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Orexin A promotes progesterone secretion in luteinized granulose cells of Mongolian *Ovis aries* ovary by *PRRT2* and *ABCG1* genes

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Research Article

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Summary

To study the role of orexin A in the reproductive regulation of Mongolian sheep, ovine ovarian granulosa cells were cultured *in vitro*. The cells were divided into groups after luteinization, the experimental group was given orexin A and the transcriptome was sequenced together with that of the control group. The different genes related to reproduction were screened out. qRT-PCR, western blot and enzyme-linked immunosorbent assay (ELISA) were used to verify the selected genes and detect the effect on progesterone secretion. In total, 123 differentially expressed genes were obtained by sequencing. Six genes with high expression related to reproduction (PRRT2, ABCG1, SOX4, TBX3, ID1 and ATP8) were screened. The results of qRT-PCR were consistent with those of sequencing; western blot and ELISA were used to verify the protein levels of steroidogenic acute regulatory protein (StAR) and its related PRRT2 and ABCG1, and to detect their effect on progesterone secretion. Validation results were consistent with those of qRT-PCR and sequencing. The experimental group was given orexin A and compared with the control group. Expression of PRRT2 protein was significantly increased ($P < 0.05$), ABCG1 protein expression was significantly decreased ($P < 0.05$), StAR expression was significantly increased ($P < 0.05$), and progesterone secretion was significantly increased ($P < 0.05$). The results showed that orexin A promoted the expression of StAR by upregulating PRRT2 and downregulating ABCG1, therefore affecting secretion of progesterone. Gene expression characteristics of orexin A affecting progesterone secretion were preliminarily explored; this study provides a theoretical basis for further study on signalling pathways and reproductive regulation in Mongolian sheep.

Introduction

Orexin A was first found in the hypothalamus of rats. On further research, orexin A was also found to be distributed in other glandular tissues compared with the centrum, such as pituitary, adrenal glands, thyroid, testicle and ovary (Zu *et al*., 2017). As an important neuropeptide, orexin A functions through its receptor/G-protein-coupling (Dobrzyn *et al*., 2018, 2019). In recent years, orexin A's participation in reproductive activities and regulation of the cell growth cycle has become a research focus. Most of these studies have focussed on rats, mice, pigs, humans and some fish, while relatively few studies have focussed on sheep. Some studies showed that steroidogenic acute regulatory protein (StAR) was a key synthetase in progesterone synthesis. StAR can regulate conversion of cholesterol into steroid hormones and transport of cholesterol from ovarian granulosa cells to mitochondria (Dobrzyn *et al*., 2019).

In this study, Illumina sequencing technology was applied to determine the gene expression profiles of luteinized granulosa cells in the ovaries of sheep supplemented with orexin A. Our laboratory has previously demonstrated that the orexin A receptor is expressed in sheep ovarian granulosa cells and corpora lutea. The optimal dose of orexin A was 10 nmol/l, and the optimal time was 24 h (Li *et al*., 2017; Guo *et al*., 2019). We performed transcriptome screening and enrichment analysis of luteinized granulosa cells plus those treated with orexin A, and revealed the gene expression characteristics of luteinized granulosa cells with orexin A and their signalling pathways. This study serves as a preliminary exploration for further research on the reproductive regulation mechanism of orexin A in sheep. Functional verification of differential genes *PRRT2*, *KIRREL*, *ATP8*, *TBX3*, *SOX4*, *ID1* was performed using qPCR technology. The qRT-PCR results were consistent with the sequencing results, and signalling pathways were studied.

Materials and methods

Sample collection

In this study, adult Mongolian sheep ($n = 10$) were euthanized under the approval of the Animal Ethics Committee of the Inner Mongolia Agriculture University (licence no. SYXK, Inner

Mongolia, 2016-0017). After euthanizing the animals, ovaries were harvested, flushed with ice-cold physiological saline, placed in ice-cold phosphate-buffered saline (Gibco) supplemented with 5% penicillin/streptomycin (Gibco), and transported to a biosafety cabinet for processing.

Luteinization and identification of ovary granular cells in Ovis aries

The experimental protocol was approved by the Ethics Committee on the Use and Care of Animals, Inner Mongolia Agricultural University, China. Refer to the methods of Li Min *et al.* (2019) for culture of sheep ovarian granulosa cells. The cells were cultured with follicle stimulating hormone (FSH; 2.5 IU/ml), luteinizing hormone (LH; 2.5 IU/ml), and oestradiol (E2; 1 μg/ml) for 24 h. Cell supernatant was collected, then centrifuged for 10 min at 1300 rpm. The supernatant was then collected and the amount of progesterone secreted in the cells was detected using a sheep progesterone enzyme-linked immunosorbent assay (ELISA) kit.

