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Investigation of the interaction between bta-miR-222 and the estrogen receptor alpha gene in the bovine ovarium

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ABSTRACT

The aim of the present study was to investigate the posttranscriptional regulation of bta-miR-222 and the estrogen receptor (ER)-alpha gene in cows. The preovulatory follicles (POFs) were detected and ER-alpha levels in the corpus luteum (CL) were measured via immunofluorescent method. Afterwards, an ELISA test was performed to detect estradiol- and progesterone-active follicles, and the expression levels of ER-alpha, progesterone receptor (PR), and bta-miR-222 were measured via qRT-PCR. Finally, a western blot analysis was performed to determine ER-alpha protein levels. Immunofluorescence staining was highly positive in the follicular phase, showing ER-alpha and PR immunopositivity. In the luteal phase, ER-alpha immunopositivity decreased in lutein cells, whereas the PRs were observed to be similar in intensity to those in follicular phase. While the estradiol levels were higher in the POFs, the progesterone level was higher in the CL. In the CL, the transcription levels of ER-alpha and PR mRNA were observed to be the same as in the POFs; however, the expression of bta-miR-222 was lower. In the CL, the transcription level of ER-alpha mRNA was lower than that of PR mRNA, and the expression level of bta-miR-222 was higher than that of PR mRNA. The results of the western blot analysis show that the ER-alpha protein level in the CL was lower than that in the POFs. Taken together, our results here suggest that there is a negative correlation between bta-miR-222 and ER-alpha expression during follicular development in cow ovaries.

1. Introduction

Estrogen in involved in several critical biological pathways, such as secretion, cell differentiation, and necrosis in female reproductive organs [1]. Estrogen is predominantly synthesized and secreted from the granulosa cells of growing POFs [2]. Estrogen receptors have been detected during embryonic and fetal development [3]. During that development, estrogen may have a direct impact on cells expressing ERs; however, it cannot affect cells without these receptors [4]. There are two different forms of ER, which are referred to as ER-alpha and ER-beta, and each is encoded by a separate gene. The PR has three isoforms encoded by a single gene [5]. All three isoforms (PR-A, PR-B, and PR-C) have been detected in cow tissues [1]. It has been suggested that PR-A and PR-C may have prohibitive effects on the transcriptional activity of PR-B [6]. The presence of ER-alpha and ER-beta can be partially explained by the selective impact of estrogen in different target tissues, as well as in the same tissue with different physiologies [6]. Studies performed on various species have verified the differential distribution of these two receptors in the ovary; that is, ER-beta was primarily detected in granulosa cells, and ER-alpha was detected in theca cells, interstitial glands, stromal cells, and the germinal epithelium [7–11]. The mRNA expression of cow ER-beta in granulosa cells decreased with increased follicle size [11]. In contrast, the mRNA expression of ER-alpha in the theca internal tissue increased continuously during the final growth of cow follicles without granulosa cell proliferation [8]. While ER-alpha-knockout mice had acyclic, infertile, and hyperemic ovaries devoid of CL, ER-beta-knockout mice had small ovaries and retarded follicular development [12]. Although the distribution of ERs in the reproduction system and their functions, along with the associated molecular mechanisms, have been known for some time, there is no data regarding the post-transcriptional regulation of these receptors.

Small non-coding RNA molecules, also called miRNAs, belong to a class of short, non-coding, endogenously initiated RNAs that transcriptionally control gene expression by means of either translational suppression or mRNA degradation [13–15]. These miRNAs regulate gene expression and function in various tissues [16–18]. Initially, in the biogenesis of miRNAs, primary miRNA (pri-miRNA) is synthesized by RNA polymerase II. Pri-miRNA is transformed into the hairpin pre-

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miRNA precursor, which is composed of 70 nucleotides, by the Drosha-DGCR8 enzyme complex in the cell's nucleus. Then, pre-miRNA is transported from the cell's nucleus to the cytoplasm by exportin-5 protein. Cytoplasmic pre-miRNA is processed by the Dicer enzyme and transformed into double-stranded miRNA, which is composed of 20 nucleotides. Finally, miRNAs form an RNA-induced silencing complex and regulate gene expression [19]. Previous studies have reported that bta-miR-222 plays a role in oocyte maturation and fertilization in cows [20]. One study revealed that bta-miR-222 was expressed in the early luteal phase of the estrous cycle in cows [21]. Another previous study purported that miRNA sequences were highly preserved between the species and underwent tissue-specific expression [22]. Notably, although the significance of miRNAs during ovarian development has been shown via transgenic mouse models [23,24], the association between ER-alpha and bta-miR-222 has not been revealed in cow ovaries, and there are no relevant data in the literature.

Therefore, the aim is to reveal whether ER-alpha is regulated post-transcriptionally by bta-miR-222. Both ER-alpha and PR levels in the collected tissues were detected via the immunofluorescent method; then, estradiol and progesterone levels were detected via the ELISA method, and ER-alpha and bta-miR-222 were levels were measured using qRT-PCR. Finally, a western blot analysis was performed to measure ER-alpha protein levels.

2. Materials and method

2.1. Tissue collection

Ovary samples were collected from 20 Holstein cows after slaughtering in Oral Meat Entegre Facilities (Erzurum, Turkey), and the collected ovaries were sterilized in 70% ethanol. The sizes of the pre-ovulatory follicle (POF) and corpus luteum (CL) were measured using Vernier calipers. The approximate sizes of the POF and CL samples were 12.20 ± 1.4 mm and 15.32 ± 1.27 mm, respectively. In the present study, six POF and six CL samples (twelve in total) were used for each assay. Tissue samples were rapidly frozen in liquid nitrogen and stored at -80 °C. All experiments were performed in accordance with the relevant guidelines required by Atatürk University, Turkey. All experimental protocols were approved by Atatürk University, Faculty of Veterinary Medicine, Turkey (AÜHAYDEK-E.1600096331).

