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Hormonal regulation of beta-catenin during development of the avian oviduct and its expression in epithelial cell-derived ovarian carcinogenesis

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ABSTRACT

Beta-catenin (CTNNB1) is a dual function molecule that acts as a key component of the cadherin complex and WNT signaling pathway. It has a crucial role in embryogenesis, tumorigenesis, angiogenesis and progression of metastasis. Recently, it has been suggested that the CTNNB1 complex is a major regulator of development of the mouse oviduct and uterus. However, little is known about the *CTNNB1* gene in chickens. Therefore, in this study, we focused on the *CTNNB1* gene in the chicken reproductive tract and hormonal control of its expression in the chicken oviduct. *CTNNB1* was localized specifically to the luminal and glandular epithelium of the four segments of chicken oviduct and DES (diethylstilbestrol, a synthetic non-steroidal estrogen) increased its expression primarily in LE of the magnum. In addition, CTNNB1 mRNA and protein were expressed abundantly in glandular epithelium of endometrioid-type ovarian carcinoma, but not in normal ovaries. Moreover, *CTNNB1* expression was post-transcriptionally regulated via its 3′-UTR by binding with target miRNAs including *miR-217*, *miR-1467*, *miR-1623* and *miR-1697*. Collectively, these results indicate that *CTNNB1* is a novel gene regulated by estrogen in epithelial cells of the chicken oviduct and that it is also abundantly expressed in epithelial cells of endometrioid-type ovarian carcinoma suggesting that it could be used as a marker for diagnosis of ovarian cancer in laying hens and women.

1. Introduction

Beta-catenin (CTNNB1) is an intracellular protein that has multiple functions in the cell membrane, cytoplasm and nucleus. One of the well-defined roles of CTNNB1 at the cell membrane is as a subunit of the cadherin complex that mediates cell–cell adhesion. CTNNB1 also forms a complex with the cytoplasmic domain of type I cadherins and these functional complexes are essential for cell adhesion and the structural organization of cells (MacDonald et al., 2009; Fan et al., 2010; Suriano et al., 2005). Cytoplasmic CTNNB1 has a central role in transcriptional regulation in the WNT signaling pathways. Activation of the canonical WNT signaling cascade is initiated by binding secreted WNT ligand to members of the Frizzled (FZD) receptor family. This event leads to inactivation of glycogen synthase kinase 3 beta (GSK3B) and thereby permits the accumulation of activated CTNNB1 in the cytoplasm of cells. Afterward, the activated CTNNB1 translocates into the nucleus where it interacts with transcription factor/lymphoid enhancer-binding factor (TCF/LEF) to regulate transcriptional activation of multiple target genes, such as MYC and cyclin D1 (Suriano et al., 2005; van Amerongen and Nusse, 2009).

The chicken oviduct is a one of the classical models to investigate biological effects of steroid hormones on development of tissues/organs (Dougherty and Sanders, 2005). The oviduct of laying hens consists of four parts: the infundibulum (site of fertilization); magnum (production of components of egg-white); isthmus (formation of the shell membrane); and shell gland (formation of the egg shell) (Jung et al., 2011). During oviduct development, estrogen is the primary female sex hormone that induces cell proliferation, production of egg white proteins and formation of tubular glands (Kohler et al., 1969; Ylikomi and Tuohimaa, 1988; Palmiter, 1972). In the mouse uterus, estrogen stimulates the WNT signaling pathway through activation of CTNNB1 in epithelial cells (Hou et al., 2004). However, little is known about expression or functional roles of CTNNB1 in the reproductive tract of chickens

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during development or in laying hens that develop epithelial cell-derived ovarian cancer.