Total RNA extraction

For ovarian luteinized granulocytes (FLE-1, FLE-2, FLE-3) and the luteinized granulocytes (Ore-A-1, Ore-A-2, Ore-A-3) supplemented with orexin A, total RNA was extracted (RNA fast 200), and the concentration and purity were tested. Samples with A_{260}/A_{280} ratios between 1.8 and 2.2 qualified for inclusion. RNA integrity was tested on 1% agarose gels and samples were stored at −80 °C.

Illumina sequencing

For ovarian luteinized granulosa (FLE-1, FLE-2, FLE-3) and luteinized granulocytes (Ore-A-1, Ore-A-2, Ore-A-3) supplemented with orexin A, total RNA was extracted using RNA fast 200, and sent to a company for sequencing. Differentially enriched genes were analyzed.

Identification of differential genes

Quantitative reverse transcription PCR (qRT-PCR) was used to verify PRRT2, ATP8, TBX3, SOX4, KIRREL, ABCG1 and consistent expression of the β-actin gene.

Orexin A affects the expression of StAR protein through PRRT2 and ABCG1

Cells were divided into luteinized granulosa cell group, luteinized granulosa cells + orexin A (24 h) group, luteinized granulosa cells + PRRT2 inhibitor groups (100 nmol/l, 1 μmol/l, 10 μmol/l), luteinized granulosa cells + PRRT2 inhibitor groups (100 nmol/l, 1 μmol/l, 10 μmol/l) + orexin A group, luteinized granulosa cells + different concentrations of ABCG1 inhibitor group (20 nmol/l, 2 μmol/l, 200 μmol/l), luteinized granulosa cells + different concentrations of ABCG1 inhibitor group (20 nmol/l, 2 μmol/l, 200 μmol/l) + orexin A group.

The supernatant was collected, and the optimal concentrations of PRRT2, ABCG1 inhibitor and progesterone concentration were detected by ELISA. Cell protein was extracted, and the expression levels of PRRT2, ABCG1 and StAR protein were detected using western blot.

Statistical analysis of test data

GraphPad Prism 5 was used for statistical analysis, the test method was *t*-test. Gene Ontology (GO; http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway enrichment analyses were performed for all differentially expressed genes (DEGs). Each treatment was designed with three replicates and each experiment was repeated three times. The confidence level was significantly different at 95%.

Results

Ovarian granulosa cell culture and luteinized identification

Ovarian granulosa cells were cultured *in vitro*, Fig. 1(a) shows that ovarian granulosa cells were spindle shaped, and the growth was stable. As shown in Fig. 1(b), the granulocytes were stimulated with FSH, LH or E2 for 24 h. Comparing group A and group B, progesterone expression was significantly increased ($P < 0.01$), indicating that ovine ovarian granulosa cells had been luteinized.

Illumina sequencing

Luteinized granulosa cells of sheep ovaries were selected as the control group (sample numbers: FLE-1, FLE-2, FLE-3) and luteinized granulosa cells with added orexin A as the experimental group (sample numbers: Ore-A-1, Ore-A-2, Ore-A-3). These samples were used to construct and sequence transcriptome libraries.

Fragments per kilobase of transcript per million mapped reads (FPKM) represent the number of transcripts per thousand bases per million aligned fragments. The FPKM method distinguishes the effect of differences in gene length and sequencing quantity for calculation of gene expression. Calculated gene expression can be directly used to compare the difference in gene expression between different samples. Through differential gene screening ($|\log_2(FC)| > 1$, *P*-value <0.05), in total, 123 significantly DEGs were obtained, of which 54 genes were upregulated and 69 genes were downregulated (Fig. 2a). The differential gene clustering heat map in Fig. 2(b) shows hierarchically clustered gene expression patterns. Some genes with similar expression patterns may have a common function or participate in a common signalling pathway.

Differential gene enrichment analysis

The above DEGs were enriched as analyzed using GO and KEGG pathway functions using the online DAVID resource. Results of GO analysis are shown in Fig. 3(a, b). Genes with significant differences were mainly enriched in metabolic processes, biological processes regulation, transcription regulation and biological transport. The results of KEGG enrichment analysis are shown in Fig. 3(c), mainly enriched in metabolism, RAS, MAPK and NFκB signal pathways, as well as cancer, ribosome synthesis.

qRT-PCR verification results

As can be seen from Fig. 4, compared with the FLE group, expression levels of PRRT2, KIRREL and ATP8 were significantly higher in the added orexin A group ($P < 0.01$). Compared with the added orexin A group, expression levels of ID1, SOX4, TBX3, and ABCG1

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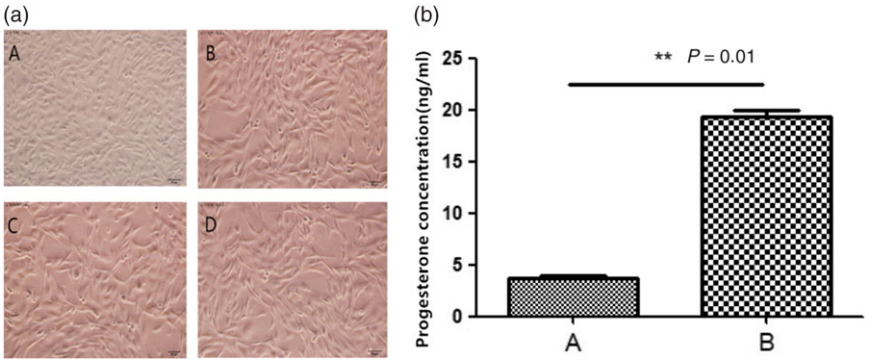

Figure 1. Culture and luteinization identification of ovarian granulosa cells in Mongolian sheep. (a, A) Primary granulosa cell; (a, B) F1 generation granulosa cell; (a, C) F2 generation granulosa cells; (a, D) F3 generation granulosa cells. (b) Group A: non-luteinized granular cells treated for 24 h; group B: luteinized granulosa cells treated for 24 h. $^{**}P < 0.01$ compared with the control group.