2.2. ELISA test

The concentrations of estradiol and progesterone were measured in the follicular liquids to determine progesterone- and estrogen-active follicles. A 17-β estradiol high-sensitivity enzyme immunoassay (EIA) kit (Cat No. ADI 901-174, Enzo Life Sciences, UK) was used for estradiol. Follicular fluid samples (15–20 µl) were diluted in assay buffer to a final volume of 150 µl and assayed in triplicate against a ten-point standard curve ranging from 1.95 to 3000 pg/ml. Plates were read on a BioTek™ ELx808™ absorbance microplate reader at 405 nm. The intra- and inter-assay coefficient variation were 18.34% and 23.11%, respectively. The detection limit of the assay was 0.9 pg/ml.

A progesterone competitive ELISA kit (Cat No. EIAP4C21, Thermo Fisher Scientific, USA) kit was used for progesterone. Follicular fluid samples (15–20 µl) were diluted in assay buffer to a final volume of 150 µl and assayed in triplicate against a ten-point standard curve ranging from 50 to 3200 pg/ml. Plates were read on a BioTek™ ELx808™ absorbance microplate reader at 450 nm. The intra- and inter-assay coefficient variation were 12.24% and 18.37%, respectively. The detection limit of the assay was 0.7 pg/ml.

To calculate the recovery, the follicular fluid samples were diluted with 1X Assay Buffer, one with slightly diluted estradiol (468.8 pg/ml) and progesterone (405.2 pg/ml) and another with a more highly diluted estradiol (1.875 pg/ml) and progesterone (1.776 pg/ml). Undiluted samples were analyzed to measure the E2 and progesterone concentrations. The concentration values were rounded to two decimal places. The recovery was calculated using the following formula: (detected concentration/expected concentration) *100. Expected concentration was calculated using the following formula: (concentration of undiluted sample + concentration of the added standard) *0.5 [25]. The recovery rates for E2 and progesterone were 107.5% and 105.4%, respectively (Tables S1 and S2).

2.3. Cumulus cell separation and culture

Healthy bovine follicular ovaries (12.20 ± 1.4 mm diameter) were obtained from a slaughterhouse. The ovaries were placed into a saline buffer at 25 °C and transferred to the laboratory within 1 h of collection. The follicular fluid was extracted from follicular ovaries and transferred into HEPES buffer (Sigma, CAS number: 7365-45-9). After approximately 36 h of in vitro maturation, we measured the cumulus cell expansion diameter and area under a microscope. This procedure was applied in accordance with previous studies [26,27]. Cumulus-oocyte complexes (COCs) were selected under a microscope. Cumulus cells (CCs) and oocytes were completely separated after digestion with 0.1% hyaluronidase (Sigma, CAS Number 37326-33-3); thereafter, opened oocytes were collected. The CCs were centrifuged at 2500 × *g* for 5 min. Later, the CCs were washed with phosphate-buffered saline (PBS) three times and subsequently collected and counted. The cells were cultured using DMEM/F12 medium (containing 10% fetal bovine serum and penicillin/streptomycin solution) and placed in a 5% CO2 incubator at 38.5 °C [28]. DMEM/F12 medium (Gibco, Cat. no:11320033); trypsin (Gibco, Cat. no: 15050065); fetal bovine serum (heat inactivated) (Gibco, Cat. no: 10082139); penicillin/streptomycin solution (Gibco, Cat. no: 15140122).

2.4. Transfection

Bta-miR-222 mimics (Cat. no: 4464066)/bta-miR-222 negative control (NC) (Cat. no: 4464058) and bta-miRNA-222 inhibitor (Cat. no: 4464084)/bta-miR-222 negative control (NC) (Cat. no: 4464076) were designed and synthesized by ThermoFisher (US). The CCs were grown in DMEM/F12 supplemented with 10% FBS and penicillin/streptomycin, which were seeded in six-well plates (3.0 × 105 cells per well) the day before transfection. After 24 h, bta-miR-222 mimics and mimic control, as well as the bta-miR-222 inhibitor and inhibitor control and the GFP plasmid for transfection efficiency, were transfected into CCs in triplicate using the TurboFect transfection reagent (ThermoFisher), following the manufacturer's protocol.

2.5. Immunohistochemistry (IHC)

The tissues were fixed in the neutral formaldehyde solution for 1 day, washed in tap water, passed through the alcohol/xylene stages, and placed in paraffin blocks. Five µm sections were taken from the paraffin blocks using a microtome and placed onto polylysine slides. After the deparaffinization of the tissues on the polylysine slide, the plates were placed in 3% H_2O_2 for 10 min to inactivate the endogenous peroxidase and then washed with PBS. To disclose the antigens in the antigen retrieval solution The tissue samples were then incubated for 10 min at 500 W and washed in PBS. To avoid nonspecific binding, a protein block solution was dripped into the tissues for 10 min and washed in PBS. Progesterone R/NR3C3 antibody (Alpha PR6) (NB120-2765), for the follicular phase, and estrogen receptor antibody (PA5-16476), for the luteal phase, were added to the tissues washed with PBS at dilutions of 1/50, where they remained at room temperature for 20 min. Then, the procedure described regarding the Expose mouse and rabbit specific HRP/DAB detection and IHC kit (abcam: ab80436) was followed. 3,3'-diaminobenzidine chromogen was used, and the slides were counterstained with hematoxylin. Positive cells were examined under light microscopy.