control, but overexpression of the AAA mutant did not restore the cell proliferation (Figure 5-6, B and C). Our findings demonstrate that the self-association of cNANOG is critical to promote both cPGC and blastoderm cell proliferation.

We further investigated the impact of the self-association of cNANOG on the transcriptional regulation of the pluripotency gene network. We employed qRT-PCR to assess the expression of pluripotency related genes such as *POUV* and *NANOG*. For *cNANOG*, we designed primers from the 5′ untranslated regions of *cNANOG* to detect endogenous *cNANOG* only, and primers from the coding region of *cNANOG* to detect the both the endogenous and exogenous *cNANOG* (Figure 5-6D). The expression of endogenous *cNANOG* significantly reduced by *cNANOG*-KD as expected, whereas the expression of c*NANOG* was upregulated upon the overexpression of WT cNANOG or AAA mutant, indicating increased expressions of exogenous *cNANOG* (Figure 5-6, E and F, *left* panels). The expression of endogenous *cNANOG* was kept repressed during the subsequent transfection and expression of exogenous *cNANOG* (Figure 5-6, E and F, *left* panels). Knockdown of endogenous *cNANOG* largely suppressed the expression of *POUV* both in cPGCs and blastoderm cells (Figure 5-6, E and F, *right* panels). Remarkably, overexpression of WT cNANOG significantly enhanced the expression of

POUV, but overexpression of the AAA mutant failed to restore the expression of *POUV* (Figure 5-6, E and F, *right* panels). Thus, the self-association of cNANOG is highly important to maintain the expression of pluripotency related *POUV* that enables the proliferation of cPGCs and blastoderm cells.

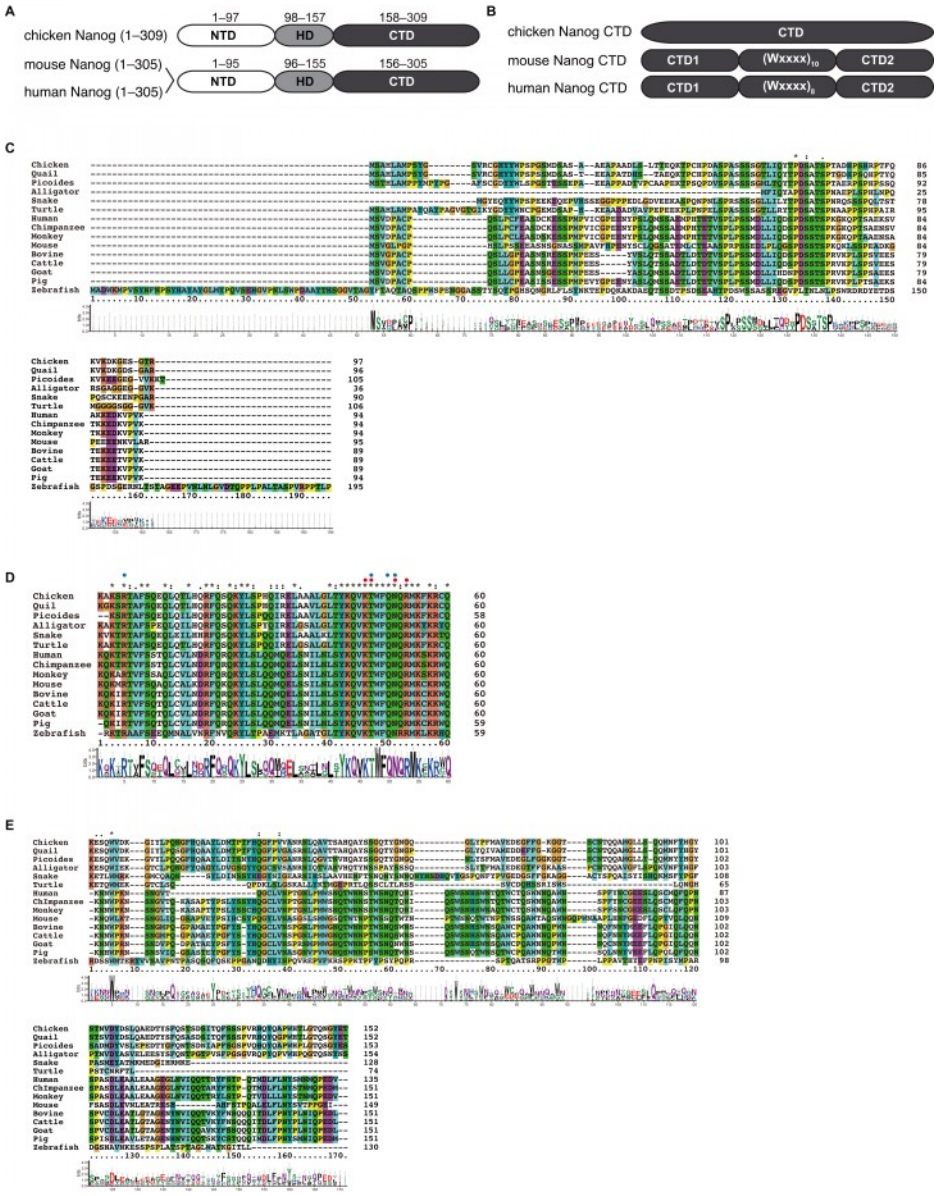


Figure 5-1. Domain organisation and multiple sequence alignment of NANOG. (A) The domain organization of the N-terminal domain (NTD), the homeodomain (HD), and the C-terminal domain (CTD) in chicken, mouse, and human NANOGs. (B) Comparison of the CTD organization between chicken, mouse, and human NANOGs. The tryptophan-repeat regions are denoted by $(Wxxxx)_{10}$ and $(Wxxxx)_8$ for mouse and human NANOGs flanked by CTD1 and CTD2 sequences. Multiple sequence alignment of (C) the NTD, (D) the HD (E)

and the CTD of NANOG from different species. The HD sequences are highly conserved across 15 species, whereas the NTD and CTD sequences are similar between avian NANOGs, but different from mammalian NANOGs in length and similarity. The NTD of avian NANOGs are longer than that of mammalian NANOGs, whereas the CTD of avian NANOGs are shorter than that of mammalian NANOGs owing to the absence of the tryptophan-repeat (WR) region. Conserved residues are annotated with asterisks (identical residues), and dots (similar residues) above the sequence alignment. Filled red circles above the HD sequence denote the OCT4 interaction sites of human NANOG, and blue circles denote Oct4-CR2 and Tcf3 interaction sites of mouse NANOG.

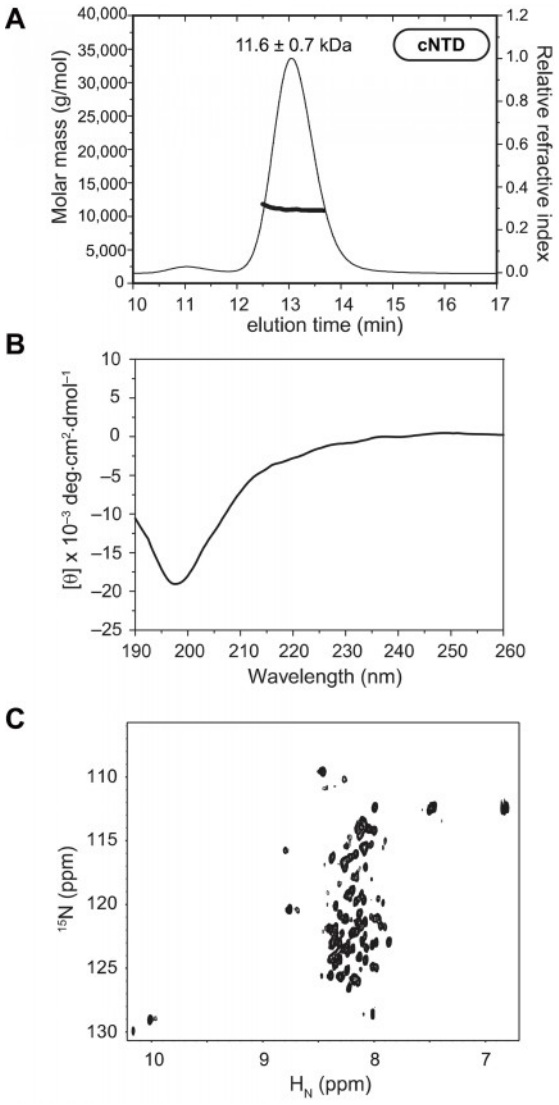


Figure 5-2. Characterisation of the N-terminal domain of chicken NANOG (cNTD). (A) Size exclusion chromatography and multi-angle light scattering data of the cNTD with the injection concentration of 0.5 mM. The molecular masses obtained from the light scattering and refractive index measurements correspond to the monomer of cNTD. (B) The far UV circular dichroism spectrum of the 5 µM cNTD in 20 mM sodium phosphate, pH 7.4, and 1 mM β-mercaptoethanol. (C) The 2D $^{1}H–^{15}N$ HSQC NMR spectrum of the 0.3 mM cNTD in 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, and 5 mM β-mercaptoethanol.

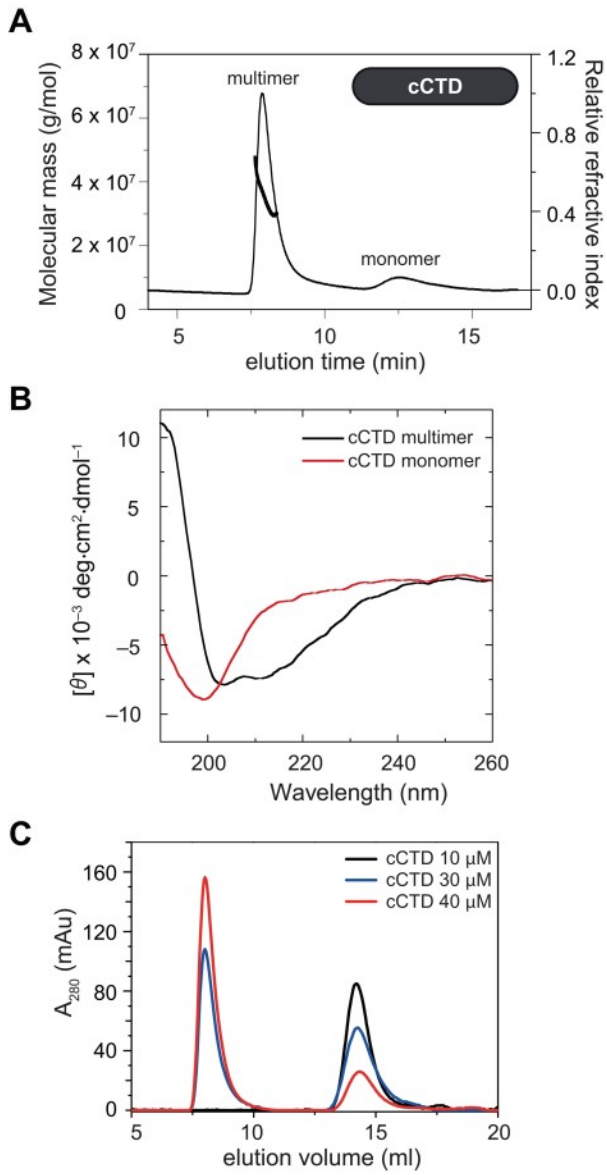


Figure 5-3. Characterisation of the C-terminal domain of chicken NANOG (cCTD). (A) Size exclusion chromatography and multi-angle light scattering data of the cCTD with the injection concentration of 70 µM. (B) The far UV circular dichroism spectra of the cCTD multimer (*black*) and the cCTD monomer (*red*). (C) Population distribution of cCTD multimers and monomers according to the injection concentrations. The injection concentrations of cCTD were 10 (*black*), 30 (*blue*), and 40 µM (*red*).

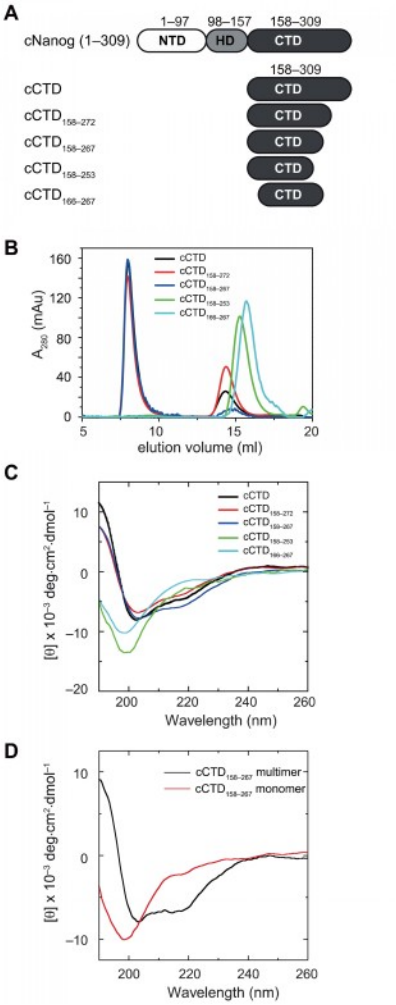


Figure 5-4. Impact of N- and C-terminal truncations on the self-association and secondary structures of cCTD. (A) Schematic diagram of cNANOG and the cCTD truncation mutants. (B) Size exclusion chromatograms using a Superdex 75 10/300 GL column for the cCTD truncation mutants. cCTD, $cCTD_{158–272}$, $cCTD_{158–267}$, $cCTD_{158–253}$, and $cCTD_{166–267}$ are coloured in *black*, *red*, *blue*, *green*, and *cyan*, respectively, as annotated. (C) Far UV circular dichroism spectra of the cCTD and cCTD truncation mutants. The protein concentration was 5 μM, and the colour scheme is the same as in (B). (D) Far UV circular dichroism spectra of the multimer (*black*) and the monomer (*red*) of $cCTD_{158–267}$. The $cCTD_{158–267}$ multimer exhibited an α-helical structure, whereas the $cCTD_{158–267}$ monomer was disordered.

