Transcriptome sequencing reveals molecular mechanisms of enhanced porcine granulosa cell activities following treatment with an anti-inhibin antibody in vitro

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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 FSH 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.

Keywords: RNA-seq, granulosa cell, immuno-neutralization, inhibin, transcriptome

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 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 throughput 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 $800\times g$ 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 μ L of culture medium containing 10% fetal calf serum (FCS) was added. The cell survival rate was $66\pm 7\%$, 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 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$. 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 $\mu\text{g/mL}$ 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 μ L 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.

2.5. RNA-Seq library construction

The four RNA-seq libraries (two RNA-seq libraries for treated granulosa cells and two RNA-seq libraries for untreated granulosa cells) were generated using an Illumina TruSeqTMRNA Sample Preparation Kit (Illumina, San Diego, USA) as per the manufacturer's recommendations; a tetrad index code was added to the sample for subsequent documentation.

The library was sequenced by pair-end sequencing on an Illumina HiSeq 2000 platform and 100 bp paired end reads were generated. The base quality distribution of different RNA-seq library was summarized. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication levels of the clean data were calculated. All the downstream analyses were based on clean data with high quality (Fig. S1).

2.6. Identification of differentially expressed genes

Based on RNA sequencing, gene expression levels were measured as the number of reads per kb of exon regions per million mapped reads (RPKM) [33]. DEGs were detected with the R package DEGseq software [34]. A corrected P-value of 0.05 and a log₂ (fold change) of 1.5 were set as the threshold for statistically significant differential expression [35]. For the genes that were differentially expressed in the granulosa cells treated with the anti-inhibin antibodies compared to untreated cells, the unigene numbers were converted to Entrez Gene ID numbers using the online g:profile conversion tool (<http://biit.cs.ut.ee/gprofiler/>).

2.7. Analysis of GO category, KEGG pathway, and Protein-Protein Interactions

To gain insights into the functional significance of the differentially expressed genes following anti-inhibin antibody treatment, we examined their known biological functions and analyzed any pathway enrichment. GO term enrichment analysis was conducted for upregulated and downregulated genes using the R package TopGO [34]. Adrian Alexi's improved weighted scoring algorithm and Fisher's exact test were used to determine the significance of GO term enrichment. GO terms with corrected P values less than 0.05 were considered significantly enriched [35]. Three structured controlled vocabularies, including cellular component, molecular function, and biological process, were used for consistent description of gene products as previously described [36]. Association of the differentially expressed genes with different pathways was computed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg>) [37,38]. Protein-protein interaction (PPI) analysis of differentially expressed genes was based on the STRING database [39]. A corrected P-value of 0.05 was set as the threshold to identify significantly different pathways [35].

2.8. Quantitative real-time RT-PCR

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, CA). The extracted product was treated with DNase (Ambion, Austin, TX) as per the

manufacturer's instructions and then quantified by UV spectrophotometry. cDNAs were reverse transcribed from 3µg of total RNA using the PrimeScript RT reagent Kit (Takara, Da Lian, China), while qRT-PCR analyses were performed using the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA). PCR was carried out in a 50 µL reaction volume with SYBR Green I Master Mix reagent (Toyobo, Osaka, Japan) and 2.5 pmol of specific primer pairs. β -actin was used as the reference gene, and all primer sequences used were listed in Table S1. Each qRT-PCR experiment was repeated three times. The relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method, while standard deviation was calculated from three biological replicates.

2.9 Statistical analysis

Differences between antibody-treated and control cells, in terms of cell number and concentration of E2 as well as differences of gene expression were analyzed by one-way analysis of variance (ANOVA). The means were compared using the least significant difference method. All values were expressed as mean $\pm$ SEM. All statistical analyses were performed with SAS software Version 8.01 (SAS Institute Inc.; Cary, NC, USA).

3. Results

3.1. Estradiol secretion and cell proliferation

We found a 10-fold increase in E2 concentration in the culture medium of granulosa cells treated with anti-inhibin α -subunit antibodies at 200 µg/mL for 48 h compared to the control cells. Moreover, the granulosa cell number was increased after the anti-inhibin antibodies treatment (Fig. 1).

3.2. Transcriptome profile of granulosa cells treated with anti-inhibin antibodies

To survey changes in the porcine granulosa cell transcriptome following treatment with anti-inhibin α -subunit antibody, we constructed two cDNA libraries of the antibody-treated granulosa cells and non-treated control cells, respectively. Clean data

mapping and annotation showed the high quality of the two libraries, with a total of 40,582,824 and 43,243,032 sequences that were matched, respectively (Table 1 and Table 2). We also examined the global gene expression values. Based on the RPKM value, it was found that 94.03% of the genes were expressed at low abundance (i.e., $0.5 < \text{RPKM value} < 60$) (Table 3).

3.3. Differential gene expression profile

A total of 476 genes were differentially expressed in granulosa cells treated with an anti-inhibin antibody compared with those in the untreated control granulosa cells (Table S2). The expression level of 236 genes was significantly upregulated, and the expression level of 240 genes was significantly downregulated ($P < 0.05$) (Fig. 2). The ten most upregulated genes were: *COL11A1*, *AEBP1*, *COL1A2*, *ITGA8*, *COL8A1*, *APOE*, *CXCL14*, *α2M*, *CCND1*, and *COL1A1*. The ten most downregulated genes were: *IL1β*, *XDH*, *IL8*, *CCL8*, *CCL3L1*, *CCL23*, *S100A9*, *CXCL2*, *ARG1*, and *GABRP* (Table 4).

3.4. GO annotation and significantly enriched analysis of DEGs

The functional classification of the top 30 GO terms for all DEGs was shown in Fig. 3. In the biological process category, the DEGs enriched in GO terms were classified to ‘immune system process’ and ‘immune response’. In the cellular component category, enriched GO terms of interest included ‘extracellular region’, ‘extracellular region part’, and ‘extracellular space’. In the molecular function category, the DEGs were enriched in GO terms centered on ‘chemokine activity’ and ‘extracellular matrix structural constituents’.

In order to better understand the biological function of all DEGs, we divided these DEGs into two groups: upregulated and downregulated (Fig.4). Upregulated and downregulated DEGs both were mainly enriched in ‘developmental process’, ‘single-organism development process’, ‘anatomical structure development’, ‘multicellular organismal development’, and ‘system development’ (Fig.4A). In the cellular component analysis, the enriched GO terms for both upregulated and

downregulated DEGs were focused on “cytoplasmic part” and “extracellular matrix”(Fig. 4B). In the molecular function category, the enriched GO terms for both upregulated and downregulated DEGs were focused on ‘protein binding’ and ‘structural molecular activity’ (Fig. 4C).

3.5. KEGG pathway enrichment analysis

A total of 476 DEGs were assigned to one or more KEGG annotations and were mapped to ten KEGG pathways. These enriched pathways included ECM-receptor interactions, focal adhesion, ribosomes, antigen processing and presentation, protein digestion and absorption, the PI3K-Akt signaling pathway, glycolysis/gluconeogenesis, the NOD-like receptor signaling pathway, regulation of actin cytoskeleton, and the TNF signaling pathway (Table 5, Fig. S1).

3.6. Real-time PCR validation

Based on sequencing data and functional analysis, 20 DEGs were selected for validation by qRT-PCR. These genes were divided into five groups. The genes of the first group are involved in sex hormone secretion (*FSHR*, *CYP19A1*, *INHA*, *INHB*, and *STAR*). The second group included growth factors and related factors (*FN1*, *TIMP*, *IGF1*, *IGFBP2*, *IGFBP5*, *THBS1*, *FGF2*, and *VEGFA*). The third group was comprised of cell cycle-related genes (*CCND1*, *CCND2*). The fourth group included apoptosis-related genes (*BCL2*, *CASPASE3*). The fifth group was made up of genes related to transcriptional regulation (*CEBP*, *FOS*, and *FOXL2*). We found that the upregulation of sixteen genes (*FSHR*, *STAR*, *INHB*, *FN1*, *TIMP*, *IGF1*, *IGFBP2*, *IGFBP5*, *FGF2*, *VEGFA*, *CCND1*, *CCND2*, *BCL2*, *CEBP*, *FOS*, and *FOXL2*) exhibited by RNA sequencing in anti-inhibin-treated cells was confirmed by qRT-PCR. The *THBS1* and *CASPASE* downregulation revealed by RNA sequencing was also confirmed by qRT-PCR. RNA sequencing demonstrated that *CYP19A1* and *INHA* genes were upregulated in porcine granulosa cells treated with anti-inhibin antibody, but qRT-PCR did not confirm the data . These results showed that differential expression was validated for eighteen of twenty genes (Fig. 5).