Epithelial cell-derived ovarian cancer (EOC) is one of the most common causes of cancer-related deaths among women due to the fact that there are no or few symptoms in the early stage of the disease (Jemal et al., 2011; Suh et al., 2012). Thus, the importance of development of effective biomarker for early detection and appropriate animal model for EOC research is strongly emphasized. Up to now, several animal models for research on EOC have been developed and, among them, the laying hen model is being recognized (Johnson, 2009). Indeed, EOC occurs spontaneously in laying hens and tumorigenesis derives from the ovarian surface epithelium as is the case for women (Shepherd et al., 2006). In addition, the histological and pathological types and stages of chicken EOC are conspicuously similar to EOC in women (Barua et al., 2009). Therefore, the laying hen is excellent animal model for studies of human ovarian cancer and for investigating novel therapeutic applications and drug development to diagnose and treat this disease.

Avian CTNNB1 expression is detected during development of vascular and neural rest cells and feather and skin morphogenesis (Nacher et al., 2005; de Melker et al., 2004; Widelitz et al., 2000). However, irrespective of its important potential role and function in the WNT signaling pathways during tissue/organ development, there has been little research on expression and hormonal regulation of CTNNB1 in the chicken reproductive tract. In addition, CTNNB1 is known to participate in development of tumors in the digestive tract, skin, testis and ovaries (Bullions and Levine, 1998; Chang et al., 2009; Bhatia and Spiegelman, 2005; Huiping et al., 2001; Palacios and Gamallo, 1998). Therefore, the hypothesis tested in the present study was that CTNNB1 plays crucial roles in differentiation, development and growth of the chick oviduct in response to estrogen and that its aberrant expression is involved in transitional events from a normal ovary to ovarian endometrioid carcinogenesis in laying hens. The objectives of this study were to: (1) evaluate tissue- and cell-specific expression of *CTNNB1* in the chicken oviduct; (2) determine whether *CTNNB1* expression is regulated by estrogen during development of the chick oviduct; (3) compare differential expression patterns of *CTNNB1* in normal and cancerous ovaries of laying hens; and (4) determine whether *CTNNB1* expression is controlled post-transcriptionally by specific microRNAs. Our novel results provide evidence that CTNNB1 has an important role(s) in development of chicken oviduct in an estrogen-dependent manner and that it is a candidate gene for EOC in laying hens and women.

2. Materials and methods

2.1. 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). All White Leghorn (WL) chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water, and managed according to standard practices for poultry husbandry.

2.2. Tissue samples

2.2.1. Study one

Following euthanasia of mature WL hens, tissue samples were collected from brain, heart, liver, kidney, small intestine, gizzard, muscle, ovary and oviduct of 1- to 2- year-old laying hens ($n = 5$). Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analysis. Frozen tissue samples were cut into 5- to 7-mm pieces, frozen in liquid nitrogen vapor, and stored at −80 °C. 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 μm.

2.2.2. Study two

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 DES and recovery of the oviduct were conducted as reported previously (Lim et al., 2011). We implanted a 15 mg DES pellet in 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 30 mg daily dose was administered for 10 additional days. Five chicks were assigned to each treatment group.

2.2.3. Study three

In this study, a total of 136 laying hens (88 over 36 months of age and 48 over 24 months of age) which had stopped laying eggs were euthanized for collection of normal and cancerous ovaries. From that population of laying hens, we obtained cancerous ovarian tissue 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., 2009; 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. Subsets of these 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. Epithelial ovarian cancers in chickens were classified based on their cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans (Barua et al., 2009).

2.3. RNA isolation

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

2.4. Semiquantitative RT-PCR analysis

The expression of *CTNNB1* mRNA in the normal and cancerous ovaries of laying hens was assessed using semi-quantitative RT-PCR as described previously. The cDNA was synthesized from total cellular RNA (2 μg) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower® RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *CTNNB1*, the sense primer (5′-GCG TTG GTT AAA ATG CTT GG-3′) and antisense primer (5′-GCT GGC TTG GAA TCT GTA AGG-3′) amplified a 412-bp product. For *GAPDH* (housekeeping gene), the sense primer (5′-TCC TTG GAT GCC ATG TGG ACC ATT-3′) and antisense primer (5′-TCC TTG GAT GCC ATG TGG ACC ATT-3′) amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted