SUMMARY

In the present study, various experiments were undertaken to gain a clearer understanding of the expression patterns and potential roles of specific genes identified in the chicken oviduct and cancerous ovaries. In particular, we focused on the potential role of genes regulated by estrogen in the chicken reproductive system. The functional roles and expression patterns of genes of interest involved in the laying cycle of domestic hens was also investigated.

The objective of the first experiment was to determine the expression and potential role of WNT4 in the reproductive system, as well as its relationship to ovarian cancer in chicken. WNT4 belongs to the WNT superfamily of signaling factors and normally functions as a growth factor. It has been shown to be associated with the developmental processes of multiple organs, including formation of the kidneys, mammary glands and adrenal glands, and also plays a role in mammalian sexual development. WNT4 expression is known to be regulated by estrogen throughout the estrous cycle in the reproductive tracts of female mice. The gene is therefore likely to play a crucial role in reproduction. Its dysregulation is associated with abnormal accumulation of the protein CTNNB1 in breast cancer, a disease often dependent on steroid hormone signaling. We thus hypothesized that estrogen-dependent increases in WNT4 expression play a role in development of the chicken oviduct, and abnormal expression of the gene induces the accumulation of CTNNB1. Such findings would have important implications for the incidence of ovarian cancer in domestic hens. The expression patterns and cell-specific localization of *WNT4* was determined by RT-PCR, qRT-PCR and *in situ* hybridization analysis in chicken oviducts, DES-treated chick oviducts, oviducts removed at different

points in the laying cycle and chicken oviducts after molting. WNT4 mRNA exhibited specific expression patterns in normal (48 weeks) and cancerous ovaries (96 weeks), but expression levels were found to be significantly lower in cancerous ovaries and no expression was detected in normal ovaries (96 weeks). In addition, *FZD2*, which is likely to act as a receptor for the WNT4 ligand, appeared to be under specific miRNA transcriptional regulation. Our study demonstrates that the WNT4 gene may play a crucial role in the development and function of the reproductive system. Expression of the gene is also likely to be involved in the development and proliferation of ovarian cancer in chicken.

Our second study focused on the analysis of differential expression patterns and the function of SLCs in the chicken oviduct between 3 and 20 h post-ovulation. Domestic hens follow a distinct ovulation cycle ranging from 24 to 28 h, and generally lay an egg each day. This cycle is heavily influenced by steroid hormones such as estrogen. Numerous reports have shown that estrogen binds to the promoter regions of the important egg proteins ovalbumin, ovomucoid and ovotransferrin. During ovulation, follicles move from the ovary to the oviduct, which consists of four segments, each with a specific function during oviposition. The SLC family of genes comprise various membrane transport proteins that traffic nutrients, amino acids and glucose. One such amino acid, arginine, is an important factor in mammalian embryo development. Similarly, glutamate and glucose are known to play crucial roles in the process of embryo development. Other materials transported by SLCs play roles in the development and survival of the embryo. We hypothesized that specific SLCs are responsible for development, growth and survival of chicken embryos through the transport of amino acids and other materials, including metal ions and glucose, to the egg yolk. The expression pattern and specific localization of SLC mRNA

were detected by *in situ* hybridization analysis, while the regulation of SLC transcription was determined by miRNA-regulatory analysis. Our results indicate that the expression of selected SLCs change in a time-dependent manner throughout the laying cycle and play a crucial role in egg formation and the development and survival of embryos in the reproductive tracts of hens.

Keywords: chicken, reproductive tract, WNT4, ovulation, SLC, microRNA, ovarian cancer

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LIST OF ABBREVIATION

cDNA: Complementary DNA

DIG: Digoxigenin

DNA: Deoxyribonucleic acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ER : Estrogen receptor

EL: Endoplasmic reticulum

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IP_3: Inositol 1,4,5-triphosphate

mRNA: Messenger RNA

PBS: Phosphate-buffered saline

RNA: Ribonucleic acid

RT-PCR: Reverse transcriptase-polymerase chain reaction

SLC : Solute carrier

SLC1A4: solute carrier family 1 (glutamate/neutral amino acid transporter), member 4

SLC4A5 : Solute carrier family 4, sodium bicarbonate cotransporter, member 5

SLC7A3: Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3

SLC13A2 : solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2

SLC35B4: solute carrier family 35, member B4

SSC: Standard saline citrate

tRNA: Transfer RNA

TGF-β: Transforming growth factor beta

WNT4: Wingless-type MMTV integration site family, member 4

WL: White Leghorn

CHAPTER 1

LITERATURE REVIEW

1. Structure of chicken oviduct

In mammals, the female embryo harbors two Müllerian ducts and undifferentiated gonads. These subsequently develop into ovaries and oviducts during later embryogenesis. The female embryos of birds also contain undifferentiated gonads and Müllerian ducts (Ha et al., 2004). However, the chicken's genital developmental process contains several different steps when compared to their mammalian counterparts. Specifically, the chicken embryo's left ovary and Müllerian duct develop into genital organs during the process of differentiation, while the right ovary and Müllerian duct regress (Ha et al., 2004). This is a unique characteristic of avian embryo development.

In the male embryo, the right and left Müllerian ducts degrade by Day 12 of incubation. The female embryo's right Müllerian duct also regresses, but the left duct develops into an oviduct, consisting of 5 segments which include the infundibulum, magnum, isthmus, shell gland and vagina (Jonchere et al., 2010). By Day 12 of incubation, the female embryo can be distinguished from that of a male embryo, due to the appearance of the Müllerian ducts (Teng, 1987).

The oviducts of laying hens are 40 to 80 cm in length and normally weigh approximately 40 grams. The infundibulum, located on the top of the oviduct, has an open structure to allow it to engulf the ovum (Shimada and Tanabe, 1982). The organ is approximately 11 cm in length and consists of a thick, wall-like tissue. The mucosa of the chick infundibulum is generated by chorionic epithelia. In addition, the outer layer of the yolk skin (also called the vitelline membrane) and chalazal layer (which appears as a white

ropelike structure on each side of the yolk) of the albumen are probably generated here.

The magnum segment of the chick oviduct is approximately 34 cm long. The organ is the longest among the 5 portions of the hen's oviduct. In this segment, egg albumen is secreted in order to cover the egg yolk. The ovum enveloped with albumen then enters the 11 cm portion of the isthmus. Then shell membranes of the egg yolk are formed to cover the egg albumen from the outside in the shell gland (10 cm long). The process of calcification for a hardened shell is completed in the genital organ (Bar et al., 1992; Lavelin et al., 2001). Epithelial cells in the shell gland secrete fluid with calcium ions at a high concentration through the inner surface of the organ. In the vagina, a protein called bloom is applied to the shell to protect it from harmful bacteria or dust entering the egg shell pores. The egg is then turned horizontally (a process called oviposition) before laying, so that the egg exits the body with the blunt end first.

2. Function of chicken oviduct

1) Ovulation cycle of domestic hens

The domestic hen's ovulation and ovipostion cycle generally ranges from slightly longer than 24 hr to 28 hr in length and can occur continuously for several days, or longer than 1 year. During ovulation, the follicle moves from the ovary to the oviduct segment. Subsequent to ovulation, the ovum is engulfed by the infundibulum (Shimada and Tanabe,

1982).

In the infundubulum, the ovum resides for 15 to 30 min. If the ovum is not received by the infundibulum, it will be reabsorbed within 24 hr. This segment of the chicken oviduct accommodates fertilization of the largest follicle if sperm is present. The infundibulum produces a small quantity of albumen before the ovum passes to the largest segment of the chicken oviduct, the magnum, where the majority of albumen is produced. The magnum secretes a large amount of calcium, although less than that secreted within the shell gland (Eastin and Spaziani, 1978). The ovum resides in the magnum for approximately 2 to 3 hr, before the largest follicle moves to the isthmus. Both outer and inner shell membranes are formed over 1 to 2 hr in the isthmus, while some reports have demonstrated that tissue formation is also initiated. Finally, the ovum moves to the shell gland, which is characterized by a prominent longitudinal muscle layer. The ovum resides in the shell gland for 18 to 26 hr for calcification and shell formation. Calcification within the shell gland correlates with stimulus initiated through ovulation and neuroendocrine factors that control the process.

A number of steroid hormones are known to induce ovulation. These steroid hormones participate in a complex interplay of signaling from follicle growth to explusion of the egg (Etches et al., 1983; Johnson et al., 1984; Lebedeva et al., 2004).

2) Fertilization of ovum in chicken

The testes of male chickens produce sperm, which is released by the cloaca of the male bird during mating. If successful, released sperm

arrive in the oviduct of the female and travel through the reproductive tract until reaching the infundibulum. The sperm pass through four segments of the chicken oviduct including the shell gland, isthmus, magnum and infundibulum over the course of a week (Bakst, 1998). Once reaching the infundibulum, the sperm can survive for a further week while waiting for the arrival of the egg mass, which will not have a shell casing. When a yolk arrives in the infundibum from the ovary, fertilization can take place. The fertilized egg mass then passes through the female oviduct to develop into its full form and receive a hardened shell. After the fertilized egg is laid, the female hens are ready to begin the formation of a new egg (Clulow and Jones, 1982; Howarth, 1983).

3. Signal transduction for development of oviduct

1) EGF & EGFR

EGF/EGFR signal transduction is known to regulate cell adhesion, proliferation, motility and invasion (Citri and Yarden, 2006). The expression of EGF, a growth factor, and EGFR in the mouse reproductive tract have been reported to play a crucial role in embryo development (Huet-Hudson et al., 1990; Paria et al., 1993; Paria and Dey, 1990). EGF signaling regulates cell function through interaction with its receptor, EGFR (Paria et al., 1994). The receptor has been identified on the surface of diverse tissues and organs, including the uterus in various species (Das et al., 1994; Mukku and Stancel, 1985). In addition, the signal pathway is known to be related to growth of the uterus and vagina in mice through the regulation of estrogen. Knockout mouse model studies have indicated that

the growth of the uterus and vagina require EGF/EGFR signaling (Hom et al., 1998). EGF may also play an important role in the process of regulating epithelial proliferation in estrogen target organs, including the reproductive tract (Paria et al., 1994).

2) Transforming growth factor-beta (TGF-β)

The TGF-β superfamily is the largest known family of secreted growth factors in vertebrates. These genes play important roles in diverse processes of physiology and pathology (Li et al., 2011). The signal pathway involves various receptors, ligands and SMAD transducers, and can be regulated by inhibitory SMADs and ligand traps (Attisano and Wrana, 2002; Derynck and Zhang, 2003). TGF-βs are also known to regulate a plethora of functions in cells and play a crucial role in control of various developmental and homeostatic processes (Attisano and Wrana, 2002). TGF-βs target different receptor types, known as TGF receptor types I and II, and involves numerous additional signaling factors including BMP, AMHR and TGF-β1 (Vilar et al., 2006). In the mouse, it has been reported that three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) are regulated at the mRNA and protein level through genes expressed in the uterus and vagina by estrogen. TGF-βs may also play an important role in the developmental processes of the uterus and vagina in mice (Takahashi et al., 1994).

3) Estrogen & Estrogen receptor

Estrogen is a steroid hormone that plays a major role in the differentiation and proliferation of various cells, as well as in regulation of the reproductive system and diseases including ovarian cancer (Dougherty

and Sanders, 2005a). Major research into the function of estrogen has been conducted using knockout transgenic mice (Hewitt et al., 2005). Estrogen signaling is mediated by the estrogen receptor, a nuclear receptor of the ligand-inducible transcription factor family. The expression and localization of the receptor is likely to be involved in important functions concerned with cell proliferation and functional differentiation of the reproductive tract (Okada et al., 2003).

The chicken oviduct is a useful model for the investigation of steroid hormone function because the organ is highly responsive to such hormones (Dougherty and Sanders, 2005b). Estrogen in the chicken ovaries confers additional functions related to reproduction (Etches, 1987). For example, the chicken oviduct's development is primarily associated with the induction of estrogen. The oviduct of immature birds can host extensive cell proliferation and synthesis of egg white protein if induced by estrogen treatment. During treatment, the oviduct of immature birds also increases in weight, with formation of egg white proteins including ovalbumin and conalbumin (Palmiter and Mulvihill, 1978). In contrast, the removal of estrogen results in cessation of egg white protein synthesis. These results clearly outline the functional mechanism of the steroid hormone action and its crucial role in the hen oviduct (Oka and Schimke, 1969b; Palmiter and Mulvihill, 1978).

CHAPTER 2

Avian WNT4 in the Female Reproductive Tracts: Potential Role of Oviduct Development and Ovarian Carcinogenesis

1. Abstract

The wingless-type MMTV integration site family of proteins (WNT) are highly conserved secreted lipid-modified signaling molecules that play a variety of pivotal roles in developmental events such as embryogenesis, tissue homeostasis and cell polarity. Although, of these proteins, WNT4 is well known to be involved in genital development in fetuses of mammalian species, its role is unknown in avian species. Therefore, in this study, we investigated expression profiles, as well as hormonal and post-transcriptional regulation of WNT4 expression in the reproductive tract of female chickens. Results of this study demonstrated that WNT4 is most abundant in the stroma and luminal epithelia of the isthmus and shell gland of the oviduct, respectively. WNT4 is most abundant in the glandular epithelium of the shell gland of the oviduct of laying hens at 3 h post-ovulation during the laying cycle. In addition, treatment of young chicks with DES stimulated WNT4 only in the glandular epithelial cells of the isthmus and shell gland of the oviduct. Moreover, results of our study demonstrated that *miR-302b* influences FZD2, a cognate co-receptor of WNT4, expression via their specific binding sites in its 3′-UTR. On the other hand, our results also indicate that WNT4 is expressed predominantly in the glandular epithelium of cancerous ovaries, but not in normal ovaries of hens. Collectively, these results indicate cell-specific expression of WNT4 in the reproductive tract of chickens and that it likely has crucial roles in development and function of oviduct as well as initiation of ovarian carcinogenesis in laying hens.

