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Disruption of follistatin by RNAi increases apoptosis, arrests S-phase of cell cycle and decreases estradiol production in bovine granulosa cells

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ABSTRACT

Follistatin (FST), a local regulator of gonadal functions is a powerful inhibitor of follicle stimulating hormone (FSH) secretion. In the present study, the expression of FST was partially silenced at both transcriptional and translational levels by RNAi-Ready pSIREN-RetroQ-ZsGreen Vector mediated recombinant pshRNA vectors in bovine granulosa cells (bGCs). The results showed that transfection with FST-1 and FST-2 vectors significantly down-regulated mRNA and protein expressions of follistatin by 51% ($P = 0.0093$) and 72% ($P = 0.0078$) respectively. After down-regulation of FST in bGCs, cell cycle was arrested at S-phase (9.2 ± 0.6 vs 12.5 ± 0.2, $P = 0.0055$), and apoptosis was significantly (21.3 ± 2.7 vs 13.9 ± 2.5, $P = 0.0051$) increased. These findings were further verified by down-regulation of protein level of B-cell leukemia/lymphoma 2 (*Bcl2*, $P = 0.0423$), and up-regulation of caspase-3 ($P = 0.0362$), p21 ($P = 0.0067$) and mRNA levels of Bcl2-associated X protein (*Bax*, $P = 0.041$). Knockdown of FST in bGCs significantly increased activin A concentration in culture medium, while level of estradiol (E2) was suppressed without affecting progesterone production. In addition, mRNA levels of all activin receptor subtypes [activin receptor types I (ACRI) and II (ACRIIA and ACRIIB)] and inhibin α-subunit were augmented ($P < 0.05$) without altering both inhibin β-subunits. These findings suggest that follistatin may participate in caspase3-dependent apoptosis through Bcl2/Bax gene family in bovine GCs, whereas, activin and its receptors are associated with its regulation. Activin-induced up-regulation of inhibin-α subunit in bGCs seems to be involved in the regulation of steroidogenesis.

1. Introduction

Follistatin (FST), an activin binding protein was first discovered in ovarian follicular fluid (Ueno et al., 1987). Its protein exists in full {315 amino acids (aa)}, intermediate (303 aa) and shortest (288 aa) forms in human ovarian tissues (Sugino et al., 1993). Of all follistatin actions described to date, follistatin is best known for its high-affinity binding to activin for inhibition of its activity (Nakamura et al., 1990). The activin signaling complex is transmitted via

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type I and two type II receptors. Activin association with its receptors includes initial attachment with two type II receptors, proposed to hold internal integrity and enables it to bind with type I receptor (Walton et al., 2012). Crystallization studies show that two FST molecules cover large surface area of the activin dimers, and completely blocks both type I and type II receptor binding sites. Moreover, C-terminus of one FST molecule contacts the N-terminus of FST molecule as if to lock them in place (Thompson et al., 2005). Through this binding, FST potentially regulates cellular differentiation and their fate (Nakamura et al., 1990).

For proper ovarian growth and maturation, the development and differentiation of GCs are of particular interest. This can be achieved by proper balance between proliferation and apoptotic activity in these cells (Wang et al., 2012). The role of FST in the ovary is remarkable in this regard, as it functions through negative feedback with activin. Activin-mediated cell growth arrest and apoptotic induction is reported in many of the previous cell models (Ying and Zhang, 1996; Zhang et al., 1997; Zhou et al., 2000). These death signaling pathways are mainly associated through ACRIIB or Smad signaling pathways (Chen et al., 2000; Lee et al., 2009).

To date, many previous studies have delineated the biological role of FST in the reproductive axis (Thompson et al., 2005). For example, the knockout FST mice were failed to survive due to multiple anomalies, including growth and muscle retardation, abnormal formation of diaphragm and craniofacial defects (Matzuk et al., 1995). On the other hand, numerous reproductive defects, including failure to induce folliculogenesis and retarded gonadal growth were recorded in overexpressing FST mice (Guo et al., 1998). The GCs specific knockdown study of FST has also been implemented in an in vivo model (Jorgez et al., 2004).