3.7. Protein-Protein Interactions Network Analysis

Five PPI sub-networks were constructed by mapping the downregulated and upregulated DEGs. Among them, networks A and D were related to ‘matrix’ and ‘extracellular matrix’, respectively (Fig. 6A and 6D). Network A consisted of twenty-one DEGs, including fibronectin 1 (*FN1*), integrin, beta 1 (*ITGB1*), and *CD61*. Network D was composed of five DEGs (*MMP2*, *MMP9*, *MMP14*, *TIMP1*, and *TIMP2*). Network B was related to the interaction of ribosomal proteins, and included sixteen ribosomal proteins (Fig. 6B). Network C was related to mitochondrial respiratory chain carbon metabolism (Fig. 6C). Finally, network E consisted of five chemokines (*AMCFII*, *CAMP-1*, *IL8*, *IL1 β* , and *JUNB*).

4. Discussion

Immunization against inhibin, which neutralizes the bioactivity of endogenous inhibin, can be used as a technique for enhancing animal ovarian follicular development. Using this approach, the researchers have successfully stimulated ovarian follicle development and improved ovulation rate [10, 11], embryo production [12, 14], and even litter size [40-42]. Apart from the mechanism of stimulating pituitary FSH secretion, it is likely that inhibin immunization enhances follicle development through a direct effect on granulosa cell proliferation and steroid hormone secretion. Such effects have been demonstrated in both bovine and porcine granulosa cells cultured *in vitro* and treated with anti-inhibin antibodies [18, 25]. In this study, we observed that treatment of porcine granulosa cells cultured *in vitro* with the anti-inhibin antibodies stimulated cell proliferation as well as E2 secretion.

Transcriptome analysis of the granulosa cells treated with the antibodies revealed 476 DEGs, including 27 novel genes. Most of these DEGs were mapped to ECM-receptor interactions, focal adhesion, ribosomes, antigen processing and presentation, protein digestion and absorption, the PI3K-Akt signaling pathway, glycolysis/gluconeogenesis, the NOD-like receptor signaling pathway, regulation of the actin cytoskeleton, and the TNF signaling pathway. These results suggest that

treatment of the anti-inhibin antibodies enhanced cultured porcine granulosa cell function by strengthening the molecular processes of chemotaxis, extracellular structural organization, cell proliferation, differentiation, and focal adhesion.

Validation of the data obtained by RNA-seq was performed using qRT-PCR. Eighteen of twenty genes selected for qRT-PCR exhibited similar changes in expression pattern observed after the RNA-seq analysis. However, some discrepancies did exist between the RNA-seq data and the qPCR results. For example, although estradiol secretion was similarly enhanced in anti-inhibin antibody-treated granulosa cells in this and in a previous study [15], we observed only a slight increase in CYP19A1 mRNA expression in the RNA-seq data, in contrast to the substantial upregulation detected by qRT-PCR in the other study [15]. These discrepancies may be caused by differences between the linear accumulation of the RNA-seq reads and the exponential expansion of PCR products. Alternatively, synthesis and secretion of E2 may also involve molecules or enzymes other than CYP19A1; one such molecule could be α 2-M. This protein has been observed to be capable of dramatically elevating ovarian follicle E2 secretion *in vivo* without affecting aromatase activity [43]. In the present study, granulosa cell α 2-M gene expression was substantially upregulated by the treatment of the anti-inhibin antibodies, and so this could also have contributed to the enhancement of E2 secretion. Alternatively, the enhanced E2 secretion could also arise as a result of a higher number of granulosa cells. To this end, anti-inhibin α -subunit antibody treatment led to enhanced cell proliferation in the present study, but was inconclusive in a previous study [44]. As additional evidence of the increase in E2 levels, several of the differentially expressed genes were estrogen-target genes, including SMAD3, FOXO1, and BMP6, among others.

Besides genes involved in the enhanced sex hormone synthesis and secretion, (such as steroid synthases and gonadotrophin receptor signal transduction pathways), the DEGs regulated by anti-inhibin antibody treatment also included immune response factors such as chemokines and interleukins. Chemokines, which are small molecular mass peptides secreted by leukocytes, have been thought to influence ovarian function by acting on genes associated with follicular atresia, ovulation, and

luteolysis [45, 46]. Because ovulation of mammalian follicles has been likened to an inflammatory response, it is perhaps not surprising that chemokines or interleukins are involved in the process of ovulation or follicle maturation. In fact, one of the C-X-C type chemokines, *IL8*, has been shown to be involved in follicle maturation and ovulation [47, 48]. *IL8* production by human granulosa cells has also been shown to be increased by hCG treatment [46, 47]. Furthermore, *IL-8* increased the ovulation rate of rabbits following hCG treatment, and this effect could be impeded using anti-*IL-8* antibody treatment [48]. Apart from these findings, other immune factors have also been demonstrated to be involvement in the regulation of follicle maturation and ovulation [49, 50]. For example, *IL-1 β* was found to promote ovulation in rats [49] and rabbits [50]. In our study, anti-inhibin antibody treatment of porcine granulosa cells downregulated *IL-1 β* and *IL8* gene expression. These data indicate that the molecular mechanism by which anti-inhibin antibodies promote oocyte maturation and ovulation is different from that of hCG-induced ovulation.

We also found that the expression levels of genes related to the extracellular matrix (ECM) were also significantly altered in granulosa cells following treatment with the anti-inhibin antibodies. The ECM is vital for supporting ovarian follicle growth and maintaining its proper function, as it constitutes the follicular basal lamina and is responsible for aggregation of mural granulosa cells. The ECM is involved in processes such as follicle development and cell migration, proliferation, and growth [51, 52]. Previously, treatment with the anti-inhibin antibodies has been shown to upregulate the expression of *FNI* and thrombospondin 1 (*THBS1*) in granulosa cells [18]. Additional ECM related genes, *-FNI* and *α 2-M* were found to be upregulated more than three-fold after anti-inhibin antibody treatment in the present study. KEGG analysis of the gene expression data pointed to several genes encoding ECM proteins such as collagens (*COL1A1* to *COL5A2*), *FNI* and fibrillin 1 (*FBN1*), and their integrin receptors (*ITGAs*, *ITGBs*). *FN1* and *FBN1* are ubiquitous ECM glycoproteins that are assembled into a fibrillar matrix in all tissues, including ovarian follicular tissues [51,53]. By binding to the transmembrane receptors such as integrins, matrix molecules such as *FN1* influence cell signal transduction and ultimately regulate

cellular signaling [54]. The protein-protein interaction network constructed from the differentially expressed genes following anti-inhibin antibody treatment also indicated genes encoding ECM proteins and their integrin receptors. These included Type I and IV collagen, fibronectin, laminin, matrigel, and other ECM proteins. This ECM protein interaction network constructed using DEGs, and these DEGs were found in bovine granulosa cells super-stimulated *in vivo* with FSH [28]. This is perhaps of no surprise, as immunization against inhibin and FSH lead both to enhancement of follicle growth and estradiol secretion [11, 55] *via* enhancement of granulosa cell proliferation, steroidogenesis, extracellular matrix remodeling, and angiogenesis [43, 56]. Therefore, these *in vivo* regulatory mechanisms leading to enhanced follicle development are also reflected *in vitro* in cultured porcine granulosa cells with treatment of the anti-inhibin antibodies.