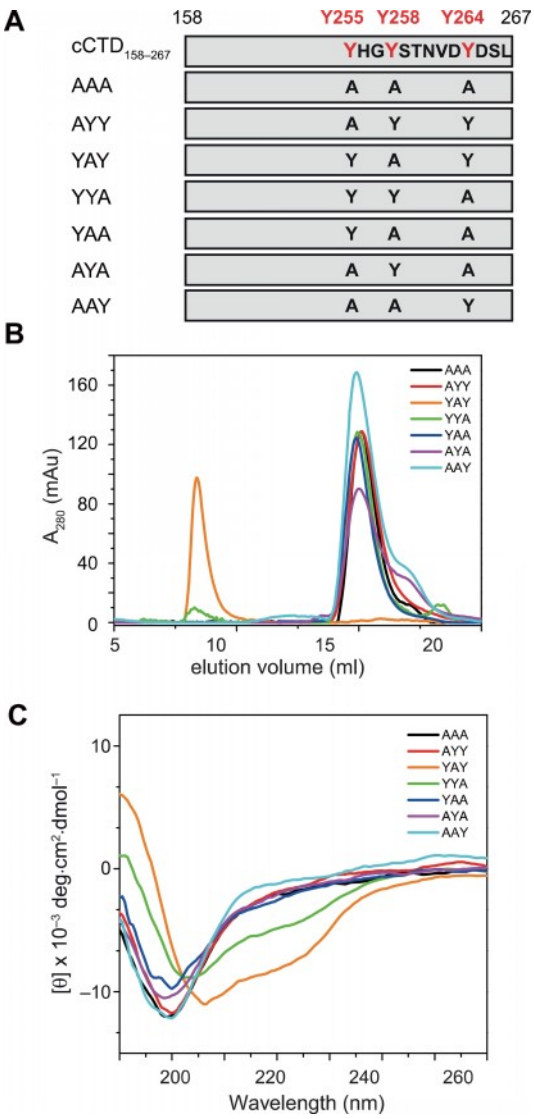


Figure 5-5. Role of conserved aromatic residues in the self-association of cCTD. (A) Schematic diagram of the cCTD mutations for three conserved tyrosine residues: Tyr255, Tyr258, and Tyr264. The triple mutant (AAA), single mutants (AYY, YAY, and YYA), and double mutants (YAA, AYA, and AAY) are described. (B) Size exclusion chromatograms using a Superdex 75 10/300 GL column for the cCTD mutants. The elution profile of the AAA mutant is shown in *black*, and the other mutants are shown in the rainbow colour scheme. (C) Far UV circular dichroism spectra of the various cCTD mutants. The ellipticity of individual mutants are drawn using the same colour scheme as in (B).

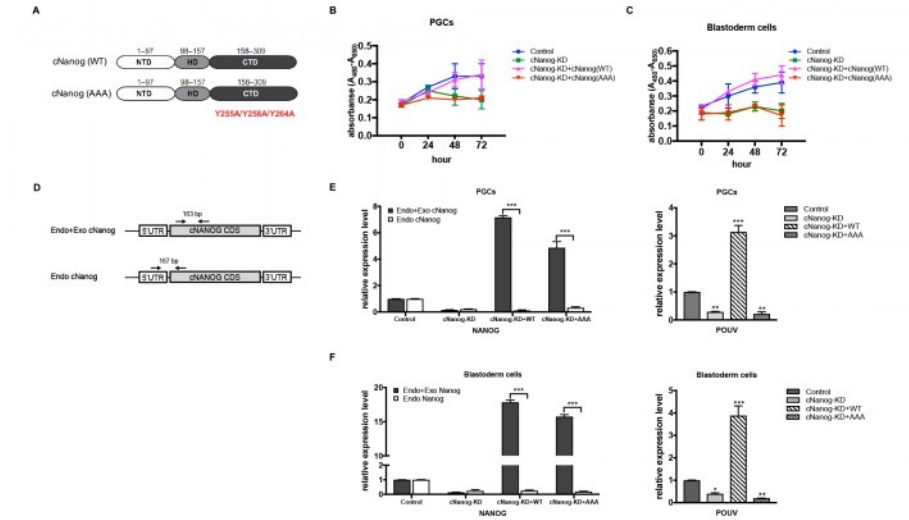


Figure 5-6. Impact of the cNANOG self-association on the proliferation of primordial germ cells (PGCs) and blastoderm cells. (A) Schematic diagram of the wild-type cNANOG (WT) and the triple mutant cNANOG (AAA; Tyr255Ala/Tyr258Ala/Tyr264Ala). WST-1 analysis of cPGCs (B) and blastoderm cells (C) transfected with control siRNA, *cNANOG* siRNA, sequentially transfected with WT or AAA for 2 days after *cNANOG* siRNA transfection for 1 day. Bar indicate the SEM or triplicate analyses. (D) Diagram showing the primers used to identify the endogenous and exogenous cNANOG. (E, F) Relative expression of endogenous and exogenous *cNANOG* (*left* panels), and *cPOUV* (*right* panels) analysed by qRT-PCR. *In vitro* cultured cPGCs (E) and blastoderm cells (F) were transfected with siRNA for 24 hr (cNANOG-KD), and sequentially transfected with expression vectors of WT cNANOG or the AAA mutant for 48 hr in fresh medium. Bars indicate the standard error of the mean (SEM) of triplicate analyses. *P<0.033, **P<0.002, and ***P<0.001 compared to the control. PGCs, primordial germ cells; Endo, endogenous; Exo, exogenous; *cNANOG*-KD, chicken *NANOG* knockdown.

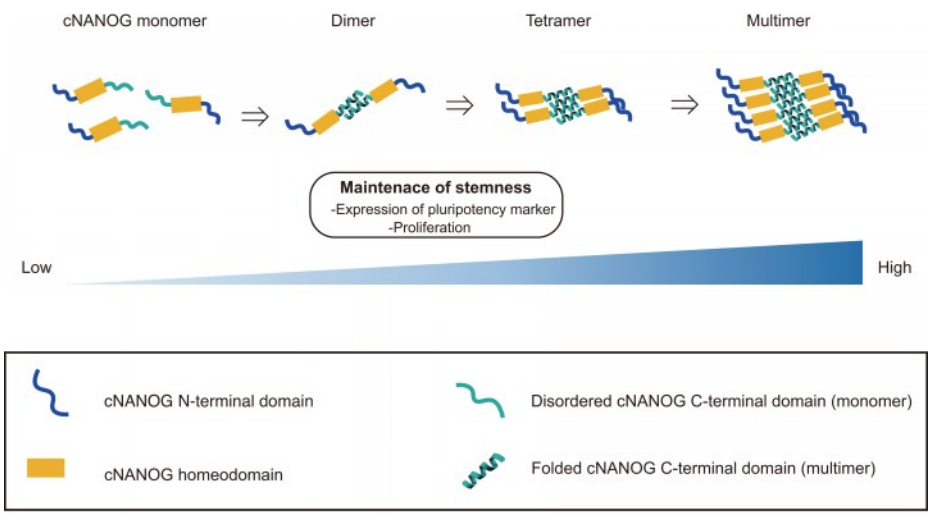
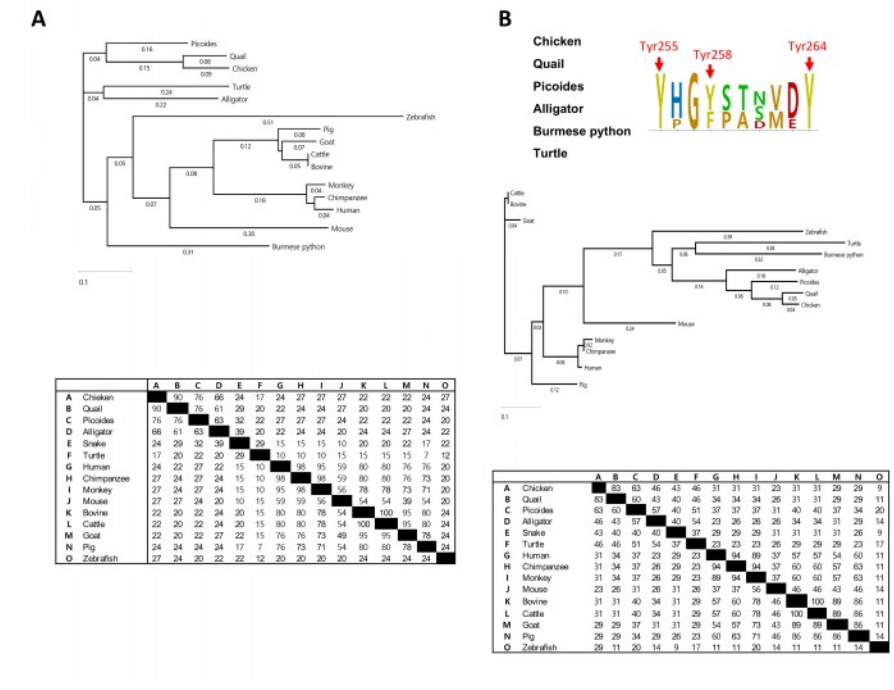


Figure 5-7. Proposed model for the molecular mechanism of cNANOG self-association. cNANOG self-associates to form a high-order multimer through hydrophobic motifs within the CTD via a folding-upon-binding mechanism. The CTD is disordered as a monomer that switches into a helical multimer upon association. The disordered NTD is shown as a blue line, and the HD is shown as an orange rectangle. The disordered CTD monomer is shown as a green line, and the helical CTD multimer is shown as a green helix.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A Chicken		90	76	66	24	17	24	27	27	27	22	22	22	24	27
B Quail	90		76	61	29	20	22	24	24	27	20	20	20	24	24
C Picoides	76	76		63	32	22	27	27	27	24	22	22	22	24	20
D Alligator	66	61	63		39	20	22	24	24	20	24	24	27	24	22
E Snake	24	29	32	39		29	15	15	15	10	20	20	22	17	22
F Turtle	17	20	22	20	29		10	10	10	15	15	15	15	7	12
G Human	24	22	27	22	15	10		98	95	59	80	80	76	76	20
H Chimpanzee	27	24	27	24	15	10	98		98	59	80	80	76	73	20
I Monkey	27	24	27	24	15	10	95	98		56	78	78	73	71	20
J Mouse	27	27	24	20	10	15	59	59	56		54	54	39	54	20
K Bovine	22	20	22	24	20	15	80	80	78	54		100	95	80	24
L Cattle	22	20	22	24	20	15	80	80	78	54	100		95	80	24
M Goat	22	20	22	27	22	15	76	76	73	49	95	95		78	24
N Pig	24	24	24	24	17	7	76	73	71	54	80	80	78		24
O Zebrafish	27	24	20	22	22	12	20	20	20	20	24	24	24	24	

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A Chicken		83	63	46	43	46	31	31	31	23	31	31	29	29	9
B Quail	83		60	43	40	46	34	34	34	26	31	31	29	29	11
C Picoides	63	60		57	40	51	37	37	37	31	40	40	37	34	20
D Alligator	46	43	57		40	54	23	26	26	26	34	34	31	29	14
E Snake	43	40	40	40		37	29	29	29	31	31	31	31	26	9
F Turtle	46	46	51	54	37		23	23	23	26	29	29	29	23	17
G Human	31	34	37	23	29	23		94	89	37	57	57	54	60	11
H Chimpanzee	31	34	37	26	29	23	94		94	37	60	60	57	63	11
I Monkey	31	34	37	26	29	23	89	94		37	60	60	57	63	11
J Mouse	23	26	31	26	31	26	37	37	37		46	46	43	46	14
K Bovine	31	31	40	34	31	29	57	60	60	78		100	89	86	11
L Cattle	31	31	40	34	31	29	57	60	60	78	100		89	86	11
M Goat	29	29	37	31	31	29	54	57	57	73	89	89		86	11
N Pig	29	29	34	29	26	23	60	63	63	71	86	86	86		14
O Zebrafish	9	11	20	14	9	17	11	11	11	20	14	11	11	11	14	

Figure S5-1. **Phylogenetic tree and sequence pair distances of the NANOG sequences of from different species.** (A) The sequence analysis of cNTD, and (B) the sequence analysis of cCTD. From the phylogenetic tree and sequence pair distances, avian and mammalian NANOGs reveal low sequence identity in the NTD and CTD. Interestingly, the tree shows highly conserved CTD homology between avian and reptilian NANOGs. The phylogenetic tree shows evolutionary relationships estimated from the multiple sequence alignment using the BioNJ algorithm, a variant of the Neighbour-Joining algorithm in MegAlign (DNAStar, USA). The bar indicates 1% divergence.

Table 5-1. chicken NANOG site-directed mutagenesis primers

Gene	Primer sequences	
	Forward (5'→3')	Reverse (5'→3')
cCTD $_{158\text{-}272}$	GACAGCTTGCAGGCAGAAGATACCTA GTAGTTCCAGAGCACCTC	GAGGTGCTCTGGAACTACTAGGTAT CTTCTGCCTGCAAGCTGTC
cCTD $_{158\text{-}267}$	CAATGTGGATTATGACAGCTTGTAGTA GGAAGATACCTACAGCTTCCAGA	TCTGGAAGCTGTAGGTATCTTCCTAC TACAAGCTGTCATAATCCACATTG
cCTD $_{158\text{-}253}$	GGTTTATTAAGTCAACAGATGAACTAG TAGCATGGCTACTCTACCAATGTGGAT T	AATCCACATTGGTAGAGTAGCCATG CTACTAGTTCATCTGTTGACTTAATA AACC
cCTD $_{166\text{-}267}$	AAAGGGATTTATCTACCACAGAATGG GTTTCATCAAGCT	GGGTGAAAACCTGTATTTTCAGGGA
AAA	TCTACCAATGTGGATGCTGACAGCTTG	CAGATGAACTTCGCTCATGGCGCC
AYY	ATGAACTTCGCTCATGGCTACTCTACC AATGTGGATTAT	ATAATCCACATTGGTAGAGTAGCCAT GAGCGAAGTTCAT
YAY	ATGAACTTCTATCATGGCGCCTCTACC AATGTGGATTAT	ATAATCCACATTGGTAGAGGCGCCA TGATAGAAGTTCAT
YYA	TACTCTACCAATGTGGATGCTGACAGC TTGCAGGCA	TGCCTGCAAGCTGTCAGCATCCACA TTGGTAGAGTA
YAA	TCTACCAATGTGGATGCTGACAGCTTG	CAGATGAACTTCTATCATGGCGCT
AYA	TCTACCAATGTGGATGCTGACAGCTTG	CAGATGAACTTCGCTCATGGCTAC
AAY	TCTACCAATGTGGATTATGACAGCTTG	CAGATGAACTTCGCTCATGGCGCC

Table 5-2. Primers used for quantitative real-time PCR

Gene	Primer sequences	
	Forward (5'→3')	Reverse (5'→3')
GAPDH	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA
Endo+Exo NANOG	ACTACTACTGGCCCTCTCCG	AGTGGCAGAGTCTGGGGTAT
Endo NANOG	GTATCTCCGTGCAGTAGGCG	GGCAGACGCGCTATCCA
POUV	TGAAGGGAACGCTGGAGAGC	ATGTCACTGGGATGGGCAGAC

4. Discussion

Transcription factors generally function as a dimer or a tetramer to achieve higher specificity. The transcription factor NANOG also forms a dimer or an oligomer to maintain the pluripotency of ES cells. In addition, pluripotent factors interaction mediated by NANOG dimerization promote proliferation (Dixon et al., 2010). Mammalian NANOG self-associates via the WR motif in the CTD (Mullin et al., 2008, Wang et al., 2008), but avian NANOG lacks the WR motif, and exhibits low sequence similarity with mammalian NANOG in the CTD (Figure 5-1A). Here we demonstrate that the avian NANOG forms a high-order multimer through its CTD via hydrophobic interactions distinct from mammalian NANOG (Figure 5-3 and Figure 5-5). Remarkably, cCTD remains disordered as a monomer, but switches into a helix upon self-association in a concentration-dependent manner (Figure 5-3 and Figure 5-4). Mutations of key aromatic residues perturbed the self-association, and the monomeric mutant failed to replace pluripotency related gene, *POUV*, in chicken PGCs and blastoderm cells (Figure 5-6). Thus, these data suggest that NANOG self-association may act important role cooperating with pluripotent factors in pluripotency networks. Our study illustrates that cNANOG forms a functional multimer via a novel hydrophobic interaction motif, extending the established self-association mechanism of mammalian NANOG.

Structural data have been scarce for NANOG except for HD as DNA binding domain (Figure 5-1D) (Jauch et al., 2008, Hayashi et al., 2015). We combined SEC, CD, and NMR spectroscopy with an extensive mutagenesis of cNANOG to define the domain length and key interfacial residues for the self-association of cNANOG. Both cNTD and cCTD belong to an intrinsically disordered protein (IDP) family. IDPs function as hub proteins in the protein–protein interaction network, and play a central role in the regulation of transcription and translation (Dunker et al., 2001, Liu et al., 2006, Kim et al.,

2008). IDPs can employ the disordered region to engage with multiple binding partners (Dunker et al., 2001), and can be promptly activated and deactivated in response to environmental changes with rapid turnover (Dyson and Wright, 2005, Gsponer et al., 2008, Tompa et al., 2008). The fact that cCTD exists as a disordered monomer at low concentration implicates that monomeric cCTD may form multiple scaffolds to recruit other proteins. The expression level of cNANOG would then determine the self-association for proper transcriptional regulation. Given that NANOG is central to the signal transduction network for the maintenance of PGCs and ES cells, the intrinsic disorder of cNTD and cCTD may facilitate the interactions with multiple partner proteins, and immediate signalling for transcriptional activation.

It is notable that cCTD self-associates to form a large multimer with an undefined stoichiometry. We speculate that individual cCTD subunits interact with one another in a head-to-tail manner by heterologous association, which was observed in the auxin-response transcription factors and repressors in plants (Han et al., 2014, Korasick et al., 2014, Nanao et al., 2014). The disorder-to-helix transition upon self-association, however, is unique to cCTD. cCTD exchanges between a large multimer and a monomer depending on concentrations, but we could not observe the intermediate-size oligomers. This strongly suggests that cCTD self-associates in a cooperative manner, such that the initial association of a few cCTD molecules may serve as the nucleation core for the kilo-subunit cCTD assembly (Figure 5-7). We note, however, that NANOG is not likely to function as a huge multimeric aggregate. NANOG may exist as a low-order oligomer at dilute cellular concentrations. Transcription factors are produced and degraded promptly according to cell signals, and do not tend to accumulate to a large quantity. Our study highlights that NANOG has a strong tendency toward self-association, and that the multimer (dimer, tetramer, etc.) does not readily dissociate when concentration drops, since the self-association is driven by favorable free energy of folding. We postulate that the

coupled folding and self-association mechanism enables NANOG to maintain a functional dimer at a very dilute concentration. The tyrosine residues critical for the self-association of chicken NANOG are highly conserved among avian and reptile species (Figure 5-5 and Figure S5-1). It is unknown whether reptile NANOG self-associates to function, but we speculate that reptile NANOG may also form a multimer via the conserved tyrosine residues in the CTD.

It has been reported that chicken NANOG can replace mouse NANOG to induce and maintain pluripotency (Theunissen, Costa et al. 2011). Given the high sequence similarity between HDs of chicken and mouse NANOGs, we infer that both NANOGs would function in a similar manner via DNA binding of the HD and self-association of the CTD. It has also been reported that the HD of mouse NANOG is sufficient to induce naive pluripotency in NANOG$^{-/-}$ somatic cells (Theunissen et al., 2011). Self-association of NANOG was prerequisite for self-renewal and pluripotency maintenance of mouse embryonic stem cells (Wang et al., 2008, Mullin et al., 2008) and cPGCs (this study). Thus, the HD was sufficient for pluripotency induction of somatic cells, but self-renewal and pluripotency maintenance of stem cells and cPGCs likely require the CTD for self-association as well as the HD. It remains to be seen whether NANOG has different functions as a monomer and as a multimer.

In summary, we have demonstrated that cNANOG self-associates to form a larger multimer via key hydrophobic interactions in the CTD, which is distinct from the self-association of mammalian NANOGs. CTD of cNANOG is a disordered as a monomer, and adopts a helical conformation upon self-association, illustrating a folding-upon-binding mechanism underlying the self-association. We have identified conserved aromatic residues required for the self-association, which is crucial for the maintenance of chicken PGCs and blastoderm cells. Given the sequence similarity, our finding may be extended to

explain the self-association mechanism of avian and reptile NANOGs in general.

4. Conclusion

NANOG plays a pivotal role in pluripotency acquisition and lineage specification in higher vertebrates, and its expression is restricted to primordial germ cells (PGCs) during early embryonic development. Mammalian NANOG self-associates via conserved tryptophan-repeat motifs in the C-terminal domain (CTD) to maintain pluripotency. Avian NANOG, however, lacks the conserved motifs, and the molecular mechanism underlying the biological function is not clearly understood. Here, using the spectroscopic and biochemical methods and cell-based assays, we report that chicken NANOG (cNANOG) oligomerises through its CTD via a novel folding-upon-binding mechanism. The CTD of cNANOG is disordered as a monomer, and associates into an α-helical multimer driven by intermolecular hydrophobic interactions. Mutation of key aromatic residues in the CTD abrogates the self-association, leading to a loss of the proliferation of chicken PGCs and blastoderm cells. Our results demonstrate that the CTD of cNANOG belongs to a novel intrinsically disordered protein that switches into a helical oligomer via self-association, enabling the maintenance of PGCs and blastoderm cells.

CHAPTER 6

Discovery of Novel Gene Expressed in Magnum Tissue of Chicken Oviduct

1. Introduction

The hen's egg is an important source of energy and nutrients. It contains an excellent value of nutrition; the proteins, lipids, vitamins, minerals. It has a crucial role in the development of embryo, as well as the protection from the bacterial and viral infection. The egg is comprised of many biotechnological components (Fujita et al., 1995, Ibrahim et al., 2000, Mine and Kovacs-Nolan, 2004). Recently, the researches on the egg component analysis have focused on the development of strategies to prepare and to use these components for biological applications (Gasparri et al., 1999, Pan et al., 2015).

The egg formation occurs in the oviduct and steroid hormones are the key regulators in the mechanisms of the egg forming process (Lague et al., 1975). Specifically, estrogen plays an important role in the development of the female reproductive system. The oviduct has several regions; the infundibulum, magnum, isthmus, shell gland and vagina. The infundibulum is place where the fertilization occurs and the magnum is responsible for the egg white protein production and secretion. Egg shell formation takes place in the shell gland. Estrogen controls folliculogenesis and production of egg white protein (Oka and Schimke, 1969). It is also an essential modulator of the oviduct development. In addition, the proliferation and differentiation of tubular glands and epithelial cells are sensitive to the estrogen treatment (Okada et al., 2005). For example, the administration of diethylstilbestrol (DES), which is a nonsteroidal synthetic estrogen, triggers the massive growth of the immature chicks (Palmiter and Wrenn, 1971). Moreover, estrogen treatment induces the increase of the egg white protein expression such as ovalbumin, conalbumin, ovomucoid, and lysozyme (Palmiter, 1972).

The previous studies on discovery of candidate genes and pathways

regulating oviduct development in chicken have shown that most of the estrogen induced genes were greatly increased in juvenile oviduct by DES treatment (Song et al., 2011). Here, we investigate the hypothesis that the novel transcripts that showed significantly high fold change post DES treatment may be the potential egg white protein genes. We examined novel transcript changes in the chick oviducts after an exposure to DES through microarray analysis. To confirm our microarray data, we performed reverse transcript PCR analysis and in situ hybridization in order to compare the expression pattern between ovalbumin and the novel transcripts. The results showed that five of the novel transcripts were specifically expressed in the oviduct. Therefore, we mainly selected and examined one of the novel transcripts that is specifically expressed in the magnum region of the oviduct and compared its expression with that of the ovalbumin gene. Our studies indicate the expression pattern of novel transcripts which is associated egg white protein genes.

2. Materials and methods

Animal care

The care and experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5) White Leghorn chickens were managed following a standard program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, estrogen treatment and surgeries adhered to the standard operating protocols of our laboratory.

DES Treatment and Oviduct Retrieval

Female chicks were selected by PCR analysis using the W chromosome specific primer sets described (Lee et al., 2009). DES treatment and oviduct retrieval were conducted as reported previously (Sanders and Mcknight, 1988, Seo et al., 2009). Briefly, a 15-mg DES pellet was implanted subcutaneously in the abdominal region of 1-wk-old female chicks for 10 days. The DES pellet was removed from half the chicks for 10 days, and a 30-mg dose was administered for 10 more days (Li and Wong, 2001)

Microarray Analysis

Microarray analysis was performed using Affymetrix GeneChip Chicken Genome Arrays (Affymetrix, Santa Clara, CA). Data were generated by SeouLin Bioscience Corporation (Seoul, Korea), and the dChip software was used for the analysis (Sanders and Mcknight, 1988). Because oviducts from the control chicks were very small, a total of nine oviducts derived from control chicks were pooled into three control samples, whereas oviducts from three DES-treated chicks were large enough to be analyzed individually. For

biological replication, total RNA was individually extracted from three pooled oviducts from control chicks and three oviducts from DES-treated chicks using TRIzol (Invitrogen, Carlsbad,CA) and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). We selected differentially expressed genes at each time point by two-sample comparisons, with a lower threshold of greater than a twofold change, a 90% confidence interval, and a group mean difference greater than 100. Differentially regulated genes identified in the microarray analyses were analyzed using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Mountain View, CA). Canonical pathway analyses identified the pathways from the IPA library of canonical pathways that were most highly represented in the data set.

Transcriptase PCR Analysis

Total RNA was extracted from the above tissues using TRIzol (Invitrogen). Complementary DNA was synthesized using a Superscript III First-Strand Synthesis System (Invitrogen). To determine the expression patterns of the chicken novel transcripts, RT-PCR analysis was performed using specific primer sets. The reactions were performed with an incubation at 94°C for 5min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The reaction was terminated by a final incubation at 72 °C for 5 min.

In Situ Hybridization Analysis

We examined using in situ hybridization, as described (Rengaraj et al., 2008). For hybridization probes, PCR products were generated from cDNA. The products were gel extracted and cloned into a pGEM-T Easy Vector (Promega, Madison, WI). After sequence verification, a DIG-labeled RNA probe was prepared using a DIG RNA Labeling kit (Roche Applied

Science, Indianapolis, IN). Frozen 10 μm sections were mounted on 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO) pretreated slides, dried on a 50°C slide warmer, and fixed in 4% paraformaldehyde in PBS. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed three times in PBS. The sections were incubated in a prehybridization mixture containing 50% formamide and 5X standard saline citrate (SSC) for 15 min at room temperature. The sections were then incubated with a hybridization mixture containing 50% formamide, 5X SSC, 10% dextran sulfate sodium salt, 0.02% bovine serum albumin, 250 μg/ml yeast tRNA, and denatured DIG-labeled cRNA probes for 18 h at 55°C in a humidification chamber. The sections were washed for stringency in a series of solutions containing 50% formamide and downgrades of SSC (5X SSC for 15 min, 2X SSC for 30 min, and 0.2X SSC for 60 min) at 55°C. After blocking with 1% blocking reagent (Roche) in a buffer containing 1 M Tris-HCl (pH 7.5) and 2.5 M NaCl, the sections were incubated at 4°C overnight with sheep anti-DIG antibody (1:5000 in 1% blocking reagent) conjugated to alkaline phosphatase (Roche). The signal was visualized after exposure to a visualization solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM levamisole (Sigma). All sections were counterstained with 1% (w/v) methyl green (Sigma), and photographs were taken using a Zeiss Axiophot light microscope equipped with an AxioCam HRc camera (Carl Zeiss, Oberkochen, Germany).