2. Introduction

The chicken oviduct is well known as a model for research on hormone action, including effects of estrogen and progesterone (Dougherty and Sanders, 2005b). Development of the chicken oviduct is induced by sex steroid hormones, including estrogen, which are associated with development processes associated with folliculogenesis, ovulation and formation of the egg in the reproductive tract (Dougherty and Sanders, 2005b; Kohler et al., 1969; Palmiter and Wrenn, 1971). Estrogen is well known as the hormone responsible for growth of the yolk and follicle, and the process of calcium metabolism for formation of the egg shell and the process of oviposition or laying of the egg (Bar, 2009; Yoshimura and Bahr, 1995). In addition, estrogen has also a crucial role in the process of the synthesis of egg white proteins in the oviduct (Palmiter, 1972). Furthermore, formation of the tubular glands and differentiation of the epithelial cells including goblet and ciliated cells in the chicken oviduct are induced by estrogen (Palmiter and Wrenn, 1971).

During mammalian embryogenesis, the wingless-type MMTV integration site family (WNT) signaling molecules are pleiotropic and involved in various biological processes such as cell proliferation, differentiation and specification of cell fate (Logan and Nusse, 2004). Of these, WNT4 is a secretory signaling protein concerned with multiple processes in organ development including formation of kidney, mammary gland and adrenal gland, as well as sexual development in mammals (Jeays-Ward et al., 2004; Kispert et al., 1998; Miyakoshi et al., 2009; Treier et al., 1998). Of particular note, WNT4 is a key player in the

development and differentiation of the female reproductive system (Miller et al., 1998; Miyakoshi et al., 2009). In mice, the Wnt4 signaling pathway participates in folliculogenesis, luteogenesis and steroidogenesis of granulosa cells and in the regulatory processes of stromal cell proliferation and differentiation for survival and development of embryos within the uterine lumen (Franco et al., 2011). Interestingly, several genes involved in the WNT signal pathway(s) are regulated by estrogen. In fact, the WNT4-FZD2 signaling pathway is activated by binding of estrogen to estrogen receptor alpha (ESR1) in the uterus (Hou et al., 2004) and in somatotrophs that produce growth hormone in the anterior pituitary gland of rodents (Miyakoshi et al., 2009). Furthermore, over-expression of WNT4 leads to the development of malignant tumors. Indeed, the elevated expression of WNT4 is frequently observed in many breast cancer patients which implies that it aberrant expression leads to abnormal cell proliferation and breast cancer in women (Huguet et al., 1994).

There is little known about the expression or function of WNT4 in the reproductive tract of female chickens. Therefore, the objectives of this study were to: 1) reveal cell-specific expression patterns of WNT4 in the chicken oviduct during the reproductive cycle; 2) determine whether estrogen regulates expression of WNT4 during development of the chick oviduct; 3) determine whether WNT4 expression is mediated through post-transcriptional activity of specific microRNAs and 4) compare the expression pattern of WNT4 between normal and cancerous ovaries. Results of the present study provide novel insights into the WNT4 gene of chickens with respect to cell-specific expression and hormonal regulation of its expression during oviduct development, the laying cycle and development of ovarian carcinogenesis in laying hens.

3. Material & methods

Experimental Animals and Animal Care

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) laying hens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea. All hens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water.

Tissue Samples

Study One. Following euthanasia, tissue samples were collected from brain, heart, liver, kidney, small intestine, gizzard, ovary, oviduct and testis of 48-week-old WL roosters (n =3) and laying hens (n= 3). The collected samples were either frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces, frozen in liquid nitrogen vapor, and stored at -80℃. The other samples were cut into 10 mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 mm.

Study Two. Female chicks were identified by PCR analysis using W chromosome-specific primer sets (Lee et al., 2009). Treatment with diethylstilbestrol (DES) and recovery of the oviduct were performed as reported previously (Sanders and McKnight, 1988). Briefly, a 15 mg DES pellet was implanted subcutaneously in the abdominal region of 1-week-old female chicks for 10 days. The DES pellet was removed for 10 days,

and then a 30 mg dose of DES was administered for 10 additional days. Five 37-day-old chicks in each group were euthanized using 60%–70% carbon dioxide. The collected samples were either frozen or fixed in 4% paraformaldehyde for further analyses. Paraffin-embedded tissues were sectioned at 5 μm.

Study Three. Hens (n=5 per time point) were euthanized at either 3 h or 20 h after ovulation using 60%–70% carbon dioxide. Samples of the magnum and the shell gland of oviducts from each hen were collected at each time point. Sampling of magnum and shell gland was at the middle of each tissue to prevent mixing with another tissue such as the infundibulum and isthmus. The tissue samples of similar size were: 1) removed and placed in Optimal Cutting Temperature (OCT) compound (Miles, Oneonta, NY); 2) frozen in liquid nitrogen and stored at -80℃; 3) fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4); or 4) frozen immediately in liquid nitrogen and stored at -80℃ until analyzed. After 24 h, tissues fixed in 4% paraformaldehyde were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany).

Study Four. A total 136 mature laying hens (88 over 36 months and 48 over 24 months of age), which had stopped laying eggs, were euthanized for collection of cancerous ovarian tissues. We obtained cancerous ovarian tissues from 10 hens and normal ovarian tissues from 10 egg-laying hens of similar age. We evaluated tumor stage of 10 hens with cancerous ovaries according to characteristic features of chicken ovarian cancer (Barua et al., 2009b; Lim et al., 2012). Three hens had stage III disease as ovarian tumor cells had metastasized to the gastrointestinal (GI) tract and liver surface with profuse ascites in the abdominal cavity. Five hens had tumor cells spread to distant organs including liver parenchyma, lung, GI tract and oviduct with profuse ascites, indicating stage IV disease. Two

hens had stage I disease as tumors were limited to their ovaries. The collected samples were fixed in 4% paraformaldehyde for further analyses. After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 µm and stained with hematoxylin and eosin (Supplementary Figure 1). Epithelial ovarian cancers in chickens were classified based on the cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans (Barua et al., 2009b).

RNA Isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Semiquantitative RT-PCR analysis

The expression of *WNT4* mRNA in chicken organs including the oviduct, ovary and cancerous ovary was assessed using semi-quantitative RT-PCR as described previously described previously (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 ug) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPowerH RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *WNT4*, the sense primer (5′- GGA GTG CCA GTA CCA ATT CC -3′) and antisense primer (5′- CGT CGA ATT TCT CCT TCA GC -3′) amplified a 491-bp product. For *GAPDH* (housekeeping gene), the sense primer (5′- TGC CAA CCC

CCA ATG TCT CTG TTG -3′) and antisense primer primer (5′- TCC TTG GAT GCC ATG TGG ACC ATT G -3′) amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted as described previously (Song et al., 2007). PCR amplification was conducted using approximately 60 ng cDNA as follows: (1) 95℃ for 3 min; (2) 95℃ for 20 sec, 60℃ for 40 sec (for *WNT4* and *GAPDH*) and 72℃ for 1 min for 35 cycles; and (3) 72℃ for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel DocTM XR+ system with Image LabTM software (Bio-Rad).

Sequence analysis

The mRNA and protein sequences of chicken WNT4 were obtained from a BLAST search of the *Gallus gallus* genome database at the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) of the Geneious Pro Version 5.04 (Drummond et al., 2010). To determine the confidence level for each internal node on the phylogenetic tree, 1,000 nonparametric bootstrap replications were used (Felsenstein, 1985).

Quantitative RT-PCR Analysis

Total RNA was extracted from each segment of oviduct and ovary using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using a Superscript® III First-

Strand Synthesis System (Invitrogen). Gene expression levels were measured using SYBR® Green (Biotium, Hayward, CA, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was analyzed simultaneously as a control and used for normalization of data. These experiments were performed in triplicate. For *WNT4*, the sense primer (5′- GGA GTG CCA GTA CCA ATT CC -3′) and antisense primer (5′- AGA GAT GGC GTA GAC GAA CG -3′) amplified a 121-bp product. For *GAPDH*, the sense primer (5′- ACA CAG AAG ACG GTG GAT GG -3′) and antisense primer primer (5′- GGC AGG TCA GGT CAA CAA CA -3′) amplified a 193-bp product. The PCR conditions were 94℃ for 3 min, followed by 40 cycles at 94℃ for 20 sec, 60℃ for 40 sec, and 72℃ for 1 min using a melting curve program (increasing the temperature from 55℃ to 95℃ at 0.5℃ per 10 sec) and continuous fluorescence measurement. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C_T value represented the cycle number at which a fluorescent signal was significantly greater than background, and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the C_T value of the control oviduct.

In Situ Hybridization Analysis

For hybridization probes, PCR products were generated from cDNA primers used for RT-PCR analysis. The products were gel-extracted and cloned into pGEM-T vector (Promega). After verification of the sequences, plasmids containing gene sequences were amplified with T7- and SP6-specific primers (T7:5’-TGT AAT ACG ACT CAC TAT AGG G-

3’; SP6:5’-CTA TTT AGG TGA CAC TAT AGA AT-3’). Then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Tissues were collected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm and sections placed on APES-treated (silanized) slides. The sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed twice in DEPC-treated PBS. The sections were then digested in 5μg/ml Proteinase K (Sigma) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37℃. After post-fixation in 4% paraformaldehyde, sections were incubated twice for 5 min each in DEPC-treated PBS and incubated in TEA buffer [0.1M triethanolamine containing 0.25% (v/v) acetic anhydride]. The sections were incubated in a prehybridization mixture containing 50% formamide and 4X standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 40% formamide, 4X SSC, 10% dextran sulfate sodium salt, 10mM DTT, 1 mg/ml yeast tRNA, 1mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe overnight at 42℃ in a humidified chamber. After hybridization, sections were washed for 15 min in 2X SSC at 37℃, 15min in 1X SSC at 37℃, 30 min in NTE buffer (10mM Tris, 500mM NaCl and 1mM EDTA) at 37℃ and 30 min in 0.1X SSC at 37℃. After blocking with 2% normal sheep serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche, Indianapolis, IN). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM

levamisole (Sigma Chemical Co., St. Louis, MO).

MicroRNA Target Validation Assay

The 3'-UTR of *FZD2* was cloned and confirmed by sequencing. The 3'-UTR was subcloned between the eGFP gene and the bovine growth hormone (bGH) poly-A tail in pcDNA3eGFP (Clontech, Mountain View, CA) to generate the eGFP-miRNA target 3'-UTR (pcDNA-eGFP-3'UTR) fusion constructs. For the dual fluorescence reporter assay, the fusion constructs containing the *DsRed* gene and either *miR-302b, miR-302c,* or *miR-302d* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3'UTR and pcDNA-DsRed-miRNA (4μg) were co-transfected into 293FT cells using the calcium phosphate method. When the DsRed-miRNA is expressed and binds to the target site of the 3'-UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistical Analyses

Differences in the variance between untreated and DES-treated oviducts were analyzed using the F test, and differences between means were detected using the Student's t test. The probability value of $P<0.05$ was considered statistically significant. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

Table 1. Pairwise comparisons of WNT4 among chicken and several other vertebrate species

Species	Symbol	Identity (%)	Genbank #
G. gallus (Chicken)	WNT4	-	NP_990114.1
vs. H. sapiens (Human)	WNT4	85.5	NP_110388.2
vs. P. troglodytes (Chimpanzee)	WNT4	79	XP_524597.3
vs. M. mulatta (Monkey)	WNT4	85.5	XP_001100814.1
vs. C. lupus (Dog)	WNT4	85.8	XP_855190.2
vs. B. taurus (Cow)	WNT4	85.8	XP_003585839.1
vs. M. musculus (Mouse)	Wnt4	84.9	NP_033549.1
vs. R. norvegicus (Mouse)	Wnt4	84.3	NP_445854.1
vs. D. rerio (Zebra fish)	WNT4	85.8	NP_001035477.1

4. Results

Expression and Localization of WNT4 in the chicken oviduct

Anatomically, the chicken oviduct consists of five segments: the infundibulum (site of fertilization), magnum (production of components of egg-white), isthmus (formation of the shell membrane), shell gland (formation of the egg shell) and vagina (deposition of antimicrobial film over shell during oviposition). Results of RT-PCR analysis indicated a high level of *WNT4* mRNA expression in the isthmus and the shell gland as compared with the infundibulum and the magnum (Figure 1A). Further, quantitative PCR analysis revealed that *WNT4* mRNA levels in the isthmus and the shell gland were 3.59- and 3.29-fold ($P < 0.01$), respectively, higher than those of the infundibulum and the magnum (Figure 1B). To determine localization of *WNT4* mRNA in the chicken oviduct, *in situ* hybridization analysis was performed (Figure 1C). The *WNT4* mRNA was most abundant in stromal cells and luminal epithelia (LE) of the isthmus and the shell gland, respectively. However, little or no mRNA was detected in the infundibulum and the magnum of the chick oviduct. These results indicate that WNT4 may have roles in the formation of the shell membrane and the egg shell in laying hens.

Expression and localization of WNT4 in the chicken oviduct at different stages of the laying cycle

We previous reported spatial and temporal changes in gene expression in the oviduct of laying hens at different stages of the laying cycle (Jeong et al., 2012a). In order to detect cell-specific localization of

WNT4 mRNA in the chicken oviduct between 3 h and 20 h after ovulation, RT-PCR, quantitative PCR and *in situ* hybridization analyses were performed. As illustrated in Figure 2A, RT-PCR analysis detected the highest level of *WNT4* mRNA expression at 3 h post-ovulation in the shell gland and lowest expression at 20 h post-ovulation in the shell gland, but little or no detectable *WNT4* mRNA in the magnum at either time point. In addition, quantitative PCR analysis revealed that expression of *WNT4* mRNA was 3.32-fold ($P < 0.001$) at 3 h than at 20 h post-ovulation in the shell gland, but changes in expression of *WNT4* mRNA were not different between 3 h and 20 h post-ovulation in the magnum. Consistent with these results, *in situ* hybridization analyses indicated that *WNT4* mRNA was predominantly localized to the glandular epithelium (GE) of the shell gland at 3 h post-ovulation and it was also detected to a lesser extent in LE of the shell gland at both time points (Figure 2C). However, there is either no or very little expression of *WNT4* in the magnum. These results suggest that the WNT4-mediated Ca^{+2} signaling pathway is activated in the GE of the chicken oviduct during egg formation and oviposition.

