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Original article

Transcriptome sequencing analysis of porcine granulosa cells treated with an anti-inhibin antibody

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ABSTRACT

Inhibin can regulate granulosa cell proliferation and function *via* direct action on granulosa cells, or indirectly through stimulation of pituitary follicle-stimulating hormone secretion. Thus far, it has not been possible to unravel or formulate the chain of molecular events that lead to enhanced granulosa cell proliferation and function using conventional gene expression analysis. The aim of this study was to examine the biological effects of immuno-neutralization of inhibin bioactivity in porcine granulosa cells using transcriptome profiling by the RNA-seq technology.

Treatment of granulosa cells with anti-inhibin α subunit antibodies increased both cell proliferation and estradiol secretion. Data revealed by RNA sequencing were subjected to bioinformatic analysis. The results showed that a total of 476 genes, including 27 novel genes, were differentially expressed in anti-inhibin antibody-treated granulosa cells compared to untreated granulosa cells. RNA sequencing data were validated by qRT-PCR which confirmed differential expression (upregulation and downregulation) of eighteen of twenty selected genes A total of 476 differentially expressed genes were enriched in processes such as matrix remodeling, chemokine activity, protein binding, and structural molecular activities, and which could be related to granulosa cell proliferation, estradiol synthesis, and ovarian follicle growth. In particular, the data emphasized the importance of extracellular matrix remodeling and the involvement of chemokines in enhanced granulosa cell function, which are important features of ovarian follicle growth, development, maturation, and ovulation. This study provided a new level of understanding of enhanced granulosa cell function and ovarian follicle development achieved through immuno-neutralization of endogenous inhibin bioactivity.

1. Introduction

Inhibin, primarily secreted by the granulosa cells of dominant or large ovarian follicles, is a dimeric glycoprotein comprised of an α- and a β-subunit [1]. Inhibin suppresses pituitary follicle-stimulating hormone (FSH) secretion through a negative feedback mechanism and inhibits development of sub-ordinate follicles through para/autocrine regulatory mechanisms [2–4]. Both these actions result in atresia of small follicles and selection of a dominant follicle. It is believed that inhibin's regulatory function is mediated by competing with activin for binding to the type 2 activin receptor (ACTRII) [5,6]. Immuno-neutralization of inhibin's ovarian follicle suppression activity, by passive or active immunization against the inhibin α subunit peptide, enhances follicle development and substantially improves ovulation rates in a variety of animals [7–15]. In addition, immunization against inhibin enhances not only follicular development, but also luteal function after ovulation of the follicles, as well as oocyte maturation and subsequent quality of embryo development [12,13,16]. These enhancements have allowed this method to develop into new reproductive techniques that improve the

Abbreviations: DMEM/F12, Dulbecco's modified eagle's medium/Ham's F-12 nutrient mixture; FSH, follicle-stimulating hormone; ACTRII, the type 2 activin receptor; E2, estadiol; DEGs, differentially expressed genes; PBS, phosphate-buffered saline; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OD, optical density; RPKM, reads per kb (of exon regions) per million mapped reads; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; PPI, protein-protein interactions; FN, fibronectin 1; 2-M, 2-Macroglobulin; FBN1, fibronectins; BCL2, B-Cell CLL/Lymphoma 2; CCND, 1cyclinD1; CCND3, cyclinD3; ECM, the extracellular matrix; hCG, human chorionic gonadotropin.

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conception rate or reproductive performance of livestock animals [17]. The fundamental mechanism by which immunization against inhibin works is through a blockade of inhibin regulation of ovarian follicle development, or more precisely, a blockade of inhibin's direct actions on granulosa cell proliferation and function [18], as well as a blockade of its effects on stimulation of pituitary FSH secretion.

Numerous studies have been carried out to study inhibin's regulation of granulosa cell development and function [18–21], while others have studied activin's regulatory abilities [22–24]. Treating granulosa cells cultured *in vitro* with an anti-inhibin α subunit antibody neutralizes and removes the negative regulatory role of endogenously produced inhibin and enhances granulosa cell proliferation as well as estradiol (E2) secretion [18,25]. In addition, the expression of a wide range of genes involved in cell cycle and proliferation, apoptosis and follicular atresia, follicle growth and angiogenesis, as well as steroid hormone synthesis were altered following anti-inhibin antibody treatment [18]. However, using a conventional gene expression assay, it is still not possible to unravel the chain of molecular events that lead to enhanced granulosa cell proliferation and function following neutralization of inhibin activity. More importantly, a conventional gene expression assay could potentially miss important genes for granulosa cell function and follicle development, especially previously unknown novel genes. Therefore, we considered it necessary to utilize a high-throughput transcriptome analysis technique as a means to investigate the mechanism of inhibin's effects on granulosa cell function.