Immunohistochemistry

Primary cultured oviductal epitheliar cells (OECs) were fixed in 4 % buffered paraformaldehyde for 10 min and then rinsed three times with 1X PBS. Cells was then permeabilized with 1% Tween-20 and rinsed with 1X

PBS. Cells were incubated with blocking solution (10% BSA and 5% goat serum in 1X PBS) for 3 hours, and then incubated with 1:1000 diluted Flag (mouse immunoglobulin [Ig] at 4°C overnight. After washing with 1X PBS, gonads were incubated with fluorescein isothiocyanate (Santa Cruz Biotechnology) conjugated goat anti-mouse IgG (diluted 1:200) at room temperature. Immunostained cells were examined under a fluorescence microscope (Nikon, Tokyo, Japan).

3. Results

Expression pattern of novel transcripts

To investigate which novel transcripts are associated with the egg white protein, first we selected the 20 candidates from the microarray analysis (Table 6-1). To detect the oviduct specific expressed genes, we examined the expression pattern by reverse transcriptase PCR form the bran, liver, muscle, spleen, ovary and oviduct (Figure 6-1A). The result was only 5 novel transcripts were detected in oviduct. Furthermore, examination of the expression pattern in 4 regions of oviduct; the infundibulum, magnum, isthmus and shell gland revealed no obvious differences with several egg proteins (Figure 6-1B). However, we observed BX261629 was expressed in the oviductal magnum.

We focused on the BX261629 and to investigate the expression pattern between the immature chicken oviduct and adult chicken oviduct we performed the reverse transcriptase RCR (Figure 6-2). In the immature chicken, no detection in ovalbumin gene and BX261629 were observed. On the other hand, we detected difference expression pattern in the adult chicken according to ovulation after 3 hours and 20 hours. Ovalbumin gene and BX261629 were stronger expressed in ovulation after 3 hours than 20 hours.

Localization of novel transcript

To investigate further the localization of the BX261629 in oviduct, we performed in situ hybridization analysis. We also compared with the expression pattern BX261629 and ovalbumin gene. As expected, we observed quite similar to expression pattern between BX261629 and ovalbumin gene in each tissue. However, cell-specific differences in the expression these genes

were observed within the chicken oviductal magnum (Figure 6-3). No detect these genes in immature chicken (18 weeks). Whereas ovalbumin gene expressed in adult chicken oviduct and BX261629 was specifically expressed in adult chicken oviductal magnum. All mRNA was localized predominantly in the tubular gland cell and, to a lesser extent, the epithelium cell of the chicken oviductal magnum. Furthermore, the expression patterns of ovulation after 3 hours and 20 hours were observed that magnum of ovulation after 3 hours was stronger than magnum of ovulation after 20 hours.

Translation of novel transcript

To validate ORF translation, we inserted a N-terminal Flag-tag at the 5' end of BX261629 ORF (Figure 6-4). We confirmed the translation of ORF in DF1 and primary OECs. BX261629 with Flag-tagged gene was stained by Flag at the cytoplasm. Immunostained for Flag in OECs showed that BX261629 is localized membrane region.

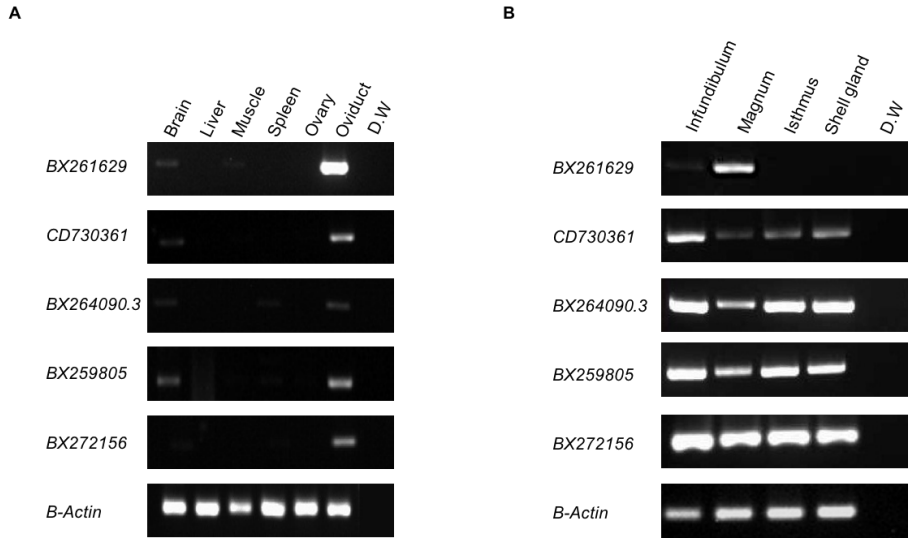


Figure 6-1. Expression pattern of novel transcripts in various tissues. (A) Expression of transcripts in the brain, liver, muscle, spleen, ovary, oviduct, control and 24 weeks hens. (B) Expression patterns and comparison between a set of candidate transcripts and egg white proteins in the infundibulum, magnum, isthmus, shell gland tissues at 24 weeks hen oviduct.

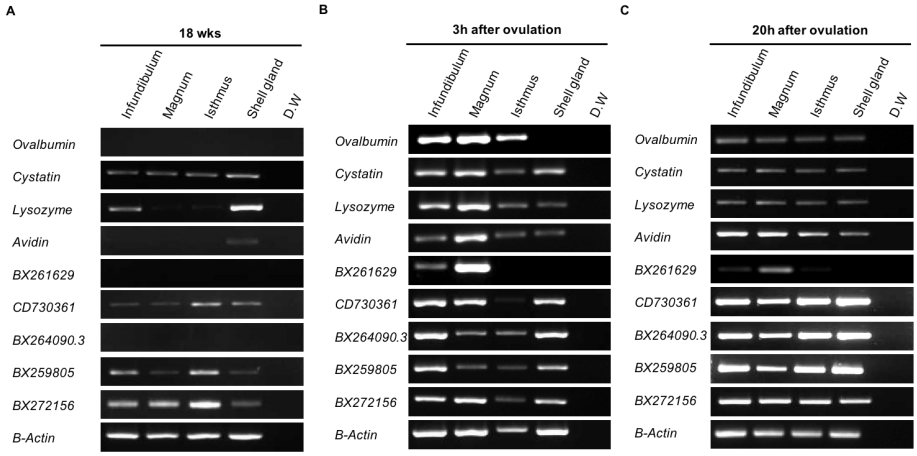


Figure 6-2. Expression pattern of novel transcripts in immature and adult oviduct. Expression patterns and comparison between a set of candidate transcripts and egg white proteins in the infundibulum, magnum, isthmus, shell gland tissues at 18weeks (A) and 24 weeks after ovulation time after 3 hours (B) and 20 hours (C) hen oviduct.

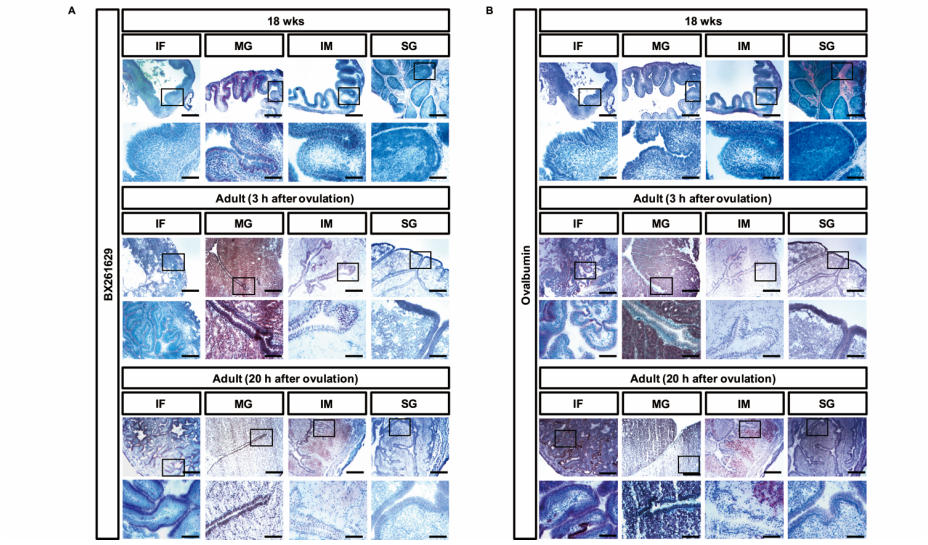


Figure 6-3. Messenger RNA localization of ovalbumin gene and BX261629 related to similar expression in chicken oviduct. In situ hybridization analyses of BX261629 (A) and ovalbumin (B) in immature and adult oviduct. The mRNA of these genes was localized predominantly to the tubular gland cell and to a lesser extent the epithelial cell of the chicken oviductal magnum.

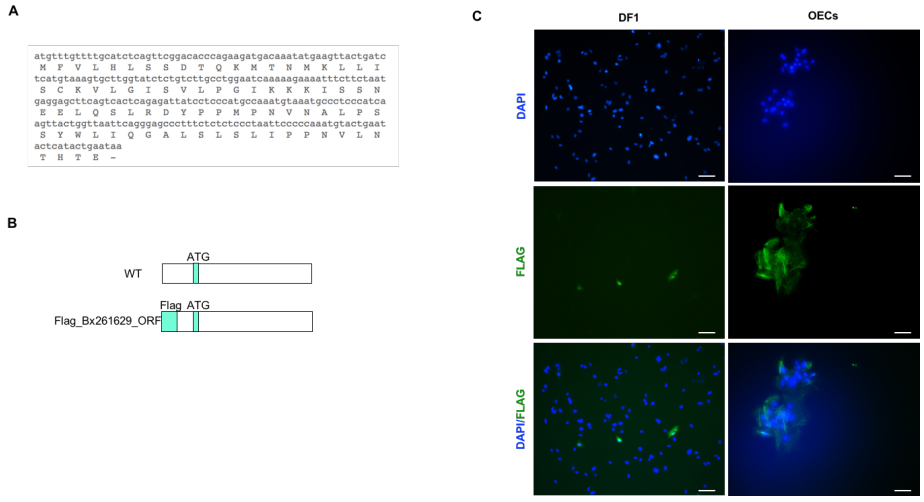


Figure 6-4. BX261629 encodes a novel polypeptide. (A) Schematic representation of BX261629 constructs used in this study. (B) Putative ORFs in BX261629. (C) Representative immunofluorescence images from primary DF1 and oviductal epithelial cells (OECs). Bar =100 µm.

Table 6-1. Candidate genes expressed in chicken oviduct

Gene Name	FC	Affimetrix ID	EST NO.
Gga.6622.1	24.76	Gga.6622.1.S1_at	BX261629
Gga.5085.1	22.39	Gga.5085.1.S1_at	J00902
RCJMB04_11e10	17.19	GgaAffx.12938.14.S1_s_at	AJ720136
LOC424557	17.08	Gga.7703.1.S1_at	BX932049
Gga.380.1	13.03	Gga.380.1.S1_at	BX257543.4
GgaAffx.171.1	10.30	GgaAffx.171.1.S1_at	
GgaAffx.2163.1	9.66	GgaAffx.2163.1.S1_at	
Gga.7493.1	9.62	Gga.7493.1.S1_at	BX258490
Gga.18428.1	8.91	Gga.18428.1.S1_at	BU335185.1
Gga.16555.1	8.22	Gga.16555.1.S1_at	BU352299
Gga.8989.1	7.88	Gga.8989.1.S1_at	CD730361.1
LOC41978	7.50	Gga.5986.1.S1_at	BX264090.3
Gga.6717.1	6.91	Gga.6717.1.S1_at	BX266116
Gga.5817.1	6.39	Gga.5817.1.S1_at	BU327761.1
Gga.7441.1	5.92	Gga.7441.1.S1_at	BX259805.2
Gga.19351.1	5.86	Gga.19351.1.S1_at	BU219778.1
LOC770777	5.35	Gga.990.1.S1_at	BX271857.4
Gga.7038.1	4.63	Gga.7038.1.S1_at	BX272156.3
Gga.6458.1	4.46	Gga.6458.1.S1_at	BX270231.3
Gga.6130.1	4.10	Gga.6130.1.S1_at	BX262892

Table 6-2. Information of primer sequence

Gene	Sequence
Bx261629	5’-ATGGCACACGGTTGGGAC-3’
	5’-CAGTAACTTGATGGGAGGGCA-3’
CD730361	5’-CCTGGTAAAGGGTCGGTTTC-3’
	5’-AACTCCTGGGTCACTCTCCA-3’
BX264090.3	5’-CAGATGTCCCCATTCTGCTG-3’
	5’-GCACCTGAACTGACACAGCC-3’
BX259805	5’-GGTCTCCCTCCTTGGTGATG-3’
	5’-TTGCAGCTTGCCAGAGTAGC-3’
B-Actin	5’-GGCTGTGCTGTCCCTGTATG-3’
	5’-ACCCAAGAAAGATGGCTGGA-3’

4. Discussion

The present study indicates that we found the novel transcripts which were induced in chick oviduct by estrogen exposure and BX261629 was specifically expressed in the oviductal magnum where the egg white proteins are mainly secreted. Until recently, DES treatment used to study of development of female reproductive cycle or effect of egg white protein (Palmiter, 1972). Interestingly, egg white proteins increase the expression level by estrogen treatment so in advance our research focused on novel transcripts changes after exposure to DES by microarray analysis.