Effects of DES on WNT4 expression in the chicken oviduct

Cell-specific expression of *WNT4* mRNA in the oviduct of mature hens suggested regulation by estrogen during development of the chicken oviduct. Because diethylstilbestrol (DES) is a synthetic estrogen that binds to estrogen receptors with similar effect of the natural estrogen 17β-estradiol (Dougherty and Sanders, 2005b; Sanders and McKnight, 1988; Song et al., 2011), we determined effects of DES and reported that DES regulates growth, development and cytodifferentiation of the immature chick oviduct (Song et al., 2011). Likewise, we examined the effects of DES on expression of *WNT4* mRNA in the chicken oviduct in the present

study. As illustrated in Figure 3A and 3B, expression of *WNT4* mRNA increased in DES-treated oviducts as compared with untreated oviducts. Further, quantitative PCR analysis confirmed that *WNT4* expression was an overall 3.8-fold ($P < 0.05$) increase in the DES-treated oviducts (Figure 3C). In addition, DES treatment stimulated 38.8- and 59.4-fold increases ($P < 0.001$) in *WNT4* mRNA in the isthmus and the shell gland, respectively (Figure 3D). To determine localization of *WNT4* mRNA in chick oviducts treated with DES, *in situ* hybridization analysis was used to determine that *WNT4* mRNA is expressed predominantly expressed in GE of the isthmus and the shell gland (Figure 3E). There was little or no detectable *WNT4* mRNA in the infundibulum and magnum. These results indicate that estrogen induces WNT4 expression during oviduct development in chicks and during formation of the shell membrane (isthmus) and egg shell (shell gland) during the laying cycle of hens.

Differential expression of WNT4 between normal and cancerous ovaries of hens

The laying hen is a unique animal model for study of human epithelia-derived ovarian cancer research. This is because they spontaneously develop ovarian cancer of the surface epithelium of the ovaries at a high rate and are useful for development of biomarkers for detection and early diagnosis of ovarian cancer, as well as for discovery of anti-cancer drugs/biomaterials (Stammer et al., 2008). In fact, in our preliminary experiments, we found abundant amounts of *WNT4* mRNA in ovaries of 48-week-old female (n= 3) chickens by RT-PCR analyses (Supplementary Figure 1). Therefore, we determined whether cell-specific WNT4 expression was detectable ovarian cancer in laying hens. As illustrated in Figure 4A, quantitative PCR revealed that *WNT4* mRNA

increased 1.74- ($P < 0.05$) and 3.35-fold ($P < 0.05$) in cancerous ovaries as compared with normal ovaries from 48-week-old and 96-week-old of laying hens, respectively. Further, *WNT4* mRNA was localized predominantly to GE of cancerous ovaries and normal ovaries from 48-week-old hens, but not in any other cells including stroma and blood vessel (Figure 4B). However, *WNT4* mRNA was not detected in normal ovaries from 96-week-old hens. These results indicate that *WNT4* mRNA is expressed at very low levels in ovaries of normal egg laying hens, but decreases to undetectable amounts as the laying hen ages. However, expression of *WNT4* increases in cancerous ovaries with advancing age of hens.

Post-transcriptional regulation of microRNA affecting FZD2

WNT4 protein mainly binds to its cognate extracellular surface receptor, frizzled family receptor 2 (FZD2) in order to activate its intracellular signal transduction cascade (Dale, 1998; Hou et al., 2004). Expression of chicken *FZD2* gene is post-transcriptionally regulated by microRNAs (miRNAs); therefore, we performed a miRNA target validation assay. We identified potential miRNA binding sites within the 3′-UTR of the *FZD2* gene using the miRNA target prediction database (miRDB; http://mirdb.org/miRDB/) which revealed three putative binding sites for *miR-302b, miR-302c, miR-302d*. Therefore, we determined whether these miRNAs influenced expression of the *FZD2* gene via its 3′-UTR. A fragment of the 3’-UTR of *FZD2* gene harboring binding sites for the miRNAs were cloned in downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3′-UTR region. After co-transfection of eGFP-3’-UTR of *FZD2* gene and DsRed-miRNA, the intensity of GFP expression and percentage

of GFP-expressing cells were analyzed by fluorescence microscopy and fluorescence activated cell sorting (FACS). As illustrated in Figures 5C and -5D, in the presence of *miR-302b*, the expression level of GFP-expressing cells decreased 24.7% ($P < 0.05$) as compared with control based on FACS and fluorescence microscopy analyses. However, in the presence of *miR-302c* or *miR-302d*, there was no significant change in expression of GFP as compared to the control (data not shown). Therefore, these results indicate that *miR-302b* directly binds to the *FZD2* transcripts and post-transcriptionally regulates expression of the *FZD2* gene.

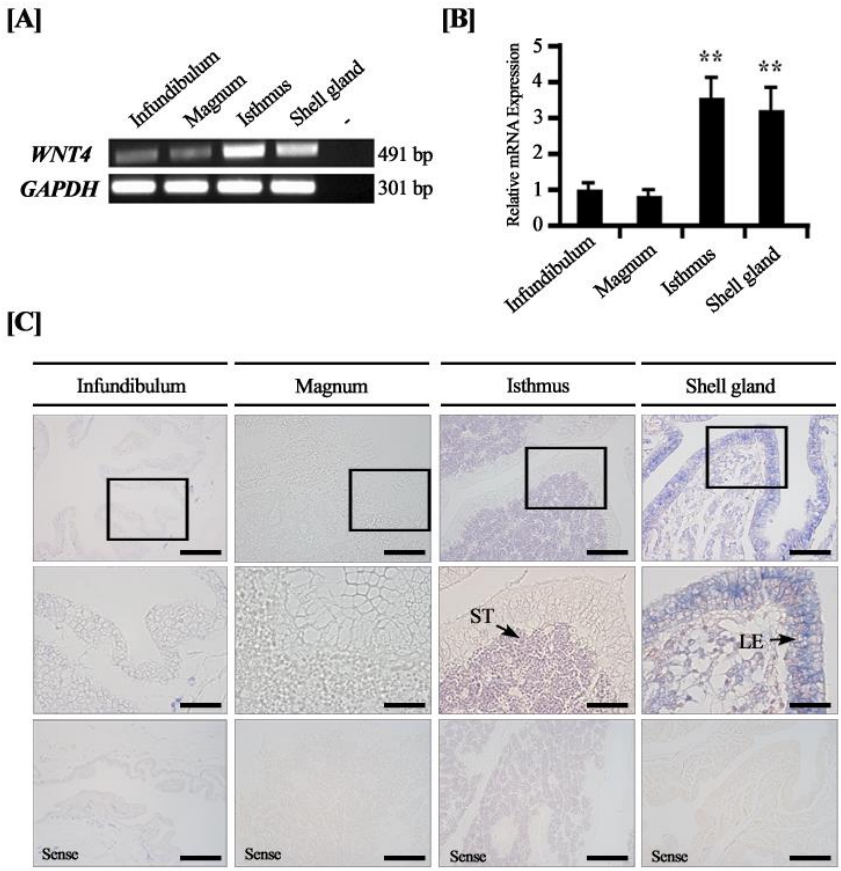


Fig.1. Expression and localization of *WNT4* in the chicken oviduct. Both RT-PCR [A] and quantitative PCR [B] analyses were performed using cDNA templates from each segment of the chicken oviduct. [C] *In situ* hybridization analysis for cell-specific changes in expression of *WNT4* in the each segment of the chicken oviduct. Legend: ST, stroma; LE, luminal epithelium. Scale bar represents 50 μm (the first horizontal panels), 20 μm (the second horizontal panel) and 100 μm (the third horizontal panel). The asterisks denote statistically significant differences (**P < 0.01).

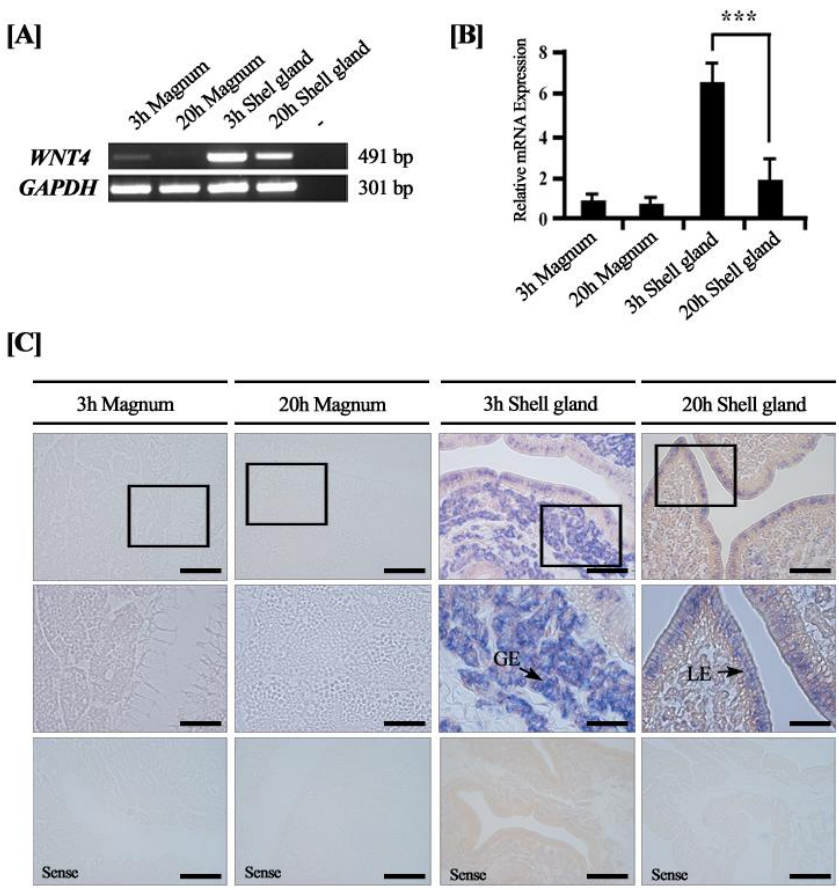


Fig.2. Expression and localization of *WNT4* in the magnum and the shell gland of the oviduct at 3 h and 20 h after ovulation. Both RT-PCR [A] and quantitative PCR [B] analyses were performed using cDNA templates from the magnum and the shell gland segment at 3 h and 20 h after ovulation. [C] *In situ* hybridization analysis for cell-specific changes in expression of *WNT4* in the magnum and the shell gland at 3 h and 20 h after ovulation. Legend: GE, glandular epithelium; LE, luminal epithelium. Scale bar represents 50 μm (the first horizontal panel), 20 μm (the second horizontal panel) and 100 μm (the third horizontal panel). The asterisks denote statistically significant differences (***$P < 0.001$).

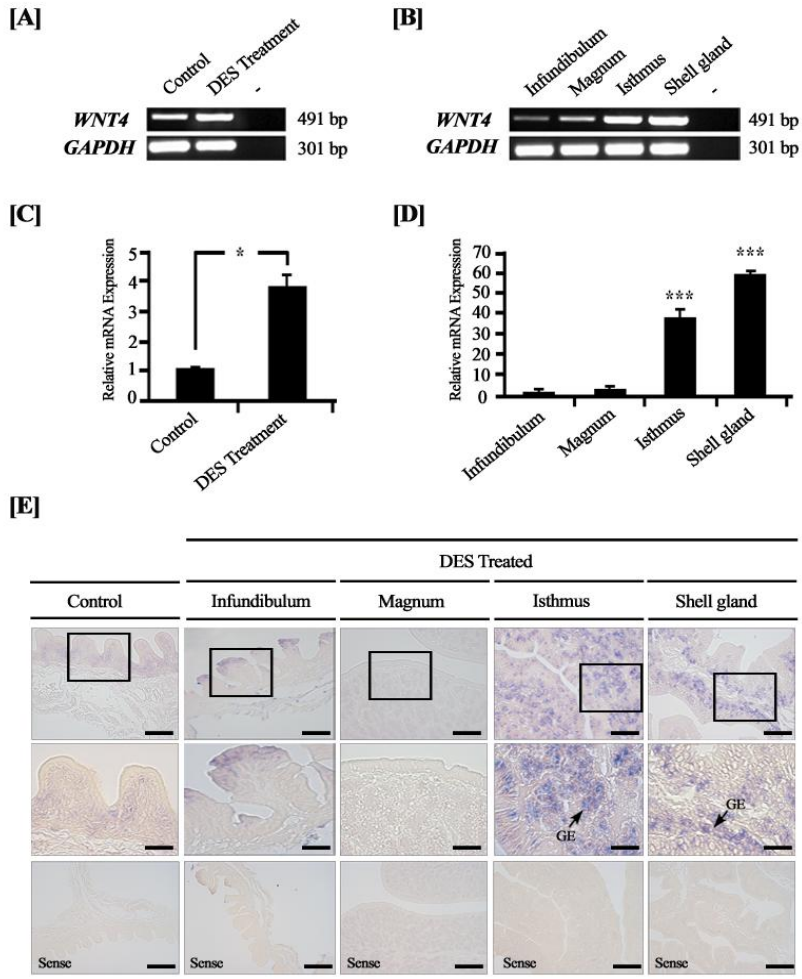


Fig.3. Effect of DES on tissue-specific expression of chicken *WNT4*. Both RT-PCR [A and B] and quantitative PCR [C and D] analyses were performed using cDNA templates from DES-treated and untreated oviducts. [E] *In situ* hybridization analyses revealed cell-specific expression of *WNT4* mRNA in oviducts of DES-treated and untreated chicks. Legend: GE, glandular epithelium. Scale bar represents 50 μm (the first horizontal panel), 20 μm (the second horizontal panel) and 100 μm (the third horizontal panel). The asterisks denote statistically significant differences (***$P < 0.001$ and *$P < 0.05$).