The anti-inhibin antibody treatment may increase growth factor expression in granulosa cells. In this study, the mRNA expression levels of the IGF1 ($\log_2\text{Fold_change} = 2.03$) and EGF ($\log_2\text{Fold_change} = 0.29$) genes were also slightly upregulated by anti-inhibin antibody treatment. IGF-I has been shown to rescue granulosa cells from apoptosis [57,58]. We also found that the mRNA expression levels of the genes encoding the anti-apoptosis proteins BCL2 and TIMP2 were also upregulated by anti-inhibin antibody treatment. FOXO1, a critical factor in promoting follicular atresia and granulosa cell apoptosis, has been shown to be suppressed by FSH treatment *in vivo* [59]. In this *in vitro* study, when FSH was not present, FOXO1 expression was also found to be downregulated by the anti-inhibin antibody treatment, but the change was not significantly (Table S2). This data suggest that treatment with anti-inhibin antibody may play a similar role to that of FSH in downregulating FOXO1 gene expression. All of the above data suggest that anti-inhibin antibody treatment promoted the expression of growth factors and at the same time suppressed apoptosis of the granulosa cells, ultimately promoting growth and survival of granulosa cells. It is proposed that this is the basis for the enhancement in ovarian follicle growth and hormone secretion observed in inhibin-immunized animals [11,13,16].

Due to extracellular matrix remodeling, changes in granulosa cell functions are fulfilled *via* upregulation or downregulation of the expression of relevant genes. Antibody treatment promoted cell division or proliferation by upregulating the expression of genes *CCND1* and *CCND3* related with the cell cycle [60, 61], which was consistent with the higher MTT values noted in cells treated with the anti-inhibin antibodies. Treatment with the anti-inhibin antibodies had also been shown to upregulate the expression of insulin growth factor binding protein genes, such as *IGFBP4*, *IGFBP5*, and *IGFBP7* [18]. Upregulation of the anti-apoptotic genes (*TIMP2*, *ADAMTS2*, *ADAMTS7*, and *ADAMTS14*) would further enhance granulosa cell viability following treatment with the anti-inhibin antibodies [62, 63]. In fact, in the current study, the number of granulosa cells at the end of the culture period was significantly increased following antibody treatment. As an inhibitor of granulosa cell apoptosis, estrogen secreted by granulosa cells works along with many other anti- and pro-apoptotic factors that are involved in promoting follicular atresia to ensure that most of the follicles undergo degeneration during growth and development [64].

Finally, as expected, the RNA-seq technique allowed also to uncover twenty seven novel differentially expressed genes which are regulated by inhibin. GO and KEGG analyses have also enriched some of these genes into important molecular functions, and biological processes of granulosa cells. However, their exact functions are unknown at present, and thus warrant further study.

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Authors' contributions

Mingming Lei and Zhendan Shi designed the study, while Hui Li and Liuping Cai isolated and cultured of porcine ovarian granulosa cells. Zhen Chen measured E2 concentration and carried out real-time PCR, Mingming Lei and Zhendan Shi prepared the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

List of 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: estradiol, 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, FN1: fibronectin 1, α 2-M: α 2-Macroglobulin, FBN1: fibronectins, BCL2: B-Cell CLL/Lymphoma 2, CCND1: cyclinD1, CCND3: cyclinD3, ECM: the extracellular matrix, hCG: human chorionic gonadotropin.

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Table 1–Summary of the transcriptome sequencing data

Sample name	Raw reads	Clean reads	clean bases	Error rate(%)	Q20(%)	Q30(%)	GC content(%)
AGC_1	26341675	25453094	2.55G	0.03	97.89	93.26	52.39
AGC_2	26341675	25453094	2.55G	0.03	97.76	93.30	52.44
CGC_1	28440824	27217762	2.72G	0.03	97.27	91.51	51.76
CGC_2	28440824	27217762	2.72G	0.03	96.75	90.72	51.83

CGC: the control granulosa cells

AGC: the granulosa cells treated with anti-inhibin antibody

Table 2 –RNA- Seq statistics of control granulosa cells (CGC) and granulosa cells treated with anti-inhibin antibody (AGC)