The result indicates that we found novel transcript as a potential egg white protein. Based on the microarray data we selected 20 candidates of novel transcripts though the bioinformatics tools which novel transcripts possible to relate with egg white proteins as table 6-1. The five novel transcripts of interest, these genes were expressed in oviduct by reverse transcriptase PCR using the brain, liver, muscle, spleen, ovary, oviduct and control. Moreover, the BX261629 transcript reveals that specifically expressed in oviductal magnum and comparison of ovalbumin and BX261629 patterns was quite closed. The result shows that these genes were not detected in immature oviduct and strongly expressed during ovulation after 3 hours than 20 hours. We suggest that BX261629 expression was associated with egg white proteins.

Comparison of these genes pattern in ovulation after 3 hours and 20 hours, expression pattern of ovalbumin and BX261629 were also similar to previous data through in situ hybridization. During the egg formation, ovum located in magnum region to produce of egg white protein and secret albumen in the ovulation after 3 to 5 hours. On the other, egg shell formation occurs in the ovulation 20 hours (Anton et al., 2006). Corollary, Egg white protein

genes remain the high level during ovulation 3 to 5 hours. Our result shows that ovalbumin and BX261629 were highly expressed in ovulation after 3 hours.

We hypothesize that the novel transcripts that showed significantly high fold change post DES treatment may be the potential egg white protein genes. We studied novel transcripts were newly detected via the analysis of mRNA expression pattern as the potential egg white protein. We will investigate further the discovery of function of novel transcript as a potential therapeutic peptides or proteins for the prevention and treatment of various diseases. Overall, these results provide important to study of utilized for production of egg-targeting bioreactor as well as finding of new biological compound.

5. Conclusion

Laying hens are related to ovulation and ovoposition and the estrogen treatment increases the expression of egg white proteins such as ovalbumin. Estrogen, as a sex hormone, regulates the ovulatory cycle and production of egg white proteins in chicken. Injections of estrogen into chickens induced the accumulation of ovalbumin mRNA. To investigate the estrogen induced genes in oviduct using microarray analysis, we performed serial treatments of DES (synthetic estrogen) in female immature chicks (4week) and sexually mature chickens (30 weeks old). Eighteen transcripts were selected via the microarray analysis. To confirm microarray and tissue-specific expression of selected transcripts, the expression patterns were analyzed. Five transcripts were expressed in oviduct and one of them was specifically expressed in magnum tissue of the oviduct. This study showed that novel transcripts, stimulated by estrogen treatment in chick oviduct, were detected and expressed in the magnum of hen oviduct, where egg white proteins are mainly secreted. Through further study, we will investigate the biological functions of these transcripts and the mechanisms of their biological activities.

CHAPTER 7

GENERAL DISCUSSION

In the first study, we established an alternative germ cell-transfer system for producing germline chimeras by replacing an original host embryo with a donor embryo, while retaining the host extraembryonic tissue and yolk, before circulation. PGCs, which are precursor cells of functional gametes, have been recognized as an alternative source of germline transmission in avian species. Of the various avian species, only chicken PGCs have shown germline competency. In this regard, we explored an alternative method to produce germline chimeras that can be applied to most avian species. To evaluate germline chimerism in the offspring of interspecies germline chimeras after embryo replacement, it would be necessary to hatch the manipulated embryos. In this study, the transplanted embryos survived until a maximum of E16.5, but no hatchlings were available. We also found that quail embryos were not able to enclose the yolk after E16.5 because the chick yolk was larger than that of the quail. Thus, the large yolk size may be a major factor preventing hatching. It was further suggested that the yolk could be surgically removed to allow hatching after embryo replacement. Through improved transplantation and culture methods, mature germ cells of endangered species can potentially be harvested from more abundant host species such as the chick or quail, thus facilitating their restoration/conservation.

The avian embryos have been used as a good model to study embryonic development. Due to its unique development in the eggshell, avian embryos can be cultured and hatch in the surrogate eggshell system. In this study, we examined the viability, normal development and hatchability of Korean Oge (KO) chicken embryos in White Leghorn (WL) surrogate eggshells. KO chicken embryos are successfully transferred and cultured in WL surrogate eggshells. However, the viability and hatchability of embryos transferred at 4-day-old were higher than that of 3-day-old. Furthermore, the embryonic development of all viable embryos was normal in our surrogate

system. Our study suggests that culturing of chicken embryos from one breed in the surrogate eggshells of others breed is highly possible. This method can be also useful for the culture of embryos produced from the transgenic bioreactor that contains foreign protein in egg white.

In the third study, we demonstrated that the avian NANOG forms a high-order multimer through its CTD via hydrophobic interactions distinct from mammalian NANOG. Remarkably, cCTD remains disordered as a monomer, but switches into a helix upon self-association in a concentration-dependent manner. The fact that cCTD exists as a disordered monomer at low concentration implicates that monomeric cCTD may form multiple scaffolds to recruit other proteins. The expression level of cNANOG would then determine the self-association for proper transcriptional regulation. Given that NANOG is central to the signal transduction network for the maintenance of PGCs and ES cells, the intrinsic disorder of cNTD and cCTD may facilitate the interactions with multiple partner proteins, and immediate signalling for transcriptional activation. Also, NANOG is not likely to function as a huge multimeric aggregate. NANOG may exist as a low-order oligomer at dilute cellular concentrations. In addition, mutations of key aromatic residues perturbed the self-association, and the monomeric mutant failed to replace pluripotency related gene, *POUV*, in chicken PGCs and blastoderm cells and proliferation. Thus, our results demonstrate that the CTD of cNANOG belongs to a novel intrinsically disordered protein that switches into a helical oligomer via self-association, enabling the maintenance of PGCs and blastoderm cells.

Estrogen, as a sex hormone, regulates the ovulatory cycle and production of egg white proteins in chicken. Injections of estrogen into chickens induced the accumulation of ovalbumin mRNA. In this study, we investigated novel transcripts which were induced in chick oviduct by

estrogen exposure and BX261629 was specifically expressed in the oviductal magnum where the egg white proteins are mainly secreted. This finding suggests that novel transcripts significantly high fold change post DES treatment may be the potential egg white protein genes or modulator of egg white protein secretion.

REFERENCES

Aigegil, V. and K. Simkiss (1991). "Sterilizing Embryos for Transgenic Chimeras." British Poultry Science 32(3): 427-438.

Andacht, T., W. Hu and R. Ivarie (2004). "Rapid and improved method for windowing eggs accessing the stage X chicken embryo." Mol Reprod Dev 69(1): 31-34.

Anton, M., F. Nau and Y. Nys (2006). "Bioactive egg components and their potential uses." Worlds Poultry Science Journal 62(3): 429-438.

Aoyama, H., K. Asamoto, Y. Nojyo and M. Kinutani (1992). "Monoclonal antibodies specific to quail embryo tissues: their epitopes in the developing quail embryo and their application to identification of quail cells in quail-chick chimeras." J Histochem Cytochem 40(11): 1769-1777.

Baltus, G. A., M. P. Kowalski, A. V. Tutter and S. Kadam (2009). "A Positive Regulatory Role for the mSin3A-HDAC Complex in Pluripotency through Nanog and Sox2." Journal of Biological Chemistry 284(11): 6998-7006.

Bernardo, A. D., Sprenkels, K., Rodrigues, G., Noce, T., Lopes, S. M. C. D (2012). "Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation." Biology Open 1: 1126-1152.

Bosselman, R. A., R. Y. Hsu, T. Boggs, S. Hu, J. Bruszewski, S. Ou, L. Kozar, F. Martin, C. Green, F. Jacobsen and et al. (1989). "Germline transmission of exogenous genes in the chicken." Science 243(4890): 533-535.

Boyer, L. A., T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. R. Zucker,

M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch and R. A. Young (2005). "Core transcriptional regulatory circuitry in human embryonic stem cells." Cell 122(6): 947-956.

Brinster, R. L. and J. W. Zimmermann (1994). "Spermatogenesis following male germ-cell transplantation." Proc Natl Acad Sci U S A 91(24): 11298-11302.

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

Carsience, R. S., M. E. Clark, A. M. Verrinder Gibbins and R. J. Etches (1993). "Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos." Development 117(2): 669-675.

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.

Chang, D. F., S. C. Tsai, X. C. Wang, P. Xia, D. Senadheera and C. Lutzko (2009). "Molecular characterization of the human NANOG protein." Stem Cells 27(4): 812-821.

Chang, I. K., A. Tajima, Y. Yasuda, T. Chikamune and T. Ohno (1992). "Simple method for isolation of primordial germ cells from chick embryos." Cell Biol Int Rep 16(9): 853-857.

Chang, I. K., A. Yoshiki, M. Kusakabe, A. Tajima, T. Chikamune, M. Naito and T. Ohno (1995). "Germ line chimera produced by transfer of cultured chick primordial germ cells." Cell Biol Int 19(7): 569-576.

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.

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.

Cheng, M. H., J. Z. Maines and S. A. Wasserman (1998). "Biphasic subcellular localization of the DAZL-related protein boule in Drosophila spermatogenesis." Dev Biol 204(2): 567-576.

Choi, J. W., S. Kim, T. M. Kim, Y. M. Kim, H. W. Seo, T. S. Park, J. W. Jeong, G. Song and J. Y. Han (2010). "Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells." PLoS One 5(9): e12968.

Clark, A. T. and R. A. Reijo Pera (2006). "Modeling human germ cell development with embryonic stem cells." Regen Med 1(1): 85-93.

Clawson, R. C. and L. V. Domm (1969). "Origin and early migration of primordial germ cells in the chick embryo: a study of the stages definitive primitive streak through eight somites." Am J Anat 125(1): 87-111.

Cooke, H. J., M. Lee, S. Kerr and M. Ruggiu (1996). "A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads." Hum Mol Genet 5(4): 513-516.

Darr, H., Y. Mayshar and N. Benvenisty (2006). "Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features." Development 133(6): 1193-1201.

De Melo Bernardo, A., K. Sprenkels, G. Rodrigues, T. Noce and S. M. Chuva De Sousa Lopes (2012). "Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation." Biol Open 1(11): 1146-1152.

Dieterlen-Lievre, F. (1975). "On the origin of haemopoietic stem cells in the avian embryo: an experimental approach." J Embryol Exp Morphol 33(3): 607-619.

Dixon, J. E., C. Allegrucci, C. Redwood, K. Kump, Y. Bian, J. Chatfield, Y. H. Chen, V. Sottile, S. R. Voss, R. Alberio and A. D. Johnson (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.

Dougherty, D. C. and M. M. Sanders (2005). "Estrogen action: revitalization of the chick oviduct model." Trends Endocrinol Metab 16(9): 414-419.

Dunker, A. K., J. D. Lawson, C. J. Brown, R. M. Williams, P. Romero, J. S. Oh, C. J. Oldfield, A. M. Campen, C. M. Ratliff, K. W. Hipps, J. Ausio, M. S. Nissen, R. Reeves, C. Kang, C. R. Kissinger, R. W. Bailey, M. D. Griswold, W. Chiu, E. C. Garner and Z. Obradovic (2001). "Intrinsically disordered protein." J Mol Graph Model 19(1): 26-59.

Dyson, H. J. and P. E. Wright (2005). "Intrinsically unstructured proteins and their functions." Nat Rev Mol Cell Biol 6(3): 197-208.

Dzierzak, E. and A. Medvinsky (2008). "The discovery of a source of adult hematopoietic cells in the embryo." Development 135(14): 2343-2346.

Eberhart, C. G., J. Z. Maines and S. A. Wasserman (1996). "Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia." Nature 381(6585): 783-785.

Eddy, E. M. and D. A. O'Brien (1998). "Gene expression during mammalian meiosis." Curr Top Dev Biol 37: 141-200.

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

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

Firket, J. (1920). "On the origin of germ cells in higher vertebrates." Anatomical Record 18(309-316).

Francoise-Lievre, F. (2005). "Commitment of hematopoietic stem cells in avian and mammalian embryos: an ongoing story." Int J Dev Biol 49(2-3): 125-130.

Fujimoto, T., Y. Miyayama and M. Fuyuta (1977). "The origin, migration and fine morphology of human primordial germ cells." Anat Rec 188(3): 315-330.

Fujimoto, T., T. Ninomyiya and A. Ukeshima (1976). "Observations of the primoridal germ cells in blood samples from the chick embryo." Dev Biol 49(1): 278-282.

Fujimoto, T., A. Ukeshima and R. Kiyofuji (1976). "The origin, migration and morphology of the primordial germ cells in the chick embryo." Anat Rec 185(2): 139-145.

Fujita, H., R. Sasaki and M. Yoshikawa (1995). "Potentiation of the antihypertensive activity of orally administered ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidylcholine." Biosci Biotechnol Biochem 59(12): 2344-2345.

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 of the United States of America 91(25): 12258-12262.

Gasparri, A., M. Moro, F. Curnis, A. Sacchi, S. Pagano, F. Veglia, G. Casorati, A. G. Siccardi, P. Dellabona and A. Corti (1999). "Tumor pretargeting with avidin improves the therapeutic index of biotinylated tumor necrosis factor alpha in mouse models." Cancer Res 59(12): 2917-2923.

Ginsburg, M. (1994). "Primordial germ cell formation in birds." Ciba Found Symp 182: 52-61; discussion 61-57.

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." J Embryol Exp Morphol 95: 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.

Grimes, S. R. (2004). "Testis-specific transcriptional control." Gene 343(1): 11-22.

Gruidl, M. E., P. A. Smith, K. A. Kuznicki, J. S. McCrone, J. Kirchner, D. L. Roussell, S. Strome and K. L. Bennett (1996). "Multiple potential germ-line helicases are components of the germ-line-specific P granules of Caenorhabditis elegans." Proc Natl Acad Sci U S A 93(24): 13837-13842.

Gsponer, J., M. E. Futschik, S. A. Teichmann and M. M. Babu (2008). "Tight regulation of unstructured proteins: from transcript synthesis to protein degradation." Science 322(5906): 1365-1368.