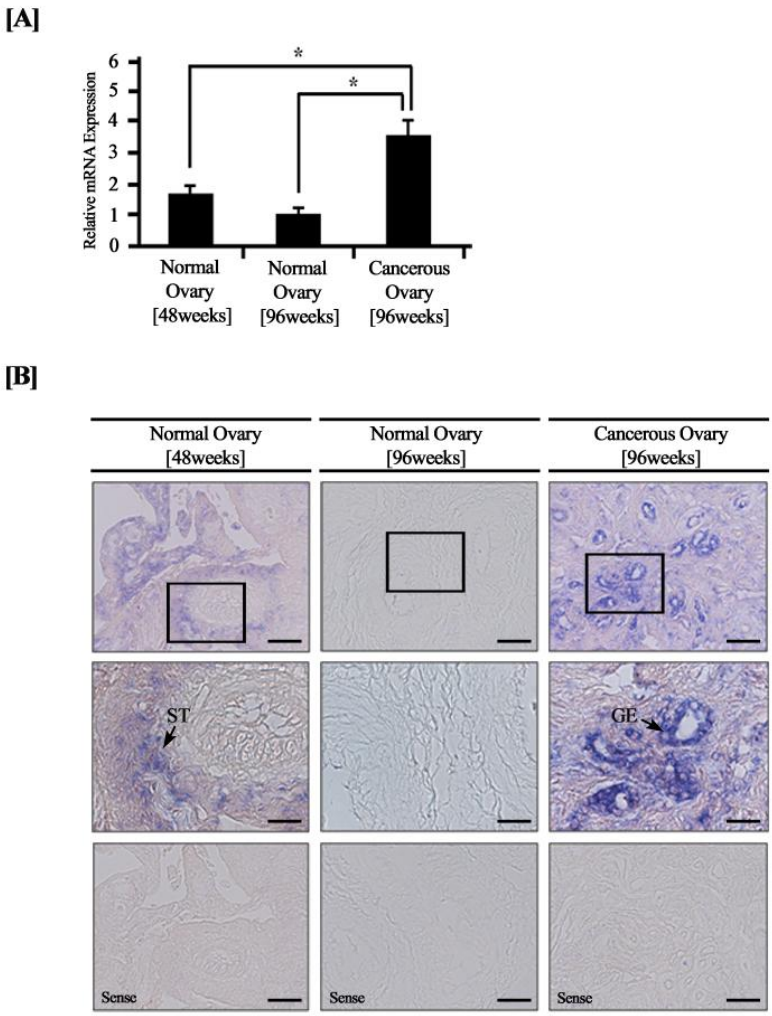


Fig. 4. Expression and localization of WNT4 between normal and cancerous ovaries. Expression and localization of *WNT4* between normal and cancerous ovaries. [A] Quantitative PCR analysis was performed using cDNA templates from normal and cancerous ovaries. [B] *In situ* hybridization analyses revealed cell-specific expression of *WNT4* mRNA between normal and cancerous ovary. Legend: GE, glandular epithelium; ST, stroma. Scale bar represents 50 μm (the first horizontal panel), 20 μm (the second horizontal panel) and 100 μm (the third horizontal panel). The asterisks denote statistically significant differences (*$P < 0.05$).

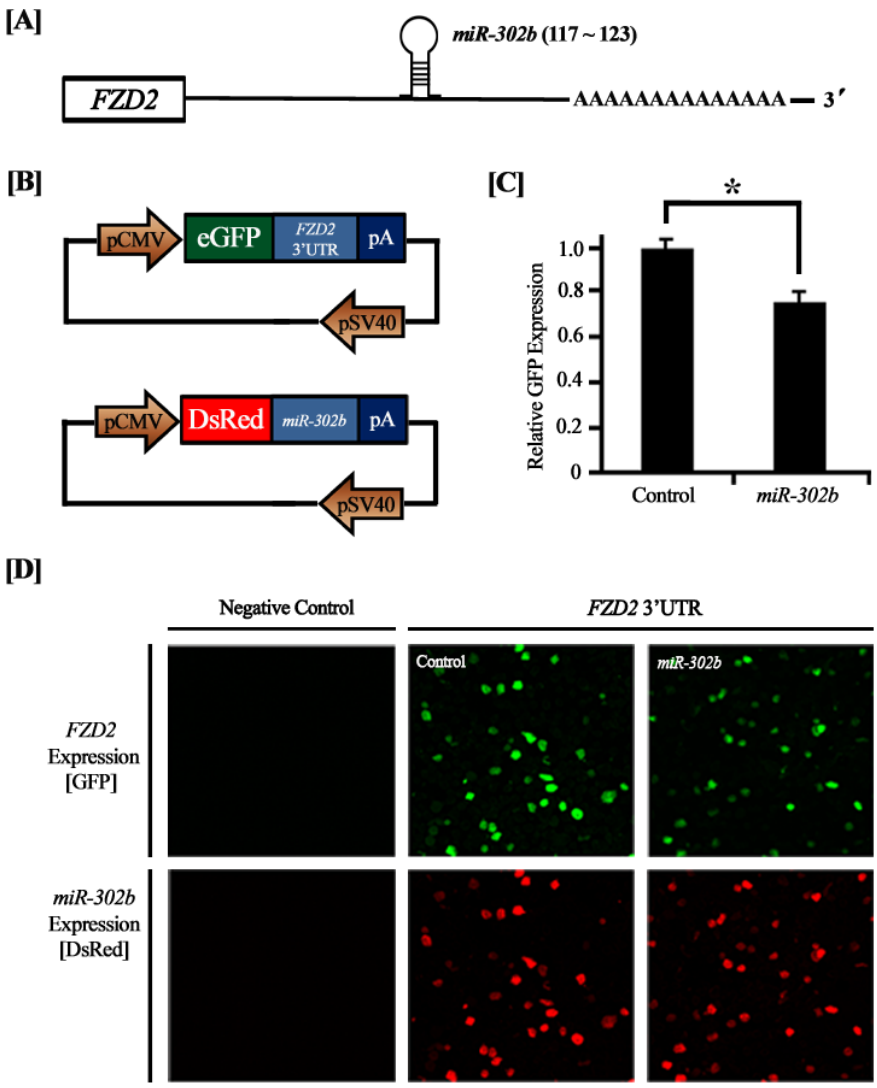
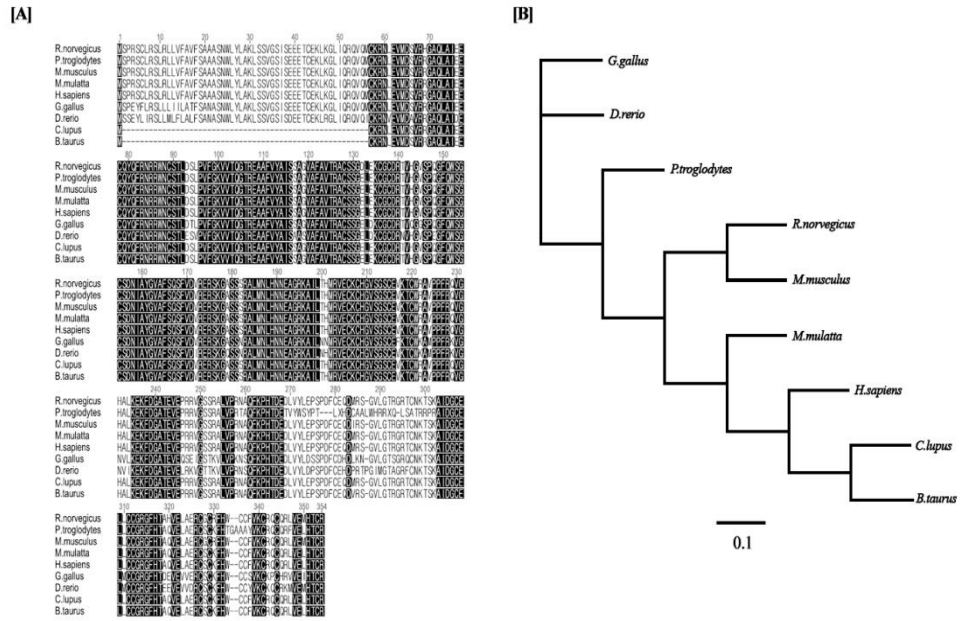
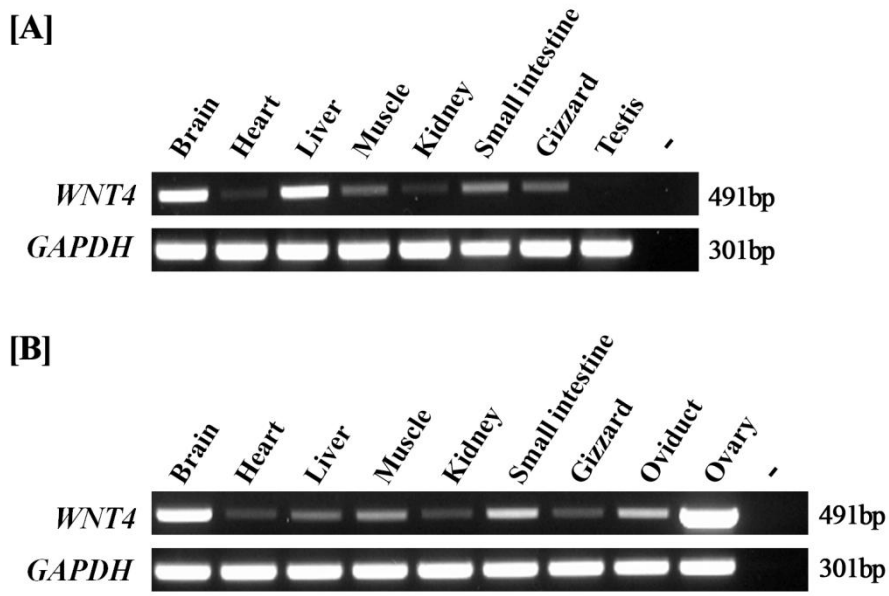


Fig. 5. *In vitro* target assay of *miR-302b* on *FZD2* transcript. [A] Diagram of *miR-302b* binding site in the *FZD2* 3'-UTR. [B] Expression vector maps for eGFP with *FZD2* 3'-UTR and Ds-Red with *miR-302b*. [C and D] After co-transfection of pcDNA-eGFP-3'UTR for the *FZD2* transcript and pcDNA-DsRed-miRNA for the *miR-302b*, the fluorescence signals of GFP and DsRed were detected using fluorescent microscopy [C] and FACS [D].



Supplementary Fig. 1. Multiple sequence alignment and the phylogenetic tree analyses of WNT4 proteins of chickens and other vertebrate species WNT4 proteins. [A] The amino acid sequences of WNT4 proteins from chicken and seven species (human, chimpanzee, monkey, dog, cattle, mouse and zebra fish) were aligned using Geneious Pro Version 5.04 with default penalties for gap and the protein weight matrix of BLOSUM (Blocks Substitution Matrix). Shaded amino acid sequences are identical among all species examined. Dashes represent gaps among the sequences. The conserved functional domains in WNT4 proteins were identified using the Pfam-A family matrix and NCBI conserved domain database. [B] The phylogenetic tree was constructed by the neighbor-joining method using the Geneious program. The numbers next to the branches indicate bootstrap values from 1000 replicates. Bar shows a genetic distance.



Supplementary Fig. 2. Expression of *WNT4* in chickens. [A and B] Expression of *WNT4* in various organs of male and female of chickens. Results of RT-PCR analysis using cDNA templates from different organs of male [A] and female [B] chickens with chicken *WNT4* and chicken *GAPDH*-specific primers.

5. Discussion

In the present study, we demonstrated that the *WNT4* gene is expressed in the isthmus and shell gland of the chicken oviduct in response to estrogen. In addition, we found increased expression of *WNT4* in cancerous ovaries of laying hens that increased with age. Moreover, expression of *FZD2*, the cognate receptor of WNT4 (Hou et al., 2004), is post-transcriptionally regulated by direct binding of *miR-302b*. These results support our hypothesis that WNT4 affects growth, differentiation and development of the chicken oviduct, and provide novel insights and concepts for further study of WNT4-related physiological processes in the laying cycle of hens and in ovarian carcinogenesis.

The WNT family includes 19 genes for secreted and locally-acting signaling glycoproteins that affect embryonic development through effects on cell fate determination, motility, polarity, primary axis formation, organogenesis and tumorgenesis (Boyer et al., 2010a; Clevers, 2006; Komiya and Habas, 2008; Liu et al., 2008; Yang et al., 2008). In fact, WNT proteins mainly relay their signals through two different signal pathways: WNT/beta-catenin (CTNNB1) signal transduction cascade known as the canonical pathway and WNT/Ca^{2+} signaling cascade or non-canonical pathway (Komiya and Habas, 2008). For instance, the WNT-CTNNB1 signaling pathway is concerned with translocation and accumulation of CTNNB1 protein in the nucleus to effect its co-transcriptional factors and cognate response elements (Gordon and Nusse, 2006). In addition, CTNNB1 protein accumulation in the nucleus is closely associated with development of various cancer types such as breast cancer, cervical cancer and ovarian carcinoma (Brown, 2001; Uren et al.,

2005; Wright et al., 1999). Among the WNT family members, WNT4 is a growth factor involved in development of a number of organs such as kidney, mammary gland and adrenal gland, as well as development of the reproductive tract in mammals (Jeays-Ward et al., 2004; Kispert et al., 1998; Miyakoshi et al., 2009; Treier et al., 1998). In mice, *Wnt4* is highly expressed in the female reproductive tract with different expression patterns depending on stage of the estrous cycle and it is also involved in stromal cell proliferation and differentiation in the uterus which is required for implantation and decidualization during early pregnancy (Franco et al., 2011). In addition, *Wnt4*$^{-/-}$ mice have an abnormal phenotype with respect to postnatal uterine development which renders female mice subfertile due to defects in implantation of blastocyst and subsequent survival, differentiation, and responsiveness to progesterone signaling by uterine stromal cells (Franco et al., 2011; Hou et al., 2004). However, WNT4 expression and function in the reproductive tract female chickens has not been reported previously.

