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Molecular cloning, expression analysis, and function of decorin in goat ovarian granulosa cells

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ABSTRACT

Decorin (DCN), a component of the extracellular matrix (ECM), participates in ECM assembly and influences cell proliferation and apoptosis in many mammalian tissues and cells. However, expression and function of DCN in the ovary remain unclear. This study cloned the full-length cDNA of goat DCN obtained from the ovary of an adult goat. Sequence analysis revealed that the putative DCN protein shared a highly conserved amino acid sequence with known mammalian homologs. The tissue distribution of DCN mRNA expression was evaluated by real-time PCR, and the results showed that DCN was widely expressed in the tissues of adult goat. Immunohistochemistry results suggested that DCN protein existed in the granulosa cells and oocytes from all types of follicles and theca cells of antral follicles. Moreover, hCG-induced DCN mRNA expression was significantly reduced by the inhibitors of protein kinase A, PI3K, or p38 kinase ($P < 0.05$), which are key mediators involved in hCG-induced DCN expression. Overexpression of DCN significantly increased apoptosis and blocked cell cycle progression in cultured granulosa cells ($P < 0.05$). Western blot analysis also showed that overexpression of DCN upregulated the expression levels of p21 protein ($P < 0.05$), whereas no effects were observed on the expression of Bax and Bcl-2 and on Bcl-2/Bax ratio ($P > 0.05$). These findings suggested that DCN regulates the apoptosis and cell cycle of granulosa cells.

1. Introduction

The extracellular matrix (ECM) is a major component of the cellular microenvironment and provides support to cells [1]. Decorin (DCN), a member of the small leucine-rich proteoglycan (SLRP) gene family, is a component of the ECM and plays a crucial role in a variety of physiological processes [2]. DCN also regulates collagen fibril formation and controls cell proliferation [3]. DCN protein is highly conserved and shares structural homologies, such as cysteine residues, leucine-rich repeats (LRRs), and at least one glycosaminoglycan side chain, with those of other species [4]. In addition, DCN is widely expressed in mammalian tissues and can directly regulate multiple growth factors, such as transforming growth factor-β1 [5,6]. DCN also antagonizes a number of receptor tyrosine kinase (RTKs), including epidermal growth factor receptor (EGFR) [7], insulin-like growth factor 1 receptor (IGF-IR) [8], and receptor for hepatocyte growth factor [9].

DCN is expressed in reproductive tissues, including ovary and uterus. In the uterus, high levels of DCN are detected, and the steroids upregulate DCN expression levels [10,11]. In the ovary, DCN is present in normal and tumorous tissues [12–14]. Moreover, overexpression of DCN in ovary cells suppresses cell proliferation [15]. A recent study has also shown that DCN is produced in follicular cells, and treatment with DCN causes rapid phosphorylation of EGFR in ovarian granulosa cells [16]. However, the roles of the DCN in female fertility and ovarian function remain largely unknown.

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Our previous studies have shown that DCN expression is upregulated in ovarian tissues of polytocous goats compared with that in monotocous goats (unpublished data), and hCG induces a rapid and transient DCN expression in cultured granulosa cells of goat [17]. These results suggested that DCN regulates the reproductive function of goats. In this follow-up study, we cloned and characterized the goat DCN gene and investigated its expression in various tissues and in follicles of goat. We also determined the regulatory mechanism of hCG toward DCN expression in goat ovarian granulosa cells and the potential function of DCN in the ovary.

2. Materials and methods

2.1. Reagents

hCG was purchased form Sigma-Aldrich (St. Louis, MO, USA). Chemicals and reagents, including H89, LY294002, and Forskolin (FSK), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and SB2035850 were purchased from Beyotime Biotechnology (Jiangsu, China). DMEM/F12 and fetal bovine serum were obtained from Life Technologies Inc (Carlsbad, CA, USA).

2.2. Tissue collection and cell culture

Healthy adult Guanzhong dairy goats (n = 4, 1–3 years old, cyclic, and in good body condition) were stunned using a captive bolt and then slaughtered to collect different tissues. Eleven tissues, including the uterus, spleen, kidney, heart, liver, lung, ovary, muscle, fat, mammary gland, and oviduct, were immediately collected, cut into small pieces of approximately 1 g in 0.1% w/v diethylpyrocarbonate in water and then immediately placed in liquid nitrogen. All animal procedures presented in this article were approved by the animal ethics committee of the Northwest A&F University (Yangling, China).

Goat granulosa cells were collected from the ovaries of Guanzhong dairy goat by using the follicle isolation method as previously described [17]. Briefly, the tissue was first washed with 75% alcohol for 1 min and then washed 3 times with PBS to eliminate alcohol. Small antral follicles (1–3 mm) were then harvested by an aseptic needle under the stereomicroscope. After washing 3 times with DMEM/F12 medium (Gibco, Grand Island, NY, USA), the small antral follicles were cut into pieces, and then an aseptic needle was used to release the granulosa cells. The COCs and ovarian tissues were discarded under the stereomicroscope. Granulosa cells were harvested by centrifuging ($800 \times g$) for 10 min and washing twice in DMEM/F12 medium. The granulosa cells were counted in a hemocytometer, the viability was determined by trypan blue exclusion, and the cells were seeded in 12-well culture plates at a density of 2×10^5/well in 1 mL of DMEM/F12 with 10% FBS, 100 IU/mL penicillin, and 50 mg/mL streptomycin. The cells were cultured at 37°C in a 5% CO_2 atmosphere. After 24 h, the cells were washed twice with PBS and changed with fresh medium (DMEM/F12 with 10-mM HEPES, 20-mM L-glutamine, 100 IU/mL penicillin, 50 mg/mL streptomycin, 0.2% BSA, 10 μg/mL transferrin, 4 ng/mL sodium selenite, and 10 ng/mL insulin) for 12 h. The cells were then treated with specific reagents at a time interval indicated in the text. When the reagents were dissolved in dimethylsulfoxide (DMSO), the same DMSO concentration was added into the medium of the control cells. The final concentration of DMSO in cultures was less than 0.05%. At the end of each culture period, the cells were collected for total RNA isolation.

2.3. Cloning, sequence alignment, and phylogenetic analyses of goat DCN

The goat DCN was cloned through RT-PCR using the cDNA obtained from the adult ovary of a goat. The primers (forward 5′-GGGCTCCAGTGGCAAATC-3′, reverse 5′-CCCGCCGTGAGTTACAGA-3′) were synthesized based on the DCN cDNA sequences of cattle (NM_173906) and sheep (GAAI01007035). The amplified fragments were visualized in a 1.5% agarose gel, and the products exhibiting the expected size were cut and purified using a TIANgel Midi Purification Kit (Tiangen Bio-tech, Beijing, China). The purified PCR products were ligated into a pMD19-T vector and then transformed into *DH5α* (*Escherichia coli*) according to the manufacturer's instructions. The full-length ORF cDNAs were finally determined through sequencing (Genewiz, Suzhou, China).

The deduced amino acid sequences were compared with the sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST) program available from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The signal peptide was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw), and a phylogenetic tree was constructed by MEGA 5.0 using the neighbor-joining method [18].

2.4. RNA isolation, reverse transcription, and real-time PCR

Total RNA was extracted from 11 different tissues (uterus, spleen, kidney, heart, liver, lung, ovary, muscle, fat, mammary gland, and oviduct) and from cells using RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the optical density (OD) at 260 and 280 nm wavelengths using an Epoch Microplate Spectrophotometer (BioTek Instruments. Inc., USA). The $OD_{260/280}$ ratios were >1.8 and <2.1 for all of the samples. The total RNAs (500 ng) were used to convert the mRNAs into cDNAs by using a PrimeScript RT reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Real-time (RT) PCR was then performed in a 20-μL reaction vol containing 10 μL of SYBR Premix Ex Taq II (TaKaRa, Dalian, China), 1 μL of template cDNA, and 1 μM of primers by using the *CFX Connect* Real-Time PCR Detection System (Bio-Rad, CA, USA). The thermal cycling conditions were 95°C for 10 min followed by 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The following primers were used for RT PCR: DCN, 5′-TGGATTGAACCAGATGATCGTC-3′ (sense) and 5′-GTCAGCAATGCGGATGTAGGA-3′ (antisense); and β-actin, 5′-TGACCCA GATCATGTTTGAGA-3′ (sense) and