Sample name	AGC	CGC
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Total reads	50906188	54435524
Total mapped	40582824 (79.72%)	43243032 (79.44%)
Multiple mapped	1657138 (3.26%)	1886231 (3.47%)
Uniquely mapped	38925686 (76.47%)	41356801 (75.97%)
Read-1	19498814 (38.3%)	20755209 (38.13%)
Read-2	19426872 (38.16%)	20601592 (37.85%)
Reads map to '+'	19465174 (38.24%)	20676351 (37.98%)
Reads map to '-'	19460512 (38.23%)	20680450 (37.99%)
Non-splice reads	25454629 (50%)	27314055 (50.18%)
Splice reads	13471057 (26.46%)	14042746 (25.8%)

Read1 refers to the left end of the measured sequence, and **read2** refers to the right end of the measured sequence in pair-end sequencing

Reads map to '+': sequence alignment to the positive chain of genome , **Reads map to '-'** : sequence alignment to the negative chain of genome

Table 3–Distribution of expression values of the differentially expressed genes

RPKM Interval	AGC	CGC
0~1	11529(46.32%)	11402(45.81%)
1~3	2469(9.92%)	2487(9.99%)
3~15	5564(22.36%)	5466(21.96%)
15~60	3840(15.43%)	4002(16.08%)
>60	1487(5.97%)	1532(6.16%)

CGC: the control granulosa cells

AGC: the granulosa cells treated with anti-inhibin antibody

Table 4–Top ten upregulated and downregulated genes in porcine granulosa cells treated with anti-inhibin antibody compared with the untreated controls

Genes	Description	Fold_change	P value
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Downregulated			
IL1 β	interleukin 1, beta	75.74	3.24E-07
XDH	xanthine dehydrogenase	28.20	8.80E-05
IL8	interleukin 8	25.17	1.97E-13
CCL8	chemokine (C-C motif) ligand 8	13.91	1.03E-05
CCL3L1	chemokine (C-C motif) ligand 3-like 1	10.38	3.57E-04
CCL23	chemokine (C-C motif) ligand 23	9.96	5.01E-06
S100A9	S100 calcium binding protein A9	9.23	9.08E-05
CXCL2	chemokine (C-X-C motif) ligand 2	8.27	7.23E-09
ARG1	Arginase 1	2.88	8.54E-04
GABRP	Gamma-Aminobutyric Acid (GABA) A Receptor, Pi	2.72	7.73E-09
SAA4	Serum Amyloid A4, Constitutive	2.64	3.22E-13
S100A8	S100 calcium binding protein A8	2.29	1.9 E-04
Upregulated			
COL11A1	collagen, type XI, alpha 1	21.45	6.44E-253
AEBP1	AE binding protein 1	12.76	1.88E-137
COL1A2	collagen, type I, alpha 2	11.50	8.37E-213
ITGA8	integrin, alpha 8	9.98	1.68E-15
COL8A1	collagen, type VIII, alpha 1	9.59	1.41E-14
APOE	apolipoprotein E	9.14	4.22E-90
CXCL14	chemokine (C-X-C motif) ligand 14 (CXCL14)	7.69	8.73E-06
α 2M	alpha-2-macroglobulin	7.39	7.61E-35
CCND1	cyclinD1	6.98	7.06E-35
COL1A1	collagen, type I, alpha 1	7.24	2.14E-05

Table 5–Canonical pathways to differentially expressed genes in granulosa cells treated with anti-inhibin antibody

Pathway ID	KEGG pathway name	No. of genes	Genes	P-Value
ssc04512	ECM-receptor interaction	17	<i>CD61, COL11A1, COL1A2, COL3A1, COL4A1, COL4A2, COL5A1, COL5A2, FN1, ITGA11, ITGA2, ITGA8, ITGAV, ITGB1, SPP1, THBS1, THBS2, Novel00619, Novel01574</i>	4.32E-11
ssc04510	Focal adhesion	22	<i>CCND1, CD61, COL11A1, COL1A2, COL3A1, COL4A1, COL4A2, COL5A1, COL5A2, FN1, ITGA11, ITGA2, ITGA8, ITGAV, ITGB1, JUNB, MYL9, MYLK, PPP1CB, SPP1, THBS1, THBS2, Novel00619 RPL7A, RPL9,RPL4, RPS26, RPL24, RPL22, RPS3, RPL3, RPL5, RPS18, RPS4, RPL30, RPL19, RPS3A, RPS23, RPS27A, RPL13A, RPL6</i>	2.63E-07
ssc03010	Ribosome	18	<i>RPS18, RPS4, RPL30, RPL19, RPS3A, RPS23, RPS27A, RPL13A, RPL6</i>	
ssc04612	Antigen processing and presentation	12	<i>SLA-I, □2M, HSPCB, SLA-II, PSME2, IFI30, CTSB, CANX, CALR, TMP-SLA-3, SLA-3</i>	7.81E-05
ssc04974	Protein digestion and absorption	10	<i>COL9A2, COL3A1, COL5A2, COL11A1, COL18A1, COL5A1, COL1A2, COL14A1, COL4A1, COL4A2</i>	0.0002
ssc04151	PI3K-Akt signaling pathway	23	<i>GNG2, TGAV, TNC, COL11A1, COL1A2, ITGA8, KIT, THBS1, FN1, CD61, COL3A1, COL5A2, ITGA11, ITGB1, COL4A2, COL4A1,</i>	0.0106