Presently, no data is available on FST knock down strategy to study the development of GCs, which is ultimately important for follicular maturation. Particularly, in bovines, the role of FST by RNAi could be served as a model for monotocous animal. We have used bovine GCs model in this study because many aspects of bovine GCs are similar with that of human, and it also offers improvements in reproductive performance of dairy cattle. The present study is, therefore, conducted to estimate bovine GCs apoptosis, growth, gene regulation and steroidogenesis by RNAi of FST.

2. Material and method

2.1. Granulosa cell cultures

Culture of GCs was performed as previously described (Sen et al., 2007; Wang et al., 2012). Bovine ovaries (Chinese Holstein dairy cows, $n = 8–10$, age ranges from 4 to 6 years) were obtained from the local slaughterhouse (Hubei, China) which were preserved within 30 min in phosphate buffered saline (PBS; Hyclone Laboratory, Inc., Logan, UT, USA) with penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}), (Pen-Strep, Invitrogen, Carlsbad, CA, USA) at 37 °C. After rinsing ovaries twice with PBS, follicular fluid was aspirated from 3 to 6 mm antral follicles through 20-gauge needle by puncture method, and was pooled together. At this step, the hemorrhagic follicles or contaminated follicular fluid was discarded. The supernatant of the follicular fluid was collected, and GCs were harvested by centrifugation at 1500 × *g* for 5 min. The remaining pellet was washed and re-suspended 4 times with PBS. The cell pellet was digested for 5 min by 0.25% trypsin (Gibco) with 0.025% EDTA (Gibco). At last, the GCs were cultured in 60 mm dish with Dulbecco' Modified Eagle Medium (DMEM, Hyclone) supplied with 10% fetal bovine serum (FBS, Hyclone Co.) and penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in an incubator containing 5% CO_2 and 95% air for 48 h. For theca cell contamination, expression of CYP11A was measured in the culture. The cell numbers and viability were estimated by trypan blue exclusion method as previously described elsewhere (Wang et al., 2012).

2.2. Extraction of RNA from GCs and qualitative transcriptional study

Total RNA was extracted from the cells after culturing using RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China). The kit was supplied with DNase I for the removal of genomic DNA contamination. After extraction, the quality of the total RNA was detected by spectrophotometric absorbance at 260 nm/280 nm. 2 μg of RNA was reverse transcribed into cDNA using first strand cDNA synthesis kit (Toyobo, Japan). For expression analysis of bovine FST, RT-PCR was executed in a total volume of 20 μl including, 10 μl PCR master mix, both forward and reverse primers for FST (1 μl, Table 1), 1 μl cDNA and 7 μl ultra pure water. Negative samples were prepared by replacing cDNA with ultra pure water. Amplification conditions include: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C (30 s), annealing at particular temperatures (Table 1, 30 s), elongation at 72 °C (30 s) followed by final extension at 72 °C for 5 min. PCR product was visualized on 1.5% agarose gel. For sequencing, PCR product was extracted from the gel and later sent to sangon sequencing company (Beijing, PR China).

2.3. Target gene selection and RNA interference vector construction

The complete coding sequence of bovine follistatin (NM_175801.2) was acquired from NCBI GenBank database. Two siRNA target sites were selected according to the siRNA online program (http://www.genscript.com) at positions of 404 and 1149 in the coding region. The oligonucleotides were annealed and inserted with the EcoRI and BamHI sites of RNA-Ready pSIREN-RetroQ-Zsgreen Vector (BD Bioscience, Clonetech, Mountain View, CA). The recombinant RNAi plasmids (silencing via shRNA) were named as FST-1 and FST-2 respectively (Table 2). A plasmid named control, containing a scrambled sequence was used as a negative control. The day before transfection, GCs were cultured in serum free conditions to get >80% of confluence and were transfected with FST-1, FST-2 and negative control vectors in 6-well plates in triplicate using the Lipofectamine LTX and PLUS Reagents according to the user's guidelines for 48 h. For maximal efficiency of these vectors,