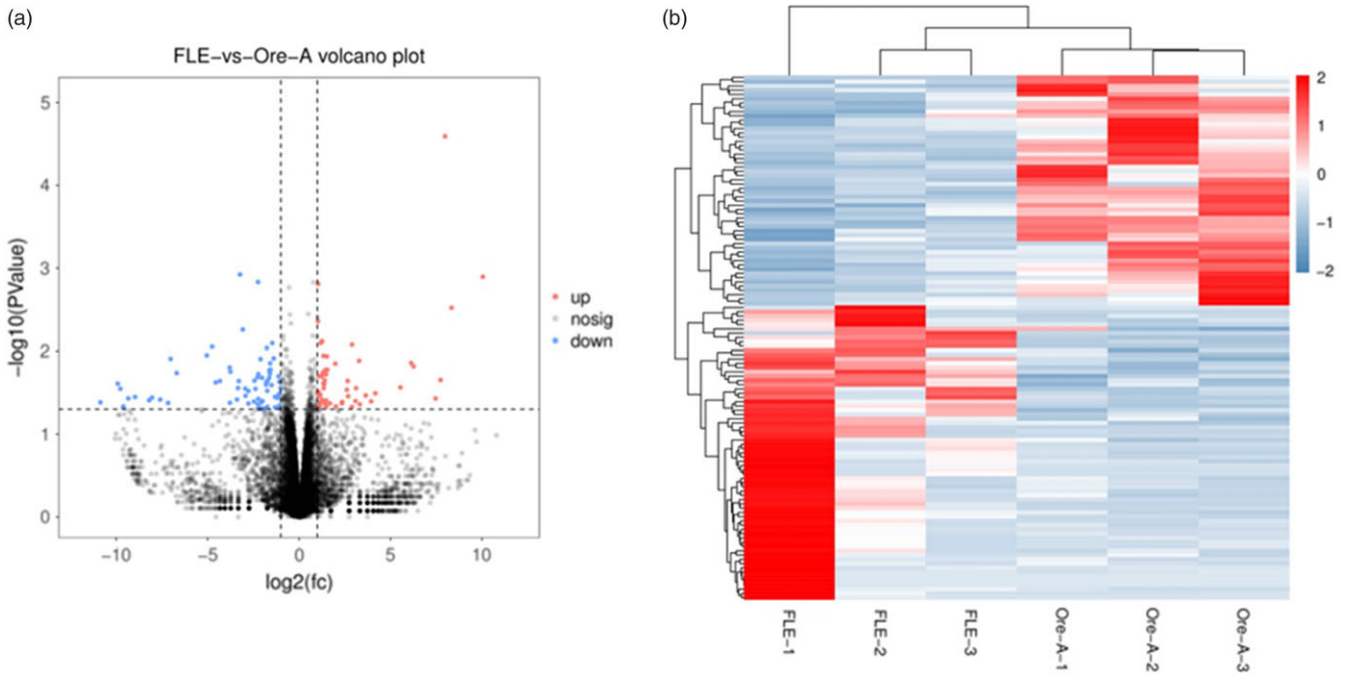

Figure 2. Volcano plot and heatmap of the luteinized granulosa group with orexin A and the control group. Red indicates upregulated expression, blue indicates downregulated expression, and black indicates no significant change in expression. FLE-1, FLE-2 and FLE-3 are groups of luteinized granulosa cells; Ore-A-1, Ore-A-2 and Ore-A-3 are groups of luteinized granulosa cells with orexin A added.

in the FLE group were significantly higher ($P < 0.01$), which was consistent with the sequencing results.

Effect of PRRT2 and ABCG1 inhibition on progesterone secretion of luteinized granulosa cells was detected by ELISA

As shown in Fig. 5(a), the progesterone content of luteinized granulosa cells in group B was significantly higher compared with that in group A ($P < 0.05$). In group C with added 10 nmol/l HY-10230, group D with added 100 nmol/l HY-10230, group E with added 1 μmol/L HY-10230 compared with the luteinized granulosa cells (group A), the concentrations of progesterone were significantly lower compared with those in group A ($P < 0.05$). In group F (luteinized granulosa cells + 10 nmol /l HY-10230 + orexin A), and group G (luteinized granulosa cells + 100 nmol/l HY-10230 + orexin A) compared with luteinized granulosa cells supplemented with orexin A (group B), the concentration of progesterone was significantly decreased ($P < 0.05$), and that in group H (luteinized granulosa cells + 1 μmol/l HY-10230 + orexin A) was significantly lower compared with that in group B ($P < 0.01$).

These results showed that orexin A could promote progesterone secretion and regulate progesterone secretion of luteinized granulosa cells by regulating PRRT2 protein. When the concentration of PRRT2 inhibitor was 1 μmol/l, the effect was the strongest.

As shown in Fig. 5(b), progesterone in group B was significantly higher compared with that in group A ($P < 0.05$), indicating that orexin A can increase the secretion of progesterone in granulosa cells of sheep ovary. Compared with group A, the secretion of progesterone in group C with added 20 nmol/l T0901317, group D with added 2 nmol/l T0901317 and group E with added 200 μmol/l T0901317 was increased, and the effect was most significant at the concentration of 20 nmol/L ($P < 0.05$). Compared with group B, the concentration of 20 nmol/l in group F (luteinized granulosa cells + 20 nmol/l T0901317 + orexin A), group G (luteinized granulosa cells + 2 nmol/l T0901317 + orexin A) and group H (luteinized granulosa cells + 200 μmol/l T0901317 + orexin A) the level was significantly increased ($P < 0.05$).

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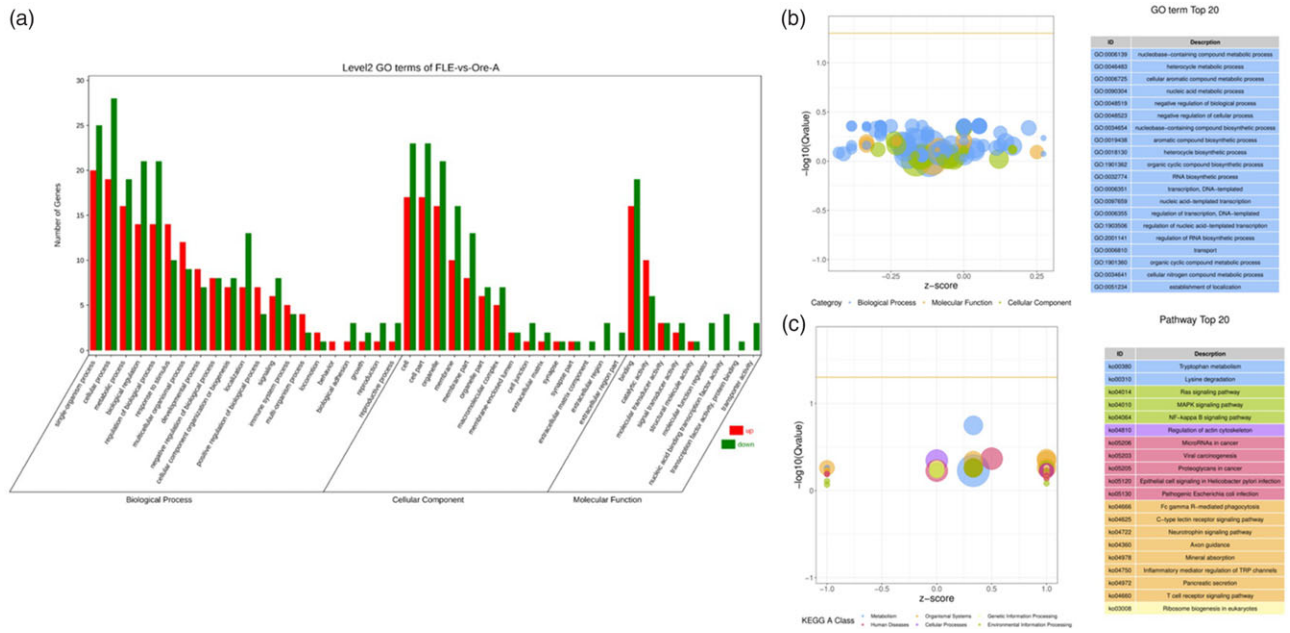

Figure 3. (a) Enrichment analysis results of GO and KEGG analysis. Red indicates upregulated expression, green indicates downregulated expression. (b, c) The vertical coordinate is $-\log_{10}$ (Q-value), the horizontal coordinate is the *Z*-score, the yellow line represents the threshold value of value = 0.05, and different colours represent different ontology groups.

These results indicated that the optimal concentration of ABCG1 inhibitor T0901317 on ovine ovarian granulosa cells was 20 nmol/l. Moreover, orexin A promoted progesterone secretion from luteinized ovarian granulosa cells, and this was regulated by ABCG1 protein.

Effect of orexin A on the expression of PRRT2 and StAR in ovarian luteinized granulosa cells

As can be seen from Fig. 6(a), the expression of PRRT2 in group B was significantly lower compared with that in group A ($P < 0.05$), indicating that orexin A can increase the expression of ABCG1 in luteinized granulosa cells of ovine ovary, which was consistent with the sequencing results and differential gene verification results. Expression of PRRT2 in group A was significantly higher compared with that in group C ($P < 0.05$); the expression of PRRT2 in group D was significantly lower compared with that in group C ($P < 0.05$), orexin A could increase the expression of PRRT2, and HY-10230 could inhibit the expression of PRRT2 in luteinized granulosa cells.

Compared with group A, the expression of StAR in group B was lower compared with that in group A, but there was no significant difference; the expression of StAR in group C was significantly higher compared with that in group A ($P < 0.01$); the expression of StAR in group D was significantly lower compared with that in group C ($P < 0.05$).

As can be seen from Fig. 6(b), the expression of ABCG1 in group B was significantly lower compared with that in group A ($P < 0.05$), indicating that orexin A can reduce the expression of ABCG1 in luteinized granulosa cells of ovine ovary, which was consistent with the sequencing results and differential gene verification results. Compared with group A, the expression of ABCG1 was significantly decreased in group C ($P < 0.05$); compared with group B, the expression of ABCG1 in group D was significantly lower compared with that in group B ($P < 0.05$).

Compared with group A, the expression of StAR in group B was significantly increased ($P < 0.05$), indicating that orexin A can increase the expression of StAR in luteinized granulosa cells of Mongolian sheep ovary; compared with group A, the expression of StAR was significantly increased in group C ($P < 0.05$), compared with group B, expression of StAR increased significantly in group D ($P < 0.05$).

In conclusion, addition of orexin A could increase progesterone secretion of luteinized granulosa cells, inhibition of PRRT2 could reduce progesterone secretion and inhibition of ABCG1 could increase progesterone secretion of luteinized granulosa cells after addition of orexin A.

Discussion

Orexin A is an important neuropeptide hormone that plays an important role in promoting physiological functions of animals (Guo *et al.*, 2019). After ovulation of mature follicles, granulocytes differentiate into granulocytes under the stimulation of LH, and mainly secrete progesterone. The granulocytes are luteinized by adding FSH, LH, and E2 *in vitro* to achieve granulocyte function and participate in embryo implantation and pregnancy to ensure the normal reproduction of sheep (Yang *et al.*, 2017). ELISA is used to detect progesterone content in the supernatant to identify if granulocytes were luteinized. Identification results were consistent with those obtained by Lu Xi (Lu, 2011), which demonstrated that Mongolian sheep ovary granulocytes have luteal bodies, to be examined in further research.

The optimal concentrations of PRRT2 and ABCG1 inhibitors in three concentration gradients were obtained. In the follow-up test, levels of StAR, PRRT2 and ABCG1 in cells were detected using western blot analysis to determine if orexin A could affect progesterone secretion through PRRT2 and ABCG1. In some cancer cells, activation of TACE/ADAM17 requires the association of PRRT2 induced cell sarcoma (c-Src) with TACE/ADAM17

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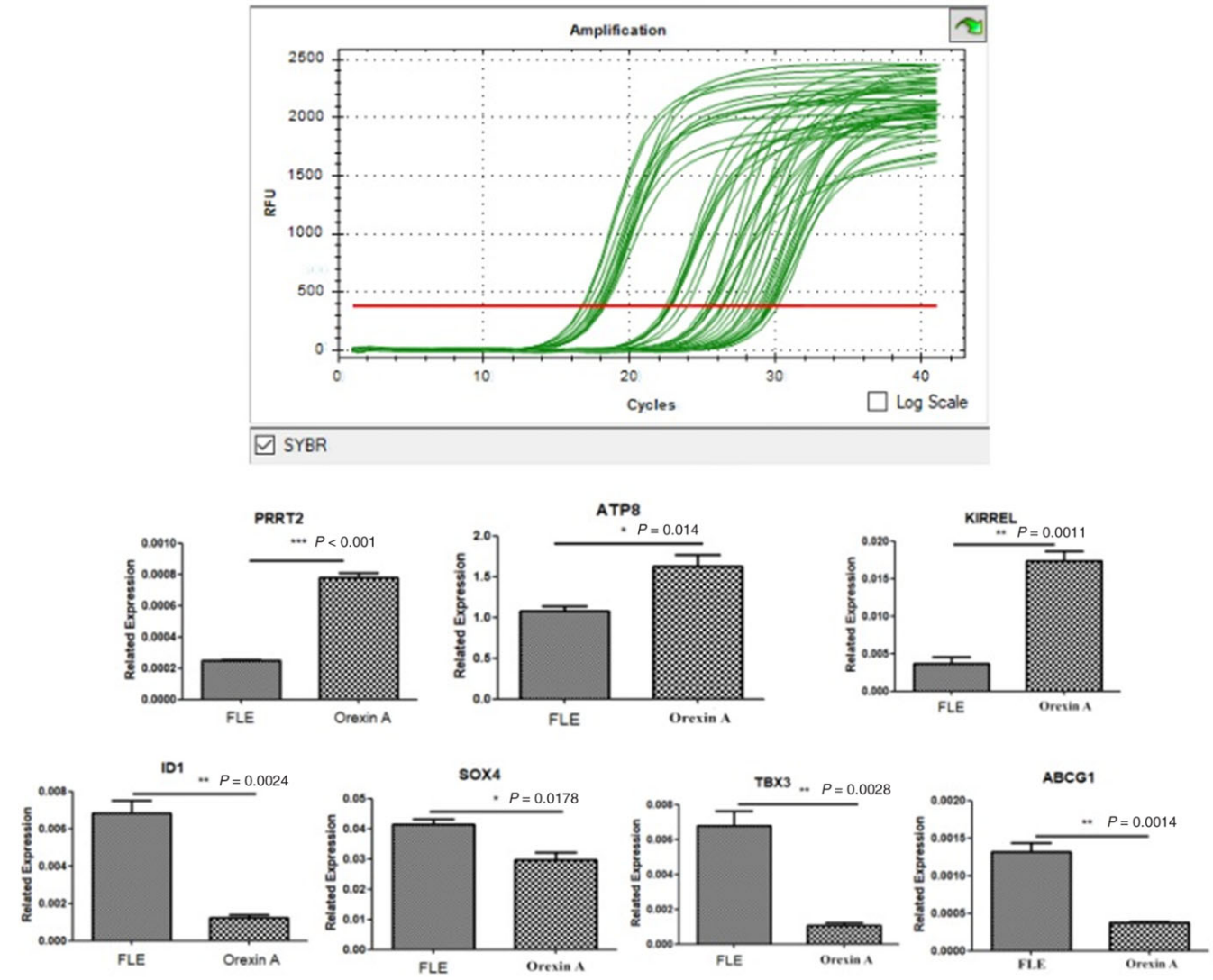

Figure 4. Amplification curve of luteinizing granulocytes and luteinizing granulocytes containing orexin A and expression of related genes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the control group.

