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Differential expression of vitelline membrane outer layer protein 1: Hormonal regulation of expression in the oviduct and in ovarian carcinomas from laying hens

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ABSTRACT

Vitelline membrane outer layer protein 1 (VMO1), a basic protein present in the outer layer of the vitelline membrane of eggs, plays essential roles in separating the yolk from the egg white and preventing infection from bacteria by forming a barrier of fibrous layers in avian eggs. Although *VMO1* is expressed in the oviduct of hens, little is known about endocrine regulation of transcription of *VMO1* in the oviduct and its expression in cancerous ovaries of laying hens. Results of present study indicated that expression of *VMO1* mRNA increased in the chick oviduct in response to diethylstilbestrol (DES, a synthetic non-steroidal estrogen). *VMO1* mRNA and protein were particularly abundant in the glandular epithelium (GE) and luminal epithelium (LE) of the magnum of the oviducts of chicks treated with DES. Also, during the regression and recrudescence phases of the oviduct during induced molting with zinc feeding, *VMO1* expression decreased as the oviduct regressed and increased with remodeling and recrudescence of the oviduct in laying hens. In addition, changes in relative expression of specific microRNAs (*miR-1623, miR-1552-3p, miR-1573, miR-22-3p, miR-124a* and *miR-1764*) regulating *VMO1* gene were detected in the oviducts during the molting period. Moreover, abundant expression of *VMO1* was found in GE of cancerous, but not normal ovaries of laying hens. Results of the present study suggest that *VMO1* is regulated by estrogen and target microRNAs in the chicken oviduct and that it is a potential diagnostic marker of ovarian cancer in laying hens.

1. Introduction

VMO1 is one of the components of the outer layer of vitelline membrane that prevents mixing of yolk and egg white and it is a component of an essential antimicrobial barrier in avian eggs. It is tightly bound to ovomucin with lysozyme and *VMO2* to construct the backbone of the outer layer membrane (Back et al., 1982; Kido and Doi, 1988; Kido et al., 1992). The chicken *VMO1* gene spans 3990 bp on chromosome 1. The gene consists of 163 amino acids with a molecular weight of 17,979 Da and chicken VMO1 protein has 59.1% homology to human VMO1 protein (Kido et al., 1995). The expression of *VMO1* mRNA is in limited parts of female reproductive organs of chickens such as infundibulum and magnum, but expression of *VMO1* was not detected in the ovary or lower part of the oviduct (Uyeda et al., 1994). The physiological function(s) of *VMO1* is unknown in the chicken.

The reproductive system of hens is composed of one functional ovary and an oviduct. The oviduct is renowned for unique biological research model with respect to hormonal regulation of gene expression, the molting process, and morphogenesis (Dougherty and Sanders, 2005). As a primary female sex hormone, estrogen plays an important role in the differentiation of tubular gland cells of the oviduct, as well as vitellogenesis and follicular development and differentiation in reproduction of hens (Hrabia et al., 2008; Palmiter and Wrenn, 1971). Artificially induced molting is useful to enhance egg production and quality which decrease in the absence of estrogen and regression of reproductive organs, but then increases with increasing concentrations of estrogen in blood and weight and size of the ovary and oviduct in hens (Jeong et al., 2013; Webster, 2003). Therefore, these studies were performed to: (1) determine tissue- and cell-specific changes in expression of *VMO1* during oviduct development in chicks treated with DES; (2) demonstrate spatial- and temporal-expression of *VMO1* in the chicken oviduct during the molting period; (3) investigate whether *VMO1* is regulated by specific microRNAs; (4) compare expression of *VMO1* in normal and cancerous ovaries from laying hens. Results of present study provide novel insights into the *VMO1* gene regarding cell-specific expression and regulation of its expression by estrogen, molting, and

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microRNAs during oviduct development and ovarian carcinogenesis in chicken.

2. Materials and methods

2.1. Experimental animals and animal care

The experimental use of chickens for this study was approved by the Animal Care and Use Committee of Korea University. White Leghorn chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water, and subjected to standard poultry husbandry guidelines.