In the present study, multiple sequence alignment analysis showed that chicken WNT4 protein has high homology to WNT4 protein of eight other vertebrate species that averages 84.6% with a range of 79 to 85.8% (Table 1 and Supplementary Figure 2A). In addition, a phylogenetic tree generated using the primary sequences indicated that chicken and zebra fish *WNT4* genes clustered together and a larger cluster is formed with the other mammalian species (Supplementary Figure 2B). These results indicate that chicken *WNT4* is likely to have similar physiological functions as for other vertebrate species, although it diverged from mammalian *WNT4* very early in its evolution. In fact, WNT4 expression is required for development of female reproductive tract during early embryogenesis, as well as functional growth of follicular and granulosa

cells, as well as their transition of luteal cells of the corpora lutea in mammals (Heikkila et al., 2002; Jeays-Ward et al., 2004; Vainio et al., 1999; Yao et al., 2004). Although WNT4 expression has been reported for embryos and developing ovarian follicles of chickens (Chue and Smith, 2011; Diaz et al., 2011; Hu and Marcucio, 2009; Sienknecht and Fekete, 2008), there is little information about expression, regulation of expression or function in the chicken oviduct. In this study, we demonstrated expression of WNT4 in glandular (GE) and luminal epithelia (LE) of the isthmus and shell gland of the chicken oviduct (Figure 1 and 2). During passage of the egg through the oviduct, several layers of egg shell membranes that surround the yolk and the white are added sequentially as the egg mass passes through successive sections of the oviduct (Lavelin et al., 2000). About 2 to 3 h after ovulation, the fertilized egg, with secretion of egg-white proteins including albumen from the magnum, passes into the isthmus which secretes various components of the soft shell membranes such as keratin-like protein, and types I, V and X collagens (Leach et al., 1981; Wang et al., 2002). The formation of the egg shell involving calcium deposition (approximately 5 to 6 g of calcium carbonate) is completed in the shell gland of the oviduct within 17 to 20 h after ovulation (Lavelin et al., 2000). Results of the present study also demonstrated that DES stimulates WNT4 expression in GE of the isthmus and shell gland of the oviduct of developing chick reproductive tract. Accordingly, estrogen from the ovarian follicles likely plays a pivotal role in development and function of the avian reproductive system as it does in the mammalian reproductive tract. For instance, DES induces massive growth of the juvenile oviduct and induces cytodifferentiation of epithelial cells into tubular gland cells, goblet cells, and ciliated cells (Dougherty and Sanders, 2005b). In mice, expression of the *Wnt4* gene is regulated by estrogen during development of the uterus (Franco et al., 2011; Hou et al.,

2004). There are changes in gene expression in the chick oviduct after exposure to DES (Song et al., 2011) as DES binds strongly to estrogen receptor alpha (ESR1) to act as an agonist with effects similar to those induced by 17β-estradiol (Dougherty and Sanders, 2005b; Kohler et al., 1969; Palmiter and Wrenn, 1971). Collectively, our results indicate that DES increases expression of WNT4 in a tissue- and cell-specific manner that is coordinate with development, differentiation and function of the various anatomical components of the chicken oviduct.

Ovarian cancer is a lethal gynecological malignancy that claims the lives of over 15,000 women and 22,000 are diagnosed with the disease annually in the US and the overall 5-year survival rate from this disease is only 30 to 40% because most cases of the ovarian cancer are not detected at an early stage which makes it difficult to apply any type of therapy (Bovicelli et al., 2011). To overcome this problem, various animal models have been developed, but they have not proven to be as useful as the laying hen. For example, genetically engineered rodent models have been developed to elucidate some aspects of the etiologies and pathogenesis of ovarian cancer; however, the non-spontaneous nature of their ovarian cancer as compared to that in women limits their clinical relevance (Barua et al., 2009b; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003a). The laying hen is recognized as the most appropriate animal model because they spontaneously develops ovarian cancer of the surface epithelium at a high rate as they age, as also occurs in women (Stammer et al., 2008). Indeed, *cathepsin B (CTSB)* (Ahn et al., 2010), *serpin peptidase inhibitor, clade B, member 11 (SERPINB11)* (Lim et al., 2012), *alpha 2 macroglobulin (A2M)* (Lim et al., 2011), and *pleiotrophin (PTN)* (Lee et al., 2012) are genes that we reported to be most abundant in the GE of ovarian cancers in laying hens. In the present study, we found that WNT4

is highly expressed in the GE of the cancerous ovaries as compared with normal ovaries of hens. These results strongly support the idea that the *WNT4* expression is associated with initiation and development of human ovarian cancer and even more so as the individual ages (Peltoketo et al., 2004). In the mouse uterus, *Wnt4* expression is regulated by estrogen in an ESR1-dependent manner (Miyakoshi et al., 2009) and involved in normal development of ovarian follicles, uterine function and fertility (Boyer et al., 2010b). Moreover, ESR1 is also abundantly expressed in human endometrial adenocarcinoma cell lines (Ali et al., 2004) and increased WNT4 expression level is initiated and regulated by estrogen and ESR1 (Miyakoshi et al., 2009). Our results showed that the WNT4 is expressed in normal ovaries and to a greater extent in cancerous ovaries of young hens (48-week-old), but only in cancerous ovaries of older hens (96-week-old). Normal ovaries of young hens have more abundant expression of ESR1 than those of old hens (Hrabia et al., 2008), and ESR1 expression is greater in ovarian carcinoma of young hens than in ovarian carcinoma of old hens (Lee et al., 2012). Therefore, we suggest that ESR1-dependent overexpression of WNT4 in cancerous ovaries induces accumulation of CTNNB1 which is closely associated with various cancer types including breast, cervical and ovarian carcinoma (Brown, 2001; Uren et al., 2005; Wright et al., 1999). Thus, ESR1-dependent WNT signaling may be inappropriate and lead to development of ovarian cancer as laying hen become older.

MicroRNAs (miRNAs) are short RNA molecules that control expression of certain genes by regulating mRNA stability and translation (Kloosterman and Plasterk, 2006). In chickens, hundreds of miRNAs have been identified, but functinons are known for only a few miRNAs. In this study, we performed a miRNA target validation assay to determine

whether specific miRNAs bind to the 3'-UTR of *WNT4* gene with the potential to affect expression. We did not discover potential miRNA binding sites within the 3′-UTR of the *WNT4* gene; therefore, we identified miRNAs with the potential to affect expression of *FZD2* since WNT4 protein mainly binds to this receptor to activate its intracellular signal transduction cascade (Dale, 1998; Hou et al., 2004). We found that *miR-302b* inhibits expression of *FZD2* in laying hens by regulating various post-transcriptional events that likely affect cellular processes in development, differentiation and oncogenesis in the reproductive tract of laying hens. Future studies will examine aberrant WNT4 signaling by loss-of-function of *miR-302b* to better understand the etiology and oncology of ovarian carcinoma in laying hens.

In summary, results of this study demonstrated that *WNT4* is an estrogen-regulated gene during growth, development and differentiation of the chicken oviduct and that is likely plays a critical role in abnormal growth and function of cancerous ovarian cells of laying hens. Furthermore, *miR-302b* may influence expression and regulation of WNT4-FZD2 signaling pathway-related genes in laying hens. Collectively, these results provide new insights into the roles of WNT4 with respect to its hormonal regulation and post-transcriptional regulation of its expression in the oviduct and in ovarian tumors of laying hen.

CHAPTER 3

Differential Expression of Select Members of the SLC Family of Genes and Regulation of Expression by MicroRNAs in the Chicken Oviduct

1. Abstract

The yolk and white of eggs from chickens contain proteins and other molecules either secreted or transported by cells of the reproductive tract or secreted by the liver and transported to the ovarian follicles of laying hens. Nutrients transported by solute carriers (SLC) include glucose, electrolytes and amino acids. Although *SLC* genes have been investigated in mammals, there are few studies of expression of *SLC* genes in the chicken oviduct. Therefore, we investigated temporal and cell specific expression of selected *SLC* genes at 3 h and 20 h post-ovulation and regulation of their expression by microRNAs (miRs). Expression of *SLC1A4* (glutamate and neutral amino acid transporter), *SLC13A2* (dicarboxylate transporter) and *SLC35B4* (UDP-xylose: UDP-N-acetylglucosamine transporter) mRNAs was limited to glandular epithelium (GE), while *SLC4A5* (sodium bicarbonate co-transporter) and *SLC7A3* (cationic amino acid transporter) mRNAs were expressed predominantly in the luminal epithelium (LE) of the magnum. Interestingly, *SLC1A4*, *SLC4A5*, *SLC13A2* and *SLC35B4* mRNAs were abundant only in GE of the shell gland, whereas *SLC7A3* was not detected in the shell gland. In the magnum, *SLC4A5*, *SLC7A3* and *SLC7A3* were expressed, but *SLC1A4* and *SLC35B4* were not expressed at 20 h post-ovulation. In the shell gland, all *SLC* mRNAs were expressed at both time points, except for *SLC7A3*. The miRNA target validation assay revealed that *miR-1764* and *miR-1700* bind directly to *SLC13A2* and *SLC35B4* transcripts, respectively, to regulate expression. Results of this study demonstrated cell-specific and temporal changes in expression of selected *SLC* genes and regulation of *SLC13A2* and *SLC35B4* expression by miRs in the oviduct of laying hens.

2. Introduction

The chicken is an established animal model for research on actions of hormones such as estrogen and progesterone on the oviduct (Dougherty and Sanders, 2005b). In addition, it can be used as a bioreactor to produce therapeutic proteins (Han, 2009). Folliculogenesis, ovulation and egg formation are in response to effects of estrogen on accumulation of components of yolk in the ovarian follicle and calcium metabolism for formation of the egg shell and oviposition (Bar, 2009; Yoshimura and Bahr, 1995). Estrogen also stimulates development of the chicken oviduct which consists of five segments: infundibulum, magnum, isthmus, shell gland and vagina (Dougherty and Sanders, 2005b). In addition, formation of tubular glands and differentiation of epithelial cells, including goblet and ciliated cells, in the chicken oviduct are induced by estrogen (Palmiter and Wrenn, 1971). Estrogen is also required for the expression of genes for synthesis of egg white proteins in the magnum of the oviduct of laying hens (Palmiter, 1972).

Concentrations of progesterone in blood of chickens are greatest between 6 h and 4 h prior to ovulation and the preovulatory surge of LH. Progesterone can induce ovulation in the absence of a preovulatory gonadotropin surge (Nakada et al., 1994). The effects of progesterone on development and function of the chicken oviduct appear to be dependent on its interaction with estrogen, including development and function glands and associated glandular epithelial cells (Nakada et al., 1994; Oka and Schimke, 1969a, c, d).

The ovulatory cycle of hens begins with ovulation of an ovum and

ends with oviposition which requires 24 to 28 h. The 24 h ovulatory cycle is characteristic of chicken and turkey as well as several other avian species including Japanese quail and bobwhite quail. After ovulation, the ovum is engulfed by the infundibulum where it resides for 15 to 30 min for fertilization (Shimada and Tanabe, 1982). The infundibulum produces the first layer of albumen and then the ovum is transported into the magnum where the majority of albumen and other proteins in egg white, as well as other components, including calcium, accumulate over a period of 2 to 3 h [8]. The egg mass is then transported into the isthmus and resides there for 1 to 2 h to acquire its outer and inner shell membranes. Finally, the egg mass moves into the shell gland (also referred to as the uterus) which is characterized by its pouch-like appearance and prominent longitudinal muscle layer, glandular epithelium and luminal epithelium. The egg resides in the shell gland for 18 to 26 h to allow for egg shell calcification. The vagina adds the egg shell cuticle protecting the egg against microorganisms and serves as conduit between the shell gland and cloaca during oviposition (Wedral et al., 1974).

The solute carriers (SLCs) are membrane proteins which transport various molecules across the cell membrane. There are 229 *SLC* genes in the chicken genome (Li et al., 2008) likely involved with transport of molecules including glucose and other sugars, amino acids, inorganic ions, and minerals such as iron and zinc (Fredriksson et al., 2008). Several *SLC* genes also affect digestive enzymes and regulatory factors (Barfull et al., 2002; Chen et al., 2005; Geyra et al., 2002; Gilbert et al., 2007). MicroRNAs (miRs), which consist of 18 to 25 nucleotides, are expressed in multicellular organisms to control gene expression by regulating mRNA stability and translation (Kloosterman and Plasterk, 2006). Therefore, miRs are regulators of a variety of biological processes, such as cell

proliferation, differentiation, development of organs and embryonic development. Hundreds of miRs have been reported in chickens, but differential expression and function is known for only a few miRs. Therefore, new knowledge of chicken miRs allows researchers to integrate and organize functional data to better understand integrated regulatory mechanisms and the role(s) of newly discovered miRs in chickens. Phenotypic changes in the reproductive tract of hens during the reproductive cycle and laying cycle reflect post-transcriptional regulation of gene expression (Carletti and Christenson, 2009). However, little is known about post-transcriptional gene regulation via miRs in reproductive tissues of chickens. Reports on the role of key modulators of gene expression for functions of the oviduct in laying hens are limited and miR-related regulation of expression of members of the SLC family of genes are not available.

In this study, we tested the hypothesis that expression of selected *SLC* genes are temporally associated with formation of the egg mass and development of the chick embryo through actions for transport of molecules including amino acids, carbohydrates, minerals and ions, into to the lumen of the reproductive tract. The expression patterns of selected members of the SLC family of genes were examined in the oviduct of laying hens at 3 and 20 h post-ovulation. Formation of the egg and embryonic development in hens occur as the yolk passes through infundibulum (15 min), magnum (3 h to 4 h), isthmus (75 min) and shell gland/uterus (18 to 20 h) before oviposition through the vagina and cloaca. Fertilization takes place in the infundibulum, the second meiotic division is completed during passage of the ovum through the magnum, and the first mitotic division occurs 3 to 5 h after the zygote enters the shell gland (Fasenko, 2007). Based on the chicken genome database and results from

high-throughput analyses using the chicken DNA microarray (Jeong et al., 2012b), this study focused on expression of the following genes: *SLC1A4* (solute carrier family 1, glutamate/neutral amino acid transporter, member 4); *SLC4A5* (solute carrier family 4, sodium bicarbonate co-transporter, member 5); *SLC7A3* (solute carrier family 7, cationic amino acid transporter, y+ system, member 3; *SLC13A2* (solute carrier family 13, sodium-dependent dicarboxylate transporter, member 2); and *SLC35B4* (solute carrier family 35, UDP-xylose/UDP-N-acetylglucosamine transporter, member B4) mRNAs.

3. Material & methods

Experimental Animals and Animal Care

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) laying hens were subjected to standard management practices at the University Animal Farm, Seoul National University, Republic of Korea. All hens were exposed to a daily light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water.

Tissue Samples

Hens (n=5 per time point) were euthanized at 3 h and 20 h after ovulation using 60% –70% carbon dioxide. Samples of the magnum and the shell gland of oviducts from each hen at each time point were collected in the same manner from the middle portion of each of the respective tissues. Tissue samples were either: 1) removed and placed in OCT embedding compound (Miles, Oneonta, NY), frozen in liquid nitrogen, and stored at -80℃; 2) for RNA isolation, frozen immediately in liquid nitrogen and stored at -80℃ until analyzed; or 3) fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). After 24 h, tissues fixed in 4% paraformaldehyde were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany).

RNA Isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

In Situ Hybridization Analysis

Total RNA was extracted from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and cDNAs were synthesized using total RNA and AccuPower® RT PreMix (Bioneer, Daejeon, Korea). The mRNA sequences of chicken SLCs were obtained from a BLAST search of the *Gallus gallus* genome database at NCBI. For *in situ* hybridization probes, PCR products were generated from each cDNA with the primers used for RT-PCR analysis (Table 1). The products were gel-extracted and cloned into pGEM-T vector (Promega). After verification of the sequences, plasmids containing gene sequences were amplified with T7- and SP6-specific primers (T7:5'-TGT AAT ACG ACT CAC TAT AGG G-3'; SP6:5'-CTA TTT AGG TGA CAC TAT AGA AT-3'). Then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Tissues were collected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5μm and sections placed on APES-treated (silanized) slides. The sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed twice in DEPC-treated PBS. The sections were then digested in 5μg/ml Proteinase K (Sigma) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37℃.

After post-fixation in 4% paraformaldehyde, sections were incubated twice for 5 min each in DEPC-treated PBS and incubated in TEA buffer [0.1M triethanolamine) containing 0.25% (v/v) acetic anhydride]. The sections were incubated in a prehybridization mixture containing 50% formamide and 4X standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 40% formamide, 4X SSC, 10% dextran sulfate sodium salt, 10mM DTT, 1 mg/ml yeast tRNA, 1mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe overnight at 42℃ in a humidified chamber. After hybridization, sections were washed for 15 min in 2X SSC at 37℃, 15min in 1X SSC at 37℃, 30 min in NTE buffer (10mM Tris, 500mM NaCl and 1mM EDTA) at 37℃ and 30 min in 0.1X SSC at 37℃. After blocking with 2% normal sheep serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche, Indianapolis, IN). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM levamisole (Sigma Chemical Co., St. Louis, MO).

Table 1. Information on forward and reverse primers use for cloning.

Gene	Sequence (5'→3'): forward and reverse	Gene Bank accession no.
SLC1A4	AGGAGTGGCACTGAAAAAGC CCATCCATGTTCACAGTTGC	XM_001232899.1
SLC4A5	GGACCCCAACATTAGAATCG AGCAAAGAGGCAAAACATGG	XM_423797.3
SLC7A3	GGACTCAGACGATCCAGAGG GTAGATCACACGTGGCATGG	HQ166627.1
SLC13A2	AGCCAACGAAATCAAACAGC ATGAAGAGGACCAGGACTGC	XM_425404.2
SLC35B4	AGCAACGTGGTGTTCTTGG TGGAAAATTCCCATTCTTGC	XM_414994.3

MicroRNA Target Validation Assay

The sequence information for the 3'-untranslated regions (3'-UTRs) of *SLC13A2* and *SLC35B4* was obtained from the NCBI sequence database. We then amplified those regions using 3'-UTR specific primers, and PCR products were generated from chicken oviduct cDNA and cloned into the pGEM-T vector (Promega). After verification of the sequences, the 3′-UTR was subcloned between the eGFP (green fluorescent protein) gene and bovine growth hormone (bGH) poly-A tail in pcDNA3eGFP (Clontech, Mountain View, CA) to generate the eGFP-miR target 3′-UTR (pcDNA-eGFP-3′UTR) fusion constructs. For the dual fluorescence reporter assay, the fusion contained the DsRed gene and either *miR-1812, miR-1620,* or *miR-1764* for *SLC13A2*. *SLC35B4* fusion constructs also contained the DsRed gene and either *miR-1555, miR-1612, miR-1741,* or *miR-1700*. The constructs were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miR). The pcDNA-eGFP-3′-UTR and pcDNA-DsRed-miR (3μg) were co-transfected into 293FT cells using the calcium phosphate precipitation method (Chen and Okayama, 1987). The 293FT cell line is commonly used because the cells are relatively easy to culture in a laboratory, and the cells allow high levels of protein to be expressed from vectors. These cells are also easily transfectable using standard methods including calcium phosphate precipitation. When the DsRed-miR is expressed and binds to the target site of the 3′-UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACS Calibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Quantitative-PCR analysis of miRNAs

Total RNA was extracted at each time point from the oviduct of chickens using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). The miRNA first strand cDNA was synthesized from total RNA (1 µg) using the miRNA first strand cDNA synthesis kit (Stratagene, CA). Total RNAs were treated with *E. coli* poly-A polymerase (PAP) to generate a poly-A tail at the 3'-end of each miRNA. Following polyadenylation, cDNAs were synthesized using the RT adaptor primer. Quantitative RT-PCR analysis for miRNA-first strand cDNAs was performed using the High-Specificity miRNA QPCR Core Reagent Kit (Stratagene) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture was prepared by adding 2.5 µL of 10X core PCR buffer, 2.75 µL of 50 mM $MgCl_2$, 10 µL of 20 mM dNTPs, 1.25 µL of 20X EvaGreen dye, 1.0 µL of 3.125 µM universal reverse primer, 1.0 µL of 3.125 µM miRNA-specific forward primers and 0.5 µL of High-Specificity polymerase in 25 µL including the prepared miRNA cDNA. Data from quantitative-PCR were normalized relative to expression of U6 snoRNA (endogenous control) for miRNA, respectively and calculated using the $2^{-\Delta\Delta Ct}$ method. These experiments were conducted in triplicate.

Statistical Analyses

Data from quantitative-PCR for miRNAs were subjected to analysis of variance (ANOVA) according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC) to determine whether effects time post-ovulation were significant. Data presented are mean ± SEM unless otherwise stated.

4. Results

Localization of SLC1A4, SLC4A5, SLC7A3, SLC13A2 and SLC35B4 mRNAs in the chicken oviduct at different stages of the laying cycle

In situ hybridization was used to determine cell-specific localization of the *SLC* mRNAs in the chicken oviduct at 3 h and 20 h after ovulation. In the magnum of the chicken oviduct, there was strong expression of *SLC1A4*, *SLC13A2* and *SLC35B4* in the glandular epithelium (GE) at 3 h, but little or no detectable expression at 20 h post-ovulation in either GE or LE (Figure 1). Expression of *SLC4A5* and *SLC7A3* mRNAs was weak in GE of the magnum at 3 h, but *SLC7A3* was more abundant in LE and GE, and *SLC4A5* was more abundant in LE of the magnum at 20 h post-ovulation. In the shell gland, *SLC7A3* was essentially undetectable at both 3 and 20 h post-ovulation, while *SLC1A4* was readily detectable at 3 h, but not at 20 h post-ovulation. Expression of *SLC4A5*, *SLC13A2* and *SLC35B4* mRNAs were expressed strongly in GE of the shell gland at both 3 h and 20 h post-ovulation (Figure 2).

Post-transcriptional function of the microRNAs on SLC13A2 and SLC35B4

Analysis of potential miR binding sites within the 3′-UTR for *SLC13A2* and *SLC35B4* utilized a miR target prediction database (miRDB; http://mirdb.org/miRDB/) revealed three (*miR-1812, miR-1620, miR-1764* for *SLC13A2*) and four (*miR-1555, miR-1612, miR-1741, miR-1700* for *SLC35B4*) to detect putative binding sites. There were no potential miR binding sites detected within the 3′-UTR for *SLC1A4, SLC4A5,* and

SLC7A3. Therefore, we focused on miRs that might affect expression of *SLC13A2* and *SLC35B4* via their 3'-UTR. A fragment of the 3'-UTR of both the *SLC13A2* and *SLC35B4* genes that contain binding sites for the miRs was cloned downstream of the GFP reading frame, thereby creating a fluorescent reporter for function of the 3′-UTR region. After co-transfection with eGFP-*SLC13A2* or e-GFP-*SLC35B4* 3'-UTR and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing cells were analyzed by fluorescence microscopy and fluorescence-activated cell sorting (FACS). As shown in Figure 3A, the presence of *miR-1764* decreased ($p < 0.01$) the intensity and percentage of GFP-expressing cells (51.5% in control vs. 6.6% in *miR-1764*). On the other hand, in the presence of *miR-1812* and *miR-1620* for *SLC13A2*, there was no significant decrease in GFP as compared to the control (data not shown). Also, expression of *miR-1764* increased in the magnum at 20 h post-ovulation as compared to 3 h post-ovulation (Figure 3B). However, the expression level of this miR was not significantly different in the shell gland between the two sampling times. In addition, as illustrated in Figure 4A, the presence of *miR-1700* decreased ($p < 0.01$) the intensity and percentage of GFP-expressing cells (59.6% in control vs. 4.9% in *miR-1700*). However, in the presence of *miR-1555*, *miR-1612* and *miR-1741* for *SLC35B4*, there was not a significant decrease in GFP as compared to the control (data not shown). The level of expression of *miR-1700* increased in the magnum at 20 h as compared to 3 h post-ovulation, but was unchanged in the shell gland between 3 and 20 h post-ovulation (Figure 4B).

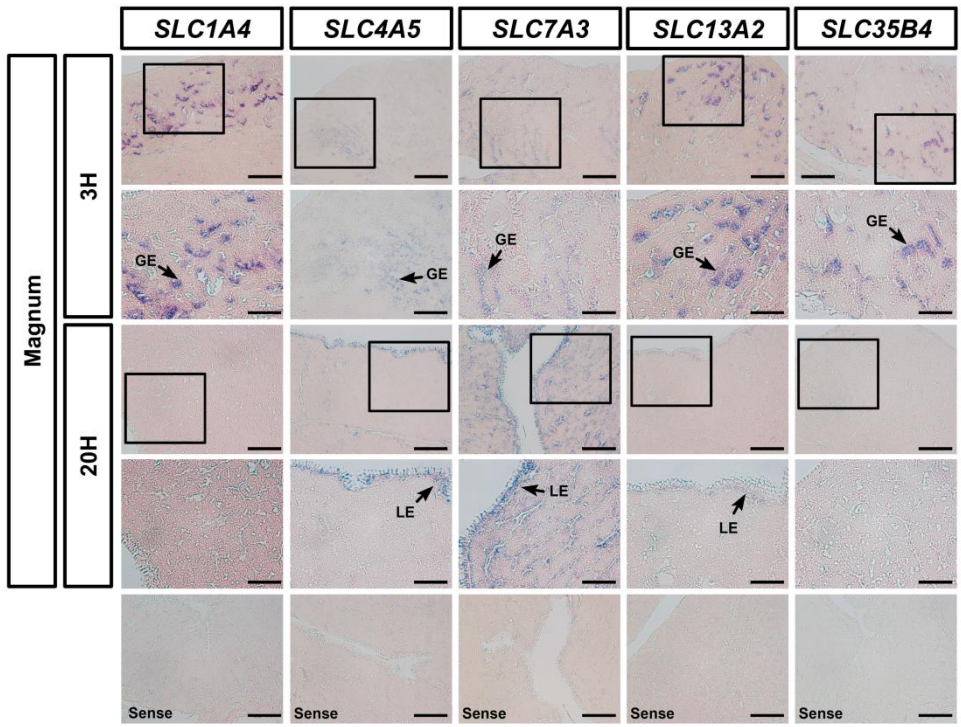


Fig.1. ***In situ*** **hybridization analysis for cell-specific changes in expression of** ***SLC1A4, SLC4A5, SLC7A3, SLC13A2*** **and** ***SLC35B4*** **mRNAs in the magnum of the chicken oviduct at 3h and 20h after ovulation.** Legend: GE, glandular epithelium; LE, luminal epithelium. Scale bar represents 100 μm (the first and third horizontal panels and sense) and 50 μm (the second and forth horizontal panels)

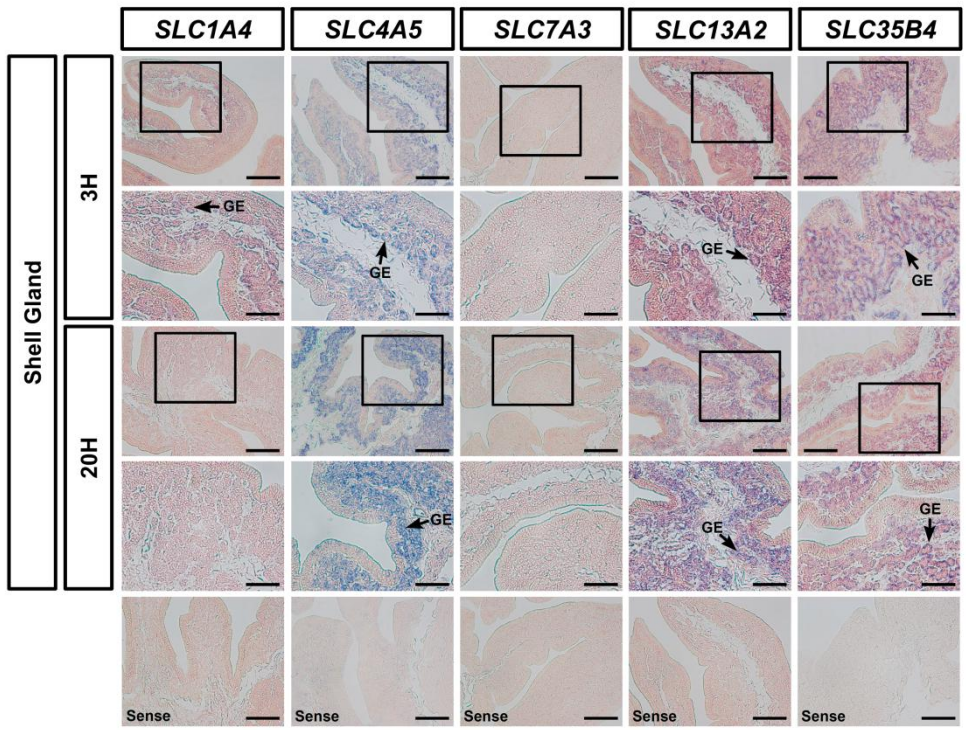


Fig.2. *In situ* hybridization analysis for cell-specific expression of *SLC1A4, SLC4A5, SLC7A3, SLC13A2* and *SLC35B4* mRNAs in the shell gland of laying hens at 3h and 20 h after ovulation. Legend: GE, glandular epithelium; LE, luminal epithelium. Scale bar represents 100 μm (the first and third horizontal panels and sense) and 50 μm (the second and forth horizontal panels)