Hall, J. M., J. F. Couse and K. S. Korach (2001). "The multifaceted mechanisms of estradiol and estrogen receptor signaling." J Biol Chem 276(40): 36869-36872.

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

Hamra, F. K., J. Gatlin, K. M. Chapman, D. M. Grellhesl, J. V. Garcia, R. E. Hammer and D. L. Garbers (2002). "Production of transgenic rats by lentiviral transduction of male germ-line stem cells." Proc Natl Acad Sci U S A 99(23): 14931-14936.

Han, J. Y., T. S. Park, Y. H. Hong, D. K. Jeong, J. N. Kim, K. D. Kim and J. M. Lim (2002). "Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained in vitro for an extended period." Theriogenology 58(8): 1531-1539.

Han, M., Y. Park, I. Kim, E. H. Kim, T. K. Yu, S. Rhee and J. Y. Suh (2014). "Structural basis for the auxin-induced transcriptional regulation by Aux/IAA17." Proc Natl Acad Sci U S A 111(52): 18613-18618.

Hart, A. H., L. Hartley, M. Ibrahim and L. Robb (2004). "Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human." Dev Dyn 230(1): 187-198.

Hatano, S. Y., M. Tada, H. Kimura, S. Yamaguchi, T. Kono, T. Nakano, H. Suemori, N. Nakatsuji and T. Tada (2005). "Pluripotential competence of cells associated with Nanog activity." Mech Dev 122(1): 67-79.

Hay, B., L. Y. Jan and Y. N. Jan (1990). "Localization of vasa, a component of Drosophila polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity." Development 109(2): 425-433.

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

Hayashi, Y., L. Caboni, D. Das, F. Yumoto, T. Clayton, M. C. Deller, P. Nguyen, C. L. Farr, H. J. Chiu, M. D. Miller, M. A. Elsliger, A. M. Deacon, A. Godzik, S. A. Lesley, K. Tomoda, B. R. Conklin, I. A. Wilson, S. Yamanaka and R. J. Fletterick (2015). "Structure-based discovery of NANOG variant with enhanced properties to promote self-renewal and reprogramming of pluripotent stem cells." Proc Natl Acad Sci U S A 112(15): 4666-4671.

Hecht, N. B. (1998). "Molecular mechanisms of male germ cell differentiation." Bioessays 20(7): 555-561.

Hewitt, S. C., J. C. Harrell and K. S. Korach (2005). "Lessons in estrogen

biology from knockout and transgenic animals." Annu Rev Physiol 67: 285-308.

Hough, S. R., I. Clements, P. J. Welch and K. A. Wiederholt (2006). "Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog." Stem Cells 24(6): 1467-1475.

Houston, D. W. and M. L. King (2000). "A critical role for Xdazl, a germ plasm-localized RNA, in the differentiation of primordial germ cells in Xenopus." Development 127(3): 447-456.

Houston, D. W. and M. L. King (2000). "Germ plasm and molecular determinants of germ cell fate." Curr Top Dev Biol 50: 155-181.

Hrabia, A., A. Lesniak-Walentyn, A. Sechman and A. Gertler (2014). "Chicken oviduct-the target tissue for growth hormone action: effect on cell proliferation and apoptosis and on the gene expression of some oviduct-specific proteins." Cell Tissue Res 357(1): 363-372.

Hudson, Q. J., C. A. Smith and A. H. Sinclair (2005). "Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary." Dev Dyn 233(3): 1052-1055.

Hwang, Y. S., M. H. Ko, Y. M. Kim, Y. H. Park, T. Ono and J. Y. Han (2016). "The avian-specific small heat shock protein HSP25 is a constitutive protector against environmental stresses during blastoderm dormancy." Sci Rep 6: 36704.

Hyslop, L., M. Stojkovic, L. Armstrong, T. Walter, P. Stojkovic, S. Przyborski, M. Herbert, A. Murdoch, T. Strachan and M. Lako (2005). "Downregulation

of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages." Stem Cells 23(8): 1035-1043.

Ibrahim, H. R., Y. Sugimoto and T. Aoki (2000). "Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism." Biochim Biophys Acta 1523(2-3): 196-205.

Ikenishi, K. and T. S. Tanaka (1997). "Involvement of the protein of Xenopus vasa homolog (Xenopus vasa-like gene 1, XVLG1) in the differentiation of primordial germ cells." Dev Growth Differ 39(5): 625-633.

Jauch, R., C. K. Ng, K. S. Saikatendu, R. C. Stevens and P. R. Kolatkar (2008). "Crystal structure and DNA binding of the homeodomain of the stem cell transcription factor Nanog." J Mol Biol 376(3): 758-770.

Jung, K. M., Y. M. Kim, T. Ono and J. Y. Han (2017). "Size-dependent isolation of primordial germ cells from avian species." Mol Reprod Dev 84(6): 508-516.

Kagami, H., T. Tagami, Y. Matsubara, T. Harumi, H. Hanada, K. Maruyama, M. Sakurai, T. Kuwana and M. Naito (1997). "The developmental origin of primordial germ cells and the transmission of the donor-derived gametes in mixed-sex germline chimeras to the offspring in the chicken." Mol Reprod Dev 48(4): 501-510.

Kamihira, M., S. Oguchi, A. Tachibana, Y. Kitagawa and S. Iijima (1998). "Improved hatching for in vitro quail embryo culture using surrogate eggshell and artificial vessel." Dev Growth Differ 40(4): 449-455.

Kanatsu-Shinohara, M., N. Ogonuki, K. Inoue, H. Miki, A. Ogura, S.

Toyokuni and T. Shinohara (2003). "Long-term proliferation in culture and germline transmission of mouse male germline stem cells." Biol Reprod 69(2): 612-616.

Kang, K. S., H. C. Lee, H. J. Kim, H. G. Lee, Y. M. Kim, H. J. Lee, Y. H. Park, S. Y. Yang, D. Rengaraj, T. S. Park and J. Y. Han (2015). "Spatial and temporal action of chicken primordial germ cells during initial migration." Reproduction 149(2): 179-187.

Kang, S. J., J. W. Choi, S. Y. Kim, K. J. Park, T. M. Kim, Y. M. Lee, H. Kim, J. M. Lim and J. Y. Han (2008). "Reproduction of wild birds via interspecies germ cell transplantation." Biol Reprod 79(5): 931-937.

Karashima, T., A. Sugimoto and M. Yamamoto (2000). "Caenorhabditis elegans homologue of the human azoospermia factor DAZ is required for oogenesis but not for spermatogenesis." Development 127(5): 1069-1079.

Kenny, D. and R. C. Cambre (1992). "Indications and Technique for the Surgical Removal of the Avian Yolk-Sac." Journal of Zoo and Wildlife Medicine 23(1): 55-61.

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, M. A., T. S. Park, J. N. Kim, H. J. Park, Y. M. Lee, T. Ono, J. M. Lim and J. Y. Han (2005). "Production of quail (Coturnix japonica) germline chimeras by transfer of gonadal primordial germ cells into recipient embryos." Theriogenology 63(3): 774-782.

Kim, P. M., A. Sboner, Y. Xia and M. Gerstein (2008). "The role of disorder in interaction networks: a structural analysis." Mol Syst Biol 4: 179.

Kohler, P. O., P. M. Grimley and B. W. O'Malley (1969). "Estrogen-induced cytodifferentiation of the ovalbumin-secreting glands of the chick oviduct." J Cell Biol 40(1): 8-27.

Korasick, D. A., C. S. Westfall, S. G. Lee, M. H. Nanao, R. Dumas, G. Hagen, T. J. Guilfoyle, J. M. Jez and L. C. Strader (2014). "Molecular basis for AUXIN RESPONSE FACTOR protein interaction and the control of auxin response repression." Proc Natl Acad Sci U S A 111(14): 5427-5432.

Kossack, N., J. Meneses, S. Shefi, H. N. Nguyen, S. Chavez, C. Nicholas, J. Gromoll, P. J. Turek and R. A. Reijo-Pera (2009). "Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells." Stem Cells 27(1): 138-149.

Kusumegi, T., J. Tanaka, M. Kawano, J. Yonemoto, C. Tohyama and H. Sone (2004). "BMP7/ActRIIB regulates estrogen-dependent apoptosis: new biomarkers for environmental estrogens." J Biochem Mol Toxicol 18(1): 1-11.

Kuwana, T., Y. Miyayama, Y. Kajiwara and T. Fujimoto (1987). "Behavior of chick primordial germ cells moving toward gonadal primordium in vitro: scanning electron microscopic study." Anat Rec 219(2): 164-170.

Lague, P. C., A. van Tienhoven and F. J. Cunningham (1975). "Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chicken [Gallus domesticus]." Biol Reprod 12(5): 590-598.

Lasko, P. F. and M. Ashburner (1990). "Posterior localization of vasa protein

correlates with, but is not sufficient for, pole cell development." Genes Dev 4(6): 905-921.

Lassila, O., C. Martin, P. Toivanen and F. Dieterlen-Lievre (1982). "Erythropoiesis and lymphopoiesis in the chick yolk-sac-embryo chimeras: contribution of yolk sac and intraembryonic stem cells." Blood 59(2): 377-381.

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." Dev Biol 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.

Lavial, F. and B. Pain (2010). "Chicken embryonic stem cells as a non-mammalian embryonic stem cell model." Dev Growth Differ 52(1): 101-114.

Lawson, K. A. and W. J. Hage (1994). "Clonal analysis of the origin of primordial germ cells in the mouse." Ciba Found Symp 182: 68-84; discussion 84-91.

Lee, H., N. Karasanyi and R. G. Nagele, Jr. (1978). "The role of the cell surface in the migration of primordial germ cells in early chick embryos: effects of concanavalin A." J Embryol Exp Morphol 46: 5-20.

Lee, H., S. Kim, T. Park, D. Rengaraj, K. Park, H. Lee, S. B. Park, S. Kim, S.

B. Choi and J. Han (2013). "Compensatory proliferation of endogenous chicken primordial germ cells after elimination by busulfan treatment." Stem Cell Res Ther 4(6): 136.

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, H. C., S. Lim and J. Y. Han (2016). "Wnt/beta-catenin signaling pathway activation is required for proliferation of chicken primordial germ cells in vitro." Sci Rep 6: 34510.

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." Proc Natl Acad Sci U S A 108(26): 10426-10431.

Lee, S. I., W. K. Lee, J. H. Shin, B. K. Han, S. Moon, S. Cho, T. Park, H. Kim and J. Y. Han (2009). "Sexually dimorphic gene expression in the chick brain before gonadal differentiation." Poult Sci 88(5): 1003-1015.

Lee, Y. M., J. G. Jung, J. N. Kim, T. S. Park, T. M. Kim, S. S. Shin, D. K. Kang, J. M. Lim and J. Y. Han (2006). "A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes." Biol Reprod 75(3): 380-386.

Li, C. and W. H. Wong (2001). "Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection." Proc Natl Acad Sci U S A 98(1): 31-36.

Li, H., L. Zhao, H. Kagami, K. Matsui and T. Ono (2001). "Identification of transferred

chicken germ cells in quail gonad and semen by amplification of chicken specific

PCR products." J Poult Sci 38: 308-316.

Li, P., C. Tong, R. Mehrian-Shai, L. Jia, N. Wu, Y. Yan, R. E. Maxson, E. N. Schulze, H. Song, C. L. Hsieh, M. F. Pera and Q. L. Ying (2008). "Germline competent embryonic stem cells derived from rat blastocysts." Cell 135(7): 1299-1310.

Li, Z. D., H. Deng, C. H. Liu, Y. H. Song, J. Sha, N. Wang and H. Wei (2002). "Production of duck-chicken chimeras by transferring early blastodermal cells." Poult Sci 81(9): 1360-1364.

Liang, J., M. Wan, Y. Zhang, P. L. Gu, H. W. Xin, S. Y. Jung, J. Qin, J. M. Wong, A. J. Cooney, D. Liu and S. Y. Zhou (2008). "Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells." Nature Cell Biology 10(6): 731-739.

Lin, T. X., C. Chao, S. 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-U180.

Lin, Y. and D. C. Page (2005). "Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice." Dev Biol 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.

Liu, C., K. A. Khazanehdari, V. Baskar, S. Saleem, J. Kinne, U. Wernery and I. K. Chang (2012). "Production of chicken progeny (Gallus gallus domesticus) from interspecies germline chimeric duck (Anas domesticus) by primordial germ cell transfer." Biol Reprod 86(4): 101.

Liu, C., J. Zu, V. Baskar, U. Wernery and I. K. Chang (2012). "Culture of chicken embryo in interspecific surrogate egg white." Poult Sci 91(11): 2866-2871.

Liu, C. H., J. Kinne, V. Baskar, Z. Guerineche, U. Wernery and I. K. Chang (2013). "Houbara bustard hatching in surrogate chicken albumen and eggshell." Avian Biology Research 6(3): 239-242.

Liu, J., N. B. Perumal, C. J. Oldfield, E. W. Su, V. N. Uversky and A. K. Dunker (2006). "Intrinsic disorder in transcription factors." Biochemistry 45(22): 6873-6888.

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.

Lorsch, J. R. (2002). "RNA chaperones exist and DEAD box proteins get a life." Cell 109(7): 797-800.

Macdonald, J., J. D. Glover, L. Taylor, H. M. Sang and M. J. McGrew (2010). "Characterisation and germline transmission of cultured avian primordial germ cells." PLoS One 5(11): e15518.

Martin, C. (1972). "Technique d'emplantation in ovo de blastodermes d'embryonsd'Oiseaux." C R Soc Biol 116: 283-285.

Martin, C., D. Beaupain and F. Dieterlen-Lievre (1978). "Developmental relationships between vitelline and intra-embryonic haemopoiesis studied in avian 'yolk sac chimaeras'." Cell Differ 7(3): 115-130.

McGrew, M. J., A. Sherman, F. M. Ellard, S. G. Lillico, H. J. Gilhooley, A. J. Kingsman, K. A. Mitrophanous and H. Sang (2004). "Efficient production of germline transgenic chickens using lentiviral vectors." EMBO Rep 5(7): 728-733.