2.2. Tissue samples

2.2.1. Study one

Fifteen female chicks were identified by PCR analysis using W chromosome-specific primer sets (F: 5'-CTA TGC CTA CCA CAT TCC TAT TTG C-3' and R: 5'-AGC TGG ACT TCA GAC CAT CTT CT-3'). Treatment with diethylstilbesterol (DES, a synthetic estrogen agonist) and recovery of the oviduct were conducted as described previously (Lim et al., 2011b). We implanted a 10 mg DES pellet into the abdominal region of 1-week-old female chicks to release the hormone for 10 days. The DES pellet was removed from all chicks for 10 days, and then a 10 mg daily dose was administered for 10 additional days. Control chicks were raised normally without any treatment for 37 days after hatching. Five 37-day-old control chicks and five 37-day-old DES-treated chicks were euthanized using 60–70% carbon dioxide to provide samples of oviduct which were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The remaining tissues were cut into 10- to 15-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 Microsystem, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 μm.

2.2.2. Study two

Hens ($n = 5$ per time point) in each subgroup including the molting group (normal feeding group, 6 days and 12 days after onset of zinc feeding) and the recrudescence group (20, 25, 30 or 35 days after onset of zinc feeding, and 8, 13, 18 or 23 days of normal feeding after cessation of egg production and removal from the high zinc diet) were euthanized using 60–70% carbon dioxide prior to collecting the oviduct on each assigned day (Jeong et al., 2013).

2.2.3. Study three

A total 136 laying hens (88 aged over 36 months and 48 aged over 24 months), which had completely stopped laying eggs were euthanized for biopsy and collection of cancerous ($n = 10$) ovaries. As a control, normal ($n = 5$) ovaries were also collected from egg-laying hens. We have examined the tumor stage in 10 hens with cancerous ovaries according to characteristic features of chicken ovarian cancer. (Barua et al., 2009; Lim et al., 2012b).

2.3. RNA isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations.

2.4. Quantitative RT-PCR analysis

Complementary DNA was synthesized using total RNA extracted from each tissues and AccuPower® RT PreMix (Bioneer, Daejeon, Korea). Gene expression levels were measured using SYBR® Green (Sigma, St. Louis, MO, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization for variation in loading. *VMO1* and *GAPDH* were analyzed in triplicate. Based on the sequence of *VMO1* mRNA (GeneBank number: NM_001167761), the sense primer (5'-TTG CCC TAC AGG GTA CTT GG-3') and antisense primer (5'-CCC CAT GAT AGT CCA TCA CC-3') amplified a 138-bp product. For *GAPDH*, the sense primer (5'-ACA CAG AAG ACG GTG GAT GG-3') and antisense primer (5'-GGC AGG TCA GGT CAA CAA CA-3') amplified a 193-bp product. Using the standard curve method, we determined the level of expression of *VMO1* gene using the standard curves and C_T values, and normalized them based on *GAPDH* expression. The PCR conditions were 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s using a melting curve program (increasing the temperature from 55 to 95 °C at a rate of 0.5 °C per 10 s) 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 statistically greater than background, and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. In situ *hybridization analysis*

For hybridization probes, PCR products were generated from cDNA with the primers used for RT-PCR analysis. The products were gel-extracted and cloned into TOPO® vector (Invitrogen). After verification of the sequences, plasmids containing the correct 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, USA). Tissues were collected and fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm 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 two times in DEPC-treated PBS. After washing in DEPC-treated PBS, they were digested with 5 μg/ml proteinase K (Sigma) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37 °C. Paraffin-embedded tissue 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 next 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, 10 mM DTT, 1 mg/ml yeast tRNA, 1 mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe overnight at 42 °C in a humidified chamber. After hybridization, sections were washed for 15 min in 2X SSC at 37 °C, 15 min in 1X SSC at 37 °C, 30 min in NTE buffer (10 mM Tris, 500 mM NaCl and 1 mM EDTA) at 37 °C and 30 min in 0.1X SSC at 37 °C. After blocking with a 2% normal sheep serum (Santa Cruz Biotechnology, INC.), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). 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).

2.6. Immunohistochemistry

The localization of VMO1 protein in the chicken oviducts and ovaries was evaluated by immunohistochemistry (IHC) using an anti-human VMO1 polyclonal antibody (catalog No., ab126510; Abcam plc, Cambridge, UK) at a final dilution of 1:200 (1 μg/ml), and antigen