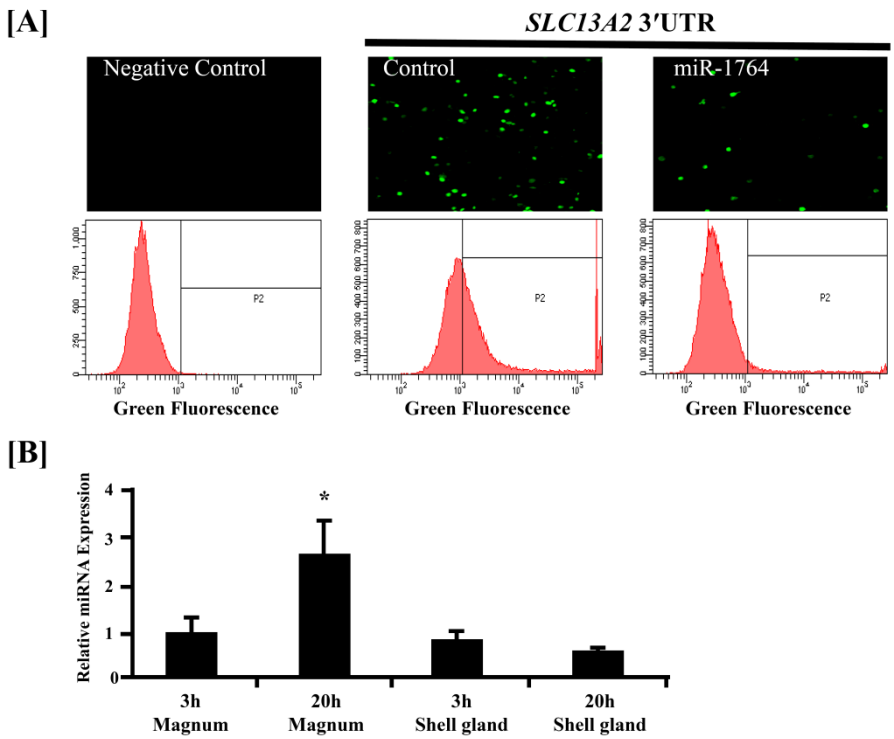


Fig. 3. *In vitro* target assay of *microRNAs* for the *SLC13A2* transcript. [A] These results are from experiments with 293FT cells co-transfected *SLC13A2* 3'UTR-eGFP and DsRed-*miR-1764* by fluorescence microscopy to determine the percent expression in the co-transfected cells following FACS analysis. [B] Quantitative expression analysis of *miR-1764* mRNA at 3 h and 20 h post-ovulation. These experiments were conducted in triplicate and normalized to control U6 snRNA expression.

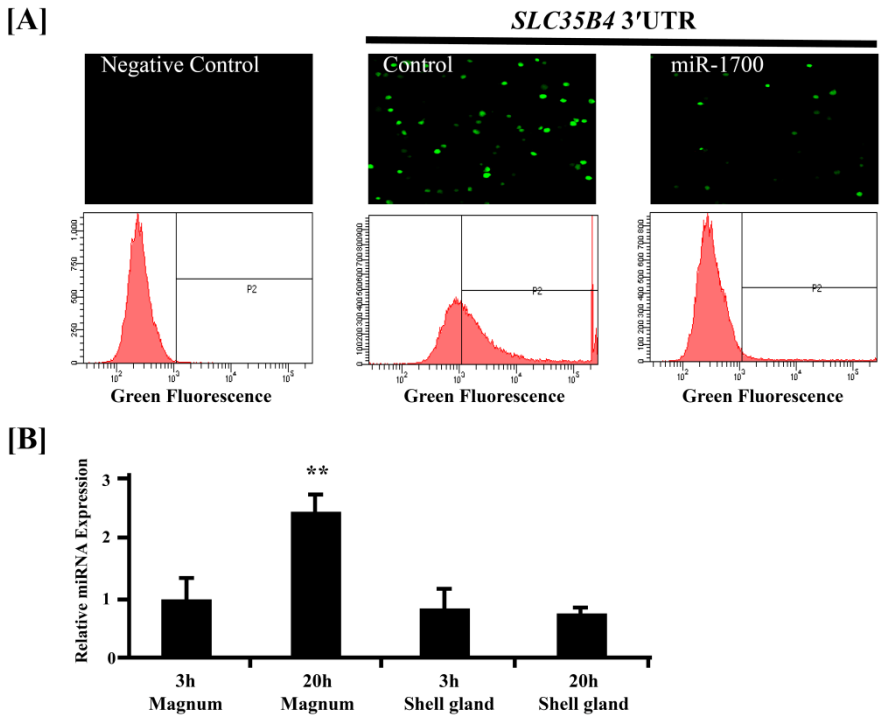


Fig. 4. *In vitro* target assay of *microRNAs* on the *SLC35B4* transcript. [A] These results are from experiments with 293FT cells co-transfected *SLC35B4* 3'UTR-eGFP and DsRed-*miR-1700* by fluorescence microscopy for determination of percent expression in the co-tranfected cells by FACS analysis. [B] Quantitative expression analysis of *miR-1700* mRNA at 3 h and 20 h post-ovulation. These experiments were conducted in triplicate and normalized to control U6 snRNA expression.

5. Discussion

Results of the present study provide evidence for differential expression of selected *SLC* mRNAs in GE and/or LE between 3 and 20 h post-ovulation when the egg mass is in the magnum and shell gland, respectively. In the magnum and shell gland of hens, many genes associated with physiological events, embryonic development, and cellular and molecular functions occur between 3 h and 20 h post-ovulation (Jeong et al., 2012b). Based on findings from studies of the mammalian reproductive tract, we hypothesized that temporal and cell-specific changes in expression of *SLC* genes during the ovulatory cycle are crucial for development of chick embryos and an environment that supports embryonic survival through transport of various molecules, including amino acids, metal ions and minerals including calcium ions. The candidate *SLC* genes have functions related to embryonic development in mammals. The results of this study support our hypothesis in that the selected *SLC* genes have different temporal and cell-specific patterns of expression in the magnum and shell gland at 3 h and at 20 h of the laying cycle which may be critical for supporting embryonic development.

The *SLC1A4* gene encodes for a Na^+-dependent neutral amino acid transporter (also called ASCT1) (Arriza et al., 1993; Shafqat et al., 1993) that regulates the exchange of neutral amino acids and is insensitive to inhibition by K^+ (Zerangue and Kavanaugh, 1996). SLC1A4 transports L-serine, L-cysteine, L-alanine and L-threonine in a stereospecific manner (Kanai and Hediger, 2004). *SLC1A4* is expressed in the ovine uterus for transport of amino acids that likely support of growth, development and survival of the conceptus (Gao et al., 2009b). For example, cysteine has an

important role in cell growth and cell morphology (Bannai and Ishii, 1982; Bird et al., 1996). In the present study, *SLC1A4* mRNA was expressed in GE of the magnum at 3 h when the fertilized ovum undergoes the second meiotic division. Along with albumen secreted by epithelium of the magnum at 3 h post-ovulation, neutral amino acids transported into the lumen of the oviduct by SLC1A4 are incorporated into the egg mass for support of growth and development of the chick embryo. In shell gland, *SLC1A4* was expressed in GE at both 3 h and 20 h which suggests that is has an important role for transport of neutral amino acids into the lumen of the shell gland to create an environment conducive to calcification of the egg shell.

The *SLC4A5* gene encodes for a protein which is an electrogenic Na^+-HCO_3^- co-transporter (Sassani et al., 2002). Bicarbonate ions have an important role in many reproductive processes including fertilization, and embryonic development in mammals. In the human, regulation of bicarbonate transport is important for maintaining the acid-base balance of tissues (Damkier et al., 2007), including the reproductive tract (Liu et al., 2012). This transporter has a specific function in the regulation of pH and transports Na^+ and HCO_3^- as an electrogenic co-transporter in the same direction (Liu et al., 2011; Stutz et al., 2009). The activity of the HCO_3^-/Cl^- exchange is especially critical to the viability of mouse embryos (Zhao and Baltz, 1996). In humans, regulation of bicarbonate ion transport affects such diverse functions such as cell division, cellular pH regulation and acid-base balance of tissues (Damkier et al., 2007). In the female genital tract, HCO_3^- induces increases in intracellular cAMP through activation of soluble adenyl cyclase which is essential in many biological systems (Chen et al., 2000). In the present study, *SLC4A5* mRNA was expressed in both the magnum and the shell gland. Based on the findings

from studies of the mammalian reproductive tract, we hypothesize that the SLC4A5 protein in the chicken oviduct may be essential for development of early stage embryos and for maintenance of an intra-oviductal environment conducive to embryonic development and for physiological events unique to the shell gland.

The *SLC7A3* gene encodes for a cationic amino acid transporter that is Na^+-independent and also called CAT3 (Closs et al., 2006). In humans, the *SLC7A3* gene is expressed moderately in testis, uterus, and mammary gland, and weakly in the ovary. *SLC7A3* is also expressed in conceptuses and uterine endometrium where its transports cationic amino acids such as arginine that enhances conceptus growth and survival (Gao et al., 2009a). In chickens, arginine accounts for 85% of guanidino compounds in egg yolk and 48% in egg white indicating that SLC7A3 is an important transporter of L-arginine in the chicken oviduct (Ramirez et al., 1970). Arginine is now considered a conditionally essential amino acid since it serves as the substrate for production of nitric oxide and polyamines that are required for embryonic development (Gao et al., 2009a). In the present study, *SLC7A3* mRNA in the magnum was weak in GE at 3 h post-ovulation but was more abundant in GE/LE at 20 h post-ovulation. We hypothesize that expression of *SLC7A3* in the magnum is important for arginine transport into the egg-mass at 3 h after ovulation for enhancing development of the embryo through production of nitric oxide and polyamines to stimulate physiological functions including vasodilation and angiogenesis within the vascular system (Gao et al., 2009a).

The *SLC13A2* gene encodes for a co-transporter of Na^+-dicarboxylate, also called NADC1 or SDCT1 (Markovich and Murer,

2004; Pajor, 1995) that regulates calcium deposition in tissues including the proximal tubules of the kidney (Bergeron et al., 2011; Pajor et al., 2000). In both mammals and birds, citrate plays a crucial role in the release of energy via the citric acid cycle and its presence in bone and blood is concerned with calcium metabolism (Taylor, 1960; Taylor et al., 1960). At high concentrations, the citrate forms a complex with calcium to form relatively insoluble calcium citrate (Harrison, 1956; Rumenapf and Schwille, 1988). In the shell gland, there is abundant calcium that complexes with citrate for calcification of the egg shell (Bar et al., 1992; Eastin and Spaziani, 1978; Parsons and Combs, 1981). In this study, *SLC13A2* mRNA was most abundant in the shell gland at 20 h as compared to 3 h post-ovulation; therefore, it likely contributes significantly to calcification of the egg shell prior to oviposition (Bar et al., 1992; Eastin and Spaziani, 1978). Functions of this member of the SLC13 family of transporters in the reproductive tract of female mammals are not well known. However, there is transport of calcium by the placentae of mammals and in species such as the pig there are calcium oxalate and calcium citrate deposits known as petrifactions (Hansard et al., 1966).

The *SLC35B4* gene encodes for a protein for transport of nucleotide sugars (Ashikov et al., 2005) such as UDP-galactose which is one of the nucleotide sugars associated with synthesis of proteoglycans via the hexosamine pathway (Ashikov et al., 2005; Maszczak-Seneczko et al., 2011). Glucosamine, the key amino sugar in the hexosamine pathway, leads to synthesis of proteoglycans that comprise the extracellular matrix (Sutton-McDowall et al., 2006). In addition, glucosamine has a crucial role in embryonic development in mice and cattle (Kimura et al., 2008; Vidal y Plana and Karzel, 1980). Proteoglycans have an important role in the development of the chick embryo including brain and cornea (Burg et

al., 1995; Connon et al., 2003; Funderburgh et al., 1986; Hennig et al., 1992). In the present study, *SLC35B4* was expressed in the magnum at 3 h post-ovulation and at 3 and 20 h post-ovulation in the shell gland. These results suggest that SLC35B4 likely affects growth and development of embryos because of its role in transport of substrates for synthesis of components of the extracellular matrix via the hexosamine biosynthetic pathway.