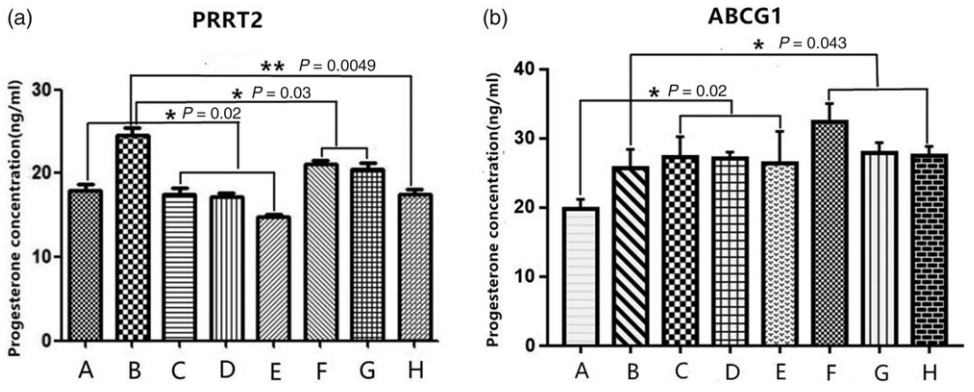

Figure 5. Effects of different concentrations of (a) PRRT22 and (b) ABCG1 inhibitors on progesterone secretion. A: luteinized granulosa cell; B: luteinized granulosa cell with orexin A; C–E: luteinized granulosa cells + different concentrations of PRRT22 and ABCG1 inhibitors; F–H: luteinized granulosa cells + different concentrations of PRRT22 and ABCG1 inhibitors + orexin A. *$P < 0.05$; **$P < 0.01$ compared with the control group.

(Desquiret-Dumas *et al.*, 2017). When granulosa cells or cumulus–oocyte complexes were cultured with FSH, PRRT2 activity and c-Src phosphorylation increased, which was related to the increase in TACE/ADAM17 enzyme activity, PRRT2 inhibitors caltroponin C (CAL C) and c-Src inhibitors [4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine (PP2)] inhibited the activity of TACE/ADAM17, but did not affect the expression of TACE/ADAM17 mRNA (Spießberger *et al.*, 2009; Groeneveled *et al.*, 2010). Immunoprecipitation analysis showed that FSH mediated the association of c-Src with TACE/ADAM17 through a PRRT2-dependent mechanism. For porcine granulosa cells *in vitro*, Calc or PP2 could inhibit the downstream signal

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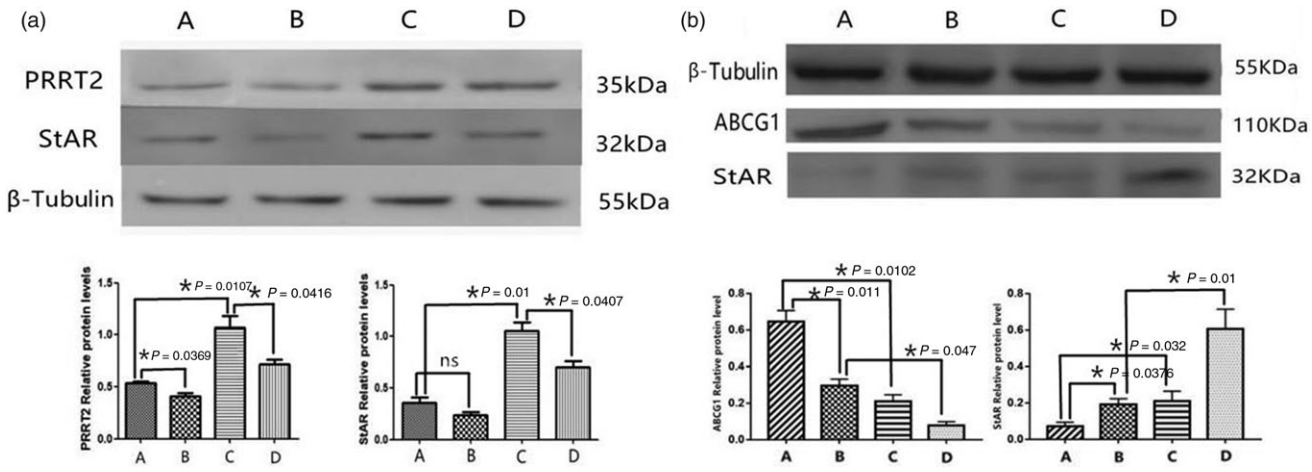