			<i>HSPCB, THBS2, ITGA2, CCND1, COL5A1, SPP1, HSP90B1, Novel00619</i>	
ssc00010	Glycolysis / Gluconeogenesis	7	<i>ACSS1, ENO3, HK1, PKM</i>	0.0149
ssc04621	NOD-like receptor signaling pathway	6	<i>CXCL8, HSPCB, IL1α, NFKB1A, HSP90B1</i>	0.0211
ssc04810	Regulation of actin cytoskeleton	16	<i>FN1, CD61, ITGA8, MYLK, ITGAV, EZR, CFL1, MYL9, ENAH, ITGA2, PIP4K2A, ITGA11, PPP1CB, ITGB1, TMSB4X</i>	0.0252
ssc04668	TNF signaling pathway	10	<i>TNFAIP3, ICAM-1, JUNB, CH242-185F9.2, CXCL2, IL1B, NFKB1A, MMP9, MMP14, VCAM1</i>	0.0295

Figure legends

Fig.1–Porcine granulosa cell number and estradiol concentration in the culture medium of porcine granulosa cells after 200 µg/mL anti-inhibin antibody treatment (mean ± SEM). A-C: $p < 0.01$.

Fig. 2–Venn diagram summarizing sequencing analysis results of granulosa cells treated with 200 µg/mL anti-inhibin antibody. The number of the upregulated genes and downregulated genes was determined using a fold change of ≥ 1.5 and a P value of ≤ 0.05 .

Fig.3–Classification of the differentially expressed genes in granulosa cells after anti-inhibin antibody treatment. CGC: Control granulosa cells, and AGC: Granulosa cells treated the 200 µg/mL anti-inhibin antibodies. The asterisks represent that DEGs are significantly enriched in the GO terms ($p < 0.05$).

Fig.4–Function analysis of the differentially expressed genes of granulosa cells after anti-inhibin antibody treatment. CGC: control granulosa cells, and AGC: granulosa cells treated the 200 µg/mL anti-inhibin antibodies. Dark box represent the number of the upregulated genes and white box represent the number of the downregulated genes.

Fig. 5–Quantification ($\log_2$ of fold change; mean ± SEM) of the mRNA profile of granulosa cells treated with anti-inhibin antibody using RNA-seq and qRT-PCR. White box represents the expression level of of the differentially expressed genes detected by the RNA-seq, while dark box represents the expression level of the differentially expressed genes obtained by qRT-PCR.

Fig. 6—Protein interaction networks of the differentially expressed genes. A and D: matrix and extracellular matrix networks; B: ribosomal protein network; C: carbon metabolism network; E: chemokine network. Nodes are labeled with black color to indicate the types of proteins represented. The node color represents the connectivity between genes. Deeper color is associated with a better connectivity between adjacent genes. Edge width represents the interaction between the two nodes. The wider edge indicates a stronger interaction. Nodes are labeled with black color to indicate the types of proteins represented. The node color represents the connectivity between genes. Deeper color is associated with a better connectivity between adjacent genes. Edge width represents the interaction between the two nodes. The wider edge indicates a stronger interaction.

Figure

Figure

The Most Enriched GO Terms (CGCsvsAGC)

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