In recent years, the high-throughput RNA sequencing technique has emerged as a powerful tool for transcriptome analysis [26], and, in particular, it has been applied to studying ovarian follicle development and maturation [27–30]. By using genome-wide transcriptome analysis, many differentially expressed genes (DEGs) encoding signaling molecules (e.g. *GDF9*, *BAX*, *BAD*, *NDUFA13* and *IFI6*, and *CAV1*) have been found to be associated with sheep fecundity [31]. Similarly, transcriptome analysis of bovine granulosa cells after pre-LH surge demonstrated an increased expression of genes regulating cellular processes important for ovulation [29] and a set of genes important for granulosa cell luteinization was also upregulated in cells after post-LH surge [32]. The results of the aforementioned studies demonstrate that high thoroughput RNA-sequencing is a comprehensive and efficient way to identify key genes that regulate prominent changes in cellular processes. Using the RNA-seq technology, we have systematically examined the changes in gene expression of porcine granulosa cells following treatment with anti-inhibin antibodies. The obtained results help to illustrate the regulatory mechanisms underlying the enhanced granulosa cell activities as well as enhanced ovarian follicle development in animals immunized against inhibin.

2. Materials and methods

2.1. Obligatory ethical approval

The experimental procedures were approved by the Research Committee of Jiangsu Academy of Agricultural Sciences and conducted with adherence to the Regulations for the Administration of Affairs Concerning Experimental Animals (Decree No. 63 of the Jiangsu Academy of Agricultural Science on July 8, 2014).

2.2. Isolation and culture of porcine ovarian granulosa cells

Ovaries from pre-pubertal gilts aged 165–180 days were obtained from a local slaughterhouse and transported to the laboratory within 2 h of isolation in a vacuum thermos flask containing sterile physiological saline at 30–37 °C. After ovaries were washed three times with sterile physiological saline at 37 °C, follicular fluid and granulosa cells were aspirated from 40 medium-sized follicles between 4 and 6 mm in diameter by using a 10 mL syringe. The cells were then transferred to a 15 mL centrifuge tube and 1 mL of 0.25% trypsin was added to disperse any cell clumps. Following incubation at 37 °C for 3–5 min, 1 mL of 10% fetal calf serum-supplemented Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F12, without phenol red) was added to the tube to terminate trypsin digestion. The cells were centrifuged at 800 × *g* for 15 min to be precipitated and then washed twice with phosphate-buffered saline (PBS).

Cells were plated into a 96-well plate at a cell density of 1×10^5 cells per well and 200 μL of culture medium containing 10% fetal calf serum (FCS) was added. The cell survival rate was 66 ± 7%, measured using the trypan blue exclusion test. The cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h, and then washed with PBS to remove any unattached cells. Previously it was shown that estradiol production in granulosa cells treated with the anti-inhibin antibodies at 200 μg/mL was significantly higher than estradiol production in untreated granulosa cells [18]. Moreover, the number of viable cells at the end of the culture period was significantly affected by the anti-inhibin antibodies. Therefore, in the present study, the culture medium was changed and replaced with new DMEM/F12 medium containing 2% FCS, 0.1 μM androstenedione with the polyclonal anti-inhibin α-subunit antibody at a final concentration of 200 μg/mL. The titer of the polyclonal anti-inhibin α-subunit antibody used was 1:51200. The cells were incubated for a further 48 h under a humidified atmosphere at 5% CO_2 and 37 °C. At the end of the incubation period, granulosa cells treated with 200 μg/mL anti-inhibin antibodies and untreated control granulosa cells were collected for RNA sequencing and qRT-PCR, and aliquots of the culture medium were collected to measure the concentration of E2.

2.3. Measurement of E2 concentration

Concentrations of E2 in the culture medium were measured by enzyme-linked immunosorbent assay (Beijing North Institute of Biological Technology; Beijing, China) according to the manufacturer's instructions. The E2 standard curve ranged from 40 to 1000 pg/mL. Cell culture media were diluted 5-, 40-, 120-, and 200-fold using FCS-free medium to ensure that the assay value fell within the detection range of the standard curve. Each sample was assayed in duplicate, and the E2 concentration was calculated by multiplying the end value by the dilution factor. The assay sensitivity, range, and intra-assay coefficient of variation were 1 pg/mL, 1–1000 pg/mL, and <15%, respectively.

2.4. Measurement of cell proliferation

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) is converted into yellow formazan after being reduced by succinate dehydrogenase, which is synthesized by the mitochondria of living cells. The production of formazan is proportional to the number of living cells. Therefore, the MTT assay was used to assess granulosa cell proliferation. Following granulosa cell culture for 48 h, 10 μL of MTT solution containing 5 mg/mL thiazolyl blue MTT was added to each well of Cell Counting Kit 8 (Shanghai QCBio Science & Technologies Co. Ltd.; Shanghai, China). Then, the cells were cultured for a further 3 h and the optical density (OD) of the yellow colored formazan was measured at 490 nm using a Biotek EON microtiter plate reader. Each treatment was repeated using two duplicate wells, and then repeated across six independent experiments.