Figure 6. Effect of orexin A on PRRT2 and StAR expression in luteinized ovarian granulosa cells. (a) A: luteinized granulosa cells; B: luteinized granulosa cells treated with HY-10230 for 5 h; C: treatment of luteinized granulosa cells with orexin A; D: after 5 h of HY-10230 treatment, luteinized granulosa cells were treated with orexin A. (b) A: luteinized granulosa cells; B: treatment of luteinized granulosa cells with orexin A; C: luteinized granulosa cells treated with T0901317 for 48 h; D: after 48 h of T0901317 treatment, luteinized granulosa cells were treated with orexin A. *$P < 0.05$ compared with the control group.

transduction pathway of EGFR (MAPK3/1) and reduce the production of progesterone (Lehoux *et al.*, 1999; Belani *et al.*, 2018).

The precursor of progesterone synthesis is cholesterol, which is converted into steroid hormone progesterone in mitochondria and endoplasmic reticulum. ABCG1 is a protein that can promote cholesterol outflow and prevent cholesterol accumulation in the cell (Guo *et al.*, 2012; Yu *et al.*, 2018). In this study, progesterone secretion from luteinized granulosa cells increased after orexin A was added. The increase in progesterone would need to use more raw materials. To increase cholesterol synthesis, ABCG1 protein transporting cholesterol to the outside of cells would be reduced (Ju *et al.*, 2014). ABCG1 can effectively promote cholesterol efflux and reverse cholesterol transport, which is beneficial to remove excess cholesterol and avoid cholesterol accumulation in cells (Yang *et al.*, 2015). As a substrate for progesterone synthesis, most cholesterol is synthesized in the liver and then transported to steroid-producing tissues in the form of lipoprotein (Marathe *et al.*, 2012; Wang *et al.*, 2006). In this study, cells were divided into luteinized granulosa cells group, PRRT2 inhibitor treatment group, ABCG1 inhibitor treatment group, orexin A treatment group, luteinized granulosa cells + PRRT2 inhibitor + orexin A, luteinized granulosa cells + ABCG1 inhibitor + orexin A. Expression levels of StAR, PRRT2 and ABCG1 in these groups were compared, PRRT2 expression was significantly increased, while expression of ABCG1 protein was significantly decreased, which was consistent with the sequencing results and qRT-PCR. StAR expression decreased after PRRT2 was inhibited, and StAR expression was increased after ABCG1 inhibition, which indicated that StAR expression was related to PRRT2 and ABCG1, corresponding to previously published results. The effect of orexin A on luteinized granulosa cells is very complicated because it does not inhibit the upstream factors. The effect of orexin A on the expression of PRRT2 and ABCG1 and then the secretion of progesterone may involve many cell information transmission systems. Therefore, the existing experimental results are likely to have an influence on cell signal transduction network, and the cascade reaction of multiple signal factors cannot be ruled out. In the future, we will further explore the mechanism of progesterone secretion by luteinized granulosa cells and explore new ways to prevent Mongolian sheep reproductive diseases and promote fertility.

In conclusion, luteinized granulosa cells could secreted progesterone 24 h after being treated with FSH (2.5 IU/ml), LH (2.5 IU/ml) and E2 (1 μl/ml). In total, 123 DEGs were identified by transcriptome sequencing, including 54 upregulated genes and 69 downregulated genes. qRT-PCR, ELISA and western blot results confirmed that orexin A could regulate the expression of StAR by affecting PRRT2 and ABCG1, therefore affecting progesterone secretion, and could provide a basis for studying the regulation mechanism of progesterone secretion.

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Conflict of interest. None

Ethical standards. In this study, adult Mongolian sheep were euthanized under the approval of the Animal Ethics Committee of the Inner Mongolia Agriculture University (Licence No. SYXK, Inner Mongolia, 2016-0017).

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