MicroRNAs (miRs) are expressed in multicellular organisms to control gene expression by regulating mRNA stability and translation (Kloosterman and Plasterk, 2006). Therefore, miRs are regulators of a variety of biological processes, such as cell proliferation and differentiation, as well as development of organs and embryonic development. Hundreds of miRs have been reported for chickens, but differential expression and function is known for only a few miRs. Therefore, new knowledge of chicken miRs allows researchers to integrate and organize functional data to better understand integrated regulatory mechanisms and the role(s) of newly discovered miRs in chickens. In fact, phenotypic changes in the reproductive tract of females are dramatic during the reproductive cycle or ovulatory cycle. For these dramatic changes, post-transcriptional regulation of gene expression is a key mechanism for coordinating changes in gene expression (Carletti and Christenson, 2009). However, little is known about post-transcriptional gene regulation via miRs in reproductive tissues of chickens. Reports on the role of key modulators of gene expression for functions of the oviduct of laying hens are limited and miR-related regulation of expression of members of the SLC family of genes are not available. It is critically important to identify and validate miR-mRNA target pairs (Kuhn et al., 2008). In the present study, we performed the miR target validation assay

to identify whether specific miRs bind and repress expression of selected members of the SLC family of genes. Results of the present study showed that the 3'-UTR of *SLC13A2* and the 3'-UTR of *SLC35B4* are repressed by *miR-1764* and *miR-1700*, respectively. *In vivo* expression profiles of these miRs and SLC transcripts were inversely related in the magnum during the laying cycle of hens. Based on these results, we hypothesize that *SLC13A2* and *SLC35B4* are important targets for *miR-1764* and *miR-1700* activity and that these specific miRs are closely related to expression and functions of *SLC13A2* and *SLC35B4* genes. *MiR-1764* and *miR-1700* targeted *SLC13A2* and *SLC35B4* via binding sites in their 3′-UTR to restrict expression. The observation that expression of *SLC13A2* and *SLC35B4* could be under miR control is important because these *SLC* genes are key transporters for molecules affecting development of the chick embryo and for calcification of the egg shell. MiR-based regulation provides the possibility to fine-tune *SLC13A2* and *SLC35B4* expression independent of transcriptional control, which can contribute to functional gene networks in the correct tissues and cells at the right time. Based on results of the present study, *miR-1764* and *miR-1700* constitute such regulators of gene expression; however, the significance of this regulation by *miR-1764* and *miR-1700* remains to be determined. Future studies will elucidate effects of loss of function of *miR-1764* and *miR-1700* in the chicken oviduct to determine effects on formation of the egg and embryonic development. Also, identification of other potential gene targets and functional studies will be undertaken to determine the roles of *miR-1764* and *miR-1700* during the egg laying cycle of hens.

In summary, results of the present study are the first to demonstrate post-transcriptional regulation of chicken *SLC13A2* and *SLC35B4* genes via miRs. The results also provide the first evidence for

temporal and cell-specific changes in expression of selected members of the SLC family of genes at 3 h and 20 h post-ovulation in hens which includes egg formation, oviposition, and embryonic development. It is probable that other members of the SLC family of genes participate in the complex events of the laying cycle of hens, but these are yet to be explored. Taken together, our findings suggest new research approaches for identification of integrated mechanisms for key events in avian reproduction and provide new insights into the post-transcriptional regulation of the SLC family of genes. To our knowledge, this study is the first report involving miR-mediated regulation of the *SLC* gene superfamily in chickens. Understanding the functions of these transporters and miRs is relevant to increasing knowledge of nutrient metabolism, embryonic development and survival, pharmacology, cancer chemotherapy and improvement of reproduction in chickens (Tizard et al., 2007). Future studies will be required to identify the integrated mechanisms of gene expression in the chicken oviduct during the ovulatory cycle.

CHAPTER 4

GENERAL DISCUSSION

Ovarian cancer in humans is the most lethal gynecological disease in women (Suzuki et al., 2010; Vanderhyden et al., 2003a). Approximately 90% of cancerous ovaries originate from the ovarian surface epithelium. Over the years, animal models have made significant contributions toward research concerning human diseases. Many laboratory animals share conserved evolutionary processes with humans. In fact, the laboratory mouse shares an estimated 97.5% of its DNA with humans. Therefore, many disease processes and novel treatment strategies can be analyzed in animal models, while due to their shorter life cycle, they can be studied relatively easily throughout an entire life span or across several generations. Smaller animals like mice and chickens are economically feasible to accommodate, and researchers can easily regulate their environment.

The chicken oviduct is regarded as an increasingly popular model for research involving hormones such as estrogen (Dougherty and Sanders, 2005b). The organ can also function as a bioreactor and can be used to produce biomaterials, with the potential for therapeutic protein production (Han, 2009). Many studies have reported that the development of the chicken oviduct is induced by estrogen, which also regulates the developmental process of folliculogenesis and the process of egg production (Dougherty and Sanders, 2005a; Hewitt et al., 2005; Okada et al., 2003; Proszkowiec-Weglarz et al., 2005). However, to date, research related to the biological and physiological changes that occur within the chicken oviduct has not been extensively conducted. In addition, only a few specific genes concerned with development and reproductive function in the chicken oviduct have been identified.

The reason for such high rates of epithelial ovarian cancer has

been suggested to be a result of incessant ovulation, which induces genetic damage in the cells of the surface epithelium (Murdoch, 2005). Domestic laying hens ovulate almost every day, mimicking such damage. Ovarian cancer in hens occurs spontaneously and shares significant similarities with human ovarian cancer (Barua et al., 2009a). Therefore, the chicken is well-suited as an animal model for studying the mechanisms responsible for human ovarian cancer (Vanderhyden et al., 2003b).

The present study was undertaken in order to clarify the expression of specific genes which are involved in developmental and functional processes in the chicken oviduct, using oviduct and cancerous ovaries. The expression and potential role of genes regulated by estrogen in the chicken reproductive system was also analyzed. We also identified expression patterns and putative functions for specific genes involved in the laying cycle of domestic hens.

Our first objective was to reveal the pattern of expression of WNT4 in the reproductive system of chickens. The WNT4 gene encodes a growth factor related to various processes including organ and sexual development in mammals (Jeays-Ward et al., 2004; Kispert et al., 1998; Miyakoshi et al., 2009; Treier et al., 1998). To achieve these outcomes, WNT4 can interact with and regulate both canonical and non-canonical pathways (Lyons et al., 2004). The expression levels of the WNT4 gene indicate abundant levels and differential patterns throughout the estrous cycle in the reproductive tracts of female mice. Therefore, the gene is likely to play important roles in the female reproductive systems of higher-order mammals (Miller et al., 1998; Miyakoshi et al., 2009). We examined the expression of WNT4 in the chicken reproductive tract including the oviduct and ovary, and observed cell-specific localization and expression

of WNT4 mRNA by *in situ* hybridization. We determined that the WNT4 gene exhibits differential expression regulated by estrogen during growth, development and differentiation of the chicken oviduct. It therefore stands to reason that the expression of the gene may play important roles in the control of abnormal growth and function in ovarian surface epithelial cells, the dysregulation of which could lead to ovarian cancer. FZD2, which is regarded as a WNT4 receptor, is associated with a specific miRNA that exerts posttranscriptional regulation via the FZD2 3‘UTR. Our study provides evidence that WNT4 expression is related to the processes of development and differentiation, as well as the function of the chicken reproductive system.

Our second study focused on examining differential expression patterns of solute carriers (SLCs) in the chicken oviduct between 3 and 20 h after ovulation, and to determine the posttranscriptional activity of SLCs. SLCs are membrane proteins which transport various molecules across the cell membrane (Li et al., 2008). Expression of the genes are related to physical transport of glucose and other sugars, amino acids, inorganic ions, and drugs across cell membranes (Fredriksson et al., 2008). The expression of several SLC genes affecting digestive enzymes and regulatory factors have been reported in hens (Barfull et al., 2002; Chen et al., 2005; Geyra et al., 2002; Gilbert et al., 2007). Our results provide the first evidence for differential expression patterns of SLCs at 3 and 20 h post-ovulation in the laying cycle of hens, which is likely to play an important role in the processes of egg formation, oviposition, and embryonic development. We also show for the first time that the chicken SLCs can be subject to miRNA regulation.

Our evidence suggests that these specific genes play crucial roles

in the development and function of the chicken reproductive system. The expression patterns of such genes that regulate and support specific processes in the chicken oviduct are relevant for future study into the development and treatment of ovarian cancer in chicken as an animal model. We anticipate that our findings will provide new insights for further study of ovarian cancer and the reproductive system of domestic hens.

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SUMMARY IN KOREAN

본 연구에서는 에스트로겐의 작용을 규명하기 위한 가장 적합한 모델인, 닭의 난관 발달 과정과 인간 난소암 연구의 훌륭한 모델로써 잘 알려진 닭의 난소암 모델에서 유전자의 발현 분석과 그 잠재적 기능을 밝히기 위한 연구를 수행하였다. 그 다음으로, 닭의 산란주기에서의 특정 시간 및 기관에서의 유전자 발현 패턴과 그리고 그 기능을 규명하기 위한 연구를 진행하였다.

Wingless-type MMTV integration site family, member 4 (WNT4)는 WNT 유전자 패밀리 중 하나의 유전자로 성장인자의 역할을 하는 것으로 알려져 있다. 그리고 포유류에서는 신장과 같은 기관 발달 과정과 젖샘, 부신의 발달 그리고 성적 발달에 대해 중요한 기능을 하는 것으로 연구되었다. 또한 마우스의 생식기관에서 발정주기에 따라 그 유전자 발현이 변화하는 것으로 알려져 졌고 그로 인해 생식기관에서 중요한 역할을 수행할 것이라 추측되고 있다. 그리고 WNT4는 에스트로겐에 의해서 조절되며, 에스트로겐 등의 스테로이드 호르몬에 큰 영향을 받는다고 알려져 있는 유방암에서 그 비정상적인 발현으로 인해 CTNNB1의 축적으로 암의 발달 및 성장에 중요한 역할을 한다고

보고되었다. 그래서 우리는 WNT4에 대한 연구가 거의 없는 닭에서 WNT4가 에스트로겐에 의한 난관 발달에서 발현이 증가함으로써 인해 그 매커니즘이 진행되어 기관 발달이나 세포 분화에 관련된 목표 유전자의 발현을 증가시켜 주는 성장인자로써의 역할을 할 것이라 생각하며 난소암에서 WNT4 유전자의 비정상적인 발현으로 인한 CTNNB1의 축적이 난소암의 발달 및 성장에 영향을 미칠 것이라고 생각하였다. 그래서 우리는 역전사효소 폴리머레이즈 연쇄반응과 양적 중합효소연쇄반응, 현장혼성화 분석 방법을 사용하여 *WNT4*의 발현 양상을 보았으며 그 mRNA이 어디 위치에서 특이적으로 발현하는지를 관찰하였다. 닭의 난관과 DES 처리된 닭, 배란 후 특정 시간대에서 그리고 환후가 유도된 닭의 난관에서 특이적 발현 패턴을 보였고 난소암에서는 정상난소를 가진 48주령 닭에서와 난소암 조직에서 발현이 되는 것을 확인하였다. 48주령 닭에서의 발현은 난소암보다 더 적은 발현 정도를 가지는 것으로 조사되었고 98주령의 정상난소에서는 발현이 없는 것을 관찰하였다. 또한, microRNA (miRNA) 조절 분석을 통해 WNT4의 수용체로 가능성이 있는 *FZD2*가 특정 miRNA에 의해 그 전사 기능이 조절되는 것을 밝혔다. 이 연구의 결과는 WNT4

유전자가 닭의 생식계의 다양한 기능 및 과정과 난소암의 발달 및 조절에 중요한 역할을 가지고 있음을 나타낸다.

달걀의 난황과 난백은 histotroph에 관련된 많은 영양소와 protein을 함유하고 있다고 잘 알려져 있고, 이 영양소와 protein들은 reproductive tract의 lumen에서 분비되어 ovum으로 전달되거나 liver에서 분비되어 ovarian follicle의 ovum에 전달되는 방식으로 난황과 난백에 위치된다고 알려져 있다. 다양하고 많은 nutrient transport systems들 중에 solute carriers (SLCs)은 glucose와 amino acids 그리고 electrolytes 등을 포함한 많은 substrate를 전달하는 system을 가지고 있다고 잘 알려져 있다. SLC 유전자들에 관해서는 mammalian에서 많이 연구되고 밝혀져 있지만 닭, 특히 닭의 난관에서는 연구된 바가 거의 없다. 따라서 우리는 닭의 난관에서 ovulation 후의 3h과 20h에서의 특정 point에서 SLC 유전자들의 발현 패턴에 대해서와 그 유전자들의 발현을 조절하는데 관련된 miRNA에 대해 연구를 진행 하였다. 이 연구에서 *SLC1A4* (glutamate and neutral amino acid transporter)과 *SLC13A2* (dicarboxylate transporter), *SLC35B4* (UDP-xylose:UDP-n-acetylglucosamine transporter)이 난관조직의 Magnum에서 glandular epithelium (GE)에서 특이적으로 발현이 되었으며 *SLC7A3* (cationic

amino acid transporter)과 *SLC4A5* (sodium bicarbonate co-transporter)는 난관조직의 Magnum의 luminal epithelium (LE)에서 특이적 발현이 관찰되었다. *SLC1A4*, *SLC4A5*, *SLC13A2* 그리고 *SLC35B4* mRNA는 난관조직 중 shell gland에서 GE에 강한 signal을 보였으며 *SLC7A3*는 흥미롭게도 발현이 나타나지 않았다. 그리고 *miR-1764*와 *miR-1700*는 각각 *SLC13A2*와 *SLC35B4* transcript에 직접적으로 반응하므로 post-transcriptional 조절이 가능할 것이라 예측된다. 결과적으로 닭의 난관에서 SLCs가 세포 특이적인 발현을 하고, ovulation 후 시간에 따라서 다르게 발현되었으며 *miRNA*를 통해 발현이 조절 가능하다고 예측을 해 볼 수 있었다. 이 연구를 통해 닭의 산란주기에서 선택된 SLC 유전자들이 그 특이적 시간에 따라 발현이 변하며 그것이 난관에서 태아의 생존과 알을 만드는 과정에 중요한 역할을 할 것임을 시사한다.

이러한 결과들을 통해서, 본 연구는 닭의 생식계의 발달 및 기능, 그리고 난소암에 대한 유전자들의 발현을 분석하여 이 과정에서의 이해를 돕는데 기여하였으며 이와 관련된 실험 방향에 대한 새로운 시각을 제시하는데 많은 도움이 될 것이다.

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