Meyer, D. B. (1960). "Application of the Periodic Acid-Schiff Technique to Whole Chick Embryos." Stain Technology 35(83-89).

Mine, Y. and J. Kovacs-Nolan (2004). "Biologically Active Hen Egg Components in Human Health and Disease." J. Poult. Sci 41: 1-29.

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.

Miyahara, D., I. Oishi, R. Makino, N. Kurumisawa, R. Nakaya, T. Ono, H. Kagami and T. Tagami (2016). "Chicken stem cell factor enhances primordial germ cell proliferation cooperatively with fibroblast growth factor 2." J Reprod Dev 62(2): 143-149.

Mohr, S., J. M. Stryker and A. M. Lambowitz (2002). "A DEAD-box protein functions as an ATP-dependent RNA chaperone in group I intron splicing."

Cell 109(6): 769-779.

Monroe, D. G., R. R. Berger and M. M. Sanders (2002). "Tissue-protective effects of estrogen involve regulation of caspase gene expression." Mol Endocrinol 16(6): 1322-1331.

Monroe, D. G., D. F. Jin and M. M. Sanders (2000). "Estrogen opposes the apoptotic effects of bone morphogenetic protein 7 on tissue remodeling." Mol Cell Biol 20(13): 4626-4634.

Mozdziak, P. E., J. Angerman-Stewart, B. Rushton, S. L. Pardue and J. N. Petitte (2005). "Isolation of chicken primordial germ cells using fluorescence-activated cell sorting." Poult Sci 84(4): 594-600.

Mullin, N. P., A. Gagliardi, L. T. P. Khoa, D. Colby, E. Hall-Ponsele, A. J. Rowe and I. Chambers (2017). "Distinct Contributions of Tryptophan Residues within the Dimerization Domain to Nanog Function." J Mol Biol 429(10): 1544-1553.

Mullin, N. P., A. Yates, A. J. Rowe, B. Nijmeijer, D. Colby, P. N. Barlow, M. D. Walkinshaw and I. Chambers (2008). "The pluripotency rheostat Nanog functions as a dimer." Biochem J 411(2): 227-231.

Murakami, K., U. Gunesdogan, J. J. Zylicz, W. W. C. 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.

Nagano, M., B. Y. Ryu, C. J. Brinster, M. R. Avarbock and R. L. Brinster (2003). "Maintenance of mouse male germ line stem cells in vitro." Biol

Reprod 68(6): 2207-2214.

Naito, M., K. Nirasawa and T. Oishi (1990). "Development in culture of the chick embryo from fertilized ovum to hatching." J Exp Zool 254(3): 322-326.

Naito, M., K. Nirasawa and T. Oishi (1995). "An in vitro culture method for chick embryos obtained from the anterior portion of the magnum of oviduct." Br Poult Sci 36(1): 161-164.

Naito, M., A. Sano, T. Harumi, Y. Matsubara and T. Kuwana (2004). "Migration of primordial germ cells isolated from embryonic blood into the gonads after transfer to stage X blastoderms and detection of germline chimaerism by PCR." Br Poult Sci 45(6): 762-768.

Naito, M., A. Tajima, Y. Yasuda and T. Kuwana (1994). "Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells." Mol Reprod Dev 39(2): 153-161.

Nakamura, Y., H. Kagami and T. Tagami (2013). "Development, differentiation and manipulation of chicken germ cells." Dev Growth Differ 55(1): 20-40.

Nakamura, Y., F. Usui, T. Ono, K. Takeda, K. Nirasawa, H. Kagami and T. Tagami (2010). "Germline replacement by transfer of primordial germ cells into partially sterilized embryos in the chicken." Biol Reprod 83(1): 130-137.

Nakamura, Y., Y. Yamamoto, F. Usui, T. Mushika, T. Ono, A. R. Setioko, K. Takeda, K. Nirasawa, H. Kagami and T. Tagami (2007). "Migration and proliferation of primordial germ cells in the early chicken embryo." Poult Sci

86(10): 2182-2193.

Nakanoh, S., K. Okazaki and K. Agata (2013). "Inhibition of MEK and GSK3 supports ES cell-like domed colony formation from avian and reptile embryos." Zoolog Sci 30(7): 543-552.

Nanao, M. H., T. Vinos-Poyo, G. Brunoud, E. Thevenon, M. Mazzoleni, D. Mast, S. Laine, S. Wang, G. Hagen, H. Li, T. J. Guilfoyle, F. Parcy, T. Vernoux and R. Dumas (2014). "Structural basis for oligomerization of auxin transcriptional regulators." Nat Commun 5: 3617.

Nasiri, Z., S. M. Hosseini, M. Hajian, P. Abedi, M. Bahadorani, H. Baharvand and M. H. Nasr-Esfahani (2012). "Effects of different feeder layers on short-term culture of prepubertal bovine testicular germ cells in-vitro." Theriogenology 77(8): 1519-1528.

Navarro, P., N. Festuccia, D. Colby, A. Gagliardi, N. P. Mullin, W. S. 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 Journal 31(24): 4547-4562.

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.

Oatley, J. M. and R. L. Brinster (2006). "Spermatogonial stem cells." Methods Enzymol 419: 259-282.

Ogawa, T., I. Dobrinski and R. L. Brinster (1999). "Recipient preparation is

critical for spermatogonial transplantation in the rat." Tissue Cell 31(5): 461-472.

Oka, T. and R. T. Schimke (1969). "Interaction of estrogen and progesterone in chick oviduct development. I. Antagonistic effect of progesterone on estrogen-induced proliferation and differentiation of tubular gland cells." J Cell Biol 41(3): 816-831.

Oka, T. and R. T. Schimke (1969). "Interaction of estrogen and progesterone in chick oviduct development. II. Effects of estrogen and progesterone on tubular gland cell function." J Cell Biol 43(1): 123-137.

Okada, A., T. Sato, Y. Ohta and T. Iguchi (2005). "Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents." J Toxicol Sci 30(2): 75-89.

Ono, T. and Y. Machida (1999). "Immunomagnetic purification of viable primordial germ cells of Japanese quail (Coturnix japonica)." Comp Biochem Physiol A Mol Integr Physiol 122(2): 255-259.

Ono, T., T. Murakami, M. Mochii, K. Agata, K. Kino, K. Otsuka, M. Ohta, M. Mizutani, M. Yoshida and G. Eguchi (1994). "A complete culture system for avian transgenesis, supporting quail embryos from the single-cell stage to hatching." Dev Biol 161(1): 126-130.

Ono, T. and N. Wakasugi (1983). "Development of cultured quail embryos." Poult Sci 62(3): 532-536.

Ono, T., R. Yokoi and H. Aoyama (1996). "Transfer of male or female primordial germ cells of quail into chick embryonic gonads." Exp Anim

45(4): 347-352.

Ono, T., Yokoi, R.,Aoyama, H (1996). "Transfer of male or female primordial germ cells of quail into chick embryonic gonads." Experimental Animals 69: 546-555.

Ono T, Y. R., Maeda S, Nishida T, Aoyama H. (1998). "Settlement of quail

primordial germ cells in chicken gonads." Anim Sci Technol 69: 546-555.

Pain, B., M. E. Clark, M. Shen, H. Nakazawa, M. Sakurai, J. Samarut and R. J. Etches (1996). "Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities." Development 122(8): 2339-2348.

Palmiter, R. D. (1972). "Regulation of protein synthesis in chick oviduct. II. Modulation of polypeptide elongation and initiation rates by estrogen and progesterone." J Biol Chem 247(21): 6770-6780.

Palmiter, R. D. and J. T. Wrenn (1971). "Interaction of estrogen and progesterone in chick oviduct development. 3. Tubular gland cell cytodifferentiation." J Cell Biol 50(3): 598-615.

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." FASEB J 20(10): 1730-1732.

Pan, J. F., N. H. Liu, L. Y. Shu and H. Sun (2015). "Application of avidin-biotin technology to improve cell adhesion on nanofibrous matrices." J Nanobiotechnology 13: 37.

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: 785.

Park, K. J., S. J. Kang, T. M. Kim, Y. M. Lee, H. C. Lee, G. Song and J. Y. Han (2010). "Gamma-irradiation depletes endogenous germ cells and increases donor cell distribution in chimeric chickens." In Vitro Cell Dev Biol Anim 46(10): 828-833.

Park, T. S. and J. Y. Han (2012). "piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens." Proc Natl Acad Sci U S A 109(24): 9337-9341.

Park, T. S., D. K. Jeong, J. N. Kim, G. H. Song, Y. H. Hong, J. M. Lim and J. Y. Han (2003). "Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos." Biol Reprod 68(5): 1657-1662.

Park, T. S., K. S. Kang and J. Y. Han (2013). "Current genomic editing approaches in avian transgenesis." Gen Comp Endocrinol 190: 144-148.

Park, T. S., M. A. Kim, J. M. Lim and J. Y. Han (2008). "Production of quail (Coturnix japonica) germline chimeras derived from in vitro-cultured gonadal primordial germ cells." Mol Reprod Dev 75(2): 274-281.

Park, T. S., H. J. Lee, K. H. Kim, J. S. Kim and J. Y. Han (2014). "Targeted gene knockout in chickens mediated by TALENs." Proc Natl Acad Sci U S A 111(35): 12716-12721.

Pearce, S. T. and V. C. Jordan (2004). "The biological role of estrogen receptors alpha and beta in cancer." Crit Rev Oncol Hematol 50(1): 3-22.

Percharde, M., F. Lavial, J. H. Ng, V. Kumar, R. A. Tomaz, N. Martin, J. C. Yeo, J. Gil, S. Prabhakar, H. H. Ng, M. G. Parker and V. Azuara (2012). "Ncoa3 functions as an essential Esrrb coactivator to sustain embryonic stem cell self-renewal and reprogramming." Genes & Development 26(20): 2286-2298.

Pereira, L., F. Yi and B. J. Merrill (2006). "Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal." Molecular and Cellular Biology 26(20): 7479-7491.

Perry, M. M. (1988). "A complete culture system for the chick embryo." Nature 331(6151): 70-72.

Petitte, J. N., M. E. Clark, G. Liu, A. M. Verrinder Gibbins and R. J. Etches (1990). "Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells." Development 108(1): 185-189.

Razandi, M., A. Pedram, I. Merchenthaler, G. L. Greene and E. R. Levin (2004). "Plasma membrane estrogen receptors exist and functions as dimers." Mol Endocrinol 18(12): 2854-2865.

Rengaraj, D., D. K. Kim, Y. H. Zheng, S. I. Lee, H. Kim and J. Y. Han (2008). "Testis-specific novel transcripts in chicken: in situ localization and expression pattern profiling during sexual development." Biol Reprod 79(3): 413-420.

Reynaud, G. (1969). "[The transfer of turkey primordial germ cells to chick

embryos by intravascular injection]." J Embryol Exp Morphol 21(3): 485-507.

Reynaud, G. (1976). "[Reproductive capacity and offspring of chickens submitted to a transfer of primordial germ cells during embryonic life]." Wilehm Roux Arch Dev Biol 179(2): 85-110.

Richardson, B. E. and R. Lehmann (2010). "Mechanisms guiding primordial germ cell migration: strategies from different organisms." Nat Rev Mol Cell Biol 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.

Roe, M., N. McDonald, B. Durrant and T. Jensen (2013). "Xenogeneic transfer of adult quail (Coturnix coturnix) spermatogonial stem cells to embryonic chicken (Gallus gallus) hosts: a model for avian conservation." Biol Reprod 88(5): 129.

Rowlett, K. and K. Simkiss (1985). "The Surrogate Egg." New Scientist 107(1469): 42-44.

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.

Ryu, B. Y., K. E. Orwig, J. M. Oatley, C. C. Lin, L. J. Chang, M. R. Avarbock and R. L. Brinster (2007). "Efficient generation of transgenic rats through the male germline using lentiviral transduction and transplantation of spermatogonial stem cells." J Androl 28(2): 353-360.

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

Sanchez-Sanchez, A. V., E. Camp, A. Leal-Tassias, S. P. Atkinson, L. Armstrong, M. Diaz-Llopis and J. L. Mullor (2010). "Nanog regulates primordial germ cell migration through Cxcr4b." Stem Cells 28(9): 1457-1464.

Sanders, M. M. and G. S. Mcknight (1988). "Positive and Negative Regulatory Elements Control the Steroid-Responsive Ovalbumin Promoter." Biochemistry 27(17): 6550-6557.

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.

Savarese, F., A. Davila, R. Nechanitzky, I. De La Rosa-Velazquez, C. F. Pereira, R. Engelke, K. Takahashi, T. Jenuwein, T. Kohwi-Shigematsu, A. G. Fisher and R. Grosschedl (2009). "Satb1 and Satb2 regulate embryonic stem cell differentiation and Nanog expression." Genes Dev 23(22): 2625-2638.

Schuff, M., D. Siegel, M. Philipp, K. Bundschu, N. Heymann, C. Donow and W. Knochel (2012). "Characterization of Danio rerio Nanog and functional comparison to Xenopus Vents." Stem Cells Dev 21(8): 1225-1238.

Seo, H., J. Park, H. Lee, D. Kim, Y. Song, J. Lim, G. Song and J. Han (2009). "Physiological effects of diethylstilbestrol exposure on the development of the chicken oviduct." Anim Sci Technol 51: 485-492.

Siu, M. K., E. S. Wong, D. S. Kong, H. Y. Chan, L. Jiang, O. G. Wong, E. W. Lam, K. K. Chan, H. Y. Ngan, X. F. Le and A. N. Cheung (2013). "Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers." Oncogene 32(30): 3500-3509.

Smith, C. L. and B. W. O'Malley (2004). "Coregulator function: a key to understanding tissue specificity of selective receptor modulators." Endocr Rev 25(1): 45-71.

Song, G., H. W. Seo, J. W. Choi, D. Rengaraj, T. M. Kim, B. R. Lee, Y. M. Kim, T. W. Yun, J. W. Jeong and J. Y. Han (2011). "Discovery of candidate genes and pathways regulating oviduct development in chickens." Biol Reprod 85(2): 306-314.

Song, Y., S. Duraisamy, J. Ali, J. Kizhakkayil, V. D. Jacob, M. A. Mohammed, M. A. Eltigani, S. Amisetty, M. K. Shukla, R. J. Etches and M. C. de Lavoir (2014). "Characteristics of long-term cultures of avian primordial germ cells and gonocytes." Biol Reprod 90(1): 15.

Stebler, J., D. Spieler, K. Slanchev, K. A. Molyneaux, U. Richter, V. Cojocaru, V. Tarabykin, C. Wylie, M. Kessel and E. Raz (2004). "Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12." Dev Biol 272(2): 351-361.

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.

Suzuki, A., A. Raya, Y. Kawakami, M. Morita, T. Matsui, K. Nakashima, F. H. Gage, C. Rodriguez-Esteban and J. C. Izpisua Belmonte (2006). "Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells." Proc Natl Acad Sci U S A 103(27): 10294-10299.

Swartz, W. J. and L. V. Domm (1972). "A study on division of primordial germ cells in the early chick embryo." Am J Anat 135(1): 51-70.

Swift, C. H. (1914). Origin and early history of the primordial germ-cells in the chick. Baltimore.

Tajima, A., M. Naito, Y. Yasuda and T. Kuwana (1993). "Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus)." Theriogenology 40(3): 509-519.

Tanaka, S. S., Y. Toyooka, R. Akasu, Y. Katoh-Fukui, Y. Nakahara, R. Suzuki, M. Yokoyama and T. Noce (2000). "The mouse homolog of Drosophila Vasa is required for the development of male germ cells." Genes & Development 14(7): 841-853.

Theunissen, T. W., Y. Costa, A. Radzisheuskaya, A. L. van Oosten, F. Lavial, B. Pain, L. F. 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. R. and M. R. Capecchi (1987). "Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells." Cell 51(3): 503-512.

Tompa, P., J. Prilusky, I. Silman and J. L. Sussman (2008). "Structural disorder serves as a weak signal for intracellular protein degradation."

Proteins 71(2): 903-909.

Torres-Padilla, M. E. and I. Chambers (2014). "Transcription factor heterogeneity in pluripotent stem cells: a stochastic advantage." Development 141(11): 2173-2181.

Trefil, P., M. R. Bakst, H. Yan, J. Hejnar, J. Kalina and J. Mucksova (2010). "Restoration of spermatogenesis after transplantation of c-Kit positive testicular cells in the fowl." Theriogenology 74(9): 1670-1676.

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., Kudo, M. & Fujimoto, T. (1987). "Relationship between Genital Ridge Formation and Settlement Site of Primordial Germ-Cells in Chick-Embryos." Anatomical Record 291: 311-314.

van de Lavoir, M. C., E. J. Collarini, P. A. Leighton, J. Fesler, D. R. Lu, W. D. Harriman, T. S. Thiyagasundaram and R. J. Etches (2012). "Interspecific germline transmission of cultured primordial germ cells." PLoS One 7(5): e35664.

van de Lavoir, M. C., J. H. Diamond, P. A. Leighton, C. Mather-Love, B. S. Heyer, R. Bradshaw, A. Kerchner, L. T. Hooi, T. M. Gessaro, S. E. Swanberg, M. E. Delany and R. J. Etches (2006). "Germline transmission of genetically modified primordial germ cells." Nature 441(7094): 766-769.

van den Berg, D. L. C., W. S. Zhang, A. Yates, E. Engelen, K. Takacs, K. Bezstarosti, J. Demmers, I. Chambers and R. A. Poot (2008). "Estrogen-

related receptor beta interacts with Oct4 to positively regulate Nanog gene expression." Molecular and Cellular Biology 28(19): 5986-5995.

Wakely, J. and M. A. England (1979). "Scanning electron microscopical and histochemical study of the structure and function of basement membranes in the early chick embryo." Proc R Soc Lond B Biol Sci 206(1164): 329-352.

Wang, J., D. N. Levasseur and S. H. Orkin (2008). "Requirement of Nanog dimerization for stem cell self-renewal and pluripotency." Proc Natl Acad Sci U S A 105(17): 6326-6331.

Wang, J., L. Ma, K. Huo, X. Yang and F. He (2006). "A protein interaction network of human liver." Molecular & Cellular Proteomics 5(10): S185-S185.

Watanabe, M., M. Kinutani, M. Naito, O. Ochi and Y. Takashima (1992). "Distribution analysis of transferred donor cells in avian blastodermal chimeras." Development 114(2): 331-338.

Wernery, U., C. Liu, V. Baskar, Z. Guerineche, K. A. Khazanehdari, S. Saleem, J. Kinne, R. Wernery, D. K. Griffin and I. K. Chang (2010). "Primordial germ cell-mediated chimera technology produces viable pure-line Houbara bustard offspring: potential for repopulating an endangered species." PLoS One 5(12): e15824.

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.

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, R. C., C. L. Smith and B. W. O'Malley (2005). "Transcriptional regulation by steroid receptor coactivator phosphorylation." Endocr Rev 26(3): 393-399.

Xu, H. Y., Z. D. Li, M. Y. Li, L. Wang and Y. H. Hong (2009). "Boule Is Present in Fish and Bisexually Expressed in Adult and Embryonic Germ Cells of Medaka." Plos One 4(6).

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.

Yamaguchi, S., K. Kurimoto, Y. Yabuta, H. Sasaki, N. Nakatsuji, M. Saitou and T. Tada (2009). "Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells." Development 136(23): 4011-4020.

Yasuda, Y., A. Tajima, T. Fujimoto and T. Kuwana (1992). "A method to obtain avian germ-line chimaeras using isolated primordial germ cells." J Reprod Fertil 96(2): 521-528.

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.

Zaccanti, F., M. Vallisneri and A. Quaglia (1990). "Early aspects of sex differentiation in the gonads of chick embryos." Differentiation 43(2): 71-80.

Zhao, D. F. and T. Kuwana (2003). "Purification of avian circulating primordial germ cells by nycodenz density gradient centrifugation." Br Poult Sci 44(1): 30-35.

SUMMARY IN KOREAN

조류는 생식기관 및 발달 생물학과 같은 기초연구나 응용 연구를 위한 적합한 모델 동물이다. 특히, 닭 원시생식세포는 초기 배자에서 쉽게 분리가 가능하고 체외에서 원활한 증식 및 인위적 유전자 조작이 용이하여, 이종간 생식선 키메라 연구 및 유전자 편집기술을 통한 형질전환 조류 생산에 중요한 세포 공급원이다. 따라서, 생식세포의 특성을 유지하지 위한 원시생식세포에 대한 폭넓은 이해는 조류생식자원을 보존하고, 생식세포의 기초적인 지식을 확립하여 조류 생식세포의 응용 가능성을 높이기 위한 학문적 연구가치가 높다. 이러한 측면에서, 원시생식세포가 갖는 독특한 이동 경로와 배아 교체 방법을 조합하여 야생 조류 및 멸종위기에 처한 조류를 보존하기 위한 새로운 접근방식의 이종간 키메라 생산 방법을 개발하였다. 또한, 조류 원시생식세포 특이적 NANOG 유전자의 분자생물학적 특성을 규명하고 원시생식세포에서의 기능 검증을 통해 생식세포의 기초적인 지식을 확립하였다. 마지막으로, 닭의 난관 발달 과정에서 에스트로겐에 의해 조절되는 난관 특이적 유전자를 조사하였다.

본 연구에서 첫 번째 주제는 조류 생식세포 특이적 이동능을 활용한 새로운 접근방식의 생식세포 매개 이종간 키메라 생산 방법 개발에 관한 연구이다. 조류 원시생식세포는 특이 항체를 이용해 생체 내에서 분리하여 이종간 키메라를 효율적으로 생산하는데 널리 사용되어왔다. 하지만, 항체에 의해 원시생식세포를 분리하는 방법은 특정 종에서만 확립이 되어 매우 제한적으로 적용되기 때문에 생식세포 매개 이종간 키메라 생산 연구를 대체할 수 있는 대안적인 방법이 필요하다. 따라서 본 연구에서는 조류 원시생식세포가 초기 배자 발달 동안 배체 외역에 머물다 혈관이 형성되면 이를 따라 생식선으로 이동하는 특성과 혈관 발달 이전 배아를 교체하는 방법을 접목하여 새로운 생식선 키메라 생산 기법을 도입하였다. 우선, 배아 교체법을 통한 이종간 키메라 생산 기법을 확립하기 위해 혈관 형성 이전의 GFP-transgenic 닭의 배아와 일반 오계계의 배아를 이용하여 배아 교체법을 시행한 후 배체 외역 공여자의 원시생식세포가 수여 배아의 생식선에 안착되었는지 관찰하였다. 그 결과 GFP-TG 배체 외역에 이식된 일반

배아(KO-TG 키메라)의 5.5일령 생식선에서 GFP를 띄는 공여자의 생식세포가 관찰되었다. 그 후, 메추리-닭 생식선 키메라를 생산하기 위해 혈관 형성 이전의 닭의 배체 외역에 메추리의 배아를 이식하여 닭의 생식세포가 메추리 생식선에 이동하였는지 종 특이적 마커와 특이 항체를 이용해 조사하였다. 메추리-닭 생식선 키메라의 5.5일령 생식선에서 genomic DNA 를 추출하여 종 특이적 마커를 이용해 메추리와 닭의 마커를 검출 한 결과 두 종의 마커가 검출 되었다. 또한, 원시생식세포의 이동을 관찰하기 위해 메추리-닭 키메라 5.5일령 생식선에서 메추리 생식세포 특이적 항체인 QCR1 과 닭 생식세포 특이적 항체인 SSEA-1을 이용한 면역 염색법을 통해 발현을 관찰하였다. 그 결과 QCR1 과 SSEA-1 두 항체가 모두 발현하는 것이 관찰되었다. 따라서, 배체외역에 존재했던 닭 생식세포가 이식한 메추리 생식선에 안착된 것을 검증하였다. 따라서서, 배아 교체법을 통해 이종간 생식선 키메라 생산 연구에 활용할 수 있음을 검증 하였다. 본 실험의 결과로 생식세포 분리가 어려운 멸종 위기 조류 종의 복원 및 야생 조류종의 복원을 위해 배아 교체법을 적용시킬 수 있다고 판단된다.

야생조류 및 멸종 위기에 놓인 조류를 보전하기 위한한 대리 난각 시스템을 최적화 하였다. 2013년도에 멸종 위기 종의 배아를 대리 난각 시스템을 이용하여 보존할 수 있다는 논문이 발표되었다. 그러나 기증자 배아를 대리 난각 시스템으로 이식시키는 시점과 부화율에 관련한 최적화된 조건은 여전히 잘 알려지지 않았다. 따라서, 본 연구에서는 3일령과 4일령의 오계 배아를 White Leghorn (WL) 난각을 이용해 이식의 최적 시기를 조사하였다. 3일령과 4일령에서 옮겨진 오계 배아는 WL 의 대리난각에서 정상적인 발달을 보여주었다. 3일령 오계 배아의 부화율은 23.1% 였고, 4일령 오계 배아의 부화율은 36.0% 로 더 높게 나타났다. 이러한 결과는 대리 난각 시스템의 최적화는 멸종 위기 종의 복원 및 보전에 기여할 수 있을 것이라 여겨진다.

다음실험으로 조류 생식세포 특이적 NANOG 유전자에 대한 분자 생물학적 특성을 규명하고, 생식세포 내에서 갖는 NANOG 단백질의 기능 검증을 수행하였다. 포유류에서 NANOG 단백질은 C-말단 영역의 tryptophan 반복(WR) 모

티프에 의해 이합체가 형성되어 줄기세포 내에서 다능성을 유지하는 중요한 역할을 한다고고 보고되었다. 하지만, 조류의 NANOG 단백질은 WR 모티프가 존재하지 않고, 조류 생식세포 내에서의 고유한 기능이 검증되지 않았다. 따라서, 본 연구에서 닭 NANOG 단백질이 갖는 기능적 역할 및 분자 생물학적 특성을 조사하였다. 우선 생화학적 방법을 통해 닭 NANOG 단백질의 C-말단 영역에서 다합체를 형성하는 것을 확인하였다. 특이적으로 단합체의 경우 구조가 없는 무질서한 상태이다가 다합체가 형성되면 α나선 구조로 변성되며 포유류와 다른 모티프를 활용해 다합체를 형성하는 것을 검증하였다. 마지막으로 생식세포와 배반엽 세포에서의 기능 검증 실험을 통해 다합체를 형성하는 모티프가 돌연변이되었을 때 세포 증식이 억제되며, POUV 와 같은 다능성 관련 유전자의 발현이 저해되는 것을 확인하였다. 따라서, 닭 NANOG 단백질은 자가 결합 시시 생식세포 내에서 세포 증식에 관여하며, 상호작용하는 유전자의 발현을 조절하고 있음을 검증하였다.

마지막 실험으로써, 에스트로겐에 의해 조절되는 난관 특이적으로 발현하는 새로운 유전자를 발굴하였다. 성 성숙 이전의 암탉에 DES 를 처리 하여 난관 특이적 새로운 유전자를 선별하고, 난백 단백질을 분비하는 magnum 조직 내의 상피세포와 관상선에서만 발현하고 있음을 확인하였다. 또한, 새로운 유전자의 ORF 가 단백질로 분비되는지를 확인하기 위해 N-말단 영역에 Flag-tag 을 삽입하여 난관 상피세포에서 단백질로 발현되는 것을 검증하였다. 추후, 기능적 검증 실험을 통해 새로운 유전자가 지니는 생물학적 의미를 규명할 것이다.